High-pressure induced gelation of globular proteins

by

Pedro Alvarez

Department of Food Science and Agricultural Chemistry

Macdonald Campus, McGill University

Montreal, Quebec

Canada

A Thesis Submitted to the Graduate and Post-Doctoral Studies Office in Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy

October 2008

Suggested short title:

HP-induced gelation of proteins

Abstract

This thesis is focused on the structural and rheological changes in globular proteins when subjected to high pressure processing (HPP), a novel non-thermal food processing technique. Structure-functionality changes associated with thermal processing has been well studied, but little is known for HPP. One of the principal objectives of this thesis was to compare protein structure-functionality relationship between thermal and high pressure processing. Three groups of proteins of varying complexity and source were studied: β-lactoglobulin, a small well understood protein from cow's milk whey (model system); porcine blood plasma proteins, and soy protein concentrate. Blood is generally considered a waste from the meat industry, plasma is obtained from centrifugation of the blood and is composed mainly of serum albumin and globulins (simplified multi-component system). Soy protein concentrate is a complex system of vegetable proteins composed of a varied mix of large glycoproteins.

In general, HP treatment affected the protein structure and functionality, but the specific effects depended on the characteristics of the protein. Pressure-independent parameters, like protein concentration and pH, exerted a major influence on protein denaturation and influenced the HP-induced gel network formation. Rheological characteristics were unaffected by pressures up to 550 MPa. At 600 MPa significant structural changes were observed and these were correlated to changes in the viscoelastic properties. A true gel was only formed after applying 650 MPa to a β-lg dispersion of 20 % concentration, but it did not follow the classic structural changes of thermal induced gel formation, resulting in a significant drop in the intensity of intramolecular β-sheet and an increase in α-helixes and random coil structures. Using mathematical models, the exponential model described reasonably well the higher end HP treatments for prediction of storage modulus; and the cubic model fitted the protein structural data related to β-structure HP-induced formation.

Protein gels of comparable strength were produced by thermal and high pressure treatments (concentration ≥ 20 %); the heat induced gels were stiffer than their pressure induced counterparts, the latter only modestly generating intermolecular β-sheets albeit a broad destruction of native structures. For heat induced gelation, the change in protein secondary structure resulting in the formation of intermolecular β-sheets in conjunction with

additional intermolecular disulfide bonds, explains the development of the three-dimensional structure responsible for the gel stiffness and viscoelastic parameters increase. On the other hand, for the pressure induced gel formation, changes in the protein secondary structure did not fully explain the creation of gel scaffold; but the augmented hydrophobic interactions due to internal molecular rearrangements creating different electronic densities in the molecule is perhaps important.

Within the secondary structure of plasma proteins, the globulin fraction appears to be more susceptible to changes in pH. At any given pH, the intensity of the native amide I' bands decreased with an increase in temperature. In addition, a decrease in pH resulted in a higher susceptibility to conformational changes during heating. A decrease in pH at room temperature enhanced the denaturation of globulin fraction while an increase in temperature first affected α-helical domains, which are predominantly associated with the serum albumin fraction.

Conclusively, the dynamic rheology indicated a strong influence of protein concentration and pH on both elastic and viscous moduli of soybean protein concentrate (SPC). The structure of the soybean proteins suffered limited changes after HP treatment: hydrophobicity increased, as well as the relative proportion of random coil, while the β-sheet content decreased. It was envisioned that HP treatment can be used to enhance the viscoelastic behavior of SPC; and the HP treated SPC can then be used to enrich both protein content and improve textural properties of foods. A wide range of gel textures, determining mouth-feel, can be obtained with varying SPC concentrations, pressure levels, holding times and processing temperatures, pH, and additives. Therefore, with a target product on hand, one can aim for specific rheological characteristics.

These results are expected to provide valuable information to the food industry for enhancing the functionality and value of common proteins through the use of HPP. Milder HP treatments promoted creation of soft gels while more severe treatments resulted in a firm gel. In addition, milder HP treatments could be used for modifying the rheological properties of foods at higher the protein content, and with more complex and higher molecular weight proteins.

Résumé

Cette thèse est concentrée sur les changements structuraux et rhéologiques des protéines globulaires une fois soumise à l'haute pression procès (HPP), une technique non thermique innovatrice de traitement des produits alimentaires. Il y a un ensemble de connaissances impressionnant sur les changements de structure-fonctionnalité liés au procès thermique mais peu est connu pour HPP. Un des principaux objectifs de cette thèse était de comparer le rapport de structure-fonctionnalité entre les deux procédés (haute pression et thermique). Trois groupes de protéines on été étudiés en variant la complexité et la provenance : la β-lactoglobuline, une petite protéine très bien connu, provenant du lait de vache (système modèle); les protéines de plasma sanguin porcine, et le concentré protéique de soja. Le sang est généralement considéré comme un dechet de l'industrie de viande, le plasma est obtenu à partir de la centrifugation du sang et se compose principalement d'albumine sérique et de globulines (système simplifié à plusieurs éléments). Le concentré protéique de soja est un système complexe des protéines végétales composées de mélange divers de grosses glycoprotéines. Généralement le traitement de HP a affecté la structure et la fonctionnalité de protéine, mais les effets spécifiques de HP sur les protéines ont dépendu des caractéristiques du système en question. Les paramètres comme la concentration en protéine et le pH, ont exercé une influence importante sur la dénaturation des protéines et ainsi que la formation de gels. Les caractéristiques rhéologiques du système n'étaient pas changées par des pressions jusqu'à 550MPa. À 600 MPa on a observé des changements significatifs qui ont été corrélés avec des changements des propriétés viscoélastiques. Un vrai gel a été formé seulement après application de 650 MPa et une concentration de 20 %, mais celui-ci n'a pas suivi les changements structurels classiques de la formation thermique de gel, ayant une baisse significative de l'intensité intramoléculaire de la feuille β et en même temps une augmentation en α-hélicoïdaux, et changements aléatoires des structures d'ensablement. Utilisant les modèles mathématiques, le modèle exponentiel a raisonnablement bien décrit les traitements de HP (hautes valeurs) pour la prédiction du module de stockage ; et le modèle cubique était adequat pour les valeurs des protéines structurales liées à la formation de structure dû au traitement HP.

Des gels de protéine de force comparable ont été produits par chaleur ou avec l'application de la haute pression (concentré plus de 20 %) ; les gels produits par la chaleur étaient plus rigides que ceux qui ont été préparés par haute pression, ce dernier produisant seulement des feuilles β intermoléculaires quoi qu'une large destruction des structures natives. Tandis que pour la formation du gel par la chaleur, le changement de la structure secondaire de la protéine quant à la formation de feuilles β intermoléculaire en même temps que la formation des liaisons intermoléculaires additionnelles de bisulfure, semblent expliquer le développement de la structure tridimensionnelle de gel par la rigidité du gel et les paramètres viscoélastiques. La formation de gel induit par la pression, et les changements de structure secondaire de la protéine n'éclairent pas complètement la formation des réarrangements du gel ; mais l'augmentation des interactions hydrophobes dues aux internes moléculaires remettre créant différentes densités électroniques dans la molécule, sont peut être importants.

Dans la structure secondaire des protéines de plasma, particulièrement sensible aux changements du pH, la fraction de globuline apparaît comme plus susceptible à ces changements. À n'importe quel pH, l'intensité des bandes indigènes I' d'amide diminue à mesure que la température augmente. D'autres diminutions du pH ont comme conséquence une susceptibilité plus élevée aux changements conformationnels pendant le chauffage. Une diminution du pH à la température ambiante incite la dénaturation de la fraction de globuline tandis qu'une augmentation de la température affect les domaines α-hélicoïdaux, qui sont principalement associés à la fraction d'albumine sérique.

D'une manière concluante, la rhéologie dynamique a indiqué une influence forte de concentration en protéine et de pH sur les modules élastiques et visqueux du concentré protéique de soja (SPC). La structure des protéines de soja a été peu affectée par les traitements de HP : l'hydrophobicité a augmenté, aussi bien que la proportion relative de l'enroulement aléatoire, alors que le contenu de feuille β a diminué.

En conclusion, on l'a envisagé que le traitement de HP peut être employé pour augmenter le comportement viscoélastique du SPC, après quoi le SPC peut être utilisé pour enrichir la teneur en protéines et les propriétés de texture des nourritures. Une grande variété de textures de gel, déterminant l'effet en bouche, peut être obtenu en variant des concentrations de SPC, niveaux de pression, temps d'entreposage et les températures du

procédé, pH, et des additifs. Par conséquent, en ayant un produit d'cible en développement, on peut viser pour des caractéristiques rhéologiques spécifiques. Ces résultats peuvent être utiles pour l'industrie alimentaire, quand le but de fabrication est d'améliorer la viscoélasticité des aliments par l'utilisation du traitement HP. Il s'avère qu'on doit appliquer des conditionnes HP plus douces pour éviter les gels très rigides. Plus la teneur en protéines des aliments est faite et plus le poids moléculaire est élevé, plus on doit appliquer un traitement de pression réduit pour obtenir des changements importants de viscosité des aliments ou de la réelle formation du gel. On s'attend à ce que ces résultats fournissent des informations valables à l'industrie alimentaire pour augmenter la fonctionnalité et la valeur des protéines par l'utilisation des HP.

to my -soon to get bigger- Family,

Love you Laura, Marco Andrés and Ana Isabel.

Acknowledgments

I would like to express my most sincere thanks to Dr. Hosahalli Ramaswamy, my thesis supervisor for his guidance and encouragement to perform with high standards. I am deeply thankful to Dr. Ashraf Ismail, my thesis co-supervisor whose expertise, friendship and wise advice was invaluable for the completion of this work. Thank you both for the financial support necessary to accomplish my degree.

I would like to express my gratitude to Dr. Elena Saguer of Universitat de Girona (Spain) for providing me with porcine plasma proteins samples, as well as for valuable discussions, and great 3 years of collaboration. I am also very thankful to Dr. Jaqueline Sedman for her editorial help of this and other manuscripts.

I would like to thank the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) for awarding me the Doctoral Research Scholarship. Also thanks to the Canadian Institute of Food Science and Technology (CIFST) for awarding me the George Weston Ltd. Graduate Student Scholarship 2008. Thanks to the Faculty of Agricultural and Environmental Sciences of McGill University for awarding me the Agri-Food travel grant. Also thanks to Macdonald Campus Graduate Student Society for their Scientific Acknowledgment Award. Thanks to McGill University for awarding me the Alma Mater Student Travel Grant and the McGill Graduate Studies Fellowship.

Thanks to Dr. Joanne Turnbull of Concordia University -Biochemistry Department- for her kind lending of the fluorescence instrument; as well as Julie and John from Dr. Turnbull's lab for technical assistance with the flourimeter. I would also like to thank Dr. Frederick van de Voort for letting us use his FT-Raman equipment.

Thanks to my colleagues in the Food Processing Group: Dr. Kevin Shao, Dr. Sonming Zhu, Elham Azarpazhooh, Jaideep Arora, Yu Liang, Mritunjay Dwivedi, Sibtain Saqib; and in the McGill-IR Group: Laura Carranza, Moeed Haq, Andrew Ghetler, Alexandra Grygorczyk, and Ahmed Ibrahim.

My deepest gratefulness to my wife Laura and my son Marco Andrés for making every day of this journey memorable, you replenish my soul a smile at a time.

Finally, I am proud very much of my parents Marco Tulio Alvarez and Blanca Isabel Blanco who raised 6 kids to be all professionals; stiff competition for me being the younger offspring.

Contributions of Authors

Parts of this thesis have been presented at international conferences and Chapter 6 has been published in a refereed journal (Journal of Food Engineering). The level of involvement of the different authors is as follows:

Pedro Alvarez is the Ph.D. candidate who planed and conducted experiments, gathered and analyzed the results, and prepared manuscripts for publication.

Dr. H.S. Ramaswamy is the thesis director, under whose guidance the overall research plan was conducted, and who assisted the candidate in planning the research especially in his area of expertise, rheology and DMA analytical techniques. He also assisted in correcting, editing and reviewing the thesis manuscript and papers derived from it.

Dr. A.A. Ismail is the thesis co-director who also provided important guidance and knowledge -for all spectroscopic techniques- in planning the research work, co-edited and reviewed the scientific papers.

Dr. E. Saguer provided the porcine plasma protein sample, helped in the acquisition of thermal data for Chapter 5, as well as provided valuable discussions and input.

Dr. J. Sedman provided editorial support throughout the thesis.

Contributions to Knowledge

The present work contributes to scientific knowledge in general by documenting from a variety of perspectives the correlations between high pressure induced changes in the molecular structure of proteins and viscoelasticity changes. For this aim, 3 different model matrices varying in protein size, complexity and source were used. Additionally, the research results represent an important addition to scientific knowledge as they also contrast the above mentioned changes to the ones observed by protein thermal gelation/denaturation. The main specific contributions of this thesis work are described below:

1. This research is the first one to evaluate the repeatability of the effects on proteins of the treatment delivered by a commercial isostatic press through the use of an established quantitative analytical technique, Fourier transform infrared spectroscopy.

2. This is the first comprehensive analysis of how beta-lactoglobulin (β-lg) viscoelasticity is affected by high pressure in a wide range of concentrations from 5 to 40 % (w/v).

3. The use of mathematical predictive models in order to explain the increase in elastic modulus and the formation of the 1627 cm^{-1} FTIR band (intermolecular β-sheet) relative to increasing pressure, is also a novel and an important contribution.

4. The thesis work is the first to document the achievement of comparable protein gel strength via thermal and pressure gelation of β-lg, albeit at high protein concentrations (> 20 %).

5. Overall, the results significantly broaden the current trends of thought regarding the HP gel network formation mechanism using commercially available isostatic press able to release high pressures (900 MPa) akin to currently used commercial processes. This is an important context for the results presented here, since most of the scientific data available in the literature up to now, has been produced using either commercial HP equipment only capable of delivering low pressures (around 400 MPa) or using diamond anvil cells. This latter type of press can differ dramatically from a real commercial HP treatment especially in terms of depressurization times.

6. This research is the first to describe the sequence of events that take place during the heat-induced unfolding and aggregation of plasma proteins under different pH conditions.

7. This work initiates the study structural changes of HP-treated plasma proteins.

8. The research presented widens the data available for viscoelasticity of soy proteins, as is the first to study this property in a wide range of protein concentrations 5 to 20 % (w/v) and pH 3 to 7.

9. Finally the present work is pioneer in investigating the relation between changes in viscoelasticity and structural changes in soy proteins subjected to HP-treatment.

Publications and Presentations from this Thesis

Selected portions of this thesis have been published or prepared for submission as follows:

Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. 2008. High pressure gelation of soy proteins: Effect of concentration, pH and additives. *Journal of Food Engineering*, 88(3), 331-340.

Alvarez, P.A., Ramaswamy, H.S., Sedman, J. & Ismail, A.A. 2008. Molecular Basis of High Pressure Induced beta-lactoglobulin Soft Gels. In preparation.

Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. 2008. Comparison of High Pressure and Heat Induced β-lactoglobulin Self-standing Gels by FTIR Spectroscopy and DMA. In preparation.

Alvarez, P.A., Saguer, E., Sedman, J., Ramaswamy, H.S. & Ismail, A.A. 2008. Heat and pressure induced gel formation of plasma proteins: new insights by FTIR 2D correlation spectroscopy. In preparation.

Selected portions of this thesis have been presented at scientific conferences:

Alvarez, P.A., Ismail, A.A. & Ramaswamy, H.S. May 2006. Effect of high pressure processing on the rheological characteristics of soy protein isolate dispersions. 2006 CIFST-AAFC joint Conference, Montreal, QC.

Alvarez, P.A., Saguer, E., Ramaswamy, H.S., Sedman, J. & Ismail, A.A. July 2008. Heat-induced gel formation of plasma proteins: New insights by FTIR 2D correlation spectroscopy. IFT conference 2008, New Orleans, LA.

Table of Contents

Abstract iii
Résumé v
Contributions of Authors xi
Contributions to Knowledge xii
Publications and Presentations from this Thesis xiv
Table of Contents xv
List of Figures xx
Index of Tables xxii
Chapter 1. General Introduction 1
Chapter 2. Literature Review 4
2.1 History of high pressure (HP) for food applications 4
2.2 Advantages and short comings of the use of HP to treat food 9
2.2.1 HP-cold pasteurization of high acid foods 13
2.2.2 Changes in quality 16
2.2.3 Changes in functional properties 20
2.2.3.1 Food rheology and texture 20
2.2.3.2 Protein foaming 22
2.2.3.3 Protein emulsifying capacity/behavior 24
2.2.3.4 Protein solubility 26
2.2.3.5 Protein digestibility 27
2.2.3.6 Protein water holding capacity (WHC) 29
2.2.4 Changes in molecular structure of proteins 29
2.2.4.1 Protein secondary level 30
2.2.4.2 Protein tertiary level 34
2.2.5 Enhance release/production of bioactives. Decrease immunoreactivity and/or elimination of antinutritional fractions 35
2.2.6 Other uses 36
2.3 Methods to study protein structure 38
2.3.1 Primary level 38
2.3.1.1 Classical protein sequencing methods 38

2.3.1.2 Mass spectrometry (MS) 39
2.3.2 Secondary level 40
2.3.2.1 Far UV circular dichroism (far UV-CD) 40
2.3.2.2 Raman spectroscopy 40
2.3.2.3 Fourier transform infrared (FTIR) spectroscopy and 2D correlation 42
2.3.2.4 Nuclear magnetic resonance (NMR) 44
2.3.3 Tertiary and quaternary levels 45
2.3.3.1 Fluorescence spectroscopy 45
2.3.3.2 Raman spectroscopy 47
2.3.3.3 Near UV-CD 48
2.3.3.4 Electrospray ionization mass spectrometry (ESI-MS) 49
2.3.3.5 Quantitative ultracentrifugation 50
2.4 Methods to study rheology/texture 51
2.4.1 Classical rheology (flow behavior) 51
2.4.2 Small amplitude oscillatory methods 53
2.4.2.1 Dynamic rheology 54
2.4.2.2 Dynamic mechanical analysis (DMA) 55
2.5 Proteins studied in this thesis 56
2.5.1 Bovine milk 56
2.5.1.1 Milk proteins 57
2.5.1.2 Whey proteins 60
2.5.2 Porcine blood 62
2.5.2.1 Blood proteins 63
2.5.2.2 Plasma 64
2.5.2.3 Use of plasma proteins to enhance texture of meat products 66
2.5.3 Soy proteins 67
2.5.3.1 Soy protein components 68
Connective statement to Chapter 3 70
Chapter 3. Molecular Basis of High Pressure Induced beta-lactoglobulin Soft Gels 71
3.1 Abstract 71
3.2 Introduction 71

3.3 Materials and Methods 73
3.3.1 High pressure treatments 75
3.3.2 Sample preparation for FTIR spectroscopy analysis 75
3.3.3 Sample preparation for rheological analysis 76
3.3.4 Statistical analysis 76
3.4 Results and Discussion 77
3.4.1 Effect of varying protein concentration and pressure level on rheological properties 77
3.4.2 FTIR spectroscopy 79
3.4.3 Effect of HP treatment level on rheological properties 83
3.4.4 Model fitting 86
3.4.4.1 Exponential fit of viscoelastic parameters 86
3.4.4.2 Cubic fit of structural parameters 86
3.5 Conclusions 88
Connective statement to Chapter 4 90
Chapter 4. Comparison of High Pressure and Heat Induced β-lactoglobulin Self-standing Gels by FTIR Spectroscopy and DMA 91
4.1 Abstract 91
4.2 Introduction 91
4.3 Materials and Methods 92
4.3.1 High pressure treatments 92
4.3.2 Heat treatments 93
4.3.3 Sample preparation for FTIR spectroscopic analysis 93
4.3.4 Sample preparation for FT-Raman spectroscopic analysis 94
4.3.5 Sample preparation for dynamic mechanical analysis (DMA) 94
4.3.6 Statistical analysis 94
4.4 Results and Discussion 95
4.4.1 Mechanical analysis 95
4.4.2 FTIR spectroscopy 98
4.4.3 FT-Raman spectroscopy 102
4.5 Conclusions 107

Connective statement to Chapter 5 108
Chapter 5. Heat and pressure induced gel formation of plasma proteins: new insights by FTIR 2D correlation spectroscopy 109
5.1 Abstract 109
5.2 Introduction 109
5.3 Materials and methods 111
5.3.1 Solution preparation 111
5.3.2 High pressure treatment of plasma 111
5.3.3 FTIR measurements 112
5.3.3.1 Generalized 2D Correlation Spectroscopic Analysis 112
5.4 Results and Discussion 113
5.4.1 Changes in 1D FTIR spectra of plasma proteins as a function of pH 113
5.4.2 Sequential events leading to pH-induced denaturation of plasma proteins by 2D COS 114
5.4.1 Changes in 1D FTIR spectra of plasma proteins upon heating as a function of pH 116
5.4.2 Thermal transitions of plasma proteins from 2D correlation plots 118
5.4.3 HP treatments of plasma proteins 123
5.5 Conclusions 128
Connective statement to Chapter 6 129
Chapter 6. High Pressure Gelation of Soy Proteins: effect of concentration, pH and additives 130
6.1 Abstract 130
6.2 Introduction 130
6.3 Materials and Methods 132
6.3.1 Rheology 133
6.3.2 Fluorescence spectroscopy using ANS probe 133
6.3.3 FTIR spectroscopy 133
6.4 Results and Discussion 134
6.4.1 Wide-range concentration and pressure effects 134
6.4.2 Narrow-range concentration effect at 250 MPa pressure 138

6.4.1 Wide-range pH effect at 250 MPa pressure, 15 % SPC concentration 141
6.4.2 Effect of additives, pH, temperature and pressure holding time 143
6.4.3 Fluorescence spectroscopy using ANS as probe 146
6.4.4 FTIR spectroscopy of SPC 149
6.5 Conclusions 153
Chapter 7. General Conclusions 154
References 158

List of Figures

Figure 3.1 Rheological measurements of different β-lactoglobulin concentrations treated at different pressure levels. 78

Figure 3.2 Amide I FTIR spectral variance of β-lactoglobulin samples exposed to 0.1 to 650 MPa of pressure. 81

Figure 3.3 FTIR spectra of β-lactoglobulin after two different pressure treatments 82

Figure 3.4 FTIR normalized spectra of β-lg dispersions of different concentrations treated with 450 MPa of pressure 84

Figure 4.1 Mechanical parameters of heat induced (95 °C) β-lactoglobulin gels prepared from dispersions of different concentrations (20, 30, and 40 %) using different holding times: 3.75, 7.5, 15 and 30 minutes 96

Figure 4.2 Mechanical parameters of pressure induced (900 MPa) β-lactoglobulin gels prepared from dispersions of different concentrations (20, 30, and 40 %) using different holding times: 3.75, 7.5, 15 and 30 minutes 97

Figure 4.3 Controls and pressure treated (900 MPa) β-lg samples at different concentration levels..... 100

Figure 4.4 Protein aggregation as measured from FTIR spectra of β-lactoglobulin after heat and pressure induced gelation 101

Figure 4.5 Tertiary structure information derived from FT-Raman spectra of heat induced (95 °C) β-lactoglobulin gels (20, 30 and 40 % protein) subjected to different holding times 104

Figure 4.6 Tertiary structure information derived from FT-Raman spectra of pressure induced (900 MPa) β-lactoglobulin gels subjected to different holding times..... 106

Figure 5.1 FSD spectra obtained at 30 ºC from plasma solutions adjusted to different pH. 115

Figure 5.2 Synchronous (a) and asynchronous (b) 2D IR correlation maps in the 1710-1590 cm^{-1} region generated from the spectra obtained in the pH range from 5.5 to 4.5 .117

Figure 5.3 Fourier self-deconvolved infrared spectra in the amide I' absorption region upon heating plasma protein solutions (7 % w/v in D_2O) from 30 ºC to 90 ºC at different pH. 120

Figure 5.4 Variation in the intensity of the aggregation band at 1617 cm^{-1} as a function of increasing temperature at selected pH. 121
Figure 5.5 The synchronous (a) and asynchronous (b) 2D IR correlation maps generated from the spectra obtained a pH 7.5 during heating (30 ºC to 90 ºC) 122
Figure 5.6 Amide I' FTIR spectra of pressure treated (600, 700, 800 or 900 MPa with 5 min holding) plasma samples..... 125
Figure 5.7 Ratio of intensities, obtained from the FTIR spectra of plasma proteins, of 1653 to 1638 cm^{-1}, assigned to α-helices and intramolecular β-sheets respectively..... 126
Figure 6.1 Rheological parameters G' (black) and G" (grey) of different concentrations of soy protein isolate when exposed to various pressures 135
Figure 6.2 Phase angle of different soy protein concentrate (SPC) dispersions subjected to various pressure treatments..... 137
Figure 6.3 Viscoelastic parameters of 15 % soy protein concentrate dispersions treated at 150 and 200 MPa. 139
Figure 6.4 Viscoelastic parameters of 14, 15 and 16 % SPC dispersions before and after 250 MPa pressure treatment..... 140
Figure 6.5 Viscoelastic parameters of 15 % SPC dispersions at different pHs, before and after 250 MPa pressure treatment 142
Figure 6.6 Rheological parameters of 15 % SPC dispersions subjected to 250 MPa with different pH, pressure holding time, calcium chloride, sucrose and processing temperature 144
Figure 6.7 Fluorescence spectra of ANS bind to soy proteins subjected to increasing pressure 148
Figure 6.8 Amide I' region of the FTIR spectra of soybean proteins. Control sample (solid line, average of 3) and pressure treated sample (dashed line, average of 3) at 250 MPa and 0.1 minutes holding time. 152

Index of Tables

Table 3.1 Composition of β-lactoglobulin sample used in this work, as per information provided by the commercial supplier. 74

Table 3.2 Band assignment of the amide I spectral region of β-lactoglobulin. 80

Table 5.1 Sequence of changes in secondary structure of plasma proteins as a function of decreasing pH from 5.5 to 4.5. 119

Table 5.2 Sequence of unfolding and aggregation events upon heating plasma proteins at pH 7.5. 127

Table 6.1 Phase angle of 15 % pH 7 and pH 3 SPC dispersions exposed to 250 MPa and different concentrations of calcium chloride and sucrose; process temperature and pressure holding time. Controls (not pressure treated) were included in the table. 145

Table 6.2 Band assignment of the amide I' spectral region of soy proteins. 151

Chapter 1. General Introduction

High pressure (HP) treatment is an emerging alternative to the more traditional thermal processing of foods. HP can kill spoilage and pathogenic bacteria, and can also be used for improving functionality of food ingredients and finished foods. The technique consists of placing a pre-packaged food in a flexible container/bag, into a fluid containing vessel and raising the pressure in the vessel by injecting more fluid using a hydraulic/high-pressure pump. The pressure is created instantaneously and acts uniformly through the vessel without pressure gradients; this property offers a direct advantage over heat processing, where there is always a temperature gradient and a cold spot in the geometry of the packaged food to be processed.

The HP treatment of foods under different product and process specific conditions can cause partial unfolding of proteins that can lead to the reversible/irreversible gelation of the product. These changes can be desirable or undesirable depending on the product and processing scenarios. The amount of protein unfolding is dependant of many parameters and can be modulated by means of altering process-related parameters like: pressure level, pressure holding time, pressure cycles, processing temperature, depressurization time; and other food intrinsic parameters also play a key role: protein nature, protein concentration, food pH and ionic strength.

Understanding the changes in protein gelation obtained after high pressure treatment will help develop specific protocols for industrial use of this alternative process. Correlating the changes in protein functionality (gelation and thickening in our case) with conformational changes will further elucidate the understanding of the processes proteins undergo when subjected to high pressure. Some researchers have worked on this subject at the structural level (e.g. Smith, Galazka, Wellner & Sumner, 2000) and others have evaluated HP effects on protein functionality (e.g. Ahmed & Ramaswamy, 2003a; Ibanoglu & Karatas, 2001); but very limited studies have looked at relating the two under commercial HP processing conditions. The published information is limited by protein or technique and is usually restricted to a maximum of 400 MPa of pressure (e.g. Lopez-Fandiño, Carrascosa & Olano, 1996). Studies dealing with changes in protein structure under heat induced gel formation are more abundant but special care on the choice of

methods to apply is of utmost importance. In the case of Liu, Zhao, Xiong, Xie & Liu (2007) a practical application was the foundation of their work on gelation of fish and pork meat mixes for surimi production; this article is a good example of a nice attempt to correlate product functionality and changes in molecular structure, but some methodological uncertainties lingers, like discrepancies in the protein concentration used for rheological determinations and for circular dichroism studies; and also differences in heating rates.

This thesis work is focused on studying the effects of high pressure on globular proteins, which are commonly used in the food industry for its functional properties including gelling, thickening and foaming abilities. The research is centered on two different perspectives: to study the changes in functional properties of the HP treated solutions, and to study the structural changes that the proteins undergo after pressure treatment.

Three groups of globular proteins were studied varying in complexity and source:

- Beta-lactoglobulin, a small (18.4 kDa) well understood protein from bovine milk whey was employed as model system. It comprises around 50 % of the whey proteins, been the most abundant of them.
- Porcine blood plasma proteins, blood is generally considered a waste from the meat industry, plasma is obtained from centrifugation of the blood and it is composed mainly of serum albumin (60 %) and globulins (36 %). This served us as a simplified multi-component system. Albumin (66 kDa) and globulins (150 kDa) have an intermediate molecular weight, but are bigger than beta-lactoglobulin.
- Soy protein concentrate, a vegetable protein composed of a varied poll of big glycoproteins presented the more complex system of all selected. Soy protein is an immense mix of different proteins varying in size, function and functionality. The most abundant soy proteins, both beta-conglycinin (150-200 kDa) and glycinin (320-350 kDa) have complex molecular arrangements of many subunits.

The objectives of my thesis are:

a) Analyze the intrinsic variability of the HP-processing repeatability;

b) To characterize the effects of high pressure treatment on globular proteins from a structural and functional point of view;
c) To compare thermal and pressure-induced protein gelation;
d) Study the effects of HP up to 650 MPa on the secondary structure of β-lg (conc. < 20 %) examined by Fourier transform infrared (FTIR) spectroscopy;
e) Investigate the changes in viscoelastic properties of β-lg, as a function of increasing protein concentration and HP level to better understand the molecular basis of HP-induced viscoelasticity changes in solution and as well as in soft (not self standing) gels;
f) Model, using statistical analysis, the response of the viscoelastic properties and secondary structure to changes in pressure treatment level;
g) Study the effect of 900 MPa and 95 °C on the secondary and tertiary structure of β-lg by FTIR spectroscopy and Fourier transform Raman (FT-Raman) spectroscopy;
h) Examine the changes in mechanical properties of β-lg (self-standing) gels by means of dynamic mechanical analysis (DMA), as a function of increasing protein concentration and treatment holding time;
i) Characterize the sequence of events leading to heat-induced and pH-induced unfolding and intermolecular association of porcine plasma proteins using generalized two-dimensional correlation spectroscopy (2D COS);
j) Investigate the structural changes of plasma proteins subjected to HP-induced denaturation;
k) Evaluate the influence of protein concentration, pH, sugar, $CaCl_2$, pressure level and holding time, and process temperature on the dynamic rheology of soybean protein solutions;
l) Study the structural changes in soybean proteins caused by HP through the use of extrinsic fluorescence of the 8-anilino-1-naphthalene sulfonic acid (ANS) and Fourier transform infrared (FTIR) spectroscopy.

Chapter 2. Literature Review

2.1 History of high pressure (HP) for food applications

The food processing and preservation industry boomed in 1809 with the first canned products, and it has evolved during two centuries into a mature industry that still relies heavily on thermal processing to feed a growing global population. The novel non-thermal alternatives include pulsed electric field (PEF) and high pressure (HP) preservation. While the PEF is mainly applied to liquids or pumpable products using a flow-through treatment chamber; the HP can be applied to either liquid or solid foods in flexible containers, and it has proved valuable for food processing and preservation.

The recent upsurge in demand for fresh and healthy food has presented challenges to the food industry, primarily regarding the need of implementing techniques that will allow to keep food fresher for longer, along with a reasonable shelf life and food safety assurance. Thanks to these changes in consumer preferences, a new impulse has been given to the development of concept-driven technologies that could potentially provide the required processing using non or mildly thermal means. Therefore, much of the recent scientific research for the food industry is focusing in non-thermal processing, with high pressure processing being one of the few that shows vast potential in commercial aspects of the industry (Alvarez, Ramaswamy & Ismail, 2008; Knorr, 1999; Rastogi, Raghavarao, Balasubramaniam, Niranjan & Knorr, 2007; San Martín, Barbosa-Cánovas & Swanson, 2002).

In 1899, Japanese researcher Hite attempted HP treatment of food for the first time. His publication represents the first reported case of reduction of spoilage by means of high hydrostatic pressure. Hite and co-workers further expanded their experiments from treating meat and milk products to fruit and vegetables, successfully preserving peaches and pears treated at about 400 MPa at room temperature for 30 minutes (Hite, 1899; Hite, Giddings & Weakly, 1914).

While working on a project that involved the generation of high pressure in 1905, Percy W. Bridgman, a doctoral student at Harvard University, frustrated by the inadequacy of the available equipment, approached the pressure sealing by designing a leak-proof sealing that ensured that the sealing always remained at a higher pressure than the materials under study. This first major change led to other changes in the pumping system, then the development of

the opposed-anvil device and further technical innovations, opening a whole new field of study of materials behavior under unprecedented high pressures. Bridgman experimented with organic compounds and biological materials, noticing the denaturation of enzymes submitted to HP (McMillan, 2005).

In 1960, Keizo Suzuki reports on the combined effect of pressure and temperature on the kinetics of protein denaturation deduced by turbidity. Suzuki calculated the thermodynamic functions in the activation process of denaturation from the relations of absolute reaction rates obtained from ovalbumin and carbonylhemoglobin (Suzuki, 1960).

Hawley in 1971, determined the pressure-temperature-reversible transition surface for chymotrypsinogen at pH 2.07 by ultraviolet difference spectra (temperature interval 8.5 to 70 °C) between atmospheric pressure and 7000 atm. (709 MPa), using the apparatus designed by Bridgman, and was able to fit the surface to a relatively simple equation of state, found to be compatible with known denaturation phenomena associated with pressure-temperature interaction. In further exploration of reversible denaturation of chymotrypsinogen under HP, the conformational relaxation occurred slowly, which allowed for the main states of the equilibrium mixture to separate by electrophoresis; he reported experimental concentration distribution patterns obtained at pH 2.03 and 20.5 °C consistent with the expected behavior from a simple two-state isomerism (Hawley & Mitchell, 1975).

Taniguchi & Suzuki (1983) examined the pressure effects on the inactivation of α-chymotrypsin (α-CHT) at different pressures up to 5 kbar (500 MPa) from 20 to 55 °C, the apparent inactivation rate increased when the temperature increased, and then suddenly dropped to zero above certain temperature, therefore reflecting the temperature dependence of the deacyclation and the heat inactivation of α-CHT. Considering the volume alterations and hydrophobic interactions, the results seemed to support a scheme where pressure inactivation is caused by the rupture of hydrophobic interactions amid nonpolar groups of α-CHT. Also in 1983, Weber and Drickamer review their ground-breaking work on the use of fluorescence as a probe of protein conformation; they established the nature of changes in protein conformation induced by temperature or pH which were invariably irreversible. Further studies have shown that, under a variety of circumstances pressure can introduce reversible changes (Drickamer, 1999).

In 1985, Heremans and Wong indicated the role of intermolecular interactions in pressure-induced protein denaturation; and later, in 1987, Kauzmann singled out the importance of high pressure in the understanding of the hydrophobic effect.

Hayashi, Kawamura, Nakasa & Okinaka (1989) applied high hydrostatic pressure to fresh egg white and yolk, further analyzing the resulting gels in regards of their texture, protease susceptibility and nutrients. The egg white set to a stiff gel at above 6000 kg/cm^2 (585 MPa), while the egg yolk reached the same state at a lower pressure of 4000 kg/cm^2 (390 MPa). In both cases, the gels had a natural taste, preserved their vitamins and showed no amino acid residue or formation of unusual compounds. The authors proposed the use of HP to minimize adverse effects in food processing and preservation.

In the early 1990s the first commercial food applications of HP technology were seen, when the Japanese company Medi-Ya launched and marketed the first high pressure processed (HPP) food, a high-acid jam (Mozhaev, Heremans, Frank, Masson & Balny, 1994). Due to the commercial success of jams, other products have since been marketed, such as HPP jellies and shellfish in Japan, oysters and guacamole in the United States, and fruit juices in France, Mexico and the United Kingdom (Smelt, 1998; Torres & Velazquez, 2005).

In 1992, the first high pressure conference in bioscience and biotechnology took place, organized by Claude Balny. This same year, Groß and Ludwig reported pressure, temperature phase diagrams for the inactivation of microorganisms, and later in 1994 Jonas and Jonas account on the first NMR study of the temperature and pressure of a protein. Also, phase diagrams were developed for synthetic polymers (Kunugi, Takano, Tanaka, Suwa & Akashi, 1997); for the gelation of starch (Rubens, Snauwaert, Heremans & Stute, 1999) and for staphylococcal nuclease denaturation using fluorescence and nuclear magnetic resonance (Royer, Hinck, Loh, Prehoda, Peng, Jonas & Markley, 1993).

High pressure treatment of milk is currently a popular research topic, and it has celebrated a centenary of the original work of Hite in 1899. Hite had succeeded in prolonging milk shelf life, but the technical difficulties of obtaining the necessary pressures caused for his work to be largely dormant and only reappeared when Japanese researchers teamed up with heavy industry who had the necessary engineering know-how. Clearly, much more is now known about the mechanism of action of high pressure. A few selected applications of high

pressure in foods have been a commercial success, among them avocado paste and raw ham, and in some dairy applications, HP processing has accomplished in eliminating yeasts in fermented products, although the high resistance of spores has proved to be a hard to solve problem (Patazca, Koutchma & Ramaswamy, 2006; Shao, Ramaswamy & Zhu, 2007; Zhang & Mittal, 2008).

Mussa & Ramaswamy (1997) studied the kinetics of microbial destruction and changes in physico-chemical characteristics of fresh raw milk caused by HP treatment which was conducted at 200-400 MPa for various holding times (5-120 min). The treatment led to an efficient destruction of microorganisms and a prolonged shelf-life of milk up to 18 days at 5 °C and 12 days at 10 °C. It was concluded that HP processing of milk may be a useful alternative for extending the shelf-life with quality advantages.

It becomes obvious that along a century of utilizing HP for food processing is only over the last 20 years that significant advances in HPP technology have been made, in the form of semi-continuous systems, to the scaling up of pilot units, to successful commercially viable processes. Present industrial HPP treatment of food is carried out using a batch or semi-continuous process; solid food can only be treated in a batch mode whereas liquid products can also be treated using a continuous or semi-continuous process (Hogan, Kelly & Sun, 2005). A main instance of the commercial significance of HPP food equipment at an industrial level is Avomex Inc., which began HP-treating avocado using a 25 L batch processing unit in 1996 and then decided to invest in another 25 L as well as a 50 L vessel as product demand expanded. By the 21st Century, the company had undergone another expansion, investing in a semi-continuous unit and a larger 215 L batch processing vessel (Torres & Velazquez, 2005). More recently, HPP has extended to include food products such as salsa, rice products, fish, meal kits (containing HP-treated cooked meats and vegetables), poultry products and sliced ready-to-eat meats (Murchie, Cruz-Romero, Kerry, Linton, Patterson, Smiddy & Kelly, 2005). HPP treatment of such foods has enabled the consumer to access foods with distinct advantages over thermally processed foods, such as minimally processed, fresh-tasting, high-quality convenient products with an extended shelf life.

Nowadays there is an important amount of scientific publications on HP related topics produced per year and the number keeps on rising. Among the different research groups on HP

processing in the world, our McGill University-Macdonald campus group lead by Dr. H.S. Ramaswamy stands out as the most complete in terms of equipment, and perhaps also the most diverse in terms of HP-related topics studied. Starting research on high pressure subjects in 1993, Dr. Ramaswamy's group has produced a vast amount of peer-review scientific publications in topics like:

a) HP destruction of spoilage and pathogenic bacteria in different foods (Basak, Ramaswamy & Piette, 2002; Basak, Ramaswamy & Smith, 2003; Mussa & Ramaswamy, 1997; Mussa, Ramaswamy & Smith, 1999a,b; Pandey, Ramaswamy & Idziak, 2003; Ramaswamy, Riahi & Idziak, 2003; Riahi, Ramaswamy & Idziak, 2003; Shao et al., 2007);

b) High pressure shifted freezing and thawing (Chen, Zhu, Ramaswamy, Marcotte & LeBail, 2007; Ousegui, Zhu, Ramaswamy & LeBail, 2006; Rouille, LeBail, Ramaswamy & LeClerc, 2002; Sequeira-Muñoz, Chevalier, Simpson, LeBail & Ramaswamy, 2005; Zhu, Bulut, LeBail & Ramaswamy, 2004; Zhu, LeBail, Chapleau, Ramaswamy & Lamballerie-Anton, 2004; Zhu, LeBail & Ramaswamy, 2003, 2006; Zhu, LeBail, Ramaswamy & Chapleau, 2004; Zhu, Ramaswamy & LeBail, 2004, 2005a,b, 2006; Zhu, Ramaswamy & Simpson, 2004);

c) Changes in quality of foods, including changes derived from HP inactivation of enzymes (Ahmed & Ramaswamy, 2006a,b; Ashie, Simpson & Ramaswamy, 1996b; Basak & Ramaswamy, 1997, 2001; Basak, Ramaswamy & Simpson, 2001; Pandey & Ramaswamy, 2004; Pandey, Ramaswamy & St-Gelais, 2003a,b; Ramaswamy & Riahi, 2003; Riahi & Ramaswamy, 2003, 2004; Sequeira-Muñoz, Chevalier, LeBail, Ramaswamy & Simpson, 2006);

d) HP-induced changes in functional properties of foods, including texture, viscoelasticity and digestibility (Ahmed, Ayad, Ramaswamy, Alli & Shao, 2007; Ahmed & Ramaswamy, 2003a,b, 2004, 2005, 2007; Ahmed, Ramaswamy, Alli & Ngadi, 2003; Ahmed, Ramaswamy & Hiremath, 2005; Ashie, Simpson & Ramaswamy, 1996a; Basak & Ramaswamy, 1998; Izquierdo, Alli, Gomez, Ramaswamy & Yaylayan, 2005; Pandey, Ramaswamy & St-Gelais, 2000; Sareevoravitkul, Ramaswamy & Simpson, 1996);

e) HP at elevated temperature inactivation of bacterial spores (Patazca et al., 2006; Shao, Zhu, Naim, Marcotte, Ramaswamy & Shao, 2008; Zhu, Ramaswamy & Marcotte, 2008);

f) HP-induced structural changes of proteins (Ahmed et al., 2007; Alvarez, Ramaswamy & Ismail, 2007; Alvarez et al., 2008).

2.2 Advantages and short comings of the use of HP to treat food

Treatment of food with HP is been primarily studied as a means to control bacterial populations with the improvement of the food's shelf life and/or increased product safety. But there are some other areas of interest to the technology to which some of the most promising are: HP-shifted freezing and thawing; HP-assisted thermal sterilization; inactivation of quality affecting enzymes; enhance activity of enzymes, as to accelerate processes like cheese ripening; enhance functionality of foods with minimal impact to nutritional/quality aspects; improve proteins digestibility; minimize allergens and antinutritional factors; enhanced release of nutraceuticals from foods.

Before we examine some of the most important applications of HP technology, we should take a look at some of the governing principles inherent to this novel process: the pressure applied to foods being processed is transmitted isostatically and instantaneously; thus the process is not dependent on the shape or size of the food. This is a major improvement, since the food is treated evenly throughout its geometry, which has regularly been problematic in thermal processing of large or bulky food products. Pressurization of liquid or solid foods at room temperature is usually accompanied by a 5-15 °C temperature increase, termed adiabatic heating, depending on the food composition (Balasubramanian & Balasubramaniam, 2003). Foods cool down to their original temperature on decompression provided no heat is lost or gained during the pressure hold time (Hogan et al., 2005).

HPP acts instantaneously and uniformly throughout a mass of food independent of size, shape, and food composition. Consequently, package size, shape, and composition are not factors in process determination. The work of compression during HPP treatment will increase the temperature of foods through adiabatic heating approximately 3 °C per 100 MPa, depending on the composition of the food. For example, if the food contains a significant amount of fat, such as butter or cream, the temperature rise can be larger. Foods cool down to their original temperature on decompression if no heat is lost to or gained from the walls of the pressure vessel during the hold time at that pressure. A uniform initial temperature is required to achieve a uniform temperature increase in a homogenous system during compression (Zhu, Ramaswamy & Le Bail, 2005).

Characteristically, food preservation techniques are for the most part evaluated based on their ability to eradicate pathogenic microorganisms, in so doing improving food safety and extending product shelf life through the inactivation of spoilage microorganisms. High pressure treatment has a distinct advantage in this respect producing foods of superior quality and nutritional value than thermally processed products (Smelt, 1998).

HPP is potentially able to produce high-quality extended-shelf life foods that exhibit characteristics of fresh products and are microbiologically safe. For today's market, HP treated foods are novel foods under the criteria of a new manufacturing process being employed in their production, and their history of human consumption, to date, being minimal. However, consumer perception of food quality depends not only on microbial quality but often and mainly on other more apparent factors such as biochemical and enzymatic reactions and structural changes, taste and visual appeal. High Pressure treatment of food can have an effect on food yield and on sensory qualities such as color and texture, and it is common knowledge that the appearance and color of food significantly influence consumer sales (Hogan et al., 2005).

During HP treatment of certain high-protein foods, some degree of protein denaturation can take place, but the resulting changes in physical functionality and/or raw product color are significantly less compared to those experienced using conventional thermal processing techniques (Hogan et al., 2005). Even further, the reversibility of HP-induced protein denaturation is determined by treatment conditions such as temperature, time and pressure, and also depends on the type of protein (Rastogi et al., 2007). By using high pressure conditions of 100-300 MPa, proteins usually, though not always, denature, dissolve or precipitate reversibly (Thakur & Nelson, 1998).

The HPP treatment could extend the shelf life of fresh meat and poultry by controlling the growth of both spoilage and pathogenic bacteria. Hugas, Garriga & Monfort (2002) report that from both a physico-chemical and microbiological point of view, cooked pork ham, dry cured pork ham and marinated beef loin, vacuum-packed and high pressure treated at 600 MPa for 10 min at 30 °C, are substantially equivalent to the same untreated products. In fresh meat and poultry, pressure-induced color changes are due to changes in myoglobin, heme displacement/release or ferrous atom oxidation, which can result in a cooked-like appearance

that prevents the product from being sold as fresh meat. On the other hand, high pressure treatment of white or cured meats hardly ever causes major color changes (Cheftel & Culioli, 1997).

Along with color, food texture has a vast impact on product sales, as even the most appealing food will be perceived as decaying or just "not good" if its textural properties are not similar to those of fresh food, e.g. soft/spongy texture. Consequently, a detailed understanding of the rheological properties of different kinds of foods is essential for product development and quality control. The physical structure of most high-moisture food products remains unchanged after HPP exposure as the pressure exerted does not generate shear forces; however, color and texture may change in gas-containing products after high pressure treatment due to gas displacement and liquid infiltration into the collapsed gas pockets from the surrounding food structure. Shape distortion and physical shrinkage can occur due to the collapse of air pockets, generally causing irreversible compression of whole foods such as fruit (Hogan et al., 2005); however, fruit fragments versus whole fruit are better suited for such treatment. Regardless of some of the objectionable textural changes, high pressure treatment can also be used to induce desirable changes in product texture and structure such as melting of Mozzarella cheese during processing (O'Reilly, Murphy, Kelly, Guinee, Auty & Beresford, 2002).

Among the main benefits of HP processing of food is the extension of shelf life while retaining the sensory characteristics of fresh food products (Lakshmanan, Patterson & Piggott, 2005). One of the most successful cases is that of fresh avocado, in regards of which, Palou, Hernández-Salgado, López-Malo, Barbosa-Cánovas, Swanson, & Welti-Chanes (2000) reported that its delicate sensory attributes could be preserved using HPP while also conferring a sensibly safe and stable shelf life. In general, HPP treatment gives a higher food product yield compared with heat treatment, with effects depending on product type and treatment intensity. This has enormous economic significance to food manufacturers (Hugas et al., 2002).

Whereas conventional thermal sterilization processes are the most frequently used methods for food preservation, and they are effective mechanisms for microbial inactivation, these techniques often lead to undesirable changes in the product quality, flavor, texture, color, and vitamin contents. In contrast, microbial inactivation provided by HPP, targets primarily cell membranes of treated cells, even though in some cases the process can cause solute loss,

protein denaturation and key enzyme inactivation. However, in this occasions when the sole use of high pressure yields to unsatisfactory results, the food treatment benefits when HP is combined with other processing techniques, such as HP with acidification, and HP with pulsed electric fields. In addition, when HP has been used concurrently with mildly thermal processes, has been found to increase the inactivation of bacterial spores (Raso & Barbosa-Canovas, 2003).

Most of the high-pressure equipment currently used is operated in the batch mode. Since pressurizing and depressurizing steps can now be rapidly accomplished, the low efficiency associated with batch processing may be minimized. Rapid pressurizing and depressurizing cycles also can cause metal fatigue and reduce the life of equipment. Above 400 MPa, the weight of equipment increases significantly, as does its cost (Torres & Velazquez, 2005).

Foods produced by HPP are already available on both the Japanese and US markets. These foods generally cannot be processed by conventional methods. Guacamole (prepared from avocado) is traditionally prepared immediately prior to consumption owing to its sensitivity to heat and other preservation methods. High pressure-processed guacamole, having a refrigerated shelf-life of several weeks, is now produced by Avomex (Avomex Inc., Keller, Texas) for the US market. Pressure-processed fruit juices with sensory qualities similar to those of the freshly prepared products are scheduled to appear in the US market in the near future. The recent application of HPP to fresh seafood allows the sale of minimally processed oysters with a level of contamination that otherwise could not be reached without affecting the sensory properties of the product. Pressure-processed fresh oysters will soon be available on the US market. The novelty of pressure-processed products justifies their relatively high price and the high processing start up costs (Palou et al., 2000; Kural & Chen, 2008).

As for the Canadian market, in 2004 Health Canada approved the sale of applesauce and applesauce/fruit blends treated by high hydrostatic pressure by Leahy Orchard Inc., Franklin Centre, Quebec. The processing consists of a 550 MPa treatment with a 1 minute hold.

2.2.1 HP-cold pasteurization of high acid foods

High pressure processing is rising as a promising alternative to the prevalent thermal methods of food processing. High pressure has a lethal effect on the viability of many microorganisms, and while its application on liquid foods is not new, more recent developments combine high pressure with other treatments with encouraging results (Phua & Davey, 2007). High pressure can be applied either as HP-cold pasteurization, or as HP-temperature pasteurization, and in both cases, the final product has the added advantage of being additive-free, but in the case of cold pasteurization, it also retains most of its original nutrients, sensory and olfactory properties (Dumay, Picart, Regnault & Thiebaud, 2006)

Like any other food processing method, HPP cannot be universally applied to all types of foods. However HPP can be used to process both liquid and solid foods. Foods with a high acid content are particularly good candidates for HPP technology. At the moment, HPP is being used in Japan, Europe, and the United States on a select variety of high-value foods either to extend shelf life or to improve food safety. Some products that are commercially produced using HPP are cooked ready-to-eat meats, avocado products (guacamole), tomato salsa, applesauce, orange juice, and oysters (Rastogi et al., 2007).

Orange juice is a highly valued source of vitamin C, during storage orange juice sustains deteriorative reactions such as ascorbic acid degradation, cloud loss, microbial spoilage, off flavor and changes in color, texture and overall appearance. The orange juice commonly marketed, is habitually obtained from frozen concentrates reconstituted and thermally pasteurized to accomplish microbial stability and extend the shelf life of the product for a few weeks under refrigeration. While conventional treatment of fruit juices has been the most extensively and efficiently used, this process has a negative effect on the sensorial and nutritional characteristics of the final product (Rassis & Saguy, 1995).

Degradation of L-ascorbic acid during the thermal processing of orange juice is an important problem associated with its quality. During storage, ascorbic acid is degraded both aerobically and anaerobically, at a pace depending on storage and packaging conditions, as well as the processing method used. In contrast, high pressure treatment alone doesn't seem to significantly change vitamin C concentration of orange juice (Polydera, Stoforos & Taoukis, 2003, 2005).

Some four years earlier Irwe and Olsson (1994) had already started investigation on the pectin methyl esterase (PME) enzyme residual activity after pressures up to 600 MPa, while Basak & Ramaswamy (1997) performed destruction kinetic studies of PME between 100 and 400 MPa.

Garcia-Graells, Hauben & Michiels (1998) reported as a reassuring observation that HP cold pasteurization even under the mildest conditions (mango juice, pH 4.0, and HEPES buffer, pH 4.0; 300 MPa), led to a 100-fold further reduction of pressure-resistant *E. coli* organisms during the first 3 days of refrigerated storage. Considering that enterohemorrhagic *E. coli* strains have a low infectious dose, this reduction may not be sufficient to provide the desired level of safety, and a higher pressure was recommended in this particular case. Assuming that an acceptable safety level requires a 105-fold reduction in numbers of organisms; this can be achieved, even in a pH 4 juice, within 2 days of refrigerated storage after a moderate pressure treatment (500 MPa, 20°C, 15 min).

