DEVELOPMENT OF BIOCATALYTIC APPROACHES FOR THE EXTRACTION OF RHAMNOGALACTURONAN I AND THE GENERATION OF PREBIOTIC OLIGOSACCHARIDES FROM POTATO CELL WALL

By

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Production of Potato Galactan-rich Rhamnogalacturonan I and Prebiotics

ABSTRACT

Potato (*Solanum tuberosum* L.) pulp rich in pectic polysaccharides is an abundant by-product of the starch and potato processing industry. The main component of potato pectic polysaccharides is rhamnogalacturonan I (RG I, 72% w/w), which is rich in β-linked galactan side chains (67% w/w). Given its structural properties, galactan-rich RG I can serve as a new source of non-digestible oligosaccharides, notable for their promising health benefits when employed as prebiotics. Conventional alkaline (NaOH and KOH), enzymatic (*Aspergillus niger* endo-polygalacturonase) and microwave-assisted alkaline (KOH) processes' efficiency in extracting galactan-rich RG I from potato cell walls were compared. While alkaline extraction led to a 5-27% degradation of side chains, enzymatic extraction had no such effect and released the highest proportion of high-molecular weight polysaccharides (>500 kDa; 62.2%). In the alkaline process, the debranching of arabinan side chains (8-27%) exceeded that of galactan side chains (5-14%). Optimization of the enzymatic process through response surface methodology identified the reaction conditions under which the greatest yield (63.9%) and final galactose (Gal) content (71.8%) were achieved. Furthermore, the optimization of microwave-assisted alkaline extraction yielded the highest Gal content of 63.1%. For this process, yield and Gal contents were most strongly influenced by the solid/liquid ratio. More soluble and producing more stable emulsions than potato galactan or orange homogalacturonan, galactan-rich RG 1 polysaccharides showed Newtonian behaviour at 25°C and 73°C, and pseudoplastic behaviour at 49°C.

A Multi-enzymatic approach was developed for generation of oligosaccharides/oligomers from the extracted RG I polysaccharides and were compared in terms of yield, molecular weight distribution and monosaccharide profile of oligosaccharides/oligomers. Yield (1.0-93.9%, w/w) and monosaccharide content of hydrolysates (0.0-34.2%, w/w) were dependent on the multi-enzymatic type and reaction conditions. Under selected reaction conditions, hydrolysates were mainly composed of oligosaccharides with a degree of polymerization (DP) ranging from 2-12 (79.8 - 100%), with Gal (58.9-91.2%, w/w) being the major monosaccharide. Resulting in the greatest oligosaccharide yield and Gal content, but the lowest release of monosaccharides, a combination of Depol 670L and Gamanase 1.5L was selected for further optimization of oligosaccharide/oligomer generation.

Enzymatic production of oligosaccharides/oligomers with defined DP and monosaccharide profile using bi-multi-enzymatic system (Depol 670L and Gamanase 1.5L) was optimized and the effect of reaction conditions (time, Depol 670L/Gamanase 1.5L ratio and substrate concentration) on yield, monosaccharide profile and DP of hydrolysates were evaluated. The results showed that the highest yields of oligosaccharides (DP of 2-6 and 7-12) and oligomers (DP of 13-70) were obtained when the ratio of Depol 670L and Gamanase 1.5L was 1:1. Longer incubation times and lesser substrate concentrations generated the greatest yield of oligosaccharides with a DP of 2-6 (11.2%), while shorter incubation times resulted in the greatest yield of oligosaccharides with a DP of 7-12 (21.9%). The prebiotic properties of potato RG I polysaccharides and their hydrolysates were assessed using continuous culture system inoculated with immobilized faecal microbiota. While both potato RG I polysaccharides and their hydrolysates were fermented by *Bifidobacterium* and *Lactobacillus*, they did not support the growth of *Bacteroides* and *Clostridium leptum* species. As compared to RG I polysaccharides, their hydrolysates were fermented more selectively and promoted a greater production of SCFA. The *in vitro* digestibility study revealed that 81.6 and 79.3% of the initial quantity of RG I polysaccharides and their corresponding hydrolysates remained unhydrolyzed, respectively, revealing their high persistence.

RÉSUMÉ

La pulpe de la pomme de terre (*Solanum tuberosum* L.) riche en polysaccharides pectiques est un sous-produit abondant de l'industrie de transformation de l'amidon. Le composant principal ces polysaccharides pectiques est la rhamnogalacturonane I (RG I, 72% p/p), qui est riche en chaînes latérales de galactanes (67% p/p). Compte tenu de ses propriétés structurelles, la RG I riche en galactanes est identifiee une nouvelle source des oligosaccharides non digestibles qui sont connus par leurs effects prébiotiques ayant des bienfaits pour la santé. Les efficacités de l'extaction alcaline (NaOH et KOH), de l'extraction enzymatique (endo-polygalacturonase de *Aspergillus niger*) et de l'extraction alcaline assistée par micro-ondes (KOH) pour isoler la RG I des parois cellulaires des pommes de terre ont été comparées. Bien que l'extraction alcaline a mené à une dégradation de 5-27% de chaînes latérales, l'extraction enzymatique n'a pas eu un tel effet et a libéré une plus grande proportion de polysaccharides de poids moléculaires élevés (> 500 kDa; 62,2%). L'optimisation du procédé enzymatique, en utilisant une méthodologie de surface de réponse, a été realisée, et les conditions de réaction pour un rendement élevé (63,9%) et une teneur en galactose élevée (71,8%) ont été obtenues. En outre, l'extraction alcaline assistée par micro-ondes a conduit à une teneur plus élevée en galactose de 63,1%. Pour ce procédé, le rendement et le contenu en galactose ont été plus fortement influencés par le rapport solide/liquide. En plus d'être soluble et de produire des émulsions plus stables que la galactane de pomme de terre ou l'homogalacturonane d'orange, la RG I rich en galactanes a montré un comportement newtonien à 25 ° C et à 73 °C, et un comportement pseudoplastique à 49 °C.

Une approche multi-enzymatique a été développée pour la production des oligosaccharides et des oligomères à partir de la RG I. Des preparations multi-enzymatiques ont été évaluées en termes de rendement, de la distribution des poids moléculaires et du profil saccharidique des oligosaccharides et des oligomères. Le rendement (1,0 à 93,9%, p/p) et la teneur en monosaccharides des hydrolysats (0,0 à 34,2%, p/p) ont été dépendants des conditions réactionnelles et du type des preparations multi-enzymatiques. Conduisant à un rendement plus élevé, à un plus large contenu des oligosaccharides riches en galactose et à une libération faible de monosaccharides, une combinaison de Depol 670l et de Gamanase 1,5 L a été choisie pour une optimisation de la production des oligosaccharides et des oligomères. En utilisant le système bi-multi-enzymatique (Depol 670l et Gamanase 1.5L), une production enzymatique des oligosaccharides et des oligomères ayant un profil bien défini a été optimisée et l'effet des

conditions de réaction (le temps, le ratio de Depol 670l/Gamanase 1.5L et la concentration de substrat) sur le rendement et sur le profil a été évalué. Les résultats ont montré que les rendements les plus élevés des oligosaccharides (DP de 2-6 et 7-12) et des oligomères (DP de 13 à 70) ont été obtenus lorsque le rapport de Depol 670l et de Gamanase 1,5 L était de 1:1. Les propriétés prébiotiques des polysaccharides RG I de la pomme de terre et leurs hydrolysats (oligosaccharides/oligomères) ont été évaluées en utilisant un système de culture en continu ensemencé avec de la flore fécale immobilisée. Bien que les polysaccharides RG I et leurs hydrolysats ont été fermentés par le *Bifidobacterium* et le *Lactobacillus*, ils n'ont pas soutenu la croissance des espèces du *Bacteroides* et du *Clostridium leptum*. Par rapport aux polysaccharides RG I, leurs hydrolysats ont pu promouvoir de façon plus sélective une plus grande production d'acides gras à courte chaîne. L'étude *in vitro* de la digestibilité a révélé que 81,6 et 79,3% de la quantité initiale des polysaccharides RG I et leurs correspondants oligosaccharides/oligomères, respectivement, sont restés intacts révélant leur grande persistance.

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CONTRIBUTION OF AUTHORS

This thesis consists of ten chapters.

Chapter I provides a general introduction and a brief literature review on the field, and outlines the objectives of the study.

A literature review on the cell wall polysaccharides from potato pulp and on the enzymatic generation of non-digestible oligosaccharides (NDOs) and oligomers from cell wall polysaccharides is presented in Chapter II. The structural properties of the cell wall polysaccharides and their interactions are provided. Different extraction methods of pectic polysaccharides and their effect on the functional properties are outlined. A brief explanation on the health promoting properties of potato pectic polysaccharides and how to assess the prebiotic properties and digestibility of carbohydrates are also reported. This chapter ends by describing some of the analytical techniques used for the structural characterization of polysaccharides, oligosaccharides and oligomers.

Chapter III to IX are presented in the form of manuscripts, and have been or will be submitted for publication. The connecting statements provide the rationale link between the different parts of this study.

In Chapter III, alkaline and enzymatic extraction of rhamnogalacturonan I (RG I) and its neutral side chains are reported. Chapter IV presents optimization of the enzymatic extraction of galactan-rich RG I polysaccharides from potato pulp by response surface methodology. Optimization of microwave-alkaline extraction of RG I polysaccharides from potato pulp and characterization of their solubility, emulsifying and viscoelastic properties are covered in Chapter V. Chapter VI reports the enzymatic generation of oligosaccharides and oligomers from potato RG I using selected multi-enzymatic preparations. Chapter VII covers the optimization of the enzymatic production of oligosaccharides/oligomers with defined degree of polymerization (DP) and sugar composition from potato RG I using multi-enzymatic preparations. Finally, Chapter VIII presents the study on prebiotic properties and digestibility of RG I polysaccharides and their oligosaccharides/oligomers using simulators of human intestinal tract.

Nastaran Khodaei, the author, was responsible for the experimental work and the preparation of the first draft of the manuscripts for publication and dissertation.

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

Dr. Valérie Orsat, the third author of manuscripts #3 (Chapter V), provided the technical support for the part related to the microwave-assisted alkaline extraction, and she was involved in the revision of manuscript #3.

Dr. Benoit Fernandez and Dr. Ismail Fliss, the second and third authors of manuscripts #6 (Chapter VIII) provided technical support and guided the research related to fermentability and digestibility and reviewed the manuscripts #6 before submission.

PUBLICATIONS

1. Khodaei, N., & Karboune, S. (2013). Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall. *Food Chemistry, 139*(1), 617-623.

2. Khodaei, N., & Karboune, S. (2014). Enzymatic extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product. *LWT-Food Science and Technology, 57*(1), 207-216.

3. Khodaei, N., Karboune, S., & Orsat, V. (2016). Microwave-alkaline extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product. *Food Chemistry, 190*(1), 495-505.

4. Khodaei, N., & Karboune, S. (2015). Enzymatic production of oligosaccharides/oligomers from potato rhamnogalacturonan I (Submitted).

5. Khodaei, N., & Karboune, S. (2015). Optimization of enzymatic production of prebiotic oligosaccharides and oligomers from potato rhamnogalacturonan I (To be submitted).

6. Khodaei, N., Fernandez, B., Fliss, I. & Karboune, S. (2015). Digestibility and prebiotic properties of potato rhamnogalacturonan I and its oligosaccharide/oligomers. *Carbohydrate Polymers*. *Under Revision.*

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NOMENCLATURE/LIST OF ABBREVIATIONS

CH	Carbohydrate
ANOVA	The analysis of variance
Ara	Arabinose
CCRD	Central composite rotatable design
CDTA	Cyclohexanediaminetetraacetic acid
Dep-based oligo-RG I	Depol 670L-generated oligosaccharides from rhamnogalacturonan I
DNS	Dinitrosalicylate
DP	Degree of polymerization
EDTA	Ethylenediaminetetraacetic acid
FOS	Fructo-oligosaccharides
Gal	Galactose
GalA	Galacturonic acid
Gal-based oligo-RG I	Endo-β-1,4-galactanase -generated oligosaccharides from rhamnogalacturonan I
Glc	Glucose
HG	Homogalacturonan
HPAEC	High pressure anion exchange chromatography
HPSEC	High pressure size exclusion chromatography
Man	Mannose
MW	Molecular weight
NDO	Non-digestible oligosaccharide
NMR	Nuclear magnetic resonance
Oligo-RG I	Oligosaccharides and oligomers generated from rhamnogalacturonan I
PAD	Pulsed amperometric detection
PCR	Polymerase chain reaction
PI	Prebiotic index
PMA	Propidium monoazide treatment
Rha	Rhamnose
RG I	Rhamnogalacturonan I
RSM	Response surface methodology
SCFA	Short chain fatty acids
Xyl	Xylose

CHAPTER I

GENERAL INTRODUCTION

There is an increasing interest in the production of novel prebiotic carbohydrates as functional ingredients. Prebiotics are non-digestible but fermentable by the intestinal microflora and selectively stimulate the growth and/or the activity of health-promoting bacteria in the gut and hence reduce the risk of colon cancer (Al-Sheraji et al., 2013; Gullón et al., 2013). Being the major component of abundant food processing by-products, cell wall polysaccharides have received special attention as potential sources of bioactive carbohydrates, including prebiotics. Indeed, prebiotic carbohydrates derived from cell wall polysaccharides would be structurally different from those currently available and might offer more prolonged modulation of gut microbiota and other functional properties (Chen et al., 2013; Mussatto & Mancilha, 2007). For instance, cell wall pectic polysaccharides and their corresponding oligosaccharides have been found to exhibit, in addition to their prebiotic activities, various biological activities including immuno-modulation, anti-cancer and anti-obesity effects (Combo et al., 2013; Gullón et al., 2013). Given its high galactan-rich RG I content (75% of pectic polysaccharides) and low homogalacturonan (HG) content compared to other food processing by-products, potato (*Solanum tuberosum* L.) pulp represents a distinct pectin-rich by-product. Comparatively, in other sources such as citrus and apple (*Malus domestica* Borkh.), RG I accounts for only ~20% of pectic polysaccharides (Axelos & Thibault, 1991; Harris, 2009). A second distinguishing structural property of potato pectin is its high proportion of (1→4)-β-galactan side chains (67% of RG I) linked to the RG I backbone (Oomen et al., 2003). Several bioactive effects of galactan-rich RG I have been put forward, in particular the fermentability of arabinogalactan (Nangia-Makker et al., 2002) and the potential of galactan side chains in stimulating the immune system and preventing the metastasis by preventing binding of galectin-3 and its receptors (Kelly, 1999; Nangia-Makker et al., 2002). Moreover, studies on the digestive and metabolic effects of potato pectic polysaccharides and its hydrolysates have shown that these compounds stimulated the growth of beneficial microflora (*e.g.*, *Bifidobacterium* spp. and *Lactobacillus* spp), but do not support the growth of *Clostridium* spp. (Lærke et al., 2007; Michalak et al., 2012; Olesen et al., 1998; Thomassen et al., 2011).

In spite of the interesting structural properties of potato pectic polysaccharides, in particular galactan-rich RG I, and their high availability, their exploration as sources of bioactive molecules has been relatively limited. Traditionally, pectic polysaccharides are extracted under acidic conditions; however, such conditions result in the degradation of the neutral side chains

(Levigne et al., 2002). Furthermore, the extraction of pectic polysaccharides using chelating agents has led to poor yields (Fischer, 1994; Oosterveld, 1996). Alkaline extraction of pectic polysaccharides can release intact RG I rich in neutral side chains through the extensive hydrolysis of HG-rich wall regions by β-elimination and oxidative peeling (Zykwinska et al., 2006). Combination of alkaline extraction with microwave heating provides advantage of increased substrate concentration and yield and decreased extraction time (Kratchanova et al., 2004; Li et al., 2012). Moreover, enzymatic extraction of RG I can be performed using selected biocatalysts (e.g., endo-polygalacturonase and pectin lyase) that hydrolyze the HG region and thereby open the pectin network for the efficient release of intact RG I region from the cell wall (Byg et al., 2012; Øbro et al., 2004; Thomassen et al., 2011). Only few studies have investigated the isolation of pectic polysaccharides from potato pulp using either alkaline (Turquois et al., 1999) or enzymatic (Byg et al., 2012; Øbro et al., 2004; Thomassen et al., 2011) processes. In addition, no comparative study for the isolation of RG I from potato cell wall by the different processes has, so far, been reported.

Most studies have focused on the generation of oligosaccharides from pectin-rich by-products mainly composed of HG polysaccharides [*e.g.*, sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Döll) pulp, citrus pulp, bergamot (*Monarda didyma* L.) peel and apple pomace (Chen et al., 2013; Gullón et al., 2011; Holck et al., 2011; Manderson et al., 2005; Martínez et al., 2009; Martínez Sabajanes et al., 2012; Olano-Martin et al., 2001). Furthermore, potato polysaccharides were mainly studied for their prebiotic properties in a native form (Lærke et al., 2007; Olesen et al., 1998; Thomassen et al., 2011) or as hydrolysates covering a wide spectrum of molecular weights (Michalak et al., 2012). Considering their structural properties, potato galactan-rich RG I type pectic polysaccharides can serve as a new source of galacto- and galacto(arabino)-oligosaccharides. Galacto-oligosaccharides are well recognized for their bifidogenic activity and their higher inhibitory effects against attachment of enteropathogenic *Escherichia coli* to HEp-2 and Caco-2 cells, compared to inulin or fructo-oligosaccharides (FOS) (Macfarlane et al., 2008; Shoaf, Mulvey, Armstrong, & Hutkins, 2006).

Because of the complexity, the diversity and the multiple side chains of potato pectic polysaccharides, their controlled hydrolysis into well-defined oligosaccharides is challenging, requiring the synergistic actions of glycosyl-hydrolase combinations identified based on the molecular structure of parent polysaccharides. In this regard, the use of commercial multi-

enzymatic preparations can be more favourable than mono-component enzymes. Indeed, selected fungi and bacteria are capable of producing more than one biocatalysts that can act synergistically for the tailored hydrolysis of polymers into oligomers (Spagnuolo et al., 1997).

The overall objective of the present study was the development of a novel biocatalytic approach for the isolation of galactan-rich RG I from potato pulp and generation of prebiotic oligosaccharides from extracted RG I.

The specific objectives of this study were as follow:

1. Investigation and comparison of the efficiency of the alkaline and enzymatic extractions of RG I polysaccharides from potato cell wall and characterization of their yield, monosaccharide profile and structure.
2. Optimization of the enzymatic extraction of RG I from potato cell wall and study of the interactive effects of reaction parameters on the yield, monosaccharide profile and debranching of the extracted RG I polysaccharides.
3. Optimization of microwave assisted-alkaline extraction of RG I from potato cell wall, highlighting the effects of extraction parameters on the yield and the structural properties of extracted polysaccharides. Assessment of emulsifying, solubility and viscoelastic properties of extracted RG I polysaccharides.
4. Investigation of the enzymatic generation of galacto-, galacto(arabino)-oligosaccharides from potato galactan-rich RG I polysaccharides using mono-component enzymes and multi-enzymatic preparations. The determination of the effect of the enzyme activity profile of multi-enzymatic preparations on the yield, the monosaccharide profile and the molecular weight (MW) of generated oligosaccharides and oligomers.
5. Optimization of a bi-enzymatic system for the generation of oligosaccharides/oligomers from galactan-rich RG I polysaccharides and investigation of interactive effects of reaction parameters on the yield, the monosaccharide profile and MW of generated oligosaccharides and oligomers.
6. Assessment of the fermentability of oligosaccharides and oligomers from potato RG I by intestinal bacteria. Determination of their selectivity, their ability to produce short chain fatty acids (SCFA) and their digestibility using simulators of the human intestinal tract.

CHAPTER II

LITERATURE REVIEW

2.1. Potato pulp

Potato pulp is a major waste product of the potato-processing industry. Approximately 0.75 kg of potato pulp is generated to produce 1.0 kg of starch (Klingspohn et al., 1993). On a dry weight basis, the non-starch components of potato pulp include fibre (88.3%), protein (6.3%), ash (5.0%) and fat (0.4%). The moisture (8.7- 87.0%) and starch content (12.0-37.0% by dry weight) of potato pulp varies depending on the starch extraction method and any further drying process (Langner et al., 2011; Mayer, 1998; Thomassen & Meyer, 2010, *www.food.lyckeby.com*). Concerns about rising production costs and environmental pollution have led to considerable efforts towards recovering and adding value to this waste. Accordingly, potato pulp has been used in a variety of ways: as cattle feed, in syrup production, as a substrate for alcohol and vitamin B12 production, and for the biogas generation (Mayer and Hillebrandt, 1997). Although potato pulp contains considerable quantities of pectic polysaccharides, it remains largely unexploited because pectin extracted from potato has low gelling properties due to its high content of neutral sugars. Pectin side chains prevent aggregation of the pectin molecules and can result in significant entanglement in concentrated solutions (Thakur, Singh, Handa, & Rao, 1997). Indeed, citrus peel and apple pomace are the major sources for the extraction of pectin (ØBro et al., 2004; Willats et al., 2006). Therefore, the development of alternative applications for the potato pectic polysaccharide is of high interest.

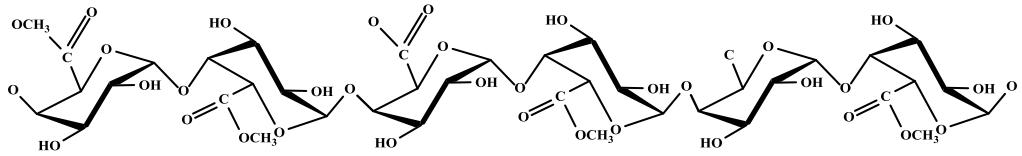
2.2. Plant cell wall

The plant cell wall consists of complex macromolecules; however, its composition and structure differs according to the plant species and functional form of the cell in question (Harris and Smith, 2006). Cell wall of dicotyledonous plants, including potato, consists of two layers: the middle lamella and the primary wall (Zykwinska et al., 2006). The middle lamella is the outer layer, which predominantly consists of pectic polysaccharides shared by adjoining cells. The internal layer is primary wall, which represents a structure that is more organized and composed mainly of polysaccharides with relatively small amounts of other compounds (*e.g.*, glycoproteins, phenolic esters, ions, enzymes and proteins) (Oechslin et al., 2003). The major polysaccharides of the dicotyledonous cell wall are pectic polysaccharides (56%), cellulose (30%) and hemicelluloses (up to 14%) (Oomen et al., 2002).

2.2.1. Pectic polysaccharides

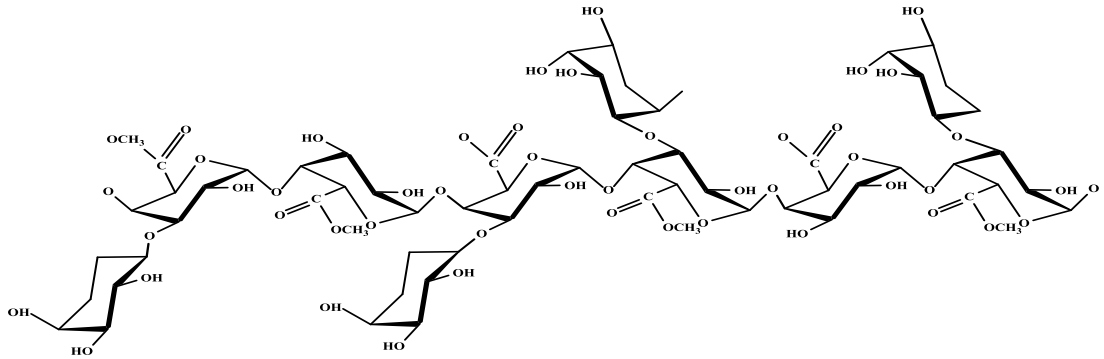
Pectic polysaccharides are complex carbohydrates rich in galacturonic acid (GalA). Four common types of pectic polysaccharides are present in the cell wall of dicotyledonous plants: HG, xylogalacturonan, RG type I and RG type II (RG II), with HG and RG I being the most abundant (Meyer et al., 2009).

- HG is a linear polymer of α-D-(1→4) GalA residues that are methylated at O-6 (C-6 carboxyl) and acetylated at O-2 or O-3 (Hoondal et al., 2002; Leroux et al., 2003; Mohnen, 2008).



Scheme 2.1 HG with methyl esterification

- Xylogalacturonan is a substituted polygalacturonan with β-D-(1→3) xylose (Xyl). Some of the linked Xyl residues are further linked to other Xyl residues at O-4 (Vincken et al., 2003; Mohnen, 2008).

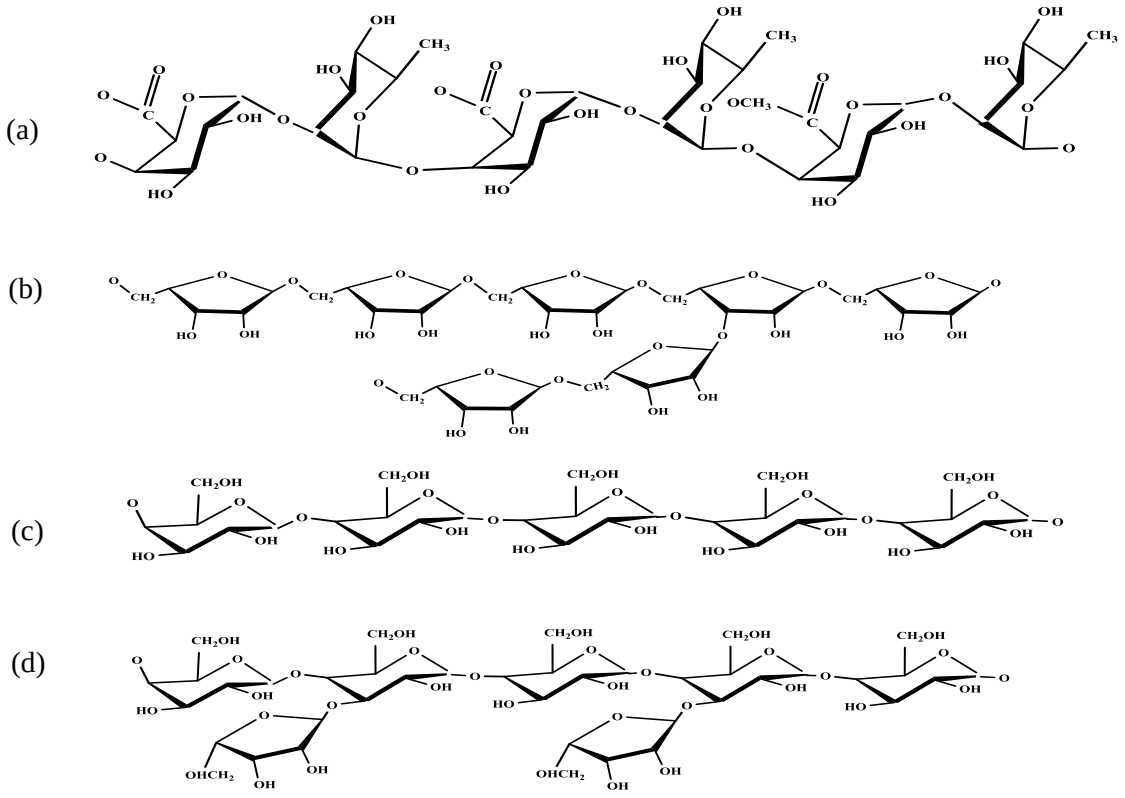


Scheme 2.2 Xylogalacturonans (HGs substituted with α-D-xylose residues at the C3 position of GalA units).

- RG I has a backbone composed of alternating disaccharide of [→2)- α-L-rhamonose (Rha)-(1→ 4)-α-D-GalA)-(1→]. The GalA residues in the backbone are partially methyl-esterified and O-acetylated at C-2 or C-3 (Vincken et al., 2003). Up to 80% of the Rha residues are substituted at O-4 with different amounts of arabinan, galactan and arabinogalactan side chains or individual α-L-arabinose (Ara) and β-D-galactose (Gal) residues (Vincken et al., 2003; Harris and Smith, 2006). Galactan side chains are polymers of (1→4) linked β-D-Gal, while arabinan side chains

have a backbone of α-L-(1→5) Ara substituted with α-L-(1→3) Ara, α-L-(1→2) Ara or chains of α-L-(1→3) Ara (Mohnen, 2008). Arabinogalactan type I consist of a backbone of β-D-(1→4) Gal with α-L- Ara or arabinan branches attached to O-3 of Gal residues, whereas arabinogalactan type II has a backbone of β-D-(1→3) Gal with β-6-linked galactan or arabinogalactan branches (Mohnen, 2008).

- RG II is another substituted galacturonan. Its complex structure consists of 12 different monosaccharides linked with 20 different linkages. It comprises about 10% of pectic polysaccharides in dicotyledons (Mohnen, 2008). RG I and RG II have different structures; indeed, RG II has a backbone of polygalacturonic acid with four different side chains attached to GalA residues (Ronald, 2001; Oomen et al., 2002).



Scheme 2.3 (a) RG I backbone (b) (1→5)-α-L-arabinans side chain (c) (1→4)-β-D-galactan side chain (d) arabinogalactan side chain.

2.2.2 Hemicellulosic polysaccharides

The main hemicellulosic polysaccharides in dicotyledonous plants are xyloglucans, heteromannans and heteroxylans, accounting for 11, 3 and <3% of total cell wall

polysaccharides, respectively (Oomen et al., 2002). Xyloglucans are composed of a backbone of (1→4)-β-D-glucose (Glc) with α-D-Xyl residues attached to O-6 of Glc residues. Heteromannans in the form of galactomannan contain a backbone of repeating β-D-mannose (Man) and β-D-Glc with Gal units linked to Man residues (Schröder et al., 2001). The heteroxylans are glucuronoarabinoxylans, which have mostly single α-L-Ara and α-D- Glc residues linked to the Xyl residues of the (1→4)-β-D-xylan backbone at O-3 and O-2, respectively (Harris and Smith, 2006).

2.2.3. Cellulose

Cellulose maintains the structural integrity of the plant cell (Imam et al., 2004) and is a linear homopolymer of 100 to 14,000 Glc units linked through β-1,4 bonds (Hon, 1994). Cellulose molecules are oriented in parallel structure with intra and intermolecular hydrogen bonds, which results in a cellulose microfibril structure (Heredia et al., 1995).

2.2.4. Cell wall polysaccharide interactions

Several linkages between cell wall components have been reported. Presence of linkages between cell wall components result in pectic polysaccharides being trapped in the cell wall structure, such that the pectin network must be disrupted to enable extraction. Coenen et al. (2007) have shown that the acid hydrolysis of pectic polysaccharides released segments of RG I covalently linked to HG and xylogalacturonan, confirming the existence of linkage between these polysaccharides. In addition, a three-dimensional pectic network is formed upon non-covalent cross-linking of some glycosyl residues in the pectin structure. For example, Ca^{2+} forms ionic cross-links between carboxyl groups of the GalA residues in HG and the methyl esterified GalA residues of HG may associate through hydrophobic interactions (O'Neill et al., 2004). Moreover, linkage of xyloglucan and neutral side chains of RG I to the surface of cellulose microfibrils has been reported. Interactions between cellulose and pectic side chains and xyloglucan are mediated by hydrogen bonds (Zykwinska et al., 2005, 2006, 2007; Popper and Fry, 2008). Zykwinska et al. (2006) have reported the presence of two fractions of arabinan and galactan side chains with different mobility in potato and sugar beet cell wall. Those with restricted mobility are tightly associated with cellulose while the others are highly mobile. They reported that about 70% of Gal in potato and 69% of Ara in sugar beet cell walls was highly mobile, while the remainder showed restricted mobility.

2.2.5. Structural properties of potato pectic polysaccharides

Potato pectic polysaccharides show significant differences, in terms of composition and structural properties, depending on the source and extraction method (Table 2.1). Such variations affect their application as a source of food ingredients, nutraceuticals and pharmaceutical molecules. While potato RG I makes up 75% of pectic polysaccharides, it only accounts for 20% on average in other species such as citrus and apple (Axelos and Thibault, 1991; Harris, 2009). Conversely, HG makes up 20% of potato pectic polysaccharides, compared to 56%, on average, for pectins from other species. Furthermore, potato RG I is highly ramified with (1→4)-β-galactan (67% of RG I) and (1→5)-α- arabinans side chains comprising 19% of RG I (hairy region) (Oomen et al., 2003). Potato pectin is also highly acetylated (15.0%) and has lower methylation degrees (17.4-29.5%) as compared to other sources (Table 2.1). The presence of hairy regions and acetyl groups reduces potato pectin gelling ability (Willats et al., 2006).

2.3. Functional and health promoting properties of pectic polysaccharides

Several studies have indicated health promoting effects of pectin derived compounds. For example oligosaccharides obtained from apple pomace, orange and bergamot peel have been reported to enhance *Bifidobacterium* and *Lactobacillus* growth while not supporting *Bacteroides* or *Clostridium* growth (Chen et al., 2013; Gullón et al., 2013; Gullón et al., 2011; Hotchkiss et al., 2003; Manderson et al., 2005). Samples tested in these studies mainly originated from the cell wall's HG region; however, neutral-sugar-rich carbohydrates have shown prebiotic properties as well. For example, fermentation of the highly branched arabinan-containing hairy region of pectin was shown to result in a greater quantity of SCFA, specifically propionate, as well as a lesser pH decrease, than that seen with commercial pectin (Gulfi et al., 2007).

Moreover Gullón et al. (2011), who tested fermentability of the different oligosaccharides obtained from apple pomace in a medium inoculated with fecal samples, reported that gluco-oligosaccharides, galacto-oligosaccharides and xylo-oligosaccharides were those most fermentable by intestinal bacteria followed by arabino-oligosaccharides and oligogalacturonides.

Table 2.1 Monosaccharide profile, degree of acetylation and methylation of acid extracted pectin from apple, citrus and beet and pectic polysaccharides from starch-free potato cell wall preparation

Pectin sources	Potato	Apple	Citrus	Beet	References
GalA [a]	29.3	85.3	94.0	75.7	(Axelos & Thibault, 1991; Vincken et al., 2000)
Rha [a]	5.3	2.7	1.7	6.6	"
Gal [a]	50.3	5.1	1.3	6.2	"
Ara [a]	14.3	2.0	0.2	0.2	"
Xyl [a]	1.0	4.9	2.8	11.3	"
Degree of acetylation (%) [b]	15.0	5.0	1.4–1.6	16.0–35.0	(Axelos & Thibault, 1991; Kravtchenko et al., 1992; Levigne et al., 2002; Turquois et al., 1999)
Degree of methylation (%) [b]	17.4-29.5	74.0	72.0	54.0	(Axelos & Thibault, 1991; Harris, 2009)

[a] Monosaccharide profile expressed in molar proportion

[a] Calculated on the basis of GalA content

2.3.1 Potato pectic polysaccharides and their health effects

The structural properties of potato pectic polysaccharides containing galactan-rich RG I, make it a new potential source of NDOs, in particular (arabino) galacto-oligosaccharides and galacto-oligosaccharides. Currently available commercial galacto-oligosaccharides are produced from lactose and therefore contain considerable amounts of Glc and lactose, making them non-consumable to consumers suffering from diabetes mellitus or lactose intolerance (Cheng et al., 2006). However, NDOs derived from cell wall polysaccharides would be structurally different from those currently commercially available and might therefore offer other interesting functional properties.

Potato pectic polysaccharides have been mainly recognized for their bioactive properties, in particular their fermentability and their bifidogenic activity. Olesen et al. (1998) showed that enzymatically solubilized potato fibres are fermentable and their consumption increased end-expiratory H_2 levels. It has also been shown that consumption of soluble potato fibers obtained by enzymatic treatment of potato pulp decreased weight gain in rats (Lærke et al., 2007). Having extracted high-MW polysaccharides (up to 400 kDa) from potato pulp using pectin lyase and polygalacturonase, Thomassen et al. (2011) found the extracted polysaccharides, assumed to be mainly RG I with galactan side chains, promoted *Bifidobacterium* and *Lactobacillus* growth. The increase in number of the *Bifidobacterium* when RG I-rich and HG-rich fractions were used was higher than results obtained for FOS (DP 2–8 Orafti®P95) and RG I-rich fraction had better bifidogenic properties than HG-rich fraction. Moreover, Michalak et al. (2012) extracted potato pulp polysaccharides through treatment with pectin lyase and polygalacturonase and used endo-1,4-β-galactanase to produce two fractions of <10 and >10 kDa. They reported that both fractions promoted the growth of *Bifidobacterium longum* and *Lactobacillus acidophilus* and generally did not support the growth of *Clostridium perfringens*. Stimulation of *B. longum* with hydrolyzed samples were significantly better than with FOS, Gal or unhydrolyzed galactan.

2.4. Extraction of pectic polysaccharides from cell wall material

2.4.1. Enzymatic extraction of pectic polysaccharides

Pectin can be solubilized from plant cell wall material using different pectic enzymes that either degrade the backbone or the substituents or side chains of the pectin structure (Ronald, 2001). Basically two types of backbone degrading pectic enzymes exist:

- *De-esterifying enzymes* (pectin esterases) catalyse the de-esterification of pectin methoxyl groups.
- *Depolymerizing enzymes* (pectinases: hydrolases and lyases) split the α-(1,4)-glycosidic bonds between galacturonic monomers in pectic substances either by hydrolysis (hydrolases) or by β-elimination (lyases). The latter results in galacturonide with an unsaturated bond between C4 and C5 at the non-reducing end of the GalA formed (Kashyap et al., 2001).

The hydrolases have been divided based on their substrate specificity to highly methylated pectin (polymethylgalacturonases), or low degrees of esterification (polygalacturonases) (Ronald, 2001). The prefixes endo- and exo- used represent whether the cleavage is random or endwise, respectively (Kashyap et al., 2001). The endo-acting enzymes break bonds in the interior of the polymer, whereas the exo-acting ones cleave monomer units from terminal ends of the polymeric chain.

Similar to hydrolases, lyases have been divided according to their preferring substrates (e.g. a lyase with an affinity for a substrate with a high degree of methylesterification: endopolymethylgalacturonate lyase). They are also classified according to whether the cleavage is random (endo-) or endwise (exo-) (Kashyap *et al.*, 2001).

2.4.2. Chelating agents-based extraction of pectic polysaccharides

Pectic polysaccharides can be extracted by treating the plant cell wall with a chelating agent such as cyclohexanediaminetetraacetic acid (CDTA) or ethylenediaminetetraacetic acid (EDTA) (Huisman et al., 1999). Indeed, Ca^{2+} in plant cell walls forms intermolecular bonds to carboxylates of polygalacturonic acid generating a three-dimensional structure that is modeled as an "egg carton." Extraction with chelating agents results in high-MW pectic polysaccharides, but a lesser yield than other extraction methods (Huisman et al., 1999; Zykwinska et al., 2006).

While Fischer (1994) reported the CDTA-soluble fraction to originate strictly from the middle lamella, Renard and Thibault (1993) have challenged the hypothesis that the CDTA-soluble fraction consists only of the middle lamella pectin, arguing that the quantities of pectin extracted by CDTA are too high to originate solely from the middle lamella. Also, the high degree of methylation in this pectic fraction does not allow the formation of stable ionic bonds with divalent calcium ions according to the 'egg box' model (Renard and Thibault, 1993). In addition,

this fraction contains high amounts of highly esterified unbranched pectin, which could have come from the primary cell wall (Fischer, 1994).

2.4.3. Acidic extraction of pectic polysaccharides

Commercial pectins are, generally, extracted by treating the raw material with hot diluted mineral acid solutions at a pH of 1-3, 50-90°C for 3–12 h (Fissore et al., 2010). Pectin solubilization takes place under the influence of different factors, mainly temperature, pH and time (Pinheiro et al., 2008). Tartaric, malic, citric, lactic, acetic and phosphoric acids have been used for pectin extraction; however, cheaper mineral acids tend to be used (Harris and Smith, 2006; Fissore et al., 2010). Among the acids, the extraction of pectins with sulfuric (Yapo et al., 2007), hydrochloric (Mesbahi et al., 2005) and nitric acids (Pagan et al., 2001) have been well investigated.

The acid extraction process generally yields pectin with a high degree of esterification (high DE pectin), approximately in equal quantity with naturally occurring pectin. High DE pectin has a degree of esterification of 50% or greater. Because of their different gelling properties and mechanisms, low and high DE pectin have different applications in foodstuffs such as gelling agent, thickener, texturizer, emulsifier, and stabilizer (Joye and Luzio, 2000). Although acid extraction is the most widely used method to obtain commercial pectin, it causes side chain degradation (Levigne and Ralet, 2002; Harris and Smith, 2006). In addition, under acid conditions, the linkages between GalA and rhamnose in RG-I blocks are much more labile than the linkages between adjacent non-methylated GalA residues in HG blocks; therefore, the RG I region is more susceptible to hydrolysis under acidic conditions (Khalikov and Mukhiddinov, 2004). Although acid extraction is a favourable extraction method when the objective is the extraction of the HG region, it can cause neutral side chain degradation, and is thus commonly used for the extraction of the HG but not RG I region (Levigne et al., 2002).

2.4.4. Alkaline extraction of pectic polysaccharides

Alkaline extraction of pectic polysaccharides can release intact RG I rich in neutral side chains through extensive hydrolysis of the HG region by β-elimination and oxidative peeling (Zykwinska et al., 2006). The alkaline extraction process yields a pectin of low degree of esterification (low DE pectin) as a result of saponification of the ester groups (Joye and Luzio, 2000).

Sakamoto & Sakai (1995) boiled sugar beet pulp with 0.1 M NaOH for 1 h to extract alkaline soluble pectin. The results showed that the extracted polysaccharides contained the hairy region of pectin with a sugar composition of Ara (72.0%), Gal (15.0%), Rha (5.0%) and GalA (8.0%).

Alkaline extraction of pectic polysaccharides from sugar beet pulp (4 M NaOH, 0.02 M $NaBH_4$, 80°C, 2 h) resulted in the recovery of HGs, RGs, arabinans and relatively small amounts of glucomannans and xyloglucans. Three populations of ramified 'hairy' regions of pectin were separated on a diethylethanolamine column. Two of these were RGs with high apparent molecular weights of 1300 and 120 kDa. These populations had high Ara content. The third population, which originated from the ramified 'hairy' regions, was a neutral population, which did not interact with the diethylethanolamine column and had a low apparent MW and a high Ara content (Oosterveld et al., 2000).

Zykwinska et al. (2005) extracted cell wall polysaccharides with 0.05-0.5 M NaOH at 4-90 °C. Their results showed that using milder extraction conditions (0.05 M, 4 °C), yield of 86% was obtained, and extracted polysaccharides contained GalA (19.9%), Rha (1.6%), Gal (28.7%), Ara (6.4%), Xyl (3.2%), Man (1.2%) and Glc (38.7%). Using harsher extraction conditions (0.5 M, 90 °C) led to a decrease in yield (11%), GalA (1.5%), Rha (0.0%), Gal (1.4%) and Ara (1.5%) contents while the proportion of Xyl (4.7%), Man (1.3%) and Glc (89.6%) increased.

Investigating the cell wall material residue after treatment for 1 h with 0.05 M, 0.275 M or 0.5 M NaOH at 40, 65 or 90°C, Zykwinska et al. (2006) showed that, in contrast to cellulosic and hemicellulosic sugars, the amount of pectins recovered in the residues varied largely with the conditions used. The NaOH concentration had a less significant effect than the temperature, greater solubilisation occurring at higher temperatures. Residues recovered at 90 °C contained only minor fragments of pectic backbone sugars (Zykwinska et al., 2006).

2.4.5. Microwave-Assisted extraction of pectic polysaccharides

Conventional heating method used for extraction can be replaced with microwave heating. Rapid microwave heating results in fewer covalent bonds suffering hydrolysis, which produces pectins of greater molar mass, viscosity and gel strength (Fishman et al., 2006). Moreover, evaporation of moisture inside the plant cell wall increases internal pressure, rupturing the cell wall and making its components more easily extractable (Mandal et al., 2007). This property of microwave heating provides the advantage of having an increased substrate concentration and a

decreased extraction time. Moreover, greater yield has been reported when using microwave extraction (Kratchanova et al., 2004; Li et al., 2012). Microwave extraction can be combined with either acidic or alkaline solutions.

2.4.5.1. Microwave-assisted acidic extraction of pectic polysaccharides

So far, most studies on microwave extraction of pectin have been performed by microwave treatment of cell wall material in acidic solutions (pH 1-3). Table 2.2 gives the experimental details of a number of studies on the extraction of pectin using a microwave-assisted acidic extraction method. A wide variety of raw materials have been studied, mainly citrus peel, sugar beet pulp and apple pomace.

The solid/liquid ratios tested in these studies ranged between 0.01 and 10.0% and used powers ranged between 50 and 1200 W, resulting in a power to substrate ratio of 15 to 1200 W/g. The yields reported in these studies covered a wide range (0.5-84.0%), with the highest yield range (59–84%) being reported by Bélafi-Bakó et al. (2012) for the extraction of pectic polysaccharides from berry fruits. However, a relatively long extraction time (30 min) was used in their study (Bélafi-Bakó et al., 2012; Fishman et al., 1999; Fishman et al., 2006; Fishman et al., 2008; Guo et al., 2012; Li et al., 2012; Liu et al., 2006; Prakash Maran et al., 2013; Wang et al., 2005).

2.4.5.2. Microwave assisted-alkaline extraction of pectic polysaccharides

Contrary to microwave-assisted acidic extraction, only few studies have investigated microwave-assisted alkaline extraction for the isolation of pectin. Fishman et al. (2009) have used microwave treatment to extract alkaline soluble polysaccharides from fresh sugar beet pulp after pectin was removed by acidic extraction. These authors reported yields of 18–33% upon treatment of sugar beet pulp at 1200 W of power for 10-30 min and a 5% solid/liquid ratio. Yeoh et al. (2008) carrying out microwave-assisted extraction of pectin from orange peel at a temperature of 120 °C for 15 min and a 6.3% solid/liquid ratio, reported a decrease in yield from 4.5 to 1.5% when pH increased from 1.5 to 10.

Table 2.2 Selected studies on microwave assisted-acidic extraction of the pectic polysaccharides

Raw material	Solid/liquid (%)	Power (W)/time (min)	Yield (%)	References
Berry fruits	2.5-10.0	50-700/30 min	59-84	(Bélafi-Bakó et al., 2012)
Orange albedo	4.0	630/2.5-8	3.3 [a]	(Fishman et al., 1999)
Lime flavedo, albedo and pulp	4.0	630/1-10	1.1-10.0	(Fishman et al., 2006)
Sugar beet pulp	5.0	1200/3-20	8.4-14.8	(Fishman et al., 2008)
Orange peel	2.0	500/21	20.0	(Guo et al., 2012)
Sugar beet pulp	0.01-0.02	150-250/2-4	5.0 – 32.4	(Li et al., 2012)
Orange peel and albedo	2.0-8.0	15 min [b]	0.5-1.6	(Liu et al., 2006)
Orange peel	3.3-10.0	160-480/1-3	7.4-18.6	(Prakash Maran et al., 2013)
Apple pomace	0.03-0.06	320-580/10.6-17.4	2.8-11.6	(Wang et al., 2005)

[a] Yield obtained for extraction time of 3 min.

[b] Temperature was maintained constant at 150 °C.

2.4.6. Extraction of pectic polysaccharides from potato pulp

Both alkaline and enzymatic extraction of pectic polysaccharides from potato pulp have been reported before in literature. Turquois et al. (1999) extracted pectin from sugar beet and potato pulp using treatment with 50 mM NaOH at room temperature for 2 h. The polysaccharides extracted from sugar beet (10% yield) were composed of GalA (76.0%), Rha (1.1%), Gal (5.1%), Ara (15.7%) and Glc (2.1%) while treatment of potato pulp with alkaline solution resulted in higher yield of 20% and monosaccharide profile of GalA (44.3%), Rha (1.2%), Gal (17.7%), Ara (4.0%) and Glc (32.8%).

Øbro et al. (2004) extracted RG I polysaccharides from de-starched potato cell wall using *Aspergillus niger* endo-polygalacturonase and achieved a 40% yield.. The major monosaccharide of the extracted RG I was Gal (62.1%) followed by Ara (18.2%). GalA and Rha composed 15.0 and 4.7% of extracted polysaccharides, respectively. Thomassen et al. (2011) released 75% (w/w) of polysaccharides from potato pulp through treatment with 1.0% (w/w, enzyme/substrate) pectin lyase from *Aspergillus nidulans* and 1.0% (w/w, enzyme/substrate) polygalacturonase from *Aspergillus aculeatus*. Extracted polysaccharides from the HG fraction (10–100 kDa) were rich in GalA (47.7%), while the RG I fraction (>100 kDa) was rich in Gal (65.5%). Moreover, Byg et al. (2012) solubilized RG I enzymatically using endo-polygalacturonase from *A. aculeatus*, achieved an extraction yield of 9-11% depending on the extraction scale (laboratory or large scale), with a monosaccharide profile of Gal (66.7-73.4%), GalA (17.1-10.3%), Ara (8.7-10.7%), Rha (5.9-4.2%) and Xyl (0.5%).

2.5. Non-digestible oligosaccharides (NDOs)

The IUB-IUPAC nomenclature classifies oligosaccharides as carbohydrates consisting of 3 to 10 monomers. NDOs are those that are resistant to digestion by upper intestine enzymes, mainly due to the presence of β-glycosidic bonds between their monosaccharide units (Mussatto and Mancilha, 2007). Given these properties NDOs can act as prebiotics, *i.e.*, "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health" (Gibson *et al.*, 1995). Therefore, prebiotics should resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, be fermented by the intestinal microflora and selectively stimulate the growth and/or activity of beneficial intestinal bacteria such as *Bifidobacterium* and

*Lactobacillu*s (Gibson et al., 2004; Macfarlane et al., 2008). In addition to stimulating growth of beneficial bacteria, prebiotics can provide other health benefits. For example it has been reported that NDOs can inhibit attachment of enteropathogenic *E. coli* to HEp-2 and Caco-2 cells, prevent acute gastroenteritis, reduce cancer risk, and improve mineral absorption and lipid regulation (Gullón et al., 2011; Macfarlane et al., 2008; Shoaf et al., 2006). There are currently different types of commercially available food-grade prebiotics, but the most important NDOs include FOS, galacto-oligosaccharides, isomalto-oligosaccharides, inulo-oligosaccharides and soybean oligosaccharides (Van Laere et al., 2000; Marshall, 2008).

