POTATO PROTEINS: ISOLATION, CHARACTERIZATION, AND THEIR APPLICATION FOR THE ENZYMATIC GENERATION OF PEPTIDES AND FOR THE DEVELOPMENT OF A REDUCED-GLUTEN COOKIE

by

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SUGGESTED SHORT TITLE

POTATO PROTEINS: ISOLATION, CHARACTERIZATION, AND THEIR APPLICATION AS HEALTH PROMOTING INGREDIENTS

ABSTRACT

Potato proteins were isolated from industrial by-products potato fruit juice (PFJ) and potato pulp, using several conventional extraction techniques namely: thermal/acidic combination, acidic precipitation, salt precipitation, ethanol precipitation, ammonium sulphate ($(NH_4)_2SO_4$) saturation, carboxymethyl cellulose complexation and a novel enzymatic approach. Of the conventional methods assessed $(NH_4)_2SO_4$ precipitation resulted in the highest protein recovery yield of 74.4 to 98.6%, with isolates enriched in non-denatured patatin, the main storage protein found in potatoes (*Solanum tuberosum*) as demonstrated by best preservation of its lipid hydrolase activity. The novel enzymatic approach began with the use of pure enzymes, specifically endo-polygalacturonase M1 and endo-β-1,4-galactanase, for the opening of the pectin network and the isolation of potato proteins with a recovery yield of 73.9%. The protein recovery yield and recovered patatin was found to be antagonistically affected by temperature and units of endo-polygalacturonase, whereas the recovered protease inhibitors was governed by pulp concentration and temperature. Similar high yields, were achieved with a more industrial appealing approach using multi-enzymatic systems, where similar enzyme activity profile led to an equivalent protein recovery yield. Of those assessed, Depol 670L and Ceremix 2XL were examined and optimized for their ability to recover protein extract enriched with patatin (up to 60.0%) and protease inhibitor recovery (up to 72.0%), respectively. Depol 670L-based enriched patatin extracts (0.51 μmol/(min. mg patatin)) greatly preserved the lipid acyl hydrolase activity emphasizing the preservation of its functionality, when compared to the employed industrial extraction thermal/acidic process (<0.04 μmol/(min. mg patatin)). Whereas Ceremix 2XL-based enriched protease inhibitor extracts (834.4 mg protease/g extract) resulted in higher preservation of trypsin inhibition when compared to the industrial technique (363.5 mg protease/g extract).

These extraction techniques were further scaled-up in a pilot plant facility, to compare their efficiencies and extracts recovered, in terms of thermal denaturation, protein profile, and functionalities. Two of these processes were based on the isolation of potato proteins from PFJ by (1) subsequent-ultrafiltration steps or (2) protein precipitation at 60% $(NH_4)_2SO_4$ saturation. The other process was performed on potato pulp, (3) involving the use of a multi-enzymatic system (Depol 670L) to degrade the plant cell wall releasing the proteins.

The need for an extracting agent was deemed necessary as the protein recovery increased from 3.79 g/kg potato (ultrafiltration) to 7.27 and 5.63 g/kg for $(NH_4)_2SO_4$ precipitation and Depol 670L, respectively. The Depol 670L-based protein concentrate had improved functionality in terms of emulsifying activity index (32.4 m^2/g protein) and foam expansion (416.4%), due to the increase presence of sugar which improved protein solubility.

Potato protein concentrate and isolate extracted by ultrafiltration (PPC) and 60% $(NH_4)_2SO_4$ precipitation (PPI) were further incorporated into a reduced gluten cookie. PPI resulted in cookies which were more texturally preferred by 25 semi-trained panelists as well as had higher fracturability, when compared to cookies prepared with PPC. A central composite rotatable design was performed with 2-variables namely, rice flour proportion and protein enrichment and 5-levels. The design was evaluated by 70 semi-trained panelists on a 5-point hedonic scale for mean quantitative descriptive scores and mean liking based scores. The attributes, which were found to be statistically relevant, were crispness, aftertaste and overall liking. Consumers generally preferred the cookie control made of wheat flour alone; however, the formulation composed of 67.88% rice flour/32.12% wheat flour with 7.20% potato protein isolate enrichment resulted in acceptable reduced-gluten cookies based on desirability plots, generated from mean liking scores. The instrumental textural analyses showed that cookies enriched with PPI were harder and more flexible; this was well correlated with the consumer's preference for the desirable crispness and adhesiveness, when compared to control rice flour.

Potato protein isolate was used as a starting material to generate peptides using four selected proteases namely, Novo Pro-D, Alcalase, Flavourzyme, and Papain. The processes were evaluated in terms of the degree of hydrolysis and end-product profile. Both, Flavourzyme and Papain exhibited a high catalytic efficiency for the hydrolysis of potato proteins, due to their dual catalytic nature possessing both endo-protease and exo-peptidase activity. This dual nature was found to greatly impact end-product generation, and the presence of the protease inhibitors present in the isolate was found to have an inhibiting effect on both endo- and exo- modes, contributing to the end-product profile. Peptide mapping was used for the identification of the generated peptides as well as for the determination of their parent potato protein fraction. Flavourzyme-based hydrolysates, contained more known exclusive unique peptides common to patatin, whereas Papain-based hydrolysates were more specific to the protease inhibitors.

RÉSUMÉ

Les protéines de pomme de terre ont été isolées à partir des sous-produits de pomme de terre préparés par imitation de techniques industrielles (jus et pulpe), en utilisant différentes techniques d'extraction conventionnelles à savoir: combinaison thermique/acide; la précipitation par un acide, le sel, l'éthanol; la complexation par carboxyméthyl cellulose et à travers une nouvelle approche enzymatique. Parmi ces procédés évalués, la précipitation par sulfate d'ammonium ($(NH_4)_2SO_4$) a abouti à un rendement élevé de récupération de protéines de 74,4 à 98,6%, sous forme d'isolats enrichis en patatine non dénaturée, la protéine de stockage principale trouvée dans les pommes de terre *(Solanum tuberosum)* telles que démontrées par la meilleure préservation de son activité enzymatique de type lipide acyl-hydrolase. La nouvelle approche enzymatique consiste à l'utilisation d'enzymes pures, en particulier l'endo-polygalacturonase M1 et l'endo-β-1,4-galactanase, pour l'ouverture du réseau de pectine et l'isolement des protéines de pommes de terre avec un rendement de récupération de 73,9%. Le rendement de récupération des protéines et la concentration de patatine récupérée ont été affectés de façon antagoniste par la température ainsi que les unités d'endo-polygalacturonase, alors que la récupération des inhibiteurs de la protéase était régie par la concentration de la pulpe et la température. Des rendements élevés similaires ont été obtenus avec une approche industrielle plus attrayante utilisant des systèmes multi-enzymatiques. Les systèmes multi-enzymatiques ayant le même profil d'activité enzymatique ont démontré un rendement de récupération de protéines semblable. Parmi ceux évalués, Depol 670L et Ceremix 2XL ont été examinés et optimisés pour leur capacité à récupérer l'extrait de protéine enrichi en patatine (jusqu'à 60,0%) et en inhibiteurs de protéases (jusqu'à 72,0%), respectivement. L'extrait enrichi en patatine (0,51 μmol/(min.mg)) a grandement conservé l'activité enzymatique du type lipide acyl-hydrolase, démontrant la préservation de la fonctionnalité de la patatine, en comparaison avec l'extraction industrielle employée: thermique/acide (<0,04 μmol/(min.mg)). L'extrait enrichi en inhibiteurs des protéases (834,4 mg protéase/g) a démontré une préservation de l'activité inhibitrice contre la trypsine par comparaison à la technique industrielle (363,5 mg protéase/g).

Ces techniques d'extraction ont été mises à l'échelle pilote afin de comparer leurs efficacités et les propriétés des extraits récupérés, en termes de dénaturation thermique, de profil

protéique, et de fonctionnalité. Deux de ces procédés ont été basés sur l'isolement de protéines de pomme de terre à partir de jus de pomme de terre par (1) des étapes ultérieures d'ultrafiltration ou (2) par précipitation par 60% $(NH_4)_2SO_4$ à saturation. L'autre processus a été réalisé sur la pulpe de pomme de terre, (3) impliquant l'utilisation d'un système multi-enzymatique (Depol 670L) pour dégrader la paroi cellulaire de la plante, libérant les protéines. La nécessité d'un agent d'extraction a été jugée nécessaire lorsque la récupération des protéines a augmenté de 3,79 g/kg de pommes de terre (ultrafiltration) à 7,27 et 5,63 g/kg par la précipitation en $(NH_4)_2SO_4$ et l'utilisation de Depol 670L, respectivement. Le concentré de protéines obtenu suite à l'utilisation de Depol 670L a démontré des fonctionnalités améliorées en termes d'indice d'activité émulsifiante (32,4 m^2/g protéines) et d'expansion de la mousse (416,4%), et ce, en raison de l'augmentation de la présence de sucre qui a amélioré la solubilité des protéines.

Un concentré et un isolat de protéines de pomme de terre extrait par ultrafiltration (PPC) et par $(NH_4)_2SO_4$ précipitation (PPI), respectivement, ont été incorporés dans un biscuit à teneur réduite en gluten. Le PPI a permis d'obtenir des biscuits dont la texture était préférée par 25 panélistes semi-formés, et ils avaient aussi une fracturabilité supérieure, par rapport aux biscuits préparés avec PPC. Une étude expérimentale a été réalisée avec deux variables, à savoir, la proportion de farine de riz et l'enrichissement en protéines à 5 niveaux. La conception a été évaluée par 70 panélistes semi-formés sur une échelle de catégorie hédonique de 5 points en utilisant des scores descriptifs quantitatifs et des scores basés sur l'appréciation. Les attributs qui ont été trouvés statistiquement pertinents étaient la texture croustillante, l'arrière-goût et une appréciation globale. Les consommateurs préfèrent généralement les biscuits faits de farine de blé seul; toutefois, la formulation composée de 67,88% de farine de riz / 32,12% de farine de blé avec 7,20% d'enrichissement en isolat de protéines de pomme de terre a donné lieu à des biscuits réduit en gluten acceptables et ce, en se basant sur des facteurs de désirabilité, générés à partir de scores d'appréciation. Les analyses instrumentales de la texture ont démontré que les biscuits enrichis avec des PPI étaient plus durs et plus flexibles; Cela a été bien corrélé avec la préférence des consommateurs pour une texture croustillante avec l'adhésivité souhaitable, lorsque comparé avec le contrôle fait de farine de riz.

L'isolat de protéines de pommes de terre a été utilisé comme matière première pour produire des peptides en utilisant quatre protéases sélectionnées, à savoir, Novo Pro-D,

Alcalase, Flavourzyme et la Papaïne. Les procédés ont été évalués en termes de degré d'hydrolyse et du profil du produit final. Les deux enzymes, Flavourzyme et Papaïne, ont démontré une efficacité catalytique élevée pour l'hydrolyse de protéines de pomme de terre, en raison de leur double nature catalytique possédant à la fois une activité endo-protéase et une activité exo-peptidase. Cette dualités est d'une grande importance influant le profil des produits finaux; et la présence d'inhibiteurs de protéases dans l'isolat s'est révélée avoir un effet inhibiteur sur les deux modes endo- et exo-activités, ce qu'a aussi contribué au profil des produits finaux. La spectroscopie de masse a été utilisée pour l'identification des peptides générés ainsi que pour déterminer leur fraction mère de protéines de pomme de terre. Des hydrolysats à base de Flavourzyme, contenaient plus de peptides uniques et exclusifs, connus de la patatine, tandis que les hydrolysats à base de Papaïne sont plus spécifiques aux inhibiteurs de protéase.

STATEMENT FROM THE THESIS OFFICE

According to the regulation of the Faculty of Graduate Studies and Research of McGill University, Guidelines for Thesis Preparation include:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

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When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

CONTRIBUTION OF AUTHORS

The present thesis consists of ten chapters.

Chapter I provides a general introduction and a short literature review of the research work, and describes the objective of the study.

Chapter II presents a comprehensive assessment of the literature on potato proteins in terms of their chemical, structural, nutritional, and functional properties, as well as, their isolation and extraction from industry by-product potato fruit juice and potato pulp. A brief description of common techniques employed to separate the potato proteins into their corresponding fractions mainly, patatin and protease inhibitors is presented along with the exploration of the potential application of potato protein for the generation of peptides using commercially available proteases.

Chapter III to VIII are presented in the form of manuscripts and either have been or are in the process of being prepared for publication. The connecting statements between the chapters provides the rational linking the different chapters. Chapter III is a comprehensive assessment of the conventional extraction techniques and effect of extracting agents on the isolated proteins. Chapter IV develops and optimizes a novel enzymatic approach using pure enzymes endo-polygalacturonase and endo-β-1,4- galactanase for the recovery of potato protein from potato pulp. Chapter V extrapolates on the novel enzymatic approach using commercially available multi-enzymatic systems containing several glycosyl-hydrolase activities of interest for the recovery of potato proteins from potato pulp. Chapter VI examines the most effective conventional and multi-enzymatic system on a pilot scale for the extraction of potato proteins from by-products, potato fruit juice and potato pulp, and the extracts are compared according to their structural effects and functional properties. Chapter VII explores the application of potato proteins in a reduced-gluten cookie formulation and analyzes the cookie instrumentally (colour and textural analysis), as well as sensorially using 70 student panelists. Finally, Chapter VIII investigated the generation of peptides from potato proteins using selected proteases.

Chapter IX provides a general conclusion to the thesis with a summary of the major findings.

Chapter X provides the contribution of this research to the field and outlines possible recommendations regarding future work related to potato protein.

Amanda Waglay, the author, was responsible for the experimental work and the preparation of the manuscripts for publication and dissertation.

Dr. Salwa Karboune, the supervisor of the author's PhD work guided all the research and critically revised the manuscripts prior to submission.

Dr. Inteaz Alli, the third author of manuscript 1 (Chapter III) reviewed the manuscript prior to submission.

Dr. Maryam Khodadadi, the third author of manuscript 3 (Chapter IV) contributed to the research work related to the optimization of potato proteins using pure enzyme and revised the manuscript.

For manuscript 5 (Chapter VI), **Dr. Allaoua Achouri** (the second author), **Dr. Mohammad Reza Zareifard** (the fourth author) and **Dr. Lamia L'Hocine** (the fourth author) contributed to the research work related to the scale-up pilot process for the extraction of potato proteins from potato by-products. **Dr. Allaoua Achouri** (the second author) also provided technical support for the structural analysis of the proteins and was involved in the revision of manuscript 4.

PUBLICATIONS

1. **Waglay, A.,** Karboune, S., & Alli, I. (2014). Potato protein isolates: Recovery and characterization of their properties. *Food Chemistry, 142*, 373-382.

2. **Waglay, A.,** & Karboune, S. (2015). Potato Proteins. In L. Kaur, & J. Singh. *Advances in Potato Chemistry* (2 ed., p, Chapter 4). Maine, USA: Elsevier Inc. *(Accepted*)

3. **Waglay, A.,** Karboune, S., & Khodadadi, M. (2015). Investigation and Optimization of a Novel Enzymatic Approach for the Isolation of Proteins from Potato Pulp. *LWT-Food Science and Technology*. http://dx.doi.org/10.1016/j.lwt.2015.07.070

4. **Waglay, A.** & Karboune, S. (2015). A Novel Enzymatic Approach Based on the Use of Multi-Enzymatic Systems for the Recovery of Enriched Patatin and Protease Inhibitors Extracts from Potato Pulp. (*To be submitted*)

5. **Waglay, A.,** Achouri, A., Zareifard, M. R., & L'Hocine, L. (2015). Pilot Plant Extraction of Potato Proteins and their Structural and Functional Properties. (*To be submitted*)

6. **Waglay, A.** & Karboune, S. (2015). Formulation of a Functional Cookie Enhanced with Potato Protein Isolates: Textural and Sensory Evaluations. (*To be submitted*)

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NOMENCLATURE/ LIST OF ABBREVIATIONS

DPPH	1,1-diphenyl 1,2-picrylhydrazyl
$(NH_4)_2SO_4$	Ammonium sulphate
ANOVA	Analysis of variance
ACE	Angiotensin converting enzyme 1 inhibition
Amu	Atomic mass unit
BCA	Bicinchoninic acid assay
BSA	Bovine Serum Albumin
BHT	Butylated hydroxyl toluene
$CaCl_2$	Calcium chloride
ΔH	Calorimetric enthalpy
CMC	Carboxymethyl cellulose
CCRD	Central composite rotatable design
CER	Ceremix 2XL
DEP	Depol 670L
DTGS	Deuterated triglycine sulfate detector
DIA	Diazyme L-200
DSC	Differential scanning calorimetry
EAI	Emulsifying activity index
ESI	Emulsion Stability Index
ARase	Endo-arabinanase
PGase	Endo-Polygalacturonase M1
GLase	Endo-β-1,4galactanase
U	Enzymatic units
$FeCl_3$	Ferric chloride
FTIR	Fourier-transform infrared spectroscopy
GAMase	Gamanase 1.5L
ha	Hectare
HEMase	Hemicellulase CE-1500
HDL	High density lipoprotein
HPSEC	High performance size exclusion chromatography
HPLC	High-performance liquid chromatography
HCl	Hydrochloric acid
IOG	Iogen HS 70
pI	Isoelectric point
LAM	Laminex DG
Leu	Leucine
LAH	Lipid acyl hydrolase
LDL	Low density lipoprotein
$MnCl_2$	Manganese chloride
MALDI-TOF-MS	Matrix laser desorption ionization- time of flight- mass spectroscopy
T_d	Maximum temperature of denaturation
Met	Methionine

MW	Molecular weight
MWCO	Molecular weight cut-off
MANOVA	Multi-variant analysis of variance
NEWase	Newlase II
N-succinyl-AAPP	*N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide
N-*α*-BAPA	*N*-α-Benzoyl-D-L-arginine 4-nitroanilide hydrochloride
T_o	Onset temperature of denaturation
ppm	Parts per million
PAT	Patatin
PEC	Pectinex Ultra SPL
H_3PO_4	Phosphoric acid
PT-1	Potamin-1
PCI	Potato carboxypeptidase inhibitor
PFJ	Potato fruit juice
PPC	Potato protein concentrate
PPC Enz	Potato protein concentrate extracted with multi-enzymatic product, Depol 670L
PPC UF	Potato protein concentrate extracted with ultrafiltration (MWCO <5 kDa)
PPI	Potato protein isolate
PPI AS	Potato protein isolate extracted with 60% ammonium sulphate saturation
PI	Protease inhibitors
PI-1	Protease inhibitors 1
PI-2	Protease inhibitors 2
RSM	Response surface methodology
rpm	Revolutions per minute
RF	Rice Flour
NaCl	Sodium chloride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide
H_2SO_4	Sulphuric acid
T_{mFTIR}	Thermal FTIR transition temperature
TBARS	Thiobarbituric acid reactive substance
VIS	Viscozyme
WF	Wheat Flour
Termamyl	α-amylase *Bacillus licheniformis*

CHAPTER I. GENERAL INTRODUCTION

Potatoes (*Solanum tuberosome*) are the fourth most significant crop after rice, wheat and corn. Potatoes have many nutritional benefits as they are composed of carbohydrates, proteins, vitamins and minerals. Of particular interest are the potato proteins as they provide many beneficial effects such as nutritional aspects, functional attributes, and various health benefits. In the past, potato proteins have not been considered commercially viable due to their low content. However, when comparing the vegetable and cereal crops produced, potatoes result in the second largest protein supplying crop per hectare grown.

Potato proteins are commonly divided into three fractions; patatin which comprises 40% and possesses a molecular weight of 39-45kDa, protease inhibitors which represents 50% with a corresponding molecular mass of 5-25kDa (Pots, et al., 1999; Pouvreau, et al., 2001), and other high molecular weight proteins which encompass the remainder. The potato proteins as a whole are known to have nutritional benefit equal to that of egg proteins (Ralet & Guéguen, 2000). Several associated health benefits such as decreased occurrence of allerginicity (Moreno, 2007), antimicrobial effects (Kim, et al., 2005), antioxidant potential (Liu, et al., 2003), regulation of blood pressure (Deveaux-Gobert, 2008; Pihlanto, et al., 2008), and blood serum cholesterol control (Liyanage, et al., 2008). In addition to these health benefits, the fractionated patatin fraction has been shown to possess excellent foaming, emulsifying abilities, and lipid acyl hydrolase and transferase activity (LAH) (Bártova & Bárta, 2009; Pots, et al., 1999; Ralet & Guéguen, 2001; van Koningsveld, et al., 2002; van Koningsveld, et al., 2006).

Potato proteins can be readily extracted from the tuber itself, although another source includes a high polluting capacity discarded waste stream from the potato starch industry known as potato fruit juice (PFJ) (Wojnowska, et al., 1981; Strolle, et al., 1973). The industrially used extraction technique to remove proteins from PFJ is a combination of thermal coagulation and acidic precipitation (Knorr, et al., 1977; Knorr, 1982), which results in proteins with poor functionality. Other techniques under investigation include acidic precipitation (Knorr, et al., 1977), salt precipitation (Bártova & Bárta, 2009), ethanol precipitation (Bártova & Bárta, 2009; van Koningsveld, et al., 2001), ammonium sulphate precipitation (van Koningsveld, et al., 2001), and carboxymethyl cellulose complexation (Gonzalez, et al., 1991; Vikelouda & Kiosseoglou, 2004). These extraction techniques have

been extensively studied on different potato cultivars from different regions, which present a range of conflicting results, as studies have shown that potato protein fractions vary among cultivars (Bártová, et al., 2015; Höfgen & Willmitzer, 1990). Therefore a comprehensive assessment on one potato cultivar would be of interest to make an adequate comparison of the conventional extracting agents.

The current proposed techniques are associated with several disadvantages such as the effects and the removal of extracting agents, maintenance of optimal extraction conditions, and loss of functionality of proteins. Therefore, the development of a novel biocatalytic approach will be of interest as it would involve a subtractive effect, degrading the polysaccharide components mainly pectic polysaccharides which surround the proteins present in the plant cell wall (Khodaei & Karboune, 2013). Due to the mild operating conditions employed with the use of enzymes, the extraction technique will be improved, preserving the proteins functionality, which will broaden their potential applications. In order to enhance the feasibility of novel extractions technique into industry, the exploration of several commercially available multi-enzymatic products with desirable specificities will be assessed. Moreover, the effectiveness of this novel technique will be determined through the preservation of the structural and functional characteristics of the extracted potato proteins.

Extracting potato proteins at the lab scale renders isolates with a high protein recovery yield, but limited protein quantity (Wu, et al., 1999). To overcome this dilemma, the most promising extraction techniques have been scaled-up to pilot plant environment. The isolates were characterized according to their protein recovery yield, protein profile, conformational changes, and functionality, specifically emulsifying ability and foaming properties. To date, limited studies are available for the recovery of potato proteins at the pilot-scale, with the exception of the use of chromatographic techniques such as expand bed absorption and specific ligand binding columns (Løkra, et al., 2008; Schoenbeck, et al., 2013; Straetkvern, et al., 1999; Zeng, et al., 2013).

Due to the above mentioned functionalities and health benefits the exploration of potato proteins for food application would be of interest. Currently, there is a heavy emphasis

from consumers to formulate snack foods which contain high protein content with limited animal protein sources, a reduced gluten content, that are nutritionally balanced and easy to consume as a snack food. This poses a concern to food developers as gluten is responsible for the desirable attributes associated with dough rheology (Marco & Rosell, 2008). However, patatin has been shown to possess excellent emulsifying abilities (van Koningsveld, et al., 2006), and therefore its incorporation into a reduced gluten cookie formulation would be desirable as it would meet all current consumer concerns. To date, no studies have examined the use of potato protein enrichment in cookie formulations to reduce the gluten content of a readily consumed snack food. Assessing the cookies by their color, textural, and sensorial evaluation will help understand the role that the proteins play in their formulation.

The literature emphasizes the benefit of protein modification to yield an end-product which has more appealing properties than the parent protein (Kamnerdpetch, et al., 2007; Cheng, et al., 2010; Miedzianka, et al., 2014). One such method involves the enzymatic hydrolysis of parent proteins to yield peptides which have the potential to possess bioactive ability towards prevention and maintenance of improved health (Udenigwe & Aluko, 2012). Potato peptides have been studied to participate in lipid cholesterol control (Liyanage, et al., 2008), involved with lipolysis-stimulating activity (Huang, et al., 2015), possess antioxidant ability and contribute in angiotensin converting enzyme inhibition (Pihlanto, et al., 2008). Currently, these limited studies generate peptides using commercially available proteases for the generation of peptides from potato proteins. However, these studies lack the proper quantification of the peptides generated solely reporting on free amino acid rendered. Furthermore no structural characterization of the hydrolysates has been conducted. In order to expand on the current explored hydrolysis methods, several commercial proteases both endo- and exo- protease will be compared as well as the generated peptides will be further characterized structurally.

The overall objective of the present research is the optimization of the extraction of potato proteins, with particular focus on a novel biocatalytic approach, and characterization of the protein isolates' structural and functional properties, for their application in the

biogeneration of peptides and incorporation in a reduced gluten cookie. It is subdivided into the following specific objectives:

1) Extraction of potato proteins: conventional extraction techniques and development of a novel enzymatic approach
2) Investigation and optimization of the use of multi-enzymatic products to extract and fractionate potato proteins
3) Pilot plant scale-up for the recovery of potato proteins from imitation by-products potato fruit juice using ultrafiltration and ammonium sulphate saturation; as well as potato pulp using commercially available multi-enzymatic products
4) Structural characterization and the assessment of the functional properties of potato protein isolates
5) Formulation of a snack food product enhanced with potato protein isolates and its analysis using sensory evaluation
6) Development of an enzymatic approach for the biogeneration and characterization of peptides from potato protein isolates

CHAPTER II. LITERATURE REVIEW

2.1. Introduction

Potatoes (*Solanum tuberosum*) are the fourth most produced crop after rice, wheat and corn (Kamnerdpetch, et al., 2007). Despite their low protein concentration of 1.7%, potatoes are the second highest protein providing crop per hectare grown following wheat. The North American consumer associates potatoes with unhealthy diets due to the poor processing techniques often employed such as frying. Contrary to this negative connotation, potatoes contain fibers, many high quality proteins, and several vitamins and minerals (Bártova & Bárta, Chemical Composition and Nutritional Value of Protein Concentrates Isolated from Potato (Solanum tuberosum L.) Fruit Juice by Precipitation with Ethanol and Ferric Chloride, 2009). These high quality proteins are composed of a high proportion of lysine (Kamnerdpetch, et al., 2007), threonine, tryptophan, and methionine, the latter an essential amino acid often lacking in cereal and vegetable crops (Kärenlampi & White, 2009). Due to their amino acid composition, potato proteins have been studied to be nutritionally equivalent to the animal protein lysozyme (Ralet & Guéguen, 2000).

Potato proteins are often classified into three groups namely patatin, protease inhibitors, and high molecular weight proteins. They are associated with several health benefits such as lower allergic response (Fu, et al., 2002), antimicrobial effects (Kim, et al., 2005), antioxidant potential (Pihlanto, et al., 2008), as well as regulation of blood pressure and blood serum cholesterol (Pihlanto, et al., 2008; Liyanage, et al., 2008) and anti-carcinogenic behavior (Blanco-Aparicio, et al., 1998). Amongst these health benefits are several other functional qualities which will help broaden their application in the food industry such as emulsification and foaming abilities.

Potato proteins have often been overlooked, but their removal from the starch industry by-product is necessary to help overcome the economic impact coupled to its high polluting capacity (Strolle, et al., 1973). Many extraction techniques have been explored in order to maintain or enhance the functional qualities possessed by the proteins (Cheng, et al., 2010). Further research is necessary to develop an industrial scale and cost efficient process for extracting the potato proteins with minimal functional loss to broaden their potential applications. This chapter will explore the protein isolates obtained using several extracting agents such as thermal and acidic precipitation, salt precipitation, ethanol precipitation,

$(NH_4)_2SO_4$ saturation, and CMC complexation. To date, many studies have been conducted on examining the recovery yield, functionality, fractionation, and structural impact of the extracting agents on the protein isolates.

2.2. Potato Proteins

2.2.1. Chemical and Structural Properties

2.2.1.1. Patatin

The major protein found in potatoes is patatin, otherwise known as tuberin. It is mostly found in the tuber or stolons of the plant, specifically in the vacuole of the parenchyma tissue (Straetkvern, et al., 1999). However, the protein has also been observed in substantial amounts in the small above ground buds that form only if the tuber and stolons of the plant have been removed. Other studies have also demonstrated that patatin can be found in small quantities in the stem of the plant (Park, 1983).

Patatin represents approximately 40% of the soluble protein found in the tuber and is a group of glycoproteins with molecular weights from 40-45 kDa (Kärenlampi & White, 2009). It has been reported that patatin is present as an 88 kDa dimer when analyzed on a native gel. However, in the presence of sodium dodecyl sulphate (SDS), the protein is broken down into its monomer units (Ralet & Guéguen, 2000). Patatin has been shown to be made up of approximately 366 amino acids. These amino acid residues possess negative and positive charges that are distributed throughout the protein's length (Pots, et al., 1998). The isoelectric point of patatin has been established to be at a pH of 4.9 (van Koningsveld, et al., 2001). Three glycosylation sites have been studied to be specific to patatin. The sites have been examined to occur at the asparagine residues, which show interesting biological function. These glycosylation sites are named asparagine-linked oligosaccharides and have been linked to signaling for intracellular targeting, protection for proteolytic breakdown, and preservation of protein stability by influencing unfolding patterns (Sonnewald, et al., 1989).

Structurally patatin is a tertiary globular stabilized protein. Pots et al. (1998) have determined patatin to contain an estimation of 45% β-strand and 33% α-helix. The tertiary structure is stable up to 45 ^{0}C; however, after this point patatin's secondary structure begins to unfold and at temperatures of 55 ^{0}C the α-helical part denatures (Pots, et al., 1998). In

comparison to other common protein vegetable sources, patatin is of equal nutritional benefit to egg albumen and has been studied to have better emulsifying properties than soy proteins (Løkra, et al., 2008).

Patatin is made up of many proteins that are represented by two multi-gene families (Pots, et al., 1999). The Class I gene family is represented in large concentrations in the tuber, whereas Class II gene family is represented in smaller concentrations throughout the potato plant (Pots, et al., 1999). The literature demonstrates that for the potato variety Bintje, patatin can be separated into four isoforms based on charge. An isoform is defined as "a protein that has the same function as another protein but which is encoded by a different gene and may have small difference in its sequence" (MedicineNet, 2004). Patatin is represented as four isoforms A, B, C, and D which are present in varying amounts: 62%, 26%, 5%, and 7%, respectively, where all four patatin isoforms are homologous in nature and possess an identical immunological response (Pots, et al., 1999). In general, isoforms vary according to differences in charge, molecular mass, or structural properties. The patatin isoforms present in the different potato varieties do fluctuate. For example cultivar (cv.) Désirée was found to have nine isoforms, whereas Bintje was found to have four (Lehesranta, et al., 2005; Pots, et al., 1999). The different patatin isoform ratios can be observed through experimental techniques that separate based on charge. This is an important parameter which can help with differentiation between patatin families for a range of potato species (Bohac, 1991).

Patatin isoforms are represented by a charge difference, which was demonstrated through anionic exchange (Pots, et al., 1999). Pots et al. (1999) have found that isoform A showed very low affinity with the anion exchange column, and it represented the shortest running distance on a native gel. Conclusions were drawn that isoform A must have the lowest surface charge when compared to isoforms B, C, and D. It was also observed that isoform A exhibited the highest isoelectric focusing and shortest capillary electrophoresis. Based on the proteins isoelectric point, at pH 3 isoform A contained the largest amount of positively charged residues and therefore in turn at pH 8 it contained the largest amount of negatively charged residues when compared to the other isoforms B, C, and D (Pots, et al., 1999). Pots et al. (1999), determined that the difference amongst the isoforms were a

variation of molecular masses of 40.4 and 41.8 kDa. An explanation for the molecular masses differences of the isoforms were compared to a previous study performed by Mignery et al. (1984) on the potato variety Superior. It was found that the isoforms contained 366 amino acids with a variation of 21 amino acids. The varying mutated amino acids between the isoforms were represented experimentally with a molar mass difference of 663 Da. However Mignery et al. (1984) calculated the theoretical difference to be 100 Da. This discrepancy demonstrates that the patatin isoforms may have undergone glycosylation between the protein and carbohydrate present in the potato. Pots et al. (1999) used similar calculations for the isoforms among the Bintje variety. According to calculations the isoforms for this variety can possess a maximum difference of 198 Da. However, no correlation was made between this calculation and their results attained from matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy (MALDI-TOF MS) (Pots, et al., 1999).

While patatin is present in the tuber as the main storage protein, studies have concluded that patatin and its isoforms must have functions in the tuber other than storage because of its presence during the developing stages of the plant. This has led to the determination that the protein possesses antioxidant activity (Wang & Xiong, 2005; Kärenlampi & White, 2009; Liu, et al., 2003) and furthermore it has been categorized as an esterase enzyme complex (Vreugdenhil, et al., 2007). Patatin demonstrates enzymatic activity toward lipid metabolism through lipid acyl hydrolases as well as acyl transferases (Höfgen & Willmitzer, 1990; Liu, et al., 2003). It has been hypothesized that these mechanisms allow patatin to participate in plant defenses (Kärenlampi & White, 2009). This lipid acyl hydrolase has been found to vary according to the potato cultivar, extraction technique, and fatty acid substrate (Table 2.1). As shown in Table 2.1, generally lipid acyl hydrolase activity was higher when the substrate *p*-nitrophenyl laurate was used, as shown by Pots et al. (1999). However when examining purified patatin and its corresponding lipid acyl hydrolase activity substrate *p*-nitrophenyl butyrate provides comparable results.

Table 2.1 Comparison of lipid acyl hydrolase (LAH) activity corresponding to patatin isolates from different sources using different extraction techniques.

Cultivar	*Patatin*	*Extraction Technique*	*LAH activity*	*Substrate*	*Authors*
Beroline	40%	0.1% polyvinyl-polypyrrolidone, DEAE-Sephacel and ConA-Sepharose chromatography	5[a]	*p*-nitrophenyl fatty acid esters (chain length 2-18)	Höfgen and Willmitzer, 1990
Désirée	20%		0.5[a]		
Bintje	purified	DEAD Sephraose CL-6B, Concanavalin A-Sephraose 4B	4.54[b]	*p*-nitrophenol butyrate	Pots et al., 1998
	patatin family	ConA, SourceQ	3.72[b]	*p*-nitrophenol laurate	Pots et al., 1999
	62% isoform A		3.66[b]		
	26% isoform B		3.55[b]		
	7% isoform D		3.80[b]		
Elkana	100.00%	Purified patatin: Source 15Q column, Superdex 75 column	1.84[b]	*p*-nitrophenyl butyrate	van Koningsveld et al., 2001
PFJ (Lyckeby Amylex, Czech Republic)	30.70%	Freeze dried PFJ	0.03[b]	*p*-nitrophenyl butyrate	Bártová and Bárta, 2009
	25.60%	Ethanol ppt	0.07[b]		
	20.30%	Ferric chloride ppt	0.03[b]		
	32.30%	Heat coagulated	0.01[b]		
	n.a	Purified patatin: Ethanol ppt, DEAE 52-cellulose servacel, ConA Sepharose 4B column, Sephadex G-25 gel filtration column	0.5[b]		

[a] Units activity is expressed in nm/min x mg patatin.[b] Units activity is expressed in μmol/min/mg protein.

This hydrolase activity is due to the central core of patatin being composed of parallel β-sheet as well as the catalytic serine being located in the nucleophilic elbow loop (Rydel, et al., 2003). This core consisting of parallel β-sheet with the catalytic residue being buried within an elbow loop are all key attributes belonging to the hydrolase family (Rydel, et al., 2003).

2.2.1.2. Protease Inhibitors

The second group of proteins found in potatoes are the protease inhibitors (tuberinin), which possess molecular weights ranging from 5-25 kDa (van Koningsveld, et al., 2002). Like patatin, these protease inhibitors represent 30- 40% of the total tuber protein (Pouvreau, et al., 2001; Jørgensen, et al., 2006). The protease inhibitors act by hindering the activity of serine protease, cysteine protease, aspartate protease, and metalloprotease (Kärenlampi & White, 2009). Through these inhibition mechanisms, the digestibility and availability of the proteins decreases (Kärenlampi & White, 2009). When patatin and the protease inhibitors are compared, protease inhibitors tend to have more hydrophilic properties, however both protein fractions tend to coagulate by heat (Kärenlampi & White, 2009).

The protease inhibitors are much more diverse than the patatin family and are able to act on a variety of proteases and other enzymes. The protease inhibitors that have been examined in great detail, a brief summary is shown in Table 2.2, the general sub-categories include protease inhibitor I (PI-I), protease inhibitor II (PI-2), potato aspartate protease inhibitor, potato cysteine protease inhibitor, potato Kunitz-type protease inhibitor, other serine protease inhibitor, and potato carboxypeptidase inhibitor (PCI) (Pouvreau, et al., 2001). Table 2.2 shows the variation according to molecular weight, isoelectric point, and their proportion within the cultivar Elkana. However studies demonstrate that these protease inhibitors vary according to chain length, amino acid composition, and inhibitory activities (Pouvreau, et al., 2001; Jørgensen, et al., 2006). PI-1 is a pentameric serine protease inhibitor composed of five 7-8 kDa isoinhibitor protomers (Pouvreau, et al., 2001). PI-1 has a strong affinity for chymotrypsin, trypsin, and human leukocyte elastase and inhibits their activities (Pouvreau, et al., 2001). PI-2 is a dimeric serine protease inhibitor composed of two 10.2 kDa proteins that are linked together by disulfide linkages (Pouvreau, et al., 2001). They have fluctuating inhibiting effects amoung the sub-categories but generally inhibit trypsin, chymotrypsin, and human leukocyte elastase (Pouvreau, et al., 2001). The potato aspartate protease inhibitors also vary in terms of inhibiting

activity among their sub-categories but generally inhibit trypsin, chymotrypsin, cathepsin D, and human leukocyte elastase (Pouvreau, et al., 2001). The potato cysteins protease inhibitors vary among sub categories but most have inhibiting activity towards trypsin, chymotrypsin, papain, and to a lesser extent human leukocyte elastase (Pouvreau, et al., 2001). Both potato Kunitz- type and other serine protease inhibitors are able to inhibit trypsin and chymotrypsin, whereas only the other serine protease inhibitors are able to inhibit human leukocyte elastase (Pouvreau, et al., 2001). Finally, PCI is a thermostable 4.3 kDa single subunit peptide (Pouvreau, et al., 2001). Jørgensen et al. (2006) examined the potato variety cv. Kuras, a variety that is commonly used in the European potato starch industry. The cv. Kuras was found to have a protease inhibitors protein fraction, which could be separated into five non homologous families. Those families were 13A Kunitz peptidase inhibitors, I13 peptidase inhibitors I, I20 peptidase inhibitors II, I25 multicystatin peptidase inhibitor, and I37 carboxypeptidase inhibitor (Jørgensen, et al., 2006).

The protease inhibitors have been studied to possess protease inhibition activity. This inhibition activity has been studied to increase with long term storage and sprouting. It is hypothesized that the inhibition activity is required to help in the breakdown of the proteins during the developing stages of the tuber. More specifically the serine protease inhibitors help in protein regulation by the removal of proteins during chloroplast ontogeny as well as assist in nitrogen mobilization during germination, and in leaf aging (Weeda, et al., 2009). The potato multicystatin have been shown *in vitro* to degrade patatin, which has been associated as a regulator in protein accumulation in the tuber (Weeda, et al., 2009).

Table 2.2 Sub-categories of potato protease inhibitors found in cv. Elkana[a]

Name	M.W	pI	PI Proportion in cv. Elkana PFJ
Potato Inhibitor I	7 683-7 873	5.1-6.3	3.9
	7 683- 7 873	7.2, 7.8	3.1
	7 683-7 873	5.1, 6.3	1.8
Potato Inhibitor II	20 279	6.5	14.5
	20 023	6	4.5
	20 273	6.1	11.3
	20 674	5.8	0.6
	20 676	5.5	2.6
	20 315	5.9	6.9
	20 265	6.9	3.7
Potato Aspartate Protease Inhibitor	19 987	6.2	0.2
	20 039	8.4	2.8
	22 025	8.6	2.8
	19 878	8.7	2.6
	20 141	7.5	2.4
	19 883	8.2	0.8
	22 755	6.7	1.5
	22 769	6.6	1.6
	22 674	5.8	2.6
Potato Cysteine Protease Inhibitor	22 773	7.1	0.9
	20 096	8	2.6
	20 127	8.6	4.1
	20 134	>9.0	3.1
	20 433	8.3	7.2
Potato Kunitz-type Protease Inhibitor	20 237	>9.0	1.8
	20 194	8	5.3
Other Serine Protease Inhibitor	21 025	8.8	1.3
	21 804	7.5	1.7
Potato Carboxypeptidase Inhibitor	4 274	n.d	1.8

[a]Adapted from Pouvreau et al., 2001

2.2.1.3. Others

The remaining fraction of proteins found in the potato consists of a variety of high molecular weight proteins (van Koningsveld, et al., 2002). This group of protein has not been extensively studied.

2.2.2. Nutritional and Health-Promoting Properties

Potatoes are often considered nutritionally beneficial for their high starch content, which supplies the body with energy along with providing proteins, vitamins and dietary fibers (Kärenlampi & White, 2009). Overall, carbohydrates represent approximately 75% of the total dry matter. This section will focus primarily on the nutritional benefit of potato proteins.

The potato tubers contain 20 g of protein per kg on a fresh weight basis (Kärenlampi & White, 2009). Potato proteins have been proven nutritionally superior to most other plant and cereal proteins and relatively close in comparison to egg protein (lysozyme) (Ralet & Guéguen, 2000). As a result, potato proteins have a high nutritional value, attributed to the amino acids that make up these proteins. Unlike most cereal and plant proteins, the potato proteins contain a high proportion of the essential amino acid lysine (Kamnerdpetch, et al., 2007; He, et al., 2013) and relatively high proportions of sulphur-containing amino acids such as methionine and cysteine (Kärenlampi & White, 2009).

The benefit in using potato proteins compared to other proteins is the lower occurrence of allergenicity (Fu, et al., 2002). Other common industry sources, including egg, gluten, soy, fish and nut proteins, are linked to allergic responses, in approximately 1-2% of the human population (Løkra, et al., 2008). Studies have shown that when 800 infants were given an allergy test only five percent showed an allergic reaction to potatoes, whereas for eggs and cow's milk the allergic reaction was fifteen and nine percent, respectively (Kärenlampi & White, 2009; He, et al., 2013). Most of the adult driven allergies to potato can be eliminated through the cooking process (Kärenlampi & White, 2009). Possible allergic response symptoms include eczema, gastrointestinal issues, urticaria and angioedema, wheezing, and anaphylaxis (Kärenlampi & White, 2009).

Protease inhibitors have recently been shown to exhibit anti-carcinogenic behavior and to possess beneficial dietary qualities. Potato protease inhibitors specifically demonstrate anti-

carcinogenic effects by three mechanisms, namely interfering in tumor-cell proliferation, hydrogen peroxide formation, and involvement in processes related to solar-UV irradiation (Pouvreau, et al., 2001). Potato protease inhibitors has also been shown to provide a positive effect on satiety through their hunger suppression effect (Schoenbeck, et al., 2013).

Kim et al. (2005) found that the Kunitz type protease inhibitors accumulated in the potato leaves and tubers help in mechanical wounding of the crop, UV-radiation, and wounds started by insects or phytopathogenic microorganisms. Previous studies on tomatoes, found that phytopathogenic microorganisms produce extracellular proteinases, which have a direct influence on the development of diseases (Kim, et al., 2005). As a defense mechanism the plant produces inhibitory polypeptides to deactivate the extracellular proteinases. In the case of potatoes the protease inhibitors of serine proteinases variety, function as the plants source of defense. The study conducted by Kim et al. (2005) examined the antimicrobial activity of the potato protease inhibitor, potamin-1 (PT-1). This peptide demonstrated antimicrobial activity towards the human pathogenic fungus *Candida albicans*, plant pathogenic fungus *Rafael Solanic*, and the pathogenic bacterium *Clavibacter Michiganense.* PT-1 was tested for its ability to function as a serine protease inhibitor; it was tested to inhibit trypsin, chymotrypsin and papain. All three serine protease enzymes were inhibited dose-dependently by PT-1, hence linking its antimicrobial effect to its serine protease inhibition (Kim, et al., 2005).

Patatin has been shown to possess antioxidant activity (Wang & Xiong, 2005). When enzymatic hydrolysis is used it results in the formation of potato protein hydrolysate, which contains many potato peptides. Wang and Xiong (2005) studied the antioxidant ability of a potato protein hydrolysate fraction in cooked beef patties with the peroxide and thiobarbituric acid reactive substances (TBARS) antioxidant assays. They concluded that potato proteins possess substantial reducing power that can be attributed to peptide cleavage, which results in a product that has a higher availability of hydrogen ions. These hydrogen ions are now able to transfer and stabilize the free radicals to a greater extent from the smaller peptide when compared to the larger protein. Amino acid composition could be strongly related to the protein's antioxidant activity as literature has shown that methionine, histidine, and lysine have been shown to inhibit lipid oxidation. These amino acids are present in potato proteins and

could be credited for the improvement in oxidative stability of the refrigerated cooked ground beef patties (Wang & Xiong, 2005).

Liu et al. (2003) have studied the antioxidative activities of purified patatin. They found that patatin exhibited a dose-dependent response when its antiradical activity was studied with the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant assay, using butylated hydroxytoluene (BHT) and reduced glutathione as controls. When the particle size was reduced to nanomole basis, purified patatin exhibited a similar response as the known antioxidant BHT and had a better antioxidant response than reduced glutathione. The same study also determined the ability of patatin to act on LDL (low density lipoproteins) peroxidation using the TBARS assay, particularly important because LDL peroxidation is associated with the development of atherosclerosis in humans. The TBARS assay showed that increasing the concentration of patatin reduced the level of oxidized LDL; therefore they concluded that patatin possesses a dose-dependent protection response against human LDL-oxidation (Liu, et al., 2003).

Bioactive peptides are attained from parent protein molecules that have undergone hydrolysis and experimental details of the biogeneration process will be explored in Section 2.8.1. These peptides usually possess better functional properties than their parent biopolymers and also help mediate the mechanisms that their parent proteins are involved in by displaying a regulatory type response (Liyanage, et al., 2008). Many studies have been performed to examine the benefits of soybean derived peptides, which includes the reduction of cholesterolemia. Liyanage et al. (2008) compared the characteristics of soybean peptides and potato peptides by examining the lipid metabolism of rats fed a cholesterol free diet. The proteins that were compared were soybean, casein, and potato protein. The rats fed solely on potato proteins seemed to exhibit the lowest weight gain, however this is possibly due to the hygroscopic ability of potato protein, which would in turn lead to reduced quality of the diet. Their results showed a correlation between the intake of potato and soy peptides and serum cholesterol levels. Potato peptides demonstrated effects similar to soy peptides, namely a low level of non-HDL-C concentration, high fecal lipid excretion, and lower apo B mRNA levels compared to the casein fed group (Liyanage, et al., 2008). Hepatic apo B mRNA is a hepatic gene which expresses the LDL receptor. When apo B mRNA is present in low levels the liver is triggered to increase clearance of LDL cholesterol, which is associated with higher excretion

levels in the feces and the reduction of serum cholesterol levels. The serum triglyceride concentration was lowest among the rats fed a potato peptide diet when compared to both the soy peptide and casein protein diets. The low concentration of serum triglycerides could have been attributed to the low ingestion of the potato peptide diet as well as the increase in total lipid fecal excretion. The study concluded that the potato peptide diet did affect the serum cholesterol levels of the related fed group based on different mechanisms according to the different lipoproteins and triglycerides (Liyanage, et al., 2008).

Studies have found that bioactive peptides can participate in a reduction of blood pressure (Liyanage, et al., 2008). The bioactive peptides are able to act through the mechanism of Angiotensin converting enzyme I (ACE) inhibition. The mechanisms to raise blood pressure can occur through two processes. The first is catalyzed by converting angiotensin I to angiotensin II which is a known vasoconstrictor that also signals to the kidneys to retain salts and water therefore increasing the extracellular fluid volume leading to an increase in blood pressure. The second is by deactivating a vasodilator bradykinin. ACE inhibition reduces blood pressure by decreasing peripheral vascular resistance and stabilizes renal function (Mäkinen, et al., 2008). Mäkinen et al. (2008) studied the effect of potato peptides and ACE inhibition. They found that hydrolysis of potato proteins resulted in increased ACE inhibition, and that autolysis of the proteins resulted in greater ACE inhibition (Mäkinen, et al., 2008).

2.2.3. Functional Properties

Important to food systems are the functional properties that proteins possess including solubility, water holding ability, gelation, foaming, and emulsification (McClements, et al., 2009). Potato proteins have been reported to possess foaming and emulsification properties (van Koningsveld, et al., 2002; van Koningsveld, et al., 2006). In industry, one difficulty is achieving these desirable characteristics at minimal cost. Potato proteins can be obtained from potato fruit juice (PFJ), which is commonly discarded from the potato starch industry (Ralet & Guéguen, 2001). When extracting the protein, it is important to use appropriate extraction methods in order to maintain the protein in its raw form, as usually once the protein is denatured it will lose all functionalities.

2.2.3.1. Solubility

Solubility is affected by many different components present in the solution, including addition of thermal and acidic treatments, dialysis, metal salts, and organic solvents (van Koningsveld, et al., 2001). Solubility is a parameter that is easily measured in single protein solutions; however the measurements become more difficult in protein mixtures because different proteins have varying solubilities. As previously mentioned, potato proteins are made up of many proteins; therefore assessing their solubility is a very difficult process. Techniques to circumvent this difficulty include relating protein solubility to the total insoluble protein (van Koningsveld, et al., 2001). In this study, van Koningsveld et al. (2001) have related the ability of the remaining precipitate to resolubilize in a given solution compared to the total protein present in the PFJ. The solubilities were tested in the presence of various acids such as sulphuric acid (H_2SO_4), hydrochloric acid (HCl), citric, and acetic acids. Of the many proteins contained in PFJ, most possess isoelectric points between pH 4.5 and 6.5 (van Koningsveld, et al., 2001).

In the presence of acid, regardless of type, the potato proteins seemed to precipitate to a greater extent at pH 3 (van Koningsveld, et al., 2001). In terms of the resolubilisation of the precipitates, the weak acids (citric and acetic) seemed to have a higher resolubility than the stronger acids (hydrochloric and sulphuric); as the weaker acids were able to resolubilize approximately 4% more of the total proteins in the PFJ than the stronger acids (van Koningsveld, et al., 2001).

When purified patatin and PFJ were compared, the purified patatin seemed to exhibit almost total solubility at pH 4, precipitate at pH 5, and undergo complete resolubilization at pH 6. Conversely, PFJ proteins demonstrated maximum precipitation at a pH less than 4, and precipitates collected at pH 5 were only partially resoluble at pH 7. They concluded that purified patatin and purified potato proteins behave differently than PFJ as the proteins present in the PFJ do not behave in relation to their isoelectric point (van Koningsveld, et al., 2001). This trend was also shown by Waglay et al. (2014) where proteins present in PFJ had low solubility at pH 3 with increasing resolubility outside that condition.

van Koningsveld et al. (2001), tested the solubility of potato protein in the presence of heat treatments. Results show that when PFJ was heated to temperatures above 40 ^{0}C the proteins

began to precipitate out of solution, it was shown that 50% precipitation occurred at 60 ^{0}C, and complete denaturation occurred at 70 ^{0}C. This was confirmed by Waglay et al. (2014) where 60 ^{0}C resulted in 50% re-solubility whereas at 70 ^{0}C less than 10% resolubility was shown. Increasing the ionic strength was found to have a slight influence on the effect of temperature. Ionic strength was varied, $(NH_4)_2SO_4$ precipitated potato protein, was found to have similar characteristics to undenatured potato protein. When the effect of temperature was tested in the presence of high ionic strength 200mM NaCl, the denaturation pattern shifted slightly by 5 ^{0}C, where complete denaturation took place at 75 ^{0}C as opposed to the 70 ^{0}C previously observed. Contrarily, decreasing the ionic strength seemed to have no influence on the solubility curves. van Koningsveld et al. (2001) also studied the solubility of the potato proteins separately, and conclusions were made that the protease inhibitors seemed to exhibit a slightly higher precipitation temperature when compared to patatin. The protease inhibitors showed insolubility at temperatures of 50-60 ^{0}C. Whereas patatin began to be insoluble at temperatures above 40 ^{0}C (van Koningsveld, et al., 2001).

2.2.3.2. Emulsifying Properties

According to Smith and Culbertson (2000), an emulsion is defined as "mixtures of at least two immiscible liquids, one of which is dispersed in the other in the form of fine droplets". In order to stabilize an emulsion it is often beneficial to add an amphiphilic surfactant such as protein. Proteins contain both hydrophobic and hydrophilic components due to their amino acids therefore when added to the emulsion they will orient themselves at the interface of the appropriate molecule based on polarity. This orientation at the interface will help stabilize the emulsion by decreasing the interfacial tension between the two immiscible liquids (Smith & Culbertson, 2000).

It is also important to note that other factors often influence the stability of the emulsion once formed. According to van Koningsveld et al. (2006), common instabilities include creaming and droplet aggregation. Creaming is the result of the low density droplets layering themselves above the higher density aqueous phase. Droplet aggregation is when the droplets group together and increase the average size of each droplet; this is described as a colloidal attraction between droplets. Other possible reasons for droplet aggregation include bridging and depletion flocculation. Bridging flocculation involves a molecule that creates a bridge between

the interface and the droplets, while depletion flocculation occurs in the presence of a high concentration of non-adsorbing polymers (van Koningsveld, et al., 2006).

In the study conducted by van Koningsveld et al. (2006), different potato protein samples were observed to establish their emulsion-forming and stabilizing effects under various conditions. PFJ was collected from the cv. Elkana potato variety. Protein samples were prepared from PFJ with different extraction techniques to yield five unique protein samples: potato protein isolate; $(NH_4)_2SO_4$ precipitate; patatin; ethanol-precipitated patatin; and protease inhibitor pool (van Koningsveld, et al., 2006). The emulsions were formed in a neutral environment and it was observed that the emulsion containing patatin showed no droplet aggregation under microscopic conditions, whereas the other protein samples showed severe droplet aggregation and therefore the emulsion was unstable.

van Koningsveld et al., (2006) determined the type of aggregation that was occurring with the protein samples. In order to decipher between the aggregation types different techniques were used. For depletion aggregation the interactions between the droplets should be quite weak, therefore with increasing shear rate the viscosity of the emulsion should decrease. When this test was conducted the potato protein isolates and protease inhibitors exhibited higher viscosity at a low shear rate and therefore depletion aggregation was concluded to not be the cause of the instability of the emulsion. Furthermore, the addition of dithiothreitol completely broke apart the aggregates, demonstrating bridging aggregation between the disulphide bonds present in the protease inhibitors and dithiothreitol. They concluded that this could be avoided by simply ensuring that there is no incorporation of air during the preliminary stages of emulsion formation (van Koningsveld, et al., 2006).

A key parameter for a protein to maintain its functional characteristics is the pH environment the protein is subjected to. When a protein is close to its isoelectric point it possesses a low solubility and therefore loses most of its emulsifying ability (Smith & Culbertson, 2000). In the study by Ralet and Guéguen (2000), the emulsion properties of different potato protein fractions over a pH range of 4 to 8 were studied. The pH range was tested with the addition and absence of NaCl at a concentration of 1%. They observed that in an acidic environment in the absence of NaCl, the patatin emulsion was slightly more stable, compared to the emulsion

in the presence of NaCl. Moreover, when the protease inhibitor fraction was examined it did not show pH dependency (Ralet & Guéguen, 2000).

2.2.3.3. Foaming Properties

According to Phillips et al. (1990), foam is defined as "a two-phase system in which a distinct gas bubble phase is surrounded by a continuous liquid lamellar phase". Proteins are often incorporated into foams to help with stabilization of the foam. The molecular characteristics of the substance that attribute to foaming are its solubility, ability to form interfacial interactions, ability to unfold at the interface, ability to react with a gaseous or aqueous phase, amount of charged and polar subunits which prevent foam formation, and steric effects (Ralet & Guéguen, 2001). When examining the foaming properties of a given protein, it is important to examine both the foam forming ability and the stability of the foam formed. These parameters are evaluated by measuring the foam volume and the amount of liquid released over time.

The extraction methods that have shown to maintain potato proteins functional characteristics in foam stabilization are ultrafiltation, carboxymethyl cellulose complexation, and anion-exchange chromatography (van Koningsveld, et al., 2002).

A study completed by van Koningsveld et al. (2002), examined the potato protein ability to both form and stabilize the foam. The foams were formed with two different methods known as the sparging and whipping techniques. The sparging technique is more desirable for highly structured proteins as it allows the rigid protein more time at the interface, which results in the release of bubbles due to buoyancy forces (van Koningsveld, et al., 2002). The whipping technique begins with beating which causes velocity and pressure fluctuations. As a result, the bubbles interact with each other, causing both the interfacial area and the surface tension to change over time. The foam will form once the whipping speed is high enough to cause the surface tension to become too great, thereby allowing the bubbles to coalesce. The addition of various proteins allows the foam to have an optimum whipping time for formation. This is beneficial as a longer whipping time will show positive correlation with foam volume, however the disadvantage is that over whipping will cause the proteins to denature to the point that they aggregate which decreases foam volume (van Koningsveld, et al., 2002).