Among the nutritional needs of humans, folates are one of the most lacking in the modern diet. These hematopoietic vitamins are especially important during pregnancy and they are present in fruits, particularly citrus, green leafy vegetables and organ meats. The loss of folate during food processing and home cooking/preparation as well as the incomplete bioavailability of folate in many human diets is also an important factor in the folate scarcity, even though common citrus fruit such as oranges are an significant source of folates. Hayashi et al., (1989) conducted high pressure studies on folates in egg white and yolk, reporting no significant vitamin loss under pressure treatment at 25 °C, in contrast with the considerable loss that occurred at 100 °C. A 2- to 3-fold increase in the proportion of folate in the monoglutamate form was reported as a consequence of the pressure treatment of leaks, cauliflower and green beans, potentially representing a better bioavailability of folates (Melse-Boonstra et al., 2002).

Butz, Serfet, Fernández García, Dieterich, Lindauer, Bognár & Tauscher (2004) reported that high pressure treatment of freshly squeezed orange juice for the purpose of cold pasteurization seemed to be feasible and effective regarding the folate retention at 25 °C. A temperature increase (80 °C) and high pressure for a short time didn't cause drastic loss of folates in natural orange juice.

In Australia, microbiological safety and quality of high pressure processed juice has been examined, as well as the physical and chemical characteristics and other quality indicators like nutrient content (vitamin C and beta-carotene), and descriptive sensory analysis and consumer acceptability testing conducted (Baxter, Easton, Schneebeli & Whitfield, 2005).

The microbiology results revealed that it was possible to extend the shelf life of orange juice using a pressure treatment of 600 MPa for 60 seconds. This treatment killed the bacteria and most of the yeasts and moulds which normally cause fresh juice to spoil very quickly. Acid foods such as fruit juices have historically been considered safe products, but acid tolerant pathogens such as *Salmonella* and *Escherichia coli* O157:H7 have been shown to be able to survive in refrigerated juices. In Food Science Australia, they were interested in looking at the level of reduction of Salmonella obtainable by high pressure treating the orange juice. By inoculating the orange juice with differing amounts of Salmonella, they were able to show that the same pressure treatment which gave orange juice a 3 month refrigerated shelf life would also kill very high levels of Salmonella in the juice. After the pressure treatment, they also held the juice at 4 °C for 4 days and found no pressure-injured Salmonella was able to recover in the juice during refrigerated storage. Bull and her team developed a mathematical model to predict inactivation of Salmonella in orange juice in terms of the pressure magnitude and hold time required to obtain a specified log reduction of *Salmonella* in orange juice. With the Salmonella inactivation model it will be possible to determine the magnitude of pressure and hold times required when processing orange juice to achieve a reduction of *Salmonella* that will meet Australian regulation requirements (Bull, Szabo, Cole & Stewart, 2005).

In an endeavor to assess the effects of HPP at various exposure times on the survival of *Cryptosporidium parvum* oocysts in apple and orange juice, Slifko, Raghubeer & Rose (2000) suspended the oocysts in a sample media, deionized water, PBS buffer, apple juice, and orange juice, and then treated with HPP at 550 MPa for 0, 30, 45, 60, 90, and 120 seconds. Oocyst viability was judged by excystation using bile salts and trypsin, while the foci detection method (FDM) was used to assess infectivity. HPP inactivated *C. parvum* oocysts by at least 3.4 log after 30 seconds of treatment in both apple and orange juice. After 60 seconds of treatment with HPP, no activity was evident in either juice sample. The oocyst infectivity of the orange juice process control was 49 % more infective than the mean infectivity of the PBS control.

However, these oocysts appeared to be more susceptible to inactivation than the oocysts in apple juice because no activity was noted after only 30 seconds exposure to HPP. The researchers propose that this difference was due to the combination of sugars and acidity in the orange juice, which enhanced excystation of the untreated oocysts and increased the percent infectivity. It appears that the FDM was capable of assessing *C. parvum* infectivity when suspended in apple and orange juice. The study also demonstrated that HHP efficiently rendered the oocysts nonviable and noninfectious after treatment at 550 MPa for 30 seconds or more. These conclusions will aid in developing a recommendation for high-pressure processing treatment conditions that will produce adequate reductions of *Cryptosporidium* outbreaks.

2.2.2 Changes in quality

According to the United Kingdom's Agri-Food and Biosciences Institute, high pressure processing can be used to give additional microbiological safety assurance to meats already treated with another preservation method without further adversely affecting eating quality. The foods are not sterile and must be refrigerated to ensure optimum quality. HPP is being used commercially in the USA and Europe to treat cooked poultry, cooked beef, vacuum packaged ham, fermented sausage and salami (Hayman, Baxter, O'Riordan & Stewart, 2004; Marcos, Aymerich & Garriga, 2005; Patterson, 2005). In the light of the unlucky events of sliced ready to eat meats in Canada involving contamination with *Listeria monocitogenes,* HP processing should become one of the alternatives to prevent such events in the future.

Regarding the HP treatment of dairy products, HPP can affect the protein structure in milk, giving the potential to produce cheese with unique, desirable characteristics. This has been used successfully in the manufacture of mozzarella cheese which has improved "meltability" and stringiness, both desirable characteristics in pizza production. Changes in the protein structure can also lead to novel gelling properties, which could be of value in the manufacture of yogurts and other dairy desserts (Sheehan, Huppertz, Hayes, Kelly, Beresford & Guinee, 2005; Walker, Farkas, Loveridge & Meunier-Goddik, 2006).

Molina, Alvarez, Ramos, Olano & López-Fandiño (2000) evaluated the suitability of high-pressure-treated milk for reduced-fat cheese production. For this purpose, pasteurized, pressurized and pasteurized-pressurized milks were compared regarding cheese-making properties, cheese composition, proteolysis during ripening and development of cheese flavor

and texture. The results obtained showed that pressurization (400 MPa, 22 °C, 15 min) of semi-skim milk prior to cheese-making increased the yield of reduced-fat cheese through an enhanced protein and moisture retention. The sequential application of pasteurization (65 °C, 30 min) and pressurization increased such effect over the pressurization or raw milk and, in addition, pressurization of pasteurized skim milk improved its coagulation properties. The cheeses made from pasteurized-pressurized and pressurized milks underwent a faster rate of protein breakdown than the cheeses made from pasteurized milk, which could have been responsible for a more rapid development of texture and flavor. The cheeses with the highest moisture content and proteolytic degradation presented the lowest hardness, as determined by both the sensory panel and instrumental analyses. In addition, the results suggested a relationship between the lower hardness and cohesiveness of the cheeses made from pasteurized-pressurized and pressurized milks, as determined instrumentally, and their higher texture scores determined by sensory analysis. Pressurization of low-fat milk prior to cheese-making improved cheese texture and thus accounted for a higher overall acceptability, except for the cheese made from pasteurized-pressurized milk at 60 days of ripening, whose acceptability score was adversely affected by bitterness. Since pressurized milk coagulates faster and produces firmer curds, the amount of coagulant used can be reduced so the amount of residual rennet can be optimized.

The development of food ingredients with novel functional properties offers the dairy industry an opportunity to revitalize existing markets and develop new ones. HPP can lead to modifications in the structure of milk components, in particular protein, which may provide interesting possibilities for the development of high value nutritional and functional ingredients. O'Reilly, O'Connor, Murphy, Kelly & Beresford (2000) reported that the application of HPP to Cheddar cheese resulted in increased levels of proteolysis, a key measure of maturity, indicating that HPP has the potential to accelerate the ripening of Cheddar. However, the level of acceleration observed was not sufficient to support the commercial exploitation of the technology.

Walker, Farkas, Loveridge & Meunier-Goddik (2006) used HPP to treat fruit yogurt in an attempt to develop a shelf stable fruit yogurt and monitor changes in color, texture and microbial content during 60 days storage at 4.4 °C and room temperature (25 °C). High

pressure treated yogurt had a noticeably smoother and thicker appearance and maintained larger viscosity at both storage temperatures over the 60 days storage period, compared with the non pressure treated control yogurt. In addition, there were no significant effects on color or pH. The HPP inactivated yeasts and moulds and no growth was observed following prolonged storage at room temperature, but the HPP also inactivated the lactic acid bacteria after 40 days, therefore not allowing for the "live culture" claim to be stated in the yogurt container.

The browning of high pressure treated fruits is a disadvantage for its marketing value. Prestamo & Arroyo (1999) assessed the effects of ascorbic acid on protecting fruit products from the browning effect produced by high-pressure treatment, contributing to a better understanding of how fruits respond to high pressure. The combination of HP and ascorbic acid was found to reduce the browning effect completely in the treated samples. Ascorbic acid treatment (20 mM) prevented the samples from browning unlike the untreated samples and samples subjected to HP alone. HP extended the shelf-life of the product, eliminating the microbial population after pressurization.

The most valuable elements in some fruit products are extremely hard to maintain throughout conventional processing methods, as is the case with broccoli's sulforaphane. Houška et al. (2006), strived to develop the optimum method to obtain broccoli juice with the highest possible content of sulforaphane. The repeated storage experiments with broccoli juice and with apple-broccoli juice flavored with frozen orange juice concentrate and acidified with lime juice showed that the high-pressure pasteurization is effective to kill the viable microorganisms naturally present in the juice. The microbial quality was excellent for 30 days of chilled storage and the challenge tests made with moulds, yeasts and coliforms demonstrated the capability of the high-pressure pasteurization to decrease the five logarithmic decades of these microbes especially the *E. coli* while maintaining a desirable sulforaphane content.

Fruit products treated with high temperatures frequently exhibit a cooked flavor, and a loss of texture, color and nutrient quality. Non-thermal high pressure processing exhibits the potential of achieving a "fresh-like" fruit product desired by consumers while inactivating pathogenic and spoilage microorganisms (Kimura, Ida, Yosida, Ohki, Fukumoto & Sakui, 1994) compared the flavor components, color, hue and nutrients of pressure-treated (400-500 MPa, 10-30 min at room temperature) and heat-treated (93 °C, 20 min) strawberry preserves

during storage at 5 and 25 °C for 1-3 months. Immediately after processing, the pressure-treated fruit preserves exhibited better fresh quality than the heat-treated fruit preserves. The quality of the heat-treated fruit preserves after 3 months of storage was maintained at both refrigerated and room temperatures. The quality of pressure-treated fruit preserves after 3 months of storage was maintained at refrigerated temperatures, but not at room temperature. The deterioration of the pressure-treated fruit preserves held at ambient temperature was attributed to the presence of dissolved oxygen and enzymes.

Given the traditional nature of the oyster industry, one of the most stimulating developments in the industry in recent years is HP processing of oysters. HP can kill *Vibrio parahaemolyticus* and other pathogenic microorganisms in these products. He, Adams, Farkas & Morrissey (2002) reported that HP reduced the initial total microbial load of oysters by 2 or 3 logs and that counts remained at a reduced level during subsequent storage (over 27 days at 3 °C). They also studied the use of HP for opening of oysters and reported that HP treatment at 241 MPa for 2 min caused detachment of adductor muscle (shucking) in 88 % of oysters, while treatment at 310 MPa, with immediate pressure release, resulted in 100 % efficiency of shucking; changes in oyster visual characteristics were observed at higher pressures.

Cruz-Romero, Kelly & Kerry (2007) studied the effects of HP treatment on the pH, color, proteins and gross composition of oysters, finding that HP treatment influenced the composition of oysters, resulting in significant changes in proximal composition compared to untreated oysters. For example, HP treatment significantly increased the pH of whole oyster tissue, compared to untreated oysters. HP treatment was also effective in detaching oysters' adductor muscles from the shell, producing a shucked oyster tissue with a good shape and appearance which looked slightly more voluminous and juicy than untreated oysters, as previously reported by López-Caballero, Pérez-Mateos, Montero & Bonderías (2000) and He, Adams, Farkas & Morrissey (2002).

The unique physical and sensory properties of pressure processed foods offer new opportunities for product development. These could include new, long-shelf-life convenience foods with fresh flavors and colors, minimally processed or raw fish and meat, new types of food gels and frozen foods with radically improved quality. Because the process is relatively expensive, products manufactured in this way will need to have inherent added value or increased profitability. It is unlikely that pressure processing will replace food canning or freezing but it could find applications for expensive foods with short shelf lives and high value

ingredients such as flavors, vitamins and functional biopolymers which are sensitive to heating (San Martín et al., 2002).

2.2.3 Changes in functional properties

High pressure processing can modify functionality of food products to a certain extent and this can be controlled by varying the pressure level, pressure holding time and depressurization time, as well as other parameters non inherent to the high pressure process such as pH, ionic strength and food composition (Messens, Van Camp & Huyghebaert, 1997).

2.2.3.1 Food rheology and texture

Modern rheology is defined as the deformation and flow of matter and food rheology is the deformation and flow of the raw materials, the intermediate products and the final products of the food industry. Rheology focuses on the relationship between a force acting on a substance and its resulting deformation; simply put, it is the study of how systems behave when work is applied (liquids might flow, solids may bend) (Walstra, 2003).

Rheological tests in food are of great value for engineering process design, since the knowledge about food flow and deformation properties makes possible the design of equipment for handling foods, such as conveyor belts, pumps, pipelines, storage containers, etc. Rheological tests allow acquiring information on the structure of the food and its macromolecular elements, as well as assessing the textural attributes of the product prior to its processing, and based on these predictions, the food processing will be oriented towards achieving a final product with the characteristics that have proven desirable for the consumer. The deformation of the food under the influence of a force is frequently used as a measure of quality, so foods that deform to a large extent are categorized as soft, flaccid or spongy, whereas those who deform to a small extent are classified as firm, hard or rigid. This doesn't mean that either group is better than the other, because the association of these characteristics with food quality depends on the desirability for the consumer regarding that specific type of food (Borwankar, 1992).

In order to obtain reproducible results in deformation tests, the geometry of the samples need to be controlled, so is customary to use specimens of standard dimensions and pre-defined geometry to perform the tests, where the force per unit area is called stress and the deformation per dimensional unit is called strain. The geometry determination is especially important in the type of foods that cannot be cut in standard-size/shape pieces (i.e. lettuce, eggs). Only

understanding how size and shape affect the deformability of the food is possible to separate the deformability measurements into differences due to changes in textural quality from those due to changes in size (Bourne, 1967).

There are a number of studies regarding rheological properties of high pressure treated foods. Arora, Chism & Shellhammer (2003) examined the effects of surfactant type and concentration, dispersed phase concentration, and the presence of a stabilizer on the rheological properties as well as physical stability of pressure-treated acidic emulsions, using materials and conditions found in commercial salad dressings. This research confirmed that pressure treatment had no significant detrimental effects on the rheological behavior as well as physical stability of acidified emulsions stabilized by whey protein isolate and polysorbate-60. Differences in the flow behavior, viscoelasticity (G' and G"), particle size distribution, and physical stability of emulsions were influenced largely by the lipid content and the type of surfactant. Emulsions stabilized by soy lecithin were inherently unstable, and this instability was further aggravated under pressure. Pressure stable oil-in-water emulsions can be formed using hydrophilic surfactants such as polysorbate-60 or whey protein. The addition of xanthan gum improved stability in systems emulsified with polysorbate-60, and this stability was further improved by the application of pressure treatment.

Ahmed, Ramaswamy, Alli & Ngadi (2003) investigated the effect of HPP on rheological characteristics of whole liquid egg (WLE), albumen and yolk, finding that all egg samples behaved as thixotropic fluid. The egg protein structure break-down was enhanced with pressure and it was complete at 300 MPa for 30 min at 20 °C. High pressure affected the protein structure of albumin and WLE; however, electrophoresis results exhibited that the protein coagulation was irreversible. The yolk behaved differently with pressure treatment. The study concludes stating that further work is needed on pressure-temperature combination and microbiological aspects of post-processed samples before implementing to industrial sector.

Ahmed, Ramaswamy & Hiremath (2005) observed improved rheological characteristics of mango pulp after HP treatment, with significant differences between canned (thermally pre-treated) and fresh mango pulp. No significant changes in color were detected after HP treatment.

The high pressure treatment also inactivates microorganisms and, therefore, this technique provides an alternative to heat treatments. In contrast, there are considerable

differences in protein denaturation and aggregation induced by high pressure compared with heat. The use of high pressure to modify the functionality of food proteins was reviewed by Heremans & Smeller (1997) and Messens et al., (1997) finding that, while blood plasma and egg white proteins are known to be sensitive to heat and readily form gel networks at moderate operating temperatures, at 80 °C (30 min), no gelation occurs if these proteins are pressurized for 30 min at a pressure of 400 MPa. This stability may be positively correlated with the high amount of disulfide bonds stabilizing the three-dimensional structure of both proteins. At the same time, β-lactoglobulin appears far more sensitive towards pressure than ovalbumin and bovine serum albumin (BSA). High pressure processing has been shown to destabilize casein micelles in reconstituted skim milk. The size distribution of the spherical casein micelles changed from ~200 nm to 120 nm after pressurization, whereas subsequent heating of skim milk at 30 °C and at atmospheric pressure restored the original size distribution.

2.2.3.2 Protein foaming

Foams are an array of bubbles dispersed within a continuous phase arranged in the form of a thin film called lamellae. In the case of water-based foams, the air bubbles are separated by an hour-glass shaped film containing dissolved solutes such as sugars, fat, ice crystals, low molecular weight emulsifiers, thickeners (non-gelling), starch, protein or a combination of these. Foaming capacity (FC) is the volume of gas-phase, and foam stability (FS) is the degree to which fluid can be retained within the foam, requiring a cohesive protein film that entraps air bubbles (Bos & van Vliet, 2001).

Foam is caused by the protein film lowering surface tension. An example would be cohesive force of water molecules that tend to collapse a bubble, and give resistance to shearing/tearing with high level of stretching capacity. So when a protein solution is whipped or stirred vigorously, air is pulled down into solution, and when it tries to escape up, the flexible protein surface forms a bubble. Not all proteins foam equally well and not all of the foams are particularly stable. So BSA is slightly below average on foaming, while casein forms allow to obtain a good foam, but not stable. Egg protein doesn't form much foam, but it is quite stable, and gelatin provides a good amount of foam that is also stable (Campbell & Mougeot, 1999; Foegeding, Luck & Davis, 2006).

Proteins are without doubt the most employed foaming agents in the food industry. They adsorb sturdily at the air-water interface, where the protein unfolds. Hydrophobic amino acid residues within the protein orient themselves toward the gas phase while hydrophilic portions of amino acids place themselves in the aqueous phase. Because proteins typically have multiple hydrophobic sites, they adsorb strongly at the interface with little likelihood of spontaneous desorption. Unfolded proteins can also interact with other protein molecules, forming a film at the interface. Measuring the strength of the film using surface rheology is helpful in predicting stability against foam destabilization mechanisms such as coalescence and disproportionation (Murray, 2007).

Rodiles-López, Jaramillo-Flores, Gutiérrez-López, Hernández-Arana, Fosado-Quiroz, Barbosa-Cánovas & Hernández-Sánchez (2008) found an increased foaming capacity and stability with HPP of α-lactalbumin (600 MPa at 55 °C for 10 min). Ibanoglu & Karatas (2001) studied the foaming capacity of whey protein isolate as affected by HP treatment; they found a positive correlation between total foam volume (FV) and pressure level applied, whereas pressure holding time did not have a significant effect over FV or foam stability.

Van der Plancken, van Loey & Hendrickx (2007) studied the foaming properties of egg white proteins affected by HP treatment; they found foams with high volume and average stability and density obtained by pressure treatment at pH 8.8 (above 500 MPa). The processing-induced changes in the foaming properties could not be attributed to the changes in a single physicochemical property; the foaming ability was in part determined by the sulfhydryl content and protein flexibility. Improved protein-protein interactions (solubility and exposed SH groups) contributed to increased foam stability of treated egg white solutions.

Lim, Swanson & Clark (2008) studied the foaming properties of fresh and commercial whey protein concentrate (WPC) after HP treatment. Solutions of WPC were treated with 300 and 400 MPa (0- and 15-min holding time) and 600 MPa (0-min holding time) pressure. After HHP, the solubility of the WPC was determined at both pH 4.6 and 7.0 using UDY and BioRad protein assay methods. Overrun and foam stability were determined after protein dispersions were whipped for 15 min. The protein solubility was greater at pH 7.0 than at pH 4.6, but there were no significant differences at different HHP treatment conditions. The maintenance of protein solubility after HHP indicates that HHP-treated WPC might be appropriate for

applications to food systems. Untreated WPC exhibited the smallest overrun percentage, whereas the largest percentage for overrun and foam stability was obtained for WPC treated at 300 MPa for 15 min. Additionally, HHP-WPC treated at 300 MPa for 15 min acquired larger overrun than commercial WPC 35. The HHP treatment of 300 MPa for 0 min did not improve foam stability of WPC. However, WPC treated at 300 or 400 MPa for 15 min and 600 MPa for 0 min exhibited significantly greater foam stability than commercial WPC 35. The HHP treatment was beneficial to enhance overrun and foam stability of WPC, showing promise for ice cream and whipping cream applications.

2.2.3.3 Protein emulsifying capacity/behavior

An emulsion is a mixture of two immiscible substances, where one of them or dispersed phase exists as discrete droplets suspended in the other or continuous phase. There is an interfacial layer between the two phases formed by some indispensable surfactant material (Schramm, 2005).

There are three main types of emulsions of high relevance to the food industry: oil-in-water emulsions (o/w), where droplets of oil are suspended in an aqueous continuous phase; water-in-oil emulsions (w/o), which depend more for their stability on the properties of the oil and the surfactant used than in the properties of the aqueous phase, and water-in-oil-in-water emulsions (w/o/w), an o/w emulsion where the oil droplets are in fact a w/o emulsion (they contain water droplets). This last type of emulsion is the most complex to produce and control, because the stability of the water droplets contained in the oil droplets, as well as the stability of the oil droplets contained in the continuous aqueous phase must be achieved (McClements, 2005).

Emulsions play an important part among the structure units within foods, as not only they impart a desirable mouth-feel to them, but also are essential ingredients in the formation of structures of certain products such as whipped toppings, ice creams and even processed cheeses (McClements, 2005).

The interfacial layer of most o/w emulsions contains proteins, often mixed with other surfactant materials. Proteins are frequently present in the raw food and they are excellent emulsifiers, but the composition of the interfacial layer if determined by what is present at the moment the emulsion is formed. If proteins are the only emulsifier, they will adsorb to the oil

interfaces in proportion to their concentration in the aqueous phase, except for some mixtures of α- and β-caseins, where there will be a preferential adsorption of β-casein (Dickinson, 1999).

Several different methods have been used to estimate the dimensions of adsorbed protein monolayers, such as dynamic light scattering, ellipsometry and neutron reflectance, showing that adsorbed layers of protein may be thick compared to molecular dimensions, one of the reasons why adsorbed proteins can stabilize emulsion droplets either by steric repulsion and electrostatic mechanisms. Most adsorbed proteins exist in conformations unlike their original state, as a result of the tendency of hydrophobic parts of the molecule to be adsorbed by the hydrophobic interface with the disruption of their secondary and tertiary structures. That is the main reason why the properties of the emulsion are not the same of those of the parent protein (Dalgleish, 1997).

In the food industry, proteins macromolecules are usually the universal stabilizer of w/o emulsions and foams. Emulsions in ice creams, toppings and creams are consistently stabilized by milk proteins, being usually casein in aggregated form the main stabilizer (Dickinson, 1999).

Regarding β-lactoglobulin emulsions, Dickinson & James (1998) reported that moderate thermal processing showed to have a far greater effect than high-pressure processing on the state of flocculation of β-lactoglobulin-coated emulsion droplets, therefore regarding HPP as a milder processing operation than thermal treatment. It was found that at neutral pH, strong emulsion gels were produced from concentrated emulsion samples (40 vol % oil + 1.0 wt % protein) following heat treatment at 70 C for 5 min. The increase in viscoelasticity caused by mild heating (i.e., 65 °C for 5 min) resembled the change in rheological behavior induced by rather severe pressure processing (800 MPa for 60 min). Even though both treatments induced droplet flocculation, the rheological properties of moderately dilute β-lactoglobulin-stabilized emulsions were unaffected by high pressure or thermal treatment, given of the insensitivity of small-deformation rheological analysis to the state of flocculation of droplets in a dilute emulsion such as the one used. Under conditions of lower pH or higher ionic strength, β-lactoglobulin-stabilized emulsions became more flocculated following temperature or pressure processing. The extent of emulsion flocculation following high pressure appeared greater when there was a larger proportion of free protein present in the aqueous continuous phase. The observation of significant levels of droplet flocculation, even in the presence of minimal unadsorbed protein, suggested that changes in adsorbed protein structure also contributed significantly to the overall effect of pressure treatment.

Parés & Ledward (2001) studied the emulsifying and gelling properties of porcine blood plasma as influenced by high-pressure processing, finding that, while high-pressure treatments up to 300 MPa did not affect the functionality of blood plasma proteins, regardless of the pH, the effects of treatments at pressures above 400 MPa caused pH-dependent changes. At acidic pH (5.5), increasing pressures led to a decrease in the emulsifying properties of plasma solutions and provoked changes in the textures of heat-induced gels, which adversely affected their water-holding capacity. Both properties were seriously affected by the changes in structure that the proteins underwent following HPP at this pH. At pH 6.5, high-pressure treatments at 400 MPa improved the emulsifying properties of plasma solutions without negatively affecting the characteristics of heat-induced gels. Treatments above 400 MPa produced solutions with reduced emulsifying activity and stability. At pH 6.5, heat-induced gels were always weaker and less elastic but, in some cases (pH 6.5), they showed improved water-holding capacity.

Rodiles-López et al., (2008) found improved emulsion activity index and emulsion stability with HPP α-lactalbumin after 600 MPa at 55 °C for 10 min at different pH (3.0-9.0). Yin, Tang, Wen, Yang & Li (2008) observed that HP treatment at 200 and 400 MPa significantly increased the emulsifying activity index (EAI) and emulsion stability index (ESI) of kidney-bean protein isolate (KPI); however, EAI was significantly decreased at 600 MPa (relative to untreated KPI).

2.2.3.4 Protein solubility

Solubility is a crucial function of proteins for the food industry. The Osborne classification of proteins such as albumins, globulins, glutelins and prolamins is based in their solubility. Albumins are those proteins that are readily soluble in water, while globulins are those that may require salt solution for solubilization. Glutelins are soluble in dilute acid or base, and prolamins require alcohol based media as solvents (Osborne & Campbell, 1898).

At the surfaces of proteins are amino acid residues that interact with water. The amino acids are referred to as hydrophilic amino acids and include arginine, lysine, aspartic acid, and glutamic acid. At pH 7 the side chains of these amino acids carry charges-positive for arginine and lysine, negative for aspartic acid and glutamic acid. As the pH increases, lysine and arginine begin to lose their positive charge, and at pHs greater than about 12 they are mainly

neutral. In contrast, as pH decreases, aspartic acid and glutamic acid begin to lose their negative charges, and at pHs less than 4 they are mainly neutral (Arakawa & Timasheff, 1985).

The surface of a protein has a net charge that depends on the number and identities of the charged amino acids, and on pH. At a specific pH the positive and negative charges will balance and the net charge will be zero. This pH is called the isoelectric point (p*I*), and for most proteins it occurs in the pH range of 5.5 to 8. A protein has its lowest solubility at its isoelectric point. If there is a charge at the protein surface, the protein prefers to interact with water, rather than with other protein molecules. This charge makes it more soluble. Without a net charge, protein-protein interactions and precipitation are more likely (Arakawa & Timasheff, 1985).

Rodiles-López et al. (2008) studied the solubility of α-lactalbumin treated with 600 MPa at 55 °C for 10 min, and found a positive effect on protein solubility after HPP, in particular at low pH values. Additionally, Yin, Tang, Wen, Yang & Li (2008) found the solubility of kidney-bean protein isolate significantly improved at pressures of 400 MPa or higher, possibly due to formation of soluble aggregates from insoluble precipitates.

2.2.3.5 Protein digestibility

Digestion typically begins in the stomach when pepsinogen is converted to pepsin by the action of hydrochloric acid, and continued by trypsin and chymotrypsin in the intestine. The amino acids and their derivatives into which dietary protein is degraded are then absorbed by the gastrointestinal tract. The absorption rates of individual amino acids are highly reliant on the protein source; for example, the digestibility of many amino acids in humans differs between soy and milk proteins and between individual milk proteins, beta-lactoglobulin and casein. For milk proteins, about 50% of the ingested protein is absorbed between the stomach and the jejunum and 90% is absorbed by the time the digested food reaches the ileum (Matthews & Laster, 1965).

The digestibility of proteins depends, among other things, on their structure. Compacting of the protein structure by cross-linking prevents unfolding of the peptide chains, which may decrease the digestibility by making some of the peptide bonds unreachable to the enzymes. Cross-linking may be caused by decrease on the rate of protein hydrolysis due to heating, as well as by interactions of amino groups with reduced saccharides or other compounds containing carbonyl groups. In food processing, these are secondary products of lipid oxidation and aldehydes contained in smoked products. As a result of several further

reactions, different unsaturated compounds are generated and interact with the amines, possibly involving different cross-linking reactions (Matthews & Laster, 1965).

The protein digestibility-corrected amino acid score (PDCAAS) method has been considered to be a straightforward and scientifically sound approach for routine assessment of dietary protein quality for humans. Major questions have, however, been raised about the validity of the PDCAAS relative to its inability to credit the extra nutritional values of proteins having scores higher than that of the reference protein, its failure to fully account for the possible adverse effects of antinutritional factors, and its assumption about complete biological efficiency of supplemental amino acids in improving quality of proteins, which may not be true in the case of poorly digestible, low quality proteins (Schaafsma, 2005).

Throughout food processing, protein sources are treated with heat, oxidizing agents such as hydrogen peroxide, organic solvents, alkalis, and acids for a variety of reasons such as to sterilize or pasteurize, to improve flavor, texture, and other functional properties, to deactivate antinutritional factors, and to prepare concentrated protein products. These processing treatments may cause the formation of Maillard compounds, oxidized forms of sulfur amino acids, D-amino acids, and cross-linked peptide chains, resulting in lower amino acid bioavailability and a decrease in protein quality (Bender, 1972).

In the journey towards a more accurate method to determine protein and amino acid digestibility of food products, several in vitro methods have been developed, simulating the physiological process of digestion. The Agri-Food Agriculture Canada center in St-Hyacinthe (Quebec) has acquired a complete system that simulates the digestive system from mastication to absorption of nutrients, this instrument is pretended to give a more holistic approach to food research.

Yin, Tang, Wen, Yang & Li (2008) studied *in vitro* trypsin digestibility of kidney-bean protein isolate and found that the protein digestibility decreased only at pressures above 200 MPa and for long holding times (up to 120 min). Chicón, Belloque, Recio & López-Fandiño (2006) previously noted an improved *in vitro* trypsin proteolysis of β-lactoglobulin as affected by pressures up to 400 MPa, regardless of the digestion carried out under pressure or over a pre-HP-treated protein. They extended their work to the pepsin digestibility of β-lg and whey protein isolate after HP treatment and found that HP treatment at 400 MPa promoted the

hydrolysis of β-lg by pepsin, but this increased susceptibility of β-lg to proteolysis was progressively lost during refrigerated storage (Chicón et al., 2008).

Izquierdo et al., (2005) reported complete in-vitro hydrolysis of β-lactoglobulin by α-chymotrypsin or pronase when using intermediate pressures (100-300 MPa), compared to partial hydrolysis by microwave or conventional heating.

2.2.3.6 Protein water holding capacity (WHC)

Water holding capacity is an important property of gelled foods, particularly in dehydrated and rehydrated products, in which the strength of the water binding to the solid matrix can be weaker than that in the original food. In some cases, water will not leak out of a solid-like food, but it is present in closed cells, in open pores in a solid matrix, or between chains of coiled polymers. Binding sites for water need not to be present for the water to be held, and this amount of held water can be large: in some gels, 1 g of polymer can hold 100 g of water, usually in the form of an aqueous solution rather than as pure water (Kneifel, Paquin, Abert & Richard, 1991).

A simple explanation for the way that water is held would be that of water as space filler. A coiled polymer molecule has a certain equilibrium conformation, and there are water molecules in the space between its segments in such a way that removal of water will cause shrinking of the coil, which costs free energy, since it implies a decrease in conformational entropy. This is also true for gels and gel-like structures, when deformation of the network needs a force and consequently, energy. The amount of held water in a gel-like system would vary with all the factors that affect the equilibrium state of swelling of the gel, such as concentration of cross-links, solvent quality, pH and ionic strength (Foegeding, 2006).

Pandey, Ramaswamy & St-Gelais (2000) studied the WHC of rennet curds obtained from HP-treated milk. They found that in general, with a decrease in pressure level, temperature and holding time, there was a decrease in water-holding capacity and an increase in the gel-strength of the produced rennet curds.

2.2.4 Changes in molecular structure of proteins

Proteins are linear condensation products of various α-L-amino acids which differ in molecular mass, charge and polar character, bound by transpeptide linkages. Proteins differ also

in the number and distribution of amino acid residues in the molecule, that is, the secondary structure containing helical regions, β-pleated sheets and β-turns, the tertiary structure or the spatial arrangement of the chains, and the quaternary structure or assembly of polypeptide chains (Richardson, 1981).

The structure of protein is an important determinant for its functionality. Therefore, changing the protein structure may improve functionality. The protein structure can unintentionally be changed during processing as in Maillard reactions and oxidation of cysteine or deliberately by modification reactions (Kester & Richardson, 1984).

Chemical modification methods that are commercially used include extensive hydrolysis and deamidation. Extensive hydrolysis by boiling with acids results in free amino acids and very small peptides, and is mainly applied for the preparation of hydrolyzed vegetable protein, frequently used to fortify and enhance the aroma of soups. Deamidation, which is the conversion of the amino acids glutamine and asparagine to their acidic counterparts, is meant to change the charge, achieving alteration and extension of the range of functional properties of wheat gluten (Vojdani & Whitaker, 1994).

Most studies on modification of protein molecular structure have focused on enzymatic modification, especially on the use of proteases and transglutaminases. There are also a number of publications on the enhancement of solubility, foaming and emulsifying properties by limited proteolysis leading to changes in charge, hydrophobicity and molecular mass in the transition from protein to peptide mixtures (Panyam & Kilara, 1996).

HPP can affect protein conformation and produce protein denaturation, aggregation or gelation, depending on the protein system, the applied pressure, the temperature and the duration of the pressure treatment. Low pressures usually induce reversible changes such as dissociation of protein-protein complexes, the binding of ligands and conformational changes, while pressures higher than 500 MPa induce, in most cases, irreversible denaturation to HP sensitive proteins (Gross & Jaenicke, 1994; Knorr, Heinz & Buckow, 2006).

2.2.4.1 Protein secondary level

The secondary structure of a segment of polypeptide chain is the local spatial arrangement of its main-chain atoms without regard to the conformation of its side chains or to

its relationship with other segments. There are three common secondary structures in proteins, namely alpha helices, beta sheets and turns. Those which cannot be classified as one of the standard three classes is usually grouped into a category called "random coil" (Richardson, 1981).

The alpha-helix and beta-structure conformations for polypeptide chains are generally the most thermodynamically stable of the regular secondary structures. However, particular amino acid sequences of a primary structure in a protein may support regular conformations of the polypeptide chain other than alpha-helical or beta-structure. Thus, whereas alpha-helical or beta-structure are found most commonly, the actual conformation is dependent on the particular physical properties generated by the sequence present in the polypeptide chain and the solution conditions in which the protein is dissolved. In addition, in most proteins there are significant regions of unordered structure in which the angles are not equal. A large proportion of helices are distorted in some way i.e. radius of curvature greater than 90 Å and deviation of axis from straight line is equal to or greater than 0.25 Å. These may be due to a number of reasons (Richards, 1977):

• CO groups form hydrogen bonds with NH groups 3 residues along the chain forming a 3_{10} helix. A substantial amount of all 3_{10} helices occur at the ends of alpha-helices. They are called 3_{10} because there are 3 residues per turn and 10 atoms enclosed in a ring formed by each hydrogen bond. Dipoles are not aligned as in a normal right-handed alpha-helix.

• Packing of buried helices against other secondary structural elements in the core of a protein can lead to distortions since the side chains are on the surface of helices.

• Proline residues induce distortions of around 20 deg in the direction of a helix. This is because proline cannot form a regular alpha-helix due to steric hindrance arising from its cyclic sidechain which blocks the main chain NH group. Proline causes 2 hydrogen bonds in the helix to be broken. Helices containing proline are usually long because shorter helices would be destabilized.

• Exposed helices are often bent away from the solvent. This is because the exposed C=O groups tend to point towards solvent to maximize their hydrogen bonding capacity i.e tend to form hydrogen-bonds to solvent as well as N-H groups. This gives rise to a bend in the helix axis.

• The pi helix is an extremely rare secondary structural element in proteins. Like the 3_{10} helix, one turn of pi helix is sometimes found at the ends of regular alpha helices. The infrequency of this particular form of secondary structure stems from the following properties: (i) the phi and psi angles of the pure pi helix (-57.1, -69.7) lie at the very edge of an allowed minimum energy region of the Ramachandran map, (ii) the pi helix requires that the angle tau (N-Calpha-C') be larger (114.9) than the standard tetrahedral angle of 109.5degrees, (iii) the large radius of the pi helix means the polypeptide backbone is no longer in van der Waals contact across the helical axis forming an axial hole too small for solvent water to fill, and (iv) side chains are more staggered than the ideal 3_{10} helix but not as well as the alpha helix (Low & Grenville-Wells, 1953; Schulz & Schirmer, 1990).

Besides the alpha-helix, beta-sheets are another major structural element in globular proteins. The basic unit of a beta-sheet is a beta strand (which can be thought of as a helix with n=2 residues/turn) with approximate backbone dihedral angles phi = -120 and psi = +120 producing a translation of 3.2 to 3.4 Å / residue for residues in antiparallel and parallel strands, respectively. The beta strand is then like the alpha-helix, a repeating secondary structure. However, since there are no intra-segment hydrogen bonds and van der Waals interactions between atoms of neighboring residues are not significant due to the extended nature of the chain, this extended conformation is only stable as part of a beta-sheet where contributions from hydrogen bonds and van der Waals interactions between aligned strands exert a stabilizing influence. The beta-sheet is sometimes called the beta pleated sheet since sequential neighboring C-alpha atoms are alternately above and below the plane of the sheet giving a pleated appearance. Beta-sheets are found in two forms designated as "antiparallel" or "parallel" based on the relative directions of two interacting beta strands (Darby & Creighton, 1993; Kabsch & Sander, 1983).

Like alpha-helices, beta-sheets have the potential for amphiphilicity with one face polar and the other apolar. However, unlike alpha-helices which are composed of residues from a continuous polypeptide segment (i.e., hydrogen bonds between CO of residue I and NH of residue I+3), beta-sheets are formed from strands that are very often from distant portions of the polypeptide sequence. Hydrogen bonds in beta-sheets are on average 0.1 Å shorter than those found in alpha-helices. The classical beta-sheets originally proposed are planar but most sheets observed in globular proteins are twisted (0 to 30 degrees per residue) (Baker & Hubbard, 1984).

Antiparallel beta-sheets are more often twisted than parallel sheets. Another irregularity found in antiparallel beta-sheets is the hydrogen-bonding of two residues from one strand with one residue from another called a beta bulge. Bulges are most often found in antiparallel sheets with ~5 % of bulges occurring in parallel strands (Chan, Hutchinson, Harris & Thornton, 1993; Richardson, 1981).

Turns are the third of the three "classical" secondary structures that serve to reverse the direction of the polypeptide chain. They are located primarily on the protein surface and accordingly contain polar and charged residues. Antibody recognition, phosphorylation, glycosylation, and hydroxylation sites are found frequently at or adjacent to turns. Turns were first recognized from a theoretical conformational analysis by Venkatachalam (1968). He considered what conformations were available to a system of three linked peptide units (or four successive residues) that could be stabilized by a backbone hydrogen bond between the CO of residue n and the NH of residue n+3. He found three general types, one of which (type III) actually has repeating, values of -60 deg, -30 deg and is identical with the 3_{10}-helix. The three types each contain a hydrogen bond between the carbonyl oxygen of residue i and the amide nitrogen of i+3. These three types of turns are designated I, II, and III. Many have speculated on the role of this type of secondary structure in globular proteins. Turns may be viewed as a weak link in the polypeptide chain, allowing the other secondary structures (helix and sheet) to determine the conformational outcome. In contrast (based on the recent experimental finding of "turn-like" structures in short peptides in aqueous solutions, turns are considered to be structure-nucleating segments, formed early in the folding process. Type I turns occur 2-3 times more frequently than type II. There are position dependent amino acid preferences for residues in turn conformations. Type I can tolerate all residues in position i to i+3 with the exception of Pro at position i+2. Proline is favoured at position i+1 and Gly is favored at i+3 in type I and type II turns. The polar sidechains of Asn, Asp, Ser, and Cys often populate position i where they can hydrogen bond to the backbone NH of residue i+2 (Dyson, Rance, Houghten, Lerner & Wright, 1988).

The practical limitations and complexities encountered in high resolution structural studies of proteins stimulated the development of low-resolution techniques such as Fourier transform infrared (FTIR) spectroscopy, which can be utilized for estimating the secondary

structure contents of proteins very rapidly. For the analysis of secondary structure of proteins from FTIR spectra, commonly, the amide I region (1700-1600 cm^{-1}) is utilized. Different conformational types, such as helix, sheet, turns etc. result in different discrete bands in amide I region, which are usually broad and overlapping. Therefore, to identify the bands from FTIR spectra, mathematical resolution enhancement techniques have to be applied. Different techniques, such as curve fitting, partial least squares analysis, factor analysis, and artificial neural networks (ANN) have been explored to predict the secondary structure of proteins from FTIR spectra by using the correlation between the FTIR spectral bands and the crystallographic data for proteins whose X-ray data is available (Byler & Susi, 1986; Jackson & Mantsch, 1995).

Native bovine serum albumin undergoes significant unfolding and aggregation following pressure treatment (Galazka, Sumner & Ledward, 1996), and a loss of secondary structure, depending on the magnitude of applied pressure (Hayakawa, Kajihara, Morikawa, Oda & Fujio, 1992).

Ahmed et al. (2007) found some limited HP-induced modification of the secondary structure of Basmati rice proteins exposed to 650 MPa of pressure. Alvarez et al. (2008) also found relatively small changes in secondary structure of soy proteins after a treatment of 250 MPa.

2.2.4.2 Protein tertiary level

Zhang, Li, Tatsumi & Kotwal (2003) investigated the influence of high pressure on conformational changes of soybean proteins by means of sulphydryl groups detection, spectrofluorimetry, ultraviolet difference spectra, circular dichroism, differential scanning calorimetry and electrophoresis. Alvarez et al. (2008) found that soy proteins became loosely folded (packed) after increasing pressure level treatments. Walker, Farkas, Anderson & Meunier-Goddik (2004) studied β-lactoglobulin tertiary structure by near-UV CD, intrinsic protein fluorescence spectroscopy, hydrophobic fluorescent probe binding, and thiol group reactivity; as affected by high-pressure/low-temperature processing, they found structural changes at all processing conditions, but larger changes at increased temperature (24 °C). Yin et al. (2008) found that HP treatment resulted in gradual unfolding of protein structure, as evidenced by gradual increases in fluorescence strength and SS formation from SH groups, and decrease in denaturation enthalpy change.

2.2.5 Enhance release/production of bioactives. Decrease immunoreactivity and/or elimination of antinutritional fractions

Bioactive peptides are extensively distributed among milk proteins. Numerous studies have shown in vitro formation of bioactive peptides from milk proteins and in some of them in vivo formation has also been found. In addition to liberation during in-vivo digestion, bioactive peptides may be liberated during the manufacture of milk products. For example, hydrolyzed milk proteins used for hypoallergenic infant formulas, clinical diets and as food ingredients, comprise exclusively peptides. Proteolysis during milk fermentation and cheese ripening leads to the formation of various peptides. Indeed, casomorphins, ACE-inhibitory peptides and phosphopeptides have been found in fermented milk products (Séverin & Wenshui, 2005; Shah, 2000).

On the other side of the spectrum we find antinutritional factors that may take place naturally or may be formed during heat processing. Some examples of naturally occurring antinutritional factors include glucosinolates in mustard and rapeseed protein products, trypsin inhibitors and hemagglutinins in legumes, phytates in cereals and oilseeds, and gossypol in cottonseed protein preparations, which could adversely affect nutrient utilization and may contribute to growth depression in animals (Francis, Makkar & Becker, 2001).

The recent evolution of HP technology has allowed the development of new processes, where an enhancement of enzymatic proteolysis can be obtained by combining with high-pressure treatment, which modifies the tertiary and quaternary structures of proteins; when the structure modification is reached in the presence of active proteases, the hydrolysis can be improved. Furthermore, by combining proteolysis and HP treatment, it is possible to produce hydrolysates with lower residual antigenicity (Chicón et al., 2006; Quirós, Chichón, Recio & López-Fandiño, 2007).

There are few published reports available on potential risks of high-pressure processing; but it is necessary to collect data for clarifying the role of HPP towards allergenicity and nutritional quality of pressurized foods. Allergenicity is a concern in the safety assessment of novel foods, and one of the major challenges of molecular allergy and food sciences is to predict the allergenic potential of a protein. Kato, Katayama, Matsubara, Omi & Matsuda (2000) reported specific protein release from rice grains during pressurization and identified the

released proteins as 16 kDa albumin, α-globulin, and 33 kDa globulin, which are known as major rice allergens.

Methods and possibilities for reduction of food allergy on the level of the proteins are as follows: to avoid allergens, production of hypoallergenic infant formula/food, heat treatment, other processes to avoid the most allergenic fractions, high-pressure, enzymatic modifications, proteolysis/fermentation, modification of epitopes (EPM), masking epitopes (transglutaminase), production of oligoantigenic peptide mixture for prevention, proposal for tolerogenic antigen peptides, among others. This is why new studies on the allergenic character of high hydrostatic pressure-treated foods are considered, in a search to find out if high-pressure processing can become a technology to obtain foods with reduced allergenicity or that proteins in foods create or unmask new immunoreactive structures (Meyer-Pittroff, Behrendt & Ring, 2007).

Hajós, Polgár & Farkas (2004) evaluated the effects of pressurization at 600 MPa as an effective microbiological decontamination treatment, encountering that, on the IgE-immunological reactivity of the raw batter's proteins of a typical Hungarian fermented sausage. High-pressure-induced denaturation/aggregation of proteins caused a decrease in the solubility of proteins also in the urea-soluble fractions of sausage batter; however, the patterns suggested that pressure-induced protein aggregations were mostly reversible. Pressurization altered the immunoreactive profiles of the urea-soluble protein fraction of sausage batter, causing complete disappearance of several proteins and also the appearance of other proteins with isoelectric points greater than 8.2. According to these results, high pressure induced conformational changes in the pork batter's proteins with alteration of some of the epitope structures. High pressure might form new protein aggregates with weak immunoreactivity. Curiously, high hydrostatic pressure at 600 MPa reduced significantly the potential allergenic character of a number of protein spots, and modified the structure/conformation of some proteins without altering the epitopes.

In a study performed by Nakamura, Sado & Syukunobe (1993), the antigenicity of whey protein hydrolysates treated with high pressure was found to be lower than that of heat-treated hydrolysates.

2.2.6 Other uses

Other potential applications of HP treatment on milk include low-temperature inactivation of enzymes and stabilization of fermented dairy products, improved coagulation of

milk, and the manufacture of dairy gels and emulsions with novel textures. Furthermore, studies have been undertaken on the effects of HP treatment on meat proteins myosin and metmyoglobin, egg white, ovalbumin and soy proteins. Additional experimental research on protein model systems and real food products is required to understand the potential of this technology in the restructuring of food proteins and stabilizing their biological activities (Alvarez, Ramaswamy & Ismail, 2007; Alvarez et al., 2008).

High pressure treatments have been used commercially in the United States to facilitate the shucking or raw oysters for several years, with the additional advantage of inactivation of *Vibrio parahemolyticus* and *V. vulnificus*. He, Adams, Farkas & Morrissey (2002) studied the use of HP for opening of oysters and reported that HP treatment at 310 MPa, with immediate pressure release, resulted in 100% efficiency of shucking or opening of the shell; changes in oyster body color and other visual characteristics were still observed at higher pressures. Cruz-Romero et al., (2007) reported that HP processing of oysters is potentially a more suitable post-harvest treatment than heat treatment, causing less negative effects on the quality attributes and resulting in significantly higher yield than untreated samples.

Another use for HP treatment is the acceleration of cheese ripening. Yokoyama, Sawamura & Motobayashi (1992) applied pressure of 10 to 250 MPa at 25 °C for 3 days to cheddar cheese, made using a 10-fold higher level of proteolytic starter, reporting that the flavor was similar to that of six-month-old Cheddar cheese. Besides, the authors treated cheese at 50 MPa and 25 °C for 3 days in combination with addition of lipase, protease and salting, reporting that the resultant cheese developed a Parmesan-like flavor equivalent to a commercial control in terms of flavor scores and levels of free fatty acids. O'Reilly et al. (2000, 2002, 2003) studied the effects of a range of HP treatment conditions on key ripening characteristics of both cheddar and mozzarella cheeses; overall, they found that HP treatment at low pressures (~50-200 MPa) combined with long processing times (up to 82 h) impacted mainly on proteolysis, while HP treatment at higher pressures (200-400 MPa) for relatively shorter processing times (~20 min) caused changes in the protein structure, which in turn improved cheese functional properties. However, the increases in proteolysis following HP treatment at low pressures were considered to be not adequate to warrant the commercial exploitation of HP treatment for acceleration of cheese ripening.

Similar to thermal treatment, HP causes destruction of microorganisms but, unlike thermal treatment, it does not inactivate certain enzymes that play an important role in cheese ripening, such as lipoprotein lipase. Trujillo, Royo, Ferragut & Guamis (1999) found higher production of free fatty acids in HP treated goat's milk compared to conventionally pasteurized milk, indicating higher lipoprotein lipase activity. The use of HP on cheese results in an increase in moisture content and pH, generating modifications to the cheese matrix and lysis of cells, which assists ripening. HPP of cheese affects the pattern of proteolysis during ripening, with an overall effect that depends on the type of cheese as well as magnitude, duration and temperature of pressure treatment.

Other studies concerning goat's cheese by Saldo, Sendra & Guamis (2000) involved pressure treatment at 400MPa for 5 min. which led to a reduction in started counts by ~6 log cycles, while treatment at 50 MPa for 72 h slightly decreased the starter count. Lysis of started by high pressure is important in cheese ripening, given that it releases intracellular enzymes into the cheese matrix, which play an important role in the breakdown of large and intermediate size peptides to small peptides and amino acids. The HP treatment resulted in an increase in pH and levels of proteolysis.

2.3 Methods to study protein structure

2.3.1 Primary level

2.3.1.1 Classical protein sequencing methods

Protein sequencing is used to determine the sequence of amino acids in a protein or a peptide. Edman degradation, involving sequential removal of marked amino acids residues from the N terminal of a protein or a peptide, is used; peptide bonds between the remaining amino acids are not disrupted. There are two basic ways of identifying N terminal amino acids. The first involves binding an appropriate substitute to the N terminal amino acid. After acidic or enzymatic hydrolysis, the amino acid marked by the substitute can be detected spectrophotometrically, fluorometrically or isotropically. The other way to determine the N terminal amino acid involves binding the amino acid residue occurring at the end of a protein phenylisothiocyanate, to an α-amine group; the result is the formation of a phenylisothiocarbamoyl derivative released from the protein in a weakly acidic medium in the

form of phenylisothiohydantoin (PTH) amino acid derivative. The peptide, shortened by one amino acid residue, can be subjected to another cycle of marking and splitting. Sequence information can also be obtained from the carboxyl terminal of proteins or peptides. The carboxyl-terminal degradation or C-alkylation method, which activated the C terminal only once at the start of the chemistry cycle, prevents the detection of background amino acids, and increases the accuracy of sequence calling. Degradation is achieved by both chemical and enzymatic techniques. Enzymatic hydrolysis involves specific exopeptidases that release a single amino acid from the polypeptide chain terminal. Aminopeptidase releases the amino acid from the N terminal, while carboxypeptidase releases it from the C terminal (Edman, 1950).

The Edman degradation method has been automated and improved so that it is possible to elucidate a sequence of about 50 amino acids from the N terminal of a protein with a pico-mole amount of the initial material. Amino acid sequence analysis in high-molecular-weight proteins usually requires that they be preliminary degraded into smaller fragments of 20 to 100 residues. The fragments are then separated and sequenced (Niall, 1973).

2.3.1.2 Mass spectrometry (MS)

The other major direct method by which the sequence of a protein can be determined is mass spectrometry. This method has been gaining popularity in recent years as new techniques and increasing computing power have facilitated it. Mass spectrometry can, in principle, sequence any size of protein, but the problem becomes computationally more difficult as the size increases. Peptides are also easier to prepare for mass spectrometry than whole proteins, because they are more soluble. One method of delivering the peptides to the spectrometer is electrospray ionization, for which Tanaka won the Nobel Prize in chemistry 2002. The protein is digested by an endoprotease, and the resulting solution is passed through a high performance liquid chromatography column. At the end of this column, the solution is sprayed out of a narrow nozzle charged to a high positive potential into the mass spectrometer. The charge on the droplets causes them to fragment until only single ions remain. The peptides are then fragmented and the mass-charge ratios of the fragments measured. It is possible to detect which peaks correspond to multiply charged fragments, because these will have auxiliary peaks corresponding to other isotopes, the distance between these other peaks is inversely proportional to the charge on the fragment. The mass spectrum is analyzed by computer and

often compared against a database of previously sequenced proteins in order to determine the sequences of the fragments. This process is then repeated with a different digestion enzyme, and the overlaps in the sequences used to construct a sequence for the protein (Steen & Mann, 2004).

