MANNOPROTEINS AND β-GLUCAN FROM *SACCHAROMYCES CEREVISIAE* YEAST-BASED PRODUCTS: ISOLATION AND CHARACTERIZATION OF THEIR PROPERTIES

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ABSTRACT

Cell wall yeast, recovered upon the production of yeast extract, was investigated as an abundant source of mannoproteins. Selected isolation methods were evaluated for the recovery of mannoproteins, in terms of yield, mannan and protein recovery yield, mannoproteins content and mannan to protein ratio in extracted mannoproteins. The results showed that heat treatment and sodium dodecyl sulfate (SDS) extraction led to lower yields compared to the enzymatic treatment; and no glycosylated proteins could be obtained upon the SDS extraction. As compared to other methods, the enzymatic approach, based on the use of Zymolyase®, exhibiting a high β-1,3-glucanase activity, resulted in the highest yield, mannoproteins content and mannan to protein ratio. A 5-level, 2-variable central composite rotatable design contributed to the better understanding of the effects of independent variables, reaction time and enzyme units, on the efficiency of the enzymatic treatment and to the better modulation of their actions. The effects of enzyme units and reaction time on the mannoproteins content and the mannan to protein ratio exhibited the same patterns. Comparison of predicted and experimental values validated the established predicted models, which can be used to identify the conditions for the isolation of mannoproteins with well-defined molecular properties.

The yields and the structural properties of mannoproteins varied depending on the isolation methods and their sources (baker's and brewer's *Saccharomyces cerevisiae* yeasts). Non-covalently-bound mannoproteins (6.5 kDa) with a mannan/protein ratio of 0.63 and 2.78 were recovered upon the heat treatment of brewer's and baker's yeasts, respectively. While SDS treatment led mainly to the release of non-glycosylated proteins. The highest yield of mannoproteins was achieved upon the enzymatic isolation with Zymolyase® from *Arthrobacter luteus.* The recovered convalently bound mannoproteins were characterized by a higher mannan/protein ratio (13.1-42.7) and a wider MW distribution (5-10 kDa;10-100 kDa;100-400 kDa). Predictive models were developed to understand and modulate the effects of isolation parameters on yield, the mannoproteins content and the mannan/protein ratio. The enzyme concentration was the most significant parameter affecting the yield, while the reaction time was the most significant parameter affecting mannan/protein ratio. Comparison of predicted and experimental values validated the established predicted models.

The broad MW distribution and the variability of mannan to protein ratio of purified mannoproteins, isolated from yeast cell walls upon the enzymatic treatment, revealed their multiplicity. The main fraction of high-MW Agrimos®-MP1 and YCW-b-MP1' contained mannoproteins with a mannan to protein ratio of 3.5 and 6.9, respectively. Low-MW YCW-b-MP2' was mainly comprised of mannan, with a ratio of 181, whereas low-MW Agrimos®-MP2 was characterized by a ratio of 12.2. The solubility of MP1/MP2 was higher than that of MP1'/MP2'. Mannoproteins showed similar or lower solubility than mannan, and they exhibited a Newtonian behavior. Sonication was the appropriate method for the formation of mannoproteins-based emulsions. Contrary to MP1/MP1'-based emulsions, MP2/MP2'-based ones showed higher affinity towards soybean oil than glyceryl-trioleate. pH affected the emulsifying ability of MP1/MP1'. MP1/MP1' showed similar or slightly inferior emulsifying properties than lecithin. This study is expected to broaden the applications of mannoproteins as value-added ingredients.

Two purified mannoprotein populations were recovered including a high molecular weight (MP1, 620-36 kDa) composed of a variety of mannoproteins with different mannan to protein ratio (2.5-10), and a low molecular weight (MP2, 2.3-6.8 kDa) being identified as a monocomponent. α-(1,6) Mannanase treatment revealed the presence of α-(1,6) mannose linkages in the high MW MP1 population, but not in the low MW MP2 population. The isolated mannoproteins MP1 were mainly O-glycosylated. Mannoprotein structures were further characterized by 1D and 2D NMR, 7 anomeric carbons were determined in Agrimos® MP1, 4 of them have very similar chemical shifts than those of mannan standard. The proportion of α-(1,6) linked mannan was found to be the highest in the Agrimos® MP1. The thermal conformational changes of mannoproteins in both solid and liquid states were revealed by FTIR.

β-glucan has been associated with several functional and health-promoting properties. An enzymatic approach, based on the use of proteases, including Flavourzyme, Novo-proD, Papain and Alcalase, was investigated for the isolation and generation of β-glucan from yeast cell wall. The combination of hot water extraction step with protease treatment was found to improve the generation efficiency of β-glucan by opening the cell wall network and improving the accessibility of proteases to the protein substrates. Alcalase and Novo-ProD show higher specificity for hydrolyzing peptide bonds that have large hydrophobic aromatic amino acids (tyrosine, tryptophan, and phenylalanine). In addition, this study revealed the synergistic actions between the

proteolytic enzymes. The supplementation of Alcalase (0.1%, v/w) with Novo-ProD (0.5%, v/w) resulted in the highest total and β-glucan content of 62.1 and 44.2% (w/w), respectively; such increase corresponds to 98% enrichment of yeast cell wall with β-glucan. The supplementation of Alcalase (0.1%, v/w) with Papain (0.5%, w/w) and Flavourzyme (0.5%, v/w) led to 76.5 and 47.3% enrichment of yeast cell wall with β-glucan, respectively. A 5-level, 3-variable central composite rotatable design (CCRD) was performed with varying concentrations of Alcalase (0-0.82% v/w), Novo-proD (0-0.82% v/w) and Flavourzyme (0-0.82% v/w); and the responses were total glucan content, β-glucan content, β-glucan enrichment, weight loss, protein loss, glucan loss and mannan loss. The developed models are expected to provide the capability to enrich β-glucan in the extract and broaden their applications as value added ingredients.

RÉSUMÉ

La paroi cellulaire de levures, récupérée lors de la production d'extrait de levure, a été étudiée comme une source abondante de mannoprotéines. Des méthodes d'isolement sélectionnées ont été évaluées pour la récupération des mannoprotéines, en termes de rendement, de rendement en mannane et en protéines, de teneur en mannoprotéines et de rapport mannane/protéine dans les mannoprotéines extraites. Les résultats ont montré qu'un traitement thermique et une extraction au dodécyl sulfate de sodium (SDS) entraînaient des rendements plus faibles par rapport au traitement enzymatique; et aucune protéine glycosylée n'a pu être obtenue lors de l'extraction par SDS. Par rapport à d'autres méthodes, l'approche enzymatique, basée sur l'utilisation de Zymolyase®, présentant une activité élevée β-1,3-glucanase, a donné un rendement, une teneur en mannoprotéines et un rapport mannane/protéine les plus élevés. Une conception centrale composite rotative à 5 niveaux et 2 variables a permis de mieux comprendre les effets des variables indépendantes, du temps de réaction et des unités enzymatiques, sur l'efficacité du traitement enzymatique et de mieux moduler leurs actions. Les effets des unités enzymatiques et du temps de réaction sur la teneur en mannoprotéines et le rapport mannane/protéine présentaient les mêmes tendances. La comparaison des valeurs prédites et expérimentales a validé les modèles prédits établis, qui peuvent être utilisés pour identifier les conditions permettant l'isolement de mannoprotéines ayant des propriétés moléculaires bien définies.

Les rendements et les propriétés structurelles des mannoprotéines variaient en fonction des méthodes d'isolement et de leurs sources (levures de boulanger et de brasseur *Saccharomyces cerevisiae*). Des mannoprotéines non liées de manière covalente (6.5 kDa) avec un rapport mannanes/protéines de 0.63 et 2.78 ont été récupérées lors du traitement thermique des levures de bière et de boulangerie, respectivement. Le traitement au SDS a principalement conduit à la libération de protéines non glycosylées. Le rendement le plus élevé en mannoprotéines a été atteint lors de l'isolement enzymatique avec Zymolyase® d'*Arthrobacter luteus*. Les mannoprotéines ont été caractérisées par un rapport mannane/protéine plus élevé (13.1-42.7) et une distribution de MW plus large (5-10 kDa; 10-100 kDa; 100-400 kDa). Des modèles prédictifs ont été développés pour comprendre et moduler les effets des paramètres d'isolement sur le rendement, la teneur en mannoprotéines et le rapport mannane/protéine. La concentration en enzyme était le paramètre le plus important pour le rendement, tandis que le temps de réaction était le paramètre le plus

important pour le rapport mannane/protéine. La comparaison des valeurs prédites et expérimentales a validée par les modèles prédits établis.

Les mannoprotéines isolées à partir de sous-produits de levures à l'aide de la méthode de traitement optimale à la Zymolyase® ont été encore purifiées à l'aide d'une chromatographie sur colonne d'affinité et d'une chromatographie d'exclusion de taille. Deux populations de mannoprotéines (MP1/MP1' et MP2/MP2') ont été obtenues, l'une avec une masse moléculaire élevée et l'autre avec une masse moléculaire faible. MP1 dérivé d'Agrimos® contenait 4 fractions de mannoprotéines, tandis que MP1' dérivé de YCW-b était composé de 2 fractions de mannoprotéines de composition différente. La solubilité de ces mannoprotéines était similaire ou inférieure à celle du mannane. Les mannoprotéines présentaient un comportement newtonien à 25, 50 et 73 °C et leur viscosité avait tendance à diminuer avec l'augmentation de la température. Le procédé de traitement par sonication s'est avéré plus approprié pour la formation d'émulsions à base de mannoprotéines que le procédé combiné de sonication/homogénéisation. Les émulsions à base de MP1/MP1' n'ont montré aucune différence significative dans l'affinité pour l'huile de soja ou le trioléate de glycérol, alors que les émulsions à base de MP2/MP2' présentaient une forte affinité pour l'huile de soja. Le pH a un effet significatif sur les émulsifiants à base de MP1/MP1. Les émulsions à base de MP1/MP1 obtenues à partir d'Agrimos® et de YCW-b ont montré des propriétés émulsifiantes similaires ou légèrement inférieures par rapport à la lécithine.

Deux populations de mannoprotéines purifiées ont été récupérées, dont une masse moléculaire élevée (MP1, 620-36 kDa) composée d'une variété de mannoprotéines avec un rapport mannane/protéine différent (2.5-10) et une faible masse moléculaire (MP2, 2.3-6.8 kDa). Le traitement à la α-(1,6) Mannanase a révélé la présence de liaisons α-(1,6) mannose dans la population de MP1 à haut MW, mais pas dans la population de MP2 à faible MW. Les mannoprotéines MP1 isolées étaient principalement des O-glycosylées. Les structures de mannoprotéines ont ensuite été caractérisées par RMN 1D et 2D, 7 atomes de carbone anomériques ont été déterminés dans Agrimos® MP1, dont 4 présentent des déplacements chimiques très similaires à ceux de la mannane de référence. La proportion de mannane liée α-(1,6) s'est avérée être la plus élevée dans Agrimos® MP1. Les modifications de conformation thermique des mannoprotéines à l'état solide et liquide ont été révélées par FTIR.

Le beta-glucane a été associé à plusieurs propriétés fonctionnelles et favorables à la santé. Une approche enzymatique, basée sur l'utilisation de protéases, incluant Flavourzyme, Novo-proD, Papain et Alcalase, a été étudiée pour l'isolement et la génération de beta-glucane à partir de la paroi cellulaire de levure. La combinaison d'une étape d'extraction à l'eau chaude et d'un traitement à la protéase a permis d'améliorer l'efficacité de la génération de beta-glucane en ouvrant le réseau de la paroi cellulaire et en améliorant l'accessibilité des protéases aux substrats protéiques. Alcalase et Novo-ProD présentent une spécificité plus élevée pour l'hydrolyse de liaisons peptidiques comportant un acide aminé aromatique hydrophobe important (tyrosine, tryptophane et phénylalanine). De plus, cette étude a révélé les actions synergiques entre les enzymes protéolytiques. La supplémentation en alcalase (0.1%, v/w) de Novo-ProD (0.5%, v/w) a donné les teneurs les plus élevées en teneur totale et en β-glucane de 62.1 et 44.2% (w/w); cette augmentation correspond à un enrichissement de 98% de la paroi cellulaire de levure en β-glucane. La supplémentation en alcalase (0.1%, v/w) en papaïne (0.5%, w/w) et Flavourzyme (0.5%, v/w) a conduit à un enrichissement de 76.5 et 47.3% de la paroi cellulaire de levure avec du β-glucane, respectivement. Une conception rotative composite centrale (CCRD) à 5 niveaux et 3 variables a été réalisée avec des concentrations variables d'Alcalase (0-0.82% v/w), de Novo-proD (0-0.82% v/w) et de Flavourzyme (0-0.82% v/w); et les réponses étaient la teneur totale en glucane, la teneur en-glucane, l'enrichissement en-glucane, la perte de poids, la perte de protéines, la perte de glucanes et la perte de mannane. Les modèles développés permettront d'enrichir le beta-glucane dans les extraits de levure et d'élargir leurs applications en tant qu'ingrédients à valeur ajoutée.

STATEMENT FROM THE THESIS OFFICE

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

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

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

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contribution of Authors" as a preface of the thesis.

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

CONTRIBUTION OF AUTHORS

This thesis consists of IX chapters.

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

Chapter II presents a comprehensive assessment of the literature on the distribution, structural properties, health-promoting properties and techno-functional properties of mannoproteins and β-glucan derived from brewer's or baker's yeast *Saccharomyces cerevisiae.* A brief description of some of the analytical techniques used for the structural characterization of mannoproteins. This chapter ends by describing common techniques employed to separate mannoproteins and β-glucan from yeast-based products.

Chapter III to VII are presented in the form of manuscripts, and will be submitted for publication. The connecting statements provide the rationale link between the different parts of this study. In chapter III, a comprehensive assessment of heat treatment, SDS extraction and enzymatic process for the isolation of mannoproteins from yeast cell wall were established, then optimization of the enzymatic extraction of mannoproteins derived from yeast cell wall was investigated by response surface methodology. In chapter IV, mannoproteins isolated from baker's and brewer's yeasts using different methods were compared, then the effects of selected reaction parameters of beta-1,3-glucan laminaripentaohydrolase from *Arthrobacter luteus* for the isolation of mannoproteins from brewer's yeasts and their interactions were investigated by response surface methodology. Chapter V provides the characterization of solubility, emulsifying and viscoelastic properties of the purified mannoproteins, which obtained through using affinity chromatography on concanavalin A and size exclusion chromatography. Chapter VI reports the structure of purified mannoproteins which studied with specific enzymatic cleavages, NMR and FTIR methods. Chapter VII used an efficient and sustainable enzymatic approach, based on the use of proteases, including Flavourzyme, Novo-proD, Papain and Alcalase, to isolate and generate non-denatured β-glucan from yeast cell wall.

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

Chapter IX provides the contribution of this research to the field and outlines possible recommendations regarding future work related to mannoproteins and β-glucan derived from yeast-based products.

Jin Li, 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.

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PUBLICATIONS

1. Li, J., & Karboune, S. (2018). A comparative study for the isolation and characterization of mannoproteins from *Saccharomyces cerevisiae* yeast cell wall. *International journal of biological macromolecules*, *119*, 654-661.

2. Li, J., Asehraou, A & Karboune, S. (2018). Mannoproteins from inactivated whole cells of baker's and brewer's yeasts (*Saccharomyces cerevisiae*): Isolation and Optimization (Submitted).

3. Li, J., & Karboune, S. (2018). Techno-functional properties of mannoproteins derived from yeast-based products with well-defined structures (Submitted).

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TABLE OF CONTENTS

ABSTRACT III
RÉSUMÉ VI
STATEMENT FROM THE THESIS OFFICE IX
CONTRIBUTION OF AUTHORS X
PUBLICATIONS XII
ACKNOWLEDGEMENTS XIII
TABLE OF CONTENTS XIV
LIST OF FIGURES AND SCHEMES XIX
LIST OF TABLES XXIII
NOMENCLATURE/LIST OF ABBREVIATIONS XXV
CHAPTER I 1
GENERAL INTRODUCTION 1
CHAPTER II 7
LITERATURE REVIEW 7
2.1 Introduction 8
2.2 Mannoproteins/Mannans 10
2.2.1 Distribution and Structural Property of Mannoproteins 10
2.2.2 Nutritional and Health-Promoting Properties 11
2.2.3 Techno-Functional Properties 14
2.3 Selected Techniques for the Characterization of Mannoprotein Structures 19
2.3.1 Mass Spectrometry 19
2.3.2 Nuclear Magnetic Resonance (NMR) 19
2.3.3 Fourier Transform Infrared Spectroscopy (FTIR) 21
2.4 β-Glucan 21
2.4.1 Structural Properties 21
2.4.2 Functional Properties 22
2.4.3 Health-promoting Properties 23
2.4.4 Application of β-Glucan 24

2.5 Extraction Techniques for Mannoproteins and β-Glucan 25
2.5.1 Hot Water Extraction 25
2.5.2 Acid-Alkaline Extraction 25
2.5.3 SDS Extraction 26
2.5.4 Enzymatic Methods 27
CONNECTING STATEMENT 1 29
CHAPTER III 30
A COMPARATIVE STUDY FOR THE ISOLATION AND CHARACTERIZATION OF MANNOPROTEINS FROM *SACCHAROMYCES CEREVISIAE* YEAST CELL WALL 30
3.1 Abstract 31
3.2 Introduction 31
3.3 Materials and Methods 33
3.3.1 Yeast-based Starting Material 33
3.3.2 Selected Isolation Methods 33
3.3.3 Optimization of Enzymatic Treatment for the Isolation of Mannoproteins . 34
3.3.4 Analytical Methods 34
3.4 Results and Discussion 36
3.4.1 Approximate Composition of Agrimos® 36
3.4.2 Isolation of Non-covalently Bound Mannoproteins 37
3.4.3 Isolation of Covalently-bound Mannoproteins 42
3.4.4 Optimization of the Enzymatic Isolation of Mannoproteins from Agrimos® 43
3.5 Conclusion 48
CONNECTING STATEMENT 2 51
CHAPTER IV 52
MANNOPROTEINS FROM INACTIVATED WHOLE CELLS OF BAKER'S AND BREWER'S YEASTS (*SACCHAROMYCES CEREVISIAE*): ISOLATION AND OPTIMIZATION 52
4.1 Abstract 53
4.2 Introduction 53
4.3 Materials and Methods 54
4.3.1 Materials 54

4.3.2 Determination of Morphological and Compositional Properties of Whole Yeasts ... 55
4.3.3 Investigation of Selected Isolation Methods ... 55
4.3.4 Purification of Mannoproteins ... 56
4.3.5 Optimization of the Enzymatic Isolation of Mannoproteins from Whole Brewer's Yeast ... 56
4.3.6 Analytical Methods ... 57
4.4 Results and Discussion ... 59
4.4.1 Cellular and Chemical Compositions of Inactivated Baker's and Brewer's Yeasts ... 59
4.4.2 Isolation of Non-covalently Bound Mannoproteins ... 62
4.4.3 Isolation of Covalently Bound Glycosylphosphatidylinositol (GPI) Mannoproteins ... 65
4.4.4 Optimization of the Enzymatic Isolation of Mannoproteins from Brewer's Yeasts ... 70
4.4.5 Effects of Reaction Parameters ... 75
4.5 Conclusion ... 79
CONNECTING STATEMENT 3 ... 80
CHAPTER V ... 81
TECHNO-FUNCTIONAL PROPERTIES OF STRUCTURALLY WELL-DEFINED MANNOPROTEINS FROM YEAST (*SACCHAROMYCES CEREVISIAE*)-BASED BY-PRODUCTS ... 81
5.1 Abstract ... 82
5.2 Introduction ... 82
5.3 Materials and Methods ... 83
5.3.1 Materials ... 83
5.3.2 Isolation and Purification of Mannoproteins ... 83
5.3.3 Structural Characterization of Purified Mannoprotein Fractions ... 85
5.3.4 Scanning Electron Microscopy (SEM) ... 85
5.3.5 Assessment of Techno-functional Properties of Mannoproteins ... 86
5.4 Results and Discussion ... 87
5.4.1 Isolation, Purification and Chemical Characterization of Mannoproteins ... 87

5.4.2 Techno-Functional Properties of Mannoproteins 92
5.5 Conclusion 105
CONNECTING STATEMENT 4 107
CHAPTER VI 108
CHARACTERIZATION OF THE STRUCTURAL PROPERTIES OF MANNOPROTEINS ISOLATED FROM SELECTED YEAST-BASED PRODUCTS BY SPECIFIC ENZYMATIC CLEAVAGES, NMR AND FTIR ANALYSIS 108
6.1 Abstract 109
6.2 Introduction 109
6.3 Materials and Methods 111
6.3.1 Materials 111
6.3.2 Enzymatic Extraction of Mannoproteins 111
6.3.3 Purification of Mannoproteins by Affinity Chromatography 112
6.3.4 Fractionation of Mannoproteins by Size Exclusion Chromatography 112
6.3.5 Analysis of Crude Mannoprotein Extract 113
6.3.6 Characterization of MW Distribution 113
6.3.7 Structural Characterization of Purified Mannoproteins 113
6.3.8 Fourier Transform Infrared Spectroscopy (FTIR) Analysis 114
6.4 Results and Discussion 115
6.4.1 Enzymatic Isolation of Mannoproteins from Selected Yeast-based Products 115
6.4.2 Structural Characterization of Glycosidic Linkages of Purified Mannoproteins 121
6.4.3 Characterization of the Secondary Structure of Mannoproteins 129
6.5 Conclusion 136
CONNECTING STATEMENT 5 137
CHAPTER VII 138
PROTEOLYTIC ENZYMES-BASED APPROACH FOR THE GENERATION OF β-GLUCAN FROM YEAST CELL WALL: A COMPARATIVE STUDY 138
7.1 Abstract 139
7.2 Introduction 139

7.3 Materials and methods 141
7.3.1 Materials 141
7.3.2 Proteolytic Activity Assay 142
7.3.3 Hot water treatment 142
7.3.4 Protease Treatment 142
7.3.5 Measurement of Total Glucan and Mannan Contents 142
7.3.6 Measurement of α-Glucan Content 143
7.3.7 Determination of Protein Content 143
7.3.8 Optimization, Experimental Design and Data Analysis 143
7.4 Results and Discussion 144
7.4.1 Efficiency of hot water treatment 144
7.4.2 Efficiency of combined hot water and protease treatments 147
7.4.3 Effect of Protease Type and Concentration 147
7.4.4 Optimization of Combined Proteases-based Treatment on β-Glucan Enrichment 153
7.5 Conclusion 159
CHAPTER VIII 161
GENERAL SUMMARY AND CONCLUSION 161
CHAPTER IX 165
CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES 165
9.1. Contribution to Knowledge 166
9.2 Recommendations for Future Research 167
REFERENCES 168

LIST OF FIGURES AND SCHEMES

CHAPTER II

Scheme 2.1. The components of *S. cerevisiae* cell walls. ……..……...22

CHAPTER III

Figure 3.1. Scanning electron micrograph (magnification 1000×) of whole baker's yeast (A) and baker's yeast cell wall (YCW) (B)**.** ……..……..……..….…38

Figure 3.2. Effect of heat treatments on the yield of recovered extract (■) (% initial cell wall, w/w) and the protein content in the recovered extract (▭) (% extract, w/w) ……..…41

Figure 3.3. Contour plots of predictive models of yield of recovered extract (%) (A), mannoproteins content (%) (B), mannan to protein ratio in mannoproteins (w/w) (C). The numbers inside the contour plots indicate the predicted values under given reaction conditions. ……..……..……..……..……..….…49

CHAPTER IV

Supplemental Figure 4.1. Scanning electron micrograph (magnification 1000×) of whole brewer's yeast ……..……..……...…….59

Figure 4.1. SDS-PAGE protein profiles of crude mannoprotein extracts after heat treatment and SDS extraction from baker's yeast and brewer's yeast (Lane 1: Standard; Lane 2: Inactivated whole baker's yeast after heat treatment; Lane 3: Inactivated whole brewer's yeast after heat treatment; 4: Inactivated whole baker's yeast after SDS extraction; Lane 5: Inactivated whole brewer's yeast after SDS extraction). ……..……..……..……..65

Figure 4.2. Molecular weight distributions of mannoproteins recovered upon zymolyase treatment from baker's yeast and brewer's yeast under selected conditions: 5-10 kDa (■), 10-100 kDa (□), 100–400 kDa (▨), >400 kDa(▩).……..……..……..….…....69

Figure 4.3. Effect of substrate concentrations on the yield of recovered extract (%, w/w initial weight) from brewer's yeast (Zymolyase® units/substrate ratio of 67 units per g of brewer's yeast; 4 h enzymatic treatment). Vertical lines indicated standard deviation……..….....71

Figure 4.4 Contour plots of predictive models of yield of recovered extract (%) (A), mannoproteins content (%) (B), mannan to protein ratio in mannoproteins (w/w) (C). The numbers inside the contour plots indicate the predicted values under given reaction conditions. ……..……..……..……..……..…..76

CHAPTER V

Figure 5.1. Purification of Agrimos® (A) and YCW-b (B) mannoproteins through size exclusion HiLoad™ column. mannan content(·····), protein content (—)……...88

Supplemental Figure 5.1. Scanning electron micrograph of original materials and mannoproteins with magnification of 1000. Yeast cell wall Agrimos® (A), YCW-b (B), Agrimos® MP1 (C), YCW-b MP1'(D), Agrimos® MP2 (E), YCW-b MP2' (F). ……...91

Figure 5.2. Effect of pH (A) and temperature (B) on the solubility of mannoproteins: Agrimos® MP1 (■), Agrimos® MP2 (〓), YCW-b MP1'(▩), YCW-b MP2'(▩), Mannan (⊡) …….92

Figure 5.3. Changes in sheer stress vs sheer rate and viscosity (at shear rate of 100 s^{-1}) as changes of temperature for solutions of 1% Agrimos MP1 (A), 1% Agrimos MP2 (B), 1% YCW-b MP1' (C), 1% YCW-b MP2' (D), 1% mannan (E), 1% whey protein (F), 5% Agrimos MP1 (G), 5% Agrimos MP2 (H), 5% YCW-b MP1' (I), 5% YCW-b MP2' (J), 5% mannan (K), 5% whey protein (L) at 25 °C (O), 50 °C (Δ) and 75 °C (□) …….94

Figure 5.4. (A) Emulsifying and stability properties of mannoproteins under different conditions, * means there is significant difference ($p<0.05$) between the two treatment methods (B) Effect of ultrasound treatment under different amplitudes on the emulsion droplet sizes: Agrimos MP1 (Δ), YCW-b MP1' (□) (C) Effect of soybean oil on the emulsifying properties of mannoproteins: Agrimos MP1 (■), Agrimos MP2 (〓), YCW-b MP1' (▩), YCW-b MP2' (⊡) ……96

(D) Effect of glyceryl trioleate on the emulsifying properties of mannoproteins: Agrimos MP1 (■), Agrimos MP2 (〓), YCW-b MP1' (▩), YCW-b MP2' (⊡)* means there is significant difference ($p<0.05$) between soybean oil and glyceryl trioleate……96

Figure 5.5. Effect of oil/mannoprotein ratio (v/w) on the emulsifying properties of mannoproteins Agrimos MP1(—●—), YCW-b MP1' (—■—)…….100

Figure 5.6. Influence of pH on the emulsifying properties and emulsion stability of MP1/MP1' derived from Agrimos® (A), YCW-b (B) and lecithin (C): 25 ^{0}C (■), 25 ^{0}C – 2h (▨), 50 ^{0}C – 2h (▣), 75 ^{0}C – 2h (▤) ……..102

CHAPTER VI

Figure 6.1. Molecular weight distributions of mannoproteins recovered upon Zymolyase® treatment of 4 yeast-based products under optimal conditions: 5-10 kDa (▩), 100-400 kDa (▨), >400 kDa (■) ……..……..……..……..……..117

Figure 6.2. Purification of mannoproteins through size exclusion chromatography (mannan content ·····, protein content —): Baker's yeast (A), Brewer's yeast (B), Agrimos® (C), YCW-b (D) ……..……..……..……..……..…118

Figure 6.3. HPSEC profiles of MP1 before and after treatment with α-1,6 Mannanase: Agrimos® MP1 UV(A), Agrimos® MP1 RI (B), YCW-b MP1 UV(C), YCW-b MP1 RI (D) ……..……..……..……..……..…121

Figure 6.4. NMR spectrum of Agrimos® MP1 and mannan: Agrimos® ^{1}H(A), Agrimos® ^{13}C (B), Mannan ^{1}H (C), Mannan ^{13}C (D) …..……..……..……..……..…….....123

Figure 6.5. Edited gradient-enhanced ^{1}H–^{13}C HSQC spectrum of Agrimos® MP1(A) and mannan standard (B). Boxed area is anomeric region……..……..……....125

Figure 6.6. (A) FTIR spectra of MP1 and MP2 derived from Agrimos® in the solid state, (B) Fourier self-deconvolved FTIR spectrum of MP1 in D_2O solution (1800-1500 cm^{-1}) ……..……..……..……..……..…128

Figure 6.7. Changes in secondary structure compositions of MP1 derived from Agrimos® and YCW-b following thermal denaturation in the solid state, as estimated from Fourier transform infrared spectra. Aggregation (■), β-sheet (▨), β-turns (▩), random coil (⊡), α-helix (▤) ……..……..……..……..……..…131

Figure 6.8. Changes in percentage intensities of amide I' band components in FTIR spectra of MP1 derived from Agrimos® (A) and YCW-b (B) in D_2O solutions heated to different

temperatures: Aggregation ( ), β-sheet ( ), β-turns ( ), random coil ( ) and α-helix ( ). ……..……..……..……..……133

CHAPTER VII

Figure 7.1. Effect of hot water treatment on the recovery of β-glucan from Agrimos®: Total glucan content (□), Mannan content (■), α-Glucan content (▨), β-Glucan content (▩), Weight loss (▩) and Crude protein loss (▨).……..……143

Figure 7.2. Effect of combined hot water and protease treatments on the β-glucan enrichment of Agrimos®: Total glucan content (□), Mannan content (■), α-Glucan content (▨), β-Glucan content (▩).……...146

Figure 7.3. Effect of combined hot water/protease treatments (▩) and heat pretreatment (■) on the enrichment level of β-glucan in Agrimos®.……...147

Figure 7.4. Effect of different protease treatments on the enrichment of β-glucan in Agrimos®: Total glucan content (□), Mannan content (■), α-Glucan content (▨), β-Glucan content (▩), Weight loss (▩) and Crude protein loss (▨).……..149

Figure 7.5. Contour plots of total glucan (AB), β-glucan (CD), weight loss (EF), and protein loss (GH) as a function of Novo-ProD concentration (v/w) and Alkalase (v/w) (ACE), as a function of Flavourzyme concentration (v/w) and Alcalase concentration (v/w) (BDFH), and as a function of Novo-ProD concentration (v/w) and Flavourzyme concentration (v/w) (G). The numbers inside the contour plots indicate the predicted values under given reaction conditions. Response: Lowest Highest. ……156

LIST OF TABLES

CHAPTER III

Table 3.1. Effect of heat treatment, SDS extraction and Zymolyase® treatments on the isolation of mannoproteins.....39

Table 3.2. Experimental design of 5-levels 2-variables central composite design and the responses.....44

Table 3.3. Analysis of variance (ANOVA) for the yield of recovered extract (%), the mannoproteins content (%) and the mannan to protein ratio (w/w) in extracted mannoproteins 46

CHAPTER IV

Table 4.1. Composition and protein profiles of inactivated whole baker's yeast and whole brewer's yeast.....60

Table 4.2. Effect of heat treatment and SDS extraction on the isolation of mannoproteins from baker's and brewer's yeast.....64

Table 4.3. Effect of Zymolyase® treatments on the isolation of mannoprotein extract from baker's and brewer's yeast.....67

Table 4.4. Experimental design of 5-levels 2-variables central composite design and the responses.....72

Table 4.5. Analysis of variance (ANOVA) for the yield of recovered extract (%), mannoproteins content (%) and mannan to protein ratio in mannoproteins (w/w) 73

CHAPTER V

Table 5.1. Composition and molecular weight of mannoproteins.....90

CHAPTER VI

Table 6.1. Isolation of mannoproteins using selected optimal enzymatic treatments on 4 yeast-based products.....116

Table 6.2. Possible Linkage of Mannoprotein Fractions and Mannan Standard Using a Web-based Version of the Computer Program CASPER.....127

Table 6.3. Band assignments for the Fourier self-deconvolved FTIR spectrum of MP1 in D_2O solution.....…..……..… .....…..……..… .....…..……..… .....…..……..……131

CHAPTER VII

Table 7.1. Central composite quadratic design of actual independent variables and the observed response……...…….153

Table 7.2. Analysis of variance (ANOVA) for total glucan, β-glucan, weight loss, and protein loss response models. ……...……..155

NOMENCLATURE/LIST OF ABBREVIATIONS

Alc	Alcalase
ANOVA	The analysis of variance
Asp	Aspartic Acid
Glu	Glutamic Acid
BSA	Bovine serum albumin
CCRD	Central composite rotatable design
C.jejuni	*Campylobacter jejuni*
CWPs	Cell wall proteins
DNS	Dinitrosalicylate
DP	Degree of polymerization
EDTA	Ethylenediaminetetraacetic acid
Flav/FLA	Flavourzyme
FSD	Fourier self-deconvolution
GLU	Glucose
GPI	Glycosylphosphatidylinositol
HPAEC	High pressure anion exchange chromatography
HPSEC	High pressure size exclusion chromatography
HT	Heat treatment/Heat treated
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Man	Mannose
MOS	Manno-oligosaccharides
MW	Molecular weight
NMR	Nuclear magnetic resonance
NT	No treatment
Nov	Novo-proD
PAGE	Polyacrylamide gel electrophoresis
Pap	Papain
Pir	Proteins with internal repeat
PT	Alcalase treatment
SDS	Sodium dodecyl sulphate
S.cerevisiae	*Saccharomyces cerevisiae*

CHAPTER I

GENERAL INTRODUCTION

Yeasts have been and are still being used in many food-biotechnological processes. Considerable quantities of yeast cell wall and of brewer's *Saccharomyces cerevisiae* yeast are generated as by-products by the yeast and brewery industry. Yeast extracts, which consist primarily of amino acids, peptides, nucleotides and other soluble components, are used as flavoring agents, and upon their production, a large amount of yeast cell wall (50-60%, w/w) are recovered (Chae et al., 2001). Yeast cell walls are structurally unique and significantly different from the plant cell wall. Their main components are β-glucan (~ 60%, w/w), mannoproteins (~ 40%, w/w), and chitin (~2%, w/w) (Kaushik et al., 2006; Couto et al., 2010).

Mannoproteins are glycoproteins and contain up to 50 to 95% polysaccharides (Lipke & Ovalle, 1998; H. Liu et al., 2018). The mannoproteins can be divided into three groups: (i) non-covalently bound, (ii) covalently bound to the structural glucan and (iii) disulfide bound to other proteins that are covalently bound to the glucan of the cell wall. Covalently bound mannoproteins, referred to as cell-wall proteins (CWPs), fall into two categories, proteins with internal repeat-CWPs (Pir-CWPs, referred as alkaline-sensitive mannoproteins) and glycosylphosphatidylinositol-CWPs (GPI-CWPs, referred as glucanase-extractable mannoproteins). GPI-CWPs are the major class of yeast cell wall proteins (Peter Orlean, 2012; Matsuoka et al., 2014). Because of their molecular and structural properties, cell wall mannoproteins exhibit many techno-functional properties that make them attractive for food applications (Caridi, 2006). For instance, cell wall mannoproteins have shown high emulsifying and stabilizing properties, making them attractive natural emulsifiers in salad dressing or mayonnaise without affecting the sensory attributes of these products (Silva Araújo et al., 2014; de Melo et al., 2015). They have also been extensively used in the enology industry due to their techno-functionalities, including the adsorption of ochratoxin A, the complexation with phenolic compounds, the increase in the growth of malolactic bacteria, the inhibition of tartrate salt crystallization, the prevention of haze formation and the reinforcement of aromatic components (Caridi, 2006). In addition, mannoproteins showed health-promoting benefits, as they stimulate the growth of lactic acid bacteria (K Newman, 1994; Ganan et al., 2012) and inhibit pathogenic bacteria (Ganan et al., 2009). The techno-functional and health promoting properties of cell wall mannoproteins were reported to be dependent on their molecular and structural properties, including the molecular weight, the monosaccharide composition and the glycosylation extent (Caridi, 2006).

Regarding the β-glucan, which is the primary component of yeast cell wall, it is composed of three main classes including: 1) insoluble β-(1,3)-glucan; 2) alkali soluble β-(1,3)-glucan; 3) highly branched β-(1,6)-glucan. Insoluble β-(1,3)-glucan as the major component of cell wall of *S. cerevisiae* is mainly composed of a backbone chain of β-(1-3) linked glucose units (240 kDa) with a low degree of inter and intramolecular branching through β-(1-6) linkages. The structures of β-(1-3)-glucan may vary considerably in the degree of branching and heterogeneity (Freimund et al., 2003). β-glucan has attracted a lot of attention due to its health promoting properties. The most important biological activity of β-glucan is its ability to stimulate the immune system (J.-N. Lee et al., 2001; Freimund et al., 2003), and many of its other effects are also related to this activity, for example, antitumor, antibacterial, antiviral, anticoagulatory and wound healing effects (Jørgensen et al., 1993; Cheung et al., 2002; Przybylska-Diaz et al., 2013). β-Glucan can also exhibit water-holding capacity, oil-binding capacity and emulsion stabilizing capacity (Petravić-Tominac et al., 2011); however, β-glucan derived from yeast-based products has been less studied compared to the ones isolated from cereals, including oat, barley and wheat.

The isolation of mannoproteins and β-glucan from yeast/yeast cell wall by-products with well-defined structures and glycosylation levels is challenging because of the heterogeneity of their structures (Orlean, 2012). Heat treatment is a simple process for the isolation of non-covalently bound mannoproteins, but the recovery yield is low (Freimund et al., 2003; H.-Z. Liu et al., 2011). Acid-alkaline methods have been widely used for the isolation of mannoproteins and β-glucan from yeasts (Kath & Kulicke, 1999; H.-Z. Liu et al., 2011; H.-Z. Liu et al., 2015). In addition, sodium dodecyl sulfate (SDS) was used for the isolation of mannoproteins that are loosely associated with the cell wall (Van der Vaart et al., 1997). However, the use of acids, alkalis and other chemical extracting agents involve many non-green steps and can result in the low recovery yield of β-glucan and mannoproteins with low purity (Freimund et al., 2003). Meanwhile, in carbohydrate chemistry, it is well known that acidic/alkaline conditions can lead to a more or less strong degradation of the glucose/mannose chains and their debranching (Young & Liss, 1978; Müller et al., 1997; Jaehrig et al., 2008). The degradation and the debranching of β-glucan and mannoproteins can affect their biological functions (Müller et al., 1997; X. Y. Liu et al., 2008). Therefore, in order to preserve the native structure of β-glucan and mannoproteins and ensure a minimum chain degradation, there is a need for milder manufacturing processes. Biocatalysis,

which encompasses the use of biocatalysts to assist in synthetic conversions, is emerging as a highly promising area of research for the development of sustainable technologies for the production of innovative structurally well-defined bioactive molecules (Karboune et al., 2005; Karboune et al., 2008; Karboune et al., 2009). The interest in biocatalysis stems from the fact that most biocatalytic processes have been proven to be highly viable alternatives to their chemical counterparts, while eliminating many of the drawbacks commonly associated with the latter. Biocatalytic processes owe their advantages to the characteristics of enzymes, in particular, their diversity, high selectivity, high turnover number and their capacity to operate under environmentally-friendly conditions (Kobayashi et al., 2001; Adamczak & Krishna, 2004). The isolation of the covalently-bound cell wall mannoproteins was achieved through the use of the enzymatic method, based on the β-1,3-glucanase activity (De Nobel, Klis, Priem, et al., 1990; van der Vaart et al., 1995); however, as far as the authors are aware, the effects of isolation parameters were not investigated, and the functional properties of purified mannoproteins were not investigated as they are related to their structural properties. Because the techno-functional properties of mannoproteins are driven by their structural properties, it is important to isolate well-defined mannoproteins to modulate their properties. On the other hand, Freimund et al. (2003) have reported a non-degrading process for the isolation of β-glucan from baker's yeast that combines gentle extractions by hot water and organic solvents with a protease treatment. Furthermore, Liu et al. (2008) have investigated a process for the isolation of β-glucan from spent yeast cells that combines induced autolysis, gentle extractions by water and organic solvents, homogenization and protease hydrolysis. These authors obtained a high yield of 91% (w/w) β-glucan of the original ratio in the yeast cell walls. Furthermore, Jaehrig et al.(2008) have included an additional lipase-catalyzed step (LipolaseTM), and reported a yield around 80% of β-glucan as end product. However, most of the studies have been concerned about the yield and the purity of isolated β-glucan, but not about their molecular properties and distributions. In addition, as far as the authors are aware, no study did investigate the synergistic actions of proteases for the efficient isolation of yeast β-glucan (Ferrer, 2006).

The overall objective of the present study was to develop enzymatic approaches aimed at the isolation of mannoproteins and β-glucan from yeast-based products using selected enzymes and study the functional and structural properties of the purified mannoproteins.

The specific objectives of this study were as follows:

1. Investigation of the isolation of mannoproteins from yeast cell wall Agrimos® by heat treatment, SDS extraction and enzymatic treatment. The efficiency of the isolation methods was assessed in terms of yield of recovered extract, mannan and protein recovery yield, mannoproteins content and mannan to protein ratio in mannoproteins. Given the dependence of the techno-functional and health promoting properties of mannoproteins on their molecular properties, the effects of the parameters of enzymatic isolation on the structural properties of mannoproteins was also investigated.

2. Investigation of the isolation of mannoproteins from baker's or brewer's *S. cerevisiae* yeasts using the conventional methods (heat treatment and SDS extraction) and the enzymatic approach. The efficiency of the isolation methods, in terms of yield of recovered extract, mannoproteins content and mannan to protein ratio, was discussed while elucidating the difference between baker's and brewer's yeast. The effects of the parameters (enzyme amount; time) of the enzymatic isolation of mannoproteins from brewer's yeast were further investigated.

3. Understanding of the techno-functional properties of mannoproteins as they are related to their structural properties. Mannoproteins extracted upon the enzymatic treatment were purified by affinity chromatography on concanavalin A column and were then fractionated on Superdex™ prep grade and prepacked HiLoad™ columns. The structural composition and the techno-functional properties of the recovered mannoprotein fractions were characterized. The solubility of mannoprotein fractions, their produced viscosity and their emulsifying properties (the droplet size and the stability) at selected pH and temperature values were also investigated.

4. Investigation of a comparative structural characterisation of purified mannoproteins from selected yeast-based products produced using the β-glucanase-based enzymatic approach. The proportion of α-(1,6) mannose residues was assessed through the hydrolysis of mannoproteins by specific α-(1,6)-mananase. Further structural characterization of the linkage types of sugar residues in the mannoproteins was investigated using 1D and 2D NMR analysis. While the thermally induced changes of the secondary structure of the protein fractions in the mannoproteins were determined by FTIR.

5. Investigation of the efficiency of the hot water and the protease treatments as well as their combination for the isolation of β-glucan from yeast cell wall. The effects of protease specificity

and their synergistic actions were elucidated through the use of selected proteases, including Flavourzyme, Novo-proD, Papain and Alcalase as a monocomponent and a mixture.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

The yeast cell wall has been regarded as a complex matrix containing glucan (47-65%), mannan/mannoproteins (40%), small amounts of chitin (1-2%) and lipids (1-7%) (Freimund et al., 2003). The yeast cell wall is located outside of the cell membrane, and consists of two layers (Cruz, 2011). The inner layer, made of β-1,3- and β-1,6-glucan linked with chitin, forms a continuous three-dimensional network and provides cell wall strength. The outer layer, consisting of mannoproteins, modulates most of the cell's surface properties, protects the yeast against enzymatic damage and acts as a filter. Additionally, mannoproteins are involved in many cell functions, including flocculation, biofilm formation and strain invasiveness (Cruz, 2011). The majority of mannoproteins are covalently linked to the inner glucan layer. Another component of the cell wall, chitin, is a linear polymer that may become glycosidically linked through its reducing end to 1,3-β-glucan. It can also be linked to β-1,6-glucan and plays an important role in shouldering the responsibility of cell wall stress (Freimund et al., 2003).

Mannoproteins have found many applications in the food industry. They are defined as glycoproteins, which have been implicated in favoring lactic acid bacteria growth while limiting the proliferation of pathogenic bacteria (Newman, 1994; Roberfroid, 2007). Mannoproteins play a very crucial role in the vinification process. Food Standards Australia New Zealand (FSANZ) approved the use of crude mannoproteins extracted from yeast cell walls as a food additive in wine to inhibit the crystallization of potassium bitartrate in 2009, while in July 2015, Health Canada had also approved the same use of crude yeast mannoproteins in the wine making process at a maximum addition of 0.04%. Mannoproteins have been shown to have a positive influence on wine polysaccharide content, polyphenolic content, and color composition (Guadalupe & Ayestarán, 2008). Furthermore, due to their chemical and physical stability, they can be used as bioemulsifiers in processed foods (Lukondeh et al., 2003). The size of mannoproteins varies dramatically among species and strains. Mannoproteins secreted into wine by yeast were reported to range from 5 kDa to more than 800 kDa (Chalier et al., 2007). The molecular weights of purified mannoproteins used as bioemulsifiers were reported as 14-15.8 kDa (Torabizadeh et al., 1996). A trace level of mannoproteins present in wine after a combination of anion exchange, Concanavalin A, cation exchange and gel permeation chromatography separation was reported to be 420 kDa (Waters et al., 1994). Different extraction and purification methods yield different sizes of

mannoproteins, subsequently affecting their structural, physicochemical and functional properties. Crude mannoproteins have been reported and used in the wine making process and in the food industry.

Many factors can affect the dry mass and polysaccharide content of the cell wall, particularly the conditions of cultivation, including the nature of the carbon source, nitrogen limitation, pH, temperature and aeration, and the mode of cell cultivation (shake flasks vs controlled fermenters). Variations in the dry mass and polysaccharide content of the cell wall have been reported to be greater than 50% due to the above factors (Aguilar Uscanga & Francois, 2003). While the effects of growth conditions on cell wall composition have been studied, the correlation of these changes with cell wall integrity was not investigated (McMurrough & Rose, 1967; Morris et al., 1986; Kapteyn et al., 2001).

A rich source of mannoproteins is spent brewers' yeast. It is an excellent source of proteins for animals, and is a certified generally recognized as safe (GRAS) food additive, with a bitter taste (Ferreira et al., 2010). Indeed, yeast biomass contains functional ingredients, including vitamins, nucleic acids, minerals, nicotinic acid, glycine, cysteine and glutamic acid (Ferreira et al., 2010). It can be used in the area of veterinary medicine, in the pharmaceutical, cosmetic and chemical industries as well as food and feed production. In animal feed, it is paramount that animal diets contain all essential nutrients required for normal physiological functioning but also serve as health provider medium to improve animals' immune system (Gatlin III, 2002). Brewer's yeast can provide two roles for animals' development, including immune stimulators and prebiotics (Ferreira et al., 2010). It has been reported that brewer's yeast can replace 50% of fishmeal protein with no negative effects in fish performance and has been included in commercial diet formulations for several fish species, including salmonids. Moreover, the inclusion of up to 30% brewer's yeast in the diet of fish has been shown to improve feed efficiency (Ferreira et al., 2010). A study has shown that when fed to rainbow trout, the disruption of the cell wall increased digestibility and conferred beneficial effects, the absorption of nitrogen increased by more than 20% compared to intact cell wall and the metabolizable energy of the yeast increased by more than 10% (G. Rumsey et al., 1991). Spent brewers' yeasts are usually found in the form of powders, flakes, tablets or in liquid form. When using spent brewers' yeasts in food industry to produce yeast protein concentrates or isolates, they can preserve most functional properties and nutritive values.

Additionally, different forms of spent brewers' yeast are included in milk, juice, soup and gravy products, or used as seasonings (Ferreira et al., 2010). Polysaccharides extracted from the yeast cell wall can absorb mycotoxins at low inclusion levels, and can be used as natural growth stimulators for animals (G Kogan & Kocher, 2007).

2.2 Mannoproteins/Mannans

2.2.1 Distribution and Structural Property of Mannoproteins

Mannoproteins are the second most important component (35 – 40%, w/w) in the yeast cell wall (Caridi, 2006). The isolation and characterization of mannoproteins is challenging because of their heterogeneity and their different glycosylation levels (Peter Orlean, 2012). Mannoproteins can be divided into three groups: (i) non-covalently bound mannoproteins, (ii) covalently bound to the matrix of amorphous β-1,3 glucan by covalent bonds and (iii) disulfide bound to other proteins that are covalently bound to the glucan of the cell wall. Covalently bound mannoproteins, also referred to as cell-wall proteins (CWPs), fall into two categories: GPI-CWPs (glycosylphosphatidylinositol cell wall proteins, referred to as glucanase-extractable mannoproteins), and Pir-CWPs (proteins with internal repeat, referred as alkaline-sensitive mannoproteins). GPI-CWPs are a major class of yeast cell wall proteins. GPI attachment sequences reveal that the *S. cerevisiae* proteome contains 60-70 potential GPI proteins, which often contain serine/threonine-rich stretches (Peter Orlean, 2012). GPI-CWPs are covalently bound to β-1,6-glucan and can be released upon the digestion of glucan with glucanase, but they are resistant to extraction in hot sodium dodecyl sulfate (Hamada et al., 1998). These GPI-CWPs are characterized by three main features: a signal sequence for secretion at the N-terminus, a serine- and threonine-rich sequence providing sites for glycosylation, and a C-terminal GPI-attachment signal. These three characteristics are not only found in yeast, but also in GPI-anchored plasma membrane mannoproteins in other organisms including mammals (Peter Orlean, 2012). GPI proteins contain serine- and threonine-rich domains, receive short mannose chains linked to the hydroxyl side chains of serine or threonine residues via an α-mannosyl bond, thus *o*-mannosylation is a common feature of GPI-CWPs. The second type of cell wall protein, Pir proteins (Pir-CWPs, proteins with internal repeats), have no GPI attachment sequence and are not linked to β-1,6-glucan; rather, they are ester-linked to β-1,3-glucan via side chains of amino acids in the repeat sequences. These Pir proteins are also rich in serine and threonine, leading to extensive O-mannosylation. Other

mannoproteins can be released from the walls of living cells with sulfhydryl reagents, which indicates that they are directly attached via disulfide bonds or retained behind a network of disulfide-linked proteins. Disulfide-linked mannoproteins are able to create a barrier protecting wall polysaccharides from externally added glycosyl hydrolases, making a mercaptoethanol and protease pretreatment necessary for spheroplasting with lytic enzymes (Peter Orlean, 2012). There is another type of mannoprotein that is found between the plasma membrane and the cell wall composed of 30%-50% protein and 50%-70% carbohydrate (Jones & Ballou, 1969; Cohen & Ballou, 1980; Alvarado et al., 1990). Mannoproteins contain a large fraction, between 50 to 95%, of hydrophilic compounds, mainly polysaccharides (van der Vaart et al., 1995; Peter Orlean, 1997; Lipke & Ovalle, 1998). In general, glucan, which accounts for 60% (w/w) of the dry weight of the yeast cell wall, determines the rigidity of the cell wall, while mannoproteins determine the degree of porosity (De Nobel, Klis, Munnik, et al., 1990). Hence, mannoproteins play an important role in controlling the leakage of periplasmic proteins and the entry of macromolecules from the environment (Caridi, 2006).