2.5.1. Production of NDOs

2.5.1.1. Direct extraction of NDOs from natural food products

Some NDOs are naturally present in foods such as milk, honey, as well as fruits and vegetables like onion (*Allium cepa* L.), leek (*Allium ampeloprasum* L.), garlic (*Allium sativum* L.), artichoke (*Cynara scolymus* L.), banana (*Musa* × *paradisiaca* L.), rye (*Secale cereale* L.), and barley (*Hordeum vulgare* L.), usually at concentrations of 0.3% and 6% of fresh weight. The concentration of the NDOs is between 5% and 10% for chicory (*Cichorium intybus* L.) and salsify (*Tragopogon porrifolius* L.) and up to 20% for Jerusalem artichoke (*Helianthus tuberosus* L.) and yacon (*Smallanthus sonchifolius* (Poepp.) H. Rob.). The NDOs in milk are mainly Gal-containing oligosaccharides, either in free form or as glycoconjugates. The major NDOs in the soybean (*Glycine max* (L.) Merr.) and other pulses and leguminous seeds are raffinose and stachyose. FOS are found in asparagus (Asparagus officinalis L.), sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat (*Triticum æstivum* L.), honey, banana, barley, tomato (*Solanum lycopersicum* L.) and rye. Moreover, honey and sugarcane (*Saccharum officinarum* L.) juice are sources of isomaltulose, while xylooligosaccharides appear naturally in bamboo shoots, fruits, vegetables, milk and honey (Mussatto & Mancilha, 2007; Rastall, 2010).

2.5.1.2. Chemical synthesis of oligosaccharides

Different glycosylation strategies have been reported for the chemical synthesis of oligosaccharides (Boons, 1996). The glycosylation reaction is achieved by an inter-glycosidic condensation between a completely protected glycosyl donor (R-OH) that has an excellent leaving group (halogenides) at its anomeric position and a glycosyl acceptor that possesses only one free hydroxyl group. Among these glycosyl donors, anomeric fluorides,

trichloroacetimidates and thioglycosides are currently being employed (Boons, 1996). The preparation of specific glycosyl donor and acceptor implies many protection and deprotection steps to combine high yields with regional stereoselectivity. With these strategies oligosaccharides of interest such as oligo-galacturonides have been synthesized (Magaud et al., 1997). These compounds used as glycosylation intermediates should permit the production of higher oligomers of D-galacturonates. Xylooligosaccharides (DP 4 up to 10) have also been generated through a complex blockwise synthesis approach (Takeo, 1995). These syntheses show that the formation of oligosaccharides is only possible when each step in the assembly of the glycosyl (donor and acceptor) is high yielding. Therefore, in spite of recent developments, synthesis of oligosaccharides by chemical glycosylations does not seem realistic at the industrial scale (Barreteau et al., 2006).

2.5.1.3. Enzymatic synthesis of oligosaccharides

In comparison to chemical synthesis, enzymatic synthesis offers regiospecificity and stereospecificity to the glycosidic linkages. NDOs can be synthesized using β-glycosidase (EC 3.2.1.) or glycosyltransferase (EC 2.4.1). For instance, galacto-oligosaccharides can be obtained through the enzymatic conversion of lactose (milk sugar) by β-galactosidase (EC 3.2.1.23), which can mediate both the hydrolysis and polymerization of β-linked sugars (Marshall, 2008). During the hydrolytic reaction, galactosidase forms an active intermediate with lactose and reacts with water to catalyze the hydrolysis of the β-galactoside (Barreteau et al., 2006). During the polymerization process, the trans-galactosylation of lactose or the growing chain of oligosaccharides as a donor, gives rise to heterogeneous mixtures of β-galacto-oligosaccharides of varying chain length and linkages (Nauta et al., 2010). The ratio of transferase and hydrolase activities of β-galactosidase affects the amount and nature of the oligosaccharides formed. The enzyme source, the concentration and nature of the substrate and the reaction conditions (pH, temperature and time) are the main key factors affecting the ratio of transferase to hydrolase activity (Martínez-Villaluenga et al., 2008).

2.5.1.4. Generation of oligosaccharides and oligomers from polysaccharides

Plant polysaccharides are often present in large amounts in fiber-rich and low-cost by-products and biomass wastes which are mainly composed of cell wall polysaccharides.

Depending on the source and the production method, oligosaccharides obtained from complex and heterogeneous cell wall polysaccharides can have various compositions and structures. Cell wall polysaccharides are therefore a good but unexplored potential source of new prebiotics (Concha Olmos & Zúñiga Hansen, 2012; Gullón et al., 2011).

Different approaches have been used for the conversion of polysaccharides into oligosaccharides, including acid hydrolysis, physical methods and enzymatic hydrolysis. For instance, orange albedo and apple pomace were investigated for the production of pectic oligosaccharides by acid hydrolysis (HNO_3, HCl and trifluoroacetic acid) (Coenen et al., 2008; Manderson et al., 2005). Rha (GalA) oligosaccharides were produced from de-esterified beet pulp using HCl (Gullón et al., 2013; Renard et al., 1997). In addition, oligosaccharides have been generated using physical methods. For example, pectic oligosaccharides from sugar beet pulp and orange peel were produced using hot/compressed water (autohydrolysis or hydrothermal treatments) with 29.7 and 24.0% yields,, respectively (Martínez et al., 2009, 2010). Chen et al. (2013) have used dynamic high-pressure microfluidization to produce pectic-oligosaccharides from apple pectin. The resulting pectic-oligosaccharides were composed of 29.6% GalA and 58.5% neutral sugars.

Enzymatic depolymerization is the main approach in preparing large amounts of oligomers. This strategy involves the use of polysaccharide-degrading enzymes such as glycosyl-hydrolases and polysaccharide lyases (Barreteau et al., 2006). Isomalto-oligosaccharides, xylo-oligosaccharides, manno-oligosaccharides and pectic oligosaccharides are examples of NDOs produced through a depolymerization approach. Isomalto-oligosaccharides are composed of Glc monomers linked by α-1,6 (and occasionally α-1,4) glycosidic linkages. Commercial products produced from cornstarch consist of isomaltose, isomaltotriose and panose. Starch-type dextrans are converted to isomalto-oligosaccharides upon the actions of three selected enzymes (Crittenden and Playne, 1996). Firstly, starch is hydrolysed to malto-oligosaccharides by α-amylase (EC 3.2.1.1) and pullulanase (EC 3.2.1.41). Then, α-glucosidase (EC 3.2.1.20) is added to catalyze a transfer reaction converting the α-1,4 linked malto-oligosaccharide into α-1,6 linked isomalto-oligosaccharide. Glc is then removed to produce a higher product concentration (Kohmoto et al., 1998). Xylo-oligosaccharides are chains of Xyl molecules linked by β-1,4 bonds and mainly consist of xylobiose, xylotriose and xylo-tetraose. Xylo-oligosaccharides are manufactured by the enzymatic hydrolysis of xylan from corn (*Zea mays* L.) cobs (Crittenden &

Playne, 1996). For the enzymatic production of xylo-oligosaccharides, enzyme complexes with low exo-xylanase and/or xylosidase activity are required to avoid the production of Xyl.

Pectic polysaccharides have also been used for the generation of NDOs. For example, Olano-Martin et al. (2001) converted low and high methoxyl pectin to pectic oligosaccharides, with yields reaching 95%, using endo-polygalacturonase. Moreover, oligo-galacturinide was obtained from polygalacturonic acid using pectate-lyase (Hotchkiss et al., 1991). Michalak et al. (2012) used endo-1,4-β-galactanase from *Emericella nidulans* (anamorph *A. nidulans*) to generate oligomers and oligosaccharides from high-MW solubilized potato pulp polysaccharides.

Efficient depolymerization of polysaccharides requires cooperative or synergistic interactions between the enzymes responsible for cleaving the different linkages. Synergy has been reported for many enzymes involved in degradation, usually between a main-chain-cleaving enzyme and one or more accessory enzymes (De Vries et al., 2001). Synergistic property can be employed for NDOs production by the use of an enzyme mixture or a multi-enzymatic preparation.

The use of a mixture of enzymes for production of pectic oligosaccharides has been reported. Bonnin et al. (2002) used a mixture of pectin-methylesterase, rhamnogalacturonase, galactanase and arabinanase activities to generate rhamno-oligosaccharides from sugar beet. Holck et al. (2011) purified homogalacturonides and rhamnogalacturonides from sugar beet pectin by sequential use of mono-component enzymes. However use of commercial multi-enzymatic preparations can be more favourable because in addition to providing the advantage of using multiple enzymatic activities and therefore developing synergistic effects amongst them, they are cheaper than mono-component enzymes and have therefore been widely used at the industrial scale. Indeed, selected fungi and bacteria are often capable of producing more than one enzyme activity (Spagnuolo et al., 1997) and their resulting multi-enzymatic preparation can be used for the production of NDOs from plant cell wall polysaccharides, such as pectic polysaccharides. Use of multi-enzymatic preparations for production of oligosaccharides from pectin has been reported before. Celluclast 1.5L from *Trichoderma reesei* and Viscozyme L from *A. aculeatus* have been used to hydrolyze orange peel waste and it was reported that 46.5–73.8% and 70.5–100% of galactan and arabinan side chains were hydrolyzed, respectively (Martínez Sabajanes et al., 2012). The same enzymes used to hydrolyse sugar beet pulp with yield of 26.7% (Martínez et al., 2009). However, the use of multi-enzymatic preparation for the generation of oligosaccharides from potato RG I has not been reported so far.

2.5.2. Assessment of prebiotic properties of oligosaccharides

The anaerobic fermentation properties of the NDOs can be tested using *in vivo* and *in vitro* methods. Laboratory animals such as heteroxenic rats harboring a human fæcal flora, livestock and human subjects have been used for *in vivo* studies by measuring concentration of breath gases (essentially hydrogen) and recovery of the tested carbohydrate and SCFA in faecal samples (Gibson et al., 2004).

Although *in vitro* studies are easier to perform, they do not take into account the interactions between gut tissues and microbiota. Moreover, these methods require anaerobic sampling followed by reliable and quantitative microbiological analysis of a wide variety of bacterial genera, *i.e*., total aerobes and anaerobes, some of which may not be culturable (Gibson et al., 2004; Macfarlane et al., 1998). *In vitro* studies are done using batch and continuous culture fermentation systems. Multi-chamber continuous culture systems have been developed to reproduce gastrointestinal regions. Each vessel is stirred and the temperature set at 37°C by a circulating water bath. Culture pH is controlled automatically and anaerobic conditions are maintained by sparging the vessels with oxygen-free nitrogen gas (Mandalari et al., 2007). Either pure culture(s) of selected species or faecal slurry can be used to assess fermentability of NDOs. Using pure cultures, the prebiotic properties of the NDOs are assessed by comparing their fermentation properties by beneficial and/or pathogenic strains of gut microorganisms. Usually, the selected strains are grown in a sugar-free, anaerobic medium, to which the NDOs are added as carbon source (Gibson et al., 2004). Inoculation with faecal slurry is preferable because pure cultures of single microbial strains do not consider bacterial interaction and selectivity of the prebiotics (Gibson et al., 2004, Thomassen et al., 2011).

2.5.3. Assessment of digestibility of NDOs

In vivo NDO digestibility can be assessed by measuring the NDOs present in the rat gastrointestinal segments and faeces. Models applicable to humans involve the measurement of breath-hydrogen and blood Glc or insulin following oral administration of the NDOs. Moreover direct recovery at the terminal ileum have been measured in ileostomy patients (Cummings et al., 2001). *In vitro* studies of digestibility includes determining resistance to acidic conditions (such as conditions in the stomach) and enzymatic (saliva, pancreatic and small intestinal) hydrolysis. These analyses are performed by measuring hydrolysis products (Gibson et al., 2004). The *in*

vitro studies can also be done in complex intestinal models such as SHIME (Simulation of the Human Intestinal Microbial Ecosystem) (Molly et al., 1993). These models are pH and temperature-controlled and simulate most of the human intestinal tract attributes (Rastall, 2010).

2.6. Structural characterization of polysaccharides and oligosaccharides

2.6.1. High-Performance liquid chromatography (HPLC)

Chromatographic methods are the most powerful analytical techniques for the analysis of polysaccharide type and concentration.. The carbohydrate separation can be based on their partition coefficients, polarities or sizes, depending on the type of column used. The weak anion exchanger column TSKgel diethylethanolamine-5PW has been used as an anion exchange column for the separation of galactorunic acid-containing carbohydrates (Massiot, 1994; Akita et al., 2002). For size exclusion analysis, TSKgel PWxl columns, composed of spherical, hydrophilic polymethacrylate beads have been used. The main application area for TSKgel PWxl columns is the analysis of water-soluble polymers (Oosterveld, 1996; Zandleven et al., 2006; Meyer et al., 2009) .

2.6.2. High Pressure anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

HPAEC-PAD is based on the fact that in a strongly alkaline environment carbohydrates will ionize, thereby rendering them amenable to separation on an ion exchange column. High pressure anion exchange chromatography (HPAEC) columns used for carbohydrates are coated with an anion exchange resin (Brummer & Cui, 2005). HPAEC systems typically use sodium hydroxide as the eluent to separate mono- and disaccharides. The detector of choice for HPAEC is the PAD (ØBro et al., 2004; Manderson et al., 2005). In general, amperometry measures the change in current resulting from the oxidation or reduction of a compound at an electrode. In PAD, it is the change in current resulting from carbohydrate oxidation at a gold or platinum electrode that is measured. The advantage of PAD is not only its low detection limits, reportedly in the picomole range, but also its suitability for gradient elution, which provides an analyst with more flexibility when optimizing separation conditions (Brummer & Cui, 2005).

2.6.3. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy can provide detailed structural information on carbohydrates, including identification of monosaccharide composition, elucidation of α- or β-anomeric configurations, establishment of linkage patterns, and sequences of the sugar units in oligosaccharides and/or polysaccharides (Duus et al., 2000). The most useful nuclei in carbohydrate research are ^{1}H and ^{13}C. Every polysaccharide has a unique spectrum in both ^{1}H and ^{13}C NMR spectroscopy. In other words, a NMR spectrum contains all of the structural information about the interested oligosaccharides or polysaccharides. Unfortunately, the signals of carbohydrates in NMR spectra are frequently crowded into a narrow region (3-5 nm), especially for the ^{1}H NMR spectrum. As a result, the interpretation of ^{1}H NMR spectra becomes difficult if a polysaccharide contains many similar sugar residues (Vandevelde and Kiekens, 2004). The most recent development in two and multi-dimensional NMR techniques has significantly improved NMR spectroscopy resolution and sensitivity. For example, homonuclear correlated spectra are extremely useful for assigning ^{1}H resonances while the complete assignment of ^{13}C - resonances is achieved by ^{1}H -^{13}C heteronuclear correlation. Long range correlation techniques, such as nuclear Overhauser enhancement (NOE) and heteronuclear multiple bond correlation (HMBC), are most useful in providing sequence information of polysaccharides (Brisson et al., 2002; Cui, 2005).

2.6.4. Mass spectroscopy

Mass spectroscopy is a technique for determining the masses of electrically charged molecules or particles. Conventional electron impact ionization mass spectroscopy (EI-MS) and chemical ionization (CI) only work for vaporized compounds. Newer ionization techniques, such as fast atom bombardment (FAB), electrospray ionization (ESI), and more recently, matrix-assisted laser desorption ionization (MALDI), have been developed for analysing non-volatile biomolecules, including oligosaccharides and some small MW polysaccharides, proteins, glycoproteins, and glycolipids. These new techniques offer broader analytical versatility, higher sensitivity, and more precise results (Cui, 2005). Conventional ESI MS produces a relatively weak ion signals for native oligosaccharides compared to those for peptides or proteins. Nano ESI produces ion signals that are comparable between the peptide and carbohydrate classes. It, therefore, appears that the hydrophilicity of oligosaccharides limits the surface activity in ESI droplets and that, with small droplets, their sensitivity is significantly enhanced and the ESI of carbohydrates appears to be more effective at the Nano scale (Zaia, 2004).

CONNECTING STATEMENT 1

A literature review on the structural properties of cell wall polysaccharides and their isolation as well as on the enzymatic generation of non-digestible oligosaccharides were presented in Chapter II. Chapter III investigates the isolation of intact rhamnogalacturonan I from potato pulp. Two approaches, including alkaline (NaOH and KOH) and enzymatic (endo-polygalacturonase from *A. niger*) extraction methods, are discussed in terms of yield, monosaccharide profile and MW distribution. Lastly, the structural properties of the three recovered fractions (neutral, weak acidic and strong acidic) by anion exchange chromatography are reported in this chapter.

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CHAPTER III

EXTRACTION AND STRUCTURAL CHARACTERIZATION OF RHAMNOGALACTURONAN I-TYPE PECTIC POLYSACCHARIDES FROM POTATO CELL WALL

3.1. Abstract

Cell wall material from potato pulp by-product was used for the extraction of galactan-rich RG-I type pectic polysaccharides using alkaline (NaOH and KOH) and enzymatic (endopolygalacturonase from *A. niger)* methods. The extraction yield increased as the concentration of alkaline solution was increased from 0.5 M (22-24%) to 2 M (53-56%). The yield of 38% obtained upon the enzymatic treatment was similar to those observed with 1 M alkaline solutions. The results reveal the high debranching of arabinan side chains of RG I as compared to the galactan ones under harsh alkaline conditions. The MW distribution shows that the enzymatic extraction led to the highest proportion of high-MW polysaccharides (>500 kDa; 62.2%). According to monosaccharide pattern, the weak acidic fractions of high alkaline (1-2 M)-based polysaccharide extracts was the most enriched with galactan-rich RG I. Using milder conditions (enzyme and weak alkaline), two RG I populations with low and high linked HG fragments were recovered in the weak and strong acidic fractions, respectively. The structure of galactan-rich RG I was confirmed by H^1 NMR spectroscopy analysis.

3.2. Introduction

There is a high interest in adding more value to the potato pulp by-product, which is a major waste from the potato processing industry. Although many value addition pathways have been investigated, potato pulp is mainly used as an animal feed ingredient and recently as a source of potato fibers. Potato pulp consists of cell wall polysaccharides (70%), starch (12%), water (9%), proteins (5%), ash (4%) and fat (0.3%) (Langner et al., 2009). Pectic polysaccharides, which are the main polysaccharides of potato cell wall (56%), have unique structures. Their main structural characteristic is their high proportion of RG I (75%) and lower proportion of HG (20%). RG I backbone is composed of altering disaccharide α-L-Rha-(1→4)-α-D-GalA, with up to 80% of Rha residues substituted at O-4 with different amounts of arabinan, galactan and arabinogalactan side chains (Naran et al., 2008; Oomen et al., 2002; Vincken et al., 2003). Contrary to the RG I from other cell wall sources (10-24%, w/w), potato RG I is rich in (1→4)-β-galactan side chains (67%) (Oomen et al., 2002; Schols et al., 1990; Yapo, Lerouge et al., 2007). Because of its high content of neutral sugars leading to low gelling properties, potato pectic polysaccharides remain largely unexploited as functional food ingredients. Therefore, the use of pectic polysaccharides from potato pulp as a source of biologically active molecules is of high interest. In this context,

Nangia-Makker et al. (2002) have reported that galactan-rich molecules originating from pectic polysaccharides inhibit tumor growth and metastasis by preventing binding of galectin-3 and its receptors. In addition, in a study on the digestive and metabolic effects of arabinogalactan of RG-I, it has been found that arabinogalactan increased the production of SCFA, stimulated the growth of beneficial microflora and immune system (Kelly, 1999). Potato galactan-rich RG I can also serve as a new source of NDO, which may offer improved prebiotic and other functional properties.

Many chemical methods have been reported for the isolation of pectic polysaccharides from cell wall. Extraction with chelating agents resulted in high-MW pectic polysaccharides, but low yields (Zykwinska et al., 2006). Acid extraction is the most widely used method to obtain commercial pectin; however, it causes neutral side chain degradation (Levigne et al., 2002). Alkaline extraction of pectic polysaccharides releases intact RG I including its neutral side chains through extensive hydrolysis of HG region by β-elimination and oxidative peeling (Zykwinska et al., 2006). In addition to these methods, an enzymatic method, based on the use of endopolygalacturonase, has been investigated to hydrolyze the HG region and open the pectin structure (Øbro et al., 2004; Schols et al., 1995; Yu et al., 2010). So far, no comparative study for the isolation of RG I from potato cell wall has been reported.

The objective of this study was to investigate the extraction of galactan-rich RG I-type pectic polysaccharides from potato cell wall using alkaline and enzymatic methods. The efficiency of the extraction methods, in terms of yield, neutral side chain debranching and sugar composition was assessed. In addition, the extracted galactan-rich RG I were purified by anionic fractionation and their structures were characterized by H^1 NMR spectroscopy.

3.3. Materials and methods

3.3.1. Preparation of potato cell wall material

Potato pulp from Lyckeby Starch AB was used to prepare cell wall according to a modified method of Jardine et al. (2002). Potato pulp (20 g) was soaked in 1 l of pre-cooled (4 °C) salt buffer at pH 5 consisting of sodium acetate (10 mM), KCl (3 mM), MgCl (2 mM) and $CaCl_2$ (1 mM) and filtered through the miracloth. The recovered residues were suspended in 400 ml of saturated phenol-acetic acid-water solution (2:1:1, v/v/v). After 2 h incubation at 25 °C, the residues were recovered by filtration. These steps were repeated three times. The residues were

then suspended in 500 ml of pre-heated buffer at pH 6.5 consisting of KH_2PO_4 (10 mM), $CaCl_2$ (1 mM) and NaN_3 (0.005 %. w/v). In order to gelatinize starch, the buffer was preheated to 95 °C before adding the residue. After gelatinizing for 30 s, thermostable α-amylase (from *Bacillus licheniformis*, Sigma-Aldrich, St-Louis, MO, USA) was added to the suspension at a ratio of 1:10 (v/w). After 15 min at 85 °C, 1 l of buffer was added to the suspension and subsequently amyloglucosidase from *A. niger* (≥300 units/ml, Sigma-Aldrich) was added at a ratio of 1:2.3 (v/w). After 16 h at 40 °C and 150 rpm, the cell wall was recovered by filtration.

3.3.2. Isolation of pectic polysaccharides from potato cell wall material

3.3.2.1. Alkaline treatment

Potato cell wall material (2%, w/v) was suspended in NaOH and KOH alkaline solutions (0.5, 1, or 2 M), containing 0.02 M $NaBH_4$; the mixtures were incubated at 60 °C for 24 h. The supernatants were recovered after centrifugation (18,500 ×g, 10 min) followed by filtration (0.22 µm). The recovered polysaccharide solutions were neutralized and dialyzed (6 - 8 kDa cutoff).

3.3.2.2. Enzymatic treatment

The enzymatic treatment was carried out according to the modified method of Øbro et al. (2004). To remove the methyl and acetyl groups, 0.1 g of cell wall was suspended in 400 ml of weak alkaline solution (0.2 M Na_2CO_3 and 10 mM $NaBH_4$). After 24 h incubation at 4 °C, plant cell wall was recovered by filtration (0.22 µm) and freeze dried. The de-esterified cell wall material (0.2%, w/v) was then suspended in sodium acetate buffer (10 mM, pH 4.0) containing 0.05% NaN_3. Endopolygalacturonase from *A. niger* (2.5 units/µl, Sigma-Aldrich) was then added at a concentration of 40 units/g cell wall. After 24 h incubation at 35 °C and 200 rpm, the suspension was boiled for 5 min and filtered. The filtrate was dialyzed (6- 8 kDa cut-off) at 4 °C.

3.3.3. Sugar Contents

Uronic acid content: Sulphamate/m-hydroxydiphenyl assay was used to determine uronic acid content (Blumenkrantz & Asboe-Hansen, 1973). *Neutral sugar content:* The phenol-sulfuric acid colorimetric assay was used for the determination of neutral sugar content (Dubois et al., 1956).

3.3.4. Fractionation of extracted polysaccharides

The extracted polysaccharides were fractionated by anion exchange chromatography on Source 15Q (2×15 cm, GE Healthcare, Qc, Canada) column equilibrated with sodium acetate buffer (5 mM, pH 5). The loaded samples were eluted at a rate of 1.5 ml/min with an isocratic flow of 5 mM sodium acetate buffer (1CV), followed by a linear gradient from 5 mM to 1.5 M sodium acetate buffer (7CV) and isocratic elution with 1.5 M sodium acetate buffer (1CV).

3.3.5. Determination of the monosaccharide composition

Prior to the determination of monosaccharide composition, pectic polysaccharides were hydrolyzed using a two-step procedure. First, the samples (28-70 mg/ml) were mixed with HCl/methanol mixture (1:4, v/v) at a ratio of 1:5 (v/v) and incubated at 60 °C. After 24 hrs, trifluoroacetic acid was added to the mixtures at a ratio of 1:8 (v/v); the resulted mixtures were boiled for 1 h and neutralized. Monosaccharide composition of the hydrolysates was determined using HPAEC-PAD (Dionex), the Chromeleon Software and a CarboPac PA20 column (3 × 150 mm) set at 32 ^{o}C. Isocratic elution was performed with 5 mM NaOH (0.5 ml/min).

3.3.6. High performance anion exchange chromatography

Quantitative analysis of pectic polysaccharides was carried out using HPAEC, according to a modification of the method of Massiot et al. (1994). A Waters HPLC system (Model 25P, Waters Corp., Milford, MA, USA) equipped with refractive index detector (Model 2414) was used. The separation was performed on a Bio Gel TSK DEAE-5-PW column (2mm × 7.5cm) at a flow rate of 0.5 ml/min, using a linear gradient from 5 to 20 mM sodium acetate (pH 5) for 12 min, followed by an isocratic elution with 20 mM sodium acetate (pH 5) for 4 min.

3.3.7. High pressure size exclusion chromatography (HPSEC)

To estimate the MW distribution of polysaccharides, HPSEC was carried out at 30 °C on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL) using Waters HPLC system. Samples were eluted with water at a flow rate of 0.5 ml/min and the eluate was monitored using a refractive index detector. Dextrans (50 to 270 kDa) were used as standards for calibration.

3.3.8. NMR analysis

H^1 NMR spectroscopy was performed on a Bruker AM 500 spectrometer (Bruker BioSpin Ltd., Milton, ON, Canada). Samples were dissolved in D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) and spectra were collected at frequency of 500 MHz and in 5 mm o.d. tube. Chemical shifts are reported in parts per million (ppm) downfield (δ-units) relative to the signal for free acetate (δ 1.908). A sweep width of 8012 Hz was used. ^1H spectrum is the accumulation of 4 transients with a 45^0 pulse width, acquisition time of 2.0 s and a recycle delay of 1 s.

3.4. Results and discussion

3.4.1. Cell wall material preparation

To isolate the plant cell wall material from potato pulp, starch and protein need to be removed with minimum structural degradation. The results (Table 3.1) show that the starch removal by the enzymatic method combined with thermal gelatinization was efficient resulting in a level less than 1% (w/w) of starch. This level is lower than that (<4.9%) reported by Jardine et al. (2002), who restricted the temperature for gelatinization to 70 °C. The results also confirm the efficiency of phenol-acetic acid-water mixture as protein extractant. The recovery of the cell wall material accounted for 48% (w/w) of the potato pulp. To determine the monosaccharide composition, a two-step method (methanol/HCl; trifluoroacetic acid) was optimized for a complete hydrolysis of cell wall (data not shown). The monosaccharide composition (Table 3.1) reveals that the main sugars of potato pulp and cell wall were Gal (31-32%), GalA (28-44%) and Glc (14-31%). Because of the starch removal, Glc was presumed to be part of cellulose and/or hemicelluloses. The results also show that GalA/Rha molar ratio of potato cell wall (12/1) is lower than that reported for apple (21/1) (Voragen et al., 2001) and citrus peel (31/1) (Yapo et al., 2007) pectin. These results can be attributed to the low proportion of HG in potato pectic polysaccharides as compared to other sources (Coenen et al., 2007). The Gal/Ara ratio of isolated potato cell wall of 6.6 is higher than those (3.0 to 4.9) reported in the literature (Meyer et al., 2009; Øbro et al., 2004; Thomassen et al., 2010). The Gal/Ara ratio confirms the high proportion of galactan side chains as compared to arabinan ones (Øbro et al., 2004; van Marle et al., 1997).

Moreover, the high Gal/Rha ratio (8.9) indicates the highly branching of pectic polysaccharides and the presence of short length RG I backbone stretches.

Table 3.1 Chemical composition of potato pulp and cell wall material.

Factor	Potoato pulp	Cell wall material
- Yield[a]	-	48 (±5.2)%
- Proteins[a]	4.0	<0.1%
- Starch[a]	13.1	<1%
- Monosaccharide composition[b,c]		
GalA	28.0	44.3
Rha	1.9	3.6
Ara	4.6	4.8
Gal	30.9	31.8
Glc	30.9	13.6
Xyl	1.7	1.5
Man	1.9	0.5
GalA/Rha	14.4	12.4
Gal/Rha	15.9	8.9
Gal/Ara	6.7	6.6

[a]Expressed as percentage of dry weight.

[b]Expressed as molar proportion (%).

[c]All measurements were carried as triplicate and relative standard deviations are less than 10%.

3.4.2. Alkaline and enzymatic extraction of RG I-type pectic polysaccharides

To isolate the RG I-type pectic polysaccharides from potato plant cell wall, alkaline and enzymatic treatments were carried out. The results (Table 3.2) show that the yield varies between 22 and 56% of the original cell wall material depending on the treatment. As an overall, the extraction yield increased as the concentration of alkaline solution was increased from 0.5 to 2 M. On the other hand, the yield obtained upon the enzymatic treatment of 38% is similar than those obtained with 1 M NaOH and KOH alkaline solutions. Comparable yield of 40% was reported by Øbro et al. (2004) upon the treatment of potato pulp with endopolygalacturonase. The results also show that the proportion of total neutral sugars increased as the concentration of alkaline solution was increased, whereas the molar proportion of uronic acid decreased. With an increase in the concentration of alkaline solution, the Gal content did not increase, indicating the extraction of other neutral sugar-rich polysaccharides. However, the enzymatic treatment resulted in polysaccharides with the highest ratio of Gal to total neutral sugar contents.

As compared to cell wall material, the high molar ratio of Gal, Ara and Rha with low levels of GalA confirmed the enrichment of the extracts with highly Gal-branched RG I type pectic polysaccharides (Table 3.2). However, pectic polysaccharides obtained upon enzymatic treatment contained slightly higher molar proportion of GalA (29%) than those recovered after alkaline treatments (10-24%). These results suggest that the HG backbone in potato cell wall was not completely degraded by polygalacturonase. As expected, the molar proportion of GalA decreased with increasing the concentration of alkaline solution, indicating the efficiency of β-elimination and oxidative peeling of HG under sever alkaline conditions. However, both smooth and strong alkaline treatments led to polysaccharide extracts with high molar proportions of Ara (12-15%) and Gal (53-67%). These results reveal the high mobility of arabinan and galactan side chains in potato cell wall. Similarly, Zykwinska et al. (2006) reported that arabinan and galactan chains in potato cell wall are loosely associated to cellulose microfibrils.

As compared to other monosaccharides, Glc, Xyl and Man represent lower molar proportions in the extracted pectic polysaccharides (Table 3.2). The enzymatic treatment resulted in the lowest Glc (0.7%), Xyl (0.2%) and Man (0.2%) molar proportions as compared to the alkaline treatments (0.2 - 6.5%).Increasing the concentration of alkaline solutions increased the molar proportion of Glc and Xyl. Hemicellulosic polysaccharides, such as xyloglucan, heteromannans and heteroxylans, are possible sources of these sugars.

Table 3.2 Alkaline and enzymatic extractions of pectic polysaccharides from potato cell wall [a]

Treatment	Yield (%)[b]	Molar proportion of sugars (%)		Monosaccharide composition (mol%)							GalA /Rha	Gal/ Ara	Gal/ GalA
		Neutral sugars	Uronic acid	Rha	Ara	Gal	Glc	Xyl	Man	GalA			
NaOH													
0.5 M	24.4	82.3	17.7	3.1	13.1	67.4	1.3	0.6	1.2	13.3	4.3	5.16	5.05
1 M	40.7	81.9	18.1	3.5	11.8	52.8	2.7	2.3	3.1	23.7	6.84	4.47	2.23
2 M	53.6	90.3	9.7	3.1	13.6	60.0	6.5	5.3	2.0	9.7	2.99	4.41	6.38
KOH													
0.5 M	22.2	77.9	22.1	2.9	12.9	62.6	1.8	1.1	1.6	17.1	5.83	4.84	3.66
1 M	34.8	77.8	22.2	3.2	14.0	57.6	1.6	0.8	2.2	20.6	6.49	4.11	2.8
2 M	55.7	88.5	11.5	5.1	15.5	52.6	5.2	3.0	2.2	16.4	3.24	3.39	3.2
Enzyme	37.9	61.7	38.3	3.5	11.2	55.0	0.7	0.2	0.2	29.2	8.35	4.92	1.88
Cell wall		55.7	44.3	3.6	4.8	31.8	13.6	1.5	0.5	44.3	12.43	6.63	0.72

[a] All measurements were run as triplicate and the relative standard deviations are less than 12%.

[b] Yield is expressed as the weight percentage of the extract.

The polysaccharide extracts obtained upon alkaline and enzymatic treatments showed lower GalA/Rha ratios (3.0-8.4) and higher Gal/GalA (1.9-6.4) as compared to the initial plant cell wall (12.4; 0.7). However, as compared to the alkaline treatments (3.2-6.8; 2.8-6.4), the enzymatic treatment resulted in higher GalA/Rha and lower Gal/GalA molar ratios of 8.4 and 1.9, respectively. Øbro et al. (2004) have reported lower GalA/Rha molar ratio of 3.2 for the potato RG I preparation obtained upon enzymatic treatment. The high GalA/Rha ratios may suggest the presence of GalA oligomeric units in the RG I obtained upon enzymatic treatment. It has been reported that *A. niger* endopolygacturonase has low catalytic efficiency for the cleavage of GalA oligomers that are smaller than tetramer (Øbro et al., 2004). Gal/Ara ratio (4.9) obtained upon the enzymatic treatment is comparable to that (3.4) reported by Øbro et al. (2004).

3.4.3. MW distribution of extracted pectic polysaccharides

To determine the MW distributions, the extracted pectic polysaccharides were characterized by size exclusion chromatography. HPSEC elution patterns showed three main MW populations of <50, 100-500 and >500 kDa (Table 3.3). The presence of high-MW population may be due to the extraction of RG I cross-linked with RG II and remaining HG (Ridley et al., 2001). In addition to these covalent interactions, covalent interactions of RG I polysaccharide with xyloglucan and cellulose have been reported (van Marle et al., 1997). The results show that the MW distribution of extract was dependent on the type of alkaline solution. Lower proportions of high-MW polysaccharide population (>500 kDa) and high proportions of low-MW polysaccharide population (<50 kDa) were obtained with NaOH alkaline solutions as compared to KOH ones. These results suggest the high degradation rate of polysaccharides in the presence of NaOH solution. As compared to alkaline extractions, enzymatic extraction led to the highest proportion of high-MW polysaccharide population (>500 kDa; 62.2%). Øbro et al. (2004) have fractionated pectic polysaccharide extracted with endopolygalacturonase into three pools including pool I (50–>500 kDa), pool II (12–50 kDa) and pool III (0.2–12 kDa). They have suggested the high-MW polysaccharide population (50->500 kDa) to correspond to RG I region of pectic polysaccharides.

Table 3.3 Molecular weight distribution of extracted pectic polysaccharides from potato cell wall material.

Treatment	Molecular weight range (kDa)		
	<50	50-500	>500
NaOH			
0.5 M	28.1	32.0	39.9
1 M	31.3	32.3	36.4
2 M	25.5	37.0	37.5
KOH			
0.5 M	24.0	31.3	44.7
1 M	20.0	26.4	53.6
2 M	18.5	40.5	41.0
Enzyme	22.1	17.3	62.2

Meyer et al. (2009) have reported four populations (710 kDa, 99 kDa, 14 kDa, and 911 Da) for the potato fiber obtained after treatment of pulp with Viscozyme® L with very high-MW weight one being identified as Gal-branched-RGI (~ MW of 710 kDa).

3.4.4. Fractionation of extracted polysaccharides

The extracted pectic polysaccharides were fractionated by anion exchange chromatography (Figure 3.1). Contrary to other extracts, the elution profiles of pectic polysaccharides, obtained with 2 M KOH and NaOH, showed major neutral polysaccharide fraction eluted at 5 mM sodium acetate buffer (Figure 3.1d and 3.1e). However, using 1 M KOH and NaOH, the more abundant fractions were weak and strong acidic ones eluted at 0.2-0.4 M and 0.4-0.5 M sodium acetate buffer, respectively (Figure 3.1a and 3.1b). Contrary to the alkaline extractions, the enzymatic extraction (Figure 3.1c) resulted in only weak and strong acidic polysaccharide fractions. The relatively high numbers of fractions obtained upon alkaline treatments may be due to the defragmentation of pectic polysaccharides.

The neutral, weak acidic and strong acidic populations were pooled and characterized for their monosaccharide composition (Table 3.4). Using harsh alkaline concentrations (1-2 M), the main population of polysaccharides was the weak acidic one (55-87%). On the other hand, the soft alkaline and enzymatic-based polysaccharide extracts showed a high strong acidic population (60-70%). The results also indicate that neutral fractions of pectic polysaccharides, obtained with 0.5 M of KOH and NaOH, were mainly composed of Gal (60-63%). However, the relative molar distribution of Gal between the three populations indicates the low recovery of Gal (5-14%) in the neutral fractions, revealing the low debranching of galactan side chains under alkaline conditions. As compared to Gal, Ara sugar exhibited higher molar distribution in the neutral fractions (8-27%) of alkaline-based polysaccharide extracts. These results reveal that arabinan side chains are more susceptible to the degradation by alkaline conditions than galactan ones. As overall, the molar distributions of Gal and Ara in the neutral fractions obtained upon extraction with strong NaOH (1-2 M) solutions are higher than those recovered with KOH ones. These results suggest that KOH may have led to less degradation of arabinan and galactan side chains.

As compared to other populations, higher relative molar distributions of hemicellulosic sugars (Glc, Xyl, Man; 64-82%) were detected in the neutral fraction of pectic polysaccharide extracts recovered using strong alkaline solutions, 2 M NaOH and KOH.

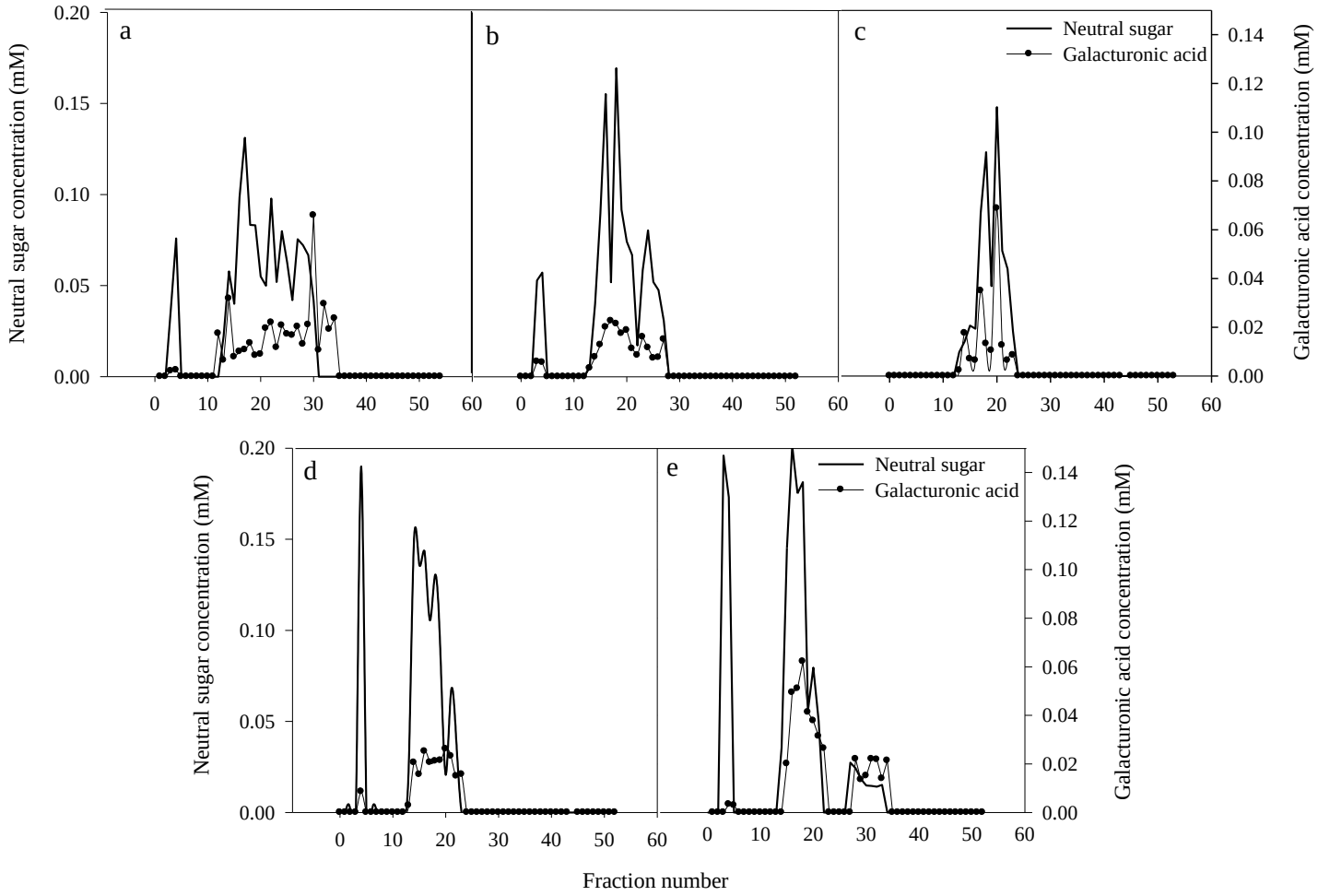

Figure 3.1 Anionic exchange chromatography profiles, on source 15Q column, of polysaccharide extracts obtained upon alkaline and enzymatic treatments: (a) 1 M KOH , (b) 1 M NaOH, (c) enzyme , (d) 2 M KOH and (e) 2 M NaOH.

Table 3.4 Monosaccharide composition of polysaccharide fractions recovered upon anionic exchange chromatography.

Treatment [a]	Fraction	Total sugar (%)	Monosaccharide composition (%)														Gal/GalA
			Rha		Ara		Gal		Glc		Xyl		Man		Gal A		
NaOH																	
0.5 M	Neutral	5.0	0.0	(0.0) [b]	19.5	(8.3)	63.6	(5.3)	4.7	(19.8)	1.7	(16.1)	5.5	(26.2)	5.0	(2.1)	12.64
	Weak acidic	25.3	2.4	(21.3)	14.8	(30.7)	67.3	(27.1)	2.6	(54.4)	1.1	(51.0)	2.3	(53.4)	9.4	(19.1)	7.16
	Strong acidic	69.6	3.6	(78.7)	11.8	(61.0)	67.8	(67.7)	0.5	(25.8)	0.3	(32.8)	0.4	(20.5)	15.6	(78.8)	4.34
1 M	Neutral	8.3	1.4	(3.2)	11.6	(8.0)	37.2	(5.7)	15.9	(47.2)	8.1	(28.0)	5.0	(13.0)	20.9	(7.2)	1.78
	Weak acidic	76.0	3.8	(81.0)	12.4	(78.2)	58.8	(82.6)	1.4	(38.8)	1.5	(48.8)	0.7	(17.9)	21.3	(66.7)	2.76
	Strong acidic	15.7	3.1	(15.8)	9.3	(13.8)	35.0	(11.6)	2.2	(14.0)	3.1	(23.2)	12.1	(69.1)	35.2	(26.1)	0.99
2 M	Neutral	24.9	0.0	(0.0)	15.7	(27.1)	36.4	(14.2)	22.1	(80.0)	18.4	(81.7)	6.5	(75.3)	0.9	(2.3)	39.93
	Weak acidic	66.0	4.1	(92.7)	13.4	(70.5)	69.7	(83.0)	1.4	(16.0)	0.9	(12.7)	0.7	(24.7)	9.7	(73.6)	7.19
	Strong acidic	9.1	4.5	(7.3)	6.6	(2.4)	32.9	(2.8)	5.2	(4.1)	5.9	(5.6)	0.0	(0.0)	44.9	(24.1)	0.73
KOH																	
0.5 M	Neutral	8.1	0.0	(0.0)	21.4	(15.0)	60.3	(8.7)	4.8	(24.0)	1.8	(15.6)	1.2	(6.6)	10.5	(5.5)	5.74
	Weak acidic	32.2	2.3	(32.9)	12.4	(40.1)	73.3	(48.8)	1.0	(23.8)	0.5	(21.3)	0.4	(11.1)	9.9	(24.2)	7.38
	Strong acidic	59.7	4.0	(67.1)	11.8	(45.0)	54.0	(42.5)	1.9	(52.2)	1.4	(63.1)	2.6	(82.3)	24.4	(70.3)	2.21
1 M	Neutral	4.2	19.5	(26.9)	0.0	(0.0)	62.7	(4.8)	3.1	(8.3)	1.5	(8.1)	3.2	(6.5)	9.9	(2.1)	6.31
	Weak acidic	54.9	2.0	(37.5)	14.4	(60.5)	60.1	(61.2)	0.9	(31.4)	1.0	(73.3)	3.0	(80.0)	18.5	(52.8)	3.24
	Strong acidic	40.9	3.1	(35.6)	15.0	(39.5)	53.0	(34.0)	2.7	(60.3)	0.4	(18.7)	0.8	(13.5)	25.1	(45.1)	2.11
2 M	Neutral	12.8	0.3	(0.6)	18.6	(13.6)	35.3	(7.6)	7.4	(16.0)	16.8	(63.9)	12.5	(64.1)	9.1	(6.3)	3.87
	Weak acidic	87.2	5.7	(99.4)	15.1	(86.4)	54.8	(92.4)	5.0	(84.0)	1.2	(36.1)	0.9	(35.9)	17.4	(93.7)	3.16
Enzyme	Weak acidic	34.5	2.2	(21.5)	13.2	(40.8)	73.2	(46.0)	0.3	(16.5)	0.1	(16.7)	0.1	(21.8)	10.8	(12.8)	6.78
	Strong acidic	65.5	4.2	(78.5)	10.1	(59.2)	45.4	(54.0)	0.9	(83.5)	0.3	(83.3)	0.2	(78.2)	38.9	(87.2)	1.17

[a]All measurements were run in triplicate and the relative standard deviations are less than 10%.

[b]Relative molar distribution of each monosaccharide between the neutral, weak acidic and strong acidic fractions.

The high molar proportion of Glc (22%) and Xyl (18%) combined with low level of GalA (0.9), found in the neutral fraction of high alkaline NaOH-based polysaccharide extract, suggest that the major part of the hemicellulose polysaccharides is made of xyloglucan and xylans. Xyloglucan consists of β-(1-4) linked Glc residues to which Xyl residues are attached to O-6 of Glc residues; while xylan are mainly made of β-(1-4) linked Xyl residues substituted at positions C2 or C3 with 4-O-arabinofuranosyl (Ebringerová, 2005). On the other hand, because of the relatively high molar proportion of Xyl (17%), Man (12%) and GalA (9%) combined with low level of Glc (7%) detected in the neutral fraction of high alkaline KOH-based polysaccharide extract, the extracted hemicelluloses are presumed to be mainly xylogalacturonan and heteromannan. In addition, Glc and Xyl were also detected in weak and strong acidic fractions, which suggest the presence of linkage between RG-I and xyloglucan in potato. The linkages between xyloglucan/HG and xyloglucan/RG I have been reported for pectins from soybean and arabidoopsis (Mohnen, 2008).

According to the monosaccharide composition (Rha, 37-99%; Gal, 60-86%; Ara, 61-92%), galactan-rich RG I was presumed to be mainly present in the weak acidic populations of high alkaline (1-2 M)-based extracts. In contrast, the strong acidic population of low alkaline NaOH-based polysaccharide extract showed the highest relative molar distribution levels of Rha (78%), Gal (68%) and Ara (61%). The enzymatic and low alkaline KOH-based extracts exhibited more or less comparable relative Gal and Ara monosaccharide distributions in the weak and strong acidic populations. These reveal the presence of two RG-I populations with high and low linked HG fragments when extraction was performed at milder conditions (enzyme and weak alkaline).

3.4.5. NMR analysis

To characterize the structure of pectic polysaccharides obtained upon enzymatic treatment, ^{1}H NMR spectroscopy analysis of the two isolated enzymatic-based RG-I populations (weak and strong acidic fractions) was performed (Table 3.5). Galactan from potato and arabinan from sugar beet were used as standard for the NMR analysis. The signal at δ 4.68 and 4.65 ppm were assigned to the anomeric protons of 1→3 and 1→4 linked β-D-Gal in the galactan standard, respectively (Bushneva et al., 2002; Habibi et al., 2004). In addition, chemical shifts for 1→2, 1→3 and 1→5 linked α-L- Ara were identified and detected in the arabinan standard (Bushneva et al., 2002; Habibi et al., 2004).

Table 3.5 ^{1}H chemical shifts (ppm) of standards and separated fractions determined at 500 MHz.