Various conditions can occur which lead to decreased stabilization of the foam. A major concern is the presence of hydrophobic molecules, as these fat molecules either coalesce, leading to a spreading of the bubbles, or their particle size is larger than the foam bubbles which enhances the foam breakdown. Another cause of foam instability is drainage, which is the result of gravity forcing the liquid out of the foam. However in the presence of proteins, drainage is greatly reduced due to the formation of stagnant layers. An additional negative impact on foam stabilization is Ostwald ripening. While the ideal foam is composed of many small bubbles, air is more soluble in a liquid phase and therefore solubility is higher around smaller air bubbles. This leads to the continual growth of larger air bubbles, the process known as Ostwald ripening (van Koningsveld, et al., 2002).

van Koningsveld et al. (2002), observed the whipping time at varying whipping speeds for different proteins such as β-lactoglobulin, β-casein, potato protein isolated using 15% ethanol, potato protein isolated using 20% ethanol, $(NH_4)_2SO_4$ potato protein isolates, patatin, patatin extracted using 20% ethanol precipitation, protease inhibitors, and protease inhibitors isolated using 20% ethanol precipitation. The results concluded that at a standard whipping time of 70 sec the protease inhibitors showed no optimal whipping speed, whereas $(NH_4)_2SO_4$ potato protein isolates, patatin extracted with 20% ethanol, and protease inhibitors extracted with 20% ethanol all showed an optimum whipping speed of 1,914*g*, while potato protein isolates extracted using 20% ethanol formed a foam at 1,077*g*. When patatin was examined it was found that using the standard whipping time (70 sec) no foam was formed, therefore the whipping time was reduced to 30 sec and the optimum speed was found to be 1,077. Overall it could be observed that for most protein samples as the whipping speeds increased and the whipping times increased the foam formation and foam volume became better to an optimum level (van Koningsveld, et al., 2002).

The foam that was formed from patatin at neutral pH was similar in appearance to the foam formed by lysozyme (egg white protein). Patatin is very structurally rigid and therefore as predicted, shorter whipping times and speeds are required for the protein to unfold at the interface. Two conclusions can be drawn about patatin with foam formation by the whipping test, either the rate of unfolding is extremely slow, or the rate of refolding is extremely fast, this would explain why longer whipping times did not improve foam formation. When the

sparging test was utilized the results were similar to that of other proteins, namely β-casein and β-lactoglobulin. As previously mentioned sparging is the result of low surface expansion rates. The conclusion of this technique in foam formation was that patatin must exhibit slow unfolding rates, and therefore the foam could not stabilize against the effects of drainage, coalescence, and Ostwald forces (van Koningsveld, et al., 2002).

In the study completed by Ralet and Guéguen (2001), raw potato proteins, a patatin fraction, and a 16-25 kDa fraction (protease inhibitors), were compared to a standard Ovomousse M (a commercial spray-dried hen egg white powder). A plastic column and a porous metal disk were used to form the foams. Air was blown through the metal disk into the column, and two electrodes located in the columns measured the drainage of the foams once formed. All the samples required the same whipping time to reach a volume of 35ml, at a neutral pH with no NaCl solution added. At these conditions the patatin fraction, raw potato protein, and Ovomousse M all exhibited the same characteristics over time as the foam structures were not significantly broken. When the three samples were compared, patatin seemed to present the most stable foam over time, while the 16-25 kDa fraction lost most of its foaming characteristics due to drainage. When salt solutions were added to the protein samples, the samples seemed to form more stable foams more quickly, and they also became pH independent. The pH of the solution is very important as protein's electrostatic charge is pH dependent. When a protein is in a solution close to its isoelectric point it should stabilize foams to a greater extent due to the decrease in electrostatic repulsion. Patatin is an exception to this rule because as observed, patatin is not as soluble at pH 4 (Ralet & Guéguen, 2001).

2.3. Potato By-Product as a Source of Potato Proteins

The industrial uses of potatoes mostly involve the extraction of the starch source, resulting in a waste product referred to as potato fruit juice (PFJ) at a rate of about 5 to 12 cubic meters for one metric ton of potatoes (Ralet & Guéguen, 2000; Miedzianka, et al., 2014). One issue that arises is the difficulty in disposing of this product as it possesses a high polluting capacity. As a result, removing its protein components is beneficial to discard the effluent as well as to obtain useful proteins that could have applications in the food industry (Ralet & Guéguen, 2001). Many studies have been performed in order to improve the removal of proteins from the

starch industry by-product because it contains the highest bioavailable oxygen demand when compared to other industry by-products (Strolle, et al., 1973).

PFJ contains many desirable components that can be extracted such as proteins, amino acids, organic acids, and potassium. Once the proteins are removed from the PFJ, the juice is subjected to ion-exchange chromatography which separates the other components. However protein concentration must be below 180 ppm (parts per million) in order to prevent the proteins from being separated in the column instead of the other minor components. Analysis of raw PFJ shows that the protein level is approximately 1500-4000 ppm (Strolle, et al., 1973) or approximately 25 g of protein per kg of PFJ (Pastuszewska, et al., 2009). The protein extracted from PFJ has been studied to be comparable to that of soy and animal proteins, as similar essential amino acid content is attained (Pastuszewska, et al., 2009). The soluble solids composition of the juice consists of 35% crude protein, 35% total sugar, 20% minerals, 4% organic acids, and 6% others (Strolle, et al., 1973). PFJ contains approximately 1.5% (w/ v) soluble proteins and most of the proteins present in PFJ are the above mentioned patatin and protease inhibitor proteins (van Koningsveld, et al., 2006).

The most common method to extract the proteins from the PFJ is precipitation with the addition of acidic and heat treatments. However, several improvements have been explored in order to improve the quality of the potato proteins, as the heat and acidic treatments denature the proteins. These denatured proteins no longer possess their functional characteristics such as foaming, emulsification, and water or oil holding capacity and therefore their use in food applications is diminished (van Koningsveld, et al., 2006; Miedzianka, et al., 2014).

The antinutritional or toxic properties that are present in the PFJ are solanidine glycoalkaloids and protease inhibitors, specifically trypsin inhibitors. A study conducted by Pastuszewska et al., (2009) found that for both solanidine glycoalkaloids and protease inhibitors the amount varied greatly and the differences were probably due to the variation in potatoes. However both amounts were found in concentrations similar to ones found in the tuber itself for solanidine glycoalkaloids, and protease inhibitors were present in similar concentrations as soybean meal (Pastuszewska, et al., 2009). This study has no mention of the beneficial effect of protease inhibitors and therefore conclusions vary greatly from other studies.

2.4. Extraction Methods of Proteins

The primary challenge is to extract the proteins from waste materials or industrial by-products without denaturing or affecting their functional characteristics. This section will focus on advantages and disadvantages of a variety of methods that have been studied for the recovery of the proteins.

2.4.1. Combination: Thermal and Acidic Precipitation

The use of acid and heat coagulation either in combination or separately has been used for many years for the recovery of proteins (Knorr, et al., 1977). Historically, these techniques were used in order to remove the protein content of the PFJ and therefore decrease the polluting capacity and economic cost. Nowadays, it is often undesirable to employ either technique as both acid and heat coagulation lead to protein denaturation. When a protein structure is altered there is often a complete loss of its functional characteristics, which therefore impedes its ability to be incorporated into other food products.

Heat coagulation is the method commonly used in the potato starch industry. Temperatures in excess of 90 ^{0}C are often used, which causes a major disadvantage with economic cost and at these temperatures the potato proteins are rendered insoluble, which in turn limits their application in other sectors of the food and pharmaceutical industries (Knorr, et al., 1977). Heat coagulation can be performed with various protocols. Strolle et al. (1973) studied steam injection heating by incorporating steam into the PFJ to increase the temperature of the juice to 104.4 ^{0}C. The juice was then flash cooled allowing the proteins to be collected. This study concluded that steam injection heating is ideal at temperatures above 99 ^{0}C with pH adjustments to 5.5. Furthermore, steam injection heating was found to be an efficient and simple method for the removal of the proteins from the PFJ. The technique was also found to be economically disadvantageous due to the energy consumption as well as the ion-exchange step required to remove the other components (organic acids, amino acids, etc.) present in the PFJ (Strolle, et al., 1973).

Acid coagulation commonly involves the use of HCl, phosphoric acid (H_3PO_4), or H_2SO_4 (Knorr, et al., 1977). The same authors (Knorr, et al., 1977) have examined the use of acid and heat coagulation of potato protein from waste effluent. Results showed that ferric chloride ($FeCl_3$) demonstrated protein recovery similar to HCl in the pH range of 2-4, whereas above

this range (pH 5-6) HCl demonstrated better results. When the PFJ proteins are recovered with either acidic or heat coagulation they commonly have a dark appearance due to the harsh environment and a strong cooked flavour. Therefore, when proteins are extracted with these techniques they are often used as animal feeds (Straetkvern, et al., 1999).

2.4.2. Precipitation Methods

2.4.2.1. Salt Precipitation

The most commonly used salt in salt precipitation of potato proteins from PFJ is $FeCl_3$ (Knorr, 1982). These salts function as extraction techniques through ionic strength adjustment (Knorr, 1981). Knorr et al. (1977) found that $FeCl_3$ was able to coagulate the potato proteins from PFJ just like the acid and heat coagulation. In this study, the protein precipitates all varied in their composition depending on the different extraction techniques, acidic (HCl), acidic/heat treatments, and $FeCl_3$ precipitation, while HCl alone seemed to extract more total solids when compared to the others (Knorr, et al., 1977). The same authors (Knorr, et al., 1977) found that when heat was not used during the extraction process, the precipitates tended to have higher vitamin C content and the precipitate extracted with $FeCl_3$ seemed to contain more iron (Knorr, et al., 1977). The study then compared the amount of protein denaturation caused by the different extraction techniques by measuring the solubility of nitrogen and relating that to the extent of denaturation, where the greater the solubility of nitrogen the less denatured the protein (Knorr, et al., 1977), hence the more functional characteristics the protein retains. Of the different techniques, $FeCl_3$ demonstrated the highest nitrogen solubility, followed by HCl, and finally HCl in the presence of heat (Knorr, et al., 1977).

$FeCl_3$ precipitation is a simple technique to precipitate the proteins out of solution, where increasing $FeCl_3$ concentrations resulted in high recovery of the proteins. However, after extraction, this technique introduces a disadvantage in measuring the protein content with simple protein determination methods, as the $FeCl_3$ precipitated proteins require a chelating agent to become soluble. Additionally, the high ferric ion content causes interference with the chelating agents and, therefore, interferes with protein determination using Bradford or BCA methods (Bártova & Bárta, 2009).

2.4.2.2. Ethanol Precipitation

Ethanol precipitation demonstrated relatively similar results to the $FeCl_3$ precipitation method. Bártova and Bárta (2009) compared the two precipitation techniques, ethanol and $FeCl_3$ precipitation. Results showed that a maximum concentration of ethanol is needed to optimally extract proteins with a high recovery rate, whereas increasing $FeCl_3$ concentrations resulted in high recovery of the proteins. They found that both methods resulted in a precipitate with a lower proportion of patatin. They theorized that the underestimation is due to poor extractability in the SDS-buffer; along with the poor thermostability of patatin when compared to the more thermostable protease inhibitors that are also present.

Both ethanol and $FeCl_3$ precipitated proteins that possess a high nutritional quality, proven by a high proportion of essential amino acids (Bártova & Bárta, 2009). When the precipitates were compared in their lipid acyl hydrolase activity (LAH), the ethanol precipitated proteins seemed to demonstrate a higher preservation of LAH activity (Table 2.1). However, the low LAH activity obtained upon $FeCl_3$ precipitation is due to the poor resolubility of the precipitated proteins in the buffer (Bártova & Bárta, 2009). They concluded that as compared to $FeCl_3$ precipitation, ethanol precipitation is easier to perform, and results in proteins with high nutritional value and retention of LAH activity, which indicates the occurrence of minimal protein denaturation (Bártova & Bárta, 2009).

2.4.2.3. Ammonium Sulphate Precipitation

Ammonium sulphate ($(NH_4)_2SO_4$) precipitation is a technique commonly employed to extract proteins based on solubility differences. This technique is normally used to recover undenatured potato proteins. van Koningsveld et al. (2001) have investigated the recovery of potato proteins from acidified PFJ (pH 5.7) with $(NH_4)_2SO_4$ at a saturation of 60%. $(NH_4)_2SO_4$ recover protein isolates are often compared to undenatured potato proteins due to the large proportion of protein extracted (75%), possessing a wide variation in molecular weight. $(NH_4)_2SO_4$ precipitation also extracts a large proportion of patatin when compared to other technique (van Koningsveld, et al., 2001).

van Koningsveld et al., (2001) have further assessed the $(NH_4)_2SO_4$ protein isolates and found that the solubility was influenced by ionic strength just like PFJ. When a high ionic strength was used, a minimum solubility was observed at pH 3; whereas when a low ionic strength was used, a broader soluble curve resulted with a minimum around pH of 5. (van Koningsveld, et al., 2001). When examining the effect of temperature, high ionic strength affected the occurrence of the denaturation temperature, with a shift upwards of 5 ^{0}C (van Koningsveld, et al., 2001).

2.4.2.4. Carboxymethyl Cellulose Complexation

Another extraction method uses a polysaccharide, carboxymethyl cellulose (CMC), as a precipitating agent. The addition of CMC to a protein solution allows the proteins to coagulate, promoting easier collection through simple centrifugation techniques. The precipitate formation is influenced by the pH of the environment, ionic strength of the protein and polysaccharide, net charges of the species, as well as the size, shape and interaction between molecules (Vikelouda & Kiosseoglou, 2004). The use of a complexing agent such as CMC has been hypothesized as a potential solution to decrease the pollution effect of waste effluents from the potato industry. Vikelouda and Kiosseoglou (2004) studied the effect of CMC extraction on the recovery of the proteins and their functional properties. The precipitation of protein by CMC is due to the electrostatic interactions occurring between the oppositely charged protein and carboxyl group of CMC. When present in an acidic environment (pH 2.5) the proteins will interact with the CMC resulting in an overall decrease in net charge of the molecule. This leads to a change in the isoelectric point of the proteins, which results in a neutral precipitate (Vikelouda & Kiosseoglou, 2004). The pH adjustment to 2.5 has been shown to affect several of the functional characteristics of the proteins. However, the addition of the polysaccharide has some benefits as it will enhance several of the functional qualities such as foaming. The protein precipitate recovered upon complexation with CMC was found to possess adequate solubility and foaming properties. Foaming properties could be influenced by the presence of CMC because the high surface activity proteins will interact with the polysaccharide which positively affects the foaming ability and stability. In relation to the emulsifying properties, CMC will adsorb at the interface, and the resulting protein/CMC complex will protrude, creating steric hindrance (Vikelouda & Kiosseoglou, 2004).

Gonzalez et al. (1991) have reported that CMC helps in the formation of a stable precipitate over a large pH range, unlike the protein solutions alone which are only insoluble at a pH of about 3. They concluded that for CMC with a lower degree of substitution, a pH of 2.5 was ideal for protein recovery, and for CMC with a higher degree of substitution, a pH of 3.5-4.0 was optimal. The CMC to protein ratio was also reported to be an important parameter as too much CMC will cause the precipitate to form incorrectly, thereby triggering the supernatant to have a cloudy appearance. Ratios of 0.05:1 and 0.1:1 (CMC to potato protein (w/ w)) were found to be optimal amounts for varying degree of substitution of the CMC (Gonzalez, et al., 1991). Indeed, when CMC is added in amounts larger than required for precipitation, the coagulation occurs in two stages (Gonzalez, et al., 1991). The first stage involves the formation of the precipitate through protein aggregation and the second stage involves the solubilisation of the aggregates. The second stage occurs when the proteins align and form a stable complex with the CMC, this allows for smaller particle sizes and more active sites on the CMC that can be hydrated (Gonzalez, et al., 1991).

Overall, CMC complexation technique is proven to be effective and simple for use in protein recovery from PFJ. However, one disadvantage is the needed acidic environment that may lead to the loss of some of the functional characteristics of proteins (Vikelouda & Kiosseoglou, 2004).

2.4.3. Ion Exchange

Løkra et al. (2008) have examined the use of chromatographic techniques to separate the water-soluble proteins from PFJ. Contrary to the most popular technique of heat coagulation, which results in the loss of most functional properties of the proteins, adsorption chromatography has many advantages, including the ability to collect specific fractions of proteins, the ease in removing other non-nutritional components, and the ability to remove any interfering components (low molecular weight components) (Løkra, et al., 2008). The expanded bed adsorption technique has been found to be effective at removing impurities from the PFJ such as fibres, minerals, and pigments (Løkra, et al., 2008). Once the protein sample is isolated using expanded bed adsorption, it is important that the proteins undergo drying techniques in order to be applied in the food industry. The disadvantage of most drying techniques is that they use

hot air which again affects the functional characteristics, and also adds a dark colour to the proteins, limiting their applications in food products. Therefore, gentle drying techniques are beneficial (Løkra, et al., 2008). According to Claussen et al. (2007) the most effective drying method is atmospheric freeze drying for potato protein concentrates.

Straetkvern et al. (1999) have studied the extraction of bioactive patatin from PFJ using slight pH adjustments and mixed mode high density adsorbent as the stationary phase. The pH adjustments (to pH 2-3) were needed to elute the patatin from the adsorbent material. The advantages to using mixed mode adsorbent over other resins include the ability to separate interfering brown products and the non-issue of ionic binding. This mixed mode adsorbent is made up of small glass beads that are coated with agarose substituted with low molecular weight affinity ligands. These ligands are composed of an inner core which is hydrophobic and made up of aromatic, heteroaromatic, or aliphatic molecules, allowing for a variety of hydrophilic or ionic molecules to attach. Among various mixed mode adsorbents, mixed mode ES and mixed mode ExF, commercial names, have shown high binding and specificity for patatin. The difference of these resins is the concentration of ligands, where ExF variety is substituted at low concentrations, whereas ES is substituted at high concentrations (Straetkvern, et al., 1999). The mixed mode ES resin exhibited binding at a pH range of 6.5-8.5, where the elution occurred at 2.3 min. The lower concentration of ligands in mixed mode ExF was more selective for patatin from the PFJ when compared to mixed mode ES. The benefit in using mixed mode ExF was due to the pH environment of 3.5-4.5, which is less acidic than the pH range required for elution with mixed mode ES, thereby maintaining a higher degree of protein functionality. The resins did not differentiate between the patatin isoforms, indicating that the elution was not solely based on charge but also on the group affinity to the column material (Straetkvern, et al., 1999).

A more recent study conducted by Zeng et al. (2013) studied the effect of continuous polymer and pore phase known as Amberlite XAD7HP. This membrane has a high surface area and an aliphatic surface, this allows for absorption of both polar and non-polar compounds depending on the system. They concluded that this method resulted in an extract primarily composed of a large proportion of protease inhibitors (Zeng, et al., 2013).

Although the chromatographic techniques showed promising results, they have two major disadvantages: the difficulty in applying them at the industrial scale and the need of high investment costs. Industrial application would be extremely difficult as the limits to this technique included the high density resins and their loss of binding sites (Straetkvern, et al., 1999), which decreases the efficiency of the column separation over time.

2.5. Separation of Bioactive Proteins and Peptides

Fractionation of the potato proteins is often based on differences in size and charge, as the patatin fraction has higher molecular weights and lower isoelectric point when compared to the protease inhibitor fraction. Fractionation to separate the different isoforms present in the patatin fraction was based on the charge as each isoform possesses a different charge (Pots, et al., 1999).

Ralet and Guéguen (2000, 2001) have isolated the patatin fraction and the protease inhibitor fractions from PFJ using anion-exchange chromatography with a DEAE-Sepharose Fast Flow column (25 mM phosphate buffer, pH 8). The 'acidic fraction' was recovered upon a linear gradient of NaCl at pH 8. While the unbound sample was further fractionated using a SP-Sepharose Fast flow (25 mM phosphate buffer, pH 8) and the 'basic fraction' was recovered using a linear gradient of NaCl at pH 8 (Ralet & Guéguen, 2000).

Jørgensen et al. (2006) have used a size exclusion column of Superdex 200 HR 10/30 to fractionate the tuber proteins (50 mM sodium phosphate buffer containing 40 mM NaCl at pH 7.0). The recovered protein fractions were further analyzed for their molecular masses using matrix assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS), and their activities were assayed using various activity tests, including protease inhibition, α-mannosidase, lipid acyl hydrolase, peroxidase, and glyoxalases I and II (Jørgensen, et al., 2006).

Racusen and Foote (1980) developed the most widely applied purification technique for the isolation of patatin. They compared the use of DEAE-cellulose to the concanavalin A-Sepharose. Concanavalin-A is tetrameric metalloprotein for which sugar molecules that contain α-D mannopyranosyl and α-D-glucopyranosyl have a strong affinity in the presence of

C-3, C-4 and C-5 hydroxyl groups which are required for concanavalin-A (GE Healthcare, 2005). They concluded that the use of the DEAE-cellulose resulted in a more pure sample with no interfering proteins, which was confirmed by their results obtained from silver stained SDS-PAGE. However, protein samples collected by concanavalin A-Sepharose resulted in the slight detection of contaminating proteins from SDS-PAGE (Racusen & Foote, 1980). Modification introduced by Bohac (1991) for the purification of potato proteins began with gel filtration using a Bio-Gel P-100 column with a mobile phase of phosphate buffer. The fractions were then subjected to anion-exchange chromatography, using DEAE-Sephacel medium with a mobile phase of linear gradient NaCl. Followed by a glycoprotein specific column, concanavalin A-Sepharose column prepared with 25 mM pH 7.0 phosphate running buffer with 0.5M NaCl. Like Racusen and Foote (1980) the purification was established using SDS-PAGE and similar conclusions were drawn, where concanavalin A- Sepharose fraction still contained many contaminating bands when run on SDS-PAGE; however affinity chromatography did have a direct beneficial effect on the specific activity (Bohac, 1991).

Patatin itself is the major storage protein found in tubers, for this reason it has been studied extensively. When patatin is run on SDS-PAGE the patatin exists as one broad band. Differences between the isoforms are exposed when patatin is studied using isoelectric focusing which separates proteins based on charge into many bands (Pots, et al., 1999). Pots et al. (1999) initially separated the patatin group following initial separation on a concanavalin A- sepharose column. Pots et al. (1999) and Bártova and Bárta (2009) suspended the protein precipitate in a 25 mM Tris-HCl buffer to a pH of 8.0. The isoelectric point of patatin is 4.9 therefore at pH 8 the patatin will possess a strong negative charge. This negative charge will interact with the positively charged column dependent on the exact charges of the isoforms. Pots et al. (1999) and Bártova and Bárta (2009) began with initial separation by anionic chromatography, followed by columns Source Q and diethylaminoethyl (DEAE) 52- Cellulose SERVACEL, respectively. Cellulose SERVACEL is a non-porous column consists of well separated charge groups that allow for minimal adsorption. As a result, the amount of sites for adsorption can be specified, which is desirable because mild conditions can be used during desorption (SERVACEL, 2007). Following anionic chromatography Bártova and Bárta (2009) separated the patatin group on a concanavalin-A sepharose column, which is in opposite order as performed by Pots et al. (1999).

2.6. Potential Application of Potato Proteins

2.6.1. Biogeneration of Peptides through Enzymatic Hydrolysis

The use of enzymatic hydrolysis has been successful in biogenerating smaller peptides, which have a tendency to possess better functional properties than larger proteins (Moreno & Cuadrado, 1993; Wang & Xiong, 2005). Taste, viscosity, whipability, emulsifying and foaming abilities are all improved when the large proteins undergo a specific set of hydrolysis steps to yield smaller peptides (Moreno & Cuadrado, 1993).

The major advantage in using proteases is that they are selective and specific. Proteases function under mild reaction conditions and are used to partially hydrolyze polypeptide chains. Proteases are classified according to their mechanisms as either endo-proteases or exo-peptidases. Endo-proteases are able to cleave peptide bonds within the protein chain. Conversely exo-peptidases cleave peptide bonds at either the C-terminal or N-terminal ends of the protein chain. In order to improve enzymatic hydrolysis, they are commonly used in combination in order to enhance the amount of terminal ends (Kamnerdpetch, et al., 2007). Understanding of the structural properties of the proteins is important for a better control of their hydrolysis into appropriate peptides. Wang and Xiong (2005) have investigated the hydrolysis of potato protein hydrolysates into potato peptides using an endo-protease (Table 2.3). As expected, the longer the reaction times, the higher degree of hydrolysis was obtained (Kamnerdpetch, et al., 2007; Wang & Xiong, 2005). As the degree of hydrolysis increased, the solubility of the resulted hydrolysates was enhanced. As peptides exhibit more charged groups than proteins, there is an enhancement of protein-water interaction, as well as increased electrostatic repulsion among the peptide molecules (Wang & Xiong, 2005). Characterization of the peptides present in the substrates was completed by SDS- PAGE. Prior to hydrolysis, the substrates displayed bands at approximately 45 kDa (patatin) and 18-25 kDa (protease inhibitors). However after 0.5 hrs of hydrolysis the patatin band had vanished and the small molecular weight bands appeared indicating the presence of smaller peptides after hydrolysis (Wang & Xiong, 2005). Kamnerdpetch et al. (2007) have also studied the enzymatic hydrolysis of potato pulp using selected proteases: two endo-proteases, (Alcalase 2.41, 415 U/mL; Novo Pro-D, 400 U/ml), one exo-protease, (Corolase LAP, 350 LAPU/g), and a combination of endo-protease and exo-peptidase, (Flavourzyme, 1000 LAPU/g) as well as their combinations were

investigated. Upon 26 hr of hydrolysis, Flavourzyme (7%, w/ w) demonstrated the greatest degree of hydrolysis of 22%, followed by Alcalase (7%, w/w), Novo Pro-D (7%, w/ w), and Corolase (7%, w/ w) with degree of hydrolysis 8, 3, and 2%, respectively. Because of the presence of both endo-protease and exopeptidase in the Flavourzyme, the terminal ends should be enhanced for the exo-peptidase to act on, yielding more cleavage of either carboxypeptidase (C terminal) or aminopeptidase (N terminal) bonds.

Table 2.3 Literature on enzymatic hydrolysis of potato proteins.

Starting Material	Enzyme Type	Enzyme Name/Combination	Incubation Time (hr)	Degree of Hydrolysis	Authors
Potato protein concentrate[a]	Endo	Alcalase (1:100 enzyme: substrate)	0.5	0.72	Wang and Xiong, 2005
			1	1.9	
			6	2.3	
Potato pulp[b]	Endo	Alcalase	26	5	Kamnerdpetch et al., 2006
		Novo Pro-D	26	2.5	
	Endo/Exo	Flavourzyme	26	20	
	Exo	Corolase	26	2	
	Mixtures	(2%Alc+5%Fla)	26	40	
		(3%Alc+4%Fla)	26	35	
		(2%Alc+5%Cor)	26	15	
		(3%Alc+4%Cor)	26	18	
		(2% NPD+5%Fla)	26	40	
		(3% NPD+4%Fla)	26	30	
		(2% NPD+5%Cor)	26	15	
		(3% NPD+4%Cor)	26	15	
Potato protein concentrate[c]	Endo	Alcalase	2	3.5	Miedzianka et al., 2014
		Alcalase	4	7.7	
Potato protein isolate[d]	Endo	Alcalase (5000ppm)	n.d.	60	Pęksa et al., 2014
	Endo/Exo	Flavourzyme (5000pp)	n.d.	n.d.	
	Mixture	1 Alc : 1 Fla	n.d.	60	

[a]Potato protein concentrate was obtained from AVEBE B.A. (Veendam, The Netherlands).
[b]Potato pulp was obtained from AVEBE B.A. (Veendam, The Netherlands).
[c]Potato protein concentrate was obtained from a starch factory in Łomża, Poland.
[d]Potato fruit juice was provided by starch factory in Niechlów, Poland and then underwent combination thermal treatment (70-80^{0}C) for 10-20 min.

When comparing the two endo-proteases, Alcalase, which is a serine alkaline protease with a higher specific activity and broader specificity, demonstrated a higher degree of hydrolysis. In all cases, the combination of proteases resulted in a higher degree of hydrolysis compared to the individual enzymes (Kamnerdpetch, et al., 2007). The hydrolysate with the highest degree of hydrolysis (44%) was obtained with 2% (w/w) Alcalase and 5% (w/w) Flavourzyme (Kamnerdpetch, et al., 2007).

Liyanage et al., (2008) found that potato peptides retrieved from potato pulp using alkaline commercial protease enzymes had a positive impact on the lipid metabolism in rats. This enzymatic hydrolysis process resulted in peptides with molecular weights varying from 700-1840 Da, the main molecular weight present (90% of the total) was 850 Da. They concluded that enzymatic hydrolysis in this fashion is an economical process which can be easily scaled up into large industrial scale processes (Liyanage, et al., 2008).

A study conducted by Miedzianka et al. (2014) studied the improvement of potato protein isolates by peptide generation, using a commercial product Alcalase (endo-protease from *Bacillus licheniformis*, specific activity of 2.4 AU/g). They used two incubation time conditions of 2 and 4 hr (Table 2.3). It was found that 2 hr was sufficient time to improve the solubility of the protein concentrate and functionalities (Miedzianka, et al., 2014).

More recently, a study conducted by Pęksa & Miedzianka (2014) have investigated the impact of the enzymatic hydrolysis on the amino acid composition of peptides obtained using two commercial proteases Alcalase, Flavourzyme, and their combination (Table 2.3). They found that a combination of both Alcalase and Flavourzyme was ideal, as Alcalase rendered more end terminals to improve the efficiency of Flavourzyme. In turn more peptide were hydrolyzed into oligopeptides and free amino acids decreasing the bitterness commonly associated with protein hydrolysates (Pęksa & Miedzianka, 2014). When Alcalase was used alone, a lower proportion of amino acids methionine and cysteine were observed (Pęksa & Miedzianka, 2014). The authors concluded that this enzymatic modification to the heat treated proteins could further take advantage of their use as a value added ingredient (Pęksa & Miedzianka, 2014).

The hydrolysis of potato proteins to potato peptides is desirable due to the improvement of their functional characteristics. Despite this advantage the process is limited due to the undesirable flavor profile that results. Research by Ney (1979) has shown that potato proteins possess high hydrophobicity due to the increased presence of hydrophobic amino acids. This hydrophobic character is undesirable as it is linked to a bitter taste that results from the peptides making the incorporation of potato peptides into food products difficult. As a result the optimal degree of hydrolysis is often limited by the degree of bitterness of these peptides. In a study performed by Ney (1979) it was found that peptides above 6000 Da seemed to not possess the bitterness that smaller peptides had. The addition of gelatin to the potato protein substrate decreased the occurrence of peptides smaller than 6000 Da. The ideal ratio to decrease the development of bitter peptides was determined to be 4 parts potato protein to 1 part gelatin (Ney, 1979).

CONNECTING STATEMENT 1

A literature review on the different extracting techniques for the recovery of potato proteins from a by-product generated from the starch industry is described, as well as an understanding of the proteins fractions functional and beneficial properties is outlined in Chapter II. A comprehensive assessment of several conventional extraction techniques and their effect on the potato protein extracts is presented in Chapter III. The isolation techniques were assessed according to protein yield, purification factor, and protein proportion. This chapter includes the evaluation of the potato protein extracts according to their structural conformation, physico-chemical properties, and functional activities based on the extracting technique used.

The results from this study were presented at the IFT Annual Meeting & Food Expo-Institute of Food Technologist and in the journal of Food Chemistry.

Waglay, A., Karboune, S., & Alli, I. (2012) Investigation of Selected Approaches for the Extraction of Potato Protein and Characterization of their Functional Properties. IFT12 Annual Meeting & Food Expo, Las Vegas, USA, June 25- June 28, 2012.

Waglay, A., Karboune, S., Alli, I. (2014) Potato protein isolates: Recovery and characterization of their properties. *Food Chemistry, 142,* 373-382.

CHAPTER III. POTATO PROTEIN ISOLATES: RECOVERY AND CHARACTERIZATION OF THEIR PROPERTIES

3.1 Abstract

An imitation of industrial potato fruit juice (PFJ) was prepared, using Canadian variety of potatoes, and was characterized of being composed of 22.9% patatin, 53.3% protease inhibitors, and 23.7% high MW proteins. To isolate potato proteins from PFJ, several extraction techniques were explored including thermal/acidic combination, acidic, $FeCl_3$, $MnCl_2$, ethanol and $(NH_4)_2SO_4$ precipitations, and carboxymethyl cellulose complexation. $(NH_4)_2SO_4$ precipitation led to the highest yield (98.6%) and to the recovery of protein isolates enriched in patatin with high resolubility. $FeCl_3$ precipitation resulted in the highest purification factor (6.2) and isolates with the lowest relative proportion of high MW proteins (<4.6 %,); however, its optimal isolate showed a wide minimum solubility pH range of 3.0 to 6.0. $FeCl_3$ and $MnCl_2$ were identified as the best precipitating agents for the enrichment of isolates with >15 kDa protease inhibitors. Trypsin inhibiting activities of protease inhibitors were highly preserved upon protein isolation more than the chymotrypsin ones. Acidic-based protein isolates showed the highest specific lipid acyl hydrolase activity of patatin towards 4-nitrophenyl butyrate, whereas the $FeCl_3$-based one exhibited the highest activity towards 4-nitrophenyl laurate.

3.2 Introduction

The potato starch industry releases large quantities of by-product, known as potato fruit juice (PFJ), which is costly to dispose of due to its high polluting effect (Vikelouda & Kiosseoglou, 2004) and it provides only marginal economic value when used as animal feed and fertilizer. The chemical composition of PFJ is interesting as it is rich in proteins, minerals and free amino acids (Wojnowska, et al., 1981). Converting this by-product into high value-added ingredients would have an important economical and environmental impact. In this context, the extraction of proteins from PFJ is of particular interest as manufacturing starch from one thousand kg of potatoes releases 5-12 m^3 of PFJ, which contains 30-41% protein from the total solids (Wojnowska, et al., 1981).

Compared to other proteins from other vegetable and cereal sources, potato proteins are considered higher quality as they contain a high proportion of lysine, which is often lacking in such crops. Potato proteins are commonly divided into three fractions, patatin (up to 40%), protease inhibitors (~50%), and other high molecular weight proteins (~10%). The patatin fraction is a dimer glycoprotein with a molecular weight of 40-45 kDa and is present as many

isoforms. In terms of beneficial properties, patatin has been shown to possess antioxidant ability (Pihlanto, et al., 2008; Liu, et al., 2003) and lipid acyl hydrolase (LAH) activity (Pots, et al., 1999). In addition, functionally, patatin has excellent foaming (van Koningsveld, et al., 2002) and emulsifying abilities (van Koningsveld, et al., 2006; Cheng, et al., 2010). The protease inhibitors, with a molecular weight ranging from 5-25 kDa, have been shown to have beneficial properties such as anti-carcinogenic (Blanco-Aparicio, et al., 1998), anti-microbial (Kim, et al., 2005), and a high satiety property by releasing the hunger suppressant cholecystokinin (Deveaux-Gobert, 2008). Functionally, the protease inhibitor fraction is soluble throughout a wide pH range, whereas patatin shows minimum solubility at pH 4 (van Koningsveld, et al., 2001).

Isolating proteins from PFJ without affecting their functional properties is challenging because of the aqueous nature of PFJ and its complex composition. Industrially the isolation of proteins from PFJ involves a combination of thermal coagulation and acidic precipitation (Cheng, et al., 2010; Miedzianka, et al., 2012). Although thermal/acidic precipitation results in a high yield of protein recovery, it often leads to complete loss of the protein functionality, which limits their application to animal feed (Cheng, et al., 2010; Miedzianka, et al., 2012). Other extraction techniques have been explored for the recovery of functional proteins, including salt, acid (Bárta, et al., 2008; Bártova & Bárta, 2009), ethanol (Bártova & Bárta, 2009), ammonium sulphate ($(NH_4)_2SO_4$) precipitations (van Koningsveld, et al., 2001), carboxymethyl cellulose (CMC) (Vikelouda & Kiosseoglou, 2004) complexation and chromatographic techniques (Bártova & Bárta, 2009). However, conflicting results have been reported on the efficiency of these techniques to isolate potato proteins from PFJ. These differences could be due to the use of varying potato cultivars and PFJ preparation methods. To our knowledge, to date, no literature is published on protein extraction from Canadian variety of potatoes. In addition, the relationship between the extracting agents and the functional properties of isolated proteins has been overlooked.

Only limited studies (Knorr, 1982; Bárta, et al., 2008; Bártova & Bárta, 2009) have evaluated these extraction techniques in terms of recovery yield, purification factor, protein profile, denaturation degree and functional properties. The present research work examines the effect of the concentration of precipitating agents (combination of thermal/acidic, acidic, ferric

chloride ($FeCl_3$), manganese chloride ($MnCl_2$), ethanol, $(NH_4)_2SO_4$, and CMC) on the recovery yield and the purification factor of the protein isolates. The extraction techniques were also evaluated based on protein profile, denaturation, resolubility at selected pH and temperatures and functional properties of isolated proteins (LAH activity; protease inhibitor effect). The overall objective of this study was to investigate and compare the effect of several extraction techniques for the isolation of potato proteins from PFJ.

3.3 Materials and methods

3.3.1. Materials

Sodium metabisulfite, sulphuric acid (H_2SO_4), $FeCl_3$, $MnCl_2$, CMC (medium viscosity), trifluoroacetic acid, hydrochloric acid, 4-nitrophenol, 4-nitrophenyl laurate, 4-nitrophenyl butyrate, α-chymotrypsin, *N*-α-Benzoyl-D-L-arginine 4-nitroanilide hydrochloride (*N*-α-BAPA), acetic acid, and *N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (*N*-succinyl-AAPP) were purchased from Sigma Chemical Co. (St-Louis, MO). Absolute ethanol was purchased from Ricca Chemical Company (Arlington, TX). Bradford reagent and SDS-PAGE Broad Molecular weight Standards were purchased from Bio-Rad (Mississauga, On). Bovine Serum albumin (BSA), acetonitrile, $(NH_4)_2SO_4$, potassium chloride, sodium citrate, citric acid, sodium acetate, Tris base, and trypsin were purchased from Fisher Scientific (Fair Lawn, NJ). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from MP Biomedicals, LLC (Solon, OH).

3.3.2. Preparation of potato fruit juice (PFJ)

PFJ was prepared according to the modified method of van Koningsveld et al. (2001). Potatoes of Russet Burbank variety were washed, chopped into large pieces and frozen at -80 ^{0}C for 30 min to decrease heat transfer during homogenization. The frozen potato samples (100 g) were suspended in sodium metabisulfite solution (26 mM) to prevent polyphenol oxidation and homogenized using a Waring Commercial Blender on low speed for 5 min. The potato slurry was subjected to cheese cloth filtration. The resulting turbid liquid was centrifuged at 8000*g* for 30 min at 4 ^{0}C using a Beckman Centrifuge Model J2-21. The supernatant was filtered using 1.2 μm GF/C Whatman filters. The clear yellowish filtrate is known to be similar to industrial PFJ.

3.3.3. Preparation of potato protein isolates

The potato protein isolates were prepared using selected extraction techniques. The protein isolates recovered by centrifugation at 8000*g* for 50 min at 4 ^{0}C were resuspended in water and analyzed along with their corresponding unprecipitated proteins of PFJ for their protein and nitrogen contents using Bradford and Dumas methods, respectively.

The yield was calculated as:

$$\text{Yield (\%)} = (\text{Total protein content}_{\text{Isolate}} / \text{Total protein content}_{\text{PFJ}}) \times 100 \quad (1)$$

The purification factor was expressed in dry weight basis and was defined as:

$$\text{Purification Factor} = \text{Protein proportion (w/w)}_{\text{Isolate}} / \text{Protein proportion (w/w)}_{\text{PFJ}} \quad (2)$$

3.3.3.1. Thermal/acidic precipitation

PFJ underwent a combination of thermal/acidic precipitation as previously described by Knorr et al. (1977). The pH of PFJ was adjusted to 4.8 and 5.5 with 0.5 M H_2SO_4. The suspensions were then incubated at 100 ^{0}C for 2 min. After 5 min of cooling on ice, the protein isolates were recovered.

3.3.3.2. Acidic precipitation

The acidic precipitation was carried out by adjusting the pH of PFJ to pH 2.5 with 0.5 M H_2SO_4 (Knorr, et al. 1977). The suspensions were stirred for 1 hr at ~25 °C.

3.3.3.3. $FeCl_3$ and $MnCl_2$ precipitation

$FeCl_3$ and $MnCl_2$ precipitations were performed according to the modified method of Bártová and Bárta (2009). $FeCl_3$ solution (1M) was added to acidified PFJ (pH 5.0) solution to yield 5, 10, 20 and 40 mM, whereas $MnCl_2$ was added to reach a concentration of 20 mM. Precipitations were performed at ~25 °C for 1 hr with constant agitation at 1000*g*.

3.3.3.4. Ethanol precipitation

Ethanol precipitation was performed according to the modified method of Bártová and Bárta (2009). Ethanol was added to the PFJ at ratios of 10, 20 and 30% (v/ v). Precipitations were performed for 1 hr at 1000*g* and 4 °C.

3.3.3.5. $(NH_4)_2SO_4$ precipitation

$(NH_4)_2SO_4$ precipitation was performed according to the modified method of van Konings-veld et al. (2001). The protein precipitation was carried out at 40, 60, and 80% $(NH_4)_2SO_4$ saturation. The mixtures were incubated for 90 min at 1000*g* and 4 °C. The recovered precipitates upon centrifugation were suspended in water and ultrafiltrated through regenerated cellulose filters with a MW cut-off of 3 kDa (Millipore, MS).

3.3.3.6. CMC complexation

CMC complexation was performed according to the modified method of Vikelouda and Kiosseoglou (2004). The CMC was added to the acidified PFJ (pH 2.5) to reach ratios of CMC: Potato proteins (w/w) of 0.1, 0.3, and 0.6. Precipitation was performed at ~25 °C with stirring at 1000*g* for 1 hr.

3.3.4. Protein content determination

Bradford assay was used to determine the content of the soluble protein. A DU 800 spectrophotometer (Beckman, CA) was used for the measurement of absorbance at 595 nm. Nitrogen content was determined using Leco® TruSpec N (Leco Corporation, MI). Nitrogen content was multiplied by a factor of 6.25 to determine the protein content (van Gelder, 1981).

3.3.5. Protein isolate resolubility

Upon precipitations, the resolubility of protein isolates was investigated at two selected pH values of 5.0 and 7.0 using the appropriate buffering system. The suspensions were shaken for 1 hr at ~25 °C and then centrifuged at 3600*g* for 15 min. The supernatants were analyzed for the resoluble protein content using Bradford assay, whereas the precipitates were analysed for the non-resoluble protein content using Dumas method.

3.3.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein isolates were analyzed by SDS-PAGE according to the method of Laemmli (1970) using 5 and 15% acrylamide content in the stacking and resolving gels, respectively and a mini protein gel apparatus (Bio-Rad, On) with a 1.5 mm-thick gel. The electrophoresis was conducted at a constant voltage of 120 mV. The analyses of the electrophoretic patterns to obtain the protein profiles were carried out using Red Imaging system equipped with Alpha-

View SA Software. The relative proportions of recovered patatin and protease inhibitors were estimated from bands corresponding to 38-45 kDa and 28-10 kDa, respectively.

3.3.7. Fluorescence spectroscopy

The fluorescence spectra of protein isolates were recorded using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon system, Les Ulis, FR). The spectra were recorded at two different pH of 4 and 9 by varying temperatures from 25 to 85 °C with a 5 °C interval increase. Excitation was at 295 nm and the resulting emission was measured at 305-450 nm with a scan speed of 120 nm/ min. Both the excitation and emission slit were set at 3.5 nm. Protein isolates were prepared using the optimal conditions: thermal/acidic (pH 4.8), acidic pH 2.5, 5 mM of $FeCl_3$, 20% (v/ v) of ethanol, 60% $(NH_4)_2SO_4$ saturation, and 0.1 of CMC (w/ w).

3.3.8. Effect of pH and temperature on the solubility of protein isolates

The effects of pH and temperature on the solubility of optimal protein isolates were investigated using a wide range of pH (2 to 9) and temperature (20 to 100 °C) values. To maintain the pH, appropriate buffering systems (50 mM) were used*:* potassium chloride (pH 2); sodium citrate (pH 3); sodium acetate (pH 4 and 5); sodium phosphate (pH 6 and 7); Tris-HCl (pH 8 and 9). The effect of the temperature was examined by incubating the protein isolates in sodium phosphate buffer (pH 7) at various temperatures for 2 hr. The suspensions were centrifuged at 3500*g* for 15 min. The supernatants were analyzed for the resoluble protein content using Bradford assay, whereas the precipitates were analysed for the non-resoluble protein content using Dumas method.

3.3.9. Protease inhibitor activity

Inhibitory activity of protein isolates towards serine proteases was investigated using trypsin and chymotrypsin proteases, according to the modified method of Pouvreau et al. (2001). Trypsin and chymotrypsin enzymatic solutions (10-20 mg/mL) were prepared in 50 mM Tris HCl buffer (pH 7.8) containing 100 mM $CaCl_2$. The protease and the potato protein isolates were mixed to achieve an equal protein concentration (1:1; w: w) and incubated at 37 °C for 15 min. The proteolytic activity of trypsin and chymotrypsin were assayed before and after incubation with potato protein isolates using *N*-α-BAPA and N-succinyl-AAPP as substrates, respectively. The reaction was initiated by mixing 0.2 mL of substrate (1.8 -1.12 mM), 0.2 mL

of the enzymatic solution and 0.6 mL of Tris-HCl buffer (50 mM, pH 7.8) in a 1 mL spectrophotometer cell. The variation in absorbance of the reaction mixtures, against a blank trial without enzyme, was continuously monitored at 410 nm, for 10 min. The proteolytic activity was estimated from the slope of the absorbance versus time curve. Standard curves were established at a wavelength of 410 nm using a 4-nitroaniline as standard. The inhibitory activity of potato protein isolates was calculated as:

$$\text{Inhibiting Activity} = ((\text{specific activity}_{\text{without inhibition}} - \text{specific activity}_{\text{inhibition}})/ \text{specific activity}_{\text{without inhibition}}) \times (\text{mg trypsin/g potato protein}) \times 100 \quad (3)$$

3.3.10. Lipid acyl hydrolase activity

LAH activity of potato protein isolates was measured spectrophotometrically using 4-nitrophenol butyrate and 4-nitrophenyl laurate as substrates, according to the modified method of Bártová and Bárta, (2009). Potato protein isolates (8-13 mg/mL) at an appropriate dilution were pre-heated at 37 °C in a 1 mL spectrophotometer cell using a DU 800 spectrophotometer equipped with a thermocontrolled sample compartment. The reactions (1 mL) were initiated by the addition of 50 and 500 µL of 4-nitrophenyl butyrate (10 mM) and 4-nitrophenyl laurate (1 mM, 0.5% DMSO), respectively, prepared in 50 mM Tris-HCl buffer at pH 7.4. The absorbance of the reaction mixtures was monitored at 410 nm for 5 min. Standard curve was established using 4-nitrophenol as the standard. One unit of LAH activity was defined as the amount of enzyme liberating one µmol of 4-nitrophenol per min. Analyses were performed in duplicate.

3.4. Results and discussion

3.4.1. Yield and purification factor of potato protein isolates from PFJ

Table 3.1 shows that combined thermal/acidic precipitation resulted in the highest yield of 90.2%; however, the protein isolates showed a very low purification factor of 0.74. These results reveal the low selectivity of thermal/acidic precipitation leading to the recovery of other non-protein components present in the PFJ. Contrarily, acidic precipitation without thermal treatment led to a lower yield of 64.7%, but a higher purification factor of 1.26. This low yield could be due to the partial denaturation of proteins, which are less likely to precipitate out of the acidic solution. A similar high yield of 86.2% was reported by Miedzianka et al. (2012)

upon thermal and acidic treatment of PFJ. In addition, Knorr et al. (1977) have obtained a lower yield of 61.5% after HCl precipitation as compared to HCl and heat precipitation (90.2%).

$MnCl_2$ precipitation resulted in the poorest yield of 16.8%, whereas $FeCl_3$ led to higher yields. Increasing $FeCl_3$ concentration from 5 to 20 mM resulted in relatively the same yield (58.8% ± 1.8). However, a further increase of $FeCl_3$ concentration to 40 mM led to higher recovery (75.2%). Similar results were previously reported by Bártová and Bárta (2009), where increasing concentrations of $FeCl_3$ increased potato protein recovery with no maximum. Contrary to the yield, the purification factor showed a dependence on the $FeCl_3$ concentration. Low $FeCl_3$ concentration (5 mM) resulted in the highest purification factor of 6.24.The low purification factors (1.61-3.36) obtained with higher $FeCl_3$ concentrations can be attributed to the competition of potato proteins with other components (i.e. Polyphenols) present in PFJ.

Ethanol precipitation resulted in relatively lower recovery yields (30.7-55.2%) and comparable purification factors (1.19 to 3.79). A maximum yield (55.2%) and purification factor (3.79) were obtained at an ethanol concentration of 20% (v/v) and increasing the ethanol concentration to 30% (v/v) decreased the protein yield to 15% and the purification factor to 1.19. Ethanol as a precipitating agent is able to penetrate proteins and compete with water, inducing protein conformation tightening (Gülseren, et al., 2012). At high ethanol concentrations, the proteins become compact and folded, which excludes ethanol leading to lower interactions and protein yield (Bárta, et al., 2008). Similarly, Bártová and Bárta (2009) have reported an increase of the protein yield to 66% at an optimum ethanol concentration (23.4%, v/v), followed by a decline. No optimum was found by van Koningsveld et al. (2001), who have reported higher yield of 90% when the ethanol concentration was increased to 45.6%.

Table 3.1 also indicates that the protein recovery yield increased from 70.1 to 98.6% by increasing the $(NH_4)_2SO_4$ saturation from 40 to 60% and remained thereafter constant (98.8%), whereas the purification factor (2.99-3.31) was approximately similar. As compared to other extraction techniques, $(NH_4)_2SO_4$ precipitation resulted in the highest yield (98.6%); however, the highest purification factor (6.24) was obtained upon $FeCl_3$ precipitation at low concentration, indicating the high affinity of potato proteins towards $FeCl_3$ as compared to $(NH_4)_2SO_4$. Indeed the hydrophobic interaction responsible for precipitating proteins out of

solution when using $(NH_4)_2SO_4$ is achieved with its dissociation from its ionic substances, which results in an increase of the protein's surface. This surface tension leads to a decrease in hydrogen bonding between the water molecules and the protein thereby promoting precipitation (Cheng, et al., 2010).

Comparable yields (62.7-75.3%) and purification factors (2.27-2.53) were obtained at CMC concentration of 0.1 and 0.3 (w: w). Increasing further CMC concentration to 0.6 (w: w) resulted in a significant decrease of yield and purification factor to 23.6% and 0.75, respectively. The protein complexation with CMC was reported to be highly pH dependent (Gonzalez, et al., 1991). A pH adjustment to 2.5 (between the pKa values of the carboxyl group on the polysaccharide and the protein's isoelectric point) ensured a good recovery of proteins. When comparing acidic precipitation with CMC complexation, the recovery yield was similar. However, the purification factor (2.2-2.5) is greatly improved for CMC, which can be explained by the selective interactions of potato proteins with CMC. Lower yields of 25 and 39.0% were reported for the recovery of potato proteins from PFJ by CMC complexation at a ratio of 0.1 and were attributed to the decreased association between the protease inhibitors and CMC (Gonzalez, et al., 1991; Vikelouda & Kiosseoglou, 2004).

3.4.2. Resolubility of potato protein isolates

The investigation of the resolubility is important as it implies the potential use of the potato proteins in food applications. Protein solubility can be affected by temperature, pH, solvent, and ionic strength (van Koningsveld, et al., 2001). As PFJ is composed of many different proteins, the optimal parameters may not occur for all the proteins. The resolubility at pH 5.0 and 7.0 is therefore not an absolute value, but an approximate one.

Figure 3.1 shows that at pH 5.0, a lower proportion of soluble protein (<15%) was obtained for potato protein isolates recovered upon combination, acidic, $FeCl_3$, ethanol and CMC precipitations. These low resolubilities may be attributed to conformational changes, cross-linking, and changes in their net charge, which may have shifted their isoelectric points (van Koningsveld, et al., 2001; Bártova & Bárta, 2009). A previous study conducted by van Koningsveld et al. (2001), resulted in similar results upon H_2SO_4 and HCl precipitations, where less than 15% of the proteins were resoluble at pH 5.0. When the same authors examined the effect of $FeCl_3$ they found that increasing concentrations (5 to 15 mM) increased the proportion

of soluble protein (15 to 40%). This relationship was not seen with our results, and these discrepancies could be due to the differences in the composition of PFJ in terms of polyphenols and minerals. Previous studies (Bártova & Bárta, 2009) have shown that the interference with $FeCl_3$ and polyphenols could explain the poor resolubility. Friedman (1997) concluded that potato protein can react with the polyphenols present in PFJ resulting in insoluble tannins, further lowering solubility.

Increasing the pH to 7.0 resulted in an increase in the proportion of soluble protein (35-93%) of isolates obtained upon $FeCl_3$ and ethanol precipitations (Figure 3.1). In contrast, no significant increase in the soluble protein proportion of thermal/acidic, acidic and CMC-based isolates was observed under the same conditions. Vikelouda and Kiosseoglou (2004) have reported a proportion of soluble proteins of 82-89% and 20% at pH 7.0 and 5.0, respectively, for protein/CMC complexes. Contrary to other extracting agents, increasing the ethanol concentration to 30% significantly decreased the resolubility of soluble protein by more than 60% at both pH values (Figure 3.1). These results confirm the denaturing effect of ethanol towards the potato proteins. Contradicting results on the ethanol precipitation were obtained by van Koningsveld et al., (2001) who began to see a decline in the resoluble protein at 58 % (v/v) ethanol concentration. The experimental findings also show that increasing $FeCl_3$ concentration has a larger effect on protein isolates resolubility at pH 5.0 than at pH 7.0.

Table 3.1 Recovery and proportion of proteins from potato fruit juice using selected precipitation techniques.

Precipitation Agents	Recovery Yield (%)[a]	Purification Factor[b]	Relative Protein Proportion (%)[c]				
			Patatin	Protease Inhibitors			High Molecular Weight
				25-21 kDa	20-15 kDa	<15 kDa	
PFJ[d]			22.9	24.6	18.4	10.3	23.7
Thermal/ Acidic [e]	90.2 ±0.22[f]	0.74	37.9	0.0	20.2	31.3	10.7
Acidic [e]	64.7 ±0.22	1.26	11.1	9.9	15.3	17.4	46.4
$FeCl_3$							
- 5 mM	60.8 ± 0.06	6.24	35.7	0.0	25.5	38.8	0.0
- 10 mM	58.1 ±0.08	2.65	37.7	14.3	25.1	18.6	4.2
- 20 mM	57.4 ±0.07	3.36	33.2	3.2	28.3	30.8	4.6
- 40 mM	75.2 ±0.44	1.61	21.7	18.7	23.2	34.3	2.0
$MnCl_2$[g]	16.8 ±0.11	1.52	20.4	0.0	30.9	44.2	4.6
Ethanol							
- 10%	30.7 ±0.11	2.50	37.7	11.7	21.3	23.9	5.5
- 20%	55.2 ±0.10	3.79	37.7	8.0	22.4	26.5	5.4
-30%	40.6 ±0.18	1.19	49.2	6.9	9.3	18.5	16.2
$(NH_4)_2SO_4$							
- 40%	70.1 ±0.19	3.31	35.5	7.1	22.0	27.0	8.4
-60%	98.6 ±0.23	3.28	36.5	5.3	20.8	26.2	11.3
-80%	98.8 ±0.21	2.99	31.1	7.6	23.7	26.3	11.3
CMC[h]							
- 0.1 CMC: PP	62.7 ±0.15	2.27	36.6	11.6	2.8	23.2	25.8
- 0.3 CMC:PP	75.3 ±0.14	2.53	36.6	1.8	10.0	22.9	28.7
- 0.6 CMC: PP	23.6 ±0.15	0.75	4.5	3.7	14.8	12.8	64.2

[a] Yield is expressed as the total protein content of the precipitate divided by the initial protein of Potato Fruit Juice.
[b] Purification factor expressed as the protein content of the extract per dry weight divided by the protein content of the PFJ per dry weight.
[c]Relative proportion obtained from Alpha View software.
[d]Potato Fruit Juice.
[e] Thermal acidic and acidic precipitations were run at pH 4.8 and 2.5, respectively.
[f]Standard deviation was calculated based on triplicate measurements.
[g] $MnCl_2$ was used at a concentration of 20 mM.
[h]Ratio of carboxymethyl cellulose (CMC) to potato protein (PP).

Unlike other isolates, the potato protein isolates obtained by $(NH_4)_2SO_4$ precipitation exhibited higher resolubility at both pH values, with the proportion of soluble protein ranging from 78 to 89%. These results may be attributed to the high ionic strength of $(NH_4)_2SO_4$, which may preserve the conformation of patatin and protease inhibitors (van Koningsveld, et al., 2001).