2.2.2 Nutritional and Health-Promoting Properties

2.2.2.1 Nutritional Property

Yeasts are often considered nutritionally beneficial due to their rich protein, soluble fiber and mineral contents (Yamada & Sgarbieri, 2005). Yeast cell wall consists of β-glucan (60%, w/w), mannoproteins (40%, w/w), and chitin (2%, w/w) (Aguilar Uscanga & Francois, 2003). The polysaccharide component is composed of a mixture of water-soluble mannan, alkali-soluble and alkali-insoluble glucan, and lesser amounts of chitin. Yeast proteins are a source of the essential amino acid lysine and the sulfur-containing amino acid tryptophan, giving them a superior nutritional profile when compared to cereal and plant proteins (Yamada & Sgarbieri, 2005). The proportion of mannoprotein in the yeast cell wall, as well as the protein to mannan proportion in mannoproteins, varies between species and is dependent on growth environment. Overall, mannoproteins represent approximately 35 – 40% of the mass of dry yeast cell wall (Caridi, 2006).

2.2.2.2 Inhibitory Effect on the Adhesion of Pathogenic Bacteria

Mannan-oligosaccharides (MOS) have been reported to be able to limit the adhesion of pathogenic bacteria onto *Campylobacter jejuni* (*C. jejuni)* and *Campylobacter coli* (Ramirez-Hernandez, 2014). A study showed that mannoproteins can inhibit adherence of *C. jejuni* to a Caco-2 tissue culture cell

line (Ganan et al., 2009). Adhesion of bacteria onto the host is the first step in the microbial invasion; therefore, hindering their adhesion can prevent and eliminate infection (G Kogan & Kocher, 2007). The cell surface of certain pathogenic bacteria, such as *Escherichia coli* and *Salmonella*, has a protein-chitin compound called lectin that can identify and bind to the "specific carbohydrate" receptor on animal intestinal wall cells, resulting in disease (Peumans & Van Damme, 1995). MOS have been reported to exhibit a strong binding capacity to lectin, inhibiting pathogenic bacteria's attachment to animal intestinal cells. Thus, the MOS fraction can limit pathogen invasion by acting at the bacteria attachment level (K Newman, 1994). As a result, they can allow beneficial flora to colonize the environment. Enhancement of probiotic bacteria growth also leads to a decrease in pH, further limiting pathogenic growth (K Newman, 1994; Roberfroid, 2007). Some commercial products, based on yeast cells and yeast cell walls derivatives or mannoproteins, have been reported to be successful in reducing the intestinal attachment of enteropathogenic bacteria. They have been found to be effective against *E. coli, S. typhimurium, Clostridium botulinum, Clostridium sporogenes, C. jejuni* and *Salmonella*. Animal studies have shown that dietary MOS can reduce larger intestinal concentrations of *Clostridium perfringens* in turkeys at week 6 (Sims et al., 2004). Another animal study conducted on the effect of MOS (1%) on senior dogs showed that MOS can decrease peripheral lymphocyte concentrations and alter fecal microbial populations, thus strengthening the immune system of senior dogs (Grieshop et al., 2004). Mannose and methyl-α-D-mannoside were reported to be the most efficient sugars in inhibiting the adherence of *Salmonella typhimurium* to chicken intestine epithelial cells *in vitro* (Oyofo et al., 1989). Supplementation with dietary Bio-Mos® demonstrated a reduction in Gram-negative antibiotic-resistant fecal bacteria in swine (Moran, 2004). In addition, branched mannan, such as α-(1,3) and α-(1,6) linked mannan, found in the outer cell wall of *S. cerevisiae*, demonstrated around 37.5 times binding capacity for *E. coli* as compared to D-mannose (Firon et al., 1983). All above studies confirmed the ability of different mannose-based sugars to inhibit pathogen attachment.

2.2.2.3 Beneficial Effect on Probiotic Bacteria

Mannan, mannoprotein or MOS have also been reported to be able to favor the growth of lactic acid bacteria. Yeast cell wall extracts have shown a beneficial effect on some probiotic bacteria, for example *Bifidobacterium longum, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus delbreukii, Lactobacillus plantarum, Lactobacillus salivarius* and *Enterococcus*

faecium (Ganan et al., 2009; Ganan et al., 2012). However, it remains unclear whether the beneficial effect of mannose-based oligosaccharides or polysaccharides on probiotic bacteria is a direct or indirect effect through pathogen control (Sefton et al., 2006).

2.2.2.4 Modification of the Morphology and Structure of the Intestinal and Gut Mucosa

There is increasing evidence that mannose-based oligosaccharides or polysaccharides can modify the morphology and structure of the intestinal and gut mucosa. A study showed an increase in jejunal villi length, RNA content of the ileal mucosal homogenate and protein/DNA ratio of the jejunal mucosal homogenate in broiler chickens fed diets containing commercial mannan oligosaccharide (Iji et al., 2001). Another study showed that inclusion of yeast cell wall at 0.2% in broiler diets can increase the villus height during the first 7 days of life, which may result in improvement of body weight gain (Santin et al., 2001). A supplemented diet with 0.2% MOS has been reported to improve gut morphology and the epithelial brush border in rainbow trout under commercial farming conditions, with increased gut absorptive surface area in the subadult trout, and an increase in microvilli length and density in the anterior and posterior intestine (Dimitroglou et al., 2009). An improvement of the intestinal mucosa with MOS may lead to a reduction of necrotic enteritis (Hofacre et al., 2003).

2.2.2.5 Immunomodulatory Effect on the Host Immune System

The immunomodulatory effect of MOS is less studied compared to that of β-glucan. The mechanism of mannose-based oligosaccharides or polysaccharides in immune modulation have not been completely elaborated, but may have a similar mechanism to β-glucan (Moran, 2004). The immunomodulatory effect of mannoproteins on the host immune system is mainly through the stimulation of neutrophils and macrophages, which then release their chemical mediators (K Newman, 1994; Biondo et al., 2005; Darpossolo et al., 2012). An in vivo study showed that plasma immunoglobulin G (IgG) and bile immunoglobulin A (IgA) antibody levels increased after feeding MOS-supplemented meals to turkeys (Savage et al., 1996). Another study showed that higher colostrum IgG and immunoglobulin M (IgM) was measured in sows fed Bio-Mos® 14 days pre-farrowing and throughout lactation compared to untreated sows (KE Newman & Newman, 2001). Other animal studies have shown phagocytic cells activity activated by Bio-Mos® and a dose dependent effect was observed between the stimulation of phagocytosis and Bio-Mos® content (Sisak, 1994). In contrast, Bio-Mos was found to have an inhibitory effect on lymphocyte numbers

with nursery pigs, which allowed for nutrients to be used for growth rather than activating the immune system (Miguel et al., 2004). Recently, some research suggested that MOS may suppress the pro-inflammatory immune response. An intraperitoneal injection of sterile *Salmonella typhimurium* strain SL 684 LPS reportedly induced a mild fever response in turkey poults, yet when fed a diet containing Bio-Mos®, a decrease in fever response compared to control group at 8 h post-injection was observed (Ferket et al., 2002). A greater control of the immune response, especially the fever response, can lead to energy saving of the host and maintenance of feed intake and stress reduction (Moran, 2004). More studies are needed in order to understand the complex and diverse effects of MOS derived from yeast cell wall on the hosts' immune system.

2.2.2.6 Other Health-promoting Activities

Although not widely studied, mannan, mannoproteins and MOS have been reported to have other health promoting activities. Yeast cell wall mannan has been shown to have antioxidant and antimutagenic activity against ofloxacin-induced DNA damage (Krizková et al., 2006). Following analysis of mannan *S. cerevisiae* conjugates with human serum albumin stained with acridine orange, their potential application as antioxidants and antimutagenic agents was demonstrated (Krizková et al., 2006). Similar preparation of mannans derived from *Candida. albicans* serotype A, *C. dubliniensis*, *C. tropicalis* also showed considerable antioxidant activities protecting liposomes against peroxidation by OH· radicals in a concentration-dependent manner (Machová & Bystrický, 2012). Another study showed that α-D mannan can also suppress toxic activity of mycotoxins through interaction with their toxic radical metabolites (G Kogan & Kocher, 2007).

2.2.3 Techno-Functional Properties

Mannoproteins have been widely investigated due to their specific techno-functionalities, including high water solubility, emulsifying property and positive effect in wine making process. The challenges of producing natural and effective additives are mainly the cost and safety of extraction methods. Mannoproteins are appealing for industrial application because of their low-cost as a by-product, non-toxic, biodegradable nature, and more importantly, they can be simply obtained through a hot water treatment followed by precipitation (Silva Araújo et al., 2014). The techno-functional properties of mannoproteins are driven by their compositional and structural properties, thus it is important to isolate well-defined mannoproteins.

2.2.3.1 Emulsifying Properties

An emulsion is defined as "mixtures of at least two immiscible liquids, one of which is dispersed in the other in the form of fine droplets" (Smith & Culbertson, 2000). In recent years, isolation and identification of new microbial emulsifiers received wide attention in the pharmaceutical, mining, food and other industries (Iguchi et al., 1969; Kaplan & Rosenberg, 1982; Cirigliano & Carman, 1984, 1985; Barriga et al., 1999). In particular, mannoproteins have shown potential as novel emulsifiers. A kerosene-in-water emulsion stabilized by mannoproteins extracted from *S. cerevisiae* was stable over a broad pH from 2.0 to 11.0, with up to 5% sodium chloride or up to 50% ethanol in the aqueous phase. Under low concentration of various solutes, this emulsion was stable up to three freeze-thaw cycles (Cameron et al., 1988). It should be noted that protease treatment of mannoproteins has been shown to decrease their emulsifying capacity (Cameron et al., 1988).

A study showed that the emulsification activity of mannoproteins extracted from *S. cerevisiae* was comparable to that of sodium caseinate, with an emulsion phase of 79% compared to 77% in sodium caseinate (Torabizadeh et al., 1996). Another study showed that emulsions composed of 60% oil-in-water, 8 g/L mannoprotein as the bioemulsifier and 5–50 g/L sodium chloride can last for 3 months at 4 °C at pH 3.0–11.0 (Cameron et al., 1988). It has been hypothesized that mannoproteins can be used in mayonnaise with several different formulations to replace xanthan gum (Torabizadeh et al., 1996). Some researchers (Dikit, Methacanon, et al., 2010) reported that mannoproteins from *S. cerevisiae*, isolated by autoclaving in citrate buffer (pH 7.0) for 60 min, exhibited an emulsion activity of 65% with palm oil with a critical emulsifier concentration of 20 g/L. This mannoprotein extract showed similar emulsifying capacities as gum arabic and lecithin, and stabilized emulsions over a broad pH range from pH 5.0 to 8.0, in the presence of 3% (w/v) sodium chloride and up to 0.1% (w/v) $CaCl_2$ and $MgCl_2$ in the aqueous phase. In addition, the emulsion activity of this mannoprotein extract was not affected by temperature. There is a potential of using mannoproteins as emulsifiers and stabilizing agents to replace xanthan gum in mayonnaise (Silva Araújo et al., 2014). Different concentrations of mannoproteins were added to mayonnaise, and the results showed a constant pH throughout the 28 days refrigerated storage period. Sensory evaluation results showed that aroma, color, flavor and the overall evaluation were unaffected by replacement of xanthan gum with mannoproteins. It was concluded that substitution with mannoproteins in the mayonnaise formulation presented no negative effects on the sensory

attributes (Silva Araújo et al., 2014). Similar emulsifying and stabilizing effects were obtained upon the replacement of soy lecithin with mannoproteins in the formulation of French salad dressing, with no undesirable effects on the sensory attributes and nutritional characteristics (Silva Araújo et al., 2014).

2.2.3.2 Solubility Properties

Surprisingly perhaps, there is not much information reported in the scientific literature concerning the solubility of mannoproteins. Mannans are one of the most important components in the wall of higher plants (Shukla & Pletschke, 2013). In some plants, the solubility of mannans varies due to the presence of D-galactose side chains, which prevent alignments of macromolecules and lead to formation of strong hydrogen bonds (Shukla & Pletschke, 2013). Mannan derived from yeast *S.cerevisiae* have similar structure compared to mammalian glycoproteins and have a α-(1-6) linked backbone and α-(1-2) and α-(1-3) linked branches. Branched polysaccharides normally have a higher solubility compared to uniform polysaccharides (Whistler, 1973), which may be due to branched structure consuming more space and resulting in fluffy texture compared to uniform polysaccharides. Solubility is affected by different components present in the solution. For glycoproteins, their solubility is determined by that of the polysaccharides and proteins. While the solubility of single protein solutions can be easily measured, it is more challenging in glycoproteins as the interaction between protein and polysaccharide is environment-dependent, resulting in varying solubilities. Generally, protein is least soluble when the positive and negative charges of each protein fractions are equal, and this pH value is known as isoelectric point or isoionic point of the protein (Marchal & Jeandet, 2009). The effect of protein modification with saccharides on the protein solubility is not fully established. For example, wheat germ protein glycated with dextran showed an improvement in protein solubility, but the improvement of protein solubility through glycation also depended on the degree of glycation and the carbohydrate length (Niu et al., 2011). Conversely, the solubility of chicken myofibrillar proteins decreased when conjugated with glucose (Nishimura et al., 2011).

2.2.3.3 Prevention of Haze and Precipitation

During the wine making process, grape proteins are slowly denatured and lead to protein aggregation and flocculation, resulting in a visually unappealing cloudiness. In addition, other factors, like pH, concentration of metal ions, ethanol concentration and phenolic compounds, are

also likely to influence the haze formation in wine (Waters et al., 1996). The haziness in white wine is not an issue related to safety or taste but is one of aesthetics and will affect consumer's acceptability. The grape-derived proteins, which have low isoelectric points and molecular weight, are expressed throughout the ripening period, are stable at the acidic pH and resistant to enzymatic and non-enzymatic proteolysis (Waters et al., 2005). The protein level varies in white wine and can reach up to 300 mg/L (Waters et al., 2005). The traditional method for solving the haze problem in white wine is through adsorption onto bentonite, heat treatment, and ultrafiltration (Hsu, 1986; Van Sluyter et al., 2015). The procedure of removing the haziness in white wine with bentonite has an adverse effect on the quality of white wine, through the removal of color, flavor and texture compounds (Høj et al., 2001). A few studies have shown that addition of mannoproteins into white wine can decrease the extent of haziness, which may occur through the competition of mannoproteins with wine proteins for other unknown components required for the formation of large protein aggregates (Waters et al., 1994; Dupin et al., 2000; Gonzalez-Ramos et al., 2008). However, more studies are needed to explain the detailed mode of action of mannoproteins in preventing wine haziness.

Potassium bitartrate and calcium tartrate sediments in bottled wines are the common causes of wine unacceptability (Lasanta & Gomez, 2012). The most traditional method for inhibiting tartrate salt crystallization in wine is using the cold stabilization method. Its effectiveness depends on wine composition, and is time and energy consuming (Lasanta & Gomez, 2012). Electrodialysis and ion exchange treatments can be alternative methods to rectify this issue, but they require specific equipment and may lower the quality of wine by removing desired flavor compounds (Lasanta & Gomez, 2012). Studies have demonstrated that mannoproteins can be used in wine to inhibit tartrate salt crystallization (Virginie Moine-Ledoux et al., 1997; Guadalupe & Ayestarán, 2008). The mechanism of mannoproteins in lowering the growth rate of crystal in wine may be via binding to nucleation points and preventing expansion of the crystal structure (Gerbaud et al., 1997). Carboxymethylcellulose, metatartaric acid and mannoproteins were all reported to be used in wine to prevent crystallization. Compared to metatartaric acid, mannoproteins had a stronger inhibitory effect and had the greatest stability in wine (V Moine-Ledoux & Dubourdieu, 2002). The effectiveness of carboxymethylcellulose in the inhibition of tartrate salt crystallization in red wine was to a lesser degree than in white wine (Lasanta & Gomez, 2012).

2.2.3.4. Protective Effects of Mannoproteins

Mannoproteins can be used as protective agents for food colorants (Wu et al., 2015) as well as in wine (Caridi, 2006). Ochratoxin A (OTA) is a fungal secondary metabolite, a type of mycotoxin which has been reported in grapes, grape juices and wines (Zimmerli & Dick, 1996). It is disproportionately found in red wine samples, where a study reported detecting Ochratoxin A in 71% of red wine samples compared with 45% of white wine samples (Belli et al., 2002). In April 2005, the European Community limited the maximum allowable concentration of ochratoxins in wine to 2.0 μg/kg (Bayman & Baker, 2006). The occurrence of ochratoxin A is linked to the presence of *A. ochraceus*, *Aspergillus* section *Nigri* and *Penicillium. verrucosum* strains of molds on grapes (Belli et al., 2002).

Some studies reported that yeasts, yeast cell walls, yeast cell wall extracts or mannoproteins have the capacity to reduce ochratoxin A in wines or juices (Abrunhosa et al., 2002; Bejaoui et al., 2004; Moruno et al., 2005; Caridi, 2006; Caridi et al., 2006). Mannoproteins were demonstrated to have mycotoxin-binding capacity (Caridi, 2006), through a sponge-like mechanism, isolating ochratoxin A from grape juices and wines (Caridi, 2006).

Wine color, taste, and stability may be affected by phenolic compounds in enology (Gómez-Plaza et al., 2001). Yeast mannoproteins can interact with anthocyanins and tannins in wine, thus playing an important role in determining the aromatic composition in wines (Lubbers et al., 1994; Comuzzo et al., 2011; Juega et al., 2012), and stabilizing color and reducing astringency in red wines (Escot et al., 2001; Guadalupe & Ayestarán, 2008; Guadalupe et al., 2010). Mannoproteins were reported to be efficient in protecting anthocyanins from degradation at pH 7.0 during thermal stability testing at 80 and 126 °C, and no significant change in the antioxidant capacity was found (Wu et al., 2015). It can be hypothesized that mannoproteins may potentially expand the application of anthocyanins as natural colors or functional ingredients. The inclusion of mannoproteins to wine has been shown to confer additional benefits to wine products, including enhancing mouthfeel and increasing resistance against oxidation (Caridi, 2006); improving foaming properties of sparkling wines (Núñez et al., 2006); and increasing the growth of malolactic bacteria, which interact with flor wine and enrich wine during aging in fine lees (Caridi, 2006).

2.3 Selected Techniques for the Characterization of Mannoprotein Structures

Studying the structure, composition and conformation of glycoproteins is not an easy task. Monosaccharide composition, glycosaccharide composition, protein content, linkage pattern, configuration, substitution and molecular weight distribution of these compounds can all affect the structures and functional properties of glycoproteins. The commonly used techniques to determine the structure of polysaccharide include high performance size exclusion chromatography (HPSEC), high performance anion exclusion chromatograph (HPAEC), methylation analysis, 1D and 2D nuclear magnetic resonance spectroscopy, and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF-MS) (Guo, 2013).

2.3.1 Mass Spectrometry

For mannoproteins, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be used for the analysis of proteins and carbohydrates. The mass of glycoproteins can vary since N- and O-linked carbohydrate side chains of fungal glycoproteins vary in length, and the degree of occupancy of individual glycosylation sites can also differ. As a result, multiple fuzzy spots can be produced by fungal wall glycoproteins, making mass finger printing and quantitation more complex, and lowering the resolution and sensitivity of this method (Yin et al., 2008). A combination of proteolysis and liquid chromatography (LC) with tandem MS (LC/MS/MS) is a better approach for identifying multiple proteins from highly complex peptide mixtures (Insenser et al., 2010).

MALDI-TOF-MS can also be used for the structural characterization of oligosaccharides (Reis et al., 2003; Teleman et al., 2003; Ghosh et al., 2008; Peña et al., 2008). The basic experimental procedure is as follows: the analyte is first mixed with matrix compounds, followed by radiation from UV or RI laser light to excite the matrix. The sample is then charged by the exciting matrix and analyzed. The matrix compounds used are usually aromatic acids with a chromophore, such as 2,5-dihydroxybenzoic acid (2.5-DHB). Based on the specific fragmentation pattern, useful structural information in terms of sequencing, branching, linkage and profiling of oligosaccharides can be obtained (Reis et al., 2003; Teleman et al., 2003; Ghosh et al., 2008; Peña et al., 2008).

2.3.2 Nuclear Magnetic Resonance (NMR)

Mass spectrometry has been used for the characterization of glycoproteins. Nuclear magnetic resonance (NMR) spectroscopy yields the most complete picture in terms of identification of α- or

β-anomeric configuration, establishment of linkage patterns, and sequences of the sugar units (Agrawal, 1992). In addition, NMR spectroscopy can provide conformational information of molecules (Agrawal, 1992). 1D NMR includes ^{1}H and ^{13}C NMR. ^{1}H NMR is more sensitive than ^{13}C NMR due to the high abundance of ^{1}H in nature. The signals in ^{1}H spectrum are difficult to assign as most are located in a narrow region of 3~5 ppm. ^{13}C spectrum has a greater chemical shift dispersion, which increases its potential value. Homonuclear shift correlation spectrum (COSY), total correlated spectroscopy (TOCSY), heteronuclear multiple quantum correlated spectroscopy (HMQC) and heteronuclear multiple bond connectivity spectroscopy (HMBC) are commonly used 2D NMR techniques. COSY spectroscopy is used to disclose the correlations of the adjacent protons within the sugar rings. TOCSY spectroscopy is capable of showing the long range correlations of protons within sugar rings. Hence, TOCSY is usually used to confirm the assignment of ^{1}H spectrum. HMQC spectroscopy can provide the cross peaks between ^{13}C and its directly linked proton within the ring. HMBC spectroscopy can demonstrate long range coupling between proton and carbon (two or three bonds away) with great sensitivity, and can therefore be used to establish the glycosidic linkages and sequences between different sugar residues (Agrawal, 1992).

The NMR spectra of oligosaccharides in mannoproteins showed that mannan is formed mostly by α-1, 2- and α-1, 3-linked mannose residues (Valentin et al., 1984). Based on proton NMR, the signal at 5.3 ppm is typical of mannose in α-1, 2-linked side chains of two or more sub-units, and 5.1–5.2 ppm is characteristic of α-1,6-mannan in the basic chain on which the mannose side groups are suspended (Valentin et al., 1984). Furthermore, 5.02 ppm is the signal for terminal α-1, 2-linked mannose and for α-1, 2-linked mannose with a mannose substituent in the 3 position (Valentin et al., 1984). Based on ^{13}C NMR spectrum, chemical shifts between 98 and 103 ppm were a typical feature of the C-1 atoms in α-glycosidic linkages (Valentin et al., 1984). The sugar residues were mannopyranose because there was no resonance signal in 82–88 ppm (Valentin et al., 1984). The signal at 76–80 ppm could be assigned to the substituted C-2, C-3, or C-4 atoms; the signal at 70 ppm could be assigned to the substituted C-6 atom; and the signal at 61 ppm could be assigned to the unsubstituted C-6 atom (Valentin et al., 1984).Another study solved the structure of a glycoprotein from gum ghatti based on 1D and 2D NMR investigation including homonuclear 1H/1H correlations spectroscopy (COSY, TOCSY), heteronuclear $^{13}C/^{1}H$

multiple-quantum coherence spectroscopy (HMQC) and heteronuclear multiple bond correlation (HMBC) (H.-Z. Liu et al., 2015).

2.3.3 Fourier Transform Infrared Spectroscopy (FTIR)

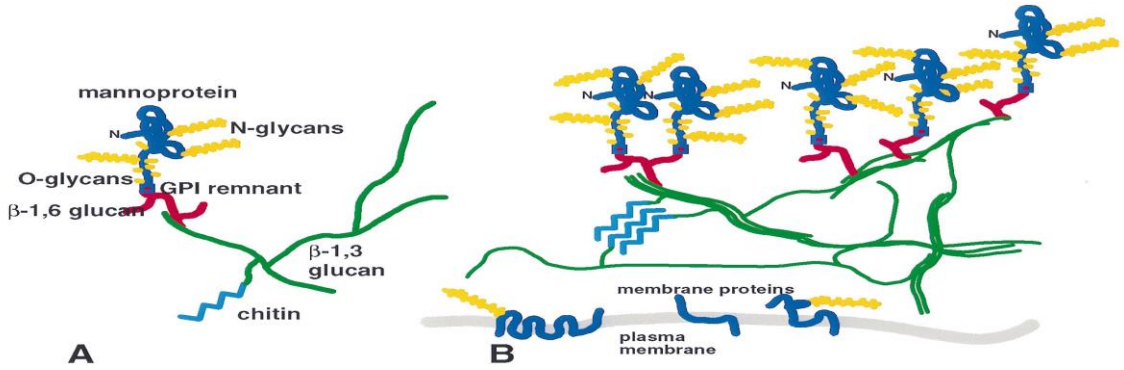
FTIR spectroscopy is a valuable tool for the examination of protein secondary structure and polysaccharide fraction in glycoproteins (Kong & Yu, 2007). This technique can be used to investigate the mannan fractions in mannoproteins derived from *S. cerevisiae*. The IR spectrum of a mannan showed OH valence vibrations appeared at 3400 cm^{-1}. The band appeared at 2900/2930 cm^{-1} was CH valence vibrations, and the band appeared at 1645/1652 cm^{-1} was consistent with acylamino, which confirmed that mannanoprotein is a glycoprotein. The C=O asymmetric stretching vibration occurred at 1635/1645 cm^{-1}; the CH deformation vibrations showed at 1454/1460 cm^{-1}. The C=O valence vibration appeared at 1000/1100 cm^{-1}, showed the presence of the pyranoid ring characteristic absorption peak. The band appeared at 820 cm^{-1} is characteristic of mannan (H.-Z. Liu et al., 2015). In addition, the change in protein secondary structure of mannoproteins can also be studied by FTIR. According to (Pots et al., 1998), the band at 1618 cm^{-1} can be assigned to strongly bonded β-strands. The remaining band assignments are: 1637 ± 3.0 and 1675 ± 5.0 cm-1 as β-sheets; 1645 ± 4.0 cm-1 as random coils; 1653 ± 4.0 cm-1 as α-helix; 1671 ± 3.0 and 1689 ± 2.0 cm-1 as β-turns (Kong & Yu, 2007).

2.4 β-Glucan

2.4.1 Structural Properties

β-glucan is polysaccharide of D-glucose linked by β-glycosidic bonds. It can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration, where it forms an amorphous fibrous network, visible by scanning electron microscopy of the inner surface of walls (Lipke & Ovalle, 1998). 85% of the yeast cell wall is composed of branched β-1,3-glucan of high molecular weight (approximately 240,000 Da), corresponding to a degree of polymerization (DP) of 1450±150. The length is around 1/10 of a cell circumference, or roughly three to six times the average wall thickness (Manners et al., 1973; Williams et al., 1991; Müller et al., 1997). β-1,3 glucan has a helical conformation, composed of either a single polysaccharide chain or of three hydrogen-bonded chains (a triple helix). This was confirmed by solid state nuclear magnetic resonance of intact yeast cells (Scheme 2.1) (Williams et al., 1991; Stokke et al., 1993; Krainer et al., 1994). The triple helical multimer provides structure and support to the yeast cell wall (Lipke

& Ovalle, 1998). The minor component, accounting for 15% of glucan, is branched β-1,6–glucan with a molecular weight of 22 kDa. Depending on the solubility properties of β-glucan, three classes of β-glucan are described in the literature: alkali-insoluble - acetic acid insoluble β-1,3 glucan; alkali-soluble β-1,3 glucan and highly branched β-1,6–glucan (Zeković et al., 2005). The alkali insoluble-acetic acid insoluble β-1,3 glucan is contributed to maintain wall mechanical strength and shape, the alkali soluble β-1,3 glucan has been proposed to provide flexibility on the cell wall; and β-1,6–glucan plays a central role in cell wall organization, interconnecting with the β-1,3 glucan, mannoproteins and chitin (Zeković et al., 2005). Glucan has been reported to be an effective immune booster in humans (Freimund et al., 2003).



Scheme 2.1. The components of *S. cerevisiae* cell walls. (A) Prototypical module with components individually labeled and colored. The mannoprotein polypeptide is shown in blue, and oligosaccharides are shown in yellow, labelled as N- or O- linked. Only a few of the branch points of the glucan are shown. Chitin can also be linked to the β-1,6 glucan. (B) Association of modules to form a wall lattice. Colors are as in panel A. The β-1,3 glucan chains are intertwined to designate triple helices, and chitin is shown as a crystalline microdomain. Cross-linking of mannoproteins through disulfide and other bonds is not depicted (Lipke & Ovalle, 1998).

2.4.2 Functional Properties

Various extraction techniques are available for β-glucan extraction. The selection of an appropriate method is critical as it may affect the functional activities of β-glucan. Yeast glucan has demonstrated water-holding, fat-binding characteristics, as well as gelling properties. The viscosity of β-glucan depends on the yeast strain used for isolation, and decreases upon heating and increases upon cooling (Moran, 2004; Thammakiti et al., 2004). In food application, β-glucan

obtained from brewer's yeast can be used as a thickening and oil-binding agent (Thammakiti et al., 2004).

2.4.3 Health-promoting Properties

β-glucan has attracted a lot of attention due to its health-promoting properties. The most important biological activity of β-glucan is its ability to stimulate the immune system, and many of its other effects are also related to this activity (Bohn & BeMiller, 1995). The immune system is a natural defense serving to protect the body from pathogenic microbes or environmental toxins and carcinogens, which may cause physical damage and disease (Brown & Gordon, 2003). To induce the immune response, glucan must be recognized by the immune system first. An in *vivo* study in animals has shown that macrophage, granulocyte and monocyte activity can be activated by oral administration of β-glucan, stimulating defense against infections, and supporting the repair of damaged tissues in the body (Rice et al., 2002; Vetvicka et al., 2007). β-glucan with a (1,3)/(1,6)-glycosidic linkage plays an important role in the immunomodulatory activity of the host and is often described as a biological response modifier (Di Luzio et al., 1979). It can stimulate immune cells such as macrophages, NK cells, T-cells and other white blood cells (Volman et al., 2008). Because β-glucan can modulate an overactive immune system, it may aid in reducing allergies and auto-immune diseases, as well as prevention of bacterial or viral infections, such as respiratory infections, chronic fatigue syndrome and other forms of chronic illnesses (Zeković et al., 2005). An in vitro study reported that between 300-3000 mg of daily β-glucan may have a positive effect against cancer cells, depending on the character and the immunostimulatory potential effect of the glucan (Zeković et al., 2005). A study suggests an intake between 300-500 mg of yeast β-(1,3)(1,6)-glucan daily during one year and 100 mg daily afterwards in order for the treatment of cancer or another serious health conditions (Mason, 2002). In addition, the results of immune response assays demonstrate that brewer's yeast can be administered for relatively long periods without causing immunosuppression (P. Li & Gatlin III, 2004). β-glucan has also shown potential as an anti-aging agent because of its inhibitory effect against free radicals (Jaehrig et al., 2008). In addition, as a soluble fiber, it has been shown to reduce low density lipoprotein cholesterol and triglycerides, decreasing the risk of cardiovascular diseases and strokes (Jamas et al., 2000). Finally, because of its satiety effect, β-glucan has been used in weight management (Zeković et al., 2005).

The primary structure, charge, solubility, conformation and particle dimensions of β-glucan were reported as the main factors affecting its biological activity (Tzianabos & Cisneros, 1996; Větvička et al., 1996; Hunter et al., 2002). The mechanism of action of β-glucan's biological activity has not been fully elucidated. The relationship between the molecular structure and biological activity has been the subject of some discrepancies (Di Luzio et al., 1979; Tokunaka et al., 2000). Some authors have reported that the triple helix is the most active conformation (Di Luzio et al., 1979; Hromádková et al., 2003), while others have claimed that the helical structure would not affect biological activity (Fleet & Manners, 1976; Kulicke et al., 1997)

2.4.4 Application of β-Glucan

Spray-dried yeast β-glucan has proven to be suitable for food formulations. They can be used as food thickeners with neutral flavor, as fat replacers to give a creamy mouthfeel, as dietary fibers, emulsifying stabilizers, film-forming agents, and water-holding/oil-binding agents (Thammakiti et al., 2004). Because of these techno-functional properties, β-glucan is applied in other industries as well, such as the animal feed, cosmetic and chemical industries.

In nonclinical applications, β-glucan has been used as an aging prevention and moisture retention agent in cosmetics. Oat β -glucan was found to be able to penetrate human skin and reduce the depth and height of wrinkles, and improve an overall roughness, though the reduced wrinkle effect was not permanent (Pillai et al., 2005). Due to its moisture-retaining capacity and anti-irritative properties, β-glucan may be a solution for sensitive and irritated skin (Wheatcroft et al., 2002). Since β-glucan is capable of stimulating epidermal macrophages (Langerhans cells) and exhibiting a free-radical scavenging effect, its application in sunscreens as a photoprotective agent was reported (Petravić-Tominac et al., 2010). Another study has described glucan's radioprotectant properties, showing enhanced host resistance to opportunistic infection in mice who were treated with irradiation (Patchen et al., 1987). Furthermore, β-glucan can serve as a prebiotic and stimulate the growth of lactic acid bacteria from genus *Lactobacillus* and *Bifidobacterium*, hence improving the intestinal tract flora (Gardiner & Carter, 2000). Other studies have also found that β-glucan may decrease indoor air-related symptoms, such as allergies and asthma (Rylander & Lin, 2000). Specifically, β-glucan consumption treatments can decrease allergic rhinitis, which is caused by allergic inflammation (Kirmaz et al., 2005).

2.5 Extraction Techniques for Mannoproteins and β-Glucan

The isolation of mannoproteins and β-glucan from the yeast and yeast cell wall by-products without denaturing or affecting their structural and functional properties is challenging. This section will focus on advantages and disadvantages of a variety of methods that have been studied for the recovery of mannoproteins and β-glucan.

2.5.1 Hot Water Extraction

Hot water extraction is an easy and simple method to extract mannoproteins from yeasts, then, via centrifugation, crude mannoproteins can be separated from β-glucan. One study obtained a crude mannoprotein yield of 17.8% following a hot water treatment of fresh bakers' yeast conducted at 121°C for 3 h at a concentration of 20% (w/v) (Cameron et al., 1988). Costa et al. (2012) studied the hot water extraction of mannoproteins from spent brewer's yeast and showed that the highest extraction yield of 4.08% was obtained after 7-9 h incubation at 95°C using 10-15% cell wall concentration. The mannoprotein extract obtained from 10% (w/v) cell wall (95°C, 9 h) was composed of 51.39% proteins, with 58 and 64 kDa peptides, and 25.89% carbohydrates (mannose and glucose) (Costa et al., 2012). Hot water extractions of mannoproteins have been performed under different temperatures (75, 85, 95 and 120 °C), durations (5, 7 and 9 h) and concentrations of yeast cell wall (10, 15 and 20% w/v), as well as combined with other purification methods (Cameron et al., 1988; Dupin et al., 2000; Núñez et al., 2006). There may be differences in chemical composition of the raw materials, resulting in different mannoprotein extraction yield under the same extraction method. Liu et al. (2008) have found that the combination of induced autolysis and hot water treatment resulted in an increased recovery of mannoproteins from 11% to 83% as compared to autolysis alone. Hot water extraction is considered the mildest treatment compared to alkaline and acid extraction methods, as it preserves the molecular structure of mannoproteins and β-glucan (H.-Z. Liu et al., 2011)

2.5.2 Acid-Alkaline Extraction

Many studies have focused on the extraction of β-glucan and mannoproteins from yeast cell walls using an acid-alkaline method (Van der Vaart et al., 1997; Freimund et al., 2003; H.-Z. Liu et al., 2011; Jin Li & Karboune, 2018). Using these methods, β-glucan is recovered in the sediment, while mannoproteins are suspended in the supernatants. After NaOH (2%, w/v) treatment on spent

brewer's yeasts in a boiling water bath under agitation of at 150 rpm/min for 2 h, the yield and the purity of the alkaline *S. cerevisiae* mannoproteins extract were 5.93 % and 88.24%, respectively (H.-Z. Liu et al., 2011). Mild alkaline treatments have been reported to degrade mannan or mannoproteins derived from *S. cerevisiae*, under conditions that lead to elimination of the substituted serine and threonine residues, releasing mannose, mannobiose, mannotriose, and mannotetraose (Nakajima & Ballou, 1974). In the presence of sodium borotritide, a strong alkaline treatment was found to degrade the mannan residue and yield a protein-free polysaccharide (Nakajima & Ballou, 1974). Masler et al. (1991) reported the method of extracting water-insoluble β-1,3-D-glucan from *S. cerevisiae* cells by using 6% (w/v) NaOH at 60℃ then followed by 4% (v/v) phosphoric acid treatment at room temperature. Similarly, Kogan et al. (1988) reported a method of obtaining glucan by means of mild alkaline extraction at increased temperature (6% NaOH, 60°C) from commercial baker's yeast. Despite achieving high yields compared to the hot water treatment, acid extraction is not an appropriate method due to the elevated salt content and release of carcinogenic compounds, including dichloropropanol (Vukašinović-Milić et al., 2007). With the alkaline extraction method, the yeast cell wall components are separated into two fractions: alkali-soluble and alkali-insoluble. The alkali-soluble fraction consists of β-1,3-glucan while the alkali-insoluble fraction is composed of β-1,6 glucan (Schiavone et al., 2014). It has been reported that 5-10% impurities, mainly proteins and mannans, were present in β-glucan extracts upon alkaline extraction. In addition, extreme conditions in the chemical extraction can severely degrade the glucan structure (Miller et al. 1993).

2.5.3 SDS Extraction

A number of covalently linked cell wall proteins are released after SDS extraction. A study showed that compared to a complete zymolyase treatment, mechanical disruption of the cells with a French pressure device treatment and autoclaving the cells, treatment with SDS resulted in the lowest yields of mannoproteins (Dupin et al., 2000). It was also reported that very low quantities of mannoproteins from three winemaking strains of *S. cerevisiae* were released*;* the mannoprotein extraction yields (w/w) were 0.2% from Maurivin PDM strain, 0.1% from AWRI 65 strain and 0.11% from AWRI strain (Dupin et al., 2000). They reported that only the surface of the cells interacts with SDS, limiting its effect (Dupin et al., 2000). Contrastingly, boiling purified yeast cell wall in 2% (w/v) SDS proved to be the best method in releasing intrinsic mannoprotein,

compared to autoclaving and zymolyase digestion methods, with a significant recovery of mannoproteins following ethanol precipitation (Valentin et al., 1984).

2.5.4 Enzymatic Methods

Because of the limitations of acid or alkaline extraction (Grigorij Kogan et al., 1988; Masler et al., 1991), biocatalytic approaches have attracted a high interest for the enrichment of cell wall with β-glucan and the extraction of mannoproteins (Freimund et al. 2003). Biocatalysis, which encompasses the use of biocatalysts to assist in synthetic conversions, is emerging as a highly promising area of research for the development of sustainable technologies for the production of innovative structurally well-defined bioactive molecules (Karboune et al., 2005; Karboune et al., 2008; Karboune et al., 2009). The interest in biocatalysis stems from the fact that most biocatalytic processes have been proven to be highly viable alternatives to their chemical counterparts, while eliminating many of the drawbacks commonly associated with the latter. Biocatalytic processes owe their advantages to the characteristics of enzymes, in particular, their diversity, high selectivity, high turnover number and their capacity to operate under environmentally-friendly conditions (Kobayashi et al., 2001; Adamczak & Krishna, 2004). Because of the complex molecular structures of enzymes, modulating their properties for specific applications, and understanding of the synergistic mechanism by which they function and the parameters that affect their thermodynamic relationships represent major scientific challenges. A study found that intact mannoproteins were hydrolyzed by Z-protease, but were resistant to β-glucanase, thereby maintaining the glucans' structure (Zlotnik, Fernandez, Bowers, & Cabib, 1984). β-glucanases are classified as exo and endo hydrolases, and are divided into three groups: β-1,3-glucanases, β-1,6 glucanases and β-1,4-glucanases, depending on the type of glucosidic linkage they are cleaving (Pang et al., 2004). β-1,3 glucanases are further classified into two groups: exo-β-1,3-glucanases (β-1,3-glucanohydrolase) (EC3.2.1.58) and endo- β-1,3-glucanases (β-1,3-glucan glucanohydrolase) (EC3.2.1.6) (Pang et al., 2004). Exo-β-1,3-glucanases release glucose units from the non-reducing end of glucan and release monosaccharides, while endo-β-1,3-glucanases hydrolyze glucose units at random sites and trigger the release of short oligosaccharides (Cheng et al., 2013). Following a zymolase (a β-1,3-glucanase) treatment, a high yield of mannoprotein emulsifier (16.7% of the weight of fresh bakers' yeast) was recovered from whole cells of fresh bakers' yeast (Cameron et al., 1988). A study showed that treatment with pronase can cleave mannoproteins and release the mannans from the mannoprotein complex (D. Li et al., 2009). A

31.8 kDa mannoprotein has been obtained industrially through commercial β-glucanase (GlucanexTM-Novo-Nordisk) enzymatic digestion of yeast cell walls (Moine - Ledoux & Dubourdieu, 1999). In an additional extraction step, Jaehring et al. (2007) have used lipase to remove lipid from the yeast cell wall. Liu et al. (2008) reported a new isolation method of β-D-glucan from spent yeast *S. cerevisiae*, which consisted of induced autolysis, water and organic solvent treatments, homogenization and protease hydrolysis. Using this multi-step method, the yield of β-glucan was estimated at 91% of the original ratio with a purity of up to 93% (w/w) (X. Y. Liu et al., 2008).

CONNECTING STATEMENT 1

A literature review on the distribution, structural properties, health-promoting properties and techno-functional properties of mannoproteins and β-glucan derived from brewer's or baker's yeast *Saccharomyces cerevisiae* were presented in Chapter II, as well as common techniques employed to isolate and generate them. Chapter III investigates a comprehensive assessment of heat treatment, SDS extraction and enzymatic process for the isolation of mannoproteins from yeast cell wall Agrimos®, then optimization of the enzymatic extraction of mannoproteins derived from yeast cell wall Agrimos® was assessed by response surface methodology.

The results from this study were presented at the IUFoST 17th World Congress of Food Science and Technology Expo and published in the journal of International Journal of Biological Macromolecules

Li, J., & Karboune, S. (2014) Investigation of Yeast Cells and Their Cell Walls as Sources of Functional Ingredients for Food Applications. IUFoST 17th World Congress of Food Science and Technology & Expo, Quebec, Canada, August 17-August 21, 2014.

Li, J., & Karboune, S. (2018). A comparative study for the isolation and characterization of mannoproteins from *Saccharomyces cerevisiae* yeast cell wall. *International journal of biological macromolecules*, *119*, 654-661.

CHAPTER III

A COMPARATIVE STUDY FOR THE ISOLATION AND CHARACTERIZATION OF MANNOPROTEINS FROM *SACCHAROMYCES CEREVISIAE* YEAST CELL WALL

3.1 Abstract

Cell wall yeast, recovered upon the production of yeast extract, was investigated as an abundant source of mannoproteins. Selected isolation methods were evaluated for the recovery of mannoproteins, in terms of yield, mannan and protein recovery yield, mannoproteins content and mannan to protein ratio in extracted mannoproteins. The results showed that heat treatment and sodium dodecyl sulfate (SDS) extraction led to a lower yield compared to the enzymatic treatment; and no glycosylated proteins could be obtained upon the SDS extraction. As compared to other methods, the enzymatic approach, based on the use of Zymolyase®, exhibiting a high β-1,3-glucanase activity, resulted in the highest yield, mannoproteins content and mannan to protein ratio. A 5-level, 2-variable central composite rotatable design contributed to the better understanding of the effects of independent variables, reaction time and enzyme units, on the efficiency of the enzymatic treatment and to the better modulation of their actions. The effects of enzyme units and reaction time on the mannoproteins content and the mannan to protein ratio exhibited the same patterns. Comparison of predicted and experimental values validated the established predicted models, which can be used to identify the conditions for the isolation of mannoproteins with well-defined molecular properties.

3.2 Introduction

There is an ever-increasing interest in exploring yeast cell wall (YCW) as an abundant source of bioactive molecules, such as mannoproteins. Indeed, a large amount of YCW is generated as a by-product of the yeast extract-producing industry, because of the high demand of yeast extract, made primarily of amino acids, peptides and nucleotides, as a flavoring agent (Chae et al., 2001). YCW is mainly used as an animal feed ingredient and is recently explored as a source of β-glucan (Borchani et al., 2014). The isolation of mannoproteins from YCW has been little investigated. Mannoproteins, which are the second most important component (40%, w/w) of the YCW (Quirós et al., 2011), are glycoproteins and contain up to 50 to 95% polysaccharides (Lipke & Ovalle, 1998; H. Liu et al., 2018). The mannoproteins can be divided into three groups: (i) non-covalently bound, (ii) covalently bound to the structural glucan and (iii) disulfide bound to other proteins that are covalently bound to the glucan of the cell wall. Covalently bound mannoproteins, referred to as cell-wall proteins (CWPs), fall into two categories, proteins with internal repeat-CWPs (Pir-CWPs, referred as alkaline-sensitive mannoproteins) and glycosylphosphatidylinositol-CWPs (GPI-CWPs, referred as glucanase-extractable mannoproteins). GPI-CWPs are the major class of

YCW proteins (Peter Orlean, 2012; Matsuoka et al., 2014). Because of their molecular and structural properties, cell wall mannoproteins exhibit many techno-functional properties that make them attractive for food applications (Caridi, 2006). For instance, cell wall mannoproteins have shown high emulsifying and stabilizing properties, making them attractive natural emulsifiers in salad dressing or mayonnaise without affecting the sensory attributes of these products (Silva Araújo et al., 2014; de Melo et al., 2015). They have also been extensively used in the enology industry due to their techno-functions in the adsorption of ochratoxin A, the complexation with phenolic compounds, the increase in the growth of malolactic bacteria, the inhibition of tartrate salt crystallization, the prevention of haze formation and the reinforcement of aromatic components (Caridi, 2006). In addition, mannoproteins showed health-promoting benefits, as they stimulate the growth of lactic acid bacteria (K Newman, 1994; Ganan et al., 2012) and inhibit pathogenic bacteria (Ganan et al., 2009). The techno-functional and health promoting properties of cell wall mannoproteins were reported to be dependent on their molecular and structural properties, including the molecular weight, the monosaccharide composition and the glycosylation extent (Caridi, 2006).

The isolation of mannoproteins from yeast/YCW by-products with well-defined structures and glycosylation levels is challenging because of the heterogeneity of their structures (Orlean, 2012). Acid-alkaline method has mainly been used for the isolation of mannoproteins from yeasts (Kath & Kulicke, 1999; H.-Z. Liu et al., 2011; H.-Z. Liu et al., 2015). A study showed that after NaOH (2%, w/v, 2 h) treatment on spent brewer's yeasts, the yield and the purity of the mannoproteins extract were 5.93% and 88.24%, respectively . Heat treatment was considered as a simple process for the isolation of non-covalently bound mannoproteins (Freimund et al., 2003; H.-Z. Liu et al., 2011). Sodium dodecyl sulfate (SDS) was also be used for the isolation of mannoproteins that are loosely associated with the cell wall, but SDS can also extract the majority of the plasma membrane proteins from yeasts (Van der Vaart et al., 1997; H.-Z. Liu et al., 2011). In addition, the enzymatic method, based on the use of β-1,3-glucanase has been investigated as a potential one for the isolation of the covalently-bound cell wall mannoproteins (De Nobel, Klis, Priem, et al., 1990; van der Vaart et al., 1995). As far as the authors are aware, no comparative study of the different methods for the isolation of cell wall mannoproteins has been conducted so far. The effects of isolation parameters of the enzymatic treatment on the structural properties of mannoproteins are still to be elucidated.

The main objective of this study was to investigate the isolation of the mannoproteins from YCW by heat treatment, SDS extraction and enzymatic treatment. The efficiency of the isolation methods was assessed in terms of yield of recovered extract, mannan and protein recovery yield, mannoproteins content and mannan to protein ratio in mannoproteins. Given the dependence of the techno-functional and health promoting properties of mannoproteins on their molecular properties, the effects of the parameters of enzymatic isolation on the structural properties of mannoproteins was also investigated.

3.3 Materials and Methods

3.3.1 Yeast-based Starting Material

Agrimos®, derived from a unique primary grown baker's yeast *S. cerevisiae*, after a specific manufacturing process to remove the cytoplasmic components, was kindly provided by Lallemand Inc. (Montreal, CA).

3.3.2 Selected Isolation Methods

3.3.2.1 Heat Treatment

Agrimos® (5%, w/v) was suspended in 50 mM phosphate buffer (pH 5, 7, 9). The suspensions were heated to 120 ºC in autoclave or to 90 and 80 °C in an oven with an erlenmeyer flask for 4 h. Later, the supernatants (enriched with non-covalently bound mannoproteins) and the precipitate (enriched with glucan), were recovered upon centrifugation at 9,800 g for 15 min and freeze-dried.

3.3.2.2 Sodium Dodecyl Sulfate Extraction

Agrimos® (5%, w/v) was suspended in 2% SDS and placed in a boiling-water bath for 5 min to extract the non-covalently bound mannoproteins (Van Rinsum et al., 1991). The soluble residues in the supernatant were -collected by centrifugation at 9 800 ×g for 15 min and then freeze-dried. Before the analysis of the SDS extracted samples by high-performance size exclusion chromatography (HPSEC), they were dialyzed using a membrane of 3 kDa molecular mass cut-off.

3.3.2.3 Enzymatic Isolation of Cell Wall Mannoproteins

The enzymatic isolation of mannoproteins from Agrimos® was carried out using a Zymolyase® enzyme (β-1,3-glucanase, Amsbio, Cambridge, MA, USA). Agrimos® (5%, w/v) was suspended in sodium phosphate buffer (50 mM, pH 6.5), and the Zymolyase® was added to yield 67-167 units of β-1,3-glucanase per g of Agrimos®. The enzymatic treatment was carried out at 45 ºC under

agitation (200 rpm) for selected reaction times. Blank reactions without Zymolyase® were run under the same conditions. After selected reaction times, the β-1,3-glucanase activity was inactivated by incubation at 60 ºC for 10 min, and the recovered mannoproteins in the supernatant were analyzed for their monosaccharide composition, protein content and molecular weight distribution.