Samples	Linkage	H1	H2	H3	H4	H5	H6
Arabinan standard	→2,3,5)-α-Ara-(1→	5.13	4.27	3.99	4.03	3.67	
	→5)-α-Ara-(1→	5.04	4.08	4.03	4.18	3.87	
	→3,5)-α-Ara-(1→	5.09	4.24	4.031	4.24	3.71	
	→2,5)-α-Ara-(1→	4.94	4.27	4.27	4.18	3.86	
Galactan standard	→4)-β-Gal-(1→	4.65	3.67	3.75	4.007	3.73	
	→ 3)β-Gal-(1→	4.68	3.75	3.91	4.46	3.73	3.75
Separated fractions	→2,5)-α-Ara-(1→	4.96	4.28	4.29	4.23	3.87	
	→4)-β-Gal-(1→	4.65	3.67	3.75	4.009	3.73	
	→ 3)β-Gal-(1→	4.69	3.75	3.92	4.45	3.73	3.75
	Rha in →4)-α-GalA(1→2)-α-L-Rha-(1→	5.16	4.06	3.87	3.34	3.78	
	GalA in →4)-α-GalA(1→2)-α-L-Rha-(1→	4.92	3.97	3.96	4.44	4.64	

In ^{1}H NMR spectra of isolated polysaccharides, the signal at δ 4.92 was most intensive in the anomeric region and was assigned to the anomeric protons of α-(1→4) linked D-GalA residues in alternating Rha and GalA structure of RG I backbone. Proton signals at δ 3.97, 3.96, 4.44 and 4.64 ppm were assigned to H-2, H-3, H-4 and H-5 of α-D-GalA. These signals were present in the two isolated fractions, but they were more intensive in the strong acidic fraction, indicating that GalA detected was mainly originated from the RG I backbone rather than the HG structure (Habibi et al., 2004). The ^{1}H NMR spectra of isolated polysaccharides also show the resonance arising from H-1 of β-D Gal residue. Signals at 4.65 and 4.68 assigned to 1→4 and 1→3 linked β-D- Gal residues, respectively, may reveal the presence of two types of arabinogalactan (Bushneva et al., 2002). The presence of two types of arabinogalactans linked to RG I has been previously reported (Habibi et al., 2004). β-D-(1→4)Gal originates from type I arabinogalactan and the presence of β-D-(1→3)Gal showed extraction of arabinogalactan type II together with other polysaccharides. The signal corresponding to β-D-(1→6)Gal wasn't detected, revealing the low quantity of this linkage. The presence of α-L-(1→5)Ara in the extracted polysaccharides was confirmed by the presence of signal at δ 4.92, which was assigned to the anomeric protons of Ara residues in the arabinan side chains; signals at 3.97, 3.96, 4.44 and 4.64 ppm were assigned to ring protons H-2, H-3, H-4 and H-5 of α-L-Ara (Habibi et al., 2004). Chemical shifts distinctive for 1→2 and 1→3 linked Ara weren't detected in any fraction, which is due to the small quantity of α-L-(1→3)Ara, α-L-(1→2)Ara linked to arabinan backbone (Bushneva et al., 2002).

3.5. Conclusion

RG-I-type pectic polysaccharides were successfully extracted from potato cell wall by alkaline and enzymatic treatments. KOH treatments have led to less degradation of arabinan and galactan side chains as compared to NaOH ones. In addition, the debranching of arabinan side chains was more significant as compared to galactan ones under harsh alkaline conditions. The MW distribution shows that the enzymatic treatment led to the recovery of intact RG-I. The NMR results confirmed the structure of galactan-rich RG-I.

CONNECTING STATEMENT 2

The efficiency of alkaline and enzymatic methods for the extraction of intact galactan-rich RG I was previously investigated and compared (Chapter III). In Chapter IV, the optimization of the enzymatic (endo-polygalacturonase from *A. niger*) extraction of the potato galactan-rich RG I polysaccharides by response surface methodology (RSM) using CCRD with three variables at five levels is studied. The effects of selected extraction parameters (cell wall concentration, enzyme amount and reaction time) on responses (yield, neutral sugar content, monosaccharide profile and weak acidic fraction proportion) and their interactions are discussed. Models were developed to relate reaction parameters to responses, and the conditions resulting in highest yield and content of Gal are identified. The analysis of glycosidic linkages of extracted polysaccharides using gas chromatography with mass spectrometry detector is also described.

The results of this chapter were presented at the *12th Annual Meeting of Institute of Food Technologists* and published in the journal of *LWT – Food Science and Technology*.

Khodaei, N., & Karboune, S. (2012) Isolation, optimization and structural characterization of rhamnogalacturonan I from potato cell wall. IFT *12th Annual Meeting of Institute of Food Technologists, Las Vegas, USA.*

Khodaei, N., & Karboune, S. (2014). Enzymatic extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product. *LWT-Food Science and Technology, 57*(1), 207-216.

CHAPTER IV

ENZYMATIC EXTRACTION OF GALACTAN-RICH RHAMNOGALACTURONAN I FROM POTATO CELL WALL BY-PRODUCT

4.1. Abstract

Potato cell wall was used as low-value source for the enzymatic extraction of galactan-rich RG I. The effects of selected reaction parameters of endo-polygalacturonase from *A. niger*-catalyzed isolation of RG I and their interactions were investigated by response surface methodology. Models were developed to relate independent parameters (cell wall concentration, enzyme amount, and reaction time) to responses (yield, neutral sugar content, saccharide molar composition, weak acidic fraction proportion). The most significant parameters that affected extracted polysaccharide yield and its Gal and Ara contents were the cell wall concentration and enzyme amount. The interaction between the cell wall concentration and the reaction time was the most determinant for the yield. However, the cell wall concentration and the enzyme amount exhibited significant interaction effect on Gal and Ara contents. Comparison of predicted and experimental values validated the established predicted models, which can be used to identify the conditions for the isolation of RG I-type pectic polysaccharides with selected structural and saccharide composition properties. The monosaccharide composition and the linkage patterns confirmed the isolation of galactan-rich RG I type pectic polysaccharides. The present study is expected to increase the capability to generate RG I targeting specific composition and functional properties.

4.2. Introduction

Potato starch industry releases large quantities of low-value pulp by-product, which is mainly used as cattle feed (Klingspohn et al., 1993). Major components of potato pulp are cell wall polysaccharides (70%, g/100g) (Langner et al., 2009). Unlike pectin from other sources, pectic polysaccharides obtained from potato cell wall (56%, g/100g) consist of high proportion of RG I (75%) and low amount of HG (20%) (Mohnen et al., 2008; Oomen et al., 2003). Although potato pectic polysaccharides have low gelling properties, their Gal-rich structural properties make them a potential source of bioactive molecules (Øbro et al., 2004; Oomen et al., 2002). Indeed, 67% (g/100g) of potato RG I consists of β-linked galactan side chains; while only 10-24% of RG I from other sources are galactan side chains (Oomen et al., 2002; Yapo et al., 2007). Olesen et al. (1998) have shown that enzymatically solubilized potato fibers were fermentable and their consumption increased end-expiratory of H_2. In particular, the fermentability of arabinogalactan of RG-I has been linked to an increase in the beneficial microflora and to the production of

SCFA (Kelly, 1999). Moreover, β-(1,4)-galactan rich potato fibers with high molecular weights, obtained upon enzymatic treatment of pulp, have shown higher bifidogenic activity than FOS (Thomassen et al., 2011). Soluble potato fibers were also reported to decrease weight gain in rats (Lærke et al., 2007). In addition, metabolic studies of galactan-rich molecules originating from pectic polysaccharides have highlighted their health beneficial effects in stimulating immune system and preventing metastasis (Kelly, 1999; Nangia-Makker et al., 2002).

The isolation of pectic polysaccharides from potato cell wall with a minimum structural degradation is particularly challenging because of the existence of linkages between RG I and xyloglucan, xylogalacturonan and cellulose microfibrils (Popper and Fry, 2008; Zykwinska et al., 2006; Zykwinska et al., 2005). In addition, because of the presence of intermolecular bonds between polyvalent ions and carboxylic group of polygalacturonic acid, the pectin network is entrapped into plant cell wall and needs to be disrupted to enable extraction of RG I. Acid extraction method is the most common method for the industrial extraction of pectic polysaccharides and results in high extraction yields; however it causes extensive degradation of neutral side chains (Levigne et al., 2002). In our previous study (Khodaei and Karboune, 2013), alkaline (NaOH, KOH) and enzymatic (endo-polygalacturonase from *A. niger*) extraction treatments have been investigated for the isolation of pectic polysaccharides from potato pulp. Both treatments resulted in extracts with high molar ratio of Gal, Ara and Rha and low levels of GalA, which confirmed the extraction of highly galactan-rich RG I-type pectic polysaccharides. However, alkaline extraction resulted in the debranching of galactan and arabinan side chains. Enzymatic treatment was identified in our previous study (Khodaei and Karboune, 2013) and in others (Byg et al., 2012; Thomassen, et al., 2011) as the most appropriate method to extract intact RG I with low neutral side chain degradation. Indeed, endo-polygalacturonase catalyzes the cleavage of α-(1-4)-D-GalA linkages of HG backbone consisting of four GalA units and more (Pařenicová et al., 2000). The efficient removal of HG backbone resulted in the opening of pectin network and the isolation of RG I region (Byg, et al., 2012; Khodaei & Karboune, 2013; Mort et al., 2008).

The functional properties of potato RG I-type pectic polysaccharides, as they are related to theirs structures, haven't been studied. However, it has been shown that the functional properties of polysaccharides with structures comparable to RG I, soluble soybean polysaccharides (Nakamura et al., 2001), were dependent on their structures and their anionic character

(Nakamura et al., 2007; Fafaungwithayakul et al., 2011). Considering that pectin subunits can show different functional properties due to the variation in their molecular weight, monosaccharide composition and anionic character, understanding the relationships between the parameters of enzymatic isolation and the structural properties of RG I is of high interest. As part of ongoing work, the objective of this study was the investigation of the interactive effects of selected parameters of enzymatic extraction of RG I-type pectic polysaccharides from potato cell wall on the yield, the saccharide profiles, the debranching extent of the side chains and the proportion of weak acidic fraction, using RSM.

4.3. Materials and methods

4.3.1. Preparation of potato cell wall material

Cell wall material was isolated from potato pulp (Lyckeby Starch AB) as previously described (Khodaei & Karboune, 2013). To remove protein, potato pulp was washed with phenol-acetic acid-water solution (2:1:1). Starch was gelatinized in buffer (10 mM phosphate buffer, 1 mM calcium chloride, pH 6.5) at 95 °C for 30 s and the resulted suspension was incubated with α-amylase from *B. licheniformis* (Sigma-Aldrich) at a ratio of 1 ml for 10 g of substrate. After 15 min at 85 °C, amyloglucosidase from *A. niger* (≥300 units/ ml, Sigma-Aldrich) was added at a ratio of 1 ml for 2.3 g of substrate. After 16 h at 40 °C, cell wall material was recovered by filtration and freeze dried.

4.3.2. Enzymatic assays

The levels of endo-polygalacturonase, galactanase and arabinanase activities present in endo-polygalacturonase product from *A. niger* (E-PGALS, Megazyme) were measured using orange polygalacturonic acid, potato pectic galactan and sugar beet arabinan as substrates, respectively. The reaction mixture (1 ml), consisting of 0.5 ml of substrate solution, 0.45 ml of buffer (0.05 mM sodium acetate buffer, pH 4.0) and 0.05 ml of appropriately diluted enzyme solution, was incubated at 35 °C for 20 min. The hydrolysis reaction was halted by the addition of 1.5 ml of dinitrosalicylate (DNS) reagent (1.0% (w/v) DNS and 1.6% (w/v) NaOH). The mixture was placed in a boiling bath for 5 min to develop the reducing sugar assay color. 0.5 ml of potassium sodium tartrate solution (50%, w/v) was then added to the mixture prior to cooling. The absorbance of the resulting mixture was read at 540 nm against blank. Standard curves, were constructed using GalA, Gal and Ara as standards (0.5-10 mM). One unit of activity was defined

as the amount of enzyme which releases one µmole of the corresponding glycoside equivalent per min per ml of enzyme.

4.3.3. Enzymatic extraction of pectic polysaccharides from cell wall

The enzymatic extraction of pectic polysaccharides was carried out according to the modified method of Øbro et al. (2004). Enzymatic extraction was initiated by adding endo-polygalacturonase from *A. niger* (5.2 – 5.5 units/µl at pH 4.0 and 35°C). Extraction of polysaccharides was performed using 80 units of enzyme per gram of de-esterified cell wall material suspended in sodium acetate buffer (10 mM, pH 4.0) containing 0.05g per 100 ml sodium azide. After incubation at 35 °C under 200 rpm agitation for selected reaction times, the filtrates containing soluble pectic polysaccharides were recovered and dialyzed against water at 4 °C using tubes with a 6-8 kDa cut-off. The recovered pectic polysaccharides were analyzed for their sugar (neutral and uronic) contents, their monosaccharide composition and their MW distribution.

4.3.4. Sugar content and monosaccharide composition analyses

Total uronic acid content was measured, according to Blumenkrantz and Asboe-Hansen (1973) method, using GalA (0.02 - 0.5 mM) as a standard. Total sugar content was assessed using the phenol-sulfuric acid colorimetric assay (Dubois et al., 1956) using Gal (0.1 - 1 mM) as a standard. To determine the monosaccharide composition, pectic polysaccharides were first hydrolyzed. HCl/methanol mixture (1:4, ml:ml) were added to polysaccharide sample (3-55 mg/ ml) at a ratio of 1:5 (ml:ml). After incubation for 24 h at 60 °C, trifluoroacetic acid was added at a ratio of 1:8 (ml:ml). Monosaccharide composition was determined using HPAEC-PAD (Dionex) and a Carbo Pac PA20 column (3 × 150 mm) set at 32 ^{o}C. Isocratic elution was performed with 5 mM NaOH (0.5 ml/min). L-Rha (2.5 - 20 µmol/l), L-Ara (2.5 - 20 µmol/l), D-Gal (25 - 200 µmol/l), D-Glc (2.5 - 20 µmol/l), D-Xyl (2.5 - 20 µmol/l) and D-Man (2.5 - 20 µmol/l) were used as standards.

4.3.5. Anionic exchange chromatography

To estimate the yield of extracted RG I, HPAEC was carried on Bio Gel TSK DEAE-5-PW column (2 mm × 7.5cm) using a Waters HPLC system (Model 25P, Waters Corp., Milford, MA) equipped with refractive index detector (Model 2414). Injections were conducted at 30 °C with a

flow rate of 0.5 ml/min. Linear gradient from 5 to 20 mM sodium acetate (pH 5) for 12 min, followed by an isocratic elution with 20 mM sodium acetate (pH 5) for 4 min were applied. Amount of RG I was estimated using a Breeze software using soybean RG I (Megazyme) as standards. Yield (%, g of extract per 100 g of substrate) was calculated as the amount of extracted RG I over the initial amount of cell wall material, multiplied by 100.

The quantification of the proportions of weak and strong acidic fractions of extracted pectic polysaccharides was assayed by anion exchange chromatography on HiTrap ™ DEAE FF column (1 ml, GE HealthCare) at a flow rate of 1 ml/min. For quantitative fractionation of pectic polysaccharides, Source 15Q (2×15 cm, GE Healthcare) column was used on a FPLC system, equipped with a LKB Controller LCC 501 at a flow rate of 1.5 ml/min. The elution for both analytic and quantitative fractionations was carried out with three successive isocratic gradients: 5 mM (1 CV), followed by 0.3 mol/l (7 CV) and subsequently 0.6 mol/l (9 CV) sodium acetate buffers at pH 5.0. Weak acidic and strong acidic fractions correspond to extracted polysaccharides eluted with 0.3 and 0.6 mol/l sodium acetate buffers, respectively. Fractions were analyzed for their total sugar content using phenol-sulfuric assay.

4.3.6. Experimental design

RSM was used to study the effects of selected reaction parameters and to maximize extraction yield of pectic polysaccharides and their neutral sugar contents. Central composite rotatable design (CCRD) with three variables at five levels was used. The independent variables and their levels were reaction time (3, 12, 30, 48, 57 h), enzyme amount (40, 80, 160, 240, 280 units/g cell wall) and cell wall concentration (0.3, 1.3, 3.1, 5.0, 5.9 mg/ ml buffer). Reaction volume was 40 ml. A total of 28 runs were performed including two replicates in factorial points (levels ± 1), axial points (levels ± α of 1.5), and six replicates in centre point. Replicates in centre points were performed to obtain pure error. In order to avoid systematic errors experiments were done in random order.

4.3.7. Statistical analysis

The analysis of variance (ANOVA) was applied to validate the model and to analyze statistical significance of data. The regression coefficients for linear, quadratic and interaction terms were determined and used to generate response surface equations using the software Design-Expert 8.0.2 (Stat-Ease, Inc., Minneapolis, MN, USA). Regression analyses were fitted

into the empirical quadratic polynomial equation (Eq. (4.1)). These equations were used to predict responses (yield, neutral sugar content, Gal, Ara, Rha, GalA contents and weak acidic fraction proportion) as function of the independent variables.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (4.1)$$

Where Y is the predicted response, β_0 the constant term, β_i the coefficient for the linear effect, β_{ij} the coefficient for the interaction effect, β_{ii} the coefficient for the quadratic effect, and X_i and X_j are the coded level of the independent variables. The contour plots were obtained using the fitted model by keeping the least effective independent variable at a constant value and changing the other two independent variables. In order to examine the overall shape of the curve and to determine whether the estimated stationary point is a maximum, a minimum, or a saddle point, canonical analysis was performed using SAS software version 9.3.

4.3.8. High performance size exclusion chromatography (HPSEC)

To determine the MW distribution, the pectic polysaccharides were analyzed by HPSEC on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL from Tosoh Bioscience, Montgomeryville, PA) using Waters HPLC system with refractive index detector. The isocratic elution was carried with water at a flow rate of 0.5 ml/min and 30 °C. Dextrans with MW of 50, 150, 270, 410 and 670 kDa were used as standards for calibration.

4.3.9. Analysis of glycosidic linkages by gas chromatography

In order to characterize glycosidic linkages of polysaccharides, carbodiimide-activated reduction, with sodium borodeuteride, followed by methylation, with methyl iodide, and acetylation, with acetic anhydride, were carried out using the modified method of Pettolino et al. (2012). Gas chromatography analysis was carried out using Varian CP-3800 GC system equipped with Varian Saturn 2000 mass spectrometry detector (Varian, Walnut Creek, CA). DB-5MS column (5% diphenyl- and 95% dimethyl-polysiloxane, 50 m x 0.2 m, 33 μm thickness, J&W Scientific, On, Canada) was used with helium as carrier gas at 1.5 ml/min. The GC oven was first set to an initial temperature of -5 °C during the first 5 min using CO_2 and then ramped at 50 °C/min to 150 °C and then ramped at 5 °C/min to 285 °C, which was hold for 20 min. The MS detector used was an ion-trap mass spectrometer. The MS transfer-line, the manifold and the

ion-trap temperatures were set at 250, 50 and 175°C, respectively. The ionization voltage of 70 eV was used, and the electron multiplier voltage was set at 1500 V.

4.4. Results and discussion

RSM approach was used in order to investigate the effects of selected parameters and their interactions on the yield, the neutral sugar content, the monosaccharide composition and the proportion of weak acidic fraction of extracted pectic polysaccharides from potato cell wall. The experimental design was performed based on the CCRD with three variables at five levels: enzyme amount (40-280 units/g cell wall), reaction time (3-57 h) and cell wall concentration (0.3-5.9 mg cell wall/ml). The levels of these selected parameters were set based on preliminary trials. Higher cell wall concentration of 10 mg/ml (1%, w/v) and enzyme amount (1g/100g substrate) were used in previous studies (Byg, et al., 2012; Thomassen, et al., 2011). However, our preliminary results have shown that above 6 mg/ml, the effect of cell wall concentration was not significant, and the amounts of enzyme used in this study did not exceed 0.01g/100g substrate).

4.4.1. Yield and neutral sugar content of extracted polysaccharides

Table 4.1 shows the experimental conditions and their corresponding responses for yield and neutral sugar content. Contrary to other studies (Byg, et al., 2012; Thomassen, et al., 2011) in which the yield was estimated as the released dry matter (g/100g substrate), yield of extracted polysaccharides (%) was quantified by HPAEC. Treatment n^{o}21 (160 units of enzyme/g cell wall; 30 h of reaction time; cell wall concentration of 0.3 mg/ml buffer) resulted in the highest extraction yield of extracted polysaccharides (69.3%) with relatively low neutral sugar content (1,638.5 µmol/g extract). This high yield at high enzyme to polysaccharide ratio may be due to the high accessibility of endo-polygalacturonase to the HG region, resulting in a significant opening of pectin network and the release of RG I polysaccharides. However, the highest neutral sugar content (4,369.6 µmol/g extract) of extracted polysaccharides was achieved with low yield (9.1%) upon the enzymatic treatment with the same enzyme amount of 160 units/g cell wall and reaction time of 30 h, but using higher cell wall concentration of 3.1 mg/ml buffer (treatment n^{o} 28). Increasing the plant cell concentration may have limited the neutral side chain debranching because of the enzyme diffusional limitations through the potato cell wall matrix.

Table 4.1 Central composite rotatable design of the independent variables and the estimated responses for yield and neutral sugar content

Std order	x_1-Time (h)	x_2-Enzyme (units/g cell wall)	x_3-Cell wall (mg/ ml)	Yield (g extract/100 g substrate)	Neutral sugar (µmol/g)
1	-1 (12)[a]	-1 (80)	-1 (1.3)	41.8	932.0
2	-1 (12)	-1 (80)	-1 (1.3)	38.3	947.5
3	1 (48)	-1 (80)	-1 (1.3)	33.5	1150.0
4	1 (48)	-1 (80)	-1 (1.3)	28.4	1556.7
5	-1 (12)	1 (240)	-1 (1.3)	50.5	798.4
6	-1 (12)	1 (240)	-1 (1.3)	48.6	778.1
7	1 (48)	1 (240)	-1 (1.3)	27.2	1529.5
8	1 (48)	1 (240)	-1 (1.3)	29.0	1750.5
9	-1 (12)	-1 (80)	1 (5.0)	3.0	1380.0
10	-1 (12)	-1 (80)	1 (5.0)	0.2	1216.9
11	1 (48)	-1 (80)	1 (5.0)	14.3	1468.0
12	1 (48)	-1 (80)	1 (5.0)	16.3	1686.8
13	-1 (12)	1 (240)	1 (5.0)	6.2	2700.0
14	-1 (12)	1 (240)	1 (5.0)	6.9	2600.7
15	1 (48)	1 (240)	1 (5.0)	12.4	2526.4
16	1 (48)	1 (240)	1 (5.0)	10.9	2860.5
17	-1.5 (3)	0 (160)	0 (3.1)	9.0	1686.7
18	1.5 (57)	0 (160)	0 (3.1)	1.6	1950.3
19	0 (30)	-1.5 (40)	0 (3.1)	8.6	1794.0
20	0 (30)	1.5 (280)	0 (3.1)	14.2	2036.0
21	0 (30)	0 (160)	-1.5 (0.3)	69.3	1638.5
22	0 (30)	0 (160)	1.5 (5.9)	20.0	3667.3
23	0 (30)	0 (160)	0 (3.1)	11.9	3699.7
24	0 (30)	0 (160)	0 (3.1)	9.5	3918.0
25	0 (30)	0 (160)	0 (3.1)	7.3	4017.0
26	0 (30)	0 (160)	0 (3.1)	10.6	3600.1
27	0 (30)	0 (160)	0 (3.1)	9.0	3871.3
28	0 (30)	0 (160)	0 (3.1)	9.1	4369.6

[a] Actual experimental conditions.

Comparing treatments no1 and 3 with 9 and 11, respectively, confirms the significant effect of plant cell wall concentration on increasing neutral sugar content and decreasing yield. A good compromise between the neutral sugar content and the yield should be achieved.

The Design-Expert software was used for the multiple regression analyses and for the determination of the best-fitting models that describe the variations of responses (yield; neutral sugar content) as function of the enzymatic extraction parameters (enzyme amount, cell wall concentration, reaction time). *F* and *P* values, lack of fit and coefficient R^2 were used to compare the significance of models (Table 4.2). The quadratic model was statistically the most significant for the description of the variations of both yield and neutral sugar content with coefficient R^2 of 0.99 and 0.95 as well as *F* value of 215.9 and 56.2, respectively. *P* value of <0.0001 for both models indicates that there is less than 0.01% of probability that the values obtained for model *F* values are because of noise. For both yield and neutral sugar content, lack of fit was not significant relative to pure error with *F* value of 1.9 and 2.7 for yield and neutral sugar, respectively (Table 4.2). These results confirm the good quadratic model fits for the endo-polygalacturonase-catalyzed isolation of pectic polysaccharides from potato plant cell wall. Similarly, Canteri-Schemin et al. (2005) have reported that the acidic extraction of the pectic polysaccharides was most suitably described by a quadratic model with R^2 of 0.93.

F and P value were used to understand the significance of the enzymatic extraction parameters and theirs interactions (Table 4.2). In terms of linear effects, cell wall concentration (x_3) was the most significant variable for both yield (*F* value of 1099.9) and neutral sugar content (*F* value of 76.6) with *P* value of <0.0001. In terms of quadratic effects, only reaction time (x_1, *F* value of 14.0; *P* value of 0.0015) and cell wall concentration (x_3, *F* value of 630.8; *P* value of <0.0001) had significant effects on the yield. However, in the neutral sugar content model, the quadratic effects of all independent variables (*F* value of 48.4-139.2; *P* value of <0.0001) were significant. Among variable interactions, interaction between reaction time (x_1) and cell wall concentration (x_3) had the most significant effect on the yield (*F* value of 151.7; *P* value of <0.0001); in addition, the positive sign of this interaction term (x_1x_3) indicates the synergistic effects of these variables (Eq 4.2). In the neutral sugar content model, enzyme amount and cell wall concentration exhibited the most significant interaction (*F* value of 21.2; *P* value of 0.0002); positive sign of this interaction term (x_2x_3) indicates that both parameters affect synergistically the neutral sugar content of extracted polysaccharides (Eq 4.3).

Table 4.2 The analysis of variance for response surface models of yield and total neutral sugar content.

Source	Yield (g extract/100 g substrate)[a]				Total neutral sugar content (μmol/g extract)[a]			
	df[b]	Mean Square	*F* Value	*P*-value[c]	df[b]	Mean Square	*F* Value	*P*-value[c]
Model	9	868.4	215.9	< 0.0001	9	3611682	56.2	< 0.0001
x_1-Time (h)	1	59.2	14.7	0.0012	1	621706	9.7	0.0060
x_2-Enzyme (unit/ g cell wall)	1	29.0	7.2	0.0151	1	1512968	23.6	0.0001
x_3-Cell wall (mg/ ml buffer)	1	4423.4	1099.9	< 0.0001	1	4916921	76.6	< 0.0001
$x_1 x_2$	1	109.5	27.2	< 0.0001	1	10231.76	0.2	0.6945
$x_1 x_3$	1	610.0	151.7	< 0.0001	1	222474	3.5	0.0791
$x_2 x_3$	1	7.3	1.8	0.1960	1	1360462	21.2	0.0002
x_1^2	1	56.2	14.0	0.0015	1	8940204	139.2	< 0.0001
x_2^2	1	2.3	0.6	0.4613	1	8111601	126.3	< 0.0001
x_3^2	1	2536.9	630.8	< 0.0001	1	3106886	48.4	< 0.0001
Residual	18	4.0			18	64210		
Lack of Fit	5	6.1	1.9	0.1653	5	117187	2.7	0.0711
Pure Error	13	3.2			13	43834		
Corr Total	27				27			

[a] R^2 of 0.99 and 0.95 were obtained for yield and neutral sugar content, respectively.

[b] Degree of freedom.

[c] $P < 0.05$ indicates statistical significance

In order to obtain final adequate predictive equation, insignificant terms were negelected. The final predictive equations are given below. Terms that were required to maintain model's hierarchy were maintained.

$$\text{Yield}\ (\%) = 8.84 + 0.920x_1 + 0.59x_2 - 14.69x_3 - 1.31x_1x_2 + 6.17x_1x_3 - 2.23{x_1}^2 + 15.26\ {x_3}^2 \quad (4.2)$$

$$\text{Neutral sugar}\ (\mu\text{mol/g extract}) = 2768.13 + 174.15x_1 + 996.37x_2 + 198.15x_3 + 145.80x_2x_3 - 903.42{x_1}^2 - 215.13\ {x_2}^2 - 532.57\ {x_3}^2 \quad (4.3)$$

The effect of the most significant interaction between reaction time and cell wall concentration on the yield of extracted polysaccharides was studied by holding the enzyme amount constant at –α, 0, +α levels (40, 160 and 280 units/ g cell wall). Contour plots (Figure 4.1a-c) generated from the predicted model display similar trends in that the predicted yield increased as the cell wall concentration and the reaction time were decreased. However, cell wall concentration seems to be a more important variable affecting significantly the predicted yield as compared to the reaction time. Indeed, at all constant enzyme amounts, significant decreases in the yield were observed with an increase in the cell wall concentration. These results can be attributed to the presence of enzyme diffusional limitations at high cell wall concentrations, limiting the access of endo-polygalacturonase to HG region and hence the opening of pectin network. However, the negative effects of reaction time on the predicted yield and its interaction with cell wall concentration were more significant at high enzyme amounts (160-280 units/g cell wall) than at low ones (40 units/g cell wall). These results may be due to the enzyme inhibition and/or to the fact that the thermodynamic reaction equilibrium of endo-polygalacturonase-catalyzed hydrolysis of HG may have been reached faster in the presence of low enzyme amount. At high enzyme amounts, the decrease in the yield with an increase in reaction time can be attributed to the extensive hydrolysis of HG region.

The contour plot graphs (Figure 4.1d-f) also show the effect of the most significant interaction between the enzyme amount and the cell wall concentration on the neutral sugar content at three different times (12, 30 and 48 h); all three plots display similar trends, and the significance of this interaction effect seems similar at all selected reaction times.

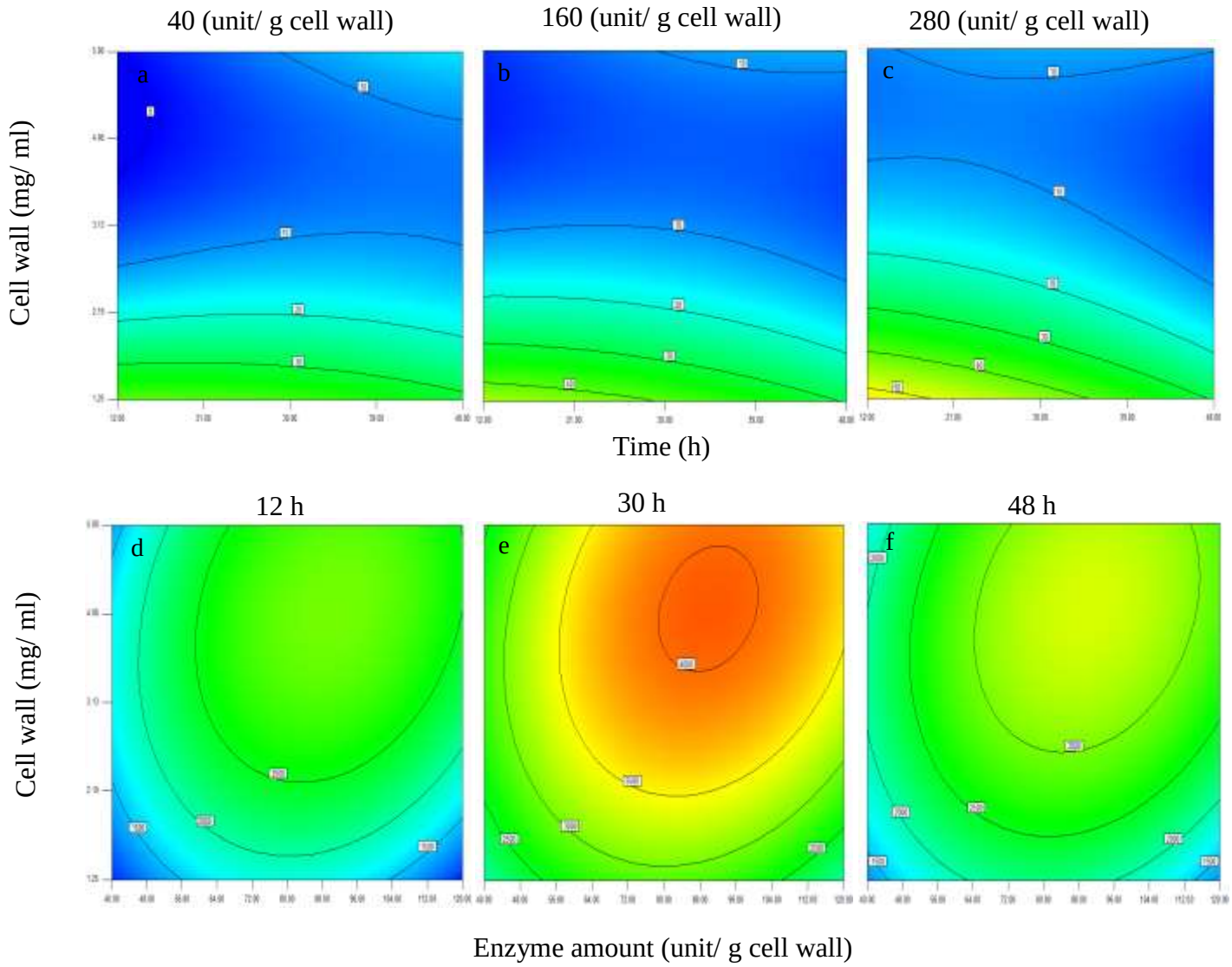

Figure 4.1 Contour plots of predictive models of yield (%) (a, b and c) and neutral sugar content (µmol/g extract) (d, e and f) of isolated RG I-type pectic polysaccharides. The numbers inside the contour plots indicate the predicted responses.

As overall, the neutral sugar content of extracted polysaccharides increased with an increase in the enzyme amount and the cell wall concentration until reaching a turning point and decreased thereafter; this last decrease is more affected by the enzyme amount as a variable than the cell wall concentration. Indeed, at high cell wall concentration to enzyme amount ratio, the decrease in the neutral sugar content beyond the optimum value is not significant. The negative effect of the enzyme amount excess on the neutral sugar content reveals the debranching of neutral side chains of RG I upon an extensive hydrolysis of HG. Similarly, Shobha et al. (2005) have reported that the polygalacturonase activity of pectinase from *A. niger* led to considerable debranching of guar galactomannan.

4.4.2. Monosaccharide composition of extracted polysaccharides

Experimental values of the molar content of main monosaccharides (Gal, Ara, Rha and GalA) in the extracted polysaccharides and of the proportion of weak acidic polysaccharides are shown in Table 4.3. Our previous study (Khodaei & Karboune, 2012) revealed the presence of weak and strong acidic galactan-rich RG I populations with short and long-linked HG fragments, respectively, upon enzymatic extraction.

The highest content of Gal (2835.1-3373.4 µmol/ g extract; 70-74%), Ara (260.9-362.4 µmol/ g extract; 7-9%) and Rha (198.3-259.4 µmol/ g extract; 5-6%) with relatively low GalA/Rha ratio of 1.9-2.5, high Gal/Ara ratio of 7.9 and Gal/Rha ratio of (13-14) were obtained upon the enzymatic treatments n° 23-28 corresponding to the center point (160 units of enzyme/g cell wall; reaction time of 30 h; cell wall concentration of 3.1 mg/ml). These results of monosaccharide composition confirm the isolation of galactan-rich RG I under the center point conditions. Comparable GalA/Rha and Gal/Rha ratios have been reported by Byg et al. (2012) for potato RG I. Depending on the enzymatic treatment conditions, GalA/Rha ratio was ranged from 1.9 to 21.2. As overall, as the Gal content in the extracted pectic polysaccharides increased, their GalA/Rha ratio decreased; these results may be due to the depolymerization of HG region and/or to the commitment increase in the Rha. On the other hand, the Gal/Ara ratio of extracted pectic polysaccharides covered a wide range from 5.8 to 14.8. The results (Table 4.3) also show that the highest percentage of weak acidic fraction (71.3 %) was obtained upon the enzymatic treatment n^{o} 4 (80 units of enzyme/g cell wall; reaction time of 48 h; cell wall concentration 1.3 mg/ml), which resulted in pectic polysaccharides with high Gal/Rha ratio of 68.2.

Table 4.3 Central composite rotatable design of the independent variables and the estimated responses for weak acidic fraction and for galactose (Gal), arabinose (Ara), rhamnose (Rha) and galacturonic acid (GalA) contents.

Std order	x_1-Time (h)	x_2-Enzyme (units/g cell wall)	x_3-Cell wall (mg/ml)	Weak acidic (%)	Gal (μmol/g)[a]	Ara (μmol/g)[a]	Rha (μmol/g)[a]	GalA (μmol/g)[a]	GalA/Rha	Gal/Rha
1	-1 (12)[b]	-1 (80)	-1 (1.3)	65.4	797.6 (76%)[c]	79.2 (8%)	30.2 (3%)	114.1 (11%)	3.8	26.4
2	-1 (12)	-1 (80)	-1 (1.3)	63.2	813.1 (77%)	81.6 (8%)	29.9 (3%)	97.6 (9%)	3.3	27.2
3	1 (48)	-1 (80)	-1 (1.3)	61.4	861.8 (65%)	54.3 (4%)	19.3 (1%)	209.4 (16%)	10.8	44.7
4	1 (48)	-1 (80)	-1 (1.3)	71.3	1356.2 (82%)	57.9 (4%)	19.9 (1%)	162.3 (10%)	8.2	68.2
5	-1 (12)	1 (240)	-1 (1.3)	59.7	668.0 (75%)	29.5 (3%)	24.2 (3%)	139.4 (16%)	5.8	27.6
6	-1 (12)	1 (240)	-1 (1.3)	54.7	639.5 (74%)	33.6 (4%)	21.3 (2%)	138.0 (16%)	6.5	30.0
7	1 (48)	1 (240)	-1 (1.3)	49.2	1322.2 (72%)	69.4 (4%)	16.2 (1%)	349.3 (20%)	21.2	81.6
8	1 (48)	1 (240)	-1 (1.3)	54.6	903.2 (65%)	74.3 (5%)	18.8 (2%)	337.2 (24%)	17.3	48.0
9	-1 (12)	-1 (80)	1 (5.0)	38.6	1176.3 (59%)	139.5 (7%)	42.2 (2%)	620.0 (31%)	14.7	27.9
10	-1 (12)	-1 (80)	1 (5.0)	34.8	1029.7 (55%)	140.5 (8%)	36.0 (2%)	634.4 (34%)	17.6	28.6
11	1 (48)	-1 (80)	1 (5.0)	41.9	980.1 (49%)	106.3 (5%)	44.8 (2%)	532.0 (26%)	11.9	21.9
12	1 (48)	-1 (80)	1 (5.0)	45.5	1424.6(69%)	12.4 (1%)	55.1 (3%)	476.3 (23%)	8.6	25.9
13	-1 (12)	1 (240)	1 (5.0)	49.0	2249.8 (73%)	263.6 (9%)	80.9 (3%)	432.6 (14%)	5.3	27.8
14	-1 (12)	1 (240)	1 (5.0)	46.0	2214.8 (72%)	264.4 (9%)	68.3 (2%)	439.2 (14%)	6.4	32.4
15	1 (48)	1 (240)	1 (5.0)	46.3	2021.1 (72%)	300.2 (11%)	116.8 (4%)	300.0 (11%)	2.6	17.3
16	1 (48)	1 (240)	1 (5.0)	46.0	2294.2 (73%)	293.5 (9%)	133.6 (4%)	351.2 (11%)	2.6	17.2
17	-1.5 (3)	0 (160)	0 (3.1)	54.2	1430.8 (66%)	163.3 (8%)	49.0 (2%)	424.8 (20%)	8.7	29.2
18	1.5 (57)	0 (160)	0 (3.1)	52.6	1592.3 (69%)	170.2 (7%)	75.3 (3%)	420.1 (18%)	5.6	21.1
19	0 (30)	-1.5 (40)	0 (3.1)	53.7	1525.5 (69%)	105.3 (5%)	43.7 (2%)	448.3 (20%)	10.3	34.9
20	0 (30)	1.5 (280)	0 (3.1)	57.1	1586.1 (70%)	208.3 (9%)	69.6 (3%)	369.2 (16%)	5.3	22.8
21	0 (30)	0 (160)	-1.5 (0.3)	47.7	1238.4 (79%)	139.0 (9%)	89.6 (6%)	91.2 (6%)	1.0	13.8
22	0 (30)	0 (160)	1.5 (5.9)	28.6	3001.9 (72%)	345.3 (8%)	205.3 (5%)	501.2 (12%)	2.4	14.6
23	0 (30)	0 (160)	0 (3.1)	45.2	3072.8 (74%)	260.9 (7%)	219.8 (5%)	500.3 (12%)	2.3	14.0
24	0 (30)	0 (160)	0 (3.1)	49.2	3207.7 (73%)	350.7 (8%)	218.3 (5%)	482.0 (11%)	2.2	14.7
25	0 (30)	0 (160)	0 (3.1)	47.0	3242.2 (72%)	356.0 (8%)	238.4 (5%)	483.0 (11%)	2.0	13.6
26	0 (30)	0 (160)	0 (3.1)	47.1	2835.1 (68%)	356.3 (9%)	198.3 (5%)	499.9 (12%)	2.5	14.3
27	0 (30)	0 (160)	0 (3.1)	47.0	2966.6 (70%)	362.4 (9%)	259.4 (6%)	493.2 (12%)	1.9	11.4
28	0 (30)	0 (160)	0 (3.1)	50.0	3373.4 (71%)	358.3 (8%)	245.9 (5%)	486.3 (10%)	2.0	13.7

[a] Molar concentration of monosaccharides is expressed in μmol per g polysaccharide extract; [b]Actual experimental conditions; [c]Relative molar proportion of monosaccharide.

However, treatments n°9 and 10 (80 units of enzyme/g cell wall; reaction time of 12 h; cell wall concentration of 5.0 mg/ml) led to the isolation of polysaccharides with the lowest proportion of weak acidic fraction (34.6-38.6%) and Gal/Rha ratio (27.9-28.6).

The best-fitting model was determined by multiple regression analyses of the experimental data. ANOVA for the responses of Gal and Ara contents as well as the weak acidic fraction proportion are summarized in Table 4.4. The quadratic model (R^2 values of 0.94, 0.95 and 0.89 for Gal and Ara content and weak acidic fraction, respectively) was the most statistically suitable for the correlation between the observed and predicted values. The non-significant "lack of fit" and the *F* values of 45.6, 56.1 and 26.4 for Gal, Ara and weak acidic fraction responses, respectively, with *P* value of >0.0001 confirm the validity of the quadratic polynomial models for representing the experimental data.

Table 4.4 shows that linear and quadratic terms of enzyme amount (x_2, *F*-value of 15.0-42.6, P≤0.0011) and cell wall concentration (x_3, *F*-value of 72.0-117.9, P<0.0001) had significant effects on Gal and Ara contents of extracted polysaccharides; for the reaction time as variable, only its quadratic terms showed significance (x_1, *F*-value of 96.6-106.3, P<0.0001). In the weak acidic fraction model, the linear effect of cell wall concentration (x_3, *F*-value of 143.3, P<0.0001) was the only significant linear term; however, the quadratic effects of all variables (x_1^2 x_2^2 x_3^2 , *F*-value of 13.9-22.3, P<0.001) were all significant terms. Among all interactions, the one between the enzyme amount and the cell wall concentration ($x_2\, x_3$, *F*-value of 24.4-51.6, P<0.0001) was the most significant in all three models. In addition, the positive sign of this interaction term ($x_2\, x_3$) indicates the positive synergistic effects of the enzyme amount and the cell wall concentration variables on all predicted responses (Eqs 4.4-4.6). The results (Table 4.4) also show that the reaction time/enzyme amount interaction ($x_1\, x_2$, *F*-value of 7.1-10.5, P<0.01) exhibits some effects in the predicted models of Ara content and weak acidic fraction proportion but not in the Gal content model. From the sign of this interaction term ($x_1 x_2$), it can be seen that the reaction time and the enzyme amount exhibited a positive synergistic effect on the predicted Ara content, but a negative adverse effect on the predicted weak acidic fraction proportion. These results reveal that a good compromise between enzyme amount and reaction time should be considered to achieve a high proportion of weak acidic fraction. Mathematical model that relates the responses and the independent variables was obtained by eliminating insignificant terms.

Table 4.4 The analysis of variance for response surface quadratic models of Gal and Ara contents as well as weak acidic fraction proportion.

Source	Gal content (μmol/ g extract)[a]				Ara content (μmol/ g extract)[a]				Weak acidic fraction (%)[a]			
	df[b]	Mean Squares	*F* - value	*P*- value[c]	df[b]	Mean Square	*F* - value	*P*- value[c]	df[b]	Mean Squares	*F*- value	*P*- value[c]
Model	9	2322306	45.6	<0.0001	9	42280	56.1	<0.0001	9	229.7	26.4	< 0.0001
x_1-Time (h)	1	161001	3.2	0.0924	1	138	0.2	0.6743	1	0.3	0.0	0.8565
x_2-Enzyme (unit/g cell	1	766579	15.0	0.0011	1	32113	42.6	<0.0001	1	6.5	0.8	0.3976
x_3-Cell wall (mg/ ml	1	3670543	72.0	<0.0001	1	88915	117.9	<0.0001	1	1247.8	143.	< 0.0001
$x_1 x_2$	1	87	0.0	0.9675	1	7935	10.5	0.0045	1	61.6	7.1	0.0160
$x_1 x_3$	1	136161	2.7	0.1195	1	1019	1.4	0.2601	1	19.8	2.3	0.1486
$x_2 x_3$	1	1246046	24.4	0.0001	1	38916	51.6	<0.0001	1	302.5	34.7	< 0.0001
x_1^2	1	5418649	106.3	<0.0001	1	72805	96.6	<0.0001	1	121.3	13.9	0.0015
x_2^2	1	5119728	100.4	<0.0001	1	80910	107.3	<0.0001	1	194.2	22.3	0.0002
x_3^2	1	2052340	40.3	<0.0001	1	25279	33.5	<0.0001	1	130.0	14.9	0.0011
Residual	18	50969			18	754			18	8.7		
Lack of Fit	5	73159	1.7	0.1984	5	276	0.3	0.9076	5	8.9	1.0	0.4390
Pure Error	13	42434			13	938			13	8.6		
Cor Total	27				27				27			

[a] R^2 of 0.94, 0.95 and 0.89 were obtained for Gal and Ara content models and weak acidic fraction model, respectively.

[b] Degree of freedom

[c] $P < 0.05$ indicates statistical significance.

Through reverse engineering, the enzymatic extraction parameters for the isolation of structurally well-defined RG I can be identified using the developed models.

$$\text{Gal (µmol/g extract)} = 2234.81 + 88.62x_1 + 780.35x_2 + 144.08x_3 + 139.53x_2x_3 - 703.34{x_1}^2 - 170.92\,{x_2}^2 - 432.85\,{x_3}^2 \quad (4.4)$$

$$\text{Ara (µmol/g extract)} = 217.12 - 24.86x_1 + 105.73x_2 + 16.54x_3 + 11.13x_1 + 24.66x_2x_3 - 81.53{x_1}^2 - 21.49\,{x_2}^2 - 48.04\,{x_3}^2 \quad (4.5)$$

$$\text{Weak acidic fraction (\%)} = 52.02 + 2.08x_1 - 4.49x_2 - 12.15x_3 - 0.98x_1x_2 + 2.17x_2x_3 + 3.33{x_1}^2 + 1.05{x_2}^2 - 3.45\,{x_3}^2 \quad (4.6)$$

The response surface contour plots presented in Figure 4.2 illustrate the interaction effects of enzyme amount and cell wall concentration on the predicted Gal and Ara contents of the extracted polysaccharides as well as on their weak acidic fraction proportion at constant reaction times. The results (Figure 4.2a-f) show that increasing enzyme up to 160 units/g cell wall resulted in an increase of predicted Gal and Ara contents in the extracted polysaccharides; however, further increase of this variable to 240 units/g cell wall led to a slight decrease of Gal and Ara contents. This decrease reveals the low debranching of the neutral side chains (> 7% galactan; > 15% arabinan) of RG I at high enzyme amounts. Such debranching may be due to the extensive opening of the pectin network and/or to the degradation of xylogalacturonan and RG-II, which are covalently linked to RG I (Vincken et al., 2003). The debranching may also be attributed to the presence of low level of contaminating galactanase and arabinanase enzymes in the endo-polygacturonase product. Indeed, the measurement of galactanase activity using potato galactan as substrate reveals the presence of negligible enzymatic units of galactanase (0.15% U/U) as compared to endo-polygalacturonase units. No arabinanase activity was reported in the investigated pure endo-polygalacturonase product from *A. niger*. Byg et al. (2012) have reported the debranching of arabinan side chain of RG I upon the use of purified endo-polygalacturonase from *A. niger* expressed in *A. aculeatus* and attributed these results to the presence of endogenous arabinanase in tubers. Zykwinska et al. (2006) have reported that arabinan and galactan side chains are highly mobile in potato cell wall. The high debranching of arabinan side chains of RG I may be due to the high mobility of the shorter arabinan side chains.

