SEPARATION AND STRUCTURAL CHARACTERIZATION OF ALPHA-LACTALBUMIN AND BETA-LACTOGLOBULIN FROM WHEY PRODUCTS

BY

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FOREWORD

The thesis is submitted in the form of original papers for journal publication. The first two sections comprise a general introduction and a literature review presenting the theory and previous knowledge on this topic. The next five sections contain the body of the thesis; each chapter represents a complete manuscript. The last section is a summary of the major conclusions. This format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines for Thesis Preparation, Thesis Specification, sections 3 entitled "Traditional and manuscript-based theses" which are as follows:

"Candidates have the option of including, as part of the thesis, the text of a paper (s) submitted, or to be submitted, for publication, or the clearly-duplicated text of published paper(s). These texts must be bound together as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation." The thesis must include: a table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a through bibliography of reference list.

Additional material must be provided where appropriate (e.g., in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an external examiner for that thesis".

Although all the work reported in this thesis is the responsibility of the candidate, the project was supervised by Dr. Inteaz Alli, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

ABSTRACT

In most food applications, whey proteins are used, rather than the individual proteins and this accounts for the high functional variability among commercially available whey protein products, and limits their applications. The overall objective of this study was to investigate the structural and thermal properties of individual α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) fractions isolated from different whey protein sources.

A common non-chromatographic process that isolate α-Lac and β-Lg, with relatively high purity and yield from liquid whey (LW), whey protein concentrate (WPC) and whey protein isolate (WPI) using different chelating agents, was developed. The use of sodium citrate (NaC) and sodium hexametaphosphate (SHMP) were more effective than other chelating agents. Yield results indicated that 47 to 69 % of β-Lg originally present in the whey preparations was recovered, with purities ranging from 84 to 95%, and protein contents ranging from 40 to 99%, while the yields of α-Lac were 23 to 89 %, with purities ranging from 83 to 90%, and protein contents ranging from 65 to 96% depending on the source of whey protein preparations and type of chelating agents.

Structural and thermal properties of β-Lg and α-Lac isolated fractions were studied using polyacrylamide electrophoresis (native and SDS), RP-HPLC, differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and electrospray ionization mass spectrometry (ESI-MS). Results showed that all β-Lg and α-Lac isolated fractions exhibit increased thermal stability and reversibility over standard proteins and difference in thermal properties were dependent on protein source. The relative intensity of the 1692 cm^{-1} band in the β-Lg isolated fractions was dependent on the nature of the chelating agent, and disappearance of this band occurred

at temperature higher than that of β-Lg standard, indicating increased thermal stability of β-Lg isolated fractions. Denaturation of apo-α-Lac was related to the gradual decrease in the α-helix band and accompanied by the gain in intensity of 1653 and 1641 cm^{-1} bands, while denaturation of holo-α-Lac was associated by breakdown of β-sheet structure and increase in turns and unordered structures.

Changes in charge state distribution (CSD), as measured by ESI-MS of β-Lg and α-Lac in response to pH and storage time, were only qualitative and were of relatively low resolution at basic pH. The hydrogen/deuterium (H/D) exchange results demonstrated that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and conformation of β-Lg variant B was more stable than β-Lg variant A. Kinetics of H/D exchange indicated that α-Lac and β-Lg fractions isolated from different whey protein sources have the same or improved conformational stabilities compared to that of α-Lac and β-Lg standard. The covalent binding of 3 or more hexose residues to α-Lac enhanced its conformational stability, but covalent binding of two hexose residues to β-Lg resulted in less stable conformation.

RÉSUMÉ

Dans la majorité des applications alimentaires, les protéines de lactosérum (concentré (WPC) ou isolat (WPI) de protéines) sont plus souvent utilisées que les protéines individuelles. Ces protéines de lactosérum sont utilisées commercialement et possèdent des propriétés fonctionnelles très variables, limitant ainsi leurs applications industrielles. Les objectifs de cette recherche étaient d'évaluer les propriétés fonctionnelles de deux protéines, soit le α- lactalbumine (α-Lac) et le β-lactoglobuline (β-Lg), présente dans trois différentes sources de lactosérum (lactosérum liquide brut, WPC et WPI). Un procédé non chromatographique a été développé afin d'isoler 1e α-Lac et le β-Lg. L'isolation de β-Lg a été possible grâce à l'utilisation d'agents chélateurs. Parmi les chélateurs étudiés, le citrate de sodium (NaC) et l'hexametaphosphate de sodium (SHMP) ont montré les meilleurs rendements d'extraction. Ces agents chélateurs permettaient une récupération de 47-69 % du β-Lg. avec un degré de pureté en protéines de 40-99%. Le taux de récupération de la protéine α-Lac était de 23-89%, avec un degré de pureté de 83-90 % et un contenu en protéines de 65-96%, dépendamment de la source de protéines de lactosérum et du type d'agents chélateurs utilisé.

Les propriétés structurelles thermales du β-Lg et du α-Lac ont été étudiées par séparation sur gel d'électrophorése, en utilisant un gel de polyacrylamide pour séparer les protéines natives et dénaturées. Une chromatographie liquide en phase inverse (RP-HPLC) a aussi été réalisée. Les protéines ont également été soumises à des analyses de calorimétrie différentielle (DSC), spectroscopie infra rouge de fourier (FT-IR) et de spectrométrie de masse par ionisation «électron spray». Les résultats ont montré que les protéines α-Lac et le β-Lg, isolées du lactosérum ont une stabilité thermale réversible et

supérieure aux protéines standards. Cette différence dépend également de la source de protéines (lactosérum liquide, WPC ou WPI). Les analyses de infra-rouge de la β-Lg montre une bande intense a 1692^{-1} et cette bande dépend de la nature de l'agent chélateur utilisé. Une disparition de cette bande est observée à une température plus élevée que celle observée pour le standard de la protéine β-Lg. Ces résultats suggèrent que la stabilité thermale de la protéine β-Lg, isolée du lactosérum est supérieure à la protéine standard. La dénaturation de apo-α-Lac étair reliée à la réduction graduelle de la bande α-hélice et à une augmentation de l'intensité des bandes 1653 et 1641 cm^{-1}. La dénaturation de holo-α-Lac était associée à la destruction de la structure des feuillers β et de l'augmentation du nombre de structures non ordonnées.

L'état de distribution des charges (CSD) de β-Lg et de α-Lac a été mesurée qualitativement par ESI-MS en fonction du pH et du temps de rétention. Les résultats ont montré une basse résolution à un pH basique. Les résultats obtenus après échange de hydrogène/deuterium (H/D) ont montré que la conformation du holo-α-Lac était plus stable que le apo-α-Lac. De plus, la conformation du β-Lg variant B était plus stable que le variant A du β-Lg. Les cinétiques d'échanges ont montré que la stabilité structurelle des protéines isolées du lactosérum étaient la même ou améliorée comparativement aux protéines standards. Des liaisons covalentes de 3 ou plus de résidues hexose dans la protéine α-Lac améliorent sa stabilité structurelle, mais une liaison covalente de résidue hexose dans la protéine de β-Lg résulte d'une stabilité structurelle moins stable.

PREFACE

CLAIMS TO ORIGINAL RESEARCH

1. This represents the first study to develop a common non-chromatographic process that isolate β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac), with relatively high purity and yield, from liquid whey, whey protein concentrate and whey protein isolate using sodium citrate and sodium hexametaphosphate.
2. This is the first study to investigate the use of charge state distribution (CSD) and hydrogen/deuterium (H/D) exchange rates by electrospray ionization mass spectrometry for determining structural stability of whey proteins and for identifying differences in conformation of β-Lg and α-Lac fractions isolated from different whey protein sources.
3. This study has shown that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and the conformation of β-Lg variant B was more stable than β-Lg variant A by probing hydrogen-deuterium exchange rates as measured by electrospray ionization mass spectrometry.

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Alomirah, H. F.; Alli, I.; Ismail, A. A.; Konishi Y. Thermal Denaturation and Conformational Stability of β-Lactoglobulin Isolated from Whey and Whey Protein Preparations. *J. Agric. Food Chem.* (In Preparation).

Alomirah, H. F.; Alli, I.; Ismail, A. A.; Konishi Y. Thermal Denaturation and Conformational Stability of α-Lactalbumin Isolated from Whey and Whey Protein Preparations. *J. Agric. Food Chem.* (In Preparation).

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ABBREVIATIONS

α-Lac	α-lactalbumin
APCI	atmospheric pressure chemical ionization
β-Lg	β-lactoglobulin
BSA	bovine serum albumin
CCP	colloidal calcium phosphate
CI	chemical ionization
CMP	caseinomacropeptide
CN	casein
CPP	casein phosphopeptide
ΔH	change in enthalpy
DH	degrees of hydrolysis
DSC	differential scanning calorimetry
E. coli	Escherichia coli spp.
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetra acetic acid
EI	electron impact
ESI	electrospray ionization
ESI-MS	electrospray ionization mass spectrometry
ESI-MS/MS	electrospray ionization tandem mass spectrometry
FAB	fast atom bombardment
FD	field desorption
FTICR	Fourier transform-ion cyclotron resonance
FTIR	Fourier transform infrared spectroscopy
H/D	hydrogen/deuterium exchange
High-M_r	high molecular mass
HPLC	high-performance liquid chromatography

LD	laser desorption
Low-M_r	low molecular mass
LW	liquid whey
m/z	mass to charge ratio
MALDI	matrix-assisted laser desorption/ionization
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
M_r	molecular mass
MS	mass spectrometry
MW	molecular weight
NaC	sodium citrate
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PD	plasma desorption
RP-HPLC	reversed-phase high-performance liquid chromatography
SBA	soybean agglutinin
SDS	sodium dodecyl sulfate
SHMP	sodium hexametaphosphate
SI	secondary ion
T_d	peak temperature of denaturation
TOF	time-of-flight
TSP	thermospray ionization
U	atomic mass units
WP	whey protein
WPC	whey protein concentrate
WPI	whey protein isolate

CHAPTER 1

INTRODUCTION

1.0 GENERAL

Currently, a variety of proteins with desirable functional properties for food products are available; these include gluten, albumin, gelatin, soyprotein, casein and whey proteins. Whey proteins (WP) are well known for their nutritional value and versatile functional properties and are widely utilized in the food industry. An estimate of the worldwide production of whey indicates that approximately 700,000 tonnes (de Wit, 1998) of true whey proteins are available annually as food ingredients; whey contains about 20-24% of total milk protein depending on the source. WPs are available commercially as whey protein isolates (WPI, 90-95 % protein) and whey protein concentrates (WPC, 35-75% protein). These whey products can be produced from whey by different methods and marketed more profitably than powder whole dry whey.

An understanding of the close structure-function relationship of protein is essential to utilize all protein resources (plant or animal origin) completely in food system. In other words, protein molecular weight (number of amino acids), type of amino acid (hydrophobic and hydrophilic), disposition of amino acid in polypeptide, protein shape (globular and fibre), structure diversity (native folded, intermediate, misfolded and denatured proteins), conformation (secondary, tertiary, quaternary) under various environmental conditions (i.e., pH, temperature, ionic strength, moisture and type of solvent) and processing treatments (i.e., mixing, centrifugation, time and storage, protein content, distribution and interaction with other food components) will control its properties (Zayas, 1997).

The heterogenous nature of WP is typical of many commercial food protein preparations. For these protein preparations, the observed functionality of the complete protein preparation is the sum of the functionality of individual proteins. The most common functional properties attributed to WPs are solubility, viscosity, emulsification, foaming, water absorption and gel formation. The main proteins in whey are: β-lactoglobulin (β-Lg, comprising 50 % of the proteins of whey), α-lactalbumin (α-Lac, 21%) and immunoglobulins (10%); bovine serum albumin (BSA, 5%) and proteose-peptones which represent minor components (Kinsella, 1984). Of these, β-Lg, α-Lac and BSA are primarily responsible for the physicochemical properties of whey proteins (Schmidt and Morris, 1984).

Although, there is a relative abundance of information in the literature on factors affecting functional properties of WPI and WPC, there is relatively limited information on the structural properties of the individual whey proteins and on the effects of these conformational structure on the functionality of whole whey; this remains a subject for future studies.

1.1 RATIONALE AND OBJECTIVES OF STUDY

In most food applications, WPs are used, rather than the individual protein and this accounts for the high functional variability among commercially available WP products and limits their applications (Morr and Ha, 1993; Mate and Krochta, 1994). This shortcoming could be addressed through the development of commercially relevant whey processing technologies and for the preparation of whey protein isolated fractions, contain mainly individual whey proteins (Smithers et al., 1996). Therefore, selective fractionation of whey major proteins may allow exploitation of their different

functional, nutritional and therapeutic properties (Bramaud et al., 1997) and may result in better understanding of the close structure-function relationship of these proteins which is essential to utilize whey protein products completely in food system.

Currently, there is growing interest in studying protein stability-function relations (PSFR) rather than traditional structure-function relations (Apenten and Galani, 1999). This is because stability is easier to quantify than structure. Although, there is a relative abundance of information in the literature on factors affecting structural stability of standard β-Lg and α-Lac, there is relatively limited information on the structural stability of new whey protein isolated fractions, contain mainly β-Lg and α-Lac, obtained from different whey protein preparation and on the effect of these β-Lg and α-Lac isolated fractions conformational structure on the functionality of whole whey.

The overall objective of this study was to investigate the structural and thermal properties of α-Lac and β-Lg fractions isolated from different whey protein sources (liquid whey (LW), whey protein concentrates (WPC) and whey protein isolates (WPI)) using different chelating agents. The specific objectives of the research were:

1. To investigate mass spectrometry in general and charge state distribution (CSD) and hydrogen/deuterium (H/D) exchange using ESI-MS in particular, to the study of food proteins and peptides.
2. To develop a common non-chromatographic process for isolating β-Lg and α-Lac in relatively pure forms from liquid whey (LW), whey protein concentrates (WPC) and whey protein isolates (WPI) using different chelating agents.

3. To investigate the thermal properties and the secondary structure of β-Lg and α-Lac fractions isolated from different whey protein sources using differential scanning calorimetry and Fourier transform infrared spectroscopy.

4. To investigate the use of charge state distribution (CSD) and hydrogen/deuterium (H/D) exchange rates by electrospray ionization mass spectrometry for determining structural stability of β-Lg and α-Lac proteins and for identifying differences in conformation of β-Lg and α-Lac fractions isolated from different whey protein sources.

CHAPTER 2

LITERATURE REVIEW

2.0 INTRODUCTION

Proteins exhibit a broad spectrum of functional properties due to their structural heterogeneity and ability to interact with other food components. The hydrophilic-hydrophobic character of proteins play a significant role in the functional properties of proteins in food; hydrophilicity is associated with protein solubility, water holding capacity and gelling capacity. Hydrophilic-hydrophobic balances are associated with emulsifying and foaming and hydrophobicity is associated with fat binding properties (Zayas, 1997). The molecular basis of hydrophilicity-hydrophobicity and therefore of functional properties are related to the structure of the protein. Protein molecular weight (number of amino acids), type of amino acids (hydrophilic and hydrophobic), disposition of amino acids in polypeptides, protein shape, structure diversity all play a role in protein functionality (Zayas, 1997). Protein functional properties are also related to protein stability as particular functional property of a protein is often governed by a specific protein conformation state and alteration of that state affect protein functionality.

2.1 PROTEIN STRUCTURE-FUNCTIONAL RELATIONSHIPS

The interrelationship between the molecular and functional properties of proteins is generally understood in a qualitative manner, quantitative prediction of the functional behavior of protein in food systems from knowledge of their molecular

properties has not been achieved and it remains a subject for investigation (Damodaran, 1994). Studies on whey protein functionality in model systems can provide valuable information. Results do not always generate data applicable in actual food systems (Zayas, 1997). Limitations associated with model system experiments can be attributed to diversity of chemical and physical properties of the protein, interactions between protein and non protein components, variation in protein content and the absence of standardized method for assessing protein functionality (Zayas, 1997).