3.3.3 Optimization of Enzymatic Treatment for the Isolation of Mannoproteins

The isolation of mannoproteins was carried out in a one-step reaction system using Zymolyase® as a biocatalyst. A five-level, 2-factor central composite rotatable design (CCRD) was employed to determine the effects of selected independent variables on the yield, the mannoproteins content in the extract, the mannan to protein ratio in the isolated mannoproteins using response surface methodology (RSM). Levels of independent variables, including time (X_1, 1-24 h) and enzyme unit (X_2, 20-160 units/g of substrate), were pre-determined. The design consisted of 4 factorial points, 4 axial points, 5 center points, leading to 21 runs. The variables were coded according to Equation (1):

$$\chi = (X_i - X_o) / \Delta X \quad (1)$$

Where χ is the coded value, X_i is the corresponding actual value, X_o is the actual value in the center point, and ΔX is the value of the step change. The relationship between variables and responses was expressed by a second order polynomial equation (2):

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \sum_j \beta_{ij} x_{ij} + \sum \beta_{ii} x_i^2 \quad (2)$$

Where Y is the measured response (yield of recovered extract, mannoproteins content and mannan to protein ratio in extracted mannoproteins), β_o is the model constant, and β_i, β_{ij} and β_{ii} are the measures of the linear, interaction, and quadratic effects of variables X_i, X_iX_j, and X^2_i, respectively. Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (Version 8.0, Stat-Ease, Inc. Minneapolis, MN, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) including lack of fit, Fisher's *F*-test (overall model significance), its associated probability *P*(*F*) and correlation coefficient R^2, to measure the goodness of fit of the selected models.

3.3.4 Analytical Methods

3.3.4.1 Protein Content

Harthree method (1972) was used as a simple and accurate assay for the estimation of the concentration of solubilized proteins. The standard curve was constructed using selected concentrations of Bovine Serum Albumin (BSA) (50 to 250 mM). The estimation of nitrogen content by Dumas method (Kirsten & Hesselius, 1983) was carried out using Leco® TruSpec N system (Leco Corporation, St-Joseph, Michigan, USA). Prior to nitrogen determination, samples were freeze-dried. Nitrogen content was multiplied by a factor of 6.25 to determine the total protein content.

3.3.4.2 Determination of Monosaccharide Composition

To determine the monosaccharide composition, the carbohydrate fractions were first hydrolyzed according to the method of Khodaei & Karboune (2013). The monosaccharides were measured using High-Pressure Anionic Exchange Chromatography equipped with Pulsed Amperometric Detection (HPAEC-PAD) (Dionex, ICS-3000) and a CarboPac PA20 column (3 × 150 mm) set at 32 °C.

3.3.4.3 Purification of Mannoproteins

Mannoproteins were separated from non-glycosylated proteins by affinity chromatography on HiTrap Con A 4B column (GE Healthcare, Piscataway, NJ, USA). The non-glycosylated proteins and other components were eluted with Tris-HCl buffer (20 mM, pH 7.4) containing NaCl (0.5 M), then 0.2 M α-D-methylglucoside was used as mobile phase to elute mannoproteins.

3.3.4.4 Characterization of Molecular Weight Distribution

To determine the molecular weight distribution, the mannoproteins were analyzed by high-performance size exclusion chromatography (HPSEC) on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL from Tosoh Bioscience, Montgomeryville, PA) using a Waters HPLC system (Model 25P, Waters Corp., Milford, MA) equipped with both refractive index (RI) and diode array (UV) detectors. The isocratic elution was carried with 0.1 M sodium chloride at a flow rate of 0.4 mL/min at room temperature. Dextrans with molecular weight of 50, 150, 270, 410 and 670 kDa were used as standards for calibration.

3.3.4.5 Characterization of Protein Profile

The protein profiles of generated extracts were analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli,

1970) slightly modified by using 5 and 20% acrylamide in the stacking and resolving gels, respectively.

3.3.4.6 Determination of Mannan Content

After the isolation of mannoproteins through HiTrap Con A 4B column, the mannan content was determined using phenol-sulfuric acid test (Dubois et al., 1956). 200 µL of phenol (5% w/v) and 1 mL of sulphuric acid were added to each aliquot of 400 µL of mannoprotein suspensions. After 15 min, the absorbance was measured spectrophotometrically (DU 800, Beckman Coulter, Fuellerton, CA) at 480 nm against a water blank. The carbohydrate content was estimated from a standard curve constructed with mannose.

3.3.4.7 Scanning Electron Microscopy (SEM)

In order to analyze the morphological properties of baker's whole yeast and Agrimos®, a Hitachi analytical desktop Microscope (TM3000, Hitachi High-Technologies Co., Tokyo, Japan) was used. The samples were fixed to a carbon conductive tab on the aluminum stub and examined at accelerated voltages of 5 and 15 kV without sputtering.

3.4 Results and Discussion

3.4.1 Approximate Composition of Agrimos®

In addition to the processing conditions, many factors can affect the composition of Agrimos®, including the nature of carbon source, nitrogen limitation, pH, temperature, oxygen and the mode of cell cultivation (Aguilar Uscanga & Francois, 2003). β-Glucans, chitin and mannans/mannoproteins are the main constitutive components of *S. cerevisiae* Agrimos®, and they are covalently linked to each other to a large extent (Lu et al., 1995; Kapteyn et al., 1997; Lipke & Ovalle, 1998; Freimund et al., 2003; Peter Orlean, 2012). Industrial Agrimos® derived from baker's yeast *S. cerevisiae*, recovered after the process of the production of yeast extract, was used as a starting material in the present study. Compared to the whole primary grown baker's yeast *S. cerevisiae*, the Agrimos® material shows clear differences in terms of protein, glucan, mannan and salt contents. As expected, the whole yeast contains significantly higher protein content (44.7%, w/w) compared to Agrimos® (19.6%, w/w). This difference is due to the removal of cytoplasmic proteins upon the production of yeast extract, thus less protein content was found in the Agrimos® by-product. However, Agrimos® material contained significantly higher amount of mannan (25.4%, w/w) compared to whole yeast (14.7%, w/w/), since mannan are mainly found

in the Agrimos® ($p<0.5$). The content of glucan was more or less similar in the whole yeast and the Agrimos® material (19.2% – 23.7% 23.7% - 19.2%, w/w). The scanning electron microscopy results (Figure 3.1) showed that Agrimos® maintained the rounded-like shape after the removal of the cytoplasmic components, but the size shrinks. A study showed that the volume of the cells changed to 1/4 of the fresh yeast cell after autolysis (X. Y. Liu et al., 2008). As expected, the proteins derived from whole baker's yeast covered a wide range, varying from greater than 180 kDa to less than 14 kDa; the most abundant proteins are those with the molecular weight between 16.4 kDa to 56.3 kDa. The protein distribution profile of the Agrimos® material revealed the presence of two major protein fractions with molecular weight higher than 100 kDa and lower than 14 kDa (data not shown). A study reported that proteins released from the baker's yeast deriving from *S. cerevisiae* covered a wider range of molecular weight, and mannoproteins possess sizes extending from 5 kDa to more than 800 kDa (Chalier et al., 2007).

3.4.2 Isolation of Non-covalently Bound Mannoproteins

In order to isolate non-covalently bound mannoproteins, Agrimos® was subjected to heat (90 ºC) treatment and SDS extraction. Heat treatment can extract non-covalently bound mannoproteins by breaking down the hydrogen bonds. While SDS as a solubilizing and denaturing reagent can breakdown the hydrogen bonds between molecules and affect the secondary and tertiary structures of the protein molecules. Table 3.1 shows that the yield of the recovered extract (11.36% - 10.89%) and the mannan recovery yield (11.75% - 11.19%) were similar upon the heat treatment and the SDS extraction. However, the protein recovery yield obtained upon the SDS extraction (45.60%) was statistically significantly higher than that obtained by the heat treatment (38.99%), thus leading to a higher relative protein proportion in the extract upon SDS extraction (79.74%) compared to the heat treatment (65.33%). The higher relative proportion of glucan/gluco-oligosaccharides obtained upon the heat treatment (17.74%) compared to the SDS extraction (8.4%) indicates the low mobility of the soluble glucan in the Agrimos®. Heat treatment led to 28.60% of mannoproteins content in the recovered extract. Meanwhile, the mannan/protein ratio

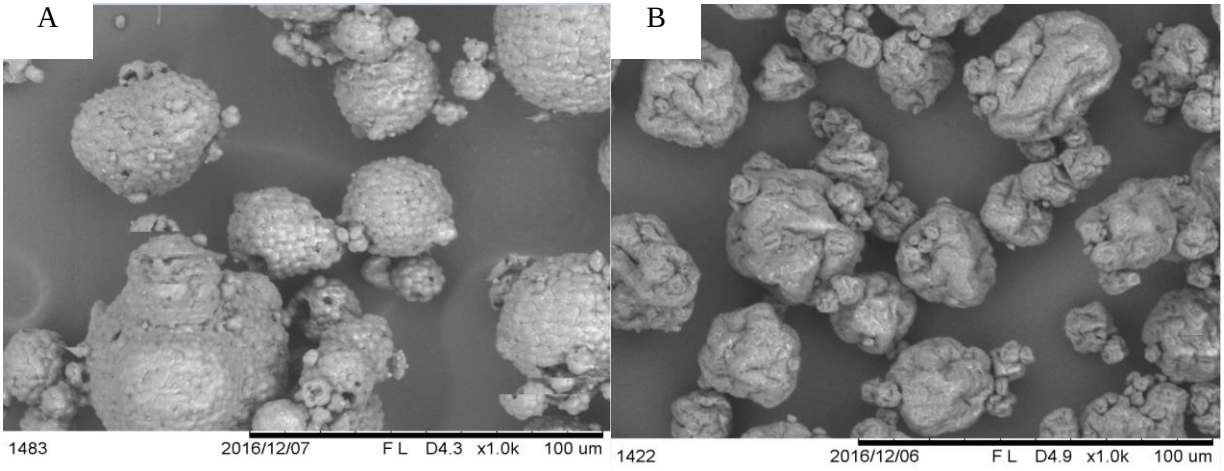


Figure 3.1. Scanning electron micrograph (magnification 1000×) of whole baker's yeast (A) and baker's yeast cell wall (YCW) (B**).**

Table 3.1. Effect of heat treatment, SDS extraction and Zymolyase® treatments on the isolation of mannoproteins

Treatments	Yield of recovered extract [a] (%)	Protein recovery yield [b] (%)	Mannan recovery yield [c] (%)	Relative protein proportion [d] (%)	Relative mannan proportion [e] (%)	Relative glucan/gluco-oligosaccharides proportion [f] (%)	Mannoproteins content [g] (%)	Mannan to protein ratio in mannoproteins [h] (w/w)
Heat treatment	11.36 (0.28)	38.99 (0.96)	11.75 (0.10)	65.33 (0.01)	16.92 (0.60)	17.74 (0.03)	28.60 (0.60)	0.64 (0.03)
SDS extraction	10.89 (0.39)	45.60 (1.03)	11.19 (0.25)	79.74 (0.06)	11.83 (0.11)	8.43 (0.12)	0.98 (0.01)	0.31 (0.01)
67 units/g-4 h	42.60 (0.27)	49.71 (4.08)	47.47 (5.29)	22.86 (1.73)	30.19 (3.18)	46.95 (6.51)	13.92 (3.30)	13.06 (1.20)
167 units/g-4 h	39.20 (0.42)	43.86 (0.07)	44.17 (4.43)	21.93 (0.27)	30.56 (3.39)	47.51 (4.50)	33.87 (4.20)	30.53 (3.20)
67 units/g-20h	26.72 (0.45)	61.06 (4.65)	98.20 (3.62)	22.30 (2.54)	26.64 (4.06)	51.06 (8.80)	15.61 (2.30)	11.89 (3.50)
167 units/g-20h	31.37 (0.72)	55.78 (2.23)	78.01 (9.50)	24.96 (0.11)	25.17 (2.20)	49.87 (6.08)	34.45 (4.30)	30.5 (4.50)

[a]Yield of recovered extract was obtained from the weight of extract divided by the weight of original yeast cell wall (YCW) and multiplied by 100.

[b]Protein recovery yield was obtained from the weight of protein in crude mannoprotein extracts divided by the weight of protein in original YCW multiplied by 100.

[c]Mannan recovery yield was obtained from the amount of mannan in crude mannoprotein extracts divided by the amount of mannan in YCW multiplied by 100.

[d]Relative protein proportion is the percentage of protein in the crude mannoprotein extracts.

[e]Relative mannan proportion is the amount of mannan in the crude mannoprotein extracts.

[f]Relative glucans/gluco-oligosaccharides proportion is the amount of glucan in the crude mannoprotein extracts.

[g]Mannoproteins content (%) is the proportion of mannoproteins in the extract

[h]Mannan to protein ratio in mannoproteins (w/w) is the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins

of 0.64 indicates that the non-covalently bound mannoproteins recovered upon heat treatment are mainly composed of short chain mannans. The SDS extraction led mainly to the release of mannans and proteins in the free forms; only a low mannoproteins content (0.98%) was recovered exhibiting a low mannan to protein ratio of 0.31. These results may be due to the defragmentation of the mannoproteins upon SDS treatment. Similarly, Dupin et al. (2000) reported that SDS extraction resulted in the release of very low amounts of mannoproteins from three winemaking strains of *S. cerevisiae* with a yield varying from 0.1-0.2%. These authors attributed these results to the fact that only the surface of the cells could interact with SDS, thus having a limited effect with whole cells. In addition, the limited solubility of SDS in low temperatures may have limited its extracting effect.

The molecular weight distribution of crude mannoproteins extracts was characterized by HPSEC using UV and RI detectors. The HPSEC profile of heat treatment-based extract shows peaks corresponding to glycoproteins, exhibiting a molecular weight of 6.5 kDa (data not shown). While the HPSEC profile of the SDS extraction-based extract confirms the abundance of proteins with a wide molecular weight distribution of 4-1045 kDa.

To assess the effect of temperatures on the isolation of cell wall mannoproteins, Agrimos® was subjected to selected heat treatments at temperatures varying from 80 to 120 °C (Figure 3.2). The non-covalently bound mannoproteins are released in the supernatants, while the insoluble glucan linked to chitin are enriched in the heat-treated Agrimos®. Figure 3.2 shows the yield of recovered extract and the protein proportion of the recovered mannoprotein extract. The proteins present in the recovered mannoprotein extract are in the free and glycosylated forms. Treatments at 120 °C resulted in the highest recovery yield of non-covalently bound mannoproteins of 16.13% (w/w of initial Agrimos®) at pH 7. When the treatment was undertaken at 80 °C, the yield of non-covalently bound mannoproteins decreased to 9.32% under the same pH. Heat treatment allowed the recovery of 37-51% of proteins in the supernatant. As a result, the relative protein content enhanced from a relative proportion of 28.53% in the initial Agrimos® to 42.96-60.43% in the crude mannoproteins extract. The pH change in the investigated range (5-9) did not have a significant effect on the yield of recovered extract; however, relatively higher protein content of crude mannoprotein extract could be obtained at acidic (pH 5.0) or alkaline condition (pH 9.0) compared to neutral pH. A study has examined the influence of alkali and heat treatment on yeast

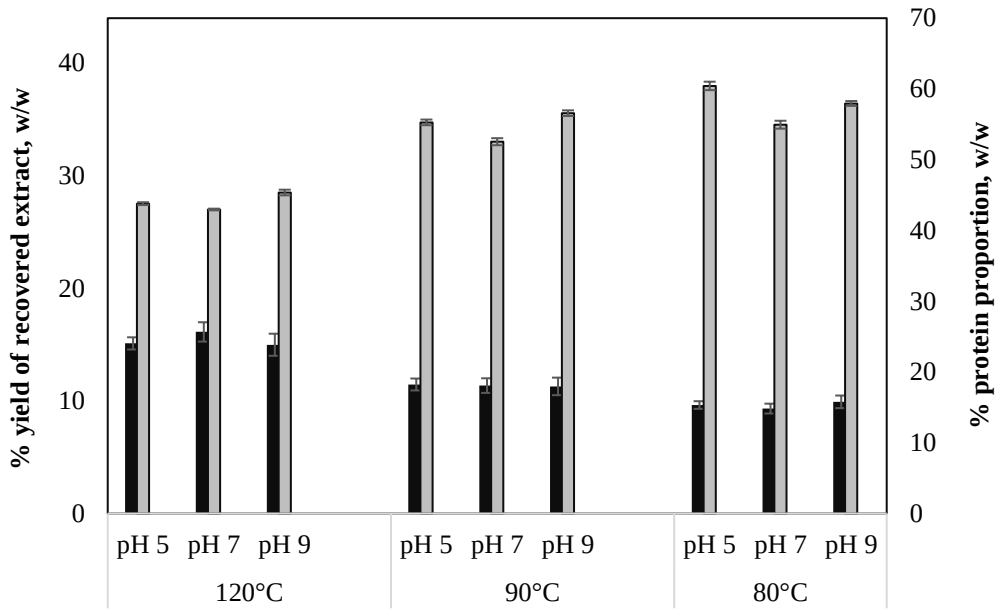


Figure 3.2. Effect of heat treatments on the yield of recovered extract (■) (% initial cell wall, w/w) and the protein content in the recovered extract (▭) (% extract, w/w)

protein and found that 42% of the nitrogen in the disintegrated cell suspension was recovered in the supernatant after heat extraction (Lindblom, 1974). This author also reported that additional 31% of the nitrogen was extracted using the alkaline condition at pH 11.5 and about 30% of the nitrogenous compounds in the yeast cell, which was bound to the subcellular structures could not be released even at higher pH values. Our results (data not shown) indicate that the precipitates, enriched with β-glucan, contain 20.11-22.56% of proteins; these results may be due to the fact that the remaining proteins are covalently linked to Agrimos® polysaccharide and may only be extracted after breaking covalently linked bonds (De Groot, Ram, & Klis, 2005).

3.4.3 Isolation of Covalently-bound Mannoproteins

The covalently-bound cell wall mannoproteins are anchored to the plasma membrane at an intermediate stage in their biogenesis via a glycosylphosphatidylinositol (GPI) moiety before they become anchored to the wall glucan via a 1,6-glucan linkage (Pastor et al., 1984; Peter Orlean, 2012). Zymolyase® from *Arthrobacter luteus* exhibits strong lytic activity against living Agrimos®, in particular high level of β-1,3-glucan laminaripentaohydrolase. However, besides the β-glucanase activity responsible for the degradation of β-glucan fibrils in the cell wall, the Zymolyase® expressed other minor side activities (mannanase, proteinase, amylase, xylanase and phosphatase).

Table 3.1 shows the effect of Zymolyase® treatments on the isolation of mannoproteins from Agrimos® under different conditions (67-167 units/g; 4-20 h). Compared to heat treatment (11.36 %) and SDS extraction (10.89%), Zymolyase® treatments led to two- to four- fold higher yield of recovered extract (26.7-42.6%). The results also show that the yield of recovered extract as well as the mannan and protein recovery yields were dependent on the Zymolyase® units. Increasing Zymolyase® units from 67 to 167 units/g resulted in a decrease in the yield of recovered extract and in the mannan and protein recovery yields (Table 3.1). As expected, this decrease was found to be more significant at 20 h reaction time (5-20 %) for the mannan and protein recovery yields compared to 4 h reaction time (3-7%). These results may be attributed to the hydrolysis of the released mannans/proteins by the hydrolytic enzymes expressed in the Zymolyase®. Increasing the reaction time from 4 h to 20 h at selected Zymolyase® units resulted in an enhanced release of the targeted macrocomponents to achieve 98.2 and 78.01% of mannan recovery yield as well as 61.0 and 55.78% of protein recovery yield at 67 and 167 units/g, respectively. The high recovery

yield of mannans demonstrates the efficiency of the Zymolyase® treatment to recover intact mannoproteins.

The results (Table 3.1) also showed that mannoproteins represent around 14-34% of the extract depending on the enzymatic units and reaction times. Higher enzyme units favored the recovery of mannoproteins in the extract; indeed, as the enzyme unit increased from 67 to 167, the proportion of mannoproteins present in the extract increased from 13.92 to 33.87% and from 15.61 to 34.45% after 4 and 24 h of treatment, respectively. The mannan to protein ratio of the isolated mannoproteins varied from 11.89 to 30.53, revealing the higher proportion of mannan. The reaction time of the enzymatic treatment seems not to affect the proportion of the mannoproteins nor the mannan to protein ratio in mannoproteins.

The HPSEC profile of Zymolyase® treatment-based extract shows mainly two molecular weight distributions of glycoproteins (5-10 kDa; 100-400 kDa), depending of the treatment conditions. The Zymolyase® treatment at 67 units/g for 4 h resulted mainly in a low molecular weight distribution of mannoproteins (5-10 kDa; 77%). However, increasing the Zymolyase® enzymatic concentration to 167 units/g led to an increase in the release of high molecular weight mannoproteins (100-400 kDa; 35.9%). A shift of the high molecular weight mannoproteins (100-400 kDa) to the low molecular one was observed upon the increase in the reaction time to 20 h.

3.4.4 Optimization of the Enzymatic Isolation of Mannoproteins from Agrimos®

RSM was applied to optimize the enzymatic isolation of mannoproteins from Agrimos® and to better understand the relationships between the reaction parameters. Central composite design (CCD) was performed with two independent variables at five levels, including time and enzyme units (U). The levels of the selected parameters were set on the basis of the previous experiments. The yield of recovered extract, the mannoprotein content and the mannan to protein ratio in mannoproteins were chosen as selected responses to assess the effect of the parameters of Zymolyase® enzymatic treatment on the isolation of mannoproteins and its influence on the structure of mannoproteins. Table 3.2 shows the experimental conditions and the experimental responses. Among the various conditions, the highest yield of recovered extract of 46.76 % (run #13) was achieved upon enzymatic treatment at Zymolyase® concentration of 139.5 units/ g

Table 3.2. Experimental design of 5-levels 2-variables central composite design and the responses

Run	Independent Variables		Responses		
	X_1[a]	X_2[b]	Yield of recovered extract [c] (%)	Mannoproteins content [d] (%)	Mannan to protein ratio [e] (w/w)
1	139.5	4.37	44.93	35.58	32.55
2	20	12.5	34.41	20.57	19.11
3	40.5	20.63	39.44	21.03	19.21
4	160	12.5	45.80	29.91	29.05
5	90	1	35.80	34.79	33.51
6	90	12.5	41.22	10.09	10.24
7	40.5	4.37	36.38	26.95	24.77
8	139.5	4.37	44.22	33.68	29.13
9	139.5	20.63	44.48	27.16	22.90
10	90	12.5	41.76	11.58	9.93
11	40.5	4.37	34.35	28.95	24.26
12	90	24	43.95	25.45	20.75
13	139.5	20.63	46.76	28.09	24.06
14	90	1	37.52	36.59	32.40
15	90	12.5	40.71	13.33	11.48
16	160	12.5	43.86	27.91	23.67
17	90	12.5	41.27	11.35	13.24
18	40.5	20.63	41.62	21.83	18.83
19	90	12.5	42.91	12.36	11.88
20	90	24	45.31	26.52	29.77
21	20	12.5	34.78	21.06	18.43

[a]Enzyme units ; [b]Reaction time (h)

[c]The yield of recovered extract was obtained from the weight of extract divided by the weight of original yeast cell wall (YCW) and multiplied by 100.

[d]Mannoproteins content (%) is the proportion of mannoproteins in the extract

[e]Mannan to protein ratio in mannoproteins (w/w) is the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins.

Agrimos® for 20.63 h of reaction time. Conversely, the lowest yield of recovered extract of 34.35% (run #11) was obtained at 40.5 units/ g of Agrimos® and 4.37 h. The mannoproteins content and the mannan to protein ratio varied significantly from 10.09 to 36.59% and 9.93 to 33.51%, respectively, depending on the Zymolyase® treatment conditions. The highest mannoproteins content (36.59%) was achieved at 90 units of Zymolyase®/ g Agrimos® for 1h reaction. Increasing the reaction time to 12.5 h at Zymolyase® concentration of 90 units/ g Agrimos® (run #6 &10) resulted in the lowest mannoproteins content (10.09-11.58%) and the lowest mannan/protein ratio (9.93-10.24). The highest mannan to protein ratio of 33.51 was obtained at run #5 corresponding to Zymolyase® concentration of 90 units/ g Agrimos® and 1 h of reaction time.

3.4.4.1 Analysis of Variance and Model Fitting

By applying multiple regression analysis, the analysis of variance (ANOVA) and the adequacy of the models are summarized in Table 3.3. The 2FI model was identified as statistically significant to represent the response of yield of recovered extract (*F*-value of 52.01 and *p*-value of < 0.0001), while quadratic models were statistically significant to represent the responses of mannoproteins content (*F*-value of 276.25 and *p*-value of < 0.0001), and the mannan to protein ratio (*F*-value of 40.97 and *p*-value of < 0.0001). The lack of fit was not significant among three models, which indicates a good quality of the fit and its ability to predict within a range of variables employed. The R^2 of the fitted models were 0.9018, 0.9893 and 0.9318 for the responses of the yield of the recovered extract, the mannoproteins content and the mannan to protein ratio, respectively, indicating that only 2-10% of the total variations in these responses were not explained by the models. The fitted models for crude mannoprotein extract yield, the mannoproteins content and mannan to protein ratio in terms of coded factors are given by Equations 3, 4 and 5.

$$\text{Yield of recovered extract (\%)} = 41.02 + 3.60X_1 + 2.18X_2 - 1.03X_1X_2 \quad (3)$$

$$\text{Mannoproteins in the extract (\%)} = 11.74 + 3.04X_1 - 3.41X_2 - 0.12X_1X_2 + 6.57X_1^2 + 9.56X_2^2 \quad (4)$$

$$\text{Mannan/protein ratio in the extracted mannoproteins (w/w)} = 11.35 + 2.69X_1 - 2.97X_2 - 0.47X_1X_2 + 5.26X_1^2 + 8.53X_2^2 \quad (5)$$

Table 3.3. Analysis of variance (ANOVA) for the yield of recovered extract (%), the mannoproteins content (%) and the mannan to protein ratio (w/w) in extracted mannoproteins

Sources	Yield of recovered extract [a](%)			Mannoproteins content [b] (%)			Mannan to protein ratio (w/w) [c]		
Parameters	Mean Square	F value	Prob>F[d]	Mean Square	F value	Prob>F	Mean Square	F value	Prob>F
Model	97.33	52.01	< 0.0001	279.44	276.25	< 0.0001	213.83	40.97	< 0.0001
X_1[d]	207.14	110.70	< 0.0001	147.89	146.20	< 0.0001	115.94	22.22	0.0003
X_2[e]	76.37	40.82	< 0.0001	185.58	183.46	< 0.0001	140.79	26.98	0.0001
X_1X_2	8.47	4.53	0.0483	0.12	0.12	0.7379	1.74	0.33	0.5726
X_1^2	-	-	-	446.22	441.13	< 0.0001	286.05	54.81	< 0.0001
X_2^2	-	-	-	944.08	933.32	< 0.0001	751.63	144.02	< 0.0001
Residual	1.87			1.01			5.22		
Lack of fit	3.48	2.89	0.0613	0.18	0.14	0.9310	2.84	0.49	0.6966
Pure error	1.20			1.22			5.81		
Cor Total									

[a]R^2=0.9018; [b]R^2=0.9893; [c]R^2=0.9318; [d] p-value<0.05 =statistically significant

[d]Enzyme units

[e]Reaction time (h)

F-test and *p*-value can be used to determine the significance of each variable and of their interactions. If the *F*-value is bigger and the *p*-value is smaller, the variables are deemed more significant. The results (Table 3.3) indicate that enzyme units (X_1, *F-value* of 110.70; $p< 0.001$) and reaction time (X_2, *F-value* of 40.82; $p< 0.001$) were significant linear terms in the yield of recovered extract model. However, the effect of enzyme units on the yield of recovered extract has a more significant effect compared to reaction time. In addition, the negative coefficient (Eq#3) of the interactive effect of both terms (X_1X_2, *F-value* of 4.53; *p value of* 0.0483) indicates the antagonistic effect between enzyme and reaction time affects significantly the yield of recovered extract. Table 3.3 also indicates that the linear effect of reaction time was the most significant in the mannoproteins content model (X_2, *F-value* of 183.46; $p< 0.0001$) and in the mannan/protein ratio model (X_2, *F-value* of 26.98; $p= 0.0001$). The linear term of enzyme units also had a significant effect on the mannoproteins content model (X_1, *F-value* of 146.20; $p< 0.0001$) and mannan/protein ratio model (X_1, *F-value* of 22.22; $p= 0.0003$). The interactive effect of enzyme units and reaction time did not show a significant effect on the responses of mannoproteins content and the mannan to protein ratio. In terms of quadratic effects, time had a significant effect on the responses of mannoproteins content (X_2^2, *F-value* of 933.32; $p< 0.001$) and mannan to protein ratio (X_2^2, *F-value* of 144.02; $p< 0.001$); while enzyme units had a less significant quadratic effect on the responses of mannoproteins content in the extract (X_1^2, *F-value* of 441.13; $p< 0.001$) and mannan to protein ratio in the extracted mannoproteins (X_1^2, *F-value* of 54.81; $p< 0.001$).

3.4.4.2 Effect of Enzymatic Reaction Parameters

The relationship between the reaction parameters (i.e. independent variables) and the responses can be better understood by studying the two dimensional (2D) contour plots of fitted models. Different shapes of the contour plots indicate different interactions between the variables; an elliptical contour plot indicates that the interactions between the variables were significant, while a circular contour plot indicates otherwise (Zhao et al., 2012). The contour graphs (Figure 3.3) show the interactive effects of enzymatic units and reaction time on the investigated responses. The contour plot of the yield of the recovered extract reveals that the interactive effect of both enzymatic units and reaction time affected the isolation of crude mannoprotein extract (Figure 3.3A). This effect can be attributed to the greater extent of hydrolysis of Agrimos® glucan at higher enzyme units and longer incubation time, leading to the release of higher amounts of

mannoproteins and gluco-oligosaccharides.The contour plots of mannoproteins content (Figure 3.3B) and mannan to protein ratio (w/w) in mannoproteins (Figure 3.3C) show similar trends. The circular contour plots of mannoproteins in the extract (Figure 3.3B) and mannan to protein ratio (w/w) of the extracted mannoproteins (Figure 3.3C) indicate that the interaction between the variables of enzyme units and incubation time was negligible. At shorter reaction time (1 to 5h), the mannoproteins content was higher, varying from 39 to 46 % (w/w recovered extract) over the investigated enzyme concentrations. Increasing the reaction time from 5 h to 19 h decreased the mannoproteins content and the mannan to protein ratio of the mannoproteins. This decrease may be attributed to the hydrolysis of the mannan side of mannoproteins by the mannase present in the Zymolyase® and/or to the release of glucan/glucooligosaccharides in the extract (Figure 3.3B and 3.3C). At a longer reaction time range from 19 h to 24 h, increasing the reaction time resulted in an increase in the mannoproteins content and the mannan to protein ratio. These results reveal that the side activities of the Zymolyase® and their synergestic interactions are dependent on the available substrates. The substrate inhibition of side activities may have also contributed to this trend of increase at longer reaction time range.

The conditions leading to the high predicted values of crude mannoprotein extract yield of 46.9% and of mannoproteins content of 39.73% were identified to be 157.4 unit of Zymolyase® per g of yeast cell wall and 23.6 h. These conditions were selected to validate the models and ran in triplicate with two center points in parallel as a reference (Waglay & Karboune, 2017). The experimental results of the recovered extract yield of 47.55% (standard deviation of 0.20) and of the mannoproteins content of 38.50% (standard deviation of 3.94) fall within the probability interval range of 43.10% to 50.54% for the response of yield of recovered extract and 36.86% to 42.83% for the response of mannoproteins content. These results validate the predictive models of the recovered extract yield and of the mannoproteins content.

3.5 Conclusion

To the best of our knowledge, this is the first comparative study of the different methods for the isolation of cell wall mannoproteins. Low molecular weight (6.5 kDa) mannoproteins were obtained upon heat treatment of Agrimos®, and they were mainly non-covalently bound mannoproteins. Higher yield of the covalently bound mannoproteins were obtained upon

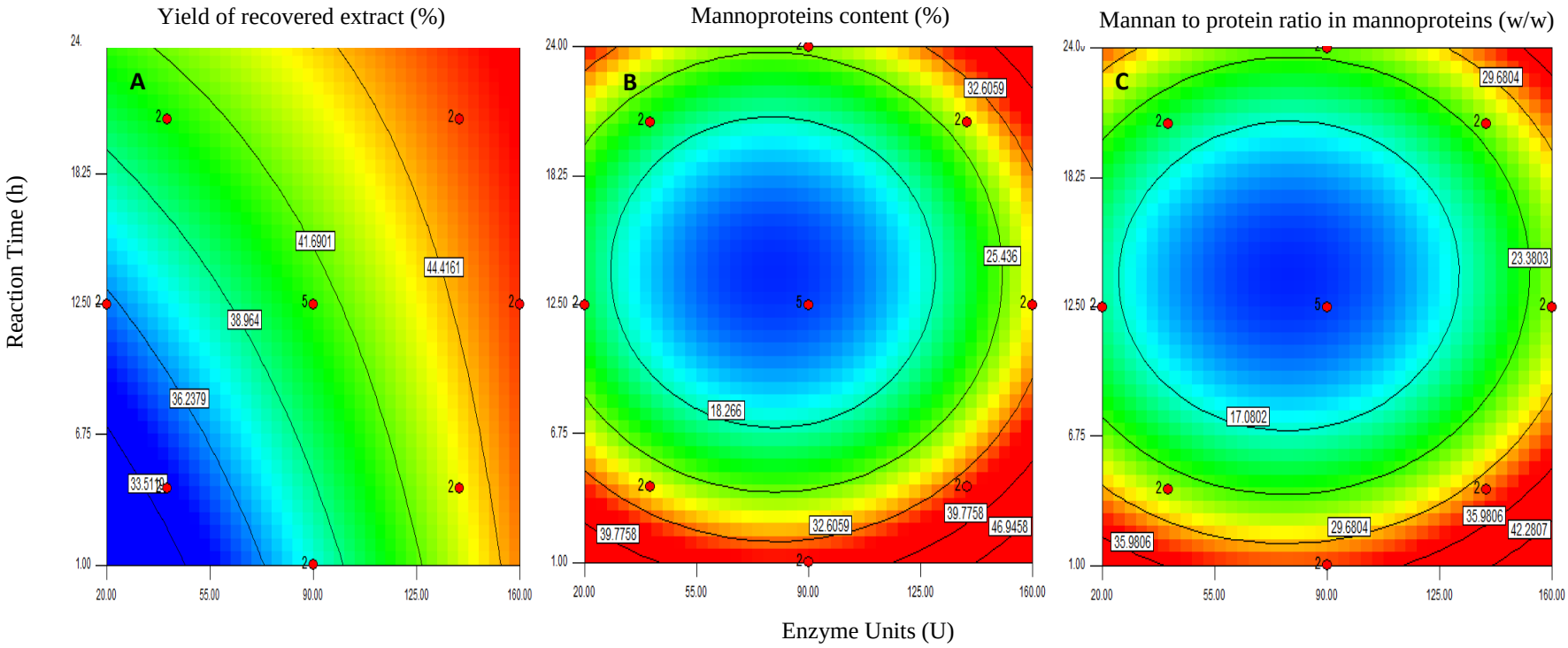


Figure 3.3. Contour plots of predictive models of yield of recovered extract (%) (A), mannoproteins content (%) (B), mannan to protein ratio in mannoproteins (w/w) (C). The numbers inside the contour plots indicate the predicted values under given reaction conditions.

Zymolyase® enzymatic treatment with molecular weight of 5 to 400 kDa. The optimization of the enzymatic isolation of mannoproteins was performed using RSM with CCD. The enzyme units and the reaction time were identified as the significant independent variables, affecting importantly yield of recovered extract, the mannoproteins content and mannan to protein ratio in mannoproteins. The interaction between the enzyme units and the reaction time displayed only significant effects on the response of yield of recovered extract. The predictive models of the mannoproteins content and the mannan to protein ratio showed the same trends with shorter/longer reaction times and lower/higher enzymatic units being more favorable for these responses. The overall findings of the present study will contribute to lay the scientific ground for the development of innovative biocatalytic process for the isolation of functional ingredients from baker's Agrimos® with many potential applications.

CONNECTING STATEMENT 2

In chapter III, the efficiency of heat treatment, SDS extraction and enzymatic process for the extraction of mannoproteins from yeast cell wall was previously investigated and compared. In chapter IV, mannoproteins isolated from baker's and brewer's whole yeasts using different methods were compared, then the effects of selected reaction parameters of beta-1,3-glucan laminaripentaohydrolase from *Arthrobacter luteus* for the isolation of mannoproteins from brewer's yeasts and their interactions were investigated by response surface methodology.

The results from this study were presented at the IFT Annual Meeting & Food Expo-Institute of Food Technologist

Li, J., & Karboune, S. (2015) Investigation of Yeast Cells and Their Cell Walls as Sources of Functional Ingredients for Food Applications. IFT15 Annual Meeting & Food Expo, Chicago, USA, July 11- July 14, 2015.

Li, J., Asehraou, A & Karboune, S. (2019). Mannoproteins from Inactivated Whole Cells of Baker's and Brewer's Yeasts (*Saccharomyces Cerevisiae*): Isolation and Optimization (Submitted).

CHAPTER IV

MANNOPROTEINS FROM INACTIVATED WHOLE CELLS OF BAKER'S AND BREWER'S YEASTS (*SACCHAROMYCES CEREVISIAE*): ISOLATION AND OPTIMIZATION

4.1 Abstract

The yields and the structural properties of mannoproteins varied depending on the isolation methods and their sources (baker's and brewer's *Saccharomyces cerevisiae* yeasts). Non-covalently-bound mannoproteins (6.5 kDa) with a mannan/protein ratio of 0.63 and 2.78 were recovered upon the heat treatment of brewer's and baker's yeasts, respectively. While SDS treatment led mainly to the release of non-glycosylated proteins. The highest yield of mannoproteins was achieved upon the enzymatic isolation with Zymolyase® from *Arthrobacter luteus.* The recovered covalently bound mannoproteins were characterized by a higher mannan/protein ratio (13.1-42.7) and a wider MW distribution (5-10 kDa;10-100 kDa;100-400 kDa). Predictive models were developed to understand and modulate the effects of isolation parameters on yield, the mannoproteins content and the mannan/protein ratio. The enzyme concentration was the most significant parameter affecting the yield, while the reaction time was the most significant parameter affecting mannan/protein ratio. Comparison of predicted and experimental values validated the established predicted models for the isolation of well-defined mannoproteins from yeast for targeted applications.

4.2 Introduction

Yeast mannoproteins as techno-functional ingredients have shown a great interest in many food applications. Indeed, because of their amphipathic structures, in which proteins are attached to the hydrophilic polymers of mannose, mannoproteins exhibit high emulsifying and stabilizing properties (de Melo et al., 2015). Furthermore, mannoproteins were found to contribute to the properties of wine during the vinification process by modulating their aromatic composition (Lubbers et al., 1994; Comuzzo et al., 2011; Juega et al., 2012), stabilizing their color and reducing astringency (Escot et al., 2001; Guadalupe & Ayestarán, 2008; Guadalupe et al., 2010), and by ameliorating the foaming properties of sparkling wines (Caridi, 2006; Núñez et al., 2006). More importantly, mannoproteins can be added to wine to prevent the crystallization of tartrate salts and the formation of protein haze (Dupin et al., 2000; V Moine-Ledoux & Dubourdieu, 2002; Bowyer & Moine-Ledoux, 2007; Gonzalez-Ramos et al., 2008). In 2015, Health Canada proposed and approved using yeast mannoproteins as a new food additive to inhibit crystal formation in wine at a maximum addition of 0.04%. In addition to these techno-functional properties, mannoproteins have shown health-promoting benefits, as they were found to stimulate the growth of lactic acid bacteria (K Newman, 1994; Ganan et al., 2012) and inhibit pathogenic bacteria (Ganan et al., 2009).

Yeast cell wall consists of two layers; the inner layer contains β-1,3-glucan (50 to 55% of the cell wall dry weight) and chitin (1 to 2%), and the external layer contains mainly mannoproteins (35 to 40%) and β-1,6-glucan (Gonzalez-Ramos et al., 2008). The cell walls of the common baker's and brewer's *Saccharomyces cerevisiae* yeasts are potential sources of mannoproteins. However, the conditions of yeast growth were reported to be determinant for the cell wall composition in terms of protein, mannan and mannoprotein contents (Freimund et al., 2003). Indeed, a study showed that the yeast source affects the amount of mannoproteins released during winemaking (Boulet et al., 2007). Previous studies revealed the dependence of the compositional and the structural properties of mannoproteins not only on the starting materials, but also on the isolation methods ((Van der Vaart et al., 1997; Freimund et al., 2003; H.-Z. Liu et al., 2011; Jin Li & Karboune, 2018)). In addition to the acid-alkaline isolation-based method, the sodium dodecyl sulfate (SDS) and heat treatments were investigated for the isolation of non-covalently bound mannoproteins from yeast cell walls (Van der Vaart et al., 1997; Freimund et al., 2003; H.-Z. Liu et al., 2011; Jin Li & Karboune, 2018). While the covalently-bound cell wall mannoproteins were isolated from yeast cell walls upon the enzymatic treatment with β-1,3-glucanase (Jin Li & Karboune, 2018). As far as the authors are aware, no comparative study of the different methods for the isolation of mannoproteins from the whole yeasts has been conducted so far. In addition, to our knowledge, no research has yet investigated the difference between mannoproteins derived from whole cells of baker’s and brewer’s yeast.

The main objective of the present research work was to investigate the isolation of mannoproteins from baker's or brewer's *S. cerevisiae* yeasts using the conventional methods (heat treatment and SDS extraction) and the enzymatic approach. The efficiency of the isolation methods, in terms of yield of recovered extract, mannoproteins content and mannan to protein ratio, was discussed while elucidating the difference between baker’s and brewer’s yeast. The effects of the parameters (enzyme amount; time) of the enzymatic isolation of mannoproteins from brewer’s yeast were further investigated. This comparative study is expected to lay the scientific ground for the isolation of well-defined mannoproteins from yeasts for targeted applications through the selection of the type of yeast and the control of the isolation conditions.

4.3 Materials and Methods

4.3.1 Materials

Two inactivated strains of *S. cerevisiae* (baker's yeast and brewer's yeast) used in this study were kindly provided by Lallemand (Montreal, CA). The baker's yeasts are grown under oxidative metabolism, while brewer's yeasts are grown under fermentative metabolism in an anaerobic environment. All other chemicals were obtained from Fisher Scientific (Mississauga, ON, Canada).

4.3.2 Determination of Morphological and Compositional Properties of Whole Yeasts

The morphological property of brewer's whole yeast was studied using a Hitachi analytical Scanning Electron Microscope (TM3000, Hitachi High-Technologies Co., Tokyo, Japan). The sample was fixed to a carbon conductive tab on the aluminum stub and examined at accelerated voltages of 5 and 15 kV without sputtering. The compositional properties of yeasts were investigated in terms of protein, glucan, mannan and fat contents. The protein content was estimated from the nitrogen content determined by Dumas method. While the mannan and glucan contents were estimated from their corresponding mannosaccharides upon chemical hydrolysis. The fat content was determined using the soxhlet method.

4.3.3 Investigation of Selected Isolation Methods

4.3.3.1 Heat Treatment

Suspensions of baker's and brewer's yeasts (5%, w/v) were prepared in phosphate buffer (50 mM, pH 7). The stirred suspensions were heated at 90 ºC in an oven for 4 h. The suspensions were then cooled to room temperature and centrifuged at 9,800 ×g for 15 min. The supernatants enriched with mannoproteins were recovered, dilalyzed (>5 kDa) and freeze-dried. The recovered crude mannoprotein extracts were analyzed for their mannan and glucan composition and their molecular weight distribution as well as their protein content. All experiments were run at least in triplicates.

4.3.3.2 Sodium Dodecyl Sulfate Treatment

To isolate the non-covalently bound mannoproteins, baker's and brewer's yeasts were suspended in 2% sodium dodecyl sulfate (SDS) at a concentration of 5% (w/v); the mixtures were incubated at 100 ºC for 5 min (Van Rinsum et al., 1991). Then the suspensions were centrifuged at 9,800 ×g for 15 min. The supernatants enriched with mannoproteins were recovered, dialyzed using a 3 kDa molecular mass cut-off membrane and freeze-dried. The recovered crude mannoprotein extracts were analyzed for their mannan and glucan composition and their molecular weight distribution as well as their protein content. All experiments were run at least in triplicates.

4.3.3.3 Enzymatic Isolation of Mannoproteins

Enzymatic isolation of mannoproteins, in particular glycosylphosphatidylinositol cell wall proteins (GPI-CWPs), from baker's and brewer's yeasts was investigated using a Zymolyase® enzyme (Amsbio, Cambridge, MA, USA) enriched with β-1,3-glucan laminaripentaohydrolase and β-1,3-glucanase. Zymolyase® enzyme was added to the suspension of yeast (5%, w/v) in a sodium phosphate buffer (50 mM, pH 7.5) to yield 1 unit of β-1, 3-glucan laminaripentaohydrolase per 6-15 mg of yeast. The enzymatic reactions were undertaken at 35 ºC under 200 rpm agitation for 4 h to 20 h. Blank reactions, without added Zymolyase®, were carried out in parallel with the reactions. After selected reaction times, the enzymatic reactions were inactivated by heating at 60 ºC for 10 min; and the recovered supernatants containing the mannoproteins were freeze-dried and analyzed for their mannan and glucan composition and their molecular weight distribution as well as their protein content. All experiments were run at least in triplicates.

4.3.4 Purification of Mannoproteins

To separate mannoproteins from other compounds, the freeze-dried extracts recovered after enzymatic treatment of baker's and brewer's yeasts were subjected to affinity chromatography using HiTrap Con A 4B column (GE Healthcare, Piscataway, NJ, USA). The non-glycosylated proteins and other components were eluted with tris-HCl buffer (20 mM, pH 7.4) containing NaCl (0.5 M), while 0.2 M alpha-D-methylglucoside was used as mobile phase to elute mannoproteins. The recovered mannoproteins were analyzed for their protein and mannan contents.

4.3.5 Optimization of the Enzymatic Isolation of Mannoproteins from Whole Brewer's Yeast

4.3.5.1 Experimental Design

The effects of selected parameters on the enzymatic isolation of mannoprotein from brewer's yeast were investigated using Response Surface Methodology (RSM). Central composite rotatable design (CCRD) with two variables at five levels was used. The independent variables and their levels were the reaction time (1, 4.36, 12.5, 20.63 and 24 h) and enzyme concentration (20, 40.5, 90, 139.5, 160 units/g of yeast). These levels were determined upon preliminary trials. A total of 21 runs were performed including two replicates in factorial points (levels ± 1), two replicated in axial points (levels ± α), and five replicates in center point. The recovered extracts (freeze-dried supernatants) upon enzymatic treatment of brewer's yeast were purified; the mannan and the protein contents of the purified mannoproteins were determined. The variables were coded according to Equation (1):

$$\chi = (X_i - X_o) / \Delta X \quad (1)$$

Where χ is the coded value, X_i is the corresponding actual value, X_o is the actual value in the center point, and ΔX is the value of the step change.

Replicates in center points were performed to obtain the pure error. In order to avoid systematic errors, experiments were done in random order, all reactions were carried as described above.

4.3.5.2 Statistical Analysis

The analysis of variance (ANOVA), including lack of fit, Fisher's *F*-test (overall model significance), its associated probability *P*(*F*) and correlation coefficient R^2, was applied to analyze the statistical significance of data and validate the best fitting models for the yield of recovered extract, the mannoproteins content and the mannan to protein ratio. 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. (2)). These equations were used to predict responses (yield of recovered extract, mannoproteins content and mannan to protein ratio in mannoproteins) as function of the independent variables.

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \sum_j \beta_{ij} x_{ij} + \sum \beta_{ii} x_i^2 \quad (2)$$

Where Y is the measured response, β_o is the model constant, and β_i, β_{ij} and β_{ii} are the measures of the linear, interaction, and quadratic effects of variables X_i, X_iX_j, and X^2_i, respectively.

Other statistical analyses for the comparison of the means of two groups were performed with t-test.

4.3.6 Analytical Methods

4.3.6.1 Protein Content Determination

The estimation of nitrogen content by Dumas method was carried out using Leco® TruSpec N system (Leco Corporation, St-Joseph, Michigan, USA). The total protein content was determined by multiplying the nitrogen content with a factor of 6.25. For the estimation of the concentration of solubilized proteins, Hartree method was used (Hartree, 1972). Bovine Serum Albumin (BSA) was used to establish a standard curve (50 to 250 mM).

4.3.6.2 Carbohydrate Content Determination

The total carbohydrate content was determined using the phenol-sulfuric acid test (Dubois et al., 1956). A volume of 200 µL of 5% phenol and 1 mL of sulphuric acid were added to each aliquot

of 400 μL of sample and left to stand for 15 min. The absorbance was then measured spectrophotometrically (DU 800, Beckman Coulter, Fullerton, CA, USA) at 480 nm against a water blank. Glucose with concentrations of 0.16 to 0.7 mM was used to construct a standard curve.

4.3.6.3 Determination of Mannan and Glucan Contents

The mannan and glucan contents were estimated from their corresponding monosaccharides. A two-step method was used to hydrolyze the carbohydrate polymers as described by Khodaei & Karboune (2013). A hydrochloric acid/methanol mixture (1:4, mL:mL) was added to sample (3–55 mg/mL) at a ratio of 1:5 (v:v). After incubation for 24 h at 60 °C, trifluoroacetic acid was added at a ratio of 1:8 (v:v). Monosaccharide composition was determined using high-pressure anionic exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD) (Dionex, ICS-3000) on a CarboPac PA20 column (3 × 150 mm) set at 32 °C. Isocratic elution was performed with 10 mmol/L NaOH (0.5 mL/min). D-glucose (Glc) (2.5-40 μmol/L) and D-mannose (Man) (2.5-40 μmol/L) were used as standards.

4.3.6.4 Characterization of Molecular Weight Distribution

To determine the molecular weight distribution, the mannoproteins were analyzed by high-performance size exclusion chromatography (HPSEC) using three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL (Tosoh Bioscience, Montgomeryville, PA, USA) and a Waters HPLC system (Model 1525, Waters Corp., Milford, MA, USA) equipped with refractive index (RI) detector and ultraviolet (UV) detector. The isocratic elution was carried out with 0.1 M sodium chloride at a flow rate of 0.4 mL/min at room temperature. Dextrans with molecular weight of 50, 150, 270, 410 and 670 kDa were used as standards for calibration.