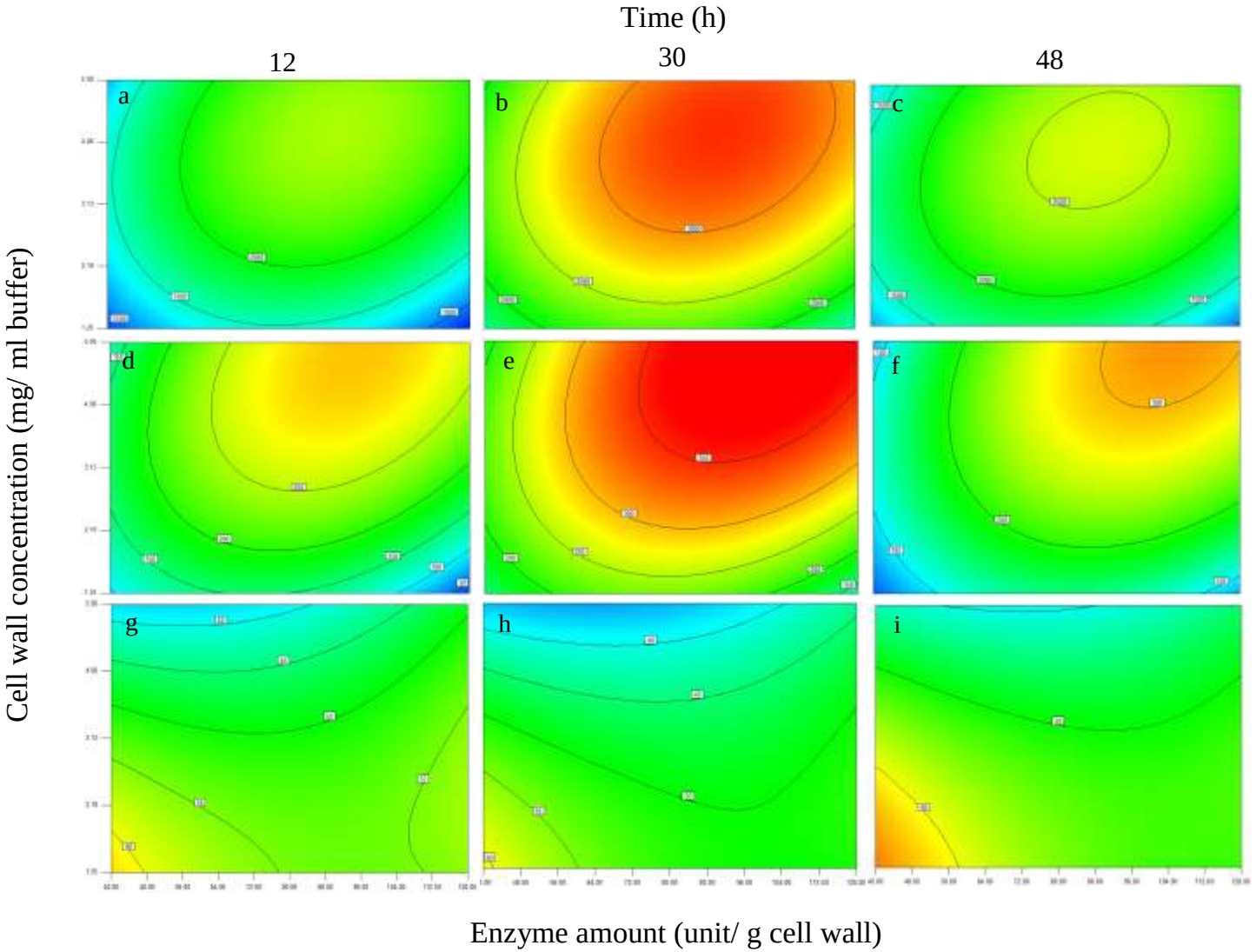

Figure 4.2 Contour plots of Gal (a, b and c) and Ara (d, e and f) contents (μmol/g extract) in isolated RG I-type pectic polysaccharides as well as of the proportion of the weak acidic fraction (%) (g, h and i). The numbers inside the contour plots indicate the predicted responses.

As expected, the results also show that the Gal and Ara contents in the extracted pectic polysaccharides increased as the cell wall concentration increased and remained constant thereafter. Such limited increase may be due to the achievement of the reaction equilibrium and/or to the enzyme inhibition. The thermal stability study of endo-polygalacturonase showed that only 13% of initial activity was lost upon incubation at 35°C for 60 h (Data not shown). The results (Figure 4.2a-f) also show that the three contour plots of predicted Gal and Ara contents at selected reaction times display similar trends, but the enzyme amount/cell wall concentration synergistic interaction was more significant at reaction time of 30 h. As a result, the highest Gal and Ara contents were achieved at 30 h. As the enzymatic extraction was proceeded up to 48 h, the predicted Gal and Ara contents in the extracted pectic polysaccharides decreased slightly.

The high proportion of weak acidic fraction may reveal the extensive hydrolysis of HG into short-linked fragments. The contour plots of the predicted weak acidic fraction (Figure 4.2g-i) show different trends as compared to those of Gal and Ara contents. The predicted proportion of weak acidic fraction decreased as cell wall concentration increases. Increasing cell wall concentration may have led to the presence of unhydrolyzed HG region linked to RG I and hence to low proportion of weak acidic RG I fraction. However, at a constant cell wall concentration, increasing enzyme amount had no significant effect on the proportion of weak acidic RG I fraction. A good compromise between low debranching of the neutral side chains of RG-I and low proportion of long-linked HG fragments can be achieved by the selection of the appropriate conditions.

4.4.3. Model validation

Adequacy of the model for predicting response values was investigated through additional runs using the optimal conditions for the yield (Opt A) and the Gal content (Opt B) estimated via the Design-Expert 8.0.2 software (Table 4.5). The output suggests that high yield of RG I-type pectic polysaccharides could be achieved upon the use of high enzyme amount to cell wall concentration ratio with short reaction time; while Gal content of isolated polysaccharides could reach its optimum value upon a prolonged reaction time and using a low enzyme amount to cell wall concentration ratio. The predicted and the experimental maximum response values are shown in Table 4.5. All experimental findings are within the statistically significant range of the estimated optimum values with 95% prediction intervals.

Table 4.5 Optimum conditions and their corresponding experimental and predicted responses.

Optimum conditions	Opt A[a]		Opt B[b]	
x_1-Time (h)	12		30.4	
x_2-Enzyme (unit/ g cell wall)	240		180.6	
x_3-Cell wall (mg/ ml buffer)	0.13		0.42	
Responses	Experimental	Predicted	Experimental	Predicted
Gal[c]	595.6 (81.2%)[d]	465.1	754.4 (71.8%)	775.8
Ara[c]	11.9 (1.6%)	18.4	82.5 (7.9%)	89.6
Rha[c]	8.7 (1.2%)	13.5	55.7 (5.3%)	55.8
GalA[c]	111.6 (15.2%)	119.2	118.8 (11.3%)	124.4
Glc[c]	3.6 (0.5%)		23.6(2.2%)	
Xyl[c]	1.1 (0.1%)		7.1 (0.7%)	
Man[c]	1.2 (0.2%)		8.1 (0.8%)	
Total neutral sugar	622.7	597.5	931.6	967.7
Yield (%)	63.955	50.2	9.5	6.6
Molecular weight range (kDa)				
<50 (%)	22.2		32.4	
50-500 (%)	15.7		22.3	
>500 (%)	62.2		42.4	

[a] Opt A corresponds to the predicted optimum conditions for the yield.

[b] Opt B corresponds to the predicted optimum conditions for the Gal content of extracted pectic polysaccharides.

[c] Monosaccharide composition is expressed in mmol of extracted sugar per 100 g cell wall.

[d] Relative molar proportion of monosaccharides.

These results indicate that there is no significant ($P < 0.05$) differences between the experimental data and the predicted values. As an overall, validation of yield and Gal content RSM models were confirmed. The conditions identified (Opt A) for the maximum yield (50-63%) resulted in a high proportion of high-MW polysaccharide population (>500 kDa; 62.2%) with GalA/Rha ratio of 12 and Gal+Ara/GalA ratio of 5.4. These results reveal the recovery of polysaccharide extracts enriched with RG I (85%), containing higher proportions of GalA oligomeric units, at Opt A conditions. On the other hand, low GalA/Rha ratio of 2.1 and high Gal+Ara/GalA ratio of 7.0 were obtained upon the use of the Opt B conditions, identified for maximum Gal content; however, at these conditions, a yield of 9.5% and a proportion of high-MW RG I-type polysaccharide population (>500 kDa) of 42.4% were obtained. At Opt B conditions, the monosaccharide profiles of isolated polysaccharides correspond to that of highly galactan-branched RG I type pectic polysaccharides with high molar proportion of Gal and low levels of Rha and GalA. Moreover, the results indicate that at both identified conditions, the Gal+Ara/Rha ratio (69.8 and 15.0 for opt A and B, respectively) was high indicating the highly branching of isolated RG I-type pectic polysaccharides and the presence of short length RG I stretches. Byg et al. (2012) have reported higher yield of 11% but the GalA/Rha ratio of isolated RG I (2.9) was higher.

The monosacharide composition (Table 4.5) also indicates the extraction of low amounts of hemicellulosic polysaccharides upon the enzymatic treatment. In particular, the presence of Xyl, Man, Glc reveals the release of heteromannan and xyloglucan, which have been speculated to be linked to RG I (Mohnen, 2008; Vincken et al., 2003). The results also show that the proportion of these released hemicellulosic polysaccharides was higher using Opt B conditions with a prolonged reaction time than Opt A ones. However, higher proportion of the hemicellulosic polysaccharides (more than 12%) have been reported upon the use of strong alkaline solutions, 2 mol/l NaOH and KOH, for the extraction of RG I (Khodaei & Karboune, 2013).

Canonical analysis was performed to confirm if the response surface has a true maximum, minimum or a saddle point within the limit of the investigated surface. The eigenvalues and eigenvectors obtained from canonical analysis were used to characterize the shape of the response surface. In terms of the Gal, all of the eigenvalues are positive, indicating that the stationary point is a maximum (data not shown). In terms of the yield, eigenvalues have both negative and positive sign, indicating that the stationary point is a saddle point. Since there was

no simple optimum point for the yield within the investigated range, ridge analysis was carried out to determine in which direction further experiments should be done to locate the optimum. Figure 4.3 shows the maximum yield when the variables are moved from centre of the experiments. It can be seen that yield can increase up to 67.4%, when reaction time decreases to 25.7 h, enzyme amount increases to 164.16 unit/ g cell wall and cell wall concentration decreases to 0.03%. This trend is in agreement with results of counterplots (Figure 4.1a-c). However, the optimum yield of 63.9% predicted by RSM software is very close to the value (67.4%) given by the ridge.

4.4.4. Linkage analysis of galactan-rich RG-I fractions

Table 4.6 shows the linkage patterns of weak and strong acidic fractions of galactan-rich RG-I obtained upon fractionation by anionic exchange chromatography. As expected, the proportion of *4*-GalA was higher in the strong acidic fraction than in the weak one. These results reveal that HG backbones in potato cell wall was not degraded with the same extent by *A. niger* polygalacturonase, resulting in two RG-I populations. Table 4.6 also show that Rha was mainly (1 → 2) linked, and branched Rha constitutes 61.9 and 64.7% of total Rha in the weak and strong acidic fractions, respectively. Comparable amount of branched Rha (50%) residues was reported by Byg et al. (2012) for potato pulp RG I. On the other hand, Harris (2005)have indicated a wide range of Rha substitution (20-80%) in RG I depening on the species. Moreover, the low proportion of terminally linked Rha suggests the low breakdown of the RG I backbone during extraction. GalA/Rha ratio was estimated at 2.7 and 3.2 for weak and strong acidic fractions of extracted RG I, respectively. Similarly, Byg et al. (2012) reported GalA/Rha ratio of 3.11. About 83.5 and 78.7% of weak and strong acidic fractions of RG I, respectively, are comprised of Gal residues. These results are in good agreement with the monosaccharide composition.

However, Byg et al. (2012) have reported lower total amount of Gal of 49% in enzymatically extracted RG I at large scale. Jarvis et al. (1981) have extracted pectic fraction from potato tuber using oxalate-citrate and sodium carbonate as extracting agents, and reported a Gal content of 61 and 81 %, respectively. On the other hand, lower total amount of Ara (5.2-5.3%) was obtained as compared to those (10-20%) reported in previous studies (Byg, et al., 2012; Jarvis, et al., 1981). These results may be due to the use of different extraction conditions. Gal and Ara residues in extracted RG I were predominantly present as linear (1→4) and (1→5) linked, respectively.

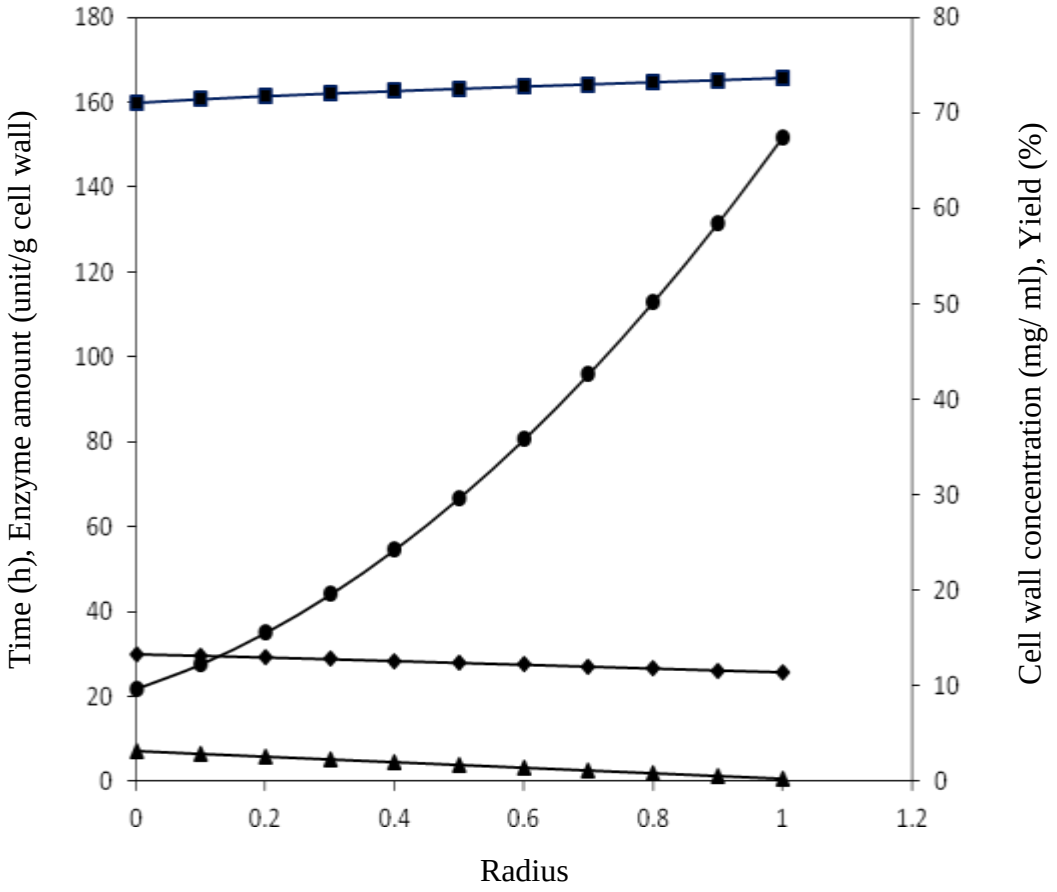

Figure 4.3 The maximum predicted value of RG I yield at increasing radii from the center of the investigated design: ♦ Time (h), ■ Enzyme (unit/g cell wall), ▲ Cell wall (mg/ml buffer), ● Yield (%).

Table 4.6 Glycosyl linkage patterns of weak and strong acidic galactan-rich RG-I obtained through methylation analysis.

Linkage type	Weak acidic RG I	Strong acidic RG I
Galactose		
4-Gal	52.7 [a]	50.7
3,4-Gal	10.1	5.8
4,6-Gal	4.9	7.8
3-Gal	0.4	0.3
6-Gal	0.1	0.2
3,6-Gal	0.2	0.2
t-Gal	15.2	13.7
Total	83.5	78.7
Arabinose		
5-Ara	1.9	2.1
2,5-Ara	0.7	0.7
2-Ara	0.8	0.9
t-Ara	1.8	1.6
Total	5.2	5.3
Rhamnose		
2-Rha	1.0	1.2
2,4-Rha	1.8	2.4
t-Rha	0.1	0.1
Total	3.0	3.8
Galacturonic acid		
4-GalA	7.8	11.9
t-GalA	0.5	0.4
Total	8.3	12.3

[a] Values presented in percentage

The results also show that branched Gal constituted 17.5-18.2% of total Gal, while branched Ara represented 14.0-12.5% of total Ara. The proportion of terminal Ara residues (30.2-34.6% of total Ara) was higher than that of terminal Gal ones (17.4-18.2% of total Gal), revealing that shorter chains of Ara units are linked to galactan side chains or RG I backbone. The ratio of linear and branched (1→3) linked to (1→4) linked Gal were 1 to 113 and 129 for weak and strong acidic fractions, respectively. Previous studies have reported a (1→3) linked to (1→4) linked Gal ratio of 1:163 for arabinogalactan (Hinz et al., 2005).

4.5. Conclusion

Enzymatic isolation of galactan-rich RG I from potato cell wall by-products was investigated using RSM as an effective tool for modeling and understanding the effects of extraction parameters and their interactions. Quadratic models were the most significant ones for the description of variations of the studied responses. Comparison of predicted and experimental values showed very good correspondence, validating the established predicted models. The monosaccharide composition and the linkage patterns confirm the isolation of weak and strong acidic galactan-rich RG I type pectic polysaccharides. The developed mathematical models are expected to provide the capability to isolate targeted RG I-type pectic polysaccharides with targeted yield and specific structural properties.

CONNECTING STATEMENT 3

Previous studies provided a good understanding of the effects of alkaline and enzymatic extractions on the yields and the structural properties of galactan-rich RG I extracts. Enzymatic extraction required longer reaction times (12-30 h) and lower substrate concentrations (0.01-2%, w/v), but it resulted in good yields and low debranching of galactan-rich RG I; while the conventional alkaline extraction led to higher yields but it promoted the neutral side chain debranching (Chapters III and IV). Combining the alkaline extraction with the microwave treatment is explored in Chapter V in order to minimize the side chain debranching and to maximize the productivity and the turnover number through an increase in the substrate concentration and a decrease in the extraction time. Using RSM and CCRD with four variables at five levels, the effects of extraction parameters, including concentration of KOH, extraction time, microwave power and solid/liquid ratio on the yield, MW distribution and monosaccharide profile of the extracted polysaccharides are discussed in this chapter. The best condition resulting in the highest content of Gal are identified. In addition, the techno-functional properties (solubility, emulsifying, and viscoelastic properties) of polysaccharides extracted under optimum conditions are examined and compared with those of potato galactan and oranges HG as standards.

The results from this chapter were presented at IFT *13th Annual Meeting of Institute of Food Technologists* and was accepted in journal of *Food Chemistry*.

Khodaei, N., Orsat, V. & Karboune, S. (2013) Investigation and optimization of microwave extraction of rhamnogalacturonan I from potato cell wall. *13th Annual Meeting of Institute of Food Technologists, Chicago, USA.*

Khodaei, N., Karboune, S., & Orsat, V. (2016). Microwave-alkaline extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product. *Food Chemistry, 190*(1), 495-505.

CHAPTER V

MICROWAVE-ASSISTED ALKALINE EXTRACTION OF GALACTAN-RICH RHAMNOGALACTURONAN I FROM POTATO CELL WALL BY-PRODUCT

5.1. Abstract

Galactan-rich RG I, exhibiting promising health benefits, is the most abundant polysaccharide in potato pulp by-product. In the present study, the microwave-assisted alkaline extraction of galactan-rich RG I was investigated. Solid/liquid ratio was identified as the most significant parameter affecting linearly yield and Gal/Rha contents. Microwave power and solid/liquid ratio exhibited a significant adverse interactive effect on the yield. Gal content of extracted polysaccharides can be modulated by compromising between KOH concentration and extraction time, which exhibited adverse interaction. Optimum conditions were identified using the established predicted models and consisted of treatment of potato cell wall at solid/liquid ratio of 2.9% (w/v) with 1.5 M KOH under microwave power of 36.0 W for 2.0 min. Yield of intact galactan-rich RG I of 21.6% and productivity of 192.0 g/l.h were achieved. The functional properties of RG I-rich polysaccharides were comparable or superior to potato galactan and oranges HG.

5.2. Introduction

Large amount of potato pulp is available as by-products of the potato processing industry. There is an increasing demand for the effective use of the potato pulp by-product as a source of functional ingredients. Potato pulp mainly consists of cell wall polysaccharides (70%, w/w) with pectic ones being the most abundant (56%, w/w) (Langner et al., 2009). However, because of the high amount of neutral side chains (67%, w/w) in potato pectic polysaccharides, they are limited in terms of their gelling properties. Potato pectic polysaccharides have been mainly recognized for their bioactive properties, in particular their fermentability and their bifidogenic activity (Kelly, 1999; Thomassen et al., 2011). In addition, they have been associated with the decrease of weight gain (Lærke et al., 2007), the stimulation of immune system and the prevention of metastasis (Kelly, 1999; Nangia-Makker et al., 2002). These bioactive properties have been linked to the unique structure of potato pectic polysaccharides. Indeed, unlike pectin from other sources, potato pectic polysaccharides consist of high proportion of RG I (75%) and low amount of HG (20%) (Mohnen, 2008; Oomen et al., 2003). In addition, potato RG I is rich in β-linked galactan side chains (67%, w/w); while RG I from other sources contain only 10-24% of galactan side chains (Yapo et al., 2007).

In our previous studies (Khodaei & Karboune, 2014, 2013), alkaline (NaOH and KOH) and enzymatic (endo-polygalacturonase from *A. niger*, E.C. 3.2.1.15) extractions were compared in terms of their efficiency to extract intact RG I with high neutral side chains. High yield (63.9%) of RG I enriched isolates was achieved upon enzymatic treatment at an enzyme amount to cell wall concentration ratio of 240.0 units/ g cell wall for 12 h; while the purity of isolated RG I reached its optimum value upon a prolonged extraction time of 30 h with a yield of 9.5% (Khodaei & Karboune, 2014). Similarly, Byg et al. (2012) have reported a yield of 9-11% for the enzymatic extraction of RG I using cell wall concentration of 1% w/v and incubation time of 18.0 h. Furthermore, Thomassen et al. (2011) have reported the extraction of fiber, containing 69% of RG I, by the enzymatic treatment of potato pulp at 1% (w/v) for shorter time of 1 min. As an overall, the enzymatic extraction of RG I-type pectic polysaccharides with high purity requires longer reaction time (18-30 h) and the use of relatively low concentration of substrate (0.04-1%, w/v) (Byg et al., 2012; Khodaei & Karboune, 2014). Contrary to the enzymatic extraction, higher substrate concentrations (2.0-3.3%, w/v) and lower incubation times (1-24 h) have been used in the alkaline extraction of RG I (Khodaei & Karboune, 2013; Zykwinska et al., 2006); however, the low turnover number and the weak productivity of enzymatic extraction limit their applications; while the conventional alkaline extraction led to high galactan and arabinan side chain degradations (Khodaei & Karboune, 2013). Combining the alkaline extraction with the microwave treatment may help to minimize the side chain debranching and to maximize the productivity and the turnover number through an increase in the substrate concentration and a decrease in the extraction time. Indeed, the microwave treatment offers a rapid heating with low covalent bonds hydrolysis (Fishman et al., 2006) and an efficient rupture of the cell wall by high internal pressure that is built as a result of the evaporation of the internal moisture (Mandal et al., 2007; Li et al., 2012). So far, most studies reported in the literature have investigated the microwave-assisted acidic extraction of pectic polysaccharides (Bélafi-Bakó et al., 2012; Fishman et al., 2008; Guo et al., 2012; Kratchanova et al., 2004; Li et al., 2012; Prakash Maran et al., 2013; Yoo et al., 2012). However, acid extraction is more suitable for the extraction of HG than RG I region because of the high neutral side chain degradation under acidic conditions (Levigne et al., 2002). As far as the authors are aware, only few studies have investigated the microwave-assisted alkaline extraction for the isolation of pectic polysaccharides from sugar beet pulp and orange peel (Fishman et al., 2009; Yeoh et al., 2008),

and the microwave-assisted alkaline extraction of potato RG I polysaccharides has not been investigated so far. The main objective of this study was to investigate the isolation of galactan rich-RG I from potato cell wall by microwave-assisted alkaline extraction. A special emphasis was put on the study of the effects of extraction parameters, including concentration of KOH (M), extraction time (min), microwave power (W) and solid/liquid ratio (%, w/v), on the yield and the structural properties of extracted polysaccharides. The functional properties (viscosity, emulsifying properties, and solubility) of extracted polysaccharides were also assessed.

5.3. Materials and methods

5.3.1. Materials

Potato galactan was obtained from Megazyme (Wicklow, Ireland). Soybean oil, KOH, sodium acetate, acetic acid, dextran standards, Gal, Ara, Rha, Glc, Xyl, Man, GalA, trifluoroacetic acid, thermostable α-amylase from *B. licheniformis*, amyloglucosidase from *A. niger* and HG from oranges were purchased Sigma Chemical Co. (St-Louis, MO). All salts were obtained from Fisher Scientific (Mississauga, ON).

5.3.2. Preparation of potato cell wall material

In order to prepare cell wall material, protein was removed from the potato pulp (Lyckeby Starch AB) as previously described (Khodaei & Karboune, 2013). Starch in the potato pulp was gelatinized at 95°C for 30 s and hydrolyzed upon treatment with thermostable α-amylase from *B. licheniformis* and amyloglucosidase from *A. niger* (≥300 units/ml) at a ratio of 1:10 and 1:2.3 (v:w) respectively. Cell wall material was recovered by filtration and freeze dried.

5.3.3. Microwave-assisted alkaline extraction of RG I from cell wall

Microwave-assisted alkaline extraction of RG I was optimized using RSM and a CCRD. Concentration of KOH (0, 0.5, 1.0, 1.5, and 2 M), extraction time (0, 2, 4, 6, and 8 min), microwave power (14, 32, 50, 68, and 86 W) and solid/liquid ratio (0.2, 1.1, 2.0, 2.9, and 3.8%, w/v) were chosen as independent variables. The volume of liquid was maintained constant at 30 ml. As a result, power to substrate ratio varied between 23.3 and 833.3 W/g cell wall. The complete design (30 experiments) includes sixteen factorial points (levels ±1), eight axial points (levels ± α), and six replicates in center point, and the experiments were run in random order.

The yield (%, w/w), the MW distribution (>600 kDa, %) and the monosaccharide profile (Gal, Ara, Rha, Glc, Xyl, Man, GalA) of extracted polysaccharides were the quantified as responses.

Isolated cell wall material was suspended in KOH solution to yield the selected solid/liquid ratio. The suspension was prepared in a cylindrical Pyrex tube and microwave heated for selected extraction time under a specific power using a Prolabo Synthewave 402 Microwave Reactor. After incubation, samples were filtered under vacuum using glass microfiber filter grade GF/D with pore size of 2.7 µm (Whatman®). pH of filtrate was adjusted to 7 using hydrochloric acid, dialyzed against water at 4 °C using membranes with a 6-8 kDa cut-off and freeze dried. Yield (%, w/w) was calculated as the amount of recovered extracts (before dialysis) over the initial amount of cell wall material, multiplied by 100. Monosaccharide composition and MW distribution of extracted polysaccharides were also analyzed.

5.3.4. Statistical analysis

Analysis of the experimental results and calculation of predicted responses were carried out using Design Expert software (Version 8.0, Stat-Ease, Inc. Minneapolis, MN, USA). The ANOVA of the model, including lack of fit, Fisher's F-test, its associated probability and correlation coefficient was investigated. The variations of the responses [yield (%, w/w), MW distribution (>600 kDa, %) and monosaccharide profile (µmol/g extract)] as function of the independent variables, including concentration of KOH (x_1), extraction time (x_2), power (x_3) and solid/liquid ratio (x_4), were explained by the following empirical quadratic polynomial equation (Eq. 5.1):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (5.1)$$

The coefficients of the polynomial equations were represented by β_0 (constant term), β_i (linear coefficient), β_{ii} (quadratic coefficient), and β_{ij} (interaction coefficient). Y represents the response and x_i and x_j represent independent variables. Response surfaces were developed using fitted polynomial equations for most significant interaction terms.

5.3.5. Sugar content and monosaccharide composition analyses

Uronic acid and total sugar contents were measured using sulfamate/m-hydroxydiphenyl and phenol-sulfuric acid colorimetric assays, respectively (Blumenkrantz & Asboe-Hansen, 1973; Dubois et al., 1956).

In order to determine the monosaccharide composition, extracted polysaccharides were first hydrolyzed using a two-step procedure as previously described by Khodaei & Karboune, (2013). Extracted polysaccharides were incubated at 60 °C for 24 h in HCl/methanol mixture (1:4, v/v) at a ratio of 0.6% (w/v) and thereafter boiled for 1 h in trifluoroacetic acid solution at a ratio of 1:8 (v/v). Hydrolyzed samples were analyzed with HPAEC-PAD (Dionex), and a CarboPac PA20 column (3 × 150 mm). Isocratic elution was performed with 5 mM NaOH (0.5 ml/min). L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl and D-Man were used as standards.

5.3.6. MW distribution

MW distribution of the extracted polysaccharides was investigated by HPSEC on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL, Tosoh Bioscience, Montgomeryville, PA) connected to a Waters HPLC system equipped with refractive index detector and Breeze software for the data analysis. NaCl (0.1M) was used as mobile phase at a flow rate of 0.5 ml/min and 30 °C. Dextrans with MW of 50, 150, 270, 410 and 670 kDa were used as standards for calibration.

5.3.7. Isolation and characterisation of RG I-enriched fraction

The extracted polysaccharides were subjected to size exclusion chromatography on Superdex 200 16/600 (GE Healthcare, NJ) column connected to ÄKTA purifier system (GE Healthcare). The isocratic elution with 0.05 M sodium acetate at pH 5 containing 0.2 M NaCl at a flow rate of 1 ml/min was used. The fractions were analyzed using phenol-sulfuric acid colorimetric assay. The recovered RG I-enriched fraction was analyzed for their monosaccharide content.

5.3.8. Assessment of functional properties of RG I-enriched polysaccharides

Extracted RG I-enriched polysaccharides were assessed for their functional properties as compared to potato galactan and orange HG as standards.

Solubility: To determine solubility, 5% (w/v) suspensions of RG I-enriched polysaccharides, potato galactan and oranges HG, in 0.1 M sodium acetate (pH 6), were prepared. Samples were incubated at 25, 42, 59, 76 and 93 °C for 2 h, and mixed at room temperature for 1 h. After centrifugation at 14,000 ×g for 15 min, the amount of total sugars in the supernatants was measured using phenol-sulfuric acid colorimetric assay. Solubility was expressed as the amount of total sugars in the recovered supernatant over its initial concentration, multiplied by 100.

Viscosity: Viscosity of solutions containing 1% (w/v) RG I-enriched polysaccharides, potato galactan or orange HG was measured at 25, 49 and 73 °C using an AR2000 controlled-stress rheometer (TA, Crawley, U.K.) equipped with 60 mm acrylic parallel plate. Samples were heated for 2 h at selected temperatures before analysis and the shear rate varied between 1 and 100 s^{-1}. Ostwald–de Waele equation (Eq. 5.2) was used in order to define the flow behavior of the liquids.

$$\tau = K\left(\frac{\partial u}{\partial y}\right)^{n} \quad (5.2)$$

In which $\partial u/\partial y$ is shear rate (s^{-1}), τ is shear stress (Pa), K is the flow consistency index ($Pa.s^{n}$) and n is flow behavior index.

Emulsifying properties: In order to assess emulsifying properties, 0.2% (w/v) solutions of RG I-enriched polysaccharides, potato galactan and orange HG were prepared and adjusted to selected pH values of 4, 6 and 8. Polysaccharide solutions were mixed with soybean oil (5%, v/v) and passed three times through a homogenizer (EmulsiFlex-C5, Avestin Inc.) at operational pressure of 5000 – 7000 kPa. Droplet size was measured using Delsa™Nano Submicron Particle Analyzer (Beckman Coulter). Measurements were performed at 25 °C immediately after homogenization and after 2 h of incubation at 25, 49, 73 and 97 °C. Droplet size distribution was expressed by relative volume (v/v, %) of particles of each size in sample. In addition, the extent of droplet size increase was estimated as the droplet diameter (nm) after incubation over the value obtained before incubation. The droplet size distribution and the extent of droplet size increase in emulsions, prepared with potato galactan and as standards, were also measured.

5.4. Results and discussion

5.4.1. Yield, MW and monosaccharide profile of extracted polysaccharides

RSM was used in order to investigate the effects of selected extraction parameters, including x_1-concentration of KOH (M), x_2-extraction time (min), x_3-power (W) and x_4-solid/liquid ratio (%, w/v), on the yield of recovered polysaccharides (%), the proportion of high-MW polysaccharides (>600 kDa, %) and the monosaccharide profile of isolated polysaccharides. The extraction of galactan-rich RG I was monitored by assessing Gal, Ara and Rha contents in the polysaccharidic extracts. While Glc, Xyl and Man monosaccharide contents were used as indicators for the extraction of hemicellulosic polysaccharides as side-products. In addition, the extent of the hydrolysis of HG region under alkaline condition was evaluated by quantifying the

GalA content in the polysaccharidic extracts (Table 5.1). Alkaline extraction of cell wall is expected to isolate intact RG I through extensive hydrolysis of HG region by β-elimination and oxidative peeling (Zykwinska et al., 2006). The experimental design was performed based on the CCRD. The range and center point values of these parameters were set based on preliminary trials. The investigated solid/liquid ratios (0.2-3.8%, w/v) were within the same range (2-4%) as most of previous studies on microwave-assisted extraction of polysaccharides (Bélafi-Bakó et al., 2012; Fishman et al., 2009; Liu et al., 2006); however, Yeoh et al. (2008) and Liu et al. (2006) have used higher solid/liquid ratios of 6.3 and 8.0%, respectively, for the microwave-assisted extraction of pectin. On the other hand, the investigated extraction times (0-8 min) were shorter than those reported in other studies (1-30 min); and the power values (14-86 W, corresponding to 23.3-833.3 W/g cell wall) were within the same range reported for the extraction of pectic polysaccharides (15 to 1200 W/g) (Bélafi-Bakó et al., 2012; Fishman et al., 1999; Fishman et al., 2008; Kratchanova et al., 2004).

Tables 5.1 summarizes the results of the yield (%, w/w), the proportion of high-MW polysaccharides (>600 kDa, %) and the monosaccharide profile upon selected microwave-assisted alkaline extractions. The highest yield of 52.0% was achieved upon treatment n^{o} 8, in which the concentration of KOH, time and power were at + 1 level of design (1.5 M, 68 W, 6 min); while solid/liquid ratio was at -1 level of design (1.1%) (Table 5.1). Therefore, harsher extraction conditions of treatment n^{o} 8, in terms of power and extraction time, combined with low solid/liquid ratio (corresponding to high power/substrate ratio of 206.1 W/g cell wall) may have led to an efficient opening of the cell wall network and hence to a higher yield.

However, the low total monosaccharide content (3,889.8 µmol/g) of the polysaccharidic extract and the low relative molar proportion of Gal (44%) associated with the high molar proportion of GalA (28%) obtained with treatment n^{o} 8, reveal the extensive defragmentation of extracted polysaccharides and the debranching of their neutral side chains (estimated at 37.8% of total extracted polysaccharides) under harsher extraction conditions; indeed, GalA/Rha and Gal/Rha ratios at treatment n^{o} 8 were only 7.5 and 11.9, respectively (Table 5.1). The results also show that with treatment n^{o} 7 (68 W, 1.1%, 6 min) in which lower KOH concentration (0.5 M) was used as compared to treatment n^{o} 8, the yield and the defragmentation/debranching of extracted polysaccharides were less significant, estimated at 41.9% and 12.1%, respectively, with GalA/Rha and Gal/Rha ratios of 8.4 and 18.6, respectively.

Table 5.1 CCRD design and responses for yield (%), high molecular weight polysaccharides (>600 kDa, %) and monosaccharide composition (µmol/g).

Std order	x_1- KOH (M)	x_2- Time (min)	x_3- Power (W)	x_4- Solid/liquid[a] (%)	Power/solid (W)/g	Yield (%)	>600 kDa (%)	Gal (µmol/g)	Ara (µmol/g)	Rha (µmol/g)	Glc (µmol/g)	Xyl (µmol/g)	Man (µmol/g)	GalA (µmol/g)
1	0.5	2	32	1.1	97.0	28.8	57.0	2590.0 (55)[b]	262.2 (6)	86.2 (2)	257.3 (5)	51.9 (1)	73.2 (2)	1392.7 (30)
2	1.5	2	32	1.1	97.0	30.3	1.0	3997.0 (60)	432.8 (6)	103.5 (2)	739.1 (11)	315.2 (5)	237.4 (4)	851.1 (13)
3	0.5	6	32	1.1	97.0	32.4	40.9	2270.0 (53)	246.0 (6)	66.0 (2)	32.1 (1)	84.0 (2)	22.4 (1)	1537.2 (36)
4	1.5	6	32	1.1	97.0	39.9	7.1	2289.0 (47)	296.0 (6)	148.0 (3)	670.0 (14)	180.0 (4)	182.6 (4)	1148.8 (23)
5	0.5	2	68	1.1	206.1	37.6	20.8	3194.4 (61)	230.4 (4)	113.3 (2)	141.7 (3)	51.0 (1)	86.9 (2)	1454.0 (28)
6	1.5	2	68	1.1	206.1	39.6	6.3	3396.4 (64)	300.5 (6)	67.0 (1)	396.6 (8)	168.3 (3)	115.0 (2)	835.0 (16)
7	0.5	6	68	1.1	206.1	41.9	13.1	3245.9 (59)	266.0 (5)	174.5 (3)	145.0 (3)	23.5 (0)	171.7 (3)	1468.3 (27)
8	1.5	6	68	1.1	206.1	52.0	11.3	1711.0 (44)	240.0 (6)	144.0 (4)	443.4 (11)	110.0 (3)	161.7 (4)	1079.7 (28)
9	0.5	2	32	2.9	36.8	33.4	27.9	2963.0 (57)	130.0 (3)	164.0 (3)	196.1 (4)	31.9 (1)	77.2 (1)	1615.4 (31)
10	1.5	2	32	2.9	36.8	27.4	8.0	5381.8 (65)	402.7 (5)	188.6 (2)	951.4 (11)	355.0 (4)	251.8 (3)	776.7 (9)
11	0.5	6	32	2.9	36.8	31.6	10.5	3173.6 (58)	226.6 (4)	121.7 (2)	182.7 (3)	21.8 (0)	107.7 (2)	1602.0 (29)
12	1.5	6	32	2.9	36.8	37.4	7.8	3807.9 (58)	365.5 (6)	180.3 (3)	983.9 (15)	226.0 (3)	241.9 (4)	802.6 (12)
13	0.5	2	68	2.9	78.2	31.2	8.4	3326.3 (57)	223.6 (4)	179.0 (3)	283.0 (5)	24.2 (0)	191.9 (3)	1614.2 (28)
14	1.5	2	68	2.9	78.2	20.2	6.7	4558.0 (58)	513.0 (6)	107.0 (1)	1241.7 (16)	437.0 (6)	176.2 (2)	865.3 (11)
15	0.5	6	68	2.9	78.2	27.6	1.5	4482.0 (60)	392.4 (5)	215.8 (3)	638.0 (9)	37.4 (1)	436.7 (6)	1211.5 (16)
16	1.5	6	68	2.9	78.2	30.1	20.9	3424.9 (46)	629.6 (8)	147.8 (2)	1818.4 (24)	370.0 (5)	408.5 (5)	692.3 (9)
17	0.0	4	50	2.0	83.3	37.8	23.0	2887.6 (46)	214.0 (3)	172.3 (3)	288.8 (5)	9.1 (0)	128.1 (2)	2556.2 (41)
18	2.0	4	50	2.0	83.3	42.1	2.5	3605.0 (48)	423.0 (6)	154.0 (2)	1327.6 (18)	432.16 (6)	332.7 (4)	1200.0 (16)
19	1.0	0	50	2.0	83.3	29.5	16.7	3106.0 (77)	207.0 (5)	59.0 (1)	59.5 (1)	103.0 (3)	22.2 (1)	473.0 (12)
20	1.0	8	50	2.0	83.3	41.0	8.3	2266.0 (53)	394.4 (9)	140.6 (3)	464.1 (11)	75.9 (2)	153.3 (4)	762.6 (18)
21	1.0	4	14	2.0	23.3	38.9	28.5	2831.0 (57)	142.6 (3)	67.7 (1)	152.6 (3)	43.2 (1)	57.6 (1)	1633.6 (33)
22	1.0	4	86	2.0	143.3	39.9	13.7	3175.5 (63)	268.2 (5)	135.3 (3)	238.8 (5)	47.7 (1)	160.5 (3)	982.2 (20)
23	1.0	4	50	0.2	833.3	23.7	24.3	2763.0 (57)	342.9 (7)	131.0 (3)	356.2 (7)	94.9 (2)	144.4 (3)	983.0 (20)
24	1.0	4	50	3.8	43.9	15.5	12.9	6217.4 (60)	597.9 (6)	281.9 (3)	1706.6 (16)	189.2 (2)	349.3 (3)	1079.4 (10)
25	1.0	4	50	2.0	83.3	32.6	10.5	3616.9 (52)	473.0 (7)	217.0 (3)	1098.4 (16)	164.0 (2)	305.3 (4)	1015.0 (15)
26	1.0	4	50	2.0	83.3	29.7	16.0	4052.0 (54)	476.0 (6)	218.0 (3)	1221.1 (16)	201.6 (3)	299.5 (4)	1014.0 (14)
27	1.0	4	50	2.0	83.3	27.9	13.0	4061.0 (56)	519.1 (7)	193.1 (3)	1100.0 (15)	164.0 (2)	310.6 (4)	878.7 (12)
28	1.0	4	50	2.0	83.3	30.0	10.8	4255.5 (58)	421.8 (6)	183.8 (3)	1008.3 (14)	161.6 (2)	274.5 (4)	1012.0 (14)
29	1.0	4	50	2.0	83.3	28.6	11.5	4049.0 (54)	478.0 (6)	220.0 (3)	1090.0 (15)	129.1 (2)	304.0 (4)	1176.2 (16)
30	1.0	4	50	2.0	83.3	31.2	12.4	4060.0 (54)	474.9 (6)	217.0 (3)	1267.3 (17)	146.2 (2)	322.7 (4)	1050.5 (14)

[a] Constant extraction volume of 30 ml was used.

[b] Relative molar proportion of the monosaccharides (%).

On the other hand, treatment n° 20 (1.0 M KOH, 50 W, 2.0%, 8 min), with longer extraction time and lower KOH concentration than that of n° 8, resulted in a lower yield (41.0%), but similar defragmentation/debranching of extracted polysaccharides (31.9%) with GalA/Rha and Gal/Rha ratios of 5.4 and 16.1, respectively. These results show the significant effects of extraction time and concentration of KOH on the yield of extracted polysaccharides and their defragmentation/debranching. However, the use of milder extraction conditions, corresponding to treatment n° 1 (0.5 M KOH, 32 W, 1.1%, 2 min), resulted in the highest proportion of high-MW polysaccharides (>600 kDa, 57.0%) and in GalA/Rha and Gal/Rha ratios of 16.0 and 30.0, respectively; these results reveal the lower break down of polysaccharides and the limited debranching of its neutral side chains under milder conditions. Comparing treatments n° 2/4 and 10/12 reveals the significant effect of the extraction time (2 to 6 min), under constant microwave power, on the debranching of galactan side chains ; indeed, the Gal contents at treatments n° 2 and 10 (3,997.0-5,381.8 µmol/g extract) were higher than at treatments n° 4 and 12 (2,289.0 – 3,807.9 µmol/g extract). The highest amount of Gal and Rha (6,217.4 and 281.9 µmol/g extract, respectively; 60 and 3%) and relatively high amount of Ara (597.9 µmol/g extract, 6 %) were obtained upon treatment n° 24, in which the highest solid/liquid ratio of 3.8% (w/v), power of 50 W (corresponding to 44 W/g cell wall) as well as moderate incubation time of 4 min and KOH concentration of 1.0 M were used.

The high isolation of galactan-rich RG I at treatment n° 24 was accompanied by the high recovery of the hemicellulose polysaccharides (Glu+Xyl+Man, 2245.1 µmol/g), representing 22% of total sugars, and the moderate debranching of galactan side chain (Gal/Rha of 22.1). The highest Gal/Rha ratios of 50.7-52.6 were obtained upon treatments n° 6 and 19 characterizing by shorter extraction time of 0-2 min and higher power (83.8-206.1 W/g cell wall). As shown by the hemicellulosic monosaccharide contents (Glc, Xyl and Man), the combined use of high KOH concentration (≥ 1 M) and high solid/liquid ratio (≥ 2%) (treatments n° 10, 14, 16, 18, 24-30) favored the isolation of hemicellulosic polysaccharides (1,567.7-2,596.9 µmol/g; 19-35%). On the other hand, treatment n° 17, in which only water was used, led to the highest amount of GalA (2,556.2 µmol/g extract; GalA/Rha of 14.8), indicating the importance of alkaline conditions for the removal of HG region through β-elimination reaction. When concentration of KOH was increased to 0.5 M (treatments n° 1, 3, 5, 7, 9, 11, 13, 15) and 1.0 M (treatments n° 19-30), the

content of GalA decreased to 1,211.5-1,615.4 µmol/g extract (16-36%) and 473.0 - 1633.6 µmol/g extract (10-33%), respectively. However, no significant changes in the GalA contents (692.3 - 1,148.8 µmol/g extract; 9-28%) was observed as a result of further increase of KOH concentration to 1.5 M (treatments n^{o} 2, 4, 6, 8, 10, 12, 14, 16).

5.4.2. Regression analysis

Mathematical models were fitted to the experimental data using Design-Expert software version 8.0.2. and significance and adequacy of the models were tested with ANOVA (Table 5.2). Neglecting the insignificant terms by backward elimination regression (α =0.05), ANOVA results indicate that the quadratic model was statistically significant for the description of the variations of both yield (*F-value* of 27.2 and *p-value* of < 0.0001) and the proportion of high-MW polysaccharides (*F*-value of 26.7 and *p*-value of < 0.0001). For these two responses, lack of fit was not significant relative to pure error with *F* value of 1.5 and 3.3, respectively. The significance of each coefficient was determined using the *F* value and *P* value as shown in Table 5.2. As expected, solid/liquid ratio (x_4, *F* value of 67.4; *P* value of <0.0001) and extraction time (x_2, *F* value of 48.3; *P* value of <0.0001) were the most significant linear terms for the extraction yield. High extraction yield is expected to be achieved with an increase in the incubation time. The results also show that the concentration of KOH (x_1, *F* value of 92.6; *P* value of <0.0001) and power (x_3, *F* value of 40.7; *P* value of <0.0001) were the most significant linear terms for the proportion of high-MW polysaccharides. In terms of quadratic terms, solid/liquid ratio (x_4^2, *F* value of 45.3; *P* value of <0.0001) and concentration of KOH (x_1^2, *F* value of 44.7; *P* value of <0.0001) were the most important variables for yield, whereas power (x_3^2, *F* value of 11.4; *P* value of 0.0042) was the most significant for the proportion of high-MW polysaccharides.

Among all interactive effects, interaction between power and solid/liquid ratio (x3 x4, F value of 57.7; P value of < 0.0001) seems to be the most significant in the yield model, and the negative sign of its term (Eq. 5.3) reveals its antagonistic effect. Interaction between concentration of KOH and power (x1 x3, F value of 77.5; P value of < 0.0001) was the most significant in the high-MW polysaccharides model and was of synergistic type as demonstrated by the positive sign of this interactive term (Eq. 5.4) The fitted quadratic model for yield and high-MW polysaccharides are given by Equations 5.3 and 5.4 using coded variable names.

Table 5.2 The analysis of variance for response surface model for yield, molecular weight distribution and monosaccharide content (Gal, Ara, Rha and GalA).

Source	Yield (%) [a]		>600 kDa (%) [a]		Gal (μmol/g)[a]		Ara (μmol/g)[a]		Rha (μmol/g)[a]		GalA (μmol/g)[a]	
	F Value	P Value	F Value	P Value	F Value	P Value	F Value	P Value	F Value	P Value	F Value	P Value
Model	27.2	< 0.0001	26.7	< 0.0001	25.1	< 0.0001	20.5	< 0.0001	22.6	< 0.0001	22.6	< 0.0001
x_1-KOH (M)	4.6	0.0483	92.6	< 0.0001	13.3	0.0024	65.3	< 0.0001	0.8	0.3873	164	< 0.0001
x_2-Time (min)	48.3	< 0.0001	6.3	0.0238	26.3	0.0001	7.3	0.0164	19.6	0.0005	1.5	0.2431
x_3-Power (W)	4.6	0.048	40.7	< 0.0001	1.4	0.251	11.7	0.0038	8	0.0127	9.4	0.0078
x_4-Solid/liquid (%)	67.4	< 0.0001	31.6	< 0.0001	138.5	< 0.0001	31.2	< 0.0001	78.1	< 0.0001	0.4	0.5147
$x_1 x_2$	24.5	0.0002	32.3	< 0.0001	45.8	< 0.0001	6	0.0266	3.3	0.0878	1.8	0.1956
$x_1 x_3$	0.4	0.5168	77.5	< 0.0001	28.1	< 0.0001	0.1	0.713	37.7	< 0.0001	0.4	0.553
$x_1 x_4$	14.2	0.0019	61.7	< 0.0001	8.7	0.01	16.9	0.0009	1.5	0.2417	4	0.0627
$x_2 x_3$	0	0.8442	6.2	0.0248	2.8	0.115	4.7	0.0471	13.9	0.002	2.6	0.1301
$x_2 x_4$	3.7	0.0725	0	0.8488	4.8	0.0454	10.2	0.0061	4.3	0.0547	6.9	0.0191
$x_3 x_4$	57.7	< 0.0001	8.5	0.0106	0	0.9535	25.9	0.0001	2.4	0.1445	0.4	0.5157
x_1^2	44.7	< 0.0001	0	0.9937	14.2	0.0019	25	0.0002	15.1	0.0015	82.8	< 0.0001
x_2^2	12.8	0.0028	0	0.9034	42.6	< 0.0001	31	< 0.0001	80.8	< 0.0001	21.1	0.0003
x_3^2	39.6	< 0.0001	11.4	0.0042	24.6	0.0002	74.2	< 0.0001	78.3	< 0.0001	8.4	0.0109
x_4^2	45.3	< 0.0001	5.5	0.0331	5.6	0.0324	0	0.8908	0.2	0.6993	0	0.9183
Lack of Fit	1.5	0.3438	3.3	0.0986	1.9	0.2521	2.1	0.209	1.1	0.4746	1.9	0.2476

[a] R^2 = 0.93, 0.93, 0.92, 0.90, 0.91 and 0.91 for yield, molecular weight population, Gal, Ara, Rha and GalA respectively.

$P < 0.05$ indicates statistical significance.