3.4.3. Protein profiles of potato isolates from PFJ

Table 3.1 outlines the protein profiles of PFJ and potato isolates, as determined by SDS-PAGE. PFJ was found to contain 22.9% patatin, 53.3% protease inhibitors and 23.7% high MW proteins. Selected results have been reported using both industrially (starch processing) and imitated PFJ. Ralet and Guéguen (2000) have reported a protein composition of 35% patatin, 56% protease inhibitors, and 9% high MW for PFJ prepared from the cultivar Bintje. Bárta et al. (2008) used a similar method with cultivar Tomensa, which consisted of 38% patatin, 45.6% protease inhibitors and 16.4% high MW. On the other hand, Bártová and Bárta (2009) reported industrial PFJ with a composition of 30.7% patatin, 52.7% protease inhibitors and 16.6% high MW. While protein profile can vary with cultivars and the preparation method, our prepared PFJ is not significantly different from industrial PFJ and is therefore a good analogue of the actual by-product. Combination thermal/acidic precipitation extracted a relatively high level of patatin (37.9%), and a high proportion of protease inhibitors (51.5%) (Table 3.1). Bártová and Bárta (2009) have examined the effect of heat coagulation and obtained comparable proportion of patatin (32.3%), but slightly lower proportion of protease inhibitors (39.5%). Patatin, being thermolabile, may have responded well to heat coagulation (Pots, et al., 1998). In contrast, the protease inhibitors, which are more thermostable, require acidic precipitation to increase their proportion. Table 3.1 also shows that acidic precipitation resulted in protein isolate composed of 11.1% patatin, 42.6% protease inhibitors and 46.4% high MW proteins. These results may be explained by the aggregation behavior of patatin under acidic conditions. As compared to $FeCl_3$, salt precipitations using $MnCl_2$ extracted significantly less patatin (20.4%), with more protease inhibitors (75.1%), indicating the low affinity of patatin toward $MnCl_2$ compared to the protease inhibitors. On the other hand, the relative proportion of patatin was dependent on the concentration of $FeCl_3$, where a maximum relative proportion of 37.7% was observed at 10 mM $FeCl_3$ followed by a

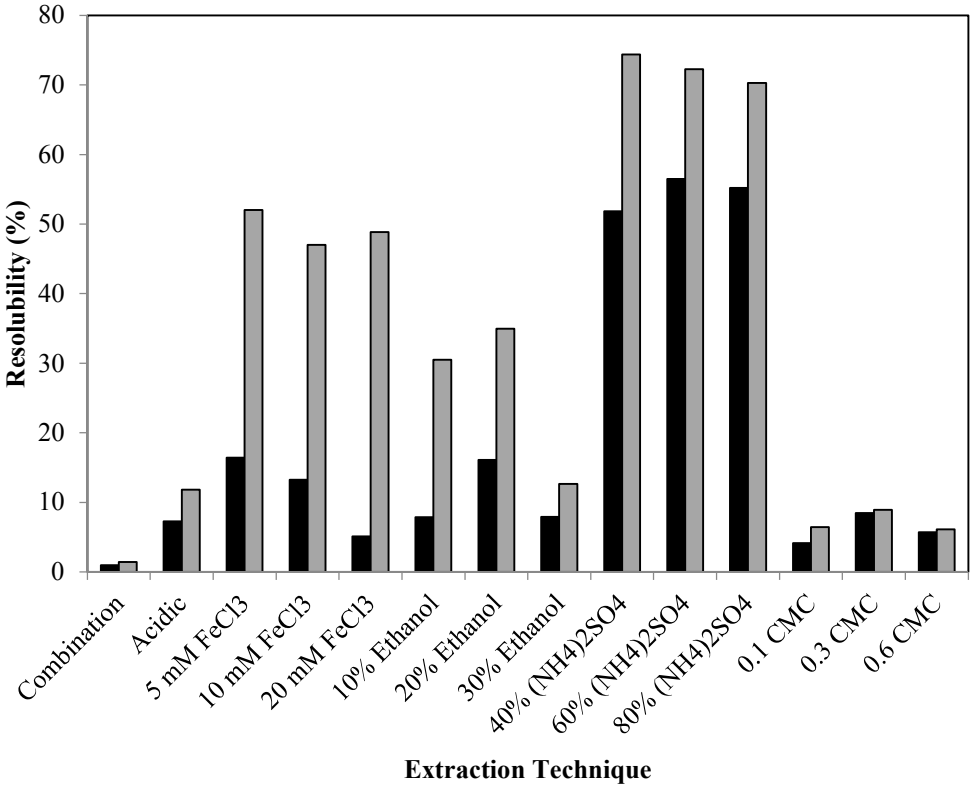

Figure 3.1 Resolubility of potato protein isolates obtained using various extraction techniques at two selected pH 5.0 (■) and 7.0 (□).

steady decrease to 21.7% at 40 mM $FeCl_3$. The 25-21 kDa and <15 kDa protease inhibitors responded well to an increase in $FeCl_3$, whereas the 20-15 kDa proteins remained relatively constant (~ 25.5%). The results also show that the high MW proteins exhibited the lowest relative proportion in the isolates obtained upon $FeCl_3$ precipitation as compared to the other techniques. Of particular interest is the high relative proportion of <15 kDa protease inhibitor proteins extracted by 5 mM $FeCl_3$ (38.8%) and 20 mM $MnCl_2$ (44.2%). Similarly, Bártová and Bárta (2009) have found that $FeCl_3$ resulted in the highest proportion of the <15 kDa protease inhibitors making it a good extracting agent to isolate potato carboxypeptidase inhibitors (8 kDa). The potato carboxypeptidase inhibitors have been known for their health benefit as antitumor compounds (Blanco-Aparicio, et al., 1998).

The results (Table 3.1) also show no significant changes in the protein profiles of the isolates obtained upon ethanol precipitation at 10 and 20% (v: v). Increasing the concentration of ethanol to 30% resulted in an increase in the relative proportions of patatin (37.7 to 49.2%) and high MW proteins (5.4 to 16.2%); however, a decrease in the relative proportion of protease inhibitors, mainly 20-15 kDa and <15 kDa ones, was observed. A study conducted by Bártová and Bárta (2009) has reported that both $FeCl_3$ and ethanol precipitation resulted in a similar patatin proportion, 20.3-25.6%. $(NH_4)_2SO_4$ precipitation showed slight concentration dependence for patatin extraction with an optimum relative proportion of 36.5% at 60% saturation. However, the relative proportions of protease inhibitors (~54%) and high MW proteins (~<11%) remained constant at the investigated $(NH_4)_2SO_4$ saturation.

Using CMC at low ratios of 0.1 and 0.3 resulted in enrichment of the isolate in patatin (36.6%) and <15 kDa protease inhibitors (23.1%). Complexation of CMC and patatin was affected by concentration where increasing the ratio to 0.6 CMC decreased patatin's relative proportion (4.5%) and increased the high MW protein proportion (64.2%) . These results reveal the effect of CMC in the aggregation of patatin at acidic conditions. van Koningsveld et al. (2001) have shown that at pH 3, weak acids greatly affect patatin's resolubility and decrease its proportion.

3.4.4. Effect of pH on the solubility of potato protein isolates

Figure 3.2 shows the effect of pH on the proportion of soluble proteins present in the isolates at a wide pH range of 2 to 9. The ionic strength of protein isolates was maintained at 50 mM, whereas PFJ was reported to be around 200 mM (van Koningsveld, et al., 2001). The results (Figure 3.2A) show that potato proteins in PFJ exhibited relatively the same soluble proportion (80.5-87.3%) in the pH range of 6 to 9. Decreasing the pH gradually decreased the soluble protein proportion to 26.6% at pH 3.0 before increasing to 68.1% at pH 2.0 (Figure 3.2A). Similar protein solubility profile of PFJ has been reported by van Koningsveld et al. (2001) with a solubility of 30% at pH 3.0. However, Ralet and Guèguen (2000) have obtained a different solubility profile of raw proteins with a minimum solubility (60%) at pH 4. These discrepancies may be attributed to the varying composition of PFJ.

The solubility profile of potato proteins as a function of pH is dependent on the precipitating agents (Figure 3.2 A-B). As compared to other techniques, combination thermal/acidic and acidic precipitations as well as CMC complexation resulted in isolates with very low soluble protein proportion (<21.9%) over the 2 to 9 pH range. The poor solubility of these protein isolates may be due to the protein denaturation under thermal and acidic treatments. Indeed, both acidic precipitation and CMC complexation were pH adjusted to 2.5 with H_2SO_4. Knorr (1982) has also reported that thermal and acidic treatments reduce the proteins solubility. Although CMC led to protein isolates with a higher purification factor (Table 3.1), the protein resolubility is lower when compared to acidic precipitation alone.

Contrary to PFJ and $(NH_4)_2SO_4$ isolate, the solubility of $FeCl_3$ and ethanol-based isolates varied strongly over the investigated pH range. These latter isolates exhibited solubility minima at pH 3.0 to 6.0 and maxima over the range 7.0 to 9.0. Minimums in the proportion of soluble proteins at pH 3.0 for $FeCl_3$ (11.0%) and ethanol (16.0%) isolates were lower as compared to that of PFJ (26.6%). However, $(NH_4)_2SO_4$ isolate assumed to contain mainly undenatured potato proteins, showed higher minimum solubility of 36.4% at pH 3.0. Figure 3.2 B shows a sharp rise of the solubility at pH 2.0 which was not observed for $FeCl_3$ (20.1%) and ethanol (17.1%) isolates as compared to PFJ (68.1%) and $(NH_4)_2SO_4$ isolate (49.2%), confirming the conformational changes of potato proteins upon $FeCl_3$ and ethanol

precipitations. Patatin was reported to have a broad minimum solubility at pH 4.5 at low ionic strength (15 mM) and to be completely insoluble at pH 3.5 at a high ionic strength (200 mM) (van Koningsveld, et al., 2001). In contrast, Ralet and Guéguen (2000) have reported a minimum solubility of a patatin-rich fraction at pH 4.0 when no salt was added and an increasing solubility with increasing ionic strength. Conflicting results were reported regarding the solubility profiles of protease inhibitor fraction as function of pH. van Koningsveld et al., (2001) have found minimum solubility of protease inhibitor fraction around pH 4.5; while Ralet and Guéguen (2000) have reported that the protease inhibitor fraction (16-25 kDa) was completely soluble over the entire pH range of 2 to 12.

3.4.5. Effect of temperature on the solubility of potato protein isolates

Figure 3.2 (C-D) shows the effect of temperature on the proportion of soluble proteins present in the protein isolates at temperatures varying from 20 to 100 °C. As shown in Figure 3.2C, PFJ maintained high resolubility (≥65%) at temperatures ranging from 20 to 40 °C. At 50 °C, a decline of 10% solubility was encountered, whereas a pronounced solubility decrease of 50% was observed at 60 °C. High temperatures of 70 to 100 °C resulted in solubilities of less than 10%. These results may be explained by the denaturation behaviors of potato proteins, as patatin is reported to unfold at 50 °C and rapidly denatures beyond that point (van Koningsveld, et al., 2001). Contrarily, the denaturation temperature of protease inhibitors is dependent on the ionic strength (van Koningsveld, et al., 2001). At low ionic strength (15 mM), the protease inhibitors unfolded between 58-71 °C, while at 200 mM, the denaturation temperature shifted to 58-75 °C (van Koningsveld, et al., 2001). There are few protease inhibitors which have been identified to be thermostable such as the carboxypeptidase inhibitors (0.9%) (Pouvreau, et al., 2001).

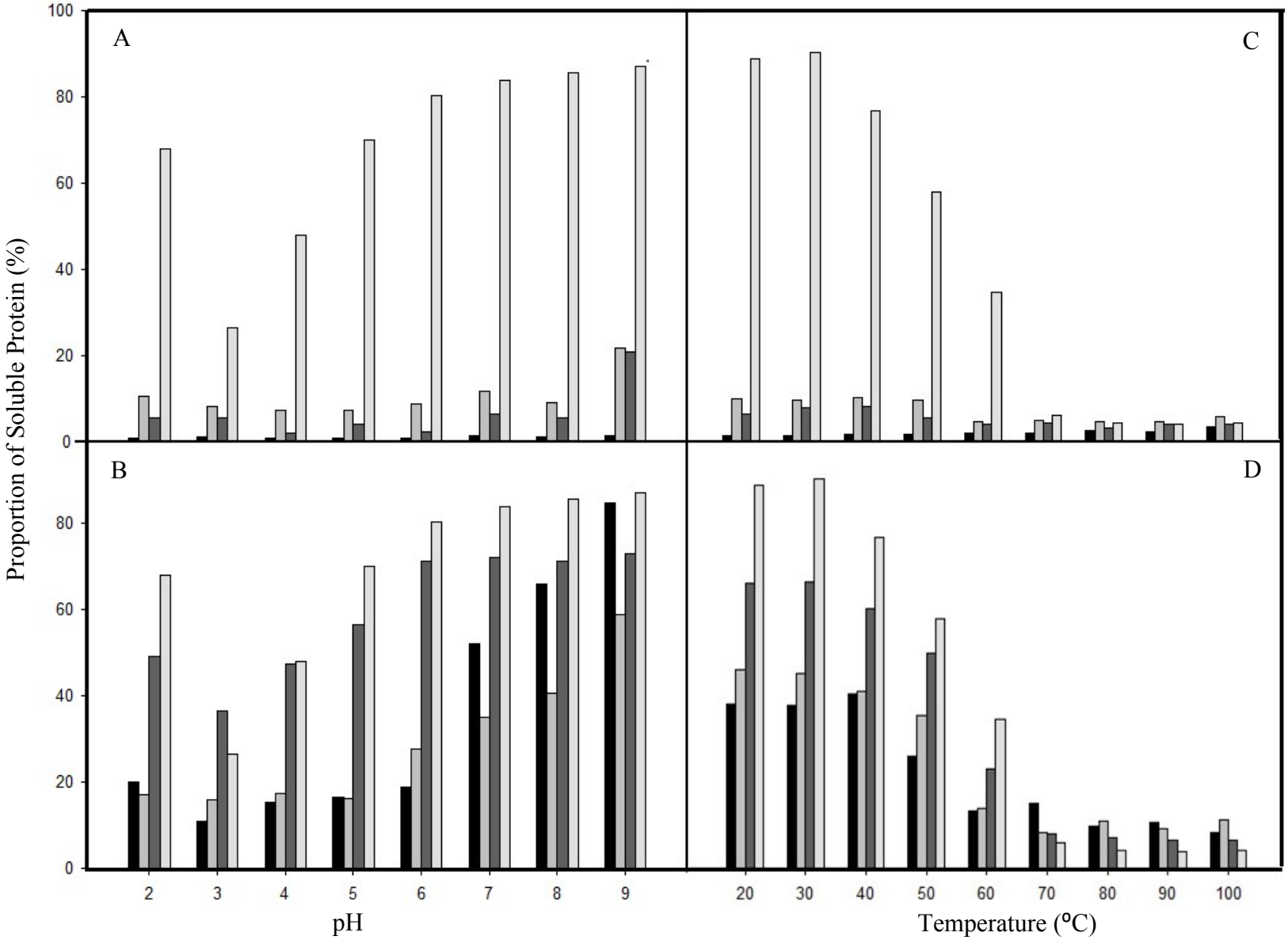

Figure 3.2 Effect of pH (A-B) and temperature (C-D) on the solubility of selected protein isolates: (A-C): thermal/ acidic precipitation combinations, (■); Acidic precipitation,(▒); CMC complexation, (▓); and PFJ, (□); and (B-D): $FeCl_3$ precipitation, (■) ; Ethanol precipitation, (▒); $(NH_4)_2SO_4$ precipitation,(▓); and PFJ,(□).

The results also show that the effect of temperature on the protein solubility is dependent on the precipitating agent used. Combination, acidic, and CMC complexation resulted in protein isolates with poor solubilities, revealing their severe conformational changes (Figure 3.2 C). As compared to acidic and CMC precipitations, combination resulted in an isolate with the lowest solubility of <4% throughout the temperature range. Acidic precipitation affected the protein isolates to a lesser extent. To our knowledge, no research is available for the effect of temperature on the protein isolates over a broad temperature range.

As shown in Figure 3.2 D, higher solubilities were obtained for protein isolates obtained upon $FeCl_3$, ethanol, and $(NH_4)_2SO_4$ precipitations. As compared to other isolates, $(NH_4)_2SO_4$ based isolate retained the highest solubility (> 60%) at a temperature range of 20-40 °C, followed by a decrease reaching 25% soluble protein at 60 °C. Above 70 °C, the proportion of soluble proteins in $(NH_4)_2SO_4$ isolate was less than 10%. As expected, $(NH_4)_2SO_4$ isolate showed a temperature profile comparable to that of PFJ. When compared to $FeCl_3$, the ethanol isolate had slightly higher proportions of soluble proteins below 50 °C. At 70 °C, a relatively higher proportion (14.9%) of soluble protein was obtained in protein isolates extracted by $FeCl_3$ when compared to other techniques. These results may be due to the change in the conformation of protease inhibitors upon $FeCl_3$ precipitation, which may have shifted their denaturation point to an upper limit of 75 °C (van Koningsveld, et al., 2001).

3.4.6. Fluorescence spectroscopy

To study the structural stability of potato protein isolates, fluorescence spectroscopy was used to monitor conformational changes of the aromatic acid environment as a function of temperature. The wavelength of the maximum intensity varied from 340-360 nm for all protein isolates at ~25 °C (data not shown). The results (Figure 3.3) show a gradual decrease in the maximum fluorescence intensity of protein isolates with increases in temperature at both pHs (4.0 and 9.0). This decline in fluorescence intensity may be attributed to the exposure of tryptophan residues to a polar environment resulting in the subsequent quenching of fluorescence (Ma & Harwalker, 1988).

At both pH 4.0 and 9.0, the thermal conformational changes of protein isolates obtained by $(NH_4)_2SO_4$ and CMC precipitations showed a linear behavior with a decrease in fluorescence intensity as the temperature increases. However, with increasing temperature the conformational changes of the tryptophan environment of $(NH_4)_2SO_4$ and CMC-based isolates was less extensive. In contrast, the isolates recovered after ethanol, acidic, thermal/acidic and $FeCl_3$ precipitations showed an exponential behavior, with a linear decrease of the maximum tryptophan fluorescence intensity up to 45 °C, followed by a decrease with lower rate; these results suggest that a major change in the protein molecular conformation took place at a temperature below 45 °C. Pots et al. (1998) have reported that patatin began unfolding at 46-55 ^{0}C, whereas above 55 ^{0}C the polarity surrounding the tryptophan residue was unchanged, as they are not available to water. Results also indicate that protein isolates recovered upon ethanol and thermal/acidic precipitations showed the highest substantial changes in tryptophan fluorescence intensity at pH 4.0 and 9.0, respectively. These results confirm the influence of the ethanol and thermal/acidic conditions on the protein stability. According to the initial linear rate of intensity changes, the heat seems to have more structural unfolding effects on the tryptophan environment of the proteins isolated by thermal/acidic at pH 9.0 than at pH 4.0, whereas it induced more changes at pH 4.0 for the protein isolates obtained by ethanol, acidic, and $FeCl_3$ precipitations. At pH 4.0, the protein isolates recovered after combination, acidic, and $FeCl_3$ precipitation thermally unfolded at comparable initial rates. Results of fluorescence intensity were expressed per mg soluble protein. At pH 4.0 (close to patatin's isoelectric point), most patatin may be insoluble, the differences in intensity at this pH can be attributed to the thermal stability of the protease inhibitors. On the contrary because pH 9.0 is within the range of the isoelectric point of the protease inhibitors, it can be assumed that the recorded thermal unfolding is related to a greater extent to the soluble patatin.

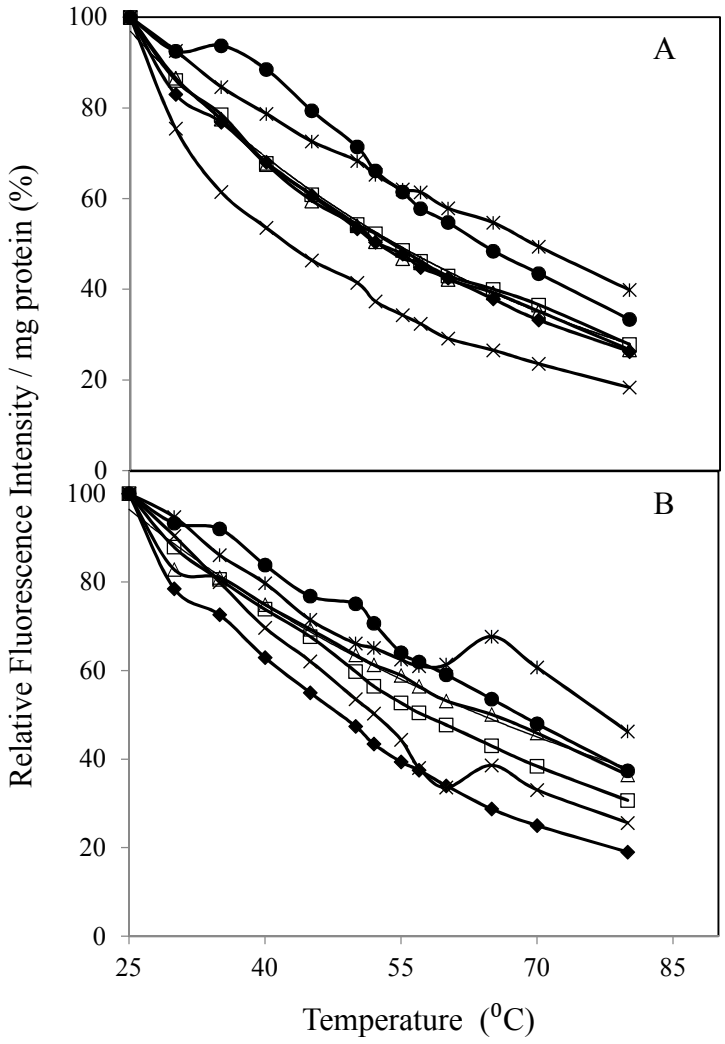

Figure 3.3 Sigmoidal transition curve of relative fluorescence intensity per milligram protein versus temperature for various protein isolates using different extracting agents. (A) pH 4 and (B) pH 9; thermal/acidic combination ( ), acidic ( ), ethanol ( ), $(NH_4)_2SO_4$ ( ), and CMC ( ) isolates.

3.4.7. Protease inhibiting activity of potato protein isolates

The protease inhibiting activity of potato protein isolates against trypsin and chymotrypsin was investigated. The inhibitory property of protease inhibitors present in PFJ has regained a great interest with potential applications as anticarcinogenic and satiety agents (Pouvreau, et al., 2001). The results (Table 3.2) show that with the exception of the thermal/acidic protein isolate, all potato protein isolates exhibited higher protease inhibiting activities against trypsin than chymotrypsin. Similarly, Pouvreau et al. (2001) have reported that 26 out of 29 protease inhibitor subcategories, recovered upon fractionation of PFJ, exhibited higher protease inhibiting activities against trypsin than chymotrypsin. The distribution of the protease inhibiting activities carried out by the same authors revealed that 82 and 50% of total trypsin and chymotrypsin inhibiting activities, respectively, were attributed to the protease inhibitor II (~20 kDa) subcategory.

When comparing all potato protein isolates, thermal/acidic protein isolates showed the lowest (363.5 mg inhibited protease/g protein) and the highest (427.2 mg inhibited protease/g protein) inhibiting activity against trypsin and chymotrypsin, respectively. The low trypsin inhibiting activity of thermal/acidic protein isolate may be attributed to the conformational changes of protease inhibitor II, (20-15 kDa; yield of 18.2%) upon their recovery under thermal (100 °C, 2 min) and acidic conditions (pH 2.5). The low solubility of thermal/acidic protein isolates can also explain the low trypsin inhibiting activity. However, the high recovery of potato inhibitors I (<15 kDa; highest absolute yield of 28.2%), which have a high affinity towards chymotrypsin, may explain the high chymotrypsin inhibiting activity of the thermal/acidic protein isolate. In contrast, van Koningsveld et al. (2001) found that most of the inhibitor activities towards trypsin and chymotrypsin were lost after heat treatments for 15 min between 55 and 70 °C.

The results (Table 3.2) also indicate that the $(NH_4)_2SO_4$ based isolate showed similar trypsin inhibiting activity as compared to the PFJ, confirming the isolation of non-denatured protease inhibitor II; however, a lower chymotrypsin inhibiting activity was found although the potato inhibitors I (<15 kDa) were recovered with high yield (26%). The experimental findings also show that the acidic and CMC isolates exhibited lower protease inhibiting activities expressed by mg potato protein isolates; these results can be explained by the low

proportions (35-37%) of protease inhibitor fraction recovered in these isolates. However, the inhibiting activities of acidic and CMC-based isolates expressed per mg protease inhibitors were comparable to those of other isolates, revealing the non-significant conformational changes of protease inhibitors. Low recovery yield (27%) of protease inhibitors (20-15 kDa; <15 kDa) by ethanol precipitation resulted in isolates with relatively low trypsin inhibiting activity (769.9 mg protease/g protein isolates) as compared to $FeCl_3$ and $(NH_4)_2SO_4$ precipitations (928.8-957.6 mg protease/g protein isolates). However, trypsin inhibiting activity expressed per g protease inhibitors of ethanol and $FeCl_3$-based isolates were comparable and slightly lower than that of the $(NH_4)_2SO_4$ –based isolate. $FeCl_3$ showed the lowest chymotrypsin inhibiting ability (474.9 mg protease/ g protease inhibitors) despite the higher yield (23.6 %) of <15 kDa protease inhibitors. These results may be attributed to the conformational changes induced by $FeCl_3$ on the active site of these protease inhibitors. Ethanol and $(NH_4)_2SO_4$ precipitations (548.8 and 572.5 mg protease/g protease inhibitors) maintained 67% of the initial chymotrypsin inhibiting activity of PFJ.

3.4.8. Lipid acyl hydrolase activity expressed in potato protein isolates

To assess the effect of precipitation techniques on the functionality of isolated patatin, LAH activity of protein isolates was investigated. As shown in Table 3.3, higher LAH activities were obtained using 4-nitrophenyl laurate as substrate for all protein isolates and PFJ, indicating the high specificity of patatin towards the long chain fatty acids. Compared to other protein isolates, the ones obtained upon acidic and $(NH_4)_2SO_4$ precipitations exhibited high specific activities of LAH towards both substrates. These results indicate that the acidic and $(NH_4)_2SO_4$ precipitations retained patatin's conformational structure to a greater extent compared to the patatin present in PFJ. However when examining the total units, $(NH_4)_2SO_4$ precipitation resulted in higher total units corresponding to a higher proportion of patatin extracted, unlike acidic precipitation (Table 3.1). Using the substrate 4-nitrophenyl laurate, Bohac, (1991) reported a 1.3 increases in the LAH activity of patatin extracted with $(NH_4)_2SO_4$ at 40-70% saturation. Table 3.3 shows that low to no detectable LAH activity could be detected with thermal/acidic and CMC-based protein isolates.

Table 3.2 Inhibiting protease activities, against Trypsin and Chymotrypsin of protein isolates from potato fruit juice (PFJ)

	Inhibiting Protease Activity[a]			
	Trypsin		Chymotrypsin	
	mg Protease/g Potato Protein Isolates[b]	mg Protease/g Protease Inhibitors[c]	mg Protease/g Potato Protein Isolates[b]	mg Protease/g Protease Inihibitors
PFJ	960.8	1801.6	431.7	809.5
Thermal/Acidic Combination	363.5	705.5	427.4	829.4
Acidic Precipitation	493.6	1299.6	224.3	590.6
$FeCl_3$ Precipitation[d]	957.6	1489.1	305.4	474.9
Ethanol Precipitation[e]	769.9	1399.2	302.0	548.8
$(NH_4)_2SO_4$ Saturation[f]	928.8	1802.5	294.9	572.5
CMC Complexation[g]	535.8	1527.2	227.6	648.7

[a]Inhibiting protease activity against trypsin and chymotrypsin was estimated from the decrease in the protease activity in the presence of potato isolates containing protease inhibitors.
[b]Inhibiting protease activity is expressed as mg protease inhibited by g potato protein isolates.
[c]Inhibiting protease activity is expressed as mg protease inhibited by g protease inhibitors present in the potato protein isolates. The weight percentage of the protease inhibitors was estimated by electrophoresis analysis and Alpha View software.
[d]$FeCl_3$ as extracting agent was used at 5 mM.
[e]20% (v/v) of Ethanol as extracting agent was used at 20% (v/v).
[f]60% $(NH_4)_2SO_4$ at 60% saturation was used.
[g]Complexation with CMC was investigated at 0.1(w/v).

Table 3.3 Lipid acyl hydrolase activity expressed in protein isolates from potato fruit juice obtained using selected extraction techniques.

Extraction Techniques	Lipid Acyl Hydrolase Activities[a]			
	p-Nitrophenyl Butyrate		p-Nitrophenyl Laurate	
	Specific Activity (μmole/(min. mg patatin))	Total Units[b]	Specific Activity (μmole/(min. mg patatin))	Total Units[b]
PFJ[c]	1.17 (±0.28)[d]	0.27 (±0.07)	1.76 (±0.24)	0.40 (±0.06)
Thermal/Acidic Combination	<0.04		<0.04	
Acidic Precipitation	0.67 (±0.5)	0.07 (±0.05)	0.97 (±0.1)	0.10 (±0.01)
$FeCl_3$ Precipitation[e]	0.15 (±0.03)	0.05 (±0.01)	1.55 (±0.22)	0.55 (±0.08)
Ethanol Precipitation[f]	0.15 (±0.03)	0.05 (±0.01)	0.52 (±0.05)	0.19 (±0.02)
$(NH_4)_2SO_4$ Saturation[g]	0.47 (±0.11)	0.17 (±0.04)	1.15 (±0.22)	0.41 (±0.08)
CMC Complexation[h]	<0.04		0.2 (±0.03)	0.07 (±0.01)

[a]Lipid acyl hydrolase activity was investigated using p-nitrophenyl butyrate and p-nitrophenyl laurate as substrates.
[b]Total lipid acyl hydrolase units (μmole/min) expressed in 1 mg of potato protein isolates
[c]Potato fruit juice.
[d]Standard deviation was calculated from triplicate trials.
[e]$FeCl_3$ as extracting agent was used at 5 mM.
[f]20% (v/v) of Ethanol as extracting agent was used at 20% (v/v).
[g]60% $(NH_4)_2SO_4$ at 60% saturation was used.
[h]Complexation with CMC was investigated at 0.1(w/v).

These results may be attributed to the low solubility of these isolates, thermal denaturation, and conformational changes of patatin upon complexation with CMC. Previous studies have shown that temperatures ranging from 46-55 ^{0}C initiates unfolding of patatin's secondary structure and complete unfolding occurs at 70^{0}C (Pots, et al., 1998). The recovery of LAH activity by ethanol and $FeCl_3$ precipitations was substrate dependent. Using 4-nitrophenyl laurate, 30 and 80% of the specific LAH activity of PFJ was expressed in ethanol and $FeCl_3$-based isolates, respectively, whereas, with 4-nitrophenyl butyrate, only 13% of specific activity of PFJ was recovered in these isolates. As compared to other isolates, $FeCl_3$-based isolates exhibited the highest specific LAH activity towards 4-nitrophenyl laurate of 1.55 μmol/ min mg patatin. These results reveal that the binding of short chain fatty acids to the active site of patatin was more affected by conformational changes induced by ethanol and $FeCl_3$ precipitations than longer chains. Contradictory results were reported by Bártová and Bárta, (2009) where towards 4-nitrophenyl butyrate, ethanol and $FeCl_3$ isolates showed 2.1 times higher and similar specific activity than PFJ, respectively. These discrepancies could be attributed to different concentrations of the precipitating agents.

3.4. Conclusion

To the best of our knowledge, this study is the first on the comparison of various protein extraction techniques using Canadian potatoes. The variation in extracting agent greatly impacted the protein isolates in terms of their physico-chemical and structural characteristics. As compared to the current traditional thermal and acidic precipitations, $(NH_4)_2SO_4$, ethanol, and $FeCl_3$ precipitations resulted in higher purification factors and had minimal deleterious effect on their isolates towards pH and thermal adjustments. Patatin's LAH activity was best preserved in $(NH_4)_2SO_4$ isolates, whereas $FeCl_3$ isolates possessed greatest protease inhibiting activity. The understanding of the relationships between the extracting agents and the functional properties of isolates is expected to provide the capability to generate potato proteins targeting specific composition and functional properties. As overall, this comprehensive comparison study will help with the improvement of the extraction of minimally-modified proteins from PFJ and contributes to the conversion of this by-product into value-added ingredients.

CONNECTING STATEMENT 2

A comprehensive assessment based on the protein recovery of several extraction techniques and their effects on the potato protein extracts is presented in Chapter III. Chapter IV focuses on the development of a novel enzymatic approach using pure enzymes endo-polygalacturonase and endo-β-1,4-galactanase for the extraction of potato proteins from potato pulp. The enzymatic approach was further optimized by response surface methodology (RSM) using a 5-levels, 5-factors central composite rotatable design (CCRD). The five factors studied were reaction temperature, incubation time, pulp concentration, units of endo-polygalacturonase, and units of endo-β-1,4-galactanase, and their effects and interactions were evaluated. The responses assessed and optimized were recovered patatin and recovered protease inhibitors.

The results from this study were presented at the IFT Annual Meeting & Food Expo-Institute of Food Technologist and in the journal of LWT-Food Science and Technology.

Waglay, A., Khodadadi, M., & Karboune, S. (2013) Investigation and Optimization of a Novel Enzymatic Approach for the Extraction of Protein from Potato Pulp. IFT13 Annual Meeting & Food Expo, Chicago, USA, July 13- July 17, 2013.

Waglay, A., Khodadadi, M., & Karboune, S. (2015) Investigation and Optimization of a Novel Enzymatic Approach for the Extraction of Protein from Potato Pulp. *LWT- Food Science and Technology*, http://dx.doi.org/10.1016/j.lwt.2015.07.070.

CHAPTER IV. INVESTIGATION AND OPTIMIZATION OF A NOVEL ENZYMATIC APPROACH FOR THE EXTRACTION OF PROTEIN FROM POTATO PULP

4.1. Abstract

Potato proteins have been associated with several functional and beneficial properties. A novel enzymatic approach, based on the use of combinations of polysaccharide degrading biocatalysts, was investigated for the isolation of non-denatured proteins from potato pulp. The removal of starch was found to be essential for the effectiveness of the enzymatic approach, as protein recovery increased from 47% to 63-75% with starch removal. Contrary to endo-arabinanase (Arase), endo-1,4-β-galactanase (GLase) and endo-polygalacturonase MI (PGase) improved the protein recovery. A 5-level, 5-variable central composite rotatable design (CCRD) was performed with varying temperature (°C; 30.0, 33.8, 37.5, 41.3, 45.0), time (h; 1.50, 9.00, 16.5, 24.0, 31.5), potato pulp concentration (kg/m^3; 80.0, 110, 140, 170, 200), PGase units (U; 1.5, 11.0, 20.5, 30.0, 39.5), and GLase units (U; 1.5, 11.0, 20.5, 30.0, 39.5); and the responses were protein recovery yield, recovered patatin concentration, and recovered protease inhibitors concentration. For yield and recovered patatin, the most significant interaction was that between temperature and units of PGase with an antagonistic relationship. The most significant interaction for recovered protease inhibitors was found to be that between pulp concentration and temperature, showing a positive correlation. Comparison of predicted and experimental values validated the established predicted models, which can be used to identify the conditions for the isolation of potato proteins with selected composition.

4.2. Introduction

Globally, potatoes are one of the staple crops, used for human consumption, industrial processing, and/or agricultural regimes (Kamnerdpetch, et al., 2007). Industrial processing of potatoes generates low value by-products known as potato fruit juice and potato pulp, which contain a crude protein content of 50% (Vikelouda & Kiosseoglou, 2004) and 74% w/w (dry weight), respectively (Kamnerdpetch, et al., 2007). These proteins have been shown to be of high nutritional quality as they contain a high proportion of lysine, an essential amino acid often lacking in vegetable and cereal proteins (Ralet & Guéguen, 2001; van Koningsveld, et al., 2001).

Potato proteins are commonly divided into three main fractions namely, patatin (40%), protease inhibitors (up 50%) and high molecular weight proteins (Pots, et al., 1998; Bártova

& Bárta, 2009; Ralet & Guéguen, 2001). Together, these fractions have been reported to possess several beneficial characteristics such as lower allergenicity (Moreno, 2007), high antioxidative activity (Liu, et al., 2003; Kudo, et al., 2009), and abilities to modulate lipid metabolism (Liyanage, et al., 2008). Along with these health promoting qualities, potato proteins, more specifically patatin, exhibit foaming and emulsifying abilities. Indeed, potato protein isolates create more stable foams when compared to β-lactoglobulin and β-casein (van Koningsveld, et al., 2002). In addition, emulsions formed with potato protein isolates prevail those obtained with soy protein isolates (van Koningsveld, et al., 2006).

In terms of structure, patatin is present as a dimer in its native form (Ralet & Guéguen, 2000); however, once denatured, it breaks down to its monomer units consisting of a single polypeptide with up to two carbohydrate chains resulting in isoforms with molecular weights ranging from 40 to 45 kDa (Pots, et al., 1999). Contrarily, the protease inhibitors, represent a heterogeneous group, which varies according to their molecular mass, inhibitory activities, and amino acid sequence (Pouvreau, et al., 2001).

Until now, the application of potato proteins has been quite limited due to the lack of non-denaturing extraction techniques. Industrially, the conventional extraction technique used is a combination of severe thermal (100 °C) and acidic treatment (pH 4.5-5.0) (Knorr, et al., 1977). These conditions lead to the extensive denaturation of the proteins as patatin has been shown to begin unfolding at 45 °C (Waglay, et al., 2014), whereas the protease inhibitors denature between 55-70 °C (van Koningsveld, et al., 2001). Therefore, in order to explore the techno-functionalities and the health benefits associated with potato proteins, improvement of the extraction method is required. Investigation of the release of cell wall protein from potato, using several glycosyl-hydrolysing enzymes, revealed that the degradation of the galacturonide linkages are crucial for the protein recovery (Strand, et al., 1976). To our knowledge no studies have taken advantage of this subtractive approach to isolate plant proteins with retained functionality for their use as health promoting ingredients.

The present study is aimed at the development of a novel enzymatic approach for the isolation of non-denaturing and highly functional proteins from potato pulp. This approach

relies on the degradation of the polymers surrounding the proteins for efficient release of proteins from the cell network with minimal deleterious effects. It will begin by removing starch to allow for easier access of the polysaccharide hydrolyzing biocatalysts, namely endo-polygalacturonase M1 (PGase), endo-β-1,4-galactanase (GLase), and endo-arabinase (ARase) to the plant cell wall components. The effects of reaction parameters (temperature, time, pulp concentration, units of PGase and GLase) on the protein recovery yield and the extracted amount of patatin and protease inhibitors were investigated using response surface methodology (RSM). The relationships between these selected parameters were examined. Preserving the proteins' structure and their functional and beneficial effects is expected to increase their application.

4.3. Materials and Methods

4.3.1. Preparation of potato pulp

Potato pulp was prepared with potatoes of Russet Burbank variety. The potatoes were washed and finely chopped into 500 kg/m^3 samples. The potato pieces were ground with a mortar and pestle for 1 min with 0.25 kg/m^3 sodium metabisulfite. The ground pieces were homogenized using a Warring commercial Blender at low speed for 1 min.

4.3.2. Starch removal

Two selected α-amylases (Sigma Chemical Co.) from *Bacillus licheniformis* (Termamyl) and *Bacillus sp*. were evaluated for the removal of starch. Dried potato pulp (150 kg/m^3) was consistently weighed and suspended in 2.12 kg/m^3 potassium phosphate buffer at pH 6.5. Selected units of α-amylase were added to the potato pulp suspension to yield 0-2.98 U/mg pulp. Reactions were carried out at 40 ^{0}C with constant stirring at 5*g* for 16 hr. The remaining starch was determined using potassium iodide colorimetric method.

4.3.3. Enzymatic approach for protein recovery

Destarched potato pulp was (25-160 kg/m^3) suspended in 8.20 kg/m^3 sodium acetate buffer pH 5.0. The enzymatic reactions were initiated by adding ARase (0.1-0.3 U/mg pulp, Megazyme), PGase (0.008-0.5 U/mg pulp, Megazyme) and GLase (0.008-0.5 U/mg pulp, Megazyme) from *Aspergillus niger* to the destarched potato pulp suspension. The reaction mixtures were incubated at 40 °C for selected reaction times of 6-48 hr. Variant

enzyme/pulp ratios (0.008-0.5 U/mg potato pulp) and pulp concentrations (28.6-200.0 kg potato pulp/m^3) generated by RSM, were investigated. After incubation, the reaction mixtures were vacuum filtered (1.2 μm) and the supernatant containing proteins were recovered and dialyzed. The protein content of the recovered pulp after enzymatic treatment and supernatant were determined using Leco® TruSpec N (Leco Corporation, St-Joseph, MI). Nitrogen content was multiplied by a factor of 6.25 to determine the protein content (van Gelder, 1981). Two blanks, without potato pulp or without enzymes, were conducted in tandem of the trials. All assays were run in duplicate. The protein yield was estimated as the recovered proteins in the supernatant over the initial proteins present in potato pulp, multiplied by 100.

4.3.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Protein extracts were analyzed by SDS-PAGE according to the method of Laemmli (1970) using 50 and 150 kg/m^3 acrylamide content in the stacking and resolving gels, respectively. Sample loading was achieved in a mini protein gel apparatus (Bio-Rad, On) with a 1.5 mm-thick gel. The electrophoresis was conducted at a constant voltage of 120 mV. The analyses of the electrophoretic patterns to obtain the protein profiles based on protein proportions, were carried out using Red Imaging system equipped with Alpha-View SA Software. The recovered patatin and protease inhibitors are expressed as g of extracted patatin or protease inhibitor per g of initial pulp and were calculated according to the following calculation:

G patatin/g pulp protein = ((g pulp x yield/100) * (relative proportion patatin/100)) / g pulp *(4)*

4.3.5. Structural characterization using fluorescence spectroscopy

The fluorescence spectra of protein extracts were recorded using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon system, Les Ulis, FR). The spectra were recorded at pH 4 and 9 while varying temperatures from 25 to 85 ^{0}C with a 5 ^{0}C interval increase. Excitation was at 295 nm and the resulting emission was measured at 305-450 nm with a scan speed of 120 nm/min. Both the excitation and emission slits were set at 3.5 nm. Protein extracts were prepared using a conventional thermal/acidic (pH 4.8) extraction method and enzymatic extraction method (150 kg pulp/m^3, 0.994 U/mg pulp Termamyl, 5.56 U/g pulp each of PGase and GLase). Following enzymatic hydrolysis, protein extracts were dialysed

for 48 hr against Millipore water using a 2000 Da molecular weight benzoylated dialysis tubing to remove any interfering low molecular weight sugars.

4.3.6. Experimental design

Optimization of the protein yield (%), the recovered patatin (g per g of potato pulp protein), and the recovered protease inhibitors (g per g of potato pulp protein) was investigated using response surface methodology (RSM). A five-level, five variable central composite rotatable design (CCRD) was used. The five independent variables with their corresponding levels consisted of x_1 temperature (30.0, 33.8, 37.5, 41.3, 45.0 °C), x_2 time (1.5, 9.0, 16.5, 24.0, 31.5 hr), x_3 pulp concentration (80.0, 110, 140, 170, 200 kg/m^3), x_4 PGase units (1.5, 11.0, 20.5, 30.0, 39.5 U), and x_5 GLase units (1.5, 11.0, 20.5, 30.0, 39.5 U). x_4 and x_5 were also reported in Table 2 as enzyme concentration per g pulp in order to assess the effect of enzyme to pulp ratio. The full design consisted of 16 factorial points, 10 axial points, and 9 center points, resulting in 35 sets of experiments. The experimental runs were divided into two blocks, where the first block had sixteen factorial points and six center points, whereas the second block contained the ten axial points with three center points. Blocking was performed in order to account for possible differences due to the use of two batches of potato pulp.

4.3.7. Statistical analysis

For approximating response surface, the obtained yield (%), proportion of patatin and proportion of protease inhibitors based on the described design, was fitted to the general model Equation 5 using the software Design-Expert 8.0.2 (Stat-Ease, inc. Minneapolis, MN, USA):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i+1 \le j \le k}^{1 \le i \le k} \beta_{ij} X_i X_j \qquad (5)$$

β_o, X_is (i=1-5), $\beta_{i,,}$ β_{ii}, and β_{ij} are the constant coefficient, coded independent variables, the coefficient for the linear effect, the coefficient for the quadratic effect, and the coefficient for the interaction effect, respectively.

4.4. Results and Discussion

4.4.1. Effect of starch removal on the isolation of potato proteins

The investigated enzymatic approach is based on the degradation of the plant cell wall polysaccharides for the efficient release of the non-denatured proteins. Starch removal is an essential step to allow polysaccharide degrading enzymes to have an increased access to the potato cell wall network (Thomassen & Meyer, 2010). α-Amylases from *B. licheniformis* (Termamyl) and *Bacillus sp*. were evaluated for the removal of the starch. Contrary to the AOAC method and the improved one developed by Thomassen & Meyer (2010), lower temperature of 40 °C was used to prevent the thermal denaturation of the potato proteins. The necessity of starch removal is better emphasized in Table 4.1. At low pulp concentration of 75 kg/m^3, the treatment involving no starch removal and the use of high PGase/GLase enzymatic units resulted in a relatively higher yield of 74.6% than with starch removal with *B. licheniformis* (66.4 %) and *Bacillus sp.* (70.8%) amylases. These results reveal that starch removal may not be required with the use of low potato pulp concentration and high PGase/GLase enzymatic units. However increasing the pulp concentration to 150 kg/m^3 and decreasing PGase/GLase enzymatic units to 16.66 U/g pulp led to a decrease in the protein yield to 46.8% with no starch removal, whereas with starch removal with *B. licheniformis* and *Bacillus sp.* amylases, protein yield reached 75.6 and 63.2%, respectively.

The effect of starch removal efficiency on the effectiveness of protein recovery by the enzymatic treatment at high potato pulp concentration was investigated by varying the enzymatic units of amylase. As shown in Figure 4.1, α-amylase from *B. licheniformis* resulted in the highest protein recovery yield at 0.99 to 2.98 U/mg pulp. The removal of starch with α-amylase from *Bacillus sp*. resulted in an increase in the relative protein recovery yield upon the enzymatic treatment to reach 96.2% of the maximum value at 0.99 U/mg pulp; while above this concentration, a sharp decline of the relative protein recovery yield was observed. A previous study conducted by Thomassen & Meyer (2010) found that enzyme units, amount of dry matter, and enzyme-enzyme interactions can have a significant impact on the starch removal yield. This sharp decline of protein recovery could be attributed to the interactions between the α-amylase *Bacillus sp.* at higher concentrations.

Table 4.1 Effect of starch removal on protein recovery yield (%).

Pulp concentration kg/m^3	No Starch	α-amylase *Bacillus licheniformis*[c]	α-amylase *Bacillus sp.*
75[a]	74.6	66.4	70.8
150[b]	46.8	75.6	63.2

[a] Polygalacturonase and endo-1,4-β-galactanase is 33.33 U/g pulp
[b] Polygalacturonase and endo-1,4-β-galactanase is 16.66 U/g pulp
[c] Commercial name: Termamyl

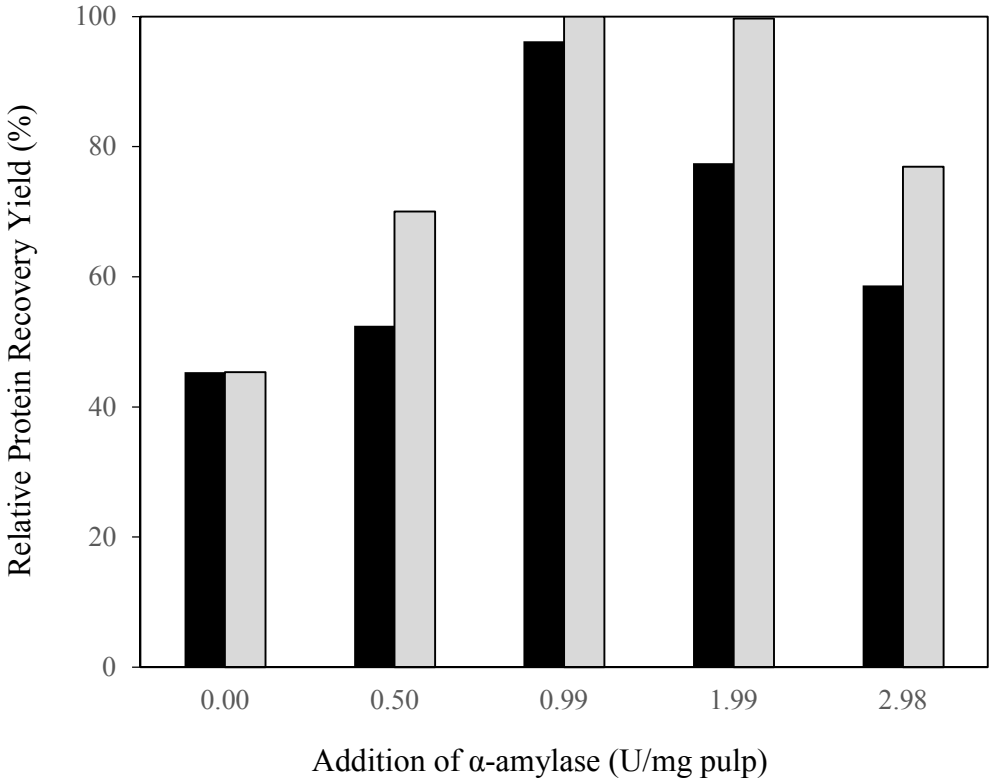

Figure 4.1 Effect of starch removal from potato pulp using two α-amylases: (■) Bacillus sp. and (▭) Bacillus licheniformis on the recovery of proteins upon the enzymatic treatment with Polygalacturonanase M1 (16.66 U/g pulp) and endo-1,4-β-D galactanase (16.66 U/g pulp).

Therefore, α-amylase from *B. licheniformis* was proven to be more effective for an improved efficiency of the polysaccharide-degrading enzymes.

4.4.2. Enzymatic isolation of potato proteins and their structural characterization

Following starch removal, the plant cell wall is mostly composed of polysaccharides, cellulose, hemicellulose, and pectin (Gilbert, 2010). The pectic polysaccharides are present within the plant cell wall to maintain cell placement and to glue together all the elementary fibers (Willats, et al., 2001). It is hypothesized that the degradation of pectic polysaccharides can lead to an efficient recovery of potato proteins. In particular, potato plant cell wall is composed of 60-65% (w/w) non-starch polysaccharide material, where approximately 38-40% (w/w) consisting of pectic polysaccharides (Meyer, et al., 2009). These pectic polysaccharides are the main components, which are mainly composed of rhamnogalacturonan I (75% w/w) and homogalacturonan (20% w/w) (Khodaei & Karboune, 2013). Of particular importance, potato rhamnogalactrunan I is composed of high proportion of (1→4)-β-galactan side chains, which is not present in other cell wall sources (Khodaei & Karboune, 2013). Based on the structural properties of potato pectic polysaccharides, three enzymes, PGase, GLase, and ARase, were deemed necessary for the effective protein recovery. The use of ARase with PGase/GLase showed a negative correlation on the protein recovery (data not shown). This decrease in the protein yield in the presence of ARase was seen with all conditions, where no starch removal had a 3.5 fold decrease, and with starch removal showed a 1.2-1.7 fold decrease. These results reveal the inhibitory effect of ARase on the PGase/GLase. ARase was not further optimized.

To study the effect of the extraction method (thermal/acid combination; enzymatic approach) and the structural stability of proteins extracted, fluorescence spectroscopy was used to monitor the tertiary conformational changes of the aromatic acid environment as a function of temperature. Results of relative fluorescence intensity were expressed per mg soluble protein. At pH 4.0 (close to patatin's isoelectric point), most patatin may be insoluble, the differences in intensity at this pH can be attributed to the thermal stability of the protease inhibitors. On the contrary because pH 9.0 is within the range of the isoelectric

point of the protease inhibitors (pH 7 to 9), it can be assumed that the recorded thermal unfolding is related to a greater extent to the soluble patatin.

Patatin contains two tryptophan (Trp 279, Trp 284) residues in the primary sequence that can be monitored for analyzing the protein tertiary structure. When excited at 295 nm, enzymatic-based protein extract exhibited a fluorescence wavelength of maximum emission intensity (λ_{max}) of 345 nm at pH 4.0-9.0, while the thermal/acid-based protein extract showed a λ_{max} of 360 nm, at pH 4.0-9.0 (data not shown). Seo et al. (2014) has reported a λ_{max} of 335 nm for native patatin. On the other hand, Koppelman et al., (2002) reported that patatin exhibits two λ_{max} of 336 and 347 nm corresponding to buried and more exposed Trp residues. In addition, a study conducted by Pots et al. (1998) concluded to a λ_{max} of 335 nm for patatin. Pouvreau et al. (2005) examined the most abundant group of protease inhibitors found in the tuber, the potato serine protease inhibitors, which demonstrated λ_{max} at 347 nm. Indeed, the high λ_{max} obtained with thermal/acid-based protein extract reveals that large conformational changes of potato proteins have occurred around the tryptophan residues resulting in their increased exposure to the polar environment. On the other hand, enzymatic-based protein extract resulted in a bathochromic and slight hypsochromic shift (345 nm) of λ_{max} as those reported for native patatin (335 nm) and potato serine protease inhibitors (347 nm), respectively. Indicative that the enzymatic treatment did not significantly affect the protein unfolding and the exposure of the tryptophan residues.

As expected, a gradual decrease in the fluorescence intensity, upon the increase of the temperature, was observed revealing tertiary conformational changes of potato proteins (Figure 4.2). At both pH 4.0 and 9.0 (Figure 4.2 A-4.2 B), the tertiary conformational changes of protein extract obtained by PGase/GLase enzymatic-based approach showed a linear behavior, at the initial stage, with a decrease in fluorescence intensity as temperature increases. In comparison, the conformational changes of the tryptophan environment exhibited an exponential behavior for the protein extract obtained by combination thermal/acidic treatment. In addition, the enzymatic-based protein extract had a lower initial velocity of 1.4 and 1.8 fold compared to the thermal/acid-based protein extract where a reduction of 3.4 and 4.3 fold resulted, for pH 4.0 and 9.0, respectively. These results

emphasize the minimal deleterious effect of the enzymatic isolation technique on the thermal stability of potato proteins compared to the industrially performed thermal/acidic method. The thermal conditions seem to have more structural unfolding effect on the tryptophan environment of the proteins isolated by thermal/acidic at pH 9.0 than at pH 4.0. Interestingly for the enzymatic based-protein extract, at pH 9.0 (Figure 4.2 B) representative of more soluble patatin, there is a small shift to a lower rate at 50 °C, followed by a gradual decrease in velocity with increasing temperatures until 70 ^{0}C. These results suggest that a major change in the molecular conformation took place at a temperature above 50 °C. Pots et al. (1998) have reported that patatin began unfolding at 46-55 ^{0}C, whereas above 55 ^{0}C the polarity surrounding the tryptophan residue is unchanged, as they are not available to water. A larger shift observed at pH 9.0 (Figure 4.2 B) and 70 ^{0}C, where the velocity decreases significantly, could be indicative of the tertiary conformation of the protease inhibitors present. A study conducted by van Koningsveld et al. (2001) indicated that potato protease inhibitors begin to unfold around 58-71 ^{0}C. This decrease in velocity was not exhibited at pH 4.0 (soluble protease inhibitors) for the enzymatic based-protein extract, which could be due the heterogeneity of the protease inhibitor group, leading to varying conformational changes.

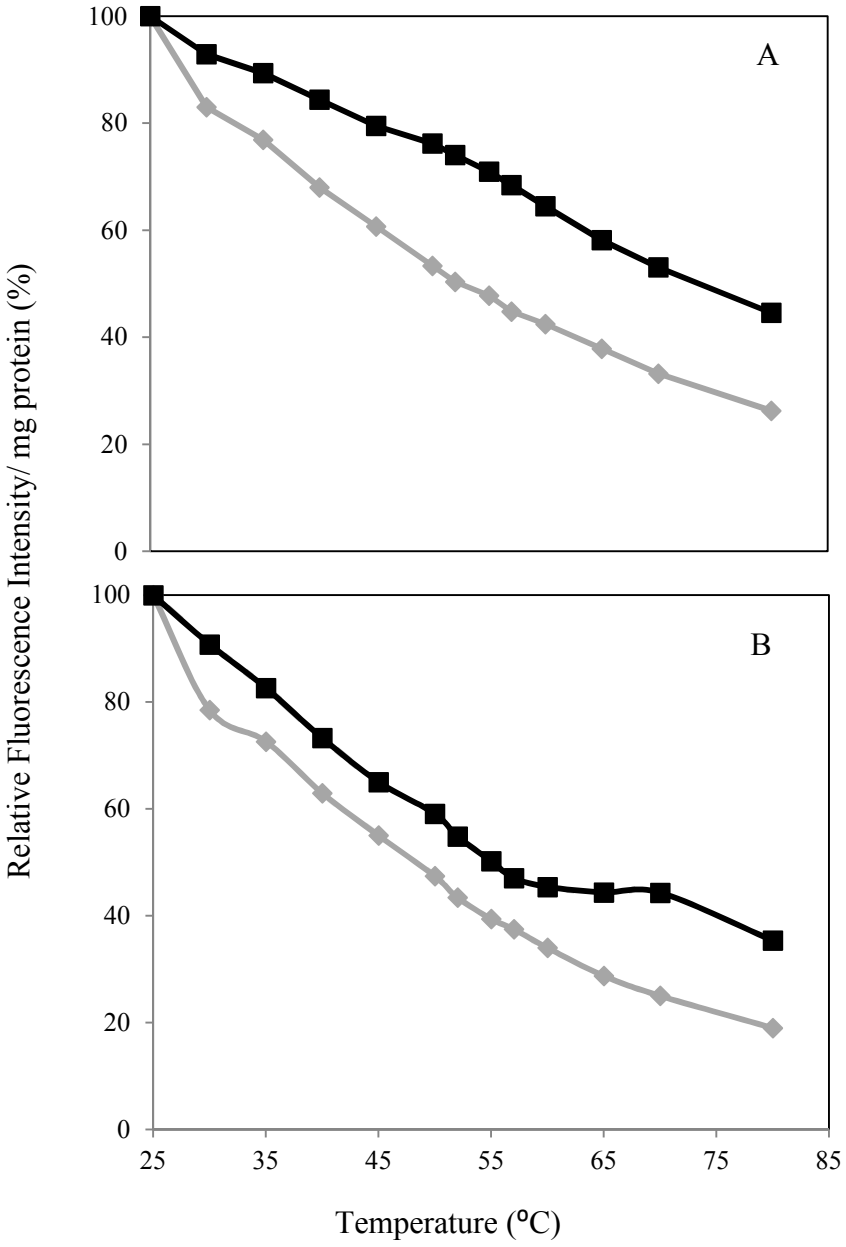

Figure 4.2 Effect of extracting agent on sigmoidal transition curve of relative fluorescence intensity per milligram protein versus temperature for protein extracts (A) pH 4 (B) pH 9. Combination thermal/acidic (—◆—) and enzymatic-based protein extracts using enzymes polygalacturonase M1 and endo-β-1,4-galactanase (—■—).