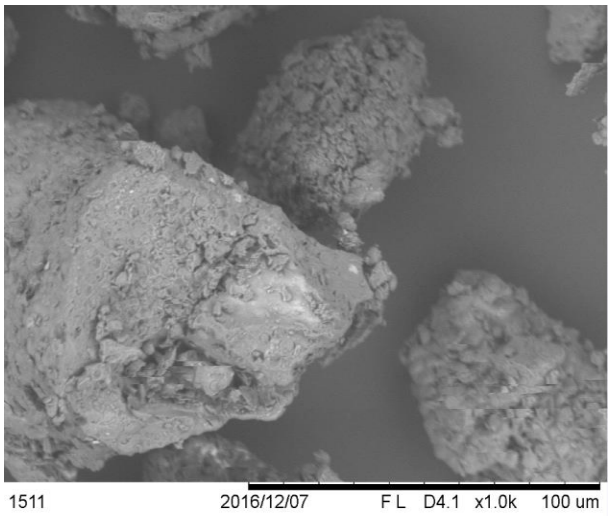
4.3.6.5 Characterization of Protein Profiles

The protein profiles of the recovered extracts were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) modified by using 50 and 200 kg/m^3 acrylamide content in the stacking and resolving gels, respectively. Samples were loaded in a mini protein gel apparatus (Bio-Rad, On) with a 1.5 mm thick gel. A constant voltage of 120 mV was applied during the electrophoresis process. Two protein standards with broad range (14 to 180 kDa and 6.5 to 250 kDa, Thermo Fisher Scientific) were used. The determination of protein electrophoretic profiles was carried out by using Red Imaging system equipped with Alpha-View SA Software (version 3.3.1, Cell Biosciences, Santa Clara, CA, USA).

4.4 Results and Discussion

4.4.1 Cellular and Chemical Compositions of Inactivated Baker's and Brewer's Yeasts

Both brewer's and baker's yeasts were inactivated by pasteurization and heat-roller drying. Scanning electron microscopy was used to investigate the morphology of brewer's yeast (Supplemental Figure 4.1). If yeasts from the same strain are grown under the same conditions, they will most likely have similar morphology, in terms of size and shape (Kondorosi et al., 2000; Suzuki et al., 2004). However, the baker's yeasts are grown under oxidative metabolism, while brewer's yeasts are grown under fermentative metabolism in an anaerobic environment. Our previous results showed that baker's whole yeast had a lemon shape (Li & Karboune, 2018), while the budding of brewer's yeast was less obvious and the size of brewer's yeast seems to be larger (Supplemental Figure 4.1). In addition, the surface of baker's yeast was smoother compared to that of brewer's yeast. The cellular composition, in terms of the yeast envelope, the cytoplasm, the cell skeleton and the nucleus, did show some differences depending on the strains (Feldmann, 2011). Cellular structures may suggest the presence of different mannoproteins. The chemical composition of brewer's and baker's yeasts is summarized in Table 4.1. As expected, the proteins constitute the main components of the yeasts; no significant difference in protein content between the two yeasts was obtained. Because of the high protein content of both yeasts, they have been used as protein sources in animal feed (G. L. Rumsey et al., 1991; Martins et al., 2014). The results (Table 4.1) also show that the brewer's yeasts contained 15.73% more glucan content as compared to the baker's yeasts; while the mannan content of baker's yeasts was slightly higher as compared to that of the brewer's yeasts. The fat content of the baker's yeasts was significantly higher than that of the brewer's yeasts. A previous study showed that the lipid composition varied among strains of *S. cerevisiae* from different origins, e.g., wine, beer and baking products (Redón et al., 2011). Another study compared the lipid compositions between aerobic baker's yeast and anaerobic brewer's yeast, their results showed that the lipid composition of both cell walls was



Supplemental Figure 4.1. Scanning electron micrograph (magnification 1000×) of whole brewer's yeast

Table 4.1. Composition and protein profiles of inactivated whole baker's yeast and whole brewer's yeast

	Protein content (%)	Glucan content (%)	Mannan content (%)	Fat (%) *	Salt (%)	Relative protein proportion (%)							
						75.5 kDa	63.4 kDa	55.1 kDa	44.9 kDa	37.7 kDa	21.7 kDa	16.7 kDa	12.4 kDa
Baker's yeast	44.65	19.17	14.37	5.5	<1	34.84	9.93	5.17	4.57	13.76	4.51	10.86	16.36
Brewer's yeast	41.72	22.75	12.71	1.5	<1	37.1	12.49	21.6	--	10.74	--	11.98	6.08

* Significant difference ($p<0.05$) between the compositions of the yeast samples

relatively similar, consisting mainly of triacylglycerols and lower acylglycerols. Indeed, the plasma membranes contained a considerable amount of triacylglycerols, while the amount of lower acylglycerols was clearly higher in the anaerobic plasma membrane (Nurminen et al., 1975). Table 4.1 also shows the protein profiles of untreated inactivated whole baker's and brewer's yeasts. Both yeasts showed the presence of proteins with molecular weights of 75.5, 63.4, 55.1, 37.7, 16.7 and 12.4 kDa with different proportion; however, baker's yeasts contained two additional proteins (44.9 and 21.7 kDa) not found in brewer's yeasts (Table 4.1). The difference in the protein profiles between baker's and brewer's yeasts may be due to the difference in their metabolism. Indeed, the oxidative metabolism of the baker's yeasts is occurring in low concentrations of carbon source, while fermentative metabolism of brewer's yeasts is occurring in an environment rich of sugars and ethanol. This may explain the high fat contents obtained in baker's yeasts as a protective agent against high water content, and the wide molecular weights of proteins (from 12.4 to 75.5 kDa).

4.4.2 Isolation of Non-covalently Bound Mannoproteins

The non-covalently bound mannoproteins were isolated from inactivated yeasts through heat and SDS treatments. Heat treatment can resolubilize soluble macromolecules present in the yeast, including non-covalently bond mannoproteins, by affecting the inter-hydrogen bonds (Freimund et al., 2003; X. Y. Liu et al., 2008; Silva Araújo et al., 2014). The results (Table 4.2) show that both heat and SDS extraction methods led to higher yields of extract and protein/mannan recovery on brewer's yeast than baker's yeast ($p<0.05$). As compared to heat treatment, SDS extraction led to a lower yield of recovered extract and mannan recovery. The highest extract yield of 38.5%, the protein recovery of 48.0% and the mannan recovery of 40.5% were achieved upon the use of brewer's yeast as starting material with heat treatment. The low yields obtained with baker's yeast may be attributed to their rigid cellular structure that may have limited the opening of the cell network, thereby reducing the release of non-covalently bound mannoproteins. In addition, the high fat content (5.5%, w/w) of the baker's yeast may have favored the interaction and the entrapment of mannoproteins into the cell walls and plasma membranes, limiting their recovery. Indeed, a total of 33 fatty acids ranging from C_8 to C_{26} have been found in *S. cerevisiae*; however, a minor polyethenoid acid component and C_8 to C_{12} fatty acids have a greater occurrence in brewer's yeast as compared to baker's one (Rattray et al., 1975). In addition of being strain-specific, another study showed that the degree of lipid unsaturation was influenced by the growth

Table 4.2. Effect of heat treatment and SDS extraction on the isolation of mannoproteins from baker's and brewer's yeast

Treatments		Yield of recovered extract [a] (%)	Protein recovery yield [b] (%)	Mannan recovery yield [c] (%)	Relative protein proportion [d] (%)	Relative mannan proportion [e] (%) *	Mannoproteins content [f] (%)	Mannan to protein ratio in mannoproteins [g] (w/w) *
Heat treatment	Baker's yeast	19.12 (0.85)	10.20 (1.10)	10.33 (0.12)	43.39 (0.31)	7.45 (0.09)	27.63 (4.32)	2.78 (0.25)
	Brewer's yeast	38.49 (1.24)	48.00 (1.05)	40.52 (0.92)	54.53 (0.00)	12.78 (0.01)	26.00 (2.32)	0.63 (0.03)
SDS extraction	Baker's yeast	14.12 (0.26)	14.28 (0.03)	3.81 (0.07)	46.15 (0.24)	3.71 (0.00)	0.77 (0.02)	1.14 (0.02)
	Brewer's yeast	26.28 (0.35)	35.53 (1.21)	24.65 (0.68)	59.09 (0.72)	11.37 (0.04)	1.01 (0.02)	0.12 (0.01)

[a]Yield of recovered extract was obtained from the weight of extract divided by the weight of original yeast and multiplied by 100.

[b]Protein recovery was obtained from the weight of protein in crude mannoprotein extracts divided by the weight of protein in original yeast and multiplied by 100.

[c]Mannan recovery was obtained from the amount of mannan in crude mannoprotein extracts divided by the amount of mannan in original yeast and multiplied by 100.

[d]Relative protein proportion is the percentage of protein in the crude mannoprotein extracts.

[e]Relative mannan proportion is the amount of mannan in the crude mannoprotein extracts.

[f]Mannoproteins content (%) is the proportion of mannoproteins in the extract

[g]Mannan to protein ratio in mannoproteins (w/w) is the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins.

* Significant difference ($p<0.05$) compared to zymolyase® treatments (see Table 4.3).

temperature in *S. cerevisiae* strains (Torija et al., 2003). The protein recovery yield obtained upon the SDS extraction of yeast cell wall (45.60%) was higher as compared to those achieved with the whole baker's yeast (14.28%) and brewer's yeast (35.53%) (Jin Li & Karboune, 2018); these results may be due to the lower protein content in the original yeast cell wall material (19.6%) compared to baker's (44.65%) and brewer's (41.72%) yeasts.

The results (Table 4.2) also show that the mannoproteins content in the recovered extracts obtained upon the heat treatment of baker's yeast (27.63%) and brewer's yeast (26.00%) were more or less similar. While SDS treatment led mainly to the release of non-glycosylated proteins with mannoprotein contents of 0.77 and 1.01%, respectively in baker's and brewer's yeasts. Mannoproteins usually contain a large fraction of mannose polymers, varying from 50 to 95%, corresponding to mannan to protein ratio between 1 and 19 (van der Vaart et al., 1995; Peter Orlean, 1997; Lipke & Ovalle, 1998). Table 4.2 indicates that the use of brewer's yeast as a starting material led to the recovery of mannoproteins with low values of mannan to protein ratio of 0.12 (SDS) and 0.63 (heat); these results reveal that the non-covalently bound mannoproteins from brewer's yeast are mainly composed of short chains of mannan polymers attached to the peptide polymers. In contrast, the mannan to protein ratios of the non-covalently bound mannoproteins isolated from baker's yeast by heat and SDS treatments were of 2.78 (heat) and 1.14 (SDS), indicating that mannan accounts for at least 50% of the mannoproteins. Similarly to the whole yeasts, only a low mannoprotein content (0.98%) was recovered upon SDS extraction in yeast cell wall, and the extracted mannoprotein exhibited a low mannan to protein ratio of 0.31 (Jin Li & Karboune, 2018).

Some discrepancies regarding the efficiency of SDS treatment to isolate mannoproteins were reported in the literature. Indeed, a study showed that SDS extraction could solubilize up to 80% of crude mannoproteins from purified cell walls of *S. cerevisiae,* releasing 30 different mannoproteins in the extract, as revealed by SDS-PAGE electrophoresis (Pastor et al., 1984). Similarly, in another study, a treatment with 2% (w/v) SDS under boiling conditions resulted in the recovery of more than 70% of the total mannoproteins from the yeast *Candida albican,* corresponding to over 40 different bands (from 15 to 80 kDa) on SDS-PAGE gel electrophoresis (Elorza et al., 1985). In contrast, Dupin et al. (2000b) reported that SDS treatment led to the release of only very low quantity of mannoproteins from three winemaking strains of *S. cerevisiae*, with yields varying from 0.1% to 0.2%. These last authors reported that only the surface of the cells

interacted with SDS leading to a limited effect on whole cells (Dupin et al., 2000). It is important to note that mannoproteins were not purified and assessed with accuracy in all reported studies.

SDS-PAGE electrophoresis results (Figure 4.1) reveal the different protein profiles of the crude mannoproteins extracts recovered after heat and SDS treatments. Heat treatment mainly released small molecular weight proteins (<14 kDa) from both baker's and brewer's yeasts; while SDS treatment led to the isolation of a variety of proteins from both baker's and brewer's yeasts, covering a wide range of molecular weights varying from 2 to 100 kDa. Size exclusion chromatography (HPSEC) was also used in order to determine the mannoprotein molecular weight profiles. Heat treatment resulted in baker's and brewer's yeast-based mannoproteins with a molecular weight of 6.5 kDa (data not shown). While no peak was detected by RI detector in the HPSEC profiles of SDS treatment-based extracts; Indeed, the three eluted peaks, with a molecular weight distribution varying from 4 -1000 kDa, were only detected by UV detector. These results corroborate those obtained upon mannoprotein purification on a HiTrap Con A 4B affinity column, confirming the release of mainly short non-covalently bound mannoproteins upon the heat treatment and non-glycosylated proteins upon the SDS treatment. Similarly to the whole yeasts, heat treatment released small molecular weight mannoproteins (6.5 kDa) from yeast cell wall, while SDS extraction led to the recovery of abundant proteins with a wide molecular weight distribution of 4-1045 kDa (Jin Li & Karboune, 2018).

4.4.3 Isolation of Covalently Bound Glycosylphosphatidylinositol (GPI) Mannoproteins

Glucanase-extractable mannoproteins are anchored to the plasma membrane at an intermediate stage in their biogenesis via a GPI moiety before they become anchored to the cell wall glucan via a β-1,6-glucan linkage (Pastor et al., 1984; Montijn et al., 1994; Muñoz-Dorado et al., 1994; Peter Orlean, 2012). Zymolyase® from *Arthrobacter luteus* expresses the β-1,3-glucan laminaripentaohydrolase and β-1,3-glucanase activity, which can hydrolyze β-glucan at β-1,3-linkages and releases mannoproteins from yeast. Table 4.3 shows the effect of Zymolyase® treatment on the isolation of mannoproteins from inactivated whole baker's and brewer's yeasts. Similarly to SDS and heat treatments, the enzymatic treatment led to higher ($p<0.05$) recovery yields (31.4-40.2%) with the brewer's yeasts as compared to the baker's yeasts (25.0-32.9%) . However, for baker's yeasts, Zymolyase® treatments produced a higher yield of

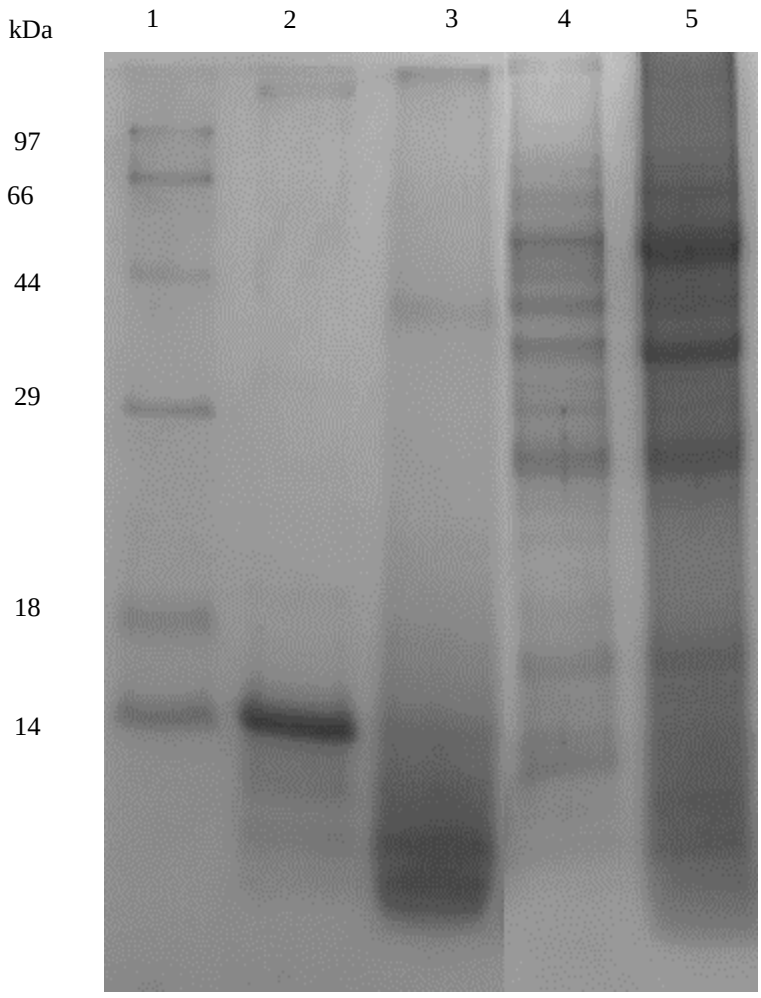


Figure 4.1. SDS-PAGE protein profiles of crude mannoprotein extracts after heat treatment and SDS extraction from baker's yeast and brewer's yeast (Lane 1: Standard; Lane 2: Inactivated whole baker's yeast after heat treatment; Lane 3: Inactivated whole brewer's yeast after heat treatment; 4: Inactivated whole baker's yeast after SDS extraction; Lane 5: Inactivated whole brewer's yeast after SDS extraction).

Table 4.3. Effect of Zymolyase® treatments on the isolation of mannoprotein extract from baker's and brewer's yeast

Treatments		Yield of recovered extract [a] (%)	Protein recovery yield [b] (%)	Mannan recovery yield [c] (%)	Relative protein proportion [d] (%)	Relative mannan proportion [e] (%) *	Mannoproteins content [f] (%)	Mannan to protein ratio in mannoproteins [g] (w/w)*
67 units/g-4 h	Baker's yeast	25.01 (0.28)	20.05 (0.33)	32.90 (4.04)	35.80 (0.10)	18.88 (2.12)	35.44 (3.55)	16.07 (1.67)
	Brewer's yeast	40.19 (0.20)	37.04 (0.60)	67.18 (6.85)	38.45 (0.64)	21.26 (2.16)	35.17 (3.24)	13.06 (1.20)
167 units/g-4 h	Baker's yeast	26.72 (0.45)	61.06 (4.65)	98.2 (3.62)	22.30 (2.54)	26.64 (4.06)	38.48 (4.12)	36.37 (2.01)
	Brewer's yeast	31.37 (0.72)	55.78 (2.23)	78.01 (9.50)	24.96 (0.11)	25.17 (2.20)	36.91 (2.58)	42.69 (3.53)
67 units/g-20 h	Baker's yeast	27.57 (0.17)	30.83 (0.79)	54.92 (0.40)	49.91 (1.58)	28.60 (0.39)	32.6 (2.9)	12.37 (1.25)
	Brewer's yeast	34.98 (0.46)	38.34 (5.96)	49.15 (0.89)	45.64 (6.50)	17.87 (0.56)	35.35(3.08)	16.39 (1.61)
167 units/g-20 h	Baker's yeast	32.92 (3.28)	35.75 (3.90)	65.91 (10.28)	48.73 (3.93)	28.11 (3.09)	28.89 (2.28)	15.88 (1.3)
	Brewer's yeast	38.01 (0.71)	40.86 (0.66)	62.70 (7.36)	44.87 (1.57)	20.92 (2.08)	33.29 (3.22)	13.05 (1.41)

[a]Yield of recovered extract was obtained from the weight of extract divided by the weight of original yeast and multiplied by 100.

[b]Protein recovery yield was obtained from the weight of protein in crude mannoprotein extracts divided by the weight of protein in original yeast and multiplied by 100.

[c]Mannan recovery yield was obtained from the amount of mannan in crude mannoprotein extracts divided by the amount of mannan in original yeast and multiplied by 100.

[d]Relative protein proportion is the percentage of protein in the crude mannoprotein extracts.

[e]Relative mannan proportion is the amount of mannan in the crude mannoprotein extracts.

[f]Mannoproteins content (%) is the proportion of mannoproteins in the extract

[g]Mannan to protein ratio in mannoproteins (w/w) is the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins.

* Significant difference ($p<0.05$) compared to heat treatment and SDS extraction methods (see Table 4.2).

recovered extract (25.0-32.9%) as compared to heat (19.1%) and SDS (14.1%) treatments. For brewer's yeast, the yield of the recovered extract obtained upon the enzymatic Zymolyase® treatment (31.4-40.2%) was close to that achieved with the heat treatment (38.5%), but relatively higher than that obtained upon SDS extraction (26.28%). The overall results confirm the high accessibility and mobility of the cellular structure and network of brewer's yeasts over that of the baker's yeasts. Table 4.3 also shows that the mannan recovery was higher upon Zymolyase® treatment than heat and SDS treatments for both yeasts; as a result, the relative mannan proportion in the extracts recovered upon Zymolyase® treatment was significantly higher. Indeed, more or less similar proportion of mannan (20.9-28.6%) were obtained under all treatment conditions, except at low enzymatic unit concentration (67 units/g) resulting in mannan proportion of 17.9-18.8%.

Mannoproteins recovered upon Zymolyase® treatment were further separated from non-glycosylated proteins using HiTrap Con A 4B column. The results indicate that the proportion of mannoproteins in the baker's yeast-based extract varied from 28.8 to 38.5%; however, the mannoprotein content was not proportionally dependent on the protein and mannan recovery yields. Mannan to protein ratio of mannoproteins increased significantly upon Zymolyase® treatment (13.06-42.69) compared to heat (0.63-2.78) and SDS (0.12-1.14) treatments. Such results demonstrate that the mannoproteins isolated upon the Zymolyase® treatment contained less than 8% peptide polymers attached to their mannose polymers. These results are well aligned with our previous findings with yeast cell walls as sources of mannoproteins (Jin Li & Karboune, 2018).

The results also showed that the increase in the Zymolyase® units didn't enhance significantly the yield of the recovered extract, but it did increase the mannan and protein recovery; for instance, the increase in the enzyme units from 67 to 167 units at 4 h treatment, resulted in an increase of the mannan recovery from 32.9- 67.2 to 78.1-98.2 % and of the protein recovery from 20.1-37.0% to 55.8-61.1%. These results may be explained by the release of glucan oligomers at low enzymatic units, contributing to the apparent high recovered extract yield. Overall, the highest yield of recovered extract (40.2%) was achieved at 67 units of Zymolyase®/ g brewer's yeasts for 4 h reaction, while the highest mannoproteins content (38.5%) was achieved at 167 units of Zymolyase®/ g baker's yeasts for 4 h reaction.

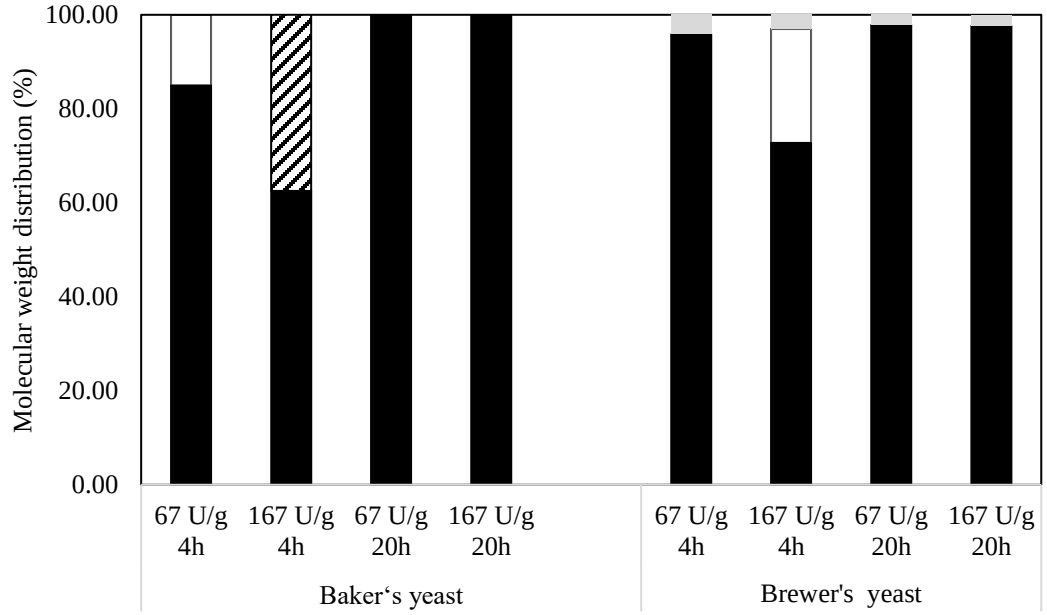


Figure 4.2. Molecular weight distributions of mannoproteins recovered upon Zymolyase® treatment from baker's yeast and brewer's yeast under selected conditions: 5-10 kDa (■), 10-100 kDa (□), 100–400 kDa (▨), >400 kDa(▩).

Molecular weight distributions of mannoproteins recovered upon Zymolyase® treatments are shown in Figure 4.2. The results indicate that Zymolyase® treatment resulted in the recovery of a large amount (62.5 -100% relative proportion) of mannoproteins with a molecular weight of 6 kDa. For baker's yeasts, 15.11% of mannoproteins with a molecular weight between 10-100 kDa were released upon 4 h treatment at 67 units/g; when the enzyme units was increased to 167 units per g of baker's yeasts, a higher proportion of mannoproteins (37.48%) with a higher molecular weight range (100-400 kDa) was isolated. However, increasing the treatment time to 20 h led to the shift in the molecular weight distribution of mannoproteins to lower range (<10 kDa). These results can be attributed to the hydrolysis of higher molecular weight mannoproteins by mannanase or proteinase expressed in Zymolyase®. Contrary to baker's yeasts, mannoproteins with molecular weights higher than 400 kDa were isolated from brewer's yeasts under different conditions, even after 20 h of the enzymatic treatment. The Zymolyase® treatment at 167 units/g for 4 h on brewer's yeasts resulted in three populations of mannoproteins: 72.7% of low MW mannoprotein fraction (<10 kDa), 24.4% of mannoprotein fraction ranging from 10-100 kDa and 3.1% of large MW (>400 kDa) mannoprotein fraction. The Zymolyase® treatment on yeast cell wall led to different MW distributions of mannoproteins, revealing the dependence of their multiplicity not only on the reaction conditions but also on the starting materials (Jin Li & Karboune, 2018). Overall, the Zymolyase® treatment released mannoproteins that could not be recovered by heat treatment and SDS extraction; the overall results reveal the potential of the biocatalytic approach for the recovery of covalently bound mannoproteins from the selected whole baker's and brewer's yeasts.

4.4.4 Optimization of the Enzymatic Isolation of Mannoproteins from Brewer's Yeasts

Optimization of the enzymatic isolation of mannoproteins from inactivated whole brewer's yeasts was investigated using RSM to better understand the relationships between the reaction parameters and modulate the structural properties of mannoproteins. This study was investigated using a yeast substrate concentration of 20% (w/v). This substrate concentration was identified as the most appropriate one in terms of compromising the recovered extract yield, the substrate diffusional limitations and the productivity. Indeed, increasing the substrate concentration from 5% to 10% resulted in a decrease in the yield of recovered extract from 40.2% to 30.8%; while further increase of the substrate concentration up to 20% didn't affect significantly the yield of recovered extract (Figure 4.3). The Central composite rotatable design (CCRD) was performed with two independent variables at five levels, including the time of reaction and the enzyme concentration.

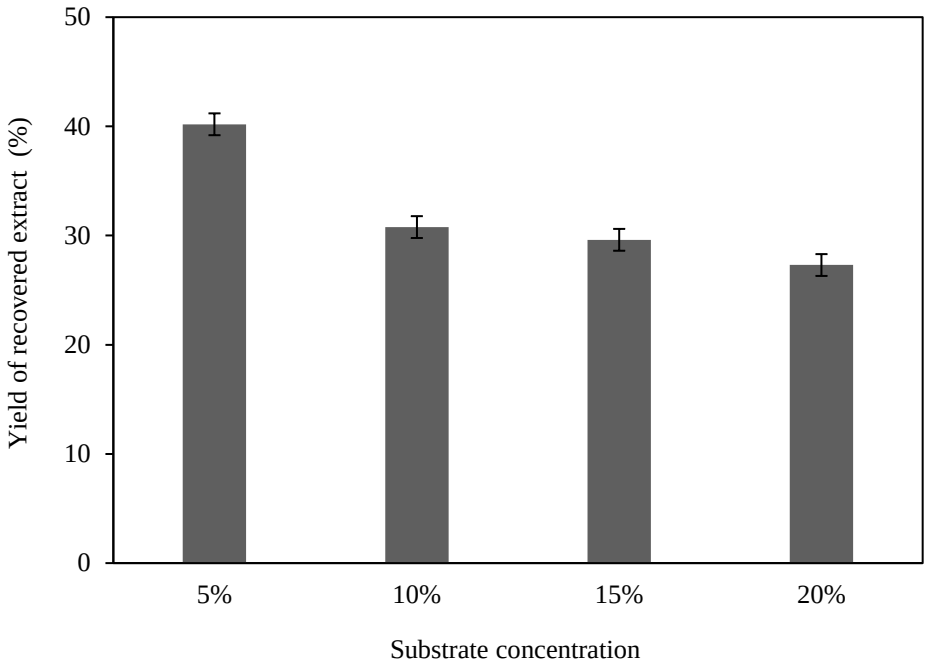


Figure 4.3. Effect of substrate concentrations on the yield of recovered extract (%, w/w initial weight) from brewer's yeast (Zymolyase® units/substrate ratio of 67 units per g of brewer's yeast; 4 h enzymatic treatment). Vertical lines indicated standard deviation

Table 4.4. Experimental design of 5-levels 2-variables central composite design and the responses

Run	Independent Variables		Responses		
	X_1[a]	X_2[b]	Yield of recovered extract [c] (%)	Mannoproteins content [d] (%)	Mannan to protein ratio [e] (w/w)
1	139.5	4.37	50.85	38.36	30.73
2	20	12.5	36.40	20.11	15.92
3	40.5	20.63	44.80	17.12	15.00
4	160	12.5	55.88	15.69	12.99
5	90	1	56.41	72.97	61.73
6	90	12.5	64.63	19.25	16.01
7	40.5	4.37	44.51	78.17	66.07
8	139.5	4.37	46.13	45.58	42.01
9	139.5	20.63	67.85	23.30	21.33
10	90	12.5	59.16	11.11	10.42
11	40.5	4.37	44.80	65.27	57.26
12	90	24	63.87	37.39	33.20
13	139.5	20.63	59.34	13.51	12.05
14	90	1	51.66	72.58	59.85
15	90	12.5	60.44	11.18	9.42
16	160	12.5	50.75	15.38	13.52
17	90	12.5	59.66	11.11	9.00
18	40.5	20.63	41.91	17.26	14.70
19	90	12.5	62.66	11.76	9.65
20	90	24	66.86	25.74	22.63
21	20	12.5	36.40	25.83	22.64

[a]Enzyme concentration (Zymolyase® units/g of inactivated yeast). [b]Reaction time (h).

[c]Yield of recovered extract was estimated as the weight of extract divided by the weight of original yeast and multiplied by 100.

[d]Mannoproteins content (%) refers the proportion of mannoproteins in the extract.

[e]Mannan to protein ratio in mannoproteins (w/w) was calculated as the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins.

Table 4.4 shows the experimental conditions and the data obtained for the yield of recovered extract, the mannoproteins content, and the mannan to protein ratio in mannoproteins. Among the various conditions, the highest yield of recovered extract of 67.85% (run #9) was obtained at the Zymolyase® concentration of 139.5 U/g of brewer's yeasts at 20.63 h. The lowest yield of recovered extract of 36.40% (run #2 and #20) was obtained at a Zymolyase® concentration of 20 U/g of brewer's yeasts at 12.5 h. The isolation of extract with the highest mannoproteins content (72.58-78.17%) and mannan to protein ratio (59.8-61.73, w/w) were achieved upon the treatment with Zymolyase® at 40.5 U/g of brewer's yeasts for 4.37 h (run #7) or at 90 U/g of brewer's yeasts for 1 h (runs #5, 14). Increasing the reaction time to 12.5 h (runs #10, 17, 19) at Zymolyase® concentration of 90 U/g led to the lowest mannoprotein content of 11.1-11.8% and mannan to protein ratios of 9.0 to 10.42. Compared to yield of recovered extract, the variations of the mannoproteins content and mannan to protein ratio were largely distributed. Contrary to the whole brewer's yeasts, the use of yeast cell walls as starting materials led to lower mannan to protein ratios up to a maximum of 33.5 (Jin Li & Karboune, 2018).

By applying multiple regression analysis using the software Design-Expert, the quadratic models were identified as statistically more suitable to represent the three investigated responses. The analysis of variance (ANOVA) and the adequacy of the models are summarized in Table 4.5. The *F-value* for the models of yield of recovered extract, mannoproteins content and mannan to protein ratio were 43.1, 60.9 and 48.4, respectively, and all three models exhibit a *p-value* lower than 0.0001, with a correlation coefficient R^2 of 0.9349, 0.9531 and 0.9417, respectively. For all the 3 predicted models, the R^2 values have reasonable agreement with the adjusted and predicted R^2 values. The overall results of ANOVA analysis indicate the significance of the predictive models. The effects of the variables (enzyme concentration X_1; reaction time X_2) are also shown in Table 4.5. The *F*- and *p*- values can be used to verify the significance of each variable and of their interactive effects. For yield predictive model, the results indicate that the enzyme concentration (X_1) was the most significant linear term (*F-value* of 70.25; $p< 0.0001$) as compared to that of time (X_2, *F value of 27.14, p* of *0.0001*). In addition, the interactive term (X_1X_2, *F value of 16.41, p* of *0.001*) and the quadratic term of variable enzyme units (X_1^2, *F-value* of 97.29; $p< 0.0001$) also affected significantly the yield of recovered extract. Considering the significant terms, the response of yield of recovered extract can be described by the following quadratic equations in terms of coded variables:

Table 4.5. Analysis of variance (ANOVA) for the yield of recovered extract (%), mannoproteins content (%) and mannan to protein ratio in mannoproteins (w/w)

Sources	Yield of recovered extract [a](%)			Mannoproteins content [b] (%)			Mannan to protein ratio [c] (w/w)		
	Mean Square	F value	Prob>F[d]	Mean Square	F value	Prob>F	Mean Square	F value	Prob>F
Model	353.27	43.10	< 0.0001	1960.90	60.97	< 0.0001	1397.08	48.42	< 0.0001
X_1	575.79	70.25	< 0.0001	381.09	11.85	0.0036	255.64	8.86	0.0094
X_2	222.46	27.14	0.0001	4649.45	144.56	< 0.0001	3190.96	110.59	< 0.0001
X_1X_2	134.47	16.41	0.0010	479.24	14.90	0.0015	368.04	12.76	0.0028
X_1^2	797.44	97.29	< 0.0001	132.91	4.13	0.0602	105.96	3.67	0.0746
X_2^2	19.32	2.36	0.1455	4148.00	128.97	< 0.0001	3070.36	106.41	< 0.0001
Residual	8.20			32.16			28.85		
Lack of fit	7.19	0.85	0.4922	63.26	2.59	0.1010	57.70	2.67	0.0951
Pure error	8.45			24.39			21.64		
Cor Total									

[a]R^2=0.9349; [b]R^2=0.9531; [c]R^2=0.9417; [d] p-value.

Yield of recovered extract (%) =61.31+6.00 X_1+3.73 X_2+4.10X_1X_2-8.79 X_1^2 (3)

For the mannoprotein content model, reaction time exhibited the most significant linear (*X_2, F-value* of 144.56; *p< 0.0001*) and quadratic (X_2^2, *F-value* of 128.97; *p<0.0001*) effects, affecting highly the mannoproteins content in the extract. While the contributions of linear (X_1 *F-value* of 11.85; *p of 0.0036*) and quadratic (X_1^2, *F-value* of 4.13; *p of* 0.0602) terms of enzyme concentration to the predictive model of mannoproteins content are less significant compared to reaction time. However, the interactive effect of both variables (X_1X_2, *F value of 14.90, p* of *0.0015*) showed a positive effect on the mannoproteins content. The final predictive equation in terms of coded factors for the response of mannoproteins content is as given below:

Mannoproteins content (%) =12.88-4.88X_1-17.05X_2+7.74X_1X_2+3.59X_1^2+20.05X_2^2 (4)

For the response of mannan to protein ratio (w/w) model, reaction time exhibited the most significant linear (*X_2, F-value* of 110.59; *p< 0.0001*) and quadratic (X_2^2, *F-value* of 106.41; *p< 0.0001*) effects, affecting highly the mannan to protein ratio. While the contributions of linear (*X_1, F-value* of 8.86; *p of 0.0094*) and quadratic (X_1^2, *F-value* of 3.67; *p of* 0.0746) terms of enzyme concentration to the predictive model of mannan to protein ratio (w/w) are less significant compared to the reaction time. However, the interactive effect of both variables (X_1X_2, *F value of 12.76, p* of *0.0028*) showed a positive effect on the mannan to protein ratio (w/w). The similarity of the results of the analysis of variance (ANOVA) between the response of the mannoproteins content and the mannan to protein ratio in mannoproteins may indicate that the recovery of mannan content was the main determinant for the recovery of mannoproteins, and the recovery of proteins attached to the mannan polymers shows a minor effect. The final predictive equation, in terms of coded factors for the response of mannan to protein ratio (w/w) in mannoproteins, is as given below:

Mannan to protein ratio in mannoproteins = 10.90 - 4.00 X_1 -14.12 X_2 + 6.78 X_1X_2 + 3.20 X_1^2 + 17.25 X_2^2 (5)

4.4.5 Effects of Reaction Parameters

The relationship between the reaction parameters (i.e. independent variables) and the responses can be better understood by studying the two dimensional (2D) contour plots of fitted models.

Different shapes of the contour plots indicate different interactions between the variables; an elliptical contour plot indicates that the interactions between the variables were significant, while a circular contour plot reveals the absence of significance (Zhao et al., 2012). In the 2D contour plot, each contour represents a specific value for the height of the surface and the curves of equal response values are drawn on a plane (Tian et al., 2014). The contour graphs of Figure 4.4 show the effect of enzyme concentration (U/g of yeasts) and reaction time (h) and their mutual interactions on the yield of recovered extract (A), the mannoproteins content (B) and the mannan to protein ratio (C). Figure 4.4A shows that at low enzyme concentrations (<55.6 U/g yeasts), the yield was independent on the reaction time; while at a high enzyme concentration, the yield of the recovered extract increased with the reaction time up to a maxium value, and remained constant thereafter (Figure 4.4A). These results may be attributed to the achievement of the reaction equilibrium at low enzyme concentrations and/or to the substrate inhibition. In addition, the elliptical contour plot of the yield of the recovered extract revealed the positive interactions between enzyme concentration and time. Contrary to the predictive contour plot of yield, those of the mannoproteins content and the mannan to protein ratio show nearly horizontal lines, confirming the less significant effect of the enzyme concentration on the mannoproteins content and the mannan to protein ratio compared to the reaction time (Figure 4.4B & 4.4C). Indeed, lower enzyme concentrations (< 90 U/g yeasts) and shorter reaction times (< 6 h) favored the production of extracts with high mannoproteins content (up to 90.5%) and mannan to protein ratio (77.3%, w/w). The reaction time exhibited a significant effect on the mannoproteins content as well as on the mannan to protein ratio. When the reaction time was increased, the mannoproteins content and the mannan to protein ratio decreased, but it increased thereafter. These results can be attributed to the fact that the extent of the hydrolysis of mannoproteins by mannanase and protease present in the Zymolyase® was dependent on the reaction components and the reaction time course. Indeed, as the reaction proceeded, the actions of the side activities were not significant because of their denaturation and/or their substrate/product inhibition. The elliptical contour plots of the responses of mannoproteins content and the mannan to protein ratio in mannoproteins reveal the positive interactions between the enzyme concentration and the reaction time.

As shown in Table 4.6, two optimal conditions were selected to isolate mannoproteins with different mannan to protein ratios in order to validate the models. Run 1 corresponds to the optimal

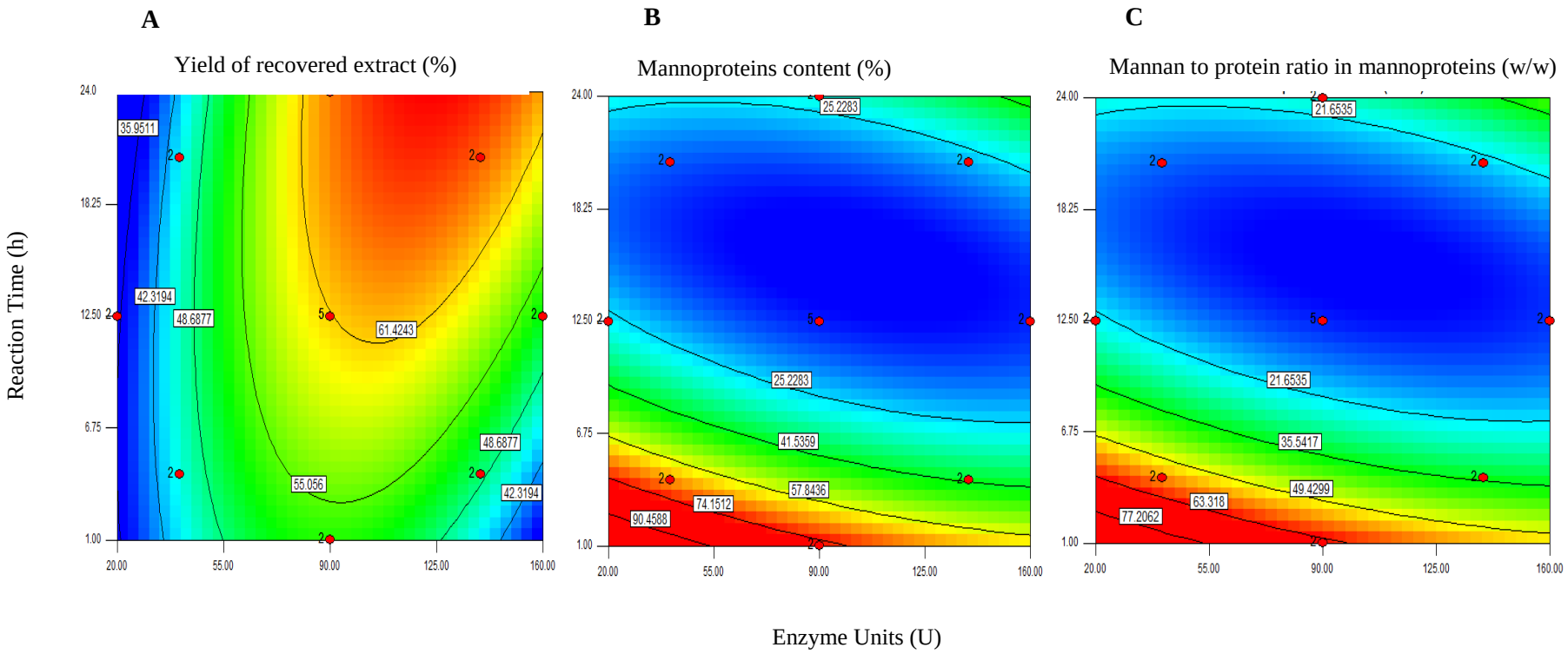


Figure 4.4. Contour plots of predictive models of yield of recovered extract (%) (A), mannoproteins content (%) (B), mannan to protein ratio in mannoproteins (w/w) (C). The numbers inside the contour plots indicate the predicted values under given reaction conditions.

Table 4.6. Model validation of the optimal conditions for the brewer's yeast yield of recovered extract (%) and mannoproteins content (%)

Optimal Conditions		Predicted			Actual			Probability Interval		
$X_{1:}$ Enzyme units	$X_{2:}$ Time (h)	Yield of recovered extract[a] (%)	Mannoproteins content[b] (%)	Mannan to protein ratio[c] (w/w)	Yield of recovered extract (%)	Mannoproteins content (%)	Mannan to protein ratio (w/w)	Yield of recovered extract (%)	Mannoproteins content (%)	Mannan to protein ratio (w/w)
Run 1										
118.44	20.63	64.98	22.33	20.01	65.31 (0.33)	17.86 (0.15)	15.44 (0.80)	57.99-71.97	8.48-36.18	6.90-33.13
Run 2										
77.31	4.37	55.15	54.45	45.24	60.51 (0.16)	60.78 (3.21)	40.10 (5.12)	48.56-61.73	40.41-66.48	32.89-57.59

[a]Yield of recovered extract was calculated as the weight of extract divided by the weight of original yeast and multiplied by 100.

[b]Mannoproteins content (%) refers to the proportion of mannoproteins in the extract.

[c]Mannan to protein ratio in mannoproteins (w/w) was calculated as the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins.

conditions for maximizing the yield of recovered extract; while Run 2 maximizes both the yield of recovered extract and the mannoproteins content. These optimal conditions were run in triplicate, with duplicate center points as a reference. As shown in Table 4.6, run 1 resulted in an actual yieldof recovered extract of 65.3%, mannoproteins content of 17.9% and mannan to protein ratio of 15.4 in mannoproteins; while run 2 resulted in an actual yield of recovered extract of 60.5%, but a higher mannoproteins content (60.8%) and mannan to protein ratio in mannoproteins (40.1) ($p<0.05$). As an overall, the yield of recovered extract, the mannoproteins content, as well as the mannan to protein ratio in mannoproteins fall into the predicted interval range, confirming the validity of the established predictive models (Table 4.6). Our previous study found that the optimal conditions for the isolation of mannoproteins from yeast cell wall required higher units of Zymolyase® (157.4 units/ g of yeast cell wall) and longer reaction time of 23.6 h (Jin Li & Karboune, 2018).

4.5 Conclusion

To the best of our knowledge, this is the first study that investigated the difference between mannoproteins derived from baker's and brewer's yeasts. The yields and the structural properties of mannoproteins (molecular weight; mannan to protein ratio) were not only dependent on the yeast source but also on the isolation methods. Non-covalently bound mannoproteins, isolated upon the heat treatment of brewer's and baker's yeasts showed different mannan to protein ratio. While SDS extraction method was not suitable for the isolation of mannoproteins, higher yields of covalently-bound mannoproteins, characterized by a wider molecular weight distribution and a higher mannan to protein ratio, were obtained upon Zymolyase® enzymatic treatment. The optimization of the production of mannoproteins from brewer's yeasts was performed using RSM with CCRD. The enzyme units, the reaction time and their interaction displayed significant effects on the responses of the yield of recovered extract, the mannoproteins content as well as the mannan to protein ratio in mannoproteins. The predictive models of the mannoproteins content and the mannan to protein ratio showed similar trends with shorter/longer reaction times and lower/higher enzymatic units being more favorable for these responses. The overall findings of the present study will contribute to lay the scientific ground for the development of innovative biocatalytic process for the isolation of functional ingredients from inactivated whole baker's and brewer's yeast cells with many potential applications.

CONNECTING STATEMENT 3

Previous studies showed that Zymolyase®-based enzymatic approach resulted in a high crude mannoproteins extract yield and a high proportion of mannoproteins in the extract, while limiting the debranching of mannoproteins (Chapters III and IV). In chapter V, mannoproteins were purified by affinity chromatography on concanavalin A and size exclusion chromatography. This chapter also provides the characterization of solubility, emulsifying and viscoelastic properties of the purified mannoproteins.

The results from this study were presented at the IFT Annual Meeting & Food Expo-Institute of Food Technologist

Li, J., & Karboune, S. (2016) Enzymatic Isolation of Mannoproteins from Brewer's and Baker's Whole Yeasts and Yeast Cell Walls and Their Structural Properties. IFT16 Annual Meeting & Food Expo, Chicago, USA, July 11- July 14, 2016.

Li, J., & Karboune, S. (2018). Techno-functional properties of mannoproteins derived from yeast-based products with well-defined structures (Submitted).

CHAPTER V

TECHNO-FUNCTIONAL PROPERTIES OF STRUCTURALLY WELL-DEFINED MANNOPROTEINS FROM YEAST (*SACCHAROMYCES CEREVISIAE*)-BASED BY-PRODUCTS

5.1 Abstract

The broad MW distribution and the variability of mannan to protein ratio of purified mannoproteins, isolated from yeast cell walls upon the enzymatic treatment, revealed their multiplicity. The main fraction of high-MW Agrimos®-MP1 and YCW-b-MP1' contained mannoproteins with a mannan to protein ratio of 3.5 and 6.9, respectively. Low-MW YCW-b-MP2' was mainly comprised of mannan, with a ratio of 181, whereas low-MW Agrimos®-MP2 was characterized by a ratio of 12.2. The solubility of MP1/MP2 was higher than that of MP1'/MP2'. Mannoproteins showed similar or lower solubility than mannan, and they exhibited a Newtonian behavior. Sonication was the appropriate method for the formation of mannoproteins-based emulsions. Contrary to MP1/MP1'-based emulsions, MP2/MP2'-based ones showed higher affinity towards soybean oil than glyceryl-trioleate. pH affected the emulsifying ability of MP1/MP1'. MP1/MP1' showed similar or slightly inferior emulsifying properties than lecithin. This study is expected to broaden the applications of mannoproteins as value-added ingredients.

5.2 Introduction

A large amount of yeast cell wall is available as a by-product upon the production of yeast extract, which is made primarily of amino acids, peptides and nucleotides, and can be used as a flavoring agent. In addition, the brewing industry generates a high amount of brewers' yeast. Because of the low-cost, the non-toxicity and the compositional properties of these by-products, there is an increasing demand for their effective conversion into added-value functional ingredients (da Silva Araújo et al., 2014; Jin Li & Karboune, 2018; Jin Li et al., 2019). Mannoproteins account for 40% of the total mass of yeast cell wall by-products. However, the isolation of mannoproteins has been less studied as compared to the glucan (60% of the total yeast cell wall) (Aguilar Uscanga & Francois, 2003). The structural properties and the techno-functional attributes of mannoproteins depend on many factors, including starting materials, type of extraction methods, technological processes, and the operating conditions (extraction time, temperature, pH) (Caridi, 2006; Jin Li & Karboune, 2018). Structurally, mannoproteins derived from yeast-based products are highly glycosylated in the form of proteoglycans, containing 50 to 95% of carbohydrate. The yeast-based mannoproteins have been reported to be an effective bioemulsifier (de Melo et al., 2015; McClements et al., 2017) and have been recognized for their beneficial winemaking properties (Dupin et al., 2000; Guadalupe et al., 2007). They have also been associated with the health-promoting properties of prebiotic ingredients as they were found to stimulate the growth of

probiotic lactic acid bacteria (Diez et al., 2010) and inhibit the growth of pathogenic bacteria (K Newman, 1994). In our previous study, heat treatment, SDS extraction and enzymatic-based approach were investigated for the isolation of mannoproteins from the yeast cell walls and the whole yeasts (Jin Li & Karboune, 2018; Jin Li et al., 2019). Higher yield of crude mannoprotein extract (46.9%) and mannoprotein content (39.8%) were achieved upon the enzymatic treatment of yeast cell wall at 157.4 units of Zymolyase® per g of yeast cell wall and for 23.6 h (Jin Li & Karboune, 2018). However, as far as the authors are aware, the understanding of the techno-functional properties of mannoproteins as they are related to their structural properties still needs to be investigated. In the present study, mannoproteins extracted upon the enzymatic treatment were purified by affinity chromatography on concanavalin A column, and then fractionated by size exclusion chromatography. The structural and the techno-functional properties of the purified mannoprotein fractions were characterized. The solubility of mannoprotein fractions, their produced viscosity and their emulsifying properties (the droplet size and the stability) at selected pH and temperature values were investigated.

5.3 Materials and Methods

5.3.1 Materials

Two types of yeast cell wall (YCW) products (Agrimos® and YCW-b, derived from a unique primary grown baker's yeast *S. cerevisiae*, after a specific manufacturing process to remove the cytoplasmic components) were kindly provided by Lallemand Inc. (Montreal, CA). Affinity chromatography media Con A Sepharose 4B with immobilized Concanavalin A and HiLoad 16/600 Superdex 200 pg prepacked XK column were obtained from GE Healthcare Life Sciences (Mississauga, ON, Canada). Zymolyase®-20T from *Arthrobacter luteus* was purchased from Amsbio (Cambridge, Massachusetts, USA). All other salts and chemicals were obtained from Fisher Scientific (Mississauga, ON, Canada).