Additionally, tandem MS-MS spectrometry can be performed coupled with a collision cell for peptide sequencing (Syka, Coon, Schroeder, Shabanowitz & Hunt, 2004).

2.3.2 Secondary level

2.3.2.1 Far UV circular dichroism (far UV-CD)

Secondary structure can be determined by CD spectroscopy in the "far-uv" spectral region (190-250 nm). At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. The approximate fraction of each secondary structure type that is present in any protein can thus be determined by analyzing its far-uv CD spectrum as a sum of fractional multiples of such reference spectra for each structural type (Douette, Navet, Bouillenne, Brans, Sluse-Goffart, Matagne & Sluse, 2004).

Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population. Thus, while CD can determine that a protein contains about 50% alpha-helix, it cannot determine which specific residues are involved in the alpha-helical portion. Far-uv CD spectra require 20 to 200 µl of solution containing 1 mg/ml to 50 µg/ml protein, in any buffer which does not have a high absorbance in this region of the spectrum; high concentrations of DTT, histidine, or imidazole, for example, cannot be used in the far-uv region (Johnson, 1990).

2.3.2.2 Raman spectroscopy

Raman spectroscopy is based on the discrete vibrational transitions that take place in the ground electronic state of molecules. In Raman spectroscopy, the sample is radiated with a monochromatic visible or near infrared light from a laser. This brings the vibrational energy levels in the molecule to a short-lived, high-energy collision state, which returns to a lower energy state by emission of a photon. Normally the photon has a lower frequency than the laser

light, and the difference in frequency of the laser and that of the scattered photon is called the Raman shift ($\Delta \nu$ in cm^{-1}). Therefore, the Raman spectrum consists of a plot of the intensity of scattered light as a function of wavenumber (cm^{-1}) shift from the laser excitation wavelength (Ismail, Cocciardi, Alvarez & Sedman, 2005).

The vibrational Raman bands observed in the spectra provide information about the vibrational motions of the molecule consisting of the absorption of energy as a function of frequency. The appearance of bands with some vibration frequencies in the Ramam spectrum depends on the molecular geometry and the chemical composition of the substance to study. Therefore, the Raman spectroscopy appears as a suitable technique to determine the protein chemical composition and its molecular geometry structure (Ismail et al., 2005).

The determination of the molecular functional group of compounds is made by the use of call frequencies of the group. These functional groups cause bands with characteristic frequencies within defined frequencies intervals, therefore, from one Raman spectrum the functional groups existing in a protein molecule and the amount of these can be known. The frequencies or characteristic bands have denominations such as stretching, bending, and twisting (Thomas, 1999).

One of the greatest advantages of Raman spectroscopy is that protein structural information can be obtained non-invasively, without the need of any preliminary sample manipulation. Also, Raman spectroscopy allows the study of the nature of protein interactions and conformational changes directly in the food system reducing the possibility of producing artifacts, which may occur during the preparation of isolate protein model systems (Thomas, 1999).

Raman spectroscopy has found extensive application in the study of proteins and involves particularly useful techniques for the elucidation of protein secondary structure. The spectra of polypeptides and proteins exhibit nine amide bands that represent different vibrations of the peptide linkage. Obtaining information pertaining to protein secondary structure from infrared and Raman spectra is based on empirical correlations between the wavenumbers of certain of these bands and the various conformations adopted by the polypeptide chain. The amide I band (in the range of 1700-1600 cm^{-1}) is the most frequently employed in IR spectroscopy, and characteristic amide I band positions have been identified for α-helices, 3_{10}-

helices, parallel or antiparallel β-sheets, turns, and unordered or irregular structures. Using these band assignments, secondary structures of proteins can be deduced, although it is generally advisable to confirm the results with a second technique such as circular dichroism. The amide III band (in the range of 1300-1190 cm^{-1}) can similarly be employed for the estimation of the relative proportions of the secondary-structure components. However, in some cases, the amide III band can be overlapped by bands due to side-chain vibrations of particular amino acid residues (or absorptions from other biomolecules present, for example, in a food matrix) and consequently one must be cautious when assigning bands in this region. In IR spectra, the amide III band is much weaker than the amide I band and hence is much less frequently employed in secondary-structure investigations. However, both the amide I and amide III bands are commonly employed in Raman spectroscopy, as their Raman intensities are comparable. The Raman spectra of proteins can also be recorded in aqueous solution but the spectra obtained from solid samples are generally of much higher quality and exhibit sharper bands (Ismail et al., 2005).

2.3.2.3 Fourier transform infrared (FTIR) spectroscopy and 2D correlation

Fourier transform infrared spectroscopy (FTIR) has been accessible to scientists since the early 1970s. FTIR is based on interferometry, which uses a beam splitter to divide the infra-red radiation into two beams: one beam is reflected to a fixed mirror and the other to a moving mirror. The two beams endure destructive and constructive interference as they recombine at the beam splitter, due to the path difference between the two mirrors. Fluctuation in the intensity of the energy reaching the detector are digitized using a real time data acquisition system and results in an interferogram containing the spectral information linked to the sample. The interferogram is converted into a conventional spectrum using the Fourier transform algorithm. This calculation requires considerable computing power, so the fast development of FTIR technology is largely due to the advances on computer technology (Ismail et al., 2005).

FTIR spectroscopy supplies information about the secondary structure content of protein by shining infrared radiation on a sample and seeing which wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. Each compound has a particular set of absorption bands in its infrared spectrum. Characteristic bands found in the infrared spectra of proteins and polypeptides include amide I and amide II, both arising from the amide

bonds that link the amino acids. The absorption associated with the amide I bands leads to stretching vibrations of the C=O bond of the amide, while the absorption associated with the amide II band leads mostly to bending vibrations of the N-H bond. Given that both the C=O and the N-H bond are involved in the hydrogen bonding that takes place between the different elements of the secondary structure, the location of both the amide I and amide II bands are sensitive to the secondary structure content of proteins (Haris & Chapman, 1995).

Studies with proteins of known structure have been used to correlate systematically the shape of the amide I band to secondary structure content, while the amide II band, even being sensitive to secondary structure content is not as good a predictor for the quantification of the secondary structure of proteins (Byler & Susi, 1986).

One complexity in the analysis of the amide I band for secondary structure is that its shifts are small compared to the intrinsic width of the band, so in place of a series of resolved peaks for each type of secondary structure, one broad lumpy peak is observed. Several numerical methods are used to increase the apparent resolution of the Amide I band so that estimates can be made of the secondary structure content (Ismail et al., 2005).

For infrared studies involving examination of the secondary-structure-sensitive amide I band, proteins are commonly dissolved in D_2O because H_2O has a strong band in the amide I region. Since D_2O has no absorptions that overlap with the amide I band (which, by convention, is termed the amide I' band when D_2O is the solvent), the use of D_2O solutions allows the protein absorptions to be observed without the need for subtraction of the spectrum of the solvent. More importantly, it also makes it permissible to use much longer pathlengths (40-80 μm vs. 10 μm for aqueous solutions), thereby yielding a higher signal-to-noise ratio. An additional advantage of dissolving proteins in D_2O is the resulting ability to study the rate at which the hydrogens of the amide groups exchange with the D_2O solvent due to the ~100-cm^{-1} shift of the amide II band (from 1560-1520 to 1460-1420 cm-1) that occurs upon H-D exchange. This rate is indicative of the compactness of the protein since amide groups exposed to the solvent undergo H-D exchange faster than those in the interior of the protein. Thus, increases in the rate of H-D exchange as a result of variations in physicochemical parameters such as pH, temperature, and pressure can be interpreted in terms of the extent of protein unfolding (Ismail et al., 2005).

The theory of generalized two-dimensional (2D) correlation spectroscopy was developed by Noda in 1993, as a further elaboration of original 2D infrared (IR) correlation spectroscopy proposed by him. In the 2D analysis two kinds of correlation maps, synchronous and asynchronous ones are generated based upon a set of dynamic spectra calculated from perturbation-induced dynamic fluctuations of spectroscopic signals. The new 2D method can handle signals fluctuating as an arbitrary function of time, or any other physical variables such as temperature, pressure, and even concentration. The extension to various kinds of spectroscopies such as FTIR is also rather uncomplicated for the generalized 2D correlation analysis method. The key advantages of 2D correlation analysis lay on the simplification of complex spectra consisting of overlapped peaks; improvement of spectral resolution by scattering peaks over the second dimension; and providing interaction and dynamic information of functional groups in the system (Noda, 2006).

2D correlated FTIR technique has been reported to be useful in investigating the dynamic variations. The generalized 2D correlation spectroscopy proposed by Noda, has in recent times been proved as a powerful tool in studying the molecule-molecule interactions in some particular systems (Ismoyo, Wang & Ismail, 2000).

2.3.2.4 Nuclear magnetic resonance (NMR)

Protein nuclear magnetic resonance is performed on aqueous samples of highly purified protein. Usually the sample consists of between 300 and 600 microlitres with a protein concentration in the range 0.1-3 millimolar. The source of the protein can be either natural or produced in an expression system using recombinant DNA techniques through genetic engineering. Recombinantly expressed proteins are usually easier to produce in sufficient quantity, and makes isotopic labelling possible. Nuclear magnetic resonance of proteins from natural sources is restricted to utilizing nuclear magnetic resonance based solely on protons. However the less common isotopes, carbon-13 and nitrogen-15, have a net nuclear spin of 1/2, a simpler case making them suitable for nuclear magnetic resonance, and therefore labeling the proteins with these compounds opens up possibilities for doing more advanced experiments which also detect or use these nuclei. Isotopic labeling is done by growing the expression host in a growth media enriched with the desired isotopes. The purified protein is usually dissolved in a buffer solution and adjusted to the desired solvent conditions (Cavanagh, Fairbrother, Palmer & Skelton, 1996).

Protein NMR utilizes multidimensional nuclear magnetic resonance experiments to obtain information about the protein. Ideally, each distinct nucleus in the molecule experiences a distinct chemical environment and thus has a distinct chemical shift by which it can be recognized. However, in large molecules such as proteins the number of resonances can typically be several thousand and a one-dimensional spectrum inevitably has incidental overlaps. Therefore multidimensional experiments are performed which correlate the frequencies of distinct nuclei. The additional dimensions decrease the chance of overlap and have larger information content since they correlate signals from nuclei within a specific part of the molecule. Magnetization is transferred into the sample using pulses of electromagnetic (radiofrequency) energy and between nuclei using delays; the process is described with so-called pulse sequences. Pulse sequences allow the experimenter to investigate and select specific types of connections between nuclei. The array of nuclear magnetic resonance experiments used on proteins fall in two main categories: one where magnetization is transferred through the chemical bonds; and the other where the transfer is through space, irrespective of the bonding structure. The first category is used to assign the different chemical shifts to a specific nucleus, and the second is primarily used to generate the distance restraints used in the structure calculation, and in the assignment with unlabelled protein (Jonas, Ballard & Nash, 1998).

In order to analyze the nuclear magnetic resonance data, it is important to get a resonance assignment for the protein. That is to find out which chemical shift in each dimension corresponds to which atom. Several different types of experiments have been invented to achieve this. The procedure depends on whether the protein is isotopically labeled or not, since a lot of the assignment experiments rely on carbon-13 and nitrogen-15 (Rule & Hitchens, 2006).

2.3.3 Tertiary and quaternary levels

2.3.3.1 Fluorescence spectroscopy

Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined will have a ground electronic state of interest, and an excited electronic state of higher energy. Within each of these electronic states are various vibrational states. In fluorescence spectroscopy, the species is first excited, by absorbing a

photon of light, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state. The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analyzing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined (Ross, Szabo & Hogue, 1997).

In a typical experiment, the different frequencies of fluorescent light emitted by a sample are measured, holding the excitation light at a constant wavelength. This is called an emission spectrum. An excitation spectrum is measured by recording a number of emission spectra using different wavelengths of excitation light. One important characteristic is the sensitivity of fluorescence spectroscopy, since only the emitted photons are counted and it is possible to detect on concentrations in the order of nM. Further, since few molecules are naturally fluorescent it is easier to determine where the signals are coming from. The down side of this is that is only possible to gain information for those parts of the system that are fluorescent. Another important characteristic of fluorescence spectroscopy is the sensitivity of the fluorophore to the environment. The fluorescence lifetime, quantum yield, and emission spectra of a fluorophore can be sensitive to the local environment. The effect of the environment on the excited state is pronounced with fluorescence spectroscopy because of the additional time available to sample/modify the environment of the excited chromophore. These changes in the properties of fluorescence make fluorescence spectroscopy a widely used method of detecting a change in the environment of the fluorophore. Also, the coupling between the transition dipoles of fluorescent molecules provides a mechanism to measure the distance between these groups on larger macromolecules. The distances that can be measured are on the order of 20-70 Å, making fluorescence spectroscopy unique in this application (Simonian & Smith, 2006).

Sharma & Kalonia (2003) attempted to obtain information about protein tertiary structure in solid state by using steady state tryptophan (Trp) intrinsic fluorescence emission

spectroscopy on protein powders. Beta-lactoglobulin and interferon alpha-2a powder samples were studied by fluorescence spectroscopy using a front surface sample holder. The researchers reported that Trp fluorescence spectroscopy provided a simple and reliable approach to characterize Trp microenvironment in protein powders that is related to the tertiary conformation of proteins in the solid state. This study showed that the use of fluorescence spectroscopy of proteins can be extended from simple protein aqueous solutions to protein powders, precipitates, and semidried protein samples to gain understanding of protein tertiary structure in these physical states.

Fluorescence spectroscopy is one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics today. Although fluorescence measurements do not provide detailed structural information, the technique has become quite accepted because of its acute sensitivity to changes in the structural and dynamic properties of proteins and protein complexes. Like most biophysical techniques, fluorescence spectroscopic studies can be carried out at many levels ranging from simple measurement of steady-state emission intensity to quite sophisticated time-resolved studies. The information content increases dramatically as various fluorescence observables are time resolved and combined in global analyses of the phenomena of interest. Nonetheless, quite a large amount of information is available from steady-state measurements for which the requirements in instrumentation are modest. Consequently, steady-state fluorimeters are routinely used to measure complexation and conformational phenomena of proteins (Hazra, Chakrabarty, Chakraborty & Sarkar, 2004).

2.3.3.2 Raman spectroscopy

The applicability of Raman spectroscopy to both solid and liquid samples makes it a useful tool for investigating protein structural changes in situ during denaturation and aggregation/gelation (Li-Chan, 1996). The foundation of Raman spectroscopic analysis is the inelastic scattering of photons, as a consequence of vibrational transitions of functional groups of a molecule. Both the frequency and intensity of molecular vibrations are sensitive to chemical changes and environment of the functional groups, reflected by changes in the Raman spectrum. This makes Raman spectroscopy suitable for studying protein conformation in different buffers. Moreover, Raman spectroscopy can also be used to monitor changes in the polypeptide backbone conformation or microenvironment of protein side chains when proteins become insoluble as a result of processing or modifications. The technique can therefore be

used to monitor the aggregation process, or to study food proteins in situ without extraction (Careche & Li-Chan, 1997).

Meng, Ma & Phillips (2003) studied red bean globulin (RBG) conformation under different environment conditions and changes in conformation during thermal denaturation/aggregation by FT-Raman spectroscopy, reporting that Raman spectroscopy is a useful technique for studying the secondary and tertiary structures of plant proteins such as RBG. They deemed it applicable both for studying protein structure in various aqueous environments and for monitoring the conformational changes during heat treatment.

2.3.3.3 Near UV-CD

The CD spectrum of a protein in the "near-UV" spectral region (250-350 nm) can be sensitive to certain aspects of tertiary structure. At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce are sensitive to the overall tertiary structure of the protein. Signals in the region from 250-270 nm are ascribed to phenylalanine residues, whereas signals from 270-290 nm are credited to tyrosine, and those from 280-300 nm are attributable to tryptophan. Disulfide bonds give rise to broad weak signals all through the near-UV spectrum (Kelly & Price, 2000).

When a protein retains secondary structure but no defined three-dimensional structure as it happens in an incorrectly folded or "molten-globule" structure, the signals in the near-uv region will be nearly zero. On the other hand, the presence of significant near-uv signals is a good indication that the protein is folded into a well-defined structure. The signals obtained in the 250-300 nm region are due to the absorption, dipole orientation and the nature of the surrounding environment of the phenylalanine, tyrosine, cysteine (or S-S disulfide bridges) and tryptophan amino acids. Unlike in far-UV CD, the near-UV CD spectrum cannot be assigned to any particular 3D structure. Near-UV CD spectra can also provide structural information on the nature of the prosthetic groups in proteins, such as heme groups e.g. in hemoglobin and cytochrome c (Strickland & Beychok, 1974).

The near-uv CD spectrum can be sensitive to small changes in tertiary structure due to protein-protein interactions and/or changes in solvent conditions. The signal strength in the near-uv CD region is much weaker than that in the far-uv CD region. Near-uv CD spectra require about 1 ml of protein solution with an OD at 280 nm of 0.5 to 1 (which corresponds to 0.25 to 2 mg/ml for most proteins) (Kelly & Price, 2000).

2.3.3.4 Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS has been employed for the determination of the changes in the tertiary structure of protein. For example, changes in the structure of ferricytochrome c caused by increasing concentrations of hydrochloric acid in different concentrations of methanol were examined by Konermann and Douglas (1997). In 3 % methanol the decrease in pH causes a cooperative unfolding at pH 2.6 with a loss of the native secondary and tertiary structure of the protein. At 50 % methanol concentration the unfolding transition occurs at pH 4.0 with only a breakdown in the tertiary structure, with the secondary structure largely unaffected. Babu, Moradian & Douglas (2000) studied the methanol-induced conformational changes under acidic conditions of β-lactoglobulin, cytochrome c, and ubiquitin. They found partially unfolded intermediates with induced α-helix structures. These intermediates were formed at 35 % methanol concentration. The authors contrasted their findings with previous results from optical methods like circular dichroism. Anderegg & Wagner (1995) studied the stability of a recombinant src homology 2 (SH-2) domain from pp60c-src protein kinase bound to a variety of synthetically produced ligands. They characterized the different effects of the ligands on the hydrogen-deuterium exchange of the complex. Slanger & Visser (1999) studied the nonglycosylated and glycosylated forms of α-lactalbumin; they identified the nonglycosylated fraction with a mass of 14178 Da and for the glycosylated fraction they found a variety of components (as much as 14 types) with mass values from 15840 to 16690 Da. Sanglier, Leize & van Dorsselaer (2003) compared the oligomeric state of hemocyanins (Hc) from crabs living at different sea depths. The results indicate that for *Bythograea thermydron*, a deep sea crab, its Hc was arranging in as many as 30 subunits. The oligomeric state of the Hc is then directly related to the amount of oxygen found in that particular biotope. Stevens, Kem & Prokai (2002) isolated and analyzed various cytolysin proteins from the sea anemone *Stichodactyla helianthus*; they also studied peptides obtained by the trypsin digestion of the proteins. Nilsson, Cooper, Håkansson, Marshall, Östberg, Lavrinovicha & Bergström (2002) characterized the highly hydrophobic outer membrane component P13 from *Borrelia burgdorferi* and some mutant forms of this protein.

The different quaternary structures of concanavalin A were investigated by Light-Wahl, Winger & Smith (1993); in a later publication the same group expanded their work

investigating avidin and human hemoglobin (Light-Wahl, Schwartz & Smith, 1994). The structural characterization of the synthetic peptide from the sequence 1-30 of Par j 1.0101 a major allergenic protein found in the pollen of *Parietaria judaica* was made by Cunsolo, Foti, Saletti, Ceraulo & Di Stefano (2001); they successfully identified and mapped the disulphide bonds present in the peptide. Hooke, Eyles, Miranker, Radford, Robinson & Dobson (1995) studied the molecular folding of hen lysozyme using hydrogen-deuterium exchange and ^{15}N isotopic labelling. The effect of trifluoroethanol on a synthetic myoglobin peptide and cytochrome c at low pH was investigated by Grandori, Matecko & Müller (2002); they found ESI-MS to be insensitive to changes in the secondary structure of the molecule, but capable to detect changes in the tertiary structural level. Mohimen, Dobo, Hoerner & Kaltashov (2003) developed a chemometric protocol to analyze information from ESI-MS relative to multiple protein conformers in solution. The signals from the different states of a protein in the same sample often overlap yielding a confusing spectrum. The use of modern chemometrics helped to overcome this limitation.

In general terms MS study the weight of molecules, but with ESI one can also observe and quantify the population of charged species formed by the ionization process. In the traditional analysis the data is used to calculate, as accurate as possible, the mass of the sample molecule. But there is another analysis that is becoming popular and it include the study of the third dimensional structure of proteins interpreting in a new fashion the data from ESI-MS. The principle of the analysis is that the more flexible (relaxed structure) the protein is, the easier will be to charge groups in the interior of the molecule at the ionization stage. In this sense, the more relaxed the protein a higher proportion of the population of molecules will display higher charges (Alomirah, Alli & Konishi, 2000). The chart displaying the different charged states of the molecule of study is called charged-state-distribution (CSD). A protein in its native state will show a CSD inclined to the range of lower charges or a higher mass-over-charge (m/z) ratio. An unfolded protein will have a larger m/z ratio as previously buried groups in the structure get ionized by exposing them to an acidic solvent (Alvarez et al., 2007).

2.3.3.5 Quantitative ultracentrifugation

Quantitative ultracentrifugation is an extremely versatile and powerful technique for characterizing the solution-state behavior of macromolecules. Analytical ultracentrifugation is

also a means for determining molecular weight and the hydrodynamic and thermodynamic properties of a protein or macromolecule. Sample purity, molecular weight determination in the native state, analysis of associating systems, determination of sedimentation and diffusion coefficients and ligand binding can all be determined using this versatile instrument (Lebowitz, Lewis & Schuck, 2002).

The instrument itself spins a rotor at a controlled speed and temperature under vacuum while recording the concentration distribution at set times. The rotor may spin at speeds up to 60000 rpm (250000 x g). Special cells that can withstand the high gravitational field and allow the passage of light through the sample are used. The sample itself is contained within a sector-shaped cavity sandwiched between two windows of quartz or sapphire. This cavity is contained within a centerpiece of aluminum alloy, reinforced epoxy, or a polymer (Kel-F). Double sector cells are also used so that the absorbing components of the sample solvent can be taken into account. These cells also allow the measurement of sedimentation coefficient differences and of diffusion coefficients. These centerpieces can have a pathlength of 3 to 12 mm which combined with selectable wavelengths, allows the examination of a wide range of sample concentrations. The data obtained from the ultracentrifugation is a record of the concentration distribution. This is accomplished by obtaining a set of concentration measurements at different radial positions and at a given time, using either refractometric methods or photoelectric absorption measurements (Schuck, 2003).

2.4 Methods to study rheology/texture

Fundamental rheological properties should be independent of the instruments used to measure them, so different methods should yield to the same results. In reality, different instruments rarely lead to one and the same results. Nevertheless, the accuracy of different methods should relay in their ability to distinguish true rheological material properties from subjective material characterizations which, if frequently useful, don't belong within the rheology field (Borwankar, 1992).

2.4.1 Classical rheology (flow behavior)

As has been stated before in this work, rheology is the study of deformation and flow of foods under well defined conditions, which could be defined in terms of their rate of deformation or in terms the magnitude of the stress of the strain applied. Foods with different

internal structure and bonding will react in different ways to the same applied conditions. In a system designed to apply a controlled rate of deformation to a fluid, the simplest case would be that where the shear stress developed in the fluid is directly proportional to the rate of deformation or the rate of strain, and that would apply for Newtonian fluids obeying the relationship $T=\mu\gamma$ where T is the shear stress and γ is the shear rate. The constant proportionality between the shear stress and the shear rate is the viscosity of the fluid, and from the original definition of a fluid by Pascal is considered a measure of internal friction, or ability to resist motion when a shearing stress is applied. While the modeling of fluid behavior has progressed immensely since the early Pascal model, the basic principle of many instruments still use the hypothetical two-surface concept, where the fluid is placed between two parallel infinite plates with a stationary bottom plate and an upper plate moving in the x-direction at a fixed velocity (Rao, 1977).

In a Newtonian fluid there is a fixed proportionality between shear stress and the applied shear rate, and given the relatively simple flow curve, such liquids can be characterized by a single term, or the constant proportionality of the viscosity. Furthermore, a single experiment measuring the shear stress at one surface at a single shear rate might be sufficient to quantify the rheological characteristics of the fluid. However, most food fluids are non-Newtonian and present curves known as pseudoplastic, dilatant and others exhibiting a yield stress (Nguyen & Boger, 1992; Keentok, 1982).

Several factors influence the selection of the rheological model used to describe flow behavior of a particular fluid. Many of them such as Bingham plastic and Herschel-Bulkley models, in addition to the power-law, have been used to represent the flow behavior of non-Newtonian fluids. The Cross, Reiner-Philippoff, Van Wazer and Powell-Eyring methods are useful in modeling pseudoplastic behavior over low, middle and high shear rate ranges (Rao, 1977).

Non Newtonian fluids can not be characterized by a measurement at a single shear rate, and in some food liquids the shear stress is not only determined by shear rate but is also time dependent, therefore demanding their own unique measuring system. Many foods are pseudoplastic and their response to an applied deformation varies with the rate of application of the deformation. Such fluids are typically represented by plots or flow curves, and the slope of

the curve decreases as the shear rate increases in a phenomenon often called “shear thinning” as it can be observed in solutions of concentrated molecules such as several fruit juices. The behavior of other types of foods is represented by dilatant or “shear thickening” curves, as it is observed in concentrated suspension of starch granules (Barnes, 1999).

Finally, there are the foods exhibiting a yield stress, which must be exceeded before any deformation or flow can occur. These materials behave like solids under low stress and like liquids under high stress, and knowledge of their behavior is essential for certain food processes. In fact, in absence of rapid crystallization or solidification of a coating, the yield stress will determine its thickness on a vertical surface. If the shear stress exerted by the coating itself exceeds the yield stress, then the coating will flow out of the product. On the contrary, if the yield stress exceeds the shear stress, the coating neither flow or deform and will stay on the product (Yoshimura, Prud’homme, Princen & Kiss, 1987).

Another factor to take into consideration is the dependence of the rheological behavior of fluids on temperature. As internal friction is a molecular phenomenon, the alteration of molecular movement will influence internal friction; with the consequence that rheology of most foods is highly temperature dependant (Marcotte, Taherian Hoshahili & Ramaswamy, 2001).

2.4.2 Small amplitude oscillatory methods

Many complex structures food materials display both viscous and elastic properties. Unlike purely elastic substances, a viscoelastic material has an elastic component and a viscous component. The viscosity of a viscoelastic substance gives the substance a time-dependant strain rate and it loses energy when a load is applied, then removed. Hysteresis is observed in the stress-strain curve, with the area of the loop being equal to the energy lost during the loading cycle. Since viscosity is the resistance to thermally activated plastic deformation, a viscous material will lose energy through a loading cycle. Plastic deformation results in lost energy, which is uncharacteristic of a purely elastic material's reaction to a loading cycle. Since viscoelasticity is a molecular rearrangement, if stress is applied to a viscoelastic material such as a polymer parts of the long polymer chain change position. This movement or rearrangement is called creep. Polymers remain a solid material even when these parts of their chains are rearranging in order to accompany the stress, and as this occurs, it creates a back stress in the

material. When the back stress is the same magnitude as the applied stress, the material no longer creeps. When the original stress is taken away, the accumulated back stresses will cause the polymer to return to its original form. Linear viscoelasticity is the simplest viscoelastic behavior in which the ratio of stress to strain is a function of time alone and not of the strain or stress magnitude, while nonlinear viscoelasticity determines the display of mechanical properties that are a function of time and the magnitude of stress used (Gallegos & Franco, 1999).

Among many rheological measurements, small amplitude oscillatory shear has been one of the most widely used methods, because it is sensitive to the microstructure and has solid mathematical background. Often it is possible to distinguish structural difference even in nanometer scale or through any small structural change that may have evolved during the processing. An advantage of the small amplitude oscillatory squeeze flow is that there is a theoretical support developed from first principles and an equation linking the displacement sinusoid and the force sinusoid. Essentially, the squeeze tests involve sandwiching the sample between two parallel plates, where the upper plate oscillates at a small amplitude about the mean gap position, and a force transducer detects the force signal that arises from the sample. This makes the technique particularly useful in the detection of viscoelastic properties (Taherian, Fustier & Ramaswamy, 2008).

The upper plate can be oscillated in two different ways, the first would be a single frequency sinusoid, or a band-limited pseudorandom noise signal, which is a synthetic signal that comprehends a range of frequencies earlier telescoped into a single excitation sequence. In this case, the consequential force is also random in nature, but on-line Fast Fourier analysis will break this signal into the individual frequency components, allowing calculation of the dynamic viscoelastic properties. Several small oscillatory methods are used to measure linear viscoelastic properties of a range of foods, such as oils, gels, concentrated suspensions of particles dispersed in water, and emulsions (Alvarez et al., 2008; Murray, 2002).

2.4.2.1 Dynamic rheology

Dynamic rheology is a form of rheology where the samples are subjected to small sinusoidal varying loads in which either the shear stress or strain are controlled. The magnitude of these deforming loads is small, and they are chosen so the structure of the material is not

damaged. Under such conditions, the viscoelastic properties of the sample become evident. Dynamic rheology can be illustrated by imagining a slab-shaped volume between two parallel rectangular plates, in which the lower plate is fixed and the upper plate is allowed to move back and forth in a horizontal direction. In a control strain test, the strain is applied by presetting the path, and the volume is submitted to a force of shear stress. In control stress systems, the oscillating stress from the force means that the volume element undergoes a strain. With controlled strain instruments the strain curve as a function of time is given by $\gamma = \gamma 0 \sin(\omega\tau)$, where $\gamma 0$ is the amplitude of the strain equal to the distance from center moved by the plate divided by the distance separating the plates, and ω is the frequency expressed in rads/s. Consequently, the magnitude of the strain is determined by the amplitude and the frequency (Lucey, Teo, Munro & Singh, 1997; Steffe, 1996).

Independently of whether a controlled stress or controlled strain system is used, in absolutely elastic substances the strain and the stress waves will be in phase with each other, while in solely viscous fluids the strain and the stress will be exactly 90° out of phase with each other. For viscoelastic substances the phase angle will lie in the range of $0° < \delta < 90°$. From the recorded sinusoidal curve the storage modulus and the loss modulus can be calculated. The first represents the elastic behavior of a sample as its magnitude represents the strain energy, which is reversibly stored and recovered from the substance; while the latter represents the quality of energy irreversibly released by the substance to the environment and characterizes the viscosity of the sample (Steffe, 1996).

Both the storage (G') and loss (G") moduli can be combined to give a single figure called the tan δ which provides a ratio between the amount of energy stored and lost in a cycle, and hence a relationship between the elastic and viscous portions of the sample. The tan δ goes from zero to infinity, with higher values in Newtonian fluids and lowest in substances resembling hookean solids (Lucey, Johnson & Horne, 2003).

2.4.2.2 Dynamic mechanical analysis (DMA)

Dynamic mechanical analysis systems include mechanical spectroscopy and several dynamic mechanical analysis methods, the basic principle of the measurements is to apply an oscillating mechanical stress at a given frequency to cause a nondestructive strain in the sample

and detect its recovery. A portion of the applied energy is stored in the material and used in recovery, whereas some of the applied energy is converted to heat and lost. The sample deforms under the load, so the stiffness of the sample can be determined, and the sample moduli can be calculated. By measuring the time pause in the displacement compared to the applied force it is possible to determine the damping properties of the material. The time holdup is reported as a phase lag, which is an angle (Menard, 1999).

Viscoelastic materials such as polymers typically exist in two distinct states. They display the properties of a glass, known as high modulus, at low temperatures and the properties of a rubber, or low modulus, at higher temperatures. By monitoring the temperature during a DMA experiment, it is possible to observe this change of state, the glass transition or alpha relaxation. The glass transition temperature (Tg) is often measured by differential scanning calorimetry (DSC), but the DMA technique is more sensitive and yields more easily interpretable data. DMA can also be used to investigate the frequency and consequently the time-dependent nature of the transition, since the degree of dependence is specific to the transition type. DMA also allows to quantify moduli values (Nielsen & Landel, 1994).

2.5 Proteins studied in this thesis

2.5.1 Bovine milk

Milk and dairy products are some of the most important components of the human diet, providing close to 30 % or dietary proteins and lipids and about 80 % of dietary calcium. The vast majority of milk is processed to some extent, even though some fluid milk is still consumed. Liquid milk is a widespread item in all developed dairying countries taking approximately 40 % of total milk production. The remainder percentage is processed into a massive number of products, making out of the dairy industry one of the most diverse and flexible sectors of the food industry. Milk as a raw material presents a flexibility that resides in the uniqueness of many of its chemical and physico-chemical properties of its components. The main constituents of milk can be modified by enzymatic, chemical or physical methods leading to the production of different items for consumption or further processing (DePeters & Cant, 1992; Jenness, 1980; Kinsella, Patton & Dimick, 1967).

Nevertheless, the proportions and properties of milk contituents are variable and for this reason, the processability of milk and the properties of dairy products would be inconsistent if

not for the ability of the modern processing technologies to minimize that variability and, therefore, achieve consistently good quality dairy products. To achieve this consistency all the way through the processing, storage, distribution and consumption, it is necessary to understand the properties of the milk ingredients at the molecular level. Bovine milk is a raw material from which several thousand types of dairy products are produced around the world in a wide diversity of flavors and forms, including more than a thousand varieties of cheese, but also butter, whole milk powder, skimmed milk powder, concentrated milk products, fermented milk products, casein and infant formulae. This diversity is in part due to the ease with which the principal constituents of milk are isolated, leading to the production of valuable food ingredients, and also because milk is free of off-flavors, pigments and toxins (Jenness, 1999).

Bovine milk contains the nutrients needed for growth and development of the calf, and is a source of lipids, proteins, amino acids, vitamins and minerals. It contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides. The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein and salts, primarily calcium. Lactose and most minerals are in solution. Milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder (Jenness, 1999).

2.5.1.1 Milk proteins

Bovine milk contains about 32 g protein/L. The milk protein has a high biological value, and milk is therefore a good source for essential amino acids. In addition, milk contains a wide array of proteins with biological activities ranging from antimicrobial ones to those facilitating absorption of nutrients, as well as acting as growth factors, hormones, enzymes, antibodies and immune stimulants. The nitrogen in milk is distributed among caseins, whey proteins and non-protein nitrogen (Bassette & Acosta, 1999).

The casein content of milk represents about 80 % of milk proteins. Caseins biological function is to carry calcium and phosphate and to form a clot in the stomach for efficient digestion. The milk whey proteins are globular proteins that are more water soluble than caseins, and the principal fractions are beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin and immunoglobulins. The rate at which the amino acids are released during digestion

and absorbed into the circulation may differ among the milk proteins, and whey proteins are considered as rapid digested protein that gives high concentrations of amino acids in postprandial plasma. The most important casein fractions are alpha(s1)- and alpha(s2)-caseins, β-casein, and kappa-casein. The distinctive property of all caseins is their low solubility at pH 4.6. The general compositional factor is that caseins are conjugated proteins, most with phosphate groups esterified to serine residues. These phosphate groups are important to the structure of the casein micelle. Calcium binding by the individual caseins is proportional to the phosphate content (Bassette & Acosta, 1999).

Some of the milk proteins such as secretory immunoglobulin A, lactoferrin, 1-antitrypsin, β-casein and α-lactalbumin may be relatively resistant to digestive enzymes, and the whole protein or peptides derived from it, may exert their function in the small intestine before being fully digested. As several bioactive proteins and peptides derived from milk proteins are potential modulators of various regulatory processes in the body, some of these are produced on an industrial scale, and are considered for application as ingredients in both functional foods and pharmaceutical preparations. Although the physiological significance of several of these substances is not yet fully understood, both the mineral binding and cytomodulatory peptides derived from bovine milk proteins are now claimed to be health enhancing components that can be used to reduce the risk of disease or to enhance a certain physiological function (Whitney, 1999).

The conformation of caseins is very similar to that of denatured globular proteins. The high number of proline residues in caseins causes particular bending of the protein chain and inhibits the development of close-packed, ordered secondary structures. Caseins contain no disulfide bonds, and the lack of tertiary structure accounts for the stability of caseins against heat denaturation because there is very little structure to unfold. Without a tertiary structure there is considerable exposure of hydrophobic residues, this feature, and the strong association reactions of the caseins, renders them insoluble in water. Within the group of caseins, there are several distinguishing features based on their charge distribution and sensitivity to calcium precipitation (Whitney, 1999):

• Alpha(s1)-casein: molecular weight 23000; 199 residues, 17 proline residues; two hydrophobic regions, containing all the proline residues, separated by a polar region, which

contains all but one of eight phosphate groups and can be precipitated at very low levels of calcium.

• Alpha(s2)-casein: molecular weight 25000; 207 residues, 10 prolines; concentrated negative charges near N-terminus and positive charges near C-terminus that can also be precipitated at very low levels of calcium.

• β-casein: molecular weight 24000; 209 residues, 35 prolines; highly charged N-terminal region and a hydrophobic C-terminal region. This is an amphiphilic protein that acts like a detergent molecule. Self association is temperature dependant and will form a large polymer at 20 °C but not at 4 °C. It is less sensitive to calcium precipitation.

• kappa-casein: molecular weight 19000; 169 residues, 20 prolines, very resistant to calcium precipitation, stabilizing other caseins. Rennet cleavage at the Phe_{105}-Met_{106} bond eliminates the stabilizing ability, leaving a hydrophobic portion, para-kappa-casein, and a hydrophilic portion called kappa-casein glycomacropeptide (GMP), or more accurately, caseinomacropeptide (CMP).

The caseins behave differently than most proteins. They form extremely flexible structures and for some time they were described as being essentially random. There is no crystal data for the caseins because the molecules do not form crystals. This has been cited as evidence that they do not have well defined three-dimensional structures. The most detailed evidence for secondary and tertiary structure is obtained from x-ray diffraction studies of crystalline molecules, although it is possible to make estimates of secondary structure from molecules in solution. The open and flexible nature of the caseins makes them unusual. While they may have a preferred secondary and tertiary structure, they are often in other conformations. These other structures expose hydrophobic groups to contact with water, but the structures attained by the caseins can accommodate this contact. For most proteins, unfolding and exposure of hydrophobic groups to water results in unstable structures, as they refold to lower the contact with water, often resulting in precipitation, but because the caseins exist in open structures to begin with, they are not as sensitive to structural alterations. Also, the caseins are very resistant to temperature changes. They may be exposed to boiling for extended periods of time without alteration of their solubility, which is an extremely useful property for food products that will be subjected to severe heat treatment (Swaisgood, 1993).

2.5.1.2 Whey proteins

The proteins that remain in solution after removal of casein are by definition termed whey proteins. The most prevalent protein in whey is β-lactoglobulin. It comprises 10 % of the total milk protein or about 58 % of the whey protein. It contains 162 amino acids with a molecular weight of about 18300. There are two main genetic variants, A and B that differ in the substitution of a glycine in variant B for an aspartic and in variant A. The molecule contains two disulfide and 1 free sulfhydryl groups and no phosphorus. Below pH 3.0 and above pH 8.0, β-lactoglobulin exists as a monomer. Between pH 3.1 and 5.1 at low temperatures and high protein contents, it associates to form an octamer. This polymerization seems to be mediated through the action of carboxyl groups and thus the A variant forms better octamers than does the B variant. At other pH values, including the pH of milk, beta-lactoglobulin tends to be found as a dimer. These dimers are spherical with diameters of about 18 Å. The complex association-dissociation behavior of beta-lactoglobulin has been the subject of extensive study. Beta-lactoglobulin is synthesized specifically in the mammary gland for inclusion in milk where its role is unknown. All ruminant milk contains β-lactoglobulin while the milk of almost all non-ruminants does not. While biological functions have been speculated to exist for β-lactoglobulin, to date none have been fully accepted. The molecule has a very hydrophobic region that is quite effective in binding retinol. Some speculate that the binding of vitamin A may have a regulatory role in the mammary gland. Because of its prevalence in bovine milk, to a large extent the properties of whey protein concentrates are in effect, the properties of β-lactoglobulin. The secondary structure of β-lactoglobulin is homologous to that of retinol-binding proteins. It contains 9 strands of beta structure, 8 of them arranged to form a β-barrel. The lone helix is located on the surface of the molecule. The center of the barrel is hydrophobic and can be involved in the binding of hydrophobic molecules (Jost, 1993; Kontopidis, Holt & Sawyer, 2004; Sawyer & Kontopidis, 2000; Whitney, 1999).

The second most prevalent protein in whey is α-lactalbumin which comprises about 2 % of the total milk protein and about 13 % of the total whey protein. The molecule consists of 123 amino acids and has a molecular weight of 14146. The molecule contains 4 disulfide linkages and no phosphate groups. Alpha-lactalbumin has been shown to modify the activity of the enzyme galactosyl transferase. In the absence of α-lactalbumin, this enzyme adds UDP-galactose to N-acetyl glucosamine groups that are attached to proteins. It can transfer the UDP galactose to glucose, but the K_m for glucose is 1400 mM and thus, the reaction proceeds slowly, if at all. Alpha-lactalbumin serves to lower the K_m for glucose to 5 mM and the enzyme

complex now will add UDP-galactose to glucose to produce lactose and UDP. Thus, the milk of all mammals containing lactose also contains α-lactalbumin. The α-lactalbumin of any species isolated so far will serve to modify galactosyl transferase activity. When the sequences of α-lactalbumin and lysozyme are compared, about 40 % of the residues are found to be the same, including all the cysteine residues. Another 20 % of the residues have similar structures. This information coupled with the fact that α-lactalbumin helps to synthesize the same linkage that lysozyme cleaves, suggests that the molecules are closely related. In fact, knowledge of the three-dimensional structure of lysozyme has been utilized to predict the three-dimensional structure of α-lactalbumin. Regardless of their similarity, they do not work on the same substrates and are not related ontogenetically. The site of synthesis of α-lactalbumin like beta-lactoglobulin is the mammary gland. Alpha-lactalbumin is different in that the molecule is more stable to heat in the presence rather than the absence of calcium. Most proteins show increased heat sensitivity in the presence of calcium, probably due to the ability of calcium to promote the formation of ionic intermolecular cross-links with most proteins. These cross-links hold the molecules in proximity and increase the likelihood of aggregation upon heating. Alpha-lactalbumin, in contrast, uses calcium to form intramolecular ionic bonds that are apt to make the molecule resistant to thermal unfolding. Under favorable conditions of calcium and pH, α-lactalbumin can remain soluble after exposure to temperatures as high as 100 °C (Heine, Klein & Reeds, 1991; Permyakov & Berliner, 2000).

The Bovine Serum Albumin (BSA), isolated from milk, is identical to the blood serum molecule. Thus, BSA is not synthesized in the mammary gland, but rather into the milk through passive leakage from the blood streams. This protein has a molecular weight of 69000, contains no phosphorus, and it is conformed by 17 disulfides and 1 free sulfhydryl group. In blood plasma albumin is a carrier of free fatty acids. The molecule has specific binding sites for hydrophobic molecules and may bind them in milk as well (Carter & Ho, 1994; Whitney, 1999).

The immunoglobulins include at least 2 % of the total milk protein. There are four classes of immunoglobulins found in milk: 1gG1, lgG2, lgA and lgM. All of these molecules have a similar basic structure composed of 2 light chains with molecular weights of 20000-25000 and two heavy chains with molecular weights of 50000-70000. These molecules are not synthesized in the mammary gland and consequently must first enter into the gland and then be transported through it to be able to enter the milk composition. In the case of at least one class of antibodies, lgG1, a specific receptor site has been located on the membrane of the cells of the

mammary gland that facilitates the entry of this protein into the gland. The immunoglobulins supply passive immunity to the calf when provided in the colostrum. This protection lasts until the animal is old enough to begin synthesis of its own antibodies (Whitney, 1999).

At last, proteose-peptone have been defined as those proteins that remain in solution after milk has been heated at 95 °C for 20 minutes and then acidified to pH 4.7, and are precipitated by 12 % trichloroacetic acid. This fraction can be divided into 4 major components while other minor ones are documented. Proteose peptone component 3 is found only in whey and is not associated with casein. This protein contains over 17 % carbohydrate and has a molecular weight of 20000. Antibody to proteose peptone component 3 will cross-react with fat globule membrane and it has been suggested that this component is of membrane origin. Proteose peptone component 5 has a molecular weight of 13000 and is associated with both the whey and casein fractions of milk. The molecule contains phosphorus and has been shown to consist of the N-terminal 107 amino acids of β-casein that arrive from the proteolytic cleavage that yields the γ-caseins. Similarly, proteose peptone component 8 fast with a molecular weight of 3900 represents the N terminal 28 amino acids released from the cleavage of β-casein. The remaining proteose peptone component, 8 slow, with a molecular weight of 9900, has not yet been shown to have been derived by the proteolysis of any milk proteins. As a group, the proteose peptones are by definition resistant to heating. They are also very surface active due in part to their low molecular weights and also to the carbohydrate associated with component 3. About 1.1 % of the total milk proteins consist of proteose peptone. As some of these molecules are derived from the proteolysis of beta-caseins, it can be predicted that their concentration in milk will increase with time (Sørensen & Petersen, 1993; Whitney, 1999).

2.5.2 Porcine blood

The exploitation of different sources of food proteins coming from the meat industry by-products could, at a low cost, improve the nutritional status of the population, particularly in poor countries. In doing so, animal blood could turn into an exceptional quality raw source to be used. However, the usage of blood in food processing for human diet has been neglected. Regardless of the low cost of animal blood such as porcine blood, the fact that it possesses high nutritional and functional quality proteins, and that it is an excellent source of highly available iron in the diet, it is little used in the food industry because of the dark color it conveys and

because this color is passed onto the products to which it is added. For this reason, most of the industrial use of animal blood has been restricted to production of animal feed, fire extinguishers foams, fertilizers, cosmetics formulations and microbial media, among others (Pearson & Dutson, 1988; Wismer-Pedersen, 1988).

Pigs are a significant protein source in many countries, and more than 400 million pigs are slaughtered annually as meat supply, which go together with the generation of approximately 1 million tons of blood in China alone. From this by-product the red blood cell (RBC) fraction represents 40 % of blood and hemoglobin (Hb) corresponds to 80 % of the protein content of blood. The high iron and protein contained in porcine blood, as well as its superior functional properties, make RBC interesting as a food ingredient, provided that the problems of the dark color and blood flavor, which appear when it is added to food products, are solved. However, the lack of effective use of animal blood, especially blood Hb, generates a surplus volume that is usually discarded causing severe environmental problems due to the associated high organic pollutant (biochemical oxygen demand) and microbial loads (Yike, Jianen, Yuji, Xuefang, Yuguang & Bingcheng, 2006).

Most of porcine slaughterhouse blood is generally wasted, creating a serious environmental contamination problem. A nominal part of this blood is processed into food products by extensive heat treatment, which degrades its value, destroying some of the protein functional value and results in products of inconsistent nutritional usefulness. Because of the use of blood in food processing results in the product being dark and often unpalatable, and because plasma has a more desirable color and functional properties, plasma is the portion of blood that obtains more attention from the food industry (Toldrá, Busquets, Saguer, Parés & Carretero, 2002).

2.5.2.1 Blood proteins

Blood contains highly functional proteins such as hemoglobin, albumin and immunoglobulin, and also has a valuable essential amino acid profile. Proteins such as those found in blood play an important part in the functionality of foods and pharmaceuticals, in addition to biological systems. The amino acid composition of blood proteins is close to that of proteins which are considered well balanced from a nutritional point of view, or at least this is the case with the essential amino acids. Nonetheless, the His and Met concentration is lower than that of the milk proteins, while the Lys and Thr concentration is higher (Tybor, Dill & Landmann, 1975).

Porcine blood produced during slaughtering is a valuable protein source, and has been found to produce several bioactive peptides. Some bioactive peptides isolated from the hydrolysate of blood hemoglobin exhibit opioid, bacterial growth stimulating, ACE inhibiting, and antioxidant activities (Teschemacher, 2003; Yike et al., 2006).

Porcine red blood cells (RBC) share a number of common characteristics with human RBC. Pig RBC diameters are 4 to 8 μm, and RBC counts in porcine RBC is 5.7 to 6.9 million, similar to human RBC. Although, RBC average lifespan is rather shorter in pigs: 86 days vs. 120 days in humans. The most closely studied pig blood group system is the A–O (H) system, but herds have been developed that are uniformly of blood type O. Porcine hemoglobin shares 85 % sequence identity with its human counterpart, and it has also been reported a similar three-dimensional structure at 2.8 Å resolution (by X-ray diffraction). Furthermore, human hemoglobin has been expressed in transgenic pigs, with normal post-translational modifications and biological function (Cooper, 2003).

Ramos-Clamont, Fernández-Michel, Carrillo-Vargas, Martínez-Calderón & Vázquez-Moreno (2003) separated red cell concentrate, serum, albumin and immunoglobulin from porcine blood, examining functional and microbiological properties; finding that both red cell concentrate and serum isolate were high in protein but limiting in methionine and isoleucine. Porcine serum and albumin were found to have emulsifying properties comparable to the same bovine blood fractions, while porcine immunoglobulins were 96 % soluble. All fractions were of outstanding microbiological quality, indicating the potential functionality of porcine protein fractions.

2.5.2.2 Plasma

Plasma has been widely used in the meat industry because of its excellent functional properties. Plasma contains one-third of the total blood protein, and it consists of 7 % to 8 % protein and 91 % water. After the separation of plasma from the red cells, it is usually cooled, frozen or spray dried. The red cells, consisting of 34 % to 38 % proteins and 62 % water, are dried to form a meal or the heme group is removed to obtain globin. Anticoagulants are normally used in collecting whole blood and are injected via hollow knife if a vacuum system transport is adopted. In this process, continuous blood-separation equipment is used and the separation of the fractions is accomplished with a high speed centrifuge or separator. After

separation, the plasma is frozen or spray dried at low temperature in order to maintain its functionality and solubility. To freeze blood plasma, it is normally placed on a vertical rotating drum at a temperature between -10 °C and -40 °C, and then the frozen plasma is scraped from the surface in the form of a flake. When blood is dried, great care must be exercised to prevent denaturation of the protein, and consequent low quality of the dried fraction. The first step for the blood plasma drying process in most plants is concentration, and it is generally accomplished by membrane filtration and evaporation. The drying process of the concentrated plasma is then finished by a spray drying system or fluidized drying system. A dried blood plasma can be produced with 96.4 % protein and 2.4 % moisture by this processing method (Hermansson, 1982; Parés et al., 1998).

Plasma is a naturally occurring protein mix, which can be arranged into three major groups according to a relevant content for functionality purposes: albumin, globulins and fibrinogen. Albumin is a globular protein with a molecular weight of 66 kDa and an isoelectric point around 4.8. This is the most abundant of plasma proteins, representing up to a 60 % of the total protein content. The three-dimensional configuration of serum albumin is composed of three homologous domains: I, II and III, mainly helical and extensively cross-linked by a number of disulfide bridges. It has a low content of tryptophan and methionine, and a high content of cystine and charged amino acids such as aspartic and glutamic acids, lysine and arginine. The primary level structure of albumin is responsible for its high solubility, and this protein is formed by circa 580 amino acids in a single chain with 17 disulfide bridges in its sequence and one free cysteine in position 34 (Pearson & Dutson, 1988).

Another of the plasma proteins is globulin, further subdivided in α, β and γ fractions, including a diverse group of globular proteins that comprise a variety of enzymes, carrier and antigenic proteins, and represents 40 % of the plasma protein content. The most abundant of the globulin subgroups is the inmunoglobulins. The molecular weight of these proteins range from a few to hundreds of kDa and they also display a variety of isoelectric points (p*I*) between 5 and 7. The typical tertiary structure of inmunoglobulins is a Y-shaped congregation of two pairs of light and heavy chains stabilized by disulfide bridges, with an antigen binding site at the edge. Of the globulins, the most numerous is immunoglobulin G (IgG), followed by α1-antitrypsin and transferring (Putnam, 1965).

In the third category, there is a single protein that represents 3 % of the total content: fibrinogen, a multidomain fibrous protein weighing 340 kDa and p*I* of about 5.5, which plays an important role in blood coagulation, given that its main function is the formation of the 3-dimensional network of fibrin fibers during the blood clotting process. In the fibrinogen structure, there are 3 pairs of polypeptide chains: Aα, Bβ and γ which form two identical subunits which, as the chains, are linked together by 29 disulfide bonds that form a symmetrical trinodular structure (Pearson & Dutson, 1988).