Factors which are known to contribute to protein functionality can be classified into intrinsic and extrinsic factors. Intrinsic factors are related to hydrodynamic (e.g., protein shape and size) and thermodynamic (e.g., energy required to expose reactive site during unfolding) properties of individual proteins which are related to the amino acid sequence (Boye, 1995). Therefore, the proportion of polar, non-polar, positively and negatively charged amino acids and their distribution along the chain can determine certain properties such as solubility, surface hydrophobicity and the ability to stabilize foam and emulsion (Hall, 1996). They also determine the favored (or native) folded secondary structure of protein by determining the rotation of the amino acid about the single-bonded backbone of protein until the side chains reach thermodynamically stable distribution (Hall, 1996). Three-dimensional twisting of the linear chains of primary and secondary structures forms the tertiary structure while association of two or more protein chains into an oligomeric unit forms the quaternary structure. These complex levels of structure give proteins a great variety of forms and functions, and retention or loss of this structure will determine food functional application. It has been recognized that determination of protein hydrodynamic and thermodynamic properties do not necessarily mean that a positive correlation with protein functionality can be

established, but rather provide an indication of potential functionality (Patel and Fry, 1982).

Extrinsic factors can be divided into two areas of influence, those which are related to environmental conditions and processing treatments. Temperature, pH, ionic strength, moisture and type of solvent are examples of environmental conditions. Mixing, centrifugation, time and storage, protein content and amounts of distribution and interactions with other food components are examples of processing treatments. These extrinsic factors affect protein structure and behavior.

2.1.1 Physicochemical and Conformational Properties of β-Lg

Table 2.2 shows the composition and some physicochemical properties of whey proteins. β-Lg is well characterized with respect to its molecular and physicochemical properties. Characterization of β-Lg by amino acid composition, sequencing and isoelectric focussing have demonstrated the existence of seven genetic polymorphs referred as the A, B, C, D, E, F and G forms, the most prevalent being the A and B forms (Ng-Kwai-Hang and Grosclaude, 1992). Comparison of the sequences of the purified β-Lg from milks of several different animal species have revealed an average of 55 amino acid residues common to all members suggesting that these residues are decisive to the structure and conformation of the protein (Godovac-Zimmermann et al., 1988). The amino acid sequence in the β-Lg family has revealed that the highest degree of sequence homology among the proteins occurs in the amino terminal region of each protein molecule and that the position of disulfide bonds formed between Cys160-Cys66, Cys119-Cys106 and the free thiol group on Cys121 are consistent (Godovac-Zimmermann and Shaw, 1987; Godovac- Zimmermann et al., 1988).

β-Lg contains 6% α-helix, 43.8% β-sheet, 12.3% β-turns and the remaining 37.7% represent amino acid residues in random coil arrangement (Papiz et al., 1986; Creamer et al., 1983). X-ray crystallography studies indicated that β-Lg (Figure 2.1) is made up of 9 antiparallel β-strands, eight of which wrap round to create β-barrel, with an helix at the external face, that has the ability of binding and transporting apolar, lipophilic or labile compounds (Papiz et al., 1986; Sawyer, 1987).

2.1.2 Physicochemical and Conformational Properties of α-Lac

α-Lac is the second major protein component in the whey of milks from various mammalian species, constituting 20% of the proteins in bovine whey, and serves as lactose synthase regulatory protein. The primary structure of this globular protein consists of 123 amino acid residues with MW of 14,147 Da for genetic variant A and 14,175 Da for variant B both contain four intramolecular disulfide bonds and contain no free thiol groups (Table 2.1). This macromolecular structure of α-Lac has a high degree of homology with lysozyme. Out of a total of 123 residues in α-Lac, 54 are identical to corresponding residues in lysozyme and a further 23 residues are structurally similar (e.g. Ser/Thr, Asp/Glu) (Fox and McSweeney, 1998). Crystallographic studies reported that the secondary structure of α-Lac consist of an α-helical content of 30%, a 3_{10} helices content of 20% with β-structure content of 6% and the remaining 44% represent amino acid residues in a random coil arrangement while the tertiary structure α-Lac is made up of a large α-domain which consists of four α-helices and two short 3_{10} helices and of a small β-domain which consists of a triple-stranded antiparallel β-sheet, a 3_{10} helix and series of loops (Figure 2.2) (Acharya et al., 1989; Pike et al., 1996). α-Lac

Table 2.1: Composition and some physicochemical properties of major whey proteins.

Properties	β-Lg (B)	α-Lac (B)	BSA	IgG
Concentration (g/L of milk)	3.2	1.2	0.4	0.8
Approximate % of total whey protein	45	20	5	10
MW (Da)	18,363	14,176	66,267	150,000-1,000,000
Amino acids/mol	162	123	582	>1000
Genetic Variants	7	3	1	-
Cysteine/mol	5	8	35	-
Disulfide bonds/mol	2	4	17	-
% Charge residues/mol	30	28	34	-
Isoelectric point (P*I*)	5.3-5.4	4.2-4.5	5.1	5.5-8.3
Average hydrophobicity (kJ/residue)	5.1	4.7	4.3	4.6

Modified from Cayot and Lorient, 1997; de Wit, 1998; Fox and McSweeney, 1998; Belitz and Grosch, 1999.

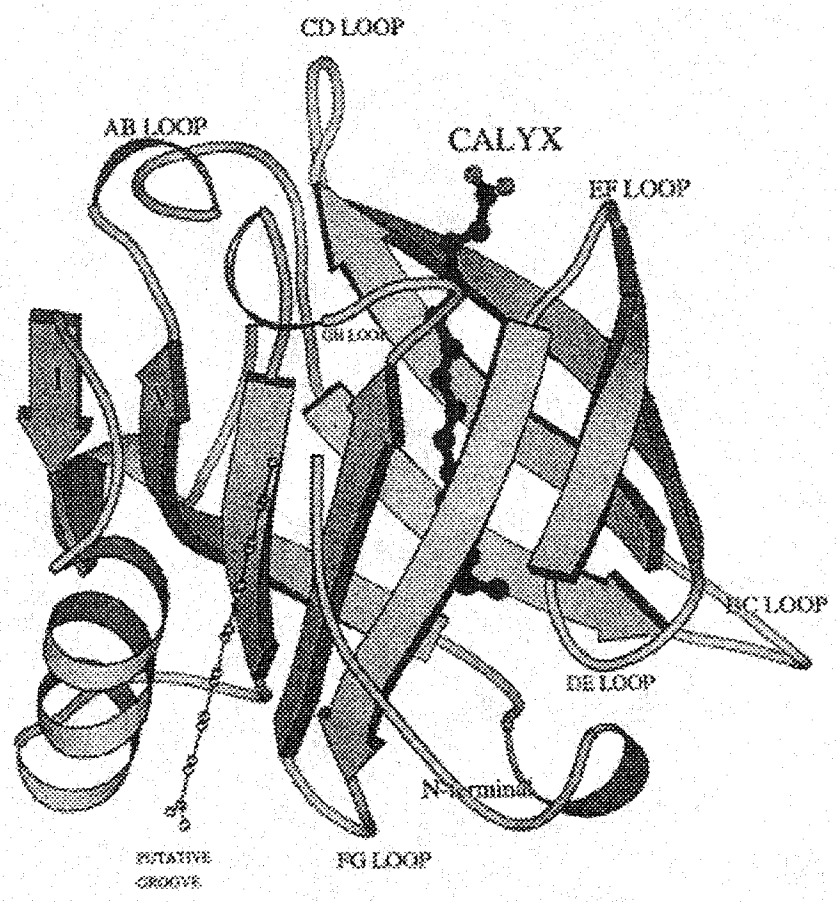

Figure 2.1: A general view of β-lactoglobulin. The binding site (filled atoms is shown in the central calyx, and the putative binding site (open atoms) is indicated on the outer service of the protein (Wu et al., 1999).

contains a single tightly bound calcium (Ca^{2+}), the binding site being essentially identical in all the known α-Lac crystal structure (Pike et al., 1996). The binding site for the Ca^{2+} is located in a loop between the 3_{10} helix (β-domain) and an α-helix (α-domain). The Ca^{2+} is co-ordinated by the side chain β-carboxylate groups of three aspartic acid residues (Asp 82, Asp 87, Asp 88), two backbone carbonyl oxygen atoms (Lys 79 and Asp 84) and two water molecules, which contribute two oxygen ligands (Pike et al., 1996).

The binding of Ca^{2+} stabilizes the structure of native α-Lac (Hiraoka et al., 1980). The apo-form (Ca^{2+}-free) and holo-form (Ca^{2+} -bound) of α-Lac has peak temperature (T_d) at 35 and 64 °C corresponding to the denaturation, respectively (Boye et al., 1997a; Boye and Alli, 2000). Although the apo-form is less stable than the holo-form and its refolding is much slower (Hiraoka and Sugai, 1984; Kuwajima et al., 1989; Forge et al., 1999), the folded conformation of the two native forms are similar (Kuwajima et al., 1986, 1989; Forge et al., 1999); these CD and proton NMR studies demonstrated that the stability of molten globule (native-like structure in partially folded protein) formed under refolding conditions is enhanced by Ca^{2+}. When α-Lac is subjected to various physicochemical conditions such as low pH values, temperature above 50 °C and binding of ions, the native state (N) of α-Lac can undergo various conformational transition to form the molten globule state (A), which is a stable, partially folded state (Kronman, 1989). Under these conditions the protein will aggregate and precipitate.

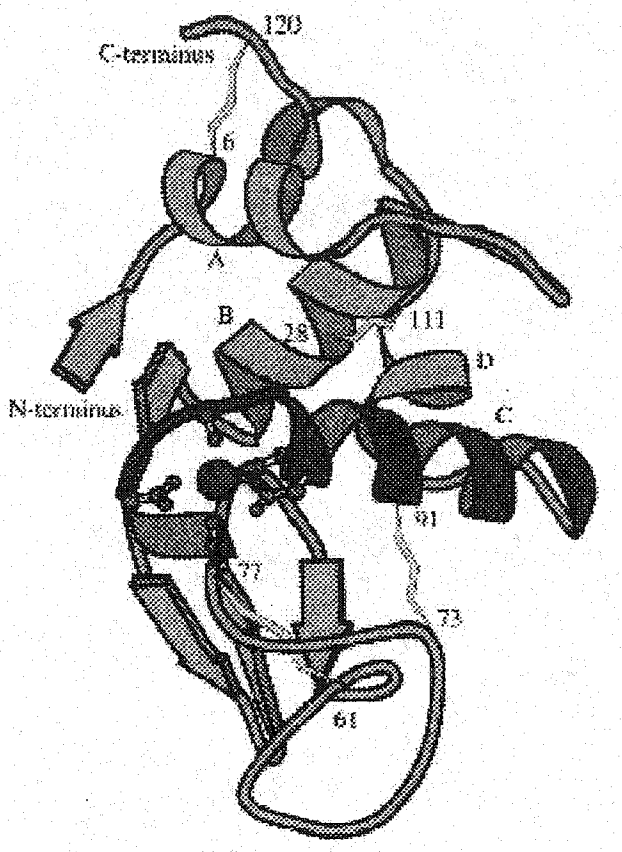

Figure 2.2: Structure of holo-α-lactalbumin with the Ca^{2+}-binding region highlighted. The C-helix, the 3_{10} helix and the loop linking them are shown in dark blue. The Ca^{2+}-binding residues, Lys79, Asp82, Asp84, Asp87 and Asp88, are shown in a ball-and-stick representation in green. The Ca^{2+} is shown in red and the four disulphide bonds are in yellow. The four α-helices are labeled (Pike et al., 1996).

2.1.3 Effect of Extrinsic Factors on the Structure and Function of β-Lg and α-Lac

β-Lg and α-Lac structures are profoundly influenced by environmental factors. Minor changes in pH, ionic strength, temperature and the presence of denaturants often result in alteration of β-Lg and α-Lac conformation and functional behavior. Thus, changes in pH, ionic strength, or the addition of other solutes generally influences β-Lg and α-Lac unfolding and resulting in changes in solubility, heat denaturation profile, aggregation behavior and spectral properties (Paulsson et al., 1985, Phillips et al., 1994).

2.1.3.1 Effect of pH

β-Lg is one of relatively few globular proteins which remains in solution near its isoelectric point (p*I*). The fact that β-Lg is soluble even when electrostatic repulsion is low or absent suggest that other solution forces are more dominant under these conditions (Phillips et al., 1994). Hydrophobic interactions can affect solubility and therefore, the protein structure at various pH values. Although β-Lg is a relatively hydrophobic protein, the relative absence of nonpolar groups, particularly aromatic amino acids, on the molecular surface of β-Lg explains why this protein remain soluble in aqueous solution, even when electrostatic repulsive forces are low or absent (Swaisgood, 1982; Hayakawa and Nakai, 1985).

β-Lg (MW 18, 300 Da) exists as a dimer (36, 700 Da) between pH 5 and 6.5 as a result of electrostatic interactions between Asp 130 and Glu 134 of one monomer with Lys 135, Lys 138 and Lys 141 of another monomer (Creamer et al., 1983). Above pH 6.5, the dimer dissociates due to excessive electrostatic repulsion (Brown and Farrell,

1978). At pH 4 to 5 and above 8, the dimer self-associate to form an octamer (Pessen et al., 1985). It has been speculated that β-Lg undergoes specific structural transitions characterized by a tighter, less elastic conformation at acidic pH, compared with highly flexible, more hydrophobic molecules at values above 7.5 (Kella and Kinsella, 1988b). The conformation stability and flexibility of β-Lg at various pH values was assessed by studying the equilibrium thermal unfolding curve of the protein in the temperature range of 64 °C to 84 °C (Kella and Kinsella, 1988a). The transition temperature progressively increased with a decrease in pH from 7.5 to 6.5 and 3.0 to 1.5, indicating an increase in protein stability. The more stable conformation of β-Lg at acidic pH can be attributed to its ability to carry approximately 18 positive charges; protonation of certain carboxylic groups can contribute to extra hydrogen bonding (Kella and Kinsella, 1988a). The decrease in temperature of denaturation as the pH is raised is indicative of the β-Lg molecules becoming more thermolabile and is consistent with exposure of non polar groups to the solvent during the heat induced transition from native to the unfolded state (Waissbluth and Grieger, 1974).

At pH around 4, α-Lac loses the bound Ca^{2+}, resulting in conformational changes that make the protein more sensitive to heat denaturation and enzymatic treatment (Cayot and Lorient, 1997). Heating at pH 7 or 9 increases the foaming capacity and stability of α-Lac. However, heating at pH 2 (apo-form) or at pH 5 (near p*I*) has a negative effect on foaming properties of α-Lac, by inducing hydrophobic aggregation which is detrimental to stability of foams (Cayot and Lorient, 1997). In solution, α-Lac undergoes a rapid reversible association at acidic pH, very little

association between pH 6 and 8.5 and expansion without aggregation at pH above 9.5 (Boye et al., 1997a).

2.1.3.2 Effect of Ions

The type and concentration of ions in the medium greatly influence protein solubility and thermal stability. Salts at low to moderate concentration generally counteract the effect of the electric double layer that surrounds protein molecules in solution. Increasing the ionic strength of the protein solution masks charged groups that becomes accessible through heat–induced conformational rearrangements, thus enhancing hydrophobic interactions (Sawyer and Puckeridge, 1973). β-Lg in solution (0.05 to 0.1 M NaCl), heated to 90 °C for varying lengths of time (0 to 60 min) unfold, yet remain soluble (Harwalkar and Kalab, 1985). However, at higher ionic strengths (> 0.15 M NaCl), β-Lg in solution becomes turbid after 5 to 10 min of heating (Harwalkar and Kalab, 1985). Differential scanning calorimetry (DSC) studies on the effect of NaCl and $CaCl_2$ on the thermal transition characteristics of β-Lg showed that both salts increased temperature of denaturation whereas the enthalpy was not significantly affected (Harwalkar and Ma, 1987).