5.3.2 Isolation and Purification of Mannoproteins

5.3.2.1 Preparation of Crude Yeast Cell Wall Extract

The yeast-based products contain salts, vitamins, sugars, and free proteins. To prepare the crude cell wall materials, the yeast-based products were dialyzed using a 6-8 kDa molecular weight (MW) benzoylated dialysis tubes and then centrifuged (8000×g, 30 min) to recover the precipitate

enriched with the yeast-cell wall materials. Soluble free proteins (30 to 50% of proteins) with MW higher than 8 kDa were kept in the supernatant.

5.3.2.2 Enzymatic Isolation of Mannoproteins

Zymolyase®- based enzymatic treatment of crude cell wall materials was conducted using the identified optimal reaction conditions in our previous study (Jin Li & Karboune, 2018). A mass of 30 g of yeast cell wall was suspended in 200 mL sodium phosphate buffer (50 mM, pH 6.5), and the Zymolyase® was added to yield 157.4 units of β-1,3-glucanase per gram of yeast cell wall; then, the enzymatic treatment was performed at 45ºC under agitation (200 rpm). After the 23.6 h of reaction, the Zymolyase® was inactivated by incubation at 60 ºC for 10 min.

5.3.2.3 Isolation of the Mannoproteins on Concanavalin A Sepharose Column

Mannoproteins recovered after Zymolyase® treatment were purified on Concanavalin-A sepharose affinity chromatography column using an ÄKTA purifier system (GE Healthcare). The crude extracts were loaded onto the column using a binding buffer made of 20 mM Tris-HCl buffer at pH 7.4 containing 0.5 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ at a flow rate of 0.5 mL/min. After washing the column with 5 bed volume of binding buffer to remove unbound material, the mannoproteins were recovered by elution with 0.2 M alpha-D-methylglucoside buffer solution. The elution was monitored using UV-Vis detector. The fractions corresponding to the peak at an absorbance at 280 nm were collected and then freeze-dried.

5.3.2.4 Fractionation of Mannoproteins on Superdex™ 16/ 200.

Purified mannoprotein extract (10%, w/v) was loaded into a Hiload 16/60 Superdex 200 column (GE Healthcare Life Sciences) using a FPLC system (GE Healthcare) equipped with a UV-900 detector. The elution was carried out with water at a flow rate of 1 mL/min. The eluted fractions (3 mL) were analyzed for the carbohydrate and protein contents. The carbohydrate content in each fraction was determined by phenol-sulfuric acid colorimetric assay (Dubois et al., 1956). The protein content was estimated from the 280 nm absorbance based on the calibration curve built from a series of bovine serum albumin (BSA) standard solutions (0.5, 1, 3, 5, 7, and 10 mg/mL). In order to determine the MW distribution of mannoproteins, myoglobulin (17 kDa), ovalbumin (44 kDa), human albumin (66 kDa), immunoglobulin G (158 kDa) and ferritin (440 kDa) were used as protein standards to construct the calibration curve. The calibration curve was drawn by plotting the log of the MW of the standards versus the elution volume.

5.3.3 Structural Characterization of Purified Mannoprotein Fractions

5.3.3.1 Determination of Monosaccharide Composition

The monosaccharide composition of the purified mannoprotein fractions was determined after their hydrolysis with a two-step method using HCl and trifluoroacetic acid (TFA). Mannoprotein fractions (40-50 mg/mL) were first suspended in methanol/HCl mixture (2:1, v/v) solutions and incubated at 60 ºC for 24 h. The hydrolysates were boiled for 1 h to evaporate the methanol. Then, 3 mL of deionized water and 0.5 mL of TFA were added to the hydrolysates and boiled for 1 h. The pH of the hydrolysate solutions was adjusted with sodium hydroxide to 6.5-8.0 and then centrifuged. The recovered supernatants were analyzed by high performance anion exchange chromatography (HPAEC), equipped with a pulse amperometric detector (PAD) and a CarboPac PA20 column (3 × 150 mm) set at 32 °C (Dionex-5500, Dionex Corporation, Sunnyvale, California, USA). Sodium hydroxide solution (10 mM) was used as an isocratic eluent at a flow rate of 0.5 mL/min. D-glucose (Glc) (2.5–40 μmol/L) and D-mannose (Man) (2.5–40 μmol/L) were used as standards.

5.3.3.2 Determination of Protein Content

To determine the protein content, the purified mannoprotein fractions were solubilized in water and their protein content was measured by Hartree method at 650 nm using UV/Visible spectrophotometer (DU 800, Beckman Coulter, Fullerton CA, USA) (Hartree, 1972). The standard curve was constructed using BSA solutions ranging between 50 and 250 mM.

5.3.3.3 Determination of the MW Distribution of Mannoproteins

To reveal the MW distribution of mannoproteins, high-performance size exclusion chromatography (HPSEC) on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL from Tosoh Bioscience, Montgomeryville, PA) using a Waters HPLC system (Model 25P, Waters Corp., Milford, MA) equipped with both refractive index (RI) and diode array (UV) detectors was used. The isocratic elution was passed with 0.1 M sodium chloride at a flow rate of 0.4 mL/min at room temperature. Dextrans with MW of 50, 150, 270, 410 and 670 kDa were used as standards for calibration.

5.3.4 Scanning Electron Microscopy (SEM)

A Hitachi analytical desktop Microscope (TM3000, Hitachi High-Technologies Co., Tokyo, Japan) was used to analyze the morphological properties of the starting yeast cell wall materials (Agrimos® and YCW-b) and of the resulting mannoprotein extracts. A carbon conductive tab on

the aluminum stub was used to fix the samples, which were examined afterward by means of accelerated voltages of 5 and 15 kV without sputtering.

5.3.5 Assessment of Techno-functional Properties of Mannoproteins

5.3.5.1 Mannoprotein Solubility

The mannoprotein solubility was determined at selected pH values. A solution of 10 mg/mL of mannoproteins was prepared in 50 mM buffer solution (potassium chloride at pH 2.0, citric acid-sodium citrate at pH 3.0, 4.0, 5.0 and 6.0, sodium phosphate at pH 7.0, 8.0 and 9.0), thoroughly mixed and centrifuged at 3,500 ×g for 15 min. The amount of total carbohydrate content in the supernatants was measured using phenol-sulfuric acid colorimetric assay (Dubois et al., 1956), while their total protein content was measured using the Hartree method (Hartree, 1972). Mannoprotein solubility was expressed as the percentage of total protein content and the total polysaccharide content in the supernatant with respect to the total weight.

5.3.5.2 Mannoprotein Viscosity

The viscosity of solutions containing 1% or 5% mannoproteins, mannan and whey protein was measured at 25, 50, and 75 °C using an AR2000 controlled-stress rheometer (TA, Crawley, U.K.) equipped with a 40 mm cone plate geometry, and a 2-degree angle at 56 micro gap size was used. Samples were heated for 2 h at selected temperatures before the analysis, and the shear rate varied between 1 and 100 s^{-1}. Ostwald–de Waele equation (Eq. 5.1) was used to define the flow behavior of the liquids.

$$\boldsymbol{\tau} = K\left(\frac{\partial u/\partial y}{\partial y}\right)^{n} \qquad (5.1)$$

Where, $\partial u/\partial y$ is shear rate (s^{-1}), τ is shear stress (Pa), K is the flow consistency index ($Pa.s^n$) and n is the flow behavior index. Assays were conducted at least in triplicate.

5.3.5.3 Mannoprotein Emulsifying Property

A suspension of mannoproteins (1.25%, w/v) was prepared in 50 mM buffer solutions (potassium chloride at pH 2.0, citric acid-sodium citrate at pH 3.0, 4.0, 5.0 and 6.0, sodium phosphate at pH 7.0, 8.0 and 9.0). After thorough mixing, 1 mL of soybean oil or glyceryl trioleate was added into the suspension to form 20% oil-in-water (v/v) emulsion. Emulsification was performed by sonication method or a combination of sonication and homogenization method. Emulsions produced by the sonication method were performed using an ultrasonic liquid processor (Misonix, Farmingdale, NY) comprised of a generator, a converter, and a horn tip. The converter, which was immersed in the emulsion, could vibrate at a peak-to-peak amplitude of 100 μm at full power

(100%). All experiments were performed in 50 mL centrifuge tubes. For the combined sonication and homogenization method, after the sonication step, the emulsion mixture was further homogenized at full speed for 3 min using a homogenizer (PowerGen 125, Fisher Scientific, Fairlawn, NJ). Dynamic light scattering (DLS) was utilized to determine the droplet size of the oils in the emulsions at 25 ^{0}C as a mean of size distribution obtained from 70 measurements using Delsa Nano C (Beckman Coulter, Fullerton, CA).

5.4 Results and Discussion

5.4.1 Isolation, Purification and Chemical Characterization of Mannoproteins

The mannoproteins were isolated from the *S. cerevisiae* yeast cell wall preparations, Agrimos® and YCW-b, using the Zymolyase®-based enzymatic approach and the optimal conditions identified in our previous study (Jin Li & Karboune, 2018). The Concanavalin A affinity column, which specifically binds molecules containing α-D-mannose and sterically related residues with available C-3, C-4, or C-5 hydroxyl groups, was used for the purification of the isolated mannoproteins. Several studies have reported the high efficiency of Concanavalin-A sepharose affinity chromatography column to purify mannoproteins (Gonçalves et al., 2002; Levitz & Specht, 2006; Shokri et al., 2008). In the present study, mannoproteins were eficiently eluted with the buffer containing alpha-D-methylglucoside (data not shown). The purified mannoproteins were further fractionated based on their MW on Superdex™ 16/ 200 size exclusion column. Figure 5.1 shows the elution profiles of mannoproteins. The results show the recovery of two main populations of mannoproteins from Agrimos® (MP1, MP2) and YCW-b (MP1', MP2') (Figure 5.1). MP1 derived from Agrimos® contained 4 fractions (MP1a, MP1b, MP1c, MP1d), while MP1' derived from YCW-b contained 2 fractions (MP1'a, MP1'b). The recovered populations were further injected into high-performance size exclusion chromatography (HPSEC) on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL) to confirm their glycosylated form and their MW distribution. All eluted peaks were simultaneously detected by UV detector (280 nm) and RI detectors, confirming that the recovered populations correspond to glycoproteins. In addition, both MP1 and MP1' showed a very broad MW distribution. Indeed, a variety of mannoprotein populations have been reported in the literature varying from 5 to 800 kDa (Doco et al., 2003). The multiplicity of mannoproteins was attributed to the presence of different numbers of the mannan moieties attached to protein (Gonçalves et al., 2002).

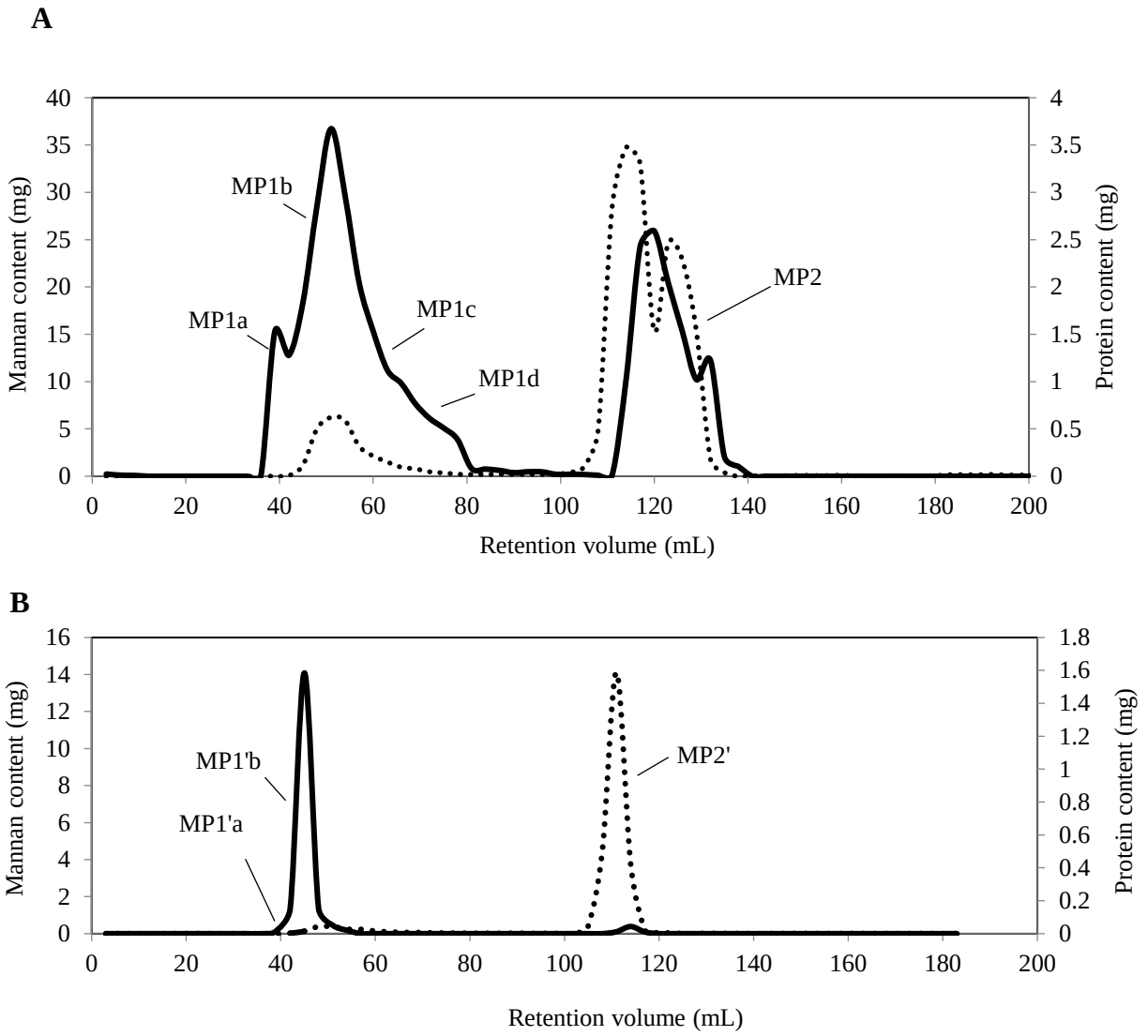


Figure 5.1. Purification of Agrimos® (A) and YCW-b (B) mannoproteins through size exclusion HiLoad™ column. mannan content(·····), protein content (—)

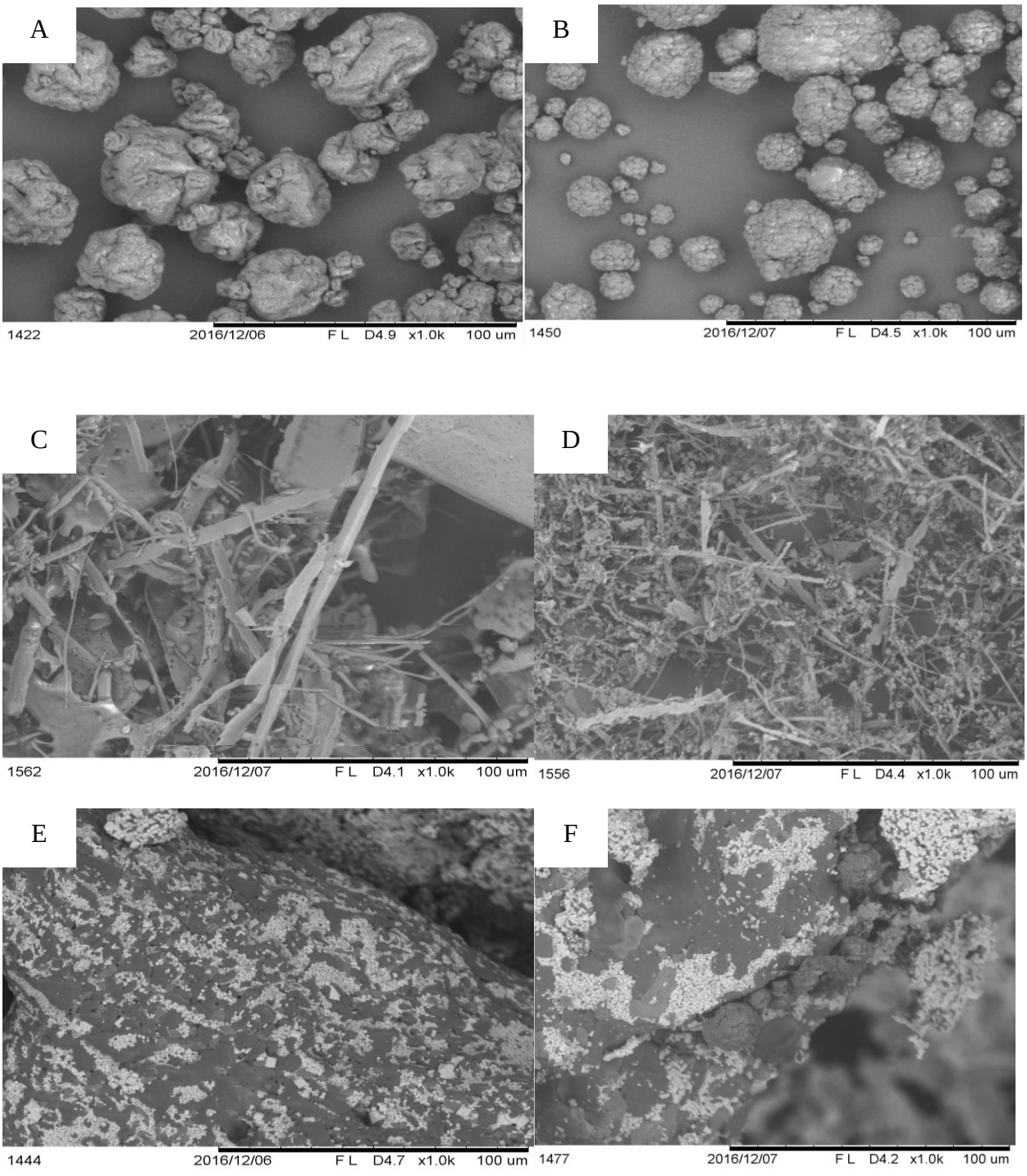
Table 5.1 lists the chemical composition of the recovered fractions of mannoproteins derived from yeast cell wall Agrimos® and YCW-b. The high MW mannoproteins (MP1, MP1') populations exhibit different chemical compositions compared to the low MW (MP2, MP2') ones. For both yeasts, the high MW mannoproteins (MP1, MP1') populations contain lower mannan to protein ratio compared to the low molecular one (MP2, MP2'). Similarly, it has been reported that the high molecular weight mannoproteins derived from white wine and red wine showed a high protein content(Gonçalves et al., 2002). The results (Table 5.1) also show that MP1 and MP1' populations are mainly made of a mannan homopolymer, attached to protein. Four fractions could be identified for the high MW MP1 from Agrimos®, including 5% of 620 kDa-mannoproteins with a mannan to protein ratio of 2.5, 26% of 242 kDa-mannoproteins with a mannan to protein ratio of 4, 60% of 90 kDa-mannoproteins with a mannan to protein ratio of 3.5, and 9% of 36 kDa-mannoproteins with a mannan to protein ratio of 10. On the other hand, two fractions could be identified for the high MW MP1' from YCW-b, including 17% of 453 kDa-mannoproteins with a mannan to protein ratio of 3 and 83% of 90 kDa- mannoproteins with a mannan to protein ratio of 6.9. The results also show that MP2' derived from YCW-b were essentially comprised of polysaccharide polymers, with a mannan to protein ratio of 181; while MP2 derived from Agrimos® contained a mannan to protein ratio of 12.2. The presence of glucose residue (8.9-10.5% of the total sugar residues) in the MP2 and MP2' fractions is probably due to the non-completed hydrolysis of the glucan attached to the mannoproteins and/or due to the recovery of glucooligosaccharides and glucans (Gonçalves et al., 2002). Supplemental Figure 5.1 shows the scanning electron micrograph of original yeast cell walls and the isolated mannoproteins. The micrographs of the starting materials, Agrimos® (Supplemental Figure 5.1A) and YCW-b (Supplemental Figure 5.1B), showed a typical cell wall structure with ovoid and round shapes. However, the surface of Agrimos® was smoother as compared to that of YCW-b. The results also show that high MW mannoproteins (MP1) exhibited more fluffy and porous structure (Supplemental Figures 5.1C, 5.1D) than the low MW mannoproteins (MP2) (Supplemental Figures 5.1E, 5.1F). These experimental findings indicate that the micro-structure of mannoproteins is dependent on their chemical composition.

Table 5.1. Composition and molecular weight of mannoproteins

	Fractions	Proportion(%)	Sugar residues[a]		Mannan/protein ratio [b]	Molecular weight (kDa)
			Glucose	Mannose		
Agrimos MP1	MP1a	5		100	2.5	620
	MP1b	26		100	4	242
	MP1c	60		100	3.5	90
	MP1d	9		100	10	36
YCW-b MP1’	MP1’a	17		100	3	453
	MP1’b	83		100	6.9	80
Agrimos MP2			10.5	89.5	12.2	2.3
YCW-b MP2’			8.9	91.1	181	3.1

[a] Percentage of sugars in the polysaccharide

[b] Mannan to protein ratio in mannoproteins (w/w) is the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins



Supplemental Figure 5.1. Scanning electron micrograph of original materials and mannoproteins with magnification of 1000. Yeast cell wall Agrimos® (A), YCW-b (B), Agrimos® MP1 (C), YCW-b MP1'(D), Agrimos® MP2 (E), YCW-b MP2' (F).

5.4.2 Techno-Functional Properties of Mannoproteins

5.4.2.1 Temperature - pH Dependence of Solubility of Mannoproteins

The solubility of purified mannoproteins and mannan standard at selected pH and temperature values is shown in Figure 5.2. The solubility of polysaccharides is generally determined by their structures and their self-assembly, with branched polysaccharides being more soluble than uniform polysaccharides; while the solubility of proteins is highly dependent on their amino acid compositions and their three-dimensional conformations (Tornberg, 2005). The results indicate that mannan standard exhibited a solubility of 86.9-96.2% at a pH range of 2.0 to 8.0, and it was totally soluble (100%) at pH 9 (Figure 5.2A). Indeed, mannan derived from *S. cerevisiae* yeast is structurally similar to the carbohydrate fraction of mammalian glycoproteins. N-linked glycoproteins have an α-(1-6) linked mannose backbone with α-(1-2) and α-(1-3) branch linkages, while for the O-linked glycoproteins, the Man0–2Man-Ser/Thr core structure is common to all fungi studied to date (Gemmill & Trimble, 1999).

Many factors can affect the solubility of glycoproteins, including molecular weight, glycation extent, conformations and charges of glycoproteins (J. Liu et al., 2012). The results (Figure 5.2A) show that compared to mannan, Agrimos® MP1 showed slightly higher solubility at the pH range of 4.0 to 8.0; this result may due to the change in the self-assembly of mannan upon their attachment to proteins. Most of reported studies focused on the improvement of protein solubility through glycation, which was found to be dependent on the degree of glycation and the carbohydrate length (Niu et al., 2011; Seo et al., 2013). For instance, wheat germ protein glycated with dextran showed an improvement in its solubility, which was attributed to the stabilizing effect of dextran, dispersing the protein molecules from self-association (Niu et al., 2011). Figure 5.2A also shows that, at pH range of 3 to 9, the solubility of MP1 and MP2 populations isolated from Agrimos® was higher than MP1' and MP2' ones from YCW-b. The higher solubility of Agrimos® MP1 and MP2 may be attributed to their higher protein content, contributing to the loose network and porous structure depicted by the scanning electron micrographs (Supplemental Figures 5.1C, 5.1D). While at pH 2, the solubility of MP1 from Agrimos® (74.2%) was lower than that of MP1' from YCW-b (83.8%). This dependence of the solubility of MP1 on the pH in the acidic region reveals the contribution of the amino acid charges to the solubility (Tornberg, 2005; Fuchs et al., 2006).

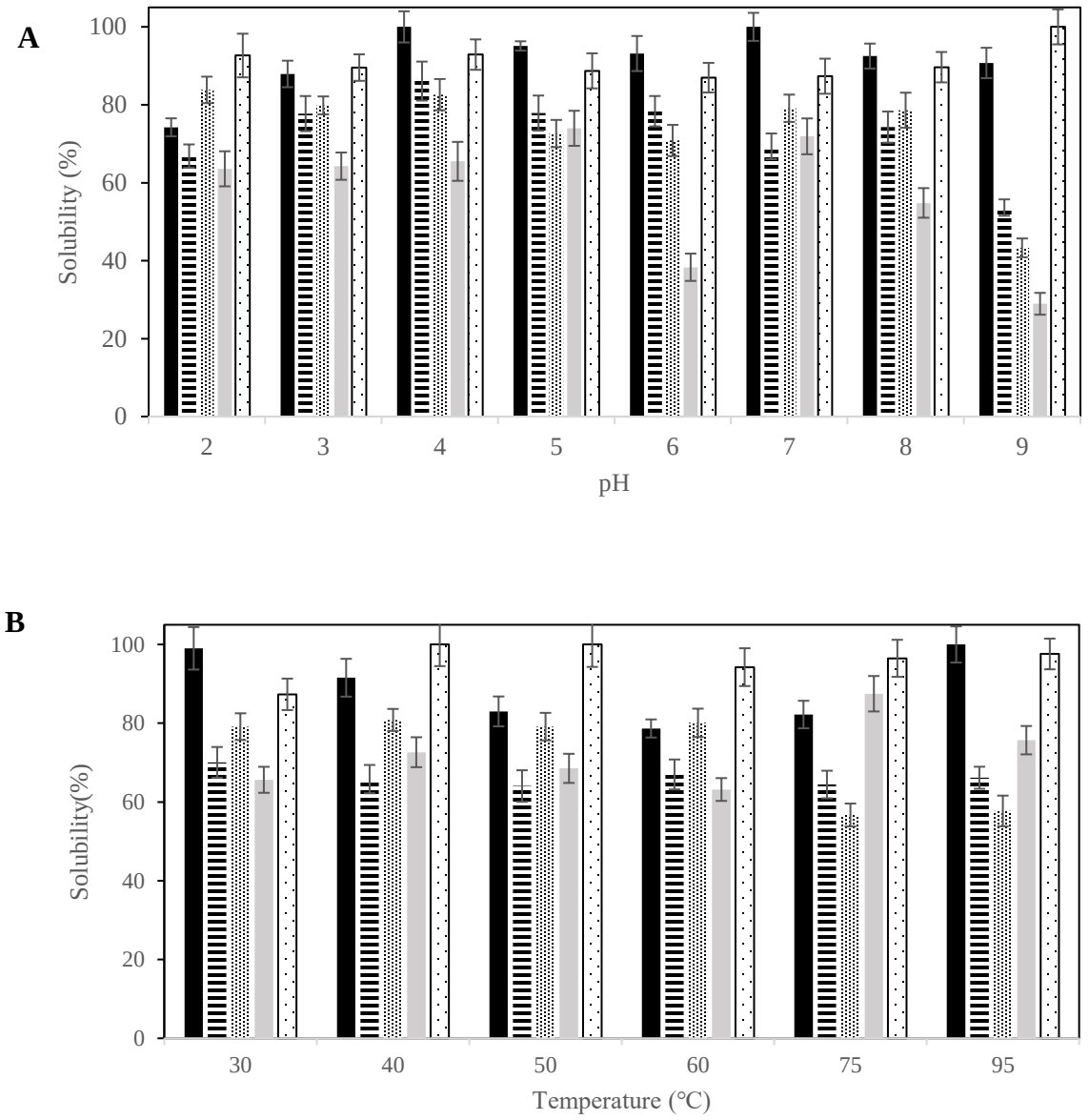


Figure 5.2. Effect of pH (A) and temperature (B) on the solubility of mannoproteins: Agrimos® MP1 (■), Agrimos® MP2 (═), YCW-b MP1'(▒), YCW-b MP2'( ), Mannan (⊡)

The results also indicate that MP1 and MP1' from both yeast cell materials exhibited a higher solubility (43.3-100%) than MP2 and MP2' (28.9-86.1%). These results were supported by the scanning electron micrograph, revealing the fluffier structure of MP1 compared to MP2. The low solubility of MP2 and MP2' may be attributed to their weak heterogeneity and their limited branching compared to MP1 and MP1'.

Figure 5.2B displays the solubility of mannoproteins and mannan standard at selected temperatures (30 to 95 °C). Mannan exhibited a solubility of 87.3% at 30 °C and was almost totally soluble (96.5-100%) at a temperature range of 40 to 100 °C. The solubility of Agrimos® MP1 decreased, when the temperature was increased from 30 to 60 °C. This decrease can be attributed to the denaturation of proteins and their aggregation. However, at higher temperatures varying from 60 to 100 °C, the solubility of Agrimos® MP1 increased; this can be explained by the improvement of the solubility of mannan proportion as the temperature increased from 60 to 100 °C. MP1' derived from YCW-b showed different solubility-temperature profile with a constant solubility of 80.0% at 30 to 60 °C and a decreased solubility thereafter to 56.7-57.7 % at 75-95 ^{0}C. These results show that MP1 and MP1' populations derived from Agrimos® and YCW-b exhibited different protein denaturation temperatures. This may indicate that MP1 and MP1' fractions not only exhibit different protein contents but also differ in their amino acid composition and structure. Contrary to MP1 and MP1' fractions, the solubility of MP2 and MP2' fractions derived from Agrimos® (64.1-70.1%) and YCW-b (63.2-75.7%) did not show a significant change within the temperature range of 30 to 95 °C, except that YCW-b has an increased solubility at 75 °C of 87.5%. The low protein content can explain the non-dependence of MP2 and MP2' solubility on the temperature, in which less protein denaturation and aggregation occurred with the increase of temperature.

5.4.2.2 Viscosity of Mannoproteins

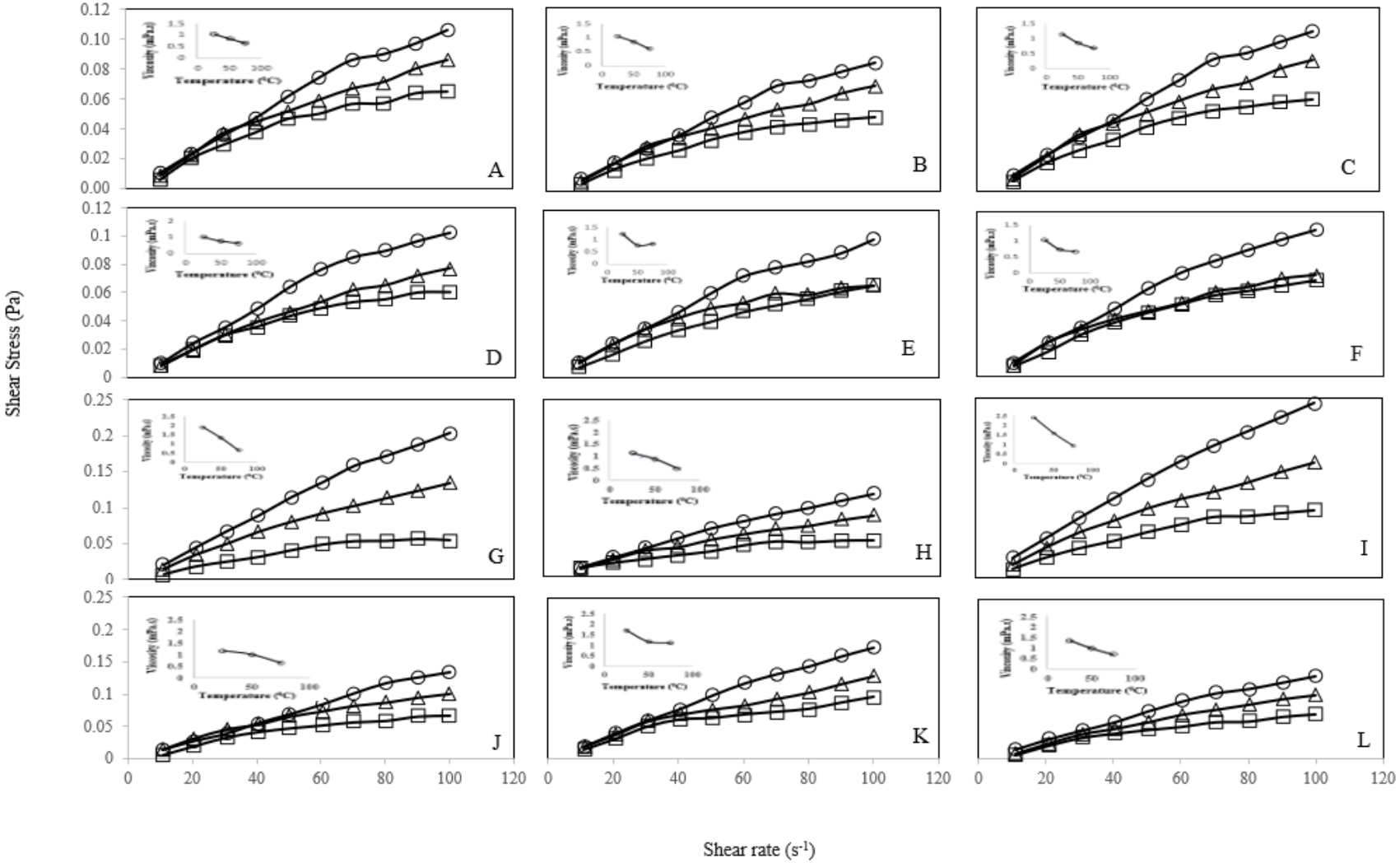
Supplemental Figure 5.2 shows the changes in shear stress (Pa) with varying shear rate (s^{-1}) from 1 to 100 for solutions containing 1% or 5% of Agrimos® MP1, Agrimos® MP2, YCW-b MP1', YCW-b MP2', mannan and whey protein at 25 °C, 50 °C and 75 °C. For all investigated mannoproteins, the shear stress was directly proportional to the shear rate at the investigated temperatures (25-75 °C) indicating their Newtonian behavior. Mannan and whey protein standards showed similar behavior. The flow behavior index (n) of all the samples was close or equal to 1 (data not shown). It was reported that at a concentration below 10% of edible yeast, the viscosity was constant with shear rate; however, at concentrations exceeding 10%, the apparent viscosity

increased rapidly and showed pseudoplastic non-newtonian behavior (Labuza et al., 1970). Another study showed that yeast behaved as Newtonian liquids with suspensions in the range of 2.5-20 % (w/v) (Mancini & Moresi, 2000). The results show that as the concentration was increased from 1 to 5%, the viscosity of MP1 and MP1' from Agrimos® and YCW-b, respectively, increased significantly from 1.05 to 1.91 mPa.s^{-1} and from 1.14 to 2.41 mPa.s^{-1}, respectively. The viscosity of the MP2 and MP2' derived from Agrimos® and YCW-b, respectively, remained more or less constant, when the concentration was increased. The viscosity of mannan standard increased from 1.23 to 1.71 mPa.s^{-1} as the concentration was increased from 1 to 5%; while the viscosity of whey protein increased from 1.04 to 1.37 mPa.s^{-1}. As expected, the viscosity of all investigated suspensions was inversely related to temperature. Indeed, the viscosity of all 1% samples decreased from 1.04 - 1.23 mPa.s^{-1} to 0.74 - 0.86 mPa.s^{-1}, as the temperature increased from 25 to 50 ˚C. Further increase of the temperature to 75 ˚C resulted in a decrease in the viscosity to 0.61 - 0.69 mPa.s^{-1}, except for the viscosity of mannan which increased to 0.84 mPa.s^{-1}. The increase of the viscosity of mannan with the temperature from 50 to 75 ˚C may due to the decrease in the solubility of mannan, as a result of its limited rotation. On the other hand, the viscosity of all 5% samples decreased from 1.14 - 2.41 mPa.s^{-1} at 25 ˚C to 0.90 - 1.60 mPa.s^{-1} at 50 ˚C then to 0.51- 0.93 mPa.s^{-1} at 75 ˚C. As an overall, when the temperature was increased, the viscosity decreased more significantly for the high MW MP1 and MP1' suspensions than the low MW MP2 and MP2' ones. It has been reported that the glycation of protein results in an increase in the viscosity or the viscoelastic property, hence improving the gelling capacity (J. Liu et al., 2012).

5.4.2.3 Emulsifying Property of Mannoproteins

5.4.2.3.1 Effect of Homogenization Types on the Particle Size Distribution of Emulsion

Emulsions can be formed through a variety of methods, generally classified as high energy or low energy approach (S. J. Lee & McClements, 2010; Qian & McClements, 2011). The high-energy approach includes high-pressure homogenization, microfluidization, and sonication; while the low energy approach mainly achieved through a change in the spontaneous curvature of the surfactant (Sole et al., 2006). Depending on the homogenization conditions, emulsions, with different droplet size, can be obtained, including micro-emulsions (10-100 nm), nano-emulsions (100-1000 nm) and macro-emulsions (0.5-100 μm) (Jafari et al., 2008). Figure 5.3A shows the effect of sonication alone and combined with a homogenization step on the droplet size distribution of mannoprotein-based emulsions.



Supplemental Figure 5.2. Changes in sheer stress vs sheer rate and viscosity (at shear rate of 100 s^{-1}) as changes of temperature for solutions of 1% Agrimos MP1 (A), 1% Agrimos MP2 (B), 1% YCW-b MP1' (C), 1% YCW-b MP2' (D), 1% mannan (E), 1% whey protein (F), 5% Agrimos MP1 (G), 5% Agrimos MP2 (H), 5% YCW-b MP1' (I), 5% YCW-b MP2' (J), 5% mannan (K), 5% whey protein (L) at 25 °C (O), 50 °C (Δ) and 75 °C (□).

Mannoproteins were able to form nano-emulsions (168.7 nm to 720.9 nm), and macro-emulsions (4.3 μm to 15.6 μm) via high-energy sonication method and a combination of sonication/homogenization method (Figure 5.3A). Using sonication, mannoproteins derived from Agrimos® led to smaller emulsion droplet size (168.7 nm for MP1 ; 4,350.1 nm for MP2) as compared to those from YCW-b (351.4 nm for MP1` ; 9,320.0 nm for MP2`). These results may due to the higher protein content of Agrimos®-based mannoproteins compared to YCW-b-based ones. Compared to the low MW mannoproteins, the high MW ones showed higher emulsifying properties (with smaller droplet size), which may be attributed to their higher protein content and solubility. Indeed, the presence of protein backbone covalently attached to the hydrophilic mannan polymers enhances the amphiphilic character of mannoproteins and hence their ability to decrease the surface tension at the interface of the two immiscible phases (Cameron et al., 1988; Seo et al., 2013). However, the mannan to protein ratio determines the hydrophilic-lipophilic balance (HLB) of mannoproteins and their amphiphilic character (Pacwa-Płociniczak et al., 2011). Furthermore, the ability of the glycated proteins to enhance the repulsive steric forces between the oil droplets may have also contributed to the emulsifying properties of mannoproteins (Darewicz & Dziuba, 2001).

Figure 5.3A shows that the combination of sonication/homogenization led to an increase in the droplet size distribution of the emulsions. These results reveal that the "over-processing" through the addition of the homogenization step have caused the coalescence of the newly formed emulsion droplets. In addition, for sonication-based emulsification, increasing the energy input (by increasing sonication time) led to "over processing" as depicted by the increase of the emulsion droplet sizes (data not shown). Figure 5.3B displays the effect of sonication treatment under different amplitudes on the emulsion droplet size formed with MP1 and MP1' derived from Agrimos® and YCW-b, respectively. High amplitudes of ultrasonic waves produce high power outputs in the medium. A previous study showed that the output power increases exponentially with increased amplitude (Raso et al., 1999). Figure 5.3B shows a large decrease in the mean particle size (from 487.5 nm to 151.4 nm) of Agrimos® MP1- based emulsion, when the amplitude was increased from 5 to 40; thereafter, the droplet size increased from 151.4 to 194.1 nm as the amplitude continued to increase from 40 to 60. A similar trend was also observed in YCW-b MP1`-based emulsions, except that the smallest droplet sizes of 289.4 nm was achieved at 50 amplitude.

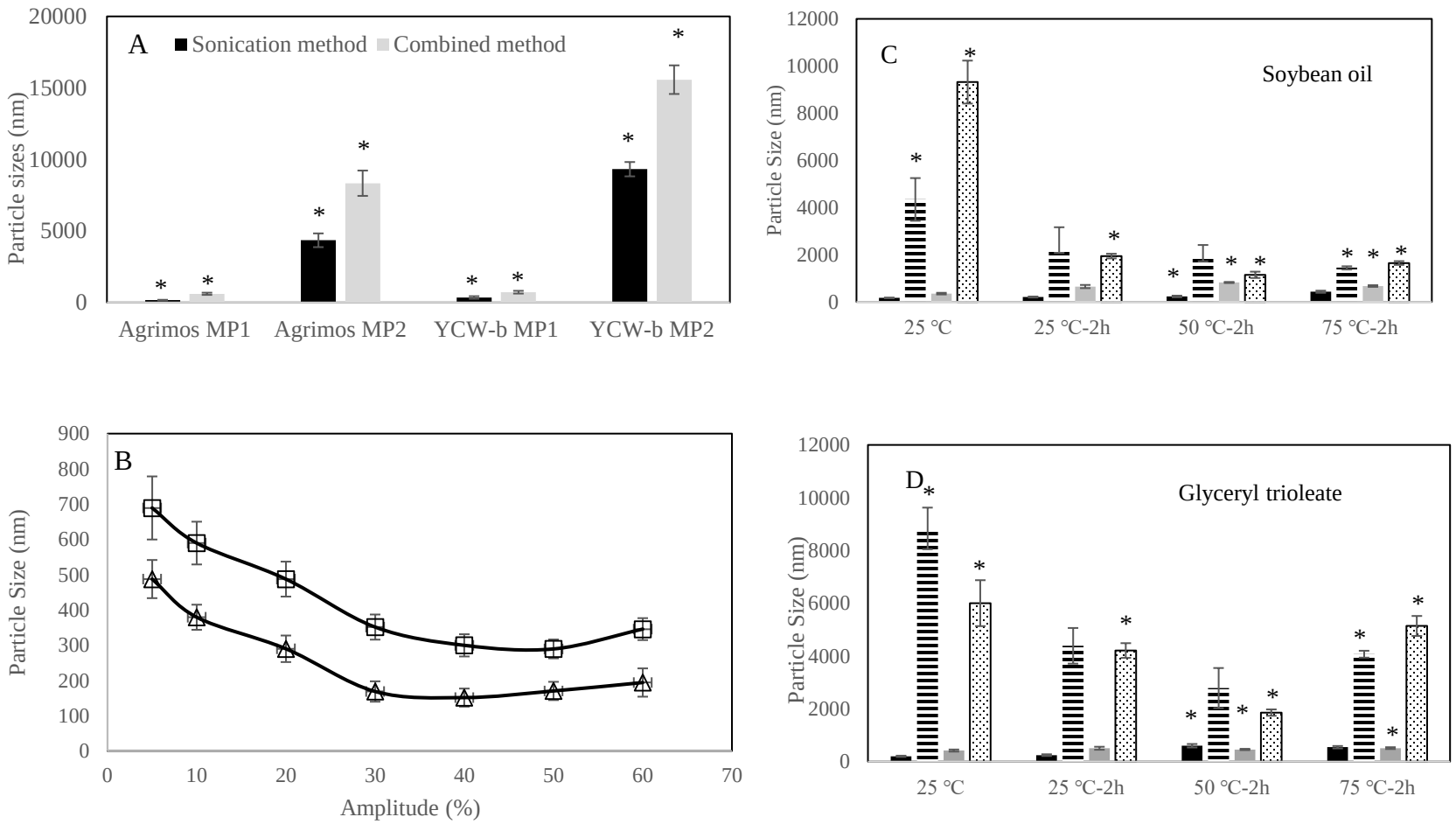


Figure 5.3. (A) Emulsifying and stability properties of mannoproteins under different conditions, * means there is significant difference ($p<0.05$) between the two treatment methods (B) Effect of ultrasound treatment under different amplitudes on the emulsion droplet sizes: Agrimos MP1 (Δ), YCW-b MP1' (□) (C) Effect of soybean oil on the emulsifying properties of mannoproteins: Agrimos MP1 (■), Agrimos MP2 (〓), YCW-b MP1' (■), YCW-b MP2' (⊡);

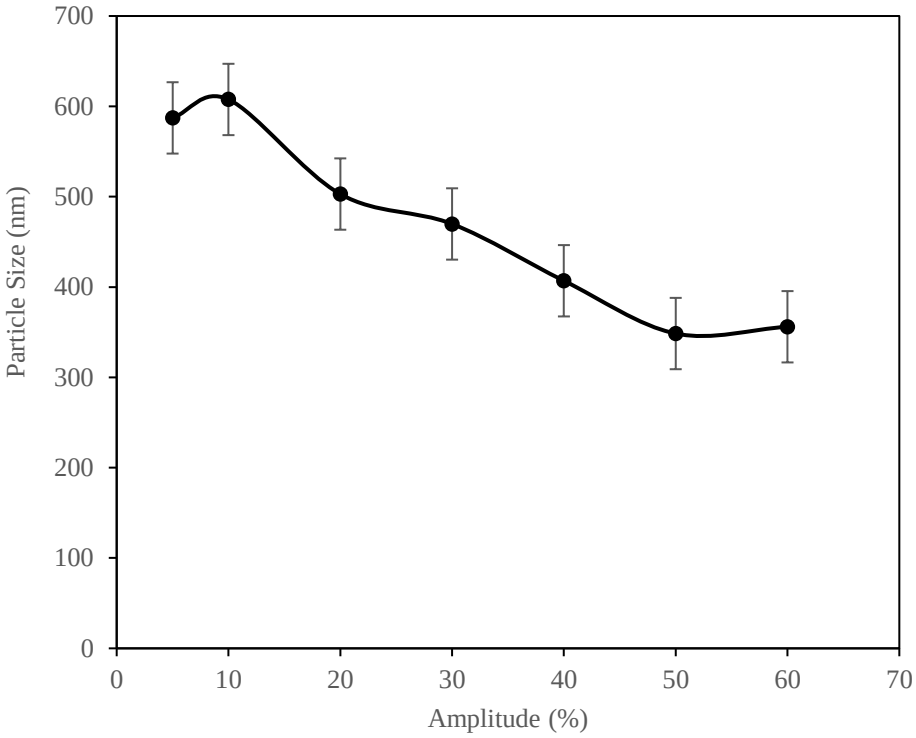
(D) Effect of glyceryl trioleate on the emulsifying properties of mannoproteins: Agrimos MP1 (■), Agrimos MP2 (〓), YCW-b MP1' (■), YCW-b MP2' (⊡)* means there is significant difference ($p<0.05$) between soybean oil and glyceryl trioleate

As an overall, MP1 and MP1' derived from Agrimos® and YCW-b, respectively, show better emulsifying properties with smaller droplet sizes compared to the whey protein under different amplitude treatments (Supplemental Figure 5.3).

5.4.2.3.2 Effect of Properties of Oil Phases on the Emulsifying Properties of Mannoproteins

Figure 5.3C and 5.3D display the effect of the types of oil substrates on the emulsion. Soybean oil and glyceryl trioleate were used as substrates to assess the emulsification ability of mannoproteins. The droplet size distribution of the emulsions was measured immediately at 25 °C; while to assess the stability, the emulsions were incubated for 2 h at 25, 50 or 75 °C. Figures 5.3C and 5.3D shows that except for YCW-b MP2', smaller droplet sizes of mannoproteins-based emulsions were obtained with soybean oil (168.7 - 4350 nm) than with glyceryl trioleate (201.2 - 8835.6 nm) at 25 °C. YCW-b MP2' led to a smaller droplet size in the presence of glyceryl trioleate (6004.8 nm) compared to that in soybean oil (9320 nm). However, there was no significant difference ($p>0.05$) between the MP1 and MP1'-based emulsions formed with soybean oil and glyceryl trioleate; while a significant difference ($p<0.05$) between the MP2 and MP2'-based emulsions were obtained with these two oil phases at 25 °C. Glyceryl trioleate is a monoacid triglyceride, with three units of the unsaturated oleic acid and has a molar mass of 885.4 g/mol. Soybean oil was reported to comprise about 16 grams of saturated fat, 23 grams of monounsaturated fat, and 58 grams of polyunsaturated fat per100 grams (Dahiya et al., 2016). A study showed that soybean oil contains 88.5% of C_{18} triglycerides fatty acids, 10.5% of C_{16} triglycerides fatty acids, 1.1% of C_{20} triglycerides fatty acids and 0.1% of C_{14} triglycerides fatty acids (F.-J. Chen & Patel, 2004). In general, the results reveal that mannoproteins-based emulsifiers have stronger affinity towards soybean oil compared to glyceryl trioleate. No mannoprotein-based emulsifier HLB value has been reported in the literature; however, it is expected that the HLB values of mannoproteins will be highly dependent on the ratio of hydrophilic mannan polymers to hydrophobic proteins.

The stability of YCW-b MP1'-based emulsion at 25, 50 and 75 °C was more pronounced (with small change in droplet size) with glyceryl trioleate (419.3 to 511.7 nm) compared to soybean oil (351.4 to 824 nm). The droplet size of the emulsions is expected to increase upon the incubation and the increase in the temperature (G. Chen & Tao, 2005; Ee et al., 2008).



Supplemental Figure 5.3. Effect of ultrasound treatment under different amplitude on the emulsion droplet size formed with whey protein.

Different from MP1 and MP1'-based emulsions, after 2h of incubation at 25 ºC and 50 ºC, MP2 and MP2' derived from Agrimos® and YCW-b, respectively, led to a significant decrease in the droplet size of the emulsions made of both soybean oil and glyceryl trioleate as compared to their initial values. This decrease in the droplet sizes upon incubation at high temperatures, can be explained by the flow-driven coalescence effect, where both viscosity and interfacial tension decrease when the temperature increases (G. Chen & Tao, 2005; Dahiya et al., 2016). A study used a low-energy method with a vortex at a high speed to form emulsions with mannoproteins of yeast isolated from sugar palm wine (Dikit, Maneerat, et al., 2010). These authors found that mannoproteins were able to form stable emulsions with various vegetable oils, and that the emulsification performances (emulsification activity and stability) were high with palm oil and poor with rice bran oil, suggesting that mannoproteins extract had more affinity/specificity for palmitic acid (Dikit, Maneerat, et al., 2010).