4.4.3. Study and optimization of the enzymatic isolation of potato proteins

RSM was used to investigate the effects of selected reaction parameters on the isolation of proteins from destarched potato pulp using the enzymatic glycosyl-hydrolases (PGase/GLase)-based approach. The experimental design was performed based on CCRD. Five reaction parameters, including temperature (^{0}C, x_1)), time (hr, x_2), pulp concentration (kg/m^3, x_3), units of PGase (U, x_4), and units of GLase (U, x_5), were assessed. The levels of each reaction parameters were chosen based on preliminary trials. Table 4.2 shows that the protein yield ranged from 42.41-73.91%. The lowest protein yield resulted from the use of low temperature (x_1) (-1), low time (x_2) (-1), high pulp concentration (x_3) (+1), low units of PGase (x_4) (-1) and GLase (x_5) (-1), whereas highest protein yield was obtained at the center point temperature (x_1) (0), mid time (x_2) (0), lowest pulp concentration (x_3) (-2), the center point units of PGase (x_4) (0) and GLase (x_5) (0). The relative proportion of patatin was found to vary between 26.0 and 65.5% (Table 4.2). Higher patatin (65.5%) was extracted with lowest temperature (x_1) (-2) and midpoint values for the remaining factors level (0), corresponding to the experimental run 1, with a corresponding high protein recovery yield of 71.0%. When increasing temperatures (x_1) to the highest axial point of 45 ^{0}C, while maintaining center points for all other factors level, patatin extraction decreased by 1.24 fold from 65.5%. This could be due to the unfolding of patatin which begins at 45 ^{0}C rendering it insoluble therefore remaining in the degraded pulp and in turn not recovered. However, this occurrence was limited as temperature was set to not exceed 45 ^{0}C to preserve patatin's structure, and buffering systems were maintained at 50 mM. van Koningsveld et al. (2001) have reported that increasing salt concentrations from 15 to 200 mM increased the denaturation temperature for some potato proteins by shielding the protein from electrostatic repulsive forces. The relative proportion of protease inhibitors recovered varied from 20.1 to 74.8% (Table 4.2). Contrarily to patatin recovery, lowest relative proportion of protease inhibitors (20.1%) was attained for experimental run 1 where lowest temperature (x_1) (-2) and constant midpoint values for the remaining factors. As shown in Table 4.2, the highest relative proportion of protease inhibitors was obtained for experimental run 18, at the factorial point, high temperature (x_1) (+1), low time (x_2) (-1), high pulp concentration (x_3) (+1), high units of PGase (x_4) (+1) and low GLase (x_5) (-1), the

Table 4.2 Central composite rotatable design (CCRD) arrangement of the actual and coded experimental variables and the observed protein recovery and protein proportions obtain from enzymatically treated potato pulp.

Run no.	x_1 Temperature (ºC)	x_2 Time (hr)	x_3 [Pulp] (kg/m^3)	x_4 Polygalacturonase (Units/g pulp)	x_5 Endo-galactanase (Units/g pulp)	Protein Yield (%)	Extracted Patatin Recovery	Extract Protease Inhibitors Recovery
1	30 (-2)	16.5 (0)	140 (0)	18.3 (0)	18.3 (0)	71.02	(65.5)[a] 0.458[b]	(20.1) 0.143
2	33.75 (-1)	9 (-1)	110 (-1)	12.5 (-1)	34.09 (+1)	54.22	(31.8) 0.172	(47.4) 0.257
3	33.75 (-1)	9 (-1)	110 (-1)	22.06 (+1)	12.5 (-1)	61.50	(41.1) 0.230	(43.3) 0.267
4	33.75 (-1)	24 (+1)	110 (-1)	12.5 (-1)	12.5 (-1)	54.03	(26.0) 0.140	(47.5) 0.257
5	33.75 (-1)	9 (-1)	170 (+1)	0.74 (-1)	0.74 (-1)	42.41	(33.8) 0.143	(40.0) 0.170
6	33.75 (-1)	24 (+1)	110 (-1)	34.09 (+1)	34.09 (+1)	58.36	(32.9) 0.192	(57.2) 0.334
7	33.75 (-1)	24 (+1)	170 (+1)	8.09 (-1)	22.06 (+1)	49.99	(28.0) 0.140	(57.9) 0.289
8	33.75 (-1)	24 (+1)	170 (+1)	22.06 (+1)	8.09 (-1)	56.85	(35.5) 0.202	(38.1) 0.216
9	33.75 (-1)	9 (-1)	170 (+1)	22.06 (+1)	22.06 (+1)	50.16	(39.3) 0.197	(49.6) 0.249
10	37.5 (0)	16.5 (0)	140 (0)	1.34 (-2)	18.30 (0)	69.71	(51.5) 0.309	(46.5) 0.324
11	37.5 (0)	1.5 (-2)	140 (0)	18.30 (0)	18.30 (0)	52.84	(49.8) 0.263	(32.2) 0.170
12	37.5 (0)	16.5 (0)	80 (-2)	32.03 (0)	32.03 (0)	73.91	(42.9) 0.317	(36.6) 0.270
13	37.5 (0)	16.5 (0)	140 (0)	18.30 (0)	35.68 (2)	61.47	(46.3) 0.284	(38.5) 0.237
14	37.5 (0)	16.5 (0)	140 (0)	18.30 (0)	1.34 (-2)	68.88	(49.4) 0.340	(40.5) 0.279
15	37.5 (0)	16.5 (0)	200 (+2)	12.81 (0)	12.81 (0)	57.31	(46.8) 0.268	(45.3) 0.260
16	37.5 (0)	16.5 (0)	140 (0)	35.27 (+2)	18.30 (0)	61.49	(40.7) 0.250	(33.2) 0.204
17	37.5 (0)	31.5 (+2)	140 (0)	18.30 (0)	18.30 (0)	54.72	(45.9) 0.251	(43.9) 0.240
18	41.25 (+1)	9 (-1)	170 (+1)	22.06 (+1)	8.09 (-1)	55.01	(41.9) 0.230	(74.8) 0.411
19	41.25 (+1)	9 (-1)	110 (-1)	34.09 (+1)	34.09 (+1)	58.21	(38.8) 0.226	(50.4) 0.294
20	41.25 (+1)	24 (+1)	170 (+1)	22.06 (+1)	22.06 (+1)	59.14	(38.1) 0.225	(73.3) 0.433
21	41.25 (+1)	24 (+1)	110 (-1)	12.5 (-1)	34.09 (+1)	71.12	(28.7) 0.204	(32.7) 0.233
22	41.25 (+1)	24 (+1)	170 (+1)	8.09 (-1)	8.09 (-1)	64.23	(31.9) 0.205	(54.6) 0.351
23	41.25 (+1)	9 (-1)	170 (+1)	8.09 (-1)	22.06 (+1)	63.05	(28.0) 0.177	(50.4) 0.318
24	41.25 (+1)	24 (+1)	110 (-1)	34.09 (+1)	12.5 (-1)	69.23	(31.2) 0.216	(45.8) 0.317
25	41.25 (+1)	9 (-1)	110 (-1)	12.5 (-1)	12.5 (-1)	70.90	(36.7) 0.260	(48.5) 0.344
26	45 (+2)	16.5 (0)	140 (0)	18.30 (0)	18.30 (0)	68.85	(52.8) 0.363	(34.7) 0.239
CP	37.5 (0)	16.5 (0)	140 (0)	18.30 (0)	18.30 (0)	62.97±9.17	(36.7) 0.22±0.07	(45.5) 0.27±0.04

[a]Relative proportion (%) obtained from Alpha View software

[b]Experimentally calculated g of patatin/g of potato pulp

corresponding protein recovery yield was relatively low at 55.0%. These two extreme conditions emphasize the need for higher temperatures, adequate substrate concentration, as well as the necessity in achieving the adequate enzyme ratio (PGase/GLase) for the effective recovery of protease inhibitors.

4.4.4. Model fitting and analysis of variance

Multiple regression analysis and backward reduction algorithm was used to evaluate the best fitting model for the protein recovery yield (%), the recovered patatin (g/g pulp protein), and the recovered protease inhibitors (g/g pulp protein). For the protein recovery yield and the recovered patatin, the quadratic model was selected as the appropriate significant model. While for recovered protease inhibitors, the most significant model was 2FI. For all predicted models, all R^2 and predicted R^2 are in reasonable agreement with each other, which confirms that the fraction of variation of the response with the model agrees with the fraction of variation of the response predicted by the model (Akoh, et al., 2007).

Table 4.3 shows the analysis of variance (ANOVA) for the CCRD. For all responses, protein recovery yield, recovered patatin and recovered protease inhibitors, all selected models are statistically significant with F-values of 5.18, 5.16, and 4.94 and *p*-values 0.00120, 0.000700, and 0.00230, respectively. Furthermore, the non-significant lack of fit with an *F*-value of 0.440 (*p*-value 0.929), 0.690 (*p*-value 0.756), and 1.02 (*p*-value 0.526) for all models confirms the validity of the models. The predicted model equation for the coded factors are given below:

$$\text{Protein Recovery Yield (\%)} = 62.97 + 3.29x_1 + 1.30x_2 - 3.75x_3 - 0.75x_4 - 2.41x_2^2 - 3.37x_1x_4 \quad (6)$$

$$\text{Recovered Patatin (g/ g pulp protein)} = 0.25 + 0.21x_1 - 0.0066x_2 - 0.010x_3 + 0.0076x_4 - 0.0095x_5 - 0.010x_2^2 - 0.012x_1x_4 + 0.011x_2x_3 \quad (7)$$

$$\text{Recovered Protease Inhibitors (g/ g pulp protein)} = 0.27 + 0.036x_1 + 0.0048x_3 - 0.00043x_5 + 0.032x_1x_3 - 0.023x_1x_5 \quad (8)$$

The significance of each variable was determined using the *F*-test and *p*-value. The variables are deemed more significant if the *F*-value is bigger and the *p*-value is smaller. As shown in Table 4.3, for the protein recovery yield, the two significant linear terms include pulp concentration (x_3, *F*-value of 10.31, *p*-value of 0.00340) and incubation

temperature (x_1, F-value of 7.96, p-value of 0.00880). Lower pulp concentrations (x_3) resulted in higher protein recovery yield, which could be attributed to the higher substrate accessibility and hence increased enzyme/substrate interactions (Table 4.3). Both enzyme units (PGase/GLase) (x_4 and x_5) had no significant linear effect on the protein recovery yield within the investigated range. For the recovered patatin response, the linear terms with the largest effects were incubation temperature (x_1, F-value 17.30, p-value 0.0003), pulp concentration (x_3, F-value 3.96, p-value 0.0577) and to a lower extent the GLase units (x_5, F-value 3.53, p-value 0.0721). In addition, the negative sign of the coefficients of pulp concentration and GLase units indicate the adverse effect of these variables on the recovery of patatin (Eq. 7). This can be attributed to (a) the occurrence of mass transfer limitations at high pulp concentration and/or (b) to the presence of protein interactions (GLase/patatin), which may have limited the catalytic efficiency of GLase at high enzymatic units. Contrarily to the recovered patatin, the only linear significant term for the recovered protease inhibitor included incubation temperature (x_1, F-value of 13.4, p-value of 0.00100). For all responses, incubation temperature (x_1) exhibited a positive effect (Eq. 6, 7, 8), where increased temperature (x_1) improved protein yield and profile recovery.

In terms of quadratic effect, incubation time was found to have a significant negative quadratic effect towards the protein yield (x_2^2, F-value of 5.58, p-value of 0.0256) and the recovered patatin (x_2^2, F-value of 5.20, p-value of 0.0314) (Table 4.3, Eqs. 6&7), and not towards the recovered protease inhibitors. This quadratic effect could be due to the thermal denaturation of proteins and/or to the increase of protein/protein interactions over prolonged time limiting their solubility and hence their recovery. The interaction between incubation temperature and PGase showed significant effect on the protein recovery yield (x_1x_4, F-value of 5.56, p-value of 0.0258) and the recovered patatin (x_1x_4, F-value of 3.94, p-value of 0.0583). In the recovered protease inhibitor model, the most significant interactions are the ones between incubation temperature/pulp concentration (x_1x_3, F-value of 7.68, p-value of 0.0653) and incubation temperature/GLase (x_1x_5, F-value of 3.68, p-value of 0.0653).

Table 4.3 Analysis of variance results: the parameters and the significant interactions on the protein recovery, gram patatin/gram pulp, and gram protease inhibitor/gram pulp

	Protein Recovery (%)[b]		Gram Patatin/ gram pulp[c]		Gram Protease Inhibitor/ gram pulp[d]	
Parameters and interactions (x)	F-value	P	F-value	P	F-value	P
Model	5.18	0.0012	5.16	0.0007	4.94	0.0023
Incubation temperature (x_1)	7.96	0.0088	17.30	0.0003	13.42	0.0010
Incubation time (x_2)	1.25	0.2743	1.70	0.2045		[a]
Pulp/ Buffer (x_3)	10.31	0.0034	3.96	0.0577	0.24	0.6277
Polygalacturonase (x_4)	0.41	0.5281	2.24	0.1471		[a]
Endo-Galactanase (x_5)		[a]	3.53	0.0721	0.001946	0.9651
x_1x_4	5.56	0.0258	3.94	0.0583		[a]
x_1x_3		[a]		[a]	7.68	0.0653
x_1x_5		[a]		[a]	3.68	0.0653
x_2x_3		[a]	3.45	0.0750		
$(x_2)^2$	5.58	0.0256	5.20	0.0314		[a]
Lack of fit	0.44	0.9289	0.69	0.7564	1.02	0.5262

[a] Not significant
[b] α-value 0.05
[c] α-value 0.1
[d] α-value 0.1

Incubation temperature/GLase or PGase interactions exhibited opposite effects in all models, as shown by the negative sign of their coefficients (Eqs. 6, 7, 8). Table 4.3 also shows that potato pulp concentration had a significant additive interactive effect with incubation time (x_2x_3, *F*-value of 3.45, *p*-value of 0.0750) and incubation temperature (x_1x_3, *F*-value of 7.68, *p*-value of 0.0653) for the recovered patatin and the recovered protease inhibitors models, respectively.

4.4.5. Effect of reaction parameters

Contour plots were generated from the predictive models to outline the significant interactions. Figure 4.3 a shows the interactive effect of temperature with units of PGase (x_1x_4) on the protein yield. When examining the surface plot (Figure 4.3 a), we can see that the center point is a saddle point where a combination of high temperature (x_1)/low units of PGase (x_4) and low temperature (x_1)/high units of PGase (x_4) resulted in high protein yields. These results confirm that both temperature and PGase adversely affect the yield. This adverse effect can be attributed to the dependence of activation/denaturation effects of incubation temperature on the amounts of PGase. The presence of protein/protein interaction at high PGase units may also have contributed to this effect. The increase of temperature seems to have more effect on the yield than the PGase units. A previous study confirms the necessity in hydrolysing the galacturonide segments to release cell-wall proteins which was previously conducted with polygalacturonase purified from *Verticillium alboatrum* (Strand, et al., 1976).

Like protein yield, extracted patatin was also affected by the interaction of units of PGase and temperature (x_1x_4, Table 4.3 and Figure 4.3 b). When examining the surface plot in Figure 4.3 b, similar to protein recovery yield, high temperatures (x_1) in combination with low units of PGase (x_4) extracted a high proportion of patatin. Compared to protein yield, the saddle point was more shifted towards high temperature and high PGase units. To preserve patatin, extracted temperatures were maximally set at 45 °C as patatin begins to unfold at these conditions (Waglay, et al., 2014). This denaturation effect could lead to patatin being retained in the degraded pulp instead of filtering into the supernatant.

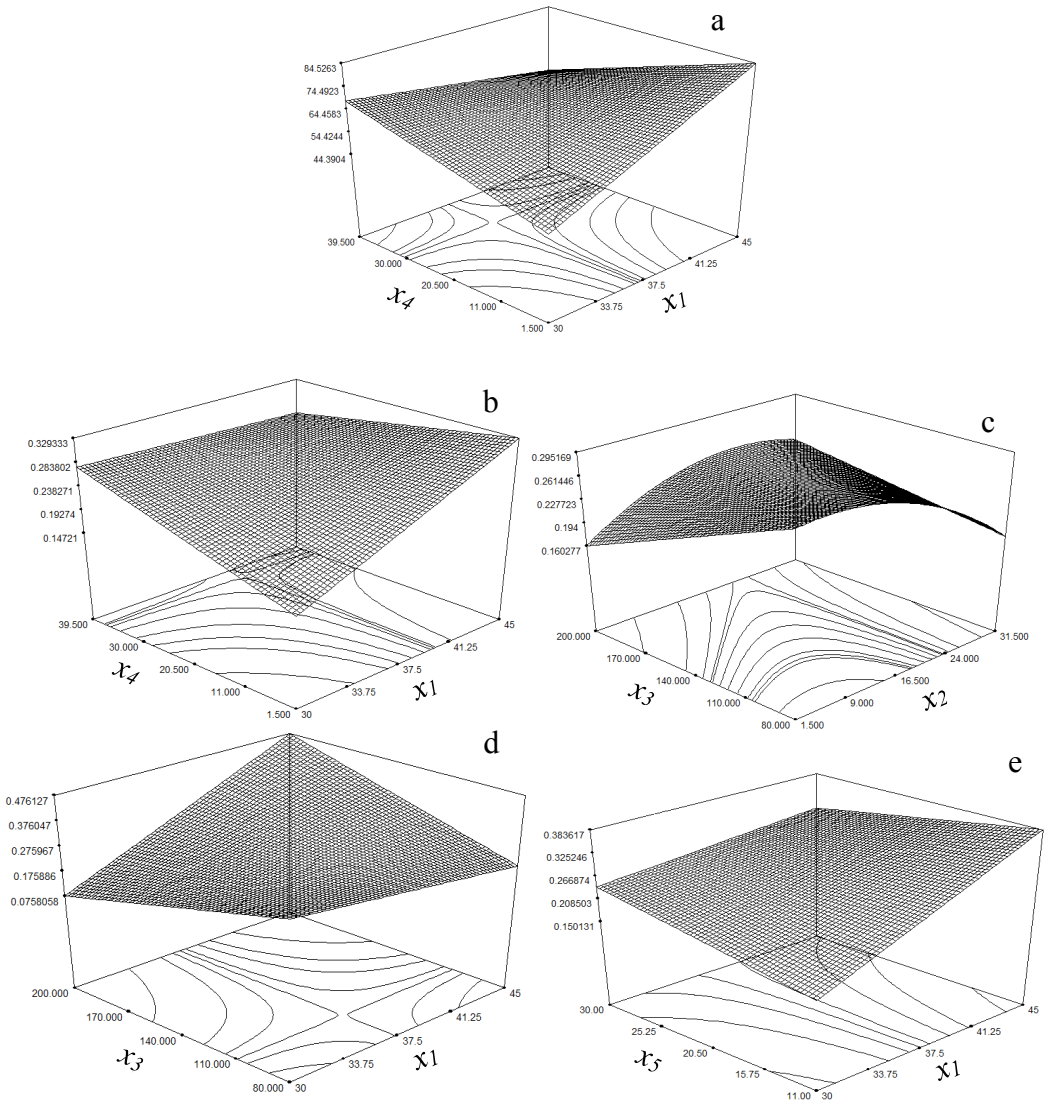

Figure 4.3 Response surface plots for the significant interactions of protein recovery yield, recovered patatin, recovered protease inhibitors as affected by temperature (x_1, ^{0}C), time (x_2, hr), pulp concentration (x_3, kg/m^3), units of Polygalacturonase (x_4, U), units of endo-β-1,4-galactanase (x_5, U) : (a) Recovery yield (x_1x_4) (b) Recovered patatin (x_1x_4) (c) Recovered patatin (x_2x_3) (d) Recovered protease inhibitors (x_1x_3) (e) Recovered protease inhibitors (x_1x_5).

As shown in Figure 4.3 c, extracted patatin recovery was significantly affected by the interaction of pulp concentration and incubation time (x_2x_3), where low pulp concentrations (x_3) and short time (x_2) resulted in high patatin recovery. The low recovery of patatin at high incubation time occurred beyond the center point; below this point, the incubation time had no significant effect nor enhanced the recovered patatin depending on the pulp concentration. As expected, the potato pulp concentration exhibited more effect on the recovered patatin at the initial stage of the reaction (shorter incubation time). As previously mentioned, low pulp concentrations (x_3) leads to increased diffusion between enzymes, whereas short time (x_2) will limit the protein/protein interactions.

The extraction of protease inhibitors was affected by the interaction of pulp concentration and temperature (x_1x_3), as shown in the contour plots, (Figure 4.3 d) where increasing both factors resulted in high protease inhibitor recovery. However at two extremes, with either low incubation temperature (x_1)/ high pulp concentrations (x_3) or high incubation temperature (x_1)/ low pulp concentrations (x_3), a lower amount of protease inhibitors was recovered. Therefore, ensuring adequate substrate concentration with temperature was crucial for protease inhibitor recovery. The second significant interaction, as shown in Figure 4.3 e the interactions of incubation temperature and units of GLase (x_1x_5), high recovery of protease inhibitors resulted when temperature (x_1) was high and units of GLase (x_5) were low. Although, at lower temperature (x_1) and low units of GLase (x_5), extremely low recovery of protease inhibitors resulted. Therefore potentially for improved protease inhibitor recovery the exploration of a higher temperature range would be of interest.

4.1.1. Optimal Conditions and Model verification

As shown in Table 4.4, two optimal conditions corresponding to high yield with high patatin and protease inhibitor recovery were selected in order to validate the models. These optimal points were run in triplicate, with duplicate center points as a reference. Both conditions were chosen for their high predicted protein recovery yields of 79.4 and 73.9%, along with their high predicted protease inhibitors and patatin recovery 0.355 g protease inhibitor/ g pulp protein and 0.303 g patatin/g pulp protein, respectively. As shown in Table 4.4 both reactions' run 1 and 2 resulted in actual yields of 67.0 and 59.5%, respectively.

Table 4.4 Model validation of the optimal conditions found for the protein recovery yield, recovered patatin and protease inhibitors.

		Predicted			Actual			Probability Interval		
		Yield (%)	g patatin/ g pulp	g protease inhibitor/ g pulp	Yield (%)	g patatin/g pulp	g protease inhibitor/ g pulp	Yield (%)	g patatin/ g pulp	g protease inhibitor/ g pulp
Run 1										
x_1[a]	42.6									
x_2[b]	21.0									
x_3[c]	126.4	79.4	0.291	0.355	67.0±7.8	0.325±0.05	0.268±0.03	64.0-94.9	0.204-0.379	0.226-0.485
x_4[d]	1.89									
x_5[e]	1.5									
Run 2										
x_1	43									
x_2	19.72									
x_3	182.6	73.9	0.303	0.433	59.5±6.6	0.217±0.03	0.328±0.08	57.6-90.1	0.214-0.392	0.305-0.561
x_4	1.53									
x_5	8.888									

[a] Incubation temperature (°C)
[b] Incubation time (hr)
[c] Pulp concentration (kg/m^3)
[d] Units of polygalacturonase M1 (U)
[e] Units of endo-β-1,4-galactanase (U)

Run 1 resulted in a higher extraction of patatin recovered of 0.325 g patatin/ g pulp protein whereas run 2 resulted in higher recovery of protease inhibitors 0.328 g protease inhibitors/ g pulp protein. These yields and extracted patatin and protease inhibitor recovery can be accepted as they fall into the predicted interval range (Table 4.4). Therefore the actual obtained results confirm the validity in the model.

4.2. Conclusion

An optimized enzymatic approach, based on the use of PGase and GLase as degrading plant cell wall biocatalysts, was developed in order to isolate non-denatured proteins from potato pulp. The developed enzymatic approach has the potential to isolate potato proteins with minimal deleterious effects, due to the mild reaction conditions along with the specificity and selectivity of the enzymes. This process required an initial step to remove starch using α-amylase from *B. licheniformis* as it was proven to be more effective. Quadratic models were the most significant ones for the description of variations of the protein recovery yield and the recovered patatin. While for recovered protease inhibitors, the most significant model was 2FI. The developed models are expected to provide the capability to isolate potato proteins with targeted composition and broaden their applications as value added ingredients.

CONNECTING STATEMENT 3

An optimized method for the extraction of potato proteins from potato pulp was achieved using the pure enzymes endo-polygalacturonase and endo-β-1,4-galactanase (Chapter IV). Chapter V focuses on the extrapolation of this method for the development of a novel enzymatic approach using ten commercial multi-enzymatic products namely Gamanase 1.5L, Depol 670L, Ceremix 2XL, Hemicellulase CE 1500, Iogen HS 70, Viscozyme, Pectinase, Newlase II, Diazyme L-200, and Laminex DG for the isolation of potato proteins from potato pulp. The two best multi-enzymatic products Depol 670L and Ceremix 2XL for recovered patatin and recovered protease inhibitors, respectively, were further optimized by response surface methodology (RSM) using a 5-level, 2-factor central composite rotatable design (CCRD). The two factors studied were incubation time and units of selected multi-enzymatic product.

The results from this study were presented at the IFT Annual Meeting & Food Expo-Institute of Food Technologist and IUFoST 17th World Congress of Food Science and Technology Expo.

Waglay, A., & Karboune, S. (2014) A Novel Enzymatic Approach based on the Use of Multi-Enzymatic Systems for the Recovery of Enriched Patatin and Protease Inhibitors Extracts from Potato Pulp. IFT14 Annual Meeting & Food Expo, New Orleans, USA, June 21- June 24, 2014.

Waglay, A., & Karboune, S. (2014) Optimization for the Recovery of Potato Proteins using a Novel Approach based on Multi-Enzymatic Products. IUFoST 17th World Congress of Food Science and Technology & Expo, Quebec, Canada, August 17-August 21, 2014.

Waglay, A., & Karboune, S. (2015) A Novel Enzymatic Approach Based on the Use of Multi-Enzymatic Systems for the Recovery of Enriched Patatin and Protease Inhibitors Extracts from Potato Pulp. *(To be submitted)*

CHAPTER V. A NOVEL ENZYMATIC APPROACH BASED ON THE USE OF MULTI-ENZYMATIC SYSTEMS FOR THE RECOVERY OF ENRICHED PATATIN AND PROTEASE INHIBITORS EXTRACTS FROM POTATO PULP

5.1. Abstract

Ten commercially available multi-enzymatic systems have been explored for the efficient recovery of patatin and protease inhibitors from potato pulp. Their enzyme activity profile was characterized and correlated to their efficiency to isolate potato proteins. The results confirmed the significance of the opening of the pectin network by pectin-hydrolyzing biocatalysts for the efficient recovery of potato proteins. The multi-enzymatic systems with the same enzyme activity profile led to a similar protein recovery yield. Of those assessed, Depol 670L (DEP) and Ceremix 2XL (CER) were efficient for the recovery of protein extract enriched with patatin (up to 60.0%) and protease inhibitors (up to 72.0%), respectively. The efficiency of DEP was significantly dependent on the interactive effect of enzymatic units and incubation time, while CER was found to be affected by the quadratric effect of both variables without any significant interactive effect. Compared to the industrial technique of a combination of thermal/acidic precipitation, the enriched patatin DEP-based protein extract possessed higher lipid acyl hydrolase activity emphasizing the preservation of its functionality. On the other hand, the enriched protease inhibitors CER -based protein extract resulted in higher trypsin inhibiting activity (1817.9 mg protease/g potato protein inhibitors), when compared to the industrial recovery method. Patatin and aspartic protease inhibitors present in DEP and CER-based protein extracts were structurally identified by peptide mass mapping.

5.2. Introduction

Potato crops are the second highest protein providing crop per hectare grown (0.3 kg protein/ ha) when compared to wheat (0.69 kg protein/ ha), rice (0.29 kg protein/ ha), and corn (0.15 kg protein/ ha). Potato proteins are of great interest as food ingredients due to their high nutritional quality (Bártova & Bárta, 2009), their antioxidant potential (Kudo, et al., 2009), and their techno-functional properties (Creusot, et al., 2010). Potato processing industry releases two by-products rich in proteins namely, potato fruit juice (PFJ) and potato pulp, in large quantities. Recovering proteins from these by-products constitutes a great avenue as they are coupled with high polluting capacity (Wojnowska, et al., 1981).

Potato proteins comprise of three main fractions namely, patatin, protease inhibitors, and high molecular weight proteins. Patatin is the major storage protein found in the tuber which represents approximately 40% of the protein. It is known as a glycoprotein, studied as a

homogeneous group of isoforms possessing molecular weight ranging from 39-45 kDa (Pots, et al., 1999). The use of patatin as a food ingredient is of great interest due to its nutritional quality, which is similar to egg protein (Ralet & Guéguen, 2001), its antioxidative activity (Liu, et al., 2003), its anti-proliferative activity against mouse melanoma B16 (Sun, et al., 2013), and its lower allerginicity (Koppelman, et al., 2002). In addition, patatin offers many techno-functional properties such as foam forming, gelling and emulsifying properties (Ralet & Guéguen, 2001; Creusot, et al., 2010; van Koningsveld, et al., 2006). These properties are primarily governed by patatin's low denaturation temperature of 50-55 ^{0}C (Pots, et al, 1998). Therefore, mild reaction conditions are necessary to preserve patatin and take advantage of its functional and beneficial properties. On the other hand, the protease inhibitors are a heterogenous group varying according to their molecular mass ranging from 5-25 kDa, amino acid composition, and their inhibitory activity (Pouvreau, et al., 2001). Six main groups of protease inhibitors have been identified, including potato inhibitor I, potato inhibitor II, potato aspartate protease inhibitors, potato cysteine protease inhibitor, potato Kunitz-type protease inhibitor, and potato carboxypeptidase inhibitor (Pouvreau, et al., 2001). Each group has been shown to possess several beneficial properties such as the protease inhibitor I and II, potato cysteine inhibitors, and Kunitz-type have been studied to possess anti-microbial activity (Kim, et al., 2005; Kim, et al., 2013). Contrarily, the potato carboxypeptidase and potato cysteine inhibitors have been shown to inhibit tumor cell growth and decrease reactive oxygen species, respectively (Blanco-Aparicio, et al., 1998; Frenkel, et al., 1987). Unlike patatin, protease inhibitors exhibit higher denaturing temperatures of 55-70 ^{0}C and form unstable foams and emulsions (van Koningsveld, et al., 2001).

In our previous (Waglay, et al., 2014) and other studies (Bártova & Bárta, 2009; Knorr, et al., 1977), the efficiency of selected extraction techniques to isolate potato proteins has been studied and compared. Thermal/ acidic precipitation as the industrially employed technique resulted in high yields (> 85%), with limited applications due to loss of functionalities (Knorr, et al., 1977). Ferric chloride has been shown to have a strong affinity towards potato proteins resulting in protein recovery yields ranging from 75.2- 86.5% (Bártova & Bárta, 2009; Waglay, et al., 2014). Ammonium sulphate saturation resulted in a positive concentration dependent yield recovery, with a corresponding negative impact on

purification factor (Waglay, et al., 2014). Complexation with carboxymethyl cellulose led to the recovery of protein extracts with enhanced functionality (Vikelouda & Kiosseoglou, 2004). However, limited patatin extraction was obtained due to the strong pH adjustment necessary for protein-polysaccharide interaction (Waglay, et al., 2014).

A novel enzymatic approach was proven to be efficient for the isolation of proteins with retained functionality from potato pulp (Waglay, et al., 2015). This two-step enzymatic approach begins by removing starch using amylase, Termamyl followed by degrading the cell wall pectin network by glycosyl-hydrolases for an efficient recovery of proteins. Starch removal was found to allow for easier access of the polysaccharide-hydrolyzing enzymes to plant cell wall components. The combined use of endo-1,4-β-galactanase and endo-polygalacturonase MI as mono-component biocatalysts improved the potato protein recovery, while endo-arabinanase did not contribute significantly to this recovery. In order to broaden this enzymatic approach and make it more industrially appealing, the use of multi-enzymatic products, which contain several glycosyl-hydrolases activities, have been explored. The efficiency of ten multi-enzymatic systems namely, Gamanase 1.5L (GAMase), Depol 670L (DEP), Ceremix 2XL (CER), Hemicellulase CE-1500 (HEMase), Iogen HS 70 (IOG), Viscozyme (VIS), Pectinex Ultra SPL (PEC), Newlase II (NEWase), Diazyme L-200 (DIA), and Laminex DG (LAM) to recover the proteins was assessed using the potato pulp as starting material. The effect of the enzyme activity profile of the multi-enzymatic systems on the protein recovery yield, the extracted amount of patatin, and protease inhibitors were discussed. It is hypothesized that multi-enzymatic systems with the same enzyme activity profile can lead to similar protein recovery efficiency. Using DEP and CER-based recovery processes, the effects of endo-β-1,4-galactanase unit level and incubation time on the protein recovery and fractionation were further investigated using response surface methodology (RSM). The improved biocatalytic approach is expected to preserve the structure of potato proteins and their functional and beneficial effects hence increasing their applications.

5.3. Materials and Methods

5.3.1. Materials

Fresh potatoes of Russet Burbank variety were purchased from a local supermarket. Sodium metabilsulfite, sulphuric acid (H_2SO_4), trifluoroacetic acid, hydrochloric acid were purchased from Sigma Chemical Co. (MO, USA). Bradford reagent and SDS-PAGE Broad Molecular weight standard were obtained from Bio-Rad (On, Ca). Bovine serum albumen (BSA), Tris base and potassium phosphate dibasic were acquired from Fisher Scientific (NJ, USA). Potassium phosphate monobasic was purchased from MP Biomedicals, LLC (OH, USA). GAMase from *Aspergillus niger* and CER from *Bacillus spp.* were obtained from Novo Nordisk Bioindustrials (Novo Alle, DE), while DEP from *Trichoderma reesei,* was provided from Biocatalyst Limited (Mid Glamorgan, UK). On the other hand, VIS, and PEC from *Aspergillus aculeatus* were obtained from Novozyme Canada (Qc, Ca), HEMase and LAM from *Trichoderma reesei* were obtained from Genencor (CA, USA). IOG from *Rhizopus niveus* was obtained from Iogen Bio-Products, while NEWase from *R. niveus*, was provided by Amano Enzyme (USA); DIA from *A. niger*, was obtained from Solvay Enzyme Products Incorporated (On, Ca). Potato protein extract enriched with patatin was provided by Solanic (NL).

5.3.2. Preparation of potato pulp

Potato pulp was prepared with potatoes of Russet Burbank variety. The potatoes were washed and finely chopped into 0.5 g/mL samples. The potato pieces were ground with a mortar and pestle for 1 min with 1.315 mM sodium metabisulfite. The ground pieces were homogenized using a Warring commercial Blender on low speed for 1 min.

5.3.3. Characterization of enzyme activity profile of multi-enzymatic system

5.3.3.1. Proteolytic activity assay

The presence of proteolytic activity in the multi-enzymatic systems was assessed using potato proteins as substrate. Potato protein extracts (1%, w/v) in potassium phosphate buffer (50 mM, pH 6.5) was added to the multi-enzymatic systems at appropriate dilutions to yield a final concentration of 0.5% (w/v). Reaction mixtures were incubated for 20 min at 40 °C. A blank substrate and blank enzyme were run in parallel. The released amino acids were

quantified using ninhydrin assay. 200 µL of reaction mixture were mixed with 100 µL of 10 mM sodium cyanide suspended in 2.65 M sodium acetate buffer at pH 5.4 in a ratio 1: 49 (v/v) and 100 µL of 3% (w/v) ninhydrin solution in 2-methoxyethanol. The ninhydrin reaction mixture was incubated for 15 min at 100 °C. After cooling to room temperature with the addition of 1 mL *n*-propanol diluted with distilled water (1: 1 v/v), the absorance of the mixture was measured at 570 nm. Standard curve was developed using leucine as a free α-amino group.

5.3.3.2. Glycosyl-hydrolase activity assay

Selected multi-enzymatic systems were assessed for their levels of endo-polygalacturonase, endo-1,4-β-galactanase, rhamnogalacturonase, and endo-α-1,5-arabinanase activities using orange polygalacturonic acid, potato galactan, soybean rhamnogalacturonan, and sugar beet arabinan as substrates, respectively. The reaction was initiated by adding 25 µL of multi-enzymatic system at appropriate dilutions to 0.475 mL of substrate solution (0.25%, w/v, for polygalacturonic acid and 0.55%, w/v for galactan, rhamnogalacturonan, and arabinan) in 50 mM sodium acetate buffer at pH 5.0. The reaction mixtures were incubated at 40 °C for 20 min. Following incubation, the addition of 750 µL of dinitrosalicylate (DNS) reagent (DNS, 1.0 g/100mL and NaOH, 1.6 g/100 mL) was quickly added to terminate the hydrolysis reaction. The reaction mixtures were then boiled for 5 min; thereafter, 250 µL of potassium sodium tartarate solution (50 g/100mL) was added to stabilize the reducing sugar assay colour. Absorbance of the mixture was measured at 540 nm. Standard curves were constructed using galacturonic acid, galactose, rhamnose, and arabinose as standard. One unit of activity was defined as the amount of enzyme which releases one µmole of the corresponding glycoside end per min of reaction. The specific activity was expressed as enzymatic activity unit per mg protein.

5.3.4. Enzymatic approach for protein recovery

The enzymatic approach consists of a two-step process in which the potato pulp was first treated with α-amylase followed by the enzymatic treatment with the multi-enzymatic system. The α-amylases from *Bacillus licheniformis* (Termamyl) was used for the removal of starch. Dried potato pulp (29.2% w/v) was suspended in 10 mM potassium phosphate

buffer at pH 6.5. Selected units of α-amylase were added to the potato pulp suspension to yield 1 U/mg pulp. Reactions were carried out at 40 ^{0}C with constant stirring at 5*g* for 16 hr. Following starch removal, destarched potato pulp (16.2% w/v) was suspended with 100 mM sodium acetate pH 5.0 to a final volume of 7 mL. The enzymatic reactions were initiated with the addition of 3.42 U of endo-polygalacturonase/g pulp for GAMase, CER, HEMase, IOG, and LAM-based reaction systems to the destarched potato pulp suspensions; while for DEP, VIS, PEC, NEWase, and DIA ones, 6.84 endo-1,4-β-galactanase U/g pulp was added. The reaction mixtures were incubated at 42.6 °C for selected reaction times of 10 and 20 hr. After incubation, the reaction mixtures were vacuum filtered using 1.2 μm GF/C Whatman filters and the supernatants containing proteins were recovered. The supernatants were further dialyzed and lyophilized, whereas the degraded pulps were oven dried at 105 °C. The protein contents of the pulp and the supernatant were determined using Dumas method as described by Kirsten and Hesselius (1983). Nitrogen content was multiplied by a factor of 6.25 to determine the protein content (van Gelder, 1981).

5.3.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Protein extracts were analyzed by SDS-PAGE according to the method of Laemmli (1970) using 5 and 15% acrylamide content in the stacking and resolving gels, respectively. Sample loading was achieved in a mini protein gel apparatus (Bio-Rad) with a 1.5 mm-thick gel. The electrophoresis was conducted at a constant voltage of 120 mV. The gels were then stained for 2 hr with a staining solution of Coomassie brilliant blue R250 (1 %, w/v) in methanol: water: acetic acid (45: 45: 10, v/v/v), followed by destaining in methanol: acetic acid: water (1: 1: 8, v/v/v). The analyses of the electrophoretic patterns to obtain the protein profiles were carried out using Red Imaging system equipped with Alpha-View SA Software. The relative proportions of recovered patatin and protease inhibitors were estimated from bands corresponding to 38-45 kDa and 28-10 kDa, respectively. Low molecular weight bands <5 kDa were not considered as part of the protease inhibitors as they may contain the peptides generated by the proteolytic activity of multi-enzymatic systems. The calculated extracted patatin or protease inhibitor amount per g of initial pulp protein, as well as their corresponding purity are as follows:

Recovered patatin amount (g patatin/ g pulp protein) = (g isolated proteins * relative proportion patatin/100))/ initial g pulp protein *(9)*

Purity = g patatin /g isolated proteins *(10)*

5.3.6. Experimental design

Optimization of the protein recovery using DEP- and CER-based processes was investigated using response surface methodology (RSM). The quantified responses were the protein yield (%), the recovered patatin amount (g patatin/g pulp protein) and the recovered protease inhibitors amount (g protease inhibitors/g pulp protein). A five-level, two-variable central composite rotatable design (CCRD) was used for each multi-enzymatic system-based process. The full designs consisted of 4 factorial points, 4 axial points, and 6 center points, resulting in 14 sets of experiments for each multi-enzymatic system-based process. The two variables consisted of x_1 units of enzyme (for DEP 2.77, 4.62, 9.24, 13.86, 15. 71 units endo-β-1,4-galactanase/g pulp; and for CER 60.01, 75.81, 113.94, 151.84, 167.63 units endo-β-1,4-galactanase/g pulp), and x_2 time (0.96, 2.0, 4.5, 7.0, 8.04 hr for DEP; and 0.65, 5.0, 15.5, 26.0, 30.35 hr for CER). The experimental runs were run in duplicate and divided into two blocks, where the first block had four factorial points and three center points, whereas the second block contained the four axial points with three center points. Blocking was performed in order to account for possible differences in preparation of the two batches of potato pulp. All reaction points were run in duplicate and averages were taken as the experimental findings.

5.3.7. Statistical analysis

For approximating response surface, each of the obtained yield, recovered patatin amount, and the recovered protease inhibitors amount was fitted to the general model Equation 11 using the software Design-Expert 8.0.2 (Stat-Ease, inc. Minneapolis, MN, USA):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i+1 \le j \le k}^{1 \le i \le k} \beta_{ij} X_i X_j \quad (11)$$

β_o, X_is (i=1-5), $\beta_{i,,}$ β_{ii}, and β_{ij} are the constant coefficient, coded independent variables, the coefficient for the linear effect, the coefficient for the quadratic effect, and the coefficient for the interaction effect, respectively. Contour plots were obtained from the fitted model

by keeping the independent variables at a constant value while changing the other two variables.

5.3.8. Lipid acyl hydrolase activity of recovered protein extracts

LAH activity of potato protein extracts recovered using DEP-based enzymatic process and combination of thermal/acidic precipitation were measured spectrophotometrically according to the modified method of Bártová and Bárta (2009) using 4-nitrophenol butyrate and 4-nitrophenyl laurate as substrate. Potato protein extracts (0.92-13 mg/mL) at an appropriate dilution were pre-heated at 37 °C in a 1 mL spectrophotometer cell using a DU 800 spectrophotometer equipped with a thermocontrolled sample compartment. The reaction (1 mL) were initiated by the addition of 50 and 500 µL of 4-nitrophenyl butyrate (10 mM) and 4-nitrophenyl laurate (1 mM, 0.5% DMSO), respectively, prepared in 50 mM Tris-HCl buffer at pH 7.4. The absorbance of the reaction mixture was monitored at 410 nm for 5 min. Standard curves was established using 4-nitrophenol as standard. One unit of LAH activity was defined as the amount of enzyme liberating one µmol of 4-nitrophenol per min. The standard deviation was determined on duplicate reactions.

5.3.9. Protease inhibiting activity of recovered protein extract

Protease inhibiting activity of protein extracts recovered using CER-based enzymatic process and combination of thermal and acidic precipitation towards serine proteases (trypsin and chymotrypsin) was investigated according to the modified method of Pouvreau et al., (2001). Trypsin and chymotrypsin enzymatic solutions (10-20 mg/mL) were prepared in 50 mM Tris-HCl buffer (pH 7.8) containing 100 mM $CaCl_2$. The protease and potato protein extracts were mixed to achieve an equal protein concentration (1:1; w:w) and incubated at 37 °C for 15 min. The proteolytic activity of trypsin and chymotrypsin were assayed before and after incubation with potato protein extracts using *N*-α-Benzoyl-D-L-arginine 4-nitroanilide hydrochloride (*N*-α- BAPA) and *N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (*N*-succinyl-AAPP) as substrates, respectively. The reaction was initiated by mixing 0.2 mL of substrate (1.8-1.12 mM), 0.2 mL of enzymatic solution and 0.6 mL of Tris-HCl buffer (50 mM, pH 7.8) in a 1 mL spectrophotometer cell. The variation in absorbance of the reaction mixtures, against a blank trial without enzyme was continuously monitored at 410 nm, for 10 min. The proteolytic activity was estimated from the slope of

the absorbance versus time curve. Standard curves were established at a wavelength of 410 nm using a 4-nitroaniline as standard. The protease inhibiting activity of potato protein extracts was calculated as:

$$\text{Protease inhibitory Activity (\%)} = ((\text{specific activity}_{\text{without inhibition}} - \text{specific activity}_{\text{inhibition}})/ \text{specific activity}_{\text{without inhibition}}) \times (\text{mg trypsin/ g potato protein}) \times 100 \quad (12)$$

5.3.10. Structural characterization of potato proteins

5.3.10.1. Protein in-gel digestion

Structural characterization of potato proteins was performed at Centre Protéomique at Universite de Laval. Tryptic digestion was performed according to the method described by Shevchenko et al. (1996) with slight modification performed according to Havlis et al. (2003) using a MassPrep liquid handling robot (Micromass, MCH, UK). In summary, SDS-PAGE gels were run as described above for the optimal conditions of the enriched patatin and enriched protease inhibitor extracts, isolated by DEP and CER, respectively. One protein band corresponding to patatin (43 kDa) from the DEP enriched patatin extract was collected. In addition, one protein band corresponding to protease inhibitor with molecular weights of 23.2 kDa was collected and analyzed. All protein bands were reduced with 10 mM DDT and alkylated with 55 mM iodoacetamide. Trypsin digestion was initiated with 105 mM of modified porcine trypsin (sequencing grade, Promega, WI, USA) at 58 ºC for 1 hr. Recovery of the generated peptides were performed using 1% formic acid/2% acetonitrile followed by 1% formic acid/50% acetonitrile. Peptides extracts were pooled and solvent was removed by vacuum centrifugation. Prior to mass spectroscopy, dried peptide extracts were suspended in 10 µL of 0.1% formic acid.

5.3.10.2. Mass spectrometry

Mass spectrometry was performed using a Thermo Surveyor MS pump connected to a LCQ Deca XP mass spectrometer (Thermo Electron, CA, USA) supplied with a nanoelectrospray ion source (Thermo Electron, CA, USA). Peptide extracts were separated by online reverse-phase nanoscale capillary liquid chromatography and analyzed by electrospray mass spectrometry. Initially, a cap trap (Michrom Bioresources, CA, USA) bound the peptide extracts at 10 µL/min following with chromatographic separation using a PicoFrit column BioBasic C18, 10 cm x 0.075 mm internal diameter (New Objective, MA, USA) using a

linear gradient of 2 to 50% acetonitrile and 0.1% formic acid, for 30 min, at a flow rate of 200 nL/min (obtained by flow splitting). Xcalibur software version 1.2 in the data-dependent acquisition mode was used to collect mass spectra. The three most intense ions as determined in the full scan mass spectra (400-2000 *m/z*) underwent collision-induced dissociation, where dynamix exclusion was enabled (30 s exclusion duration) and relative collisional fragmentation energy was set at 35%.

5.3.10.3. Database searching

The MS/MS samples were analyzed using Mascot (Matrix Science, LND, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Assuming trypsin digests, X! and Mascot were set to search the UR12_5_SolanumTub_20120718 database (unknown version, 2615 entries). The fragment ion mass tolerance and parent ion tolerance were set to 0.100 Da. A fixed modification was established in Mascot and X! Tandem for the carbamidomethyl of cysteine. The n-terminus was dehydrated, Glu→ pyro-Glu, ammonia loss of the n-terminus, Gln → pyro-Glu of the n-terminus, deamidated of asparagine and glutamine, and oxidation of methionine.

5.3.10.4. Amino acid sequencing for protein identification

Validation of MS/MS based peptide and protein identification was performed using Scaffold (version Scaffold_3.6.2, Proteome Software Inc., OR, USA). A 95.0% or greater probability determined by Peptide Prophet algorithm allowed for accepting the peptide identification and protein identification. Protein identification was accepted based on two conditions, 95% or greater probability as well as a minimum of 2 identified peptides. The principles of parsimony were used to group similar peptides belonging to different proteins, in which case MS/MS analysis were unable to detect differences. The amino acid sequence of patatin and protease inhibitors was determined using the FASTA format. The most prominent accession numbers were Q2MY36 and API11_SOLTU (UniProtKB), for patatin and protease inhibitors, respectively.

5.4. Results and Discussion

5.4.1. Investigation of the efficiency of multi-enzymatic systems

In our previous study (Waglay, et al., 2015), we have shown that the hydrolysis of pectic polysaccharides, which act as binders in the cell wall, by endo-polygalacturonase and endo-β-1,4-galactanase improves the recovery of proteins from potato pulp. In order to enhance the industrial appeal of the enzymatic approach as a great potential for the isolation of highly functionalized proteins, the exploration of the use of multi-enzymatic systems instead of monocomponent biocatalysts was investigated. Ten multi-enzymatic systems, namely GAMase, DEP, CER, HEMase, IOG, VIS, PEC, NEWase, DIA, and LAM, were selected and characterized for their proteolytic activity towards potato proteins, and for their pectic polysaccharide-hydrolyzing activities, including endo-polygalacturonase, endo-β-1,4-galactanase, rhamnogalacturonase, and endo-α-1,5-arabinanase. These multi-enzymatic systems are known for expressing selected glycosyl-hydrolase activities including: β 1-4 mannase and xylanase for GAMase (Redgwell, et al., 2005); β-glucanase, arabinase, endo-glucanase, xylanase, polygalacturonase, and feruloyl esterase for DEP (Vafiadi, et al., 2008); α-amylase, β-glucanase, and protease for CER (Kawa-Rygielska, et al., 2012); cellulase, xylanase, and β-glucosidase activity for HEMase (Tangnu, et al., 1981); xylanase for IOG ; β-glucanase, xylanase, cellulase, and hemicellulase for VIS (Sørensen, et al., 2003); pectinesterase, polygalacturonase, pectintranseliminase, and cellulase for PEC (Soffer & Mannheim, 1994); glucoamylase, xylanase, carboxymethyl cellulase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase for DIA; cellulase and β-glucosidase for LAM (Philippidis, et al., 1993).

As shown in Table 5.1, GAMase, DEP, HEM, VIS, PEC, and NEWase demonstrated non-significant proteolytic activity towards potato proteins, whereas CER, DIA, and LAM exhibited some proteolytic activity. Contrarily, IOG showed the highest proteolytic activity towards potato proteins. In order to prevent the hydrolysis of potato proteins, the level of the proteolytic activity needed to be limited. As shown in the literature, CER has been reported to possess proteolytic activity, which aids in its use for reducing the fermentation

Table 5.1 Characterization of Enzyme Activity Profile of Selected Multi-Enzymatic Systems.

Multi-Enzymatic System	Proteolytic Activity	Endo-polygalacturonase (U[c]/g pulp)	Endo-1,4-β-Galactanase (U/g pulp)	Rhamnogalactanase (U/g pulp)	Endo-α-1,5-Arabinanase (U/g pulp)	Endo-polygalacturonase/ endo-1,4-β-Galactanase[a]	Endo-1,4-β-Galactanase+Endo-α-1,5-Arabinanase/ Endo-polygalacturonase+ Rhamnogalactanase[b]
Gamanase 1.5L	---	3.42	8.63	0.26	0.06	0.40	2.4
Depol 670L	---	0	6.84	0.06	0.06	0.00	115.0
Ceremix 2XL	+	3.42	152.02	1.82	4.76	0.022	29.9
Hemicellulase CE-1500	---	3.42	57.57	6.16	3.15	0.10	6.30
Iogen HS 70	++++	3.42	70.08	2.55	3.15	0.049	12.30
Viscozyme	---	7.78	6.84	0.32	0.12	1.10	0.90
Pectinex Ultra SPL	---	9.06	6.84	0.14	0.08	1.30	0.80
Newlase II	---	30.12	6.84	0.53	0.14	4.40	0.20
Diazyme L-200	+	0	6.84	4.15	0.96	0.00	1.90
Laminex DG	++	3.42	6.94	1.19	0.84	0.50	1.70

[a]Endopolygalacturonase/ Endo-1,4-β-Galactanase ratio is representative of the hydrolysis of the polysaccharide backbone.
[b]Galactanase+Arabinase/ Endopolygalacturonase+Rhamnogalactanase ratio is representative of the hydrolysis of the polysaccharide branching.
[c]Units (U) are expressed in μmol/min.

time of waste generated from wheat-rye bread mashes (Kawa-Rygielska, et al., 2012).

Table 5.1 also shows the enzyme activity profile of selected multi-enzymatic systems. Indeed, endo-polygalacturonase and rhamnogalacturonase hydrolyse the backbones of homogalacturonan and rhamnogalacturonan I pectic polysaccharide components (Khodaei & Karboune, 2013). On the other hand, endo-1,4-β-galactanase and endo-α-1,5-arabinanase cleave the galactan and arabinan side chains of rhamnogalacturonan I (Zykwinska, et al., 2006). The results indicate that all multi-enzymatic systems possessed high levels of endo-polygalacturonase and endo-β-1,4-galactanase when compared to levels of rhamnogalacturonase and endo-α-1,5-arabinanase. VIS, PEC, NEWase exhibited higher hydrolytic activities towards the backbone of pectic polysaccharides with a ratio of endo-1,4-β-galactanase+endo-α-1,5-arabinanase/endo-polygalacturonase+ rhamnogalacturonase of 0.2 to 0.9. While higher hydrolytic activities towards the side chains were expressed by GAMase, DEP, CER, HEMase, and IOG exhibiting high ratios of endo-1,4-β-galactanase+endo-α-1,5-arabinanase/ endo-polygalacturonase+ rhamno-galacturonase varying between 2.4 to 115.0 (Table 5.1). On the other hand, DIA and LAM had more or less similar enzyme activity profile in which the levels of side-chain hydrolyzing activities were 1.7 to 1.9 higher as compared to those of the backbone ones (Table 5.1).

Figure 5.1 shows that the protein recovery yield was dependent on the enzyme activity profile of the multi-enzymatic system. The highest yield of 72.9% was achieved with the use of DEP (Figure 5.1 A). Indeed, DEP exhibited no significant endo-polygalacturonase activity and the highest galactanase activity level with a ratio of endo-β-1,4-galactanase+endo-α-1,5-arabinanase/endo-polygalacturonase+rhamnogalacturonase activity of 115.0, revealing its higher hydrolyzing activity toward side chains of pectic polysaccharides as compared to their backbones. On the other hand, NEWase, which had the highest endo-polygalacturonase activity level and endo-polygalacturonase/endo-β-1,4-galactanase activity ratio of 4.4, also resulted in a higher protein recovery yield of 70.7%. These results reveal that the hydrolysis of galactan side chains and/or homogalacturonan backbone favored the opening of the pectin network and the recovery of potato proteins.

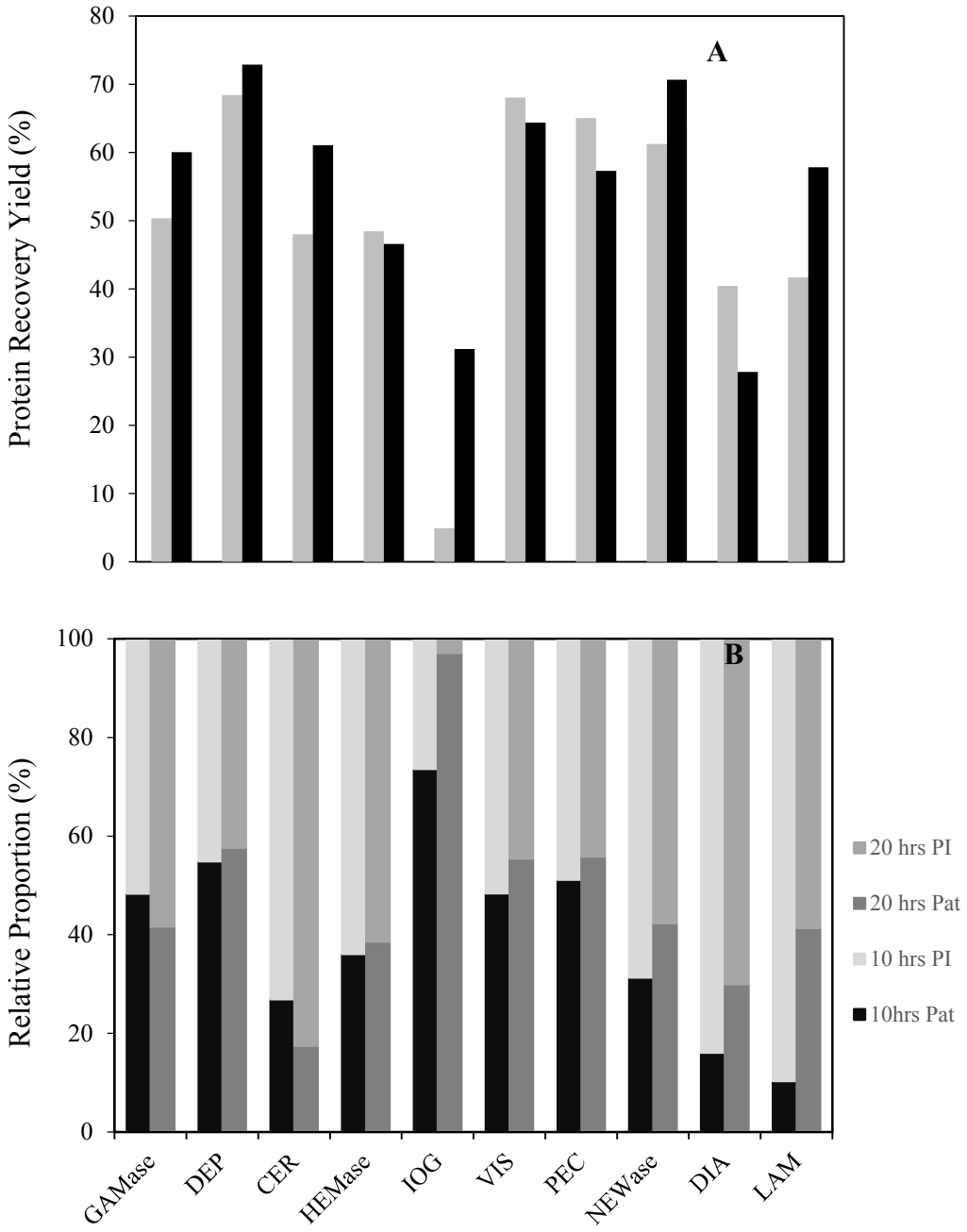

Figure 5.1 (A) Protein recovery yield and (B) relative proportion of patatin (Pat) and protease inhibitors (PI) recovered upon enzymatic treatment of potato pulp with selected multi-enzymatic systems for 10 and 20 hr: GAMase, Gamanase 1.5L; DEP, Depol 670L; CER, Ceremix 2XL; HEMase, Hemicellulase CE-1500; IOG, Iogen HS 70; VIS, Viscozyme; PEC, Pectinex Ultra SPL; NEWase, Newlase II; DIA, Diazyme L-200; and LAM, Laminex DG.