α-Lac shows high affinity for Ca^{2+} ($K=2.5x10^8$) and a number of other cationic metal ions, including Mg^{2+}, Na^+, K^+ and Zn^{2+}, (Permyakov and Berliner, 2000). DSC data showed that the apo-form (Ca^{2+}-free) and holo-form (Ca^{2+} -bound) of α-Lac has peak temperature (T_d) at 35 and 64 °C corresponding to the denaturation, respectively (Boye et al., 1997a; Boye and Alli, 2000). In the absence of Ca^{2+}, binding of Mg^{2+}, Na^+ and K^+ increases the thermal stability of α-Lac but binding of Zn^{2+} ions to holo-α-Lac

decreases the thermal stability of the protein, causing aggregation and increases its susceptibility to enzymatic treatments (Permyakov and Berliner, 2000).

2.1.3.3 Effect of Denaturants

Urea, guanidine hydrochloride and alcohol are common denaturing agents employed to study transitions between native and unfolded states of proteins. Alcohols denature proteins by diminishing hydrophobic interaction while enhancing hydrogen bonding and electrostatics; that is, the balance of forces is shifted in favor of hydrogen bonding and helix formation with increasing mole fraction of alcohol (Philips, 1994). In contrast, urea and guanidine hydrochloride induce denaturation by weakening hydrophobic interaction, without appreciable alteration of hydrogen bonding (Edelhoch and Osborne, 1976). CD spectral analysis showed that α-helical structure increased in β-Lg suspended in alcohol solution (1% W/V) (Mattarella et al., 1983). The denaturation of β-Lg in 6 M urea at pH 3.0 is completely reversible (Pace and Tanford, 1968). Philips (1992) showed that under extreme denaturation conditions (6M urea, pH 7 and 14 mM DTT) a portion of the globular structure of β-Lg remained intact. In the presence of guanidine hydrochloride, α-Lac conformation undergoes a three-stage denaturation mechanism ($N \rightleftharpoons A \rightarrow D$) which include destruction of tertiary structure (transition A) that can be restored by binding of monovalent cations (Na^+ and K^+), followed by destruction of secondary structure (transition D) and complete unfolding of the protein (Wong et al., 1996).

2.1.3.4 Effect of Temperature:

Globular proteins display a wide range of heat-induced behavior, which reflect their inherent differences in molecular and physical structures and intermolecular interactions (Philips et al., 1994). Thermal denaturation of β-Lg follows a series of unfolding and association steps as indicated by a significant increase in the amount of β-structure as the temperature is progressively increased from 60 to 80 °C. Irreversible conformation changes of β-Lg occur at a temperature around 70 °C. At this temperature, β-Lg aggregates due to spontaneous interaction arising from partial unfolding of the molecule, which releases previously committed hydrogen bonded protein groups for alternative interaction, although the specific structure of aggregates is unknown (Sawyer et al., 1971). FTIR experiments demonstrated the effect of elevated temperature on the secondary structure of β-Lg (Boye, 1995). On denaturation, the major changes were a decrease in α-helical structure and an increase in β-sheet structure. On aggregation, the major changes observed were, an increase in intermolecular hydrogen-bonded β-sheet structure, slight decrease in number of turns and further loss of α-helical structure (Boye, 1995).

Heating of α-Lac (0.7%) in phosphate buffer (0.1 M, pH 7) for 10 min at 100 °C results in no gel formation which is not the case for β-Lg and BSA (Relkin, 1996). The absence of gel formation in α-Lac is due to the absence of a free sulfhydryl group that can induce thiol-thiol or thiol-disulfide interchange upon heating (Gezimati et al., 1997). The binding of Ca^{2+} increases the thermal stability and reversibility of α-Lac (Boye et al., 1997a) and heating in the presence of β-Lg, increases the thermal stability of apo-α-Lac (Boye and Alli, 2000).

2.2 PROTEIN FOLDING AND STABILITY

The thermodynamic hypothesis proposed by Anfinsen (1973) indicated that in order for proteins to fold spontaneously into their native state, the native state must assumes the global minimum in Gibbs energy and native and unfolded states are in equilibrium. The equilibrium between native (*N*) and unfolded (*U*) states is defined by the equilibrium constant, *K*, as:

$$K = [U]/[N] \qquad \text{(Eqn 1)}$$

The stability of a protein molecule is described by the difference in free energy (ΔG) between the unfolded and folded states and can be expressed as:

$$-RT \ln K = \Delta G = \Delta H - T\Delta S \qquad \text{(Eqn 2)}$$

Where R is the universal gas constant, T is the absolute temperature (in Kelvin), ΔH is the enthalpy change and ΔS is the entropy upon unfolding. From equation (2), it can be observed that protein stability result from a balance between large and opposing entropic and enthalpic effect, both of which are highly temperature dependent. Dill (1990) hypothesized that folding of a protein is driven by hydrophobicity which is defined in terms of the transfer of nonpolar amino acids from H_2O into a medium that is nonpolar and preferably capable of hydrogen bonding. Nevertheless, steric constraints in compact chain are also supposed to be responsible for their considerable internal architecture (Dill, 1990). The folded states of proteins are only marginally more stable than the fully unfolded state, even under optimal conditions, with a net stability about 21-63 kJ/mol equivalent to few hydrogen bonds (Phillips et al., 1994). This explains why slight changes in environmental conditions can cause a protein to undergo conformational changes (Bryant and McClements, 1998). In native protein

conformation, the stabilizing contributions that arise from various molecular interactions which includes steric interactions (overlap of electron clouds); Van der Waals forces (interactions between fixed and induced dipoles); electrostatic interactions (charges); hydrogen bonding (resulting from the sharing of a hydrogen between electronegative atoms); hydrophobic effect; disulfide bridges and are largely offset by the destabilizing configurational (local and non-local) entropy (Damodaran, 1997; Murphy, 2001). The general characteristics of the molecular interactions between protein molecules are summarized in Table 2.2.

2.3 TECHNIQUES FOR STUDYING CONFORMATIONAL CHANGES

Various analytical techniques including, optical rotation spectrophotometry, viscosimetry, fluorescence, circular dichroism, infrared absorption and nuclear magnetic resonance (NMR) of hydrogen/deuterium (H/D) exchange are commonly employed to monitor conformational changes of food proteins.

A change of state of a substance is accompanied by changes in energy level. Thermodynamic properties of proteins can be studied by differential scanning calorimetry (DSC). DSC can provide data for measuring sample purity, reaction kinetics and transition temperature (Kilara and Harwalkar, 1996). However, due to the relatively low sensitivity of commercially available DSC instruments, together with the fact that proteins usually exist in low concentration in many foods and have small denaturation enthalpy values, the use of DSC for studying food proteins has been somewhat limited (Kilara and Harwalkar, 1996).

Table 2.2: General characteristics of molecular interactions between two similar protein molecules in aqueous solution. (Bryant and McClements, 1998)

Type	Sign	Strength	Range	pH	I.S.	Temperature
Hydrophobic	Attractive	Strong	Long	No	No	Increase
Electrostatic	Repulsive	Weak → Strong[a]	Short → Long[a]	Yes	Decreases	Increase
Hydrogen bonding	Attractive	Weak	Short	No	No	Decrease
Hydration	Repulsive	Strong	Short	No[b]	No[b]	Decrease
Van der Waals	Attractive	Weak	Short	No	No	-
Steric repulsion	Repulsive	Strong	Short	No	No	-
Disulfide bonds	Attractive	Very Strong	Short	Yes	No	-

[a] Depends on pH and ionic strength (I.S.).
[b] Indirectly depends on pH and I.S. because these factors influence the degree of protein hydration.

Visible and ultraviolet spectroscopic techniques are based on absorption of light and emission of radiation, by tryptophan, tyrosine and to some extent phenylalanine and cystine (i.e. disulfide bond) in the UV range (250-300 nm). Although changes in absorption spectra in UV range are useful to observe structural changes, many difficulties are associated with this technique; these include the presence of prosthetic groups, spectral sensitivity to type and accessibility of solvent and the presence of acid and alkali. Nevertheless, there are studies that have reported the use of UV spectroscopy to monitor food protein conformational changes (Matsuda et al. 1981a, b; Hermansson, 1979; Ma and Harwalkar, 1988).

Circular dichroism (CD) and optical rotation dispersion (ORD) are two optical activity techniques which are used to monitor conformational changes in proteins. They are based on the ability of the protein to rotate a plane polarized light. CD bands originate from the differences of an absorption of left and right handed circularly polarized light. This result from optically active asymmetric molecules at far UV/ amide regions (170-250 nm) due to peptide bonds and at near UV (250-300 nm) due to immobilized aromatic amino acids. The CD technique is useful for studying the secondary structure of proteins and to determine the relative properties of α-helicity, β-sheet and random coil (Wagner and Anderegg, 1994). However, this technique has limited applications in monitoring conformational changes in food proteins. (Bembers and Satterlee, 1975; Matsuda et al., 1981a, b; Kato et al., 1981).

Fluorescence emission is observed when excited electrons return from the first excited state back to the ground state. Fluorescence emission originates from the aromatic amino acids. Fluorescence is an excellent tool to investigate conformational

changes of proteins; however, it is affected by the nature and concentration of the buffer, presence of fluorescence impurities and dust (Schmid, 1997). Although structural changes of protein have been studied by tryptophan fluorescence (Lin et al., 1994), localized structural changes may not be detected since tryptophan exist less commonly in many proteins. Examples of the application of fluorescence techniques to food proteins are illustrated by Yamagishi et al. (1982) and Ma and Harwalkar (1988).

Fourier transform infrared (FTIR) spectroscopy technique has become widely accepted for studying protein structure. This technique is based primarily on the examination of C=O stretching vibrations of amide groups in the region between approximately 1600 and 1700 cm^{-1}. Many studies have used FTIR to monitor changes in secondary structure of food proteins under various physicochemical factors in β-lactoglobulin (Boye et al., 1996b), in BSA (Boye et al., 1996a) and in α-lactalbumin (Boye et al., 1997a). IR spectroscopy has proved useful for 1) assessment of protein-polypeptide secondary structure from the analysis of amide bonds (I and II), 2) mechanistic information about protein function/structure and protein-ligand interaction and 3) determining the accessibility of entire protein backbone to hydrogen-deuterium (H/D) (Surewicz and Mantsch, 1996). However, IR provides little information about the levels of H/D content in a specific region of a protein (Osborne and Nabedryk-Viala, 1982) and whether H/D exchange is complete or not (Surewicz and Mantsch, 1996). Moreover, IR spectroscopy provides little information on the tertiary structure of a protein and requires relatively high protein concentration (Havel, 1996). The absorption of side chains can also affect the spectral properties, since almost 20% of the protein absorption in the amide region is caused by the side chains (Rahmelow et al., 1998).

More detailed insight into the kinetics of structure formation may be gained from nuclear magnetic resonance-deuterium exchange technique. Deuterium exchange studies have been used in combination with NMR to determine protein conformation (Englander and Mayne, 1992), to characterize the fluctuations between different protein conformation (Rohl et al., 1992) and to monitor the changes in protein conformation due to ligand binding (Mayne et al., 1992). However, obtaining these information is restricted to highly soluble proteins with molecular weight of less than 20 kDa (Zhang et al., 1996). Moreover, many amide hydrogens are not included in hydrogen exchange studies. NMR is not capable of measuring the exchange rate of the surface amide hydrogen because either their resonances have not been assigned or they exchange too quickly, before the analysis is complete and consequently cannot be determined (Marmorino et al., 1993). Although much useful information has been obtained through numerous applications of these methods, they give little information about specific regions undergoing structural or dynamic changes and require relatively high concentration and large quantities of purified protein.

2.4 DETECTION OF PROTEIN CONFORMATIONAL CHANGES USING ESI-MS

2.4.1 Charge State Distribution

The utility of electrospray ionization mass spectrometry (ESI-MS) lies in its ability to generate multiply charged gas phase ions from protein molecules in solution. The number of basic residues (Arg, Lys and His) plus the N-terminal amino group in a protein was reported by Loo et al. (1988 and 1990) and Covey et al. (1988) to be

directly related to the charge state of the analyte species observed in ESI mass spectra. However, the shape and placement of this distribution of charge state along the *m/z* axis of mass spectrum depend on the accessibility of these basic sites (Wang and Cole, 1997). Changes in protein conformation from folded, native structure to extended denatured one, make charge sites more accessible (Wang and Cole, 1997). Many reports have appeared on monitoring conformational changes in chicken egg lysozyme induced by altering the pH (Loo et al., 1991), temperature (Le Blance et al., 1991; Mirza et al., 1993) and disulfide bridges (Loo et al., 1990). However, CSD is sensitive to tuning conditions in the mass spectrometer, to slight variation in pH and to counter-ion effect (Robinson, 1996). Therefore, more reliable methods for detecting protein conformational stability are required.

2.4.2 Hydrogen-Deuterium Exchange

The rate at which deuterium exchanges with hydrogen located on the peptide bond has been used to detect structural changes in protein for over thirty years and often measured by NMR and FTIR (Smith et al., 1997). Hence, amide hydrogen exchange rate can be a sensitive probe for detecting changes in protein conformation. Increased or decreased rates of isotopic change can contribute to loosening and tightening of folded structure. Interest in amide hydrogen change as a tool for investigating protein structure, accelerated dramatically with the use of electrospray mass spectrometry (ESI-MS) because of its accurate determination of deuterium levels in peptide and proteins (Smith et al., 1997). The technique is based on the mass spectrometric measurement of the extent of H/D exchange that occurs in different protein conformers over defined periods of time (Katta and Chait, 1991, 1993). This technique is relatively easy to execute, can

monitor exchange simultaneously of global or local regions of protein molecules, and has little or no effect on protein conformation (Sivaraman and Robertson, 2001).

2.4.2.1 Amide Hydrogen Exchange Rates: influencing Factors and Kinetics

Exchange rates of protein and peptide amide hydrogens are sensitive to pH, neighboring side-chain and conformation; detailed studies (Smith et al., 1997) have indicated that amide hydrogen exchange rate (k_{ex}) is catalyzed by acid and base, and can be expressed as the sum of rate constant for acid (k_H) and base (k_{OH}), as indicated in the following equation:

$$k_{ex} = k_H [H^+] + k_{OH} [OH^-] \qquad \text{(Eqn 3)}$$

Bai et al. (1993) measured the isotopic exchange rate (k_{ex}) in polyalanine model as a function of pH (Figure 2.3). They concluded that isotopic exchange must be preformed under pH control. Sensitivity of isotopic exchange of protein and peptide amide hydrogens to neighbouring side-chains has been quantitatively assessed using model dipeptides of the naturally occurring side-chain (Bai et al., 1993).

Higher order structural (2^o, 3^o) features of proteins and peptides can affect amide hydrogen exchange rate drastically, thereby forming the basis for estimating, detecting and locating conformational changes. Various kinetic models have been developed to describe isotopic exchange of amide hydrogen in proteins and peptides (Miller and Dill 1995); most of the results for amide hydrogen exchange in folded protein can be explained by the following equations:

$$NH_{(cl)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} NH_{(op)} \xrightarrow{k_{rc}} ND \qquad \text{(Eqn 4)}$$

Where k_{op} and k_{cl} are the rate constants for structural opening and closing, respectively, and $NH_{(op)}$ is the open form that exchange with solvent at intrinsic rate constant k_{rc}, where rc stands for random coil (Huyghues-Despointes et al., 2001). The measurable rate constant for isotopic exchange k_{ex} is given by:

$$k_{ex} = (k_{op} \cdot k_{cl})/(k_{op} + k_{cl} + k_{rc}) \qquad \text{(Eqn 5)}$$

If the protein is present mainly in the open form ($k_{op} >> k_{cl}$), the isotopic exchange rate constant, k_{ex} is measured as follow:

$$k_{ex} = k_{rc} \qquad \text{(Eqn 6)}$$

The rate constant for isotopic exchange (k_{ex}) in the process described by equation (6) is designated for folded proteins. In this process, isotopic exchange of many amide hydrogen occurred without large structural changes of the native protein (Smith et al., 1997). Isotopic exchange of amide hydrogen located on the surface of folded proteins and peptides; or near open channels, dominates this process. However, unfolding, which include breaking intermolecular hydrogen bonds, is required for isotopic exchange of amide hydrogen that are involved in the secondary structure (e.g., in α-helix and β-sheets).