Figure 5.4 illustrates the effect of the oil/mannoprotein ratio on the droplet size distribution of mannoprotein-based emulsions. As the oil/mannoprotein ratio increased from 5:1 to 50:1 (v/w) by maintaining the same mannoprotein-based emulsifier concentration, an increase in the droplet size of the emulsion was observed. A similar trend was reported for the emulsifiers made with soybean soluble polysaccharide-based emulsifiers (Nakamura et al., 2004). When emulsions were prepared at a constant concentration of soybean soluble polysaccharide-based emulsifiers (4%), the average droplet size of the emulsions increased gradually from 0.52 to 10.5 µm with increasing oil content (10% to 50%) (Nakamura et al., 2004). In the present study, the average droplet size of the emulsions made with MP1 derived from Agrimos® ranged from 118.5 nm to 315.5 nm, while the average droplet size of the emulsions made with MP1' derived from YCW-b ranged from 348.1 nm to 747 nm under different oil/mannoprotein ratio. These results suggest that smaller droplet size emulsions can be obtained with MP1 derived from Agrimos® compared to MP1' derived from YCW-b. This may be attributed to the higher protein content and the lower polysaccharide content Agrimos® MP1. These findings are consistent with the previous study on soybean soluble polysaccharide-based emulsifiers (Nakamura et al., 2004). The use of the appropriate emulsifier concentration is crucial to fully occupy the oil-water interface and stabilizes the emulsion droplets from coalescence (Nakamura et al., 2004; G. Chen & Tao, 2005; Cambiella et al., 2006; Nakauma et al., 2008; Sun & Gunasekaran, 2009).

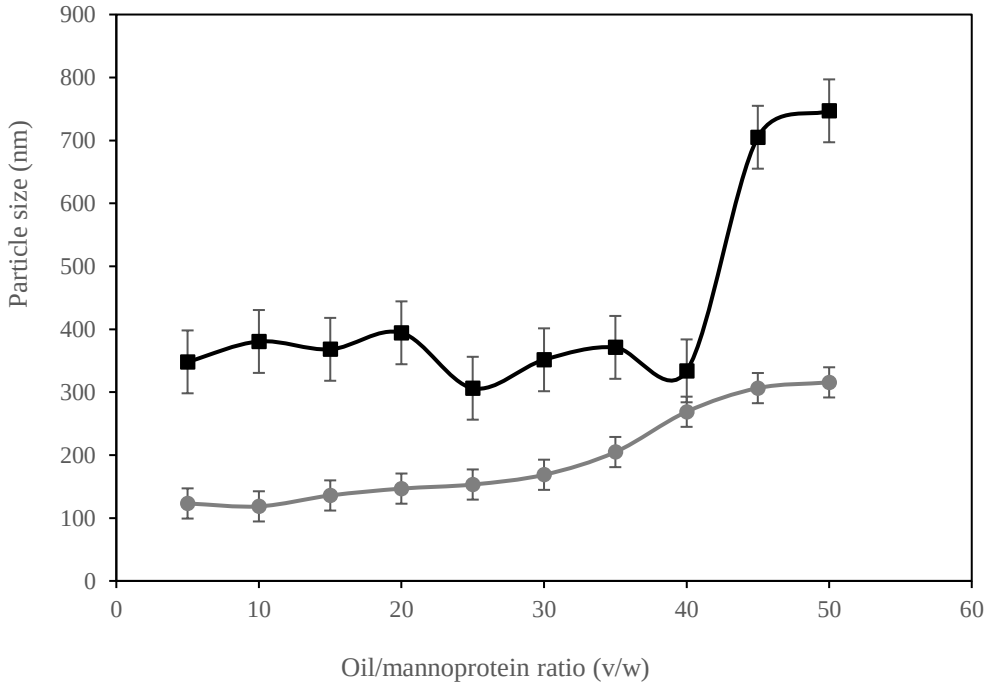


Figure 5.4. Effect of oil/mannoprotein ratio (v/w) on the emulsifying properties of mannoproteins Agrimos MP1(—●—), YCW-b MP1' (—■—)

Nakamura et al. (2004) reported that 4% of soybean soluble polysaccharide can cause the formation of fine emulsion that can last for 30 days (with 20% oil). With a critical emulsifier concentration of 2%, mannoproteins extracted from a yeast, isolated from sugar palm wine, showed 65% of emulsion activity towards palm oil (Dikit, Maneerat, et al., 2010). According to another study (S. J. Lee & McClements, 2010; McClements et al., 2017), mannoproteins can form stable emulsions at relatively high concentrations exceeding 2%. Most of studies on the mannoproteins reported higher critical concentrations to form stable emulsions. In the present study, the emulsions formed with 1% of MP1 and MP1' from Agrimos® and YCW-b (oil/mannoprotein ratio of 20:1) maintained the same appearance, when left at room temperature for up to 60 days; no discernable oil layer at the top was observed.

5.4.2.3.3 Influence of pH on the Emulsifying Properties and Stability of Mannoproteins

The emulsifying properties of Agrimos® MP1, YCW-b MP1' and lecithin were assessed immediately at 25 °C after sonication (30 amplitude, 1 min) and after 2h of incubation at 25, 50 and 75 °C (Figure 5.5). Lecithin contains both polar phosphoric acid moiety head group and two nonpolar fatty acids tail groups on the same molecule (McClements et al., 2017). Lecithin is able to form small droplet emulsions via high-energy sonication method (Figure 5.5C). The emulsion droplet size ranged between 102.9 to 745.1 nm with 1% lecithin at pH values between 2.0 and 9.0, at different incubation temperatures. At pH 2 and 3, the stability of lecithin emulsion became very unstable as evidenced by an almost complete separation of the oil layer from buffer solution after incubation at 50 or 75 °C for 2 h. Meanwhile, the emulsion droplet size increased from 253.9 and 244.2 nm (measured immediately) to 745.1 and 520.6 nm (75 °C incubation for 2 h) at pH 2.0 and pH 3.0, separately. Lecithin was reported to be unstable under acidic conditions (pH < 3.0) (McClements et al., 2017), which corroborates our results. However, minor changes in the droplet sizes of lecithin-based emulsions were recorded at a pH range of 4 to 9 and after incubation at 25 °C and 50 °C for 2 h; meanwhile no discernible oil layer was observed under these conditions (Figure 5.5C). The results also show that there is no significant difference ($p>0.05$) in the droplet sizes of lecithin-based emulsions in the pH ranges of 2 to 6 (220 - 254 nm), and of 7-9 (103 - 131 nm). However, lecithin shows better emulsifying properties at pH 7-9 as depicted by the low oil particle sizes.

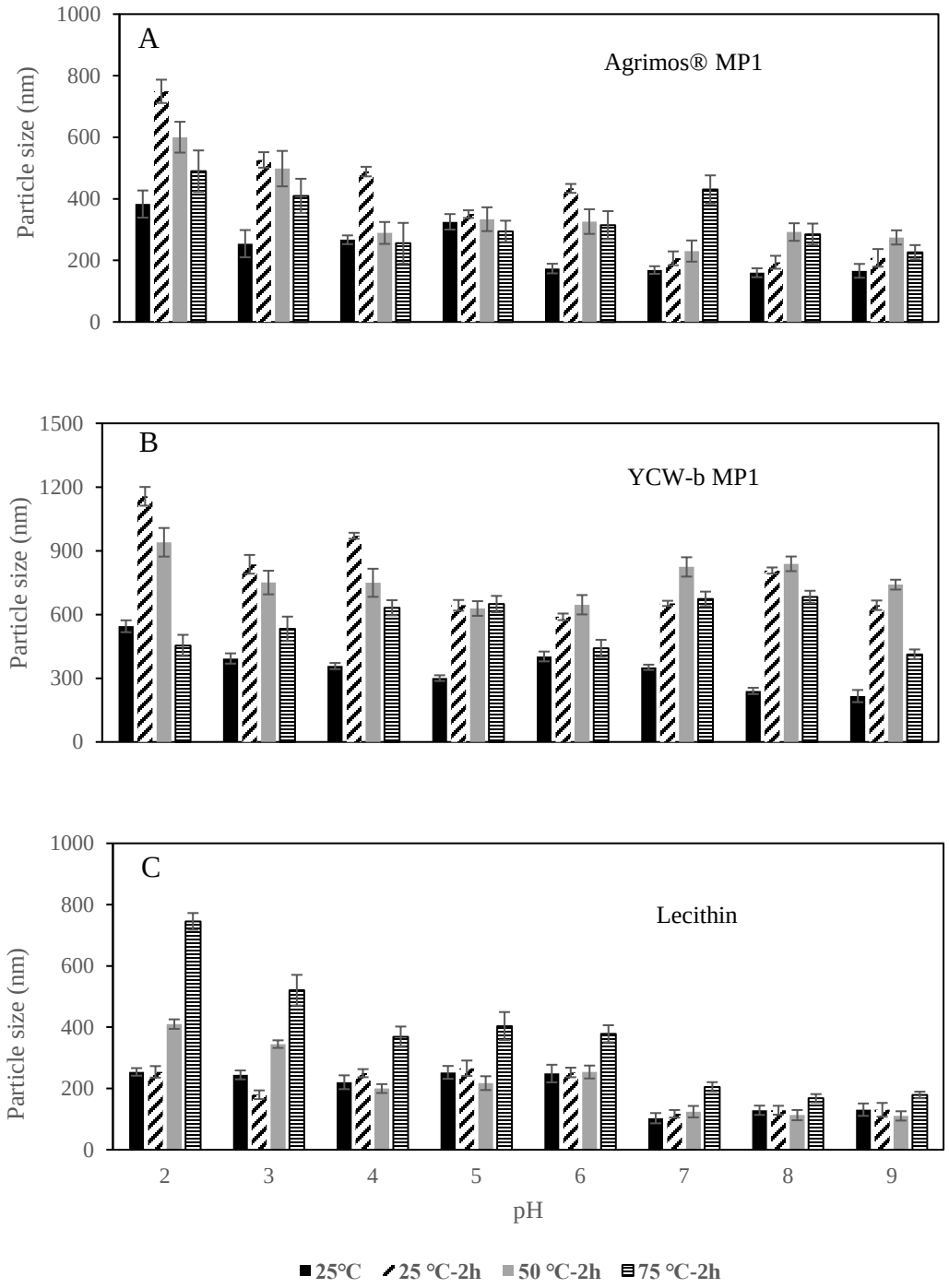


Figure 5.5. Influence of pH on the emulsifying properties and emulsion stability of MP1/MP1' derived from Agrimos® (A), YCW-b (B) and lecithin (C): 25 ^{0}C (■), 25 ^{0}C – 2h (▨), 50 ^{0}C – 2h (▩), 75 ^{0}C – 2h (▤)

Compared to lecithin, MP1 and MP1' derived from Agrimos® and YCW-b, respectively, showed comparable or slightly lower emulsification ability. When assessing the emulsions immediately after sonication at pH 2 to 9, the emulsion droplet size varied from 159.9 to 383 nm in Agrimos® MP1-based emulsion and from 215.9 to 544.7 nm in YCW-b MP1'-based one, compared to the relatively lower size range (130.7 - 253.9 nm) in lecithin-based emulsions. However, the MP1 and MP1' derived from Agrimos® and YCW-b, respectively, were uniformly dispersed under the investigated conditions (data not shown). The results also show that Agrimos® MP1-based emulsion droplet sizes varied from 267 to 383 nm and 159.9 to 173.5 nm at the pH range of 2 to 5 and 6 to 9, respectively, revealing no significant difference ($p>0.05$) at these pH ranges; as depicted by the lower particle sizes, Agrimos® MP1-based emulsifier shows significantly better emulsifying properties at pH 6 to 9. Similarly, improved emulsifying properties of YCW-b MP1' were obtained at higher pH values of 8 to 9 (215.9-240.0 nm) compared to lower pH values of 2 to7 (300.0 - 544.7 nm). An increase in the droplet sizes for the MP1 and MP1' mannoproteins-based emulsifiers was observed upon incubation for 2 h at 25 °C. Meanwhile, incubation at higher temperatures (50 °C or 75 °C) for 2 h led to a decrease in the droplet size. This is probably due to the denaturation of proteins (unfolded structure), which might have changed the amphiphilic property of the mannoproteins, thus affecting their emulsifying behavior. It has been reported in some studies that emulsions made with mannoproteins derived from *S. cerevisiae* and *S. uvarum* from baker, spent beer or wine yeasts were stable at a broad pH range (3.0 to 11.0) (Cameron et al., 1988; Silva Araújo et al., 2014); while other studies reported the high pH sensitivity of mannoproteins (McClements et al., 2017). Mannoproteins-based emulsions can also be stable under different salt conditions, such as solutions of 5% sodium chloride or 0.1% calcium chloride and magnesium chloride (McClements et al., 2017). Many factors would affect the stability of mannoproteins-based emulsions, including the strains, the pH values, other compounds present in the mannoprotein extract, protein to mannan ratio, salt content. As the purified mannoproteins were used in our study, the results reflect their appropriate emulsifying properties as they vary with their structural composition.

5.5 Conclusion

Mannoproteins derived from Agrimos® and YCW-b, obtained through Zymolyase® enzymatic treatment, were further purified using concanavalin affinity and Superdex™ size exclusion chromatography. Two populations of mannoproteins (MP1/MP1' and MP2/MP2') were recovered

and characterized. As an overall, the solubility of mannoproteins was similar or lower than that of mannan. The mannoproteins showed Newtonian behavior at 25, 50, and 75 °C, and their produced viscosity tended to decrease with increased temperatures. The sonication alone was found to be a more appropriate method for the formation of the mannoproteins-based emulsions than the combined sonication/homogenization method, as it yielded emulsions with significant smaller droplet sizes ($p<0.05$). Moreover, there was no significant difference ($p>0.05$) between the MP1/MP1'-based emulsions formed with soybean oil or glyceryl trioleate; while the MP2/MP2'-based emulsions were dependent on the type of the oil phase at 25 °C. Both MP1 and MP1'-based emulsions obtained from Agrimos® and YCW-b, respectively, showed higher emulsifying properties at higher pH values. MP1/MP1'-based emulsions obtained from Agrimos® and YCW-b showed similar or slightly inferior emulsifying properties compared to commercial lecithin.

CONNECTING STATEMENT 4

Chapter V investigated the techno-functional properties of the purified mannoproteins. Four types of yeast-based products were subjected to optimal Zymolyase® enzymatic hydrolysis to obtain mannoproteins in Chapter VI, two mannoprotein populations were recovered including a high molecular weight (MP1, 620-36 kDa) composed of a variety of mannoproteins with different mannan to protein ratio (2.5-10), and a low molecular weight (MP2, 2.3-6.8 kDa) being identified as a monocomponent. α-(1,6) mannanase treatment analysis was used to assess the presence of α-(1,6) mannose linkages. The structure of these two types of mannoproteins were studied using NMR spectroscopy with a web-based version of the computer program CASPER and FTIR methods.

Li, J., Karboune, S., Sedman, J., Ismail, A. (2018). Characterization of the structural properties of mannoproteins isolated from selected yeast-based products by specific enzymatic cleavages, NMR and FTIR Analysis (To be submitted).

CHAPTER VI

CHARACTERIZATION OF THE STRUCTURAL PROPERTIES OF MANNOPROTEINS ISOLATED FROM SELECTED YEAST-BASED PRODUCTS BY SPECIFIC ENZYMATIC CLEAVAGES, NMR AND FTIR ANALYSES

6.1 Abstract

Four types of yeast-based products (baker's yeast, brewer's yeast, Agrimos® and YCW-b) were subjected to optimal Zymolyase® enzymatic hydrolysis to obtain mannoproteins. The efficiency of the enzymatic approach was evaluated based on the yield of recovered extract, the mannan recovery yield, the protein recovery yield, the mannoproteins content and the mannan to protein ratio. After further purification steps, two mannoprotein populations were recovered including a high molecular weight (MP1, 620-36 kDa) composed of a variety of mannoproteins with different mannan to protein ratio (2.5-10), and a low molecular weight (MP2, 2.3-6.8 kDa) being identified as a monocomponent. α-(1,6) Mannanase treatment revealed the presence of α-(1,6) mannose linkages in the high MW MP1 population, but not in the low MW MP2 population. The isolated mannoproteins MP1 were mainly O-glycosylated. Mannoprotein structures were further characterized by 1D and 2D NMR, 7 anomeric carbons were determined in Agrimos® MP1, 4 of them have very similar chemical shifts than those of mannan standard. The proportion of α-(1,6) linked mannan was found to be the highest in the Agrimos® MP1. The thermal conformational changes of mannoproteins in both solid and liquid states were revealed by FTIR.

6.2 Introduction

There is a high interest in valorizing and adding more value to yeast-based products, which are generated by the brewing industry and the yeast extract-producing one. Many value addition pathways have been investigated; however, yeast by-products are mainly explored as animal feed ingredients and as a source of β-glucan. Yeast cell wall consists of β-glucan (around 60%, w/w), mannoproteins (around 40%, w/w), and chitin (2%, w/w) (Aguilar Uscanga & Francois, 2003) . In particular, mannoproteins have unique structures composed of mannan (50 to 95 %, w/w) linked to proteins (5 to 50 %, w/w) (Lipke & Ovalle, 1998; Jin Li & Karboune, 2018). Compared to other plant-based proteins (cereal, such as wheat, barley and rice), yeast proteins are rich in essential amino acid lysine and sulfur-containing amino acid tryptophan, which make them nutritionally superior (Yamada & Sgarbieri, 2005). The most limiting amino acid in the whole yeast cells was identified to be leucine. However, the amino acid scoring of whole yeast cells and phosphorylated yeast protein concentrate were reported to be high and comparable to that of casein (Yamada & Sgarbieri, 2005). Due to the high hydrophilic nature of mannans linked to proteins, mannoproteins exhibit high emulsifying and emulsion-stabilizing properties (da Silva Araújo et al., 2014; Jin Li et al., 2019). Our previous study has demonstrated that mannoproteins exhibit high emulsifying

and emulsion-stabilizing properties under pH range of 6-9, which were comparable to the commercial emulsifier lecithin (Jin Li et al., 2019).

Comparative studies have examined varying extraction techniques for the recovery of mannoproteins from yeast products (Cameron et al., 1988; Dupin et al., 2000; Núñez et al., 2006; Nunez et al., 2008; Jin Li & Karboune, 2018; Jin Li et al., 2019). Hot water extraction of mannoproteins can preserve the molecular structure of non-covalently bound mannoproteins but the recovery yield was low (Costa et al., 2012; Jin Li & Karboune, 2018). Furthermore, sodium dodecyl sulfate (SDS) as a solubilizing and denaturing reagent was reported to release a very limited amount of mannoproteins (0.1-0.2%) (Dupin et al., 2000). Alkaline extraction is the most widely used method to obtain mannoproteins from *S. cerevisiae*; however, it causes degradation of the substituted serine and threonine residues, releasing mannose, mannobiose, mannotriose, and mannotetraose (H.-Z. Liu et al., 2011). Despite the high yield of acid extraction, it was reported to be an inappropriate method to obtain mannoproteins due to the release of toxic compounds, such as dichloropropanol (Vukašinović-Milić et al., 2007). Nevertheless, an enzymatic method, based on the use of β-glucanases, was proven in our study and others to be efficient for the isolation of the covalently-bound cell wall mannoproteins from yeast cell wall with high extract yield, mannoproteins content and mannan to protein ratio; this enzymatic method relies on the hydrolytic activity of β-glucanases to hydrolyse β-glucan, to open the yeast cell wall structure and then to release the mannoproteins (Cameron et al., 1988; Moine ‐ Ledoux & Dubourdieu, 1999; Jin Li & Karboune, 2018).

Mannoproteins from yeast can be used as value added ingredients in food industry, but their techno-functional properties are greatly affected by their structure. Determination of mannoprotein structure is becoming increasingly important in aiding to understand how these molecules function in biological environments and their application in the food industry. The objective of the present study was to investigate a comparative structural characterisation of mannoproteins from selected yeast-based products produced using the β-glucanase-based enzymatic approach. The recovered extracts were first characterized in terms of yield, mannoproteins content and mannoprotein molecular weight (MW) distribution. The isolated mannoproteins were further purified by affinity chromatography followed by size exclusion chromatography. N-linked sugar structures of mannoproteins are comprised of α-(1,6)-linked mannose-based polymers to which short branches

of α-(1,2) and α-(1,3)-linked mannoses are attached; while the O-linked glycoproteins, oligomannosides are attached to glycoproteins via peptidyl-serine or threonine (De Groot et al., 2005). The proportion of α-(1,6) mannose residues was assessed through the hydrolysis of mannoproteins by specific α-(1,6)-mananase. Further structural characterization of the linkage types of sugar residues in the mannoproteins was investigated using 1D and 2D NMR (COSY, TOCSY, HMQC and HMBC) analyses, while the thermally induced changes of the secondary structure of the protein fractions in the mannoproteins were determined by FTIR. Understanding the linkage patterns and the structural properties of mannoproteins is a key step for the better modulation of their structural and functional properties.

6.3 Materials and Methods

6.3.1 Materials

Two inactivated whole yeasts (baker's yeast and brewer's yeast from *S. cerevisiae*) and two types of yeast cell wall products (Agrimos® and YCW-b derived from a unique primary grown baker's yeast *S. cerevisiae*, after specific manufacturing process to remove the cytoplasmic components) were kindly provided by Lallemand. Broad range SDS-PAGE standard was purchased from Bio-Rad (Philadelphia, PA). Affinity chromatography media Con A Sepharose 4B with immobilized Concanavalin A resin (100 mL) and HiLoad 16/600 Superdex 200 pg prepacked XK column were purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada). Zymolyase® - 20T from *Arthrobacter luteus* was purchased from Amsbio (Cambridge, Massachusetts, USA). α-Mannanase 76A from *Bacteroides thetaiotaomicron* was purchased from Creative enzymes (New York, NY, USA). All other salts and chemicals were obtained from Fisher Scientific (Mississauga, ON, Canada).

6.3.2 Enzymatic Extraction of Mannoproteins

Prior to enzymatic extraction, the yeast-based products were subjected to dialysis using a 6-8 kDa cut-off tubes in order to remove low MW sugars, small proteins, vitamins and salts. In addition, the soluble proteins (30-50%, w/w) were removed by centrifugation (8000 ×g, 30 min). The enzymatic isolation of mannoproteins from the recovered yeast-based products were conducted under the selected optimal reaction conditions identified in our previous studies (Jin Li & Karboune, 2018; Jin Li et al., 2019). For yeast cell wall-based products, yeast cell wall suspensions (15%, w/v) were prepared in sodium phosphate buffer (50 mM, pH 6.5), and the Zymolyase® (expressing β-1,3-glucan laminaripentaohydrolase and β-1,3-glucanase) was added to yield 154.01

units of β-1,3-glucanase per g of yeast cell wall; the enzymatic extraction was carried out at 45 ºC under agitation (200 rpm) for 23.84 h. For the whole inactivated yeast cells (baker's yeast and brewer's yeast), suspensions (15%, w/v) were made using 50 mM of sodium phosphate buffer at pH of 7.5; after adding Zymolyase® to yield 118.43 units of β-1,3-glucanase per g of whole yeasts, the reaction suspensions were incubated for 20.63 h at 35 ºC. After the incubations, the enzymes were inactivated by incubation at 60 ºC for 10 min. Then, the suspensions were centrifuged at 9,800 ×g for 15 min. The supernatants enriched with mannoproteins were recovered, freeze-dried and then analyzed for their relative monosaccharide composition, protein content and mannoprotein proportion in the extract.

6.3.3 Purification of Mannoproteins by Affinity Chromatography

Mannoproteins recovered after Zymolyase® treatment were purified using Concanavalin-A sepharose affinity chromatography column (100 ml) and an ÄKTA FPLC system (GE Healthcare). Concanavalin-A, tetrameric metalloprotein, has a strong affinity for the sugar molecules in the presence of C-3, C-4 and C-5 hydroxyl groups. The non-glycosylated proteins and other components were eluted with tris-HCl buffer (20 mM, pH 7.4, 5 column volume) containing NaCl (0.5 M) $MnCl_2$ (1 mM) and $CaCl_2$ (1 mM), while 0.2 M alpha-D-methylglucoside was used as a mobile phase to elute mannoproteins at a flow rate of 0.5 mL/min. The elution was monitored at 280 nm using UV-900 detector. The peaks corresponded to mannoproteins were recovered, collected and analyzed for their protein and mannan contents.

6.3.4 Fractionation of Mannoproteins by Size Exclusion Chromatography

Purified mannoprotein extract (10%, w/v) was loaded into a Hiload 16/60 Superdex 200 column using an ÄKTA FPLC system (GE Healthcare) equipped with a UV-900 detector. Tris-HCl buffer (pH 7.4, 20 mM) containing 0.15 M of sodium chloride was used as the elution buffer at a flow rate of 1 mL/min. The eluted fractions (3 mL) were assayed for their carbohydrate and protein contents. The carbohydrate content was assessed by phenol-sulfuric acid colorimetric assay (Dubois et al., 1956). While the protein content was estimated from the 280 nm absorbance using bovine serum albumin (BSA) as a standard. In order to determine the MW distribution of mannoproteins, myoglobulin (17 kDa), ovalbumin (44 kDa), human albumin (66 kDa), immunoglobulin G (158 kDa) and ferritin (440 kDa) were used as protein standards. The calibration curve was drawn by plotting the log of the MW of the standards versus the elution volume.

6.3.5 Analysis of Crude Mannoprotein Extract

6.3.5.1 Determination of Protein Content

Dumas method was conducted using Leco® TruSpec N system (St-Joseph, Michigan, USA) for the quantitative determination of nitrogen content of the starting yeast-based products and the recovered enzymatic-based extracts. The total protein content was estimated by multiplying the nitrogen content by a factor of 6.25.

6.3.5.2 Determination of Monosaccharide Composition

The carbohydrate fractions were first hydrolyzed according to the method of Khodaei & Karboune (2013) to determine the monosaccharide composition. The monosaccharides were then measured by High-Pressure Anionic Exchange Chromatography equipped with Pulsed Amperometric Detection (HPAEC-PAD) (Dionex, ICS-3000) and a CarboPac PA20 column (3 × 150 mm) set at 32 °C. D-glucose (Glc) (2.5–40 μmol/L) and D-mannose (Man) (2.5–40 μmol/L) were used as standards.

6.3.6 Characterization of MW Distribution

In order to determine the MW distribution, the crude mannoprotein extracts were analyzed by high-performance size exclusion chromatography (HPSEC) on three columns in series (TSK G3000 PWXL, TSK G4000 PWXL and TSK G5000 PWXL) using a Waters HPLC system (Model 25P, Waters Corp., Milford, MA) equipped with both refractive index (RI) and diode array (UV) detectors. The isocratic elution was carried with 0.1 M sodium chloride at a flow rate of 0.4 mL/min at room temperature. Dextrans with MW of 50, 150, 270, 410 and 670 kDa were used as standards to construct the calibration curve.

6.3.7 Structural Characterization of Purified Mannoproteins

6.3.7.1 Determination of the proportion of α-(1,6) mannose residues

Mannoprotein fractions (2%, w/v) were dissolved in 50 mM citrate buffer (pH 7.0), and subjected to the enzymatic hydrolysis with 2% or 5% (v/w) α-(1,6) mananase. The enzymatic hydrolysis was carried at 37 ℃ for 24 h, in tandem with blank reactions without α-(1,6) mannanase. The reactions were terminated by heating at 100 ℃ for 10 min. Thereafter, methanol was added to the reaction mixtures at a ratio of 1:1 (v/v) to precipitate the unhydrolysed mannoproteins and its corresponding high MW fractions enriched with α-(1,2) and α-(1,3) mannose residues. These products were analyzed by high performance size exclusion chromatography (HPSEC) coupled

with refractive index (RI) and diode array (UV) detectors. In addition, a HPSEC equipped with a PAD detector (Dionex-5500) was used to determine the mannose released from α-(1,6) linked mannose residues.

6.3.7.2 NMR Analysis

All NMR experiments were performed on a Bruker AVII 700 MHz spectrometer with an H/C cryoprobe (Chemistry, Université de Montréal). Samples were dissolved in 600 μL D_2O. 1D 1H spectra were acquired using a 30-degree tip-angle pulse and signal-averaged over 32 transients. Multiplicity-edited 1H HSQC were acquired with spectral widths of 8403 (1H) and 31698 (13C). The experiment consisted of a 1200 × 400 data matrix, which was zero-filled to 2048 × 2028 before Fourier Transformation. 1D 13C experiments were acquired using a spin-echo pulse sequence employing an adiabatic inversion pulse to minimize baseline distortion. NMR data were processed and analyzed using Topspin software (Bruker Biospin AG, Switzerland) as well as a web-based version of the computer program CASPER. This program was facilitating the determination of structure of mannan and glycoproteins derived from Agrimos®, which made the structure determination faster and simpler (Jansson et al., 1991).

6.3.8 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

6.3.8.1 Thermally Induced Changes of Freeze-dried Mannoproteins

Purified freeze-dried mannoproteins were heated at 90 °C for 2 weeks in an oven. The absorptions in the spectral region of the amide I of the non-heated freeze-dried mannoproteins and heated ones were obtained using an FTIR spectrometer (system FTS 3000) equipped with a deuterated triglycine sulfate (DTGS) detector (Bio-rad Laboratories, MA, USA). Each spectrum was obtained by 256 scans at 4 cm^{-1} resolution to maximize signal-to-noise ratio. Fourier self-deconvolution (FSD) in the amide I′ region (between 1800 and 1600 cm^{-1}) was performed using Omnic 6.0 software (Thermo Electron Corporation, Madison, WI), with a half-bandwidth of 16 cm^{-1} and an enhancement factor of 3.

6.3.8.2 Thermally Induced Changes of mannoproteins suspensions

Mannoproteins suspensions (6%,w/v) were prepared by dissolving the purified mannoproteins in D_2O as it offers a greater transparency in the infrared amide I′ region compared to H_2O (Susi & Byler, 1983). In order to investigate the thermally induced changes in the secondary structure of the purified mannoproteins, suspensions were heated from 35 to 95 °C in 10 °C increments using

an IR cell with a 25 μm path length, between two CaF_2 windows regulated by an Omega temperature controller (Omega Engineering, Laval, Qc, Canada). 3 μL of solution was spotted directly onto the CaF_2 window and carefully spread using a pipette tip to ensure that the entire volume covered the sampling window with no spillage for all samples. At each temperature, the sample was equilibrated for 10 min before the spectrum was obtained at 25 °C with an Excalibur FTIR spectrometer (system FTS 3000) equipped with a deuterated triglycine sulfate (DTGS) detector (Bio-rad Laboratories, MA, USA). The spectrometer was continuously purged with dry air from Balston dryer (Balston, Haverhill, MA). The spectrum and fourier self-deconvolution was performed under the same condition as described previously. Differently, the FSD spectra were devoid of water vapor absorptions by subtracting the spectra recorded with D_2O blank control.

6.4 Results and Discussion

6.4.1 Enzymatic Isolation of Mannoproteins from Selected Yeast-based Products

Selected yeast-based products, including whole inactivated yeasts and yeast cell walls, were investigated as starting materials for the isolation of mannoproteins. As shown in Table 6.1, yeast-based products showed some differences in their composition in terms of protein, glucan and mannan contents. For the primary grown baker's and brewer's whole yeast *S. cerevisiae*, their relative contents of protein (44.6 - 41.7%, w/w), glucan (19.2% - 22.7%, w/w) and mannan (14.4% - 12.7%) were more or less similar. After the removal of cytoplasmic proteins to produce the yeast extracts from baker's yeasts under different processing methods, the resulted Agrimos® and YCW-b showed clear differences in terms of protein, glucan and mannan contents. Agrimos® showed significantly higher glucan (23.7%, w/w) and mannan contents (25.4%, w/w) compared to those of YCW-b products (10.0% of glucan & 15.6% of mannan); while its protein content (19.6%, w/w) was significantly lower than that of YCW-b product (35.3%, w/w). These results reveal the significant effects of the processing conditions and the yeast types on the compositional characteristics of the yeast-based products.

In our previous comparative studies, the results demonstrated the higher efficiency of the enzymatic treatment, based on the hydrolysis of glucans by Zymolyase®, to isolate covalently bound mannoproteins compared to heat treatment and SDS extraction methods (Jin Li & Karboune, 2018; Jin Li et al., 2019). Among the important parameters affecting the efficiency of the enzymatic treatment, enzyme concentration was the most significant parameter affecting the yield,

while the reaction time was the most significant parameter affecting mannoproteins content and mannan/protein ratio for both brewer's yeast and Agrimos®. However, they had shown different optimization models, specifically, 2FI model was represented for the response of yield of recovered extract in Agrimos®, while quadratic models were statistically significant to represent mannoproteins content and mannan/protein ratio responses for Agrimos® and all responses for brewer's yeast (Jin Li & Karboune, 2018; Jin Li et al., 2019).

Table 6.1 also shows that the highest yield of recovered extract was obtained upon the use of the brewer's whole yeast (65.3%, w/w); while similar yields of recovered extract were obtained with baker's whole yeast (48.3%, w/w) and Agrimos® (47.6%, w/w). YCW-b, composed of the lowest glucan and high mannan contents, resulted in the lowest yield of 30.6% (w/w). Indeed, mannoproteins are divided into three groups based on the different linkages that attach them to the cell wall, including the non-covalently bound, the covalently bound to the structural glucan and the disulfide bound to other proteins that are covalently bound to the glucan of the cell wall. Covalently bound mannoproteins, fall into two categories, alkaline-sensitive mannoproteins and glucanase-extractable ones, with the latter ones being the major ones (Peter Orlean, 2012). Zymolyase® contains β-1,3-glucan laminaripentaohydrolase and β-1,3 glucanase, which can isolate the major covalently bound mannoproteins by hydrolyzing the glucans. Many factors can affect the yield of extract recovered upon the Zymolyase® treatment, including the accessibility of enzymes to substrates and the debranching/hydrolysis of mannoproteins by the secondary enzymes. Indeed, Zymolyase® expresses also low levels of mannanase and protease, which may hydrolyze the isolated mannoproteins.

Table 6.1. Isolation of mannoproteins using selected optimal enzymatic treatments on 4 yeast-based products

Treatments	Protein (% in initial weight)	Glucan (% in initial weight)	Mannan (% in initial weight)	Yield of recovered extract [a] (%)	Protein recovery yield [b] (%)	Mannan recovery yield [c] (%)	Relative protein proportion [d] (%)	Relative mannan proportion [e] (%)	Relative glucan/gluco-oligosaccharides proportion [f] (%)	Mannoproteins content [g] (%)	Mannan to protein ratio in mannoproteins [h] (w/w)
Baker's yeast	44.65 (2.45)	19.17 (1.38)	14.37 (1.45)	48.29 (2.49)	34.00 (0.13)	51.20 (1.10)	37.67 (0.14)	15.72 (0.31)	46.62 (0.21)	38.91 (0.43)	30.30 (0.14)
Brewer's yeast	41.72 (1.23)	22.75 (0.34)	12.71 (1.67)	65.31 (0.33)	40.13 (1.12)	46.81 (0.32)	38.34 (0.76)	13.26 (0.13)	48.41 (0.77)	33.26 (0.54)	17.86 (0.15)
Agrimos®	19.60 (0.39)	23.7 (0.56)	25.4 (0.19)	47.55 (0.20)	52.36 (2.13)	72.42 (0.55)	15.92 (0.13)	36.54 (0.54)	47.54 (0.34)	38.50 (3.94)	22.90 (0.75)
YCW-b	35.29 (1.24)	9.96 (0.32)	15.64 (0.11)	30.63 (0.7)	22.75 (0.33)	50.80 (2.11)	32.52 (0.42)	36.67 (0.21)	29.81 (0.55)	24.02 (0.33)	24.22 (0.36)

[a]Yield of recovered extract was obtained from the weight of extract divided by the weight of original yeast cell wall (YCW) and multiplied by 100.

[b]Protein recovery yield was obtained from the weight of protein in crude mannoprotein extracts divided by the weight of protein in original YCW multiplied by 100.

[c]Mannan recovery yield was obtained from the amount of mannan in crude mannoprotein extracts divided by the amount of mannan in YCW multiplied by 100.

[d]Relative protein proportion is the percentage of protein in the crude mannoprotein extracts.

[e]Relative mannan proportion is the amount of mannan in the crude mannoprotein extracts.

[f]Relative glucans/gluco-oligosaccharides proportion is the amount of glucan in the crude mannoprotein extracts.

[g]Mannoproteins content (%) is the proportion of mannoproteins in the extract.

[h]Mannan to protein ratio in mannoproteins (w/w) is the amount of mannan in mannoproteins divided by the amount of protein in mannoproteins.

Among all investigated yeast-based products, Agrimos® led to the highest protein (52.4%, w/w) and mannan (72.4%, w/w) recovery yields. These results reveal despite of its lowest initial protein content, the high accessibility of β-(1,3)-glucan laminaripentaohydrolase expressed in the Zymolyase® product to the glucans attached to the mannoproteins present in the Agrimos® yeast cell wall helped to obtain the highest protein and mannan recovery yields. On the other hand, the high protein content in the YCW-b product probably hindered the intermolecular interactions between glucan and β-(1,3)-glucan laminaripentaohydrolase, hence limiting the recovery yields. Zymolyase® treatments led to a similar mannoproteins content of extract isolated from baker's yeast and Agrimos® (38.5-38.9%) as starting materials; but the lowest mannoproteins content was obtained upon the use of YCW-b (24.0%). Mannan to protein ratio are high in the four yeast-based products (17.9-30.3), which corroborates our previous results (Jin Li & Karboune, 2018).

The MW distribution of the extracted mannoproteins were characterized by size exclusion chromatography (HPSEC). Our previous study has shown that 5-10 kDa mannoproteins were the dominant ones, and different incubation time and Zymolyase® concentration led to mannoproteins with different MW distributions (Jin Li & Karboune, 2018). In this study, three main MW populations of 5-10 kDa, 100-400 kDa and >400 kDa were obtained (Figure 6.1). Similar to our previous findings, mannoproteins with a low MW were the dominant ones recovered from the four yeast-based products (76.5-97.2%). In addition to the low MW mannoproteins, YCW-b and baker's yeast also contained 12.7% and 7.4% of 100-400 kDa mannoproteins, respectively. The highest MW distribution of mannoproteins (>400 kDa) was isolated from brewer's yeast (2.8%) and Agrimos® (18.5%).

Figure 6.2 shows the elution profiles of mannoproteins on Superdex™ 16/ 200 size exclusion column. The chromatograms showed the recovery of two main populations of mannoproteins (high MW-MP1 and low MW-MP2) from the four yeast-based products. Although baker's yeast resulted in 4 protein peaks, 2 of them (unlabeled ones) were minimally glycosylated. For the MP1 mannoproteins populations, the presence of different numbers of the mannan moieties attached to protein (mannan to protein ratio of 2.5-10) contributes to its heterogeneity. Several factors can explain the heterogeneity of MP1 population, including the structural difference in the yeast-based starting materials, the variability in the Zymolyase® treatment conditions (time, Zymolyase®, pH and temperatures) to responses.

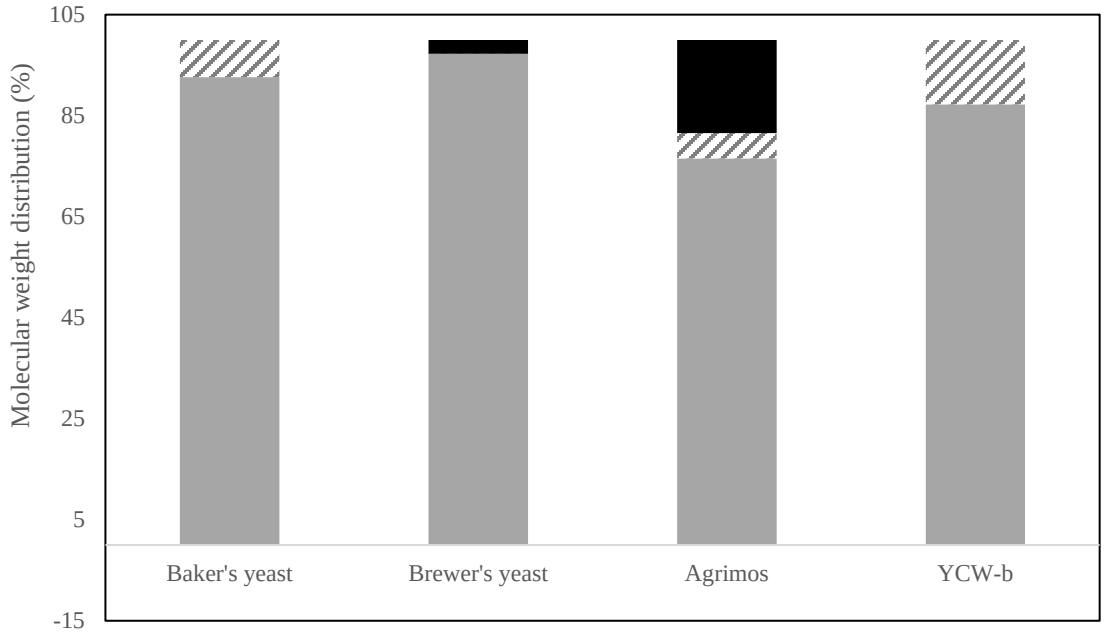


Figure 6.1. Molecular weight distributions of mannoproteins recovered upon Zymolyase® treatment of 4 yeast-based products under optimal conditions: 5-10 kDa (▒), 100-400 kDa (▨), >400 kDa (■)

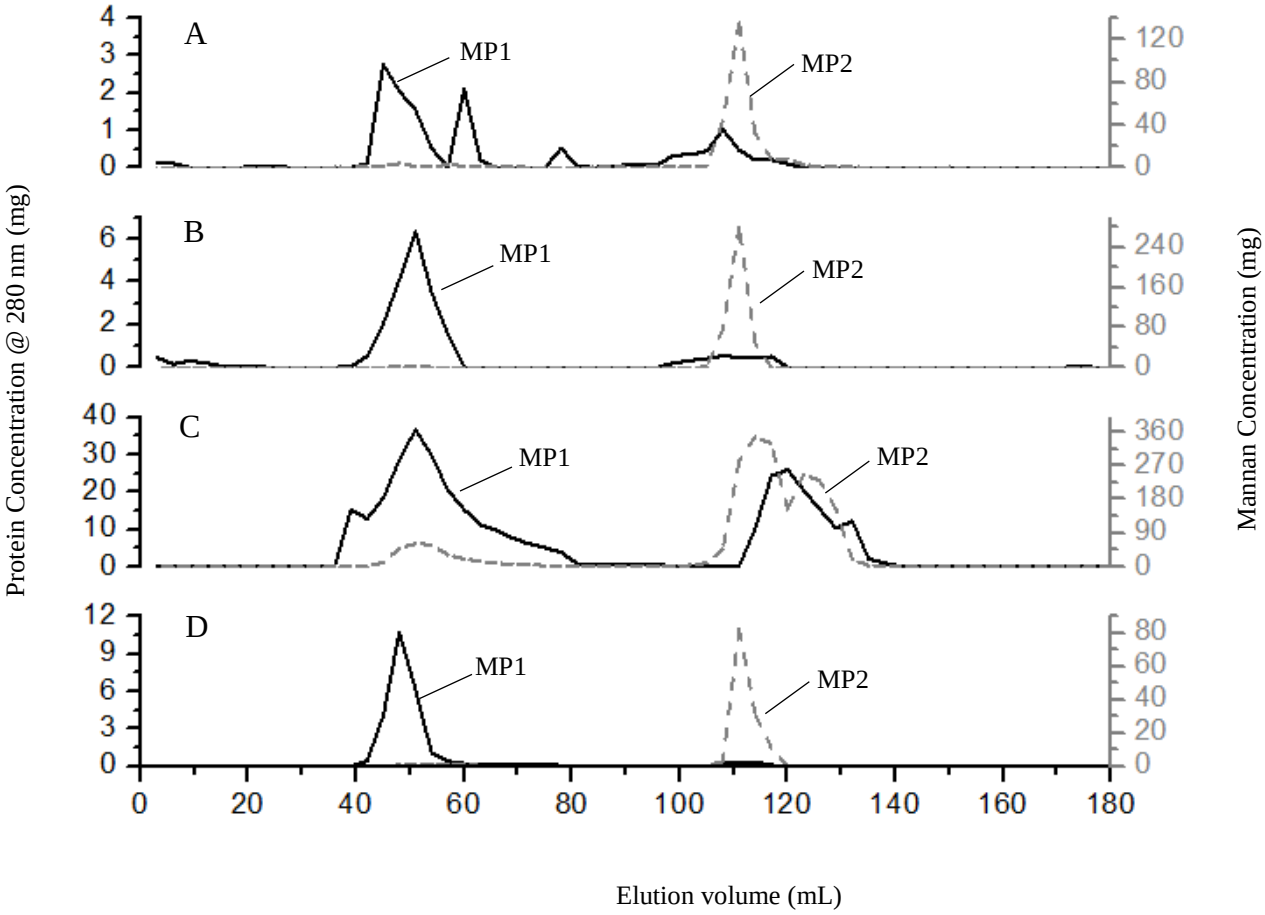


Figure 6.2. Purification of mannoproteins through size exclusion chromatography (mannan content ·····, protein content —): Baker's yeast (A), Brewer's yeast (B), Agrimos® (C), YCW-b (D)

However, the dominant mannoproteins in the MP1 population have similar MW (75-90 kDa) within 4 yeast-based products, with 60% in Agrimos® (90 kDa; mannan to protein ratio of 3.5), 83% in YCW-b (90 kDa, mannan to protein ratio of 6.9), 69% in baker's yeast (84 kDa, mannan to protein ratio of 7.3) and 81% in brewer's yeast (75 kDa, mannan to protein ratio of 7.5) (data not shown). MP2 population showed a monocomponent low MW mannoproteins in all the four yeast-based products (2.3-6.8 kDa). The mannan to protein ratio of Agrimos®-based MP2 was 12.2, whereas MP2 from the three other yeast products varied between 175-249 (data not shown).

6.4.2 Structural Characterization of Glycosidic Linkages of Purified Mannoproteins

The most abundant mannoproteins are joined to the polysaccharide network in the yeast cell wall through a lipidless remnant of a glycosylphosphatidylinositol (GPI) anchor. These anchors have been identified as signals for the incorporation of proteins in the cell wall after transit through the plasma membrane (De Groot et al., 2005). Two types of glycosylation can be found in mannoproteins, N-linked and O-linked. In *S.cerevisiae*, N-linked carbohydrate side chains of fungal glycoproteins consist of a core structure with a α-(1,6)-mannosyl backbone heavily substituted with short α-(1,2) and α-(1,3)-linked mannosyl side chains (De Groot et al., 2005). In addition, some degree of phosphorylation in the form of phosphodiester bonds can be found in N-glycan side chains (De Groot et al., 2005). For the O-linked glycoproteins, short oligomannosides are attached to glycoproteins via peptidyl-serine or threonine, and the O-linked glycans might also contain phosphodiester-linked mannose residues (De Groot et al., 2005).

To assess the presence and the abundance of the α-(1,6)-mannose linkages in the isolated mannoproteins from Agrimos® and YCW-b, mannoproteins were subjected to the hydrolysis by a specific α-1,6 mannanase from *Bacteroides thetaiotaomicron.* This type of mannanase, containing endo-α-(1,6)-mannosidase (EC 3.2.1.101), exo-β-(1,6)-mannanase, endo-α-(1,6)-D-mannanase, endo-β-(1,6)-mannanase, mannan endo-β-(1,6)-mannosidase, and α-(1,6)-D-mannan mannanohydrolase, can catalyze the hydrolysis of α-(1,6)-D-mannosidic linkages in (1,6)-mannans. A high performance anion exchange chromatograph (HPAEC) was used to determine the α-(1,6) linked mannose proportion compared to the whole mannan content in derived mannoprotein fractions. The results (data not shown) showed that the α-(1,6) linked mannose accounted for 46.51 and 46.01% of total mannan for MP1 derived from Agrimos® and YCW-b, respectively. The presence of α-(1,6) linked mannose was not obviously found in MP2 derived

from Agrimos® and YCW-b. It can be hypothesized that mannoproteins MP2 are mainly composed of protein chains attached to residues of mannose linked by α-(1,2) linkages.

As shown in the HPSEC-UV elution profile of Agrimos®-based MP1 hydrolysates, the main peak with a wide MW distribution of mannoproteins of 620 to 36 kDa was shifted into a shorter MW of 331 to 5 kDa (Figure 6.3A). Similar shift of this main peak was observed in the RI elution profile (Figure 6.3 B). These results suggest the released α-(1,6)-mannose and protein fragments. In addition, the results (Figure 6.3A & 6.3B) show that the peak area of mannoproteins with MW of 0.85 kDa (retention time of 54 min) increased under UV and RI detection, confirming that the newly released protein groups are covalently linked to the α-(1,6) mannose residues. However, the fact that there was no excessive hydrolysis of Agrimos®-based mannoproteins MP1 into many fragments reveals the preservation of mannoproteins backbone after α-(1,6) mannanase treatment. It can be speculated that most of the extracted mannoproteins are not N-linked glycoproteins. Indeed, the N-linked glycoproteins, which contain a α-(1,6)- mannosyl backbone heavily substituted with short α-(1,2) and α-(1,3)-linked mannosyl side chains, can be greatly hydrolyzed by α-(1,6) mannanase and won't maintain the main structure. Both N-linked and O-linked glycoproteins have been reported in *S. cerevisiae* (Sharma et al., 1991; Abe et al., 2016; Hernández et al., 2017); however, one study has reported that glycoproteins in *S. cerevisiae* contain exclusively O-linked oligosaccharides (P Orlean et al., 1986). Since GPI proteins contain serine/threonine-rich domains, which receive short mannose chains onto the hydroxyl side chains of serine or threonine residues through an α-mannosyl bond, O-mannosylation is a common feature of GPI dependent cell wall proteins (Lesage & Bussey, 2006). For MP1 derived from YCW-b, similar results can be observed in the HPSEC profile (Figure 6.3C and 6.3D). Before degradation of MP1 from YCW-b, one broad peak with MW of 453-90 kDa (retention time of 30 to 46 min) was observed in both UV and RI elution profiles (Figure 6.3C & 6.3D). Within the identified mannoproteins population, 17% of mannoproteins (453 kDa) was characterized by a mannan to protein ratio of 3, and 83% of mannoproteins (90 kDa) had a mannan to protein ratio of 6.9. After hydrolysis with α-1,6 mannanase, a broad peak (retention time at 31-46 min) with MW of 94 and 50 kDa was observed.