2.5.2.3 Use of plasma proteins to enhance texture of meat products

Blood in food is used as an emulsifier, stabilizer, color additive and nutritional component. Most of the blood is used in livestock feed in the form of blood meal and used as protein supplement, milk substitute, lysine supplement or vitamin stabilizer, being an excellent source of trace minerals. Blood plasma has gel-forming ability because it contains 60 % albumin, and is the best water and fat binder of this blood fraction. Plasma gels appear very similar to cooked eggs whites, and it forms thermally-induced gels at protein concentrations of 4 % to 5 %, with its gel strength increasing as the concentration increases. In addition to the gel forming, blood plasma also has outstanding foaming capacity. So much so, that it could be used to replace egg whites in the baking industry (Hermansson, 1982; Howell & Lawrie, 1984; Lee & Hirose, 1989).

The application of transglutaminase (TGase) from porcine blood to products in the meat industry is one of the most investigated applications in food processing. Blood factor XIII is a TGase that appears as an enzymogen in plasma, placenta and platelets. The reaction catalyzed by Ca^{+2} dependent factor XIIIa involves the formation of a ε-(γ-glutamyl)-lysyl bond between an acycle donor (glutaminyl residue) and an acyl receptor (lysyl residue) of the proteins fibrin and fibronectin, fibrin and actin, myosin and fibronectin and myisin and actin. Consequently, this enzyme catalyzes conversion of soluble proteins to insoluble high-molecular polymers through formation of covalent cross-links. In a similar manner, transglutaminase extracted from blood is used to improve the binding ability of fresh meat products at chilling temperature and it shows how myosin is cross-linked with TGase. An important property of the TGase reaction has been documented after finding the cross-linking between myosin and proteins such as soy, casein and gluten, commonly used in meat processing. Furthermore, the

restructured meat products without heating and decreased with salt and phosphates, can be made by addition of TGase from plasma (Motoki & Seguro, 1998).

2.5.3 Soy proteins

Soybeans are typically processed into 3 major groups: soy flours with a minimum protein content of 50 %; soy protein concentrates with 65 %, and isolated soy proteins with 90 % in a dry basis. Soy flours are manufactured as fine powders or grits with a particle size ranging from 0.2 to 3 mm. They can be produced by using minimal heat to maintain the inherent enzyme activity of the soybean, or by toasting to variable degrees if the desired result is the reduction or elimination of the enzymatic activity and improvement of the product flavor, soy flours and grits have been used in the baking industry for a long time. Soy protein concentrates are usually manufactured by using alcohol to remove the soluble carbohydrates from the de-oiled soy flakes, resulting in a protein with low solubility and a product with water holding capacity, but incapable to gel or emulsify fat. Traditional alcohol-washed soy concentrates are used for protein fortification of foods as well as in the production of textured soy concentrates; whereas functional soy concentrates are produced from the traditional soy concentrate by using heat and homogenization followed by spray drying, or by using a water-wash process at an acid pH to remove the soluble sugars followed by neutralization, thermal processing, homogenization and spray drying. Functional soy concentrates hold water, emulsify fat and form a gel upon heating; they are widely used in the meat industry and are also efficient for the stabilization of high fat soups and sauces (Berk, 1992).

Soy proteins isolates are manufactured from de-fatted soy flakes by separation of the soy protein from both the soluble and the insoluble carbohydrate fractions of the soybean. In a development throughout more than 60 years, the soy protein isolates produced today are bland in flavor, light in color and have a number of functional properties such as gelation, viscosity, emulsification, water holding capacity, and even some foaming and whipping ability. In order to take full advantage of these functional properties, it is necessary for isolated soy proteins to reach a high degree of solubility, and also to be properly hydrated. Isolated soy proteins are integrated in food systems for a diversity of reasons. Sometimes they are used for simple protein fortification purposes, others for their functional properties or for the health benefits associated with the consumption of soy protein. Nevertheless, the selection of the appropriate soy protein isolate is decisive for an accomplished product development (Berk, 1992).

2.5.3.1 Soy protein components

The main content of protein in soy is found in storage sites called protein bodies or aleuronic grains, which are subcellular structures that range in diameter from 2 μm to 20 μm. The protein bodies have been reported to contain approximately 10 % nitrogen, 0.8 % phosphorus, 8.5 % sugar, 7 % ash, 0.5 % RNA, 4.5 % lipid and 2 % phospholipid. The protein bodies are close to 75 % protein and the globular reserve proteins make up about 80 % of the soy seed protein, while the remaining 20 % is constituted by biologically active proteins such as enzymes, enzyme inhibitors and lectins. The soybean storage proteins were extracted and characterized for the first time by Osborne & Campbell in 1898, and they named the extracted protein glycinin. It was later noticed that this protein was heterogeneous and when ultracentrifugated at pH 7.6 and 0.5 ionic strength, the fractions 2S, 7S, 11S and 15S were given. Catsimopoolas (1969) proposed a classification of soy protein components based on an immunochemical reference system. Since, four inmunochemically distinctive globulins have been identified: glycinin, matching the 11S globulin and not the same as the Osborne & Campbell's glycinin; α-conglycinin, that is a part of the 2S globulin fractions, β-conglycinin and γ-conglycinin, these two latter being part of the 7S fraction. The vast majority of the native soy storage protein is composed of glycinin (11S globulin) and β-conglycinin (7S globulin). Soy protein has a relatively well-balanced amino acid composition (Mendel & Fine, 1911; Wolf, Babcock & Smith, 1961).

One of the methods used to test protein quality is based on feeding the protein product to rats, and thus determine the protein efficiency ratio (PER), which has been found to be lower than that of animal proteins, but upon fortification with sulfur-containing amino acids it reaches a similar PER level. More recently, the quality of soy protein assessed by the protein digestibility corrected amino acid score PDCAAS is deemed comparable to that of animal protein (Schaafsma, 2005).

Other than the amino acid composition, there are factors that affect the nutritional quality of soy protein, including treatment of soy protein by heat and the means of application of these types of treatments, as well as the modification of soy globulin structures to diminish or eliminate their antigenicity using alcohol and heat. In addition, there are possibly antinutrients and allergens present at biologically active levels within the soy protein matrix, which are

destroyed by heat treatment or aqueous alcohol extraction. The raw soybean contains numerous antinutrients that can be reduced by the processing method, but not completely eliminated. Some of these antinutrients are phytic acid, which binds and prevents mineral absorption of zinc, calcium, and magnesium; hemagglutinins, which have an ability to agglutinate the red blood cells in humans and in other animal species, and significantly suppress growth in laboratory rats; and trypsin inhibitors, which inhibits the action of the digestive enzyme trypsin (Anderson & Wolf, 1995; Gao et al., 2007; Nordlee, Taylor, Townsend, Thomas & Bush, 1996; Sharon & Lis, 2004). The possibility of using HPP to inactivate some of these problematic factors in soy was explored by van der Ven, Matser & van den Berg (2005).

Connective statement to Chapter 3

The importance of studying the effect of high pressure treatment on proteins was highlighted in the previous chapter, as well as the need for continued research in this area. In this chapter the effect of HP up to 650 MPa on the secondary structure of bovine β-lactoglobulin (β-lg) was examined by Fourier transform infrared (FTIR) spectroscopy as well as the resulting influence on viscoelastic properties of β-lg, as a function of increasing protein concentration and HP level to better understand the molecular basis of changes in the viscoelasticity properties of the protein resulting in a soft gel. Beta-lactoglobulin (β-lg) is the most simple protein selected in this study and has been well studied with respect to its structure and properties.

Based on results from Chapter 3, a mauscript has been prepared for publication: Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. 2008. Molecular Basis of High Pressure Induced beta-lactoglobulin Soft Gels. This research work was completed by the Ph.D. candidate under the supervision of Dr. H.S. Ramaswamy and Dr. A.A. Ismail.

Chapter 3. Molecular Basis of High Pressure Induced beta-lactoglobulin Soft Gels

3.1 Abstract

There is increasing commercial and consumer interest in high-pressure (HP) processing as an alternative to heat processing. This has resulted in an increased interest in understanding the effect of HP on the molecular structure and functionality of protein-based gels. In this work, the effects of different pulse pressure treatments (pressures ranging between 100 and 650 MPa with a holding time of 0.1 min) on beta-lactoglobulin (β-lg) dispersions were examined by Fourier transform infrared (FTIR) spectroscopy and dynamic rheology. Results indicated a direct relationship between protein concentration, treatment pressure level and viscoelastic properties (expressed as an increase in both elastic (G') and loss modulus (G") and a decrease in phase angle), as well as changes in the amide I' bands of the protein as a function of HP treatment. The rheological characteristics of 15 % β-lg dispersions (pH 6.9) was unaffected by pressures up to 550 MPa, but 600 MPa and above, significant changes in viscoelastic properties of the protein solutions were observed. HP-induced gelation of 15 % β-lg solutions was achieved at 600 MPa. Examination of the FTIR spectra in the amide I' absorption region, of HP-induced gels and heat induced gels of β-lg, were markedly different. Thermally induced gels formed extensive anti-parallel intermolecular β-sheets accompanied by a marked decrease in the native secondary structure of the protein. HP-induced gels, on the other hand, were formed with minimal amount of intermolecular β-sheet; furthermore, the native secondary structure of the protein also remained unaffected to a large extent, showing a minor change in the relative ratio of α-helical+random to β-sheet structures.

3.2 Introduction

High pressure (HP) processing is being increasingly used as an alternative to the classical thermal processing of foods. Applying ultra high hydrostatic pressures ranging from 100 to 900 MPa has been shown to make foods safer and extend their shelf life while allowing the product to retain many of its organoleptic and nutritional attributes. This meets consumer demands for freshness without the consumer related concerns of

other methods such as irradiation. HP processing has been used for many products to achieve a variety of goals, such as: inactivation of food-borne pathogens (Mussa, Ramaswamy & Smith, 1999a,b; Riahi et al., 2003; Ritz, Tholozan, Federighi & Pilet, 2002; Shao, Ramaswamy & Zhu, 2007), bacterial spores (Delacour, Clery, Masson & Vidal, 2002), enhancement (Jung, de Lamballerie-Anton, Taylor & Ghoul, 2000) or inhibition of selected enzymes (Ashie et al., 1996b; Basak & Ramaswamy, 1997, 2001; Basak, Ramaswamy & Simpson, 2001; Garcia-Palazon, Suthanthangjai, Kajda & Zabetakis, 2004; Pandey & Ramaswamy, 2004; Ramaswamy & Riahi, 2003; Riahi & Ramaswamy, 2003, 2004), tenderize meat (Suzuki, Kim, Honma, Ikeuchi & Saito, 1992), shuck oysters (San Martin, Barbosa-Canovas & Swanson, 2002), extend shelf life (Mussa & Ramaswamy, 1997; Lee, Cha, Hwang & Park, 2003), promote ripening of cheeses (Pandey, Ramaswamy & St-Gelais, 2003b; Saldo, Sendra & Guamis, 2000), and minimize oxidative browning (Hong & Kim, 2001). High pressure in conjunction with elevated temperatures can also be employed for the sterilization of many food products (Clery-Barraud, Gaubert, Masson & Vidal, 2004; Patazca, Koutchma & Ramaswamy, 2006; Spilimbergo, Elvassore & Bertucco, 2002; Zhu, Naim, Marcotte, Ramaswamy & Shao, 2008). Additionally, recent studies have revealed that pressurization of a protein solution causes partial protein unfolding that can lead to the irreversible process of gelation (Kanno, Mu, Hagiwara, Ametani & Azuma, 1998). A number of variables such as the nature of the protein, its concentration, the level of pressure applied and the holding time, the number of pressure cycles, temperature, pH, and ionic strength all play significant roles in governing the rate and extent of protein denaturation (Iametti et al., 1997; Kanno et al., 1998; Keim & Hinrichs, 2004; San Martin et al., 2002; Van Camp & Huyghebaert, 1995; Van Camp, Feys & Huyghebaert, 1996) and the rheological properties of the pressure treated protein solutions (Ahmed & Ramaswamy, 2003a; Ahmed & Ramaswamy, 2003b; Alvarez et al., 2008). Recent studies have demonstrated that applied pressure can also affect the nutraceutical properties of whey protein isolate (Hosseini-Nia, Kubow & Ismail, 2001).

Whey protein isolate (WPI) is obtained by concentrating whey (a by-product of the cheese manufacturing process) after removal of lactose and other low-molecular-

weight constituents. The relative ratios of the different protein components of WPI will be dominated by the specific manufacturing process (Alvarez, Ramaswamy & Ismail, 2007). In general, WPI contains beta-lactoglobulin, alpha-lactalbumin, glycomacropeptides and bovine serum albumin; in that order of abundance. There is much interest in WPI and its individual components as functional ingredients in foods (Boye, Alli, Ismail, Gibbs & Konishi, 1995) and, more recently, in the bioactive peptides that can be obtained from them, which can act as nutraceuticals (FitzGerald, Murray & Walsh, 2004). As previously mentioned, applied hydrostatic pressure causes partial protein denaturation and, thus, high-pressure processing can provide a means of altering the functional properties of WPI (López-Fandiño, 2006). The relationship between pressure-induced structural changes that lead gelation is still under investigation.

In this study, the effect of HP up to 650 MPa on the secondary structure of β-lactoglobulin (β-lg) was examined by Fourier transform infrared (FTIR) spectroscopy. Some results were used to analyze the intrinsic variability of the HP-processing repeatability. Also the changes in viscoelastic properties of β-lg, as a function of increasing protein concentration and HP level was investigated to better understand the molecular basis of changes in the viscoelastic properties of this protein in solution and in soft (not self standing) gels. Statistical analysis was used to model the response of the viscoelastic properties and secondary structure to changes in pressure treatment level.

3.3 Materials and Methods

The protein used in this study was 97 % (dry basis) β-lg protein powder, obtained from Davisco Foods International (Eden Prairie, MN, USA) and used without further purification. Electrospray ionization mass spectrometry analysis showed a pure mix of A and B genetic variants of β-lg with no other protein component (Alvarez et al., 2007). The composition of the β-lg sample, as per information provided by the suppliers, is given in Table 3.1. Deuterium oxide (D_2O 99.9 % D) was purchased from Aldrich (St. Louis, MO, USA).

Table 3.1 Composition of β-lactoglobulin sample used in this work, as per information provided by the commercial supplier.

Constituent	Amount
Protein (%), dry basis (N×6.38)	97.0 ± 0.1
Ash (%)	2.5 ± 0.2
[a]Calcium	25.0 mg
Moisture (%)	5.2 ± 0.5
Fat (%)	0.3 ± 0.1

[a]Amount per 100 g of product.

3.3.1 High pressure treatments

All HP treatments were given in triplicate in a high-pressure isostatic press (ACIP6500, ACB High Pressure Systems, Nantes, France), with a chamber volume of 5 L. The pressure transfer medium used was water. The maximum operational pressure of 650 MPa was reached in approximately 2 min; the depressurization time was approximately 15 s. Samples were introduced into the high-pressure machine at 10 °C and reached a maximum of 25 °C during pressurization owing to adiabatic heating. This rise in temperature can be considered to have a negligible thermal effect because thermal denaturation of the whey proteins occurs well above ambient temperature (Boye et al., 1995; Boye, Ismail & Alli, 1996). Samples were treated at pressure levels of 100, 200, 300, 400, 450, 500, 550, 600, and 650 MPa with a holding time of 0.1 minutes constituting a pressure pulse treatment; and at selected concentration levels of 5, 10, 15 and 20 % (w/v). Control samples were noted as "control" or 0.1 MPa of pressure (normal atmospheric pressure).

3.3.2 Sample preparation for FTIR spectroscopy analysis

Solutions of β-lg were prepared (concentration range between 5 to 20 %) in D_2O and left to stand for 48 h (at 4 °C) to allow H-D exchange. 500 μL aliquots were subsequently sealed in polyethylene bags for HP treatment. Sample bags were submerged in the water-containing pressure chamber and subjected to the pressure treatment (100 to 650 MPa with 0.1 min holding time). The FTIR spectrum of each sample was immediately recorded after treatment using a Nicolet 8210E FTIR spectrometer (Thermo Electron Corporation, Madison, WI, USA) equipped with a deuterated triglycine sulphate (DTGS) detector. The spectrometer was continuously purged with dry air from a Balston dryer (Balston Products, Haverhill, MA, USA).

For the FTIR spectroscopy analyses, approximately 8 μL of each pressure-treated sample was placed between two CaF_2 windows separated by a 25 μm thick Teflon TM spacer. The temperature of the cell was regulated by an Omega temperature controller (Omega Engineering, Stamford, CT, USA). A total of 512 scans were co-added at 4 cm^{-1} resolution. The absorbance spectra were subjected to band narrowing techniques using Fourier self deconvolution (FSD) employing a bandwidth of 20 cm^{-1} (w) and enhancement

factor of 2.4 (k) followed by a two-point baseline correction starting at 1710 and ending at 1590 cm^{-1} and normalization using Omnic 6.0 software (Thermo Electron Corporation, Madison, WI, USA).

3.3.3 Sample preparation for rheological analysis

Solutions of β-lg protein were prepared as above using H_2O in place of D_2O, pH of the protein dispersion was 6.9. 10 mL aliquots were sealed in plastic bags for HP treatment and treated at pressures between 100 and 650 MPa as before. Rheological evaluations were made immediately after HP treatment using an AR-2000 rheometer (TA Instruments. New Castle, DE, USA), with similar settings as reported by Ahmed, Ramaswamy, Ayad, Alli & Alvarez (2007). Employing parallel plates geometry of 60 mm dia., 1000 μm gap, constant angular frequency of 1 Hz (0.6284 rad/sec) and temperature controlled at 20 °C. Measurements were recorded in the linear viscoelastic region previously tested for each sample.

3.3.4 Statistical analysis

In order to investigate the intrinsic (HP machine-process dependant) variability associated with the HP treatments on changes associated with proteins, three sets of samples were prepared at three pressure levels: control; true replicates (R), where three sample bags were pressure treated in different pressure runs; and pseudo-replicates (PR), where three sample bags were pressure treated in the same run. A one-way ANOVA was performed between control, 200 MPa PR, 200 MPa R, 650 MPa PR and 650 MPa R groups.

Non-linear regression curve fitting techniques were used to correlate the viscoelastic parameter storage modulus (G') of samples; and the FTIR spectroscopy secondary feature peak height at 1627 cm^{-1} (β-structure) to the pressure level used. The statistical analyses were carried out using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Effect of varying protein concentration and pressure level on rheological properties

The storage modulus (G') and loss modulus (G") measure the elasticity and viscosity of the sample solutions, respectively. Hence, a larger G' represents more elastic or gel like properties, while a larger G" represents greater viscous or liquid like properties. Significant amounts of G' and G" signifies viscoelastic nature of the sample. A larger G' combined with a smaller G" shifts properties of the sample more toward gel-like while the reverse shifts it toward more liquid like behavior. This shift is measured in terms of the phase angle. Phase angle values give an indication of the general viscoelasticity of the material; values higher than 45 degrees indicate a substance with liquid-like behavior, and values lower than 45 degrees show solid-like behavior.

A small increase in both G' and G" was observed as a function of increasing protein concentration from 5 to 20 % (Fig. 3.1a). This indicates the gel-sol network is getting strengthened with an increase in protein concentration which is expected since more protein is available to participate. This was also characterized by a minor decrease in phase angle (Fig. 3.1b). Concentration within this range did not have a major influence in the viscoelastic properties. After the samples were pressure treated, a significant increase in the viscoelastic parameters was observed with a dramatic drop in phase angle at each concentration level, thus the rheogram got shifted with the control. The higher the level of treatment pressure (650 MPa vs. 600 MPa), the higher the magnitude of both G' and G", and lower the associated phase angle. Consequently, the HP treatment contributed to building up of the viscosity and gelling behavior of the protein. The formation of a true gel, where the elastic component (G') is greater than the viscous component (G") and where the phase angle is lower than 45 degrees, is reached only at the highest pressure (650 MPa) and a protein concentration of 20 %. Although, an increase in viscoelastic parameters was observed for a dispersion of 15 % protein concentration treated at 650 MPa. These results contrast with the ones obtained by Olsen, Ipsen, Otte & Skibsted (1999) who reported gelation at 450 MPa and 5 % protein concentration; however they utilized purified β-lg B and 30 min pressure holding time. All subsequent investigations were carried out with a 15 % protein concentration.

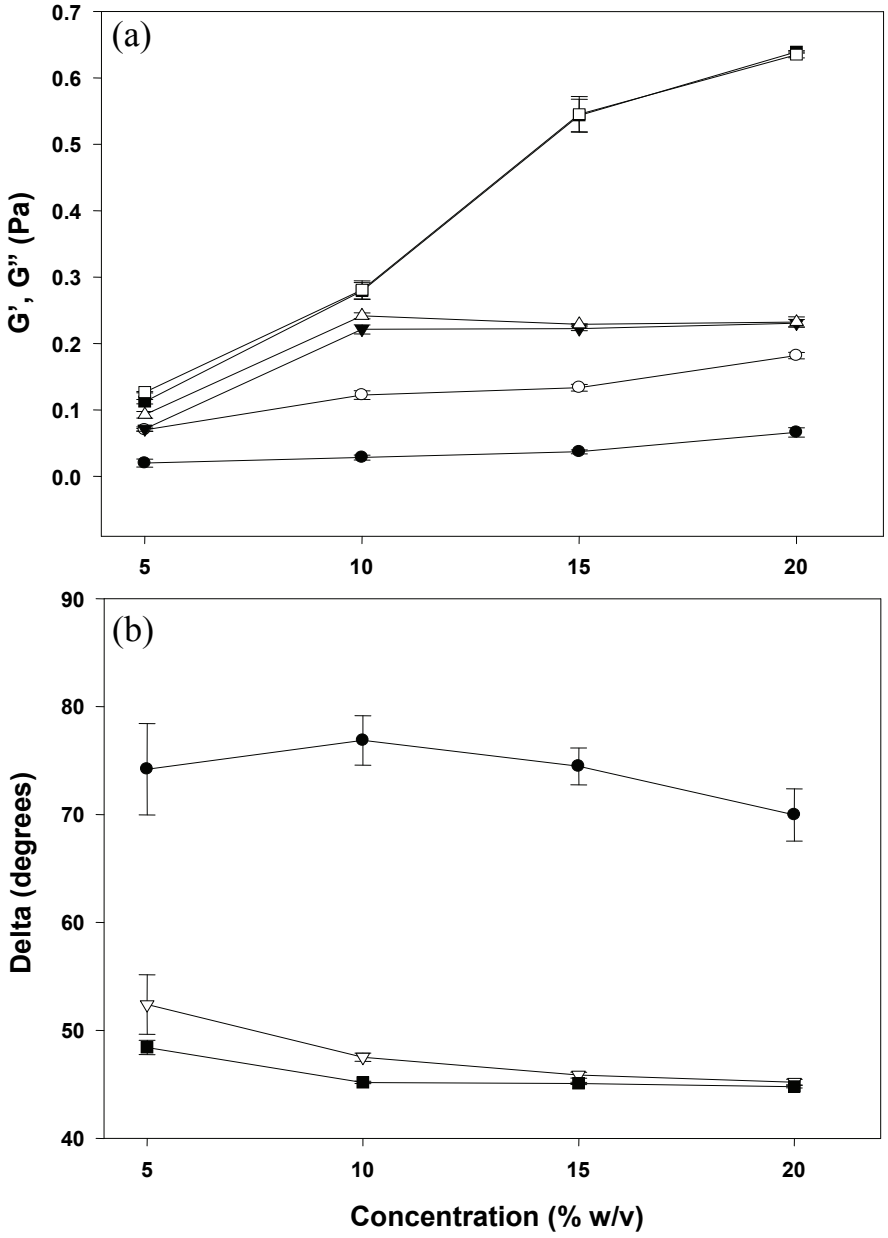

Figure 3.1 Rheological measurements of different β-lactoglobulin concentrations (5, 10, 15, and 20 %) treated at different pressure levels: 0.1 (circles), 600 (triangles), 650 MPa (squares). (a) Viscoelastic parameters of samples exposed to 0.1 MPa (● G', ○ G"), 600 MPa (▼ G', △ G"), and 650 MPa (■ G', □ G"). (b) Phase angle of protein samples of different concentrations exposed to various pressure levels (● 0.1, ▽ 600, ■ 650 MPa).

3.4.2 FTIR spectroscopy

The amide I absorption region (1700-1600 cm^{-1}) in the infrared spectrum of a protein is one of the most useful for secondary structure elucidation (Susi & Byler, 1988). The amide I band assignments of β-lg are summarized in Table 3.2 and are based on previous works (Allain, Paquin & Subirade, 1999; Boye et al., 1996; Dong, Matsuura, Manning & Carpenter, 1998; Hong and Creamer, 2002; Hosseini-Nia, Ismail & Kubow, 1999; Lefevre & Subirade, 2000; Lefevre & Subirade, 2001; Panick, Malessa & Winter, 1999; Spilimbergo, Elvassore & Bertucco, 2002; Susi & Byler, 1988).

The spectral variance of samples exposed to different pressure treatments was obtained by the statistical analysis of 24 (8 in triplicates) FTIR spectra recorded after 0.1, 100, 200, 300, 400, 500, 600 and 650 MPa of pressure (Fig. 3.2). It can be observed from figure 3.2 that the peaks at 1627 and 1635 cm^{-1}, assigned to β-sheet structures, were the two principal contributors to sample variability. Therefore the peak height at 1635 cm^{-1} was selected to assess the repeatability of pressure treatment on the protein solutions.

The irreversible changes in the secondary structure of β-lg subjected to different pressure levels are shown in Fig. 3.3a. The extensive H-D exchange is evident in all the spectra by the lack of band at 1692 cm^{-1} assigned to the H-bond amide groups of a β-sheet buried in the interior of the protein, inaccessible to the solvent under ambient conditions (Boye et al., 1996). Samples were treated in five groups: control; 200 MPa R; 200 MPa PR; 650 MPa R; and 650 MPa PR. In this case the pseudo-replicates served as an internal sample variability gauge to the actual pressure delivered by the isostatic press. After exposure to 200 MPa of pressure the secondary structure of β-lg exhibited very small changes; the most significant was a decrease in the peak at 1635 cm^{-1} corresponding to antiparallel β-sheet. When exposed to 650 MPa the secondary structure resulted in a drop in the intensity of 1635 cm^{-1} band, accompanied with an increase at the 1645 cm^{-1} band assigned to α-helix and unordered structures. Panick et al. (1999) did not find any indication of intermolecular β-sheet formation when working with pressures up to 1 GPa and 5 % protein concentration. On the other hand, Hosseini-Nia et al. (1999) did find some indication of the typical aggregation peaks (1684 and 1614 cm^{-1}) when working with a real-time pressure FTIR spectroscopy cell and pressures up to 1.2 GPa (at 10 % w/v). Both studies found some conversion of the intramolecular β-sheet structure to α-helices and random coil.

Table 3.2 Band assignment of the amide I spectral region of β-lactoglobulin.

Band position (cm^{-1})	Assignment
1692	Hidden antiparallel β-sheet
1684	Antiparallel β-sheet (aggregation)
1680-1676	β-structure
1645	α-helix and unordered
1635	Antiparallel β-sheet
1627	β-structure
1617	Intermolecular β-sheet (aggregation)

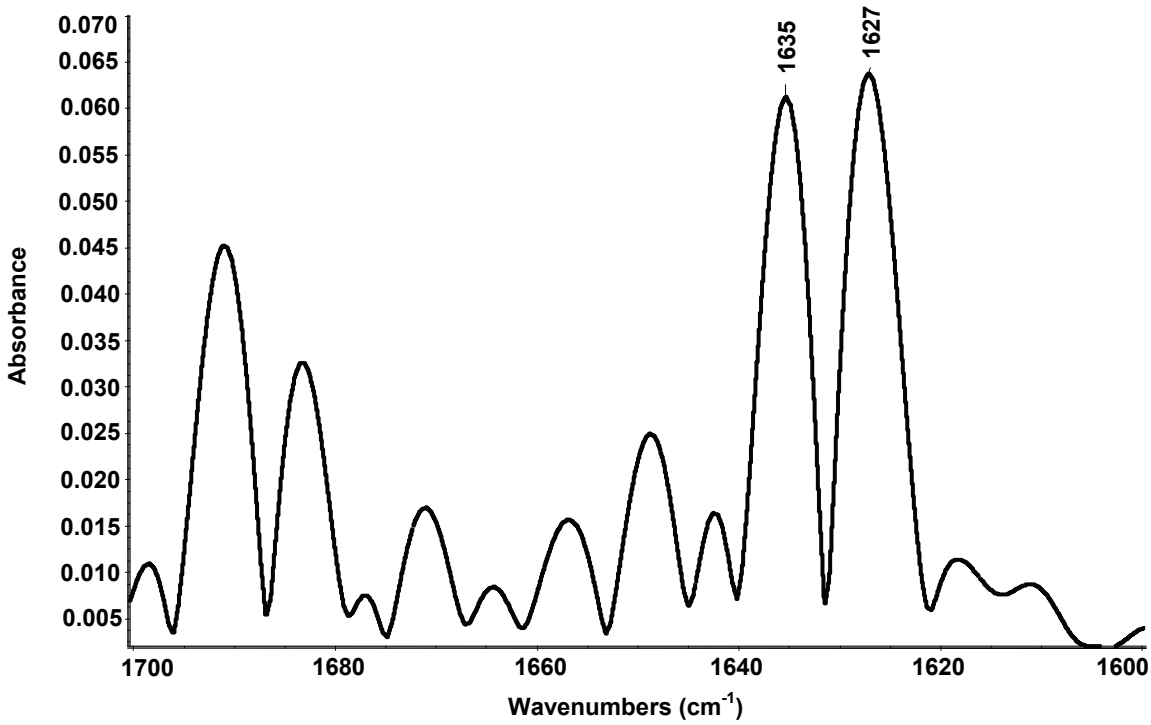

Figure 3.2 Amide I FTIR spectral variance of β-lactoglobulin samples exposed to 0.1 to 650 MPa of pressure.

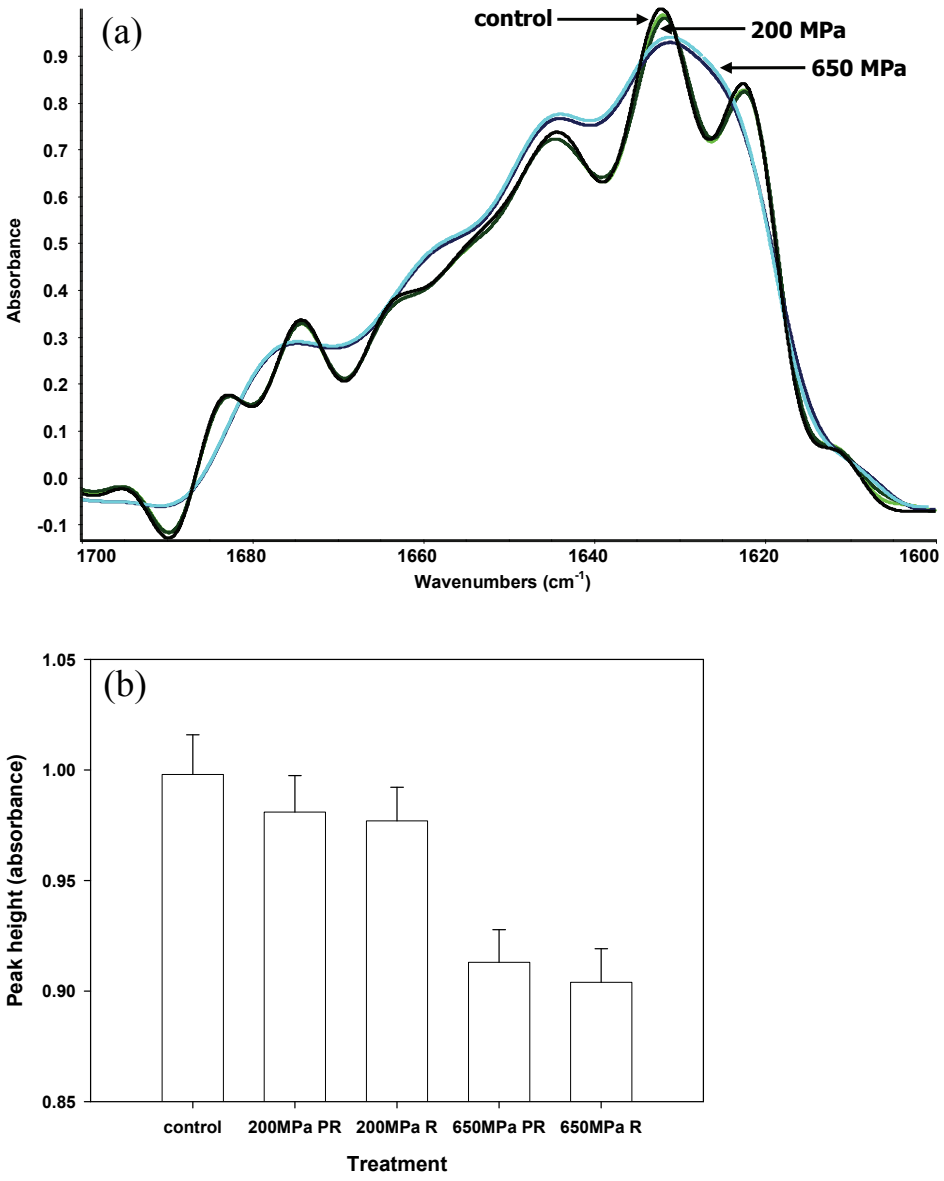

Figure 3.3 FTIR spectra of β-lactoglobulin after two different pressure treatments. (a) Black line is the control sample (non treated); light green line is the pseudo-replicates (PR) exposed to 200 MPa; dark green line is the real replicates (R) exposed to 200 MPa; light blue line is the PR exposed to 650 MPa; and the dark blue line is the R exposed to 650 MPa. Lines are average of 3 deconvolved (w=20.0 cm^{-1}, k=2.4) samples. (b) Average peak height at 1635 cm^{-1} (antiparallel β-sheet) of the two different pressure treated β-lactoglobulin FTIR spectra. SD indicated.

In order to address the changes in the intramolecular antiparallel β-sheet band from a statistical point of view, a one-way ANOVA test was performed on the peak height at the 1635 cm^{-1} band that resulted in highly significant ($p<0.01$) differences for 650 MPa and control or 200 MPa; but no statistical differences among control and 200 MPa, nor between the replicates to the pseudo-replicates. These results are in good agreement with the study by Subirade, Loupil, Allain & Paquin (1998) who reported no differences in the FTIR spectra of β-lg samples subjected to pressures up to 140 MPa. Figure 3.3b shows the average peak height and standard deviation at the 1635 cm^{-1} band for the five different groups.

The fact that, both replicates and pseudo-replicates are statistically equal is a good indication that the error produced by the isostatic press at delivering the required pressure, falls into the error of the FTIR spectrometer instrumental error. Therefore we assumed that the HP-press is delivering consistent pressure through different machine runs.

In order to verify that changes in protein concentration (up to 20 %) does not affect the final protein conformation after pressure treatment, additional FTIR spectroscopy experiments were carried out as a function of increasing concentration (5, 10, 15 and 20 %) with a pressure treatment of 450 MPa and resulted in not significant differences between samples (Fig. 3.4).

3.4.3 Effect of HP treatment level on rheological properties

Since the secondary structure of the protein molecule was unaffected by pressure of 200 MPa, it was decided to move up to higher pressures (450, 550, 600 and 650 MPa) and track the changes in the viscoelastic properties of the protein solutions after pressurization.

The pressurization of 15 % β-lg sample dispersions created almost no change in the viscoelastic properties of the protein solutions up to 550 MPa of pressure (Fig. 3.5). When pressure is raised to 600 and 650 MPa there was a significant change characterized by an increase in both elastic and viscous moduli. At the same time there was also a decrease in the phase angle from the high 70's to close to 45 degrees.

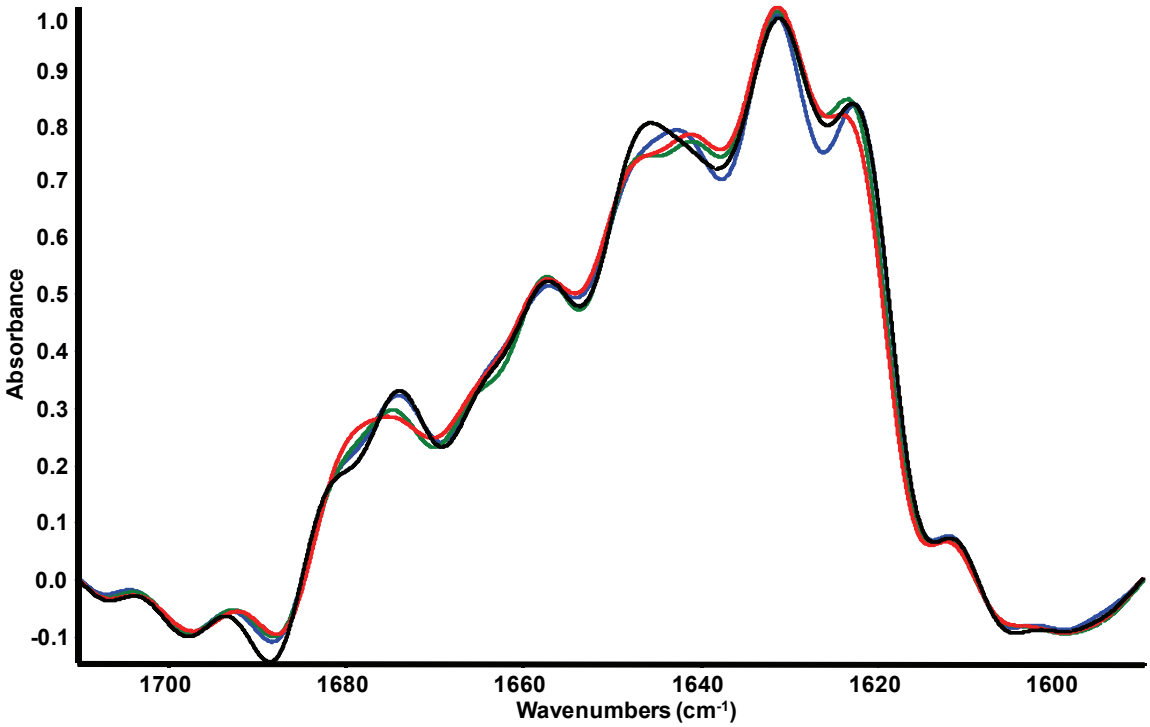

Figure 3.4 FTIR normalized spectra of β-lg dispersions of different concentrations treated with 450 MPa of pressure. Averages of 3 samples; black 5 %, blue 10 %, green 15 %, and red 20 % concentration. Readings with variable pathlengh between samples was employed to equalize absorbances.

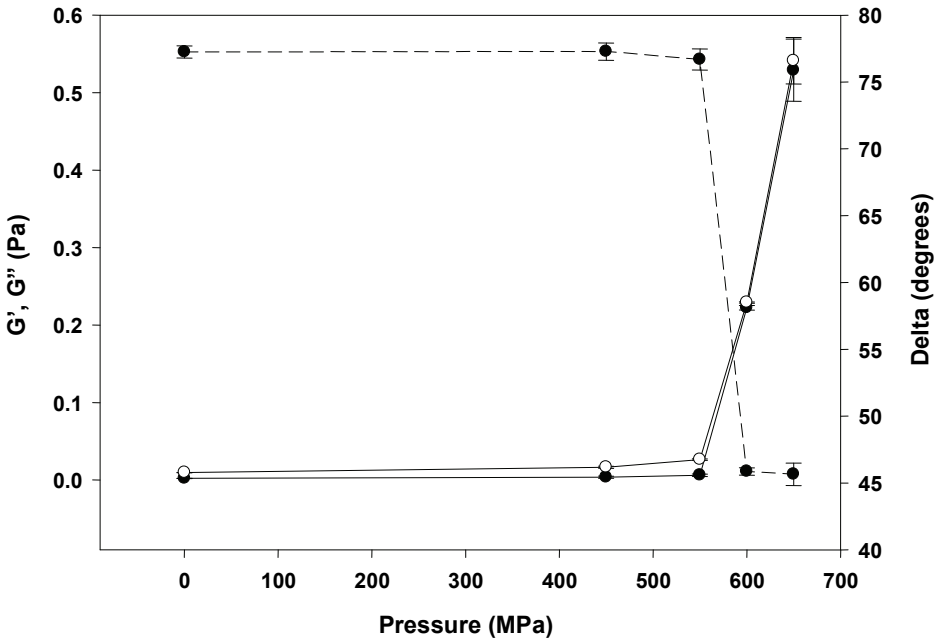

Figure 3.5 Rheological measurements of 15% β-lactoglobulin dispersions exposed to different pressure levels. Dashed line is the phase angle (delta); open circles (○) are the loss modulus (G"); and solid circles (●) are the storage modulus (G').

This decrease is indicative of the solution changing from a liquid-like consistency to a gel-like consistency. The formation of a true gel is still to be accomplished at these pressures and protein concentration levels. Similar findings were achieved by Fertsch, Muller & Hinrichs (2003).

Accordingly, the minimal changes in the molecular structure reflected in the infrared spectra of the samples are commensurate with absolute small changes in functionality, although there is a significant relative change in viscoelasticity. These findings are also in good agreement with our previous work using electrospray ionization mass spectrometry (ESI-MS) (Alvarez et al., 2007).

3.4.4 Model fitting

Non-linear regressions were performed to correlate the viscoelastic parameter storage modulus (G') of samples; and the FTIR spectroscopy secondary feature peak height at 1627 cm^{-1} (β-structure) to pressure treatment (Fig 3.6).

3.4.4.1 Exponential fit of viscoelastic parameters

Owing to the very low response of the elastic modulus (G') to pressures up to 450 MPa, these data points were excluded for model curve estimation. For the remaining dataset (500 to 650 MPa) the exponential equation $y = 2.17x10^{-10}e^{0.033x}$ being pressure the independent variable and G' the dependent variable, was found to have an R^2 of 0.860. The regression curve and actual data points for this correlation are shown in Fig.3.6a. ANOVA of the model's coefficients indicated a highly significant ($p<0.01$) estimation for both the constant and the variable coefficient, with very low standard errors (< 0.4 %).

Although the model seriously underestimates G' obtained after 600 MPa of pressure, it can still estimate the remaining 3 points reasonably well. Molecular interactions responsible for the sample's viscoelastic development seem to occur only when a certain pressure (entropy level) is reached, follow by an exponential increase.

3.4.4.2 Cubic fit of structural parameters

A cubic equation was developed for the estimation of β-structure formation (peak height at 1627 cm^{-1}) relative to increasing pressure treatment in the range of 0.1 to 650

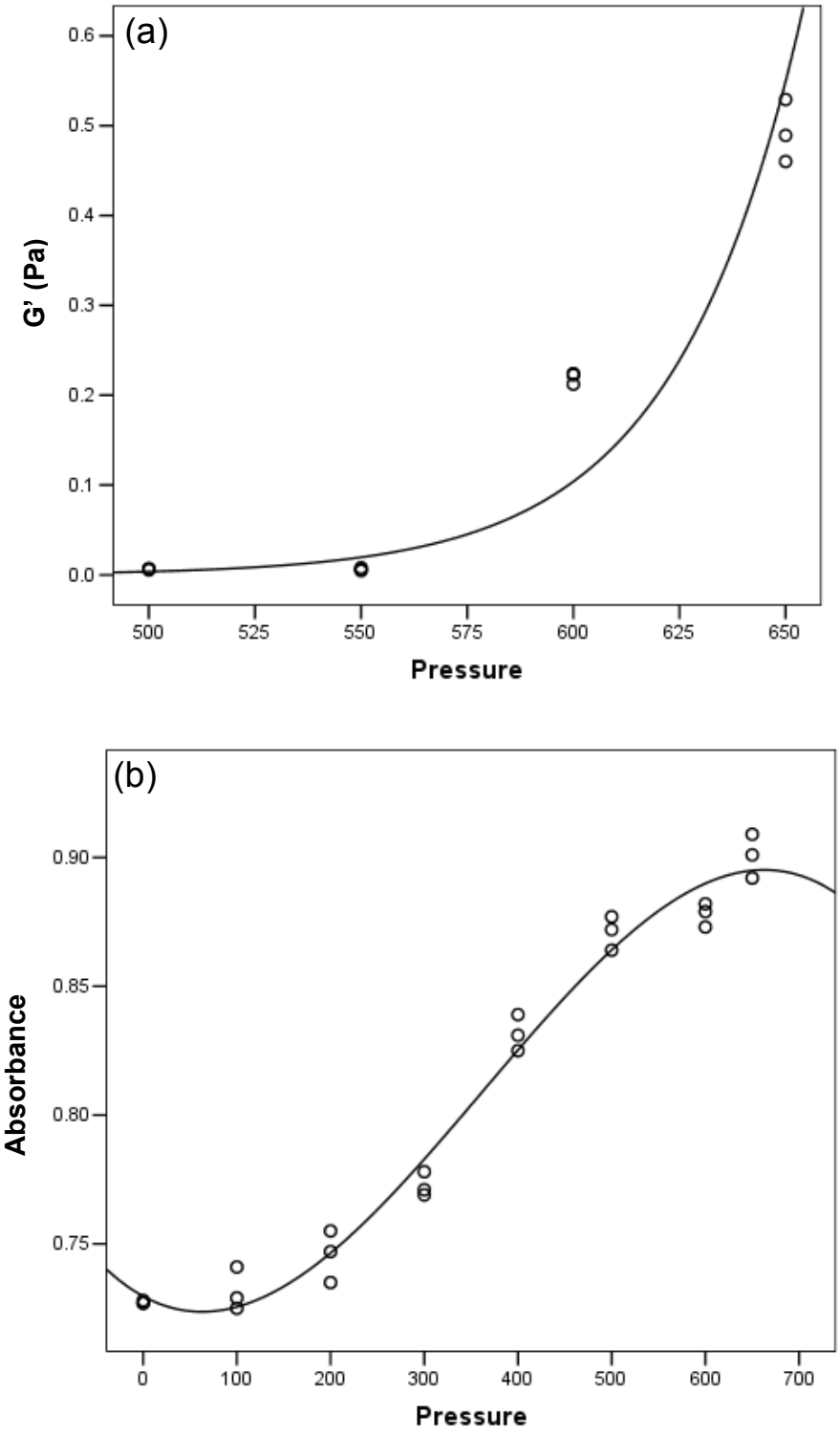

Figure 3.6 Curve fit for (a) elastic modulus G' and (b) peak height at 1627 cm^{-1} wavenumbers relative to pressure treatment level.

MPa. We obtained the cubic equation $y = -1.59x10^{-9}x^3 + 1.73x10^{-6}x^2 + 0.73$ which scored an R^2 of 0.981, with pressure as independent variable and peak height at 1627 cm^{-1} as the dependent variable. The regression curve and actual data points can be found in Fig. 3.6b. ANOVA of the model's coefficients show a highly significant ($p<0.01$) estimation for the constant and the cubic and quadratic coefficients; but only statistically significant ($p<0.05$) for the linear coefficient estimation, which was found to be zero. Standard errors for the estimated coefficients were lower than 0.6 %.

The β-structure pressure-induced formation followed classical behavior for protein structure alteration (e.g. de Jongh et al. 2001); where there is a slow formation of the secondary moiety in the lower range of the perturbation (pressure), followed by a rapid formation in the middle portion; and a saturation-like slowdown towards the higher end of the perturbation range. Likewise destruction of secondary features follow the inverse function described, as with the 1635 cm^{-1} band assigned to antiparallel β-sheets.

Correlation of β-structure formation to G' development gave a poor linear relationship with R^2 of 0.756. Therefore direct estimations of storage modulus using structural information seem not reliable, undermining the idea of using an online-coupled infrared detector to estimate real-time changes in viscoelastic parameters.

3.5 Conclusions

Within the range of experimental conditions used in this study, the rheological characteristics of 15 % β-lg dispersions were unaffected by pressures up to 550 MPa. At 600 MPa significant structural changes were observed and these were correlated to changes in the viscoelastic properties. A true gel was only formed after applying 650 MPa to a β-lg dispersion of 20 % concentration. The isostatic press delivered consistent pressure, as observed by the statistical analysis of FTIR spectroscopy measurements.

The HP-induced gels of 15 % β-lg did not follow the classic structural changes of thermal induced gel formation which involve extensive changes in the native secondary structure of the protein accompanied by the formation of anti-parallel intermolecular β-sheets (Boye et al., 1996). HP-induced gels result in a significant drop in the intensity of intramolecular β-sheet accompanied by an increase in α-helices and random coil

structures. Gel formation may result from self-association of the modified proteins with each other and the surrounding solvent.

Mathematical models fit the viscoelastic and structural information and serve to describe the behavior of the data, ultimately serving as a prediction tool for rheology or the degree of protein unfolding. The exponential model described reasonably well the higher end pressure treatments for prediction of storage modulus; and the cubic model fitted the protein structural data related to β-structure pressure-induced formation.

Connective statement to Chapter 4

After looking into the reproducibility of the pressure process and the rheological and structural changes of β-lg dispersions of up to 20 % concentration exposed to pressures of up to 650 MPa, it was decided to take on the task of producing firm protein gels by pressure and heat means, but possessing comparable viscoelastic properties. To achieve the required analogy on gels mechanical parameters, it was necessary to increase protein concentration and pressure level to match a heat process of 95 °C. From the structural data analyzed in Chapter 3, it was suspected larger changes occur at the protein's tertiary level; therefore Raman spectroscopy measurements were included in this study. The mechanism of gel network formation via pressure protein denaturation was still not fully evident, and some good observations were made from the destruction of the 1635 cm^{-1} band, but the full implications of the 1627 cm^{-1} band formation was still elusive, additional information was gathered in the following chapter which helped us complete the picture.

From the methodological point of view, studying protein conformation of hard gelled samples brings about many important challenges, e.g.: circular dichroism and fluorescence spectroscopy are not suitable since they both suffer from inner filter effect of opaque samples, this usually occurs at high protein concentration and/or when protein precipitation is present in the sample. On the other hand, FT-Raman spectroscopy measurements of liquid or semi-solid samples of β-lg exhibited a relatively low signal, which was overcomed by letting the sample air dry for some time before analysis. Infrared spectroscopy was the best suited alternative to study protein structure under these circumstances; its many different accessories allows for finding the appropriate tool of study (ATR-FTIR).

Based on results from Chapter 4, a mauscript has been prepared for publication: Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. 2008. Comparison of High Pressure and Heat Induced β-lactoglobulin Self-standing Gels by FTIR Spectroscopy and DMA. This research work was completed by the candidate under the supervision of Dr. H.S. Ramaswamy and Dr. A.A. Ismail.

Chapter 4. Comparison of High Pressure and Heat Induced β-lactoglobulin Self-standing Gels by FTIR Spectroscopy and DMA

4.1 Abstract

In this work the effect of 900 MPa HP and 95 °C on the secondary and tertiary structure of β-lactoglobulin (β-lg) was examined by Fourier transform infrared (FTIR) spectroscopy and Fourier transform Raman (FT-Raman) spectroscopy. Also the changes in mechanical properties of β-lg self-standing gels, as a function of increasing protein concentration and treatment holding time was investigated to better understand the molecular basis of gel formation (hard gels). Applying 900 MPa for the shortest holding time of 3.75 minutes to the β-lg dispersion of the lowest concentration (20 %) resulted in translucent self-standing gels with good cohesiveness and no apparent syneresis. Protein gels with strength of the same order of magnitude were produced by heating β-lg dispersions to 95 °C or applying 900 MPa pressure; the obtained heat-induced gels resulted 33 % stiffer than their pressure induced counterparts. Examination of the FTIR spectra in the amide I absorption region, of pressure induced gels and heat induced gels of β-lg were markedly different, even though the gel strengths were comparable. Thermally induced gels were formed through extensive formation of anti-parallel intermolecular β-sheets accompanied by a marked decrease in the native secondary structure of the protein. Pressure induced gels, on the other hand, were formed with minimal amount of intermolecular β-sheet, even though the native secondary structure of the protein was also largely destroyed. Extensive changes at the protein tertiary level were observed for the pressure induced gels. The mechanism of pressure induced gelation of protein may proceed through hydrophobic interactions between solvent exposed outer domains of the proteins with each other.

4.2 Introduction

Many food products consist of a complex three-dimensional network of several biopolymers. The microstructure influences the consistency of the food material, and it is important to know how to control the textural properties of the final product. The network of the gel makes the system stay together, but the role of the other ingredients in the

system has to be elucidated as well. In the case of protein rich foods, texture will be largely determined by the many parameters affecting the protein fraction of the product i.e. protein concentration, pH, ionic strength, temperature, etc. Conversely, reports indicate that high pressure pre-process does not improve the thermal gelation of relatively low (5 %) concentration β-lactoglobulin dispersions (Olsen, Ipsen, Otte & Skibsted, 1999) or plasma proteins at 7 % concentration (Parés, Saguer, Toldrá & Carretero, 2000).

As previously documented, applied hydrostatic pressure causes partial protein denaturation and, thus, high-pressure processing can provide a means of altering the functional properties of whey proteins (López-Fandiño, 2006). The relationship between pressure-induced structural changes that lead to gelation is still a matter of considerable investigations.

In this work the effect of high pressure treatment (900 MPa) on the secondary and tertiary structure of β-lactoglobulin (β-lg) at high concentration (≥ 20 %) was examined by Fourier transform infrared (FTIR) and Fourier transform Raman (FT-Raman) spectroscopic techniques; and compared to thermal processing (95 °C). The changes in mechanical properties of β-lg (self-standing) gels, as a function of increasing protein concentration and treatment holding time was investigated using a dynamic mechanical analyzer (DMA) to better understand the molecular basis of gel formation (hard gels).