If the protein is present mainly in the closed form (native), one can usually assume that $k_{cl} >> k_{op}$, in which case equation (5) simplified as follows:

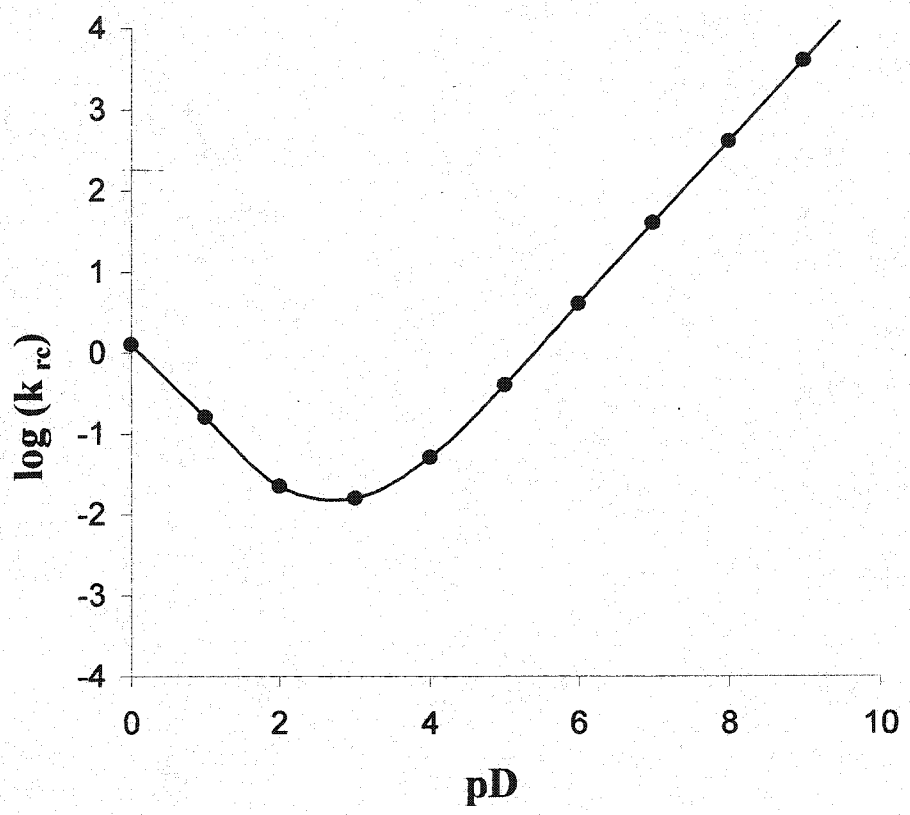

Figure 2.3: Rate constant for isotopic exchange of hydrogen located on peptide amide linkage in polyalanine. (Bai et al., 1993).

$$k_{ex} = (k_{op} \cdot k_{cl})/(k_{cl} + k_{rc}) \qquad \text{(Eqn 7)}$$

From equation (7), two mechanisms could potentially occur, depending on conditions, either as two distinct processes or in combination (Huyghues-Despointes et al., 2001). For most proteins at neutral pH, and in the absence of denaturants, $k_{cl} >> k_{rc}$ and thus the measured rate constant for isotopic exchange is given by:

$$k_{ex} = (k_{op}/k_{cl}) \cdot k_{rc} = K_{eq} \cdot k_{rc} \qquad \text{(Eqn 8)}$$

Where K_{eq} is equilibrium constant describing the opening process; this kinetic mechanism is often referred as EX2 (uncorrelated exchange or biomolecular reaction) (Miller and Dill, 1995; Huyghues-Despointes et al., 2001; Sivaraman and Robertson, 2001). Furthermore, for most proteins at extreme pH and temperature, and in the presence of denaturants, $k_{rc} >> k_{cl}$, the isotopic exchange rate constant, k_{ex} is measured as follows:

$$k_{ex} = k_{op} \qquad \text{(Eqn 9)}$$

This kinetic mechanism is often referred as EX1 (correlated exchange or monomolecular) (Miller and Dill, 1995; Huyghues-Despointes et al., 2001; Sivaraman and Robertson, 2001). For both EX2 and EX1 type exchange, apparent first-order kinetics are expected. The value of k_{ex} can thus be derived by fitting the experimental proton occupancy as a function of time to a simple exponential function (Sivaraman and Robertson, 2001).

CHAPTER 3

APPLICATIONS OF MASS SPECTROMETRY TO FOOD PROTEINS AND PEPTIDES

3.0 CONNECTING STATEMENT

Mass spectrometry (MS) has become an important analytical tool in biological and biochemical research with the development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques. Their speed, accuracy and sensitivity are unmatched by conventional analytical techniques. Characterization of food of proteins and peptides is a rapidly growing field in food chemistry. The work described in this chapter addresses the contributions of MS to determination of the structure of proteins and peptides in some common protein foods such as milk and dairy products, meat and fish products, eggs, legumes, cereal and cereal products and addresses the first objective discussed in the "Rationale and Objectives of Study" section of Chapter 1.

Note: This chapter is the text of a paper which has been published as follows:

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Contribution of co-authors: Alli, I. (thesis supervisor); Konishi, Y. (provided expertise in MS data interpretation).

3.1 ABSTRACT

The application of mass spectrometry (MS) to large biomolecules has revolutionized in the past decade with the development of electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) techniques. ESI and MALDI permit solvent evaporation and sublimation of large biomolecules into the gaseous phase, respectively. The coupling of ESI or MALDI to an appropriate mass spectrometer has allowed determination of accurate molecular mass and detection of chemical modification at high sensitivity (picomole to femtomole). The interface of mass spectrometry hardware with computers and new extended mass spectrometric methods has resulted in the use of MS for protein sequencing, post-translational modifications, protein conformations (native, denatured, folding intermediates), protein folding/unfolding, and protein-protein or protein-ligand interactions. In this review, applications of mass spectrometry, particularly electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), to food proteins and peptides are described.

3.2 INTRODUCTION

Until the middle of 1980's, the analysis of proteins and large polypeptides by mass spectrometry (MS) could not be accomplished because of their polarity and their non-volatile nature (Mann and Wilm, 1995; Siuzdak, 1994). Initially, electron impact (EI) (Biemann and Vetter, 1960) and chemical ionization (CI) (Munson and Field, 1966) were used for small molecules which could be vaporized without decomposition. In these ionization techniques, fragments and ions other than intact molecular ions, appeared in the mass spectra. The development of desorption techniques represented the first major breakthrough for the formation of gaseous protein or large peptide ions without fragmentation. These desorption techniques include field desorption (FD) (Beckey, 1977), plasma desorption (PD) (Bueler et al., 1974; Torgerson et al., 1974), laser desorption (LD) (Karas et al., 1985), secondary ion (SI) and fast atom bombardment (FAB) (Barber et al., 1982). However, experimental difficulties and limited range of molecular masses (M_r), together with low sensitivity at high molecular mass (high-M_r), limited the application of these desorption techniques (Fenn et al., 1989; Smith et al., 1990). Subsequently, atmospheric pressure chemical ionization (APCI) (Horning et al., 1974) and thermospray ionization (TSP) (Blakley and Vestal, 1983) techniques were introduced primarily for on-line coupling of MS to separation techniques like high-performance liquid chromatography (HPLC). Although APCI and TSP advanced the analysis of proteins and peptides, experimental difficulties, such as low sensitivity and limited mass range due to non-volatility and polarity of the samples were still encountered (Gaskell, 1997). Therefore, there was a need of new ionization methods with improved sensitivity and a wider range of applications.

In the early 1980s, two ionization techniques, electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were almost simultaneously emerged for the analysis of large biomolecules. Although the concepts associated with these ionization techniques dated back at least two decades (Bailey, 1988), it was Yamashita and Fenn (Yamashita and Fenn, 1984a, b) and Aleksandrov et al. (Aleksandrov et al., 1984a, b) who successfully coupled an electrospray ion source to a quadrupole and a magnetic sector mass analyzer, respectively. Karas and Hillenkamp developed the MALDI-TOF-MS technique in which relatively large numbers of intact protein ions were generated by laser desorption of a matrix containing protein molecules (Karas and Hillenkamp, 1988). Both ESI-MS and MALDI-TOF-MS now extensively support research of proteins and peptides, each having unique capabilities, as well as some fundamental similarities (Siuzdak, 1994). Their common features include ionization without fragmentation, accurate mass determination, picomole-to-femtomole sensitivity, and broad applicability (Mann and Wilm, 1995; Siuzdak, 1994; Zaluzec et al., 1994; Chait and Kent, 1992); however, induced fragmentation during MS analysis is desirable for sequence analysis of peptides. They are the foundation of the new field of biological MS, a tool that can determine M_r (Alli et al., 1994), conformation changes (Chowdhuray et al., 1990; Katta and Chait, 1991), molecular interaction (Loo and Ogorzalek Loo, 1997), sequence N-terminally blocked protein (Biemann, 1988), define N and C terminals sequence heterogeneity (Loo et al., 1990), locate and correct errors in DNA (Nordhoff et al., 1996), identify sites of deamination and isoasparty formation (Siuzdak, 1994), of phosphorylation (Liao et al., 1994), of oxidation, of disulfide bone

formation (Zaluzec et al., 1994), and glycosylation (Alli et al., 1994; Morgan et al., 1997).

While the principles of MS of proteins are broadly applicable, the focus of this review paper is primarily on applications to food proteins and peptides. During the past two decades, considerable effort has been devoted to structural characterization of food proteins and peptides. Although much useful information has been obtained through numerous spectroscopic methods, they give no information about specific regions undergoing structural or dynamic changes and require relatively high concentration and large quantities of purified protein and long analysis time. In contrast, MS can accurately and precisely probe the conformational changes of large protein M_r in relatively short analysis time (10-90 min). Food proteins whose M_r have been determined by MS are shown in Table 3.1. Comprehensive reviews of the recent theory, instruments and applications of ESI and MALDI have appeared in the literature (Gaskell, 1997; Nordhoff et al., 1996; Nguyen et al., 1995; Kaufmann, 1995; Sporns and Abell, 1996; Gross and Strupta, 1998). This review addresses the contributions of MS to determination of the structure of proteins and peptides in some common protein foods such as milk and dairy products, meat and fish products, eggs, legumes, cereal and cereal products, although the principles applies to other proteins as well.

Table 3.1: Report of Some Food Proteins and Peptides Molecular Mass by ESI and MALDI-MS

Proteins/Peptides	Note	MW (Da)	References
Animal Proteins			
Egg Proteins			
Lysozyme	Hen eggs	14305	Mirza et al., 1993
Ovotrasferrin	Hen eggs	77500	Smith et al., 1990
Ovalbumin	Hen eggs	43300-44585	Smith et al., 1989
	Hen eggs	44630	Deighton et al., 1995
Conalbumin	Turkey eggs	77500-77650	Loo et al., 1989
	Hen eggs	77790	Deighton et al., 1995
Milk Proteins			
Bovine serum albumin	Monomer	66646	Loo et al., 1991
	Dimer	133460	deFrutos et al., 1998
	Trimer	200188	deFrutos et al., 1998
	Tetramer	266329	deFrutos et al., 1998
	Pentamer	332800	deFrutos et al., 1998
β-lactoglobulin	B variant (Bovine)	18277	Morgan et al., 1997
	A variant (Bovine)	18363	Leonil et al., 1997
	Buffalo	18306	Angeletti et al., 1998
	Ewe	18102	Fanton et al., 1998
α-lactalbumin	Bovine	14175	Smith et al., 1990
	Buffalo	14270	Angeletti et al., 1998
	Ewe	14139	Fanton et al., 1998
Casein Subunits Bovine	α_{S1} phosphorylated	23725-23682	Alli et al., 1998
	α_{S1} dephosphorylated	23005	Alli et al., 1998
	α_{S2}-CN	25241	Alli et al., 1998
	β-CN	24085	Alli et al., 1998
	κ-CN	19125	Alli et al., 1998
	γ_1-CN	20085	Alli et al., 1998
	γ_2-CN	11856	Alli et al., 1998
	γ_3-CN	11591	Alli et al., 1998
Casein Subunits Buffalo	α_{S1}-CN	23406	Angeletti et al., 1998
	β-CN	24066	Angeletti et al., 1998
	κ-CN	19192	Angeletti et al., 1998
	γ_1-CN	20090	Angeletti et al., 1998
	γ_2-CN	11850	Angeletti et al., 1998
	γ_3-CN	11588	Angeletti et al., 1998
Ovine (α_{S1})			
Variant A	9P (199 amino acid)	23478	Ferranti et al., 1995
	9P (191 amino acid)	22467	Ferranti et al., 1995
Variant C	8P (199 amino acid)	23412	Ferranti et al., 1995
	8P (191 amino acid)	22390	Ferranti et al., 1995
Variant D	4P (199 amino acid)	23188	Ferranti et al., 1995

Proteins/Peptides	Note	MW (Da)	References
	4P (191 amino acid)	22194	Ferranti et al., 1995
Caprine (α_{S1})			
Variant A	8P (199 amino acid)	23362	Ferranti et al., 1997a
	8P (198 amino acid)	23239	Ferranti et al., 1997a
	8P (191 amino acid)	22351	Ferranti et al., 1997a
Variant B	8P (199 amino acid)	23345	Ferranti et al., 1997a
	8P (198 amino acid)	23215	Ferranti et al., 1997a
	8P (191 amino acid)	22333	Ferranti et al., 1997a
Variant C	8P (199 amino acid)	23267	Ferranti et al., 1997a
	8P (198 amino acid)	23141	Ferranti et al., 1997a
	8P (191 amino acid)	22252	Ferranti et al., 1997a
	dephosphorylated	21770	Ferranti et al., 1997a
Proteoso peptone p.p.8.1	Bovine	9170	Angeletti et al., 1998
	Buffalo	8670	Angeletti et al., 1998
Meat Proteins			
Pepsin	Porcine	34584	Loo et al., 1992
Rennin	Bovine	35646	Loo et al., 1992
Actin		42000	Smith et al., 1990
Plant Proteins			
Legume Proteins			
Trypsin inhibitor	Soybean	20097	Mann et al., 1989
	P. vulgaris	8406	Bergeron and
		8957	Nielsen, 1993
α-Amylase	Bacterial source	54700	Fenn et al., 1989
	Kidney beans	54857	Gibbs and Alli, 1998
Concanavalin A	Jack bean	25573	Loo et al., 1992
	A1 fragment	12937	Loo et al., 1992
	A2 fragment	12653	Loo et al., 1992
Phaseolin polypeptides	*P. vulgaris*		
	Fraction 1	49615	Alli et al., 1993
	Fraction 2	48075	Alli et al., 1993
Phaseolin polypeptides	*P. lunatus*		
	Fraction 1	26240	Alli et al., 1994
	Fraction 2	26113	Alli et al., 1994
	Fraction 3	24249	Alli et al., 1994
Soybean Agglutinin (SBA)	Glycoprotein		
SBAI	β subunit	28000	Mandal et al., 1994
	α subunit	29437	Tang et al., 1994
SBAII	β subunit	28000	Mandal et al., 1994
	γ subunit	28327	Tang et al., 1994
	γ′ subunit	28627	Mandal et al., 1994
	α′ subunit	29325	Tang et al., 1994
	α subunit	29437	Mandal et al., 1994