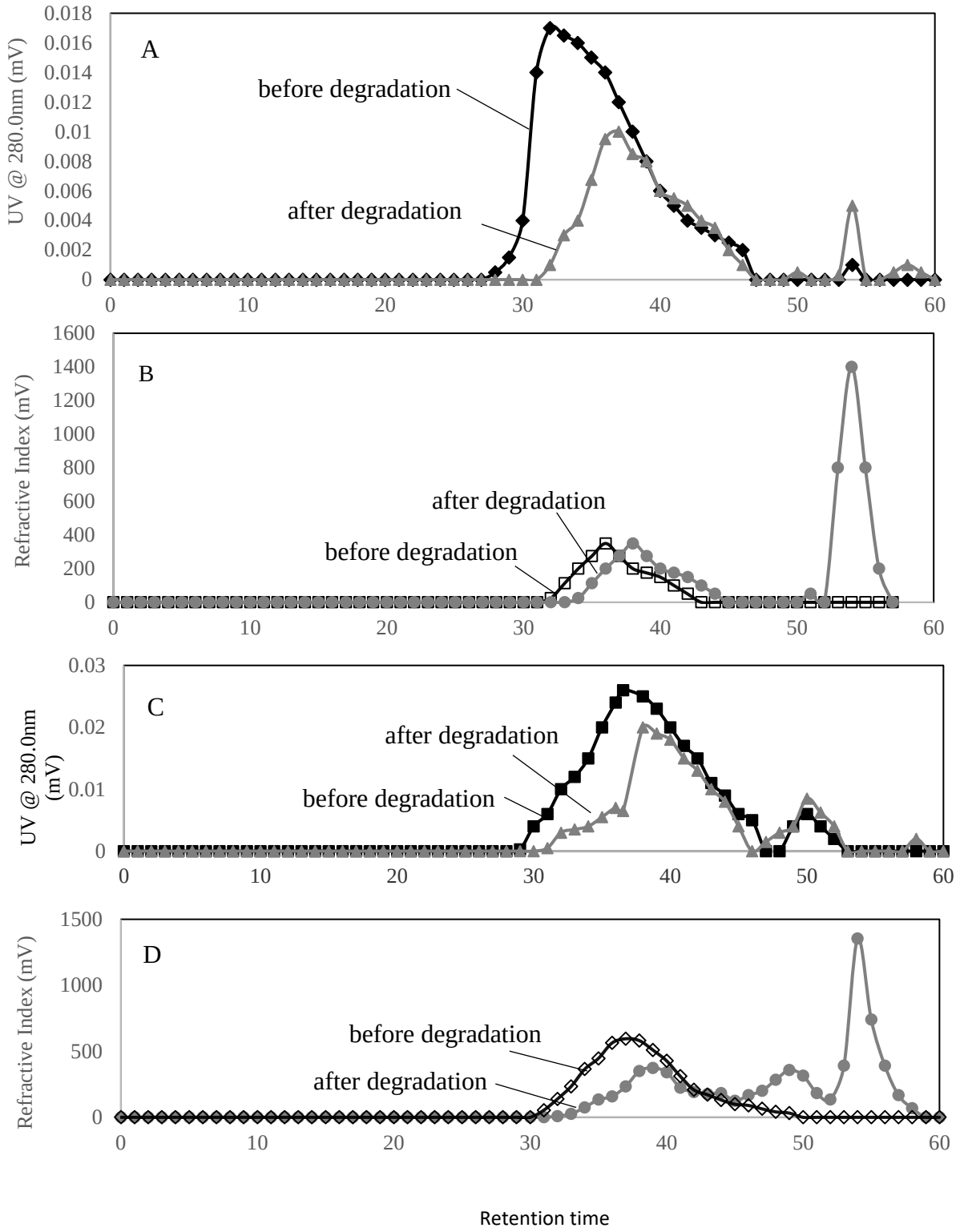


Figure 6.3. HPSEC profiles of MP1 before and after treatment with α-1,6 Mannanase: Agrimos® MP1 UV(A), Agrimos® MP1 RI (B), YCW-b MP1 UV(C), YCW-b MP1 RI (D)

The generation of a new peak (retention time of 50 min) with MW of 1.45 kDa in RI and UV elution profiles of YCW-b hydrolysate indicates the release of some protein fragments covalently linked to the α-1,6 linked mannose residues. The released of 2 units of oligosaccharides from YCW-b (retention time at 55 min, MW of 0.34 kDa) was not associated with any protein moiety (Figure 6.3D). The results (data not shown) also show that MP2 derived from Agrimos® and YCW-b have a MW of 2.3-6.8 kDa. No difference was observed in the HPSEC profiles after α-1,6 mannanase treatment of MP2. These results are in accordance with the HPAEC results.

To further characterize the structures of mannoproteins, 1D and 2D NMR (COSY, TOCSY, HMQC and HMBC) spectroscopy analyses for MP1 and MP2 derived from Agrimos® and YCW-b were performed (without α-1,6 mannanase treatment). Mannan from yeast *S. cerevisiae* was used as standard. The similarity of ^{1}H and ^{13}C NMR line-shapes between mannoproteins from Agrimos® and YCW-b indicates the similarity of their structural characteristics, in terms of linkage patterns. Therefore, only the structural properties of MP1 and MP2 derived from Agrimos® will be discussed. Figure 6.4 shows the 1D ^{1}H and 1D ^{13}C NMR spectra of Agrimos® MP1 (Figure 6.4A, 6.4B) and mannan standard (Figure 6.4C, 6.4D). There are noticeable differences in 1D ^{1}H and 1D ^{13}C NMR spectra between Agrimos® MP1 and mannan standard. The mannan structural complexity is illustrated by more than 20 signals in 1D ^{1}H NMR spectra. Broader peaks can be found in the Agrimos® ^{1}H NMR spectrum compared to mannan standard due to the presence of protein content. In the literature, the signal of anomeric proton of the glycosidically linked mannopyranose at the position of glycosidic linkage is usually used as characteristic proton chemical shift for determining the type of linkage. The proton chemical shifts were reported to be 5.05 ppm for α-(1,2) and 5.13 ppm for α-(1,2) structures (Lowman et al., 2011). However, it is not reliable to confirm the linkage type only based on the presence of proton chemical shift signal. A study has found that 5.14 ppm signal of chemical shift also represents (1,2) mannobiose with β configuration on the reducing end (Bystrický et al., 2017). Due to the structural motifs in mannan side chains, it has been reported that the signal at 5.34 ppm is typical for mannose in α-(1,2)-linked side chains of two or more sub-units, and 5.21 ppm is the typical for α-(1, 6)-mannan in the basic chain on which the mannose side groups are suspended (H.-Z. Liu et al., 2015). Furthermore, 5.03 ppm was also reported to correspond to the signal for terminal α-(1, 2)-linked mannose and for α-(1, 2)-linked mannose with a mannose substituent in the 3 positions (H.-Z. Liu et al., 2015).

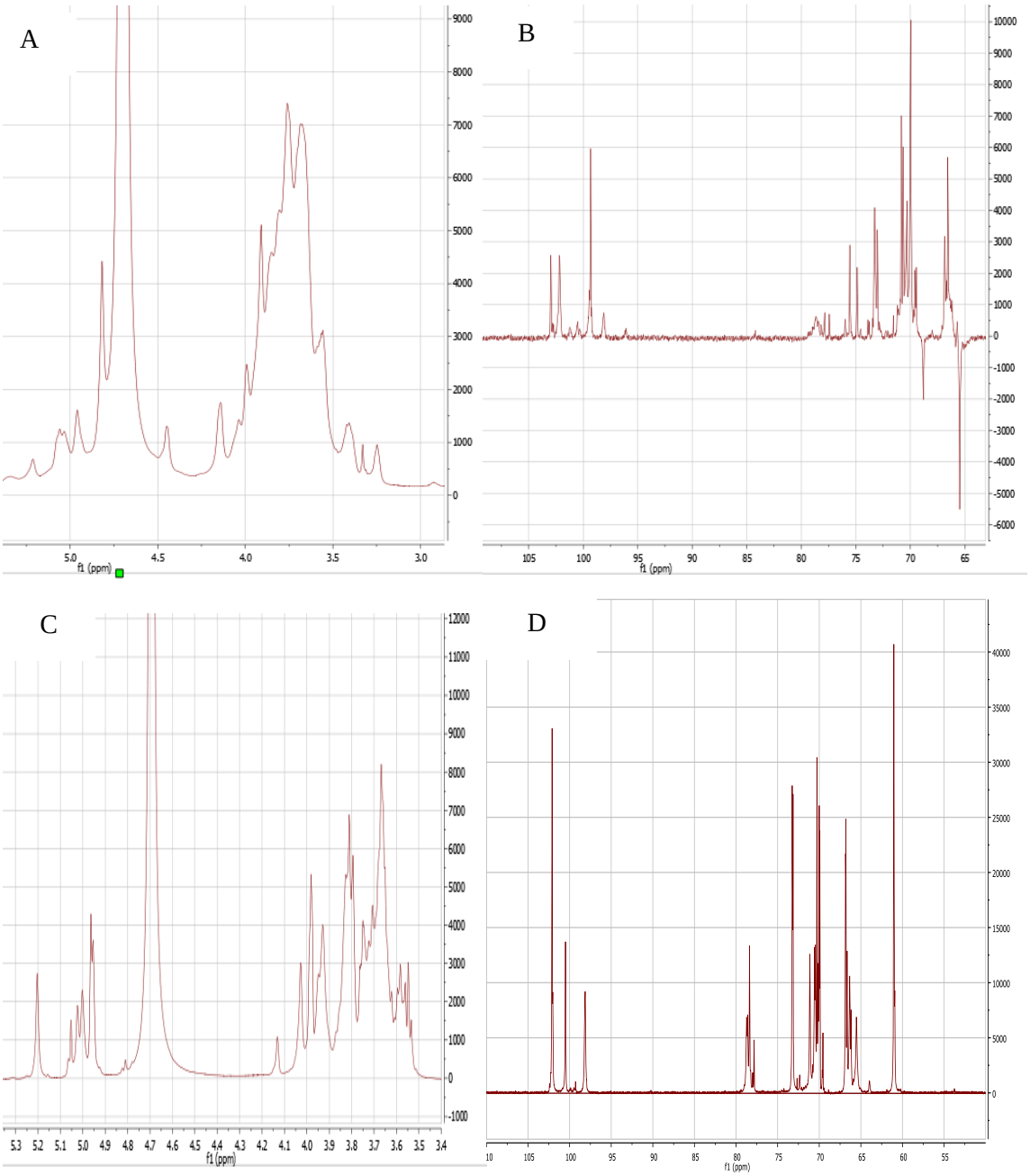


Figure 6.4. NMR spectrum of Agrimos® MP1 and mannan: Agrimos® ^{1}H(A), Agrimos® ^{13}C (B), Mannan ^{1}H (C), Mannan ^{13}C (D)

Figure 6.5 shows the edited gradient-enhanced $^{1}H–^{13}C$ HSQC spectrum of Agrimos® MP1 (Figure 6.5A) and mannan standard (Figure 6.5B). Generally, heteronuclear $^{1}H–^{13}C$ HSQC spectrum of hexoses displays three distinguishable regions: a well recognized anomeric region at 90–105 ppm for ^{13}C chemical shifts, narrow region at ^{13}C 68–80 ppm of methine groups of pyranose rings which could be partially overlapped and sometimes rather difficult to resolve, and well separated signals of C6 methylene at ^{13}C 60–65 ppm (Bystrický et al., 2017). The program CASPER was used to analyze structural properties of oligosaccharides based on comprehensive NMR database. The data were derived empirically from numerous experimental studies to improve the prediction accuracy (Bystrický et al., 2017). With the use of CASPER, we identified 4 anomeric carbons in mannan and 7 anomeric carbons in Agrimos® MP1 (Table 6.2). δ_{Rel} reflects the agreement between the submitted experimental data and the calculated chemical shifts, in that the highest ranked structure always has δ_{Rel}=1.00 and the lower δ_{Rel} corresponds to the better agreement. Our results show that in mannan standard NMR spectrum, the chemical shifts of 5.01/98.2 ppm, 5.05/102.2 ppm and 5.20/100.5 ppm correspond to those of α-(1,6), α-(1,3) and α-(1,2) linkages, respectively, all of three with δRel=1.00. The chemical shifts of 4.95/102.1 represent also α-(1,3) structure, with δRel=1.21 in mannan standard. The identification of two chemical shifts 5.05/102.2 ppm and 4.95/102.1 for the α-(1,3) linkages in mannan standard may be due to the steric and electronic proximity effect of the linked mannose. Indeed, many studies have focused on characterization of the structural motifs in mannan side-chains (Bystrický et al., 2017). Within the 7 anomeric carbons determined in Agrimos® MP1, 4 of them have very similar chemical shifts with those in mannan standard. In addition, chemical shifts 4.44/102.9 corresponds to α-(1,3) structure, while other 2 types of chemical shifts of 4.72/99.2 and 4.82/99.3 represent the α-(1,6) structure. It is worth noting that the protein residues may also affect the chemical shifts of mannoproteins.

From the intensity of NMR results, it can be seen that α-(1,2) linked mannose residues account for 4.3% of total mannan structure in Agrimos® MP1, while α-(1,3) and α-(1,6) ones correspond to 41.2% and 47.8% of total mannan structure, respectively. The high proportion of α-(1,6) linked mannan in Agrimos® MP1 is in agreement with our previous findings of α-(1,6) mannanase treatment, where α-1,6 linked mannan accounted for 46.51% of total mannan. In addition, the MP1 derived from Agrimos® does give a phosphorus peak, at -1.9 ppm (data not shown). The phosphate group is 2-3 bonds away from the proton at 5.34 ppm.

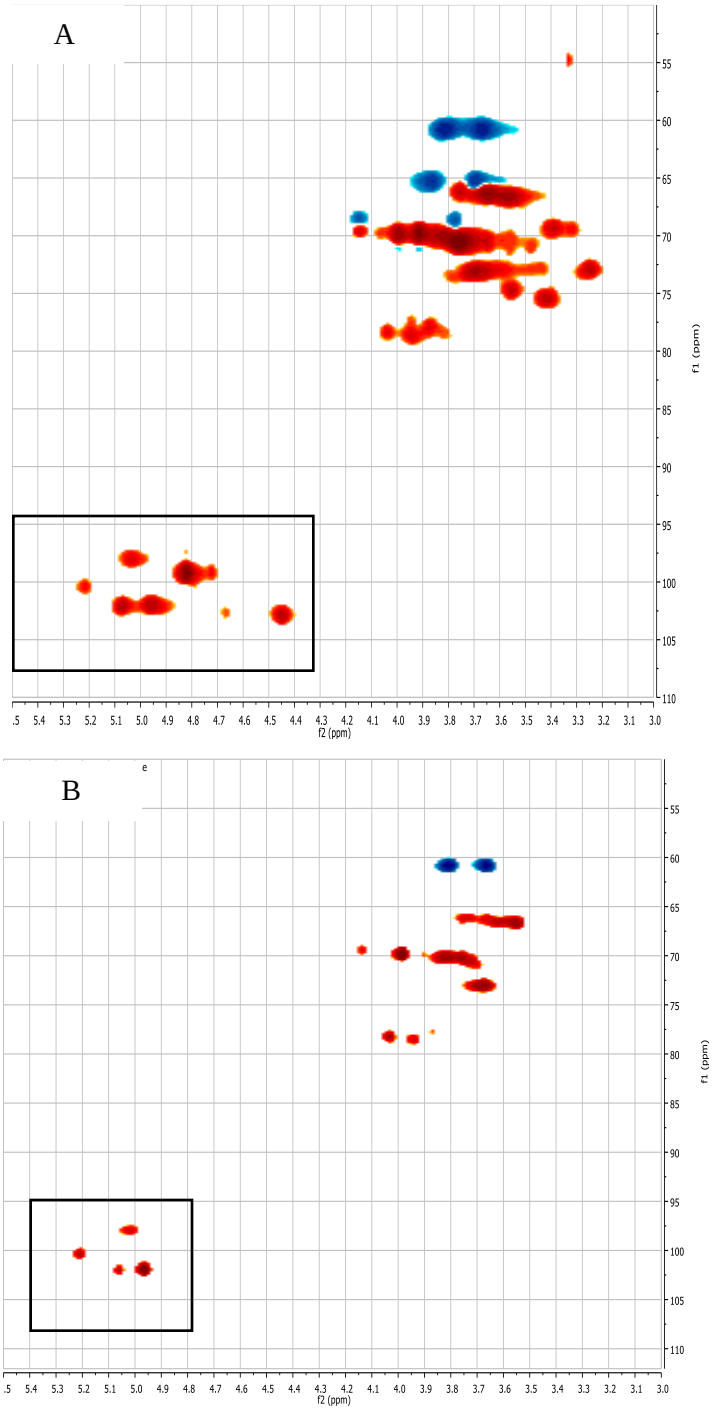


Figure 6.5. Edited gradient-enhanced $^{1}H–^{13}C$ HSQC spectrum of Agrimos® MP1(A) and mannan standard (B). Boxed area is anomeric region

Table 6.2. Possible Linkage of Mannoprotein Fractions and Mannan Standard Using a Web-based Version of the Computer Program CASPER

Samples	Peak	H1 (ppm)	C1 (ppm)	Intensity	Linkage	δ_{Rel}[a]
MP1	1	4.7176	99.2172	579677.97	→6)-α-d-Man-(1→	1.25
	2	5.2098	100.4457	612831.59	→2)-α-d-Man-(1→	1.00
	3	5.0281	98.0764	965314.94	→6)-α-d-Man-(1→	1.00
	4	5.0633	102.2007	1743964.66	→3)-α-d-Man-(1→	1.00
	5	4.4422	102.9027	1885672.5	→3)-α-d-Man-(1→	1.13
	6	4.9519	102.1129	2173711.31	→3)-α-d-Man-(1→	1.00
	7	4.8172	99.3049	6170930.53	→6)-α-d-Man-(1→	1.00
Mannan	1	5.0104	98.1642	8373981.38	→6)-α-d-Man-(1→	1.00
	2	5.0514	102.2007	10088500	→3)-α-d-Man-(1→	1.00
	3	5.2038	100.5334	11695708.9	→2)-α-d-Man-(1→	1.00
	4	4.9577	102.1129	34688738.5	→3)-α-d-Man-(1→	1.21
MP2	1	5.2098	100.4457	612831.59	→2)-α-d-Man-(1→	1.00

[a] The highest ranked structure always has δRel=1.00 and the lower δRel the better agreement between the submitted experimental data and the calculated chemical shifts.

In *S. cerevisiae* mannoproteins, the phosphate content is less than 0.1%, while the reported phosphate content in *C. albicans* wall was higher, between 0.2-1% (Gorin, 1973). MP2 shows similar chemical shift pattern (5.01, 98.2 ppm) with one of the anomeric carbons in MP1, which represents α-(1,2) structure. Only one anomeric carbon was determined in MP2, revealing the absence of side-chains in MP2 (data not shown).

6.4.3 Characterization of the Secondary Structure of Mannoproteins

The secondary structures of mannoproteins in the native and heat-denatured forms both in the solid state and in D_2O solution were investigated by FTIR spectroscopy. Three amide bands in protein spectra have been utilized in the study of protein secondary structure: amide I at ~1650 cm^{-1}, amide II at ~1550 cm^{-1}, and amide III at 1400 cm^{-1} (Boulet et al., 2007). Among these, the amide I band is the most extensively studied in spectra of protein solutions; because the amide I band overlaps with an absorption band of H_2O, D_2O solutions are employed. The solid-state spectra of MP1 and MP2 derived from Agrimos® are presented in Figure 6.6A. The most intense peaks in these spectra are observed in the region 1200-950 cm^{-1}, generally known to be a typical characteristic of polysaccharides. The intense peak at around 1650 cm^{-1}, corresponding to the amide I band, is also typical of acylamino groups, as reported previously for mannoproteins (H.-Z. Liu et al., 2015). Absorption bands at ~1550 cm^{-1} and ~1400 cm^{-1} observed for mannoproteins can be attributed to the amide II band and side-chain absorption bands (symmetric ν_{COO}- stretching vibration of Glu and Asp), respectively. There are differences in the relative intensities of the carbohydrate and protein bands in the spectra of MP1 and MP2 derived from Agrimos®; MP1 has stronger protein absorption bands due to its higher protein-to-sugar ratio. The spectrum of MP1 derived from Agrimos® dissolved in D_2O was subjected to Fourier self-deconvolution (FSD) between 1800 and 1500 cm^{-1} to mathematically enhance the spectral resolution and observe the individual contributions of secondary-structure elements to the protein amide I band (Figure 6.6B). The assignments of the bands in the FSD spectrum are shown in Table 6.3. The absorption band at 1743 cm^{-1} is assigned to the C=O stretching vibration of the methyl-esterified carboxyl group, while the bands at 1713-1704 cm^{-1} are assigned to the protonated carboxylic acid groups of Asp and Glu side chains (Boulet et al., 2007). The bands at 1583 and 1561 cm^{-1} are assigned to the asymmetric ν_{COO}- stretching vibration of Asp and Glu, respectively. MP1 also displays a distinct absorption band at 1514 cm^{-1} associated with tyrosine side chains.

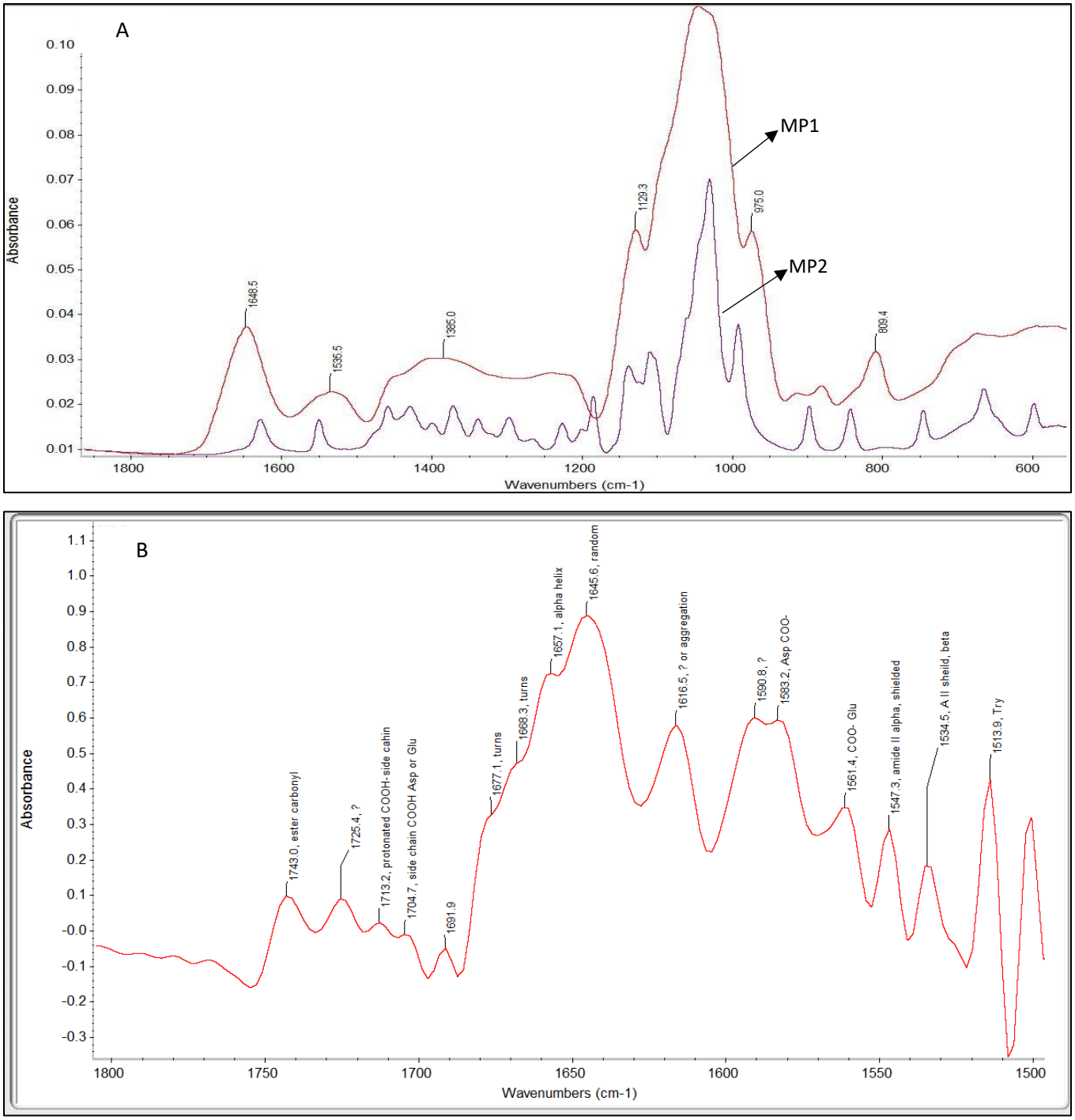


Figure 6.6. (A) FTIR spectra of MP1 and MP2 derived from Agrimos® in the solid state, (B) Fourier self-deconvoluted FTIR spectrum of MP1 in D_2O solution (1800-1500 cm^{-1})

Absorption bands at 1547 and 1534 cm^{-1} are amide II band components assigned to α-helical and β-sheet structures, respectively. The secondary structures of mannoproteins derived from Agrimos® and YCW-b before and after thermal treatment at 90 ℃ for 2 weeks in the solid state were studied (Figure 6.7). The components of the amide I band in the spectra of the samples before thermal treatment were assigned as follows. The band at 1618 cm^{-1} was assigned to intermolecular anti-parallel β-sheets indicative of protein aggregation (Pots et al., 1998). The bands at 1624 and 1642 cm^{-1} were assigned to β-sheets and unordered structures, respectively (Lewis et al., 2013). The band at 1671 ± 3.0 may be assigned to β-turns, while the band at 1689 ± 2.0 cm^{-1} is associated with β-sheet structure (Kong & Yu, 2007). The band centered around 1656 cm^{-1} was assigned to α-helical structure (Lewis et al., 2013). Finally, the absorption band at 1650 cm^{-1} was assigned to unordered structures (Lewis et al., 2013). MP2 derived from Agrimos® and YCW-b had a major absorption band at 1624 cm^{-1}, assigned to β-sheets. No significant changes in the spectra of MP2 derived from Agrimos® and YCW-b were observed following thermal treatment at 90 ℃ for 2 weeks (data not shown), which might be due to their low protein content (<7%) or higher thermal stability. In the case of MP1 derived from Agrimos®, the secondary structure of the native protein, as estimated from the relative intensities of the amide I band components, consisted of 2.6% aggregated strands, 66.1% β-sheet, 42.6% β-turns, 17.5% α-helix and 31.5% random coil. The FTIR spectrum of the heated Agrimos® MP1 showed an increase in β-sheet (17.4%), β-turns (15.7%), and aggregated strands (≈6%) and a decrease in random coils (21.7%) and disappearance of α-helical structure, which indicates that the mannoprotein's secondary structure changed upon incubation at 90 ℃ for 2 weeks. Similarly, a solid-state FTIR study of lysozyme showed that when the protein was heated above its denaturation temperature (T_d) in the solid state, the random coil signal significantly decreased while β-sheet increased (Cruz‐Angeles et al., 2015).

Table 6.3. Band assignments for the Fourier self-deconvoluted FTIR spectrum of MP1 in D_2O solution

Absorption band (cm^{-1})	Assignment
1743	C=O stretch, carbonyl and ester groups
1713.2	protonated COOH, side chain
1704.7	COOH side chain, Asp or Glu
1677.1	turns
1666.3	turns
1657.1	alpha-helix
1645.6	random coil
1616.5	Intermolecular β-sheet (protein aggregation)
1583	COO^-, Asp
1561.4	COO^-, Glu
1547.3	amide II, alpha-helix shielded
1534.5	amide II, beta-sheet shielded
1513.9	tyrosine

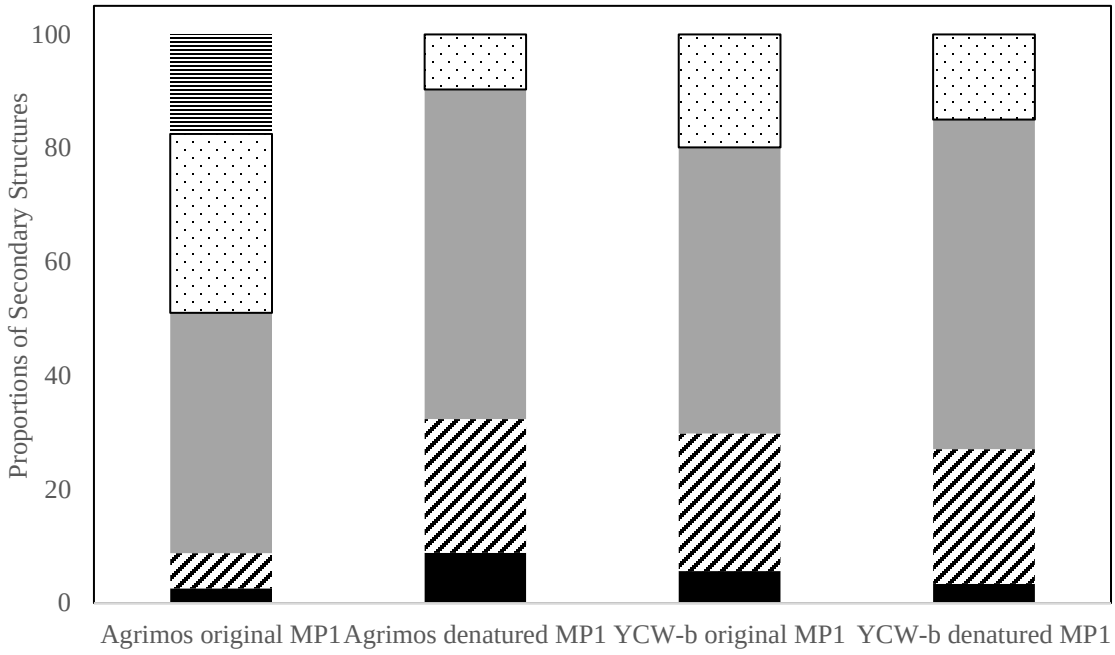
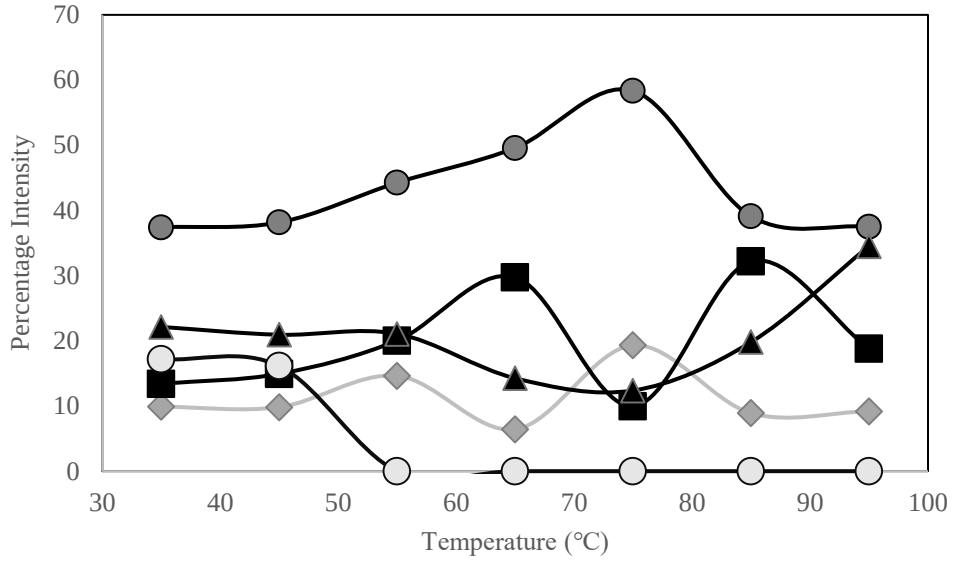


Figure 6.7. Changes in secondary structure compositions of MP1 derived from Agrimos® and YCW-b following thermal denaturation in the solid state, as estimated from Fourier transform infrared spectra. Aggregation (■), β-sheet (▨), β-turns (▩), random coil (⬚), α-helix (▤)

The FTIR spectrum of native MP1 derived from YCW-b indicated that the secondary structure consisted of 6% aggregated β-strands, 24% β-sheet, 58% β-turns, and 20% random coils (Figure 6.7). The changes in the secondary structure composition of MP1 derived from YCW-b upon heat denaturation in the solid state, as estimated from the FTIR spectra, were minor compared to those observed for MP1 derived from Agrimos® and consisted of a small decrease in aggregated strands (≈2%), β-sheet (0.5%) and random coils (5%) and a small increase in β-turns (8%). This difference may be attributed to different protein-to-sugar ratios in MP1 derived from Agrimos® and YCW-b and differences in protein profiles. The secondary structure compositions in aqueous-D_2O solution measured at 35 ℃ differed from those in the solid state, owing to the interaction of the mannoproteins with water (Figure 6.8). Again, due to the low protein content in MP2 derived from Agrimos® and YCW-b, no spectral changes indicative of changes in secondary structure were observed in the temperature range of 35-95 ℃ and the predominant secondary structure was β-sheet (data not shown). The secondary structure of Agrimos® MP1 consisted of 10% aggregated strands, 37% β-sheet, 13% β-turns, 17% α-helix and 22% random coils at 35 ℃. Upon heating of the solution to 45 ℃, minor conformational changes occurred, while at 55 ℃, a pronounced reduction in the proportion of α-helical structure occurred and was accompanied by a 5% increase in intermolecular β-sheets, characteristic of protein aggregation, as well as a 6% increase in intramolecular β-sheet structure and a 5% increase in β-turns. These results suggest that Agrimos® MP1 in aqueous solution begins to denature at 55 ℃. To date, no other study has examined the structural changes of mannoproteins in the solid state or in aqueous solution. Moreover, the denaturation temperature of mannoproteins has not been investigated. A study showed that patatin, a major protein in potatoes, has a denaturation temperature of 50-55 ºC, and secondary structure changes in this temperature range were observed in FTIR spectra (Pots et al., 1998). As the temperature increased from 55 to 95 ℃, the heat-induced rearrangement of the secondary structure continued to occur, which may be due to the unfolding of the protein with subsequent exposure of hydrophobic groups to the polar environment, allowing them to interact with mannan or form aggregates. The secondary structure composition of YCW-b MP1 differed from that of Agrimos® MP1, consisting of 6% aggregated strands, 41% β-sheet, 4% β-turns, 6% α-helix and 41% random coils at 35 ℃. Again, the difference might be due to differences in protein content and protein profiles as well as differences in the interactions between the proteins and the D_2O solvent.

A



B

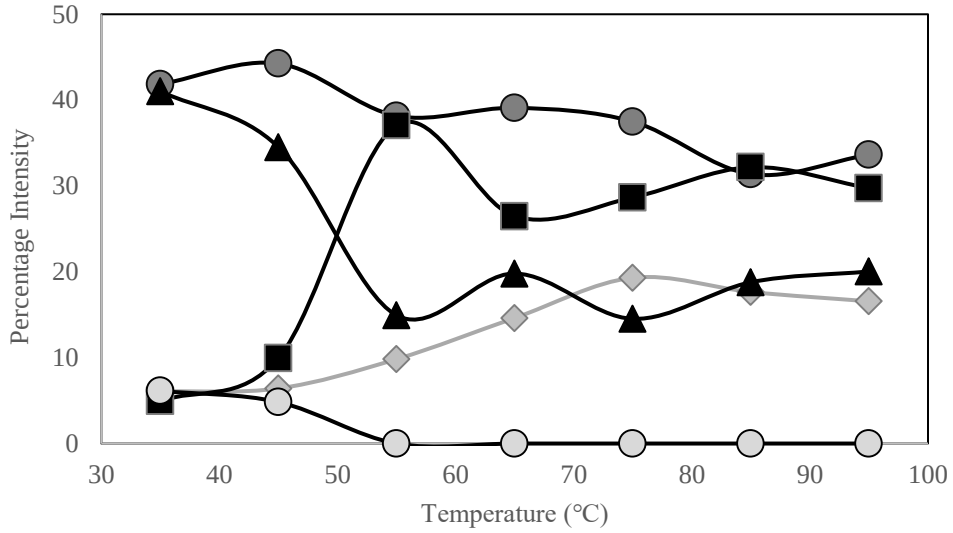


Figure 6.8. Changes in percentage intensities of amide I' band components in FTIR spectra of MP1 derived from Agrimos® (A) and YCW-b (B) in D_2O solutions heated to different temperatures: Aggregation ( ), β-sheet ( ), β-turns ( ), random coil ( ) and α-helix ( ).

At 55 ℃, disappearance of α-helical structure and increased aggregation occurred. At 65 to 75 ℃, more aggregation occurred. The pattern of conformational changes for YCW-b MP1 was similar to that for Agrimos® MP1 in terms of the decrease of α-helical structure and increased aggregation as the temperature increased from 35 to 55 ℃, but was different in terms of the changes in β-sheet structure and β-turns.

6.5 Conclusion

In present study, two main populations of mannoproteins (MP1, MP2) were isolated. To assess the presence of α-1,6 mannose linkages, α-1,6 mannanase treatment analysis was used. α-(1,6) mannose linkages were detected in the high MW MP1 population, but not in the low MW MP2 population. The extracted mannoproteins were mainly O-glycosylated. Further NMR analysis indicates that within the 7 anomeric carbons determined in Agrimos® MP1, 4 of them have very similar chemical shifts than those of mannan standard. The proportion of α-(1,6) linked mannan was found to be the highest in Agrimos® MP1, which agrees well with the results of α-(1,6) mannanase treatment. Furthermore, heat inducing methods were used to analyze the secondary structure changes of mannoproteins in both solid and liquid state using FTIR.

CONNECTING STATEMENT 5

The development and optimization of Zymolyase® enzymatic approach for the isolation of mannoproteins from yeast-based products were investigated in chapters III-VI. The techno-functional and structural properties of the purified mannoproteins were also reported in these chapters. Chapter VII focuses on the development of an efficient and sustainable enzymatic approach, based on the use of proteases, to isolate and generate β-glucan from yeast cell wall. The use of proteolytic enzyme combinations consisting of Alcalase, Novo-proD and Flavourzyme were optimized using response surface methodology RSM and a central composite rotatable design (CCRD).

Li, J., & Karboune, S. (2018). Proteolytic enzymes-based approach for the generation of β-glucan from yeast cell wall: A comparative study (To be submitted).

CHAPTER VII

PROTEOLYTIC ENZYMES-BASED APPROACH FOR THE GENERATION OF β-GLUCAN FROM YEAST CELL WALL: A COMPARATIVE STUDY

7.1 Abstract

β-glucan has been associated with several functional and health-promoting properties. An enzymatic approach, based on the use of proteases, including Flavourzyme, Novo-proD, Papain and Alcalase, was investigated for the isolation and generation of β-glucan from yeast cell wall. The combination of hot water extraction step with protease treatment was found to improve the generation efficiency of β-glucan by opening the cell wall network and improving the accessibility of proteases to the proteins substrates. Alcalase and Novo-ProD showed higher specificity for hydrolyzing peptide bonds that have large hydrophobic aromatic amino acid (tyrosine, tryptophan, and phenylalanine). In addition, this study revealed the synergistic actions between the proteolytic enzymes. The supplementation of Alcalase (0.1%, v/w) with Novo-ProD (0.5%, v/w) resulted in the highest total and β-glucan content of 62.1 and 44.2% (w/w), respectively; such increase corresponds to 98% enrichment of yeast cell wall with β-glucan. The supplementation of Alcalase (0.1%, v/w) with Papain (0.5%, w/w) and Flavourzyme (0.5%, v/w) led to 76.5 and 47.3% enrichment of yeast cell wall with β-glucan. A 5-level, 3-variable central composite rotatable design (CCRD) was performed with varying concentrations of Alcalase (0-0.82% v/w), Novo-proD (0-0.82% v/w) and Flavourzyme (0-0.82% v/w); and the responses were total glucan content, β-glucan content, β-glucan enrichment, weight loss, protein loss, glucan loss and mannan loss. The developed models are expected to provide the capability to enrich β-glucan in the extract and broaden their applications as value added ingredients.

7.2 Introduction

Over the past years, extensive nutritional and epidemiological research has established strong evidence of the important role of a healthy diet in preventing chronic diseases (Organization, 2003). Therefore, functional ingredients are becoming of primary importance with the consumer's growing health consciousness, the aging of the population, and the increasing cost of health care. In this context, the saccharide and the compositional biodiversity of yeast cell wall is of high interest as it would allow for access to a broad spectrum of β-glucan with structural and functional properties different from those currently available commercially (Kogani et al., 2008). Despite the easy acquisition and relatively low cost of yeast cell wall as an abundant affordable source, so far its use as a source of functional food ingredients has been little explored.

β-Glucan is one of the major components and represents around 50–60% of the yeast cell walls (Lipke & Ovalle, 1998). The structure of β-glucan derived from yeasts differs from the ones obtain from cereal, such as oat, barley and wheat. In the yeast cell wall, the major component (about 85 %) is branched β-1,3-glucan of high molecular weight (~240kDa), corresponding to a degree of polymerization (DP) of 1450, while the minor component is branched β-1,6–glucan, which only accounts 15% of the glucan and has a molecular weight of 22 kDa (Manners et al., 1973; Bohn & BeMiller, 1995; Kollár et al., 1997; Freimund et al., 2003). In contrast, β-glucan derived from oat and barley cell walls normally contained unbranched β-glucan with 1,3 and 1,4 β-linked glycopyranosyl residues (Volman et al., 2008). Contrary to water-soluble oat β-glucans, yeast cell wall β-glucans are covalent linked to chitin, which accounts for 1-2% of the yeast cell wall; this facilitates their recovery upon isolation. However, after removal of chitin from yeast cell wall, the solubility of β-glucan increases dramatically (Sietsma & Wessels, 1979; Grün, 2003). β-Glucan derived from different sources can also vary in molecular mass, tertiary structure, degree of branching, polymer charge and conformation (triple or single helix or random coil) (Volman et al., 2008). Some studies have shown that primary structure, charge, solubility, conformation and particle dimensions of β-glucan are the main factors affecting the β-glucan biological activity (Tzianabos & Cisneros, 1996; Větvička et al., 1996; Hunter et al., 2002), and hence their functional and health-promoting properties. In addition to β-glucan, α-glucan was also found in the insoluble fraction of yeast cell wall and was described as "difficult to wash away" yeast glycogen (Kwiatkowski et al., 2009; Kwiatkowski & Kwiatkowski, 2012). Yeast cell wall α-(1,4)-glucan consists of a branched structure with 10–14 residues of α-D-glucose (Kwiatkowski et al., 2009). The α-(1,4)-glucan content of yeast cell wall varied, from 1 to 29% of the dry weigh depending on the cell wall preparation method, the environmental conditions, the nutritional status of the cells and the time the cells were harvested (Kwiatkowski et al., 2009).

The isolation of glucans from yeast cell wall without affecting their structural and functional properties is particularly challenging because of the existence of linkages between glucan and other cell wall components (Ahmad et al., 2010). Many studies have investigated the isolation of β-glucan from yeast cell wall using hot alkali, acids or acid-alkaline methods (Grigorij Kogan, Alföldi, & Masler, 1988; Masler, Šandula, Ferenčík, & Repáš, 1991; Jamas, Rha, & Sinskey, 1993). Despite the high yield of acid extraction of β-glucan compared to the hot water treatment, it was reported of being non appropriate because of the high salt content and the release of carcinogenic

compounds, such as dichloropropanol (Vukašinović-Milić et al., 2007). With the alkaline extraction method, the yeast cell wall components were recovered into two fractions: alkali-soluble β-glucan and alkali-insoluble β-glucan (Schiavone et al., 2014). However, 5 to 10% of impurities, mainly proteins and mannans, were present in β-glucan extracts upon alkaline extraction. In addition, the use of drastic conditions in the acid-alkaline extractions can degrade the glucan' structure severely (Müller et al., 1997; Jaehrig et al., 2008), while hot water extraction has the advantage to preserve the molecular structure of β-glucan (H.-Z. Liu et al., 2011). Because of the specificity and the mild conditions of the enzymatic extraction, it has been identified as a promising method for the isolation of β-1,3-glucan (Freimund et al., 2003). So far, only limited studies did investigate the efficiency of proteases on the β-1,3-glucan isolation (Ferrer, 2006). As far as the authors are aware, no study did investigate the synergistic actions of proteases for the efficient isolation of yeast β- glucan. The main objective of the present study was to investigate the efficiency of the hot water and the protease treatments as well as their combination for the isolation of β-glucan from yeast cell wall. The effects of protease specificity and their synergistic actions were elucidated through the use of selected proteases, including Flavourzyme, Novo-proD, Papain and Alcalase as a monocomponent and a mixture.

7.3 Materials and methods

7.3.1 Materials

Dried yeast cell wall (Agrimos®, from *S. cerevisiae*) was a gift from Lallemand Inc. (Montreal, QC). The proteases, including Flavourzyme from *Aspergillus oryzae*, Novo-proD from *Bacillus sp*., and Alcalase from *Bacillus licheniformis*, were acquired by Novozymes (Bagsværd, Denmark), while Papain from papaya (>46000 USP/NF U/mg) was obtained from Acros Organics (NJ, USA). Standards such as mannan (from *S. cerevisiae*), D-(+)-Glucose, D-(+)-Mannose, and glycogen (from oyster) were purchased from Sigma-Aldrich (Saint Louis, MO). Chemical reagents including sulphuric acid, barium hydroxide monohydrate, potassium hydroxide, sodium hydroxide and anhydrous sodium acetate were also obtained from Sigma-Aldrich. Amyloglycosidase (3260 U/mL, E-AMGDF) and β-glucan from barley (medium viscosity) were obtained from Megazyme (Bray, Ireland).

7.3.2 Proteolytic Activity Assay

The proteolytic activity of the selected proteases was assessed using a chromogenic azocasein as substrate. The assays were initiated by the addition of 350 µL of the diluted enzyme solution to 300 µL of 0.4% (w/v) azocasein in 50 mM potassium phosphate buffer (pH 8) and incubated at 50°C for 20 min. Afterwards, the reaction mixture was then halted by the addition of 150 µL ice-cold 30 % (w/v) trichloroacetic acid solution. The unhydrolyzed azocasein proteins were recovered by centrifugation at 2000 rpm for 10 min. 500 µL of the obtained supernatant was mixed with 500 µL of 2.5 M KOH solution, and the absorbance of the released azo-dye was measured at 440 nm. The assay was carried out in duplicate for all samples. Substrate and enzyme blanks were run in tandem with the proteolytic assays.

7.3.3 Hot water treatment

A 10 % (w/v) suspension of Agrimos® was dissolved in MilliQ water or adjusted to pH 7 using NaOH. The suspension was heated at 80, 100, 110 and 120˚C at varying time intervals of 2, 6, 30, 240 min. 110 and 120˚C was achieved by using autoclave (above atmosphere pressure). Afterwards, the suspension was cooled to 45˚C. The insoluble residues, containing glucan, were recovered by centrifugation at 8000 rpm for 15 min and freeze dried. The recovered crude glucan extracts were analyzed for their glucan, mannan and protein contents.

7.3.4 Protease Treatment

The efficiency of protease treatment to isolate β-glucan was investigated using selected proteases, including Alcalase, Flavourzyme, Novo-proD, and Papain. A 10 % (w/v) suspension of Agrimos® at pH 8.0 was prepared and subjected to protease treatment (0.1-1% v/w). The mixtures were incubated at 60˚C for 5 h with an agitation speed of 150 rpm. In tandem with the reactions, blanks containing deactivated proteases (treated at 100˚C for 20 min) and Agrimos® suspension were run. After 20 min of protease treatment, the insoluble residues, containing β-glucan, were recovered by centrifugation at 8000 rpm for 15 min. The recovered enriched β-glucan extracts were analyzed for their glucan, mannan and protein contents.

7.3.5 Measurement of Total Glucan and Mannan Contents

To assess the efficiency of the chemical hydrolysis methods and estimate the correction factors, mannan and β-glucan were hydrolyzed along with the samples, as both internal and external standards. Moreover, glucose and mannose standards were also subjected to the acid chemical

hydrolysis assays to determine their stability.To determine the monosaccharide composition, the acid chemical hydrolysis method is based on a two-step procedure according to the method of Khodaei & Karboune (2013). The monosaccharides were measured using High-Pressure Anionic Exchange Chromatography equipped with Pulsed Amperometric Detection (HPAEC-PAD) (Dionex, ICS-3000) and a CarboPac PA20 column (3 × 150 mm) set at 32 °C. Standard curves of D-glucose and D-mannose were constructed in the range of 5–40 mM each time HPAEC analysis of samples was carried out.

7.3.6 Measurement of α-Glucan Content

To quantify the α-glucan content, original Agrimos® samples and the recovered β-glucan extracts (0.05 g/ml) were suspended in 2 M KOH. After 20 min of incubation, 1.2 M sodium acetate buffer (pH 3.8) was added to yield β-glucan suspension at a concentration of 0.01g/ml. Then, amyloglycosidase (32 U/mL) was added to the mixture and incubated at 40˚C for 30 min. The supernatants recovered upon centrifugation (1,500 rpm, 10 min) were analyzed by HPAEC to quantify the released glucose. Glycogen standard was hydrolyzed as an external standard and used for the quantification of α-glucan.

7.3.7 Determination of Protein Content

The yeast cell wall and the recovered glucan extracts were analyzed for their crude protein contents. Total nitrogen was determined by dry combustion according to the Dumas method (Kirsten and Hesselius (1983) using a NA1500 Nitrogen analyser (CE Instruments, Milan, Italy), and the crude protein was calculated as the total nitrogen content multiplied by 6.25.

7.3.8 Optimization, Experimental Design and Data Analysis

In order to optimize the production of β-glucan-enriched extract, a set of enzyme combinations consisting of Alcalase (0-0.82% v/w), Novo-proD (0-0.82% v/w) and Flavourzyme (0-0.82% v/w) were evaluated with response surface methodology RSM and a central composite rotatable design (CCRD). A 10 % (w/v) suspension of Agrimos® at pH 8.0 was prepared. Then protease combinations at selected concentrations were added to the yeast cell wall suspension. The mixtures were incubated at 60˚C for 5 h with an agitation speed of 150 rpm. In tandem with the reactions, blanks containing deactivated proteases (treated at 100˚C for 20 min) and yeast cell wall suspension were run. After stopping the reaction by heat enzyme denaturation, the insoluble residues, containing β-glucan, were recovered by centrifugation at 8,000 rpm for 15 min. The

relationship between variables and responses was expressed by a second order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \sum_j \beta_{ij} x_{ij} + \sum \beta_{ii} x_i^2$$

Where Y is the measured response (glucan, mannan, protein contents), βo is the model constant, and βi, βij and βii are the measures of the linear, interaction, and quadratic effects of variables Xi, XiXj, and X2i, respectively. Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (Version 8.0, Stat-Ease, Inc. Minneapolis, MN, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) including lack of fit, Fisher's F-test (overall model significance), its associated probability P(F) and correlation coefficient R, and determination coefficient R^2 to measure the goodness of fit of the quadratic model.

7.4 Results and Discussion

7.4.1 Efficiency of hot water treatment

The hot water treatment can open the yeast cell wall network and enhance the removal of water-soluble components of the yeast cell wall, such as soluble mannoproteins. Figure 7.1 shows the effect of the hot water treatment at selected temperatures (80-120˚C) and times (2-240 min) on the total glucan recovery yield and protein loss. Overall, the percentage of protein loss increased from 37.9 to 51.3%, when the temperature was increased from 80 to 120°C. Contrary to the temperature, the incubation time didn't have a significant effect on the percentage of protein loss. The total glucan recovery yield was used as an indicator for the degradation and/or the β-elimination of polysaccharides from yeast cell wall. As an overall, the total glucan recovery yield was incubation time dependent at 100 and 120 °C and much less at 110°C. The highest glucan recovery yields of 92-94% were obtained upon hot water treatments at 80 and 120°C for 4 h; these results reveal the minimal structural degradation of glucan at these conditions. However, lower glucan recovery yields of 63- 73% were obtained with other treatments at 110-120°C. The high loss of glucan upon the heat treatment at 120˚C for shorter times (2, 6 and 30 min) may be attributed to the use of low pressure by autoclaving and/or to the formation of glucan agglomerate (cluster). It is known that heat treatment at high pressure leads to low β-elimination of polysaccharides from cell wall as compared to low pressure (J. Chen et al., 2012).