4.3 Materials and Methods

The protein used in this work was pure β-lg, obtained from Davisco Foods International (Eden Prairie, MN, USA) and used without further purification. Electrospray ionization mass spectrometry analysis showed a pure mix of A and B genetic variants of β-lg with no other protein component (Alvarez, Ramaswamy & Ismail, 2007). For the composition of the β-lg sample, as per information provided by the suppliers was previously shown in Table 3.1. Deuterium oxide (D_2O 99.9 % D) and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.3.1 High pressure treatments

All the HP treatments were given in triplicates in a high-pressure isostatic press (ACIP9000, ACB High Pressure Systems, Nantes, France), with a chamber volume of 3

L. The pressure transfer medium used was glycerine. The maximum operational pressure of 900 MPa was reached in approximately 2 min; the depressurization time was approximately 15 s. Samples were introduced into the high-pressure machine at 10 °C and reached a maximum of 35 °C during pressurization owing to adiabatic heating. This rise in temperature can be considered to have a negligible thermal effect because thermal denaturation of the whey proteins occurs well above ambient temperature (Boye et al., 1995; Boye, Ismail & Alli, 1996). Samples were treated at 900 MPa with holding times of 3.75, 7.5, 15 and 30 min; and concentrations of 20, 30 and 40 % (w/v).

4.3.2 Heat treatments

Samples were prepared in triplicates using protein concentrations of 20, 30 and 40 % (w/v), and exposed to 95 °C for geometrically increasing times of 3.75, 7.5, 15 and 30 min, using a computer controlled Haake DC10-P5/U circulating water bath (Thermo Fisher Scientific,Waltham, MA, USA). Immediately after treatment, each sample was allowed to cool down for 5 minutes by submersion in a room-temperature water bath prior to subsequent analysis.

4.3.3 Sample preparation for FTIR spectroscopic analysis

Solutions of β-lg were prepared (20, 30 and 40 % w/v) in D_2O with 2 mg/ml sodium azide as preservative; and left to stand for 96 hours at room temperature to allow H-D exchange. Deuterium oxide is necessary to avoid the strong absorbance of water in the same region as amide I, therefore obscuring the results. 500 μL aliquots were subsequently sealed in plastic bags for HP or heat treatment. The FTIR spectrum of each sample was immediately recorded after treatment using a Bio-Rad FTS 3000MX FTIR spectrometer (Varian Scientific, Palo Alto, CA, USA) equipped with a deuterated triglycine sulphate (DTGS) detector and fitted with a Golden Gate diamond IRE ATR accessory. The spectrometer was continuously purged with dry air from a Balston dryer (Balston Products, Haverhill, MA, USA).

Thin slices of the protein gels were pressed against the ATR accessory crystal to collect a total of 512 scans at 4 cm^{-1} resolution. The absorbance spectra were subjected to

band narrowing techniques using Fourier self deconvolution (FSD) employing a bandwidth of 20 cm^{-1} (w) and enhancement factor of 2.4 (k) followed by a two-point baseline correction starting at 1710 and ending at 1590 cm^{-1} and normalization using Omnic 6.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

4.3.4 Sample preparation for FT-Raman spectroscopic analysis

Gels formed from the protein samples were allowed to air-dry for 4 days before FT-Raman spectral acquisition, to improve the sample signal. FT-Raman spectra were collected using a Varian Excalibur FT-IR spectrometer coupled with an FT-Raman module (Varian Scientific, Palo Alto, CA, USA), using a 1064 nm Nd:Yag laser (near-infrared). Incident laser power was limited to approximately 750 mW to avoid sample degradation. A total 512 co-added scans at 4 cm^{-1} spectral resolution were recorded for each sample. Spectra were normalized using the intensity of the 1005 cm^{-1} band (phenylalanine ring breathe) which is insensitive to changes in molecular structure (Li-Chan, 1996).

4.3.5 Sample preparation for dynamic mechanical analysis (DMA)

Solutions of β-lg protein were prepared as above using H_2O in place of D_2O, and pH of the protein dispersion was 6.9. Aliquots of approximately 3 mL were sealed in 5 mm diameter plastic straws for HP (900 MPa) and heat (95 °C) treatments. The resulting self-standing protein gels were cut to 60 mm which allowed for consistently clamping to the instrument's geometry for gel strength determination. Mechanical parameters measurements were evaluated using a Q-800 DMA (TA Instruments, New Castle, DE, USA), employing dual cantilever bending clamp, constant deformation frequency of 1 Hz (0.6284 rad/sec), 100 μm amplitude and temperature controlled at 20 °C.

4.3.6 Statistical analysis

Two-way ANOVA studies were carried out with protein concentration and processing holding time as factors and its effects on the mechanical gel properties as well as on the secondary structure of β-lg gels were determined. The statistical analyses were carried out using SPSS 12.0 for Windows (SPSS Inc, Chicago, IL, USA). In all cases, data were subjected to ANOVA using the general linear model procedure (Proc GLM).

Post hoc pairwise Ryan-Einot-Gabriel-Welsch multiple *F* (REGWF) test was used to compare between groups means. The significance level for all tests was α=0.05.

4.4 Results and Discussion

4.4.1 Mechanical analysis

DMA measures the magnitude and phase of the displacement of a sample in response to an applied oscillatory force. The storage modulus (E') and loss modulus (E") measure the elastic and viscous response of the sample respectively, in this sense DMA is analogous to dynamic rheology and their results could be compared in a general manner. Our previous work with β-lactoglobulin (β-lg) concentrations up to 20 % and pressures up to 650 MPa (0.1 min holding) resulted in soft gels with elastic and viscous moduli around 0.65 Pa (Chapter 3). By contrast, in this work, applying 900 MPa for the shortest holding time of 3.75 min to the β-lg dispersion of the lowest concentration (20 %) resulted in translucent self-standing gels with good cohesiveness and no apparent syneresis.

Heat induced β-lg gels (Fig. 4.1) showed slightly higher mechanical parameters which reached close to 20 million Pa for E'; compared to their pressure induced counterparts which attained almost 15 million Pa for the same modulus E' (Fig. 4.2). A T-test performed between these groups showed this difference to be statistically significant ($p<0.01$).

In the case of β-lg thermal gelation, mechanical parameters adjusted very well to the linear response model with R^2=0.983 for elastic modulus, R^2=0.992 for viscous modulus and R^2=0.999 for gel strength (stiffness). The protein concentration played a very important role in determining the gel stiffness and the related elastic and viscous moduli, for all these cases. The results from the REGWF test indicated a very significant difference between protein concentration levels, with a probability of 1 (α=0.05) for the means of the three groups been different. Protein concentration variation resulted in a significantly higher response than that of the process holding time variation (Fig 4.1). For E', the REGWF test revealed the means for holding times 3.75 and 7.5 min to be not significantly different as well as for the means for 15 and 30 min holding time (Fig 4.1a).

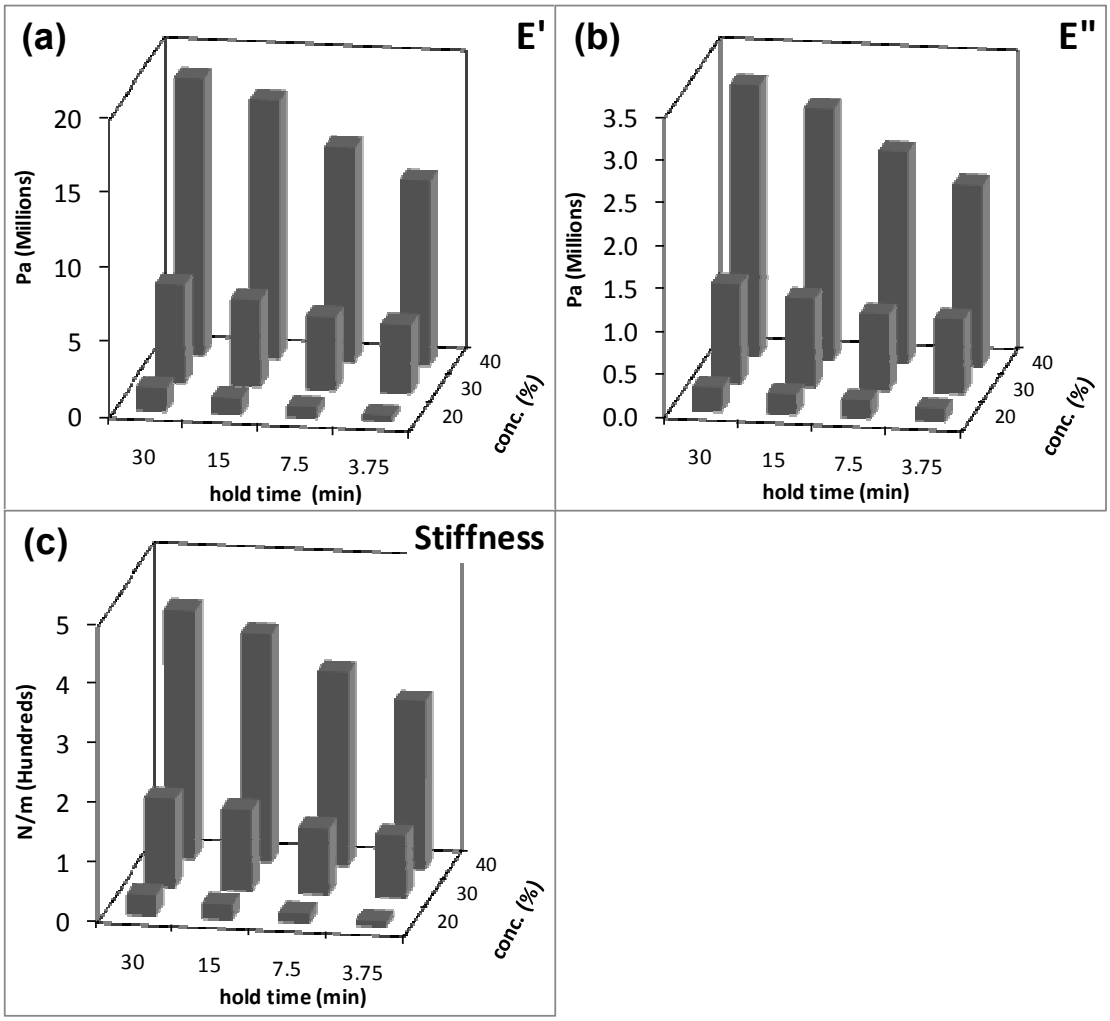

Figure 4.1 Mechanical parameters of heat induced (95 °C) β-lactoglobulin gels prepared from dispersions of different concentrations (20, 30, and 40 %) using different holding times: 3.75, 7.5, 15 and 30 min. (a) Storage modulus (E'). (b) Loss modulus (E"). (c) Stiffness. Bars are averages of 3 replicates per experimental condition.

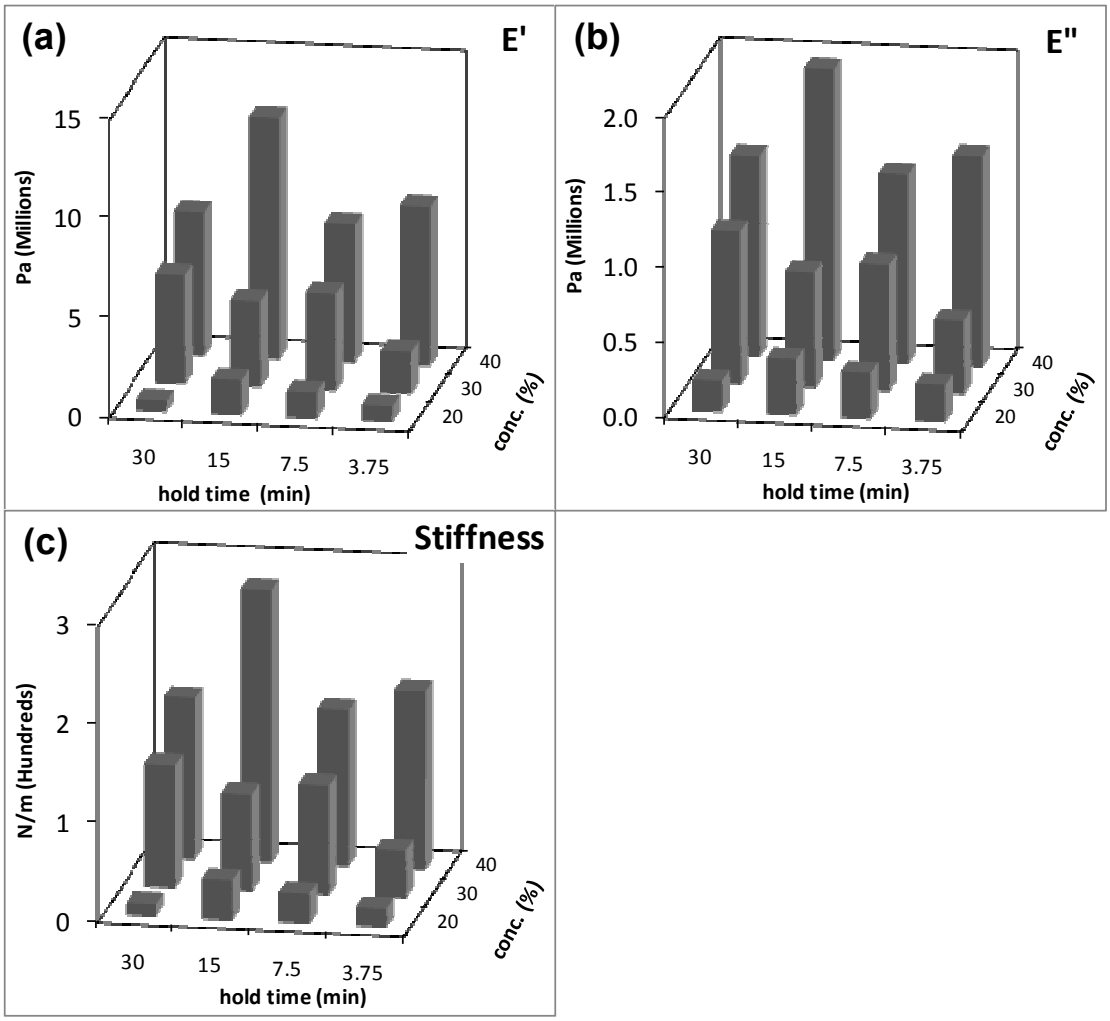

Figure 4.2 Mechanical parameters of pressure induced (900 MPa) β-lactoglobulin gels prepared from dispersions of different concentrations (20, 30, and 40 %) using different holding times: 3.75, 7.5, 15 and 30 min. (a) Storage modulus (E'). (b) Loss modulus (E"). (c) Stiffness. Bars are averages of 3 replicates per experimental condition.

On the other hand, REGWF test for E" and gel stiffness showed all holding times to be statistically different (Fig 4.1b and 4.1c).

Mechanical parameters of pressure induced β-lg gels (Fig. 4.2) were considerably less affected by changes in protein concentration when compared to the heat induced gels, although it also resulted in statistically different means as reflected by the REGWF test of the pressure induced gels data for the three different concentration levels. Generally, in both heat and pressure induced gels, the longer holding time of the treatment resulted in stiffer, more elastic and viscous gels. However, an interesting phenomenon occurred at 15 min holding for 900 MPa pressure and this resulted in a significantly higher response for E', E" and gel stiffness maybe related to molecular rearrangement to the optimal level (time modulated) that resulted in augmented gels' mechanical parameters. The REGWF test for different pressure holding times resulted in no statistical difference between 7.5 and 30 min holding for elastic and viscous moduli, as well as, gel stiffness. Results for heat induced gelation with stiffness reaching close to 500 N/m are comparable to Rejaei, Ismail, Ramaswamy & Jézéquel (1996); although, pressure induced gelation produced gels to a maximum close to 300 N/m for the tested conditions, these results are comparable to those of Kanno, Mu, Hagiwara, Ametani & Azuma (1998) for whey protein concentrate.

Mechanical property evaluations were also made for heavy water (D_2O) prepared β-lg dispersions for identical concentrations of that of the water dispersed counterparts, in order to establish the role of the deuterium atom present in the heavy water molecule. Results indicated similar mechanical parameters for both pressure and heat induced protein gels (data not shown). Therefore the strength of the hydrogen bonding altered by the presence of the heavier deuterium atom does not seem to play an important role in determining the viscoelastic properties of the resulting gels, regardless of gelation mechanism.

4.4.2 FTIR spectroscopy

Thermal denaturation of β-lg has been described using infrared spectroscopy by two prominent peaks at around 1684 and 1617 cm^{-1}, these peaks has been assigned to intermolecular antiparallel β-sheets and commonly utilized as measurements for degree of protein denaturation, aggregation or gelation (Allain, Paquin & Subirade, 1999; Boye et

al., 1996; Dong, Matsuura, Manning & Carpenter, 1998; Hong and Creamer, 2002; Hosseini-Nia, Ismail & Kubow, 1999; Lefevre & Subirade, 2000; Lefevre & Subirade, 2001; Panick, Malessa & Winter, 1999; Spilimbergo, Elvassore & Bertucco, 2002; Susi & Byler, 1988). Even some mathematical correlation through partial least squares (PLS) was found between the 1617 cm^{-1} band intensity and thermally induced gel strength (Rejaei et al., 1996). These same bands were found for the thermal gelation (95 °C) of β-lg at different concentration levels (20, 30 and 40 %) and holding times (3.75, 7.5, 15 and 30 min).

A different scenario was found for the pressure gelation (900 MPa) of β-lg. The typical intermolecular antiparallel β-sheets peaks found by protein thermal denaturation resulted shifted to 1626 and 1677 cm^{-1} for the HP induced gelation of β-lg. The higher wavenumber (frequency) of the low-frequency integrant is indicative of stronger hydrogen bonding, because of the increased energy required for the molecular vibration to occur, when compared with the thermal mechanism; but this observation is not sustained by increased gel strength. The FTIR band at 1626 cm^{-1} was already observed by our group for the HP denaturation of β-lg at 15 % concentration and maximum pressure of 650 MPa; an increasing band at 1627 cm^{-1} (well within the 4 wavenumber resolution of the method) was observed, but we were unable to properly assigned it at that time due to the extensive overlapping specially with the decreasing band at 1635 cm^{-1}. In this case all the pressure treated (gelled) samples exhibited the conspicuous two component intermolecular β-sheet formation spectra (Fig. 4.3). We observed no gradual appearance of the bands with these experimental conditions which contrast with the steady increase of the 1627 cm^{-1} band in our previous work under different conditions (Chapter 3).

The percentage of change of peak height at 1617 cm^{-1} (thermal aggregation) relative to the corresponding β-lg control solution for all the thermally gelled protein samples is shown in Figure 4.4a. The REGWF test by protein concentration showed significant differences (α=0.05) for the means of the three groups, and also significant differences were found for the means of the four groups by process holding time. We observed an increase of the thermal aggregation band with the increase in process holding time, which in turn relates to gain in gel strength; but when the data was observed in

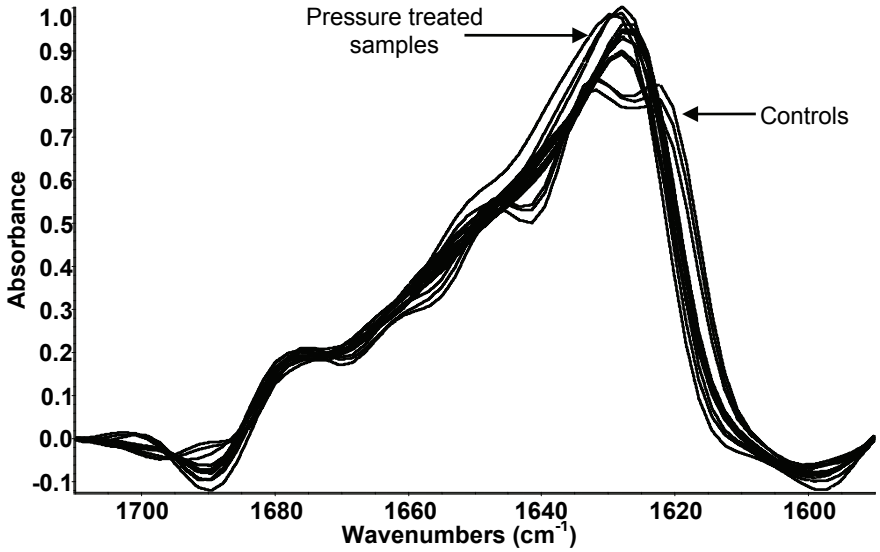

Figure 4.3 Controls and pressure treated (900 MPa) β-lg samples at different concentration levels.

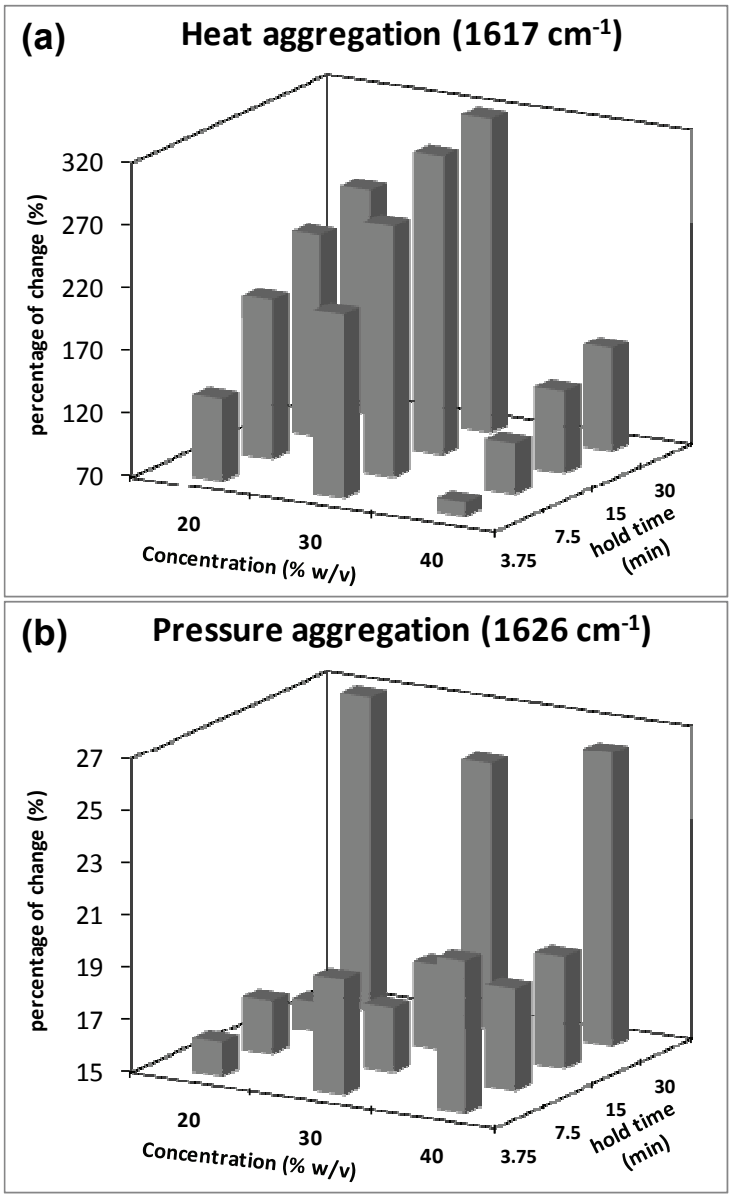

Figure 4.4 Protein aggregation as measured from FTIR spectra of β-lactoglobulin after heat and pressure induced gelation. (a) Percentage of change of the peak height at 1617 cm^{-1} compared to each corresponding control per concentration level of the heat induced (95 °C) β-lg gels. Samples were treated at different concentrations and holding times; bars are averages of 3 replicates. (b) Averages (n=3) peak heights at 1626 cm^{-1} percentage of change for the different pressure (900 MPa) treated β-lg samples.

terms of increasing protein concentration we noticed an increase in aggregation as of 1617 cm^{-1} peak height between 20 and 30 % concentration, but a large decrease for 40 % β-lg concentration. This pattern relates partially to gel strength, but the 40 % series deviates from it; of the possible causes of this behavior we discarded a difference in heat transfer coefficient among the three different protein concentration samples, although there must be a difference (untested) due to the variation in heat capacity of water and protein, the samples were exposed to a big thermal difference and the cross section of the mold used to create the gels was sufficiently small (5 mm dia) to make heat transfer differences negligible. The more plausible thesis appears to be the reduced presence of water in the 40 % protein sample which accounted for a diminished hydrogen bonding, although this idea does not eliminate the possibility of a combined effect of reduced heat transfer and lessened H-bonding at high protein concentration.

A different situation was found for the pressure induced gelation of β-lg (Fig. 4.4b), the percentage of change is dramatically less by pressure than by heat denaturation as confirmed by a T-test with $p<0.01$. This difference may be due to the mechanism of gel formation, we suggest that (as previously proposed by many authors) under heat denaturation extensive intermolecular H-bonding needs to occur in order to create the three-dimensional association necessary for network stabilization; whereas, under pressure denaturation both intermolecular β-sheet formation and hydrophobic interactions enhanced by changes in the tertiary structure of the protein, coordinate to generate the three-dimensional gel network, this thesis is proposed for the first time in this study. In this regard, we observed an increase in the 1626 cm^{-1} band with increasing concentration, but the REGWF test proved only 40 % concentration to be statistically different, with no significant difference between the 20 and 30 % concentration means. When the means of the different pressure holding times were tested, only 30 minutes resulted significantly different to the rest of the groups.

4.4.3 FT-Raman spectroscopy

The FT-Raman spectra of all the samples were collected in order to gather information of changes in tertiary structure of the β-lg molecule, the amount of sulfhydryl residues and disulphide bonds. This information is typically obscured in FTIR

spectroscopic analysis and even when the bands can be observed, they are of a very low intensity thus reducing sensitivity considerably.

As characterized by Li-Chan (1996) the ratio of intensities of the Raman spectroscopy bands at 850 and 830 cm^{-1} (I_{850}/I_{830}) gives an indication of the chemical microenvironment the tyrosine residues (as an average) in the protein are exposed to, in this sense, a I_{850}/I_{830} ratio around 1 implies that the tyrosine residues are exposed to a polar environment; a ratio below 0.5 entails the tyrosine residues acting as hydrogen bond donors; and a ratio above 2.5 means that the tyrosine residues are acting as H-bond acceptors. The ratio of Raman intensities between 1360 and 1340 cm^{-1} (I_{1360}/I_{1340}) gives a suggestion of the chemical microenvironment of the tryptophan residues in the protein molecule, therefore, a ratio lower than 1 means that the tryptophan molecules are H-bonding in a hydrophilic environment; and a I_{1360}/I_{1340} ratio higher than 1 means that the tryptophan residues are H-bonding in a hydrophobic milieu (Li-Chan, 1996).

From information collected by X-ray diffraction, out of the 162 amino acid residues in the β-lg sequence, there are a couple of disulphide bonds $Cys_{66\text{-}160}$ and $Cys_{106\text{-}119}$, plus a free thiol Cys_{121}. Also, there are tyrosine $Tyr_{20,42,99,102}$ and tryptophan $Trp_{19,61}$ (Qin, Bewley, Creamer, Baker, Baker & Jameson, 1998).

The structural information derived from FT-Raman spectra of heat induced β-lg gels is presented in Figure 4.5. The tyrosine residues in heat gelled β-lg were exposed to a polar environment as the ratio I_{850}/I_{830} around 1 suggests, this situation is standard considering that the solvent used was water. Experimental FT-Raman results for the lyophilized control β-lg sample were not included in the graphs to avoid confusion; in the case of Tyr the ratio I_{850}/I_{830} the control averaged a ratio of 1.03, very close to the rest of the averages for the lower concentration level. The different holding times did not account for changes in the microenvironment of Tyr for the 20 % concentration level. Moving up in concentration we found some drop in the ratio, not enough to be considered as Tyr acting as H-bond donor, but maybe indicative of a reduced H-bonding with the more scarce solvent. The presence of many Tyr residues per molecule probably accounted for a more averaged (buffered) response to heating times, due to some chromophores migrating to hydrophobic regions while others moving towards exposed situations and cancelling out the net effect (Fig 4.5a).

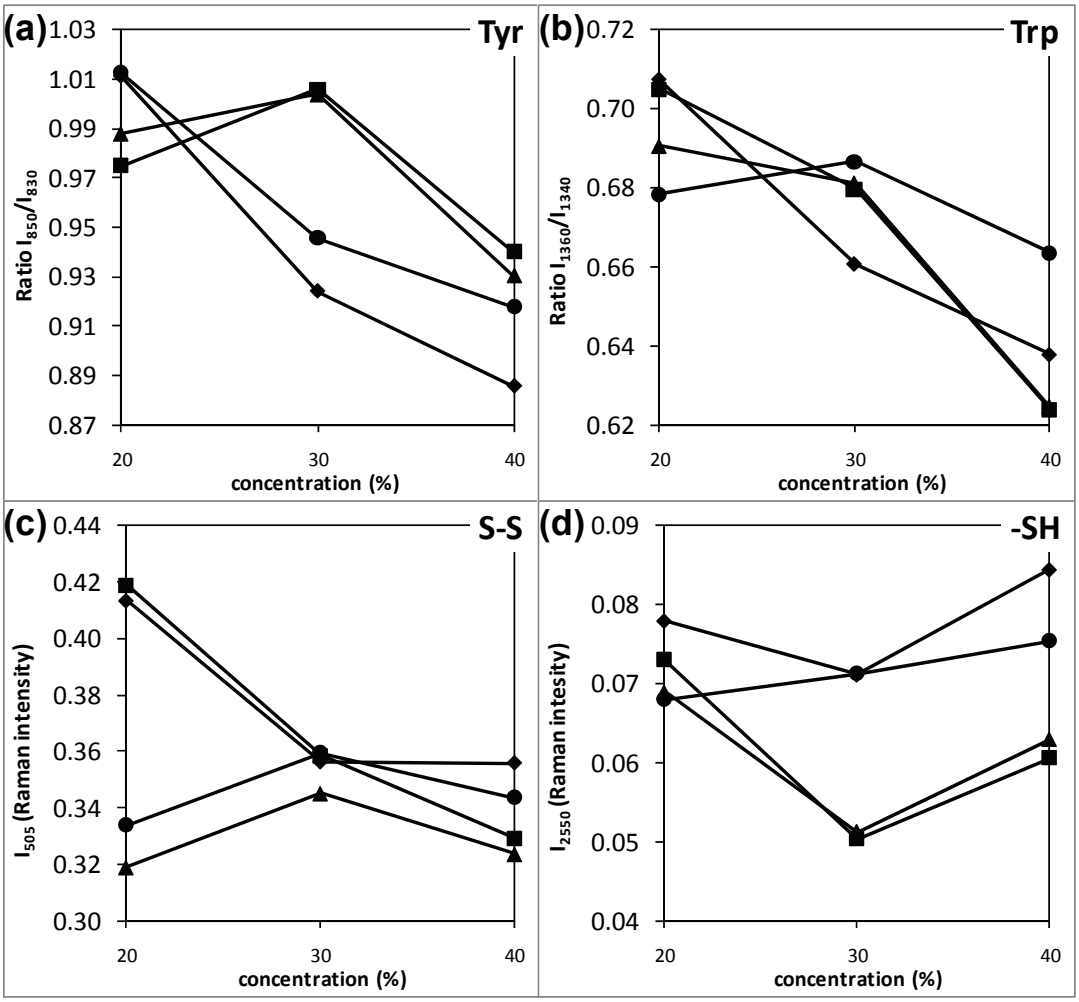

Figure 4.5 Tertiary structure information derived from FT-Raman spectra of heat induced (95 °C) β-lactoglobulin gels (20, 30 and 40 % protein) subjected to different holding times (◆ 3.75, ■ 7.5, ▲ 15, and ● 30 min). Data points are averages of 3 replicates. (a) Tyrosine chemical microenvironment as reflected by the ratio I_{850}/I_{830} from FT-Raman spectra of different heat induced β-lg gels. (b) Tryptophan chemical microenvironment as reflected by the ratio I_{1360}/I_{1340} from the FT-Raman spectra. (c) Cystine abundance (I_{505}). (d) Thiol quantity (I_{2550}).

On the contrary, for the fewer tryptophan residues (Fig 4.5b) the response to heat was clearer. From the crystallographic data both tryptophan residues appear to be allocated into exposed regions of the molecule, and this was corroborated with the I_{1360}/I_{1340} ratio lower than 1, which is indicative of tryptophan residues H-bonding in a hydrophilic environment, the average ratio for the control sample was 0.63 and no significant difference was found among groups. Figure 4.5c shows the intensity of the 505 cm^{-1} peak, direct measurement of cystine, for the thermally gelled β-lg samples. The control averaged 0.36 of Raman intensity (arbitrary units) for this band, and all heating times for the 20 % concentration samples grouped with this value. Only the longest heating at the highest concentration level showed an increase in cystine which is in agreement with previously proposed mechanism of heat gel formation (Monahan, German & Kinsella, 1995; Alting, Hamer, deKruif, Paques & Visschers, 2003). Finally, we measured the intensity of the 2550 cm^{-1} peak, indicative of thiol residues (Fig 4.5d); the control sample measured 0.075 Raman intensity which remained unchanged after heating at 20 % concentration. At 30 % concentration there is no change, and the drop observed at 40 % concentration and longer heating, correlates with the formation of new disulfide bonds.

Figure 4.6 shows the structural information extracted from the FT-Raman spectra of pressure induced β-lg gels. The tyrosine probe ratio I_{850}/I_{830} for the pressure treated samples followed a similar behavior compared to the heat induced gels; all samples indicate Tyr residues exposed to a polar environment, but not significant differences among samples (Fig 4.6a). The tryptophan ratio (Fig 4.6b) shows no statistical difference between the control and the pressure treated samples at 20 and 30 % concentration, but a significant difference for 15 minutes pressure at 40 % concentration, which correlates with increased gel stiffness and mechanical parameters. The disulphide bond abundance (intensity at the 505 cm^{-1} peak) did not change significantly with pressure holding, or concentration (Fig 4.6c). Finally, the sulfhydryl content of pressure treated samples (2550 cm^{-1} intensity) only increased significantly for the highest concentration level and holding time, but since high hydrostatic pressure can not disrupt covalent bonds as to free up cysteines already compromised in disulphide bonds (Gross & Jaenicke, 1994); a feasible explanation for this observation is that the free thiol residue already in a hydrophobic environment (Cys_{121}) is getting buried deeper into the protein and exposed to a more hydrophobic surrounding, this effect would account for an increased molar extinction coefficient as described by Koziński, Garrett-Roe & Hamm (2008), which explains the higher value at extreme conditions (Fig 4.6d).

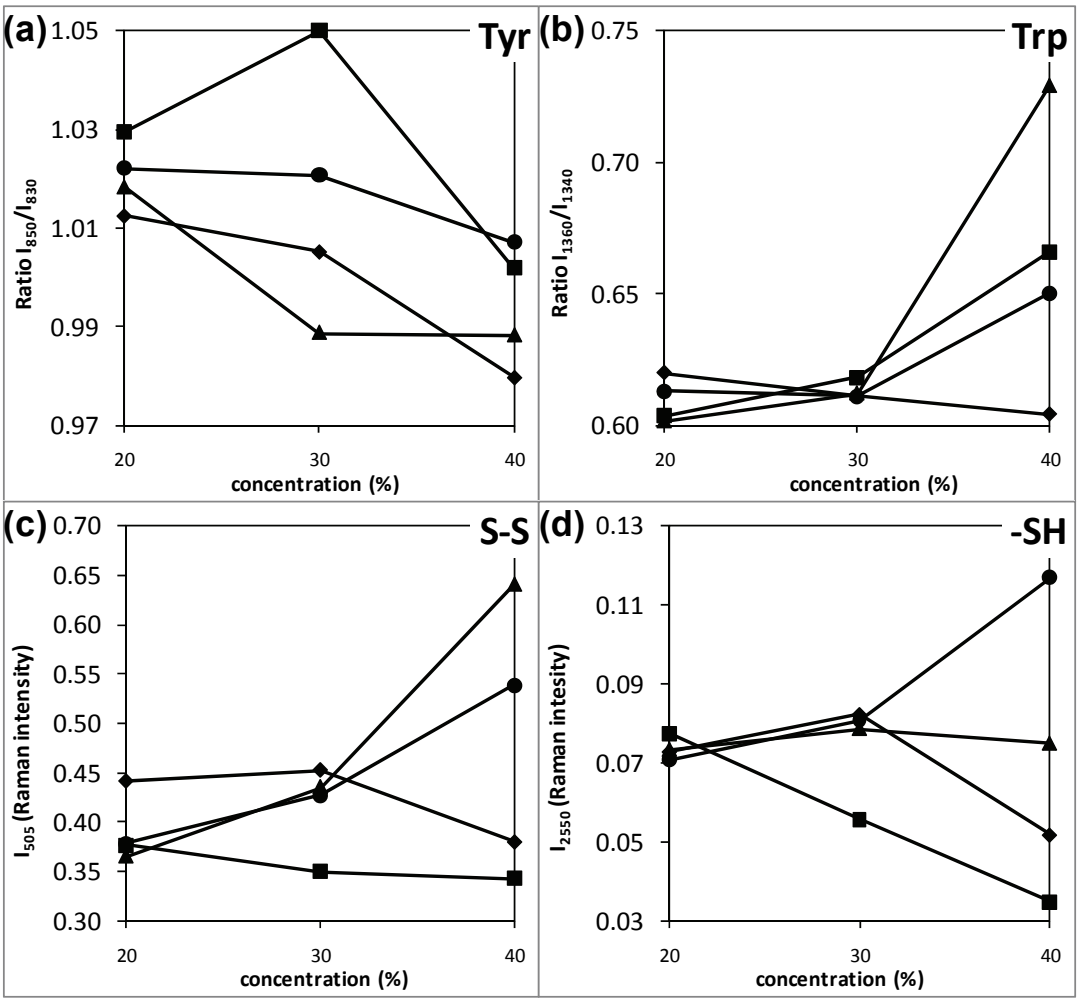

Figure 4.6 Tertiary structure information derived from FT-Raman spectra of pressure induced (900 MPa) β-lactoglobulin gels subjected to different holding times (◆ 3.75, ■ 7.5, ▲ 15, and ● 30 min). Data points are averages of 3 replicates. (a) Tyrosine chemical microenvironment as reflected by the ratio I_{850}/I_{830} from FT-Raman spectra of different heat induced β-lg gels. (b) Tryptophan chemical microenvironment as reflected by the ratio I_{1360}/I_{1340} from the FT-Raman spectra. (c) Cystine abundance (I_{505}). (d) Thiol amount (I_{2550}).

The change in tryptophan and thiol chemical microenvironment indicate a amino acid migrations with modification of the protein tertiary structure; our previous work using electrospray ionization mass spectrometry (ESI-MS) (Alvarez et al., 2007) showed also a high degree of tertiary structure changes for the pressure treated β-lg samples. It appears that the formation of intermolecular β-sheets is not the main mechanism of pressure induced protein gel formation, at least immediately after pressure treatment; there is a possibility that these changes in tertiary structure would slowly evolve to organize as intermolecular β-sheets, in particular when an important amount of intermolecular β-sheets was only formed with a 30 minutes pressure holding.

4.5 Conclusions

Protein gels with strength of the same order of magnitude were produced by heating β-lg dispersions to 95 °C or applying 900 MPa pressure; the obtained heat induced gels resulted 33 % stiffer than their pressure induced counterparts. There was no previous indication to which temperature could be comparable to which pressure level as to produce the gels of the same mechanical properties. In fact, these comparable results are only valid for elevated protein concentration, higher than 20 %.

The pressure induced gels of 20 and 30 % β-lg did not follow the classic secondary structure changes of thermal induced gel mechanism which involve the creation of extensive anti-parallel intermolecular β-sheets in expense of other native features (Boye et al., 1996). Pressure induced gels resulted in only a modest generation of intermolecular β-sheets albeit a broad destruction of native structures.

For the heat induced gelation, the change in protein secondary structure as to the formation of intermolecular β-sheets in conjunction with formation of additional intermolecular disulfide bonds, appears to explain the development of the three-dimensional gel structure probed by the gel stiffness and viscoelastic parameters. Conversely, for the pressure induced gel formation the protein secondary structure changes (emergence of intermolecular β-sheets) does not completely explain the creation of the gel scaffold explained by an increased stiffness and mechanical parameters; but the augmented hydrophobic interactions due to internal molecular rearrangements which created different electronic densities in the molecule, should also be taken into account.

Connective statement to Chapter 5

The model system of a single protein dispersion (pure β-lg) was studied in the previous two chapters and in this chapter it has been, expanded into a more complex system. The protein matrix from porcine blood plasma was selected for this study. Plasma contains a complex mix of several proteins, but with two major fractions: serum albumin (60 %) and globulins (36 %). These two proteins differ on their thermal stability, as well as their secondary structure, the second property easily monitored by decomposing an infrared spectrum of whole plasma into the components albumin and globulins. Plasma proteins served as a good model for protein-protein interactions that can play a very important role in protein denaturation pathway and gel network formation.

On the other hand, porcine blood from which plasma is obtained after removal of the cellular fraction by centrifugation is treated as a waste of the slaughter process in many countries around the world. Besides the environmental impact of this practice, this protein rich fluid is discarded despite its good functional properties among which is its excellent gelling and thickening abilities.

Part of the results from this study has been presented in a scientific conference: Alvarez, P.A., Saguer, E., Ramaswamy, H.S., Sedman, J. & Ismail, A.A. July 2008. Heat-induced gel formation of plasma proteins: New insights by FTIR 2D correlation spectroscopy. IFT conference 2008, New Orleans, LA. Based on results from Chapter 5, a mauscript has been prepared for publication: Alvarez, P.A., Saguer, E., Sedman, J., Ramaswamy, H.S. & Ismail, A.A. 2008. Heat and pressure induced gel formation of plasma proteins: new insights by FTIR 2D correlation spectroscopy. This research work was completed by the candidate under the supervision of Dr. H.S. Ramaswamy and Dr. A.A. Ismail; Dr. E. Saguer provided the plasma proteins, helped in the acquisition of thermal data and provided valuable discussions and input; Dr. J. Sedman provided editorial support.

Chapter 5. Heat and pressure induced gel formation of plasma proteins: new insights by FTIR 2D correlation spectroscopy

5.1 Abstract

Generalized 2D correlation spectroscopy (2D COS) has been applied to FTIR spectra of porcine plasma proteins to elucidate the sequence of events leading to pH and/or thermal-induced protein unfolding and aggregate formation; additionally, structural changes of plasma proteins were studied after high pressure (HP) treatments. Changes in the amide I' region of the infrared spectra (in the pH range between 7.5 and 4.5, at 0.5 pH intervals) at 30 ºC were especially evident as the pH approached the p*I* of serum albumin (4.8). The serum albumin fraction in the plasma proteins was found to undergo pH-induced aggregate formation prior to the globulin fraction. The effect of increasing temperature (from 30 ºC to 90 ºC, each 5 ºC interval) at selected pH on the secondary structure of the plasma proteins revealed that a decrease in α-helical structures is accompanied by an increase in unordered structures which associate to form intermolecular β-sheet. Further increase in temperature and a decrease in the pH of the protein solution resulted in destabilization of the intramolecular β-sheet with subsequent increase in aggregate formation. HP treatments up to 900 MPa (5 min) was found to induce a number of structural changes to plasma proteins (7 % conc), namely an increase in ratio α-helices (1653 cm^{-1}) to intramolecular β-sheets (1638 cm^{-1}); and a raise of intensity of the peaks at 1683 and 1618 cm^{-1} assigned to intermolecular β-sheets indicative of protein aggregation.

5.2 Introduction

Heat-induced gelation of proteins is considered to occur via complex multi-step events; while high pressure (HP) induced denaturation is still under study. Both the shape and the size of the protein are only mildly altered during heating (Matsumoto & Inoue, 1991; Tobitany & Ross-Murphy, 1997; Clark, Kavanagh & Ross-Murphy, 2001; Baier & McClements, 2005), but the secondary structure is often disrupted (Clark, Saunderson & Suggett, 1981; Bouraoui, Nakai & Li-chan, 1997; Qi, Holt, McNulty, Clarke, Bownlow & Jones, 1997; Belloque & Smith, 1998; Clark et al., 2001). The way in which each protein

unfolds during heating is unique and is affected by environmental factors such as pH, temperature and time, among others. The extent of protein aggregation during subsequent network formation affects the rheological properties of the gel (Tobitani & Ross-Murphy, 1997). A means of studying these events in order to gain an improved understanding of heat-induced gelation processes is provided by FTIR spectroscopy owing to the sensitivity of the infrared spectra of proteins to changes in secondary structure and protein aggregation (Byler & Susi, 1986; Ismail, Mantsch & Wong, 1992; van Stokkum, Linsdell, Hadden, Haris, Capman & Bloemendal, 1995). An even better understanding may be achieved through the application of generalized two-dimensional correlation spectroscopy (2D COS) (Noda, 1993) in conjunction with FTIR spectroscopy. Because 2D COS analysis spreads the spectral data over a second dimension (e.g., increasing temperature or heating time), spectral changes not readily observable by conventional one-dimensional (1D) FTIR spectroscopy may be clearly discerned. Furthermore, the synchronous and asynchronous 2D correlation plots generated by 2D COS analysis of FTIR spectra recorded as a function of temperature or heating time provide a means of investigating the sequence of events that result in heat-induced protein unfolding and aggregate formation (Ismoyo, Wang & Ismail, 2000; Filosa, Wang & Ismail, 2001).

In the present study, generalized 2D COS was used to characterize the sequence of events leading to heat-induced unfolding and intermolecular association of porcine plasma proteins. In a previous FTIR study in which we examined the thermal behavior of porcine plasma proteins, the spectra recorded under conditions leading to heat-induced gelation indicated that while the proteins retained a substantial amount of native secondary structure after heating, new intermolecular hydrogen-bonded β-sheet structures were formed (Saguer, Fort, Alvarez, Sedman & Ismail, 2008). Several authors maintain that the formation of such intermolecular hydrogen-bonded β-sheet structures is essential for the formation of the gel network (Allain, Paquin & Subirade, 1999). Taken together with the limited extent of protein unfolding observed in our work, this suggests that the particular secondary structure domains that unfold as well as the rate and extent of the subsequent formation of intermolecular hydrogen-bonded β-sheet structures could be important in governing the network integrity and, consequently, the rheological properties

of the gel. Further information is provided by the important role of pH, which is evidenced by the finding that at pH values far above the p*I* of serum albumin (~4.8), the most abundant plasma protein, porcine plasma proteins form gels that have excellent texture and water retention capacity (Parés, Saguer, Saurina, Suñol & Carretero, 1998; Saguer et al., 2008). Thus, the present 2D COS study was undertaken to elucidate the sequence of events leading to heat-induced gelation of porcine plasma proteins under different pH conditions. In addition, the HP-induced structural changes to plasma proteins were also studied by the means of FTIR spectroscopy.

5.3 Materials and methods

Spray-dried porcine plasma (AP920) was provided by American Protein Corporation (Ames, Iowa) and used as received. Protein and water contents were 92.5 ± 0.5% (w/w) and 4.9 ± 0.1% (w/w), respectively.

5.3.1 Solution preparation

To determine the effect of pH/temperature treatments on secondary structure, 7 % (w/v) protein solutions were prepared in D_2O (99.9 % D, Sigma-Aldrich, St. Louis, MO, USA) from spray-dried plasma and adjusted to different pH values (from 7.5 to 4.5, in increments of 0.5 pH units) using DCl (99 % D, Sigma-Aldrich St. Louis, MO, USA). The pH values were directly those as given by pH-meter. Samples were stored at 4 °C overnight to allow for full protein hydration and some H-D exchange to take place.

5.3.2 High pressure treatment of plasma

Plasma samples pH 7, prepared as discussed above, were subjected to 600, 700, 800 and 900 MPa with 5 min holding time. The HP treatments were carried out in triplicates using an ACIP9000 (ACB High Pressure Systems, Nantes, France) high-pressure isostatic press, with a chamber volume of 3 L. The pressure transfer medium used was glycerine. The maximum operational pressure of 900 MPa was reached in approximately 2 min; the depressurization time was approximately 15 s. Samples were introduced into the high-pressure machine at 10 °C and reached a maximum of 35 °C during pressurization owing to adiabatic heating. This rise in temperature can be considered to have a negligible effect because thermal denaturation of plasma proteins occurs well above ambient temperature as observed in this study.

5.3.3 FTIR measurements

FTIR spectra were recorded on a Nicolet 8210 FTIR spectrometer (Thermo Electron Corporation, Madison, WI, USA) equipped with a deuterated triglycine sulphate (DTGS) detector. The spectrometer was continuously purged with dry air from a Balston dryer (Balston, Haverhill, MA, USA). Small absorbance contributions from water vapor were subtracted by recording a background spectrum of the empty cell (no sample) before recording the sample spectra. The samples (8 μL) were placed in a thermostated IR transmission cell between two CaF_2 windows separated by a 50-μm Teflon^{TM} spacer. To study the effect of temperature on secondary structure, the protein solutions were heated from 30 to 90 °C in 5 ºC increments in the own IR-cell. At each temperature, the sample was equilibrated for 5 min prior to recording of its spectrum. The temperature of the cell was regulated by an Omega temperature controller (Omega Engineering, Stamford, CT, USA). The reported temperatures are accurate to within ±0.5 °C. Each spectrum was obtained by co-addition of 512 scans at 4 cm^{-1} resolution. Fourier self-deconvolution (FSD) was performed in the amide I' region (1700-1600 cm^{-1}) using Omnic 6.0 software (Thermo Electron Corporation, Madison, WI, USA), with a bandwidth of 27.2 cm^{-1} and a resolution enhancement parameter of 2.6. The deconvoluted spectra were normalized to unit area of the amide I' band to compensate for minor pathlength variations.

5.3.3.1 Generalized 2D Correlation Spectroscopic Analysis

Two-dimensional correlation (2D COS) analysis was performed on the FSD spectra. Synchronous and asynchronous correlation intensities were computed from the spectra recorded as a function of decreasing pH or increasing temperature. 2D COS analysis was carried out using the KG2D program (written by Y. Wang and described by Wang et al., 1998), which was implemented within Grams/32 software (Version 4; Thermo-Galactic, Salem, New Hampshire, USA); to facilitate examination of weak features, the 2D correlation maps were also displayed as 3D contour maps. The synchronous map consists of two types of peaks: auto-peaks, on the diagonal, which are always positive and are indicative of changes in band intensity induced by the pH or temperature changes, and cross-peaks, at the off-diagonal positions, which indicate that the changes in band intensity at the corresponding wavenumbers on the x and y axes are

correlated; a positive cross-peak indicates that the intensities of the two bands change in the same direction (e.g., both bands increase or decrease with increasing temperature), while a negative one indicates that the changes are in opposite directions. On the asynchronous map, auto-peaks are not observed, and the presence of cross-peaks indicates that changes in the correlated bands are taking place out-of-phase, i.e., accelerated or delayed with respect to each other. A positive sign of the synchronous and asynchronous cross-peaks at wavenumbers $x = \nu_1$ and $y = \nu_2$ indicates that the intensity change at ν_1 occurs prior to that at ν_2; if the change at ν_1 takes place after that at ν_2, the sign of the cross-peak on the asynchronous map is negative. If the cross-peak on the synchronous map is negative, the sign convention is reversed. Thus, the sequence of events is established by comparing the signs of cross-peaks in the synchronous and asynchronous maps (Noda, 1993).

5.4 Results and Discussion

5.4.1 Changes in 1D FTIR spectra of plasma proteins as a function of pH

The FSD spectra of plasma proteins in the amide I' absorption region as a function of increasing pH at 30 ºC are shown in Figure 5.1. As discussed in a previous publication (Saguer et al., 2008), the relative proportions of the serum albumin fraction, comprising ~60% of the plasma proteins, and the globulin fraction, composed primarily of immunoglobulins and representing ~36% of the plasma proteins, can be ascertained from the FSD-FTIR spectra. More specifically, the two strong bands at 1653 and 1638 cm^{-1} observed in the spectrum recorded at pH 7.5 are respectively assigned to α-helical structures, which are predominant in the serum albumin fraction (Carter & Ho, 1994; Peters, 1996), and intramolecular antiparallel β-sheet structures, mainly present in the globulin fraction of plasma (Saguer et al., 2008). Accordingly, the differences in the relative intensities of these bands in Figure 5.1 as compared to the corresponding spectra in the previous publication (Saguer et al., 2008) may be attributed to a slightly higher proportion of the globulin fraction in the porcine plasma samples examined in the latter work (which were from a different source). Apart from these variations in relative intensity, the spectra in Figure 5.1 are similar to those presented previously and, accordingly, the spectral changes observed as a function of pH will only be summarized

here (for additional information and discussion of band assignments, see Saguer et al., 2008). An examination of Figure 5.1 reveals no discernible changes in secondary structure between pH 7.5 and 6.0. In contrast, below pH 6 an increase in intensity at 1644 cm^{-1}, assigned to unordered or random structures, relative to that at 1653 and 1638 cm^{-1}, is observed. Accordingly, as the pH approaches the p*I* of serum albumin (4.8), both the serum albumin and the globulin fraction undergo gradual unfolding. The spectrum recorded at pH 5 is indicative of the loss of additional secondary structure domains in the proteins, as evidenced by the loss of band intensity at 1668 cm^{-1}, frequently assigned to turns, and at 1676 cm^{-1}, which may be assigned to the high-frequency amide I' component of intramolecular antiparallel β-sheet structure or to turns. A shoulder at 1630 cm^{-1} clearly appears at pH 4.5, which has been observed in spectra of denatured proteins in D_2O but it is not common for native proteins (Naumann, Schultz, Görne-Tschelnokow & Hucho, 1993; Arrondo & Goñi, 1999). This band has been traditionally associated with β-sheet structure, although there is some controversy around this issue being also attributed to solvated helical structures (Surewicz, Mantsch & Chapman, 1993; Reisdorf & Krimm, 1996; Gilmanshin, Williams, Callender, Woodruff & Dyer, 1997) and to the hydrogen bonded extended chains connecting helical segments (Byler and Susi, 1986; Yan, Wang, He, Hu, Zhang & Zhou, 2003; Murayama & Tomida, 2004). Finally, a shoulder located at 1617 cm^{-1}, assigned to intermolecular β–sheet structure, is clearly discerned at pH 4.5 and is indicative of partial aggregation of the plasma proteins (Damaschun et al., 2000).