Insignificant terms were dropped except for those that were necessary for maintaining the model's hierarchy.

$$\text{Yield } (\%) = 30.0 + 0.9x_1 + 2.8x_2 + 0.9x_3 - 3.3x_4 + 2.5x_1x_2 - 1.9x_1x_4 - 3.8x_3x_4 + 2.5{x_1}^2 + 1.4{x_2}^2 + 2.4{x_3}^2 - 2.6{x_4}^2 \quad (5.3)$$

$$> 600\ kDa\ (\%) = 13.1 - 7.2x_1 - 1.8x_2 - 4.3x_3 - 3.2x_4 + 4.1x_1x_2 + 6.9x_1x_3 + 6.1x_1x_4 + 2.2x_3x_4 + 2.18\,{x_3}^2 + 1.5\,{x_4}^2 \quad (5.4)$$

The results of ANOVA for the monosaccharide contents of extracted polysaccharides are shown in Table 5.2. Quadratic model was the most significant for Gal (*F*-value of 25.1 and *p*-value of < 0.0001), Ara (F-value of 20.5 and p-value of < 0.0001), Rha (*F*-value of 22.6 and *p*-value of < 0.0001) and GalA (*F*-value of 22.6 and p-value of < 0.0001) contents. Lack of fit was not significant with *F* value of 1.9, 2.1, 1.1 and 1.9 for Gal, Ara, Rha and GalA contents respectively, suggesting a good fit. Solid/liquid ratio was the most significant linear term in the Gal (x_4, *F* value of 138.5; *P* value of <0.0001) and Rha (x_4, *F* value of 78.1; *P* value of <0.0001) content models. In the Ara (x_1, *F* value of 65.3; *P* value of <0.0001) and GalA (x_1, *F* value of 164.0; *P* value of <0.0001) content models, concentration of KOH was the most significant linear term. The variable with the largest quadratic effect on Gal (x_1^2, *F* value of 42.6; *P* value of <0.0001) and Rha (x_1^2, *F* value of 80.8; *P* value of <0.0001) contents was the extraction time; while for Ara (x_3^2, *F* value of 74.2; *P* value of <0.0001) and GalA (x_4^2, *F* value of 82.8; *P* value of <0.0001) contents, power and concentration of KOH had the most significant quadratic effect respectively. The most important interactions for the Gal, Ara, Rha and GalA contents were the ones between KOH concentration/ time ($x_1\ x_2$, *F* value of 45.8; *P* value of < 0.0001), power/ solid to liquid ratio ($x_3\ x_4$, *F* value of 25.9; *P* value of < 0.0001), KOH concentration/Power ($x_1\ x_3$, *F* value of 37.3; *P* value of < 0.0001) and time/ solid to liquid ratio ($x_2\ x_4$, *F* value of 6.9; *P* value of 0.0191), respectively. These interactive effects were of antagonistic types in the Gal, Rha and GalA content models (Eq. 5.5, 5.7 and 5.8), whereas that of Ara content model (Eq. 5.6) exhibited a synergistic effect. Quadratic models were simplified by removing terms which were not statistically significant and can be described by the following equations using coded terms.

$$\text{Gal (µmol/g)} = 4015.7 + 198.2x_1 - 278.4x_2 + 64.8x_3 + 638.9x_4 - 449.9x_1x_2 - 352.3x_1x_3 + 195.9x_1x_4 + 145.1x_2x_4 - 191.2{x_1}^2 - 331.3{x_2}^2 - 252.0{x_3}^2 + 119.7{x_4}^2 \quad (5.5)$$

$$\text{Ara (µmol/g)} = 472.6 + 67.5x_1 + 22.6x_2 + 28.5x_3 + 46.6x_4 - 25.2x_1x_2 + 42.1x_1x_4 + 22.1x_2x_3 + 32.7x_2x_4 + 52.1x_3x_4 - 38.9\,{x_1}^2 - 43.4\,{x_2}^2 - 67.2\,{x_3}^2 \quad (5.6)$$

$$\text{Rha (µmol/g)} = 206.7 - 3.0x_1 + 14.7x_2 + 9.4x_3 + 29.3x_4 - 24.9x_1x_3 + 15.1x_2x_3 - 11.9{x_1}^2 - 27.7{x_2}^2 - 27.3{x_3}^2 \quad (5.7)$$

$$\text{GalA (µmol/g)} = 1021.7 - 314.8x_1 + 29.9x_2 - 75.4x_3 - 16.4x_4 - 79.0x_2x_4 + 209.7\,{x_1}^2 - 105.4\,{x_2}^2 + 67.1\,{x_3}^2 \quad (5.8)$$

These results indicate that the Gal content or the debranching of galactan side chains can be modulated by compromising between the concentration of KOH (x_1) and the extraction time (x_2), which exhibited an adverse interaction in the Gal content model. The concomitant increase of solid/liquid ratio (x_4) and power (x_3) is expected to accelerate the physical rupture of cell wall and increase concentration of arabinan side chains released into the liquid phase. Rha content, which is found in the backbone of RG I, is mainly affected by concentration of KOH (M) and power (W); indeed, increasing power and KOH concentration at the same time accelerates hydrolysis of RG backbone resulting in lower amount of Rha. Although the KOH concentration exhibited the most important linear effect on the GalA content, it didn't show any statistically significant interactive effects. However, a good compromise between solid/liquid ratio (x_4) and extraction time (x_2) needs to be considered to achieve an efficient removal of HG region.

5.4.3. Effects of microwave assisted-alkaline extraction parameters

2D contour plots (Figure 5.1a-c) generated from the predicted model of yield (%, w/w) at KOH concentration of 1.5 M (a factorial point) show the interactive effect of solid/liquid ratio and power upon selected extraction times. The extraction yield increases as the power increases and the solid/liquid ratio decreases; and increasing extraction time resulted in higher yields. The effect of power on the yield can be attributed to the efficient rupture/opening of cell wall under high microwave energy. Higher extraction time may have also led to a better interaction between the extracting agent and the cell wall material and to a high thermal accumulation. In addition,

the suspension of the cell wall in a high volume of alkaline solution (corresponding to smaller solid/liquid ratio) may have improved the diffusion rate of KOH solution into the cell wall structure and hence the absorption of microwaves by the swollen cell wall (Prakash Maran et al., 2013). Indeed, the efficient swelling of the cell wall particles suspended in the ionic solution can improve the direct absorption of the microwaves by the cell wall polysaccharides (Prakash Maran et al., 2013). An increase in the yield by increasing power and time and decreasing solid/liquid ratio has been reported in other studies (Kratchanova et al., 1996; Li et al., 2012; Prakash Maran et al., 2013; Wang et al., 2005; Yeoh et al., 2008). However, other few studies have shown a different pattern. For example, Liu et al. (2006) have reported an increase in the extraction yield of pectin from orange peels with the increase of solid/ liquid ratio from 2, 4 and 8%. On other hand, Prakash Maran et al. (2013) have found that increasing heating time up to 2.1 min and solid/liquid ratio up to 6.3% improved the extraction yield; and beyond this time and solid/liquid ratio, the extraction yield decreased. These authors have attributed this decrease to the degradation of pectin as a result of the excessive time exposure of cell wall to the microwave field and/or to the mass saturation affecting negatively the mass transfer rate of pectin into the extracting solution.

Figure 5.1d-f displays 2D contour plots of the predicted Ara content illustrating the interactions between solid/liquid ratio and power at a KOH concentration of 1.5 M. As expected, increasing the solid/liquid ratio increased significantly the absolute amount of arabinan side chains in the extracted polysaccharides. Increasing power up to 45-50 W and time up to 4 min increased the Ara content in the polysaccharide extracts; however, further increase of power to 68 W and extraction time to 8 min led to a decrease in the content of Ara. These results reveal the debranching of arabinan side chains, when time and power were increased. Zykwinska et al. (2006) have reported that arabinan side chains are highly mobile in potato cell wall.

The interactive effect of KOH concentration and extraction time on the predicted Gal content at a microwave power of 50 W exhibited a hyperbolic trend (Figure 5.1g-i). The horizontal lines of this hyperbolic trend reveal the pronounced effect of KOH concentration on the Gal content of the extracted polysaccharides at shorter extraction times (<4 min); indeed in this investigated space, increasing concentration of KOH resulted in a commitment increase of the Gal content of extracted polysaccharides. These results can be attributed to the ability of alkaline solution to open the cell wall structure and to solubilize the galactan-rich RG I.

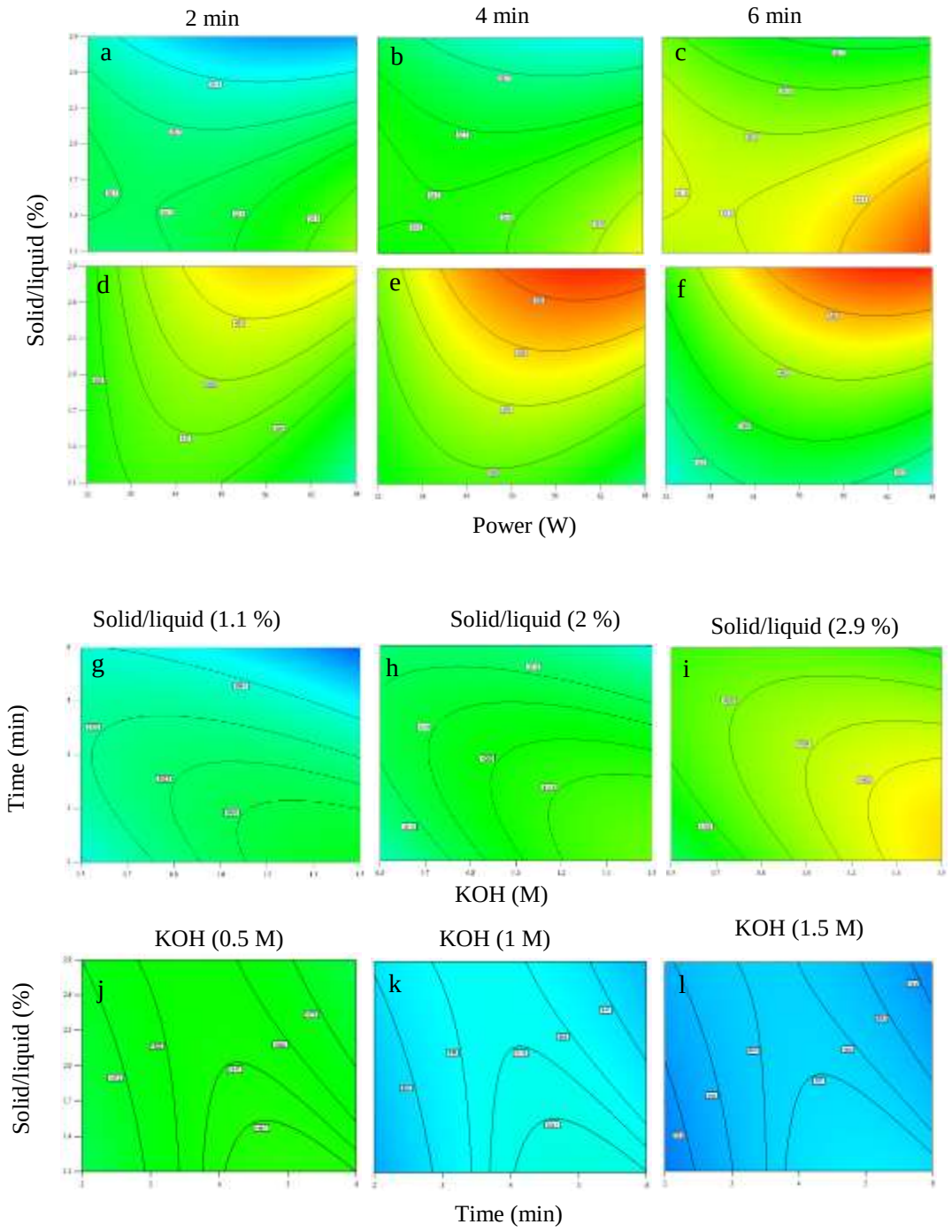

Figure 5.1 Contour plots of predictive models for yield (%) (a, b and c) and Ara content (µmol/g extract) (d, e and f) at KOH concentration of 1.5 M and for Gal (g, h and i) and GalA (j, k and l) content (µmol/g extract) at power of 50 W. The numbers inside the contour plots indicate the predicted responses.

At longer extraction times, no significant effect of the KOH concentration on the Gal content was observed. The vertical lines of the hyperbolic curves showed the significant negative effect of extraction time on the Gal content of extracted polysaccharides at higher KOH concentrations (>1.2 M). The results also indicate that the extraction time exhibited no or little effect on the Gal content of the extracted polysaccharides within the shorter time range (>3.0 min). The decrease in the Gal content with an increase in the extraction time at higher KOH concentrations can be attributed to the debranching of galactan side chains and/or to the defragmentation of RG I into oligomers. This negative effect of extraction time seems to be less pronounced at high solid/liquid ratios. These results can be attributed to the lower steric mobility of galactan side chain and/or to the higher diffusional mass limitations at high solid/liquid ratio (2.9%) as compared to low ratio.

Figure 5.1j-l shows the 2D contour plots of GalA content illustrating the interaction between extraction time and solid/liquid ratio at a microwave power of 50 W. Increasing extraction time up to 4-5 min increased the GalA content of extracted polysaccharides up to a maximum value, which decreased thereafter. These results can be explained by the fact that at shorter extraction times, the rate of the release of cell wall HG polysaccharides was higher as compared to the rate of their hydrolysis by β-elimination. However, further exposure of extracted HG to KOH caused their defragmentation. As expected, the higher is the KOH concentration, the lower is the GalA content of the extracted polysaccharides. Effect of solid/liquid ratio on GalA content seems to be dependent on the extraction time used. At shorter times (>4 min), GalA content of the extracted polysaccharides did not increase significantly with the solid/liquid ratio. However, at higher extraction time, GalA content as indicator of HG presence decreased with the increase of solid/liquid ratio. This effect may be explained by the presence of polymer/polymer interactions at high solid/liquid ratio, which may have favored the elimination of HG.

5.4.4. Optimum conditions for extraction of Gal

Using the predictive models, the optimum extraction conditions, which result in the highest amount of Gal and the lowest amount of GalA in the polysaccharide extracts (RG-enriched fraction), were identified. The identified optimum conditions corresponded to KOH concentration at + 1 level (1.5 M), solid/liquid ratio at + 1 level (2.9%), extraction time at – 1 level (2.0 min) and power close to – 1 level (36 W, corresponding to 41.4 W/g cell wall) of

design. As far as the authors are aware, optimization of microwave-assisted alkaline extraction of pectic polysaccharides has not been reported so far. Prakash Maran et al. (2013) have optimized microwave-assisted acidic extraction of pectin from dried orange peel using Box–Behnken response surface design. These authors have identified the optimum conditions being similar extraction time of 2.8 min, higher power of 422.0 W/g cell wall and higher solid/liquid ratio of 5.9% as compared to those obtained in this study. Moreover, Wang et al. (2005), who have used central composite design for the microwave–acidic extraction of pectin from dried apple pomace, have achieved optimum yield upon longer extraction time of 20.8 min at higher power of 499.4 W (249.7 W/g cell wall) and lower solid/liquid ratio of 0.069%. Comparing experimental and predicted values at the identified optimal conditions can be used to validate the predictive models (Table 5.3). These results indicate that there is no significant ($P < 0.05$) difference between the experimental data and the predicted values, and all experimental results are within the range of the estimated values with 95% prediction intervals. Using the identified optimal conditions, yield and productivity of RG-enriched polysaccharides were estimated at 21.9% (w/w) and 192 g/l.h. The extracted polysaccharides contained 71.0% of monosaccharides originating from RG I (Gal, Ara, Rha). In our previous study, lower yield of 9.5% and productivity of 1.2 mg/l.h were obtained upon the enzymatic extraction of RG I from potato cell wall with a purity of 85.0% (Khodaei & Karboune, 2014). However, similar yields of 18- 33% were reported for the microwave-assisted alkaline extraction of soluble polysaccharides from sugar beet pulp (Fishman et al., 2009). In contrast, Yeoh et al. (2008) have reported a low yield of 1.5% for the extraction of pectin from orange peel through microwave assisted-alkaline extraction. Furthermore, similar yields (15.7-32.4%) were obtained upon the microwave-assisted acidic extractions of pectin from orange peel (Guo et al., 2012; Prakash Maran et al., 2013), sugar beet pulp (Li et al., 2012) and apple pomace (Wang et al., 2005). In contrast, Bélafi-Bakó et al. (2012) have achieved higher yields of 59 - 84% upon the microwave-assisted acidic extraction of pectin from berry fruits.

Under the identified optimal conditions, no depolymerisation of extracted polysaccharides into oligomers was detected. The MW distribution of extracted polysaccharides showed two major populations (>600 kDa and <600 kDa) with higher proportion of 78.0% for the high-MW polysaccharides (>600 kDa). Lower proportions of high molecular-weight RG I-type polysaccharide population (>500 kDa) of 42.4-62.2% were obtained upon the enzymatic treatment of potato cell wall (Khodaei & Karboune, 2014).

Table 5.3 Optimum condition and corresponding responses, and sugar composition of the fractions.

x_1-KOH (M)	1.5			
x_2-Time (min)	2.0			
x_3-Power (W)	36.0			
x_4-Solid/liquid (%)	2.9			
Power (W)/g cell wall	41.4			
Responses				
	Experimental	Predicted	Fraction 1	Fraction 2
Yield (%)	21.9	26.8		
>600 kDa (%)	78.0	56.7		
<600 kDa (%)	19.1	14.1		
Polysaccharide (%)	100			
Gal [a]	4802.5(63.1)[b]	5383.8 (63.3)	4538.2 (75.3)	264.3 (17.5)
Ara	434.8 (5.7)	431.6 (5.1)	380.7 (6.3)	54.1 (3.6)
Rha	170.0 (2.2)	186.8 (2.2)	156.1 (2.6)	13.9 (0.9)
Glc	1000.9 (13.2)	1097.0 (12.9)	414.6 (6.9)	586.3 (38.8)
Xyl	373.5(4.9)	359.2 (4.2)	289.8 (4.8)	83.7 (5.5)
Man	226.7 (3.0)	245.9 (2.9)	160.5 (2.7)	66.2 (4.4)
GalA	597.1 (7.9)	806.3 (9.5)	84.7 (1.4)	443.3 (29.3)

[a] Monosaccharide composition is expressed in μmol per g of extract

[b] Relative molar proportion of monosaccharides.

Fishman et al. (2006) have reported that the MW of pectin extracted from lime flavedo/albedo/pulp decreased from 559 to 13 kDa, when the microwave-assisted acid treatment time was increased from 2.5 to 10 min. Pectin isolated from sugar beet by microwave-assisted acid extraction exhibited also high MW of 532- 1200 kDa (Fishman et al., 2008). However, low MW of alkaline-soluble pectin from sugar beet extracted with microwave method (83 - 299 kDa) was obtained (Fishman et al., 2009).

The results also indicate that the identified optimal conditions resulted in high content of Gal (4802.5 µmol/g extract, 63.1% mol/mol) in the extracted RG I-enriched polysaccharides. Similar Gal content of 66.7-71.8% has been reported for RG I polysaccharides obtained though enzymatic treatment (Byg et al., 2012; Khodaei & Karboune, 2014). RG-enriched fraction contained hemicellulosic polysaccharides, representing 21.1% of total molar monosaccharide content. The high molar proportions of Glc (13.2%), Xyl (4.9%) and Man (3.0%) suggest the extraction o, xyloglucan, heteromannans and heteroxylans as hemicellulosic polysaccharides. Higher molar proportions of hemicellulosic-based monosaccharides (Glc, 22%; Xyl, 18%; Man, 12%) were obtained upon the conventional alkaline extraction of potato RG I from cell wall (Khodaei & Karboune, 2013). In addition, the low molar proportion of GalA (7.9%) in the RG I extract confirms the efficient removal of HG region by microwave-assisted alkaline extraction as compared to the enzymatic extraction (GalA of 11.3-15.2%) (Khodaei & Karboune, 2014).

Extracted polysaccharides, under identified optimum conditions, were fractionated on preparative scale size exclusion chromatography (Table 5.3). Distribution of polysaccharides was similar to results obtained with HPSEC (two populations of polysaccharides i.e. >600 kDa and <600 kDa). High (fraction#1) and low (fraction#2) MW polysaccharides were collected and analyzed for their monosaccharide profile. High-MW fraction #1 exhibited higher molar proportions of monosaccharides originating from RG I, including Gal (75.3%), Ara (6.3%) and Rha (2.6%) as compared to low-MW fraction#2 (17.5, 3.6 and 0.9%, respectively). The high Gal content in the high-MW fraction#1 confirmed that major part of galactan side chains were not debranched and were linked to the backbone of RG I. Low-MW fraction#2 consisted of higher molar proportions of Glc (38.8%), Xyl (5.5%) and Man (4.4%) as compared to high-MW fraction#1 (6.9, 4.8 and 2.7%, respectively). These results reveal that most of hemicellulosic polysaccharides were present in low-MW fraction#2. The high molar proportion of GalA in low-MW fraction#2 (29.3%) reveals the extensive break down of HG.

5.4.5. Functional properties of extracted polysaccharides

5.4.5.1. Solubility and viscosity

Figure 5.2a displays the solubility of extracted RG-enriched polysaccharides and of standards (potato galactan and oranges HG) at selected temperatures. RG-enriched polysaccharides exhibited a solubility of 68.8% at 25 °C and 100% at 42-93 °C. The solubility of RG-enriched polysaccharides was higher than that of galactan (53.2-93.7%) and HG (12.8-19.0%). The solubility of polysaccharides is, generally, dependent on their structures. Uniform polysaccharides exhibit low solubility, while branched polysaccharides are more soluble (Whistler, 1973). Lower solubility of HG may be attributed to the fact that it is a linear polysaccharide with one type of the linkage and monosaccharide unit (Mohnen, 2008). RG-enriched polysaccharides with heterogeneous structure, consisting of different monosaccharide units and linkages, showed the best solubility.

Figure 5.2b_1-b_3 shows changes in shear stress (Pa) by changing shear rate (s^{-1}) from 1 to 100. For all samples (1% w/v) at all tested temperatures (25-73 °C), the flow behavior index (n) was close to one revealing their Newtonian behavior, except for RG-enriched polysaccharides at 49 °C which exhibited a pseudoplastic behaviour with flow behavior index of n<1 (0.6). Bélafi-Bakó et al. (2012) have reported that pectin gels, prepared with 40% saccharose and 5% (w/w) berry pectin obtained by conventional extraction, exhibited a Newtonian behavior, while those extracted with microwave method showed a flow behavior index of 0.7. On the other hand, Awasthi (2011) have shown that pectin had a Newtonian behavior at low concentration, and its pseudoplastic nature increased when its concentration was increased from 7.5 to 12.5.

In terms of the effect of temperature on the viscosity, it can be seen (Figure 5.2b_1-b_3) that galactan and RG–enriched polysaccharides showed similar pattern as their viscosity increased by increasing temperature to 49 °C and decreased at higher temperature of 73 °C . In contrast, HG showed the opposite trend as the viscosity of solution decreased slightly by increasing temperature to 49 °C and increased by further increase of temperature to 73 °C. In addition, RG-enriched polysaccharides resulted in the highest viscosity followed by galactan and HG. Owens et al. (1944) have reported that the relative viscosity of apple and citrus pectin at concentration of 0.05 – 0.5% didn’t vary with temperature; while at concentration of 0.5- 0.75%, viscosity decreased by increasing temperature from 0 to 50 °C.

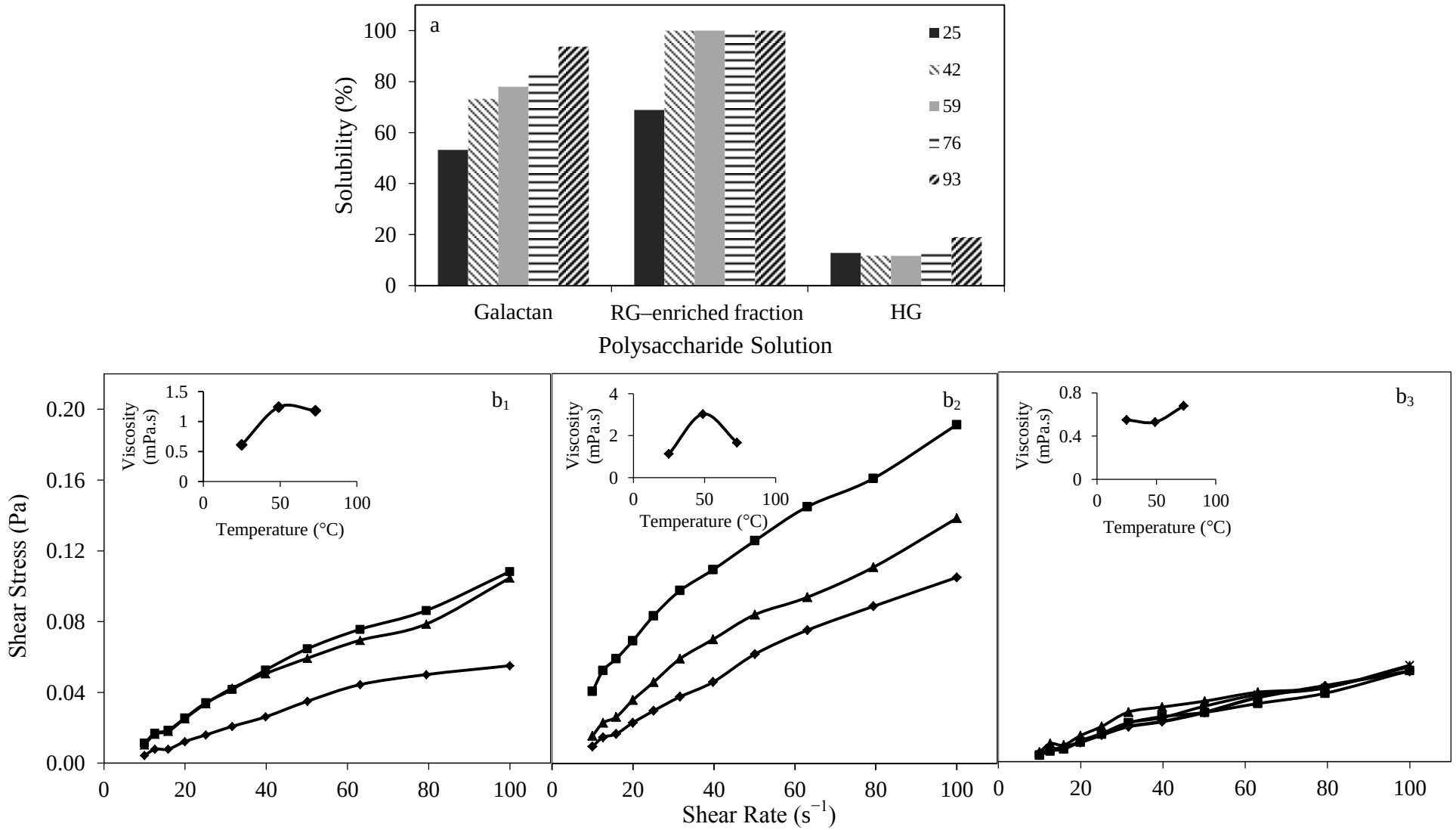

Figure 5.2 Solubility of galactan, RG-enriched polysaccharides and HG (5% (w/v)) (a) and changes in sheer stress as function of sheer rate and viscosity as function of temperature for solutions containing 1% of galactan (b_1), RG-enriched polysaccharides (b_2) and HG (b_3) at 25 °C ◆, 49 °C ■ and 73 °C ▲ and for water (25 °C) ✳. Chart equations at 25, 49 and 73 °C were $y=0.0004x^{1.0997}$, $y=0.0014x^{0.9603}$ and $y=0.0014x^{0.9469}$ for galactan, $y=0.001x^{1.0265}$, $y=0.01x^{0.6462}$ and $y=0.0022x^{0.9149}$ for RG–enriched polysaccharides, and $y=0.0005x^{1.024}$, $y=0.0004x^{1.0871}$, $y=0.0009x^{0.9274}$ and $y=0.0004x^{1.0822}$ for HG, respectively.

Moreover, Bélafi-Bakó et al. (2012) have reported that pectin obtained by microwave extraction produced higher viscosity at lower temperatures as compared to the pectin extracted with conventional extraction method.

5.4.5.2. Emulsifying properties

Droplet size distribution and stability of emulsions prepared with RG-enriched polysaccharides were assessed and compared with commercial potato galactan and oranges HG as standards. Galactan and HG were selected in order to highlight the effect of neutral sugar and GalA contents on the emulsifying properties. Because of deproteination and deacetylation/demethylation during extraction, the measured emulsifying properties can only be correlated to polysaccharides. Leroux et al. (2003) have reported that the pectins linked to protein and the acetylated ones exhibit higher emulsifying properties. Figure 5.3a-c shows droplet size distribution in the polysaccharide–enriched emulsions at selected pHs. At pH 6 and 8, RG-enriched emulsion showed sharper peaks with more homogeneous droplet size distribution (polydispersity index of 0.03- 0.04); as compared to pH 6 (943.3 nm) and 8 (900.2 nm), the diameter of the oil droplets of the RG-enriched emulsion at pH 4 (670.2 nm) was lower with a polydispersity index of 0.29. However, the HG-enriched emulsion showed lower droplet diameter distributions at pH 6 and 8 (447.8-594.7 nm, polydispersity index of 0.27-0.29) as compared to pH 4 (984.8 nm, polydispersity index of 0.33). These results may be explained by the higher charge of HG at neutral pH reducing the self-aggregation of HG chains. As expected, the pH did not affect significantly the droplet size distribution of galactan-enriched emulsions; smaller droplets of 460 to 508.3 nm were obtained with polydispersity index of 0.04-0.28.

Similarly, Nakauma et al. (2008) have reported that droplet diameters of soybean soluble polysaccharide and gum arabic-based emulsions were almost independent of pH varying from 3 to 6; however, an increase was observed at pH 6 for sugar beet pectin. These authors have shown that sugar beet pectin (550 nm) had higher emulsifying properties followed by soybean soluble polysaccharide (660 nm) and gum arabic (820 nm) using emulsifier/oil ratio of 0.1, 0.3 and 0.7 g/ml and two passes through a high-pressure homogenizer at 50000 kPa . Although lower emulsifier/oil of 0.04 g/ml and three passes at pressure of 5000 – 7000 kPa were used in the current study, the droplet diameter of RG–enriched emulsion at pH 4 (670.2 nm) was comparable to those reported for sugar beet pectin (550 nm) and soybean soluble polysaccharide (660 nm) (Nakauma et al., 2008).

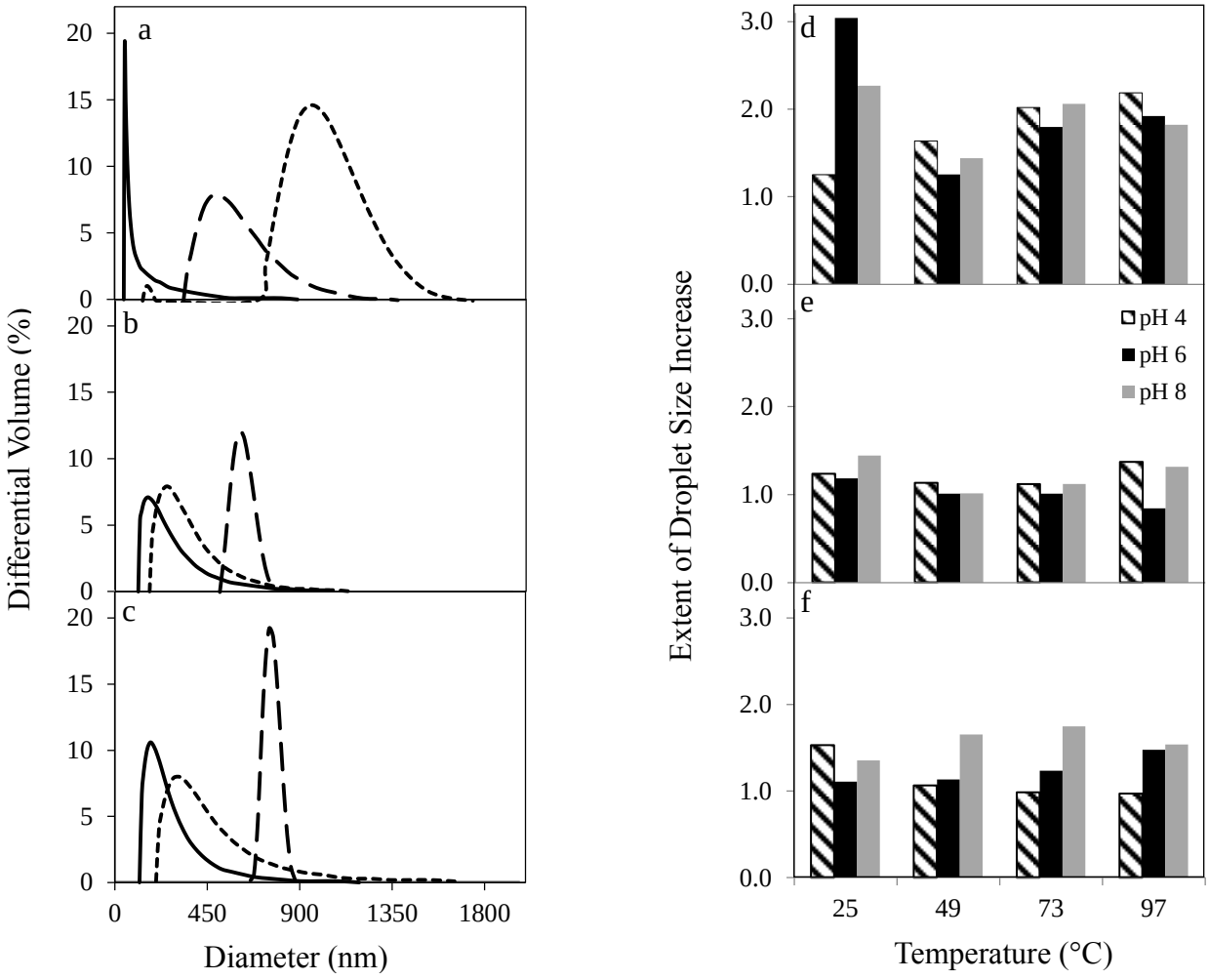

Figure 5.3 Particle size distribution for emulsions prepared at pH 4 (a), pH 6 (b) and pH 8 (c) containing galactan ——, RG-enriched polysaccharides — - and HG - - - - as emulsifier. Increase in particle diameter upon incubation of emulsions containing galactan (d), RG-enriched polysaccharides (e) and HG (f) for 2 h at 25, 49, 73 and 97 °C.

Leroux et al. (2003) have reported smaller droplet diameter of 310 nm for gum arabic-based emulsion at emulsifier/oil ratio of 2.5 and higher droplet diameter of 800 nm for citrus pectin-based emulsion at emulsifier/oil ratio of 0.4 at pH of 3.5. Figure 5.3d-f shows the extent of droplet size increase at different pHs and temperatures. As compared to galactan, heat treatment of RG-enriched emulsions resulted in lower increases of droplet diameters, indicating their high stability. HG-enriched emulsions were highly affected by the heat treatment at pH 8 and 6. These overall results show that the polysaccharide composed of either neutral sugar or GalA can only maintain the stability of emulsion at lower extent than polysaccharides containing both fractions. Highly branched structure of RG-enriched polysaccharides, promoting the steric hindrance, can also explain the stability of their emulsions. Effect of the polysaccharide stuctures on the emulsion stability was studied by Nakauma et al. (2008) who obtained a decrease in emulsion stability upon removal of neutral side chains (arabinan and galactan) of pectic polysaccharides. These authors attributed the effect of neutral side chain loss to the interfacial layer thickness reduction and hence the steric repulsion decrease between oil droplets.

5.5. Conclusion

Microwave-assisted alkaline extraction of galactan-rich RG I from potato cell wall by-products was investigated. Effects of four independent variables, including KOH (M), extraction time (min), power (W) and solid/liquid ratio (%, w/v), were studied using RSM with 5-level and 4-factor CCRD. The effects of extraction parameters and their interactions were elucidated through the developed quadratic mathematical models. A compromise between the combined effects of high KOH concentration/solid to liquid ratio and low power/extraction time was found to be a key for the efficient extraction of galactan-rich RG I and the limitation of their debranching. Using the identified optimal conditions, high yield and productivity of RG-enriched isolates were obtained. RG-enriched isolates was fractionated into two populations of polysaccharides, high (>600 kDa) and low (<600 kDa) MW ones. Galactan-rich RG I was recovered in the high-MW fraction (78%). Galactan-rich RG I polysaccharides exhibited higher solubility and emulsifying stability as compared to potato galactan and oranges HG. In addition, galactan-rich RG I polysaccharides showed Newtonian behavior at 25 and 73 °C and pseudoplastic behavior at 49 °C.

CONNECTING STATEMENT 4

In Chapters III, IV and V, alkaline (NaOH and KOH, 0.5, 1, or 2 M), enzymatic (endo-polygalacturonase from *A. niger*) and microwave-assisted alkaline extraction of potato galactan-rich RG I polysaccharides were studied and optimized. In Chapter VI, the enzymatic generation of oligosaccharides/oligomers from potato galactan-rich RG I using selected multi-enzymatic preparations was investigated at various reaction conditions. Yield, MW distribution and monosaccharide profile of generated oligosaccharides are discussed as a function of enzymeactivity profiles of the multi-enzymatic preparations. The multi-enzymatic preparations and the reaction conditions leading to the highest yield and Gal content as well as the lowest monosaccharide content are identified in this chapter.

The results from this chapter were presented at IFT *14th Annual Meeting of Institute of Food Technologists* and submitted to a scientific journal.

Khodaei, N., & Karboune, S. (2014) Enzymatic production of prebiotic oligosaccharides from potato rhamnogalacturonan I. IFT *14th Annual Meeting of Institute of Food Technologists, New Orleans, USA.*

Khodaei, N., & Karboune, S. (2015). Enzymatic production of oligosaccharides/oligomers from potato rhamnogalacturonan I (Submitted).

CHAPTER VI

ENZYMATIC PRODUCTION OF OLIGOSACCHARIDES / OLIGOMERS FROM POTATO RHAMNOGALACTURONAN I

6.1. Abstract

Because of the structural properties of potato galactan-rich RG I from potato pulp by-product, it can serve as a new source of NDOs with great potential as prebiotics. In the present study, the efficiency of selected multi-enzymatic preparations to generate oligosaccharides/oligomers from potato RG I was evaluated at various reaction conditions. Yield of oligo-RG I (oligosaccharides and oligomers) varied between 1.0 and 93.9% (w/w), and the monosaccharide content of final products was 0.0-34.2% (w/w), depending on the enzyme activity profile of the multi-enzymatic preparations, the reaction time and the enzymatic units. Oligo-RG I products were composed of oligosaccharides with a DP of 2-12 (79.8 - 100%), and oligomers with a DP of 13-70 (0.0 - 20.2 %). Highest oligo-RG I yield of 93.9 % was achieved using multi-enzymatic preparation with higher hydrolyzing activity toward side chains of RG I as compared to its backbone (Depol 670L), and Gal (58.9-91.2%, w/w) was the main monosaccharide of the oligo-RG I products followed by GalA (0.9-34.6%) and Ara (0.0-12.1%).

6.2. Introduction

In recent years, NDOs have emerged as an important dietary carbohydrate class because of their health benefits, in particular their prebiotic activity. Indeed, prebiotics can selectively stimulate the growth of beneficial bacteria in the gut and reduce the risk of colon cancer (Al-Sheraji et al., 2013; Gullón et al., 2013). As a consequence, there is a great interest in the development of novel NDOs with improved prebiotic activity and other physiological properties. The plant cell wall polysaccharides have been identified as potential sources for the production of novel prebiotics. So far, the use of these sources has been little-explored, despite their high availability and relatively low cost. Indeed, plant cell wall polysaccharides are often present in large amounts in fiber-rich by-products. In addition, prebiotics derived from cell wall polysaccharides would be structurally different from those currently commercially available and might offer stronger modulation of gut microbiota (Gullón et al., 2013).

Most studies (Chen et al., 2013; Olano-Martin et al., 2001) have, so far, focused on the production of oligosaccharides from cell wall pectic polysaccharides, specifically HG. For instance, Olano-Martin et al. (2001) have reported the enzymatic conversion of low and high methoxyl pectin to pectic oligosaccharides with a yield of 95% using endo-polygalacturonase. Cell wall pectic polysaccharides (56%, w/w) in the potato pulp by-product is mainly composed

of RG I (75%, w/w) that is highly ramified with β-linked galactan side chains (67%, w/w) (Mohnen et al., 2008; Oomen et al., 2003). Potato cell wall polysaccharides has been little explored as a source of prebiotic oligosaccharides. However, high-MW soluble pectic polysaccharides from potato cell wall were found to exhibit promising prebiotic activity as they selectively stimulated the growth of beneficial microflora and produced SCFA upon their fermentation (Lærke et al., 2007; Olesen et al., 1998; Thomassen et al., 2011). Furthermore, Michalak et al. (2012) have generated a spectrum of hydrolysates from potato pulp polysaccharides (<10 and >10 kDa) and potato galactan (<3, 3-10 and 10-100 kDa), using endo-1,4-β-galactanase from *E. nidulans* as biocatalyst; these hydrolysates were found to promote the growth of *B. longum* and *L. acidophilus* more efficiently than FOS, Gal and unhydrolyzed galactan, while they did not support the growth of *C. perfringens* in single culture fermentations.

Considering their structural properties, it can be hypothesized that potato galactan-rich pectic polysaccharides can serve as new sources of galacto- and galacto(arabino)-oligosacharides/oligomers. Galacto-oligosaccharides are well recognized for their bifidogenic activity and their higher inhibitory effects against attachment of enteropathogenic *E. coli* to HEp-2 and Caco-2 cells as compared to inulin or FOS (Macfarlane et al., 2008; Shoaf et al., 2006). Currently commercial galacto-oligosaccharides Glc α1–4[β Gal 1–6]n, in which n = 2–5) are produced by the transgalactosylation of lactose by β-galactosidase (Crittenden & Playne, 1996; Rastall, 2010). To favor β-galactosidase-catalyzed transgalactosylation reactions, high substrate concentrations are required. Because of the low solubility of lactose, the yield of galacto-oligosaccharides was limited varying between 10 and 50% (Torres et al., 2010).

Considering the complex structure of cell wall polysaccharides, their efficient hydrolysis into oligosaccharides/oligomers requires well-defined glycosyl-hydrolases as biocatalysts that can act synergistically. A combination of mono-component enzymes (pectin-methylesterase, rhamnogalacturonase, galactanase, arabinanase) has been used in one-step reaction or sequentially for the production of rhamno-oligosaccharides, homogalacturonides and rhamnogalacturonides from sugar beet (Bonnin et al., 2002; Holck et al., 2011). Contrary to mono-component enzymes, the natural multi-enzymatic preparations act more synergistically and are cheaper. The use of the multi-enzymatic preparations, Celluclast 1.5L from *T. reesei* and Viscozyme L from *A. aculeatus*, for the production of oligosaccharides from orange peel waste (Martínez Sabajanes et al., 2012) and sugar beet pulp (Martínez et al., 2009; Martínez Sabajanes

et al., 2012) has been reported. However, the multi-enzymatic preparations have not been explored, so far, for the generation of non-digestible oligosaccharides/oligomers from potato cell wall. As part of an ongoing research, this study was aimed at the investigation of the enzymatic generation of galacto-, galacto(arabino)-oligosaccharides from potato galactan-rich RG I-type pectic polysaccharides using pure mono-component enzymes and multi-enzymatic preparations. The yield, the monosaccharide profile and the DP of generated oligosaccharides were determined, and the effect of the enzyme activity profiles of mono-component enzymes and multi-enzymatic preparations was discussed.

6.3. Materials and methods

6.3.1. Materials

Gamanase 1.5L was obtained from Novo Nordisk Bioindustrial Inc. (Danbury, CT). Depol 670L was kindly provided by Biocatalysts Ltd. (Mid Glamorgan, UK). Iogen HS 70 was obtained from Iogen Bio-Products, while Newlase II were from Amano Enzyme (USA). Pectinex Ultra SPL and Viscozyme L were from Novozymes, (Ca) and endo-polygalacturonase, endo-1,4-β-galactanase, endo-1,5-α-arabinanase, soybean RG and potato pectic galactan were from Megazyme (Wicklow, Ir). Arabinan, polygalacturonic acid from oranges, KOH, sodium acetate, acetic acid, dextran standards, Gal, Ara, Rha, Glc, Xyl, Man, GalA, trifluoroacetic acid, thermostable α-amylase from *B. licheniformis* and amyloglucosidase from *A. niger*, were purchased from Sigma Chemical Co. (St-Louis, MO, USA). 1-kestose, nystose and 1F-fructofuranosyl- nystose were obtained from Wako Pure Chemical (Japan). All salts were obtained from Fisher Scientific (Fair Lawn, NJ).

6.3.2. Preparation of RG I polysaccharides from potato cell wall material

Prior to the extraction of pectic polysaccharides, protein and starch were removed from potato pulp as described previously by Khodaei & Karboune (2013). Microwave-assisted alkaline extraction method was used for the isolation of galactan-rich RG I-type pectic polysaccharides from potato cell wall using the previous identified optimized conditions (Khodaei et al., 2013d). Isolated cell wall material was suspended in 1.5 M potassium hydroxide solution to yield solid/liquid ratio of 2.9% (w/v). The suspension was heated for 2 min under power of 36 W in a cylindrical Pyrex tube in a Prolabo Synthewave 402 Microwave Reactor. Solubilized polysaccharides were recovered upon filtration through Glass microfiber filter grade GF/D with

pore size of 2.7 µm (Whatman®). Filtrate was neutralized using hydrochloric acid, dialyzed against water at 4 °C using tubes with a 6-8 kDa cut-off and freeze dried. Extracted polysaccharides were analyzed for their MW distribution by HPSEC.

6.3.3. Assessment of glycosyl-hydrolase activities of selected multi-enzymatic products

Selected multi-enzymatic preparations (Depol 670L, Gamanase 1,5L, Iogen HS 70, Newlase II, Pectinex Ultra SPL and Viscozyme L) were assessed for their pectinase, rhamnogalacturonase, galactanase and arabinanase activities. 0.025 ml of multi-enzymatic product was added to 0.475 ml of substrate solution of 0.25% polygalacturonic acid, 0.55% soybean RG, 0.55% potato pectic galactan or 0.55% arabinan for pectinase, rhamnogalacturonase, galactanase and arabinanase activity, respectively. Substrate solutions were prepared in 50 mM sodium acetate buffer, pH 5.0 at concentration of 0.5% (w/v), and the reaction mixtures were incubated at 40 °C for 20 min. Two blank tests were also performed without substrate or without enzyme. The reducing ends were quantified using DNS assay and the enzymatic activity unit (U) was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

6.3.4. Enzymatic hydrolysis of galactan-rich RG I-type pectic polysaccharides

The time course for the hydrolysis of galactan-rich RG I-type pectic polysaccharides was carried out over a reaction time of 24 h. Pectic polysaccharides suspensions in sodium acetate buffer (50 mM, pH 5.0) at selected concentrations of 0.2, 0.5 and 1% (w/v) were prepared. The multi-enzymatic products (Depol 670L, Gamanase 1,5L, Iogen HS 70, Newlase II, Pectinex Ultra SPL and Viscozyme L) or mono-component enzymes (endo-1,4-β-galactanase and endo-1,5-α-arabinanase) were added to the pectic polysaccharides suspensions to yield final enzyme/substrate ratio of 0.1, 0.2 and 0.4 galactanase U/mg substrate. After incubation at 40 °C, the reaction mixtures were boiled for 5 min in order to inactivate enzyme. Unhydrolyzed polysaccharides were precipitated by adding equal volume of ethanol and removed by centrifugation (10000 ×g, 10 min). The progress of the hydrolysis was monitored by measuring the release of reducing sugars using DNS assay in which 0.75 ml of DNS reagent (1% (w/v) DNS in 1.6% (w/v) sodium hydroxide) was added to 0.5 ml of reaction samples. After boiling the mixtures for 5 min, 0.25 ml of potassium sodium tartrate (50%, w/v) was added, and the absorbance was read at 540 nm. Standard curve was plotted using selected concentrations of Gal.

Oligosaccharides (DP of 2 to 12) and oligomers (DP of 3 to 70) were also quantified by HPSEC. Yield was defined as the amount of generated oligosaccharides/oligomers over the initial amount of potato RG I-type pectic polysaccharides. The MW distribution of generated oligosaccharides was also determined by HPSEC, and their monosaccharide profile was determined by HPAEC.

6.3.5. Characterization of structural properties

The MW distribution of carbohydrates was carried out by HPSEC using Waters HPLC system equipped with refractive index detector and Breeze software for the data analysis. For the analysis of the MW distribution of pectic polysaccharides, three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL (Tosoh Bioscience, Montgomeryville, PA) were used. Isocratic elution with 0.1 M sodium chloride was applied at flow rate of 0.5 ml/min; dextrans with MW of 50, 150, 270, 410 and 670 kDa were used as standards. To determine the yield and the MW distributions of oligosaccharides, hydrolysates were separated on TSKgel G-Oligo-PW column (Tosoh Bioscience, Montgomeryville, PA). Isocratic elution with 0.1 M sodium chloride was applied at flow rate of 0.5 ml/min; and Gal, sucrose, 1-kestose, nystose and 1F-fructofuranosyl- nystose were used as standards for calibration.

To determine the monosaccharide profile, the carbohydrates (oligosaccharides, oligomers, polysaccharides) were hydrolyzed using a two-step procedure, according to the method of Khodaei & Karboune (2013). They were first hydrolyzed with HCl/methanol mixture (1:4, v/v) at 60 °C for 24 h, and then trifluoroacetic acid was added at a ratio of 1:8 (v/v). After 1 h boiling, the monosaccharide profile of hydrolyzed samples was measured by HPAEC using Dionex system equipped with a PAD and a CarboPac PA20 column (3 × 150 mm). Mobile phase of 5 mM sodium hydroxide was used at an isocratic flow of 0.5 ml/min. L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl and D-Man were used as standards. GalA content was measured using sulphamate/m-hydroxydiphenyl colorimetric assay (Blumenkrantz & Asboe-Hansen, 1973).