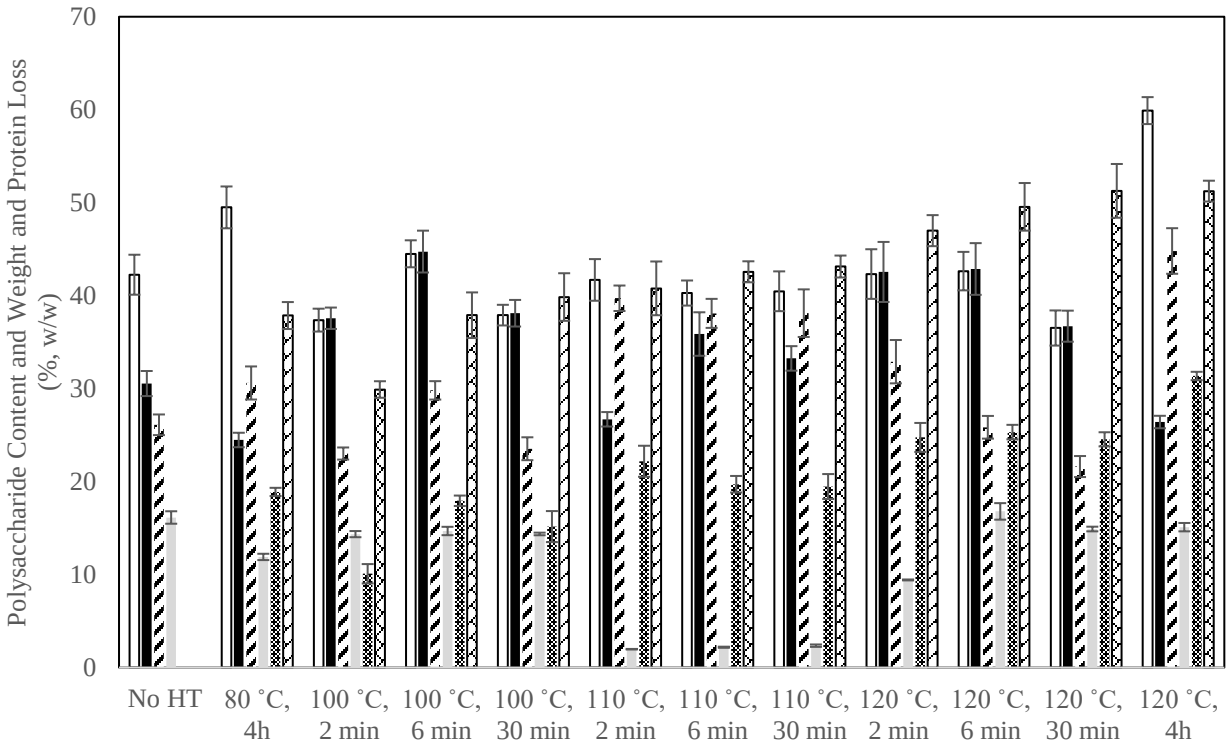


Figure 7.1. Effect of hot water treatment on the recovery of β-glucan from Agrimos®: Total glucan content (□), Mannan content (■),β-Glucan content (▨), α-Glucan content (▩), Weight loss (▩) and Crude protein loss (▨).

Abbreviations: *No Heat treatment (No HT)*

Figure 7.1 also shows total glucan, β-glucan, mannan and α-glucan contents of yeast cell wall upon selected hot water treatments. In comparison with the non-treated samples, the highest total glucan and β-glucan contents of 59.9 and 44.8 % (w/w) respectively, were obtained upon hot water treatment at 120˚C for 4 h; indeed, one third of the weight loss of the cell wall was obtained upon this treatment, which can be attributed mainly to mannoprotein and protein losses, as proven by the high protein loss (51.2%, w/w) and lowered mannan (26.4%, w/w) content (Figure 7.1). Similarly, a study has reported a thermal pretreatment of 125˚C and a 5 h stirring time to be the optimum conditions for the enrichment of glucan; this treatment resulted in a loss of weight of about one third, which was attributed mainly to the losses of mannoproteins and other proteins (Freimund et al., 2003). On the other hand, the yeast cell wall samples treated at 120˚C for 2, 6 and 30 min, did not show an increase in the total glucan content and a decrease in the mannan content, when compared to the non-treated samples (Figure 7.1). In addition, the mannan content was even higher than the non-treated samples, although the crude protein loss was found to be 49.27%, which is very close to the value obtained at 120˚C for 4 h. Indeed, non-covalent attached cell wall proteins are sequestered in the cell wall, either by protein-protein or protein-polysaccharide interactions. It has been reported that these non-covalent attached cell wall proteins interact with β-1,3-glucan by strong non-specific hydrogen-bonds (Teparić et al., 2010). The low removal of mannan in spite of a higher protein removal may also reveal the presence of high proportion of low mannosylated non-covalent cell wall proteins. On the other hand, the increase in glucan loss with an increase in mannan content may reveal the thermal sensitivity of β-1,3-glucan (attached directly to the proteins) as compared to β-1,6-glucan (attached to the reducing end mannose of mannoproteins) by β-elimination (Gemmill & Trimble, 1999). These results may be due to the difference in the molecular vibration and collisions of β-1,3 and β-1,6 linkages under high temperatures (Kivelä et al., 2011).

The use of 110˚C treatment for 2, 6 and 30 min led to 45.9 to 52.1% enrichment with β-glucan (contents of 38.1-39.8%, w/w) as compared to initial cell wall material; the results show that this enrichment was mainly attributed to the removal of protein (loss of 42.16%) and the degradation of α-glucan (loss of 86.5%). At 110 ˚C, the treatment time did not significantly affect the total glucan, β-glucan, α- glucan and mannan contents. The results also show that the hot water treatment at 100˚C for the time intervals of 2 and 30 min did not lead to higher total and β-glucan contents as compared to the non-treated samples. While the use of 6 min treatment at 100˚C seems

to compromise between the isolation efficiency and the glucan degradation by resulting in higher total glucan (44.5%, w/w) and β-glucan (29.8%, w/w) contents than the non-treated samples. With the treatment of 80˚C for 4 hours, a higher total glucan (49.5%, w/w), lower mannan (24.5%, w/w), slightly higher β-glucan (30.6%, w/w) and lower α-glucan (11.9%, w/w) as compared with non-treated samples were observed (Figure 7.1).

7.4.2 Efficiency of combined hot water and protease treatments

The hot water treatment may enhance the protease treatment by opening the cell wall network and improving the accessibility of proteases to the proteins substrates. The effect of Alcalase protease treatment on the β-glucan enrichment was investigated without and with hot water-treatment. Figure 7.2 shows the total glucan, β-glucan, mannan and α-glucan contents of the enriched β-glucan extracts recovered upon protease treatment and combined hot water/protease treatments. Protease treatment alone leads to a moderate β-glucan enrichment of 13.4% with a total glucan and β-glucan contents of 46.92 and 32.0% (w/w), respectively. The highest total glucan (61.3%, w/w) and β-glucan (47.1%, w/w) contents, and the lowest mannan content (21.2%, w/w) were achieved upon hot water treatment at 120˚C for 4 h followed by protease treatment, with β-glucan enrichment of 66.5% (w/w). The combination of a hot water treatment at 80˚C for 4 h and the protease one resulted in an enriched extract with 54.8% (w/w) of total glucan and 40.1% (w/w) of β-glucan contents. However, because of the higher loss of α-glucan in the cell wall treated at 110˚C, a high β-glucan content of 43.93% (w/w) was obtained upon hot water/protease treatment (110°C, 6 min). Although no β-glucan enrichment was observed upon the hot water treatment at 100°C for 2 min, this treatment did improve the efficiency of protease treatment with 17.5% β-glucan enrichment (Figure 7.3). Overall, it can be concluded that the contribution of the hot water treatments to the β-glucan enrichment was much higher than that of the protease treatment, expected at 100°C.

7.4.3 Effect of Protease Type and Concentration

The efficiency of selected proteases (Novo-proD; Flavourzyme; Papain) alone and in combination with Alcalase to enhance the β-glucan enrichment of yeast cell wall was studied. Proteases are subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their cleavage site.

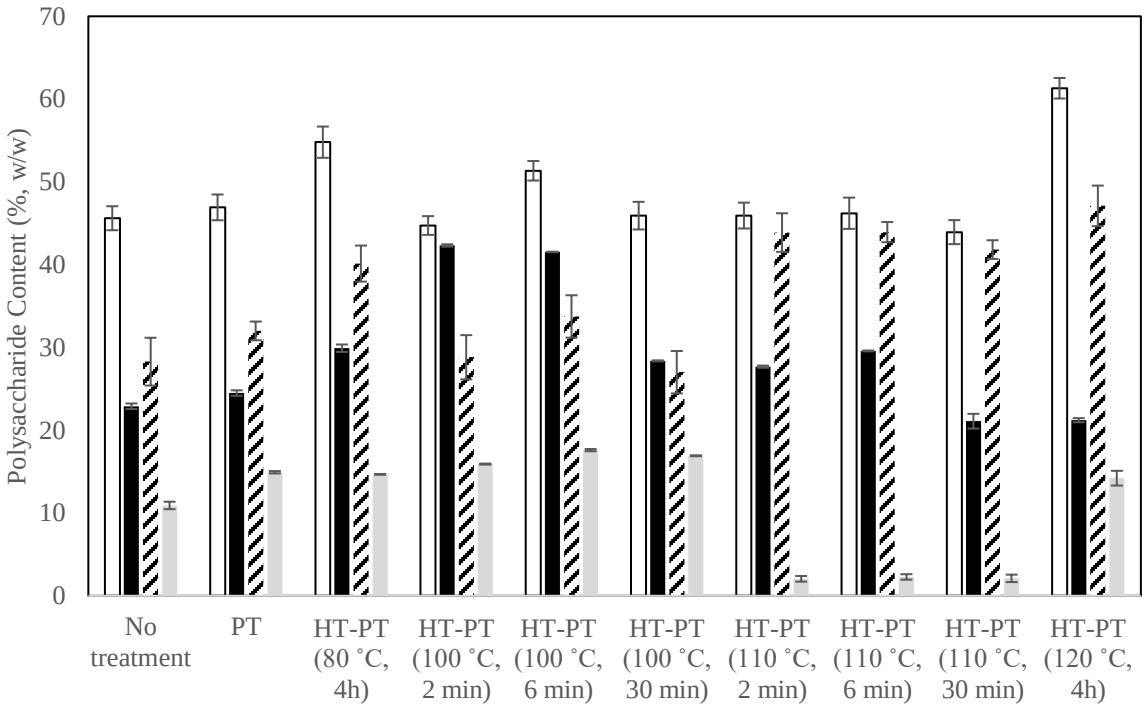


Figure 7.2. Effect of combined hot water and protease treatments on the β-glucan enrichment of Agrimos®: Total glucan content (□), Mannan content (■), β-Glucan content (▨), α-Glucan content (▩).

Abbreviations: *Alcalase treatment (PT), hot water treated (HT)*

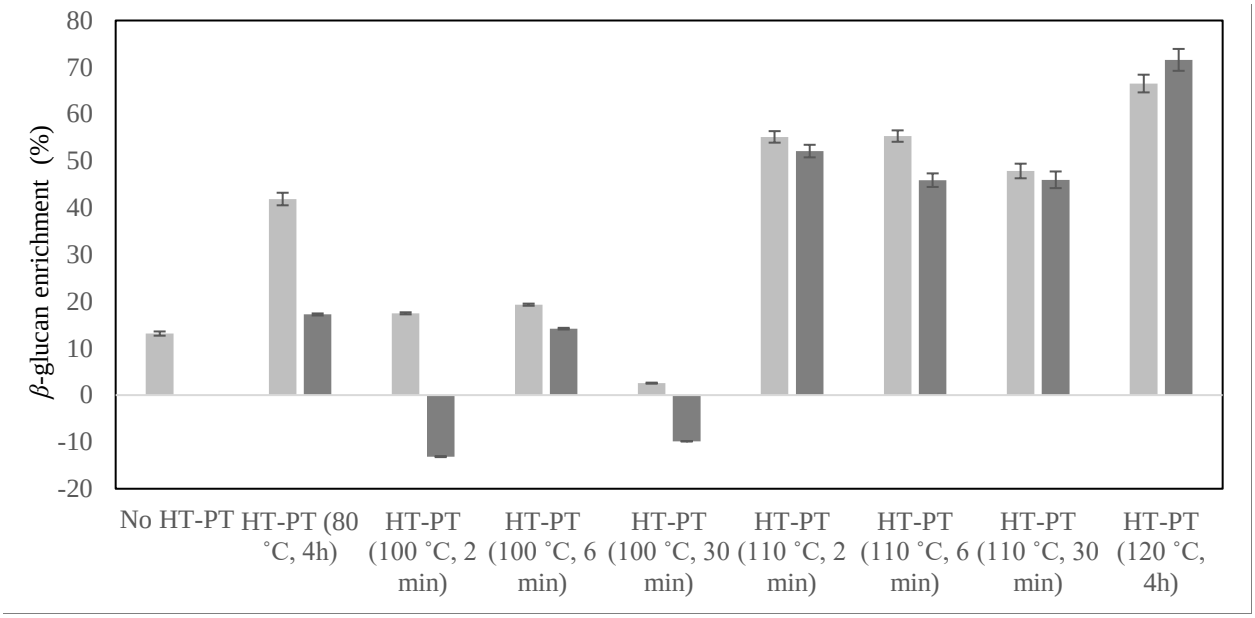


Figure 7.3. Effect of combined hot water/protease treatments (▒) and heat pretreatment (▒) on the enrichment level of β-glucan in Agrimos®.

Abbreviations: *Alcalase treatment (PT), hot water treated (HT)*

Exopeptidases cleave the peptide bonds at the terminal amino or carboxy groups leading to the release of amino acids, whereas endopeptidases cleave peptide bonds of nonterminal amino acids, leading to the release of peptides. Proteases (endo-type) are further divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases (Rao et al., 1998). Among the investigated proteases, only Flavourzyme is an aminopeptidase (exo-type). Alcalase and Novo-ProD are serine proteases that fall into the category of subtilisin-like proteases based on their structure. In general, serine proteases can be divided into two categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. Subtilisin-like proteases have generally broad specificity profiles and often display a preference for hydrolyzing peptide bonds that have large hydrophobic aromatic amino acid (tyrosine, tryptophan, and phenylalanine) (Waglay & Karboune, 2016). On the other hand, papain is a cysteine endo-protease and has been reported to be a mixture of cymopapain and lysozyme. Based on their side chain specificity, cysteine proteases are broadly divided into four groups: (i) papain-like, (ii) trypsin-like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others (Rao et al., 1998). Papain-like proteases exhibit broad specificity, with a preference for cleaving peptide bonds involving the carbonyl end of α-NH_2 substituted basic amino acids, arginine and lysine, and to a lesser extent, histidine, glycine, glutamine and tyrosine; Phenylalanine in a position Phe-X- enhances the susceptibility of a peptide to hydrolysis at the C-terminal end of X (Berger & Schechter, 1970).

Figure 7.4 shows the total, β- and α-glucan contents as well as the mannan content of the enriched cell wall obtained after selected protease treatments. Overall, the efficiency of Alcalase treatment was dependent on its concentration. Increasing the amount of Alcalase from 0.1 to 1% (v/w) resulted in an increase of the total and β-glucan contents from 39.6 to 56.8% and from 22.9 to 40.9%, respectively. The low efficiency at low Alcalase concentration of 0.1% (v/w) can be attributed to the low hydrolysis and/or solubilization of proteins. At a similar concentration of 0.5% (v/w), the efficiencies of Papain, Flavourzyme and Novo-proD treatments for the enrichment of yeast cell wall with total glucan were higher as compared to that of Alcalase. However, Novo-ProD treatment (~8%) resulted in a more or less similar enrichment level of cell wall with β-glucan as compared to Alcalase (~6%). On the other hand, Flavourzyme and Papain treatments resulted in 34.2 and 55.8% enrichment of cell wall with β-glucan, respectively, as compared to the initial cell wall (Figure 7.4).

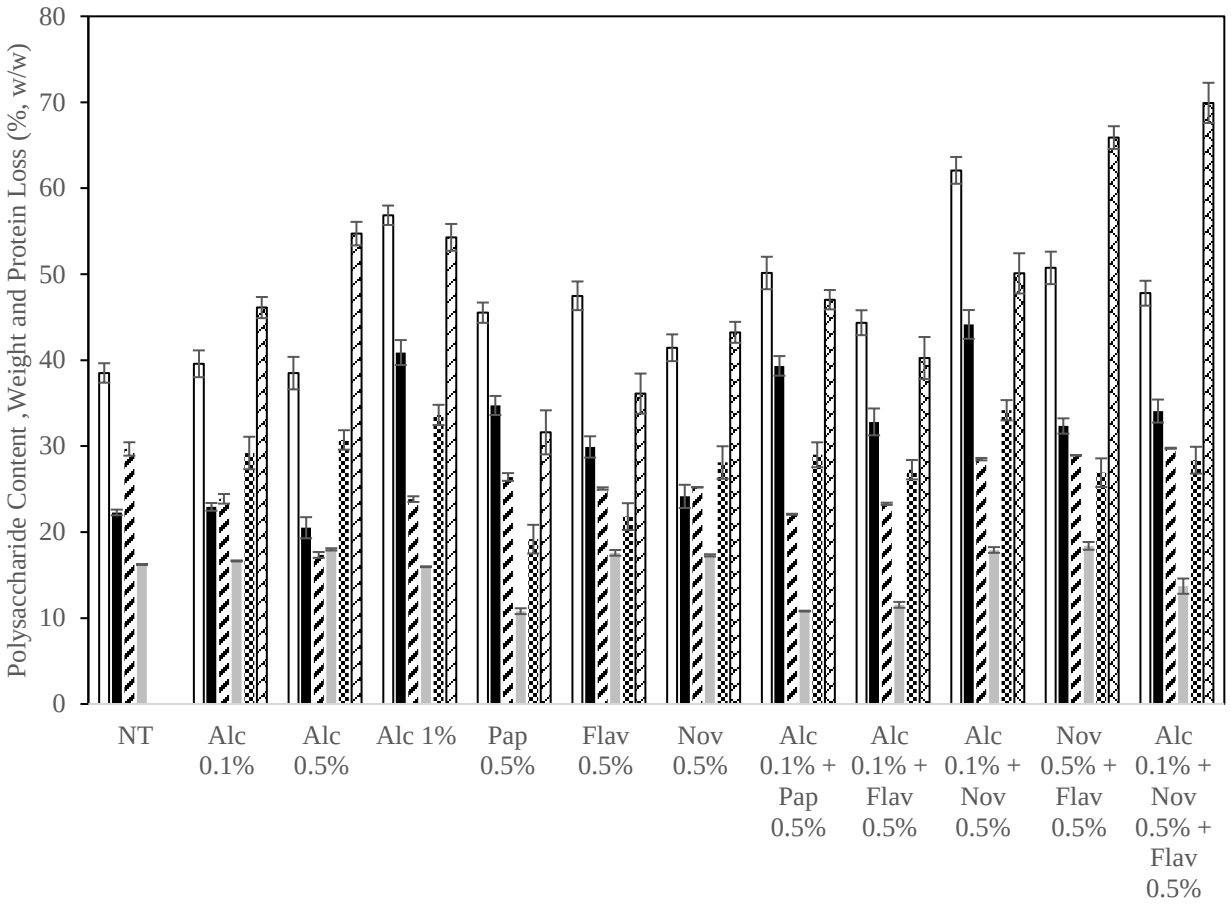


Figure 7.4. Effect of different protease treatments on the enrichment of β-glucan in Agrimos®: Total glucan content (□), Mannan content (■),β-Glucan content (▨), α-Glucan content (▩), Weight loss (▩) and Crude protein loss (▨).

Abbreviations: *No treatment (NT), Alcalase (Alc), Papain (Pap), flavourzyme (Flav), Novo-proD (Nov)*

The results (Figure 7.4) also indicate the hydrolysis and/or the solubilisation of 31.6, 36.1 and 43.2% of total proteins upon treatments of cell wall using Papain, Flavourzyme and Novo-proD, respectively. The high β-glucan enrichment upon papain treatment cannot be attributed to the efficiency of the removal of protein, but to the decrease in the α-glucan content. Indeed, it has been reported that papain contains lysozyme, which is an efficient enzyme for the hydrolysis of peptidoglycan (Uhlig, 1998). Many yeast cell wall mannoproteins have been reported to carry N-linked glycans with a core structure of $Man_{10–14}GlcNAc_2$-Asn (Peter Orlean, 1997). The efficiency of Alcalase and Novo-ProD for the removal of proteins may be attributed to their high proteolytic activity (data not shown) and/or to their high specificity for hydrolyzing peptide bonds that have large hydrophobic aromatic amino acid (tyrosine, tryptophan, and phenylalanine). Indeed, serine and threonine residues were reported to be clustered within the sequences of cell wall mannoproteins (van der Vaart et al., 1995; Lesage & Bussey, 2006). Different combinations of proteases were examined in order to assess their efficiency and synergistic interactions. All protease combinations exhibited higher efficiency for the enrichment of yeast cell wall with β-glucan as compared to the use of each mono-components of protease separately (Figure 7.4). These results reveal the synergistic interaction of the investigated proteases and the efficiency of the combinations for improving the enrichment with β-glucan. Indeed, the supplementation of Alcalase (0.1%, v/w) with Novo-ProD (0.5%, v/w) resulted in the highest total and β-glucan content of 62.1 and 44.2% (w/w), respectively; such increase corresponds to 98% enrichment of yeast cell wall with β-glucan. The supplementation of Alcalase (0.1%, v/w) with Papain (0.5%, w/w) and Flavourzyme (0.5%, v/w) led to 76.5 and 47.3% enrichment of yeast cell wall with β-glucan. As compared to Novo-ProD, the low efficiency of Papain and Flavourzyme in acting synergistically with Alcalase may be due to their action mode and specificity. Flavourzyme is an exo-type protease, while Papain is an endo-protease having a preference for cleaving peptide bonds of involving basic amino acids, arginine and lysine (Waglay & Karboune, 2016). Novo-ProD/ Flavourzyme combination (65.9% protein loss) resulted in higher solubilisation or hydrolysis of proteins as compared to Alcalase/ Flavourzyme (40.2% protein loss) (Figure 7.4). However, the efficiency of both Novo-ProD/ Flavourzyme and Alcalase/ Flavourzyme for the enrichment of yeast cell wall with β-glucan were similar. Such findings may be attributed to the efficiency of Alcalase/Flavourzyme in decreasing the mannan content of enriched yeast cell wall. The results also show that although the additional substitution of Alcalase/Novo-ProD with Flavourzyme

resulted in the highest solubilisation and/or hydrolysis of proteins, it decreased the extent of the enrichment of yeast cell wall with β-glucan from 98 to 52%. Such results may be due to the loss of glucan, which was significant upon Alcalase/Novo-ProD/Flavourzyme treatment (20%) than Alcalase/Novo-ProD treatment (11.1%) (data not shown).

The best identified protease combination for an efficient enrichment of yeast cell wall with β-glucan was Alcalase /NovoproD, which could be attributed to both their high proteolytic activity (data not shown) and their synergistic effect. Freimund (2003) reported the hydrolysis of 75% of the remained proteins after hot water extraction upon the treatment with Savinase. Savinase was reported to be an endopeptidase with broad specificity (Georgieva et al., 2001). In addition, X. Y. Liu. et al. (2008) investigated the protease treatment of yeast cell wall primarily subjected to multiple extraction steps of hot water extraction, homogenization and solvent extraction; the investigated proteases included, protease 1398, Protamex, FM.2.0L and Neutrase, and they reported that Protamex was the most efficient enzyme with an optimum condition of pH 7.5, temperature of 55˚C and an incubation time of 5h. Following the Protamex treatment, the content of β-glucan was reported to have increased to 93.12% (X. Y. Liu et al., 2008).

7.4.4 Optimization of Combined Proteases-based Treatment on β-Glucan Enrichment

To investigate the synergistic actions of Alcalase (X_1, serine type endo-protease)/Novo-ProD (X_2, endo-protease and exo-peptidase)/Flavourzyme (X_3, endo-protease and exo-peptidase) proteases combination, RSM approach was applied (Table 7.2). The experimental design was performed based on the central composite design and the levels of the selected parameters were set based on preliminary trials, where one factor at a time was varied (data not shown). The contents of total glucan, β-glucan, and the losses in weight, protein, glucan, and mannan were chosen as the elected responses to evaluate the efficiency of β-glucan enrichment. Table 7.2 shows the experimental conditions and their corresponding experimental results.

Table 7.2. Central composite quadratic design of actual independent variables and the observed response

Run	Alcalase X_1[a]	Novo-ProD X_2[a]	Flavourzyme X_3[a]	Total glucan [b]	β-glucan [b]	β-glucan Enrichment	Weight loss[c]	Protein loss[c]	Glucan Loss	Mannan Loss
1	0.52	0.52	0.52	50.48	32.57	37.75	32.21	67.05	14.41	43.18
2	0.52	0.52	0.52	47.99	28.66	19.93	34.02	68.19	21.25	50.31
3	0.52	0.52	0.52	51.57	28.25	18.20	33.54	67.63	13.56	38.09
4	0.52	0.52	0.52	55.31	33.13	38.60	33.09	67.56	11.32	50.73
5	0.52	0.52	0.52	50.78	32.97	37.95	32.31	67.15	14.51	43.28
6	0.52	0.52	0.52	51.50	32.97	37.95	32.65	68.47	13.74	51.88
7	0.52	0.52	0.52	50.51	32.77	37.12	31.55	67.15	12.36	47.77
8	0.52	0.52	0.52	51.61	35.42	48.20	31.09	65.67	24.50	59.61
9	0.52	0.52	0.52	48.64	31.70	32.62	31.79	65.74	16.24	50.32
10	0.52	0.52	0.52	48.09	29.57	23.74	32.79	68.42	19.62	48.02
11	0.52	0.52	0.52	50.86	32.43	35.70	32.66	67.79	18.01	40.96
14	0.52	0.52	0.52	54.87	31.10	30.12	33.51	68.17	9.26	51.91
15	0.52	0.52	0.52	56.08	26.75	31.91	32.79	68.33	15.87	48.66
16	0.82	0.21	0.82	57.56	42.32	77.09	33.03	64.45	6.69	36.64
17	0.82	0.21	0.82	55.67	38.70	61.93	31.71	64.99	5.44	35.27
18	0.82	0.21	0.82	56.64	41.74	74.64	32.86	64.45	5.41	36.91
19	0.21	0.21	0.21	48.38	31.23	30.68	23.84	63.49	12.36	38.05
20	0.21	0.21	0.21	51.00	33.17	38.81	22.70	67.02	4.56	53.42
21	0.21	0.21	0.21	47.24	30.93	29.42	28.19	66.65	8.56	48.21
22	0.21	0.82	0.82	56.62	42.66	78.48	33.11	66.49	9.28	34.45
23	0.21	0.82	0.82	54.79	39.82	62.42	32.63	66.59	8.20	44.83
24	0.21	0.82	0.82	57.71	41.19	72.34	33.30	67.23	9.53	40.25
25	0.82	0.82	0.21	46.22	23.82	1.30	34.66	66.69	22.25	61.15
26	0.82	0.82	0.21	45.10	23.20	1.93	34.12	65.34	26.10	69.67
27	0.82	0.82	0.21	43.94	22.20	1.12	31.50	66.47	25.13	58.96
28	0.82	0.82	0.82	44.41	24.33	1.8	34.66	65.70	24.92	57.13
29	0.82	0.82	0.82	43.72	22.96	0.53	35.21	65.43	29.54	53.46
34	0.52	0.52	0.22	48.77	39.21	64.08	25.44	67.07	23.65	54.92
35	0.52	0.52	0.22	53.60	37.66	57.59	24.06	66.72	22.65	52.15
36	0.52	0.52	0.22	49.48	32.86	37.49	24.77	67.29	26.30	52.90
37	0.21	0.21	0.82	49.53	33.34	39.49	31.47	65.34	9.86	63.28
38	0.21	0.21	0.82	52.81	34.87	45.92	32.37	65.22	10.70	67.75
39	0.21	0.21	0.82	53.60	37.27	55.95	31.47	65.34	8.56	55.48
40	0.82	0.21	0.21	54.05	39.51	65.29	25.37	70.96	6.46	42.64
41	0.82	0.21	0.21	49.39	38.01	59.04	26.24	68.95	5.47	64.37
42	0.82	0.21	0.21	58.03	41.77	74.76	24.11	66.67	1.00	39.65
43	0.52	0.01	0.52	49.22	30.41	27.25	31.60	66.57	16.26	48.70
45	0.52	0.01	0.52	52.12	32.36	35.39	31.35	66.47	11.01	51.49
46	0.52	1.03	0.52	53.56	27.04	13.13	34.01	63.77	12.09	44.10
47	0.52	1.03	0.52	48.30	28.94	21.10	34.84	63.45	21.73	62.96
48	0.52	1.03	0.52	55.52	35.55	48.76	31.73	63.95	7.88	54.63
50	0.52	0.52	0.01	53.14	27.60	15.48	31.35	67.11	0.01	35.27
53	0.01	0.52	0.52	57.81	36.60	53.12	31.09	65.66	0.01	34.50
54	0.01	0.52	0.52	52.37	33.89	41.79	31.79	65.76	4.18	35.22
57	1.03	0.52	0.52	51.66	33.70	40.99	34.39	67.74	10.91	51.70
58	0.52	0.52	1.03	49.85	41.04	71.72	33.87	65.45	13.33	33.89

[a] Concentration (v/w).[b] Responses are expressed in percentage (w/w) as compared to the enzymatically treated cell wall powder.[c] Responses are expressed in percentage (w/w) as compared to the initial cell wall-enzymes mixture.

7.4.4.1 Analysis of Variance and Model Fitting

Model fitting was performed to the observed data of total glucan, β-glucan, weight loss, protein loss, glucan loss, mannan loss (Table 7.3). Neglecting the insignificant terms by backward elimination regression (α out = 0.05), the results of analysis of variance (ANOVA) indicate that the two-factor interaction model was statistically significant for the description of the variations of the total glucan (*F*-value of 16.86 and *p*-value of < 0.0001), whereas the linear model was significant for the description of the weight loss (*F*-value of 39.78 and *p*-value of < 0.0001).

On the other hand, the quadratic model was statistically significant for the description of the variations of the β-glucan content (*F*-value of 20.85 and *p*-value of < 0.0001) and the loss in protein content (*F*-value of 7.51 and *p*-value of 0.0002). The lack of fit was not significant relative to pure error with *F*-value of 0.42-0.90 and *p*-value of 0.56–0.86; these results indicate a good quality of the fit and its ability to predict within a range of variables employed. In addition, the R^2 of the fitted models ranged from 0.83 to 0.95 indicating that only 5-17 % of the total variations were not explained by the model. No statistically significant model was found to describe the effects of Alcalase, Novo-ProD, and Flavourzyme concentrations on glucan loss and on mannan loss. The fitted models for total glucan, β-glucan, weight loss, and protein loss in terms of coded factors are given in Equations 1-4.

$$Total\ glucan = 44.39 - 1.52X_1 + 0.57X_2 - 1.06X_3 - 4.57X_1X_2 + 1.27X_1X_3 - 1.11X_2X_3 \quad (1)$$

$$\beta\text{-}glucan = 22.70 + 0.04X_1 - 1.79X_2 + 2.49X_3 - 3.93X_1X_2 - 1.58X_1X_3 + 1.84X_1^2 + 1.46X_3^2 \quad (2)$$

$$Weight\ loss = 33.04 + 0.74X_1 + 0.78X_2 \quad (3)$$

$$Protein\ loss = 67.17 + 0.83X_1 - 0.77X_2 - 0.49X_3 - 1.17X_1X_3 + 1.30X_2X_3 - 0.66X_2^2 - 0.36X_3^2 \quad (4)$$

The variable with the largest effect on the total glucan and the β-glucan was the interactive effect between the concentrations of Alcalase and Novo-ProD (X_1X_2, *F*-value of 95.85 and 53.99, respectively, *p*-value of < 0.0001). The negative sign of the term of this interaction reveals the antagonistic interactive effect of Alcalase and Novo-ProD on the total glucan and the β-glucan. Although lesser in importance, the interactive effect between the concentrations of Alcalase and Flavourzyme (X_1X_3, *F*-value of 7.92, *p*-value of 0.0075) and between the concentrations of Flavourzyme and Novo-ProD (X_2X_3, *F*-value of 5.81, *p*-value of 0.0205) on total glucan was statistically significant.

Table 7.3. Analysis of variance (ANOVA) for total glucan, β-glucan, weight loss, and protein loss response models

	Total glucan			β-glucan			Weight loss			Protein loss		
	Sum of square	*F* value	Prob>*F*	Sum of square	*F* value	Prob>*F*	Sum of square	*F* value	Prob>*F*	Sum of square	*F* value	Prob>*F*
Model	476.35	16.86	<0.0001	630.40	20.85	<0.0001	25.80	39.78	<0.0001	34.32	7.51	0.0002
Alcalase X_1	66.65	14.15	0.0005	0.04	0.01	0.9283	8.36	25.79	<0.0001	8.17	12.51	0.0022
Novo-ProD X_2	12.31	2.61	0.1136	59.04	13.67	0.0012	13.07	40.31	<0.0001	8.66	13.26	0.0017
Flavourzyme X_3	37.17	7.89	0.0076	117.46	27.20	<0.0001				4.03	6.18	0.0224
X_1X_2	451.41	95.85	<0.0001	233.14	53.99	<0.0001						
X_1X_3	37.28	7.92	0.0075	33.19	7.68	0.0108				8.26	12.65	0.0021
X_2X_3	27.37	5.81	0.0205							10.02	15.35	0.0009
X_1^2				100.72	23.32	<0.0001						
X_2^2										13.00	19.91	0.0003
X_3^2				42.48	9.84	0.0046				3.01	4.60	0.045
Residual	193.09			99.33			7.13			12.40		
Lack of fit	27.44	0.59	0.7962	12.76	0.42	0.8572	3.05	0.90	0.5627	2.81	0.63	0.7012
Pure Error	165.65			86.58			4.08			9.60		
Cor Total	669.45			729.73			32.93			46.72		

The variable with the second largest effect on the total glucan was the linear term of the concentration of Alcalase (X_1, *F*-value of 14.15, *p*-value of 0.0005), whereas the variable with the second largest effect on the β-glucan was the linear term of the concentration of Flavourzyme (X_3, *F*-value of 27.20, *p*-value of < 0.0001). This result demonstrates the selective action of Flavourzyme on proteins and towards β-glucan release. The interaction between Alcalase and Flavourzyme also significantly affected the extractability of the β-glucan (X_1X_3, *F*-value of 7.68, *p*-value of 0.0108). The results also show that the concentration of Novo-ProD was the most important variable affecting the weight loss (X_2, *F*-value of 40.31, *p*-value of < 0.0001) followed by the concentration of Alcalase (X_1, *F*-value of 25.79, *p*-value of < 0.0001). As expected, all three protease preparations significantly affected the protein loss. The quadratic term of the concentration of Novo-ProD (X_2^2, *F*-value of 19.91, *p*-value of 0.0003) was the most important affecting the protein loss, followed by the interaction between the concentrations of Novo-ProD and Flavourzyme (X_2X_3, *F*-value of 15.35, *p*-value of 0.0009) and by the linear term of Novo-ProD (X_2, *F*-value of 13.26, *p*-value of 0.0017). The interaction between the concentration of Alcalase and Flavourzyme (X_1X_3, *F*-value of 12.65, *p*-value of 0.0021) was also statistically significant in affecting the protein loss.

7.4.4.2 Contour Plots of Responses Obtained upon Proteases Treatment

The 2D contour plots generated from the predicted model of the total glucan show the interactive effect of the Alcalase and Novo-ProD (Figure 7.5A). This interactive effect on the total glucan content was particularly important at lower concentrations of Alcalase (<0.51%) and higher concentrations of Novo-ProD (>0.51%), where higher percentage of total glucan (>50%) could be obtained. At higher concentrations of Alcalase and Novo-ProD, lower total glucan contents were obtained. The interactive effect between Flavourzyme and Alcalase (Figure 7.5B), in the predictive total glucan content model, occurred at protease concentration lower than 0.75%; higher total glucan (>53%) was achieved as lower concentrations of both Flavourzyme and Alcalase proteases were used. The interaction between the concentrations of Novo-ProD and Flavourzyme affecting the total glucan (data not shown) occurred at higher Novo-ProD (>0.41%) and at lower Flavourzyme concentrations (<0.51%). Higher total glucan was obtained as the concentration of Novo-ProD increased and as the concentration of Flavourzyme decreased.

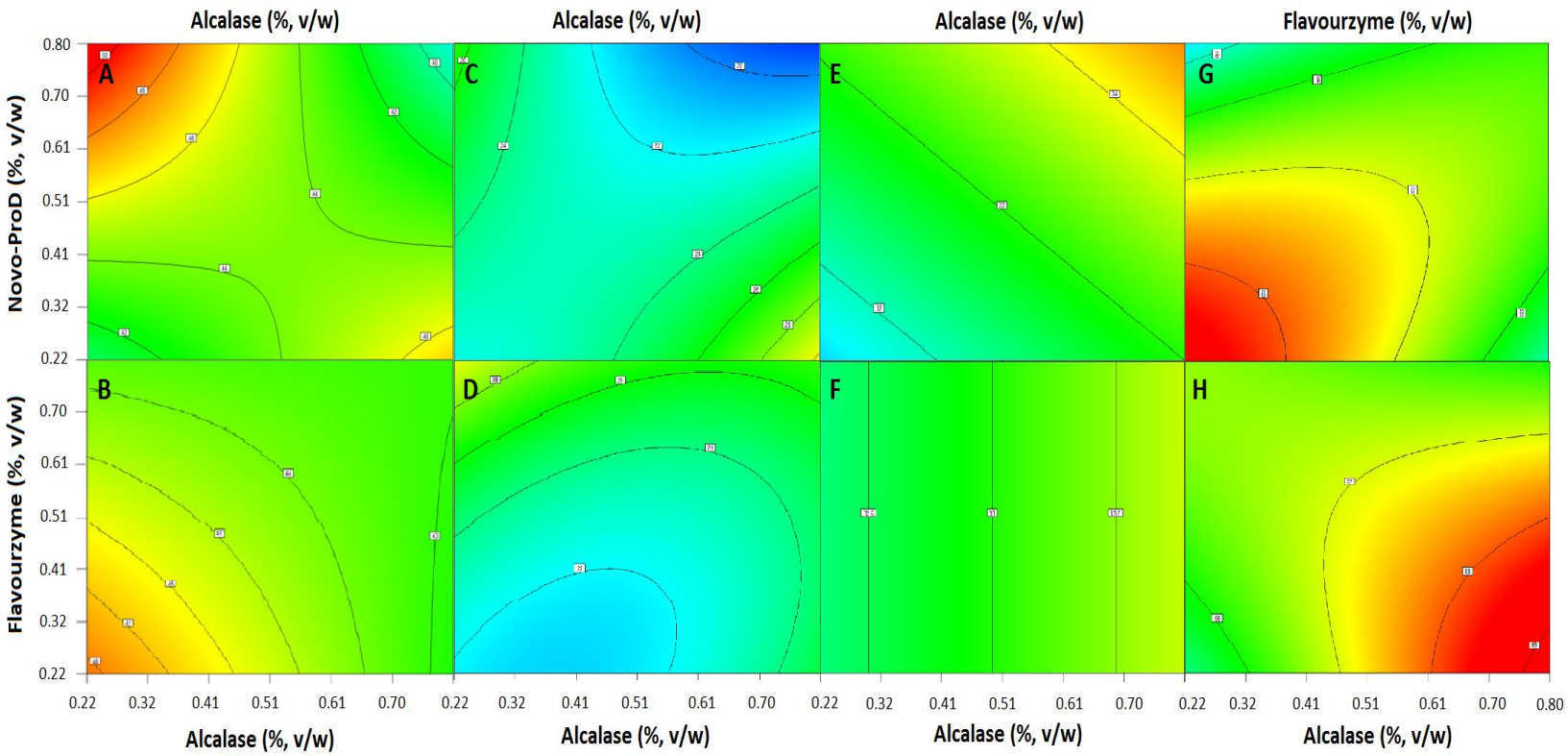


Figure 7.5. Contour plots of total glucan (AB), β-glucan (CD), weight loss (EF), and protein loss (GH) as a function of Novo-ProD concentration (v/w) and Alkalase (v/w) (ACE), as a function of Flavourzyme concentration (v/w) and Alcalase concentration (v/w) (BDFH), and as a function of Novo-ProD concentration (v/w) and Flavourzyme concentration (v/w) (G). The numbers inside the contour plots indicate the predicted values under given reaction conditions. Response: Lowest Highest.

For β-glucan model, the interaction between Alcalase and Novo-ProD (Figure 7.5C) occurred when higher concentrations (>0.41%) of either one of the enzyme was present; however, higher concentrations of Alcalase (>0.70%) and lower concentrations of Novo-ProD (<0.35%) was required to obtain the highest percentage of β-glucan within the investigated range. This trend is the opposite of the one observed for total glucan content, in which lower concentrations of Alcalase (<0.51%) and higher concentrations of Novo-ProD (>0.51%) led to higher total glucan content. The overall results show clearly that the Alcalase and Novo-ProD act synergistically when one protease is in excess as compared to the other one. Although the ellipsoidal lines demonstrating the interactions between the concentrations of Flavourzyme and Alcalase affecting the extraction of β-glucan (Figure 7.5D) were present across the investigated range, the highest percentage of β-glucan was obtained as the concentration of Flavourzyme and of Alcalase were increased.

Figure 7.5E demonstrates the linear effect of Novo-ProD and Alcalase on the weight loss. The highest weight loss (>34%) was reached when the concentrations of both enzymes were high (>0.70%). The change in concentration of Flavourzyme did not lead to any change in weight loss at different Alcalase concentrations (Figure 7.5F). The interactive effects of Novo-ProD and Flavourzyme concentrations on protein loss (Figure 7.5G) were observed as the concentrations of both Novo-ProD and Flavourzyme were low (<0.41%). The highest protein loss (>68%) was achieved at both low Novo-ProD and Flavourzyme concentrations. On the other hand, the interactive effect of Alcalase and Flavourzyme on protein loss (Figure 7.5H) occurred at lower enzyme concentrations of Flavourzyme (<0.51%) and at higher concentrations of Alcalase (>0.61%) where most loss in protein (>68%) was also observed.

7.5 Conclusion

The present study assessed the efficiency of selected proteases namely, Alcalase, Flavourzyme, Novo Pro-D, and Papain, for the generation of β-glucan through the hydrolysis and the solubilization of proteins and mannans from yeast cell wall. Combining the hot water extraction step with protease treatment was found to enhance the protease action efficiency by opening the cell wall network and improving the accessibility of proteases to the proteins substrates. The highest efficiency of Alcalase and Novo-ProD for the removal of proteins was attributed to their high proteolytic activity and/or to their high specificity for hydrolyzing peptide bonds that have large hydrophobic aromatic amino acid (tyrosine, tryptophan, and phenylalanine). Furthermore, in

order to explore the synergistic actions of proteases, the effect of the combined use of Alcalase, Novo-proD and Flavourzyme, on the generation efficiency of β-glucan was investigated and optimized.

CHAPTER VIII

GENERAL SUMMARY AND CONCLUSION

The present study investigated the efficiency of selected extraction methods for the production of mannoproteins from yeast cell wall by-product, including heat treatment, SDS extraction and enzymatic treatment. Low molecular weight (6.5 kDa) mannoproteins were obtained upon heat treatment of Agrimos®, and they were mainly non-covalently bound mannoproteins. Higher yield of the covalently bound mannoproteins was obtained upon Zymolyase® enzymatic treatment with molecular weight of 5 to 400 kDa. The optimization of the enzymatic isolation of mannoproteins was performed using RSM with CCD. The enzyme units and the reaction time were identified as the significant independent variables, affecting importantly the yield of the recovered extract, the mannoproteins content and the mannan to protein ratio in mannoproteins. The interaction between the enzyme units and the reaction time displayed only significant effects on the response of yield of recovered extract. The predictive models of the mannoproteins content and the mannan to protein ratio showed the same trends with shorter/longer reaction times and lower/higher enzymatic units being more favorable for these responses. The overall findings of the present study will contribute to lay the scientific ground for the development of an innovative biocatalytic process for the isolation of functional ingredients from baker's Agrimos® with many potential applications.

For the isolation of mannoproteins from baker's and brewer's yeasts derived from the same species of *Saccharomyces cerevisiae*, selected extraction approaches were used and the difference of the extracted mannoproteins was compared. The yields and the structural properties of mannoproteins (molecular weight; mannan to protein ratio) were not only dependent on the yeast source but also on the isolation methods. Non-covalently bound mannoproteins, isolated upon the heat treatment of brewer's and baker's yeasts showed different mannan to protein ratio. While SDS extraction method was not suitable for the isolation of mannoproteins, higher yields of covalently-bound mannoproteins, characterized by a wider molecular weight distribution and a higher mannan to protein ratio, were obtained upon Zymolyase® enzymatic treatment. The optimization of the production of mannoproteins from brewer's yeasts was performed using RSM with CCRD. The enzyme units, the reaction time and their interaction displayed significant effects on the responses of the yield of recovered extract, the mannoproteins content as well as the mannan to protein ratio in mannoproteins. The predictive models of the mannoproteins content and the mannan to protein ratio showed similar trends with shorter/longer reaction times and lower/higher enzymatic units

being more favorable for these responses. The overall findings of the present study will contribute to lay the scientific ground for the development of an innovative biocatalytic process.

Mannoproteins derived from Agrimos® and YCW-b, obtained through optimal Zymolyase® treatment methods, were further purified using concanavalin affinity column and Superdex™ prep grade and prepacked HiLoad™ column. Two populations of mannoproteins (MP1/MP1' and MP2/MP2') were obtained from two yeast-based by-products and their composition was determined. The solubility of the four mannoproteins was similar or lower than that of mannan. The mannoproteins showed Newtonian behavior at 25, 50, and 73 °C, and their viscosity tended to decrease with increased temperature. The sonication alone was found to be a more appropriate method than the combined sonication/homogenization method, as it yielded mannoprotein-based emulsions with significant smaller droplet sizes ($p<0.05$). Moreover, there was no significant difference ($p>0.05$) between the MP1/MP1'-based emulsions formed with soybean oil or glyceryl trioleate, while there was significant difference ($p<0.05$) between the MP2/MP2'-based emulsions formed with these two oil phases at 25 °C. Sonication treatment conditions and oil/mannoprotein ratio were critical factors in forming fine mannoprotein-based emulsions. Both MP1/MP1'-based emulsions obtained from Agrimos® and YCW-b showed significantly better emulsifying properties at higher pH compared to lower pH, but their optimal pH range were different. Compared to lecithin, MP1/MP1'-based emulsions obtained from Agrimos® and YCW-b showed similar or slightly inferior emulsifying properties compared to commercial lecithin.

Two main populations of mannoproteins (MP1/MP1', MP2/MP2') were isolated from 4 yeast-based products. To assess the presence of α-1,6 mannose linkages, α-1,6 mannanase treatment analysis was used. α-(1,6) Mannose linkages were detected in the high MW MP1/MP1' populations, but not in the low MW MP2/MP2' populations. The extracted mannoproteins were mainly O-glycosylated. Further NMR analysis indicates that within the 7 anomeric carbons determined in Agrimos® MP1, 4 of them have very similar chemical shifts than those of mannan standard. The proportion of α-(1,6) linked mannan was found to be the highest in Agrimos® MP1, which agrees well with the results of α-(1,6) mannanase treatment. Furthermore, heat inducing methods were used to analyze the secondary structure changes of mannoproteins in both solid and liquid state using FTIR.

The present study assessed the efficiency of selected proteases namely, Alcalase, Flavourzyme,

Novo Pro-D, and Papain, for the generation of β-glucan through the hydrolysis and the solubilization of proteins and mannans from yeast cell wall. Combining the hot water extraction step with the protease treatment was found to enhance the protease action efficiency by opening the cell wall network and improving the accessibility of proteases to the proteins substrates. The highest efficiency of Alcalase and Novo-ProD for the removal of proteins and hence the generation of high yield of β-glucan were attributed to their high proteolytic activity and/or to their high specificity for hydrolyzing peptide bonds that have large hydrophobic aromatic amino acid (tyrosine, tryptophan, and phenylalanine). Furthermore, in order to explore the synergistic actions of proteases, the effect of the combined use of Alcalase, Novo-proD and Flavourzyme, on the generation efficiency of β-glucan was investigated and optimized.

CHAPTER IX

CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

9.1. Contribution to Knowledge

The major contributions to knowledge of this study are:

1. This is the first study that compared heat treatment, SDS extraction and enzymatic treatment for the isolation of mannoproteins from yeast cell wall. The crude mannoprotein extracts were compared according to the extracting agents' effects on yield of recovered extract, mannan and protein recovery yield, mannoproteins in the extract and mannan/protein ratio in extracted mannoproteins.
2. To the authors knowledge, this is the first study being conducted for the comparison of mannoproteins extracted from baker's and brewer's yeasts derived from the same species of *Saccharomyces cerevisiae* using different methods. the study of the enzymatic extraction of mannoproteins derived from brewer's whole yeast by response surface methodology contributes to the understating of the isolation parameters on the isolation efficiency and to its optimization.
3. The solubility, the viscosity, the emulsifying property and the morphology of the purified mannoproteins were, for the first time, studied. Most of the studies reported in the literature did use non-purified mannoproteins. In our study, the production and the purification of mannoproteins achieved through a sequence of steps that first consisted of dialysis for the removal of salt, vitamins, and small soluble proteins from the starting yeast cell wall materials; then, Zymolyase® enzymatic treatment was applied for the release of mannoproteins from yeast cell wall, followed by the affinity chromatography on concanavalin A to purify mannoproteins and the size exclusion chromatography on Superdex™ and HiLoad™ columns to fractionate the purified mannoproteins.
4. For the first time, the structural properties of two types of purified mannoproteins were studied with NMR and FTIR methods, including MP1 population characterized by higher molecular weight of 36-620 kDa and the mannan to protein ratio of 2.5-10, and a MP2 monocomponent with a low molecular weight of 2.3-6.8 kDa and mannan to protein ratio of 12.2-181.0.
5. This is the first study that reported the generation of β-glucan from yeast cell wall using a set of proteolytic enzyme combinations consisted of Alcalase, Novo-proD and Flavourzyme. The synergistic actions of these protease combinations were investigated by response surface methodology RSM and a central composite rotatable design (CCRD) for the first time.

9.2 Recommendations for Future Research

- It has been suggested that mannoproteins and β-glucan, as well as their corresponding oligosaccharides/oligomers, contain a lot of health promoting properties, these properties, including digestibility, prebiotic property, anti-carcinogenic activity and anti-adhesive effects against pathogens should be investigated.
- The optimization of the processes of large-scale production of mannoproteins and β-glucan should be investigated.
- Determination of the efficiency of the techno-functional properties of mannoproteins and β-glucan in food systems through food formulations.

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