In our previous study (Waglay, et al., 2015), the combined use of pure enzymes, endo-polygalacturonase M1 and endo-β-1,4-galactanase, resulted in a similar maximum protein recovery of 73.9% as the multi-enzymatic systems (DEP ; NEWase). The comparable efficiency of the multi-enzymatic systems and the monocomponent biocatalysts confirm the significance of pectin-hydrolyzing biocatalysts for the efficient recovery of potato proteins as compared to other glycosyl-hydrolases present in the multi-enzymatic systems. In contrast, a previous study conducted by Strand, et al. (1976) reported that pectic polysaccharide degrading enzymes released less than half the proteins emphasizing the need for hydrolyzing other cell wall components.

The use of GAMase, HEMase, and CER, expressing an enzyme activity profile with high specificity toward the side chains than the backbones, led to a protein recovery yield of 60.0, 48.5, and 61.1%, respectively. Although IOG exhibited a high endo-β-1,4-galactanase+endo-α-1,5-arabinanase /endo-polygalacturonase+ rhamnogalacturonase activity ratio of 12.3, it resulted in a low yield (31.2%). This result can be attributed (a) to the presence of the proteolytic activity, which may have hydrolyzed the recovered proteins, (b) to the high substrate inhibition and/or (c) to enzyme denaturation due to protein/protein or protein/carbohydrate interactions. Contrary to other multi-enzymatic systems, VIS and PEC showed more or less similar enzyme activity profile characterizing by close hydrolytic activities towards the side chains and the backbones of pectic polysaccharides, with an endo-polygalacturonase/ endo-β-1,4-galactanase activity ratio of 1.1-1.3 and an endo-β-1,4-galactanase+endo-α-1,5-arabinanase/endo-polygalacturonase+rhamnogala-cturonase activity ratio of 0.8-0.9; as a result of this similar enzyme activity profile, they led to similar protein recovery yield of 65.1-68.1%. On the other hand, the comparison of the efficiency of DIA (40.4%) and LAM (57.8%) as a function of their enzyme activity profile indicates the importance of endo-polygalacturonase activity as compared to rhamno-galacturonase for the efficient opening of the pectin network and the high recovery of potato proteins. Indeed, DIA and LAM exhibited more or less similar levels of endo-β-1,4-galactanase and endo-α-1,5-arabinanase; however, DIA showed higher level of rhamnogalacturonase, whereas LAM expressed higher endo-polygalacturonase activity.

Figure 5.1 A shows that a significant increase in the protein recovery yield was attained after 20 hr of reaction time when compared to 10 hr in the presence of CER, IOG and LAM. As shown in Table 5.1, CER, IOG and LAM exhibited a proteolytic activity, which may have hydrolysed the potato proteins at the initial stage of the reaction and limited their recovery; however, as the potato protease inhibitors were released, they may have inhibited the proteolytic activity, resulting in the apparent significant increase in the protein recovery at extended reaction time of 20 hr. In contrast, DIA, which also expressed a proteolytic activity, led to a decrease in the protein recovery yield at 20 hr; this result may be attributed to the low inhibiting effect of potato protease inhibitors toward the proteolytic activity expressed in DIA. On the other hand, the increase in the protein recovery yield with the reaction time in the presence of GAM, DEP, and NEWase may be attributed to their limited arabinanase activity. It has been reported that the hydrolysis of arabinan can improve the access to galactan side chains (Hinz, et al., 2005).

The major associated advantage to the investigated enzymatic approach is the ability to enrich the protein extract with either patatin or protease inhibitors, as the result of the presence of highly specific proteolytic activity. It is important to consider, that the proteolytic activity belonging to certain multi-enzymatic systems could exhibit a highly specific proteolytic activity towards patatin yielding peptides. These peptides, polypeptides, or derived protein could potentially be the same mass (28 to 10 kDa) as protease inhibitors, leading to an apparent increase in their proportion; however, this hypothesis was not confirmed through peptide mapping and MS analysis, as described later. Any peptides with less than <5 kDa were not included as part of protease inhibitors as they may be overalapped with those generated from the protein hydrolysis.

As shown in Figure 5.1 B, no enrichment was obtained with GAMase, VIS, and PEC multi-enzymatic systems, which were able to isolate both fractions, patatin and protease inhibitors in about the same proportions. These results can be attributed to the presence of non-significant proteolytic activity toward potato proteins in GAMase, VIS, and PEC multi-enzymatic systems at the investigated concentrations (Table 5.1). DEP and IOG were found to both have an enrichment effect on the recovery of patatin at both incubation time. However, the recovered patatin (73.5-97.1%) achieved with IOG was very high as compared to DEP (54.8-57.6%; Figure 5.1 B). This difference is due to the high proteolytic

activity expressed in IOG at the investigated concentration. Regardless of IOG achieving higher patatin recovery with time, the substantially lower protein recovery rendered IOG inadequate when compared to DEP. Therefore, DEP was further optimized for the recovery of patatin. On the other hand, the use of CER, HEMase, NEWase, DIA, and LAM led to more enrichment of the protein extract with protease inhibitors (>61.4%; Figure 5.1 B). These multi-enzymatic systems have shown different levels of proteolytic activity with DIA, LAM and CER expressing the highest ones. Of those multi-enzymatic systems the one which consistently at both incubation times extracted more protease inhibitors was CER, indicating the high specificity of its proteolytic activity towards the hydrolysis of patatin. All others had an effect on the enrichment with less protease inhibitors with time. Therefore, CER was further optimized for the recovery of extract enriched in protease inhibitors.

To our knowledge no studies are available on the use of multi-enzymatic systems for the recovery of proteins from potato pulp. The better understanding of this subtractive technique is desirable as it takes an industrially available by-product and results in proteins with retained functionality and ability to fractionate the components, resulting in value added food ingredients.

5.4.2. Study and Optimization of Enzymatic Isolation of Potato Proteins

Optimization of the enzymatic isolation of proteins from destarched potato pulp was investigated using selected multi-enzymatic systems: DEP to isolate a fraction enriched with patatin and CER to isolate a fraction enriched with protease inhibitors. The effects of two reaction parameters, including endo-β-1,4-galactanase units (U/g pulp) and reaction time (hr), were assessed. The levels for each parameter were chosen based on the preliminary trials and on the previous study using pure enzymes (Waglay et al., 2015). RSM was used because of its ability to assess the effect of many parameters in combination or alone, collecting sufficient information for statistically significant results with less experimental runs (Akoh et al., 2007). Table 5.2 shows the experimental conditions and their corresponding observed responses.

Multiple regression analysis and backward reduction algoritm was used to evaluate the best fitting models for protein recovery yield, recovered patatin amount (g/g pulp protein),

Table 5.2 Central composite rotatable design (CCRD) arrangement of the actual experimental variables and the observed protein recovery yield, protein proportion, and purity obtained from (A) Depol 670L and (B) Ceremix 2XL treated potato pulp.

A Run no.	x_1 endo-β-1,4-Galactanase Units of multi-enzymatic system/ g pulp	x_2 Time	Protein Recovery Yield (%)	Recovered Patatin Amount (g/g pulp protein)[a]	Purity of Patatin[b]
1	2.77	4.5	59.9	0.339	0.57
2	4.62	7	67.5	0.384	0.57
3	4.62	2	50.3	0.245	0.49
4	9.24	0.96	79.8	0.36	0.45
5	9.24	8.04	51.1	0.25	0.49
6	13.86	2	78.1	0.471	0.60
7	13.86	7	64.5	0.224	0.35
8	15.71	4.5	62.2	0.257	0.41
CP 1	9.24	4.5	74.60±8.19	0.41±0.05	
CP 2	9.24	4.5	54.97±3.36	0.23±0.004	

B Run no.			Protein Recovery Yield (%)	Recovered Protease Inhibitors Amount (g/ g pulp protein)[a]	Purity of Protease Inhibitors[b]
1	60.01	15.5	44.5	0.215	0.48
2	75.81	5	57.4	0.257	0.45
3	75.81	26	54.2	0.267	0.49
4	113.94	0.65	27.6	0.122	0.44
5	113.94	30.35	51.5	0.361	0.70
6	151.84	5	48.5	0.252	0.52
7	151.84	26	67.9	0.488	0.72
8	167.63	15.5	41.9	0.22	0.53
CP 1	113.94	15.5	57.25±4.50	0.240±0.05	
CP 2	113.94	15.5	29.06±1.08	0.14±0.04	

[a] Relative proportion (%) was converted to extracted fraction by g fraction/g pulp protein

[b]Purity was calculated as the recovered patatin or protease inhibitors per total recovered proteins.

and recovered protease inhibitors amount (g/g pulp protein) (Design-Expert version 8.0.2). The results of the analysis of variance (ANOVA) indicate that the reduced quadratic model was statistically significant for the description of the variations of the DEP-based recovered patatin amount (*F*-value of 6.64 and *p*-value of 0.0071), and the CER-based recovered protease inhibitors amount (*F*-value of 5.61 and *p*- value of 0.0124; Table 5.3). As well as, the non-significant lack of fit for these responses with *F* values of 0.72-1.78 and *p*- values of 0.3423-0.6575 shows statistical significance of the models. The coefficient of determination (R^2) of all the statistically fitted models and their predicted R^2 were slightly different; this could be due to the block effect, which was seen between the two pulp preparations. Similar results were also encountered by Kumar et al. (2008) which attributed the differences to a large block effect. To verify the accuracy of all statistically significant models to predict the responses of the predicted models, the normal distribution plot and predicted versus actual (data not shown) for all responses had linear distribution which confirm good fitting models. The residual versus predicted plot were randomly distributed outlining the variance of the original observation was constant for all values of the responses.

Considering the significant terms, the responses of the DEP-based recovered patatin amount and the CER-based recovered protease inhibitors amount can be described by the following quadratic equations in terms of coded variables:

$$\text{Recovered Patatin/g pulp protein} = 0.35 - 0.031\, x_1 - 0.043\, x_2 - 0.062\, (x_1)^2 - 0.061\, (x_1)(x_2) \quad (13)$$

$$\text{Recovered Protease Inhibitors/g pulp protein} = 0.20 - 0.0014\, x_1 + 0.075\, x_2 + 0.053\, (x_1)^2 + 0.065\, (x_2)^2 \quad (14)$$

As shown in Table 5.3, for DEP-based recovered patatin model, a quadratic effect of the endo-β-1,4-galactanase units (x_1^2, F-value 11.85, *p*-value 0.0063) was the most significant one. In terms of linear terms, DEP-based recovered patatin was affected by both endo-β-1,4-galactanase units and incubation time (Eq. 13). In addition, the interactive effect of both variables (x_1x_2, F-value 8.96, *p*-value 0.0135) on the recovered patatin was significant and antagonist as shown by the negative sign of its term (Eq. 13). According to the CER-based recovered protease inhibitors model, incubation time (x_2) was the significant linear term, affecting the amount of protease inhibitors (Table 5.3). While both endo-β-1,4-galactanase units (x_1^2, F-value 4.89, *p*-value 0.0515) and incubation time (x_2^2, F-value 7.38, *p*-value

0.0217) exhibited a positive quadratic effect on the recovered protease inhibitors amount. The interactive effect of both variables did not have a significant effect of the amount of the recovered protease inhibitors.

To validate the model of the recovery of patatin extracted using DEP, one condition was selected, with a high predicted patatin recovery 0.34 g patatin/g pulp protein (Table 5.4 A). This point was ran in triplicate with two centerpoints run in parallel as a reference. As observed the actual obtained results for recovered patatin was 0.38 g patatin/g pulp protein, which falls into the probability interval range of 0.20-0.48 g patatin/g pulp protein. Therefore the predictive model was accepted and validated. For the recovery of protease inhibitors extracted using CER, one condition was selected with a high predicted protease inhibitor recovery 0.39 g protease inhibitors/g pulp protein (Table 5.4 B). The conditions were ran in triplicate with two center points run in parallel as a reference. As shown the actual results for recovered protease inhibitors was 0.35 g protease inhibitors/g pulp protein, which recovered protease inhibitors fell into the probability interval and therefore the predictive model was validated and accepted.

Table 5.3 Analysis of variance for responses: recovered patatin amount from Depol 670 L-based treated potato pulp; recovered protease inhibitors amount from Ceremix 2XL-base treated potato pulp.

	Recovered Patatin Amount (g/ g pulp protein)		Recovered Protease Inhibitors Amount (g/ g pulp protein)	
	F-value	*p*-value	F-value	*p*-value
Model	6.64	0.0071	5.61	0.0124
Endo-β-1,4-Galactanase units of Multi-enzymatic system/ g pulp (x_1)	3.57	0.088	0.003786	0.9522
Incubation Time (x_2)	7.39	0.0216	11.85	0.0063
$(x_1)^2$	11.85	0.0063	4.89	0.0515
$(x_2)^2$	----[a]	----[a]	7.38	0.0217
(x_1x_2)	8.96	0.0135	-----[a]	-----[a]
Lack of fit	0.72	0.6575	1.78	0.3423

[a]Not significant

Table 5.4 Model Validation for (A) Depol 670L (DEP)-based recovered patatin amount and (B) Ceremix 2XL (CER)-based recovered protease inhibitors amount.

		Predicted	Actual	Probability Interval
A *DEPOL 670L*		g patatin/ g pulp protein	g patatin/g pulp protein	g patatin/ g pulp protein
Units (endo-β-1,4-galactanase units/ g pulp)	4.62	0.34	0.38	0.20-0.48
Time (hr)	7			
B *CEREMIX 2XL*		g PI[a]/ g pulp protein	g PI/g pulp protein	g PI/ g pulp protein
Units (endo-β-1,4-galactanase units/ g pulp)	151.84	0.39	0.35	0.22-0.56
Time (hr)	26			

[a]Abbreviation PI, protease inhibitors

5.4.2.1. Effect of reaction parameters

The relationships between the reaction parameters and the responses can be better understood by studying the response surface plots of the fitted models. The response surface plots presented in Figure 5.2 illustrates the interaction effect of endo-β-1,4-galactanse units (expressed in the multi-enzymatic system) and incubation time on the predicted DEP-based recovered patatin (A) and the CER-based recovered protease inhibitors (B). The DEP-based recovered patatin amount was found to vary between 0.224 (purity of 0.49) and 0.471 (purity of 0.6) g patatin/g pulp protein (Table 5.2 A). When examining the surface plot Figure 5.2 A, a convex peak maximum is shown at relatively high endo-β-1,4-galactanase units of DEP (x_1-13.86 endo-β-1,4-galactanase units of DEP g pulp) and short time (x_2-0.96 hr). Increasing endo-β-1,4-galactanase units of DEP (x_1) to 15.71 U/g pulp and maintaining shorter reaction time (x_2) at 0.96 hr resulted in a relatively high recovery of patatin. While increasing reaction time (x_2) and/or lowering endo-β-1,4-galactanase units (x_1) led to low recovered patatin. This phenomenon could be due to the fact that increased incubation time may have led to small structural changes of patatin and to its unfolding. A result of this denaturation effect, patatin may have been retained in the degraded pulp instead of suspending into the supernatant, resulting in an apparent decrease of the recovered patatin. However, to preserve the extracted patatin temperatures were maximally set at 45 ºC as patatin begins to unfold above these conditions (Waglay, et al., 2014). Moreover, short times will tend to potentially limit (a) the negative interaction effect between starch removing and polysaccharide degrading enzymes and (b) the inhibiting effect of potato protease inhibitors.

The extracted protease inhibitors recovered varied from 0.122 (purity of 0.44) to 0.488 (purity of 0.72) g protease inhibitors/g pulp protein (Table 5.2 B). The surface plot shows (Figure 5.2 B) decreasing both variables to minimum values (x_1- 60.0 endo-β-1,4-galactanase units of CER/g pulp and x_2-0.65 hr) or increasing both variables to maximal values (x_1 - 167.6 endo-1,4-β-galactanase units of CER/g pulp, x_2- 30.3 hr) resulted in high protease inhibitors recovery. A concave peak minimum was exhibited around the centerpoint for both variables x_1- 113.94 endo-β-1,4-galactanase units of CER/g pulp and x_2-15.5 hr.

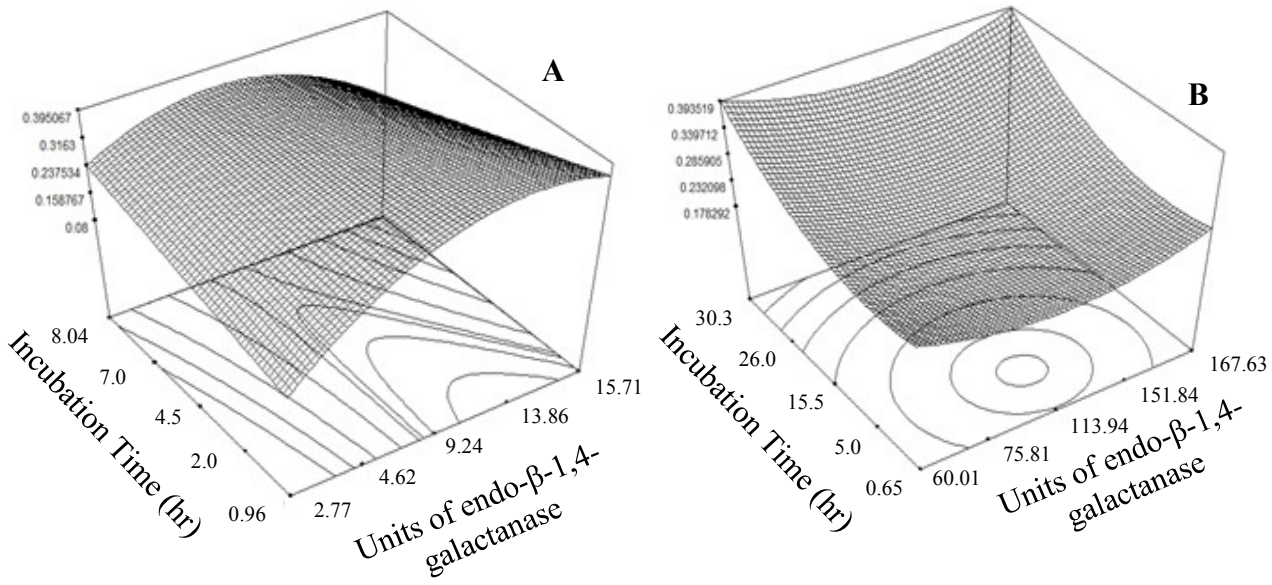

Figure 5.2 Response surface plots of recovered proteins from potato pulp as affected by reaction time and units of endo-β-1,4-galactanase expressed in Depol 670L (DEP) and Ceremix 2XL: (A) DEP-based recovered patatin amount and (B) CER-based recovered protease inhibitors amount.

To our knowledge, no literature is available characterizing the proteins recovered using enzymatic isolation, other than our previous findings, using pure enzymes endo-polygalacturonase and endo-β-1,4-galactanase. Similaraly, extracts contained a higher recovered patatin upon the use of shorter time (19.72 hr) and high endo-β-1,4-galactanase units (8.88 U) (Waglay, et al., 2015), whereas extracts with higher recovered protease inhibitors were obtained with longer time (21.0 hr) and lower endo-β-1,4-galactanase units (1.5 U) (Waglay, et al., 2015).

5.3.1. Functional Properties of Enriched Potato Protein Extracts

The LAH activity of the isolated patatin-enriched extract recovered upon enzymatic treatment of potato pulp with DEP multi-enzymatic system was assessed and compared to the currently employed industrial technique, a combination of thermal/acidic treatment. Table 5.5 shows that thermal/acidic treatment, resulted in low to no detectable LAH. These results may be attributed to the poor solubility, thermal denaturation, and conformational changes of patatin. Pots et al. (1998) reported that temperatures ranging from 46-55 ^{0}C initiates unfolding of patatin's secondary structure and complete unfolding occurs at 70 ^{0}C. The results also show that the DEP-based patatin enriched protein extract exhibited high specific activities of LAH. These results indicate that the enzymatic approach retained patatin's conformational structure to the greatest extent compared to patatin recovered by the currently employed industrial technique. As well as, these findings may be attributed to the enrichment achieved with the enzymatic approach. In addition, using 4-nitrophenyl laurate as substrate resulted in higher LAH activities (Table 5.5), indicating the high specificity of patatin towards this long chain fatty acid. Similar findings were encountered by Bohac, (1991) where using the substrate 4-nitrophenyl laurate a 1.3 increases in the LAH activity of patatin extracted with $(NH_4)_2SO_4$ at 40-70% saturation. Moreover, our previous findings (Waglay, et al., 2014) found a 2.4 higher LAH activity toward 4-nitrophenyl laurate than 4-nitrophenyl butyrate for 60% $(NH_4)_2SO_4$ saturation-based protein isolate. Comparable LAH activity was achieved for DEP-based patatin enriched protein extract using 4-nitrophenyl butyrate as a substrate. However using 4-nitrophenyl laurate resulted in roughly half the LAH activity for DEP-based patatin enriched protein extracts.

Table 5.5 Lipid acyl hydrolase (LAH) activity expressed in the protein extract, recovered upon combined thermal/ acidic treatment of potato fruit juice, and in the DEP-based patatin enriched extract, obtained upon enzymatic treatment of potato pulp with Depol 670L.

	Lipid acyl hydrolase activity[a]			
Extraction Techniques	p-Nitrophenyl Butyrate		p-Nitrophenyl Laurate	
	Specific Activity (μmole/(min. mg patatin))	Total Units[b] (per 1 mg protein extract)	Specific Activity (μmole/(min. mg patatin))	Total Units[b] (per 1 mg protein extract)
Thermal/Acidic Combination[c]	<0.04		<0.04	
DEP-based Multi-enzymatic recovery[d]	0.31 (±0.10)[e]	0.16 (±0.05)	0.51 (±0.04)	0.27 (±0.02)

[a]Lipid acyl hydrolase activity was investigated using p-nitrophenyl butyrate and 4-nitrophenyl laurate as substrates.
[b]Total lipid acyl hydrolase units (μmole/min) expressed in 1 mg of potato protein extracts.
[c]Combination: thermal and acidic precipitation involved heat treatments up to 100^0C with pH adjustment to 2.5.
[d] DEP-based system for the isolation of a fraction enriched with patatin.
[e]Standard deviation was calculated from duplicate trials.

The protease inhibiting activity of thermal/acidic protein extract and CER-based protease inhibitor enriched extract against trypsin and chymotrypsin was investigated. The results (Table 5.6) indicate that protease inhibitor enriched extract exhibited higher protease inhibiting activities against trypsin than chymotrypsin, whereas thermal/acidic protein extract showed an opposite trend. A previous study conducted by Pouvreau et al. (2001) recovered mainly the protease inhibitor II (~20 kDa) which have elevated (82%) trypsin protease inhibiting activity, whereas protease inhibitor I (< 15 kDa) have around 50% chymotrypsin inhibiting activity.

Table 5.6 compared both potato protein extracts, and the thermal/acidic-based protein extract showed the lowest (363.5 mg inhibited protease/g protein) and the highest (427.2 mg inhibited protease/g protein) inhibiting activity against trypsin and chymotrypsin, respectively. The low trypsin inhibiting activity of thermal/acidic protein extract may be due to the conformational changes and low solubility of the protease inhibitor II fraction by their recovery under thermal (100 °C, 2 min) and acidic conditions (pH 2.5), as potato protease inhibitors begin to denature between 55-70 °C (van Koningsveld, et al., 2001). Contrarily, the high recovery of potato inhibitors I (<15 kDa), which have a high affinity towards chymotrypsin, in thermal/acidic-based potato protein extract can be due to their better structural conservation in thermal environments. The results (Table 5.6) also indicate that multi-enzymatic system CER based-recovered protein extract showed high trypsin inhibiting activity, confirming the isolation of non-denatured protease inhibitor II.

Table 5.6 Inhibiting protease activities against Trypsin and Chymotrypsin of the protein extract, recovered upon combined thermal/ acidic treatment of potato fruit juice, and of the CER-based protease inhibitor enriched extract obtained upon enzymatic treatment of potato pulp with Ceremix 2XL.

	Inhibiting Protease Activity[a]			
Extraction Techniques	Trypsin		Chymotrypsin	
	(mg Protease/g potato protein extract)[b]	(mg Protease/g potato protein inhibitors)[c]	(mg Protease/g potato protein extract)[b]	(mg Protease/g potato protein inhibitors)[c]
Thermal/Acidic Combination[d]	363.5	705.5	427.4	829.4
CER-based Multi-enzymatic recovery	834.4	1817.9	168.9	368

[a]Inhibiting protease activity against trypsin and chymotrypsin was estimated from the decrease in the protease activity in the presence of potato extracts containing protease inhibitors

[b]Inhibiting protease activity is expressed as mg protease inhibited by g potato protein extracts

[c]Inhibiting protease activity is expressed as mg protease inhibited by g protease inhibitors present in the potato protein extracts. The weight percentage of the protease inhibitors was estimated by electrophoretic analyses

[d]Combination: thermal and acidic precipitation involved heat treatments up to 100^{0}C with pH adjustment to 2.5, results previously reported in Waglay et al., 2014.

5.3.2. Structural Characterization of Enriched Potato Protein Extracts

The structures of patatin and protease inhibitors recovered by DEP and CER-based enzymatic treatment of potato pulp, respectively, were characterized by peptide mass mapping, consisting of the tryptic hydrolysis of proteins followed by mass spectrometric analysis of the released peptides. Tables 5.7 and 5.8 show the peptides masses following tryptic hydrolysis of the DEP and CER-based protein extracts corresponding to those of patatin (accession number: Q2MY36, molecular weight: 42, 617.3 Da) and protease inhibitors (API11_SOLTU, 20, 590.3 Da), respectively.

The SDS-PAGE analysis of enriched patatin DEP-based protein extract reveals an abundant band with an approximate molecular weight of 43 kDa (data not shown). The peptide mapping revealed 76% homology of enriched patatin DEP-based band with that corresponding to accession number Q2MY36 with a molecular weight of 42, 617.3 Da. The DEP-based protein extract was a mixture of proteins, matched varying proteins with differing homologies. Such as patatin with accession number Q2MY44, molecular weight 42, 650.1 Da, accession number Q42502, molecular weight 42, 467.0 Da, accession number Q3YJT4, molecular weight 41, 316.4 Da, and accession number Q2MY48, molecular weight 42, 557.8 Da.. Patatin has been identified to be a dimer with a molecular weight around 88 kDa (Ralet & Guéguen, 2000). The encountered homology differences could be due to the cultivar variation, as well as to the presence of different isoforms with selected glycosylation (Pots, et al., 1999). Indeed, slightly lower molecular weight of patatin from cv. Bintje of 40, 345-41, 590 Da were determined by Pots et al., (1999) using matrix-laser desorption ionization time-of-flight mass spectroscopy.

Potato protease inhibitors are of high interest as they have been studied to possess anticarcinogenic ability as they can interfere in tumor-cell growth, peroxide formation, and processing involving solar-UV irraditation (Blanco-Aparicio, et al., 1998; Frenkel, et al., 1987). In addition, they have been shown to participate in the cholecystokinin pathway, which provides a satiety affect (Deveaux-Gobert, 2008). One protein band with approximate molecular weights of 23 kDa from the CER-based enriched protease inhibitor extract was analyzed by mass mapping. The most prominent protease inhibitors as determined by peptide mapping consisted of aspartic protease inhibitor 11 (accession number API11_SOLTU) and cysteine protein inhibitor 10 (accession number

CPI10_SOLTU). The highest homology was for protease inhibitors accession number API11_SOLTU with a molecular weight of 20, 590.3 Da, which was detected with 82% homology. This protease inhibitor mass corresponded to the most abundant group (relative proportion of 14%) as determined by SDS-PAGE analysis of CER-based enriched protease inhibitor extract (data not shown).

Table 5.7 Peptide composition of enriched extract of patatin isolates using Depol 670L.

Residues		Sequence	Number of Peptides	Actual Mass (amu)
25	40	(K)LEEMVTVLSIDGGGIK(G)	8	1675.87
41	62	(K)GIIPAIILEFLEGQLQEVDNNK(D)	5	2452.33
41	65	(K)GIIPAIILEFLEGQLQEVDNNKDAR(L)	29	2794.49
66	100	(R)LADYFYVIGGTSTGGLLTAMITTPNENNEFAAAK(D)	79	3641.8
129	137	(K)YLLQVLQEK(L)	72	1132.65
143	158	(R)VHQALTEVAISSFDIK(T)	132	1756.94
159	167	(K)TNKPVIFTK (S)	3	1046.61
180	234	(K)MYDICYSTAAAPIYFPPHHFVTHTSNGATYEFNLVDG GVATVGDPALLSLSVATR(L)	23	5929.85
235	246	(R)LAQEDPAFSSIK(S)	66	1304.66
247	268	(K)SLDYKQMLLLSLGTGTNSEFDK(T)	1	2459.23
252	268	(K)QMLLLSLGTGTNEFDK(T)	65	1852.92
252	277	(K)QMLLLSLGTGTNSEFDKTYTAEEAAK(W)	79	2833.37
269	277	(K)TYTAEEAAK(W)	18	982.46
269	282	(K)TYTAEEAAKWGPLR(W)	1	1591.8
283	310	(R)WMLAIQQMTNAASSYMTYYISTVFQAR(H)	25	3306.53
311	318	(R)HSQNNYLR(V)	3	1030.49
319	351	(R)VQENALTGTTTEMDDASEANMELLVQVGETLLK(K)	19	3581.7
357	367	(K)DSPETYEEALK(R)	9	1280.58
357	368	(K)DSPETYEEALKR(F)	31	1436.68

Table 5.8 Peptide composition of enriched extract of protease inhibitor isolated using Ceremix 2XL.

Residues		Sequence	Number of Peptides	Actual Mass
1	14	(-)ESPLPKPVLDNGK(E)	6	1494.8
1	23	(-)ESPLPKPVLDTNGKELNPNSSYR(I)	59	2538.27
15	23	(K)ELNPNSSYR(I)	6	1079.49
30	40	(R)GALGGDVYLGK(S)	33	1048.56
30	54	(R)GALGGVYLGKSPNDAPCPGVFR(Y)	10	2548.2
41	54	(K)SPNDAPCPGVFR(Y)	63	1517.66
55	67	(R)YNSDVGPSGTPVR(F)	65	1347.65
92	100	(K)LCVSYTIWK(V)	5	1168.59
101	109	(K)VGNLNAFR(T)	10	1052.54
110	128	(R)TMLLETGGTIGQADSSYFK(I)	17	2033.97
132	154	(K)LSNFGYNLLYCPITPPFLCPFCR(D)	2	2848.36
162	170	(K)LSNFGNLLYCPITPPFLCPFCRDDNFCAK(V)	1	3698.68
162	170	(K)VGVVIQNGK(R)	11	913.53
162	171	(K)VGVVIQNGKR(R)	9	1069.62
172	188	(R)RLALVNENPLDVLFQEV(-)	3	1968.07
173	188	(R)LALVNENPLDVLFQEV(-)	2	1811.97

According to Pouvreau et al. (2001), the potato aspartate protease inhibitors (12%) are the third most abundant group in PFJ prepared from cultivar Elkana following potato inhibitor II and potato cysteine protease inhibitors. This group is subcategorised by different molecular mass, isoelectric points, inhibiting activity, and proportion present in PFJ (Pouvreau, et al., 2001). According to their findings the two most abundant subcategories include those with molecular weight ranging from 20 039-22 025 Da (Pouvreau, et al., 2001), which confirms the molecular weight determined 20 590.3 Da and 82% homology.

5.4. Conclusion

This study screened several commercially available multi-enzymatic systems containing several glycosyl-hydrolase activities for an efficient isolation of potato proteins by degrading plant cell wall constituents. This process was further optimized in order to have the ability to improve patatin recovery and protease inhibitors recovery using DEP and CER, respectively. DEP was found to be significantly affected by the interaction effect of addition of units of enzyme and incubation time in addition to the quadratic effect of the addition of enzyme. While CER was found to be affected by the quadratic effect of both variables addition of enzyme and incubation time. The developed enzymatic approach has the potential to isolate potato proteins and fractionate the proteins to their corresponding fraction namely patatin and protease inhibitors with minimal deleterious effects, as indicated by preservation of LAH activity and protease inhibiting activity specifically, trypsin inhibition. This approach will help broaden their applications as value added ingredients taking advantage of their associated functional and health benefits.

CONNECTING STATEMENT 4

Optimized methods for the extraction of potato proteins, with a high proportion of patatin or protease inhibitors from potato pulp was achieved using commercial enzymes Depol 670L and Ceremix 2XL, respectively (Chapter V). Chapter VI compares the conventional extraction technique with 60% ammonium sulphate saturation and ultrafiltration techniques with the novel multi-enzymatic method using Depol 670L. These techniques were scaled up to a pilot scale environment using 170 kilograms of potato. The extracts were compared according to their protein and extract recovery yield, protein proportion, structural changes, and functional properties.

The results from this study were presented at the 12th International Congress on Engineering and Food.

Waglay, A., Achouri, A., Karboune, S., Zareifard, M.R., & L'Hocine, L. (2015) Pilot Plant Extraction of Potato Proteins and their Application as a Value Added Ingredient. ICEF12, Quebec, Canada, June 14- June 18, 2015.

Waglay, A., Achouri, A., Karboune, S., Zareifard, M.R., & L'Hocine, L. (2015) Pilot Plant Extraction of Potato Proteins and their Structural and Functional Properties. *(To be submitted).*

CHAPTER VI. PILOT PLANT EXTRACTION OF POTATO PROTEINS AND INVESTIGATION OF THEIR STRUCTURAL AND FUNCTIONAL PROPERTIES

6.1. Abstract

In the present study, three processes were scaled up to pilot scale environment for the isolation of potato proteins from imitation by-products of potato fruit juice (PFJ) and potato pulp. Two of these processes were based on the isolation of potato proteins from PFJ by (1) subsequent-ultrafiltration steps (PPC UF) or (2) protein precipitation at 60% ammonium sulphate ($(NH_4)_2SO_4$) saturation (PPI AS). The other process was performed on potato pulp, (3) involving the use of a multi-enzymatic system (Depol 670L) (PPC Enz) to degrade the plant cell wall for an efficient release of proteins. The extraction techniques were compared according to their protein recovery yield, protein profile, conformational changes, and functional properties. PPI AS and PPC Enz-based processes resulted in improved protein recovery yield from 21.6 (PPC UF) to 44.6% and 29.1%, respectively. In terms of protein profile, all extracts showed similar recovery of patatin, with a wide difference in recovered protease inhibitors. Structurally, all extracting agents had an effect on the secondary structure of the isolated proteins, where the presence of carbohydrates in PPC UF and PPC Enz provided a greater preservation against denaturation and structural reversibility after heat treatments. PPC Enz had improved functionality in terms of emulsifying activity index and foam expansion, whereas, emulsion and foam stability were better preserved with PPC UF and PPI AS.

6.2. Introduction

The most important macronutrient for human consumption is believed to be proteins (Han, et al., 2015). There is ongoing debate as to which source from either animal or vegetable protein is superior. However, with the growing population, plant proteins offer a more sustainable and cost effective source (Pimentel & Pimentel, 2003). To that end, potatoes are among the largest protein supplying crop per hectare grown, when compared to other vegetables, cereals, and seed sources (Desborough & Weiser, 1974). The use of potato proteins as a functional ingredient is an attractive source due to their promising functionalities, including foaming (Partsia & Kiosseoglou, 2001; Ralet & Guéguen, 2001; van Koningsveld, et al., 2002), emulsifying (Romero, et al., 2011; van Koningsveld, et al., 2006), and gelling abilities (Creusot, et al., 2010).

Several studies (Bártova & Bárta, 2009; Gonzalez, et al., 1991; Ralet & Guéguen, 2000; Knorr, et al., 1977; Vikelouda & Kiosseoglou, 2004; Miedzianka, et al., 2014) have examined varying extraction techniques for the recovery of potato proteins from waste streams generated from the starch industry known as potato fruit juice (PFJ) and potato pulp; these by-products contain a crude protein content of 50% (Vikelouda & Kiosseoglou, 2004) and 74% w/w (dry weight) (Kamnerdpetch, et al., 2007), respectively. The investigated extraction techniques include thermal and acidic coagulation, salt precipitation, alcohol extraction, complexing agents, enzymatic isolation, and column chromatography. Thermal and acidic coagulation resulted in higher yields (>85%) (Knorr, et al., 1977; Miedzianka, et al., 2012; Waglay, et al., 2014), when compared to acidic precipitation alone (~60%) (Knorr, et al., 1977; Waglay, et al., 2014). Ferric chloride precipitation showed conflicting results. Indeed, Bártová and Bárta (2009) reported an increase in the recovery yield of potato proteins with increasing concentration, while Waglay et al. (2014) showed an opposite dependence on concentration, where low concentrations resulted in better protein purification. Ammonium sulphate ($(NH_4)_2SO_4$) precipitation demonstrated the highest yield of 98% (Waglay, et al., 2014). The complexing agent carboxymenthyl cellulose requires a pH adjustment to 2.5 which is between the pKa value of the polysaccharide and the protein's isoelectric point in order to promote the interaction between polysaccharide and protein (Gonzalez, et al., 1991). However, this pH adjustment has a negative effect with limited patatin being extracted (Waglay, et al., 2014). Recently, our group has developed an enzymatic approach for potato protein isolation using pure enzymes and commercially available multi-enzymatic systems possessing glycosyl-hydrolase activity (Waglay, et al., 2015). Due to the selectivity and specificity of the enzyme actions, the potato proteins are able to be isolated with different proportions of their fractions, namely patatin and protease inhibitors. In addition, to the ability to fractionate the proteins, this process is able to extract the proteins with retained functionality, due to the mild operating conditions required such as low temperature (<45 °C) and pH 5 (Waglay, et al., 2015). All these techniques with the exception of chromatography have been limited to laboratory scale isolation, which results in high yields but minimal protein quantities. Recovery of potato proteins using chromatographic techniques on a pilot scale examined the use of expanded bed adsorption for the recovery of patatin, which resulted in a recovery

ranging from 37-50%; however, this process is associated with a large operating cost (Løkra, et al., 2008). While simulated moving bed separation resulted in an 82% protein recovery and lower purity with increasing passes through the process (Andersson, et al., 2008).

Currently, limited studies are available on the scale-up for the recovery of potato proteins from by-products PFJ and potato pulp. The present study is aimed at the investigation of the efficiency of three selected extraction methods, namely ultrafiltration, $(NH_4)_2SO_4$ precipitation, and enzymatic approach (multi-enzymatic Depol 670L), for the isolation and the recovery of potato protein isolate and concentrate from imitation industrial by-products, either PFJ or potato pulp, at a pilot-scale level. The structural and functional characteristics of the protein isolate and concentrates produced using the three selected pilot-scale extraction procedures were also studied.

6.3. Materials and Methods

6.3.1. *Materials*

Fresh potatoes of Russet Burbank variety were purchased from a local supermarket. Sodium metabisulfite and Termamyl alpha-amylase (*Bacillus licheniformis)* were purchased from Sigma Chemical Co. (MO,USA). Food grade sodium sulfite and ammonium sulphate was purchased from Quadra Ingredients (Qc, CA). Bradford reagent and SDS-PAGE broad molecular weight standard were obtained from Bio-Rad (On, CA). Tris base and potassium phosphate dibasic were acquired from Fisher Scientific (NJ, USA). Potassium phosphate monobasic was purchased from MP Biomedicals, LLC (OH, USA). Commercial multi-enzymatic system Depol 670L from *Trichoderma reesei,* was provided by Biocatalyst Ltd (IL, USA).

6.3.2. *Potato protein isolation on lab-scale*

6.3.2.1. Ammonium sulphate precipitation

PFJ was prepared according to the modified method of van Koningsveld, et al. (2001). Potatoes of Russet Burbank variety were washed and chopped into large pieces. Potato samples (100 g) were suspended in 50 mL sodium sulfite solution (1 g/L) to prevent polyphenol oxidation and homogenized using a Waring Commercial Blender on low speed for 5 min, after which the potato slurry was subjected to cheese cloth filtration. The resulting

turbid liquid was centrifuged at 8000*g* for 30 min at 4 °C using a Beckman Centrifuge Model J2-21. The yellowish filtrate is known to be similar to industrial PFJ, and was lyophilized prior to use. Lyophilized PFJ was then suspended in water and subjected to 60% $(NH_4)_2SO_4$ saturation with constant stirring for 1.5 hr at 4 °C. The precipitate was recovered by centrifugation (8000*g*, 50 min). The recovered protein precipitate was then dialysed for 2 days with a molecular weight cut off of 3000-6000 Da. The protein content of the recovered precipitate and supernatant were determined in triplicate.

6.3.2.2. Enzymatic-based isolation

Potato pulp was prepared with Russet Burbank potatoes. The potatoes were washed and finely chopped into 0.5 g/mL samples. The potato pieces were ground with a mortar and pestle for 1 min with 1.315 mM sodium metabisulfite. The ground pieces were homogenized using a Waring commercial Blender on low speed for 1 min. Termamyl was added to the slurry to yield 1220.63 U of α-amylase/g potato pulp. Reactions were performed in duplicate. Reactions were stirred for 17 hr at 25 °C. Following the incubation with the starch degrading enzyme, Depol 670L multi-enzymatic preparation (2.03 U of galactanase/g potato pulp) was added, and the mixtures were stirred for 5 hr at 25 °C. After incubation, the reaction mixtures were vacuum filtered using 1.2 μm GF/C Whatman filters and the supernatant containing proteins were recovered. The protein content of the recovered degraded pulp and supernatant were determined in triplicate.

6.3.3. *Potato protein isolation on pilot-plant scale*

6.3.3.1. Ultrafiltration and ammonium sulphate precipitation

To prepare the PFJ, potatoes were washed and chopped into large (1 cm^3) pieces using a Big Chop MC15 (Stephan Mikrocut, Hamelan, GE) (Figure 6.1). The entire potato sample (170 kg) was suspended in 98 L of sodium sulfite solution (6.1 mM) to prevent polyphenol oxidation and homogenized to cubes of 1 mm^3, using a chopper GK (Urshel Laboratories Inc., IN, USA). The potato slurry was subjected to constant stirring for 1 hr at 2 °C. The resulting turbid liquid was decanted at a speed of 2,756*g* for 20-30 sec, with a feed rate of 380 L/hr using a CBB Decanter s.r.1 DR250-EF (Drycake, Sr, CA). The opaque juice was then subjected to centrifugation with speed of 14,475*g* and feed rate of 200 L/hr using a

Dexter MiSR 1010 (SRS A USI Company, MI, USA). The clear yellowish filtrate is known to be similar to industrial PFJ. A fraction of PFJ was ultrafiltrated using A Koch Hollow Fiber Cartridge (Koch Membrane Systems Inc., MS, USA) with a molecular weight cut off of 5000 Da. Following the ultrafiltration, the concentrate (four times) was diafiltered two times with equal water additions. The collected retentate was lyophilized and the protein content of the retentate was determined, in triplicate. The freeze dried powder of potato protein isolated by ultrafiltration of PFJ is further abbreviated as PPC UF.

The other fraction of PFJ was subjected to 60% saturation with $(NH_4)_2SO_4$ at 2 °C with constant stirring for the precipitation of proteins. The precipitation occurred for 17 hr, after which the suspension was centrifuged with a speed of 14,475*g* and feed rate of 200 L/hr using a Dexter MiSR 1010 (SRS A USI Company, MI, USA). A subsequent ultra and dia-filtration (3 times) steps were performed, on the recovered precipitate, using a Koch Hollow Fiber Cartridge (with a molecular weight cut off of 5000 Da). The retentate was collected on ice and lyophilized. The protein content of the recovered precipitate was determined, in triplicate. The freeze dried powder of potato protein isolated by 60% $(NH_4)_2SO_4$ saturation is further abbreviated as PPI AS.

6.3.3.2. Enzymatic-based isolation

Potatoes of Russet Burbank were used to prepare potato slurry as described above. Starch degrading enzyme, Termamyl (α-amylase) (250 mL), was added to the slurry, and the mixture was incubated at 25 ^{0}C for 17 hr. This was followed by the addition of 2.03 galactanase units/g of potato of Depol 670L, after which the mixture was incubated for 5 hr at 25 ^{0}C, with constant stirring (1*g*). The resulting turbid slurry was decanted at a speed of 2,756*g* for 20-30 sec, feed rate 200 L/hr using a CBB Decanter s.r.1 DR250-EF. The opaque juice was then subjected to centrifugation with speed of 14,475*g* and feed rate of 200 L/hr using a Dexter MiSR 1010. A Koch Hollow Fiber Cartridge (Koch Membrane Systems Inc., MS, USA) was used with a molecular weight cut off of 5,000 Da for the ultra and dia-filtration steps. The collected supernatant was ultrafiltered to concentrate the retentate 2.4 times, which was followed by diafiltration steps with 3 times equal volume additions with water. The recovered supernatant and degraded pulp were lyophilized and

their protein content were determined, in triplicate. The freeze dried powder of potato protein isolated by enzymatic method is further abbreviated as PPC Enz.

6.3.4. *Compositional analysis of potato protein isolate and concentrates*

6.3.4.1. Protein content

Nitrogen content was determined according to the modified method of Kirsten & Hesselius (1983) using Leco® TruSpec N (Leco Corporation, MI, USA). Prior to nitrogen determination, samples were freeze dried and stored at -80 °C. Nitrogen content was multiplied by a factor of 6.25 to determine the total crude protein content (van Gelder, 1981). Based on the protein content, the yield was calculated as:

$$\text{Yield (\%)} = (\text{Total protein content}_{\text{isolate or concentrate}} / \text{Total protein content}_{\text{PFJ or pulp}}) \times 100 \quad (15)$$

6.3.4.2. Carbohydrate content

Carbohydrate content of the potato protein isolate and concentrates was determined using phenol-sulfuric acid colorimetric assay (Dubois, et al., 1956). The molecular weight distribution of the carbohydrates was analyzed by high-performance size exclusion chromatography using a Waters HPLC system Model 25P (Waters Corporation, Maine, USA) equipped with a refractive index detector. Three columns were connected in series at 25 °C (TSK G3000 PWXL, TSK G4000 PWXL, and TSK G5000 PWXL, Tosoh Bioscience, Montgomeryville, PA) An isocractic elution at a flow rate of 0.4 mL/min using 0.1 M sodium chloride was employed. A standard curve was developed using dextrans with known molecular weights of 50, 150, 270, 410, and 670 kDa.

6.3.5. *Structural characterization of potato protein isolate and concentrates*

6.3.5.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a Protean II electrophoresis apparatus (Bio-Rad Laboratories, CA, USA), and Ready Gel precast gels (Any kDa) polyacrylamide Tris-HCl. Protein samples (PPC UF, PPI AS, and PPC Enz) of 5 mg/mL were dispersed in 1 mL of Laemmli sample buffer (Bio-Rad, On, Ca) with the addition of 2% SDS and 5% β-mercaptoethanol and vortexed. After loading, gels were run at constant voltage (200 V) in Tris-glycine buffer containing 0.1% SDS. Gels were stained with Coomassie Brillant

Blue R-250. A Precision Plus Protein Dual Xtra Standards (Biorad, On, Ca) was used as a molecular marker, ranging in molecular weights from 2 to 250 kDa.

6.3.5.2. High performance size exclusion liquid chromatography

High performance size exclusion chromatography (HPSEC) was conducted as previously described by Achouri et al. (2010) for the analysis of potato protein isolate and concentrates, using an Agilent Bio SEC-3 column (7.8 x 300 mm) connected to an Agilent-1200 Series HPLC system (Agilent Technologies, On, CA). The elution was carried out with 10 mM phosphate buffer containing 150 mM NaCl (pH 7.8) at a flow rate of 1 mL/min. The detection was monitored at 280 nm. Mixed gel filtration standards comprising thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa) were used to estimate the molecular masses.

6.3.5.3. Differential scanning calorimetry

Calorimetric measurements were taken using a 2910 modulated differential scanning calorimeter (TA Instruments Inc., DE, USA). The instrument was calibrated using indium as a standard. Potato protein samples (10 mg) were dispersed in D_2O-phosphate buffer prepared at pH 7, all analyses were performed in triplicate. Aliquots (20 µL) of the solutions were hermetically sealed in aluminum DSC pans and heated at a scan rate of 10 °C/ min under helium through the range of 20 to 110 °C. A pan filled with 20 µL D_2O-phosphate buffer prepared at pH 7 was used as a reference. Data were analyzed using the Universal Analysis software from TA Instruments. Transition temperatures (T_o, onset temperature of denaturation; T_d, maximum temperature of denaturation) and enthalpies (ΔH; areas below the endothermic curves in joules per g of dry weight) were measured. All samples were run in triplicate.

6.3.5.4. Fourier transform infrared spectroscopy

For FTIR analysis, potato protein dispersions were prepared by dissolving PPC UF, PPI AS, and PPC Enz (10 mg protein) in D_2O-phosphate buffer prepared at pH 7. D_2O was used instead of H_2O because of its greater transparency in the infrared amide I' region (Susi & Byler, 1983). Aliquots (10 µl) of each sample were held in an IR cell with a 25 µm

pathlength, between two CaF_2 windows and infrared spectra were recorded at 25 °C with an Excalibur FTIR spectrometer (system FTS 3000) equipped with a deuterated triglycine sulfate (DTGS) detector (Bio-rad Laboratories, MA, USA). The spectrometer was purged with dry air for 10 min before recording the spectrum. A total of 256 scans were averaged at 4 cm^{-1} resolution. The signal-to-noise ratio was > 20000:1. Deconvolution was performed using the Digilab Merlin FTIR software (version 3.4) with a half-bandwidth of 13 cm^{-1} and an enhancement factor of 2.4 (Kauppinen, et al., 1981). The spectra were baseline-corrected between 1750 and 1595 cm^{-1} and normalized by dividing the absorbance value at each wavenumber in this range by the integrated area over this range, with the use of OMNIC software (Nicolet, Thermo Electron Cooperation). Band assignment was assigned according to Kong and Yu (2007), where bands at 1637± 3.0 and 1675 ± 5.0 cm^{-1} as β-sheets, 1645 ± 4.0 cm^{-1} as random coil, 1653 ± 4.0 cm^{-1} as α-helix, and 1671 ± 3.0 cm^{-1} and 1689 ± 2.0 cm^{-1} as β-turns. According to Pots et al. (1998), the band at 1618 cm^{-1} was assigned to intermolecular β-sheet which is associated with aggregation.

6.3.6. *Functional assessment of pilot-plant potato protein isolate and concentrates*

6.3.6.1. Emulsifying activity

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined according to the turbidimetric method of Cameron et al. (1991). 1.5 mL of corn oil was added to 4.5 mL of 0.5% (w/ v) potato protein solution prepared in 0.01 M phosphate buffer (pH 7), after which the mixture was homogenized at 47,850*g* at room temperature for 1 min with a PT 2100 Polytron homogenizer (Kinematica AG, Littau-luzern, CH). 250 µL of the emulsion was taken out from the bottom at different times (0 and 15 min) and diluted with 50 mL of 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was, then, determined at 500 nm with a Cary 300 Bio, UV–Visible Spectrophotometer (Varian Canada Inc., Qc, CA). All measurements were assessed in triplicate. Analysis of variance was performed on the three trials for each extract. The Emulsifying activity index (EAI) and Emulsifying stability index (ESI) were calculated using the following equations:

$$EAI\ \left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A_o \times dilution\ factor}{c \times l \times (1-\varphi) \times 10\,000} \quad (16)$$

$$\text{ESI}\ (min) = \left(\frac{A_o}{A_o - A_{15}}\right) \text{x}\ t \qquad (17)$$

Where, c is the initial protein concentration in potato protein isolate and concentrates solution (g protein/mL), l is the optical path (0.01 m), φ is the oil volume fraction used to form the emulsion, dilution factor is 200, t is 15 min, and A_0 and A_{15} are the absorbance of the diluted emulsions at 0 and 15 min, respectively.

6.3.6.2. Foaming ability

The procedure proposed by Waniska and Kinsella (1979) with some modifications was used for measuring foaming properties. The protein was dispersed in 0.01 M phosphate buffer (pH 7) at concentration of 0.5% (w/ v) with stirring for 10 min at room temperature (25 °C). The protein solution (15 mL) was then injected into the sparging chamber of a water-jacketed glass condenser via the septum-stoppered inlet. Nitrogen gas was sparged into the protein solution until the foam chamber (55 mL) was filled with foam, while simultaneously maintaining the volume of liquid in the sparging chamber by addition of protein solution. The required time to form 55 mL of foam, and the volume of protein solution added were recorded. After 5 min, the volume of liquid drained from the foam was also noted. All analyses were performed in triplicate. Analysis of variance was performed on the three trials for each extract. Several parameters were used to predict foaming properties as follows: Gi (%), which is the percent of gas entrapped in the foam; FE (%), which is the percent foam expansion and R5 (%), which represents the percent of liquid retained in the foam after 5 min. The following equations were used to determine the foaming parameters:

$$\text{G}_\text{i} = \frac{(55 - \text{V}_\text{i})}{(\text{FR})(\text{T}_\text{f})} \text{ x } 100 \qquad (18)$$

$$\frac{\text{G}_\text{i}}{\text{mL sample}} = {}^{\text{G}_\text{i}}/_{\text{V}_\text{i}} \qquad (19)$$

$$\text{FE} = \frac{7\ \theta - \text{V}_\text{o}}{\text{V}_\text{i}} \text{ x } 100 \qquad (20)$$

$$\text{V}_\text{r} = \text{V}_\text{i} - \text{V}_\text{d} \qquad (21)$$

$$\text{R}_5 = \frac{\text{V}_\text{r}}{\text{V}_\text{i}} \text{ x } 100 \qquad (22)$$

Where, G_i is percentage of gas entrapped in 55 mL of foam, FR is gas flow rate (mL/ min nitrogen), T_f is time to fill the column with foam, V_o is the initial volume of sample in the jacketed condenser (15 mL), V_i is the volume of liquid injected, V_d is the volume of liquid drained from the foam after 5 min, V_r is the volume of liquid retained in the foam after 5

min, R_5 is the percentage of liquid retained from the foam after 5 min, FE is the percent of foam expansion, and the total volume of the jacketed glass condenser is 70 mL.

6.4. Results and Discussion

6.4.1. *Recovery of potato proteins using laboratory and pilot-plant scale up isolation*

The three selected extraction methods were performed in a continuous flow pilot plant facility, commencing with the preparation of imitation industry by-products potato fruit juice (PFJ) and potato pulp (Figure 6.1). However, it should be noted that in real applications these by-products would be supplied. Figure 6.1 shows the conversion of 170 kg of potatoes to either PFJ or potato pulp. The PFJ is an imitation of the starch industry by-product, while potato pulp corresponds to the potato processing industry by-product.

PFJ was subjected to ultrafiltration and several diafiltration steps (Figure 6.1), in order to isolate and concentrate the proteins. This resulted in an extract (PPC UF) with a carbohydrate content of 8%, specifically of the oligomers consisting of approximately 40-subunits of mainly glucose (data not shown). As expected, a substantial loss of proteins was found with each ultra- and dia-filtration step. Therefore, precautions should be taken in establishing a compromise between removing interfering sugars and salts with protein loss.

PFJ underwent 60% $(NH_4)_2SO_4$ saturation, with constant gentle stirring at 2 ^{0}C (Figure 6.1). The stirring process needed to be kept minimal and at low temperatures in order to preserve the proteins structure as well as to decrease the occurrence of foaming. Foaming was a prominent occurrence throughout the extraction process, which in turn may have led to the protein loss. Similar to, substantial protein loss resulted from the ultra- and dia-filtration steps, where again interference resulted from the $(NH_4)_2SO_4$ present in the waste-stream permeate. In order to remove the interfering $(NH_4)_2SO_4$, ultrafiltration and extensive diafiltration steps were required. In industrial applications, this waste stream could be used to recover the salt, thereby decreasing associated cost. The third process consisted of the use of commercially available multi-enzymatic system Termamyl (α-amylase) and Depol 670L (glycosyl-hydrolase enzymes), for starch hydrolysis and pectin-network opening, respectively, for an efficient release of proteins. The optimal conditions used were identified in our previous study (Waglay, et al., 2015).