Proteins/Peptides	Note	MW (Da)	References
SBA III	β subunit	28000	Tang et al., 1994
	γ subunit	28327	Mandal et al., 1994
	α′ subunit	29325	Tang et al., 1994
	α subunit	29437	Mandal et al., 1994
Coconut Proteins			
Coconut milk	Fraction 1	46640	Sumual, 1994
	Fraction 2	50359-51209	Sumual, 1994
	Fraction 3	35574 & 4769	Sumual, 1994
Insoluble solids	Fraction 1	46640-46861	Sumual, 1994
	Fraction 2	50376-51100	Sumual, 1994
	Fraction 3	49040	Sumual, 1994
Acid precipitate	Fraction 3	48861-49142	Sumual, 1994
Cereal Proteins			
Glutenin (HMW Subunits)			
Sicco isogène	1Ax1	87500	Hickman et al., 1995
Chinese spring	1Dx2	88379	Hickman et al., 1995
Cheyenne	1Dx5	88930	Hickman et al., 1995
Cheyenne	1Bx7	83500	Hickman et al., 1995
Cheyenne	1By9	72500	Hickman et al., 1995
Cheyenne	1Dy10	68360	Hickman et al., 1995
Chinese spring	1Dy12	69520	Hickman et al., 1995
Katepwa	Subunit 2	86202 & 87936	Dworschak et al., 1998
Katepwa	Subunit 7	82279	Dworschak et al., 1998
Katepwa	Subunit 9	73308	Dworschak et al., 1998
Katepwa	Subunit 10	67280	Dworschak et al., 1998
Agglutinin	Wheat Germ	17090	Yamashita and Fenn, 1994a

3.3 ANIMAL PROTEINS AND PEPTIDES

3.3.1 Eggs

MS has established the heterogeneity of proteins from eggs and determined the M_r of the various egg protein components without purification. The principal proteins of egg, ovalbumin, conalbumin and lysozyme have all been characterized by ESI-MS; M_r have been determined for ovalbumin (44 300), conalbumin (77 500) and lysozyme (14 305) (Deighton et al., 1995). ESI-MS has also shown that irradiation of egg white resulted in an increase in the M_r of ovalbumin to 44 630 and conalbumin to 77 790 while the M_r of lysozyme was not affected (Deighton et al., 1995). Lysozyme, in particular, has been the subject of numerous MS studies on protein conformational changes and changes in charge-state distribution. Loo et al. observed that the charge state of lysozyme was shifted from 12+ to 14+ by changing pH from 3.3 to 1.6, reflecting the conformational change from compact native state to disordered state (Loo et al., 1991). A similar shift was observed in thermal denaturation of lysozyme (Le Blanc et al., 1991; Mirza et al., 1993). A further shift from 15+ of denatured lysozyme to 20+ was observed by reducing four disulfide bridges (Loo et al., 1990). Konermann and Douglas similarly observed more net negative charge upon unfolding of lysozyme in negative mode (Konermann and Douglas, 1998). The results indicated that the protein was charged, positively or negatively depending on the positive or negative ionization mode, respectively, and the number of net charge predominantly reflected the protein conformation.

The ability of ESI-MS to resolve mass differences as small as a single proton was used by Katta and Chait to study conformational changes in egg lysozyme by hydrogen/deuterium (H/D) exchange ESI-MS as each deuteration increases the protein mass by 1 u (Katta and Chait, 1991, 1993). The extent of H/D exchange occurred in different protein conformers over defined time periods differed widely depending on the conformation (Katta and Chait, 1991). Katta and Chait demonstrated, on the basis of both the changes in charge state distribution and H/D exchange rates, that reduced lysozyme exists in an unfolded state (Katta and Chait, 1993). The mechanism of folding in egg lysozyme was investigated using H/D exchange technique both with MS and NMR (Miranker et al., 1993).

3.3.2 Milk and Dairy Products

The proteins of milk and dairy products have been the subjected of numerous MS investigations; these include identification of milk protein variants and glycoforms, fingerprinting, degree of glycoforms, detection of milk adulteration and identification of peptides in dairy products.

3.3.2.1 Whey Proteins

ESI-MS studies with purified β-lactoglobulin (β-Lg) from bovine milk revealed the presence of multiple M_r species of β-Lg (A, B and C variants) (Burr et al., 1996). In addition, the existence of multiple glycosylation of these bovine β-Lg variant have been identified by several ESI-MS studies (Morgan et al., 1997; Leonil et al., 1997; Morgan et al., 1998; Jones et al., 1998; Slangen and Visser, 1999); the results indicated that nonenzymatic lactosylation of β-Lg occurs under mild heat treatment. ESI-MS has also

been used to rapidly characterize the complex mixture of glycoforms of α-lactalbumin (α-Lac) without the need of further purification of these forms (Slangen and Visser, 1999). Alli et al. used ESI-MS to detect the presence of glycoforms in both β-Lg (Figure 3.1a) and α-Lac (Figure 3.1b), in whey protein concentrate (WPC) and in lyophilized whey; the presence of at least three glycoforms of β-Lg and one glycoforms of α-Lac was detected (Alli et al., 1999). MALDI-TOF-MS has also been used to detect nonenzymatic glycosylation of several peptides; these researchers concluded that MALDI-TOF-MS was helpful in conforming that amino acid residues, other than lysine, are glycosylated. (Kim et al., 1997).

Two-dimensional electrophoresis coupled to MALDI-TOF-MS were used to detect the C-terminal of truncated forms of β-Lg in whey from Romagnola cow's milk (Zappacosta et al., 1998). The result clearly shows that two of minor components were related to β-Lg A variant and two to β-Lg B variant. Hu et al. used ESI-MS to determine calcium-binding stoichiometry for calcium-binding proteins (Hu et al., 1994). They found that bovine α-Lac binds specifically to one Ca^{2+} ion and suggested that ESI-MS can be used to determine the number and type of metal ions that bind to protein.

MALDI-TOF-MS was used to study the effect of different chromatographic conditions on the elution of bovine serum albumin (BSA) in reversed-phase high-performance liquid chromatography (RP-HPLC) system (deFrutos et al., 1998); multiple peaks of BSA were observed when shallow gradients were used for elution. MALDI-TOF-MS revealed that these RP-HPLC multiple peaks were aggregated forms of BSA (deFrutos et al., 1998).

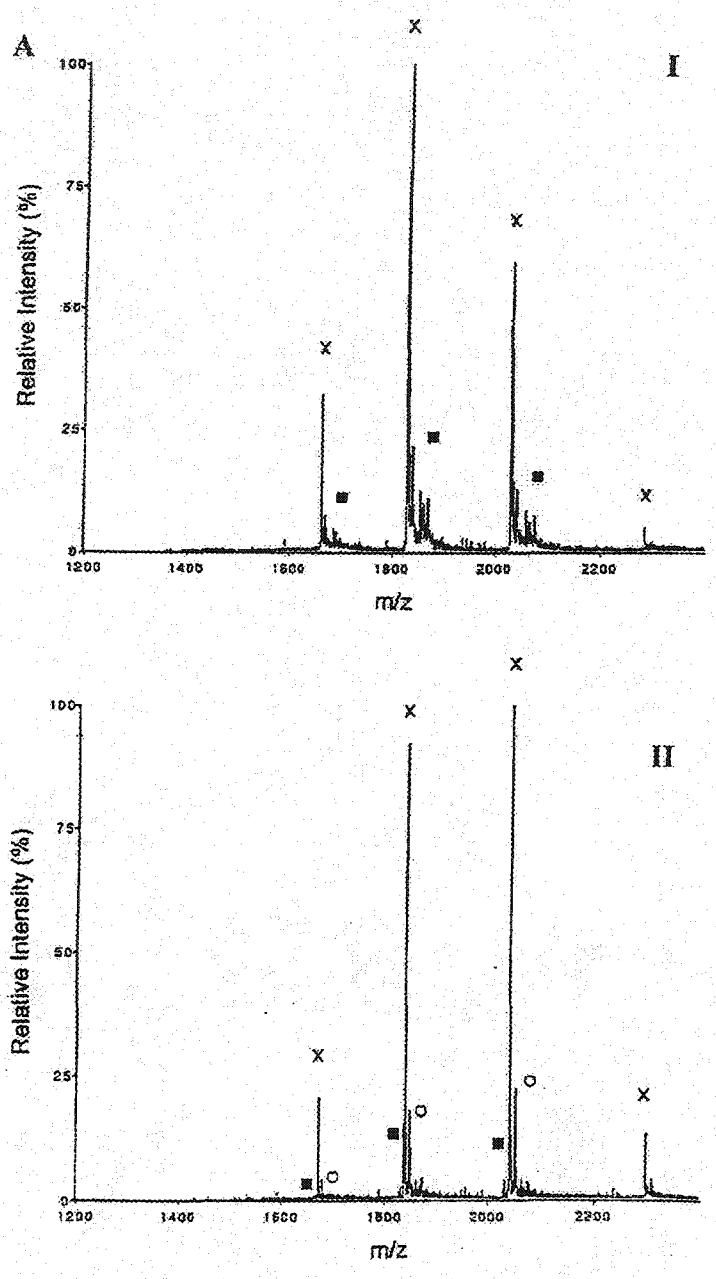


Figure 3.1: (A) Interpreted mass spectra of (I) fraction β-F1 (x= 18 279; ■= 18 606) and (II) fraction β-F2 (x= 18 366; ○= 18 464; ■= 18 692) from RP-HPLC of commercial β-Lg and (B) interpreted mass spectrum of fraction α-F from RP-HPLC of commercial α-Lac (x= 14 181; ○= 14 278; ■= 14 504). Reprinted with permission from (Alli et al., 1999).

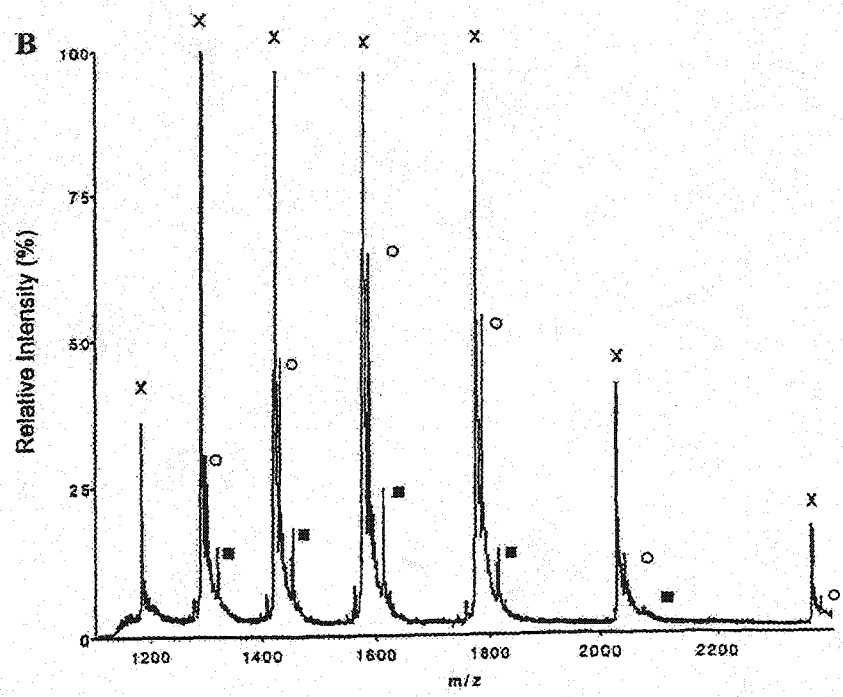


Figure 3.1: (continued).

RP-HPLC of acid hydrolysates of whey protein concentrate (WPC) identified three fractions in the unhydrolyzed WPC and three other fractions after 18 h hydrolysis (Pasdar, 1995). Each fraction was a mixture of a few to several peptides and their M_r was determined by ESI-MS. The results confirmed that organic acid hydrolysis of WPC resulted in peptides that were smaller than those in unhydrolyzed WPC (Pasdar, 1995).

3.3.2.2 Casein proteins

MS has established the heterogeneity of proteins from casein (CN) and determined the M_r of the various casein protein components. Casein is made up of several components; the main molecular subunits are α_{S1}-, α_{S2}-, β-, κ- and γ-CN (Table 3.1).

Combined use of ESI-MS and FAB-MS confirmed the primary structure of mature ovine α_{S1}-CN as well as the amino acid substitutions in variants A, C and D, and identified the phosphorylation sites (Ferranti et al., 1995). The mature protein of each variant was found to be mixture of two subunits, both with multiple phosphorylated forms of the same protein; one subunit was full-length (199 amino acid residues), which accounted for about 80%, the other was the deleted form which lacked segment 141-148 of the mature protein and represented about 20% of the α_{S1}-CN (Ferranti et al., 1995). MS analysis also revealed that the differences among the three genetic variants (A, C, and D) were simple silent substitutions, which involved the degree to which the protein was phosphorylated (Ferranti et al., 1995). Similarly, MS study revealed that mature goat α_{S1}-CN exists as a mixture of at least four variants (A, B, C and D) which differ in peptide chain length and in the number of phosphorylated serine residues (Ferranti et al., 1997a). Variant A is the main component (48%) corresponds to full-length of α_{S1}-CN

(199 amino acid). The three short forms of the protein are variant B (198 amino acid, 29%) missing Gln 78, variant C (191 amino acid, 16%) missing residues 141-148 and subunit D (191 amino acid, 7%) missing residues 110-117 (Ferranti et al., 1997a).

MS was used to resolve the heterogeneity of caseinomacropeptide (CMP), a polypeptide of 64 amino acids which is released from bovine κ-casein by the action of chymosin during the primary phase of milk clotting (Mollé and Léonil, 1995). This study described the ability of RP-HPLC coupled with ESI-MS to characterize a complex mixture like CMP. MS was also used to identify the preferential cleavage sites of recombinant chymosin on purified κ-CN (A variant) over the pH range 6.6-2.6; the rate and extent of hydrolysis of κ-CN and its macropeptide moiety increased with decreasing the pH to a maximum at 3.6 (Reid et al., 1997).

3.3.2.3 Dairy products application

MALDI-TOF-MS was used to detect thermal degradation and to determine the protein content of milk samples obtained with different conditions of pasteurization (70-90 °C for 10-30 s) and sterilization (140-150 °C for 2-5 s) (Catinella et al., 1996a; b). The capability of MALDI-TOF-MS to characterize protein profile from several cow milks was also studied (Catinella et al., 1996b); it was found that the protein fingerprint of milk from four different breeds of cows at the same lactation stage and under the same feeding system were different in their MALDI spectra (Catinella et al., 1996b). MALDI-TOF-MS was also used to evaluate the protein profile of cow's milk after different enzymatic and/or thermal treatments of eleven infant milk formulas; the results demonstrated the degree of hydrolysis of different protein hydrolysate formulas (Sabbadin et al., 1999). Similarly MALDI-TOF-MS was used to evaluate the effect of

diet and pathological states on the protein profile of human milk (Catinella et al., 1999). MALDI-TOF-MS has also been used to determine the effects of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, on milk proteins during yogurt preparation (Fedele et al., 1999a); the results indicated that fermentation with the former bacteria resulted in the breakdown of high molecular mass (high-M_r) casein with the production of a low molecular mass (low-M_r) peptide (3 850) while the latter bacteria did not exhibit any proteolytic activity. Proteolysis was enhanced when milk is incubated with a mixture of the two bacteria (Fedele et al., 1999a). Furthermore, MALDI mass spectra of different strains of the same bacteria species indicated differences in proteolytic activity (Fedele et al., 1999a, b).