5.4.2 Sequential events leading to pH-induced denaturation of plasma proteins by 2D COS

2D COS analysis was carried out on the FSD spectra presented in Figure 5.1. A weak band at 1612 cm^{-1} was not taken in consideration as it corresponds to an amino acid side chain vibration, and the band at 1667 cm^{-1}, assigned to turns, was also omitted owing to its fluctuating behaviour with changes in pH, which made it difficult to interpret the 2D COS analysis results. The synchronous correlation map is dominated by three

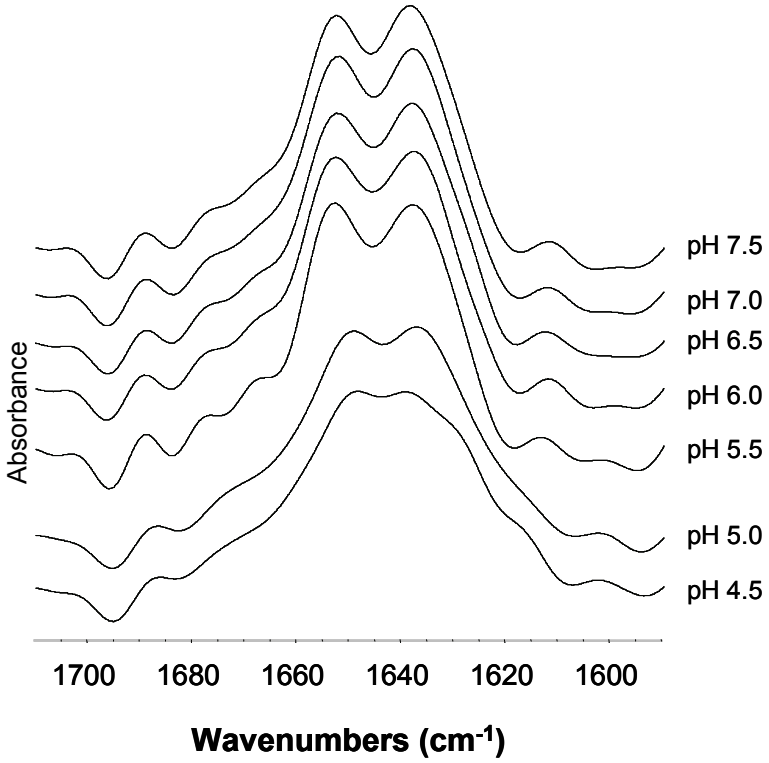

Figure 5.1 FSD spectra obtained at 30 ºC from plasma solutions adjusted to different pH.

prominent auto-peaks at 1618, 1638 and 1654 cm^{-1} (Figure 5.2a). Positive cross-peaks are seen at 1653/1638, 1676/1653, 1683/1618, 1689/1638 and 1689/1653 cm^{-1} while negative cross-peaks appear at 1638/1618, 1653/1618, 1676/1618, 1683/1653 and 1689/1618 cm^{-1}. The asynchronous correlation map (Figure 5.2b) shows positive cross-peaks at 1644/1618, 1653/1618, 1676/1638, 1676/1653, 1683/1638, 1683/1653, 1683/1676, 1689/1638, 1689/1644 and 1689/1653 cm^{-1} and negative ones at 1638/1618, 1644/1638, 1653/1638, 1653/1644, 1676/1618, 1683/1618, 1689/1618, 1689/1676 and 1689/1683 cm^{-1}. A summary of the sequence of changes in band intensity based on the signs of the cross-peaks in the synchronous and asynchronous correlation maps is presented in Table 5.1.

Based on the results in Table 5.1, the sequence of changes in the secondary structure as a function of decreasing from pH 7.5 to pH 4.5 at ambient temperature (30 ºC) is as follows: (i) increase in unordered structure (1644 cm^{-1}), suggesting that a more flexible and loose structure is adopted as a consequence of the decrease in charge repulsion as the pH is reduced; (ii) decrease in turns/β-structure (1676 cm^{-1}); (iii) decrease in intramolecular β-sheet structure (1689 cm^{-1}); (iv) decrease in intramolecular antiparallel β-sheet structure (1638 cm^{-1}); (v) formation of intermolecular β-sheets (1618 cm^{-1}), indicative of protein aggregation; and (vi) decrease in α-helical structure (1653 cm^{-1}). The final spectral change that occurs with a further reduction in pH is the appearance of a second band characteristic of aggregation (1683 cm^{-1}). Given that intramolecular antiparallel β-sheets and α-helices are mainly present in the globulin and serum albumin fractions, respectively (see above), these results indicate that denaturation commences in the globulin fraction at a higher pH than in the serum albumin fraction. However, ultimately, the pH-induced denaturation and aggregation of plasma proteins is characterized by the loss of both β-sheets and α-helices, indicating that both the globulin and serum albumin fractions are involved.

5.4.1 Changes in 1D FTIR spectra of plasma proteins upon heating as a function of pH

The changes in the FSD spectra in the amide I' absorption region upon heating of plasma protein solutions from 30 ºC to 90 ºC are shown in Figure 5.3 for solutions of four different pHs.

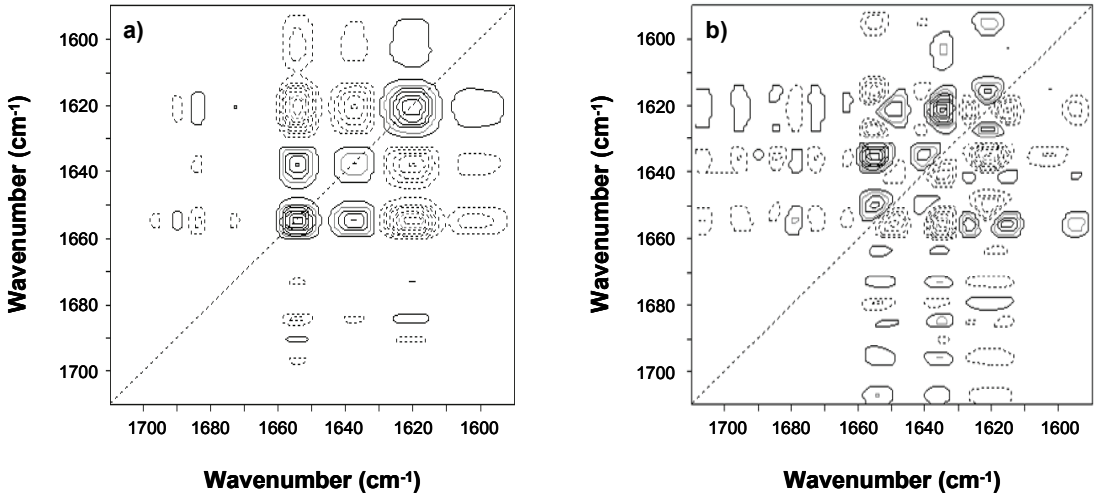

Figure 5.2 Synchronous (a) and asynchronous (b) 2D IR correlation maps in the 1710-1590 cm^{-1} region generated from the spectra obtained in the pH range from 5.5 to 4.5. All spectra were obtained at 30 ºC. Solid and dashed lines correspond to positive and negative correlation peaks, respectively.

This study was carried out over the pH range from 7.5 to 4.5 (in 0.5 pH increments). No differences in the temperature-induced spectral changes were observed between pH 7.5 and 6.0, and accordingly only the spectra recorded at pH 7.5 are shown in Figure 5.3. In contrast, major changes in thermal behavior were observed when the pH was reduced from pH 5.5 to 4.5. In all cases, the intensity of the amide I' band decreased as temperature was increased, and this decrease was accompanied by the appearance of two new peaks at 1683 and 1618 cm^{-1}, attributed to intermolecular β-sheet and related to aggregate formation, along with a shoulder at 1670 cm^{-1}, related to turns. However, with decreasing pH in the pH range of 5.5-4.5, these changes began at lower temperatures, indicating that plasma proteins are more susceptible to conformational changes during thermal treatment under acidic conditions.

Figure 5.4 shows the variation in the intensity of the band at 1618 cm^{-1} during heat treatment as a function of decreasing pH. At pHs 7.5 to 6.0, the 1618 cm^{-1} band intensity starts to increase at temperatures between 55 and 60 ºC and increases rapidly with subsequent increase in temperature. At pH 5.5 the increase in the intensity of the aggregation band begins at lower temperatures (~ 50 ºC), and plateaus at ~ 80 ºC in a sigmoidal manner. At pH 5.0 and 4.5 (near the pI of fibrinogen and serum albumin, respectively), the 1618 cm^{-1} band is already evident at 30 ºC and continues to rise in a sigmoidal fashion with increasing temperature. These results, taken together with those of the studies of pH-induced changes at ambient temperature discussed above, are consistent with the findings of Arrondo and Goñi (1999), who observed that partially unfolded proteins undergo aggregation at lower temperatures.

5.4.2 Thermal transitions of plasma proteins from 2D correlation plots

Figure 5.5 illustrates synchronous and asynchronous 2D correlation maps corresponding to the thermal treatment of plasma proteins at pH 7.5 from 30 ºC to 90 ºC. The synchronous map (Figure 5.5a) shows two prominent auto-peaks on the diagonal at approximately 1638 and 1618 cm^{-1} as well as two weak auto-peaks at approximately 1653 and 1683 cm^{-1}. Positive cross-peaks are observed at 1653/1638,

Table 5.1 Sequence of changes in secondary structure of plasma proteins as a function of decreasing pH from 5.5 to 4.5.

cm^{-1}	1689 ↓	1676 ↓	1653 ↓	1644↑	1638 ↓	1617 ↑
1602 ↑	≈	≈	- - ←	+ + ←	- + →	+ + ←
1617 ↑	- + →	- + →	- - ←	+ + ←	- + →	
1638 ↓	+ + ←	+ - →	+ + ←	- + →		
1644↑	≈	≈	≈			
1653 ↓	+ - →	+ - →				
1676 ↓	≈					

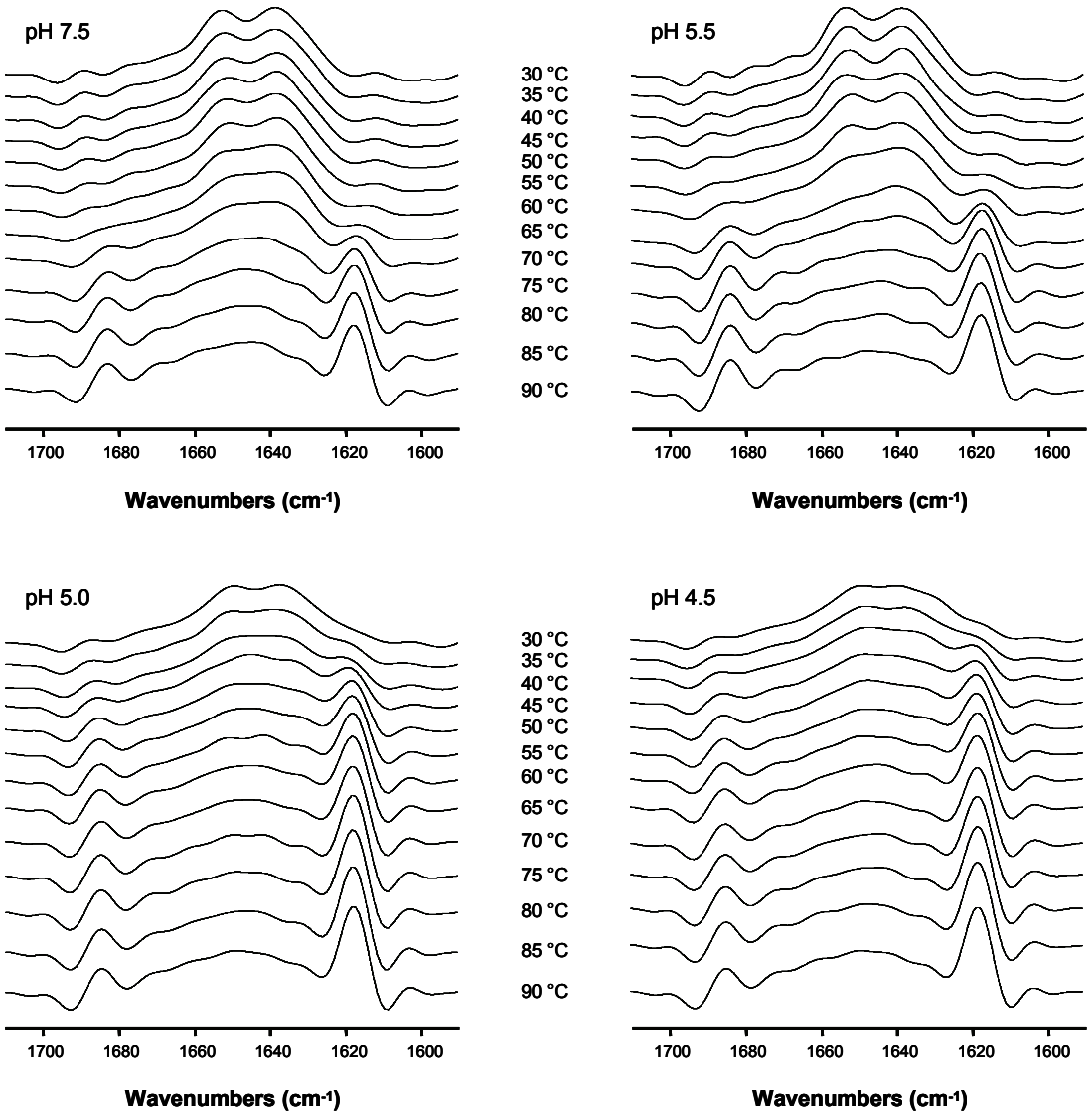

Figure 5.3 Fourier self-deconvolved infrared spectra in the amide I' absorption region upon heating plasma protein solutions (7 % w/v in D_2O) from 30 ºC to 90 ºC at different pH.

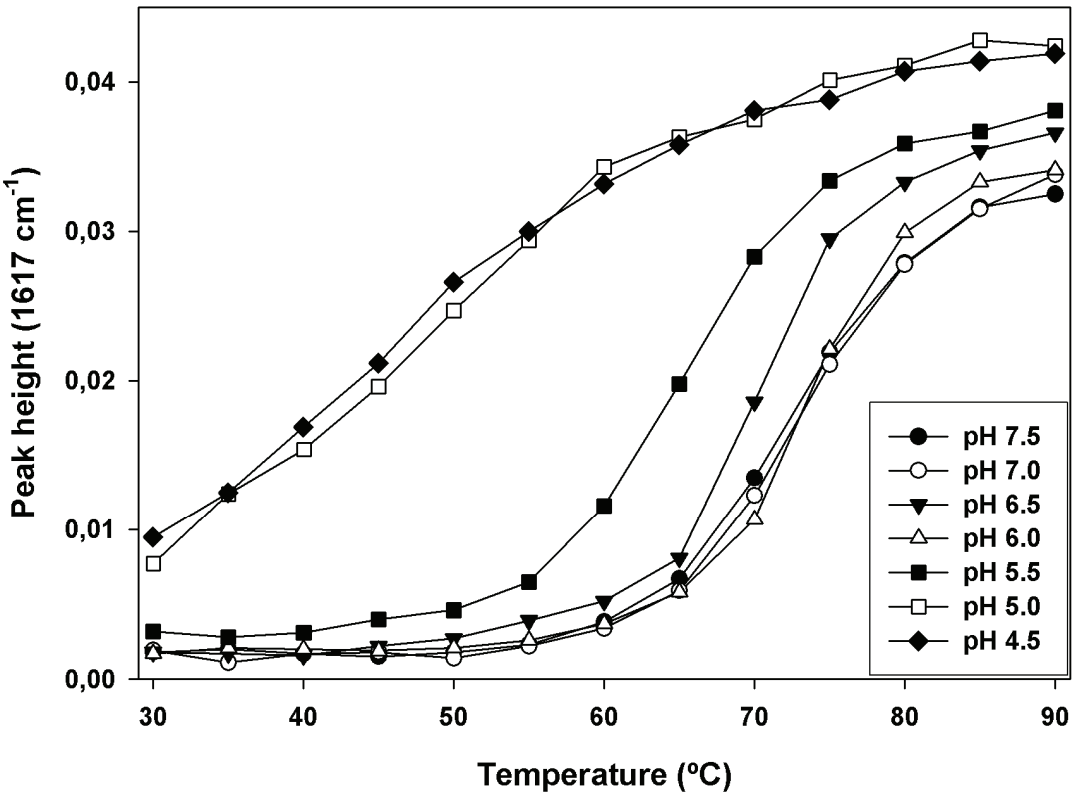

Figure 5.4 Variation in the intensity of the aggregation band at 1617 cm^{-1} as a function of increasing temperature at selected pH.

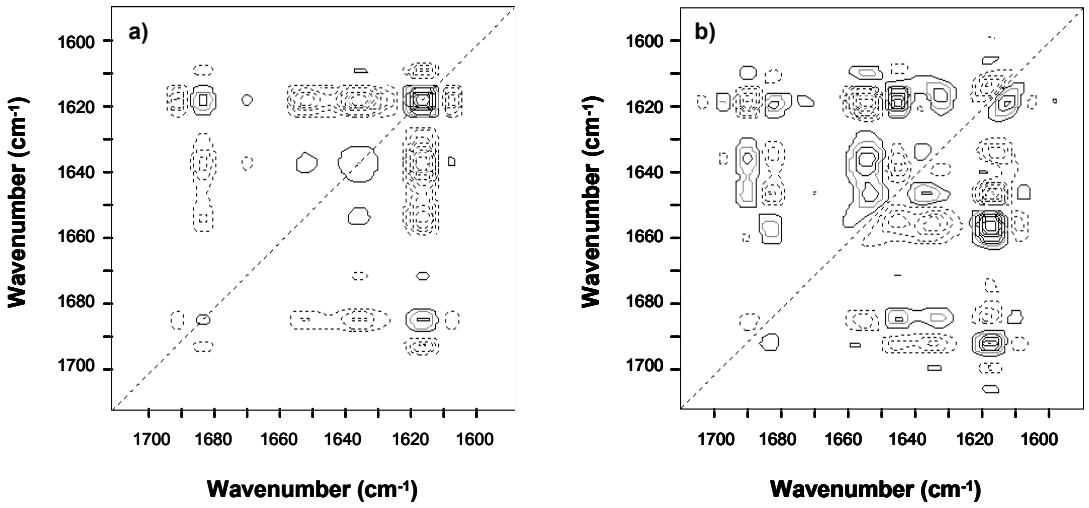

Figure 5.5 The synchronous (a) and asynchronous (b) 2D IR correlation maps generated from the spectra obtained a pH 7.5 during heating (30 ºC to 90 ºC). Solid and dashed lines are positive and negative correlation peaks, respectively.

1670/1618, 1683/1618 and 1689/1638 cm^{-1}, while negative ones appear at 1638/1618, 1644/1618, 1653/1618, 1670/1638, 1670/1653, 1683/1638, 1683/1653, 1689/1618 and 1689/1683 cm^{-1}. From the corresponding asynchronous map (Figure 5.5b), several positive cross-peaks can be identified at 1638/1618, 1644/1618, 1653/1638, 1653/1644, 1670/1618, 1670/1653, 1683/1618, 1683/1653, 1689/1638, and 1689/1644 cm^{-1}. Negative cross-peaks are observed at 1638/1618, 1644/1612, 1644/1638, 1653/1618, 1670/1644, 1683/1612, 1683/1638, 1683/1644, 1689/1618, 1689/1653 and 1689/1683 cm^{-1}. A summary of the sequence of changes in band intensity based on the signs of the cross-peaks in the synchronous and asynchronous correlation maps is presented in Table 5.2.

Based on the results in Table 5.2, the sequence of events occurring during heating at pH 7.5 is as follows: (i) decrease in α-helical structures (1653 cm^{-1}); (ii) decrease in native β-structure (1689 cm^{-1}): (iii) increase in intermolecular β-sheet structure (1683 cm^{-1} band); (iv) increase in turns (1670 cm^{-1}), together with decreases in intramolecular β- sheet structures (1638 and 1676 cm^{-1}); (v) increase in intermolecular β-sheets (1618 cm^{-1} band); and (vi) decrease in unordered structure (1644 cm^{-1}). These results seem to agree Dàvila, Parés and Howell (2007), who reported that the globulin fraction of porcine plasma possesses a higher thermal stability than the albumin fraction characterized by intramolecular β-sheet structure.

A similar sequence of heat-induced protein unfolding and aggregate formation was also observed at pHs in the range between 7.0 and 6.0. Heat-induced protein unfolding was not studied at lower pHs owing to the extensive loss of native structure at ambient temperature.

5.4.3 HP treatments of plasma proteins

Plasma samples of 7 % concentration and pH 7 were placed under high pressure treatment with 600, 700, 800 and 900 MPa for 5 min and the FTIR spectra at 30 °C was recorded immediately after treatment (Figure 5.6). Although plasma samples did not form firm gels after pressure treatment, some structural changes to plasma proteins were induced. A raise in intensity of the peaks at 1683 and 1618 cm^{-1} assigned to intermolecular β-sheets indicative of protein aggregation was observed as

the pressure increased. Also an increase in the ratio of α-helices (1653 cm^{-1}) to intramolecular β-sheets (1638 cm^{-1}) was observed with increased pressure (Figure 5.7). As the pressure increased, the ratio shifted from an almost balanced proportion of α-helices to β-sheets to a spectrum dominated by intramolecular β-sheets. These results suggest a different involvement of the two major plasma fractions albumin and globulins, but additional information is needed in order to clarify the situation.

Parés, Saguer, Toldrá & Carretero (2000) reported HP-induced structural changes to plasma proteins probed by calorimetry and solubility, of samples treated to 450 MPa for 15 min, but they reported no change in the functional properties of the proteins, namely: water holding capacity and hardness of the heat-set gels of pressurized plasma samples.

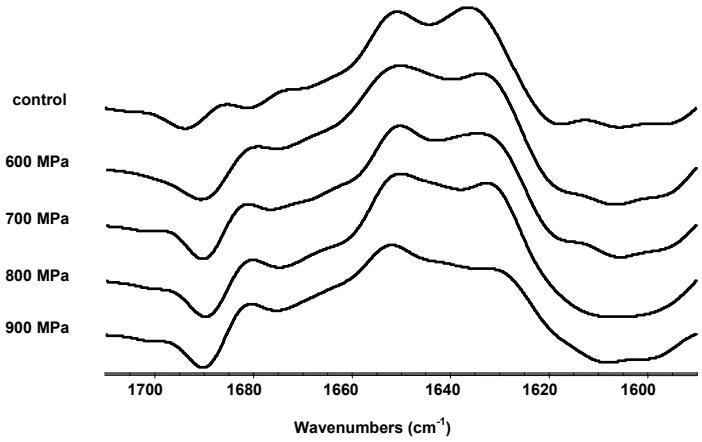

Figure 5.6 Amide I' FTIR spectra of pressure treated (600, 700, 800 or 900 MPa with 5 min holding) plasma samples.

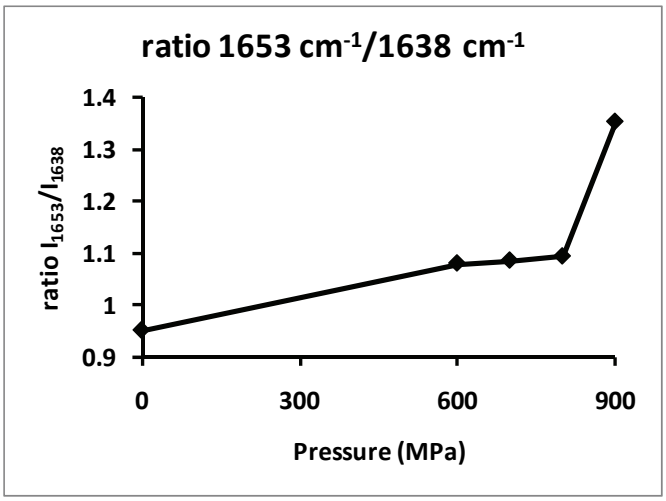

Figure 5.7 Ratio of intensities, obtained from the FTIR spectra of plasma proteins, of 1653 to 1638 cm^{-1}, assigned to α-helices and intramolecular β-sheets respectively.

Table 5.2 Sequence of unfolding and aggregation events upon heating plasma proteins at pH 7.5.

cm^{-1}	1689 ↓	1683 ↑	1653 ↓	1644 ↑	1638 ↓	1617 ↑
1612 ↓	+ + ←	- - ←	+ + ←	- - ←	≈	- + →
1617 ↑	- - ←	+ + ←	- - ←	+ + ←	- - ←	
1638 ↓	+ + ←	- - ←	+ + ←	- - ←		
1644 ↑	- + →	+ - →	- + →			
1653 ↓	≈	- + →				
1683 ↑	- - ←					

5.5 Conclusions

The secondary structure of plasma proteins is especially sensitive to changes in pH as the pH of the protein solution approaches the p*I* of serum albumin, with the globulin fraction appearing as more susceptible to changes in pH. At any pH, the intensity of the native amide I' bands decreases as temperature increases with a concomitant formation of intermolecular β-sheets. Although no important structural changes in the thermal behavior of plasma proteins are evident from physiological to slightly acidic conditions (pH 6.0), further decreases in pH result in a higher susceptibility to conformational changes during heating. 2D COS analysis proved to be a valuable tool in the elucidation of the sequence of events leading to protein unfolding and aggregate formation as a function of decreasing pH or increasing temperature. A decrease in pH at ambient temperature provokes initially the denaturation of the globulin fraction while an increase in temperature appears to first affect α-helical domains, which are predominantly associated with the serum albumin fraction. Therefore, both globulin fraction and serum albumin contribute to the aggregate/gel formation, although at different rates.

High pressure treatments were found to induce important structural changes to plasma proteins, including aggregation and a ratio of α-helices to β-sheets shifted towards an increased proportion of intramolecular β-sheets. These results suggest a different involvement of albumin and globulins in the HP-induced gelation of plasma, but additional experiments are needed to clarify the situation.

Connective statement to Chapter 6

In this final chapter, an even more complex protein model system was used represented by soy protein concentrate (SPC). Although the bulk of the soy proteins (70 %) is comprised of β-conglycinin (7S) and glycinin (11S), both these storage proteins are glycated and of high molecular weight; also, the sugar fraction comprised in their structures is of variable length. These facts, and the presence of other protein components in soy (2S and 15S), give the system a superior complexity level.

Despite the implicit challenges working with such a poll of big proteins, soy proteins have an important niche in the food industry due to their good emulsifying and gelling properties. One of the primary purposes with this final group of proteins employed in this study was to explore the possibility of using soy proteins as functionality-enhancing ingredients with the use of high pressure to facilitate a structure-altering process with potential applications in the beverage industry as a thickening agent or rheology-modifier.

Part of the results from this study has been presented in a scientific conference: Alvarez, P.A., Ismail, A.A. & Ramaswamy, H.S. May 2006. Effect of high pressure processing on the rheological characteristics of soy protein isolate dispersions. 2006 CIFST-AAFC joint Conference, Montreal, QC. Parts of the results in Chapter 6 have been published: Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. 2008. High pressure gelation of soy proteins: Effect of concentration, pH and additives. *Journal of Food Engineering*. 88(3), 331-340. This research work was completed by the candidate under the supervision of Dr. H.S. Ramaswamy and Dr. A.A. Ismail.

Chapter 6. High Pressure Gelation of Soy Proteins: effect of concentration, pH and additives

6.1 Abstract

The global demand for soybean protein has increased dramatically over the last few years due to its versatility. High pressure (HP) processing is emerging as an effective alternative to thermal processing of foods. The HP treatment of protein solutions at different process conditions can cause partial unfolding of proteins that can lead to the irreversible gelation of the product. In this study the influence of protein concentration (5 to 20 % w/v), pH (3 to 7), sugar (5 % w/v), $CaCl_2$ (5 % w/v), pressure level (up to 650 MPa) and holding time (0.1 and 10 min), and process temperature (20 and 40 °C) on the dynamic rheology of soybean protein concentrate (SPC) solutions was evaluated. Furthermore, the protein structural changes caused by HP were studied, through the use of the extrinsic fluorescence of the probe 8-anilino-1-naphthalene sulfonic acid (ANS) and Fourier transform infrared (FTIR) spectroscopy. Results indicated a strong influence of protein concentration on both elastic (G') and viscous (G") moduli, increasing with concentration. Increase in pressure and holding time produced an increase on both G' and G" for SPC concentrations higher than 10 %; at 15 % SPC concentration, a relatively low pressure treatment of 250 MPa achieved the cross-over of G' over G". The structure of the soybean proteins suffered limited changes after HP treatment; hydrophobicity increased, as well as the relative proportion of random coil, while the β-sheet content decreased. HP treatment can be used to enhance the viscoelastic behavior of SPC after which SPC can be used to enrich both protein content and textural properties of foods.

6.2 Introduction

Soybean is an industrial crop extensively cultivated for its oil and protein content. The global demand for soy protein has increased dramatically over the last few years. Recent publications refer to the widespread use of soy protein in the manufacture of many goods, mainly for its gelling behavior and emulsification properties (Maltais, Remondetto, Gonzalez & Subirade, 2005, Molina, Papadopoulou & Ledward, 2001, Renkema, Knabben & van Vliet, 2001). Most authors agree that there is a need for more

research to exploit the possibilities of the soybean protein as a functionality-enhancing ingredient. One venue of interest in soy research is the study of soybean protein fractions. Soybean proteins are composed of four major protein fractions, known as 2S, 7S, 11S and 15S, characterized by differences in sedimentation coefficients. The 11S and 7S fractions constitute about 70 % of the total protein in soybeans. The ratio 11S/7S may vary from 0.5 to 3 (Wolf, Babcock & Smith, 1961).

The changes that soy proteins may undergo during and after the variety of processing conditions found in the food industry is of particular interest to researchers in the field. There has been considerable interest in high pressure (HP) treatment which is emerging as an alternative to the more traditional thermal processing of foods. HP treatment can kill spoilage and pathogenic bacteria, and can also be used for improving functionality of food ingredients and finished foods. During HP treatment, the pressure creation is very rapid and the pressure remains uniform throughout the pressure-vessel; this characteristic offers a direct advantage over thermal processes. Additionally, HP processing can help improve the enzymatic hydrolysis of soy proteins (Peñas, Préstamo & Gomez, 2004), with the possible enhanced release of bioactive peptides.

Pressure treatment of protein solutions at different conditions, can cause partial unfolding of proteins. This protein unfolding can lead to the reversible or irreversible gelation of the product, with repercussions on the viscoelastic characteristics of the protein solution (Ahmed et al., 2003). Many articles have addressed the thermal gelation of soy proteins (Babajimopoulos, Damodaran, Rizvi & Kinsella, 1983, Hsu, 1999, Hua, Cui, Wang, Mine & Poysa, 2005, O'Kane, Happe, Vereijken, Gruppen & Van Boekel, 2004, Renkema et al., 2001, Renkema & van Vliet, 2004); and most look into textural characteristics of self-supporting gels. Some others have focused on high pressure induced gel formation of soy proteins (Apichartsrangkoon & Ledward, 2002, Apichartsrangkoon, 2003, Maltais et al., 2005, Molina, Defaye & Ledward, 2002) and even the combination of HP and temperature (Dumoulin, Ozawa & Hayashi, 1998, Molina & Ledward, 2003). In soy protein beverages, where there is great industrial interest, even just a modulation of the rheological parameters of the protein solution without the formation of hard gels is desirable (Tiziani & Vodovotz, 2005).

A number of earlier publications have shown that studying the structural changes that proteins undergo when subjected to process-specific conditions can facilitate the general understanding of the process (Mills, Marigheto, Wellner, Fairhurst, Jenkins, Mann & Belton, 2003, Nagano, Akasaka & Nishinari, 1994, Zhang, Li, Tatsumi & Isobe, 2005).

The objective of this study was to evaluate the influence of protein concentration, pH, sugar, $CaCl_2$, pressure level and holding time, and process temperature on the dynamic rheology of soybean protein solutions. A subsequent objective was to study the structural changes in proteins caused by HP through the use of extrinsic fluorescence of the 8-anilino-1-naphthalene sulfonic acid (ANS) and Fourier transform infrared (FTIR) spectroscopy.

6.3 Materials and Methods

Commercially available and organically produced soy protein concentrate (SPC) containing 83% protein content (Nx6.25) was used to make aqueous dispersions containing 5 to 20 % (w/v) SPC powder (Bonard Holdings, Saint-Hyacinthe, QC). Deuterium oxide (D_2O 99.9 % D) was purchased from Aldrich (St. Louis, MO). 8-anilino-1-naphthalene sulfonic acid (ANS) ammonium salt for spectroscopic purposes was purchased from Fluka (owned by Sigma-Aldrich. St. Louis, MO). Reagent grade sucrose, calcium chloride and sulfuric acid were obtained from Fisher Scientific (Springfield, NJ).

All the HP treatments were carried out in triplicate using an ACIP6500 (ACB High Pressure Systems, Nantes, France) high-pressure isostatic press, with a chamber volume of 5 L. Water was used as the pressure-transfer medium. The maximum operational pressure was 650 MPa and was reached in approximately 2 min; the depressurization time was approximately 15 s. The sample was introduced into the high-pressure chamber at 15 °C and reached a maximum of 25 °C during pressurization as a result of adiabatic heating. This rise in temperature can be considered to be negligible because thermal denaturation of the soy proteins occurs well above ambient temperature (Ahmed, Ramaswamy & Alli, 2006).

Protein dispersions were exposed to HP up to 650 MPa for different holding times (0.1 and 10 min) at initial temperatures of 20 and 40 °C with pH 3 to 7; further, sucrose

and calcium chloride were added (0 and 5 % w/v). In the case of multiple parameters per sample, the following notation was used as needed: A-B-C-D, where A is the percentage (w/v) of $CaCl_2$, B the percentage (w/v) of sucrose, C the initial processing temperature (°C) and D the pressure holding time (min). Protein dispersions at different pH values were prepared using dilute sulfuric acid (4 N).

6.3.1 Rheology

Rheological parameters, using small amplitude oscillatory shear (SAOS) measurement, were recorded immediately after HP treatment using an AR-2000 rheometer (TA Instruments. New Castle, DE,) employing parallel plate geometry, 60 mm diameter, 1000 μm gap, constant angular frequency of 1 Hz (0.6284 rad/s) and temperature controlled at 20 °C. Measurements were performed in the linear viscoelastic region.

6.3.2 Fluorescence spectroscopy using ANS probe

SPC dispersions of 1 % concentration were subjected to 250, 450 and 650 MPa of pressure. After pressure, the emission spectra of ANS bounded to soy proteins (0.3 mM protein solution containing 3 mM ANS in H_2O) was placed in a 10 mm path-length quartz cuvette. An excitation wavelength of 380 nm and a 2 nm slit was employed; the spectra were recorded from 400 to 600 nm using an Aminco-Bowman AB 2 spectrofluorimeter (Spectronics Instruments. Rochester, NY).

6.3.3 FTIR spectroscopy

FTIR spectra of SPC dispersions were recorded right after HP treatment (250 MPa, no holding time) using a Nicolet 8210E FTIR spectrometer (Thermo Electron Corporation, Madison, WI, USA) equipped with a deuterated triglycine sulphate (DTGS) detector. The spectrometer was continuously purged with dry air from a Balston dryer (Balston Products, Haverhill, MA, USA).

Sample dispersions (15 % SPC) were prepared in D_2O to shift away the strong absorbance of water over the amide I band of proteins. Approximately 8 μL of sample was placed between two CaF_2 windows separated by a 15 μm thick Teflon ^{TM} spacer. The temperature of the cell was regulated by an Omega temperature controller (Omega

Engineering, Stamford, CT, USA). A total of 512 scans were co-added at 4 cm^{-1} resolution. The absorbance spectra were subjected to the band narrowing technique of Fourier self deconvolution (FSD) employing a bandwidth of 20 cm^{-1} (w) and enhancement factor of 2.4 (k), followed by a two-point baseline correction starting at 1710 cm^{-1} and ending at 1590 cm^{-1}, and normalization (peak height over peak area) using Omnic 6.0 software (Thermo Electron Corporation, Madison, WI, USA).

6.4 Results and Discussion

The pressure treatment of protein solutions for different product and process specific conditions causes partial unfolding of proteins that can lead to changes in rheology with the possible gelation of the product. Results for SPC indicated a strong influence of protein concentration and pH on both elastic (G') and viscous (G") moduli.

6.4.1 Wide-range concentration and pressure effects

An increase in pressure level produced an increase in both G' and G" for SPC at concentrations higher than 10 % (Fig. 6.1). At 5 and 10 % concentration (Fig. 6.1a and 6.1b) there was very little change in viscoelastic parameters due to pressure treatment and the actual viscosity and elasticity moduli were extremely low. The magnitude of G" of the 5 % SPC dispersion (Fig. 6.1a) was found to be almost close to that of distilled water (1.2×10^{-2} Pa), recorded with the same instrument and under the same experimental conditions; as for the elastic modulus (G'), it was to be roughly two orders of magnitude higher than that of distilled water (7.9×10^{-4} Pa). Values for the 10 % SPC dispersion were around one order of magnitude higher than that of the 5 % SPC dispersion (Fig. 6.1b).

When the SPC concentration was elevated to 15 % (Fig. 6.1c), an increase in the viscoelastic parameters of approximately one order of magnitude was observed, when compared to the parameters of the 10 % SPC dispersion. It was also observed that a relatively low pressure treatment of 250 MPa at 15 % SPC concentration achieved a

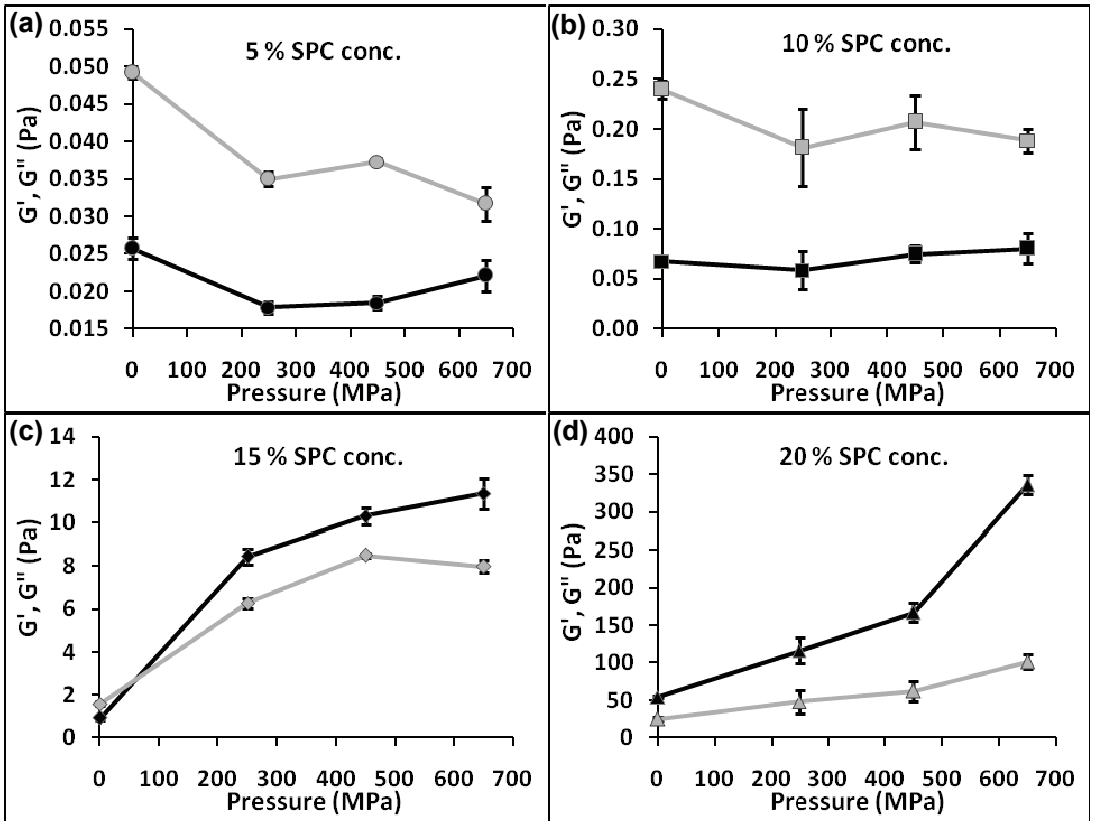

Figure 6.1 Rheological parameters G' (black) and G" (grey) of different concentrations of soy protein isolate when exposed to various pressures. Small error bars (SD) are hidden behind point markers (n=3).

greater increase of the storage modulus (G') when compared to the loss modulus (G"); this cross-over of G' over G" can also be referred to as the point where a true gel is formed. This phenomenon could be explained by the physical proximity of the protein molecules at higher concentrations, which favors intermolecular interactions, thus causing the formation of an elastic gel structure. Similarly, Maltais et al. (2005) found the creation of a three-dimensional network for dispersions of soy protein isolate in water at concentrations higher than 10 % (w/w), and Ahmed, Ayad, Ramaswamy, Alli & Shao (2007) found a very important correlation between protein concentration and viscoelastic behavior of pressure-induced soy protein isolate gels, using a different commercial brand of soy protein isolate. HP treatment of 15 % SPC at higher pressure levels caused a continuous increase of both G' and G".

Fig. 6.1d shows the effect of pressure on the 20 % SPC dispersion. Once again, both numerical values were in the region of one order of magnitude higher than those of the 15 % SPC dispersion. Also, the data exhibited an elastic modulus higher than the viscous modulus even for the control sample, although both parameters continued to increase with increasing pressure.

When the phase angle of the pressure treated samples was examined (Fig. 6.2), a shift from liquid-like behavior to solid-like behavior was found only for the 15 % SPC dispersion (Fig. 6.2a). Delta was 60 degrees for the 15 % SPC control (0.1 MPa-atmospheric pressure), and changed to around 40 degrees after an HP treatment of 250 MPa.

Solid-like behavior is defined as a delta of less than 45 degrees, while for liquid-like behavior the delta is more than 45 degrees. Plotting the same data but in terms of concentration (Fig. 6.2b), suggested that all three high-pressures achieved approximately the same phase angle; the largest change however, occurred for the 15 % SPC dispersion. At this concentration, all pressure levels produced a similar delta of around 40 degrees; as for all the rest of the experiments (5, 10 and 20 % SPC), very little change in delta was found after HP.

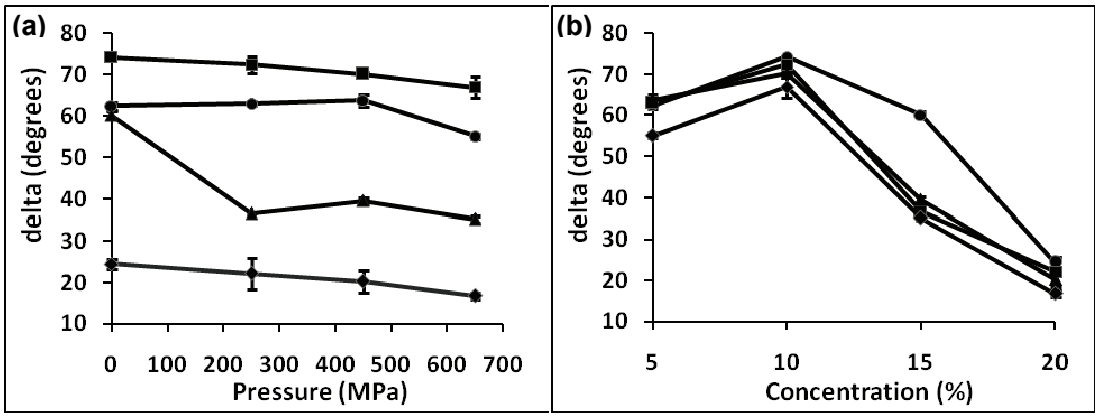

Figure 6.2 Phase angle of different soy protein concentrate (SPC) dispersions subjected to various pressure treatments. **(a)** Phase angle in terms of sample SPC concentration (5 ●, 10 ■, 15 ▲ and 20% ♦). **(b)** Phase angle in terms of processing pressure (0.1 to 650 MPa, increasing grey intensity). Small error bars (SD) are hidden behind point markers (n=3).

Dispersions of 5 and 10 % SPC concentration showed deltas of liquid-like behavior around 65 degrees and 75 degrees respectively. Although the phase angle of the 5 % SPC dispersion was lower than that of the 10 % dispersion, and this could lead one to believe that the % 5 dispersion has more rheological structure, one should keep in mind that the absolute values of G' and G" were of one order of magnitude higher for the higher concentration dispersion (10 %). As a comparative measure, distilled water recorded in the same instrument and under the same experimental conditions showed a delta of 86 degrees. Finally, the highest SPC concentration (20 %) displayed a phase angle close to 20 degrees for the control, but with a small decrease with increasing pressure.

6.4.2 Narrow-range concentration effect at 250 MPa pressure

Since there was a very interesting phenomenon occurring at 15 % SPC concentration, and all pressure levels studied (250, 450, 650 MPa) produced a similar delta (Fig. 6.2a), it was decided to study this particular point further at a fixed pressure treatment of 250 MPa. Pressure levels lower than 250 MPa (150 and 200 MPa) were also included, but they failed on achieving the objective of crossing G' over G" (Fig. 6.3).

Fig. 6.4 shows the viscoelastic parameters of SPC dispersions of 14, 15 and 16 % concentrations before (Fig. 6.4a) and after (Fig. 6.4b) 250 MPa pressure. Only when the 15 % SPC dispersion was pressure treated, did the cross-over of G' over G" occur; the 14 % SPC dispersion did not demonstrate the cross-over of G' over G" (Fig 6.4b). The 16 % SPC dispersion exhibited a storage modulus already higher than the loss modulus for the control sample (before pressure treatment) (Fig. 6.4a), even though both viscoelastic parameters were increased somewhat after pressure treatment.

The phase angle of these three samples (Fig. 6.4c) confirmed the large change in viscoelastic properties at 15 % SPC concentration with a drop of about 20 degrees, from around 60 degrees (higher than 45 degrees) to around 40 degrees (lower than 45 degrees) after pressure treatment. The 14 % SPC dispersion also showed a decrease in delta following pressure treatment, from around 60 degrees to a little below 50 degrees, but both values were within the range of liquid-like behavior. The phase angle of the 16 % SPC dispersion showed solid-like characteristics (delta=34 degrees) for the sample before pressure treatment, and this value did not change much after pressurization (delta=33 degrees).

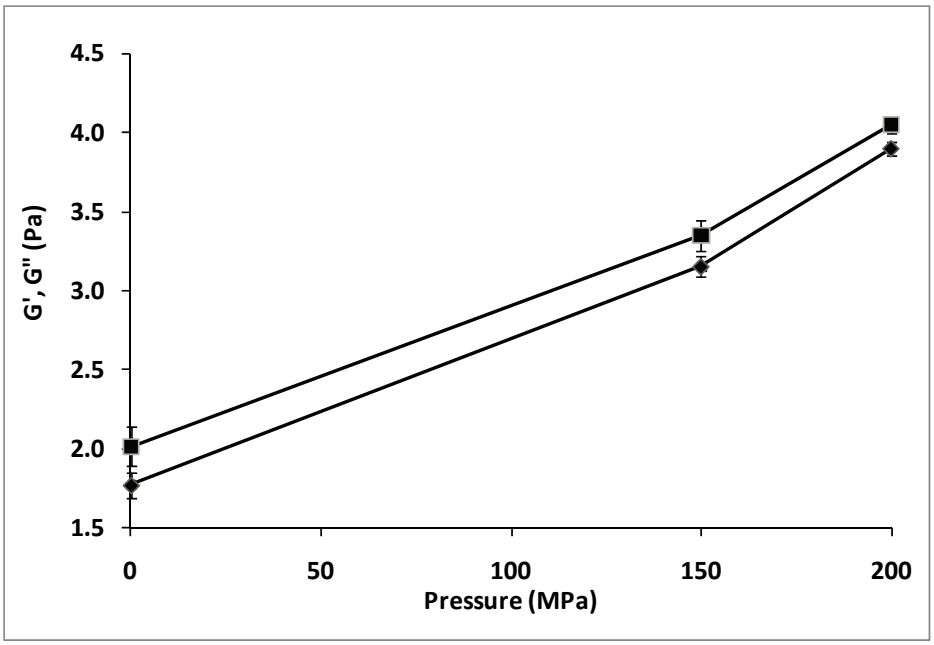

Figure 6.3 Viscoelastic parameters G' ◆ and G" ■ of 15 % soy protein concentrate dispersions treated at 150 and 200 MPa.

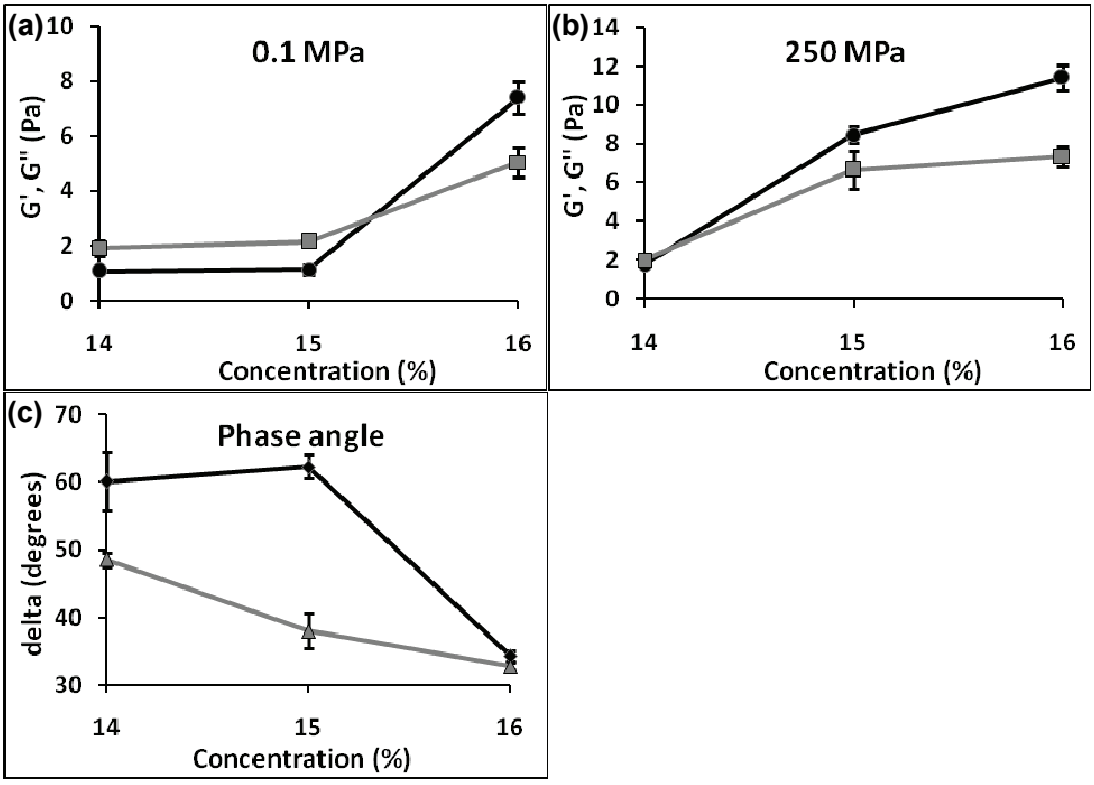

Figure 6.4 Viscoelastic parameters (G' ●; G" □) of 14, 15 and 16 % SPC dispersions before and after 250 MPa pressure treatment. **(a)** Storage and loss modulus of SPC dispersions at atmospheric pressure (0.1 MPa). **(b)** Storage and loss modulus of SPC dispersions after 250 MPa pressure. **(c)** Phase angle of SPC dispersions before (◆) and after (▲) 250 MPa pressure. Small error bars (SD) are hidden behind point markers (n=3).

6.4.1 Wide-range pH effect at 250 MPa pressure, 15 % SPC concentration

To address the effect of pH on the rheological characteristics of SPC dispersions, we decided to study the effect of 250 MPa of pressure to 15 % SPC dispersions on the neutral to acidic range of pH, 7 to 3.

The semi-logarithmic plot of pH versus G' and G" showed evidence of the dramatic loss of viscoelastic properties when the pH of the SPC dispersion is decreased (Fig. 6.5a). In the case of the control samples, both viscous and elastic moduli decreased slightly when pH was dropped from 7 to 6, but decreased a dramatic tenfold when pH was decreased from 6 to 5, and remained low for pHs of 4 and 3. This loss of viscoelastic parameters is in direct correlation with protein precipitation, which occurs when the pH of the solution is decreased and gets closer to the isoelectric point of the soy proteins (pH ~ 5.5).