The profile of generated oligosaccharides was also analyzed by HPAEC-PAD on CarboPac PA200 column. For the elution of oligosaccharides, gradient of 0 to 100% of 200 mM sodium acetate in 100 mM NaOH for 20 min was used as a mobile phase. Sucrose, raffinose, 1-kestose, nystose and 1F-fructofuranosyl- nystose were used as internal standards to identify peaks.

6.4. Results and discussion

6.4.1. Structural properties of extracted galactan-rich RG I-type pectic polysaccharides

In our previous study (Khodaei et al., 2013d), the optimum conditions for microwave-assisted alkaline extraction of galactan-rich RG I-type pectic polysaccharides were identified to be KOH concentration of 1.5 M, extraction time of 2.0 min, microwave power of 36.0 W and solid/liquid of 2.9% (w/v). Table 6.1 shows the monosaccharide profile and the MW distribution of extracted polysaccharides. The results show that 71% of monosaccharides in the extracted polysaccharides are those originating from galactan-rich RG I (Gal, Ara and Rha) with Gal being the major monosaccharide (63.1%, mole/mole). In previous studies, polysaccharide extracts from potato pulp were reported to be composed of 77.2% (Michalak et al., 2012) and 69.0% (Thomassen et al., 2011) of RG I. The experimental findings also indicate that extracted polysaccharides contained low amounts of Glc (13.2%), Xyl (4.9%) and Man (3.0%) originating from hemicellulosic polysaccharides. In addition, low content of GalA in the extract (7.9%) reveals the high hydrolysis rate of HG by β-elimination during the microwave-assisted alkaline extraction. The MW distribution indicates the presence of two polysaccharide populations (>600 kDa; <600 kDa) with the high-MW fraction being the most abundant one (80.4%, w/w). Since the polysaccharide extract was dialyzed using 6-8 kDa cut-off, no oligosaccharide was detected.

6.4.2. Characterization of the activity profile of selected multi-enzymatic preparations

The efficiency of the synergistic actions of glycosyl- hydrolase enzymes present in a multi-enzymatic preparations was reported to be higher than that of their individual action (Karboune et al., 2009). Considering the structural characteristics of potato RG I-type pectic polysaccharides, pectinase (EC. 3.2.1.15), rhamnogalacturonase (EC 3.2.1.171), endo-1,4-β galactanase (EC 3.2.1.89) and endo-1,5-α arabinanase (EC 3.2.1.99) were identified as the appropriate glycosyl-hydrolases for the tailored hydrolysis of potato pectic polysaccharides into oligosaccharides and oligomers. Indeed, up to 80% of Rha residues of potato RG I are substituted at O-4 with galactan, arabinan and arabinogalactan (Harris, 2009). The levels of the selected glycosyl hydrolase activities were assessed in six multi-enzymatic preparations: Depol 670L from *T. reesei*, Gamanase 1,5L from *A. niger*, Iogen HS 70 and Newlase II from *Rhizopus niveus* and Pectinex Ultra SPL and Viscozyme L from *A. aculeatus*.

Table 6.1 Sugar composition and molecular weight of extracted RG I using microwave assisted -alkaline extraction method (KOH concentration: 1.5 M, time: 2.0 min, power: 36.0 W, solid/liquid: 2.9%).

RG properties	Values
>600 kDa (%)	78.0
<600 kDa (%)	19.1
Polysaccharide (%)	100.0
Gal [a]	4802.5(63.1)[b]
Ara	434.8 (5.7)
Rha	170.0 (2.2)
Glc	1000.9 (13.2)
Xyl	373.5(4.9)
Man	226.7 (3.0)
GalA	597.1 (7.9)

[a] Monosaccharide composition is expressed in μmol per g of extract

[b] Relative molar proportion of monosaccharides.

Different glycosyl-hydrolase activities were reported to be expressed in the selected multi-enzymatic preparations: Depol 670L (β-glucanase, arabinanase, endoglucanase, xylanase, polygalacturonase) (Vafiadi et al., 2008), Gamanase 1,5L (β-1-4 mannanase, xylaanase) (Wang et al., 2015), Iogen HS70 (xylanase), Pectinex Ultra SPL (polygalacturonase, cellulose) (Soffer & Mannheim, 1994) and Viscozyme L (β-glucanase, xylanase, cellulase, hemicellulase). Mono-component enzymes (endo-1,4-β-galactanase and endo-1,5-α-arabinanase) were also investigated in order to compare their efficiency for the generation of oligosaccharides and oligomers with those of multi-enzymatic preparations. The specific activity of mono-component enzymes were 506.0 and 15.1 U/mg for endo-1,4-β-galactanase and endo-1,5-α-arabinanase from *A. niger*, respectively. As shown in Table 6.2, some of the investigated multi-enzymatic preparations showed higher enzymatic activities toward side chains of RG I (galactan and arabinan), while other exhibited higher levels of pectin backbone-hydrolyzing activities (RG I and HG regions). Depol 670L expressed the highest level of galactanase activity (6751.1 U/ml, 230.9 U/mg protein) and the highest enzymatic ratio of galactanase+arabinanase/rhamnogalacturonase (121.6), revealing its high hydrolyzing effectiveness toward side chains of RG I rather than its backbone. Other multi-enzymatic preparations expressing higher enzymatic activity toward galactan side chains include Gamanase 1.5L and Iogen HS 70 with a galactanase+arabinanase/ rhamnogalacturonase ratio of 33.0. These results also reveal the high relative proportion of the rhamnogalacturonase enzymatic units in Gamanase 1.5L and Iogen HS 70 as compared to Depol 670 L. However, both Gamanase 1.5L and Iogen HS showed a low catalytic efficiency for hydrolyzing arabinan side chains with an arabinanase activity of 0.1 U/mg protein and a galactanase/arabinanase ratio of 32.0-166.0. No arabinanase and rhamnogalacturonase activities were detected in the Newlase II, while the levels of pectinase and galactanase present in this multi-enzymatic preparation were similar (10.8 U/ mg protein). Viscozyme L showed the highest level of arabinanase activity (200.0 U/ml, 11.2 U/mg protein) and the lowest galactanase/arabinanase ratio (13.2). As expected Pectinex Ultra SPL had the highest pectinase activity level (1600.0 U/ml, 226.2 U/mg protein) and relatively high arabinanase one (63.0 U/ml, 8.9 U/mg protein).

Table 6.2 Pectin-hydrolyzing enzymatic activities expressed in multi-enzymatic preparations [a]

Multi-enzymatic preparations	Enzymatic Activity (U [b,c]/ml)				Enzymatic Activity Ratio (U/U)		
	Pectinase	Rhamogalacturonase	Galactanase	Arabinanase	Galactanase/ Arabinanase	(Galactanase+Arabinanase) /Rhamnogalacturonase	Pectinase/ Galactanase
Depol 670L	0.0 (0.0) [d]	56.0 (1.9)	6751.0 (230.9)	57.0 (1.9)	118.4	121.6	0.0
Gamanase 1,5L	66.0 (4.4)	5.0 (0.3)	166.0 (11.0)	1.0 (0.1)	166.0	33.4	0.4
Iogen HS 70	2.0 (0.2)	1.0 (0.1)	32.0 (2.8)	1.0 (0.1)	32.0	33.0	0.1
Newlase II [e]	34.0 (10.8)	0.0 (0.0)	34.0 (10.8)	0.0 (0.0)	-	-	1.0
Pectinex Ultra SPL	1600.0 (226.2)	90.0 (12.7)	1210.0 (171.1)	63.0 (8.9)	19.2	14.1	1.3
Viscozyme L	2996.0 (167.5)	218.0 (12.2)	2636.0 (147.4)	200.0 (11.2)	13.2	13.0	1.1

[a] All measurements were run in triplicates and the relative standard deviations were less than 10%.

[b] Each unit of enzymatic activity (U) was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

[c] Pectinase, rhamogalacturonase, galactanase and arabinanase activities were measured using polygalacturonic acid, soybean RG, potato pectic galactan and arabinan as substrates, respectively.

[d] Specific activity per milligram of total protein (expressed in U/mg protein).

[e] Enzymatic activity was expressed in U/mg of enzyme.

6.4.3. Enzymatic generation of potato oligo-RG I

The hydrolysis of potato RG I-type pectic polysaccharides by selected multi-enzymatic preparations was investigated, over a 24 h time course using well-defined galactanase units (1-2 U/ g substrate), by the estimation of the released reducing ends. The results (data not shown) showed that the maximum hydrolysis yields were achieved at 7 h, and no significant change was observed thereafter. In addition, the initial RG I-type pectic polysaccharide concentration of 0.5% (w/v) resulted in the highest hydrolysis rate as compared to 0.2 and 1%. The low hydrolysis rate at 1% may be attributed to the presence of polysaccharide/polysaccharide and/or polysaccharide/enzyme interactions limiting the access of the enzyme's active site to the substrate. Therefore, the reaction times of 0.5, 4 and 7 h as well as a substrate concentration of 0.5% (w/v) were subsequently used. These reaction times and substrate concentration fall within the same range as those reported in the literature for the enzymatic generation of oligosaccharides. Michalak et al. (2012) have used reaction times of 0.25, 1.0 and 4.5 h for the hydrolysis of galactan and potato pulp polysaccharides with endo-1,4-β-galactanase. Bonnin et al. (2002) have investigated the hydrolysis of sugar beet pectin into rhamno-oligosaccharides by a mixture of pectin-methylesterase, rhamnogalacturonase, galactanase and arabinanase activities using a substrate concentration of 0.8% and a reaction time of 1.5 h. Moreover, Holck et al. (2011) have isolated homogalacturonides and rhamnogalacturonides from sugar beet pectin by the sequential use of mono-component enzymes at substrate concentrations of 0.75-3% and reaction times of 2-16 h. However, higher substrate concentration of 8.3% and similar reaction times of 4-16 h have been used by Martínez et al. (2009) and Martínez Sabajanes et al. (2012) who have investigated the hydrolysis of orange peel waste and sugar beet pulp by Celluclast 1.5L from *T. reesei* and Viscozyme L from *A. aculeatus*, respectively.

Table 6.3 summarizes the yield of oligo-RG I (oligosaccharides, oligomers) and their MW distribution as well as the yield of monosaccharides. Because monosaccharides can be faster metabolized as an energy source and increase the caloric content of final product, their presence in prebiotic products is not favorable. In addition, it has been shown that hydrolyzed galactans and solubilized potato polysaccharides showed better prebiotic properties than pure Gal (Michalak et al., 2012). Commercially available galacto-oligosaccharides were reported to be composed of higher amounts of monosaccharide (Glc, 20%) and lactose (~20%) with an oligosaccharide content of more than 55% (Sako et al., 1999).

Table 6.3 Yield and MW distribution of generated hydrolysates [a]

Multi-enzymatic preparation	Time	0.1 U [b]/mg substrate				0.2 U/mg substrate			
		Yield (%,w/w) [c]		MW distribution (%, w/w)		Yield (%,w/w)		MW distribution (%, w/w)	
		Oligo-RG I [d]	Monosaccharide	Oligosaccharide (DP 2-12)	Oligomer (DP 13-70)	Oligo-RG I	Monosaccharide	Oligosaccharide (DP 2-12)	Oligomer (DP 13-70)
Depol 670L	0.5	3.1	0.0	90.2	9.8	3.4	0.0	89.8	10.2
	4	16.7	0.2	90.0	10.0	93.9	2.3	89.7	10.3
	7	60.2	4.0	97.5	2.5	82.3	14.0	79.8	20.2
Gamanase 1,5L	0.5	1.7	0.0	93.2	6.8	1.0	0.0	92.6	7.4
	4	21.9	0.2	92.0	8.0	43.4	1.5	92.1	7.9
	7	24.6	0.5	91.7	8.3	41.9	4.9	93.7	6.3
Iogen HS 70	0.5	43.9	5.0	97.9	2.1	48.7	2.9	99.9	0.1
	4	26.4	22.7	93.7	6.3	26.3	26.6	100.0	0.0
	7	23.5	25.8	100.0	0.0	27.6	29.4	100.0	0.0
Newlase II	0.5	3.2	0.1	97.0	3.0	26.7	0.9	98.7	1.3
	4	3.0	0.3	100.0	0.0	17.3	10.4	99.7	0.3
	7	3.2	0.3	96.6	3.4	22.4	13.7	98.9	1.1
Pectinex Ultra SPL	0.5	5.2	0.1	94.1	5.9	54.5	5.7	94.4	5.6
	4	24.8	0.6	95.0	5.0	30.1	30.8	88.1	11.9
	7	39.7	1.2	95.0	5.0	27.3	34.2	89.0	11.0
Viscozyme L	0.5	60.8	0.7	94.0	6.0	65.6	4.8	93.2	6.8
	4	41.2	21.2	95.3	4.7	61.9	10.3	91.9	8.1
	7	40.6	23.4	93.2	6.8	52.3	20.6	99.3	0.7
Endo-1,4-β-galactanase	0.5	17.6	0.4	94.0	6.0	24.7	1.5	93.7	6.3
	4	56.6	7.9	93.6	6.4	66.2	9.8	93.2	6.8
	7	52.5	15.8	92.7	7.3	45.4	32.2	90.9	9.1
Endo-1,5-α-arabinanase + endo-1,4-β-galactanase [e]	0.5	10.2	1.7	99.0	1.0	27.8	5.1	98.8	1.2
	4	16.5	6.9	98.7	1.3	23.0	10.5	99.4	0.6
	7	20.4	10.6	99.1	0.9	13.5	20.5	100.0	0.0

[a] All measurements were run as triplicate and the relative standard deviations are less than 12%.

[b] Each unit of enzymatic activity (U) was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

[c] Yield was expressed as weight (g) of generated compounds per 100 g of substrate.

[d] Oligo-RG I includes oligosaccharides (DP 2-12) and oligomers (13-70).

[e] Endo-1,5-α-arabinanase and endo-1,4-β-galactanase was used at 1:1 (U:U) ratio.

As expected, the yield (1.0 – 93.9%) and the MW distribution of oligo-RG I were dependent on the enzyme activity profile of multi-enzymatic preparations, the number of enzymatic units and the reaction time. The main hydrolysis products were oligosaccharides with a DP of 2-12 (79.8 - 100%), while the oligomers with a DP of 13-70 comprised a smaller proportion of final oligo-RG I products (0.0 - 20.2%). The use of endo-1,4-β-galactanase as a mono-component enzyme resulted in a high yield of oligo-RG I of 66.2% (0.2 U/g substrate, 4 h) with relatively low yield of monosaccharides of 9.8%. Only 6.0 to 9.1% of the oligo-RG I generated by endo-1,4-β-galactanase were oligomers with high DP. These results reveal the significance of endo-1,4-β-galactanase for the hydrolysis of potato RG I-type pectic polysaccharides into oligosaccharides. Indeed, potato RG I consists of 67% of galactan side chains (Oomen et al., 2003). As expected, the rate of oligo-RG I generation by endo-1,4-β-galactanase was higher at enzyme concentration of 0.2 U/mg substrate than at 0.1 U/mg substrate; however, increasing the reaction time beyond 4 h led to a significant decrease in the yield of oligo-RG I to 45.4% at 0.2 U/mg substrate. This decrease was accompanied by an increase in the yield of monosaccharides, revealing the partial hydrolysis of oligosaccharides upon extended enzymatic treatment of potato RG I with endo-1,4-β-galactanase.

Table 6.3 also indicates that the highest oligo-RG I yield of 93.9% was achieved upon the treatment of potato RG I-type pectic polysaccharides with Depol 670L at an enzyme concentration of 0.2 U/g substrate for 4 h. This result reveals the higher catalytic efficiency of the synergistic actions of pectin-hydrolyzing enzymes present in Depol 670L multi-enzymatic preparation as compared to the endo-1,4-β-galactanase mono-component. Indeed, Depol 670L exhibited the highest galactanase+arabinanase/ rhamnogalacturonase ratio of 121.6 and no pectinase activity, revealing its higher hydrolyzing activity toward side chains of RG I as compared to its backbone. In addition, the monosaccharide yields (0.0-14.0% w/w) generated in the presence of Depol 670L was lower as compared to that obtained with endo-1,4-β-galactanase mono-component (0.4-32.2%); while the oligomer content (2.5-20.2%, w/w) was higher with Depol 670L. These results reveal that the pectin-hydrolyzing enzymes present in Depol 670L may have displayed an endo-hydrolyzing mode of action for the synergistic hydrolysis of potato RG I. On the other hand, Gamanase 1.5L, which exhibited galactanase+arabinanase /rhamnogalacturonase ratio of 33.4, resulted in a relatively moderate maximum yield of oligo-RG I of 24.6 and 43.4% at enzyme concentration of 0.1 and 0.2 U/mg substrate, respectively;

only low amount of monosaccharides (0.0-4.9%) was released revealing the endo-mode action of its enzymes on potato RG-I. However, Gamanase 1.5L had the highest galactanase/arabinanase ratio of 166.0, revealing the low proportion of its arabinanase activity as compared to the galactanase one.

The moderate maximum yields of oligo-RG I obtained upon enzymatic treatment with Gamanase 1.5L may be due to the enzyme inhibition and/or to the steric hindrance of its endo-1,4-β-galactanase by the non-hydrolyzed arabinan side chains. Indeed, the arabinan side chains in RG I consist of a main chain of α-1,5-linked L- arabinan with 2- and 3-linked Ara, which can also be linked to galactosyl residues of β-1,4-linked galactan side chains at O-3. It has been reported that the hydrolysis of arabinan can improve the access to galactan side chains (Hinz et al., 2005). Nevertheless, using a mixture of endo-1,4-β-galactanase and endo-1,5-α-arabinanaseat a ratio of 1:1 (U:U) as mono-component enzymes resulted in a low maximum oligo-RG I yields of 20.4 and 27.8% and a high maximum monosaccharide content of 10.6 and 20.5% at enzyme concentration of 0.1 and 0.2 U/mg substrate, respectively. In addition, the low content of oligomers (1.0 - 1.3%) confirms the high hydrolysis rate of the branches of RG I using this mixture of mono-component enzymes. The modulation of galactanase/arabinanase ratio seems to be determinant for the efficient generation of oligo-RG I and the limitation of the release of monosaccharides.

The results also show that Newlase II, which is lacking arabinanase and rhamanoglacturonase activities, hydrolyzed the potato RG I-type pectic polysaccharides with the lowest extents. Indeed, the maximum yields of oligo-RG I of 3.2 and 26.7% were obtained upon the enzymatic treatment with Newlase II for a short reaction time of 0.5 h at an enzyme concentration of 0.1 and 0.2 U/mg, respectively. Increasing the reaction time did not improve the yields, and the release of monosaccharides (0.1-0.9%) and the oligomer contents (1.3-3.0%) were not significant at 0.5 h of incubation. These results may be due to the low accessibility of galactanase expressed in Newlase II to its substrate. Although Iogen HS and Newlase II showed more or less similar galactanase activity, Iogen HS was more efficient as a multi-enzymatic preparation for the generation of oligo-RG I with a maximum yield of 43.9 and 48.7% at 0.1 and 0.2 U/g substrate, respectively, and 0.5 h. Increasing the reaction time to 4 h led to a decrease in the oligo-RG I yield to 26.4 and 26.3% with a concomitant increase in the monosaccharide yields from 5.0 and 2.9to 22.7 and 26.6%; beyond this reaction time of 4 h, the yields and the product profiles

remained constant. The high hydrolysis rate of the potato RG I by Iogen HS may be attributed (a) to the appropriate enzymatic profile of its pectin-hydrolyzing enzymes with galactanase/arabinanase ratio of 32.0 and galactanase+arabinanase/rhamnogalacturonase ratio of 33.0 and/or (b) to its high xylanase activity, which may have helped the hydrolysis of linkages between RG I and xyloglucan/ xylogalacturonan (Soffer & Mannheim, 1994), allowing easiest access of pectin-hydrolyzing enzymes to their substrates. In addition, the results also indicate the exo-hydrolyzing action of Iogen HS at extended reaction times and the importance of controlling its catalytic action in order to limit the subsequent hydrolysis of oligosaccharides.

Pectinex Ultra SPL and Viscozyme L, which exhibited similar activity profile of pectin-hydrolyzing enzymes, showed more or less similar trends for the hydrolysis of potato RG I-type pectic polysaccharides, at high enzyme concentration of 0.2 U/ mg substrate, in which the generation of oligo-RG I achieved higher yields (yield of 54.5 and 65.6% for Pectinex Ultra SPL and Viscozyme L, respectively) upon 0.5 h of reaction and decreased thereafter to 27.3 and 52.3% at 7 h as a result of their partial hydrolysis into monosaccharides (34.2 and 20.6%). As compared to Depol 670 L, the presence of branches- and backbone-hydrolyzing enzymes at low ratios in Pectinex Ultra SPL and Viscozyme L (galactanase+arabinanase/ rhamnogalacturonase ratio of 13.0-14.1) may have favored an extensive hydrolysis of potato RG I-type pectic polysaccharides into oligo-RG I at higher rate. As a result, the maximum oligomer contents of oligo-RG I, generated upon Pectinex Ultra SPL (11.9%) and Viscozyme L (8.1%) treatments, were lower as compared to that (20.2%) obtained with Depol 670 L.

Martínez et al. (2009) have reported lower yield of 26.7% for the generation of pectic oligosaccharides from sugar beet pulp using Celluclast 1.5L from *T. reesei* and Viscozyme L from *A. aculeatus*. Higher pectic oligosaccharide yield of 95% was reported by Olano-Martin et al. (2001) upon the conversion of low and high meyhoxyl pectin from citrus and apple by endo-polygalacturonase from *Aspergillus pulverulentus*. Holck et al. (2011), who have used a mixture of β-galactosidase from *A. niger*, β-galactosidase from *Kluyveromyces lactis*, galactanase, arabinofuranosidase and arabinanase (0.2-4.7% enzyme/ substrate) to hydrolyze side chains of sugar beet pectin, have converted 38% and 44% of arabinan and galactan chains, respectively, to hydrolysis product with MW of <3kDa. Furthermore, Martínez Sabajanes et al. (2012) have investigated the use of Celluclast 1.5L from *T. reesei* and Viscozyme L from *A. aculeatus* for the conversion of orange peel waste into mono- and oligosaccharides; these authors have reported

the solubilization of 46.5-73.8% of galactan to Gal and galacto-oligosaccharide and 70.5–100% of arabinan to Ara and arabino-oligosaccharides.

6.4.4. MW distribution and monosaccharide profile of generated oligo-RG I

The MW distribution of generated oligosaccharides, being the major products, and the monosaccharide profile of the whole oligo-RG I (oligosaccharides, oligomers) were characterized. HPAEC-PAD elution profiles (Figure 6.1) indicate the heterogeneous nature of generated oligosaccharides. The elution profiles of the reaction components of Pectinex and Viscozyme-catalyzed hydrolysis reactions were similar with four abundant peaks#2,5,6 and 7 at retention times of 7.2, 8.6, 10.7 and 11.9 min, respectively. Indeed, Pectinex Ultra SPL and Viscozyme L exhibited similar activity profile of pectin-hydrolyzing enzymes. These results reveal the importance of the enzyme activity profile and the substrate specificity of the multi-enzymatic preparation rather than their microbial sources. Depol 670L preparation showed similar end-product profiles of low (DP of 3-4) to medium (DP of 5-6) MW oligosaccharides (Peaks#2-5 and 6-7, respectively) than that of Gamanase 1.5L; however, the molecular species detected beyond a retention time of 12 min were different for both multi-enzymatic preparations. As compared to the multi-enzymatic preparations, the elution profile of endo-1,4-β-galactanase mono-component showed relatively one abundant peak#2 with retention time of 7.2 min. The limited heterogeneity of reaction components of endo-1,4-β-galactanasemay be due its substrate specificity towards galactan. The limited number of peaks detected in the elution profiles of the reaction components of Iogen HS (peak# 1 and 3) and NewLase II-catalyzed hydrolysis (peak# 5) may be attributed to their low galactanase/arabinanase enzymatic ratio.

The percentage distribution of the selected heterogeneous pools (DP of 2-3; 4-5; 6-10) of generated oligosaccharides were determined and reported in Figure 6.2. The results show that the MW distribution of oligosaccharides (42.0-54.8% of 2-3 DP; 45.2-58.0% of 4-5 DP) generated by endo-1,4-β-galactanase was not significantly affected by the enzyme amount nor by the reaction time, expect at high enzyme concentration of 0.2 U/mg and longer reaction time of 7 h (19.9% of 2-3 DP; 80.1% of 4-5 DP). These results can be explained by the mono-component behaviors of endo-1,4-β-galactanase acting on the same glycoside sites of RG I.

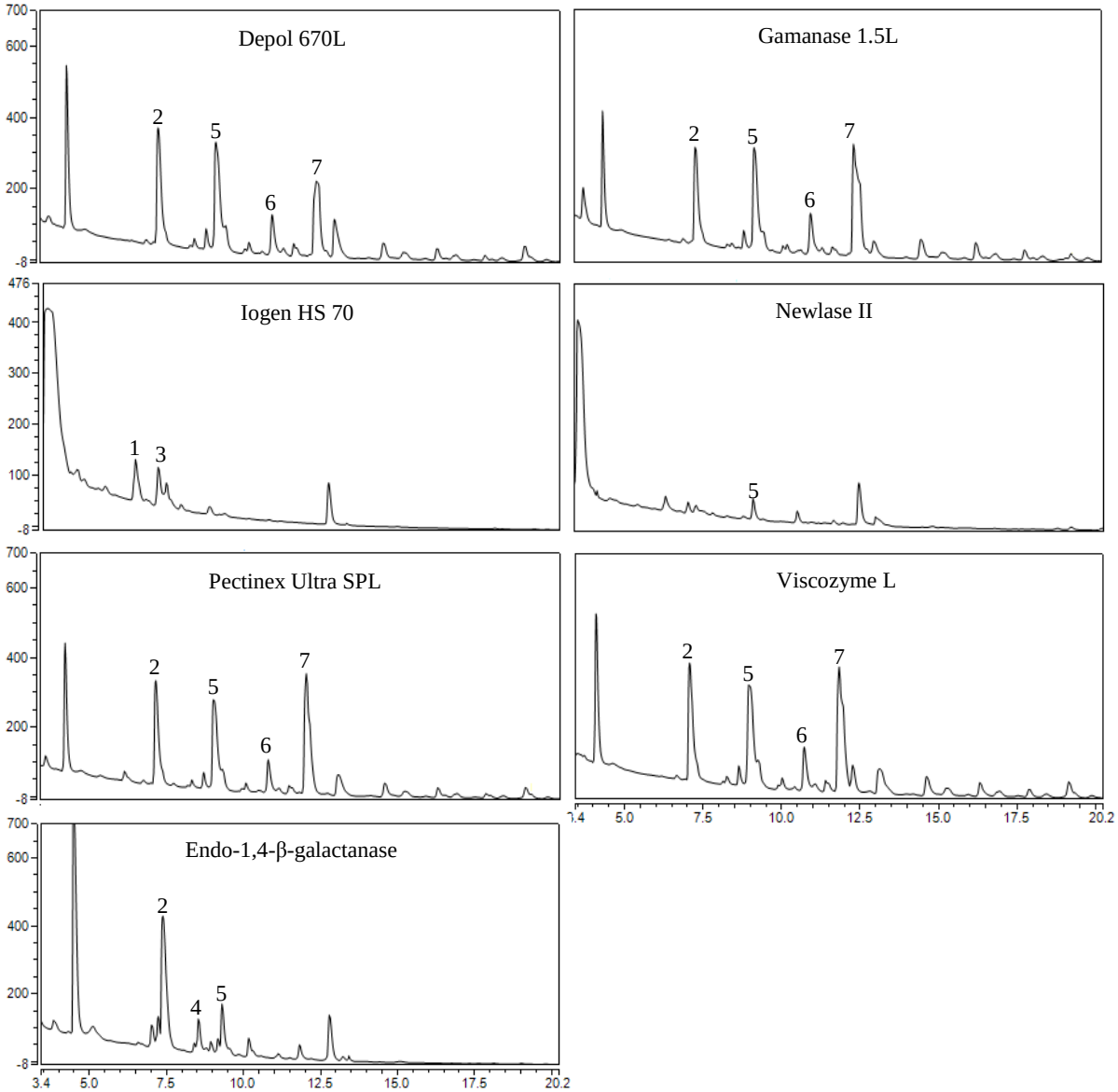

Figure 6.1 Molecular weight distribution of generated hydrolystaes.

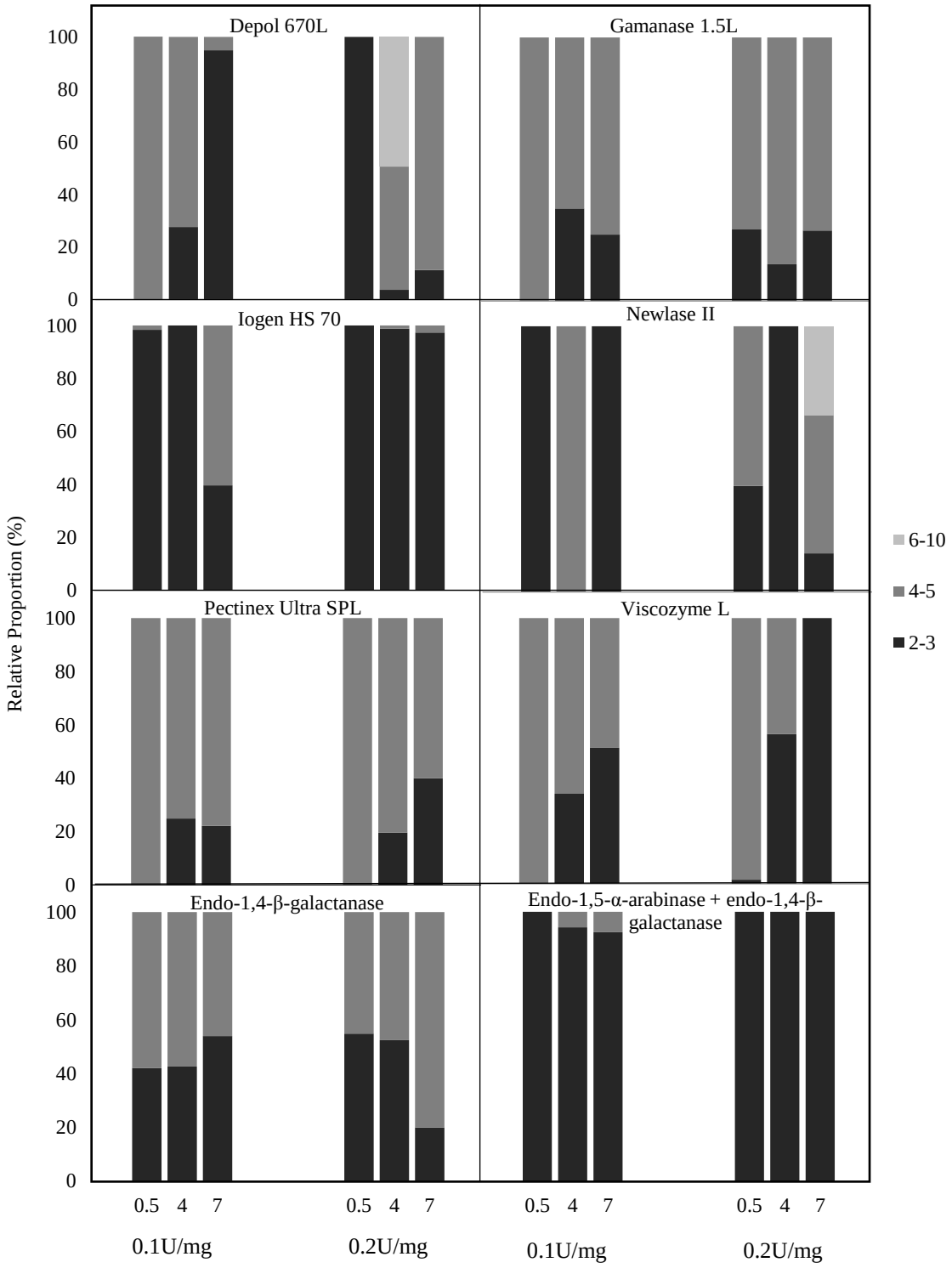

Figure 6.2 Degree of polymerization of generated oligosaccharides (%), each unit of enzymatic activity (U) defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

Supplementing the endo-1,4-β-galactanase with endo-1,5-α-arabinanase (1:1, U:U) resulted in the release of mainly low-MW oligosaccharides (92-100% of 2-3 DP), confirming the extensive hydrolysis of RG I branches as depicted by the monosaccharide content (Table 6.2). Øbro et al. (2004) have investigated the synergy between endo-1,4-β-galactanase and endo-1,5-α-arabinanase for the hydrolysis of side chains of potato RG I and reported that this synergistic action was more efficient towards the degradation of arabinans than galactans. Figure 6.2 also shows a shift in the MW distribution of oligosaccharides generated by Viscozyme L towards low-MW one (2-3 DP) upon the increase of the enzyme concentration and the reaction time to reach 100% of di/trisaccharides at 0.2 U/mg substrate and 7 h. In the presence of Pectinex Ultra SPL, this shift was less pronounced resulting in only 22.1 and 40.0% of low-MW oligosaccharides (2-3 DP) at 0.1 and 0.2 U/mg substrate, respectively. However, as compared to Viscozyme L, Pectinex Ultra SPL led to a rapid release of monosaccharides within the investigated reaction time course, revealing its pronounced exo-mode action (Table 6.2). Iogen HS 70 generated mainly low-MW oligosaccharides (39.7-100.0% of 2-3 DP) over the reaction course. While the most abundant oligosaccharides generated by Gamanase 1,5L are tetra/pentaoligosaccharides (4-5 DP) representing 65.0-100.0%. The high-MW oligosaccharides (6-10 DP) were released by Depol 670L (49.4%) and Newlase II (33.6%) at a high enzyme concentration of 0.2 U/mg substrate after 4 and 7 h reaction, respectively. From these results, we hypothesized that an enzymatic profile of multi-enzymatic preparation with relatively low or no arabinanase activity or high galactanase to rhamnogalacturonase ratio can favor the production of high-MW oligosaccharides.

The reaction conditions that can lead to the generation of high proportion of high-MW oligosaccharides and/or to high yield of oligo-RG I were identified to be : Depol 670L (0.2U/mg, 4h-7h), Gamanase 1,5L (0.2U/mg, 4h), Pectinex Ultra SPL (0.2U/mg, 0.5h), Viscozyme L (0.2 U/mg, 0.5h), endo-1,4-β-galactanase (0.1-0.2 U/mg, 4h), Iogen HS 70 (0.1-0.2U/mg, 0.5h), Newlase II (0.2U/mg, 0.5h-7h). Monosaccharide profile of oligo-RG I obtained using the identified conditions was characterised (Table 6.4). Gal was the major monosaccharide for all generated oligo-RG I (58.9-91.2%, w/w). Ara content of oligo-RG I varied between 0.0 and 12.1% depending on the type of biocatalysts and the reaction time. Newlase II, expressing no arabinanase and rhamnogalacturonase activities, released oligo-RG I with no Ara and Rha residues.

Table 6.4 Sugar composition of generated oligosaccharides (g/100g of oligosaccharide product)[a]

Enzyme/incubation condition	Rha	Ara	Gal	Glc	Xyl	Man	GalA
Depol 0.2U [b] /mg, 4h	0.9	8.5	78.0	4.7	0.0	0.5	6.6
Depol 0.2U/mg, 7h	1.9	12.1	78.1	5.6	0.0	0.0	0.9
Gamanase 0.2U/mg, 4h	0.9	12.0	77.8	3.2	1.9	2.3	1.9
Iogen 0.1U/mg, 0.5h	0.0	0.0	73.0	3.7	2.7	0.0	20.6
Iogen 0.2U/mg, 0.5h	0.0	0.2	74.3	3.7	3.2	0.0	18.6
Newlase 0.2U/mg, 0.5h	0.0	0.0	63.8	3.8	1.0	0.0	31.4
Newlase 0.2U/mg, 7h	0.0	0.0	58.9	4.7	1.9	0.0	34.6
Pectinex 0.2U/mg, 0.5h	0.9	6.6	77.4	3.8	1.9	0.0	9.4
Viscozyme 0.2U/mg, 0.5h	1.1	8.7	76.1	5.4	2.2	1.0	6.5
Viscozyme 0.2U/mg, 7h	1.9	10.2	72.4	5.3	1.9	1.0	8.3
Endo-1,4-β-galactanase 0.1U/mg, 4h	0.0	2.9	91.2	2.1	0.9	0.0	2.9
Endo-1,4-β-galactanase 0.2U/mg, 4h	0.0	3.8	88.0	3.3	1.1	0.0	3.8

[a] All measurements were run as triplicate and the relative standard deviations are less than 10%.

[b] Each unit of enzymatic activity (U) was defined as the amount of enzyme that produces 1 μmole of reducing ends per min of reaction.

As an overall, the reaction time and the enzyme concentration had more significant effect on the Ara content than Gal one of oligo-RG I. This can be attributed to the low enzymatic units of arabinanase present in the multi-enzymatic products. The generated oligo-RG I end-products with high Ara content were also characterized by high Gal Content. These results reveal the importance of synergism for the efficient hydrolysis of arabinan and galactan side chains of RG I and the presence of Ara substitutions in the galacto-oligosaccharides. Indeed, despite the low arabinanase activity of Gamanase 1.5L, it generated oligo-RG I with high Ara (12.0%) and Gal (77.8%) contents. Øbro et al. (2004) have also reported that the treatment of potato RG I with endo-galactanase released mainly galacto-oligosaccharides with containing small amount of Ara (Gal/Ara ratio of ratio of 20/1).

Rha content of pectic polysaccharides was 2.2%, while its content in most generated oligosaccharides/oligomers was 0.0-1.1%. Most of RG I backbone remained therefore intact upon enzymatic treatment. Iogen HS 70 and Newlase II resulted in the highest content of GalA in the oligo-RG I (18.6-34.6%) indicating that these multi-enzymatic preparations hydrolyzed more efficiently the HG region rather than RG I region. About 21.1% of potato RG I-type pectic polysaccharides consisted of hemicellulosic polysaccharides as indicated by the content of Glc+ Xyl+Man. However, the proportion of monosaccharides originating from hemicellulosic polysaccharides decreased in the generated oligo-RG I to 3.0-8.6%, indicating their low release. The highest amounts of Glc+Xyl+Man was obtained through treatment with Viscozyme L (8.6%), Gamanase 1.5L (7.4%) and Iogen HS 70 (6.9%).

Michalak et al. (2012) used galactan-rich potato pulp polysaccharides to produce two fractions of <10 kDa (yield of 20.6%) and >10 kDa (yield of 65.0%) through treatment with endo-1,4-β-galactanase from *E. nidulans*. The <10 kDa fraction had very similar monosaccharide profile to sample obtained with endo-1,4-β-galactanasetreatment in our study (Rha (0%), Ara (3.6%), Gal (92.9%), Glc (0.3%), Man (0.0%) and GalA (3.2%)) and the >10 kDa fraction had lower amount of Gal (53.9%) and higher amount of Rha, Ara, GalA, Glc and Man.

6.5. Conclusion

Galactan-rich RG I was extracted from potato cell wall using microwave-alkaline extraction method. Multi-enzymatic preparations showing high hydrolysis activity toward side chains and backbone of RG I were identified in order to hydrolyse extracted RG I into

oligosaccharides/oligomers. Yield of oligo-RG I (including oligosaccharides and oligomers) varied between 1.0 and 93.9% (w/w) with monosaccharide yield of 0.0-34.2%. The main hydrolysis products were oligosaccharides with a DP of 2-12 (79.8 - 100%), while the oligomers with a DP of 13-70 comprised a smaller proportion of final oligo-RG I products (0.0 - 20.2 %). Highest oligo-RG I yield of 93.9% was achieved upon the treatment of potato RG I-type pectic polysaccharides with Depol 670L, which is the multi-enzymatic preparation with higher hydrolyzing activity toward side chains of RG I as compared to its backbone. Generated oligosaccharides composed mainly of Gal (58.9-91.2%, w/w) and other monosaccharides found in oligosaccharide products included Ara (0.0-12.1%), Rha (0-1.9%) and GalA (0.9-34.6%).

CONNECTING STATEMENT 5

The enzymatic generation of oligosaccharides/oligomers from potato galactan-rich RG I using selected multi-enzymatic preparations was investigated in the previous Chapter VI. In Chapter VII, the enzymatic production of oligosaccharides/oligomers with defined DP and sugar composition using combined multi-enzymatic preparations (Depol 670L and Gamanase 1.5L) is investigated. The interactive effects of reaction parameters (incubation time, Depol 670L/Gamanase 1.5L ratio, substrate concentration) of bi-enzymatic system on the yield, the monosaccharide profile and the DP of generated oligosaccharides (DP of 2-6 and 7-12) and oligomers (DP of 13-70) are discussed using RSM and CCRD. The optimum conditions resulting in the highest content of oligosaccharides/oligomers are identified.

The results from this chapter will be submitted to a scientific journal.

Khodaei, N., & Karboune, S. (2015). Optimization of enzymatic production of prebiotic oligosaccharides and oligomers from potato rhamnogalacturonan I (To be submitted).

CHAPTER VII

OPTIMIZATION OF ENZYMATIC PRODUCTION OF PREBIOTIC OLIGOSACCHARIDES AND OLIGOMERS FROM POTATO RHAMNOGALACTURONAN I

7.1. Abstract

A bi-enzymatic system based on the combined use of two multi-enzymatic preparations (Depol 670L and Gamanase 1.5L) was investigated for the enzymatic production of oligosaccharides/oligomers, with well-defined DP and sugar composition, from potato galactan-rich RG I. Depending on the reaction condition, yields of low (DP of 2-6) and high-MW oligosaccharides (DP of 7-12) and that of oligomers (DP of 13-70) varied between 0.1-13.9, 0.0-37.5 and 0.0-75.7%, respectively. Substrate concentration and Depol 670L/Gamanase 1.5L ratio were identified as the most significant linear terms in oligosaccharide and oligomer yield models, respectively. In terms of quadratic terms, substrate concentration was the most significant in all developed predictive yield models. Moreover, interaction between reaction time and substrate concentration had a significant effect on the yield of oligosaccharides, while interaction between reaction time and Depol 670L/Gamanase 1.5L ratio affected significantly the yield of oligomers. Higher yields of both oligosaccharides and oligomers were obtained when equal amount of Depol 670L and Gamanase 1.5L was used in combination. The generated oligosaccharides and oligomers were mainly composed of Gal.

7.2. Introduction

In response to the growing awareness of consumers about the relationship between health and diet, there has been much interest in the use of NDOs with prebiotic properties as functional food ingredients. Indeed, prebiotics can be selectively fermented by beneficial bacteria in the human colon, and promote the intestinal health (Gibson & Roberfroid, 1995). Health-promoting properties of prebiotics are dependent on their structural properties, including monosaccharide composition, DP and level of branching (Kabel et al., 2002a; Kabel et al., 2002b; Holck et al., 2011). Because of the complexity and heterogeneity of pectic polysaccharides from cell wall, they can be good candidates for the generation of new prebiotics with unique properties. In addition, these polysaccharides are present in abundant and accessible by-products from food industry, such as apple pomace, citrus peel, sugar beet and potato pulp. Among these by-products, pectic polysaccharides from potato pulp has a distinct composition and chemical structure characterized by high proportion of RG I (75% of pectic polysaccharides) and high galactan side chain branching (67% of RG I) (Oomen et al., 2003). As compared to FOS, arabinogalactan was found to be more tolerable and was mainly fermented in the distal part of

the colon (Gullón et al., 2013; Terpend et al., 2013). Moreover, other studies have shown that the colonic fermentation of Gal-containing oligosaccharides increased the number of *Bifidobacterium* and generated more lactate than fructose-containing inulin (Djouzi & Andiueux, 1997; Jaskari et al., 1998). Furthermore, comparative studies of uronic acid and neutral sugar-rich oligosaccharides have highlighted the higher prebiotic properties of neutral sugar-rich oligosaccharides (Gulfi et al., 2007; Gullón et al., 2011; Gullón et al., 2009; Thomassen et al., 2011).

As far as the authors are aware, most of the studies have investigated the isolation and the prebiotic properties of non-hydrolyzed polysaccharides from potato pulp (Lærke et al., 2007; Olesen et al., 1998; Thomassen et al., 2011). Only, Michalak et al. (2012) have used endo-1,4-β-galactanase to hydrolyze potato pulp polysaccharides into two fractions (<10 and >10 kDa) covering a wide MW distribution. The generation of tailored oligosaccharides and oligomers from pectic polysaccharides still needs to be investigated further. Considering the complex structure of cell wall pectic polysaccharides, their efficient hydrolysis into well-defined oligosaccharides requires combinations of enzymatic activities that act synergistically. Our previous study (Khodaei & Karboune, 2015a) was the first to identify the appropriate multi-enzymatic preparations, expressing selected pectin glycosyl-hydrolase activities, and to assess their efficiency for the hydrolysis of potato cell wall pectic polysaccharides. Among the investigated multi-enzymatic products, Depol 670L multi-enzymatic product was characterized by high galactanase activity of 6751.0 U/ml, galactanase/arabinanase enzymatic ratio of 118.4 and galactanase+arabinanase /rhamnogalacturonase ratio of 121.6 (Khodaei & Karboune, 2015a);while Gamanase 1,5L, exhibiting relatively high galactanase+arabinanase /rhamnogalacturonase enzymatic ratio of 33.4, was assumed to be more efficient as biocatalyst for the hydrolysis of RG I backbone than the Depol 670L. Indeed, Depol 670L resulted in the highest hydrolysate yield of 96.2%, while Gamanase 1.5L led to the low content of monosaccharide (0.0 – 10.5%) and high tetra/penta-oligosaccharides proportion of 65-100%. It can be hypothesized that Depol 670L and Gamanase 1,5L can act synergistically for the efficient hydrolysis of potato RG I-rich pectic polysaccharides. As part of the ongoing research, the combined use of Depol 670L and Gamanase 1,5L multi-enzymatic products was investigated, for the production of oligosaccharides/oligomers from potato pectic polysaccharides. The interactive effects of reaction parameters (incubation time, Depol 670L/Gamanase 1.5L ratio, substrate

concentration) of bi-enzymatic system was studied for better understanding of their effects on the yield, the monosaccharide profile and the DP (2-6, 7-12, 13-70) of generated oligosaccharides and oligomers.

7.3. Material and method

7.3.1. Materials

Gamanase 1.5L from *A. niger* was obtained from Novo Nordisk Bioindustrial, Inc. (Danbury, CT), and Depol 670L from *T. reesei* was from Biocatalysts Ltd. (Mid Glamorgan, UK). Soybean RG and potato pectic galactan were from Megazyme (Wicklow, Ireland). Arabinan, polygalacturonic acid from oranges, KOH, sodium acetate, acetic acid, dextran standards, Gal, Ara, Rha, Glc, Xyl, Man, GalA, trifluoroacetic acid, thermostable α-amylase from *B. licheniformis* and amyloglucosidase from *A. niger*, were purchased from Sigma Chemical Co. (St-Louis, MO, USA). All salts were obtained from Fisher Scientific (Fair Lawn, NJ). 1-kestose, nystose and 1F-fructofuranosyl- nystose were obtained from Wako Pure Chemical (Japan).

7.3.2. Extraction of RG I-rich pectic polysaccharides from potato pulp

Prior to the extraction of pectic polysaccharides, protein and starch were removed from potato pulp as described previously (Khodaei & Karboune, 2013). A microwave-assisted alkaline extraction method, optimized in our previous study, was used for the extraction of potato RG I-rich pectic polysaccharides (Khodaei et al., 2013d). The cell wall material was dissolved in 1.5 M KOH at solid/liquid of 2.9% w/v and heated using a power/suspension ratio of 1.2 W/ml for 2 min in a Panasonic 1200W domestic microwave. After heating, the insoluble fraction was removed by centrifugation at 8000 ×g for 25 min and the supernatant was neutralized with HCl. The recovered solution was concentrated to 25% of its original volume using ultrafiltration membrane (EMD Millipore Prep/Scale spiral-wound, TFF-2, cut-off 10 kDa) connected to an EMD Millipore Pellicon™ easy-load peristaltic pump. The high-MW polysaccharides were recovered by mixing the solution with ethanol (1:1, v:v) and centrifugation at 8000 ×g for 25 min, followed by freeze drying of the pellets.

7.3.3. Assays of glycosyl-hydrolase activities

Pectinase, rhamnogalacturonase, galactanase and arabinanase activities expressed in Depol 670L and Gamanase 1,5L were assessed using 0.5% (w/v) substrate solutions of polygalacturonic acid, soybean RG, potato pectic galactan and arabinan respectively, prepared in 50 mM sodium acetate buffer, pH 5.0. The activity was measured by adding 0.025 ml of multi-enzymatic product to 0.475 ml of substrate solution, incubated at 40 °C for 20 min. Two blank tests were also performed without substrate or without enzyme and the reducing ends were quantified using DNS assay. The enzymatic activity was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction per ml or g of multi-enzymatic products. DNS assay was done by adding 0.75 ml of DNS reagent (1% (w/v) DNS in 1.6% (w/v) sodium hydroxide) to 0.5 ml of samples. Reaction tubes were placed in boiling water for 5 min and then 0.25 ml of potassium sodium tartrate (50%, w/v) was added and absorbance of generated yellow color was measured at 540 nm. Standard curve was plotted with Gal and the enzymatic activity unit (U) was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

7.3.4. Generation of oligosaccharides/oligomers from potato RG I-rich pectic polysaccharides

Extracted RG I-rich potato polysaccharides were suspended in 50 mM sodium acetate buffer, pH 5.0. A mixture of Depol 670L and Gamanase 1,5L at selected ratios was added to the polysaccharide suspension. Total enzymatic unit (expressed in galactanase unit) was kept constant at 0.2 U/mg RG I-rich pectic polysaccharides. Incubation of reaction mixture was performed at 40 °C with an agitation at 200 rpm. After selected reaction times, the enzymes were inactivated by boiling the reaction mixture for 5 min. In order to remove unhydrolyzed polysaccharides, reaction mixtures were mixed with ethanol (1:1, v:v) and were centrifuged at 12000 ×g for 10 min after 30 min of incubation at 5 °C. Yield and MW distribution of oligosaccharides was measured using HPSEC, and the monosaccharide profile of oligosaccharides was measured using HPAEC.