Angeletti et al. examined the capabilities of MALDI-TOF-MS for the characterization of water-buffalo milk and mozzarella cheese to detect possible fraudulence in mozzarella cheese production (Angeletti et al., 1998); results indicated that buffalo milk proteoso peptone p.p. 81 and α_{S1}-CN had lower M_r (8 670 and 23 406, respectively) than that of bovine milk (9 170 and 23 682, respectively)(Figure 3.2) (Angeletti et al., 1998). Furthermore, it was determined that (a) the relative abundance of peaks due to α_{S2}-CN and β-Lg was lower in buffalo milk than in bovine milk (Figure 3.2) and (b) a peak of M_r 15 790 (protein X), which is commonly found in buffalo milk, was still detected in water buffalo mozzarella cheese, indicating that this protein is resistant to thermal and enzymatic processes (Angeletti et al., 1998). MALDI-TOF-MS was used to detect adulteration of ewe's cheese with bovine milk (Fanton et al., 1998); the mass spectrum of the ewe's milk indicated that β-Lg, γ_2-CN and α-Lac have M_r of 18 102, 11 827 and 14 139 respectively, which are all lower than those of the same proteins in bovine milk. Furthermore, the relative abundance of (a) peak at *m/z* 11 325

(due to γ_3-CN) from ewe cheese and (b) peak at *m/z* 11 869 (due to γ_2-CN) from bovine cheese, can be used to determine the percentage of bovine milk fraudulently added to ewe's milk, in the production of ewe's cheese (Fanton et al., 1998).

Both MALDI-TOF-MS and ESI-MS have been used to identify peptides in cheese (Gouldsworthy et al., 1996; Alli et al., 1998). MALDI-TOF-MS revealed that eight peptides in cheddar cheese were derived from α_{S1}-CN, seven from β-CN and one from α_{S2}-CN (Gouldsworthy et al., 1996). ESI-MS and electrospray ionization tandem mass spectrometry (ESI-MS/MS) identified a total of twenty-five peptides in mild, medium and old cheddar cheese and a commercial cheddar cheese flavor (Alli et al., 1998). Thirteen peptides were found to be derived form α_{S1}-CN, seven from β-CN and five from κ-CN. Figure 3.3 (A) and (B) shows the mass spectra of a single M_r specie (M_r 1 052) and two or more molecular species, respectively, while Figure 3.4 (A) and (B) shows the ESI-MS/MS of a singly charged specie of M_r 1 052 and a doubly charged specie of M_r of 1 366, respectively (Alli et al., 1998). MS results also revealed the presence of N-terminal segments of β-, α_{S1}- and α_{S2}-CN and α-Lac in water-soluble fractions of cheddar cheese; this was explained on the basis of known specificities of lactococcal cell envelope proteinases; such as chymosin, plasmin and phosphatase (Singh et al., 1997).

The relationship between the degrees of hydrolysis (DH) of CN using trypsin and pancreatin independently, and the release of casein phosphopeptide (CPP) has been studied by MALDI-TOF-MS (Adamson and Reynolds, 1997). Highest yields of CPP were obtained at casein DH of 17% and 19-23 % for trypsin and pancreatin, respectively. The relationship between CPP production in Grana Padano cheese and

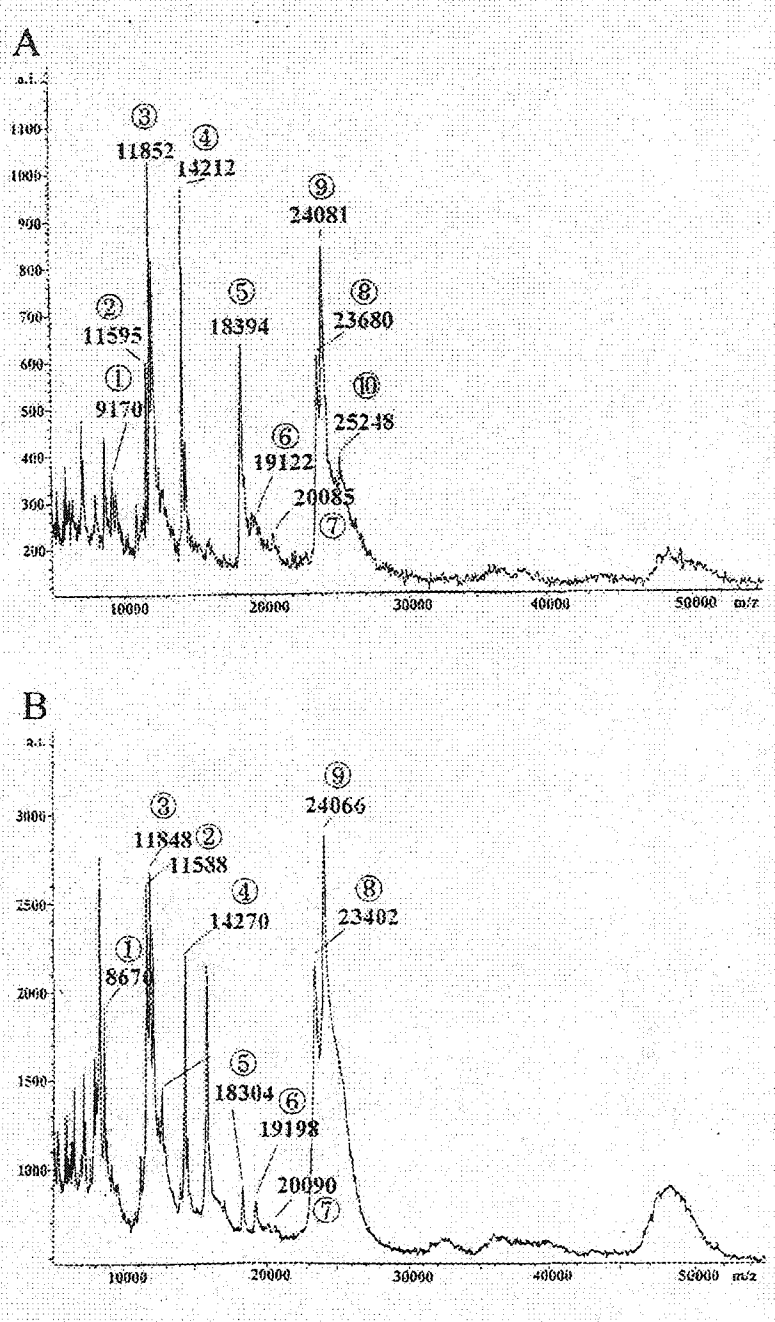


Figure 3.2: MALDI mass spectrum of bulk bovine (A) and water buffalo (B) milk. Protonated molecules of (1) proteoso peptone p.p. 8.1, (2) γ_3-CN, (3) γ_2-CN, (4) α-Lac, (5) β-Lg, (6) κ-CN, (7) γ_1-CN, (8) α_{S1}-CN, (9) β-CN and (10) α_{S2}-CN. Reprinted with permission from (Angeletti et al., 1998)

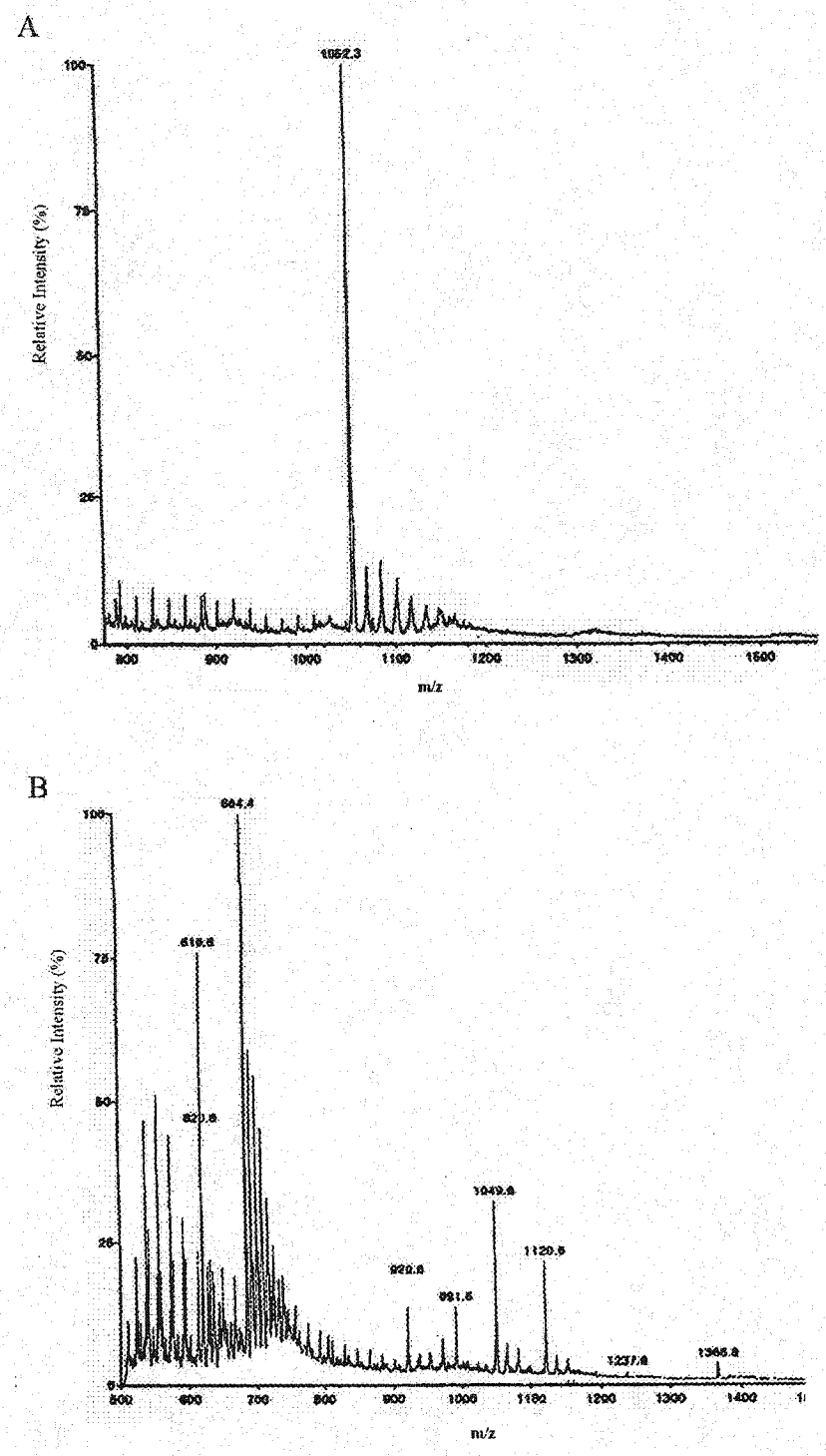


Figure 3.3: Interpreted mass spectra of a fraction containing a single M_r specie (A) and Multiple species (B). Reprinted with permission from (Alli et al., 1998).

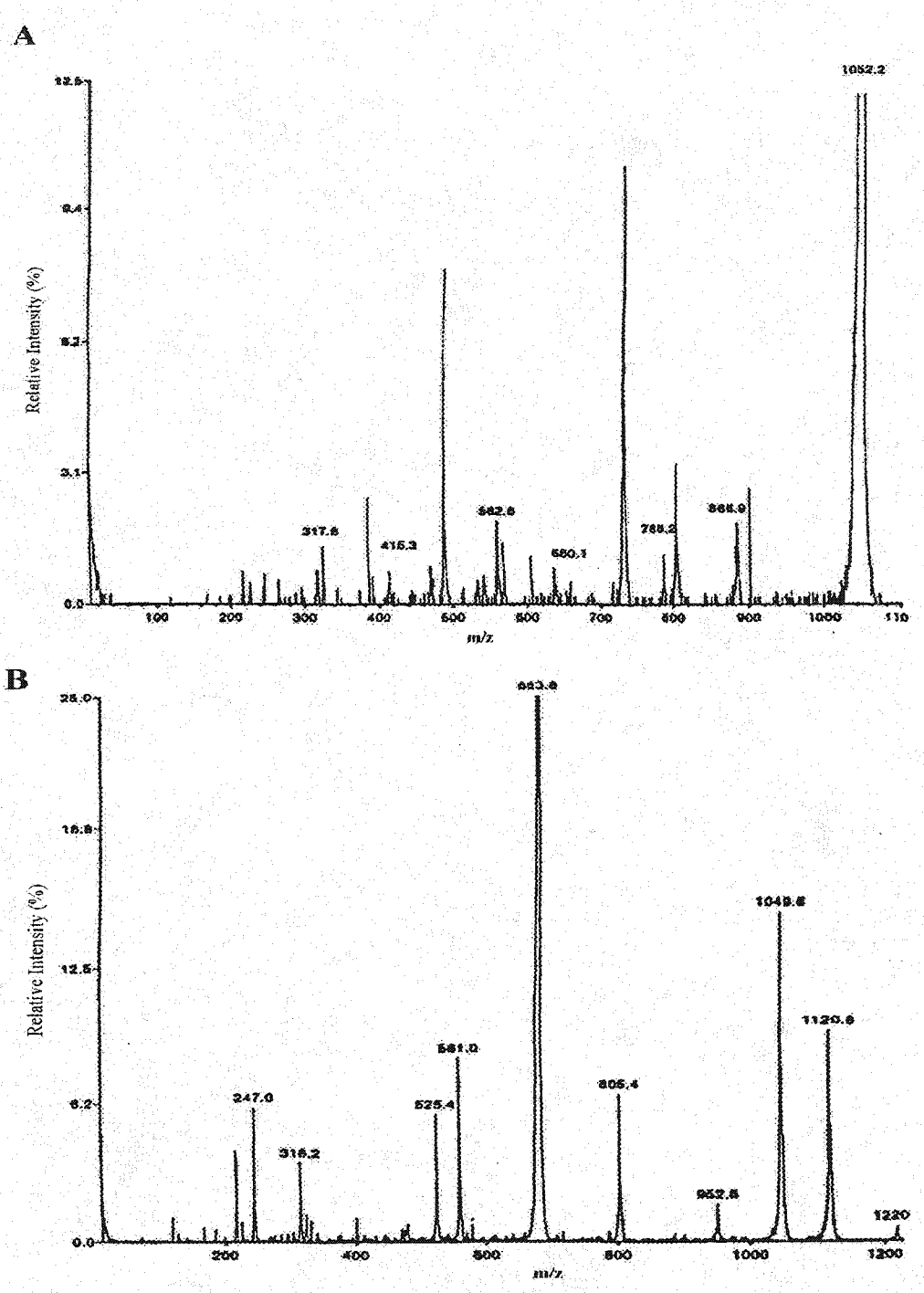


Figure 3.4: ESI-MS/MS spectra of specie (A) M_r= 1 052 (singly-charged) and (B) M_r= 1 366 (doubly-charged). Reprinted with permission from (Alli et al., 1998).

ripening (4-38 months) was also studied using FAB-MS; forty-five phosphopeptides (24 from β-CN, 16 from α_{S1}-CN and 5 α_{S2}-CN) were identified (Ferranti et al., 1997b). Moreover, CPP interaction with colloidal calcium phosphate (CCP) isolated by tryptic hydrolysis was characterized by RP-HPLC-ESI-MS (Gagnaire et al., 1996). It was shown that among the peptides produced, fourteen phosphopeptides were identified (8 α_{S1}-CN and 6 β-CN) and half of SerP cluster from β-CN and all SerP from α_{S1}-CN can interact with CCP (Gagnaire et al., 1996).