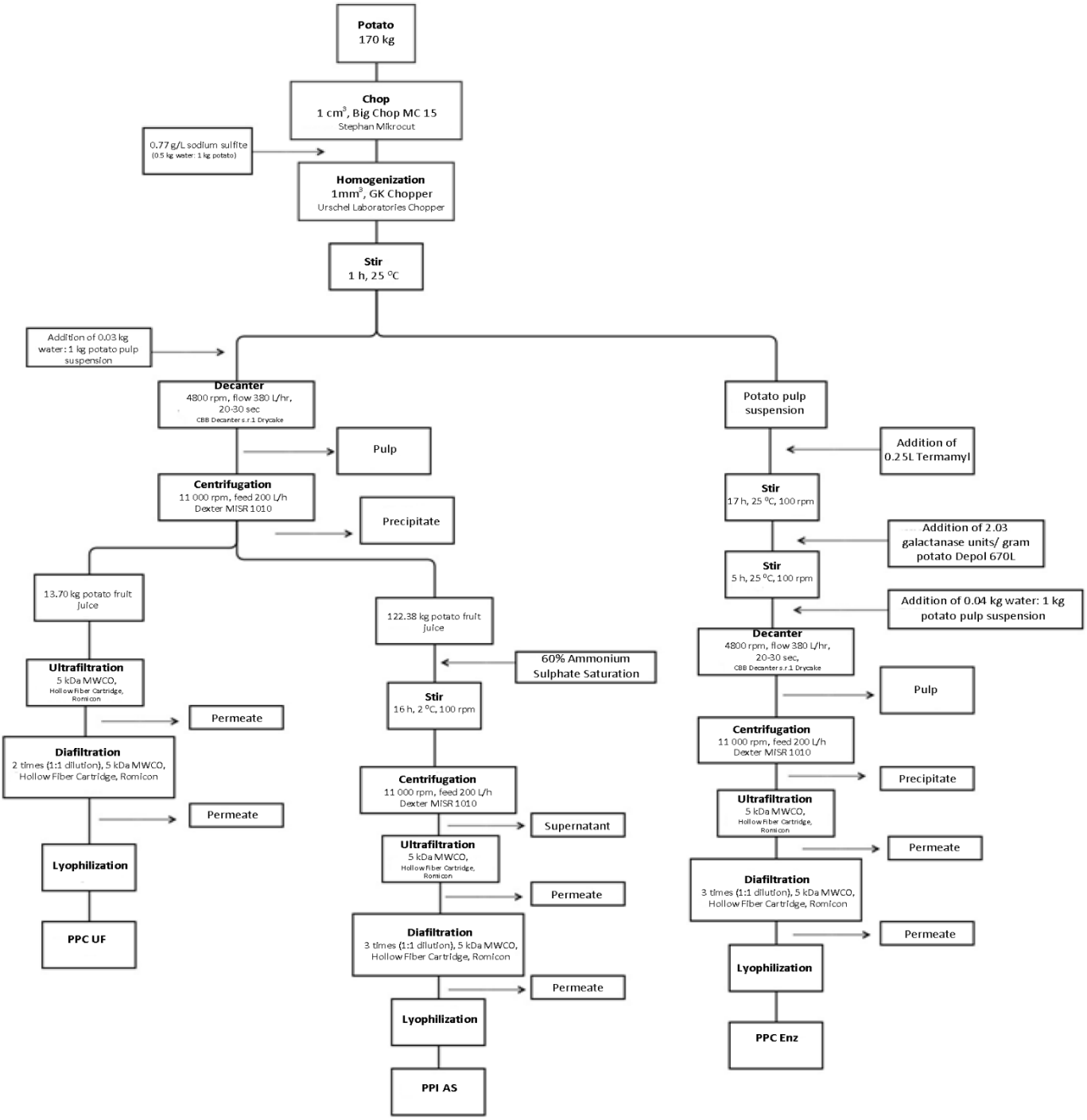

Figure 6.1 Flow diagram of pilot-scale potato protein isolation using three processes: ultrafiltration (PPC UF), 60% ammonium sulphate saturation (PPI AS), and commercial multi-enzymatic system Depol 670L (PPC Enz).

Table 6.1 Effects of extraction process scale-up of potato protein isolate and concentrates on the protein recovery yield, protein content, and relative proportion of potato protein fractions.

	Protein Recovery Yield (%)		Extract Recovery Yield (g/ kg potato)	Protein Content (%)	Relative Proportions[e]				
	Laboratory Scale	Pilot Plant	Pilot Plant	Pilot Plant	Pilot Plant				
					Patatin	Protease Inhibitors			High Molecular Weight
					35-43 kDa	21-25 kDa	15-20 kDa	<15 kDa	
PPC UF[a]	n.d.[d]	21.62	3.79	75.90	21.55	2.82	24.3	26.8	24.5
PPI AS[b]	74.37	44.56	7.27	86.47	25.57	7.19	24.5	31.7	11.1
PPC Enz[c]	50.92	29.09	5.63	40.70	24.53	0	22.6	39.5	13.4

[a]Ultrafiltration occurred on potato fruit juice with a molecular weight cut-off of 5 kDa, abbreviated PPC UF
[b]Ammonium sulphate occurred on potato fruit juice with 60% saturation, abbreviated PPI AS
[c]Multi-enzymatic system used was Depol 670L, abbreviated PPC Enz
[d]Not determined
[e]Relative proportions (%) determined by SDS-PAGE and Alpha View software.

Table 6.1 shows the recovery efficiency of protein isolate and concentrates on the lab- and pilot- scales. The pilot-scale processes were run in a continuous system, whereas at the lab-scale, they were processed as an individual batch system. The results show that the protein recovery yield obtained by $(NH_4)_2SO_4$ precipitation (74.4%) was significantly higher at the laboratory scale; these results can be attributed to the high accessibility of the salt to the proteins. In addition, the lower yield (PPI AS, 44.6%) at pilot scale could be due to losses during decanting and centrifuging as both these machines follow a continuous flow. Similarly, lower yields were previously reported during continuous pilot plant fractionation of soybean proteins when compared to laboratory extraction (Wu, et al., 1999). The enzymatic-based isolation process on pilot-scale (PPC Enz, 29.1%) resulted in more than half the protein recovery efficiency as that on the lab-scale (50.9%). These results reveal that the efficiency of both $(NH_4)_2SO_4$ and the enzymatic approach was affected by the process scale up. Interestingly, higher extract recovery yields per kg of potato of 7.3 and 5.6 g extract/kg potato were obtained for both PPI AS and PPC Enz, respectively, outlining the efficiency of both processes. Contrarily, the protein recovery yield and extract recovery yield for ultrafiltration were low 21.6% and 3.8 g extract/kg potato, respectively (Table 6.1), which emphasizes the need for the addition of extracting agents for the efficient recovery of potato protein. In comparison, PPI AS resulted in a high protein content of 86.5% and therefore is referred to as a marketable protein isolate. While the lower protein content of PPC UF and PPC Enz of 75.9 and 40.7% refers to their commercial potential as protein concentrates.

As far as the authors are aware, to date, the sole technique that has been performed on a pilot scale has been the exploration of several different resins for chromatographic separation. Straetkvern et al. (1999) explored the use of two resins that varied according to ligand concentration, mixed mode ES and mixed mode ExF with patatin recovery of 37 and 50%, respectively. This study confirmed the use of expanded bed adsorption to fractionate the patatin fraction from PFJ while retaining its functionality (Straetkvern, et al., 1999). However, the adsorbent material for this method is expensive for general processing of waste streams. To our knowledge no studies are available on the pilot plant scale up for the recovery of potato proteins from by-products (PFJ and potato pulp) using $(NH_4)_2SO_4$ and commercially available multi-enzymatic systems.

6.4.2. *Structural characterization of protein isolate and concentrates*

6.4.2.1. Molecular weight distribution of protein isolate and concentrates

The molecular weight distribution of PPI and PPCs was characterized by SDS-PAGE electrophoresis and HPSEC. The results are outlined in Table 6.1 and Figure 6.2, respectively. SDS-PAGE electrophoretic profiles indicate that the patatin content of PPI and PPCs varied from 21.5 to 25.6%, with PPI AS having the highest content, followed by PPC Enz. All three extracts of PPI and PPCs were composed of a relatively lower proportion of protease inhibitors ranging from 21-25 kDa, whereas higher proportions were obtained for both 15-20 kDa and <15 kDa (Pouvreau, et al., 2001). PPC UF, in particular, had a high proportion (24.51%) of high molecular weight proteins when compared to the other two processes. This outlines the importance for an extracting agent like $(NH_4)_2SO_4$ or enzymatic systems to increase the specificity towards the fractions of interest, namely patatin and protease inhibitors.

Our previous study outlined the protein profile of PPI AS on laboratory scale where higher proportion of patatin of 36.1% was obtained (Waglay, et al., 2014), as compared to 24.5% for the pilot scale process. A similar relative proportion was obtained for high molecular weight proteins of 11.2% and 11.1% for both laboratory and pilot plant processes. Contrary to the pilot-scale Depol 670L multi-enzymatic-based extraction, the laboratory scale extraction resulted in a protein concentrate with a higher proportion of patatin (49.78%), lower protease inhibitors (27.02%), and similar proportion of high molecular weight proteins (13%; data not shown). These differences could be attributed to the non-uniform distribution of enzyme and to the non-control of the temperature in the large batch reactions. These may have decreased the efficiency of the multi-enzymatic product, which could be overcome with increased unit additions. The multi-enzymatic product contains many glycosyl-hydrolases which act synergistically, and therefore based on varying reaction conditions can have a varying effect on the fractionation of the potato protein extracts (Waglay, et al., 2015).

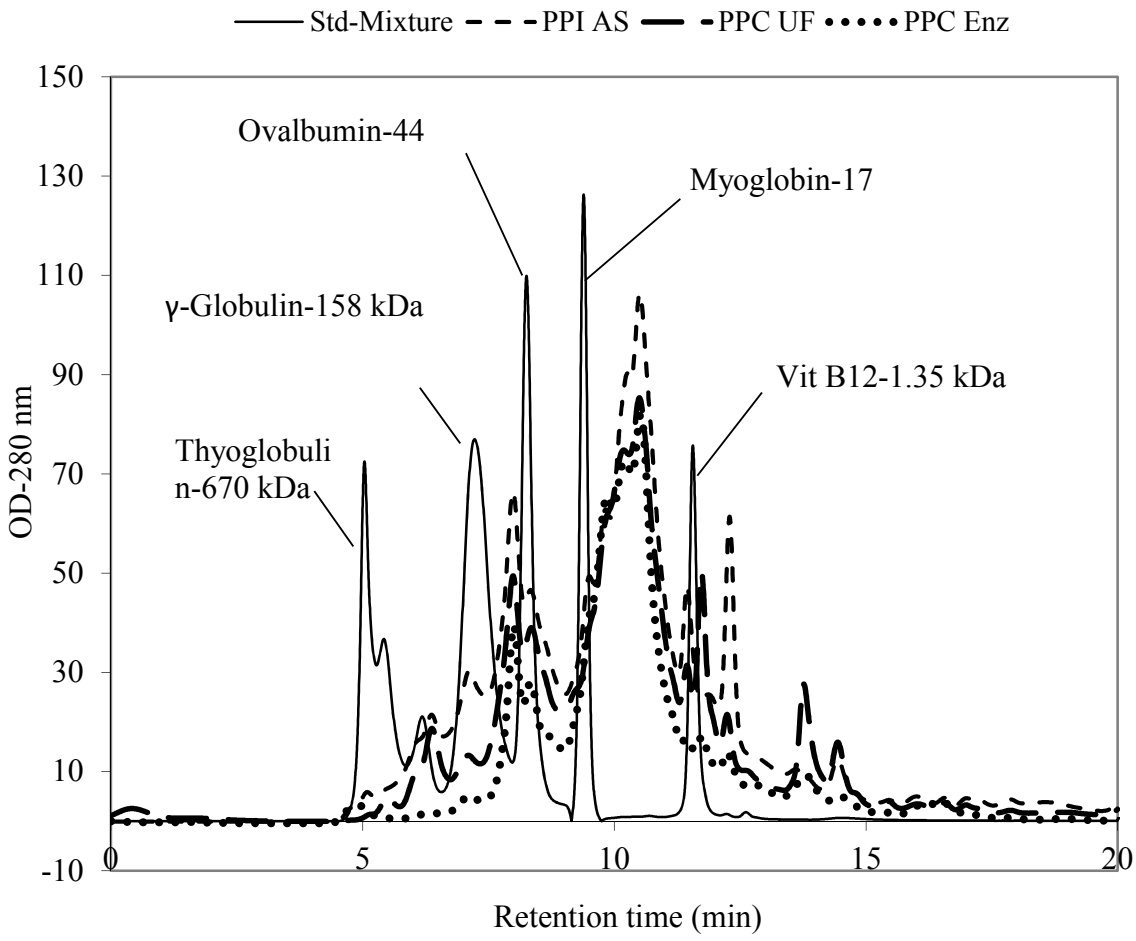

Figure 6.2 Size exclusion chromatogram of potato protein isolate (PPI AS) recovered in pilot scale by ammonium sulphate precipitation and protein concentrates extracted using ultrafiltration (PPC UF) and multi-enzymatic product Depol 670L (PPC Enz)-based processes.

For all PPI and PPCs, the HPSEC results (Figure 6.2) concorded well with those obtained by SDS-PAGE (Table 6.1), where extraction processes resulted in a relatively similar patatin profile, with large variations in the protease inhibitors being recovered. The wide variation in the profile of the protease inhibitors was observed between 1.35 and 17 kDa for PPI and PPCs. In its native form (without the presence of SDS or urea), patatin is largely present in its dimer form of 80 kDa (Pots, et al., 1998), which is likely the peak between 44 kDa and 158 kDa. All extracts contained high molecular weight proteins, as indicated by the peaks above 158 kDa. Furthermore, despite the diafiltration steps (MW cut off of 5 kDa), a few peaks were obtained at <1.35 kDa, which is indicative of peptides that have potentially interacted with polyphenols present in the by-products, which would render them insoluble, and in turn be present in the retentate (Straetkvern, et al., 1999).

6.4.2.2. Thermal and structural denaturation of protein isolate and concentrates

The thermal denaturation of PPI and PPCs were investigated. It should be noted that the structural changes are not related to a single protein, but rather a complex mixture. Moreover, several competing interactions such as with the present polysaccharides, and hydrolysed saccharides could also provide additional changes to the conformation of the protein (Boye, et al., 1996; Imeson, et al., 1977). However, an understanding of the denaturation conditions of the extracts are necessary for the evaluation of their potential application in food systems, in terms of processing conditions. Figure 6.3 shows the heat flow profiles collected from differential scanning calorimetry for PPC UF, PPC Enz, and PPI AS. As shown, PPI and PPCs exhibited a similar differential scanning calorimetry profile, with the decline around 70-75 °C. Contrarily purified patatin examined by Pots et al. (1998) showed a sharp decline in between 50-60 °C. Whereas, the most abundantly known protease inhibitors found in potato the serine protease inhibitors exhibited a denaturation temperature between 62-69 °C (Pouvreau, et al., 2005). The differences in denaturation temperature could be due to the presence of protease inhibitors, which would have a stabilizing effect on the available patatin. A previous study conducted by Koppleman et al. (2002), found that aggregated patatin in the presence of protease inhibitors have a stabilizing effect limiting the fully irreversible denaturation, which was examined in their

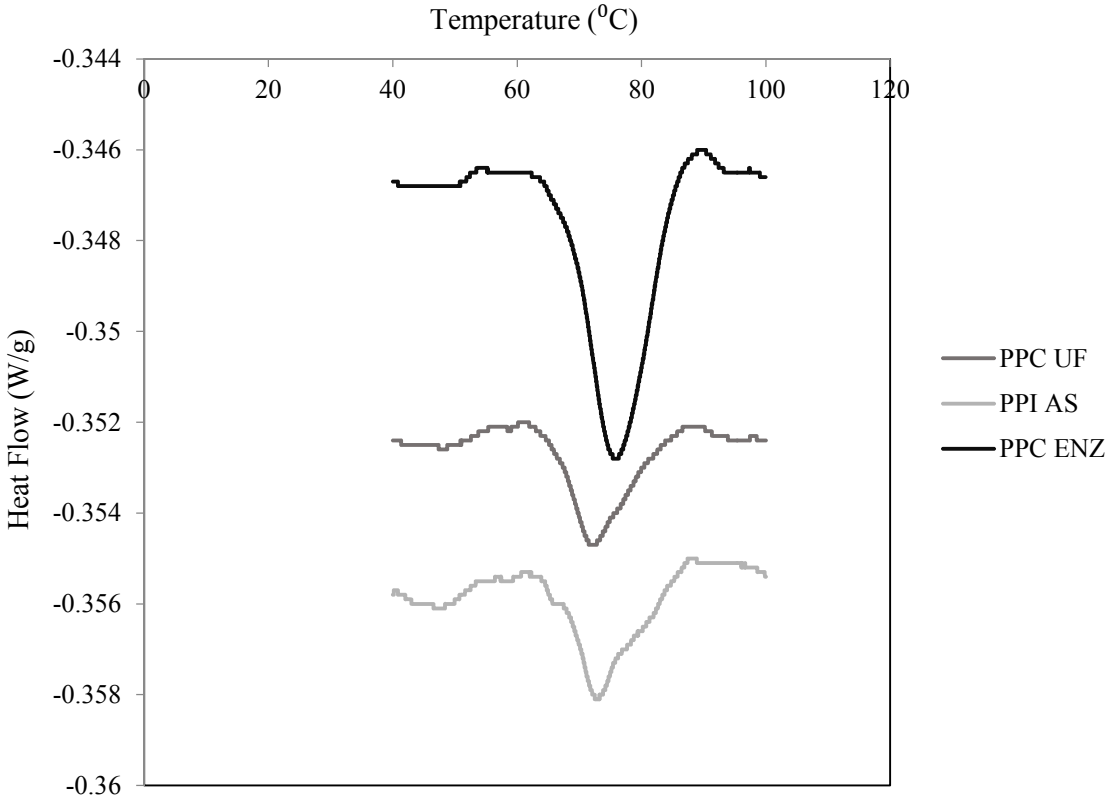

Figure 6.3 Differential Scanning Calorimetry Thermograms for potato proteins extracted using ultrafiltration (PPC UF), ammonium sulphate precipitation (PPI AS) and multi-enzymatic product Depol 670L (PPC Enz)-based processes, at pH 7.

Table 6.2 Thermodynamic data from differential scanning calorimetry profile of potato proteins extracted at a pilot plant scale by ultrafiltration (PPC UF), ammonium sulphate (PPI AS), and multi-enzymatic system (Depol 670L) (PPC Enz)-based processes.

Potato Protein Extracts	T_d (^{0}C)	ΔH (J/g)
PPC UF[a]	72.12 (±0.2)	0.40 (±0.04)
PPI AS[b]	72.81 (±0.1)	0.38 (±0.003)
PPC Enz[c]	75.86 (±0.1)	0.85 (±0.09)

[a]Ultrafiltration performed with a molecular weight cut-off of 5 kDa
[b]Ammonium sulphate performed at 60% saturation
[c]Multi-enzymatic product used was Depol 670L

absence. Table 6.2 shows the denaturation temperature (T_d) and the calorimetric enthalpies (ΔH) which were estimated from averages of triplicates measurements. PPC Enz resulted in the highest T_d of 75.86 °C, followed by PPI with T_d of 72.81 °C, whereas PPC UF resulted in the lowest T_d of 72.12 °C. van Koningsveld et al. (2001) reported that in order to inactivate most of the protease inhibitors activity within a food product, temperatures of 70 °C are required. The differences in T_d could be due to the presence of carbohydrates with varying sizes. PPC Enz contained the highest proportion of carbohydrate content of 39% followed by PPC UF with a carbohydrate content of 8% (data not shown). The presence of these sugars has been shown to effect the denaturation temperature (Boye, et al., 1996). Expectedly, PPC Enz would contain smaller sugar components when compared to PPC UF. The multi-enzymatic system Depol 670L expresses several glycosyl-hydrolases activities, which cleave the polysaccharide network surrounding the proteins within the potato cell wall releasing shorter oligomers (Waglay, et al., 2015). Contrarily, the PPC UF would contain the polysaccharide components, which were not separated during the decanting or centrifugation step. Similar to our results, varying sizes of sugars have been found to have a different stabilizing effect on bovine serum albumin by increasing the denaturation temperature as the sugar size decreases (Boye, et al., 1996). Furthermore, a study conducted by Kasapis & Al-Marhoobi (2003), found that the addition of sugar concentration from 0 to 53% in a 7% gelatin solution, resulted in a shift of the thermal denaturation temperature to higher temperature. The lowest denaturation temperature for PPC UF with the similar trend before and after denaturation could be due to the interaction between proteins and polysaccharides. Similarly, Imeson et al. (1977) found that the addition of polysaccharides with globular proteins caused the thermal denaturation temperature to be lowered.

Similar to Pots et al. (1998), a different profile before and after denaturation can be seen for PPC Enz and PPI AS indicating a difference in conformational structure following denaturation (Table 6.2). The highest enthalpy of 0.85 J/g was obtained for PPC Enz, whereas the lowest 0.38 J/g for PPI AS (Table 6.2). These differences could be explained by the conformational changes that have taken place prior to denaturation as a result of either protein-protein and/or protein-polysaccharide interactions. Indeed, aggregation and disruption of the protein's hydrophobic interactions are an exothermic process that can result in a lower enthalpy (Boye, et al., 1996).

As part of the study of the effect of the extraction techniques on the stability of PPI and PPCs, their secondary structural components were assessed from 25-95 °C using FTIR (Figure 6.4). All PPI and PPCs exhibited varying conformational profiles at 25 °C; PPC UF excessive aggregation (1618 cm^{-1}) confirms the potentially higher presence of polyphenols or polysaccharides, which could interact with the proteins leading to their aggregation (Straetkvern, et al., 1999). Contrarily, for PPC Enz a smaller but prominent aggregation peak (1618 cm^{-1}) took place prior to thermal denaturation. Possible explanations would be that the addition of the enzymes and the release of galacturonic acid oligomers led to slight pH variation during extraction, where the pH was not controlled. This could lead to ideal conditions below patatin's isoelectric point of 4.9 where the potato polysaccharides will carry an opposite charge to the protein molecule forming a complex or aggregative phase separation (Doublier, et al., 2000). Therefore, further improvements to the scale-up of the multi-enzymatic system should include pH stabilization. This aggregation band was minimally shown for PPI AS as proteins extracted in this manner have been structurally comparable to native potato protein (van Koningsveld, et al., 2001).

In summary, the C=O stretching vibrations are responsible for the absorption of energy by the amide backbone. These vibrations are influenced by hydrogen bonding and are found in the FTIR spectrum Amide I region from 1600-1700 cm^{-1}. Commonly, a second-derivative procedure is used to separate overlapping structural components found in this region (Matheus, et al., 2006; Haris, 2013). FTIR spectra (Figure 6.4 A, 6.4 B, and 6.4 C) demonstrate with all extracting agents a significant effect in the Amide I maximum shifting to higher frequencies following heating. For all PPI and PPCs, at 25 °C, a maximum was exhibited at 1638 cm^{-1}, pertaining to predominately β-sheet, followed by a peak at 1654 cm^{-1}, which is associated with α-helical structures.

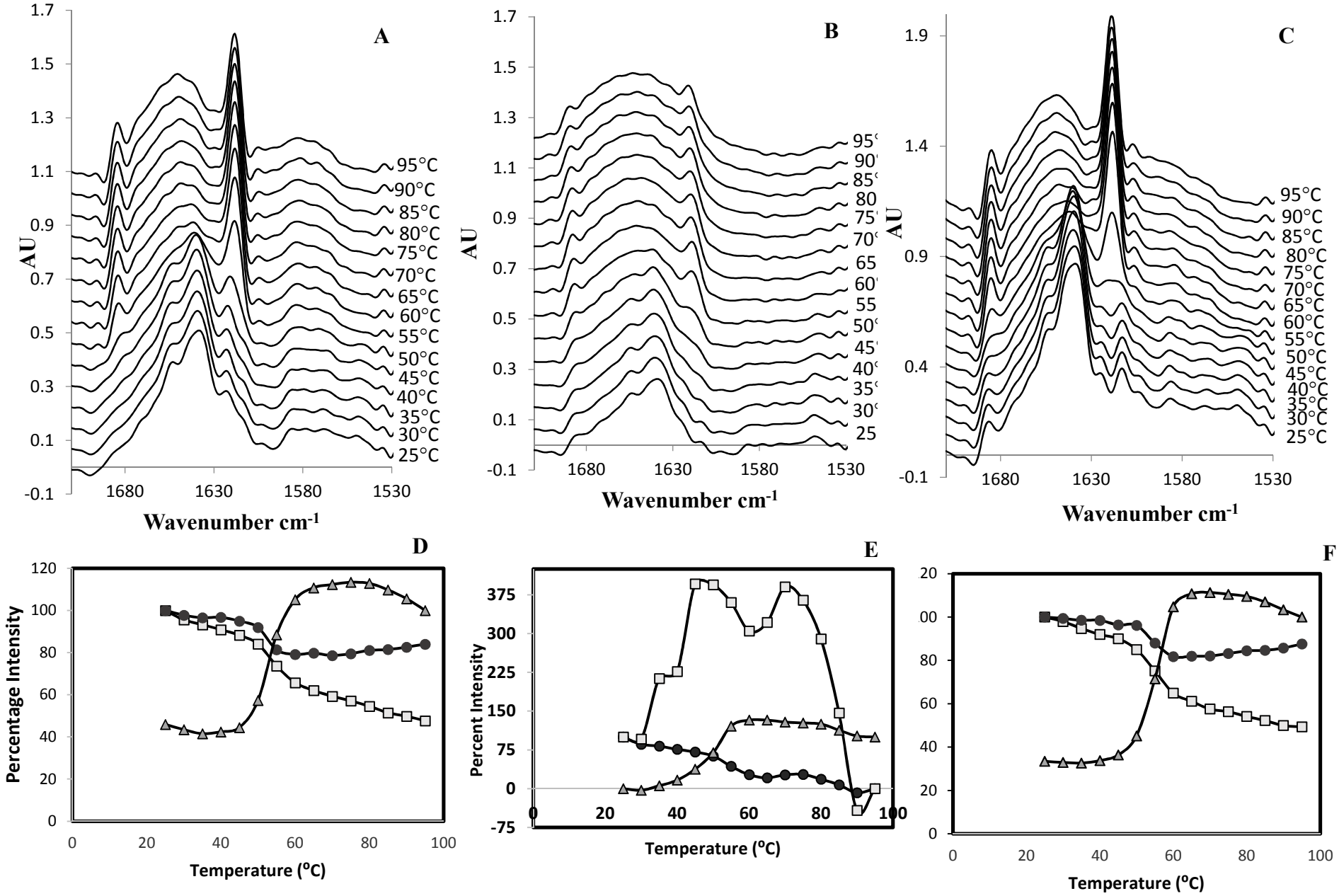

Figure 6.4 Fourier-transform infrared (FTIR) spectroscopy spectra of potato protein isolate (PPI) and concentrates (PPC) extracted on pilot plant scale using (A, D) Ultrafiltration (B, E) 60% Ammonium sulphate saturation (C, F) Multi-enzymatic system Depol 670L; Effect of extracting agent on the secondary structural changes as obtained by Fourier-transform infrared spectroscopy analysis, α-helix (—□—), intermolecular beta-sheet (—●—), and aggregation (—▲—).

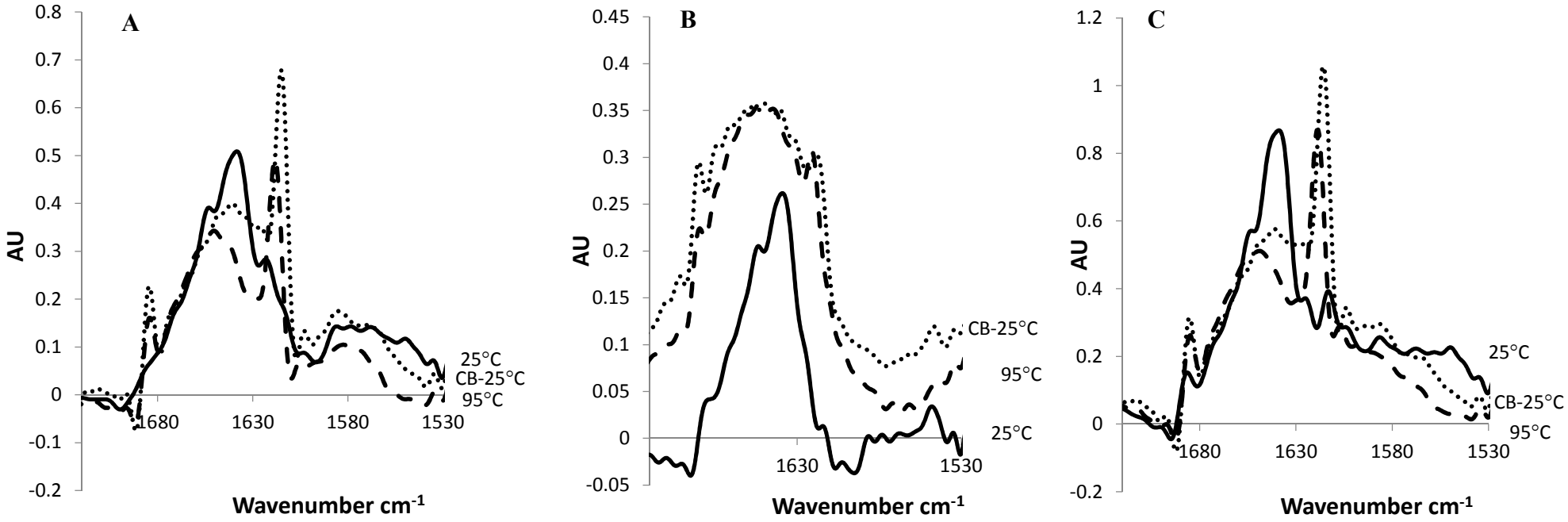

Figure 6.5 Effect of extraction process on the secondary structural changes of isolated proteins at 25^{0}C (———) , 95^{0}C (– –), and cooled back to 25°C (CB-25^{0}C, ·····) obtained by Fourier-transform infrared spectroscopy analysis : (A) ultrafiltration (PPC UF), (B) 60% ammonium sulphate saturation (PPI AS), (C) multi-enzymatic system Depol 670L (PPC Enz).

Indeed, native patatin has been shown to be composed of 45% β-strand, 33% α-helix, and 15% random coil (Pots, et al., 1998). In additional potato serine protease inhibitors, the most abundant protease inhibitor found in the tuber, have been studied to belong to β-II protein subclass, which is composed primarily of β-sheet or β-turns (Pouvreau, et al., 2005).

In all cases the thermal denaturation curves (Figure 6.4 A, 6.4 B, and 6.4 C) show that as temperatures rise above 55 ^{0}C, conformational changes of the protein mixture's secondary structure occur. Above 55 ^{0}C a pronounced aggregation peak results (1618 cm^{-1}) and a drop in peak separation ranging from 1650-1630 cm^{-1}. This is in agreement with previous findings of Pots et al. (1998) and van Koningsveld et al. (2001) where patatin begins to denature at 50-55 ^{0}C, leading to breakdown in alpha-helical (1654 cm^{-1}) and beta-stranded regions (1634 cm^{-1}) and increased occurrence of aggregation (1684 or 1618 cm^{-1}). Our DSC measurements (T_d 72.12-75.86 °C) are closely related to the reported thermal denaturation temperature (70 ^{0}C) of protease inhibitors present in the extracts (van Koningsveld, et al., 2001). Contrarily, the FTIR results are closely related to the reported thermal denaturation temperature of the patatin (55 °C) (Pots, et al., 1998) as shown by the maximum peak in the β-strand region (1634 cm^{-1}) as well as, the poor peak separation ranging from 1650-1630 cm^{-1} following heat treatments at 55 ^{0}C. The higher DSC measurements could be attributed to the predominant conformation of β-sheet structures in all protein extracts, which is often associated with a higher T_d (Shevkani, et al., 2015).

The thermal transition curves were developed by plotting the intensities of frequencies versus temperature, the intensities of frequencies consisted of: the second derivative spectrums for intensity of increasing (1618 cm^{-1}, predominantly aggregation), the second derivative spectrum for intensity of decreasing (1634 cm^{-1}, predominantly β-strand), and the second derivative spectrum for intensity of decreasing (1654 cm^{-1}, consisting of α-helical structures). The cross section of these curves could be computed as $T_{m(FTIR)\alpha\text{-helical}}$ and $T_{m(FTIR)\beta\text{-strand}}$ (Matheus, et al., 2006). PPC UF resulted in $T_{m(FTIR)\alpha\text{-helical}}$ of 51.2 °C whereas the $T_{m(FTIR)\beta\text{-strand}}$ occurred slightly higher at 53.3 °C. Thermal transition for PPI AS showed different trends with $T_{m(FTIR)\alpha\text{-helical}}$ of 86.5 °C and $T_{m(FTIR)\beta\text{-strand}}$ of 49.6 °C (Figure 6.4 E). Finally, PPC Enz resulted in a similar trend than PPC UF, with greater

$T_{m(FTIR)}$ for both structural components: $T_{m(FTIR)\alpha\text{-helical}}$ of 52.6 ^{0}C ; $T_{m(FTIR)\beta\text{-strand}}$ of 54.6 ^{o}C (Figure 6.4 F). Based on the protein profile (Table 6.1), the patatin to protease inhibitors ratio range from 0.395-0.403. A previous study demonstrated that patatin in the presence of protease inhibitors aggregate together upon heat treatments (Koppelman, et al., 2002). Other studies have further demonstrated the stabilizing effect of sugars which have been shown to shift the transition temperature by ~1.5^{o}C (Boye & Alli, 2000). In addition, large polysaccharides and protein complexes have been shown to shift the transition temperatures to higher temperatures as is the case for κ-carrageenan and soy protein isolates (Baeza, et al., 2002). To date, no study compares the structural changes of the complex mixture of potato protein isolate and concentrates based on different extracting agents. Moreover, the analysis was done on a complex mixture of proteins with varying salt and sugar concentration, where many interactions result. Therefore, these results are more representative of the denaturation behaviors of complex protein mixture for their potential application in food systems.

The reversibility of protein folding was assessed by FTIR where following heat treatment up to 95 ^{o}C, the mixtures were cooled back to 25 ^{o}C. As shown in Figure 6.5, all extracting agents resulted in severe denaturation with only minimal conformation reversibility following cooled back to 25 ^{o}C. For PPC UF as the protein is heated the intermolecular β-sheet decreases leading to protein aggregation. Interestingly, once heated PPC UF demonstrated the ability to reversibly return some of its α-helical conformation (Figure 6.5A). The ability to recover part of its structural components after heating could be due to the presence of sugar (data not shown), which has a stabilizing effect on the protein's structure (Boye, et al., 1996). Indeed, PPC Enz also had this sugar stabilizing effect, where the presence of α-helix was found following cooled back to 25 ^{o}C. As shown in Figure 6.5, PPI AS was significantly affected by heat treatments where initially at 25 ^{o}C defined peaks at 1654 cm^{-1} and 1634 cm^{-1}, however following heat treatments and cool back a broad peak results over the range of 1680-1606 cm^{-1}. To establish the reversibility, we can see that the α-helix structure (1654 cm^{-1}) was completely lost and aggregation peak was very prominent. Therefore, the proteins present in the PPI AS were the most affected by heat denaturation when compared to those recovered using the two other investigated processes.

This is indicative of the stabilizing effect of the sugars present in the isolate and concentrates, where PPI AS had the lowest polysaccharide content (2%; data not shown).

6.4.3. *Functional assessment of potato protein isolate and concentrates*

6.4.3.1. Emulsifying properties

Table 6.3 demonstrates the emulsifying properties of PPC UF, PPI AS, and PPC Enz. The emulsifying activity index (EAI) is expressed both per g of extract as well as g of protein in the extract. PPC UF and PPC Enz resulted in more or less in similar EAI (by g extract) of 13.1 and 13.4 m^2/g extract, respectively. While PPI AS exhibited an EAI of 14.0 m^2/g extract. However, when expressing the EAI per g of proteins, EAI for PPC Enz (33.5 m^2/g protein) was significantly higher than those of PPC UF (17.1 m^2/g protein) and PPI AS (16.1 m^2/g protein). This difference could be attributed to the varying percentage of protein found in each isolate and concentrate (Table 6.1). A similar trend was observed for sweet potato protein, almond protein, wheat gluten, and acidic subunits of soy 11S globulin, where it was found that the EAI decreased with increasing protein concentration (Guo & Mu, 2011; Agyare, et al., 2007; Liu, et al., 1999; Sze-Tao & Sathe, 2000). This phenomenon could be due to the ease in diffusion of the protein to the oil-water interface at low concentrations, leading to increased development of new oil droplets, thereby increasing EAI. Conversely, at higher protein concentrations diffusion is less likely to occur due to the activation-energy barrier for the protein to adsorb on the interface (Guo & Mu, 2011). In addition for PPC Enz, the presence of small sugars may have increased the protein's solubility (Arakawa & Timasheff, 1982), therefore enhancing the protein's participation to adsorb at the oil-water interface. In addition, for both PPI AS and PPC Enz a larger proportion of protease inhibitors <15 kDa is present (Table 6.1), which could lead to increase flexibility of these small proteins at the interface, leading to higher EAI (Shevkani, et al., 2015). A positive correlation can be concluded between the thermal properties (T_d and ΔH) determined by DSC and the EAI, indicative of the effect of the structural composition, where the conformational changes of the protein tertiary and quaternary structure allow for the protein to more easily absorb to the oil-water interface (Liang & Tang, 2013).

The results (Table 6.3) also show that high emulsifying stability index (ESI) values were obtained for PPC UF and PPI AS (18.6 and 18.0 min, respectively), as compared to PPC Enz (16.7 min). These results may be attributed to the low protein concentration at the oil-water interface in the case of PPC Enz, limiting the electrostatic and the steric repulsions that contribute to the strength of the interfacial layer (Guo & Mu, 2011).

As compared to other protein isolates, PPI and PPCs have better EAI (~13.5 m^2/g powder) and ESI (~17.8 min). Soy protein isolates were reported to exhibit an EAI of 10.9 m^2/g powder and ESI of 0.8 min (Achouri, et al., 2005). On the other hand, sweet potato proteins solutions were found to have an EAI of 50 m^2/g and ESI of 65 min (Guo & Mu, 2011). Conversely, sesame protein isolates have been found to have an EAI of 16.8 m^2/g and ESI of 17.4 min (Achouri, et al., 2012). Therefore potato proteins isolate and concentrates are comparable to sesame protein isolates and possess superior emulsifying properties to soy protein isolates, but are inferior to sweet potato proteins.

6.1.1.1. Foaming characteristics

Table 6.3 outlines the foaming properties obtained for PPC UF, PPI AS, and PPC Enz. Elevated G_i and FE are correlated to increased foam capacity and expansion, whereas increased R_5 is related to foam stability. PPC Enz resulted in the highest G_i and FE revealing its high foam capacity and expansion, whereas PPC UF had the highest foaming stability as indicated by R_5, 61.7%. This higher foam capacity and expansion versus stability for PPC Enz and PPC UF, respectively, could be due to the increased presence of sugars. The presence of sugars within the suspension leads to increase solubility of the protein, therefore, rendering the protein more available to participate in foaming. This phenomenon was also encountered by Partsia and Kiosseoglou (2001), who reported that the presence of complexing agent carboxymethyl cellulose with potato proteins decreased surface tension at the air and water interface.

Table 6.3 Emulsifying properties and foaming characteristics of potato protein isolates extracted at pilot plant scale by ultrafiltration, ammonium sulphate saturation, and multi-enzymatic system Depol 670L.

	Emulsifying Properties[j]		
	PPC UF[f]	PPI AS[g]	PPC Enz[h]
EAI[a] (m^2/g extract)	13.1 (±0.7)[i]	14.0 (±0.3)	13.4 (±1.5)
EAI[a] (m^2/g protein)	17.1 (±0.9)[h]	16.1 (±0.4)	32.4 (±3.8)
ESI[b] (min)	18.6 (±1.3)	18.0 (±0.5)	16.7 (±0.8)
	Foaming Characteristics[k]		
G_i[c] (%)	52.8 (±0.2)	53.3 (±1.7)	54.8 (±0.6)
G_i/mL sample injected	3.7 (±0.2)	3.6 (±0.3)	4.2 (±0.3)
R_5[d] (%)	61.7 (±3.3)	53.9 (±3.4)	59.3 (±3.5)
FE[e] (%)	384.1 (±14.2)	375.5 (±16.3)	416.4 (±29.1)

[a]Emulsifying activity index
[b]Emulsifying stability index
[c]Pergentage of gas entrapped in 55 mL of foam
[d]Percentage of liquid retained from the foam after 5 min
[e]Percent of foam expansion
[f]Ultrafiltration performed with molecular weight cut off of 5 kDa
[g]Ammonium sulphate performed at 60% saturation
[h] Multi-enzymatic product used was Depol 670L
[i]Standard deviation taken of triplicates.
[j]Results obtained were statistically significant for EAI (m^2/g extract) F-value 0.39 *p*-value 0.8076, EAI (m^2/g protein) F-value 21.01 *p*-value 0.0060 and ESI F-value 14.95 *p*-value 0.0113.
[k] Results obtained were statistically significant for G_i (%) F-value 17.26 *p*-value 0.0087, R_5 F-value 2.16 *p*-value 0.2371, and FE F-value 12.08 *p*-value 0.0166.

This allowed for more air to be incorporated as well as enabled the polysaccharides to help with foam stability by decreasing the occurrence of drainage (Partsia & Kiosseoglou, 2001). Another explanation for the differences of foaming characteristics among PPI and PPCs is the proportion of patatin and protease inhibitors. Ralet and Guéguen (2001) found that patatin was able to form very stable and resistant foams; conversely, protease inhibitors (16-25 kDa) formed very unstable foams which broke rapidly (Ralet & Guéguen, 2001). PPI AS resulted in relatively high proportion of patatin (25.57%) and 16-25 kDa protease inhibitors (31.69%), comparatively to PPC UF and PPC Enz, which contained 21.55-24.53% and 22.6-27.12% for patatin and protease inhibitors (16-25 kDa), respectively. Therefore, possible explanation for the lower foaming expansion and stability for PPI AS could be due to the higher presence of protease inhibitors (16-25 kDa), leading to foam breakage, despite the relatively high proportion of patatin. Foaming properties are also governed by the secondary conformation of the proteins, where the ease in the protein unfolding revealing its hydrophobic nature at the air-water interface will help in the foam development (Kinsella , 1981). PPC UF showed the lowest foam capacity (lowest G_i and lower FE) and low thermal properties.

Overall, PPI and PPCs have weaker foam expansion (approximately 392%) and better foam stability (about 58.3%) as compared to soy protein isolates (foam expansion and stability of 532% and 41%, respectively) (Achouri, et al., 2005). On the other hand, sesame proteins are superior for both foam expansion (537%) and foam stability (87.8%) (Achouri, et al., 2012).

6.5 Conclusion

In the present study, three processes were scaled up in a pilot plant facility to isolate potato proteins from imitation PFJ and potato pulp. Two of these processes were based on the isolation of potato proteins from PFJ by subsequent ultrafiltration steps and by 60% $(NH_4)_2SO_4$ saturation. The third process involved the use of a multi-enzymatic system on potato pulp. $(NH_4)_2SO_4$ precipitation resulted in a PPI with high protein content of 86.5%, with a high recovery yield of 44.6%. Conversely, the ultrafiltration and multi-enzymatic system-based processes produced PPCs with low protein contents of 75.9 and 40.7%, respectively and protein recovery yields of 21.6 and 29.1%. All extracting agents had an

effect on the secondary structural components of PPI and PPCs. Both PPCs contained a high polysaccharide content, which had a preservation effect against the protein extracts thermal denaturation. PPC Enz obtained by multi-enzymatic system-based process had improved functionalities in terms of emulsifying activity index and foam expansion. Conversely, emulsion and foam stability was better preserved with the PPI AS and PPC UF. This study outlined an improved scaled-up method for potato protein recovery. The prepared PPI and PPCs have great potential for functional use in food systems.

CONNECTING STATEMENT 5

Pilot-plant scale up resulted in a large quantity of potato protein isolates with a good understanding of their structural changes and functional properties (Chapter VI). Chapter VII examines the application of potato protein isolate in the development of a health promoting ingredient in a functionally enhanced cookie snack food. The cookie formulation was further optimized to assess the effect of the proportion of rice flour and the potato protein enrichment, for the reduction of gluten within the cookie formulation. The composite flours were compared according to their water absorption capacity and protein content. The cookies were also assessed according to their geometrical measurements, textural analysis, colour analysis, and sensory evaluations.

The results from this study were presented at the 12th International Congress on Engineering and Food and at the IFT Annual Meeting & Food Expo-Institute of Food Technologist.

Waglay, A., Achouri, A., Karboune, S., Zareifard, M.R., & L'Hocine, L. (2015) Pilot Plant Extraction of Potato Proteins and their Application as a Value Added Ingredient. ICEF12, Quebec, Canada, June 14- June 18, 2015.

Waglay, A., & Karboune, S. (2015) Formulation of a Functional Cookie Enhanced with Potato Protein Isolates: Textural and Sensory Evaluations. IFT15 Annual Meeting & Food Expo, Chicago, USA, July 11- July 14, 2015.

Waglay, A., & Karboune, S. (2015) Formulation of a Functional Cookie Enhanced with Potato Protein Isolates: Textural and Sensory Evaluations. *(To be submitted).*

CHAPTER VII. FORMULATION OF A FUNCTIONAL COOKIE ENHANCED WITH POTATO PROTEIN ISOLATES: TEXTURAL AND SENSORY EVALUATIONS

7.1. Abstract

Potato protein isolate (PPI) and concentrate (PPC), recovered from potato fruit juice by-product were assessed as techno-functional ingredients for the enrichment of composite flours composed of wheat and rice flours with varying ratios. PPI resulted in cookies, which bent more on first bite than that of wheat flour alone, as well as texturally were more desired by a semi-trained panel, compared to PPC. The effect of rice flour proportion (14.6-85.4%) and PPI enrichment (1.9-23.1%) was investigated using a central composite rotatable design. Two controls of rice and wheat flour alone were also run in parallel. Wheat flour-based cookies resulted in a higher maximum peak force when compared to rice flour-based ones. However, when the composite flour consisted of 75% rice flour and increasing PPI, the cookies increased in hardness. Contrarily, wheat flour alone with increasing PPI exhibited the same hardness. The sensory results (70 consumers) revealed that for high proportion of wheat flour, the control cookie of wheat alone (100%) was the most preferred. Contrarily, for a high proportion of rice flour, the predominantly preferred cookie was composed of 25/75/5 (w/w/w) of wheat/rice/PPI. Based on statistical analysis, potato proteins can be used to enrich to 7.20% for acceptable reduced-gluten cookies prepared with a combination of 67.88% rice flour and 32.12% wheat flour, while improving the textural and sensorial characteristics when compared to rice flour alone.

7.2. Introduction

Potato proteins have long been considered not economically feasible due to the low protein content (27%, dry matter) found in tuber (*Solanum tuberosum*). However, when comparing to the major vegetable and cereal crops, potatoes rank the second largest protein supplying crop grown per hectare following wheat. In terms of phytochemical composition, potatoes are a vegetable whose high-quality proteins are underestimated. In addition to their high proportion of essential amino acids (Met, Leu) (Bártová, et al., 2015), potato proteins possess ACE-inhibitory potency (Mäkinen, et al., 2008), an ability to reduce plasma triglycerides associated with a reduced risk of atherosclerosis (Liyanage, et al., 2008), and stimulate the release of the appetite regulating hormone cholecystokinin (Deveaux-Gobert, 2008). Compared to other proteins, potato proteins have reduced allergenicity (Moreno, 2007). Currently the industrial employed isolation technique limits their food application

due to harsh acid and thermal denaturation. However, several studies have examined more promising isolation techniques such as chromatographic separation, salt precipitation, and enzymatic cell wall degradation (Løkra, et al., 2008; Bártova & Bárta, 2009; Waglay, et al., 2015). These processes maintain the proteins functionalities, which would increase their incorporation into novel functional food products.

To assess the techno-functionalities of potato proteins, cookie formulations were selected because of their high consumer acceptance as a common snack food (Singh & Mohamed, 2007). Research has shown that composite flours in cookie formulations are superior over bread products due to the cookies' longer shelf-life (Singh & Mohamed, 2007). Several studies have explored nutritionally enhancing cookies by decreasing gluten content, decreasing fat content, replacing sucrose, and increasing protein concentrations (Laguna, et al., 2013; Singh & Mohamed, 2007; Wani, et al., 2013; Sudha, et al., 2007). In this perspective, rice flour has been widely investigated as a wheat substitute because of its lack of gluten, low levels of proteins, and a neutral flavour (Sivaramakrishnan, et al., 2004; Gujral, et al., 2003). To overcome the lacking gluten network of rice flour, many studies have examined the addition of polysaccharides, hydrocolloids, and gums to improve the textural properties of no to low gluten food products (Sivaramakrishnan, et al., 2004). Only limited work has investigated the use of functional proteins for enhancing low gluten product quality (Marco & Rosell, 2008).

The present study is aimed at the investigation of the enrichment of reduced gluten cookie formulations composed of wheat and rice flour at selected ratios with potato proteins. It is hypothesized that the formulation with limited gluten incorporation can be improved in the presence of proteins that positively affect the mixing and pasting properties of the dough (Wani, et al., 2013). As far as the authors are aware, no study has, so far, investigated the ability of potato proteins to improve reduced-gluten formulations. By incorporating the potato proteins into this common snack food, consumers will also have the beneficial properties associated with these proteins, while appealing to present consumer trends of reduced gluten consumption. Moreover, using potato proteins from a waste stream PFJ is desirable as it takes advantage of a previously underutilized resource, which has been limited to animal feed. Several other inexpensive waste protein sources have been evaluated

for their use in enriching the protein content of cookie formulations, namely, watermelon seed protein concentrates (up to 7.5% enrichment) and fluted pumpkin seed flour (up to 15% enrichment) (Wani, et al., 2005).

The present work will contribute to the development of a cookie formulation with limited gluten incorporation, emphasizing consumer's current trend, as well as to the creation of a product with added health benefits from a novel protein source. Bridging the analytical textural properties to the sensory results of a semi-trained sensory panel is expected to contribute to the understanding of the properties that drive the overall acceptance of reduced gluten cookie formulations.

7.3. Materials and Methods

7.3.1. Pilot-plant potato protein separation procedure

The potato proteins were isolated from PFJ using two selected approaches: the ultrafiltration or the ammonium sulfate precipitation (60%, w/w). First, PFJ was prepared according to the modified method of van Koningsveld, Gruppen, de Jongh, Winjingaards, van Boekel, and Walstra (2001). Potatoes of Russet Burbank variety (130 kg) were washed, chopped into large 1 cm^3 pieces using a Big Chop MC15 (Stephan Mikrocut, DE) and suspended in sodium sulfite solution (0.83 M, 78 L) to prevent polyphenol oxidation. After homogenization to 1 mm^3 cubes, using a chopper GK (Urshel Laboratories Inc., IN, USA), the potato slurry was subjected to constant stirring for 1 hr at 2 °C, The resulting turbid liquid was decanted at a speed of 2,756*g* for 20-30 sec, feed rate 380 L/hr using a CBB Decanter (Drycake, Sr, CA). The opaque juice was then subjected to centrifugation with speed of 14,475*g* and feed rate of 200 L/hr using a Dexter MiSR 1010 (SRS A USI Company, MI, USA). The clear yellowish filtrate is known to be similar to industrial PFJ. PFJ was divided into two batches and subjected to ultrafiltration steps and salt precipitation. For the ultrafiltration, a Koch Hollow Fiber Cartridge (Koch Membrane Systems Inc., MA, USA) was used with a molecular weight (MW) cut off of 5,000 Da. The freeze dried powder of potato protein isolated by ultrafiltration of PFJ is further abbreviated as PPC.

For salt precipitation, ammonium sulphate was added to PFJ under constant stirring at 2 °C to reach 60% saturation. The precipitation occurred for 17 hr. Following precipitation, the

slurry was centrifuged with a speed of 14,475*g* and feed rate of 200 L/hr using a SR 1919 (SRS A USI Company, MI, USA). A Koch Hollow Fiber Cartridge (Koch Membrane Systems Inc., MA, USA) was used with a MW cut off of 5000 Da for the ultra and dia-filtration steps in order to remove the salt. The recovered freeze dried potato protein isolated is further abbreviated as PPI.

7.3.2. Preparation of cookies

Cookies were prepared according to AACC method 10-53 (American Association of Cereal Chemists (AACC), 2000) with slight modifications. In order to assess the effect of the potato protein enrichment, non-fat dry milk and ammonium chloride were not added to the formulations. The organoleptic properties of the cookies were enhanced by the addition of natural sweetener agave syrup (4.8%) and natural vanilla flavouring (1.0%).

Formulations began by sifting the dry ingredients, rice flour (RF, 0-100%), wheat flour (WF, 0-100%), dry sodium bicarbonate (0.3%), and salt (0.2%). The shortening (11.5%), sugar (23.1%), and agave (4.8%) were creamed as described in AACC 10-53 using a Varimixer Bear Teddy 5L with whisk attachment. Once creamed, the appropriate water, natural vanilla flavouring (1.0%), sodium bicarbonate solution (0.4%), and potato proteins (PPI or PPC, 0-23.11%) were added and whipped for 3 min with scraping of bowl sides after each minute, this allowed for the development of a uniform emulsion. The sifted flour mixture was then incorporated and mixed for corresponding development time, with scraping of bowl sides at uniform intervals. Each cookie consisted of 15 g of dough. Comparison against two controls of RF and WF alone was assessed. Baking conditions were set at 190 °C/ 9 min with 5 minutes cooling on baking tray, and 30 minutes cooling at room temperature. Following cooling, cookies were analyzed for sensory properties, geometrical, textural, and colour characteristics, all measurements were done the same day as baking.

7.3.3. Measurement of dough consistency

Water absorption of composite flours with and without protein enrichment was determined using a Brabender Farinograph (Brabender, DE). The composition of the analyzed flours were 100% WF, 100% RF, 50/50% WF: RF, 100% WF with 10% PPI enrichment, and 100% RF with 10% PPI enrichment with corresponding protein contents of 12.2, 6.3, 9.2,

19.2, 13.9%, respectively. The moisture contents of the individual flours were determined according to AOAC method 925.10. A 50 g sample was mixed under constant agitation, and the water was added until the standard parameter of 500 Farinograph units (FU) was reached. The development time was determined as the time required to reach 500 FU.

7.3.4. Evaluation of cookies

7.3.4.1. Geometrical analysis

The cookie geometry was measured according to AACC method 10-53, where the average of five cookies was measured for their corresponding cookie width (*W*) and stack height (*h*). The spread factor of the cookie was calculated as *W/h*.

7.3.4.2. Textural analysis

Textural analyses of cookies were carried out using a TA-XT*Plus* (Texture Technologies Corporation, Scarsdale, NY, USA) equipped with a 50 kg load cell, knife probe (TA-42, 45° angle tip) and custom made support for 3 point bend test using stiff metal brackets (distance between supports 39.60mm) attached to a heavy Teflon base. The support was adjusted to be equidistant from the knife probe on either side and clamped to the base (TA-90) to prevent movement during the test. The probe was calibrated at 35 mm height prior to testing. Test speed was 1 mm/s and target distance was 15 mm, enough to completely break the cookie. All measurements were repeated in triplicate.

7.3.4.3. Colour evaluation

Colour differences were assessed by a CM-500d Chroma Meter (Konica Minolta, CA) using the software Spectra magic v2.11. The CIElab 10°/ C scale was used to determine *L* (lightness scale 100= pure white, 0= black), a^* (red), and b^* (yellow). The hue angle ($\tan^{-1}(b^*/a^*)$) and intensity ($((a^{*2}+b^{*2})^{-2})$ of cookies were calculated. Colour measurements were determined of the top and bottom of the cookie. Measurements were performed in triplicate. Total colour difference (ΔE) was calculated by the equation below:

$$\Delta E = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2} \quad (23)$$

Where, $\Delta L^* = (L^*_{sample} - L^*_{standard})$, $\Delta a^* = (a^*_{sample} - a^*_{standard})$
and $\Delta b^* = (b^*_{sample} - b^*_{standard})$

7.3.5. Compositional analysis of cookie formulations

7.3.5.1. Protein content

Nitrogen content was determined using Leco® TruSpec N (Leco Corporation, St-Joseph, MI, USA) according to Dumas method described by Kirsten and Hesselius (1983). Prior to nitrogen determination individual flour, PPI, PPC, and ground cookies were dried. Nitrogen content was multiplied by a factor of 6.25 to determine the total crude protein content (van Gelder, 1981). Analysis was performed in triplicate and averages are shown.

7.3.5.2. Water activity

Water activity (A_w) was measured using a Rotronic HygroLab-2 (Rotronic Instrument Corportion, Hauppauge, NY) in quick A_w mode, which allowed estimation of the A_w in about 5 min. Analysis was performed in duplicate on crushed cookie sample at a room temperature of $20.02^0C \pm 0.32$.

7.3.6. Sensory evaluation of cookies

The cookies were analyzed for their changes in organoleptic properties using a semi-trained panel consisting of 70 McGill University Food Science students both graduate and undergraduate. The sensory attributes were evaluated using the Compusense software (Compusense Inc., On, CA) and two scales: a mean quantitative descriptive scale with well-defined descriptors (Table 7.1); a 5-point category hedonic liking scale (dislike extremely, dislike moderately, neither like nor dislike, moderately like, and like extremely).

Table 7.1 Attributes, scale extremes, and definition used in the descriptive sensory analysis for cookie formulation by a semi-trained panel.

Parameter	Attribute	Scale Extremes	Definition
Colour	Toast Intensity	White/ Brown	Evaluation of the actual perceived colour of the top of the cookie
		White/ Brown	Evaluation of the actual perceived colour of the bottom of the cookie
Manual Texture	Fracturability	Crumbly/ Brittle	Evaluation of the force with which the samples ruptures with front teeth
In-mouth texture	Crispness	Soggy/ Very crisp	Evaluation of the noise and force with which the sample breaks or fractures
	Adhesiveness	Not sticky/ Very sticky	Evaluation of the force required to remove sample from the molars
Flavour	Aftertaste	Not strong/ Very strong	Evaluation of the taste five seconds after the product is swallowed
Preference	Overall liking	Dislike extremely/ Like extremely	

The panelists are considered semi-trained, as they were familiarized with definitions and scaling of attributes prior and during testing. The defined attributes consisted of colour of the top of cookie, colour of the bottom of cookie, fracturability, crispness, adhesiveness, aftertaste, and overall liking. Panelists were also asked a binary choice question (yes/no) regarding the overall acceptance of the product.

7.3.7. Experimental Design

To assess the effects of the rice proportion and the protein enrichment level, a 5-levels, 2 variables central composite rotatable design (CCRD) was used. The full design consisted of two center points, four factorial points, and four axial points, resulting in ten formulations. The two variables with their corresponding levels consisted of x_1 rice relative proportions in the WF/RF mix (14.64, 25.00, 50.00, 75.00, and 85.36%) and x_2 protein enrichment (1.89, 5.00, 12.50, 20.00, and 23.11%, g/100 g flour). The two controls (100% Control RF; 100% Control WF) were run in parallel but were not included in the developed models. Water incorporation and dough development time corresponded to proportion of wheat to rice flour based on Farinograph measurements. Baking temperature for the experimental design was set at 205 °C for 8 min, with 5 min cooling on baking tray, and 30 min cooling at room temperature.