Pressure treatment improved significantly both G' and G" for the pH 7 sample, and also produced the cross of G' over G". Additionally, for the samples of pH 3, 5 and 6 the viscoelastic parameters improved slightly after pressure treatment. On the contrary, the SPC dispersion at pH 4 showed a slight decrease in both G' and G" after pressure treatment.

Together with the study of the viscous and elastic moduli, we focused on the phase angle of the SPC dispersions of different pHs (Fig. 6.5b). For the control samples (before pressure treatment) we observed a large drop in delta, from around 60 degrees to about 37 degrees, when the pH was lowered to 4, despite the fact that the G' and G" had already plummeted for the pH 5 sample. There was also a tendency to change from liquid-like (delta ~60 degrees) to solid-like behavior (delta ~40 degrees for pH 7 and 5, and ~53 degrees for pH 6) after pressure treatment, for samples pH 5 and higher. In opposition, samples pH 4 and 3 changed from solid-like (delta ~37 degrees) to liquid-like behavior (delta ~50 degrees) after pressure treatment. This might be due to a limited solubilization effect over the already precipitated protein, caused by the pressure treatment.

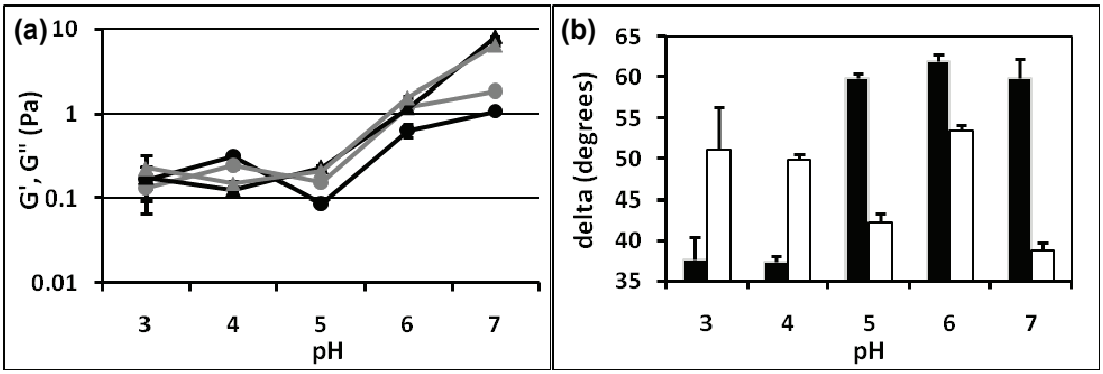

Figure 6.5 Viscoelastic parameters (G' black; G" grey) of 15 % SPC dispersions at different pHs, before and after 250 MPa pressure treatment. **(a)** Storage and loss modulus of 15 % SPC dispersions at 0.1 MPa (G' ●;G" ●) and after 250 MPa (G' ▲; G" ▲) at pH 3 to 7. **(b)** Phase angle of SPC dispersions before (black) and after (white) 250 MPa pressure at pH 3 to 7. Small error bars (SD) are hidden behind point markers (n=3).

6.4.2 Effect of additives, pH, temperature and pressure holding time

The effects of different pH, presence of calcium chloride and sucrose, as well as initial processing temperature and holding time were studied for a dispersion of 15 % SPC in water and a processing pressure of 250 MPa.

In general, for the non-HP treated controls, there was a very heavy drop in both G' and G" with the decrease in pH as observed in Fig. 6.6a and 6.6b. For the pH 7 controls, the addition of calcium chloride created a significant increase of both G' and G", whereas only a moderate increase was observed with the addition of sucrose; there was also an increase in G' and G" for the pH 3 controls with the addition of $CaCl_2$ but was less dramatic compared with the neutral pH controls. Apparently, the increase in ionic strength of the solution generated by $CaCl_2$ facilitated the protein-protein interaction, thus the raise in the elastic and viscous moduli. Similarly, Hua et al. (2005) found improved G' values for thermally-induced soy protein isolate gels in the presence of incremental amounts of NaCl. Moreover, there appears to be a competing effect of both additives, also observed by the phase angle (at pH 7) which changed from 46 to 15 degrees with the addition of $CaCl_2$, while it did not change with the combination of $CaCl_2$ and sucrose (Table 6.1). Also, the phase angle showed a change towards solid-like behavior for these samples compared to the neutral pH ones. Overall, the phase angle of the control samples showed a change towards solid- like behavior when additives were added at pH 7 but the opposite for pH 3 samples. Low pH SPC samples were very cloudy in appearance; this protein precipitation was likely to be responsible for the dramatic decrease in rheological parameters and the change towards solid-like delta.

Upon pressure treatment, samples with different pH exhibited very different rheological parameters, expressed as a percentage of change compared to its correspondent control (Fig. 6.6c and 6.6d). These differences could be the result of increased solubility under pressure for precipitated samples and the rearrangement of molecular networks, which, depending on the specific conditions, could result in an increase or decrease of both elastic and viscous moduli.

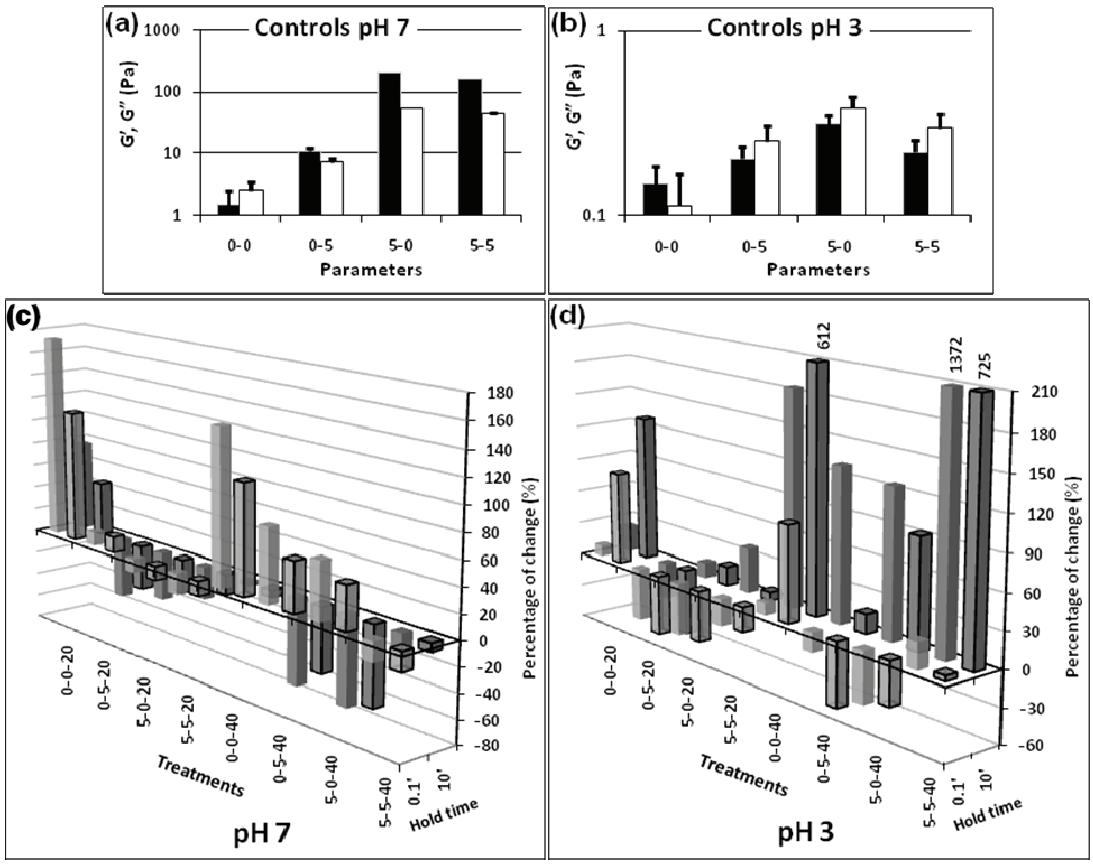

Figure 6.6 Rheological parameters of 15 % SPC dispersions subjected to 250 MPa with different pH, pressure holding time, calcium chloride, sucrose and processing temperature. **(a) and (b)**: Rheological parameters (G' black; G" white) of control (not HP treated) SPC dispersions for pH 7 and pH 3 respectively. **(c) and (d)**: Rheological parameters, expressed as percentage of change compared to the correspondent control, for differently treated SPC dispersion (G' no border; G" black border). Error bars (SD, n=3) were avoided for **(c) and (d)** to minimize confusion. Extreme values were indicated on top of bars that should go beyond the limit.

Table 6.1 Phase angle of 15 % pH 7 and pH 3 SPC dispersions exposed to 250 MPa and different concentrations of calcium chloride and sucrose; process temperature and pressure holding time. Controls (not pressure treated) were included in the table.

	pH 7		pH 3	
Treatment	Average(n=3)	Std dev	Average(n=3)	Std dev
0-0	60	0.26	37	3.14
0-5	33	1.69	51	3.46
5-0	15	0.02	50	3.61
5-5	15	0.35	54	0.98
0-0-20-0.1	39	0.47	52	1.98
0-5-20-0.1	35	1.26	48	3.01
5-0-20-0.1	15	0.08	52	1.01
5-5-20-0.1	16	0.11	54	1.87
0-0-40-0.1	38	0.57	51	3.12
0-5-40-0.1	32	2.05	34	3.54
5-0-40-0.1	14	0.05	54	2.67
5-5-40-0.1	15	0.04	49	2.78
0-0-20-10	40	0.41	55	3.55
0-5-20-10	42	1.84	37	4.87
5-0-20-10	18	0.09	52	2.87
5-5-20-10	17	0.87	46	1.81
0-0-40-10	45	0.38	62	2.54
0-5-40-10	49	3.99	32	1.94
5-0-40-10	19	0.77	46	2.61
5-5-40-10	17	0.81	37	3.11

At neutral pH (Fig. 6.6c) the longer pressure holding time had a modest difference compared to samples at low pH (Fig. 6.6d). Moreover, the longer holding time affected many of the pH 7 samples negatively, whereas it did positively to most of the pH 3 samples. This observation is also evident for the phase angle of these samples (Table 6.1).

Similar to this work, Maltais et al. (2005) found a wide variety of cold-set gels can be obtained from soy protein isolate when varying protein and calcium chloride concentrations, although they did not explore the effect of HP.

In general terms, the higher initial process temperature accounted for an increase in both G' and G" for the pH 7 short holding time samples and pH 3 long holding time samples. In the case of the pH 3 5-5-40-10 sample the longer holding time achieved absolute G' and G" values comparable to those of the pH 7 0-0 control sample, although the phase angle did not show this remarkable change. There appears to be a protection effect of the additives for the increase of the viscoelastic parameters of the pH 7 short holding time samples. A negative effect of the additives for the short holding time, but a positive for the long holding time at pH 3, was also observed. Additionally, some of the low pH samples exhibited shear thinning behavior.

Finally, the 10 min holding time produced a decrease of both G' and G" for most of the pH 7 samples with the exception of the samples with no additives, at both process temperatures. It seems that the longer holding time combined with additives resulted in a rearrangement of the gel network that resulted in a decrease of rheological parameters; this effect was amplified with the higher process temperature. Again, the phase angle did not illustrate these findings.

6.4.3 Fluorescence spectroscopy using ANS as probe

In order to investigate the molecular basis of the change on functional properties (rheology) of SPC when subjected to HP treatment, we decided to analyze the soybean protein samples using some of the structural techniques: extrinsic fluorescence and FTIR spectroscopy.

The extrinsic probe 8-anilino-1-naphthalene sulfonic acid (ANS) is a small molecule that has a relatively weak fluorescence by itself when dispersed in water, but

when it binds to hydrophobic sites or pockets in a molecule its fluorescence increases dramatically, accompanied by a blue shift of some 40 nm. The increase in fluorescence is also noticeable when ANS is dissolved in a non-polar solvent. This property is useful for the study of the changes in tertiary structure of protein molecules that leads to exposure of hydrophobic sites previously inaccessible to ANS (Boatright & Hettiarachchy, 1995, Ikeuchi, Nakagawa, Endo, Suzuki, Hayashi & Ito, 2001, Laligant, Dumay, Casas-Valencia, Cuq & Cheftel, 1991, Yang, Dunker, Powers, Clark & Swanson, 2001, Yang, Powers, Clark, Dunker & Swanson, 2003).

Dispersions of 1 % SPC concentration were first exposed to increasing ultra high pressure treatments, and later mixed with a solution of ANS, for a final ANS concentration of 3 mM and a final SPC concentration of 0.3 mM. Fluorescence experiments using samples with different pH were discarded due to protein precipitation, which causes light scattering and a large inner filter effect that confuses spectral data and creates artifacts. Fig. 6.7 shows the increasing fluorescence intensity obtained from samples exposed to increasing pressure treatments. The fluorescence intensity at λ_{max} of 466 nm was plotted against the pressure level (Fig. 6.7b). The graph shows a clear positive relationship between pressure level and fluorescence intensity. These results are similar to the ones from Ikeuchi et al. (2001), Yang et al. (2001) and Yang et al. (2003) for milk whey proteins; and Zhang, Li, Tatsumi & Kotwal (2003) for soybean proteins.

The results indicate that the soybean proteins structure was changing, becoming more loose, thereby allowing ANS molecules to enter to the hydrophobic core of the protein subunits. Another possibility is that the proteins were changing their three-dimensional structure so as to expose to the surface hydrophobic pockets previously inaccessible to solvent or ANS. This three- dimensional rearrangement of the protein molecules was apparently enough to create a different network arrangement, which in turn seems to be responsible for the increment in G' and G" discussed earlier. Although soybean proteins are big macro-molecules consisting of many subunits held together by disulphide bonds (Adachi, Takenaka, Gidamis, Mikami & Utsumi, 2001), HP processing is known to have no effect over covalent bonds; therefore a sulfhydryl interchange cannot be considered as the mechanism responsible for the change in this functional property.

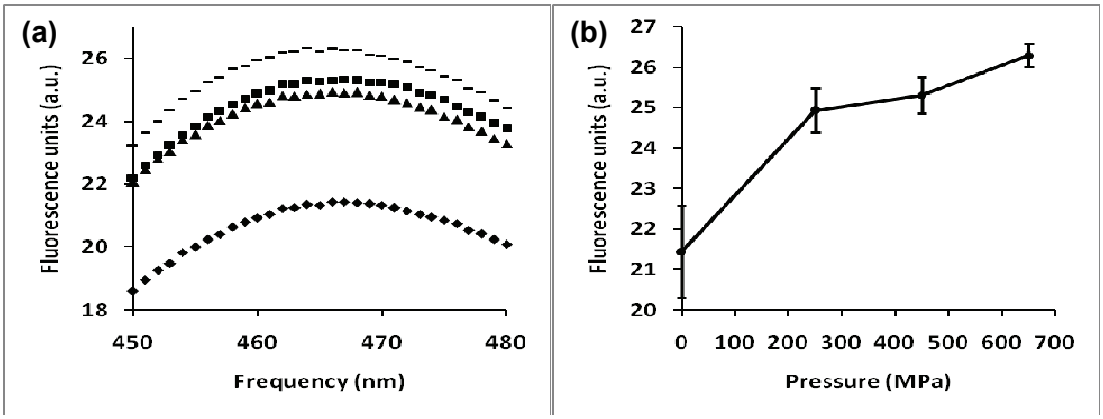

Figure 6.7 Fluorescence spectra of ANS bind to soy proteins subjected to increasing pressure. **(a)** Emission spectra of ANS using an excitation beam of 380 nm. SPC samples were subjected to different pressure treatments (◆ 0.1; ▲ 250; ■ 450; ▬ 650 MPa). **(b)** Fluorescence intensity at λ_{max} of 466 nm as function of pressure. When the pressure applied is increased, the proteins molecules relax its structure and let more ANS to bind to its hydrophobic pockets.

In addition, Catsimpoolas and Meyer (1970) already proposed that the thermally-induced gel formation had to be other than covalent bonding. Furthermore, Hua et al. (2005) found more rigid (higher G') thermally-induced soy protein isolate gels in the presence of N-ethylmaleimide (NEM), a well known reducing agent that can block the formation of new disulfide bonds; moreover, the authors found the addition of NEM facilitated the exposure of hydrophobic pockets on the protein surface, thus promoting intermolecular hydrophobic interactions (also supported by Mills et al., 2003). However, this observation contrasts with the earlier study by Babajimopoulos et al. (1983), where the authors discarded the contribution of hydrophobic interactions as a gel-promoting force.

6.4.4 FTIR spectroscopy of SPC

Lastly, we decided to study the changes in secondary structure the soybean proteins could have undergone after HP treatment. For this purpose we recorded the FTIR spectra of the 15 % SPC control and after a treatment 250 MPa pressure (0.1 min holding time) studied the changes in the amide I region.

The amide I absorption region (1700-1600 cm-1) in the infrared spectrum of a protein is one of the most useful for secondary structure elucidation (Byler & Susi, 1986, Susi & Byler, 1988). The amide I band assignments of soybean proteins are summarized in Table 6.2 and are based on previous works (Abbott, Nabetani, Sessa, Wolf, Liebman & Dukor, 1996, Byler & Susi, 1986, Susi & Byler, 1988). The irreversible changes in the secondary structure of soybean proteins subjected to 250 MPa pressure with a holding time of 0.1 min is shown in Fig. 6.8.

The secondary structure of the soybean proteins is predominantly β-sheet and unordered as observed by the bands at 1632 cm^{-1} and 1646 cm^{-1} respectively, which is in accordance with previous works by Chen, Ker & Wu (1990), Mills et al. (2003) and Nagano et al. (1994). It is important to note that this is an SPC sample therefore comprising of many proteins, although the predominant proteins are β-conglycinin (7S) and glycinin (11S); the FTIR spectrum recorded is therefore the average absorbance of

the protein concentrate. After pressure treatment, we observed limited changes in the secondary structure of the soybean proteins (Mills et al., 2003 also reported small structural changes in heat-treated glycinin samples). Some loss in the intensity of the 1692 and 1622 cm^{-1} bands was also observed, assigned to a β-turns and parallel β-sheet respectively; this change was also accompanied by an increase of the 1645 and 1632 cm^{-1} bands correspondent to random coil and β-sheet. Although both principal bands increased in intensity after HP treatment, the relative proportion shifted from an almost balanced β-sheet to random coil proportion, to a higher proportion of random coil (1645 cm^{-1}) relative to β-sheet (1632 cm^{-1}). A small band shifting was also evident and is a sign of changes in the tertiary structure of the proteins. Zhang et al. (2003) found similar observations using circular dichroism (CD) in regards to the conversion of ordered structures (α-helix and β-structure) to random coil, for a much higher pressure level (500 MPa) and holding time (10 minutes), but much lower protein concentration (0.1 %).

The changes in secondary structure of soybean proteins accompanied changes at the tertiary level, as evidenced from the fluorescence of ANS. These changes altered the protein-protein interactions and possibly the free water dynamic, therefore resulting in an increase in viscoelastic characteristics. The structural changes at the secondary level observed after pressure treatments (conversion of β-sheet to random coil) are different to the changes induced by thermal treatments; there are some discrepancies in the literature regarding the structural changes at the secondary level for soybean proteins subjected to thermal treatments, but none of the articles reported an increase in random coil upon heating.

Regarding the changes during thermal processing of soybean proteins, Chen et al. (1990) observed that the temperature increase induced a conversion of random coil (many bands assigned) to α-helix (1647 cm^{-1}), without much effect on the relative amount of β-sheets (many bands assigned). Mohamad, Maruyama, Takahashi, Yagasaki, Higasa, Matsumura & Utsumi (2004) proposed that the α-helix content decreased and β-sheet increased, while β-turn and "other" remained unchanged; this article does not mention

Table 6.2 Band assignment of the amide I' spectral region of soy proteins.

Band position (cm^{-1})	Assignment
1692	β-turns
1655	α-helix
1645	unordered
1632	Antiparallel β-sheet
1622	Parallel β-sheet

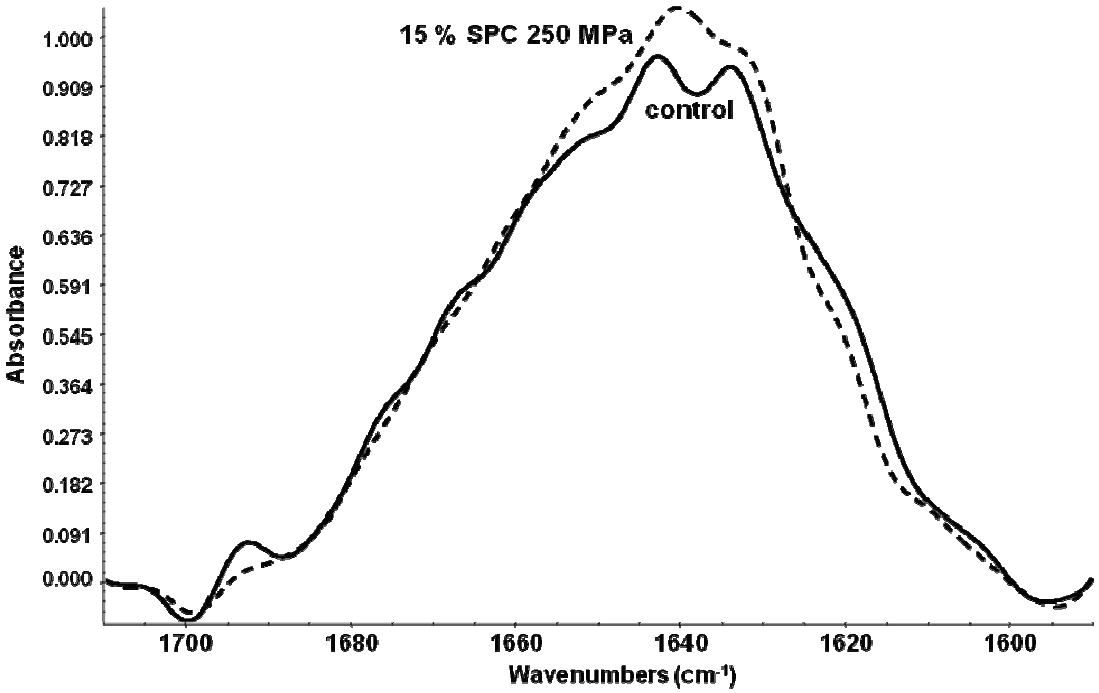

Figure 6.8 Amide I' region of the FTIR spectra of soybean proteins. Control sample (solid line, average of 3) and pressure treated sample (dashed line, average of 3) at 250 MPa and 0.1 minutes holding time.

band assignments and only recorded 16 co-added scans. Nagano et al. (1994) observed a decrease of random coil (1645 cm^{-1}), while β-sheet (1680 cm^{-1}) and intermolecular β-sheet (1618 cm^{-1}) increased; authors in this article did not apply any mathematical band-narrowing technique (e.g. FSD, first or second derivative). Mills et al. (2003) reported β-sheet (1635 cm^{-1}) and α-helix/random (1650 cm^{-1}) remained unchanged, while intermolecular β-sheet (1625 cm^{-1}) increased; these experiments were carried out on an open attenuated total reflectance (ATR) cell, which brings up questions about water evaporation (not clarified in the article). From this brief review of the literature, the debate is obvious and it is also worth noting that there is no consensus of whether the aggregation band (intermolecular β-sheet) should be considered at the 1618 cm^{-1} (as in the case of β-lactoglobulin and other proteins) or 1625 cm^{-1} position.

6.5 Conclusions

Summarizing the most relevant information obtained, it was found that the dynamic rheology indicated a strong influence of protein concentration and pH on both elastic (G') and viscous (G") moduli. A protection effect of the additives was observed against the increase of the viscoelastic parameters of the pH 7 short holding time samples. At pH 3, a negative effect of the additives for the short holding time but positive for long holding time was also observed. The structure of the soybean proteins suffered limited changes after HP treatment: hydrophobicity increased, as well as the relative proportion of random coil, while the β-sheet content decreased.

Finally, it was envisioned that HP treatment can be used to enhance the viscoelastic behavior of SPC, after which SPC can be used to enrich both protein content and textural properties of foods. A wide range of gel textures, which ultimately translates to mouth-feel, can be obtained with varying SPC concentrations, pressure levels, holding times and processing temperatures, pH, and additives. Therefore, with a target product on hand, one can aim for specific rheological characteristics.

Chapter 7. General Conclusions

The main objective of this thesis work was to characterize the effects of HP treatment on globular proteins from two different points of view: functionality and conformational; this was achieved in 3 different scenarios of proteins varying in complexity and source. In general, HP treatment affected protein structure and functionality but the specific effected result depended upon the characteristics of the system in question: for the small bovine β-lactoglobulin (β-lg) subjected to intermediate pressures (max 650 MPa) and at concentrations lower than 20 % (w/v), the secondary structure of the protein did not get largely modified, although the treatment resulted in an increase in viscoelastic parameters; when the β-lg concentration and the pressure level were increased (up to 900 MPa), it was possible to produce self standing gels with extensive modification of the protein's secondary and tertiary structures; in the simplified multi-component system of porcine blood plasma some modification in secondary structure of proteins were observed with limited changes in sample viscosity with increasing pressure treatment (up to 900 MPa) at plasma's physiological pH and concentration; in the last and more complex scenario studied of soy protein concentrate (SPC) small changes in secondary structure were found which resulted in important viscoelastic changes (250 MPa).

When dealing with proteins of different sizes and amino acid composition, which alters chromophores for spectroscopic techniques, as was the case in this work these different scenarios demanded separate analytical approaches to achieve similar goals of gathering information. As it was evident through reading the research chapters of this thesis we had to adapt our techniques to study viscoelastic changes as well as protein's conformational changes in all the different situations.

It was observed that HP-process independent parameters, like dispersion's protein concentration and pH, exerted an important influence in determining HP-induced gel network formation and the extent of protein denaturation. Also, results suggested that the bigger the protein, HP-induced gelation can take place at milder pressure conditions. This can be useful for the food industry as a general rule of thumb, when the manufacturing goal is to improve viscoelasticity of foods through the use of HP processing (HPP) it

appears that a milder HP condition is mandatory to avoid hard set gels, the higher the protein content of the food and the more complex and higher molecular weight of the proteins present, then a lower pressure should be used to achieve important changes in food thickness or actual gelation.

It was observed that although HPP and temperature have different physical mechanisms of action over proteins, they possess similar gel formation pathways; both involving the formation of intermolecular β-sheets. In the case of pressure, another stabilizing force is playing an important role in gel network formation and we suspect this force to be protein hydrophobic interactions.

The following summarizes other specific findings:

a) Statistical analysis of FTIR spectroscopy measurements of β-lg (15 %; 0.1, 200 and 650 MPa) demonstrated that the isostatic press delivered consistent/reproducible pressure. Viscoelasticity of 15 % β-lg dispersions were unaffected by pressures up to 550 MPa. 600 MPa (15 % β-lg) resulted in significant structural changes (FTIR) that correlated to changes in the viscoelasticity. A true β-lg gel was only formed after applying 650 MPa to a dispersion of 20 % concentration. The β-lg (15 %) HP-induced gels did not follow the classic structural changes of thermal induced gel formation which involve extensive changes in the native secondary structure of the protein accompanied by an important formation of anti-parallel intermolecular β-sheets; instead, HP-induced gels resulted in a significant drop in the intensity of intramolecular β-sheet accompanied by an increase in α-helices and random coil structures. At 15 % β-lg concentration gel formation appeared to result from self-association of the modified proteins with each other and the surrounding solvent. Mathematical models fit the viscoelastic and structural information and serve to describe the behavior of the data, ultimately serving as a prediction tool for rheology or the degree of protein unfolding: the exponential model described reasonably well the higher end pressure treatments (500-650 MPa) for prediction of storage modulus; and the cubic model fitted the protein structural data related to β-structure pressure-induced formation.

b) Protein (β-lg) gels of comparable strength were obtained by heating dispersions (of > 20 % concentration) to 95 °C or applying 900 MPa pressure; but the heat-induced gels resulted somewhat stiffer than the pressure-induced ones. The pressure induced gels of 20 and 30 % β-lg did not follow the classical secondary structure changes of thermal induced gel mechanism which involve the creation of extensive intermolecular β-sheets in expense of other native coils, instead HP-induced gels resulted in only a modest generation of intermolecular β-sheets albeit a broad destruction of native structures. For the heat-induced gelation, the mechanism involved the formation of intermolecular β-sheets in conjunction with creation of additional intermolecular disulfide bonds; this appears to explain the development of the 3D gel structure probed by gel stiffness and viscoelasticity measurements. Conversely, for the HP-induced gel formation the protein secondary structure changes (emergence of intermolecular β-sheets) does not completely explain the creation of the gel 3D network probed by an increased stiffness and mechanical parameters; but the augmented hydrophobic interactions due to internal molecular rearrangements which created different electronic densities in the molecule, should also be taken into account.

c) The secondary structure (from FTIR) of plasma proteins is especially sensitive to changes in pH as the pH of the solution approaches the p*I* of serum albumin (the principal component), with the globulin fraction appearing as more susceptible to changes in pH. At any pH, there was a destruction of the native structures as the temperature is increased with creation of intermolecular β-sheets. The thermal denaturation of plasma proteins was unaffected by changes in pH from 7.5 (physiological) to pH 6.0 (slightly acidic), further decreases in pH result in a higher susceptibility to conformational changes during heating. A decrease in pH at 30 °C provokes initially the denaturation of the globulin fraction while an increase in temperature appears to first affect α-helical domains (from 2D-FTIR), which are predominantly associated with the serum albumin fraction. So, both globulin fraction and serum albumin contribute to the aggregate/gel formation, although at different rates.

d) The dynamic rheology indicated a strong influence of soy protein concentration and pH on both elastic and viscous moduli. A protection effect of the salt and sugar

additives was observed against the increase of the viscoelastic parameters of the pH 7 short holding time (0.1 min) samples. A negative effect of the additives for the short holding time, but positive for long holding time (10 min) was observed at SPC dispersions of pH 3. The structure of the soybean proteins suffered limited changes after HP treatment (250 MPa): hydrophobicity increased, as well as the relative proportion of random coil, while the β-sheet content decreased. Finally, it was envisioned that HPP could be used to enhance the viscoelastic behavior of SPC, after which SPC can be used to enrich both protein content and textural properties of foods. A wide range of gel textures, which ultimately translates to mouth-feel, can be obtained with varying SPC concentration, pressure level, holding time and processing temperature, pH, and additives.

References

Abbott, T.P., Nabetani, H., Sessa, D.J., Wolf, W.J., Liebman, M.N. & Dukor, R.K. (1996). Effects of Bound Water on FTIR Spectra of Glycinin. Journal of Agricultural and Food *Chemistry*, 44(8), 2220-2224.

Adachi, M., Takenaka, Y., Gidamis, A.B., Mikami, B. & Utsumi, S. (2001). Crystal structure of soybean proglycinin A1aB1b homotrimer. *Journal of Molecular Biology*, 305(2), 291-305.

Ahmed, J. & Ramaswamy, H.S. (2003a). Effect of High-Hydrostatic Pressure and Temperature on Rheological Characteristics of Glycomacropeptide. *Journal of Dairy Science*, 86(5), 1535-1540.

Ahmed, J. & Ramaswamy, H.S. (2003b). Effect of hydrostatic pressure and temperature on rheological characteristics of alpha-lactalbumin. *Australian Journal of Dairy Technology*, 58(3), 233-237.

Ahmed, J. & Ramaswamy, H.S. (2004). Effect of high-hydrostatic pressure and concentration on rheological characteristics of xanthan gum. *Food Hydrocolloids*, 18(3), 367-373.

Ahmed, J. & Ramaswamy, H.S. (2005). Rheology of xanthan gum: effect of concentration, temperature and high-pressure. *Journal of Food Science & Technology -Mysore*, 42(4), 355-358.

Ahmed, J. & Ramaswamy, H.S. (2006a). Changes in colour during high pressure processing of fruits and vegetables. *Stewart Postharvest Review*, 5(9), 1-8.

Ahmed, J. & Ramaswamy, H.S. (2006b). High pressure processing of fruits and vegetables. *Stewart Postharvest Review*, 1(8), 1-10.

Ahmed, J. & Ramaswamy, H.S. (2007). Dynamic rheology and thermal transitions in meat based strained baby foods. *J. Food Engineering*, 78(4), 1274-1284.

Ahmed, J., Ayad, A., Ramaswamy, H.S., Alli, I. & Shao, Y. (2007). Dynamic viscoelastic behavior of high pressure treated soybean protein isolate dispersions. *Int. J. Food Properties*, 10(2), 397-411.

Ahmed, J., Ramaswamy, H.S. & Alli, I. (2006). Thermorheological characteristics of soybean protein isolate. *Journal of Food Science*, 71(3), E158-E163.

Ahmed, J., Ramaswamy, H.S. & Hiremath, N. (2005). Effect of high-hydrostatic pressure and temperature on rheological characteristics of mango puree. *International Journal Food Science & Technology*, 40, 885-895.

Ahmed, J., Ramaswamy, H.S., Alli, I. & Ngadi, M. (2003). Effect of high pressure on rheological characteristics of liquid egg. *Lebensm-Wiss Technol*. 36(5), 517-524.

Ahmed, J., Ramaswamy, H.S., Ayad, A., Alli, I. & Alvarez, P. (2007). Effect of high-pressure treatment on rheological, thermal and structural changes in Basmati rice flour slurry. *Journal of Cereal Science*, 46(2), 148-156.

Ahmed, J., Ramaswamy, H.S. & Hiremath, N. (2005). The effect of high pressure treatment on rheological characteristics and colour of mango pulp. *International Journal of Food Science & Technology*, 40(8), 885-895.

Allain, A.F., Paquin, P. & Subirade, M. (1999). Relationships between conformation of β-latoglobulin in solution and gel states as revealed by attenuated total reflection Fourier transform infrared spectroscopy. *International Journal of Biological Macromolecules*, 26(3), 337-344.

Alomirah, H.F., Alli, I. & Konishi, Y. (2000). Applications of Mass Spectrometry to Food Proteins and Peptides. *Journal of Chromatography A*, 893, 1-21.

Alting, A.C., Hamer, R.J., deKruif, C.G., Paques, M. & Visschers, R.W. (2003). Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels. *Food Hydrocolloids*, 17(4), 469-479.

Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. (2007). Effect of high pressure treatment on the electrospray ionization mass spectrometry (ESI-MS) profiles of whey proteins. *International Dairy Journal*, 17, 881-888.

Alvarez, P.A., Ramaswamy, H.S. & Ismail, A.A. (2008). High pressure gelation of soy proteins: Effect of concentration, pH and additives. *Journal of Food Engineering*, 88, 331-340.

Anderegg, R.J. & Wagner, D.S. (1995). Mass Spectrometric Characterization of a Protein-Ligand Interaction. *J Am Chem Soc*, 117, 1374-1377.

Anderson, R.L. & Wolf, W.J. (1995). Compositional Changes in Trypsin Inhibitors, Phytic Acid, Saponins and Isoflavones Related to Soybean Processing. *Journal of Nutrition*, 125(3), 581-588.

Apichartsrangkoon, A. & Ledward, D.A. (2002). Dynamic viscoelastic behaviour of high pressure treated gluten-soy mixtures. *Food Chemistry*, 77(3), 317-323.

Apichartsrangkoon, A. (2003). Effects of high pressure on rheological properties of soy protein gels. *Food Chemistry*, 80(1), 55-60.

Arakawa, T. & Timasheff, S.N. (1985). Theory of protein solubility. *Methods in Enzymology*, 114, 49-77.

Arora, A., Chism, G.W. & Shellhammer, T.H. (2003). Rheology and stability of acidified food emulsions treated with high pressure. *Journal of Agricultural Food Chemistry*, 51(9), 2591-2596.

Arrondo, J.L.R. & Goñi, F.M. (1999). Structure and dynamics of membrane proteins as studied by infrared spectroscopy. *Progress in Biophysics and Molecular Biology*, 72(4), 367-405.

Ashie, I.N.A., Simpson, B.K. & Ramaswamy, H.S. (1996a). Changes in texture and microstrusture of pressure-treated fish muscle during chilled storage. *J. Muscle Foods*, 8(1), 13-32.

Ashie, I.N.A., Simpson, B.K. & Ramaswamy, H.S. (1996b). Control of endogenous enzyme activity in fish muscle by inhibitors and hydrostatic pressure using RSM. *J. Food Sci*, 61(2), 350-356.

Babajimopoulos, M., Damodaran, S., Rizvi, S.S.H. & Kinsella, J.E. (1983). Effects of various anions on the rheological and gelling behavior of soy proteins: thermodynamic observations. *Journal of Agricultural and Food Chemistry*, 31(6), 1270-5.

Babu, K.R., Moradian, A. & Douglas, D.J. (2000). The Methanol-Induced Conformational Transitions of β-Lactoglobulin, Cytochrome c, and Ubiquitin at Low pH : A Study by Electrospray Ionization Mass Spectrometry. *Am Soc Mass Spectrom*, 12, 317-328.

Baier, S.K. & McClements, D.J. (2005). Influence of cosolvent systems on the gelation mechanism of globular protein: thermodynamic, kinetic, and structural aspects of globular protein gelation. *Comprehensive Reviews in Food Science and Food Safety*, 4(3), 43-54.

Baker, E.N. & Hubbard, R.E. (1984). Hydrogen bonding in globular proteins. *Progress in Biophysics and Molecular Biology*, 44(2), 97-179.

Balasubramanian, S. & Balasubramaniam, V.M. (2003). Compression heating influence of pressure transmitting fluids on bacteria inactivation during high pressure processing. *Food Research International*, 36(7), 661-668.

Barnes, H.A. (1999). The yield stress — a review or 'π❄υτ❄ ρει' — everything flows?. *J Non-Newtonian Fluid Mech*, 81, 133-178.

Basak, S. & Ramaswamy, H.S. (1997). Ultra high pressure treatment of orange juice: a kinetic study on inactivation of pectin methyl estease. *Food Res. Int.*, 29(7), 601-608.

Basak, S. & Ramaswamy, H.S. (1998). Effect of high hydrostatic pressure processing (HPP) on the texture of selected fruits and vegetebles. *J. Texture Studies*, 29, 587-601.

Basak, S. & Ramaswamy, H.S. (2001). Pulsed high pressure inactivation of pectin methyl esterase in single strength and concentrated orange juices. *Canadian Biosystems Engineering*, 43(3), 25-29.

Basak, S., Ramaswamy, H.S. & Piette, G. (2002). High pressure destruction kinetics of *Leucononostoc mesenteroides* and *Sachharomyces cerevisiae* in single strength and concentrated orange juice. *Innovative Food Science and Emerging Technologies*, 3, 223-245.

Basak, S., Ramaswamy, H.S. & Simpson, B.K. (2001). High pressure inactivation of pectin methyl esterase in orange juice using combination treatments. J. *Food Biochemistry*, 25(6), 509-526.

Basak, S., Ramaswamy, H.S. & Smith, J.P. (2003). High-pressure kinetics of microorganisms in selected fruits and vegetables. *Journal of Food Technology*, 1(3), 142-149.

Bassette & Acosta, (1999). Composition of Milk Products. In Wong, N.P., Jenness, R., Keeney, M. & Marth, E.H. *Fundamentals of Dairy Chemistry*, Aspen Publishers, Gaithersburg, MA. 779 pp.

Baxter, I.A., Easton, K., Schneebeli, K. & Whitfield, F.B. (2005). High pressure processing of Australian navel orange juices: Sensory analysis and volatile flavor profiling. *Innovative Food Science & Emerging Technologies*, 6(4), 372-387.

Belloque, J. & Smith, G.M. (1998). Thermal denaturation of β–lactoglobulin. A ^{1}H-NMR study. *Journal of Agricultural and Food Chemistry*, 46(5), 1805-1813.

Bender, A.E. (1972). Processing damage to protein food. A review. *J. Food Technol.*, 7, 239-250.

Berk, Z. 1992. Technology of production of edible flours and protein products from soybeans. Food and Agriculture Organization of the United Nations: Agricultural Services Bulleting No. 97. Bernan Press, USA. 187 pp.

Boatright, W.L. & Hettiarachchy, N.S. (1995). Soy protein isolate solubility and surface hydrophobicity as affected by antioxidants. *Journal of Food Science*, 60(4), 798-800.

Borwankar, R.P. (1992). Food texture and rheology: a tutorial review. *Journal of food engineering*, 16(1-2), 1-16.

Bos, M.A. & van Vliet, T. (2001). Interfacial rheological properties of adsorbed protein layers and surfactants: a review. *Advances in Colloid and Interface Science*, 91(3), 437-471.

Bouraoui, M., Nakai, S. & Li-Chan, E. (1997). *In situ* investigation of protein structure in Pacific whiting surimi and gels using Raman spectroscopy. *Food Research International*, 30(1), 65-72.

Bourne, M.C. (1967). Deformation Testing of Foods. *Journal of Food Science*, 32(5), 601-605.

Boye, J. I., Alli, I., Ismail, A.A., Gibbs, B.F. & Konishi, Y. (1995). Factors affecting molecular characteristics of whey protein gelation. *International Dairy Journal*, 5, 337-353.

Boye, J.I., Ismail, A.A. & Alli, I. (1996). Effects of physicochemical factors on the secondary structure of β-lactoglobulin. *Journal of Dairy Research*, 63, 97-109.

Bull, M.K., Szabo, E.A., Cole, M.B. & Stewart, C.M. (2005). Toward Validation of Process Criteria for High-Pressure Processing of Orange Juice with Predictive Models. *Journal of Food Protection*, 68(5), 949-954.

Butz, P., Serfert, Y., Fernandez García, A., Dieterich, S., Lindauer, R., Bognar, A. & Tauscher, B. (2004). Influence of high-pressure treatment at 25 °C and 80 °C on folates in orange juice and model media. *Journal of Food Science*, 69(3), SNQ117-SNQ121.

Byler, D.M., & Susi, H. (1986). Examination of the secondary structure of protein by deconvolved FTIR spectra. *Biopolymers*, 25(3), 469-487.

Campbell, G.M. & Mougeot, E. (1999). Creation and characterisation of aerated food products. *Trends in Food Science & Technology*, 10(9), 283-296.

Careche, M. & Li-Chan, E.C.Y. (1997). Structural changes in cod myosin after modification with formaldehyde or frozen storage. *Journal of Food Science*, 62, 717-723.

Carter, D.C., & Ho, J.X. (1994). Structure of serum albumin. *Advances in Protein Chemistry*, 45, 153-203.

Catsimpoolas, N. & Meyer, E.W. (1970). Gelation phenomena of soybean globulins 1. Protein-protein interactions. *Cereal Chemistry*, 47(5), 559-570.

Catsimpoolas, N. (1969). A note on the proposal of an immunochemical system of reference and nomenclature for the major soyabean globulins, *Cereal Chemistry*, 46, 369-372.

Cavanagh, J., Fairbrother, W.J., Palmer, A.G. & Skelton, N.J. (1996). Protein NMR spectroscopy, principles and practice. Academic Press, San Diego, Ca. 587 pp.

Chan, A.W., Hutchinson, E.G., Harris, D. & Thornton, J.M. (1993). Identification, classification, and analysis of beta-bulges in proteins. *Protein Sci.*, 2(10), 1574-1590.

Cheftel, J.C. & Culioli, J. (1997). Effects of high pressure on meat: A review. *Meat Science*, 46(3), 211-236.

Chen, C.R., Zhu, S.M., Ramaswamy, H.S., Marcotte, M. & Le Bail, A. (2007). Computer simulation of high pressure cooling of pork. *J. Food Engineering*, 79(2), 401-409.

Chen, R.H., Ker, Y.C. & Wu, C.S. (1990). Temperature and shear rate affecting the viscosity and secondary structural changes of soy 11S globulin measured by a cone-plate viscometer and Fourier transform infrared spectroscopy. *Agricultural and Biological Chemistry*, 54(5), 1165-76.

Chicón, R., Belloque, J., Alonso, E. & López-Fandiño, R. (2008). Immunoreactivity and digestibility of high-pressure-treated whey proteins. *International Dairy Journal*, 18(4), 367-376.

Chicón, R., Belloque, J., Recio, I. & López-Fandiño, R. (2006). Influence of high hydrostatic pressure on the proteolysis of b-lactoglobulin A by trypsin. *Journal of Dairy Research*, 73, 121-128.

Clark, A.H., Kavanagh, G.M. & Ross-Murphy, S.B. (2001). Globular protein gelation-theory and experiment. *Food Hydrocolloids*, 15(4-6), 383-400.

Clark, A.H., Saunderson, D.H.P. & Suggett, A. (1981). Infrared and laser-Raman spectroscopic studies of thermally-induced globular proteins. *International Journal of Peptide and Protein Research*, 17(3), 353-364.

Clery-Barraud, C., Gaubert, A., Masson, P. & Vidal, D. (2004). Combined effects of high hydrostatic pressure and temperature for inactivation of *Bacillus anthracis* spores. *Applied and Environmental Microbiology*, 70, 635-637.

Cooper, D.K.C. (2003). Porcine red blood cells as a source of blood transfusion in humans. *Xenotransplantation*, 10(5), 383-386.

Cruz-Romero, M., Kelly, A.L. & Kerry, J.P. (2007). Effects of high-pressure and heat treatments on physical and biochemical characteristics of oysters (*Crassostrea gigas*). *Innovative Food Science and Emerging Technologies*, 8, 30-38.

Cunsolo, V., Foti, S., Saletti, R., Ceraulo, L. & Di Stefano, V. (2001). Detection and Localization of Disulphide Bonds in a Synthetic Peptide Reproducing the Sequence 1-30 of Par j 1.0101 by Electrospray Ionisation Mass Spectrometry. *Proteomics*, 1, 1043-1048.

Dalgleish, D.G. (1997). Adsorption of protein and the stability of emulsions. *Trends in food science & technology*, 8(1), 1-6.

Damaschun, G., Damaschun, H., Fabian, H., Gast, K., Kröber, R., Wieske, M. & Zirwer, D. (2000). Conversion of yeast phosphoglycerate kinase into amyloid-like structure. *Proteins*, 39(3), 204-211.

Darby, N.J. & Creighton, T.E. (1993). Protein structure. IRL Press at Oxford University Press, Oxford. 99 pp.

Dàvila, E., Parés, D. & Howell, N. (2007). Studies on plasma protein interactions in heat-induced gels by Differential Scanning Calorimetry and FT-Raman spectroscopy. *Food Hydrocolloids*, 21(7), 1144-1152

de Jongh, H.H.J., Gröneveld, T. & de Groot, J. (2001). Mild Isolation Procedure Discloses New Protein Structural Properties of β-Lactoglobulin. *Journal of Dairy Science*, 84, 562–571.

Delacour, H., Clery, C., Masson, P. & Vidal, D.R. (2002). Inactivation of bacterial spores by high hydrostatic pressure. *Annales Pharmaceutiques Francaises*, 60, 38-43.

DePeters, E.J. & Cant, J.P. (1992). Nutritional Factors Influencing the Nitrogen Composition of Bovine Milk: A Review. *Journal of Dairy Science*, 75(8), 2043-2070.

Dickinson, E. & James, J.D. (1998). Rheology and flocculation of high-pressure-treated β-lactoglobulin-stabilized emulsions: comparison with thermal treatment. *Journal of Agricultural and Food Chemistry*, 46(7), 2565-2571.

Dickinson, E. (1999). Caseins in emulsions: interfacial properties and interactions. *International Dairy Journal*, 9(3-6), 305-312.

Domoulin, M., Ozawa, S. & Hayashi, R. (1998). Textural properties of pressure-induced gels of food proteins obtained under different temperatures including subzero. *Journal of Food Science*, 63(1), 92-95.

Dong, A., Matsuura, J., Manning, M.C., & Carpenter, J. F. (1998). Intermolecular β-sheet results from Trifluroethanol-induced nonnative α-helical structure in β-sheet predominant proteins: infrared and circular dichroism spectroscopic study. *Archives of Biochemistry and Biophysics,* 355(2), 275-281.

Douette, P., Navet, R., Bouillenne, F., Brans, A., Sluse-Goffart, C., Matagne, A. & Sluse, F.E. (2004). Secondary-structure characterization by far-UV CD of highly purified uncoupling protein 1 expressed in yeast. *Biochem J*, 380, 139-145.

Drickamer, H.G. (1999). Two applications of pressure tuning spectroscopy to processes in liquids. *Catalysis Today*, 53(3), 317-323.

Dumay, E., Picart, L., Regnault, S. & Thiebaud, M. (2006). High pressure–low temperature processing of food proteins. *Biochimica et Biophysica Acta - Proteins & Proteomics*, 1764(3), 599-618.

Dyson, H.J., Rance, M., Houghten, R.A., Lerner, R.A. & Wright, P.E. (1988). Folding of immunogenic peptide fragments of proteins in water solution I. Sequence requirements for the formation of a reverse turn. *J Mol Biol*, 201, 161-200.

Edman, P. (1950). Preparation of phenylthiohydantoins from some natural amino acids. *Acta Chem Scand*, 4, 277.

Fertsch, B., Muller, M. & Hinrichs, J. (2003). Firmness of pressure-induced casein and whey protein gels modulated by holding time and rate of pressure release. *Innovative Food Science & Emerging Technologies*, 4, 143-150.

Filosa, A., Wang, Y. & Ismail, A. (2001). Two-dimensional infrared correlation spectroscopy as a probe of sequential events in the thermal unfolding of cytochromes c. *Biochemistry*, 40(28), 8256-8263.

FitzGerald, R.J., Murray, B.A. & Walsh, D.J. (2004). Hypotensive peptides from milk proteins. *Journal of Nutrition*, 134, 980S-988S.

Foegeding, E.A. (2006). Food Biophysics of Protein Gels: A Challenge of Nano and Macroscopic Proportions. *Food Biophysics*, 1(1), 41-50.

Foegeding, E.A., Luck, P.J. & Davis, J.P. (2006). Factors determining the physical properties of protein foams. *Food Hydrocolloids*, 20(2-3), 284-292.

Francis, G., Makkar, H.P.S. & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199(3-4), 197-227.

Galazka, V.B., Sumner, I.G. & Ledward, D.A. (1996). Changes in protein-protein and protein-polysaccharide interactions induced by high pressure. *Food Chemistry*, 57(3), 393-398.

Gallegos, C. & Franco, J.M. (1999). Rheology of food, cosmetics and pharmaceuticals. *Current Opinion in Colloid & Interface Science*, 4(4), 288-293.

Gao, Y., Shang, C., Saghai Maroof, M.A., Biyashev, R.M., Grabau, E.A., Kwanyuen, P., Burton, J.W. & Buss, G.R. (2007). A Modified Colorimetric Method for Phytic Acid Analysis in Soybean. *Crop Sci*, 47, 1797-1803.

Garcia-Graells, C., Hauben, K.J.A. & Michiels, C.W. (1998). High-Pressure Inactivation and Sublethal Injury of Pressure-Resistant *Escherichia coli* Mutants in Fruit Juices. *Appl Environ Microbiol*, 64(4), 1566-1568.

Garcia-Palazon, A., Suthanthangjai, W., Kajda, P. & Zabetakis, I. (2004). The effects of high hydrostatic pressure on β-glucosidase, peroxidase and polyphenoloxidase in red

raspberry (Rubus idaeus) and strawberry (Fragaria × ananassa). *Food Chemistry*, 88, 7-10.

Gilmanshin, R., Williams, S., Callender, R.H., Woodruff, W.H. & Dyer, R.B. (1997). Fast events in protein folding: relaxation dynamics of secondary and tertiary structure in native apomyoglobin. *Proceedings of the National Academy of Sciences*, 94(8), 3709-3713.

Grandori, R., Matecko, I. & Müller, N. (2002). Uncoupled Analysis of Secondary and Tertiary Protein Structure by Circular Dichroism and Electrospray Ionization Mass Spectrometry. *J Mass Spectrom*, 37, 191-196.

Gross, M. & Jaenicke, R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. *Eur J Biochem*, 15(2), 617-630.

Groß, P. & Ludwig, H. (1992). Pressure-temperature-phase diagram for the stability of bacteriophage T4. *High Pressure and Biotechnology*, 224, 57-59.

Hajós, G., Polgár, M. & Farkas, J. (2004). High-pressure effects on IgE immunoreactivity of proteins in a sausage batter. *Innovative Food Science & Emerging Technologies*, 5(4), 443-449.

Haris, P.I. & Chapman, D. (1995). The conformational analysis of peptides using Fourier transform IR spectroscopy. *Biopolymers*, 37(4), 251-263.

Hawley, S.A. & Mitchell, R.M. (1975). An electrophoretic study of reversible protein denaturation: chymotrypsinogen at high pressures. *Biochemistry*, 14(14), 3257-3264.

Hawley, S.A. (1971). Reversible pressure-temperature denaturation of chymotrypsinogen. *Biochemistry*, 13(10), 2436-2442.

Hayakawa, I., Kajihara, J., Morikawa, K., Oda, M. & Fujio, Y. (1992). Denaturation of bovine serum albumin (BSA) and ovalbumin by high pressure, heat and chemicals. *Journal of Food Science*, 57(2), 288-292.

Hayashi, R., Kawamura, Y., Nakasa, T. & Okinaka, O. (1989). Application of high pressure to food processing: Presurization of egg white and yolk and properties of gels formed. *Agric. Biol. Chem.*, 53(11), 2935-2939.