3.3.2.4 Meat and fish

In comparison with the proteins of milk and egg, there have been relatively fewer MS studies on proteins of meat and fish. FAB-MS has shown that the tryptric hydrolysate of calciprotein from crayfish contains at least eight peptides with M_r ranging from 375 to 1 455 with close agreement in sequence information obtained by FAB-MS and from amino acid analysis (Aubagnac et al., 1988). ESI-MS revealed the presence of at least twenty-five polypeptides with M_r ranging from 2 000 to 42 800 in the soluble nitrogen extract of fresh carp fish (Alomirah, 1996; Alomirah and Alli, 1996); a typical interpreted ESI-MS mass spectra of fish polypeptide with M_r of 16 751 is illustrated in Figure 3.5. RP-HPLC-ESI-MS demonstrated that the sarcoplasmic protein extracted from ground and whole meat contained at least twelve polypeptides with M_r ranging from 11 000 to 42 000 (Alomirah et al., 1998). The relative peak area of 35 700 protein shown in Figure 3.6 decreased during storage of meat; this protein could be investigated as an indicator of freshness (Alomirah et al., 1998).

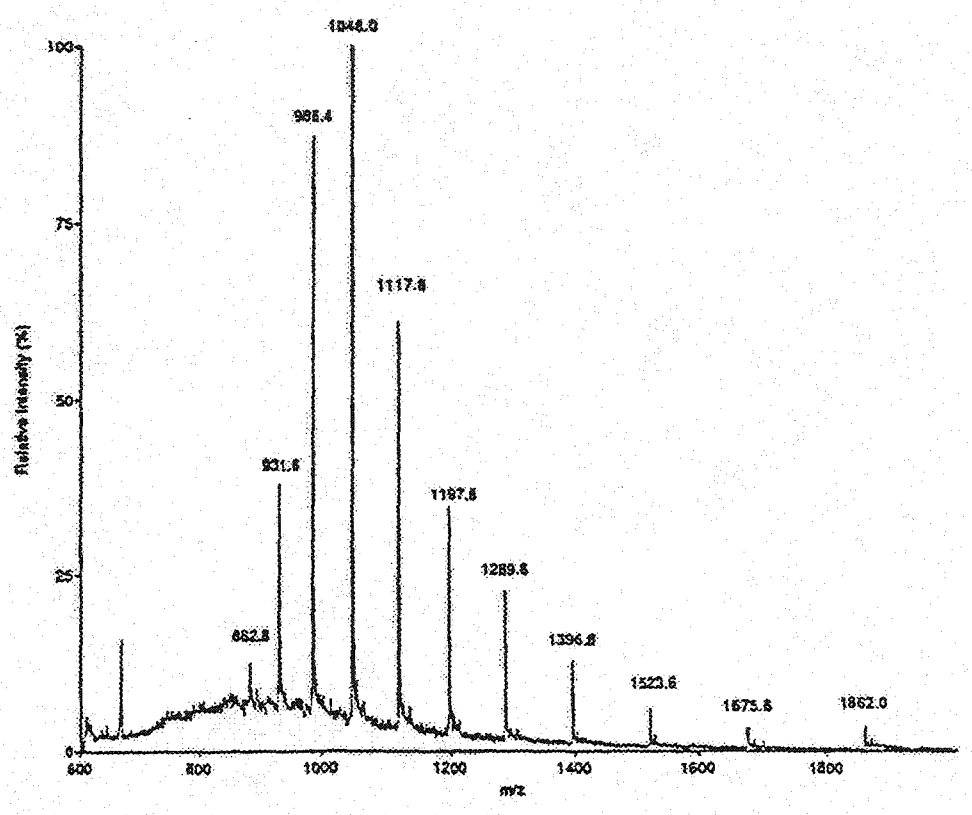


Figure 3.5: A typical interpreted ESI-MS mass spectra of carp fish polypeptide with M_r of 16 751. Reprinted with permission from (Alomirah, 1996).

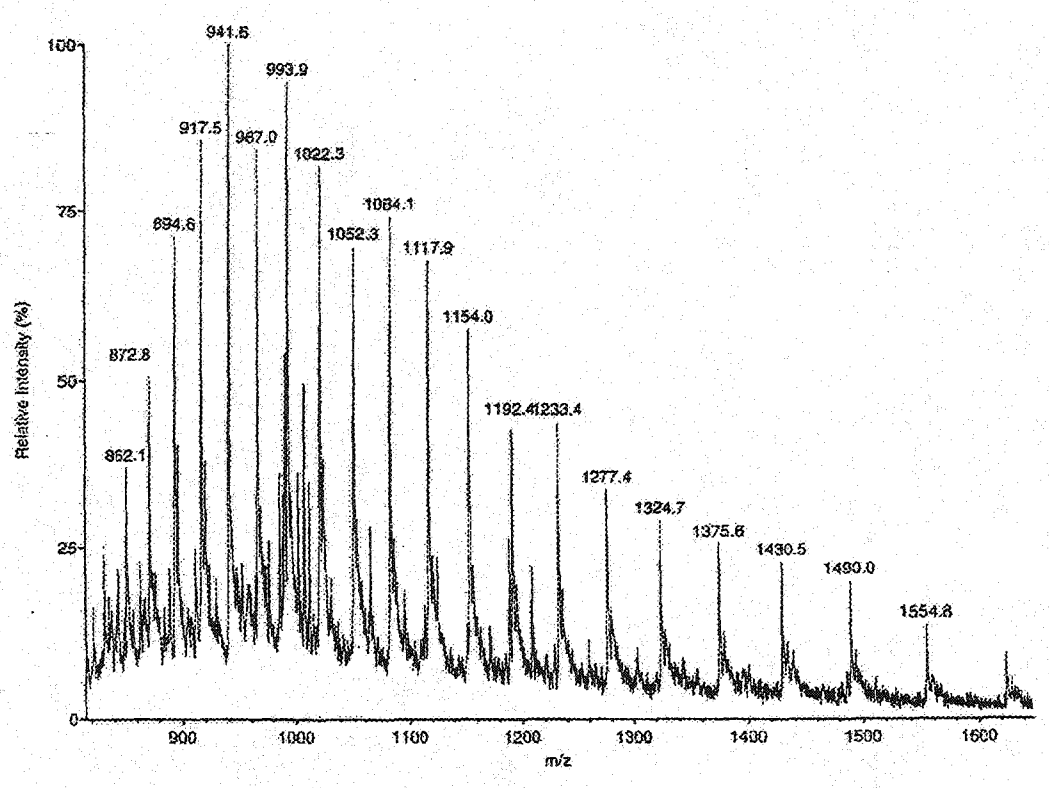


Figure 3.6: Interpreted mass spectra of sarcoplasmic protein extracted from meat with M_r of 35 740. Reprinted with permission from (Alomirah et al., 1998).

3.4 PLANT PROTEINS AND PEPTIDES

Over the years plant storage proteins have become important functional ingredients in many prepared foods. Concurrently, there has been increased interest in understanding the structure-functional relationships of these storage proteins to explain and even predict certain desired characteristics; this requires elucidation of the molecular characteristic of the proteins. To date, the use of MS for characterizing plant storage protein has been somewhat limited.

3.4.1 Cereals

Cereal grains contain complex mixtures of proteins with structurally different molecular characteristics. The classification of these proteins as globulins, albumins, glutenins and prolamins is widely accepted; each of these groups in turn, represent a complex mixture of proteins. MALDI-TOF-MS has been used in several studies to characterize the subunits of glutenin (Hickman et al., 1995; Dworschak et al., 1998). A total of seven high-M_r subunits with a M_r of 87 500, 88 379, 88 930, 83 500, 72 500, 68 360 and 69 520 have been identified (Hickman et al., 1995). In general, the M_r of these subunits are close to those calculated from gene sequence and within the range of analytical error; this study demonstrated that the high-M_r subunits are not extensively glycosylated, as previously reported (Hickman et al., 1995). Dworschak et al. reported the use of MALDI-TOF-MS for assessing the composition and mass distribution of crude and partially purified wheat gluten prolamins (gliadin) and reduced high-M_r and low-M_r glutenin subunit fractions from common and durum wheat varieties without prior separation by HPLC (Dworschak et al., 1998). Gliadins and low-M_r glutenin

subunits showed complex MALDI mass spectra with M_r ranging from 30 000 to 40 000, while the mass spectra of high-M_r glutenin subunits were fairly simple with M_r ranging from 87 936 to 71 520. The results indicated the feasibility of using MALDI-TOF-MS in wheat breeding programs for the rapid and routine identification of specific high-M_r subunits associated with superior quality (Dworschak et al., 1998).

MALDI-TOF-MS has also been used to quantify gluten gliadins in both processed and unprocessed foods (Camafeita et al., 1997a, b); the procedure is rapid and sensitive with good correlation with data from an immunological assay method. MALDI-TOF-MS can be used as a rapid screening technique for (a) the presence of gliadins in foods by monitoring the occurrence of the protonated gliadin mass pattern in the mass range from 25 000 to 40 000 and (b) the presence of other toxic gluten cereal prolamins fractions, such as barley hordeins, rye secalins and oat avenins (Camafeita and Mendez, 1998; Camafeita et al., 1998).

The major wheat flour immunoreactive proteins that are responsible for baker's asthma has been identified as a member of the α-amylase family using MS and the N-terminal amino acid sequence (Amano et al., 1998).

3.4.2 Legume Seeds

ESI-MS has been used to characterize the quaternary structure of the three isolectins of soybean agglutinin (SBA), a tetrameric glycoprotein previously reported to consist of two subunits (Mandal et al., 1994). The ESI-MS results showed that the quaternary structure composition of the three isolectins of SBA (SBA I, SBA II, and SBA III) were approximately $\alpha 2\beta 2$, $\alpha 2\beta\gamma$ (and $\alpha 2\beta\gamma'$) and $\alpha 2\gamma 2$, respectively.

Similarly, ESI coupled with time-of-flight (TOF) has been used to investigate noncovalent protein-protein interactions in SBA (Tang, 1994).

A wide range of M_rs (8 000-23 000) have been reported for trypsin inhibitors (TIs) of dry beans depending on the method used for estimating the M_r; (i.e., ultracentrifugation, size exclusion chromatography, SDS-polyacrylamide gel electrophoresis and amino acid composition). Plasma desorption mass spectrometry (PDMS), using ^{252}Cf as the ionizing source, was used in the partial characterization of TIs of great northern beans (*Phaseolus vulgaris*) (Bergeron and Nielsen, 1993). The PD-MS of TIs revealed two peaks of M_r 8 406 and 8 957, corresponding to TI chain lengths of 76 and 81 amino acid residues, respectively (Bergeron and Nielsen, 1993).

ESI-MS and ESI-MS/MS were used to investigate the extent to which the polypeptide subunits in a crystalline protein isolated by citric acid from dried seeds of white kidney beans (*Phaseolus vulgaris*) were similar to the subunits of native phaseolin through the identification and characterization of the phaseolin polypeptides (Alli et al., 1993); the isolated crystalline proteins were shown to contain polypeptides with average M_r of 49 615 and 48 075 which were similar to those reported for α-type and β-type phaseolin precursors, respectively (Alli et al., 1993). Using the same techniques (ESI-MS and ESI-MS/MS) for characterizing a crystalline protein isolated from large lima beans (*P. lunatus*), it was shown that a glycosylated phaseolin polypeptide fragment of M_r 26 240 was similar to a C-terminal segment of the phaseolin polypeptides of *P. vulgaris,* while a glycosylated subunit of M_r 26 113 and its non-glycosylated variant of M_r 24 249 were similar to an N-terminal segment of phaseolin polypeptides of *P.vulgaris*. (Alli et al., 1994) (Figure 3.7).

α-Amylase inhibitors which can effect the response of blood glucose insulin or general starch digestion and absorption in mammals has studied by RP-HPLC-ESI-MS (Gibbs and Alli, 1998). It was shown that in a crude extract prepared from white kidney beans (*P vulgaris*), a high α-amylase inhibitor activity was associated with a glycoprotein whose deglycosylated M_r was estimated as 54 857 by ESI-MS. Figure 3.8 shows the mass spectra of the purified deglycosylated (A) and glycosylated (B) inhibitors.

ESI-MS and ESI-MS/MS were used to investigate the differences in the tryptic digestion of crystalline and amorphous (non crystalline) proteins isolated from *Phaseolus* beans by comparing the peptides which resulted from the hydrolysis (Yeboah et al., 1999); the results suggested that trypsin specific peptide bonds located in the β-structure region of β-type phaseolin was resistant to trypsin hydrolysis while the most accessible region to tryptic cleavage were located within α-helix structures and in regions of interconnecting secondary structure. MS has also been used to study the proteolytic changes associated with *Rhizopus oligosporus* fermentation of soybean to produce tempe from soybeans (Ismoyo, 1995). ESI-MS showed several tempe peptides with M_r ranging from 569 to 16 688.

3.4.3 Other Plants

Proteins isolated from other plant sources such as coconut have been characterized by MS (Sumual, 1994). By use of RP-HPLC and ESI-MS proteins with M_r of 51 209, 50 359, 49 142, 49 040, 48 861, 47 679, 46 640 and 35 574 were separated and identified (Sumual, 1994). Figure 3.9. shows mass spectrum of the protein component with M_r of 46 640.

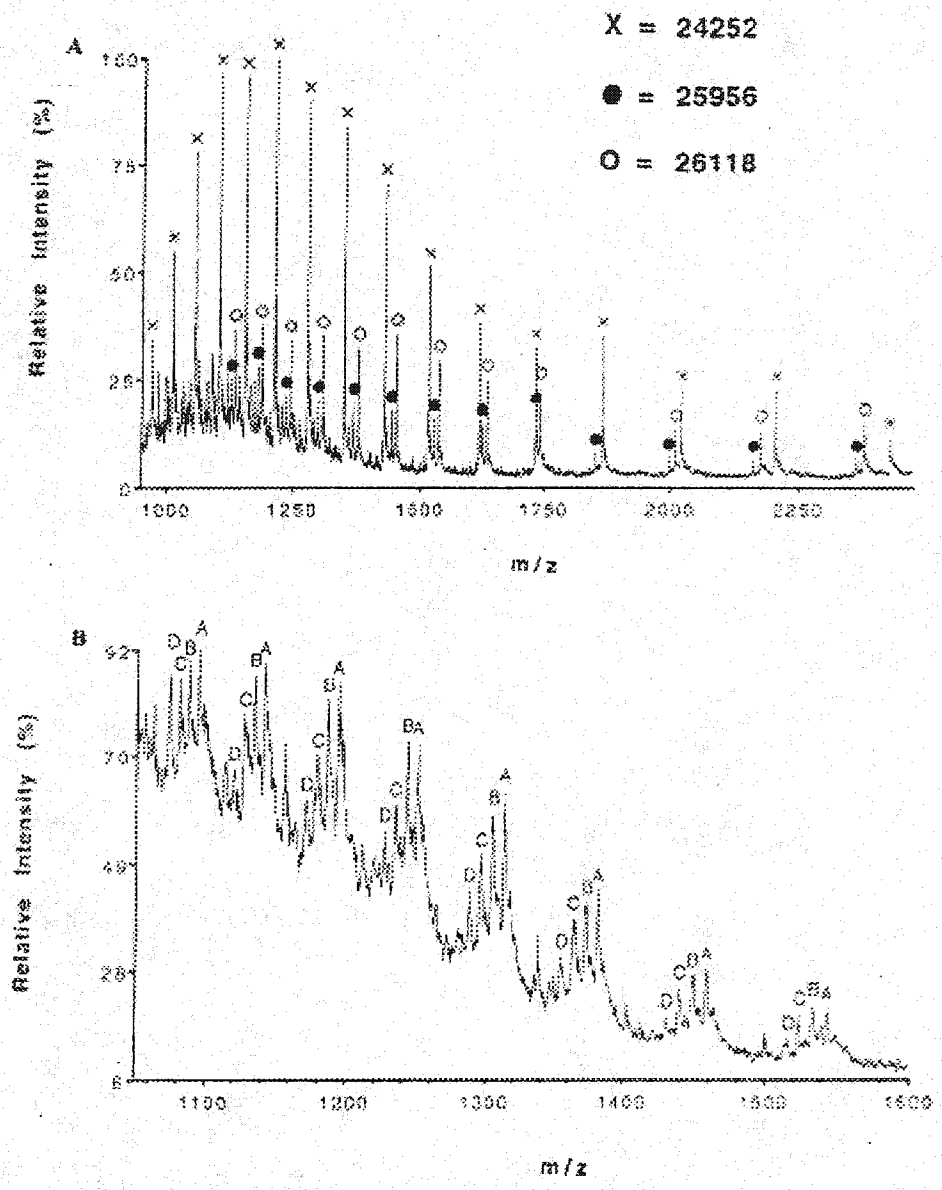


Figure 3.7: (A) Interpreted mass spectra of unfractionated crystalline protein from large lima bean and interpreted mass spectra of fractions F1 (B), F2 (C), and F3 (D) obtained from crystalline protein of large lima beans. Reprinted with permission from (Alli et al., 1994).