For approximating response surface, the obtained scores of sensory attributes according to described quantitative descriptive and liking scales were fitted to the general model Equation 24 using the software Design-Expert 8.0.2 (Stat-Ease, Inc. MN, USA):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i+1\leq j\leq k}^{1\leq i\leq k} \beta_{ij} X_i X_j \qquad (24)$$

β_o, X_is (i=1-5), $\beta_{i,,}$ β_{ii}, and β_{ij} are the constant coefficient, coded independent variables, the coefficient for the linear effect, the coefficient for the quadratic effect, and the coefficient for the interaction effect, respectively. Contour plots were obtained from the fitted models while changing the other two variables. Desirability plots were also generated by maintaining all parameters in range except for those which were found to be statistically significant as determined by Statistical Analysis Software (SAS, version 9.4).

7.3.8. Statistical Interpretation

Data was analyzed using the Statistical Analysis System (SAS version 9.4). The Proc Means was used to calculate the average and standard deviation of quantitative descriptive scores and liking scores. The Proc Can was used to assess the multivariate analysis of variance and canonical results, which were used to interpret the relationship between attributes and formulations. Consumer data were coded with a positive response denoted as 1 and a negative response as 2. The Proc Logistic was used to relate the consumer response for overall acceptance to the liking scores of attributes according to the following logistic function:

$$P\left(1/x\right) = \exp(\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n)/\{1 + \exp(\alpha + \beta_1 x_1 + \cdots + \beta_n x_n) \quad (25)$$

Where, $P\left(1/x\right)$ is the probability of overall acceptance; x_1, x_2,…, x_n are the intensities of attributes; and α, β_1, β_2,…, β_n are parameter estimates associated with the model terms (Malundo, et al., 1999).

7.4. Results and Discussion

7.4.1. Effect of potato protein (PPI, PPC) enrichment on the properties of cookies

The effect of the enrichment of selected cookie formulations (100% RF; 100% WF; 50RF/50WF) with potato proteins (5%, w/w) was investigated using PPI and PPC produced by salt precipitation and ultrafiltration, respectively (Table 7.2). PPC was composed of 75.9% proteins and 8% polysaccharides; the proteins in PPC consisted of patatin (21.6%) and protease inhibitors (53.9%) with a high presence of high MW proteins (24.5%) (data not shown). While the protein content of PPI was estimated at 86.5%, with a good distribution of patatin (25.6%), protease inhibitors (63.4%), and the high MW proteins represented only 11.1% (data not shown). The enriched cookies and the control ones were evaluated through geometrical analysis, texture analysis, colour evaluation, and sensory evaluation using 25 panelists (Table 7.2).

The cookie geometry (width and stack height) is governed by two properties, namely the leavening agent (sodium bicarbonate) and the gravitational flow. This gravitational flow is

Table 7.2 Comparison of cookie formulations enriched by potato protein isolate (PPI) or concentrate (PPC).

Formulation	Geometry[c,f]			Relative Texture[g]		Colour evaluation[h]		
	Width (*W*)	Stack Height (*h*)	Spread Factor (*W/h*)	Hardness Force (%)[d]	Fracturability (%)[e]	*L**	*a**	*b**
Control RF	58.23 ± 1.1	8.49 ± 0.5	6.86	13.62± 6.6	96.56± 0.1	73.18± 0.5	3.71± 0.9	36.81± 1.5
Control WF	56.95 ± 1.1	11.86 ± 0.4	4.80	100.0± 31.4	100.0± 0.3	71.03± 0.9	2.63± 0.1	30.05± 0.3
100 RF/ 5 PPC[a]	50.16 ± 2.2	8.61 ± 0.5	5.83	120.58± 31.5	96.30± 0.2	59.13± 1.8	6.76± 1.0	26.8± 0.6
100 WF/ 5 PPC[a]	49.45 ± 0.5	9.94 ± 0.8	4.97	182.65± 37.0	97.38± 0.3	64.63± 1.7	5.06± 1.4	27.7± 1.6
50 RF/ 50 WF/ 5 PPC[a]	51.67 ± 1.2	8.71 ± 0.08	5.93	113.94± 33.4	93.68± 0.03	62.89± 0.5	7.06± 0.6	30.82± 0.8
100 RF/ 5 PPI[b]	55.81 ± 1.3	9.96 ± 0.09	5.60	74.06± 46.8	102.49± 0.3	67.8± 2.1	2.78± 0.5	17.46± 0.7
100 WF/ 5 PPI[b]	58.65 ± 0.6	13.02 ± 0.27	4.50	73.56± 38.8	102.67± 0.1	71.13± 0.8	2.66± 0.2	19.07± 1.2
50 RF/ 50 WF/ 5 PPI[b]	53.60 ± 0.9	8.54 ± 0.2	6.28	65.14± 16.5	97.38± 0.2	71.52± 0.4	2.95± 0.04	19.04± 0.2

[a] Potato protein concentrate isolated by ultrafiltration technique from potato fruit juice

[b] Potato protein concentrate isolated by 60% ammonium sulphate saturation from potato fruit juice

[c] Measurements taken of 5 cookies

[d] Relative $texture_{Hardness\ Force}$= ($Hardness\ Force_{Actual}$/ $Hardness\ Force_{Control\ WF}$) *100

[e] Relative $texture_{Fracturability}$= ($Fracturability_{Actual}$/ $Fracturability_{Control\ WF}$) *100

[f] Results obtained were statistically significant for width F-value 15.29 *p*-value <0.0001 and height F-value 54.37 *p*-value <0.0001.

[g] Results obtained were statistically significant for Hardness Force F-value 132.08 *p*-value <0.0001 and Fracturability F-value 14.99 *p*-value <0.0001.

[h] Results obtained were statistically significant for *L** F-value 48.76 *p*-value <0.0001, *a** F-value 21.39 *p*-value <0.0001, and *b** F-value 90.59 *p*-value <0.0001.

affected by the water holding ability of the dough making it less or more viscous thereby affecting the rate at which the dough will flow. Ideally, high quality cookies are associated with a high spread factor (*W/h*) (Khouryieh & Aramouni, 2012). As shown in Table 7.2, cookies produced by RF alone resulted in a larger cookie width and spread factor when compared to WF. This could be attributed to the difference in the starch content of the two flours, where RF and WF contain 82.2% and 77.4% starch, respectively (Snow & O'Dea, 1981). A correlation has been made between higher amylose content and larger cookie diameters (Pareyt & Delcour, 2008). The low protein content of RF (6.3%) may have also contributed to the improvement of the cookie flow by lowering the water binding, thereby increasing the free available water; contrarily WF contains a higher protein content (12.2%). Table 7.2 shows that the width of the cookies was negatively affected with the addition of PPC in RF (100%) or WF (100%). However, the addition of PPI enhanced the width of cookies made of WF. The addition of PPC and PPI positively affected the height of the cookies made of RF, whereas only PPI had a positive effect on the stack height of cookies made of WF. These results may be attributed to the presence of polysaccharides in PPC affecting the viscosity and the flow rate of the dough. The results also show that the use of WF led to lower cookie spread factor of 4.8; this cookie spread was not significantly affected by the addition of potato proteins. However, when 50 WF/50 RF was used with either addition of PPC or PPI, the spread factor increased to 5.93 and 6.28, respectively, representing 23.5 and 30.8% increase as compared to 100 WF. Wani et al. (2013) reported that increasing watermelon seed protein up to 7.5% combined with wheat flour increased the spread factor, whereas enrichment up to 10% decreased the cookie spread. These authors attributed the low cookie spread at a high protein enrichment to the protein's increased hydrophilic sites, which lead to improved water binding (Wani, et al., 2013).

Textural analysis shown in Table 7.2 demonstrates that control RF (100%) resulted in a very soft cookie with a relative hardness force of 13.62 % comparatively to control WF (100.0 %). The addition of PPC increased the hardness of cookies (113.9-120.5%) well above that of control WF; conversely, the addition of PPI resulted in cookies, which were softer. PPC contained a larger amount of high molecular weight polysaccharides (8%) when compared to PPI (2%), which would affect the water binding within the cookie (Pareyt &

Delcour, 2008), thereby effecting the snap of the cookie. This phenomenon is probably linked to the effects of super-cooled sugar glass, which results in harder cookies following baking and cooling in the presence of high sugar content (Pareyt & Delcour, 2008).

The fracturability refers to the distance it takes to snap the cookies, when the distance is large; a flexible cookie will bend more when subjected to a constant force. The results show that the fracturability of the cookies was more or less similar; only small variations were associated with the addition of PPI, which produced more flexible cookies when compared to PPC. These slight differences in flexibility could be attributed to the difference in protein-protein and protein-carbohydrate interactions (Wani, et al., 2013). Table 7.2 also shows that the addition of PPC resulted in darker, more red, and more yellow cookies as shown by lower L^* values, higher a^*, and higher b^*, respectively, when compared to PPI. This could be explained by the higher presence of polysaccharides and polyphenols with PPC leading to enhanced caramelization and browning. Figure 7.1 outlines the sensorial responses of 25 panelists to the following attributes of colour, fracturability, crispness, adhesiveness, aftertaste, and overall liking. Sensory evaluation showed that the addition of PPC produced more overall preferred cookies attributed to the high scores for colour, adhesiveness, and aftertaste. However cookies which contained PPI produced texturally more preferred cookies in terms of fracturability and crispness (Figure 7.1 B), which correlated well with the highest mean quantitative descriptive scores (Figure 7.1 A) and instrumental fracturability (Table 7.2). Based on instrumental and sensory results, the addition of PPI was further investigated to produce texturally a more preferred cookie with reduced gluten. Based on several trials, the aftertaste and colour were improved by increasing the purity of PPI and the baking temperature to 205 °C.

7.4.2. Consistency of dough made of composite flours

The dough consistency is an important parameter to assess as different compositions of flour and protein can affect the water absorption and the development time and as a result the dough formation stages and the final cookie texture (Singh & Mohamed, 2007; Marco & Rosell, 2008; Wani, et al., 2013).

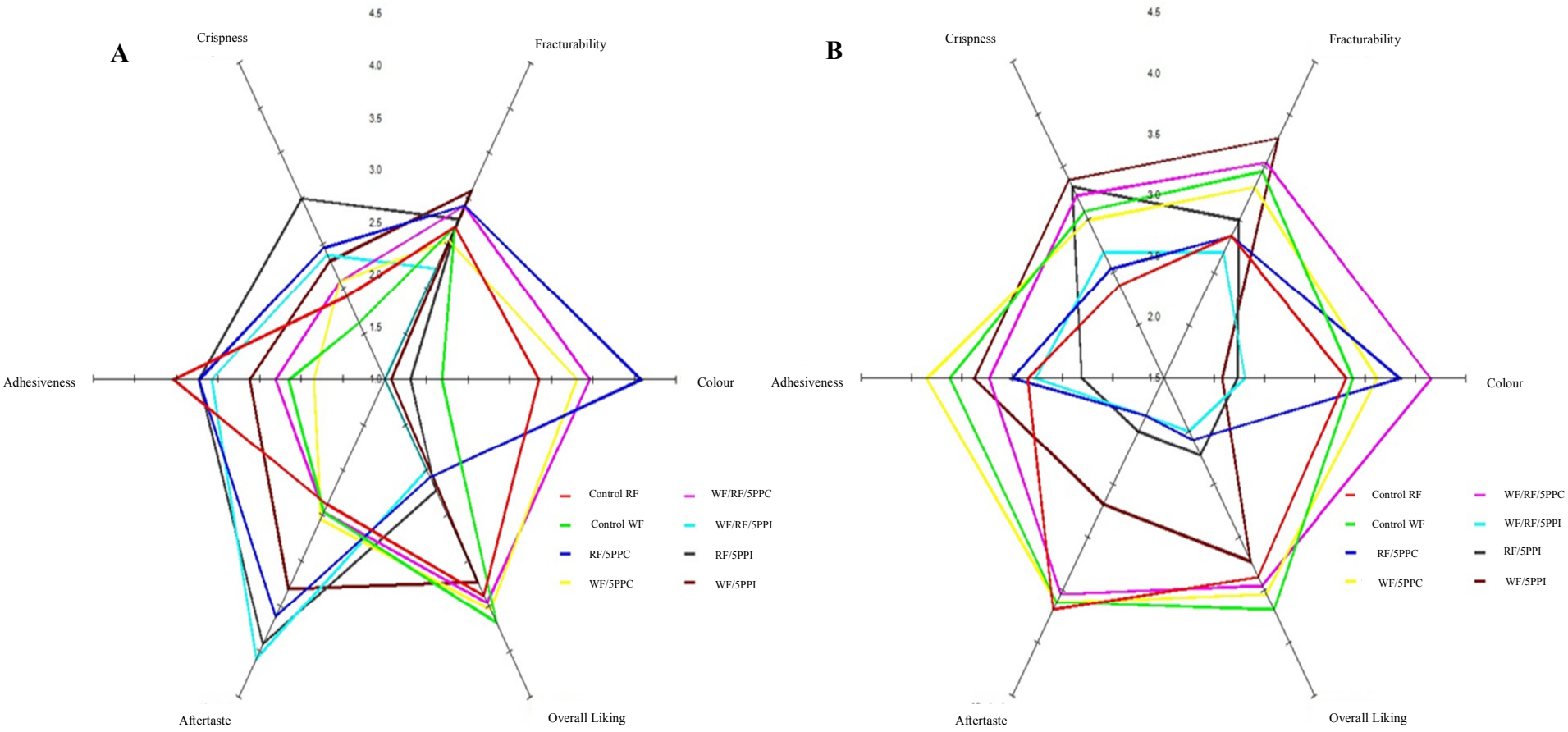

Figure 7.1 Average mean quantitative descriptive scores for cookies enriched with potato protein concentrate (PPC) or with isolate (PPI) based on specified scaling (A) or mean liking scale (B): rice flour ( ); wheat flour ( ); PPC/rice flour ( ); PPC/ wheat flour ( ); PPC/ wheat flour/ rice flour ( ); PPI/ rice flour ( ); PPI/ wheat flour ( ); and PPI/ wheat flour/ rice flour ( ).

Control RF required a higher amount of water (73.5 % relative humidity) and mixing time (17.7 min) to develop and reach the fixed dough consistency (Figure 7.3). This long developmental time is undesirable as it shows a poor dough, which is unable to relax after machinability (Sivaramakrishnan, et al., 2004) increasing past 500 FU with time (Figure 7.2 B). Conversely, Control WF showed a decrease in machinability with time, revealing the formation of a dough that is able to relax with time (Figure 7.2 A). Figure 7.2 C shows the Farinograph profile of the RF/WF (50/50%) composite, where the dough is able to reach the 500 FU faster than RF alone, as well as the dough takes the behavior of WF where it relaxes once reaching that level. The RF/WF composite flour resulted in a higher relative humidity (67%), when compared to Control WF (61.5%) but lower when compared to Control RF (73.5%). These results are not in agreement with Sivaramakrishnan et al. (2004), where their composite flour of WF and RF resulted in a lower relative humidity, as compared to pure WF. Explanation for these differences could be due to the difference in WF and RF type, where the grain and protein content have been found to influence water absorption (Sivaramakrishnan, et al., 2004).

The addition of 15% potato proteins (PPI) to RF reduced the water absorption to 62% relative humidity and developmental time to 5.7 min (Figure 7.3). Contrarily, an opposite trend was exhibited by dough made of 100% WF enriched with 15% PPI in which an increase of the relative humidity to 64% and a decrease in developmental time to 5.7 min were obtained as compared to unenriched dough (Figure 7.3). The results for increasing relative humidity in WF with protein enrichment correlate well with the literature where gluten-soy protein blends with increasing soy protein isolate and wheat with watermelon seed protein isolate and fluted pumpkin seed flour also showed an increasing water absorption (Singh & Mohamed, 2007; Wani, et al., 2013; Giami, et al., 2005). Protein enrichment of 10% and 15% resulted in the similar relative humidity (data not shown). The results also reveal that water absorption was strongly dependent on the RF to WF proportions, as shown by the significant differences in relative humidity (Figure 7.3).

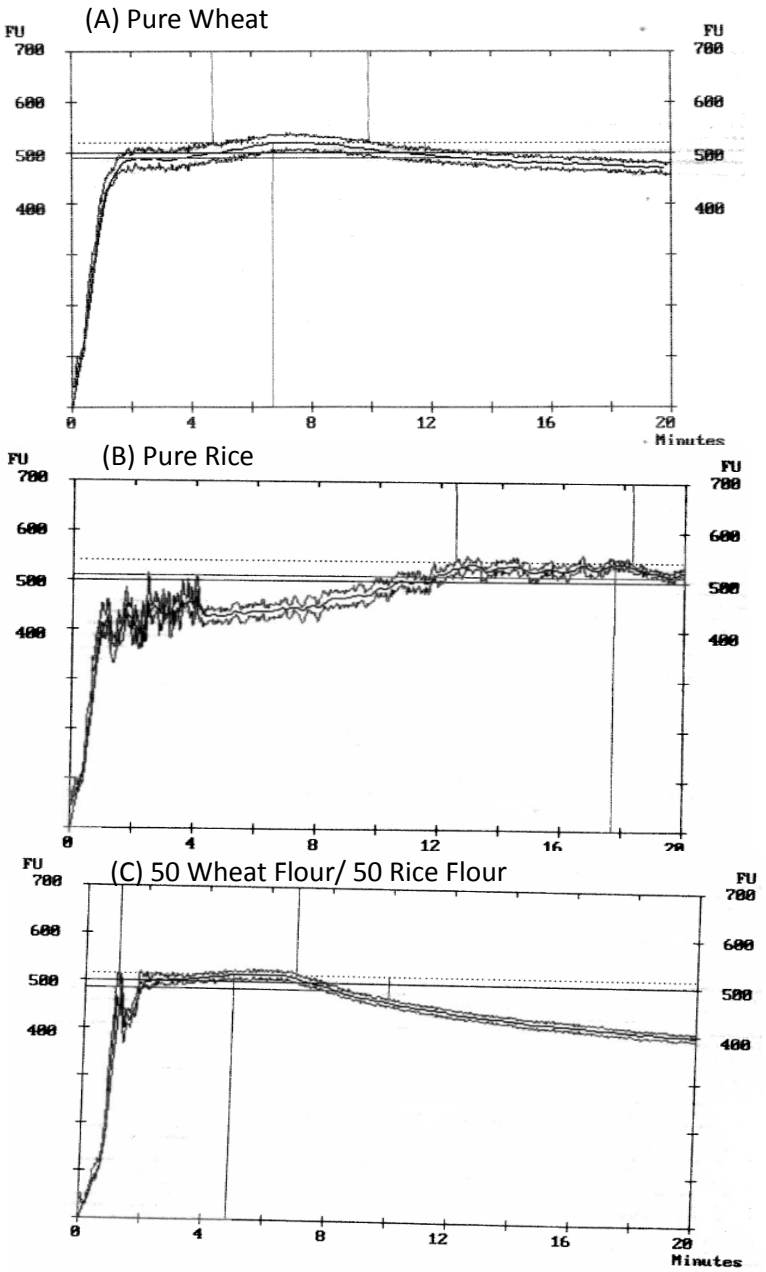

Figure 7.2 Farinograms for (A) pure wheat flour; (B) pure rice flour; (C) composite flour of 50 wheat flour/ 50 rice flour.

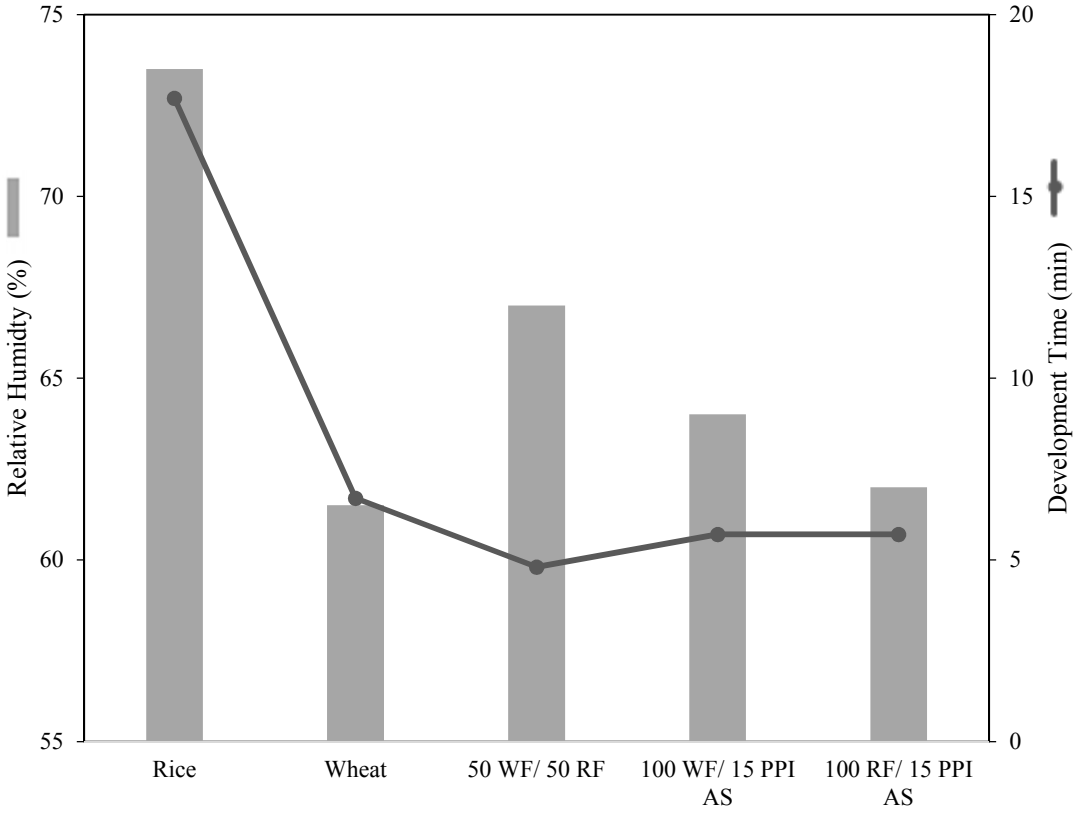

Figure 7.3 Relative humidity and development time as determined by Farinograph measurements for rice flour, wheat flour, and selected composite flours.

7.4.3. Investigation of properties of reduced-gluten cookies enriched with potato protein isolate

7.4.3.1. Compositional, geometric, textural and color properties

As shown in Table 7.3, 100% WF-based cookies contained a higher protein content (7.9%) than 100% RF-based cookies (4.1%). The protein content of low gluten cookie formulations ranged from 6.7-16.4%. These values are strongly correlated with the protein enrichment, where the highest protein content was obtained for 50 RF/50WF/23.11% PPI, whereas the lowest was obtained for 50 RF/ 50 WF/ 1.95 PPI.

The results indicate that the addition of PPI had a lower effect on the spread factor of the cookies, when compared to the ratio of RF to WF. Increasing PPI from 1.9-23.1% in 50RF/50WF-based cookies resulted in a decrease in the spread factor from 5.65 to 4.95. These results are in agreement with previous findings (Singh & Mohamed, 2007), which could be due to the proteins binding more air during the creaming stage, thereby reducing the spread. Indeed, Donelson (1988) has concluded that 3% protein is needed in gluten-free cookie formulations for a good baking quality, in terms of cookie spread (Donelson, 1988). Table 7.3 also shows that WF had a more significant effect on the height of the cookie, which yielded lower spread factors cookies. Similar results were found by Singh & Mohamed (2007), where high gluten content reduced the spread factor of the cookies. Regarding the effect of RF to WF ratio, increasing the proportion of RF from 25 to 75% in the presence of 5 PPI resulted in a decrease in the height, while the width was maintained constant; as a result, the spread factor of cookies made of 75 RF/25 WF/5 PPI was higher as compared to those with 25 RF/75 WF/5 PPI.

As shown in Table 7.3, the hardness of the cookies or maximum peak force (g force) was higher for WF-based cookies (Control WF) than RF-based cookies (Control RF). In order to examine the effect of RF to WF ratio, we can compare the formulations (85.4 RF/ 14.6 WF, 50 RF/50 WF, and 14.6 RF/ 85.4 WF) where the PPI enrichment was constant at 12.5%. These formulations show that as the RF proportion was increased, the hardness of the cookies decreased.

Table 7.3 Effect of potato protein addition on composite wheat and rice flour formulations on baking quality and textural analysis of cookies.

Formulations		Geometry[b]			Instrumental Texture[c]	
	Protein Content	Width (*W*)	Stack Height (*h*)	Spread factor	Hardness Force (g)	Fracturability Distance (mm)
Control RF	4.11± 0.11	50.67± 0.90	7.25± 0.20	6.99	684.95± 86.08	118.93± 0.42
Control WF	7.86± 0.09	52.54± 1.30	11.70± 0.10	4.49	1020.81± 147.44	123.15± 0.18
50 RF/ 50 WF/ 12.5 PPI[a]	11.43± 0.10	54.16± 0.70	10.49± 0.20	5.16	3031.27± 96.99	123.37± 0.45
85.4 RF/ 14.6 WF/ 12.5 PPI[a]	10.35± 0.12	54.10± 1.20	9.35± 0.07	5.79	2376.27± 309.07	120.97± 0.41
50 RF/ 50 WF/ 12.5 PPI[a]	11.79± 0.20	53.44± 1.20	10.76± 0.20	4.97	2677.15± 293.16	121.57± 0.18
25 RF/ 75 WF/ 20 PPI[a]	15.66±0.17	53.96± 1.10	10.71± 0.90	5.04	1278.45± 87.88	122.74± 0.20
50 RF/ 50 WF/ 23.11 PPI[a]	16.36±0.16	54.46± 0.90	10.99± 0.30	4.95	2424.50± 203.71	123.56± 0.32
75 RF/ 25 WF/ 5 PPI[a]	7.45± 0.22	51.79± 0.50	10.33± 0.07	5.01	1888.12± 216.44	121.73± 0.32
25 RF/ 75 WF/ 5 PPI[a]	9.14±0.16	51.72± 0.80	11.66± 0.09	4.44	1227.41± 11.77	122.23± 0.15
50 RF/ 50 WF/ 1.9 PPI[a]	6.71± 0.13	53.38± 0.80	9.44± 0.20	5.65	775.01± 93.65	121.57± 0.16
75 RF/ 25 WF/ 20 PPI[a]	14.18±0.06	52.10± 1.0	10.85± 0.08	4.80	2809.76± 94.57	123.42± 0.36
14.6 RF/ 85.4 WF/ 12.5 PPI[a]	13.22± 0.28	52.11± 0.5	13.42± 0.10	3.88	3595.80± 176.10	124.21± 0.23

[a] Potato protein concentrate isolated by 60% ammonium sulphate saturation from potato fruit juice.

[b] Results obtained were statistically significant for width F-value 8.36 *p*-value <0.0001 and height F-value 426.38 *p*-value <0.0001.

[c] Results obtained were statistically significant for Hardness Force F-value 54.77 *p*-value <0.0001 and Fracturability Distance F-value 18.13 *p*-value <0.0001.

Table 7.4 Instrumental colour evaluation and mean quantitative descriptive scores for experimental design cookie formulation.

Formulations	Colour Evaluation							Sensory Evaluation: Mean Quantitative Descriptive Scores					
	Top of Cookie[f]				Bottom of Cookie[g]								
	*L**	*a**	*b**	*ΔE*	*L**	*a**	*b**	Tcolour[d]	Bcolour[e]	Fracturability	Crispness	Adhesiveness	Aftertaste
Control RF[a]	52.21 ±0.74	14.77 ± 0.61	34.68± 1.51		42.75± 0.34	18.34± 0.20	29.79 ± 0.24	3.64± 0.78	3.89± 0.92	3.29± 1.27	2.86± 1.24	3.46± 1.10	2.23± 1.22
Control WF[b]	68.47± 0.13	4.57± 0.14	32.37± 0.46		50.35± 1.52	15.50± 0.59	32.47 ± 0.79	2.71± 0.85	3.71± 0.76	2.79± 1.26	1.96± 0.74	2.79± 1.03	2.96± 1.14
50 RF/ 50 WF/ 12.5 PPI[c]	55.6± 2.93	6.72± 0.2	21.72± 1.32	16.8	45.04± 4.73	12.95± 3.56	22.63 ±1.10	3.25± 0.97	3.89± 0.83	2.54± 1.17	3.04± 1.14	2.68± 1.22	3.46± 1.35
85.4 RF/ 14.6 WF/ 12.5 PPI	54.95± 0.68	5.2± 0.11	18.12± 0.23	19.3	46.92± 3.26	9.00± 3.21	19.45 ± 3.12	3.68± 1.09	4.29± 0.81	2.75± 1.29	2.57± 1.10	2.64± 1.10	3.50± 1.17
50 RF/ 50 WF/ 12.5 PPI	54.78± 1.14	6.19± 0.07	19.74± 0.26	18.7	46.62± 4.01	10.89± 2.68	20.11 ± 1.52	3.86± 1.08	3.82± 0.90	2.46± 1.10	2.29± 1.01	3.07± 1.15	3.43± 1.14
25 RF/ 75 WF/ 20 PPI	52.43± 5.16	5.43± 0.48	14.17± 2.9	24.3	54.09± 3.38	5.22± 0.85	12.48 ± 1.14	4.21±1. 40	3.96± 1.55	2.61± 1.40	1.32± 0.61	3.11± 1.31	3.82± 1.36
50 RF/ 50 WF/ 23.11 PPI	55.99± 0.47	4.77± 0.3	13.11± 0.81	23.0	53.86± 2.17	4.75± 1.27	10.93 ± 1.40	3.71± 1.36	4.04± 0.92	2.68± 0.90	2.50± 0.96	2.89± 1.03	3.82± 1.22
75 RF/ 15 WF/ 5 PPI	65.01± 0.15	5.35± 0.25	20.54± 0.34	21.3	54.28± 1.95	10.86± 1.18	19.51 ± 0.86	2.93± 0.81	3.46± 0.74	2.86± 0.85	2.86± 0.89	2.21± 0.96	2.96± 0.84
25 RF/ 75 WF/ 5 PPI	65.49± 0.8	3.6± 0.04	19.78± 0.13	13.0	57.82± 4.17	11.08± 2.32	23.73 ± 1.58	1.93±0. 90	3.07± 1.02	2.71±1.08	2.50±1.00	2.50± 1.07	2.64±0.83
50 RF/ 50 WF/ 1.9 PPI	66.98± 0.75	5.75± 0.97	26.55± 0.83	6.1	57.99± 1.98	11.05± 1.15	24.88 ± 0.57	1.86± 0.85	3.14± 0.71	2.36± 1.24	1.93± 0.90	3.07±1.02	2.89± 0.88
75 RF/ 25 WF/ 20 PPI	55.79± 2.09	4.52± 0.4	13.03± 0.43	24.2	51.05± 0.94	4.88± 1.08	10.15 ± 1.37	4.00±1. 33	4.11± 1.31	2.25± 0.80	2.36±1.03	2.61± 1.17	4.00± 1.15
14.6 RF/ 85.4 WF/ 12.5 PPI	62.62± 1.76	6.89± 0.75	20.53± 0.58	13.4	44.45± 1.49	14.17± 0.12	14.59 ± 1.33	2.61± 1.13	3.71± 0.90	2.86± 1.27	2.43± 0.92	2.43±0.96	3.00±0.90

[a]Abbreviation RF, rice flour.
[b]Abbreviation WF, wheat flour.
[c]Abbreviation, PPI, potato protein isolate extracted by 60% ammonium sulphate saturation.
[d]Colour of the top of the cookie.
[e]Colour of the bottom of the cookie.
[f] Results obtained were statistically significant for *L** F-value 48.21 *p*-value <0.0001, *a** F-value 94.21 *p*-value <0.0001, and *b** F-value 146.04 *p*-value <0.0001.
[g] Results obtained were statistically significant for *L** F-value 9.78 *p*-value <0.0001, *a** F-value 18.95 *p*-value <0.0001, and *b** F-value 69.46 *p*-value <0.0001.

Comparing 25 RF/ 75 WF/ 20 PPI and 25 RF/ 75 WF/ 5 PPI formulations reveals that the hardness was not affected by the level of PPI enrichment for high WF-based cookies; however, the addition of PPI led to higher hardness when compared to the Control WF cookie. Contradictory results were encountered by Singh and Mohamed (2007), where cookies prepared with WF, gluten and soy protein isolate blends, showed no significant textural effects, specifically peak force of the cookies (Singh & Mohamed, 2007). Contrarily, McWatters et al. (2003) reported an increase in hardness with increasing protein concentration. On the other hand, the textural effect of potato protein in high RF-based cookies (75 RF/ 25 WF/ 5 PPI; 75 RF/ 25 WF/ 20 PPI) was high showing an increase in the hardness with increasing PPI enrichment. When compared to the Control RF cookies, high RF-based cookies enriched with PPI resulted in significantly higher hardness.

The fracturability results confirm that Control WF resulted in a substantially more flexible cookie when compared to Control RF (Table 7.3). The fracturability did not vary significantly with formulation changes. Of particular interest, in the case of 75 RF/ 25 WF/ 20 PPI, 50 RF/ 50 WF/ 23 PPI, and 50 RF/ 50 WF/ 12.5 PPI formulations, the fracturability is equal to that of Control WF. The literature shows an opposite trend where enriching rice flour cookies with buckwheat flour, resulted in decreasing fracturability (Dapčević et al., 2013). Contrarily, enriching wheat flour with β-glucan rich hydrocolloids between 20-30% substitutions, resulted in increasing fracturability (Lee, et al., 2005). Previous studies (Dapčević, et al., 2013; Lee, et al., 2005; Mamat, et al., 2010) show that fracturability within the 0.65 to 0.9 mm result in enhanced consumer acceptance, however these cookies tend to have hardness within the range of 2039.4 to 3059.2 g force. In our case, cookies have hardness ranging from 684.95 and 3595.80 g force; therefore, the higher fracturability may contribute to lower crispness based on mean quantitative descriptive scores (Table 7.4). Differences to literature data on fracturability could be associated with varying formulations such as the fat to sugar ratio reported of 1.2, whereas ours was slightly higher at 2.4. In addition, cookie height (thickness) has been shown to have an influence on fracturability readings (Mamat, et al., 2010), where our cookie heights are nearly double those reported in the literature. Therefore variations in fracturability readings could be associated to the higher cookie heights. In addition, measurement differences of larger load

cell of 50 kg and slower test speed of 1 mm/sec, as opposed to the literature of a load cell of 5 kg and faster test speed of 3 mm/sec. Contrary to instrumental fracturability determinations, the mean quantitative descriptive scores of fracturability were more dependent on the type of formulations (Table 7.4). Panelists gave higher fracturability scores for 100 RF-based cookie control as compared to the 100 WF one. Moreover, panelists attributed the same mean quantitative descriptive scores for fracturability to formulations 85.4 RF/ 14.6 WF/ 12.5 PPI, 75 RF/ 15 WF/ 5 PPI, 25 RF/ 75 WF/ 5 PPI and 14.6 RF/ 85.4 WF/ 12.5 PPI, as compared to 100 WF. Limited studies, compare the texture in terms of fracturability, more generic terms such as texture are used. A previous study conducted by Arshad et al. (2007), found that WF enriched with 15% defatted wheat germ resulted in decreased texture scores associated with a crumbly texture compared to WF alone.

The results for the colour of the top of the cookie are shown in Table 7.4. As shown RF alone produced a darker, redder, with similar yellow colour cookie when compared to WF alone. In composite formulations, when RF was increased, the cookies were darker. When PPI enrichment was high, the *b** tended decreasing, thereby affecting the blue pigmentation of the cookie (Table 7.4). When examining the total colour change we can see that increasing the protein content from 1.9 to 23.1% significantly increased the total colour change from 6.12 to 22.95. This could be due to the light brown colour associated with the potato protein isolate, which is associated with the polyphenols remaining after extraction, as well as the enriched protein content could be responsible for improved Maillard reaction. Discrepancies in colour evaluation results (Table 7.2 and Table 7.4) are associated with slight changes of baking temperature and time and to further purification of PPI. Upon further comparison with the mean quantitative descriptive scores, in relation to the instrumental colour evaluation (ΔE), results correlated well with the top colour of the cookie (Tcolour; Table 7.4). The mean quantitative descriptive scores range from 1.86- 4.21, the lowest for 50 RF/ 50 WF/ 1.9 PPI, and the highest for 25 RF/ 75 WF/ 20 PPI. Therefore, descriptors were well established for the panelist and the colour evaluation.

7.4.3.2. Sensory Properties

Table 7.5 shows the mean sensory liking scores for all cookie formulations. Control WF-based formulation resulted in the most preferred cookie with the highest overall liking scores for all attributes. On the other hand, the Control RF cookie was liked fifth in aftertaste/ overall liking, third in relation to fracturability, and fourth/ fifth for colour of top and bottom of cookie, crispness, and adhesiveness. The cookies which were predominantly preferred based on all attributes were those corresponding to the 50 RF/ 50 WF/ 1.9 PPI, 75 RF/ 25 WF/ 5 PPI, and 25 RF/ 75 WF/ 5 PPI formulations. These results indicated that, 5% PPI enrichment of the composite flours of 75RF/ 25WF and 25RF/ 75WF would result in cookies with desirable characteristics, close to the preferred Control WF and much more preferred than the Control RF. The sensory liking scores of the best formulations correlate well with the mean quantitative descriptive scores shown in Table 7.4, where low top colour cookie scores (lighter), with golden cookie bottom scores, average fracturability (between crumbly and brittle), average crispness and adhesiveness, and low aftertaste were the most preferred sensory characteristics. The cookie with the least desirability was obtained with the use of 25 RF/ 75 WF/ 20 PPI formulation; these results was due to lowest sensory liking scores results obtained for most of attributes (Table 7.5), in particular for the low crispness score (soggy) and strong aftertaste.

7.4.3.3. Statistical interpretation of cookie design formulations

In order to assess the multiple variables on the single set of the cookie formulation design, the use of multivariate statistics was used (Lawless & Heymann, 2010). For the multivariate analysis of variance (MANOVA), the F-value greater than 1, and Pr -value of less than 0.05 indicate that all formulations were perceived by the panelists as different (Table 7.6). In addition, all seven attributes namely, colour of top of cookie, colour of bottom of cookie, fracturability, crispness, adhesiveness, aftertaste, overall liking were statistically different from each other (data not shown). The total-sample standardized canonical coefficient is the mean separation of the attributes (Lawless & Heymann, 2010), which indicates the attribute that is more responsible for the differences. Our results indicate from Canonical 1 (Table 7.6) that aftertaste and overall liking attributes are the most responsible for the group differences and to a smaller extent colour of the top and bottom

Table 7.5 Mean sensory liking score for cookie formulations prepared with potato protein isolate.

Formulations	Sensory Evaluation: Scaling Score						
	Tcolour[d]	Bcolour[e]	Fracturability	Crispness	Adhesiveness	Aftertaste	Overall Liking
Control RF[a]	2.39± 1.17	2.64± 1.19	2.71± 1.36	2.39± 1.23	2.64± 1.03	3.07± 1.27	2.86± 1.24
Control WF[b]	4.25± 0.93	4.00± 1.05	3.68± 1.06	3.39± 1.26	3.57± 0.88	3.96± 0.79	3.64± 1.03
50 RF/ 50 WF/ 12.5 PPI[c]	3.00± 1.33	2.75± 1.27	2.61± 1.29	2.86± 1.27	2.68± 1.02	2.25± 1.24	2.32± 1.22
85.4 RF/ 14.6 WF/ 12.5 PPI	2.61± 1.03	2.29± 0.90	2.54± 1.17	2.50± 1.07	2.93± 0.98	2.00± 0.98	2.04± 1.00
50 RF/ 50 WF/ 12.5 PPI	2.75± 0.97	2.82± 1.02	2.43± 1.17	2.61± 1.17	2.57± 1.06	2.25± 1.24	2.39± 1.20
25 RF/ 75 WF/ 20 PPI	1.96±0.92	2.36± 1.22	2.43± 1.14	2.04± 0.92	2.14± 0.93	1.43± 0.79	1.36± 0.56
50 RF/ 50 WF/ 23.11 PPI	2.29± 1.05	2.32± 1.12	2.64± 0.83	2.82± 1.09	2.82± 0.82	1.89± 0.99	1.79± 0.83
75 RF/ 25 WF/ 5 PPI	4.04± 0.92	3.89± 0.99	3.54± 1.04	3.61± 0.96	3.43± 0.74	3.64± 0.91	3.57± 1.07
25 RF/ 75 WF/ 5 PPI	3.39±1.07	3.68± 0.86	3.32±1.09	3.18±1.19	3.32± 0.94	3.39±0.79	3.32± 0.94
50 RF/ 50 WF/ 1.9 PPI	3.32± 0.90	3.93± 0.86	3.36± 0.99	3.32± 1.09	3.39±0.88	3.86± 1.01	3.61± 0.99
75 RF/ 25 WF/ 20 PPI	2.14±0.85	2.25± 0.84	2.46±1.04	2.46±1.04	2.43± 1.14	1.75± 1.04	1.61± 0.74
14.6 RF/ 85.4 WF/ 12.5 PPI	2.75± 1.08	3.00± 1.09	3.21± 0.99	2.82± 1.06	3.11±0.92	2.64±1.06	2.54± 1.00

[a]Abbreviation RF, rice flour.
[b]Abbreviation WF, wheat flour.
[c]Abbreviation, PPI, potato protein isolate extracted by 60% ammonium sulphate saturation.
[d]Colour of the top of the cookie.
[e]Colour of the bottom of the cookie.

Table 7.6 Statistical interpretation of cookie formulation design prepared with potato protein isolate.

MANOVA					
Statistic	Value	F-value	Num DF	Den DF	Pr>F-value
Wilk's Lambda	0.3623	4.59	77	1913	<0.0001
Pillai's Trace	0.8126	3.87	77	2268	<0.0001
Hotelling-Lawley Trace	1.3269	5.40	77	1296.7	<0.0001
Roy's Greatest Root	0.9762	28.75	11	324	<0.0001

Total-Sample Standardized Canonical Coefficients			
Variable	Canonical 1	Canonical 2	Canonical 3
TColour	0.3283	-1.006	0.2114
BColour	0.3773	0.2061	-0.3293
Fracturability	-0.0522	0.3054	-1.2150
Crispness	-0.2858	-0.7425	0.5316
Adhesiveness	-0.1359	-0.3433	-0.4967
Aftertaste	0.6506	0.6082	-0.5346
Overall liking	0.5688	0.4544	1.3562

Logistic Regression Analysis[a]				
	Overall Acceptance			
Variable	Estimate	χ^2	Pr> χ^2	Odd's Ratio
TColour	0.2113	0.8909	0.3452	1.235
BColour	-0.0039	0.0003	0.9868	0.996
Fracturability	-0.2700	0.9253	0.3361	0.763
Crispness	0.4711	2.7767	0.0956	1.602
Adhesiveness	-0.2638	0.8437	0.3583	0.768
Aftertaste	0.8947	9.9949	-0.0061	2.447
Overall liking	1.6936	23.7313	<0.0001	5.439

[a]Significance, α=0.1

of the cookie as well as the crispness. Logistic regression is applied when a dependent response is defined as the probability of occurrence (probability of overall acceptance of a cookie formulation) and the independent variables potentially behave according to a multivariate normal distribution (Malundo, et al., 1999). According to the results of Logistic regression analysis, aftertaste, overall liking, and crispness were found to be the most significant (α= 0.1) and determinant for the overall acceptance of reduced-gluten cookies enriched with potato proteins. Therefore, these attributes were further assessed for the optimization of the cookie formulation.

7.4.3.4. Optimization of cookie formulation

Response surface methodology (RSM) was used to assess the effects of proportion of RF (%) and PPI enrichment (%) and their interactions on the mean quantitative descriptive-based scores and the mean liking scores of crispness, aftertaste, and overall liking attributes. The last attributes were identified with the logistic regression analysis as the statistically most significant attributes driving consumer acceptance of reduced-gluten cookies enriched with PPI. Multiple regression analysis and backward reduction algorithm was used to evaluate the best fitting model for the selected sensory attributes using the software Design-Expert version 8.0.2.

For the mean quantitative descriptive- and liking-based scores of crispness, the cubic model was statistically the most significant to describe the variations of the scores with the investigated parameters. While linear and quadratic models fitted the best for the variations of mean quantitative descriptive- and liking-based scores of aftertaste, respectively. Finally, the liking-based scores of overall liking were better described by the quadratic model. All R^2 and predicted R^2 are in reasonable agreement with each other; which confirms that the fraction of variation of the response with the model agrees with the fraction of variation of the response predicted by the model (Akoh, et al., 2007). As well as the normal distribution plot (data not shown) for all responses had linear distribution which confirm good fitting models. The analysis of variance (ANOVA; Table 7.7) also confirms the statistical significant of the models with F-values of 7.34 to 84.69 and corresponding *p*-values <0.0001 (Table 7.7).

Table 7.7 Analysis of variance results: the parameters and the significant interactions for mean quantitative descriptive scores and mean liking scores for crispness, aftertaste, and overall liking.

	Mean Quantitative Descriptive Scores				Liking					
	Crispness		Aftertaste		Crispness		Aftertaste		Overall Liking	
	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value
Model	7.34	<0.0001	32.85	<0.0001	7.31	<0.0001	84.69	<0.0001	83.22	<0.0001
(x_1)	1.87	0.1730		[a]	0.52	0.4702		[a]		[a]
(x_2)	5.07	0.0252	32.85	<0.0001	17.69	<0.0001	154.64	<0.0001	159.98	<0.0001
$(x_1)^2$	0.15	0.6969		[a]		[a]		[a]		[a]
$(x_2)^2$	4.25	0.403		[a]	3.75	0.0537	13.31	0.0003	5.51	0.0196
(x_1x_2)	7.34	0.0071		[a]		[a]				
$(x_2)^3$	5.07	0.0252		[a]	7.22	0.0076		[a]		[a]
$(x_1x_2)^2$		[a]		[a]	4.26	0.0399		[a]		[a]
$(x_1^2x_2)$	5.06	0.0252		[a]		[a]		[a]		[a]
Lack of fit	0.58	0.4457	1.05	0.3952	0.33	0.8059	1.44	0.1886	1.61	0.1361

[a]Not significant

As well as the non-significant lack of fit (F-values of 0.33 to 1.61; *p*-values of 0.1361-0.8059) validates the identified models for all investigated responses.

In the crispness descriptive- and liking score-based models, the most significant linear (x_2; F-value 5.07, *p*-value 0.0252 and x_2; F-value 17.69, *p*-value <0.0001, respectively), and cubic terms (x_2^3; F-value 5.07, *p*-value 0.0252 and x_2^3; F-value 7.22, *p*-value 0.0076, respectively) were those of the PPI enrichment variable. The interaction between RF proportion and PPI enrichment also affect significantly the descriptive scores of crispness (x_1x_2; F-value 7.34, *p*-value 0.0071 and ($x_1^2\ x_2$; F-value 5.06, *p*-value 0.0252)), where increased RF proportion and PPI enrichment had a positive synergistic effect on the crispness (Table 7.7). While their quadratic interaction had an effect on the mean liking scores ($(x_1x_2)^2$; F-value 4.26, *p*-value 0.0399). This could be attributed to the potato protein improving the dough machinability (Figure 7.2), which in turn bakes a better crisp texture. Similarly, a study comparing enrichment with soybean flour, chickpea flour and lupine flour have shown that the protein enrichment affects the texture up to a specific substitution (Hegazy & Faheid, 1990).

For the mean quantitative descriptive scores of aftertaste, the most significant linear term was the PPI enrichment (x_2; F-value 32.85, *p*-value <0.0001; Table 7.6). The mean liking scores-based model reveals the most significant term to be protein enrichment both linearly and quadratically (x_2; F-value 154.64, *p*-value <0.0001 and x_2^2; F-value 13.31, *p*-value 0.0003). Indeed, as the protein concentration increases, the aftertaste attribute will become stronger (i.e higher on the described scale; Table 7.4). The aftertaste could be attributed to the bitterness of PPI which are associated with their high proportion of hydrophobic amino acids (Ney, 1979). For mean liking scores the significant linear and quadratic term was protein enrichment (x_2; F-value 159.98, *p*-value <0.0001 and x_2^2; F-value 5.51, *p*-value 0.0196, respectively). No significant interaction between the variables was detected in the overall liking for the mean liking scores-based model.

In order to understand the effect of the variables including, RF proportion and PPI enrichment on the sensory attributes, three dimensional contour plots of the significant interactions were generated from the predictive models (Figure 7.4).

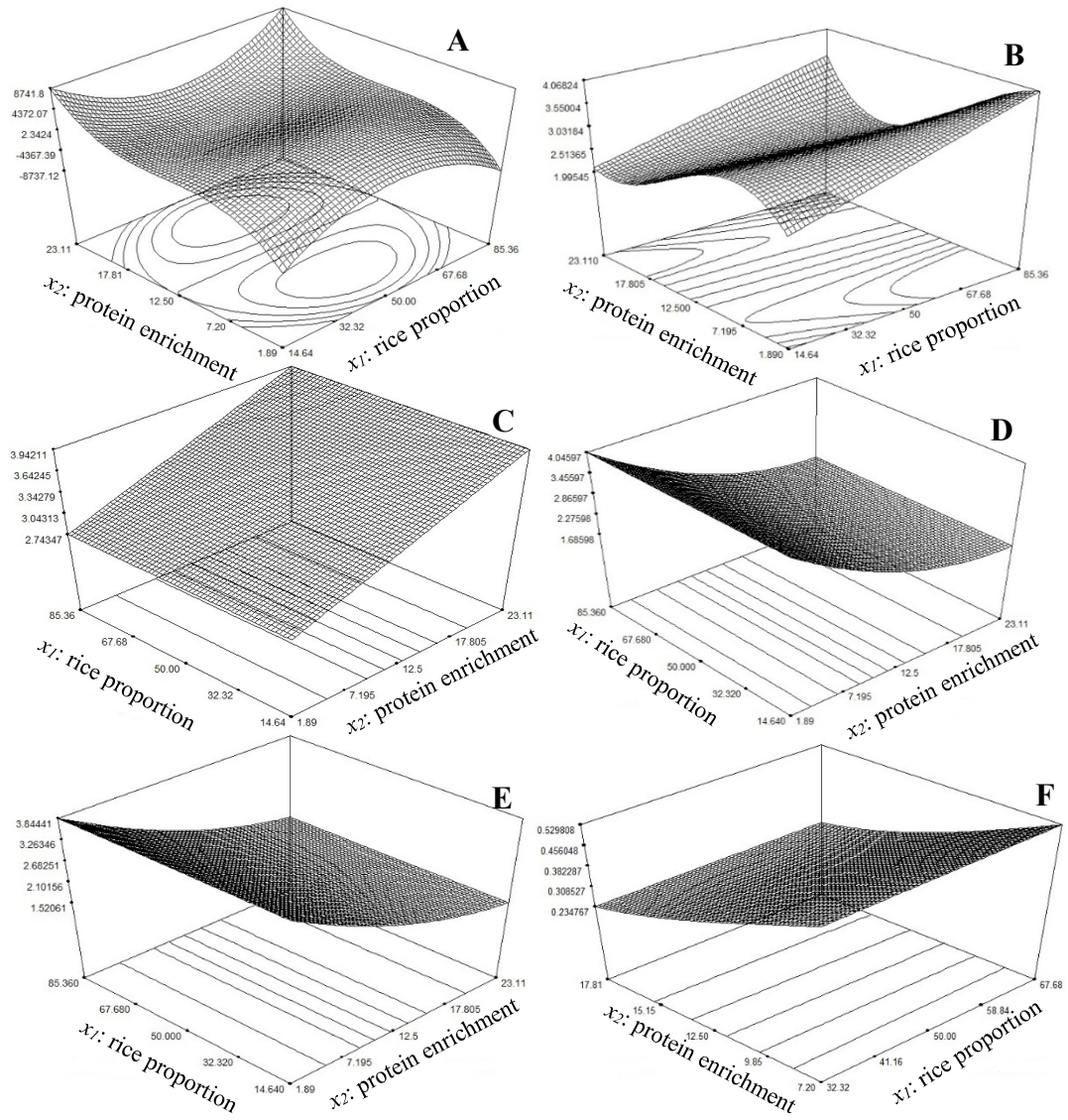

Figure 7.4 Response surface curves of mean quantitative descriptive scores for (A) Crispness, (C) Aftertaste, and mean liking scores for (B) Crispness, (D) Aftertaste, (E) Overall liking, (F) Desirability.

Following their assessment, a desirability plot (Figure 7.4 F) was generated by overlapping the contour plots of the critical attributes to identify the optimal regions for a highly preferred cookie product. Figure 7.4 A shows a trend of minimum and maximum RF proportion with maximum PPI enrichment resulted in an increase in the crispness for mean quantitative descriptive scores. Moreover, 50% rice flour proportions with 7.2% protein enrichment resulted in crisp cookies, as shown by the convex peak in the contour plot (Figure 7.4 A). However, the crispness contour plot of mean liking scores shows that the panel tended to prefer cookie crispness with a high proportion of rice flour and low protein enrichment (Figure 7.4 B).

As expected, the higher PPI enrichment resulted in higher perceived aftertaste independent on the RF proportion (Figure 7.4 C). As shown in Figure 7.4 D, consumers preferred the aftertaste of the cookie formulation which contained the lower amount of PPI enrichment, independent of the RF proportion. This is in good accordance as, higher PPI enrichment results in more prominent bitter taste (Ney, 1979). However to overcome this drawback of bitterness, the formulation can be improved in terms of addition of sweetening and flavouring at higher PPI enrichment levels.

Higher overall liking scores were achieved with low PPI enrichment, independent of RF proportions, as shown in Figure 7.4 E. Consumers did prefer low PPI enrichment compared to the Control RF as shown in Table 7.5 by the high mean overall liking scores. The desirability plot is in agreement with the overall liking plot, where higher mean liking scores were obtained at 7.20% protein enrichment with varying RF proportions (Figure 7.4 F). Where desirability point prediction indicates optimal formulation to be potato protein enrichment up to 7.20% for acceptable reduced-gluten cookies prepared with a combination of 67.88% RF and 32.12% WF.

7.5.Conclusion

Our study showed the benefit in using PPI over PPC for the development of a reduced-gluten cookie formulation. Where the cookies had an improved texture potentially due to the higher purity of the proteins extracted, and in turn lower polysaccharide content. The results also show that PPI-enriched cookie scored better for colour, fracturability, crispness, adhesiveness, aftertaste, and overall liking than the Control RF, when analyzed by a semi-

train panel of 70 McGill undergraduate and graduate students. Texturally the protein enriched-cookies are harder and more flexible which could be related to the overall desirable crispness and adhesiveness, when compared to Control RF. The statistical results show that the significant attributes for overall acceptance based on mean liking scores were crispness, aftertaste and overall liking. The desirability plot, generated from the cookie design confirm the PPI can be used to enrich cookie formulations up to 7.20% for acceptable reduced-gluten cookies prepared with a combination of 67.88% RF and 32.12% WF. Further improvement can be made to the aftertaste attributed to the added protein, by either the addition of a masking agent or by further purification to remove any of the phenolic compounds which would lead to the appearance and off-flavouring of the PPI. The use of PPI as a food ingredient is desirable, as they are recovered from industry by-products and are associated with many health benefits and functional properties.

CONNECTING STATEMENT 6

The development of a reduced-gluten cookie formulation demonstrates the potential application of potato proteins as a health-promoting ingredient (Chapter VII). Chapter VIII explores another associated application of potato protein isolates for their use as a starting material for the biogeneration of peptides. Selected proteases namely, Flavourzyme, Papain, Alcalase, and Novo Pro-D, have been examined for their effectiveness in the biogeneration of potato peptides. Time curves were established for the most promising enzymes specifically, Flavouzyme and Papain. Peptides were further characterized for their mass-to-charge profiles and identified according to their parents, patatin or protease inhibitors.

Waglay, A., & Karboune, S. (2015). Enzymatic generation of selected peptides from potato protein isolates and characterization of their structural properties. *(Submitted).*

CHAPTER VIII. ENZYMATIC GENERATION OF SELECTED PEPTIDES FROM POTATO PROTEIN ISOLATES AND CHARACTERIZATION OF THEIR STRUCTURAL PROPERTIES

8.1. Abstract

Potato protein isolate (patatin and protease inhibitors) was subjected to enzymatic hydrolysis using four proteases namely, Novo Pro-D, Alcalase, Flavourzyme, and Papain. Their catalytic efficiency was evaluated based on the degree of hydrolysis and the end-product profile. Novo Pro-D resulted in the lowest degree of hydrolysis, whereas Alcalase, Flavourzyme, and Papain exhibited a high catalytic efficiency for the hydrolysis of potato proteins. The degree of hydrolysis behaved in a concentration dependent manner. However, the end-product profile varied depending not only on the type of protease, but also on the protease concentration and the reaction time, revealing the heterogeneity of the system. The overall trend of the end-product variations was attributed to the dependence of the exo-activity on the catalytic efficiency of the endo-action of protease and to the inhibiting effect of potato protease inhibitors on exo and endo-activity to a different extent over the reaction time course. Peptide mapping results led to the identification of more unique peptides in the Papain-based hydrolysates. This can be attributed to the high exo-peptidase activity of Flavourzyme resulting in the generation of shorter peptides difficult to match. Flavourzyme produced more peptides originated from patatin fraction, whereas Papain resulted in the release of more peptides corresponding to the protease inhibitor fractions.

8.2. Introduction

Known as one of the staple crops, potatoes are readily consumed worldwide, undergo industrial processing, and/or involved in agricultural regimes (Kamnerdpetch, et al., 2007). The potato starch industry releases a by-product known as potato fruit juice (PFJ) at an exponential rate. Indeed, processing one thousand kg of potatoes releases 5-12 m^3 of PFJ, with a protein concentration of 30-41% (w/w, dry weight) (Wojnowska, et al., 1981). Potato proteins isolated from PFJ are commonly divided into three main fractions namely, patatin (36.5%), protease inhibitors (52.3%) and the remainder high molecular weight proteins when isolated using 60% ammonium sulphate $(NH_4)_2SO_4$ saturation (Waglay, et al., 2014). Removal of these proteins is necessary to overcome the large polluting capacity associated with PFJ. Currently the industry recovers the proteins by thermal coagulation and acidic precipitation (Knorr, et al., 1977), which due to the denaturation effects limits their potential applications. Therefore, modifications and improvements of potato proteins should be

undertaken in order to broaden their potential application. Indeed, potato proteins are considered of high nutritional quality being rich in lysine (7.18%) and methionine (1.06%) (Bártová, et al., 2015). Exploration of a vegetable by-product such as PFJ is both environmentally appealing as well as of great potential for fulfilling the current consumer demands for non-animal protein sources.