7.3.5. Experimental design and statistical analysis

The generation of oligosaccharides from potato RG I-rich pectic polysaccharides was optimized using RSM and a CCRD. The investigated parameters and their levels were:

incubation time (0.5, 2, 3.5, 5 and 6.5 h), Depol 670L/Gamanase 1,5L ratio (37.5/62.5, 50/50, 62.5/37.5, 75/25, and 87.5/12.5%, U/U) and substrate concentration (0.3, 0.88, 1.44, 2.0, and 2.6%, w/v). A total of 34 experiments were performed based on the CCRD with three variables at five levels. The complete design included 16 factorial points (levels ±1), 12 axial points (levels ± α), and 6 replicates in center point and experiments were done in random order.

ANOVA of obtained data was done with the Design-Expert 8.0.2 software (Stat-Ease, Inc., Minneapolis, MN, USA). Experimental data were fitted to a quadratic polynomial model, which shows behavior of responses (Yield of the oligosaccharides with DP of 2-6 and 7-12 and oligomers with DP of 13-70) when independent variables (incubation conditions) change. The general form of the quadratic polynomial model was as follows (Eq. 7.1):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \qquad (7.1)$$

The coefficients of the polynomial equations were represented by β_0 (constant term), β_i (linear coefficient), β_{ii} (quadratic coefficient), and β_{ij} (interaction coefficient). *Y* represents the response and x_i and x_j represent independent variables. Counter plots were developed for the most significant interaction terms.

7.3.6. Analytical methods

MW distribution. DP of the generated oligosaccharides/oligomers was measured using Waters HPLC system equipped with TSKgel G-Oligo-PW column (Tosoh Bioscience, Montgomeryville, PA), refractive index detector and Breeze software for the data analysis. Isocratic elution was applied with 0.1 M NaCl at flow rate of 0.5 ml/min. The overall the concentration of generated oligosaccharides/oligomers was quantified from the standard curves constructed using Gal, sucrose, trisaccharide (1-kestose), tetrasaccharide (nystose) and pentasaccharide (1F-fructofuranosyl- nystose) as standards. MW distribution of the polysaccharides was investigated using three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL, Tosoh Bioscience, Montgomeryville, PA) and 0.1 M NaCl as a mobile phase. Dextrans with MW of 50, 150, 270, 410 and 670 kDa were used as standards for calibration.

Monosaccharide profile: Generated oligosaccharides/oligomers were analyzed for their monosaccharide profile as described previously (Khodaei & Karboune, 2013). Upon the hydrolysis, the monosaccharides were analyzed using HPAEC-PAD (Dionex), and a CarboPac

PA20 column (3 × 150 mm). 5 mM NaOH with isocratic flow rate of 0.5 ml/min was used and Rha, Ara, Gal, Glc, Xyl and Man were used as standards. GalA content was measured using sulfamate/m-hydroxydiphenyl colorimetric assay (Blumenkrantz & Asboe-Hansen, 1973).

7.4. Results and discussions

7.4.1. Effect of reaction conditions on the generation of oligosaccharides and oligomers

Our previous study (Khodaei et al., 2016) has shown that 71% of polysaccharides, extracted by microwave assisted-alkaline method, corresponded to the galactan-rich RG I with a monosaccharide profile composed of Gal, Ara and Rha at a molar proportion of 63.1, 5.7, and 2.2%, respectively (Data not shown). Only low amount of hemicellulosic polysaccharides were isolated, as it was indicated by the molar proportion of Glc (13.2%), Xyl (4.9%) and Man (3.0%). The MW distribution of potato RG I-rich pectic polysaccharides was characterized by a high population of high-MW polysaccharides (>600 kDa, 80.4%, w/w), and the absence of oligosaccharides/oligomers.

The hydrolysis of potato RG I-rich pectic polysaccharides into low and high-MW oligosaccharides (DP of 2-6 and 7-12) and oligomers (DP of 13-70) was investigated using Depol 670L/Gamanase 1,5L bi-enzymatic system. In order to better understand the effects of reaction parameters and their interactions on the efficiency of bi-enzymatic system and the end-product profile, RSM has been used. Table 7.1 shows the experimental conditions and the selected responses. Yields of oligosaccharides (DP of 2-6 and 7-12) and oligomers (DP of 13-70) were selected as responses since the prebiotic property can be affected by the MW distribution. Indeed, low-MW oligosaccharides are fermented by *Bifidobacterium* at a faster rate (Al-Tamimi et al., 2006; Biedrzycka & Bielecka, 2004; Van Loo, 2004). On the other hand, higher-MW oligosaccharides and oligomers are slowly fermented, but can reach the distal region of colon and provide a more prolonged prebiotic effect in the intestine (Biedrzycka & Bielecka, 2004; Van Loo, 2004). Therefore, producing tailored mix of oligosaccharides and oligomers with well-defined MW distribution can provide the ability to modulate the intestinal health more efficiently.

Table 7.1 shows that the yields of low-MW (DP of 2-6) and high-MW (DP of 7-12) oligosaccharides and oligomers (DP of 13-70) varied between 0.1-13.9, 0.0-37.5 and 0.0-75.7% (g/ 100 g substrate), respectively.

Table 7.1 Central composite rotatable design of experiments and obtained responses.

Std order	x_1-Time (h)		x_2- Depol 670L/Gamanase 1.5L (U/U) [a,b]		x_3-Substrate concentration (%, w/v)		Monosaccharide (g/ 100 g substrate)	Oligosaccharide and oligomer yield (g/ 100 g substrate)			
								DP 2-6	DP 7-12	DP 13-70	Total oligosaccharide/oligomer
1	-1	(2.0)[c]	-1	(50.0/50.0)	-1	(0.9)	3.1	4.6	19.3	35.2	59.1
2	-1	(2.0)	-1	(50.0/50.0)	-1	(0.9)	3.0	3.7	26.2	44.2	74.1
3	1	(5.0)	-1	(50.0/50.0)	-1	(0.9)	10.3	10.3	7.7	43.9	62.0
4	1	(5.0)	-1	(50.0/50.0)	-1	(0.9)	8.3	8.5	7.7	41.1	57.3
5	-1	(2.0)	1	(75.0/25.0)	-1	(0.9)	6.3	7.3	17.0	0.0	24.2
6	-1	(2.0)	1	(75.0/25.0)	-1	(0.9)	5.4	6.2	17.0	3.7	26.8
7	1	(5.0)	1	(75.0/25.0)	-1	(0.9)	7.2	7.4	10.8	42.5	60.6
8	1	(5.0)	1	(75.0/25.0)	-1	(0.9)	7.4	7.3	6.8	23.4	37.5
9	-1	(2.0)	-1	(50.0/50.0)	1	(2.0)	3.1	3.8	0.0	34.6	38.3
10	-1	(2.0)	-1	(50.0/50.0)	1	(2.0)	3.0	3.8	0.6	34.6	38.9
11	1	(5.0)	-1	(50.0/50.0)	1	(2.0)	2.1	2.2	0.0	65.5	67.7
12	1	(5.0)	-1	(50.0/50.0)	1	(2.0)	5.0	5.2	0.0	59.7	64.9
13	-1	(2.0)	1	(75.0/25.0)	1	(2.0)	4.3	5.2	0.0	0.0	5.2
14	-1	(2.0)	1	(75.0/25.0)	1	(2.0)	2.1	2.6	0.0	0.0	2.6
15	1	(5.0)	1	(75.0/25.0)	1	(2.0)	1.0	0.9	0.0	27.3	28.2
16	1	(5.0)	1	(75.0/25.0)	1	(2.0)	1.6	1.6	0.0	47.6	49.1
17	0	(3.5)	0	(62.5/37.5)	-2	(0.3)	17.6	13.9	37.5	0.0	51.4
18	0	(3.5)	0	(62.5/37.5)	-2	(0.3)	15.9	13.9	37.5	0.0	51.4
19	0	(3.5)	0	(62.5/37.5)	+2	(2.6)	4.7	5.3	8.0	0.0	13.3
20	0	(3.5)	0	(62.5/37.5)	+2	(2.6)	4.9	5.3	6.6	0.0	11.9
21	0	(3.5)	-2	(37.5/62.5)	0	(1.4)	2.6	3.1	0.0	69.4	72.5
22	0	(3.5)	-2	(37.5/62.5)	0	(1.4)	4.5	3.7	0.0	72.9	76.6
23	0	(3.5)	+2	(87.5/12.5)	0	(1.4)	0.4	0.4	0.0	0.0	0.4
24	0	(3.5)	+2	(87.5/12.5)	0	(1.4)	0.1	0.1	0.0	0.0	0.1
25	-2	(0.5)	0	(62.5/37.5)	0	(1.4)	0.1	0.3	5.0	11.9	17.2
26	-2	(0.5)	0	(62.5/37.5)	0	(1.4)	0.1	0.3	0.0	12.8	13.1
27	+2	(6.5)	0	(62.5/37.5)	0	(1.4)	1.5	1.0	0.0	75.7	76.7
28	+2	(6.5)	0	(62.5/37.5)	0	(1.4)	2.3	2.1	0.0	74.6	76.6
29	0	(3.5)	0	(62.5/37.5)	0	(1.4)	4.4	5.1	14.1	35.1	54.3
30	0	(3.5)	0	(62.5/37.5)	0	(1.4)	4.3	5.1	7.0	41.3	53.4
31	0	(3.5)	0	(62.5/37.5)	0	(1.4)	3.9	5.1	7.0	36.3	48.4
32	0	(3.5)	0	(62.5/37.5)	0	(1.4)	4.3	5.1	7.0	37.4	49.5
33	0	(3.5)	0	(62.5/37.5)	0	(1.4)	4.1	4.5	8.2	35.4	48.1
34	0	(3.5)	0	(62.5/37.5)	0	(1.4)	4.1	4.5	8.2	32.6	45.3

[a] The enzymatic activity unit (U) was defined as the amount of enzyme that produces 1 μmole of reducing ends per min of reaction.

[b] Total enzymatic unit (expressed in galactanase unit) was kept constant at 0.2 U/mg RG I-rich pectic polysaccharides.

[c] Number in parenthesis represent actual experimental amounts.

The highest yield of low-MW oligosaccharides was obtained when the substrate concentration was at its lowest levels of -α and -1, corresponding to 0.3%, w/v (treatments n^o 17 and 18; 13.9%) and 0.9% (treatment n^o 3; 10.3%), respectively. Similarly, the highest yields of high-MW oligosaccharides were achieved upon the use of low substrate concentrations of 0.3 and 0.9%, w/v (treatment n^o 2, 17 and 18; 26.2, 37.5 and 37.5%, respectively). On the other hand, when the substrate concentration of 2.0 and 2.6% w/v (+1 and +2 level) was used the yield of both low (0.9-5.3%) and high-MW (0.0-8.0%) oligosaccharides decreased. These results indicate that higher rate and hydrolysis extent were achieved, when lower substrate concentration was used. No oligomer was accumulated upon the use of the lowest substrate concentration of 0.3%, w/v (treatments n^o 17/18) and the highest one of 2.6% (treatments n^o 19/20) using Depol 670L/Gamanase 1.5L ratio of 62.5/37.5 (U/U) and reaction time of 3.5 h. The hydrolysis of oligomers into oligosaccharides may have happened at low substrate concentration, limiting their accumulation; while the mass transfer limitations may have hindered the access of enzymes to their action sites at high substrate concentration. Comparing the end-product profiles at treatments n^o 2 and 3 reveals that increasing reaction time from 2 to 5 h, at a Depol 670L/Gamanase 1,5L ratio of 50/50 U/U, increased the yield of low-MW oligosaccharides from 3.7 to 10.3%, while that of high-MW oligosaccharides decreased from 26.2 to 7.7%. Highest yields of oligomers (69.4-75.7%) were obtained upon treatments n^o 21/22 (3.5 h; 37.5/62.5 U/U of Depol 670L/Gamanase 1.5L ratio) and 27/28 (6.5 h; 62.5/37.5 U/U of Depol 670L/Gamanase 1.5L ratio), in which a similar substrate concentration of 1.4% (w/v) was used. These treatments (21/22; 27/28) resulted in low yields of low-MW (1.0-3.7%) and high-MW (0.0%) oligosaccharides. Treatments n^o 22, 27, 28, 2, 21, and 11 led to the highest yields (67.7-76.6%) of total hydrolysates. All of these treatments resulted in higher yields of oligomers (44.2-75.7%).

Similar or lower yields have been reported in the literature when a combination of mono-component enzymes or commercial multi-enzymatic preparations were used. Martínez et al. (2009) have studied the effect of three variables including polygalacturonase to solid ratio (10-15 U/g), Celluclast 1.5L (cellulases) to Viscozyme 1.5L (endo-polygalacturonase) ratio (0-1 U/U) and reaction time (4-16 h) on the production of neutral and acidic oligosaccharides from sugar beet pulp; they have reported that 3.9-30.0, 8.2-31.2, 29.2-63.5 and 19.9-42.2% of cellulose, galactan, arabinan and HG were converted into oligosaccharides, respectively. Moreover, Holck et al. (2011) have investigated the hydrolysis of side chains of sugar beet pectin by a mixture of

enzymes, including β-galactosidase from *A. niger*, β-galactosidase from *K. lactis*, β-galactanase from *A. niger* and α-arabinofuranosidase, and α-arabinanase from *A. aculeatus* using similar substrate concentration of 1.4% (w/v) and longer incubation time of 16 h as compared to our study. Under these conditions, lower conversion yields of 38% and 44% were reported for the arabinan and galactan chains, respectively. On the other hand, Michalak et al. (2012) have studied the hydrolysis of galactan by endo-1,4-β-galactanase from *E. nidulans* and obtained yield of 28.7% and 67.0% for generation of end-products with MW of <3 kDa (60 min; enzyme/substrate ratio of 0.03% v/w) and <10 kDa (15 min; enzyme/substrate ratio of 0.3% v/w), respectively. These authors have also reported that the hydrolysis of potato pulp polysaccharides (15 min; enzyme/substrate ratio of 0.3% v/w) resulted in the release of two fractions of <10 kDa with a yield of 21% and >10 kDa with the yield of 65%. However, higher yields of 67-99.7% were reported by Olano-Martin et al. (2001) who used ultrafiltration dead-end membrane reactor to convert low and high methoxyl pectin from citrus and apple to pectic oligosaccharides using endo-polygalacturonase from *A. pulverulentus.* Substrate concentration of 1% to 5% w/v, enzyme concentration of 90 to 2700 U/l, and residence time of 40 to120 min were used by these authors.

7.4.2. Regression analysis

The multiple regression analysis was carried out using Design-Expert software version 8.0.2., and the best fitting models were selected based on *F* and *P* values (Table 7.2). The reduced quadratic model was statistically significant for all three responses, including the yield of low-MW (*F*-value of 57.4 and *P* value of < 0.0001) and high-MW (*F*-value of 56.6 and *P* valueof < 0.0001) oligosaccharides as well as that of oligomers (*F*-value of 57.7 and *P* value of < 0.0001). The coefficient of determination (R^2) of all fitted models was 0.96, indicating the appropriateness of all models to predict the responses. The lack of fit was not significant relative to pure error with *F* values of 1.3-2.7 and *P* values of 0.052-0.299; these results also indicate a good quality of the fit. Table 7.2 also shows *F* and *P* values for each coefficient. Model coefficients with *F*-value higher than 1 and *P* value less than 0.05 were considered significant.

Substrate concentration (%, w/v) was the most significant linear term in the low-MW (x_3, *F* value of 188.1; *P* value of <0.0001) and high-MW (x_3, *F* value of 267.6; *P* value of <0.0001) oligosaccharide yield predictive models.

Table 7.2 The analysis of variance for response surface model of oligosaccharides yield with DP of 2-6, 7-12 and 13-70.

Response	DP 2-6 [b]		DP 7-12 [b]		DP 13-70 [b]	
	F Value	P Value	F Value	P Value	F Value	P Value
Model	57.4	< 0.0001	56.6	< 0.0001	57.7	< 0.0001
x_1-Time (h)	5.7	0.0258	16.1	0.0005	170.0	< 0.0001
x_2- Depol 670L/Gamanase 1.5L (U/U) [a]	11.9	0.0022	0.5	0.4911	209.2	< 0.0001
x_3-Substrate concentration (%, w/v)	188.1	< 0.0001	267.6	< 0.0001	1.0	0.3174
$x_1 x_2$	19.1	0.0002	2.0	0.1664	9.5	0.0052
$x_1 x_3$	26.7	< 0.0001	20.9	0.0001	6.7	0.0166
$x_2 x_3$	2.9	0.102	0.8	0.388	1.0	0.3249
x_1^2	37.5	< 0.0001	22.2	< 0.0001	5.4	0.03
x_2^2	20.2	0.0002	29.7	< 0.0001	0.1	0.7532
x_3^2	88.1	< 0.0001	61.0	< 0.0001	69.9	< 0.0001
Lack of Fit	1.3	0.2992	2.7	0.052	2.7	0.0544

[a] $R^2 = 0.96$ for all three responses.

[b] The enzymatic activity unit (U) was defined as the amount of enzyme that produces 1 μmole of reducing ends per min of reaction.

$P < 0.05$ indicates statistical significance.

The linear term with the largest effect on the oligomer yield was Depol 670L/Gamanase 1,5L ratio (x_2, *F* value of 209.2; *P* value of <0.0001). This demonstrates the significance of the synergistic interaction between Depol 670L and Gamanase 1,5L for the hydrolysis of potato RG I-rich pectic polysaccharides. In terms of quadratic terms, substrate concentration (%, w/v, x_3^2) was the most significant in all predictive models (*F* value of 61.0- 88.1 and *P* value of <0.0001). Among all interactive effects, the most important one for the low and high-MW oligosaccharide models was the interaction between reaction time and substrate concentration ($x_1\ x_3$) (*F* value of 20.7-26.7; *P* value of <0.0001). Therefore, the time courses for the release of low and high-MW oligosaccharides seem to be more dependent on the substrate concentration than Depol 670L/Gamanase 1,5L ratio. On the other hand, the significant time/enzyme ratio interactive effect (*F* value of 9.5; *P* value of 0.0052) on the yield of oligomers reveals the dependence of their time course on Depol 670L/ Gamanase 1,5L G ratio.

The following equations (7.2, 7.3, and 7.4) show the quadratic models for the yields of low-MW and high-MW oligosaccharides as well as for that of oligomers in terms of coded factors. Model terms that were not statistically significant (p > 0.05) were removed.

$$\text{Low} - \text{MW oligosaccharides}(\text{yield}, \text{g}/100\text{g}) = 4.8 + 0.4x_1 - 0.5x_2 - 2.0x_3 - 0.9x_1x_2 - 1.1x_1x_3 - 0.9{x_1}^2 - 0.6{x_2}^2 + 1.3{x_3}^2 \quad (7.2)$$

$$\text{High} - \text{MW oligosaccharides}\ (\text{yield}, \text{g}/100\text{g}) = 8.6 - 1.8x_1 - 0.3x_2 - 7.3x_3 + 2.9x_1x_3 - 2.0{x_1}^2 - 2.3{x_2}^2 + 3.3{x_3}^2 \quad (7.3)$$

$$\text{Oligomers}(\text{yield}, \text{g}/100\text{g}) = 36.4 + 14.1x_1 - 15.6x_2 + 1.1x_3 + 4.7x_1x_2 + 3.9x_1x_3 + 2.2{x_1}^2 - 8.7{x_3}^2 \quad (7.4)$$

7.4.3. Effects of reaction parameters on the yield of oligosaccharides and oligomers

The relationships between the reaction parameters and the yields of produced oligosaccharides and oligomers can be better understood by studying the contour plots generated from the predicted models. Figure 7.1a-c and Figure 7.1d-f illustrate the interaction effect of reaction time and substrate concentration on the predicted yields of low-MW and high-MW oligosaccharides, respectively, at constant Depol 670L/ Gamanase 1,5L ratios of 50/50, 62.5/37.5, and 75/25 (-1, 0, and +1 levels).

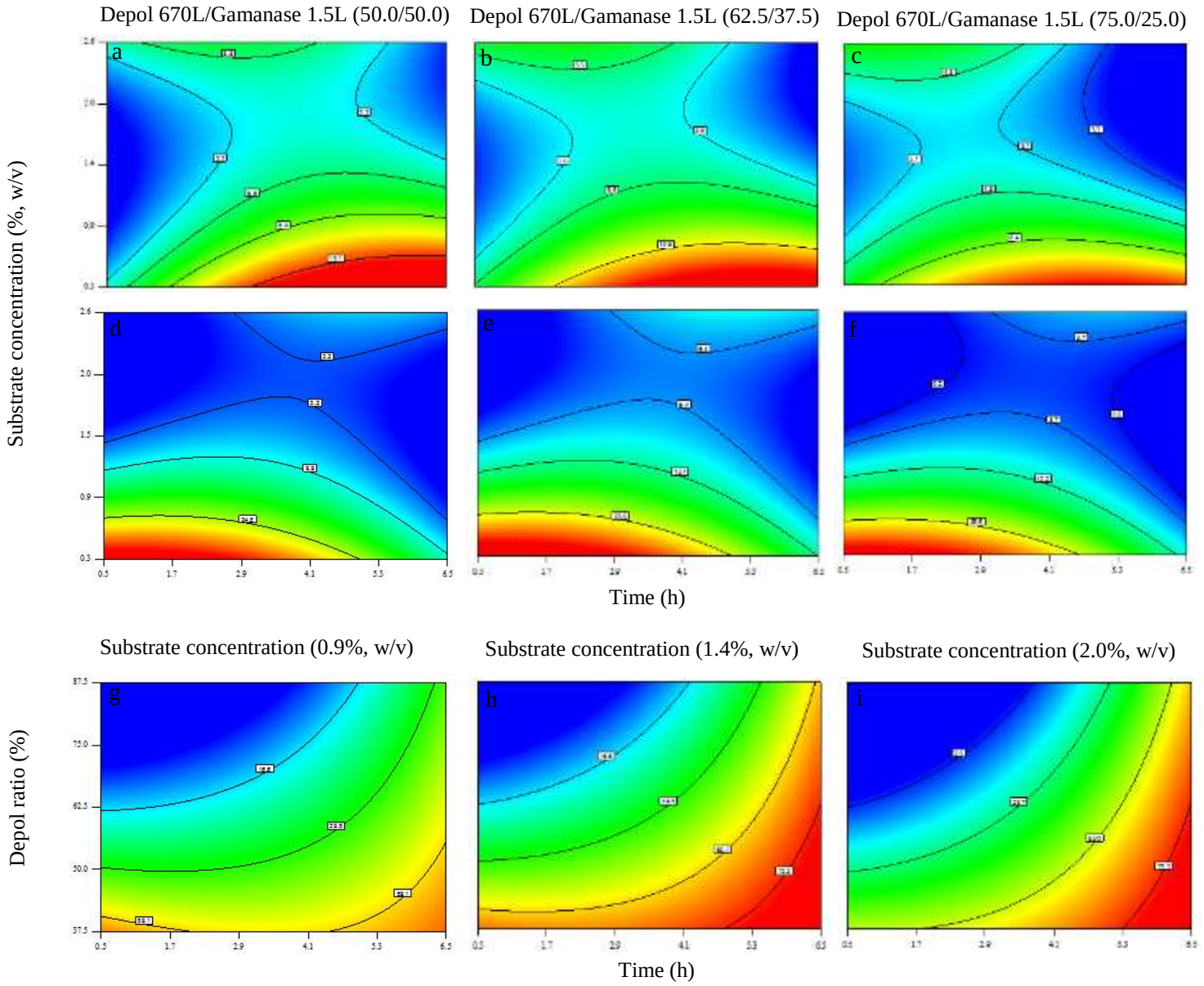

Figure 7.1 Contour plots of predictive models for yield (%) of oligosaccharides with DP of 2-6 (a, b and c) 7-12 (d, e and f) and 13-70 (g, h and i). The numbers inside the contour plots indicate the predicted responses.

The highest yields of high-MW oligosaccharides were achieved at the initial stage of reaction (< 3 h) upon the use of low substrate concentrations (<0.5%, w/v); while the low-MW oligosaccharides were dominant at the last stage of reaction (>3 h) at low substrate concentrations (<0.5%, w/v). At the low range of substrate concentration (<1.7%, w/v), the yield of low-MW oligosaccharides increases with the increase of reaction time up to 5 h and with the decrease of substrate concentration to 0.3%. These results reveal the adverse interactive effect of reaction time and substrate concentration on low-MW oligosaccharides; however, this interaction was not demonstrated at prolonged reaction times. Contrary to the low-MW oligosaccharides, the reaction time exhibited higher negative effect on the yield of high-MW oligosaccharides at prolonged reaction times (>3 h); and no or little significant positive effect was observed at the early stage. These contour plot trends may reveal the further hydrolysis of high-MW oligosaccharides, accumulated at the early stage of the reaction, into low-MW ones at the last stage. The increase in the substrate concentration had a significant negative effect on the predicted yields of both low-MW and high-MW oligosaccharides; this can be attributed to (a) the enzyme inhibition by the substrate excess, (b) the occurrence of substrate diffusional limitations and/or (c) to the presence of substrate/substrate interactions hindering their access to the enzyme's active site. Increasing Depol 670L/Gamanase 1,5L ratio from 50/50 to 75/25 (U/U) decreased the space region corresponding to the maximum yield of low-MW oligosaccharides; these results can be due to the excessive hydrolysis of low-MW oligosaccharides in the presence of higher ratio of Depol 670L as demonstrated by the released monosaccharides (data not shown). Contrary to low-MW oligosaccharides, increasing Depol 670L/Gamanase 1,5L ratio increased the space region corresponding to maximum yield of high-MW oligosaccharides. These results reveal that an excess of Depol 670L over that of Gamanase 1,5L can favor the release of high-MW oligosaccharides. Both reaction time and enzyme ratio can be controlled in order to modulate the end-product profiles of oligosaccharides.

The oligomers contour plots (Figure 7.1g-i) shows that the interaction between the enzyme ratio and the reaction time was more significant at the middle investigated ranges. As an overall, increasing Depol 670L/ Gamanase 1,5L ratio resulted in a decrease in the yield of the oligomers; this effect of enzyme ratio was more significant at the early stage of reaction. These results may be explained by the occurrence of antagonistic interaction in the presence of an excess of Depol 670L and/or by the high efficiency of glycosyl-hydrolases expressed in Gamanase 1,5L to

initiate the hydrolysis of the steric hindered regions of potato RG I-rich pectic polysaccharides. The effect of reaction time on the predicted oligomers yield was more pronounced at the last stage (> 2.9) in which an increase of the yield was observed. The hydrolysis of polysaccharide regions was enhanced at prolonged reaction times than at shorter ones. Moreover, increasing substrate concentration up to 1.44% (w/v) increased the yield of the oligomers. These results show that due to substrate inhibition, the further conversion of oligomers into oligosaccharides was limited resulting in the accumulation of oligomers. However further increases of the substrate caused slight decrease in the space region corresponding to maximum yield, which shows that substrate inhibition hinders the conversion of polysaccharide to oligomers. Substrate inhibition has been reported by Olano-Martin et al. (2001) in their studies as high substrate concentration had negative effect on product conversion when all other factors where kept constant.

7.4.4. Optimum conditions

Using Design-Expert 8.0.2 software, the developed models were used to determine the optimum conditions for the production of each of low-MW and high-MW oligosaccharides as well as oligomers. Table 7.3 shows the yield of low and high-MW oligosaccharides and oligomers as well as the monosaccharide profile of hydrolysates produced under the identified optimal conditions. Predicted values obtained for the yields of oligosaccharides and oligomers were no significantly different ($P < 0.05$) from the experimental data; and all experimental findings are within the statistically significant ranges of the estimated optimum values with 95% prediction intervals, confirming the adequacy of the models (data not shown).

At all three identified optimum conditions, Depol 670L/Gamanase 1.5L ratio was more or less at equal amount (52.0/48.0; 57.7/42.3; 50.0/50.0, U/U), revealing their synergistic interaction. In contrast, treatments n^{o} 23 and 24, in which Depol 670L was the dominant biocatalyst (87.5%), resulted in a very low yield of hydrolysates (0.1-0.4%). Moreover, treatments n^{o} 21 and 22 with Gamanase 1.5L being the dominant biocatalyst (62.5%) resulted in very low yields of both low and high-MW oligosaccharides (0.0-3.7%) and high yield of oligomers (69.4-72.9%). As expected, the highest amount of low-MW oligosaccharides (11.2%) was obtained when longer incubation time (5.0 h) and smaller substrate concentration (0.9%, w/v) was used (Opt 1).

Table 7.3 Optimum conditions resulting in highest yield of oligosaccharides with DP of 2-6 (Opt 1), 7-12 (Opt 2) and 13-70 (Opt 3), corresponding responses and their monosaccharide profile.

Sample		RG[a]	Opt 1	Opt 2	Opt 3
Incubation condition	Time (h)		5	2	5
	Depol 670L/Gamanase 1.5L (U/U) [b,c]		52.0/48.0	57.7/42.3	50.0/50.0
	Substrate concentration (%,w/v)		0.9	0.9	1.7
Yield of oligosaccharides and oligomers (%)	DP2-6		11.2	3.7	2.9
	DP7-12		7.3	21.9	0.7
	DP13-70		38.3	37.9	67.0
Total oligosaccharide/oligomer (%)			56.8	63.5	70.6
Sugar composition (%)	Rha	2.2	2.3	0.0	1.7
	Ara	5.7	11.3	7.2	7.1
	Gal	63.1	72.9	77.0	75.0
	Glc	13.2	3.2	7.9	6.5
	Xyl	4.9	0.5	0.0	0.9
	Man	3.0	1.8	0.0	1.9
	GalA	7.9	8.1	7.9	6.9

[a] RG was extracted using microwave assisted -alkaline extraction method (KOH concentration: 1.5M, time: 2.0 min, power: 36.0 W, solid/liquid: 2.9 %).

[b] The enzymatic activity unit (U) was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

[c] Total enzymatic unit (expressed in galactanase unit) was kept constant at 0.2 U/mg RG I-rich pectic polysaccharides.

Higher yield of high-MW oligosaccharides was obtained upon the decrease of incubation time to 2 h (Opt 2, yield of 21.9%); while the highest yield of oligomers was obtained when the substrate concentration was increased to 1.7 % (Opt 3, 67%), indicating the reduced hydrolysis rate. Table 7.3 also shows the monosaccharide profile of the hydrolysates, obtained using the optimum conditions. The highest proportions of Rha, Ara and GalA (2.3, 11.3 and 8.1%, respectively) were obtained at Opt 1 condition identified for the optimal generation of low-MW oligosaccharides. These results reveal that higher proportion of low-MW oligosaccharides is originated from HG region and arabinan side chains as compared to high-MW oligosaccharides and oligomers. Moreover, higher proportion of Rha at Opt 1 condition indicates more breakdown of RG I backbone, when longer reaction tine is used. The highest proportions of Gal (75.0-77.0%) and Glc (6.5-7.9%) were obtained at Opt 2 and Opt 3 conditions corresponding to optimum ones for the release of high-MW oligosaccharides and oligomers, respectively. Opt 3 condition resulted in the highest total yield (oligosaccharides/oligomers); these results confirm the efficient hydrolysis of galactan-rich RG I in the presence of equal amount of Depol 670L/Gamanase 1.5L (50/50, U/U) and upon longer reaction time. As compared to potato RG I-rich pectic polysaccharides, oligosaccharides and oligomers were enriched with Ara and Gal, revealing the efficient hydrolysis of side chains of RG I. However, the proportion of hemicellulosic monosaccharides in hydrolysates was lower than that of potato RG I-rich pectic polysaccharides (21.1%), indicating the limited hydrolysis of the hemicellulosic polysaccharides.

7.5. Conclusion

A bi-enzymatic system was studied for the generation of Gal-rich oligosaccharides/oligomers with defined DP. The effects of the reaction conditions, including reaction time, Depol 670L/Gamanase 1.5L ratio and substrate concentration, on the yield, the monosaccharide profile and the DP of generated oligosaccharides/oligomers were investigated. The results showed that the highest yields were obtained for both oligosaccharides and oligomers when equal enzymatic unit of Depol 670L and Gamanase 1.5L was used in combination, confirming their synergistic interactions. The highest amount of oligosaccharides with DP of 2-6 was obtained when longer incubation time and smaller substrate concentration were used. Decreasing incubation time resulted in the highest yield of oligosaccharides with DP of 7-12 while increasing substrate concentration led to the highest yield of oligomers with of DP of 13-70.

CONNECTING STATEMENT 6

Selected multi-enzymatic preparations were evaluated for the generation of oligomers/oligosaccharides from potato galactan-rich RG I .The DP and the sugar composition of generated oligosaccharides/oligomers were previously characterized (Chapters VI and VII). In Chapter VIII, the fermentability of potato galactan-rich RG I and their corresponding hydrolysates (oligosaccharides/oligomers), generated with Depol 670L and endo-1,4-β-galactanase, is investigated using a continuous culture system inoculated with immobilized faecal microbiota to simulate the colonic ecosystem. Upon the use of the generated carbohydrates as carbon sources, the changes in bacterial population (*Bacteroides / Prevottela / Porphiromonas, C. leptum, Bifidobacterium, Blautia, Lactobacillus, Enterobacteriaceae and Enterococcus*), prebiotic index and SCFA production are discussed. Moreover, the *in vitro* digestibility of potato galactan-rich RG I and their corresponding hydrolysates (oligosaccharides and oligomers) was studied using simulator of small intestine (TIM-1) to assess their ability to reach the colon intact.

The results from this chapter were presented at IUFOST, *17th World Congress of Food Science and Technology* and was submitted to the journal of *Carbohydrate Polymers.*

Khodaei, N., Karboune, S. (2014). High scale production of prebiotic oligosaccharides from potato rhamnogalacturonan I. IUFOST, *17th World Congress of Food Science and Technology*.

Khodaei, N., Fernandez, B., Fliss, I. & Karboune, S. (2015). Digestibility and prebiotic properties of potato rhamnogalacturonan I and its oligosaccharide/oligomers. *Carbohydrate Polymers. Under Revision.*

CHAPTER VIII

DIGESTIBILITY AND PREBIOTIC PROPERTIES OF POTATO RHAMNOGALACTURONAN I POLYSACCHARIDE AND ITS GALACTOSE-RICH OLIGOSACCHARIDES/OLIGOMERS

8.1. Abstract

Galactose-rich oligosaccharides/oligomers (oligo-RG I) were produced by the enzymatic treatment of potato galactan-rich rhamnogalacturonan I (RG I) with endo-β-1,4-galactanase and Depol 670L multi-enzymatic preparation. The digestibility study revealed that 81.6 and 79.3% of RG I and its corresponding oligomers remained unhydrolyzed, respectively. The prebiotic properties of RG I and its hydrolysates were investigated using a continuous culture system inoculated with immobilized faecal microbiota. Both RG I and oligo-RG I have stimulated the growth of *Bifidobacterium spp.* and *Lactobacillus spp.*, with oligo-RG I hydrolysates being more selectively fermented by these beneficial bacteria. Furthermore, none of RG I nor its hydrolysates increased the populations of *Bacteroidetes* and *C. leptum*. Total amounts of short chain fatty acids, generated upon the fermentation of oligo-RG I, were higher than those obtained with its parent RG I and the positive control (fructooligosaccharides). The overall study contributes to the understandings of the prebiotic properties of potato RG I and of their corresponding oligosaccharides/oligomers.

8.2. Introduction

The modulation of the gut microbiota through the consumption of prebiotic ingredients is an emerging concept for promoting the intestinal health. Plant cell wall polysaccharides from food processing by-products are very attractive as raw materials for the production of novel prebiotics. Indeed, cell wall materials have been investigated for the generation of both uronic acid-rich and neural sugar-rich oligosaccharides with prebiotic properties (Chen et al., 2013; Gullón et al., 2013; Gullón et al., 2011; Hotchkiss et al., 2003; Manderson et al., 2005). However, neutral sugar-rich oligosaccharides and oligomers showed higher prebiotic properties as compared to those containing mainly uronic acid (Gullón et al., 2011; Gullón et al., 2009). On the other hand, arabinogalactan has shown higher tolerability due to the gradual fermentation and higher persistence of the prebiotic effect up to the distal colon as compared to the well-stablished prebiotic fructo-oligosaccharide (FOS) (Gullón et al., 2013; Van Loo, 2004).

Most pectin-rich by-products, such as sugar beet pulp, citrus pulp, bergamot peel and apple pomace, have been investigated as sources of uronic acid-based prebiotic oligosaccharides, originating from homogalacturonan (HG) region of the cell wall (Chen et al., 2013; Gullón et al.,

2013; Gullón et al., 2011; Hotchkiss et al., 2003; Manderson et al., 2005). Potato pulp was identified as a pectin-rich by-product being composed of mainly pectic polysaccharides (56%, w/w) (Oomen et al., 2003; Khodaei and Karboune, 2013; Khodaei et al., 2015). In contrast to other pectin-rich by-products, potato pulp is of high interest as a source of neutral sugar-based prebiotic oligosaccharides/oligomers because of its high content of galactan-rich rhamnogalacturonan I (RG I) (75% of pectic polysaccharides) (Oomen et al., 2003; Khodaei and Karboune, 2013; Khodaei et al., 2015).

Studies on the bioactive properties of potato pectic polysaccharides have shown that they were fermentable and increased the number of *Bifidobacterium* spp. and *Lactobacillus* spp (Lærke et al., 2007; Thomassen et al., 2011); however, these studies were carried out without conversion of polysaccharides to oligosaccharides/oligomers. Michalak et al. (2012) have investigated the prebiotic properties of two fractions of <10 and >10 kDa recovered upon the enzymatic treatment of potato pulp polysaccharides with endo-1,4-β-galactanase using single culture fermentation. These authors reported that both fractions promoted the growth of probiotic strains, *B. longum* and *L. acidophilus,* and did not support the growth of *C. perfringens*.

The digestibility of structurally well-defined galactose (Gal)-rich oligosaccharides/ oligomers from potato RG I and their fermentability by microbiota as well as their ability to produce short change fatty acid (SCFA) still need to be investigated. In the present study, Gal-rich oligosaccharides/oligomers from potato RG I were produced by the enzymatic treatment with endo-β-1,4-galactanase as mono-component enzyme (Gal-based oligo-RG I) and Depol 670L as multi-enzymatic preparation (Dep-based oligo-RG I). The *in vitro* digestibility of potato RG I and of its corresponding well-defined oligo-RG I was investigated using TIM-1 model in order to assess their ability to reach the colon intact. Moreover, their fermentation was studied using a continuous culture system inoculated with immobilized faecal microbiota to simulate and mimic the colonic environment. The fermentation model of the human gut microbiota using immobilized cell technologies was developed by Cinquin et al. (2004) and has been proven to be a useful tool for the study of interaction between prebiotics (Cinquin et al., 2006; Tanner et al., 2014) and probiotics (Zihler et al., 2010; Zihler et al., 2011) and antibiotic molecules (Fernandez et al., 2014). This study is expected to contribute to the understanding of the digestibility and the prebiotic properties of potato RG I and of their corresponding oligosaccharides/oligomers with well-defined molecular weight (MW) distributions and

structures. Such understanding would provide the ability to modulate the prebiotic properties by targeting specific structural properties.

8.3. Materials and methods

8.3.1 Materials

KOH, sodium acetate, acetic acid, peptone, L-cysteine HCl, Tris-EDTA, dextran standards, Gal, arabinose (Ara), rhamnose (Rha), glucose (Glc), xylose (Xyl), mannose (Man), galacturonic acid (GalA), dextrans, gellan and xanthan gums, thermostable α-amylase from *B. licheniformis*, amyloglucosidase from *A. niger*, lysozyme, mutanolysin, proteinase K, pepsin from porcine gastric mucosa, porcine bile extract and pancreatin from porcine pancreas were purchased from Sigma-Aldrich Co. (St-Louis, MO). All salts were obtained from Fisher Scientific (Fair Lawn, NJ). Lipase from *Rhizopus oryzae* was obtained from Amano Enzyme USA Co. and human saliva was from Lee BioSolutions. Endo-β-1,4-galactanase (*A. niger)* was from Megazyme (Wicklow, Ir) and Depol 670L was from Biocatalysts Ltd. (Mid Glamorgan, UK). Propidium monoazide was obtained from Biotium, (Hayward, CA) and fast SYBR Green mastermix was from Life Technologies (Carlsbad, CA). 1-Kestose, nystose and 1F-fructosyl-nystose were obtained from Wako Pure Chemical (Japan) and FOS was from Orafti (Belgium).

8.3.2. Preparation of potato RG I-type polysaccharide extract

Potato RG I-type polysaccharides were prepared by microwave-assisted alkaline treatment of potato pulp (Lyckeby Starch AB), according to our modified method (Khodaei et al., 2015). The identified optimum conditions were: KOH concentration of 1.5 M, microwave treatment time of 2 min, power/suspension ratio of 1.2 W/mL and solid/liquid ratio of 2.9% (w/v). The recovered extracts after centrifugation (8000 ×g, 25 min) were neutralized. In order to remove starch from the recovered extract, thermostable α-amylase from *B. licheniformis* and amyloglucosidase from *A. niger* were added to yield a concentration of 1.0 and 2.2 mL per g of extract, respectively. Sodium azide (0.05%) was added to solution in order to avoid microbial growth. After incubation at 40 °C under 150 rpm agitation for 16 h, the recovered extract was ultrafiltrated through a membrane with 10 kDa cut-off using a EMD Millipore Pellicon™ peristaltic system. Additional precipitation of the polysaccharides with ethanol (1:1, v:v) was carried out. The recovered high MW polysaccharides were structurally characterized.

8.3.3. Assessment of glycosyl-hydrolase activities of selected multi-enzymatic products

Pectinase, rhamnogalacturonase, galactanase and arabinanase activities of Depol 670L and endo-β-1,4-galactanase mono-component enzyme were assessed using substrate solution of 0.25% polygalacturonic acid, 0.55% soybean RG, 0.55% potato pectic galactan or 0.55% arabinan, respectively. The substrate solutions were prepared in 50 mM sodium acetate buffer, pH 5.0 and the assays were carried out by adding 0.025 ml of enzyme to 0.475 ml of substrate solutions followed by incubation at 40 °C for 20 min. Two blank assays were also performed without substrate or without enzyme. The reducing ends were quantified using dinitrosalicylic acid (DNS) assay and the enzymatic activity unit (U) was defined as the amount of enzyme that produces 1 µmole of reducing ends per min of reaction.

8.3.4. Generation of oligosaccharides from potato RG I-type polysaccharides

Potato RG I-type polysaccharide suspension (3%, w/v) in sodium acetate buffer (50 mM, pH 5.0) was subjected to the enzymatic treatment with endo-β-1,4-galactanase as mono-component enzyme or Depol 670L as a multi-enzymatic preparation at 0.2 units of endo-β-1,4-galactanase /mg polysaccharide. After incubation at 40 °C for 4 h, the unhydrolyzed polysaccharides were precipitated by ethanol (1:1, v:v). The hydrolysates, recovered upon centrifugation (8,000 ×g, 25 min) and freeze drying were analyzed for their MW distribution and monosaccharide composition.

8.3.5. Analysis of monosaccharide profile

The monosaccharide profile of potato RG I-type polysaccharides and their hydrolysates was analyzed as described by Khodaei & Karboune (2013) by high performance anionic exchange chromatography equipped with pulsed amperometric detector (HPAEC-PAD, Dionex), and a CarboPac PA20 column. GalA content was measured using sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973).

8.3.6. Determination of MW distribution

MW distribution of the potato RG I-type polysaccharides and their hydrolysates were analyzed using Waters HPLC system equipped with refractive index detector. Oligosaccharides (DP of 2-12), oligomers (DP of 13-70) and polysaccharides (DP > 70) fractions were separated and quantified. For the polysaccharide fractions, three columns in series (TSK G3000 PWXL,

TSK G4000 PWXL and TSK G5000 PWXL) were used; while the oligosaccharides were analyzed on a TSKgel G-Oligo-PW column. All columns were from Tosoh Bioscience (Montgomeryville, PA). Isocratic elution with 0.1 M sodium chloride was applied at flow rate of 0.5 mL/min. Dextrans with MW of 50, 150, 270, 410 and 670 kDa were used for polysaccharide/oligomer analysis; while Gal, sucrose, 1-kestose, nystose and 1F-fructofuranosyl-nystose were used as the standards for the oligosaccharide analysis.

8.3.7. Evaluation of digestibility of RG I and generated oligosaccharides

The TIM-1 system (TNO, Nutrition and Food Research Institute, Zeist, Ne.) was used in order to assess the digestibility of the RG I-type polysaccharides and Depol-based RG I oligomers under intestinal conditions (pH and enzymes) (Minekus et al., 1995) (Figure 8.1). The model consists of four compartments simulating stomach, duodenum, jejunum and ileum working under controlled temperature of 37°C. Initial pH in the stomach was 5.5, which was dropped gradually to 2.7, 2.2 and 1.8, by injecting 1 M hydrochloric acid, after 40, 60 and 90 min of digestion, respectively, and remained constant at 1.7 from 120 to 300 min. The pH values in duodenum, jejunum and ileum were maintained at 6.5, 6.8 and 7.2, respectively, using 1 M sodium bicarbonate solution. Gastric secretion consisted of pepsin (0.19 g/L, ≥3200 units/mg solid) from porcine gastric mucosa and lipase (0.25 g/L, 150 units/mg protein), both in an electrolyte solution (NaCl, 4.8 g/L; KCl, 2.2 g/L; $CaCl_2$, 0.3 g/L; $NaHCO_3$, 1.25 g/L; pH 4.0) delivered at flow rates of 0.25 mL/min. Duodenal secretion was composed of 21 % (w/w) pancreatin solution in distilled water and 4% (0-30 min) or 2 % (30-300 min) (w/w) porcine bile extract as well as small intestine electrolyte solution (NaCl, 5.0 g/L; KCl, 0.60 g/L; $CaCl_2$, 0.30 g/L; pH 7.0). Contents of the jejunum and ileum were dialyzed through hollow fiber membranes (cut-off 11.8 kDa) against small intestinal electrolyte solution and the compounds that passed through membrane were collected (jejunum and ileum dialysates).

The residue that exited from ileum (effluent) and solution remaining in the duodenal, jejunal and ileal compartments at the end of the digestion (chyme) were collected and considered to be indigestible. The chymes and effluent residues were heated (75°C/5 min) immediately following removal from TIM-1 and all collected samples after 300 min of digestion were freeze dried and analyzed for their MW distribution and sugar composition.

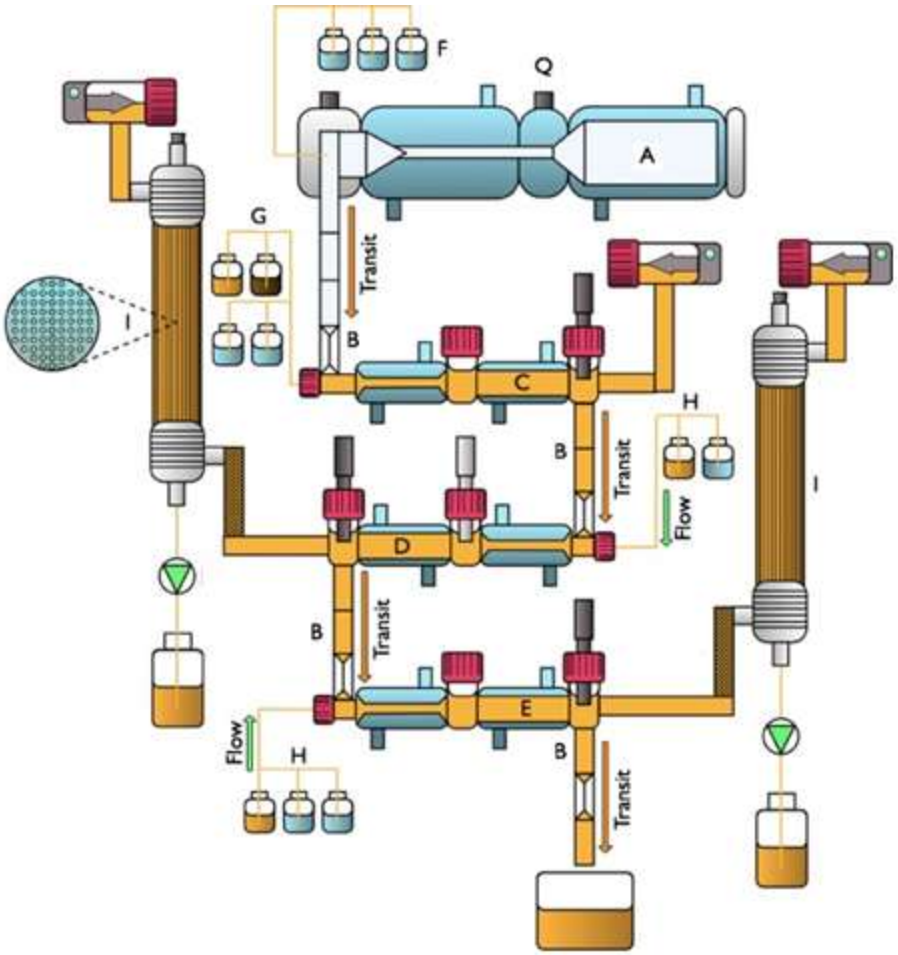

Figure 8.1 Schematic representation of TIM-1 system (Dickinson et al., 2012). A: Stomach compartment (Final pH 1.7, 300 min), B: Peristaltic valve, C: Duodenum compartment (pH 6.5, 300 min), D: Jejunum compartment (pH 6.8, 300 min), E: Ileum compartment (pH 7.2, 300 min), F: Stomach secretion (Pepsin and lipase in electrolyte solution), G: Duodenum secretion (Pancreatin solution, porcine bile extract and small intestine electrolyte), H: 1 M sodium bicarbonate solution to adjust pH, I: Hollow fiber membrane.