Hayman, M.M., Baxter, I., O'Riordan, P.J. & Stewart, C.M. (2004). Effects of high-pressure processing on the safety, quality, and shelf-life of ready-to-eat meats. *Journal of Food Protection*, 67, 1709-1718.

Hazra, P., Chakrabarty, D., Chakraborty, A. & Sarkar, N. (2004). Probing protein-surfactant interaction by steady state and time-resolved fluorescence spectroscopy. *Biochemical and biophysical research communications*, 314(2), 543-549.

He, H., Adams, R.M., Farkas, D.F. & Morrissey, M.T. (2002). Use of high-pressure processing for oyster shucking and shelf-life extension. *Journal of Food Science*, 67(2), 640-645.

Heine, W.E., Klein, P.D. & Reeds, P.J. (1991). The importance of alpha-lactalbumin in infant nutrition. *J Nutr*, 121(3), 277-283.

Heremans, K. & Smeller, L. (1997). Pressure versus temperature behaviour of proteins, *Eur. J. Solid State Inorg. Chem.*, 34, 745-758.

Heremans, K. & Smeller, L. (1998). Protein structure and dynamics at high pressure. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1386(2), 353-370.

Heremans, K. & Wong, P.T.T. (1985). Pressure effects on the Raman spectra of proteins: Pressure induced changes in the conformation of lysozyme in aqueous solutions. *Chemical Physics Letters*, 118(1), 101-104.

Hermansson, A.M. (1982). Gel characteristics - structure as related to texture and waterbinding of blood-plasma gels. *Journal of Food Science*, 47(6), 1965-1972.

Hite, B.H. (1899). The effect of pressure in the preservation of milk. *Bulletin of West Virginia University of Agriculture Experimental Station Morgantown*, 58, 15-35.

Hite, B.H., Giddings, N.J. & Weakly, C.E. (1914). The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables, *Bulletin of West Virginia University of Agriculture Experimental Station Morgantown*, 146, 1-67.

Hogan, E., Kelly, A.L. & Sun, D-W. (2005). High pressure processing of foods: an overview, in Sun, D-W. (Ed.), *Emerging technologies for food processing* (pp. 3-32). Elsevier Academic Press, San Diego, CA.

Hong, S.I. & Kim, D.M. (2001). Storage quality of chopped garlic as influenced by organic acids and high-pressure treatment. *Journal of the Science of Food and Agriculture*, 81, 397-403.

Hong, Y-H. & Creamer, L.K. (2002). Changed protein structures of bovine β-lactoglobulin B and α-lactalbumin as a consequence of heat treatment. *International Dairy Journal,* 12(4), 345-359.

Hooke, S.D., Eyles, S.J., Miranker, A., Radford, S.E., Robinson, C.V. & Dobson, C.M. (1995). Cooperative Elements in Protein Folding Monitored by Electrospray Ionization Mass Spectrometry. *J Am Chem Soc*, 117, 7548-7549.

Hosseini-Nia, T., Ismail, A.A. & Kubow, S. (1999). Pressure-induced conformational changes of β-lactoglobulin by variable-pressure Fourier transform infrared spectroscopy. *Journal of Agricultural Food Chemistry*, 47, 4537-4542.

Hosseini-Nia, T., Kubow, S. & Ismail, A.A. (2001). Hyperbaric pressurization of proteins and therapeutical uses of pressurized proteins thereof. Patent Cooperation Treaty (PCT) International Application. PCT/CA2001/000031.

Houška, M., Strohalm, J., Kocurová, K., Totušek, J., Lefnerová, D., Tříska, J., Vrchotová, N., Fiedrleová, V., Holasova, M., Gabrovská, D. & Paulíčková, I. (2006). High pressure and foods-fruit/vegetable juices. *Journal of Food Engineering*, 77(3), 386-398.

Howell, N.K. & Lawrie, R.A. (1984). Functional aspects of blood plasma proteins. II. Gelling properties. *Journal of Food Technology*, 19(3), 289-295.

Hsu, S. (1999). Rheological studies on gelling behavior of soy protein isolates. *Journal of Food Science*, 64(1), 136-140.

Hua, Y., Cui, S.W., Wang, Q., Mine, Y. & Poysa, V. (2005). Heat induced gelling properties of soy protein isolates prepared from different defatted soybean flours. *Food Research International*, 38(4), 377-385.

Hugas, M., Garriga, M. & Monfort, J. M. (2002). New mild technologies in meat processing: high pressure as a model technology. *Meat Science*, 62(3), 359-371.

Iametti, S., Transidico, P., Bonomi, F., Vecchio, G., Pittia, P., Rovere, P. & Dall'Aglio, G. (1997). Molecular modifications of β-lactoglobulin upon exposure to high pressure**.** *Journal of Agricultural and Food Chemistry*, 45, 23-29.

Ibanoglu, E. & Karatas, S. (2001). High pressure effect on foaming behaviour of whey protein isolate. *Journal of Food Engineering*, 47(1), 31-36.

Ikeuchi, Y., Nakagawa, K., Endo, T., Suzuki, A., Hayashi, T. & Ito, T. (2001). Pressure-induced denaturation of monomer β-lactoglobulin is partially irreversible: comparison of monomer form (highly acidic pH) with dimer form (neutral pH). *J. Agric. Food Chem*., 49(8), 4052-4059.

Irwe, S. & Olsson, I. (1994). Reduction of pectinesterase activity in orange juice by high pressure treatment, in Singh, R.P. & Oliveira, F.A.R. (Eds), *Minimal processing of foods and process optimization - an interface* (pp. 35-42). CRC Press, Boca Raton, FL.

Ismail, A.A., Cocciardi, R.A., Alvarez, P. & Sedman, J. (2005). Infrared and Raman Spectroscopy in Food Science, in Hui, Y.H. (Ed.), *Handbook of Food Science, Technology, and Engineering*, Marcel Dekker, New York, NY, 3488 pp.

Ismail, A.A., Mantsch, H.H., & Wong, P.T.T. (1992). Aggregation of chymotrypsinogen: portrait by infrared spectroscopy. *Biochimica et Biophysica Acta, Protein Structure and Molecular Enzymology*, 1121(1-2), 183-188.

Ismoyo, F., Wang, Y. & Ismail, A.A. (2000). Examination of the effect of heating on the secondary structure of avidin and avidin-biotin complex by resolution-enhanced two-dimensional correlation spectroscopy. *Applied Spectroscopy*, 54(7), 939-947.

Izquierdo, F.J., Alli, I., Gómez, R., Ramaswamy, H.S. & Yaylayan, V. (2005). Effects of high pressure and microwave on pronase and α-chymotrypsin hydrolysis of β-lactoglobulin. *Food Chemistry*, 92(4), 713-719.

Jackson, M. & Mantsch, H.H. (1995). The Use and Misuse of FTIR Spectroscopy in the Determination of Protein Structure. *Critical Reviews in Biochemistry and Molecular Biology*, 30(2), 95-120.

Jenness, R. (1980). Composition and Characteristics of Goat Milk: Review 1968–1979. *Journal of Dairy Science*, 63(10), 1605-1630.

Jenness, R. (1999). Composition of Milk. In Wong, N.P., Jenness, R., Keeney, M. & Marth, E.H. *Fundamentals of Dairy Chemistry*, Aspen Publishers, Gaithersburg, MA. 779 pp.

Johnson, W.C.Jr. (1990). Protein secondary structure and circular dichroism: A practical guide. *Proteins: Structure, Function, and Bioinformatics*, 7(3), 205-214.

Jonas, J. & Jonas, A. (1994). High-pressure NMR spectroscopy of proteins and membranes. *Annu. Rev. Biophys. Biomol. Struct.*, 23, 287-318.

Jonas, J., Ballard, L. & Nash, D. (1998). High-resolution, high pressure NMR studies of proteins. *Biophysical Journal*, 75(1), 445-452.

Jost, R. (1993). Functional characteristics of dairy proteins. *Trends in Food Science & Technology*, 4, 283-288.

Jung, S., de Lamballerie-Anton, M., Taylor, R.G. & Ghoul, M. (2000). High-Pressure Effects on Lysosome Integrity and Lysosomal Enzyme Activity in Bovine Muscle. *Journal of Agricultural and Food Chemistry*, 48, 2467-2471.

Kabsch, W. & Sander, C. (1983). Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers*, 22(12), 2577-2637.

Kanno, C., Mu, T.H., Hagiwara, T., Ametani, M. & Azuma, N. (1998). Gel formation from industrial milk whey proteins under hydrostatic pressure: effect of hydrostatic

pressure and protein concentration. *Journal of Agricultural and Food Chemistry*, 46, 417-424.

Kato, T., Katayama, E., Matsubara, S., Omi, Y. & Matsuda, T. (2000). Release of allergenic proteins from rice grains induced by high hydrostatic pressure. *Journal of Agricultural Food Chemistry*, 48, 3124-3129.

Kauzmann, W. (1987). Thermodynamics of unfolding. *Nature*, 325, 763-764.

Keentok, M. (1982). The measurement of the yield stress of liquids. *Rheol Acta*, 21, 325-332.

Keim, S. & Hinrichs, J. (2004). Influence of stabilizing bonds on the texture properties of high-pressure-induced whey protein gels. *International Dairy Journal*, 14, 355-363.

Kelly, S.M. & Price, N.C. (2000). The Use of Circular Dichroism in the Investigation of Protein Structure and Function. *Current Protein and Peptide Science*, 1(4), 349-384.

Kester, J.J. & Richardson, T. (1984). Modification of Whey Proteins to Improve Functionality. *Journal of Dairy Science*, 67(11), 2757-2774.

Kimura, K., Ida, M., Yosida, Y., Ohki, K., Fukumoto, T. & Sakui, N. (1994). Comparison of keeping quality between pressure-processed jam and heatprocessed jam: changes in flavor components, hue, and nutrients during storage. *Bioscience, Biotechnology and Biochemistry*, 58(8), 1386–1391.

Kinsella, J.E., Patton, S. & Dimick, P.S. (1967). The flavor potential of milk fat. A review of its chemical nature and biochemical origin. *Journal of the American Oil Chemists' Society*, 44(7), 449-454.

Kneifel, W., Paquin, P., Abert, T. & Richard, J-P. (1991). Water-Holding Capacity of Proteins with Special Regard to Milk Proteins and Methodological Aspects-A Review. *Journal of Dairy Science*, 74, 2027-2041.

Knorr, D. (1999). Novel approaches in food-processing technology: new technologies for preserving foods and modifying function. *Current Opinion in Biotechnology*, 10(5), 485-491.

Knorr, D., Heinz, V. & Buckow, R. (2006). High pressure application for food biopolymers. *BBA - Proteins & Proteomics*, 1764(3), 619-631.

Konermann, L. & Douglas, D.J. (1997). Acid-Induced Unfolding of Cytochrome c at Different Methanol Concentrations: Electrospray Ionization Mass Spectrometry Specifically Monitors Changes in the Tertiary Structure. *Biochemistry*, 36, 12296-12302.

Kontopidis, G., Holt, C. & Sawyer, L. (2004). Invited review: beta-lactoglobulin: binding properties, structure, and function. *J Dairy Sci*, 87, 785-96.

Koziński, M., Garrett-Roe, S. & Hamm, P. (2008). 2D-IR Spectroscopy of the Sulfhydryl Band of Cysteines in the Hydrophobic Core of Proteins. *The Journal of Physical Chemistry*, 112, 7645-7650.

Kunugi, S., Takano, K., Tanaka, N., Suwa, K. & Akashi, M. (1997). Effects of Pressure on the Behavior of the Thermoresponsive Polymer Poly(*N*-vinylisobutyramide) (PNVIBA). *Macromolecules*, *30*(15), 4499-4501.

Kural, A.G. & Chen, H. (2008). Conditions for a 5-log reduction of Vibrio vulnificus in oysters through high hydrostatic pressure treatment. *International Journal of Food Microbiology*, 122(1-2), 180-187.

Lakshmanan, R., Patterson, M.F. & Piggott, J.R. (2005). Effects of high-pressure processing on proteolytic enzymes and proteins in cold-smoked salmon during refrigerated storage. *Food Chemistry*, 90(4), 541-548.

Laligant, A., Dumay, E., Casas-Valencia, C., Cuq, J.L. & Cheftel, J.C. (1991). Surface hydrophobicity and aggregation of β-lactoglobulin heated near neutral pH. *Journal of Agricultural and Food Chemistry*, 39, 2147-2155.

Lebowitz, J., Lewis, M.S. & Schuck, P. (2002). Modern analytical ultracentrifugation in protein science: A tutorial review. *Protein Science*, 11, 2067-2079.

Lee, J.W., Cha, D.S., Hwang, K.T. & Park, H.J. (2003). Effects of CO_2 absorbent and high-pressure treatment on the shelf-life of packaged kimchi products. *International Journal of Food Science and Technology*, 38, 519-524.

Lee, J.Y. & Hirose, M. (1989). Gelation of porcine plasma by glutathione. *Agricultural and Biological Chemistry*, 53(10), 2839-2840.

Lefevre, T. & Subirade, M. (1999). Structural and interaction properties of β-Lactoglobulin as studied by FTIR spectroscopy. *International Journal of Food Science and Technology*, 34, 419-428.

Lefevre, T. & Subirade, M. (2000). Molecular differences in the formation and structure of Fine-Stranded and Particulate β-lactoglobulin gels. *Biopolymers*, 54, 578-586.

Lefevre, T. & Subirade, M. (2001). Molecular structure and interaction of biopolymers as viewed by Fourier transform infrared spectroscopy: model studies on β-lactoglobulin. *Food Hydrocolloids*, 15, 365-376.

Li-Chan, E.C.Y. (1996). The Applications of Raman Spectroscopy in Food Science. *Trends in Food Science Technology*, 7, 361-370.

Light-Wahl, K.J., Schwartz, B.L. & Smith, R.D. (1994). Observation of the Noncovalent Quaternary Associations of Proteins by Electrospray Ionization Mass Spectrometry. *J Am Chem Soc*, 116, 5271-5278.

Light-Wahl, K.J., Winger, B.E. & Smith, R.D. (1993). Observation of the Multimeric Forms of Concanavalin A by Electrospray Ionization Mass Spectrometry. *J Am Chem Soc*, 115, 5869-5870.

Lim, S.-Y., Swanson, B.G. & Clark, S. (2008). High Hydrostatic Pressure Modification of Whey Protein Concentrate for Improved Functional Properties. *J Dairy Sci*, 91, 1299-1307.

Liu, R., Zhao, S., Xiong, S., Xie, B. & Liu, H. (2007). Studies on Fish and Pork Paste Gelation by Dynamic Rheology and Circular Dichroism. *Journal of Food Science*, 72 (7), E399–E403.

López-Caballero, M.E., Pérez-Mateos, M., Montero, P. & Bonderías, A.J. (2000). Oyster preservation by high-pressure treatment. *Journal of Food Protection*, 63, 196-201.

López-Fandiño, R. (2006). Functional improvement of milk whey proteins induced by high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition*, 46, 351-363.

López-Fandiño, R., Carrascosa, A.V. & Olano, A. (1996). The Effects of High Pressure on Whey Protein Denaturation and Cheese-Making Properties of Raw Milk. *Journal of Dairy Science*, 79, 929-936.

Low, B.W. & Grenville-Wells, H.J. (1953). Generalized Mathematical Relationships for Polypeptide Chain Helices. The Coordinates of the Π Helix. *Proceedings of the National Academy of Sciences of the United States of America*, 39(8), 785-802.

Lucey, J.A., Johnson, M.E. & Horne, D.S. (2003). Perspectives on the Basis of the Rheology and Texture Properties of Cheese. *J Dairy Sci*, 86, 2725-2743.

Lucey, J.A., Teo, C.T., Munro, P.A. & Singh, H. (1997). Rheological properties at small (dynamic) and large (yield) deformations of acid gels made from heated milk. *Journal of Dairy Research*, 64, 591-600.

Maltais, A., Remondetto, G.E., Gonzalez, R. & Subirade, M. (2005). Formation of soy protein isolate cold-set gels: protein and salt effects. *Journal of Food Science*, 70(1), C67-C73.

Marcos, B., Aymerich, T. & Garriga, M. (2005). Evaluation of high pressure processing as an additional hurdle to control Listeria monocytogenes and Salmonella enterica in low-acid fermented sausages. *Journal of Food Science*, 70(7), M339-M344.

Marcotte, M., Taherian Hoshahili, A.R. & Ramaswamy, H.S. (2001). Rheological properties of selected hydrocolloids as a function of concentration and temperature. *Food Research International*, 34(8), 695-703.

Matsumoto, T. & Inoue, H. (1991). Effect of heat denaturation on the structure and rheological properties of ovalbumin aqueous colloids. *Journal of the Chemical Society. Faraday Transactions,* 87(20), 3385-3388.

Matthews, D.M. & Laster, L. (1965). Absorption of protein digestion products: a review. *Gut*, 6(5), 411-426.

McClements, D.J. (2005). Food Emulsions Principles Practices and Techniques. CRC Press, Boca Raton, FL. 609 pp.

McMillan, P. (2005). Pressing on: The legacy of Percy W. Bridgman. *Nature Materials*, 4, 715-718.

Melse-Boonstra, A., Verhoef, P., Konings, E.J.M., Van Dusseldorp, M., Matser, A., Hollman, P.C.H., Meyboom, S., Kok, F.J. & West, C.E. (2002). Influence of processing on total, monoglutamate and polyglutamate folate contents of leeks, cauliflower, and green beens. *Journal of Agricultural and Food Chemistry*, 50(12), 3473-8.

Menard, K.P. (1999). Dynamic mechanical analysis: a practical introduction. CRC Press, Boca Raton, FL. 208 pp.

Mendel, L.B. & Fine, M.S. (1911). Utilization of Legume Proteins. *J Biol Chem*, 433-458.

Meng, G., Ma, C-Y. & Phillips, D.L. (2003). Raman spectroscopic study of globulin from *Phaseolus angularis* (red bean). *Food Chemistry*, 81(3), 411-420.

Messens, W., Van Camp, J. & Huyghebaert, A. (1997). The use of high pressure to modify the functionality of food proteins. *Trends in Food Science & Technology*, 8(4), 107-112.

Meyer-Pittroff, R., Behrendt, H. & Ring, J. (2007). Specific immuno-modulation and therapy by means of high pressure treated allergens. *International Journal of High Pressure Research*, 27(1), 63-67.

Mills, E.N.C., Marigheto, N.A., Wellner, N., Fairhurst, S.A., Jenkins, J.A., Mann, R. & Belton, P.S. (2003). Thermally induced structural changes in glycinin, the 11S globulin of soya bean (*Glycine max*)-an in situ spectroscopic study. *Biochimica et Biophysica Acta*, 1648(1-2), 105-114.

Mohamad, R.B.M.S., Maruyama, N., Takahashi, K., Yagasaki, K., Higasa, T., Matsumura, Y. & Utsumi, S. (2004). Gelling properties of soybean β-conglycinin

having different subunit compositions. *Bioscience, Biotechnology, and Biochemistry*, 68(5), 1091-1096.

Mohimen, A., Dobo, A., Hoerner, J.K. & Kaltashov, I.A. (2003). A Chemometric Approach to Detection and Characterization of Multiple Protein Conformers in Solution Using Electrospray Ionization Mass Spectrometry. *Analytical Chemistry*, 75, 4139-4147.

Molina, E. & Ledward, D.A. (2003). Effects of combined high-pressure and heat treatment on the textural properties of soya gels. *Food Chemistry*, 80(3), 367-370.

Molina, E., Alvarez, M.D., Ramos, M., Olano, A. & López-Fandiño, R. (2000). Use of high-pressure-treated milk for the production of reduced-fat cheese. *International Dairy Journal*, 10, 467-475.

Molina, E., Defaye, A.B. & Ledward, D.A. (2002). Soy protein pressure-induced gels. *Food Hydrocolloids*, 16(6), 625-632.

Molina, E., Papadopoulou, A. & Ledward, D.A. (2001). Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. *Food Hydrocolloids*, 15(3), 263-269.

Monahan, F.J., German, J.B. & Kinsella, J.E. (1995). Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *Journal of Agricultural and Food Chemistry*, 43(1), 46-52.

Motoki, M. & Seguro, K. (1998). Transglutaminase and its use for food processing. *Trends in Food Science & Technology*, 9(5), 204-210.

Mozhaev, V.V., Heremans, K., Frank, J., Masson, P. & Balny, C. (1994). Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends in Biotechnology*, 12, 493-501.

Murayama, K. & Tomida, M. 2004. Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry*, 43(36), 11526-11532.

Murchie, L.W., Cruz-Romero, M., Kerry, J.P., Linton, M., Patterson, M.F., Smiddy, M. & Kelly, A.L. (2005). High pressure processing of shellfish: A review of microbiological and other quality aspects, *Innovative Food Science and Emerging Technologies*, 6, 257-270.

Murray, B.S. (2002). Interfacial rheology of food emulsifiers and proteins. Current *Opinion in Colloid & Interface Science*, 7(5-6), 426-431.

Murray, B.S. (2007). Stabilization of bubbles and foams. *Current Opinion in Colloid & Interface Science*, 12(4-5), 232-241.

Mussa, D.M. & Ramaswamy, H.S. (1997). Ultra High Pressure Pasteurization of Milk: Kinetics of Microbial Destruction and Changes in Physico-chemical Characteristics. *Lebensmittel-Wissenschaft und-Technologie*, 30(6), 551-557.

Mussa, D.M., Ramaswamy, H.S. & Smith, J.P. (1999a). High pressure destruction kinetics of Listeria monocytogenes in milk. *Food Res. Int.*, 31(5), 343-350.

Mussa, D.M., Ramaswamy, H.S. & Smith, J.P. (1999b). Ultra high pressure destruction kinetics of Listeria monocytogenes in pork. *J. Food Protection*, 62(1), 165-170.

Nagano, T., Akasaka, T. & Nishinari, K. (1994). Dynamic viscoelastic properties of glycinin and β-conglycinin gels from soybeans. *Biopolymers*, 34(10), 1303-1309.

Nakamura, T., Sado, H. & Syukunobe, Y. (1993). Production of low antigenic whey protein hydrolysates by enzymatic hydrolysis and denaturation with high pressure. *Milchwissenschaft*, 48(3), 141-144.

Naumann, D., Schultz, Ch., Görne-Tschelnokow, U. & Hucho, F. (1993). Secondary structure and temperature behaviour or the acetylcholine receptor by Fourier transform infrared spectroscopy. *Biochemistry*, 32(12), 3162-3168.

Nguyen, Q.D. & Boger, D.V. (1992). Measuring the flow properties of yield stress fluids. *Ann Rev Fluid Mech*, 24, 47-88.

Niall, H.D. (1973). Automated Edman degradation: the protein sequenator. *Meth Enzymol*, 27, 942-1010.

Nielsen, L.E. & Landel, R.F. (1994). Mechanical properties of polymers and composites. CRC Press, Boca Raton, FL. 557 pp.

Nilsson, C.L., Cooper, H.J., Håkansson, K., Marshall, A.G., Östberg, Y., Lavrinovicha, M. & Bergström, S. (2002). Characterization of the P13 Membrane Protein of Borrelia burgdorferi by Mass Spectrometry. *J Am Soc Mass Spectrom*, 13, 295-299.

Noda, I. (1993). Generalized two-dimensional correlation method applied to infrared, Raman, and other types of spectroscopy. *Applied Spectroscopy*, 47(9), 1329-1336.

Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. & Bush, R.K. (1996). Identification of a Brazil-Nut Allergen in Transgenic Soybeans. *The New England Jounal of Medicine*, 334(11), 688-692.

O'Kane, F.E., Happe, R.P., Vereijken, J.M., Gruppen, H. & Van Boekel, M.A.J.S. (2004). Heat-induced gelation of pea legumin: Comparison with soybean glycinin. *Journal of Agricultural and Food Chemistry*, 52(16), 5071-5078.

Olsen, K., Ipsen, R., Otte, J. & Skibsted, L.H. (1999). Effect of high pressure on aggregation and thermal gelation of beta-lactoglobulin. *Milchwissenschaft-Milk Science International*, 54(10), 543-546.

O'Reilly, C., O'Connor, P., Murphy, P., Kelly, A. & Beresford, T. (2000). The effect of exposure to pressure of 50 MPa on Cheddar cheese ripening. *Innovative Food Sci. & Emerging Technologies*, 1(2), 109-117.

O'Reilly, C.E., Kelly, A.L., Oliveira, J.C., Murphy, P.M., Auty, M.A.E. & Beresford, T.P. (2003). Effect of varying high-pressure treatment conditions on acceleration of ripening of cheddar cheese. *Innovative Food Science and Emerging Technologies*, 4, 277-284.

O'Reilly, C.E., Murphy, P.M., Kelly, A.L., Guinee, T.P., Auty, M.A.E. & Beresford, T.P. (2002). The effect of high pressure treatment on the functional and rheological properties of Mozzarella cheese. *Innovative Food Science & Emerging Technologies*, 3(1), 3-9.

Osborne, T.B. & Campbell, G.F. (1898). Proteids of the soy bean. (*Glycine hispida*), *J. Am. Chem. Soc.*, *20*, 419-428.

Osborne, T.B. & Campbell, G.F. (1898). Proteins of the pea, *Journal of American Chemical Society*, 20, 348-362.

Ousegui, A., Zhu, S., Ramaswamy, H.S. & LeBail, A. (2006). Modelling of a high pressure calorimeter - Application to the measurement of the latent heat of a model food (tylose). *J. Thermal Analysis and Calorimetry*, 86(1), 243-248.

Palou, E., Hernández-Salgado, C., López-Malo, A., Barbosa-Cánovas, G.V., Swanson, B.G. & Welti-Chanes, J. (2000). High pressure-processed guacamole. *Innovative Food Science and Emerging Technologies*, 1(1), 69-75.

Pandey, P.K. & Ramaswamy, H.S. (2004). Effect of high pressure treatment of milk on lipase and γ-glutamyl transferase activity. *J. Food Biochem.*, 28, 449-462.

Pandey, P.K., Ramaswamy, H.S. & Idziak, E. (2003). High pressure destruction kinetics of indigenous microflora and Escherichia coli in raw milk at two temperatures. *J Food Process Eng*, 26(3), 265-283.

Pandey, P.K., Ramaswamy, H.S. & St-Gelais, D. (2000). Water-holding capacity and gel strength of rennet curd as affected by high-pressure treatment of milk. *Food Research International*, 33(8), 655-663.

Pandey, P.K., Ramaswamy, H.S. & St-Gelais, D. (2003a). Effect of high-pressure processing on rennet coagulation properties of milk. *Innovative Food Science and Emerging Technologies*, 4, 245-256.

Pandey, P.K., Ramaswamy, H.S. & St-Gelais, D. (2003b). Evaluation of pH change kinetics during various stages of Cheddar cheese-making from raw, pasteurized, micro-filtered and high-pressure-treated milk. *Lebensm.-Wiss. u. Technol.*, 36(5), 497-506.

Panick, G., Malessa, R. & Winter, R. (1999). Differences between the pressure- and temperature-induced denaturation and aggregation of β-lactoglobulin A, B, and AB

monitored by FT-IR spectroscopy and small-angle X-ray scattering. *Biochemistry*, 38, 6512–6519.

Panyam, D. & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology*, 7(4), 120-125.

Parés, D. & Ledward, D.A. (2001). Emulsifying and gelling properties of porcine blood plasma as influenced by high-pressure processing. *Food Chemistry*, 74(2), 139-145.

Parés, D., Saguer, E., Saurina, J., Suñol, J.J. & Carretero, C. (1998). Functional properties of heat induced gels from liquid and spray dried porcine blood plasma as influenced by pH. *Journal of Food Science*, 63(6), 958-961.

Parés, D., Saguer, E., Toldra, M. & Carretero, C. (2000). Effect of high pressure processing at different temperatures on protein functionality of porcine blood plasma. *Journal of food science*, 65(3), 486-490.

Patazca, E., Koutchma, T. & Ramaswamy, H.S. (2006). Inactivation kinetics of Geobacillus stearothermophilus spores in water using high-pressure processing at elevated temperatures. *Journal of Food Science*, 71, M110-M116.

Patterson, M.F. (2005). Microbiology of pressure treated foods. *Journal of Applied Microbiology*, 98(4), 541-548.

Pearson, A.M. & Dutson, T.R. (1988). Edible meat by-products. Elsevier Applied Science. London, UK.

Peñas, E., Préstamo, G. & Gomez, R. (2004). High pressure and the enzymatic hydrolysis of soybean whey proteins. *Food Chemistry*, 85(4), 641-648.

Permyakov, E.A. & Berliner, L.J. (2000). alpha-Lactalbumin: structure and function. *FEBS Lett*, 473(3), 269-274.

Peters, T. Jr. (1996). All about Albumin: Biochemistry, Genetics, and Medical Applications. Academic Press. New York, USA, pp: 9-75.

Phua, S.T.G. & Davey, K.R. (2007). Predictive modelling of high pressure (≤700 MPa)–cold pasteurisation (≤25 °C) of *Escherichia coli*, *Yersinia enterocolitica* and *Listeria*

monocytogenes in three liquid foods. *Chemical Engineering and Processing*, 46(5), 458-464.

Polydera, A.C., Stoforos, N.G. & Taoukis, P.S. (2003). Comparative shelf life study and vitamin C loss kinetics in pasteurised and high pressure processed reconstituted orange juice. *Journal of Food Engineering*, 60(1), 21-29.

Polydera, A.C., Stoforos, N.G. & Taoukis, P.S. (2005). Quality degradation kinetics of pasteurised and high pressure processed fresh Navel orange juice: Nutritional parameters and shelf life. *Innovative Food Science & Emerging Technologies*, 6(1), 1-9.

Prestamo, G. & Arroyo, G. (1999). Protective effect of ascorbic acid against the browning developed in apple fruit treated with high hydrostatic pressure. *Journal of Agricultural and Food Chemistry*, 47(9), 3541-3545.

Putnam, F.W. (1965). The heterogeneity of serum globulins (invited discussion). *Journal of Cellular and Comparative Physiology*, 47(S1), 17-20.

Qi, X.L., Holt, C., McNulty, D., Clarke, D.T., Bownlow, S. & Jones, G.R. (1997). Effect of temperature on the secondary structure of β–lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis. *Biochemical Journal*, 324(1), 341-346.

Qin, B.Y., Bewley, M.C., Creamer, L.K., Baker, H.M., Baker, E.N. & Jameson, G.B. (1998). Structural basis of the Tanford transition of bovine beta-lactoglobulin. *Biochemistry*, 37, 14014-14023.

Quirós, A., Chichón, R., Recio, I. & López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry*, 104(4), 1734-1739.

Ramaswamy, H.S. & Riahi, E. (2003). High-pressure inactivation kinetics of polyphenoloxidase in apple juice. *Applied Biotechnology, Food Science and Policy*, 1(3), 189-197.

Ramaswamy, H.S., Riahi, E. & Idziak, E. (2003). High-pressure destruction kinetics of E-coli(29055) in apple juice. *J FOOD SCI*, 68(5), 1750-1756.

Ramos-Clamont, G., Fernández-Michel, S., Carrillo-Vargas, L., Martínez-Calderón, E. & Vázquez-Moreno, L. (2003). Functional properties of protein fractions from porcine blood, *Journal of Food Science*, 68(4), 1196-1200.

Rao, M.A. (1977). Rheology of Liquid Foods - A Review. *Journal of Texture Studies*, 8(2), 135-168.

Raso, J. & Barbosa-Cánovas, G.V. (2003). Nonthermal Preservation of Foods Using Combined Processing Techniques. *Critical Reviews in Food Science and Nutrition*, 43(3), 265-285.

Rassis, D. & Saguy, I.S. (1995). Kinetics of aseptic concentrated orange juice quality changes during commercial processing and storage. *International Journal of Food Science & Technology*, 30(2), 191-198.

Rastogi, N., Raghavarao, K., Balasubramaniam, V., Niranjan, K. & Knorr, D. (2007). Opportunities and Challenges in High Pressure Processing of Foods. *Critical Reviews in Food Science & Nutrition*, 47(1), 69-112.

Reisdorf, W.C. Jr. & Krimm, S. (1996). Infrared amide I' band of the coiled coil. *Biochemistry*, 35(5), 1383-1386.

Rejaei, A.R., Ismail, A.A., Ramaswamy, H.S. & Jézéquel, V. (1996). Corrélation des spectres infra-rouge de gels protéiques aux mesures rhéologiques de la force de ces gels. *Ind. Aliment. Agric.*, 133, 858-863.

Renkema, J.M.S. & van Vliet, T. (2004). Concentration dependence of dynamic moduli of heat-induced soy protein gels. *Food Hydrocolloids*, 18(3), 483-487.

Renkema, J.M.S., Knabben, J.H.M. & van Vliet, T. (2001). Gel formation by β-conglycinin and glycinin and their mixtures. *Food Hydrocolloids*, 15(4), 407-414.

Riahi, E. & Ramaswamy, H.S. (2003). High-pressure processing of apple juice: Kinetics of pectin methyl esterase inactivation. *BIOTECHNOL PROGR*, 19(3), 908-914.

Riahi, E. & Ramaswamy, H.S. (2004). High pressure inactivation kinetics of amylase in apple juice. *Journal of Food Engineering*, 64(2), 151-160.

Riahi, E., Ramaswamy, H.S. & Idziak, E. (2003). High-pressure destruction kinetics of Leuconostoc mesenteroides, Pichia membranaefaciens and Zygosaccharomyces bailii in apple juice. *Applied Biotechnology, Food Science and Policy*, 1(1), 47-54.

Richards, F.M. (1977). Areas, Volumes, Packing, and Protein Structure. *Annual Review of Biophysics and Bioengineering*, 6, 151-176.

Richardson, JS. (1981). The anatomy and taxonomy of protein structure. *Adv Protein Chem*, 34, 167-339.

Ritz, M., Tholozan, J.L., Federighi, M. & Pilet, M.F. (2002). Physiological damages of Listeria monocytogenes treated by high hydrostatic pressure. *International Journal of Food Microbiology*, 79, 47-53.

Rodiles-López, J.O., Jaramillo-Flores, M.E., Gutiérrez-López, G.F., Hernández-Arana, A., Fosado-Quiroz, R.E., Barbosa-Cánovas, G.V. & Hernández-Sánchez, H. (2008). Effect of high hydrostatic pressure on bovine α-lactalbumin functional properties. *Journal of Food Engineering*, 87(3), 363-370.

Ross, J.B., Szabo, A.G. & Hogue, C.W. (1997). Enhancement of protein spectra with tryptophan analogs: fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. *Methods Enzymol*, 278, 151-190.

Rouille, J., LeBail, A., Ramaswamy, H.S. & LeClerc, L. (2002). High pressure thawing of fish and shellfish. *J FOOD ENG*, 53(1), 83-88.

Royer, C.A., Hinck, A.P., Loh, S.N., Prehoda, K.E., Peng, X., Jonas, J. & Markley, J.L. (1993). Effects of amino acid substitutions on the pressure denaturation of staphylococcal nuclease as monitored by fluorescence and nuclear magnetic resonance spectroscopy. *Biochemistry*, 32, 5222-5232.

Rubens, P., Snauwaert, J., Heremans, K. & Stute, R. (1999). In situ observation of pressure-induced gelation of starches studied with FTIR in the diamond anvil cell. *Carbohydrate Polymers*, 39, 231-235.

Rule, G.S. & Hitchens, T.K. (2006). Fundamentals of protein NMR spectroscopy (focus on Structural Biology). Springer, Berlin, 530 pp.

Saguer, E., Fort, N., Alvarez, P., Sedman, J. & Ismail, A.A. (2008). Structure-functionality relationships of porcine plasma proteins probed by FTIR spectroscopy and texture analysis. *Food Hydrocolloids*, 22(3), 459-467.

Saldo, J., Sendra, E. & Guamis, B. (2000). High hydrostatic pressure for accelerating ripening of goat's milk cheese: proteolysis and texture. *Journal of Food Science*, 65(4), 636-640.

San Martín, M.F., Barbosa-Cánovas, G.V. & Swanson, B.G. (2002). Food Processing by High Hydrostatic Pressure. *Critical Reviews in Food Science and Nutrition*, 42(6), 627-645.

Sanglier, S., Leize, E. & van Dorsselaer, A. (2003). Comparative ESI-MS study of ~2.2 MDa Native Hemocyanins from Deep-Sea and Shore Crabs: from Protein Oligomeric State to Biotope. *J Am Soc Mass Spectrom*, 14, 419-429.

Sareevoravitkul, R., Ramaswamy, H.S. & Simpson, B.K. (1996). Comparative properties of bluefish (Pomatomus saltatrix) gels formulated by high hydrostatic pressure and heat. *J. Aquatic Food Product Technology*, 5, 65-79.

Sawyer, L. & Kontopidis, G. (2000). The core lipocalin, bovine beta-lactoglobulin. *Biochim Biophys Acta*, 1482, 136-148.

Schaafsma, G. (2005). The Protein Digestibility-Corrected Amino Acid Score (PDCAAS)—A Concept for Describing Protein Quality in Foods and Food Ingredients: A Critical Review. *J AOAC Int.*, 88(3), 988-994.

Schramm, L.L. (2005). Emulsions Foams and Suspensions Fundamentals and Applications. Wiley-VCH, Verlag GmbH & Co. KGaA, Weinheim. 463 pp.

Schuck, P. (2003). On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. *Analytical Biochemistry*, 320(1), 104-124.

Schulz, G.E. & Schirmer, R.H. (1990). Principles of Protein Structure, 2nd edn. Springer-Verlag, New York.

Sequeira-Muñoz, A., Chevalier, D., LeBail, A., Ramaswamy, H.S. & Simpson, B.K. (2006). Physicochemical changes induced in carp (Cyprinus carpio) fillets by high pressure processing at low temperature. *Innovative Food Science & Emerging Technologies*, 7(1-2), 13-18.

Sequeira-Muñoz, A., Chevalier, D., Simpson, B.K., Le Bail, A. & Ramaswamy, H.S. (2005). Effect of pressure-shift freezing versus air-blast freezing of carp Cyprinus carpio) fillets: A storage study. *Journal of Food Biochemistry*, 29(5), 504-516.

Séverin, S. & Wenshui, X. (2005). Milk Biologically Active Components as Nutraceuticals: Review. *Critical Reviews in Food Science and Nutrition*, 45(7), 645-656.

Shah, N.P. (2000). Effects of milk-derived bioactives: an overview. *British Journal of Nutrition*, 84(1), S3-S10.

Shao, Y., Ramaswamy, H.S. & Zhu, S. (2007). High pressure destruction kinetics of spoilage and pathogenic bacteria in raw milk cheese. *Journal of Food Process Engineering*, 30(3), 357-374.

Shao, Y., Zhu, S., Ramaswamy, H. & Marcotte, M. (2008 online). Compression Heating and Temperature Control for High-Pressure Destruction of Bacterial Spores: An Experimental Method for Kinetics Evaluation. *Food and Bioprocess Technology*, DOI: 10.1007/s11947-008-0057-y.

Sharma, V.K. & Kalonia, D.S. (2003). Steady-state tryptophan fluorescence spectroscopy study to probe tertiary structure of proteins in solid powders. *Journal of Pharmaceutical Sciences*, 92(4), 890-899.

Sharon, N. & Lis, H. (2004). History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology*, 14(11), 53R-62R.

Sheehan, J.J., Huppertz, T., Hayes, M.G., Kelly, A.L., Beresford, T.P. & Guinee, T.P. (2005). High pressure treatment of reduced-fat Mozzarella cheese: Effects on functional and rheological properties. *Innovative Food Science & Emerging Technologies*, 6(1), 73-81.

Simonian, M.H. & Smith, J.A. (2006). Spectrophotometric and colorimetric determination of protein concentration. *Curr Protoc Mol Biol*, 10, 10.1A.

Slangen, C. & Visser, S. (1999). Use of Mass Spectrometry to Rapidly Characterize the Heterogeneity of Bovine α-Lactalbumin. *J Agric Food Chem*, 47, 4549-4556.

Slifko, T.R., Raghubeer, E. & Rose, J.B. (2000). Effect of High Hydrostatic Pressure on *Cryptosporidium parvum* Infectivity, *Journal of Food Protection*, 63(9), 1262-1267.

Smelt, J.P.P.M. (1998). Recent advances in the microbiology of high pressure processing. *Trends in Food Science & Technology*, 9(4), 152-158.

Smith, D., Galazka, V.B., Wellner, N. & Sumner, I.G. (2000). High pressure unfolding of ovalbumin. *International Journal of Food Science and Technology*, 35, 4, 361-370.

Sørensen, E.S. & Petersen, T.E. (1993). Purification and characterization of three proteins isolated from the proteose peptone fraction of bovine milk. *J Dairy Res*, 60(2), 189-197.

Spilimbergo, S., Elvassore, N. & Bertucco, A. (2002). Microbial inactivation by high-pressure. *Journal of Supercritical Fluids*, 22, 55-63.

Steen, H. & Mann, M. (2004). The abc's (and xyz's) of peptide sequencing. *Nature Reviews Molecular Cell Biology*, 5, 699-711.

Steffe, J.F. (1996). Rheological Methods in Food Process Engineering. Freeman Press, East Lansing, MI. 418 pp.

Stevens, S.M.Jr., Kem, W.R. & Prokai, L. (2002). Investigation of Cytolysyn Variants by Peptide Mapping Enhanced Protein Characterization Using Complementary Ionization and Mass Spectrometric Techniques. *Rapid Commun Mass Spectrom*, 16, 2094-2101.

Strickland, E.H. & Beychok, S. (1974). Aromatic Contributions to Circular Dichroism Spectra of Proteins. *Critical Reviews in Biochemistry and Molecular Biology*, 2(1), 113-175.

Subirade, M., Loupil, F., Allain, A.F. & Paquin, P. (1998). Effect of dynamic high pressure on the secondary structure of β-lactoglobulin and on its conformational

properties as determined by Fourier transform infrared spectroscopy. *International Dairy Journal*, 8, 135-140.

Surewicz, W.K., Mantsch, H.H. & Chapman, D. (1993). Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment. *Biochemistry*, 32(2), 389-394.

Susi, H. & Byler, D.M. (1988). Fourier transform infrared spectroscopy in protein conformation studies. In Cherry, J.P., Barford, R.A. (Eds.), *Methods for Protein Analysis* (pp. 235-250). American Oil Chemists Society, Champaign, IL.

Suzuki, A., Kim, K., Honma, N., Ikeuchi, Y. & Saito, M. (1992). Acceleration of meat conditioning by high pressure treatment. *Colloque INSERM*, 224, 219-27.

Suzuki, K. (1960). Studies on the kinetics of protein denaturation under high pressure. *The Review of Physical Chemistry of Japan*, 29(2), 91-98.

Swaisgood, H.E. (1993). Review and Update of Casein Chemistry. *Journal of Dairy Science*, 76(10), 3054-3061.

Syka, J.E.P., Coon, J.J., Schroeder, M.J., Shabanowitz, J. & Hunt, D.F. (2004). Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *PNAS*, 101(26), 9528-9533.

Taherian, A.R., Fustier, P. & Ramaswamy, H.S. (2008). Steady and Dynamic Shear Rheological Properties, and Stability of Non-Flocculated and Flocculated Beverage Cloud Emulsions. *International Journal of Food Properties*, 11(1), 24-43.

Taniguchi, Y. & Suzuki, K. (1983). Pressure inactivation of α-chymotrypsin. *Journal of Physical Chemistry*, 87, 5185-5193.

Teschemacher, H. 2003. Opioid receptor ligands derived from food proteins. *Curr Pharm Des*, 9(16), 1331-1344.

Thakur, B.R. & Nelson, P.E. (1998). High pressure processing and preservation of food. *Food Reviews International*, 14(4), 427-447.

Thomas, G.J.Jr. (1999). Raman Spectroscopy of Protein and Nucleic Acid Assemblies. *Annual Review of Biophysics and Biomolecular Structure*, 28, 1-27.

Tiziani, S. & Vodovotz, Y. (2005). Rheological effects of soy protein addition to tomato juice. *Food Hydrocolloids*, 19(1), 45-52.

Tobitany, A. & Ross-Murphy, S.B. (1997). Heat-induced gelation globular proteins. I. Model for the effects of time and temperature on the gelation time of BSA gels. *Macromolecules*, 30(17), 4845-4854.

Toldrá, M., Busquets, A., Saguer, E., Parés, D. & Carretero, C. (2002). Effects of High Hydrostatic Pressurised Treatment on Porcine Blood Red Cells Fraction. *Food Science and Technology International*, 8(1), 41-48.

Torres, J.A. & Velazquez, G. (2005). Commercial opportunities and research challenges in the high pressure processing of foods. *Journal of Food Engineering*, 67(1-2), 95-112.

Trujillo, A.J., Royo, C., Ferragut, V. & Guamis, B. (1999). Ripening profiles of goat cheese produced from milk treated with high pressure. *Journal of Food Science*, 64(5), 833-837.

Tybor, P.T., Dill, C.W. & Landmann, W.A. (1975). Functional Properties of Proteins Isolated from Bovine Blood by a Continuous Pilot Process. *Journal of Food Science*, 40(1), 155-159.

van Camp, J. & Huyghebaert, A. (1995). High pressure-induced gel formation of a whey protein and Haemoglobin protein concentrate. *Lebensm.-Wiss. u.-Technol*, 28, 111-117.

van Camp, J., Feys, G. & Huyghebaert, A. (1996). High pressure-induced gel formation of haemoglobin and whey proteins at elevated temperatures. *Food Science & Technology*, 29, 49-57.

van der Plancken, L., van Loey, A. & Hendrickx, M.E. (2007). Foaming properties of egg white proteins affected by heat or high pressure treatment. *Journal of food engineering*, 78(4), 1410-1426.

van der Ven, C., Matser, A.M. & van den Berg, R.W. (2005). Inactivation of Soybean Trypsin Inhibitors and Lipoxygenase by High-Pressure Processing. *J Agric Food Chem*, 53(4), 1087-1092.

van Stokkum, I.H.M., Linsdell, H., Hadden, J.M., Haris, P.I., Capman, D. & Bloemendal, M. (1995). Temperature-induced changes in protein structures studied by Fourier transform infrared spectroscopy and global analysis. *Biochemistry*, 34(33), 10508-10518.

Venkatachalam, C.M. (1968). Stereochemical criteria for polypeptides and proteins. V. Conformation of a system of three linked peptide units. *Biopolymers*, 6, 1425-1436.

Vojdani F. & Whitaker, J.R. (1994). Chemical and Enzymatic Modification of Proteins for Improved Functionality. In Hettiarachchy, N.S. & Ziegler, G.R. (Eds), *Protein Functionality in Food Systems*, Marcel Dekker, 261-310 pp.

Walker, M.K., Farkas, D.F., Anderson, S.R. & Meunier-Goddik, L. (2004). Effects of High-Pressure Processing at Low Temperature on the Molecular Structure and Surface Properties of β-Lactoglobulin. *J Agric Food Chem*, 52(26), 8230-8235.

Walker, M.K., Farkas, D.F., Loveridge, V. & Meunier-Goddik, L. (2006). Fruit yogurt processed with high pressure. *International Journal of Food Science & Technology*, 41(4), 464-467.

Walstra, P. (2003). *Physical chemistry of foods*. Marcel Dekker, New York. 807pp.

Wang, Y., Palmer, R.A., Schoonover, J.R. & Aubuchon, S.R. (2006). Dynamic mechanical analysis and dynamic infrared linear dichroism study of the frequency-dependent viscoelastic behavior of a poly(ester urethane). *Vibrational spectroscopy*, 42(1), 74-77.

Wang, Y., Tsenkova, R., Amari, M., Terada, F., Hayashi, T., Abe, A. & Ozaki, Y. (1998). Potential of two-dimensional correlation spectroscopy in analyses of NIR spectra of biological fluids. I. Two-dimensional analysis of protein and fat concentration-dependent spectral variations of milk. *Analysis Magazine*, 26(4), M64-M69.

Weber, G. & Drickamer, H.G. (1983). The effect of high pressure upon proteins and other biomolecules. *Quart. Rev. Biophys.*, 16, 89-112.

Whitney, R. (1999). Proteins of Milk. In Wong, N.P., Jenness, R., Keeney, M. & Marth, E.H. *Fundamentals of Dairy Chemistry*, Aspen Publishers, Gaithersburg, MA. 779 pp.

Wismer-Pedersen J. (1988). Use of haemoglobin in foods – a review. *Meat Science*, 24(1), 31-45.

Wolf, W.J., Babcock, G.E & Smith, A.K. (1961). Ultracentrifugal differences in soybean protein composition. *Nature*, 191, 1395-1396.

Yan, Y.-B., Wang, Q., He, H.W., Hu, X.-Y., Zhang, R..Q. & Zhou, H.-M. (2003). Two-dimensional infrared correlation spectroscopy study of sequential events in the heat-induced unfolding and aggregation process of myoglobin. *Biophysical Journal*, 85 (3), 1959-1967.

Yang, J., Dunker, A.K., Powers, J.R., Clark, S. & Swanson, B.G. (2001). β-lactoglobulin molten globule induced by high pressure. *J. Agric. Food Chem.*, 49(7), 3236-3243.

Yang, J., Powers, J.R., Clark, S., Dunker, A.K. & Swanson, B.G. (2003). Ligand and flavor binding functional properties of β-lactoglobulin in the molten globule state induced by high pressure. *Journal of Food Science*, 68(2), 444-452.

Yike, Y., Jianen, H., Yuji, M., Xuefang, B., Yuguang, D. & Bingcheng, L. (2006). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin. *Peptides*, 27(11), 2950-2956.

Yin, S.W., Tang, C.H., Wen, Q.B., Yang, X.Q. & Li, L. (2008). Functional properties and *in vitro* trypsin digestibility of red kidney bean (*Phaseolus vulgaris* L.) protein isolate: Effect of high-pressure treatment. *Food Chemistry*, 110(4), 938-945.

Yokoyama, H., Sawamura, N. & Motobayashi, N. (1992). Method for accelerating cheese ripening. *European Patent Application EP 0469857 A1*.

Yoshimura, A.S., Prud'homme, K., Princen, H.M. & Kiss, A.D. (1987). A comparison of techniques for measuring yield stresses. *J Rheol*, 31(8), 699-710.

Zhang, H. & Mittal, G.S. (2008). Effects of High-Pressure Processing (HPP) on Bacterial Spores: An Overview. *Food Reviews International*, 24(3), 330-351.

Zhang, H., Li, L., Tatsumi, E. & Isobe, S. (2005). High-pressure treatment effects on proteins in soy milk. *Lebensm.-Wiss. u.-Technol.*, 38(1), 7-14.

Zhang, H., Li, L., Tatsumi, E. & Kotwal, S. (2003). Influence of high pressure on conformational changes of soybean glycinin. *Innovative Food Science & Emerging Technologies*, 4(3), 269-275.

Zhu, S., Bulut, S., LeBail, A. & Ramaswamy H.S. (2004). High-Pressure Differential Scanning Calorimetry (DSC): Equipment and Technique Validation Using Water-Ice Phase Transition Data. *J. Food Proc. Eng.*, 27, 359-376.

Zhu, S., Le Bail, A. & Ramaswamy, H.S. (2006). High-pressure differential scanning calorimetry: Comparison of pressure-dependent phase transition in food materials. *Journal of Food Engineering*, 75(2), 215-222.

Zhu, S., LeBail, A. & Ramaswamy, H.S. (2003). Ice crystal formation in pressure shift freezing of Atlantic salmon (Salmo salar) as compared to classical freezing methods. *Journal of Food Processing and Preservation*, 27(6), 427-444.

Zhu, S., LeBail, A., Chapleau, N., Ramaswamy, H. & deLamballerie-Anton, M. (2004). Pressure shift freezing of pork muscle: Effect on color, drip loss, texture and protein stability. *Biotechnology Progress*, 20(3), 939-945.

Zhu, S., LeBail, A., Ramaswamy, H.S. & Chapleau, N. (2004). Characterization of ice crystals in pork muscle formed by pressure shift freezing as compared to classical freezing methods. *Journal of Food Science*, 69(4), E190-197.

Zhu, S., Ramaswamy, H. & LeBail, A. (2005). High-pressure calorimetric evaluation of ice crystal ratio formed by rapid depressurization during pressure-shift freezing of water and pork muscle. *Food Research International*, 38(2), 193-201.

Zhu, S., Ramaswamy, H.S. & Le Bail, A. (2005). Ice-crystal formation in gelatin gel during pressure shift versus conventional freezing. *Journal of Food Engineering*, 66(1), 69-76.

Zhu, S., Ramaswamy, H.S. & LeBail, A. (2004). High-Pressure Differential Scanning Calorimetry (DSC): Evaluation of Phase Transition in Pork Muscle at High Pressures. *J. Food Proc. Eng.*, 27, 377-391.

Zhu, S., Ramaswamy, H.S. & LeBail, A. (2006). HP Calorimetry and pressure-shift freezing of different food products. *Food Science and Technology International*, 12 (3), 205-214.

Zhu, S., Ramaswamy, H.S. & Simpson, B.K. (2004). Effect of high-pressure versus conventional thawing on color, drip loss and texture of Atlantic salmon frozen by different methods. *Lebensm.-Wiss. U.-Technol.*, 37(3), 291-299.

Zhu, S.M., Naim, F., Marcotte, M., Ramaswamy, H.S. & Shao, Y. (2008 online). High-pressure destruction kinetics of Clostridium sporogenes spores in ground beef at elevated temperatures. *International Journal of Food Microbiology*, DOI: 10.1016/j.ijfoodmicro.2008.05.009.