Protein modification through enzymatic hydrolysis using proteases has been identified as a promising approach to improve the solubility, nutritional values and functional properties of proteins (Kamnerdpetch, et al., 2007; Cheng, et al., 2010; Miedzianka, et al., 2014). Several studies have highlighted the beneficial properties of potato protein hydrolysates including their participation in angiotensin converting enzyme inhibition (Pihlanto, et al., 2008), their antioxidant potential (Wang & Xiong, 2005), their lipolysis-stimulating activity (Huang, et al., 2015), and their effect on serum cholesterol levels (Liyanage, et al., 2008). Most of these studies have primarily used one protease, Alcalase, and have evaluated the degree of hydrolysis based on free amino acids and not on the generated peptides. In addition, no information is available on the structural properties of the generated peptides from potato proteins as a function of the type of proteases. In general, the catalytic efficiency of proteases varies according to their origin, substrate specificity, active site, catalytic mechanism, pH and temperature optimum (Sumantha, et al., 2006; Kamnerdpetch, et al., 2007). The substrate specificity is indicative of the preferred amino acid where the proteases cleave the peptide bond (Sumantha, et al., 2006). Their catalytic mechanism is the point of cleavage, which can either be endo-proteases, acting within the protein chain and resulting in peptides, or exo-protease hydrolysing at the C- or N-terminal ends releasing amino acids (Kamnerdpetch, et al., 2007). As far as the authors are aware, only Kamnerdpetch et al. (2007) have explored a combination of endo- and exo-proteaese with selected specificities for the efficient hydrolysis of potato proteins.

As part of ongoing research, the present study was aimed at the investigation of the enzymatic generation of hydrolysates from potato proteins isolated from PFJ. The efficiency of selected proteases, namely, Flavourzyme, Alcalase, Papain, Novo Pro-D, was assessed in terms of the degree of hydrolysis, and the composition of hydrolysates (peptides and free amino acids). The time course of the hydrolysis reaction was also determined using

the identified promising systems, and the structures of the generated peptides were further characterized according to peptide mapping. These findings could result in improved modification of a potato by-product to generate more value added ingredients with promising beneficial properties. The approach is industrially advantageous as it can be easily controlled with mild reaction conditions, due to the high specificity and selectivity of proteases.

8.3. Materials and Methods

8.3.1. Materials

Fresh potatoes of Russet Burbank variety were purchased from a local supermarket. Sodium metabilsulfite, sulphuric acid (H_2SO_4), phosphotungstic acid, trifluoroacetic acid, hydrochloric acid were purchased from Sigma Chemical Co. (St-Louis, MO). Ammonium sulphate crystals, Tris-base and potassium phosphate dibasic were acquired from Fisher Scientific (Fair Lawn, NJ). Potassium phosphate monobasic was purchased from MP Biomedicals, LLC (Solon, OH). Commercial proteases Alcalase 2.41 from *Bacillus licheniformis*, Flavourzyme from *Aspergillus oryzae*, and Novo Pro-D from *Bacillus sp.* were provided by Novo Nordisk (Bagsvaerd, DE), whereas enzyme Papain isolated from papaya was purchased from Sigma Chemical Co. (St-Louis, MO).

8.3.2. Isolation of potato proteins from potato fruit juice

8.3.2.1. Preparation of potato fruit juice

PFJ was prepared according to the modified method of van Koningsveld, Gruppen, de Jongh, Winjingaards, van Boekel, and Walstra (2001). Potatoes of Russet Burbank variety were washed, chopped into large pieces and frozen at -80 °C for 30 min to decrease heat transfer during homogenization. The frozen potato samples (100 g) were suspended in 100 mL sodium metabisulfite solution (26 mM) to prevent polyphenol oxidation and homogenized using a Waring Commercial Blender on low speed for 5 min. The potato slurry was subjected to cheese cloth filtration. The resulting turbid liquid was centrifuged at 8000*g* for 30 min at 4 °C using a Beckman Centrifuge Model J2-21. The supernatant was filtered using 1.2 μm GF/C Whatman filters. The clear yellowish filtrate is known to be similar to industrial PFJ.

8.3.2.2. Preparation of potato protein isolate

$(NH_4)_2SO_4$ precipitation was performed according to the previous reported method by Waglay et al. (2014). The mixtures were saturated with 60% ammonium sulphate $(NH_4)_2SO_4$ and incubated for 90 min at 1000*g* and 4 °C. The recovered precipitates upon centrifugation were suspended in water and dialysed for 3 days through regenerated cellulose filters with a MW cut-off of 3-6 kDa (Fisher Scientific, NJ, USA).

8.3.3. Determination of proteolytic activity of selected commercial proteases

Proteolytic activity was determined using the azocasein assay according to the modified method of Trengove et al. (1999). 350 µL of commercial protease at appropriate dilutions in sodium acetate buffer (20 mM, pH 5.0) was added to 300 µL of 0.4% (w/v) azocasein suspended in same buffer. The reaction mixture was incubated for 20 min at 50 °C, the reaction was halted with the addition of 150 µL of 30% (v/v) tricholoroacetic acid. The mixture was centrifuged at 2000*g* for 10 min. The supernatant was mixed in equal volume with 2.5 M potassium hydroxide. The absorbance was read at 440 nm, against two blanks, which were run in tandem, blank buffer and blank substrate. One unit of protease activity was defined as the amount of enzyme which gives rise to an increase in absorbance at 440 nm per min.

8.3.4. Enzymatic generation of hydrolysates from potato protein isolates

The enzymatic generation of hydrolysates (peptides and amino acids) from potato protein isolates was performed using selected proteases as biocatalysts, including endo-protease, Alcalase, Novo ProD, Papain, and an exo-peptidase, Flavourzyme. 2% (w/v) of potato protein isolate suspension was prepared in 0.05 M potassium phosphate buffer pH 8.0. The reactions were initiated by adding the selected protease at 2, 10, and 20 U/mL, based on their respective proteolytic activities. Blank enzyme, blank buffer, and blank substrate were all run in parallel. Reactions were incubated at 50 °C for 30 min to 24 hr. Following incubation, reactions were boiled (100 °C) for 10 min to deactivate the enzymes. After centrifugation (11,963*g*, 5 min), supernatants were recovered. To remove the unhydrolyzed proteins, trichloroacetic acid and phosphotungstic acid were added to the supernatants to yield a final concentration of 20 and 30%, respectively. The suspensions were centrifuged at 11,963*g* for 5 min, resulting in a clear hydrolysate solution consisting of enzymatic

generated peptides and amino acids. The supernatants were collected and frozen at -20 °C, until further analysis.

8.3.5. Degree of hydrolysis

Degree of hydrolysis was calculated by determining the total amino acid content of potato proteins and of the generated hydrolysates (peptides and free amino acids) as described by Nielsen et al. (2001):

$$DH = \frac{h}{h_{tot}} \; x\, 100 \qquad (26)$$

Where, h_{tot} is the total number of peptide bonds per potato protein and h is the number of hydrolyzed bonds. h_{tot} is dependent on the amino acid composition of the potato pulp material.

The amino acid content was determined upon the acid hydrolysis of initial proteins and generated hydrolysates (Miedzianka et al., 2014). 0.5 mL of initial protein suspension or of hydrolysates were added to 1.5 mL of 6 N HCl. After incubation for 48 hr at 110 °C, the suspensions were recovered and neutralized with NaOH. The amino acids generated upon acid hydrolysis were determined using Ninhydrin assay. All degree of hydrolysis were corrected according to blank enzyme, blank substrate, and blank buffer. Subtracting the total amino acid content of the hydrolysates (peptides and free amino acids) from that of the free amino acid provides the concentration of peptides.

8.3.6. Ninhydrin assay

The modified method of Rosen (1957) was used for the determination of free α-amino acid. 200 µL of reaction were taken and mixed with 100 µL of 10 mM sodium cyanide suspended in 2.65 M sodium acetate buffer at pH 5.4 in a ratio 1: 49 (v/v) and 100 µL of 3% (w/v) ninhydrin in 2-methoxyethanol. Ninhydrin reaction was incubated for 15 min at 100 °C. Immediately the reaction mixtures were cooled to room temperature with the addition of 1mL *n*-propanol diluted with distilled water (1: 1 v/v). Absorbance was measured at 570 nm. Standard curve was developed using Leucine as a free α-amino group.

Peptide proportion= $((h_{\text{chemically hydolyzed}} * \text{volume}_{\text{reaction}})-h_{\text{potato protein isolates}}) - ((h_{\text{hydrolysate}}-((h_{\text{blank}} * \text{volume}_{\text{blank}}) + h_{\text{potato protein isolates}})$ *(27)*

8.3.7. MS/MS analysis of peptides

Structural characterization of potato hydrolysates was performed at Centre Protéomique at Université de Laval. Generated peptides were desalted to remove any interfering components and separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES- MS/MS). Analyses were performed with a Thermo Surveyor MS pump connected to an LCQ Deca XP mass spectrometer (Thermo Electron, CA, USA) supplied with a nanoelectrospray ion source (Thermo Electron, CA, USA). Peptides were bound on a cap trap (Michrom Bioresources, CA, USA) at 10 µl/min after which chromatographic separation took place using a PicoFrit column BioBasic C18 (10 cm × 0.075 mm) (New Objective, MA, USA) set with a linear gradient from 2 to 50% of solvent B (acetonitrile, 0.1% formic acid) for 30 min, at 200 nL/min (obtained by flow-splitting). Mass spectra were acquired using a data-dependent acquisition mode using Xcalibur software version 1.2. Each full scan mass spectra (400-2000 *m/z*) was followed by collision-induced dissociation of the three most intense ions. The dynamic exclusion (30 sec exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

8.3.7.1. Database searching

All MS/MS profiles were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search the TAX_SolaTube_4113_20150112 database (unknown version, 55764 entries) assuming the digestion enzyme was non-specific. X! Tandem was set up to search a subset of the TAX_SolaTube_4113_20150112 database also assuming non-specific. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 0.100 Da. Dehydrated of the n-terminus, glu →pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln →pyro-Glu of the n-terminus, deaminated of asparagine and glutamine and oxidation of methionine were specified in X! Tandem as variable modification. Glu →pyro-Glu of the n-terminus, gln →pyro-Glu of the

n-terminus, deaminated of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications.

8.3.7.2. Criteria for peptide identification

Scaffold (version Scaffold_4.3.2, Proteome Software Inc., OR, USA) was used to validate MS/MS based peptide identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide Probabilities from X! Tandem were assigned by the Scaffold Local FDR algorithm. Peptide Probabilities from Mascot were assigned by the Peptide Prophet algorithm (Keller, et al., 2002) with Scaffold delta-mass correction. Protein identification were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, et al., 2003). Protein that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The amino acid sequence of the peptides generated were determined using the FASTA format, with the most prominent accession number PATB1_SOLTU, TUB8_SOLTU, A0A097H141_SOLTU, AP18_SOLTU, and CPI1_SOLTU (UniProtKB).

8.4. Results and Discussion

8.4.1. Efficiency of proteases for generation of peptides and amino acids

Four proteases, including Novo Pro-D, Alcalase, Flavourzyme, and Papain, were examined for their effectiveness at hydrolyzing potato protein isolates into peptides and amino acids. All proteases showed optimal temperature ranges from 50-65 °C, with optimal pH conditions ranging from 5.0-9.0 (McDonagh & FitzGerald, 1998), except for Novo Pro-D which was reported to exhibit maximum catalytic activity at pH 5.3 (U.S.A Patent No. US6787168 B1, 2004) and pH 9 (Rojas, et al., 2014) depending on the substrate. The use of different substrates results in small variations in optimal conditions (Aspmo, et al., 2005), and therefore a more basic pH was selected, to maintain uniformity among proteases examined. Temperature was maintained at 50 °C, in order to increase the solubility of potato protein isolate and limit aggregation, as pure patatin has been shown to begin to denature at temperatures higher than 50 °C (Pots, et al., 1998).

Table 8.1 shows the proteolytic activity and the degree of hydrolysis of potato protein isolates as well as the proportion of each of generated peptides and amino acids. As examined, both Novo Pro-D from *Bacillus sp.* and Alcalase from *B. licheniformis* exhibited the highest proteolytic activities. It should be noted that the amount of the protease was adjusted in order to yield the same enzymatic units per mL of reaction mixture (2 U/mL). Both Novo Pro-D and Alcalase are classified as serine proteases, where Novo Pro-D possesses a preferred cleavage at the aromatic and sulphur containing amino acids namely, histidine, phenylalanine, tryptophan, and tyrosine; in contrast, Alcalase has a broad specificity, with preferential cleavage at the aromatic amino acids (Tavano, 2013). On the other hand, Papain is classified as a cysteine protease (Liggieri, et al., 2009), with a preferred cleavage at basic amino acids like arginine and lysine (IUBMB Enzyme Nomenclature, 2000). Papain has also been shown to possess a small ability to act as an exo-peptidase cleaving the histidine and threonine from the carboxyl-terminal end (Moorhouse, et al., 1997). Flavourzyme from *A. oryzae* contains both endo-protease and exo-peptidase activities namely aminopeptidase and carboxypeptidase (ThiQuynhHoa, et al., 2014).

The results show that lower degree of hydrolysis (7.17%, Table 8.1) was achieved using Novo Pro-D. This result may be attributed to the narrow specificity of Novo Pro-D towards limited specific cleavage sites (Tavano, 2013) and/or to the use of basic pH. Similarly, Kamnerdpetch et al. (2007) have reported low hydrolysis yield of 3% for the generation of free amino acids from potato pulp by Novo Pro-D-serine protease type at pH 9. The results also show that higher degree of hydrolysis was obtained with Alcalase, Flavourzyme and Papain. The profile of potato protein isolated from PFJ using 60% $(NH_4)_2SO_4$ showed a high relative proportion of patatin (36.5%), and good distribution of protease inhibitors (52.3%) (Waglay, et al., 2014). Patatin was reported to contain approximately 10.01 and 9.25% arginine and lysine residues, respectively (Bártová, et al., 2015). Therefore, the high degree of hydrolysis achieved with papain is due to the presence of the desirable cleavage sites.

Table 8.1 Catalytic efficiency of selected proteases for the generation of peptides and amino acids from potato proteins.

Enzyme[a]	Proteolytic Activity[b] (U/mL)	Degree of hydrolysis (%)	Relative Proportion of Peptides (%)	Relative Proportion of Amino Acids (%)
Novo Pro-D	6467.67	7.17	44.22	55.78
Alcalase	9060.70	100.00	83.02	16.98
Flavourzyme	153.15	100.00	15.80	84.20
Papain	138.98	100.00	85.76	14.24

[a] Reactions were carried out using 2 U of protease activity /mL reaction mixture at 50°C and 16 hr reaction time.

[b]The proteolytic activity was assessed using azocasein as substrate. One unit of protease activity is defined as the amount of enzyme which gives rise to an increase in 1 absorbance at 440 nm per min

Because of the broader specificity of Alcalase, it also resulted in a high degree of hydrolysis, with a slightly higher release of free amino acids (16.9%) generated when compared to Papain (14.2%). Because of the endo-type mode of action of Alcalase and Papain, they led to the release of higher proportion of peptides (83.0-85.8%). A previous study (Miedzianka, et al., 2014) showed that Alcalase resulted in 3.5 and 7.7% degree of hydrolysis of potato protein concentrate to free amino acids after 2 and 4 hr incubation, respectively. On the other hand, Flavourzyme resulted in a complete degree of hydrolysis; and as expected because it contains both an endo- and exo-protease, it resulted in the large production of amino acids (84.2%; Table 8.1). Similarly, higher degree of hydrolysis to free amino acids (22.5%) was obtained, in a previous study, when Flavourzyme was compared to other enzyme preparations with only either endo- or exo-protease activity (7.5%) (Kamnerdpetch, et al., 2007). Further modulation of the reaction parameters (time, enzyme amount) of the process using Flavourzyme would be desirable to establish a balance between peptides and amino acids generated.

8.4.2. Effects of protease concentration and reaction time

Both enzymatic units and reaction time can affect the catalytic efficiency of the enzymes and are important to study in order to modulate the proportion of each of the peptides generated as well as the amino acids. The literature emphasizes the fact that an ideal molecular size or chain length of the peptides effects the functional properties, where limited hydrolysis generally results in improved functionality (Klompong, et al., 2007). A compromise must be established with increased degree of hydrolysis and peptides chain length, as smaller peptides result in an end-product with severe bitterness, attributed in large to the free amino acids generated (Ney, 1979). The chain length of peptides are classified as small <7 amino acids, medium 7-25 amino acids, and large >25 amino acids, where generally, proteases produce peptides ranging from 7-25 amino acid units (Li-Chan, 2015).

Figure 8.1 shows that, as enzyme concentration increased (0.05-2 U/mL), the degree of hydrolysis increased for both Flavourzyme and Papain-catalyzed reactions, from 25.5-100.0% and 11.3-100.0%, respectively. This is in agreement with previous findings where

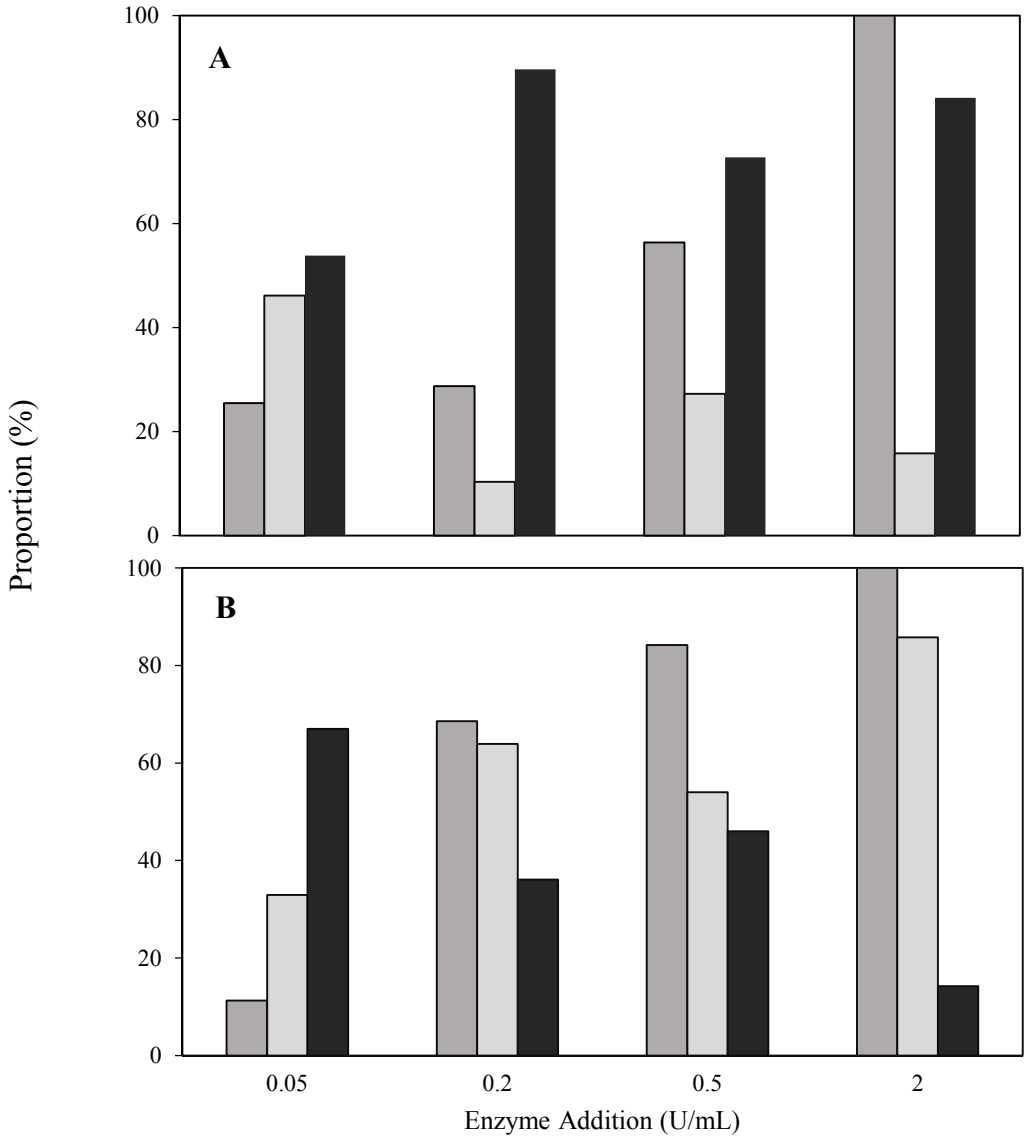

Figure 8.1 Effect of enzyme unit addition on the hydrolysis efficiency of potato proteins using (A) Flavourzyme and (B) Papain: degree of hydrolysis (▭), proportion of peptide generated (▭), and proportion of amino acid generated (▭).

increased enzyme concentration increases degree of hydrolysis, assuming substrate concentration is not lacking (Kamnerdpetch, et al., 2007). Increasing Flavourzyme concentrations from 0.05 to 0.2 U/mL resulted in an increase in the relative proportion of generated amino acids from 53.8 to 89.7%; at 0.5 U/mL, the relative proportion of generated amino acids and peptides was estimated at 72.7 and 27.3 %, respectively. However, further increase in Flavourzyme concentration to 2 U/mL led to a higher relative proportion of amino acids of 84.2%; this indicates the dependence of the end-product profile (peptides vs amino acids) on the enzyme concentration. This trend may be attributed (1) to the dependence of the exo-peptidase activity expressed in Flavourzyme on the availability of peptides released by endo-protease and/or (2) to the inhibiting effect of the potato protease inhibitors, which may have been stronger at advanced reaction and higher towards the endo-protease activity of Flavourzyme, when compared to its exo-peptidase activity.

As for Flavourzyme, the selectivity of Papain to generate amino acids and peptides was dependent on its concentration. At a low enzyme concentration (0.05 U/mL), higher relative proportion of amino acids (67%) was released with only 11% of degree of hydrolysis. As the reaction was extended with the increase in the Papain concentration of 0.2 to 2 U/mL, more peptides (54.0-85.8%) were generated. These results may be attributed (a) to the low catalytic efficiency of endo-protease activity of papain on the high-molecular weight protein than on its fragments, (b) to the difference in the inhibitory effect of potato protease inhibitors towards the endo and exo-activities. As revealed in the structural characterization study, potato protease inhibitors were partially hydrolyzed at high Papain concentration, which may have limited their inhibitory effect on the endo-activity of papain and resulted in the generation of more peptides at high Papain concentration.

Reaction time courses were obtained for both proteases, Flavourzyme and Papain as shown in Figure 8.2 A. An increase in the degree of hydrolysis was observed at the initial stage of the reaction to reach 46.5 and 17.5% at 2.25 hr for Flavourzyme and Papain, respectively. This sharp rise was achieved at a rate of 20.61 and 8.26 μmol/mL.min for Flavourzyme and Papain, respectively (data not shown). These results imply that Flavourzyme has a higher initial hydrolytic rate when compared to Papain. Similar findings were reported by Kamnerdpetch et al. (2007), where Flavourzyme resulted in the sharpest rise of degree of

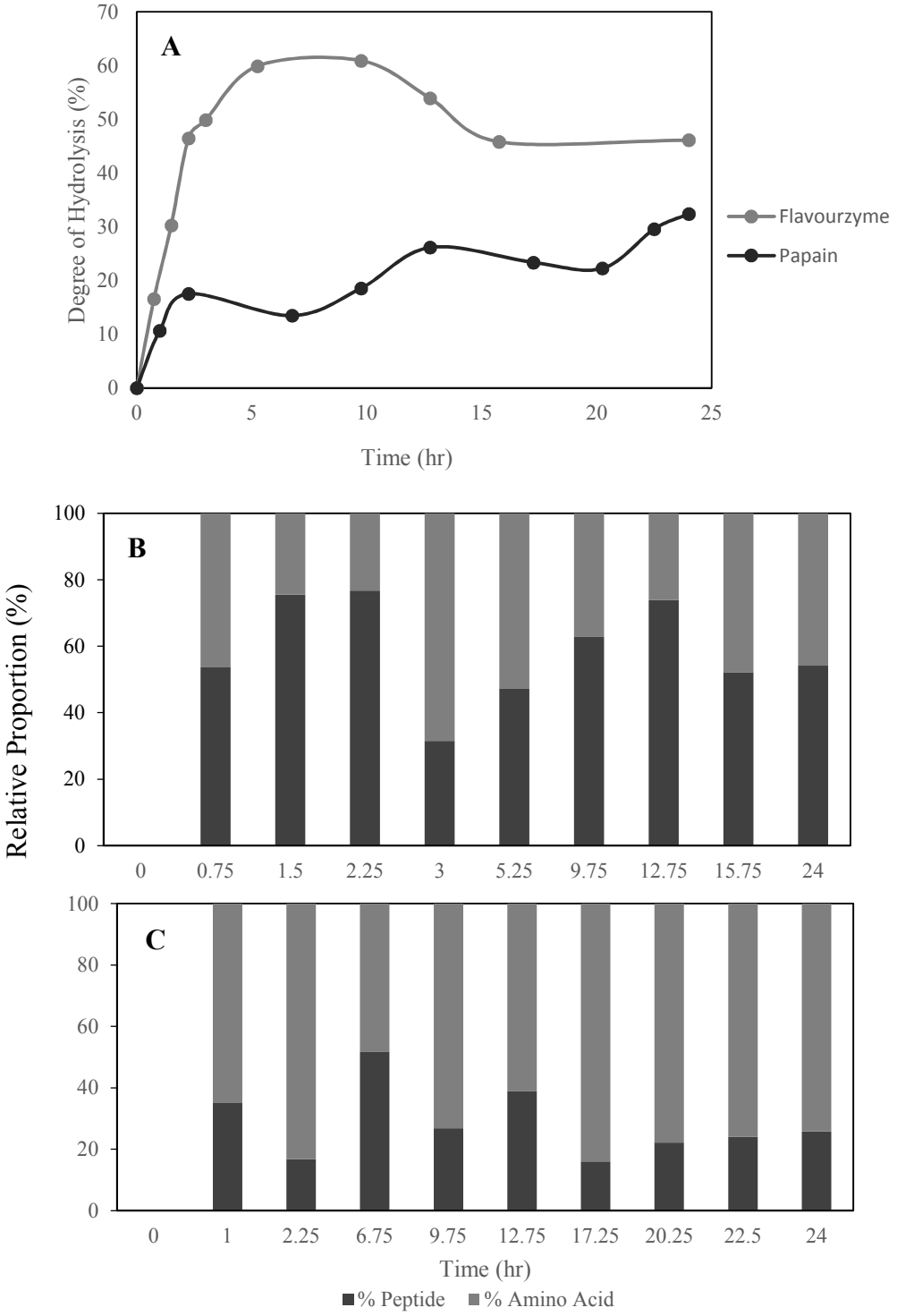

Figure 8.2 Time courses (A) from 0-24 hr for the hydrolysis reaction of potato proteins by proteases, Flavourzyme (B) and Papain (C).

hydrolysis with time, when compared to other commercial proteases, namely, Corolase, Novo Pro-D, and Alcalase (Kamnerdpetch, et al., 2007). These authors attributed this result to the endo- and exo- action mode of Flavourzyme. After reaching the maximum value of 60 and 32% at 5.25 and 24 hr in the presence of Flavourzyme and Papain, respectively. The degree of hydrolysis remained more or less constant up to 9.75 hr for Flavourzyme. The limited increase of the degree of hydrolysis was more pronounced with Papain than Flavourzyme. These results may be due to the weaker presence of exo-peptidase activity in Papain, to the denaturation of enzymes, and/or to the strong inhibition of its endo-activity by protease inhibitors. Increasing the release of products may have also competed with protein substrate at the enzyme active site thereby preventing the enzyme-substrate complex from forming (Liese, et al., 1996). Unlike typical hydrolysis time courses, a decrease in the degree of hydrolysis was observed after 9.75 hr in the Flavourzyme time course (Figure 8.2 A); this decrease may be apparent due to an increase in the solubility of potato proteins in the blank substrate as the reaction was extended.

The results also show that proportion of each of peptides and amino acids varies within the reaction time course (Figure 8.2 B). In the Flavourzyme time course, where initially with the rise in the degree of hydrolysis, there is an increase in peptide concentrations; however at 3 hr there is a sharp decline. This could be due to the higher peptide substrate availability, promoting the exo-peptidase action and/or the effect of potato protease inhibitors, which would have affected the endo-protease activity. As the reaction was proceeded to 12.75 hr, Flavourzyme favored the release of peptides to reach a proportion of 73.1%. It should be noted that because of the increase in the solubility and the mass action effect, the concentration of potato proteins available to protease may have increased as the reaction was proceeded. Papain resulted in a production of a smaller amount of peptides at the investigated conditions when compared to Flavourzyme. As shown Figure 8.2 C, generally papain generated around 16.7 to 51.8% peptide concentration, with maximum values of 51.8 and 38.8%, at 6.75 and 12.75 hr incubation time. Possible explanations for the maximum peaks include (1) the effect of the potato protease inhibitors on endo- and exo-action of Papain to a different extent and (2) the hydrolysis of protease inhibitors over the reaction time.

8.4.3. Characterization of generated peptides

As shown in Figure 8.3, both Flavourzyme and Papain generated many unique peptides which corresponded to varying known potato protein fraction, namely patatin and protease inhibitors. As shown, Papain generated more unique peptide sequences with homology assessment matching several potato proteins when compared to Flavourzyme. The low numbers of unique matched peptides identified in the presence of Flavourzyme could potentially be due to its endo- and exo-action, which would yield smaller peptides without unique homology to parent proteins; indeed, the algorithm only recognizes peptides of 8 or more residues. Therefore, these unique peptides matches are described as a qualitative comparison among generated hydrolysates and do not translate the effectiveness of either protease. In the presence of both Flavourzyme and Papain, shortest reaction time of 7.5 hr and 4.5 hr, respectively, resulted in higher number of generated unique peptides of 66 and 137, respectively. While when compared to 24 hr reaction time, Flavourzyme and Papain generated 57 and 129, respectively (Figure 8.3). Flavourzyme hydrolysates generally resulted in more unique peptides, which originated from patatin, than from the protease inhibitors fraction (Figure 8.3). The most prominent patatin source included Patatin-B1 OS with accession numbers PATB1_SOLTU with corresponding molecular weight of 42 610.4 Da. Only, fewer peptides were generated in the presence of Flavourzyme belonging to the protease inhibitors fractions. However, the most prominent group for both enzymes, in terms of unique peptides generated belonged to the Kunitz-type inhibitors B OS (accession number: A0A097H141_SOLTU), with molecular weight of 23 155.1 Da. On the other hand, Papain generated more unique peptide matching from the protease inhibitor fraction when compared to patatin. This could be attributed to the high efficiency of Papain on smaller size of protease inhibitors (5-25 kDa) over patatin (39-43 kDa).

The peptide sequences belonging to the highest percent coverage for patatin (PATB1_SOLTU) and protease inhibitors (Kunitz-type, A0A09H141_SOLTU) were further examined after low and high reaction time of 7.5 and 24 hr in the presence of Flavourzyme. Table 8.2 shows that for Flavourzyme, several peptide sequences increased in peptide number with time. The sequence which increased with longer incubation time included DDASEANMELL, DGGVATVGDPALL, DSPETYEEALK, and TGTTTEMDDASEANMELL. While several were present at the shorter incubation time

and disappeared with increasing time. This is due to the exo-peptidase activity of Flavourzyme, which would break apart the peptide sequence to amino acid units. In addition, several sequences which were not present at the shorter time, were hydrolysed and present at the longer time, indicative of the dual hydrolytic nature of Flavourzyme. While for the Kunitz-type protease inhibitor (Table 8.2) initially at 7.5 hr incubation, there were many peptides which were present in large numbers such as sequences GDVYLGKSPN, GGDVYLGKSPN, LPSDATPVLDVT, and LPSDATPVLDVTG, whereas with time, these sequences decreased in numbers. In addition, several other sequences appeared, which were not initially present. Possible explanations include the longer incubation time which allows for the enzyme to use its dual catalytic mechanism, which would hydrolyse peptides present in large numbers, as well the endo-protease activity could continue to cleave other active sites which are more available with increased reaction time. The identified Kunitz-type protease inhibitors fractions have been reported to possess anti-fungal ability (Park, et al., 2005) and represent around 22% of the potato protein (Pouvreau, et al., 2003).

Table 8.3 outlines the peptide sequences belonging to the highest percent coverage for patatin (PATB1_SOLTU) and protease inhibitors (Aspartic protease inhibitor, API8_SOLTU), with molecular weights of 42 610.4 Da and 24 191.1 Da, respectively, upon low and high reaction time of 7.5 and 24 hr, in the presence of Papain. Table 8.3 indicates that for Papain, several peptide sequences increased in peptide number with time for patatin. The sequence which increased with longer incubation time included EVDNNKDARLA, KDSPETY, KDSPETYE, KDSPETYEEAL, KDSPETYEEALK, KLEEMVTVL and SPETYEEALK. This could be associated with the protein relaxing at the 50 ^{0}C, as patatin begins to unfold at these conditions (Pots, et al., 1998). Patatin unfolding could lead to more active sites being

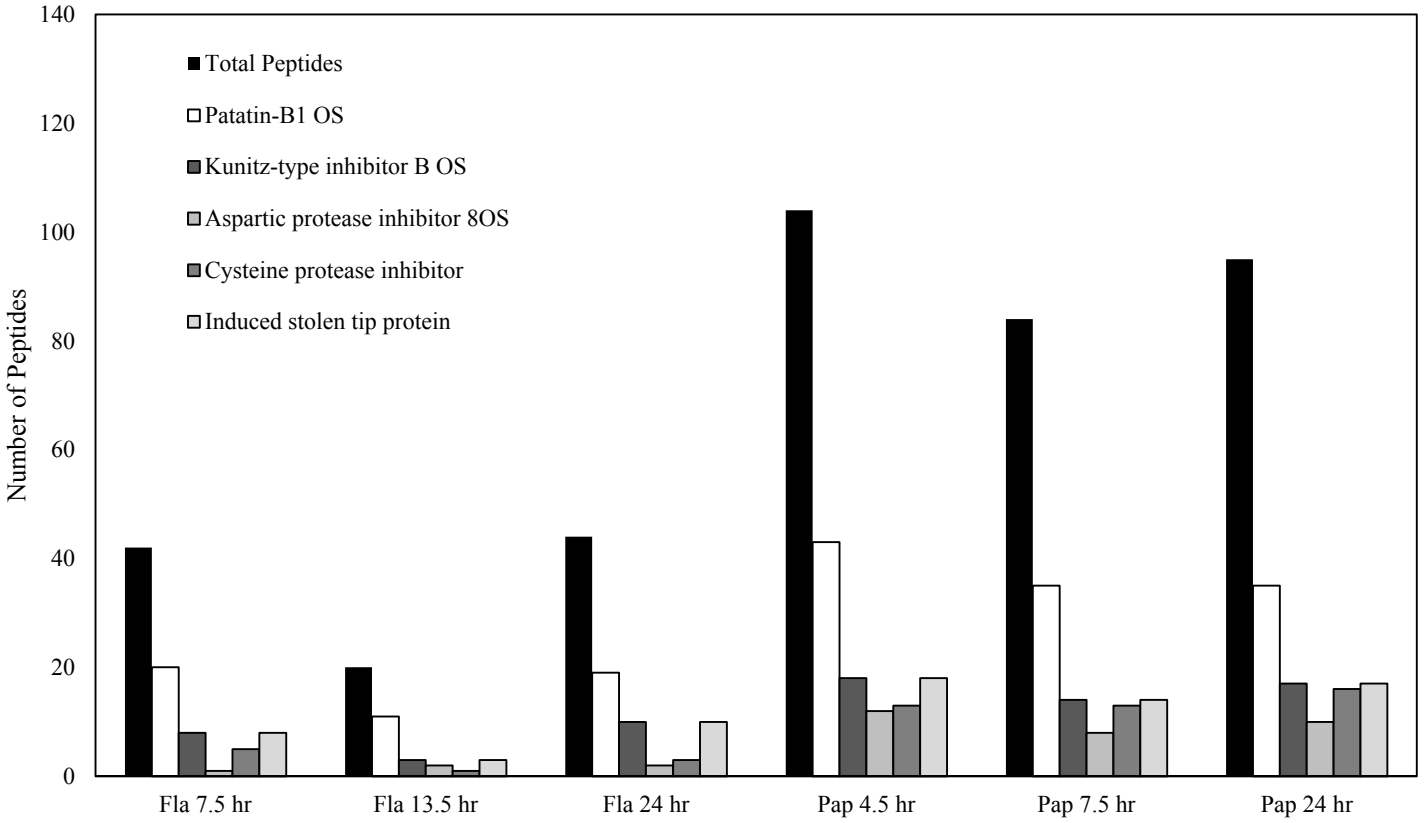

Figure 8.3 The number of exclusive unique peptides, corresponding to accession named proteins, present in potato protein hydrolysates produced by Flavourzyme and Papain.

Table 8.2 Peptide composition of potato protein hydrolysates using commercial protease Flavourzyme.

Patatin[a]		Protease Inhibitors[b]	
7.5 hr	24 hr	7.5 hr	24 hr
(Number of Peptides) Sequence (Actual Mass)			
(1) (L)AQEDPAFSSIK(S) (1191.60)	-	(2) (K)DNPLDVSFK(Q) (1033.52)	-
(3) (M)DDASEANMELL(V) (1206.53)	(9) (M)DDASEANMELL(V) (1206.53)	(19) (G)GDVYLGKSPN(S) (1048.53)	(1) (G)GDVYLGKSPN(S) (1048.53)
(2) (I)DGGGIKGIIP(A) (925.53)	-	(44) (L)GGDVYLGKSPN(S) (1105.56)	(1) (L)GGDVYLGKSPN(S) (1105.56)
(2) (I)DGGGIKGIIPA(I) (996.57)	-	(1) (N) LPSDATPVL(D) (911.51)	(1) (N) LPSDATPVL(D) (911.51)
(3) (V)DGGVATVGDPALL(S) (1183.63)	(4) (V)DGGVATVGDPALL(S) (1183.63)	(7) (N) LPSDATPVLDVT(G) (1226.66)	(2) (N) LPSDATPVLDVT(G) (1226.66)
(1) (V)DGGVATVGDPALLS (L) (1270.66)	-	(8) (N) LPSDATPVLDVTG(K) (1283.69)	-
(32) (K)DSPETYEEALK(R) (1280.60)	(41) (K)DSPETYEEALK(R) (1280.60)	(2) (P)SDATPVLDVTGKE(L) (1330.69)	(2) (P)SDATPVLDVTGKE(L) (1330.69)
(2) (Q)EDPAFSSIK(S) (992.49)	-	(1) (N) SDVGPSGTPVR (F) (1070.55)	-
(2) (Q)EVDNNKDARLA(D) (1244.61)	-	-	(4) (S) DATPVLDVTGK (E)
(1)(S)KDSPETYE(E) (967.43)	-	-	(3) (L)DVTGKELDP (R) (972.48)
(1)(F)LEGQLQEVDNNKD(A) (1501.72)	-	-	(6) (P)SDATPVLDVTGK(E) (1201.60)
-	(8) (L) EGQLQEVDNNKD(A) (1388.60)	-	(1)(S)SDDQFCLKV(G) (1053.48)
-	(1)(E) GQLQEVDNNKD(A) (1259.53)	-	(1)(N)SDVGPSGTPVR(F) (1070.53)
-	(1)(P) ETYEEALK (R) (981.47)		
(1)(S)PETYEEALK(R) (1078.52)	-		
(8) (A)QEDPAFSSIK(S) (1103.54)	-		
(13) (L)QEVDNNKDARLA(D) (1354.68)	(7) (L)QEVDNNKDARLA(D) (1354.68)		
(14) (V)QVGETLLK(K) (869.49)	(11) (V)QVGETLLK(K) (869.49)		
-	(1) (S)NLAKSPELD(A) (986.49)		
(14) (K)SPELDAKM(Y) (905.43)	(1) (K)SPELDAKM(Y) (905.43)		
-	(1) (D)SPETYEEAL(K) (1037.46)		
(11) (D)SPETYEEALK(R) (1165.57)	(10) (D)SPETYEEALK(R) (1165.57)		
-	(21)(T)TEMDDASEANMEL(L) (1814.77)		
(3)(L)TGTTTEMDDASEANMEL(L) (1814.77)	(16)(L)TGTTTEMDDASEANMEL(L) (1470.55)		
-	(1)(T)GTTTEMDDASEANMELL(V) (1842.75)		
-	(1)(L)TGTTTEMDDASEANMELL(V) (1943.80)		
-	(9)(G)TTTEMDDASEANMEL(L) (1656.65)		
-	(1)(Q) ENALTGTTTEMDDASEANM(E) (2000.79)		
(1)(T)TPNENNRPFA(A) (1159.55)	-		
(2) (L)VDGGVATVGDPALL(S) (1183.63)	-		
-	(1)(E)TYEEALK(R) (852.42)		

[a]Patatin, accession number PATB1_SOLTU, molecular weight 42610.4 Da. [b]Kunitz-type inhibitor, accession number A0A097H141_SOLTU, molecular weigh 23 155.1Da

Table 8.3 Peptide composition of potato protein hydrolysates using commercial protease Papain.

Patatin[a]		Protease Inhibitors[b]	
7.5 hr	24 hr	7.5 hr	24 hr
(Number of Peptides) Sequence (Actual Mass)			
(11) (K)DSPETYEEALK (R) (1280.58)	(5) (K)DSPETYEEALK (R) (1280.58)	(6) (L)DTNGKELNPDSSYR(I) (1595.71)	(7) (L)DTNGKELNPDSSYR(I) (1595.71)
(10) (Q)EVDNNKDARLA(D) (1244.61)	(14) (Q)EVDNNKDARLA(D) (1244.61)	-	(6) (L)DTNGKELNPDSSYRII(S) (1821.91)
(2) (D)GGGIKGIIPA(I) (881.53)	-	-	(6) (L)DTNGKELNPDSSYRIIS(I) (1908.91)
-	(1)(L)SIDGGIKGIIÂ(I) (1196.70)	(20) (S)ESPLPKPVL(D) (978.58)	(46) (S)ESPLPKPVL(D) (978.58)
(5) (A)ISSFDIK(T) (808.43)	(1) (A)ISSFDIK(T) (808.43)	-	(1)(S)ESPLPKPVLD(T) (1093.60)
(4) (A)ISSFDIKT(N) (909.48)	(2) (A)ISSFDIKT(N) (909.48)	(3) (Q)FNIPTVK(L) (817.47)	(5) (Q)FNIPTVK(L) (817.47)
(2) (A)ISSIFDIKTN(K) (1023.53)	-	(1) (N) GKELNPDSSYR(I) (1264.61)	-
-	(1)(T)TPNENNRPF(A) (1088.49)	(2) (G)QADSSYFKIVK(L) (1267.65)	(5) (G)QADSSYFKIVK(L) (1267.65)
-	(7)(T)TPNENNRPFA(A) (1159.53)	(4) (P)SESPLPKPVL(D) (1065.61)	(10) (P)SESPLPKPVL(D) (1065.61)
(1)(M)ITTPNENNRF(A) (1302.62)	-	(15) (E)SPLPKPVL(D) (849.53)	(50) (E)SPLPKPVL(D) (849.53)
(7) (M)ITTPNENNRFA(A) (1373.66)	(4) (M)ITTPNENNRFA(A) (1373.66)	(2) (L)VNENPLDVL(F) (1011.53)	(1) (L)VNENPLDVL(F) (1011.53)
-	(1)(A)AKDIVPFY(F) (951.56)		
(3) (A)KDIVPFY(F) (880.47)	(1) (A)KDIVPFY(F) (880.47)		
(1)(S)KDSPETY(E) (838.37)	(2)(S)KDSPETY(E) (838.37)		
(3) (S) KDSPETYE(E) (967.41)	(4) (S) KDSPETYE(E) (967.41)		
(11) (S)KDSPETYEEAL(K) (1280.58)	(12) (S)KDSPETYEEAL(K) (1280.58)		
(44) (S)KDSPETYEEALK(R) (1408.67)	(47) (S)KDSPETYEEALK(R) (1408.67)		
-	(4)(K)DSPETYEEALKR(F) (1436.68)		
-	(12)(S)KDSPETYEEALKR(F) (1564.77)		
-	(2)(A)KLEEMVTV(L) (947.50)		
(2)(A)KLEEMVTVL(S) (1076.58)	(4)(A)KLEEMVTVL(S) (1076.58)		
-	(1)(L)SIDGGGIKG(I) (802.42)		
(5)(V)LSIDGGGIK(G) (858.48)	-		
(1)(V)LSIDGGGIKG(I) (915.50)	-		
(37)(L)LSLSVATR(L) (845.50)	(13)(L)LSLSVATR(L) (845.50)		
(6)(S)PETYEEALK(R) (1078.52)	(2)(S)PETYEEALK(R) (1078.52)		
(6)(L)QEVDNNKDARLA(D) (1355.64)	(2)(L)QEVDNNKDARLA(D) (1355.64)		
(1)(L)SIDGGGIKG(I) (802.42)	-		
(1)(S)SIKSLDYK(Q) (952.53)	-		
-	(1)(V)SKDSPETYEEAL(K) (1367.61)		
(5)(V)SKDSPETYEEALK(R) (1495.70)	(5)(V)SKDSPETYEEALK(R) (1495.70)		
(4)(K)SPELDAKM(Y) (889.42)	(1)(K)SPELDAKM(Y) (889.42)		
(6)(K)SPELDAKMY(D) (1052.49)	(1)(K)SPELDAKMY(D) (1052.49)		
(11)(D)SPETYEEALK(R) (1165.55)	(13)(D)SPETYEEALK(R) (1165.55)		
-	(2)(D)SPETYEEALKR(F) (1321.65)		
(3) (G)TGTNSEFDKTY(T) (1261.55)	-		
-	(1)(G)TGTNSEFDKTYT(A) (1362.59)		
(1)(G)TGTNSEFDKYTYTAE(E) (1562.68)	-		
(5)(G)TGTNSEFDKTYTAEEAAK(W) (1961.88)	(1)(G)TGTNSEFDKTYTAEEAAK(W) (1961.88)		
-	(1)(G)TNSEFDKTY(T) (1104.46)		
(1)(G)TNSEFDKTYTAE(E) (1404.61)	-		
(5)(G)TNSEFDKTYTAEEAAK(W) (1803.82)	-		
(2)(A)TRLAQEDPAFS(S) (1234.60)	-		
(1)(E)TYEEALK(R) (852.42)	(1)(E)TYEEALK(R) (852.42)		
(18)(E)VAISSFDIKTN(K) (1193.63)	(4)(E)VAISSFDIKTN(K) (1193.63)		
-	(1)(T)EVAISSFDIKTN(K) (1322.68)		
(6)(T)VLSIDGGGIK(G) (957.55)	(1)(T)VLSIDGGGIK(G) (957.55)		
(1)(T)VLSIDGGGIKG(I) (1014.57)	-		

[a]Patatin, accession number PATB1_SOLTU, molecular weight 42610.4 Da; [b]Aspartic protease inhibitor, accession number API8_SOLTU, molecular weigh 24 191.1Da.

available for protease action. Similar to Flavourzyme, several peptide sequences were present at the shorter incubation time and disappeared with increasing reaction time with Papain. This can be attributed to their subsequent hydrolysis by the exo- peptidase activity belonging to Papain. The results (Table 8.3) also show that initially at 7.5 hr incubation, few peptides originated from Aspartic protease inhibitor were present in large numbers such as sequences DTNGKELNPDSSYR, ESPLPKPVL, and SPLPKPVL; with the reaction time, these sequences increased significantly. Possible explanations include the longer incubation time allowing for increased substrate accessibility. Aspartic protease inhibitors, have been studied to be present in PFJ in about 6% (Pouvreau, et al., 2001).

As shown in Tables 8.2 and 8.3, both Flavourzyme and Papain resulted in high homology coverage of 28 and 33% for Patatin B1-OS, at 7.5 hr incubation. Several sequences were similar to both hydrolysates, which included DSPETYEEALK, EVDNNKDARLA, TPNENNRPFA, KDSPETYE, PETYEEALK, QEVDNNKDARLA, SPELDAKM, SPETYEEALK, and TYEEALK.

To our knowledge, no studies to date have characterized the peptides generated from potato protein isolates by selected proteases. All previous studies assessed the amino acid composition of the protein hydrolysates following complete acid hydrolysis (Kamnerdpetch, et al., 2007; Miedzianka, et al., 2014). Our study provides an indication of the origin of the peptide generated as related to known potato protein parent molecules.

8.5. Conclusion

The present study assessed the efficiency of selected proteases namely, Alcalase, Flavourzyme, Novo Pro-D, and Papain, in terms of their degree of hydrolysis, and the relative proportions of generated peptides and amino acids. The end-products profile (peptides vs amino acids) was not only dependent on the type of protease, but also on the protease concentration and reaction time. The systems with Flavourzyme and Papain were further investigated for their time course to assess how the hydrolysis reactions function over 24 hr. The generated peptides were characterized by MS/MS to determine their corresponding parents, patatin or protease inhibitors, and their amount through peptide mapping. Interestingly, Flavourzyme hydrolysates, contained more known unique peptides

common to patatin, whereas Papain hydrolysates were more specific to the protease inhibitors, in particular high coverage homology to Aspartic protease inhibitors. As a future work, the structurally well- defined generated peptides will be assessed for their functional properties such as antioxidant ability, angiotensin converting enzyme inhibition properties, and anti-microbial activities.

CHAPTER IX. GENERAL SUMMARY AND CONCLUSIONS

This research focuses primarily on the isolation of potato proteins using conventional and novel methods from imitation by-products potato fruit juice and potato pulp, respectively. Removal of these proteins is of high interest, as these by-products are associated with a large polluting capacity. The conventional extracting agents include combination: thermal/ acidic, acid, $FeCl_3$, ethanol, $(NH_4)_2SO_4$, and CMC, were compared according to their protein recovery yield, protein profile, and purification factor. The effect of the extracting agent was evaluated according to the structural, physico-chemical, and functional properties of the extracts. Moreover, the novel approach commenced with the exploration of pure enzyme, endo-polygalacturonase and endo-β-1, 4-galactanase. This method was optimized using RSM and CCRD with 5-levels and 5-variables, namely, incubation temperature, time, pulp concentration, units of endo-polygalacturonase, and units of endo-β-1,4-galactanase. Results showed that potato protein recovery and recovered patatin was governed by incubation temperature and unit addition of endo-polygalacturonase. Contrarily, recovered protease inhibitors was significantly affected by pulp concentration and incubation temperature.

In order to make this approach more industrially appealing the exploration of ten multi-enzymatic systems containing desirable glycosyl-hydrolase activities were explored. The overall benefit to this technique are associated to the different ratios of enzymatic activities including minimal proteolytic activity, which results in high protein recovery and the ability to fractionate between patatin and protease inhibitors. Depol 670L and Ceremix 2XL were deemed superior for the recovery of patatin and protease inhibitors, respectively, with high protein recovery yields. The two multi-enzymatic systems were further optimized for the recovery of patatin and protease inhibitors, respectively using RSM with CCRD using two variable units of multi-enzymatic systems and incubation time. Depol 670L was significantly affected by the interaction of unit addition and incubation time, conversely, Ceremix 2XL was significantly affected by the quadratic effect of both variables. The Depol-based protein extract possessed higher lipid acyl hydrolase activity compared to the currently employed industrial technique of combination: thermal/ acidic, emphasizing the preservation of functionality. In addition Ceremix-based protein extract resulted in higher trypsin inhibiting activity when compared to the industrial technique. These extracts are of

great importance as both patatin and protease inhibitors have been associated with several beneficial and functional properties.

The most promising conventional method $(NH_4)_2SO_4$ precipitation, ultrafiltration, and the novel multi-enzymatic approach using Depol 670L were further scaled-up in a pilot plant facility. $(NH_4)_2SO_4$ precipitation resulted in an extract with the highest protein content with a potential to be marketed as a potato protein isolate, whereas both ultrafiltration and multi-enzymatic system resulted in potato protein concentrates The concentrates seemed to have a greater thermal preservation effect coupled to a higher presence of polysaccharides. In addition, multi-enzymatic-based potato protein concentrate resulted in improved functionalities specifically emulsifying activity index and foam expansion. Plausible explanations include the presence of sugars which may have improved the protein solubility. The potato protein concentrates and isolates have great interest as functional ingredients due to their desirable emulsifying and foaming abilities.

Taking advantage of the functionalities of the potato protein isolate and concentrate, the development of a reduced-gluten cookie formulation was investigated. The potato protein isolate produced a cookie, which was evaluated both mechanically and sensorially to be texturally more desirable than those with potato protein concentrate. The formulation was further optimized using RSM and CCRD with 5-levels and 2-variable, specifically rice proportion and potato protein isolate enrichment. Sensory evaluation consisted of seven attributes related to the cookie namely, colour of the top, colour of the bottom, fracturability, crispness, adhesiveness, aftertaste, and overall liking. The attributes were evaluated according to mean quantitative descriptive scores and mean liking scores. According to statistical analysis the three attributes which were found to be statistically significant were crispness, aftertaste and overall liking for both scales. Generally, when proportion of wheat flour was high, consumers tended to prefer low protein substitutions of 1.9%. However when rice flour proportion was high, consumers preferred substitutions between 7.2 and 12.5%. Therefore, the enrichment of potato protein, to a reduced-gluten formulation was proven effective in overcoming the lacking gluten network, thereby positively affecting the texture and spread of the cookies.

The production of potato peptides was generated using selected proteases Flavourzyme, Alcalase, Papain, and Novo Pro-D. The results showed that Flavourzyme, Alcalase, and Papain showed the highest catalytic efficiency for the hydrolysis of potato proteins. However, the end-products profile of peptides to free amino acids was not only dependent on the catalytic action of protease, specifically endo-protease or exo-peptidase, but also on the reaction time and on the inhibitory activity of generated protease inhibitors. The generated peptides were further characterized by peptide mapping, where Papain generated more exclusive unique peptide matching parent molecules of protease inhibitor fraction. Whereas Flavourzyme exhibiting higher exo-peptidase action generated shorter peptides which were difficult to match, but those assessed were more specific to the patatin fraction. These generated peptides have the potential to have bioactive abilities.

Overall, this research contributes to the scientific knowledge for the effective isolation of potato proteins from industrial by-products. Developed and optimized methods led to the preservation of the functional properties of the extracts, which increases their potential application as functional food ingredients. The understanding of the complex kinetic of the hydrolysis reaction of potato proteins catalyzed by proteases in which potato protease inhibitors contributed will allow the generation of potentially bioactive peptides.

CHAPTER X. CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

10.1. Contributions to Knowledge

The major contribution to knowledge of this study are:

1. A comparative study examined several extraction techniques on one potato cultivar from one region namely Russet Burbank, from Quebec, Canada. To the authors knowledge this is the first study being conducted on a Canadian variety of potatoes. The potato protein isolates were compared according to the extracting agents' effects on protein recovery yield, purification factor, protein proportion, physico-chemical effects, structural changes, and functional properties.
2. For the first time, the development of a novel enzymatic approach for the isolation of potato proteins from an imitation by-product potato pulp was developed. It consisted of the use of two pure enzymes endo-β-1,4-galactanase and endo-polygalacturonase for the efficient opening of the pectin network and the release of potato proteins. This study contributes to the understanding of the effects and interactions of the parameters that govern the efficiency of the enzymatic approach.
3. For the first time, the extrapolation of the use of pure enzymes resulted in the background knowledge to be able to extract the proteins in a more industrially appealing manner using commercially available multi-enzymatic products. Ten multi-enzymatic products, which contained glycosyl-hydrolase activities, were evaluated for their effectiveness at recovering potato protein from potato pulp. This study contributes to the understanding of the efficiency of the multi-enzymatic products as it is related to their enzyme activity profiles. This provides the fundamental base for the application of this enzymatic approach. The patatin and protease inhibitors enriched isolates were further characterized for their functional properties, and mass to charge ratio was confirmed through peptide mapping.
4. This is the first study, which compares at a pilot plant level several methods, namely ultrafiltration and 60% ammonium sulphate saturation with a novel multi-enzymatic extraction technique, for the extraction of potato proteins from imitation by-products. The limitations and the effects of the extraction methods were elucidated in this study by comparing the protein recovery yield, protein proportion, structural effects, and functional properties.

5. The effect of the addition of potato proteins in a reduced gluten cookie formulation consisting of wheat and rice flour, in reference to the dough rheology, textural, colour, and sensory analysis, was studied for the first time. Bridging the analytical textural properties to the sensory results of a semi-trained sensory panel is also expected to contribute to the understanding of the properties that drive the overall acceptance of the reduced gluten cookie formulations.
6. This study screened four commercially available proteases and their use in producing peptides from potato protein isolates. For the first time, the peptides generated were characterized by peptide mapping for their sequence and reference to parent protein fraction. The understanding of the complex kinetic of the hydrolysis reaction of potato proteins catalyzed by proteases in which potato protease inhibitors contributed will allow the generation of potentially bioactive peptides.

10.2. Recommendations for Future Research

- Assessment of well-defined mixtures of patatin and protease inhibitors varying according to their proportions in order to have an understanding of their mutual stabilization or destabilizing behaviors.

- Investigation of the complete fractionation of the potato proteins so that the structural and functional properties can be assessed for each individual protein fraction.

- Currently, there is no commercially available patatin fraction alone, therefore interest would lie in optimizing the fractionation process using commercially available enzyme, Depol 670L. Developing substantial quantity of isolated patatin would be desirable to market and sell this beneficial glycoprotein of great potential not only for food applications but also for pharmaceutical applications.

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