8.3.8. Immobilization of fecal inoculum in gel beads

Human faeces was collected from one volunteer, who has not been on antibiotics for at least 3 months and had no history of gastrointestinal disorders. The fecal sample was suspended in peptone water (0.1 %, w/v) containing 0.05% (w/v) L-cysteine HCl at 37 °C. The fecal inoculum was entrapped into gum-based beads according to the dispersion method of Cinquin et al. (2004).

Briefly, the fecal inoculum (2%, w/v) was added to sterilized gum solution, made of gellan (2.5%, w/v) and xanthan gum (2.5% w/v), in sodium citrate solution (0.2%, w/v). Then the inoculated gum suspension was added to a hydrophobic phase (commercial canola oil) at a ratio of 1:2 (v/v). The beads were suspended in autoclaved 0.1 M calcium chloride solution in order to harden them. The beads with diameter of 1-2 mm were separated by sieving and washed with a saline solution (0.27 M potassium chloride and 0.03 M calcium chloride).

8.3.9. Fecal culture fermentation

A stirred glass bioreactor (BIOSTAT® Qplus, Sartorius AG, Goettingen, Germany) was used to investigate the fermentability of potato RG I-type polysaccharides and their corresponding hydrolysates by the immobilized fecal inoculum (62.5 g of gel beads for 187.5 mL of broth). The colonic broth developed by Macfarlane et al. (1998), supplemented with vitamin solution (0.5 mL/L), 0.05% L-cysteine-HCl (16.0 mL/L) and bile salt (2.0 g/L), was continuously fed into the bioreactor with a flow rate of 0.35 mL/min resulting in a retention time of 12 h. Carbon source of the Macfarlane medium included pectin (citrus), arabinogalactan, xylan, guar gum, inulin and starch (potato) (2, 2, 2, 1, 1, 5 g/L, respectively). The vitamin solution was composed of pyridoxine HCl (20 mg/L), p-aminobenzoic acid (10 mg/L), nicotinic acid (10 mg/L), biotin (4 mg/L), folic acid (4 mg/L), vitamin B12 (1 mg/L); thiamine (8 mg/L) ; riboflavin (10 mg/L); menadione (2 mg/L); vitamin K_1 90.005 mg/L); pantothenate (20 mg/L) (Gibson & Wang, 1994). Culture temperature and pH were maintained at 37 °C and 6.2, respectively.

Anaerobic conditions were kept by constant flow of oxygen free carbon dioxide gas in the reactor. After 16 days of stabilization with regular Macfarlane medium, 75% of carbohydrate source in Macfarlane medium was substituted with potato RG I-type polysaccharides, Dep-based oligo-RG I, Gal-based oligo-RG I and FOS as standard reference. Fermentation of each samples was carried out for four days. A fermentation with only 25% carbohydrate was also run as a negative control. The experimental set-up design is shown in Figure 8.2. Each sample was tested twice in random order and averages of results were used in the study.

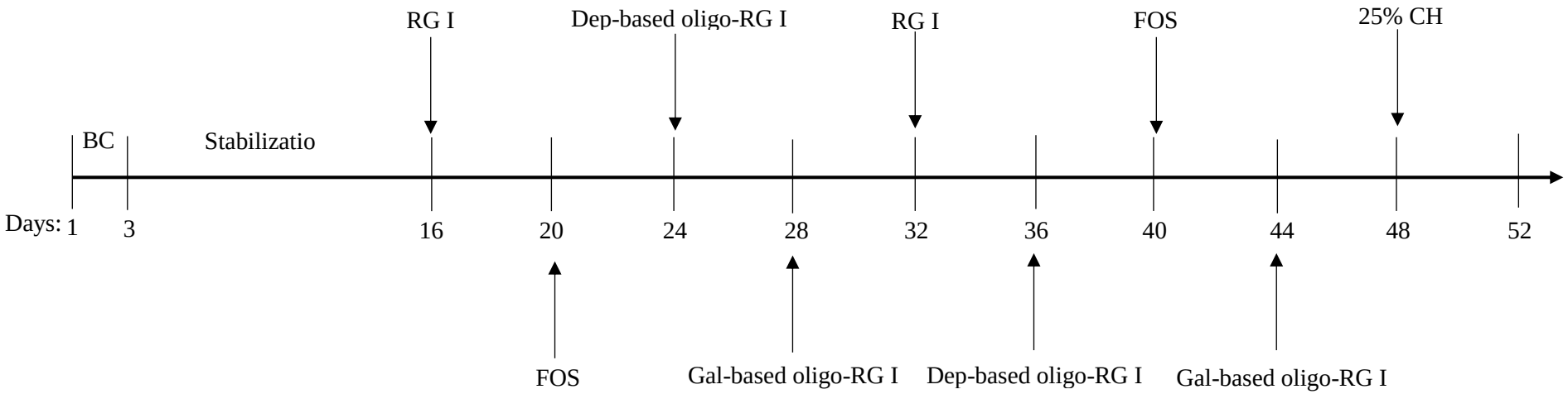

Figure 8.2 Schedule of the continuous fermentation steps, the numbers indicate days of the fermentation (BC: Beads colonization, RG I: Extracted polysaccharides from potato pulp using microwave-assisted alkaline treatment, Dep-based oligo-RG I: Generated oligosaccharide from potato RG I upon treatment with Depol 670L, Gal-based oligo-RG I: Generated oligosaccharide from potato RG I upon treatment with endo-β-1,4-galactanase, FOS: Fructo-oligosaccharide, 25% CH: Fermentation with 25% of carbohydrate source in the medium as a negative control).

8.3.10. Bacterial enumeration

8.3.10.1 Bacterial strains and growth conditions of reference strain

E. coli (ATCC 25922) and *Enterococcus faecalis* (ATCC 27275) were grown aerobically at 37 °C for 24 h in brain-heart infusion broth and tryptic soy broth supplemented with 0.6 % yeast extract, respectively. *Bifidobacterium adolescentis* (ATCC 15703), *Bacteroides thetaiotaomicron* (ATCC 29741), *C. leptum* (ATCC 29065) and *B. cocoides* (ATCC 29236) were cultured at 37 °C; MRS broth supplemented with 0.05 % L-cysteine-HCl was used for *Bifidobacterium*; while brain-heart infusion broth supplemented with 0.05 % L-cysteine-HCl was used for *Bacteroides* and *B. cocoides.* For *C. leptum,* a modified chopped meat medium with maltose was selected. All the bacterial strains were obtained from the American Type Culture Collection (Rockville, MD, USA), and sub-cultured at least three times before use.

8.3.10.2. Sample collection and DNA extraction

The enumeration of the target fecal bacterial groups was performed by quantitative polymerase chain reaction coupling with propidium monoazide treatment (PMA-qPCR). Fermentation samples were collected every day from bioreactor and 2.5 µl of propidium monoazide solution (20 mM in 20 % dimethylsulfoxide) was added to 1 mL of diluted sample. The samples were incubated for 5 min in the dark followed by incubation for 5 min under halogen light Fujimoto, Tanigawa, Kudo, Makino, & Watanabe, 2011). DNA was extracted as described by Ahlroos & Tynkkynen (2009) using genomic DNA Purification Kit (Promega, WI, USA) with some modifications. Diluted (10x) recovered DNA (5 µl) was added to the reaction mixture (20 µl) consisted of 12.5 µl of SYBR green, 1 µl of each forward and reverse primer at 5 µM and 5.5 µl of DNase free water.

List of the specific primers used for the quantification of bacteria is presented in Table 8.1. Analysis was performed on an ABI 7500 fast real-time PCR system (Applied Biosystems, ON, Ca) in 96-well plates. Prebiotic index (PI) was estimated as follow (Manderson et al., 2005):

$$\mathrm{PI} = \frac{(\Delta \log_{10} Bifidobacterium)_{t_x - t_0}}{(\Delta \log_{10} total\ counts)_{t_x}} + \frac{(\Delta \log_{10} Lactobacillus)_{t_x - t_0}}{(\Delta \log_{10} total\ counts)_{t_x}} - \frac{(\Delta \log_{10} Bacteroides)_{t_x - t_0}}{(\Delta \log_{10} total\ counts)_{t_x}} - \frac{(\Delta \log_{10} Closterdia)_{t_x - t_0}}{(\Delta \log_{10} total\ counts)_{t_x}}$$

Where t_0 is last the day of stabilization (day 16) and t_x is last day of each fermentation period (days 20, 24, 28, 32, 36, 38, 40, 44, 48, 52 and 56).

Table 8.1 Specific primers used for quantification of bacteria.

Species	Gene	Direction	Sequence 5'-3'	Amplicon (pb)	References
Bacteroides / Prevottela / Porphiromonas	16S	F	GGTGTCGGCTTAAGTGCCAT	140	(Rinttilä et al., 2004)
		R	CGGA(C/T)GTAAGGGCCGTGC		
Clostridium leptum group	16S	F	GCACAAGCAGTGGAGT	208	(Kanno et al., 2009)
		R	CTTCCTCCGTTTTGTCAA		
Bifidobacterium spp.	16S	F	TCGCGTC(C/T)GGTGTGAAAG	243	(Rinttilä et al., 2004)
		R	CCACATCCAGC(A/G)TCCAC		
Blautia cocoides	16S	F	AAATCACGGTACCTGACTAA	440	(Matsuki et al., 2002)
		R	CTTTGAGTTCATTCTTGCGAA		
Lactobacillus spp.	16S	F	TGGATGCCTTGGCACTAGGA	92	(Wang et al., 2012)
		R	AAATCTCCGGATCAAAGCTTACTTAT		
Enterobacteriaceae	16S	F	CAT GCC GCG TGT ATG AAG AA	96	(Huijsdens et al., 2002)
		R	CGG GTA ACG TCA ATG AGC AAA		
Enterococcus spp.	16S	F	CCCTTATTGTTAGTTGCCATCATT	144	(Rinttilä et al., 2004)
		R	ACTCGTTGTACTTCCCATTGT		

8.3.11. SCFA analysis

Culture samples recovered at selected fermentation period (16-56 days) were centrifuged (10,000 ×g, 10 min, 4 °C) and analyzed for their SCFA contents using a HPLC system (Waters, Milford, MA, USA) equipped with an ICSep-Ion-300 (300 x 7.8 mm) column (Transgenomics, San Jose, CA, USA) and a differential refractometer detector (Model 2142, LKB, Bromma, Sweden). The eluent was 0.0065 N (or 3.25 mM) sulfuric acid at a flow rate of 0.4 mL/min.

8.4. Results and discussion

8.4.1. Properties of RG I and generated oligo-RG I

Pectinase (polygalacturonase), rhamogalacturonase, endo-β-1,4-galactanase and arabinanase specific activities expressed in Depol 670L multi-enzymatic product were estimated at 0.0, 1.9, 230.9 and 1.9 U/mg protein, respectively; while the specific activity of endo-β-1,4-galactanase mono-component enzyme was determined to be 431.0 U/mg protein. The structural properties of the potato RG I and its hydrolysates (Dep-based oligo-RG I; Gal-based oligo-RG I) are reported in Table 8.2. As expected, the major part of the RG I extract was polysaccharides (90.8%). Dep-based oligo-RG I and Gal-based oligo-RG I did not contain any polysaccharides, but they were composed of 42.5 and 50.6% of oligomers (DP of 13-70), respectively.

Major oligosaccharides (DP of 2-12) in the Dep-based extract were those with DP of 5 (26.3%) and 6 (24.9%); while the Gal-based oligosaccharide proportion was mainly composed of oligosaccharides with DP of 3 (19.0%), 5 (10.6%) and 8 (12.6%). As far as the authors are aware, only one study (Michalak et al., 2012) has investigated the production of prebiotic compounds, by endo-1,4-β-galactanase from *Emericella nidulans*, from enzymatically solubilized β-1,4-galactan-rich potato pulp polysaccharides (>100 kDa). This study has reported the recovery of two main fractions of less (20.6%, w/w) and more than 10 kDa (65.0%, w/w) characterized by a Gal molar proportion of 92.9 and 53.9 %, respectively.

Because of the efficient removal of hemicellulosic fragments by ultrafiltration, RG I extract exhibited lower molar proportions of (Glc, Xyl, Man) monosaccharides (5.3%) originating from the hemicellulosic polysaccharides as compared to that obtained in our previous study (21.1%) (Khodaei et al., 2015).

Table 8.2 Molecular weight distribution and monosaccharide profile of potato Rhamnogalacturonan I (RG I)a and of its hydrolysates (Depb and Gal-based oligo-RG I).

Samples	Molecular Weight Distribution (% w/w)										
	Oligosaccharides –DP								Oligosaccharide	Oligomers	Polysaccharides
	1	2	3	4	5	6	7	8	(DP 2-12)	(DP 13-70)	
RG I	2.7	-	2.9	-	0.8	0	-	1.4	5.1	1.4	90.8
Dep-based oligo-RG I	6.3	-	-	-	26.3	24.9	-	0.0	51.2	42.5	-
Gal-based oligo-RG I	6.1	1.1	19	-	10.6	-	-	12.6	43.3	50.6	-

	Sugar Composition (% w/w)						
	Rha	Ara	Gal	Glc	Xyl	Man	GalA
RG I	1.9	5.5	79.9	3.4	0.7	0.9	7.7
Dep-based oligo-RG I	1.6	8.8	72.7	6.0	1.7	0.5	8.8
Gal-based oligo-RG I	1.6	9.8	70.0	12.1	1.6	1.0	3.8

[a] Extracted polysaccharides from potato pulp using microwave-assisted alkaline treatment.

[b] Generated oligosaccharide from potato RG I upon treatment with Depol 670L.

[c] Generated oligosaccharides from potato RG I upon treatment with endo-β-1,4-galactanase.

The results also show that Gal was the main monosaccharide (70.0-79.9%) in the RG I, Dep-based oligo-RG I and Gal-based oligo-RG I. Higher proportion of Gal in oligo-RG I as compared to RG I (*F*-value of 37.7 and *p*-value of 0.0004) may be attributed to the incomplete hydrolysis of galactan side chains. Indeed, endo-β-1,4-galactanase catalyzes selectively the hydrolysis of β-(1-4)-D-Gal linkages of galactan branches with less steric hindrance (de Vries et al., 2002). Moreover, the relative proportion of Ara in the oligo-RG I extracts (8.8-9.8%) was slightly higher as compared to that of RG I (5.5%); this can be attributed to the efficient hydrolysis of the arabinan side chains. Because the endo-β-1,4-galactanase mono-component enzyme exhibited low pectinase activity, the resulted Gal-based oligo-RG I had low GalA proportion (3.8%) as compared to RG I (7.7%) and the Dep-based oligo-RG I (8.8%).

8.4.2. Assessment of digestibly of RG I polysaccharides and generated oligo-RG I

In order to determine the colonic persistence, the digestibility of a mixture of RG I and Dep-based oligo-RG I (1:1, w:w) was assessed using the TIM-1 model. The MW distributions and the monosaccharides composition of the initial fed (stomach) and digesta are reported in Table 8.3. The mixture characterized at the stomach level corresponds to the properties of the initial product. The initial mixture contained 28.2% and 22.0% of oligosaccharides (DP of 2-10) and oligomers, respectively (mostly originating from Dep-based oligo-RG I) and 45.4% of polysaccharides (originating from RG I).

As expected, the monosaccharide composition of the mixture shows the abundance of Gal (75.3 %) followed with lower amount of GalA (8.3 %) and Ara (7.7 %). It was calculated that 86% of the initial amount (31.3 g) fed to the TIM-1 system (stomach) were recovered in the digesta, including jejunum/ileum dialysates, chyme (remaining solution in duodenal/jejunal/ileal compartments) and effluent; the remaining amount (14-16%) may have been lost during handling and/or left in the tubing. Considering the 11.8 kDa cut-off in hollow fiber membranes, oligosaccharides part of the mixture would enter jejunum and ileum dialysates regardless of their hydrolysis rate. However, the low recovery extent of larger oligomers (DP of 13-70) and polysaccharides in the chyme and effluent can provide an indication of their hydrolysis. The initial carbohydrate mixture contained 6.9 and 14.2 g of oligomers (DP of 13-70) and polysaccharides, respectively, corresponding to 22.0 and 45.4% of initially fed carbohydrate.

Table 8.3 Molecular weight distribution and sugar composition of carbohydrates (stomach: a mix of RG I[a] and Dep-based oligo-RG I[b], 1:1) fed to the TIM-1 system and their recovered digesta (jejunum and ileum dialysate, chyme[c], and effluent[d]).

Samples		Molecular Weight Distribution (expressed in g)															
		Oligosaccharides – DP										Oligosaccharide (DP 2-12)		Oligomer (DP 13-70)		Polysaccharide	
		1	2	3	4	5	6	7	8	9	10						
Stomach		0.4	-	0.5	-	3.8	3.9	-	0.2	-	-	8.8	(28.2)[e]	6.9	(22.0)	14.2	(45.4)
Small Intestine	Chyme	0.1	0.1	-	-	-	-	0.1	-	0.1	-	0.4	(1.4)[f]	2.2	(8.2)	2.6	(9.7)
	Jejunum	0.4	4.3	-	0.1	-	-	0.4	-	-	0.3	5.1	(19.2)	-	-	-	-
	Ileum	-	5.6	-	-	-	-	-	-	-	-	5.7	(21.1)	-	-	-	-
	Effluent	0.1	0.2	-	0.1	-	-	0.2	-	-	-	0.5	(1.8)	2.6	(9.7)	7.1	(26.3)

		Sugar Composition (expressed in g)													
		Rha		Ara		Gal		Glc		Xyl		Man		GalA	
Stomach	Total fed	0.5	(1.8)[g]	2.2	(7.2)	23.9	(76.3)	1.5	(4.7)	0.4	(1.2)	0.2	(0.7)	2.6	(8.3)
	Polysaccharide/ oligomer	0.4	(1.8)	1.4	(6.6)	16.4	(77.5)	0.9	(4.2)	0.2	(1.0)	0.2	(0.8)	1.7	(8.1)
	Oligosaccharide	0.2	(1.6)	0.9	(8.8)	7.4	(72.7)	0.6	(6.0)	0.2	(1.7)	0.1	(0.5)	0.9	(8.8)
Small Intestine	Chyme	0.0	(0.0)	0.0	(0.0)	4.6	(88.5)	0.3	(5.8)	0.0	(0.0)	0.0	(0.0)	0.3	(5.8)
	Ileum	0.1	(1.7)	0.4	(6.9)	4.4	(75.9)	0.3	(5.2)	0.1	(1.7)	0.0	(0.0)	0.5	(8.6)
	Jejunum	0.1	(1.7)	0.4	(6.7)	4.5	(75.0)	0.3	(5.0)	0.1	(1.7)	0.1	(1.7)	0.5	(8.3)
	Effluent	0.3	(3.2)	1.0	(10.8)	6.7	(72.0)	0.4	(4.3)	0.1	(1.1)	0.1	(1.1)	0.7	(7.5)

[a] Extraction of RG I polysaccharides from potato pulp was carried out by microwave-assisted alkaline treatment. [b] Generated oligosaccharides/oligomers from potato RG I upon treatment with Depol 670L. [c] Chyme refers to the suspensions remained in duodenal, jejunal and ileal compartments at the end of the digestion. [d] Effluent refers to the residue exited from ileum. [e] Molecular weight distribution of the sample fed to the TIM-1 expressed in weight percentage of initial amount (stomach, 31.3 g). [f] Molecular weight distribution of digestas expressed as weight percentage of total recovered digesta (chyme + ileum dialysate + jejunum dialysate + effluent, 26.8 g). [g] Monosaccharide composition of the samples expressed in weight percentage.

While the total amount of oligomers (DP of 13-70) and polysaccharides recovered in the chyme and the effluent was 4.8 and 9.7 g, representing 17.9 and 36.1% of recovered digesta, respectively.These results show that during digestion, 81.6 and 79.3% of the oligomers (DP of 13-70) and polysaccharides remained unhydrolyzed, respectively. These results reveal that most of the RG I and oligo-RG I remained undigested. The observed partial hydrolysis of the oligomers and polysaccharides may be due to the low stomach pH values. Similarly, it has been reported that when inulin or oligofructoses were fed to ileostomy subjects, 86-89% of the original compounds were recovered at the terminal ileum (Cummings et al., 2001). Moreover, Hernández-Hernández et al. (2012), who have studied the ileal digestibility of two galacto-oligosaccharides derived from lactulose (DP >2, 14.0% trisaccharides) and lactose (DP >3, 35.1% trisaccharides) in growing rats, have reported that 12.5% and 52.9% of the trisaccharide fraction of lactulose and lactose-derived oligosaccharides were hydrolyzed, and the disaccharide fraction of lactulose derived oligosaccharides was fully resistant to the hydrolysis of digestive tract.

Table 8.3 also indicates that the amount of monosaccharides in the total recovered carbohydrates (0.63 g, 2.4%) did not increase significantly as compared to the initial amount (0.4 g, 1.3%); these results reveal that major part of hydrolyzed polysaccharides and oligomers were converted into oligosaccharides, which themselves have prebiotic properties. Total amount of oligosaccharides (DP of 2-12) in the initial mixture was 8.8 g (28.1%) and was increased to 11.7 g (43.7%) in the total recovered carbohydrate. Due to large cut-off in membrane of TIM 1 (11.8 kDa), these oligosaccharides were absorbed through jejunum and ileum dialysates; however, in human intestine, all carbohydrates such as sucrose, lactose, and starch must be hydrolyzed to monosaccharides before they can be absorbed in the small intestine (Rhoades, 2013). Disaccharides constituted the major part of the oligosaccharides collected from jejunum (4.3 g, 16%) and ileum (5.6 g, 20.9%) dialysates.

The monosaccharide profile of the initial fed (stomach) shows that the contents of Rha, Ara, Gal, Glc+Xyl+Man and GalA in the polysaccharide/oligomer fraction were 0.4, 1.4, 16.4, 1.3 and 1.7 g, respectively (Table 8.3). The recovered polysaccharide/oligomer fraction in the chyme/effluent (representing unhydrolyzed fraction after digestion) was composed of 0.3, 1.0, 11.3, 0.9 and 1.0 g of Rha, Ara, Gal, Glc+Xyl+Man and GalA, respectively. Considering 16% loss of sample during handling, it can be estimated that of homogalacturonan (24.7%, GalA loss)

was the most hydrolyzed upon digestion, while RG I backbone (8.5%) was the least hydrolyzed. Arabinan and galactan side chains and hemicellulosic polysaccharides exhibited more or less similar hydrolysis extent (12.1-14.7%) upon digestion.

8.4.3. Prebiotic properties of RG I and generated oligo-RG I

The prebiotic activities of potato pectic-type RG I, Dep-based oligo-RG I and Gal-based oligo-RG I were assessed by measuring their abilities to support the growth of mixed bacterial populations from human faecal inocula. Figure 8.2 shows the scheme of the continuous fermentations in which 75% of carbohydrate source of the medium was substituted with RG I, oligo-RG I and FOSs. Changes in bacterial population were daily determined using PMA-qPCR. *Bifidobacterium* and *Lactobacillus* spp. consist of 4% and less than 2% of the total intestinal microbiota in adults, respectively; the effect of increasing their numbers, as a result of consuming prebiotics, on the intestinal health is well documented (Kleerebezem & Vaughan, 2009). Well-established prebiotic FOS was selected as positive control because it is well known to increase growth rate of *Bifidobacterium* and *Lactobacillus* spp. and decrease that of *Clostridium* (Michalak et al., 2012).

Table 8.4 shows the bacterial counts at the last day of fermentation and the changes occurring as result of the substitution by each of the investigated carbohydrate sources. Changes smaller than 0.5 log was not considered significant.

Looking at the beneficial bacteria, the populations of both *Bifidobacterium* and *Lactobacillus* spp. increased significantly during all fermentations (*F*-value of 78.7 and *p*-value of 0.0000 for *Bifidobacterium* and *F*-value of 220.8 and *p*-value of 0.0000 for *Lactobacillus* spp.). Indeed, *Bifidobacterium* and *Lactobacillus* spp. were reported to be able to express β-galactosidase (EC 3.2.1.23), β-1,3-galactosidase (EC 3.2.1.145), and endo-β-1,4-galactanase (EC 3.2.1.89) activities (Thomassen et al., 2011). As an overall, the increase in the bacterial population of *Lactobacillus* (0.8-2.2 log) was higher as compared to that of *Bifidobacterium* (0.6-1.5 log). For *Bifidobacterium*, the highest increase was achieved with the Dep-based oligo-RG I (1.4 log) and FOSs (1.5 log) followed by the Gal-based oligo-RG I (1.3 log). These results reveal that oligo-RG I can be considered bifidogenic under the investigated conditions. On the other hand, Gal-based oligo-RG I resulted in higher increase in *Lactobacillus* (2.2 log) as compared to the Dep-based oligo-RG I (2.0 log) and FOS (2.0 log).

Table 8.4 Bacterial populations at the last day of fermentation for each sample (Log cells/ml) and change in bacterial count during fermentation (difference between last day of each sample's fermentation and stabilization period).

Bacterial species	Bacterial populations (Log cells/ml)						Change in Bacterial Count			
	Fecal sample	Stabilization	RG I[a]	Dep-based oligo-RG I[b]	Gal-based oligo-RG I[c]	FOS[d]	RG I	Dep-based oligo-RG I	Gal-based oligo-RG I	FOS
Bacteroides / Prevottela / Porphiromonas	10.02 ± 0.08	9.36 ± 0.04	9.7 ± 0.07	9.4 ± 0.13	9.3 ± 0.07	9.4 ± 0.17	0.3	0.1	0.0	0.0
Clostridium leptum group	9.57 ± 0.07	8.06 ± 0.07	6.9 ± 0.12	7.3 ± 0.12	7.3 ± 0.16	7.4 ± 0.05	-1.2	-0.7	-0.7	-0.6
Bifidobacterium spp.	9.12 ± 0.07	5.54 ± 0.05	6.2 ± 0.18	7.0 ± 0.13	6.8 ± 0.07	7.0 ± 0.14	0.6	1.4	1.3	1.5
Blautia cocoides	9.19 ± 0.1	6.67 ± 0.07	7.3 ± 0.17	7.3 ± 0.03	7.3 ± 0.05	7.3 ± 0.13	0.7	0.6	0.6	0.6
Lactobacillus spp.	8.34 ± 0.06	4.80 ± 0.1	5.6 ± 0.14	6.8 ± 0.13	7.0 ± 0.12	6.8 ± 0.04	0.8	2.0	2.2	2.0
Enterobacteriaceae	7.93 ± 0.07	7.55 ± 0.07	8.3 ± 0.04	7.7 ± 0.14	7.9 ± 0.12	7.6 ± 0.07	0.8	0.2	0.3	0.1
Enterococcus spp.	5.94 ± 0.05	4.78 ± 0.09	6.1 ± 0.05	6.7 ± 0.02	6.8 ± 0.05	6.3 ± 0.11	1.3	1.9	2.0	1.5
Prebiotic index (PI)							0.3	0.5	0.5	0.5

[a] Extracted polysaccharides from potato pulp using microwave-assisted alkaline treatment.

[b] Generated oligosaccharide from potato RG I upon treatment with Depol 670L.

[c] Generated oligosaccharides from potato RG I upon treatment with endo-β-1,4-galactanase.

[d] Fructo-oligosaccharide.

However, the use of non-hydrolyzed RG I resulted in an increase in *Bifidobacterium* (*F*-value of 28.8 and *p*-value of >0.005) and *Lactobacillus* (*F*-value of 101.4 and *p*-value of >0.005) populations with a smaller extent (0.6 log) as compared to their corresponding hydrolysates. This difference between RG I and its oligosaccharides/oligomers may be due to the complex structure of RG I and its steric hindrance, limiting the access of the bacterial enzymes to the hydrolysis sites (Van Loo, 2004). Indeed, more enzymes are needed for the breakdown of the complex polysaccharides (El Kaoutari et al., 2013). It has been reported that low-MW oligosaccharides were more selectively fermented by *Bifidobacterium* than their parent high-MW polysaccharides (sugar beet arabinan and fructans) (Al-Tamimi et al., 2006; Van Loo, 2004). In addition, RG I backbone may be less fermentable as compared to RG I side chains. Van Laere et al. (2000) have reported that oligosaccharides derived from side chains of RG I ((arabino)galacto-oligosaccharides and arabino-oligosaccharides) were more fermented by *Bifidobacterium* than those from backbone of RG I (rhamnogalacturono-oligosaccharides and galacturono-oligosaccharides).

The results of bacterial populations (Table 8.4) also show that *Enterococcus* exhibited a significant increase in its population, with 1.9 to 2.0 log unit increment (*F*-value of 130.0 and *p*-value of 0.0000), over the fermentation process of the investigated hydrolysates as carbohydrate sources. For Dep- and Gal-based oligo-RG I, the increase in the *Enterococcus* was higher than that of *Bifidobacterium* and lower than that of *Lactobacillus.* On the other hand, the fermentation of FOS led to similar increase (1.5 log) of *Enterococcus* population as compared to *Bifidobacterium* (1.5 log) and to lower increase as compared to *Lactobacillus* (2.0 log). Potato RG I resulted in a higher increase of *Enterococcus* (1.3 log) population as compared to *Bifidobacterium* and *Lactobacillus.* Table 8.4 also indicates that *B. cocoides* population increased to equal extent when RG I (0.7 log), Dep- and Gal-based oligo-RG I and FOSs (0.6 log) were used (*F*-value of 22.0 and *p*-value of 0.0001). The MW of the investigated substrates seems not to affect the growth of *B. cocoides* (*F*-value of 0.0 and *p*-value of 1.0000). *B. cocoides* metabolizes H_2/CO_2 to acetate which decreases the total gas volume in the colon and produces a non-gaseous metabolite used as an energy source for eukaryotic cells (Bernalier et al., 1996). *Enterobacteriaceae* number increased when RG I was used (0.8 log) (*F*-value of 259.6 and *p*-value of 0.0001), while there was no significant change in their populations during the fermentation of oligo-RG I and FOS I (0.1-0.3 log) (*F*-value of 5.5 and *p*-value of 0.02).

The results also show that the proliferation of *Bacteroidetes* and *C. leptum* was not supported by the investigated carbohydrates, and their populations either remained constant (*Bacteroidetes*: *F*-value of 4.2 and *p*-value of 0.07) or decreased (*C. leptum*: *F*-value of 42.8 and *p*-value of 0.0000). More specifically, *Bacteroides/Prevottela /Porphiromonas* did not show significant changes in their populations except for RG I with a small increase of 0.3 log. Similar results have been reported in the literature demonstrating that *Bacteroides* are able to use polysaccharides, while *Bifidobacterium* prefer low-MW carbohydrates (Al-Sheraji et al., 2013; Al-Tamimi et al., 2006; Cummings et al., 2001; Van Laere et al., 2000). Moreover, Van Laere et al. (2000) have found that *Bacteroides* are able to ferment rhamnogalacturono-oligosaccharides, arabinogalactans and galacto-oligosaccharides and concluded that *Bacteroides* can express a wide variety of glycanases and glycosidases. These results are supported by our study as the number of *Bacteroides* spp. didn't decrease during fermentation. Indeed, *Bacteroides* comprise one of the major phylum in the human gut flora (approximately 25%) (Salyers, 1984) and the balance between *Bacteroidetes* and *Firmicutes phyla* can affect obesity and intestinal inflammation (Thomassen et al., 2011). It has been reported that relative proportion of *Bacteroidetes* is decreased in obese people by comparison with *Firmicutes* (Ley et al., 2006). On the other hand, the results (Table 8.4) reveal that *C. leptum* population decreased significantly with more than one log in the presence of RG I and with 0.6-0.7 log with oligo-RG I. The low affinity of *Clostridium spp*. for rhamnogalacturono-oligosaccharides has also been reported by Van Laere et al. (2000). However, these authors have found that *Clostridium* spp. were able to utilize galacto-oligosaccharides as a carbohydrate source in a single culture fermentation. The decrease in the number of *Clostridium* population in our study may be the result of growth and production of secondary metabolite by other species. Gibson & Wang (1994) who studied the fermentation of oligofructose by *Bifidobacterium infantis*, *E. coli*, and *C. perfringens* reported that *Bifidobacterium* growth inhibited that of *E. coli* and *C. perfringens* (Cummings et al., 2001). Decrease in number of *Clostridium* while maintaining the number of *Bacteroides/Prevottela /Porphiromonas* can be advantageous as it has been shown that decrease in *Clostridium* and increase in *Bacteroides* number is associated with weight loss in obese adolescents (Nadal et al., 2009).

Despite differences in the DP between the Gal- and Dep-based oligo-RG I, their effects on the selective bacterial growth were more or less similar with less than 0.2 log unit difference. The

results also show that there is no significant difference (≤0.5 log) in the bacterial counts between FOSs and oligo-RG I. In contrast, Rycroft et al. (2001) have reported that galactose-containing oligosaccharides, namely lactulose, galacto-oligosaccharides and soybean oligosaccharides, increased *Bifidobacterium* numbers more than fructose-containing inulin and FOS. Moreover, Gullón et al. (2011), who have investigated the fermentability of selected oligosaccharides obtained from apple pomace by faecal microbiota, have reported that gluco-oligosaccharide, galacto-oligosacchairdes and xylo-oligosaccharide were first fermented followed by arabino-oligosaccharides and oligogalacturonides.

Selective fermentation of potato derived polysaccharides and oligosaccharides by *Bifidobacterium* and *Lactobacillus* have been reported previously using single culture fermentation. For instance, Michalak et al. (2012) have reported that hydrolyzed galactan (<3, 3-10 and 10-100 kDa) and potato pulp polysaccharides (<10 and >10 kDa) promoted the growth of probiotic strains of *B. longum* and *L. acidophilus* and generally did not support the propagation of *C. perfringens* in single culture fermentations. The author also reported that stimulation of *B. longum* growth in the presence of hydrolyzed galactan was significantly higher than with FOS, galactose and unhydrolyzed galactan. Moreover, Thomassen et al. (2011) have obtained an increase in the numbers of *Bifidobacterium* spp. and *Lactobacillus* spp, upon the fermentation of high-MW polysaccharides (up to 400 kDa) from potato pulp by faecal microbiota. The same authors have reported that the increase in number of the *Bifidobacterium* spp. was higher when RG I-rich and HG-rich fractions were used as carbohydrate sources than FOS (DP 2–8 Orafti®P95), and RG I-rich fraction had better bifidogenic property than HG-rich fraction.

Table 8.4 also shows PI of investigated carbohydrates. Gal- and Dep-based oligo-RG I and FOS exhibited more or less similar PI of 0.46-0.5, while RG I resulted in lower PI of 0.2. Manderson et al. (2005) have reported that orange peel pectic oligosaccharides, characterized by a monosaccharide profile of Glc, Ara, Gal, GalA, Xyl and Rha of 48.1, 31.2, 9.6, 6.3, 2.4 and 2.1%, respectively, resulted in a lower PI than FOS. In contrast, Mandalari et al. (2007) have shown that bergamot oligosaccharides obtained from alcohol insoluble residue, with a monosaccharide profile of GalA, Ara, Glc, Gal, Man, Rha and Xyl of 48.2, 10.5, 5.8, 5.7, 2.1, 1.9 and 1.3%, respectively, exhibited a greater PI value than FOS.

8.4.4. The profile of SCFAs produced during fermentation

The major end products of prebiotic metabolism are SCFAs (acetate, formate, propionate and butyrate), the gases (hydrogen, carbon dioxide), and bacterial cell mass (Cummings et al., 2001; Macfarlane et al., 2008). Increased amount of total SCFA is desirable since low pH and high SCFA amount suppress pathogenic bacteria. More specifically, propionate and formate were reported to reduce the activity of *E. coli* and *Salmonella* at pH 5 (Gullón et al., 2011; Topping & Clifton, 2001). Table 8.5 summarizes the profile of SCFAs produced during fermentation of RG I, oligo-RG I and FOSs as control. The SCFA concentration was measured at the end of the four day fermentation period for each carbohydrate source.

The results show that the concentration of total SCFAs produced at the end of oligo-RG I fermentations (167.8-173.1 mM) was slightly higher than those produced in the presence of RG I and FOSs (163.6- 164.4 mM) (*F*-value of 11.59 and *p*-value of 0.0028). Similarly, Manderson et al. (2005) have reported that after 24 h fermentation, pectic oligosaccharides from orange peel (Glc 48.12, Ara 31.19, Gal 9.59, GalA 6.29%) resulted in higher total SCFA as compared to FOS and orange albedo. Moreover, Chen et al. (2013) have also reported that parent polysaccharide (apple pectin) resulted in less total SCFA as compared generated oligomers (5000 Da), and the parent pectin produced less acetate and more butyrate than pectic oligosaccharides, which is similar to results of the current study.

For all tested carbohydrates, acetate production was the dominant one as compared to other SCFAs. However, the fermentation of oligo-RG I (Gal- and Dep-based ones) induced more production of acetate (100.7-104.4 mM) than unhydrolyzed RG I (79.8 mM) and FOSs (69.2 mM) (*F*-value of 72.9 and *p*-value of 0.0000). Other studies on the prebiotic properties of pectic polysaccharides, their oligosaccharides and FOS have also reported the production of acetate as a dominant SCFA. For instance, the fermentation of oligomeric saccharides derived from apple pectin has resulted in the production of mainly acetate and butyrate (Gullón et al., 2011; Takahashi et al., 2008).

The high acetate production upon all fermentations is consistent with *Bifidobacterium* and *Lactobacillus* population increases. Acetate can be formed by many anaerobic bacteria from the human gut (Macfarlane & Gibson, 1997); however, acetate is typically generated via *bifidus* pathway, and more specifically it is a major end product of *Bifidobacterium* fermentation (Chen et al., 2013; Gullón et al., 2011).

Table 8.5 Short chain fatty acids (SCFA) released at the end of the fermentation of selected carbohydrates.

SCFA (mM)	RG I[a]	Dep-based oligo-RG I [b]	Gal-based oligo-RG I[c]	FOS[d]	25% CH[e]
Acetate	79.8 (± 0.62)	104.3 (± 1.9)	100.7 (± 0.63)	69.2 (± 0.5)	68.1 (± 1.48)
Propionate	46.8 (± 0.35)	36.0 (± 0.88)	30.4 (± 0.49)	35.8 (± 1.83)	27.4 (± 1.42)
Butyrate	31.3 (± 0.50)	26.5 (± 0.5)	30.6 (± 0.88)	51.9 (± 1.08)	16.9 (± 0.84)
Valerate	6.5 (± 0.15)	6.3 (± 0.15)	6.1 (± 0.12)	6.7 (± 0.33)	10.8 (± 0.73)
Total SCFA	164.4 (± 2.7)	173.1 (± 0.92)	167.8 (± 1.39)	163.6 (± 3.04)	123.2 (± 1.71)

[a] Extracted polysaccharides from potato pulp using microwave-assisted alkaline treatment.

[b] Generated oligosaccharide from potato RG I upon treatment with Depol 670L.

[c] Generated oligosaccharides from potato RG I upon treatment with endo-β-1,4-galactanase.

[d] Fructo-oligosaccharide.

[e] Fermentation with 25% of carbohydrate source in the medium as a negative control.

This can explain the higher production of acetate upon the fermentation of oligo-RG I as compared to the parent RG I polysaccharides stimulating less increase in the *Bifidobacterium* and *Lactobacillus* populations. However, although the fermentation of FOSs supported the growth of *Bifidobacterium* and *Lactobacillus* populations with the same extents as the oligo-RG I, it induced less production of acetate. These results may indicate the higher activity of *Bifidobacterium* in the presence of the generated oligo-RG I. Fukuda et al. (2011) have studied the effect of *Bifidobacterium* on promoting host defense against infection and showed that increased production of acetate can protect mice from enterohaemorrhagic *E. coli* O157:H7 and improve defense function of epithelial cell in intestine. In addition, acetate and propionate have shown to have anti-cholesterolemic effect (Patel & Goyal, 2011). Acetate may also be used by microbial populations to produce propionate or butyrate through metabolic cross-feeding between *Bifidobacterium* and butyrate-forming bacteria (Belenguer et al., 2007; Gullón et al., 2011).

The results (Table 8.5) also show that the amount of valerate was small and similar for both FOS and RG I derived samples (*F*-value of 4.7 and *p*-value of 0.04). Oligo-RG I maintained more or less the production of similar concentrations of butyrate (26.5-30.6 mM) and propionate (30.4-36.0 mM); while the fermentation of FOSs as a control led to higher production of butyrate (51.9 mM) followed by propionate (35.8 mM). On the other hand, the fermentation of unhydrolysed RG I resulted in higher concentration of the propionate (46.8 mM) as compared to butyrate (31.3 mM). Higher concentration of propionate resulted in fermentation of RG I is consistent with the larger increase in the number of *Bacteroides/Prevottela/Porphiromonas* upon fermentation of RG I since these microorganisms are known for their ability to produce propionate (Gullón et al., 2011). The butyrigenicity of FOSs has been previously reported (Macfarlane et al., 2008; Topping & Clifton, 2001). Butyrate is the preferred energy source for colonocytes and plays a role in maintaining normal colonocyte population, participating in energy metabolism and protection against colorectal disease and cancer (Belenguer et al., 2007; Gullón et al., 2011; Patel & Goyal, 2011). Contrary to our results, Wang & Gibson (1993) have found that FOS resulted in higher acetate and lower propionate production as compared to arabinogalactan (Cummings et al., 2001; Wang & Gibson, 1993). On the other hand, Al-Tamimi et al. (2006), who have investigated the fermentation properties of arabino-oligosaccharides with

different DP, have reported similar trends as RG I derived samples in our study with acetate being the predominant SCFA followed by propionate and butyrate.

8.5. Conclusion

The *in vitro* digestibility study revealed the high undigestibility of RG I polysaccharide and its corresponding oligomers. Both potato RG I polysaccharide and its corresponding oligosaccharides/oligomers were fermented by *Bifidobacterium* and *Lactobacillus*; however, the oligosaccharides and the oligomers exhibited higher selective fermentability by *Bifidobacterium* and *Lactobacillus*. Despite some differences in sugar composition and MW distribution between Dep and Gal-based oligo-RG I, their effects on the bacterial flora populations were very close. On the other hand, the potato RG I polysaccharide and its corresponding oligosaccharides/oligomers did not have a negative effect on *Bacteroidetes* and seem to reduce the population of the *C. leptum*. As an overall, Dep and Gal-based oligo-RG I and FOS exhibited similar PI; while RG I resulted in lower PI. Total amounts of SCFAs, generated upon the fermentation of Dep and Gal-based oligo-RG I, were higher than those obtained with RG I and FOS. Mixing RG I parent polysaccharide and oligo-RG I may provide the combination of both advantages of selective fermentation by lactic acid bacteria and the extended effect of prebiotic activity through proximal and distal regions of colon.

CHAPTER IX

GENERAL SUMMARY AND CONCLUSION

The present study investigated the efficiency of selected extraction methods, including alkaline (NaOH and KOH), enzymatic (endo-polygalacturonase from *A. niger*) and microwave-assisted alkaline (KOH), for the isolation of galactan-rich RG I from potato cell wall. Furthermore, an enzymatic approach was developed for the generation of oligosaccharides and oligomers from the extracted galactan-rich RG I polysaccharides using selected multi-enzymatic preparations. Statistically-designed experiments were used to evaluate the individual and interactive effects of different parameters on the polysaccharide extraction and the generation of oligosaccharides/oligomers. The models developed were used to generate galactan-rich RG I and oligosaccharides/oligomers with well-defined structures and functional properties. Lastly, the assessments of the prebiotic properties and digestibility of galactan-rich RG I and selected oligosaccharides/oligomers were carried out using a continuous culture system inoculated with immobilized faecal microbiota and a simulator of the small intestine (TIM-1), respectively.

RG-I polysaccharides were successfully extracted from potato cell wall using alkaline and enzymatic extraction methods. Enzymatic extraction resulted in no side chain debranching and in the release of a high proportion of high-MW polysaccharides (>500 kDa; 62.2%). In contrast, alkaline extraction led to 5 to 27% degradation of arabinan and galactan side chains depending on the type of alkaline solution (KOH, NaOH) and its concentration. In addition, the debranching of arabinan side chains was more significant as compared to galactan ones. Optimization of the enzymatic (endo-polygalacturonase from *A. niger*) isolation of galactan-rich RG I from potato pulp was carried out, and quadratic models were developed for the prediction of yield and monosaccharide profile of extracts. Using these models, the conditions for achieving the highest yield and Gal content were identified. The most significant parameters that affected the polysaccharide yield and the Gal and Ara contents were the cell wall and enzyme concentrations. The interaction between the cell wall concentration and reaction time was the most determinant interaction with respect to yield. However, the interaction of cell wall and enzyme concentrations exhibited a significant effect on Gal and Ara contents. Alkaline extraction was combined with a microwave treatment in order to extract galactan-rich RG I from potato pulp with high productivity and turnover number. The effect of extraction parameters was studied using RSM with CCRD, and the developed quadratic models used to identify the conditions resulting in the highest Gal content (63.1%). The solid/liquid ratio was identified as the most significant parameter affecting linearly the yield and Gal contents of galactan-rich RG I polysaccharides. Microwave power and solid/liquid ratio exhibited a significant adverse interactive effect on the

yield. Gal content of extracted polysaccharides could be modulated by compromising between KOH concentration and extraction time, which exhibited an adverse interaction. Galactan-rich RG I polysaccharides exhibited higher solubility and emulsifying stability as compared to potato galactan and HG from oranges. In addition, galactan-rich RG I polysaccharides showed Newtonian behaviour at 25 °C and 73 °C and pseudoplastic behaviour at 49 °C.

Extracted RG I polysaccharides were used to generate oligosaccharides/oligomers using multi-enzymatic preparations under various conditions. Higher oligosaccharide/oligomer yield was achieved using a multi-enzymatic preparation with a greater hydrolysing activity toward side chains of RG I as compared to its backbone (Depol 670L). Hydrolysates were composed of oligosaccharides with DP of 2-12, and oligomers with a DP of 13-70, and the main monosaccharide of the hydrolysates was Gal (58.9-91.2%, w/w). The biocatalysts, resulting in the highest oligosaccharide yield and Gal content and the lowest content of monosaccharide (Depol 670L and Gamanase 1.5L), were selected for further optimization of oligosaccharide/oligomer generation.

The effects of the conditions of bi-enzymatic system, including reaction time, Depol 670L/Gamanase 1.5L ratio, substrate concentration, on the yield, the monosaccharide profile and the DP of generated oligosaccharides/oligomers were investigated. The results showed that the greatest yields were obtained for both oligosaccharides and oligomers when equal amounts of Depol 670L and Gamanase 1.5L were used in combination, confirming their synergistic interaction. The greatest amount of oligosaccharides with DP of 2-6 was obtained under longer incubation times and smaller substrate concentrations. Greater quantities of oligosaccharides with a DP of 7-12 was obtained upon the decrease of incubation time; while oligomers with higher DP of 13-70 were dominant when the substrate concentration was increased. Both RG I and its hydrolysates were fermented by *Bifidobacterium* and *Lactobacillus* and did not support the growth of *Bacteroides* or *C. leptum*. The total amount of SCFA generated upon the fermentation of RG-derived hydrolysates were greater than those obtained with RG I polysaccharides and the control (FOS). The *in vitro* digestibility study revealed the high colonic persistence of RG I polysaccharides and of their corresponding oligomers.

Overall, the experimental findings provide the scientific knowledge for the extraction of galatan-rich RG I with minimum degradation and high yield and for the conversion of extracted polysaccharides to oligosaccharides/oligomers, with well-defined structures, of great potential as prebiotics.

CHAPTER X

CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

10.1. Contribution to Knowledge

The major contributions to knowledge of this study are:

1. This is the first study that compared alkaline and enzymatic treatments for the isolation of galactan-rich RG I, in terms of extraction yield, side chain debranching, monosaccharide profile and MW distribution. The effects of the type of alkaline solution (KOH or NaOH) and their concentrations have not been previously reported.
2. For the first time, the enzymatic (endo-polygalacturonase from *A. niger*) isolation of galactan-rich RG I from potato pulp was optimized, and mathematical models were developed to identify the best conditions for the extraction of RG I-type pectic polysaccharides with targeted yield and specific structural properties. Extracted polysaccharides using these conditions were characterized for their monosaccharide profile and MW distribution.
3. Microwave-assisted alkaline extraction was used for the first time for the extraction of galactan-rich RG I from potato pulp. Using identified optimal conditions, high yield and productivity of RG-enriched isolates were obtained. Moreover, for the first time, the techno-functional properties of galactan-rich RG I were characterized which can be used to identify food industry application of the extracted polysaccharides.
4. This is the first study that reported generation of oligosaccharides/oligomers from potato RG I using multi-enzymatic preparations. Oligosaccharides/oligomers with high yield and defined MW distribution and monosaccharide profile were generated and the results were discussed as function of the enzymatic activity profile of the multi-enzymatic preparations.
5. Enzymatic production of oligosaccharides/oligomers with defined DP and monosaccharide profile using bi-multi-enzymatic system (Depol 670L and Gamanase 1.5L) was optimized, and the conditions resulting in the highest yield of oligosaccharides and oligomers (DP of 13-70) were identified.
6. For the first time, it was shown that high proportions of RG I and its hydrolysates (DP of 13-70) remained unhydrolyzed during digestion, respectively. Both potato RG I polysaccharide and its corresponding hydrolysates were proven to be selectively fermented by *Bifidobacterium* and *Lactobacillus*, while reduced the population of the *C. leptum*.

The overall research will contribute to our understanding of the relationship between the extraction parameters and the structural properties of galactan-rich RG I. It is also expected to provide the capability for the enzymatic generation of well-defined oligosaccharides/oligomers from galactan-rich RG, based on the enzyme activity profile of the multi-enzymatic preparations. The investigation of the colonic fermentability of RG I and its hydrolysates will also contribute to a better understanding of the structural characteristics that drive the health-promoting properties for the effective production of prebiotics with improved functional properties.

10.2 Recommendations for Future Research

- Optimization of the processes for the large-scale production of RG I and its corresponding oligosaccharides/oligomers.
- Investigation of other health promoting properties of RG I and its corresponding oligosaccharides/oligomers, such anti-carcinogenic activity and anti-adhesive effects against pathogens.
- Determination of the efficiency of the techno-functional properties of RG I in food systems through food formulations.

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