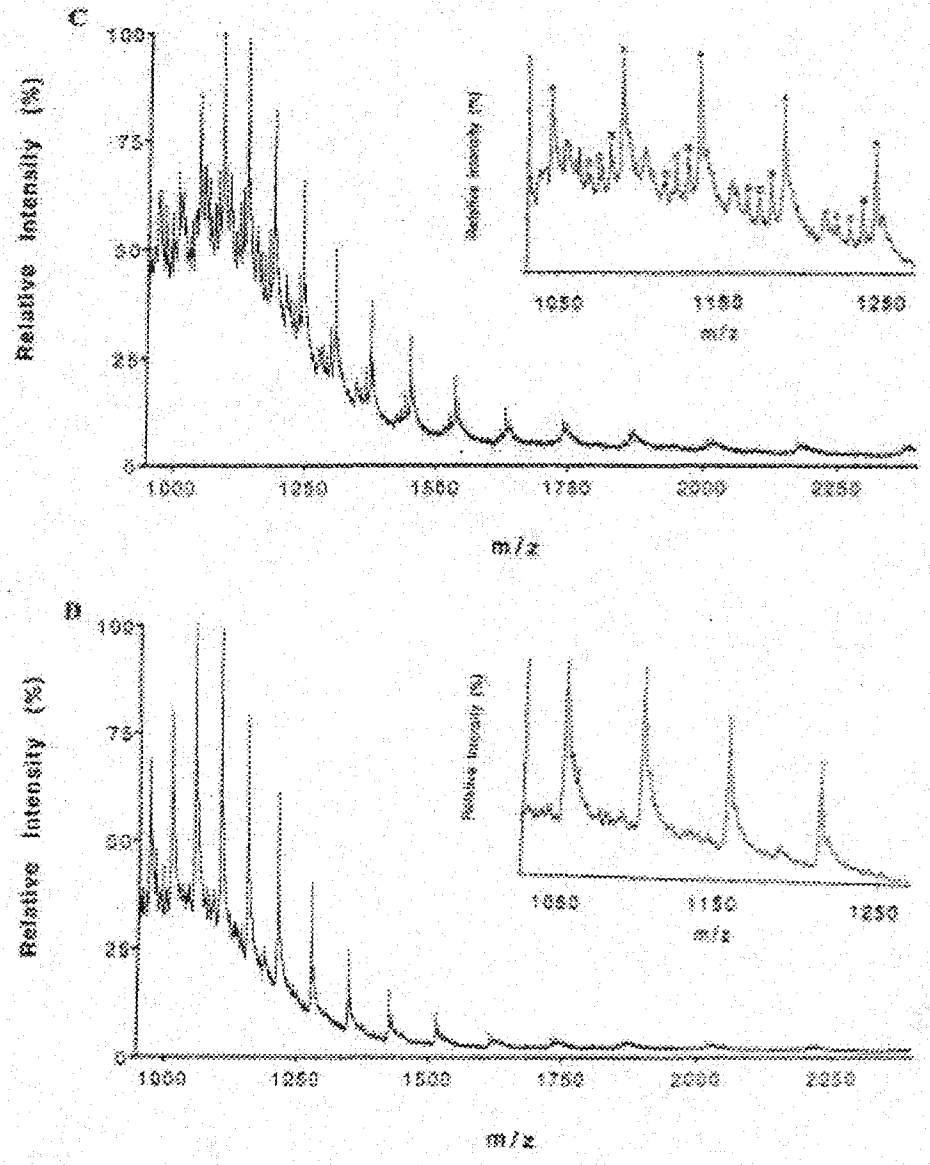


Figure 3.7: (continued).

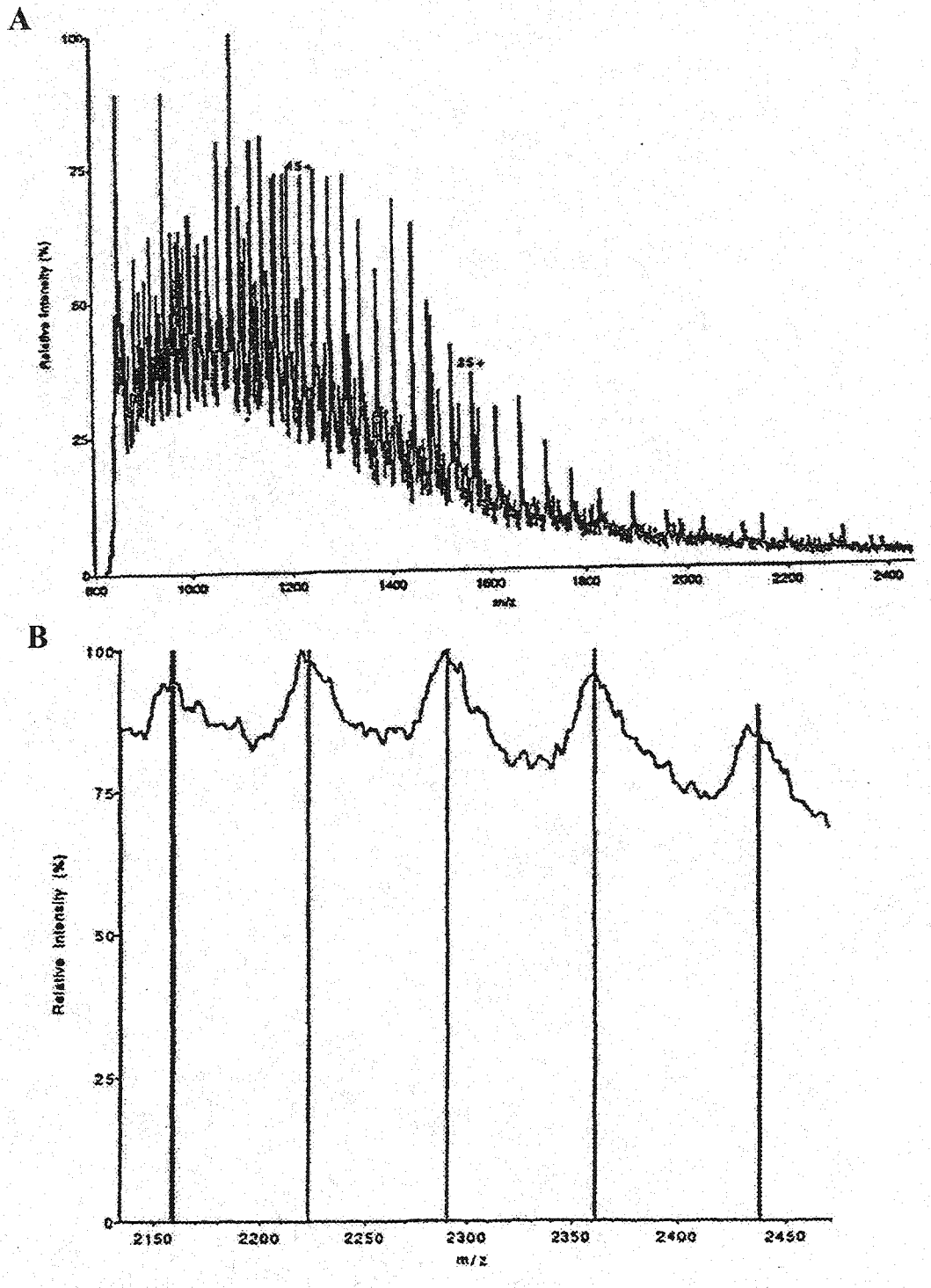


Figure 3.8: Mass spectrum of deglycosylated (A) and glycosylated (B) α-amylase inhibitor from white kidney beans. Reprinted with permission from (Gibbs and Alli, 1998).

3.5 OTHER PROTEINS

In addition to study of food proteins, MS is used increasingly to identify and characterize other proteins which are not considered food proteins but which are of interest to food scientists. MALDI-TOF-MS has been used effectively to detect bacteriocins in the culture supernatant of producer organisms (Rose et al., 1999a), while ESI-MS has been used to determine the M_r of a bacteriocin (Reutericin 6, M_r 2 400) produced by *Lactobacillus* reuteri LA6 (Kabuki et al., 1997). Both ESI-MS and MALDI-TOF-MS were used to assess the purity and stability of nisin and its degradation products (Cruz et al., 1996). In another study, nisin, its variants and degradation products were characterized and quantified using ESI-FTICR-MS (Lavanant et al., 1998); it was shown that the [nisin + 18 u] molecules present as a minor component in the mixture, was a species formed predominantly via hydration of nisin at position 33. In addition, the fate of nisin in meat products was monitored by MALDI-TOF-MS (Rose et al., 1999b). The results indicated that nisin was inactivated in raw meat, but not in cooked meat, due to enzymatic reaction with glutathione (307 u) present in raw meat (Rose et al., 1999b).

MALDI-TOF-MS analysis of whole cells has been investigated as a technique for bacterial chemotaxonomy (classification based on biochemical composition) (Holland et al., 1996); mass spectra of bacterial strains showed a few characteristic high-mass ions which are thought to be derived from specific bacterial proteins (Holland et al., 1996). MALDI-TOF-MS has also been used to study cellular proteins as biomarkers from proteins isolated from the whole cells of bacteria; the observed biomarkers facilitate the distinction between pathogenic and non-pathogenic bacteria

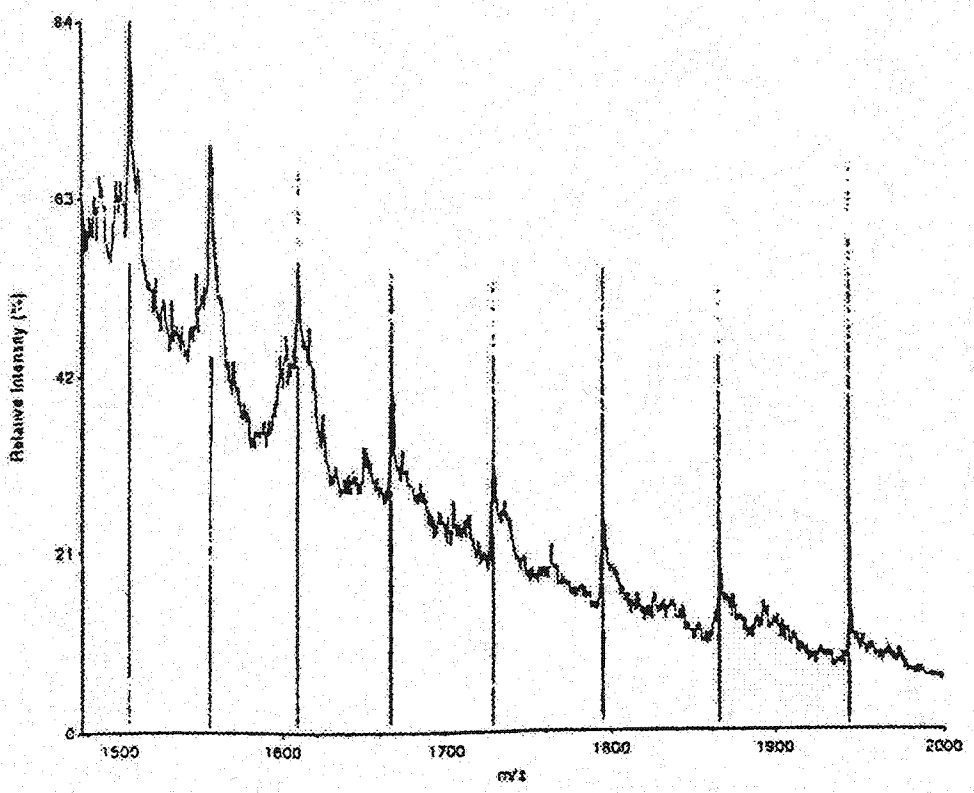


Figure 3.9: Interpreted mass spectra of coconut milk protein with M_r of 46 640. Reprinted with permission from (Sumual, 1994).

(Krishnamurthy et al., 1996) and between gram positive and gram negative bacteria (Krishnamurthy and Ross, 1996; Welham et al., 1998) and therefore allows for rapid chemotaxonomic classification of microorganisms. Furthermore, differentiation between gram positive and gram negative bacteria was also accomplished by RP-HPLC-ESI-MS (Krishnamurthy et al., 1999); the advantages of this technique over MALDI-TOF-MS technique include analysis of liquid samples, short analysis time, reproducibility and identification of the individual microorganism present in crude bacterial mixtures (Krishnamurthy et al., 1999).

Recently, the spectra reproducibility of direct analysis of cellular proteins as biomarkers has been investigated by MALDI-TOF-MS (Wang et al., 1998). It was demonstrated that although minor deviations in sample/matrix preparation procedures for MALDI resulted in significant changes in observed spectra, a number of peaks are conserved for the same bacteria and these conserved peaks are potentially biomarkers for bacterial identification (Wang et al., 1998). MALDI-TOF-MS was used to locate five family specific biomarkers for the family *Enterobacteriaceae*; these biomarkers have spectral peaks at *m/z* 4 364, 5 380, 6 384, 6 856 and 9 540 while mass peaks at *m/z* 7 324, 7 724, 9 136 and 9 253 were assigned as genus-specific biomarkers for *Salmonella* (Lynn et al., 1999). MALDI-TOF-MS and ESI-MS have also facilitated the identification of biomarkers for specific bacteria including *Escherichia coli* spp. (E. coli) (Welham et al., 1998; Wang et al., 1998; Lynn et al., 1999; Dai et al., 1999; Domin et al., 1999; Demirev et al., 1999; Chong et al., 1997; Arnold and Reilly, 1998; Saenz et al., 1999; Arnold et al., 1999), *Bacillus* ssp. (Krishnamurthy et al., 1996; Krishnamurthy et al., 1999; Wang et al., 1998; Demirev et al., 1999; Saenz et al., 1999; Birmingham et al., 1999), *Helicobacter pylori* (Nilsson, 1999), *Haemophilus* spp. (Haag et al., 1998),

Clostridium difficile (Mauri et al., 1999), *Brucella melitensis* (Krishnamurthy et al., 1996; Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1999), *Francisella tularensis* (Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1999), *Yersinia pestis* (Krishnamurthy et al., 1996; Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1999), *Staphylococcus aureus* (Welham et al., 1998), *Klebsiella aerogenes* (Welham et al., 1998) and *Proteus mirabilis* (Welham et al., 1998).

3.6 CONCLUSIONS

This article reviews the rapidly increasing use of MS for the characterization and identification of food proteins and peptides. The published work demonstrate clear advantages of ESI-MS and MALDI-TOF-MS in terms of accuracy, sensitivity, reproducibility and short analysis time for obtaining structural information. MS now serves a central role in many applications including the determination of M_r, peptide sequencing, identification of post-translational modifications (phosphorylation and glycosylation), characterization of non-covalent protein-protein or ligand complexes, identification of protein degradation products (enzymatically and chemically hydrolyzed proteins), investigation of protein folding.

CHAPTER 4

SEPARATION AND CHARACTERIZATION OF β-LACTOGLOBULIN AND α-LACTALBUMIN FROM WHEY AND WHEY PROTEIN PREPARATIONS

4.0 CONNECTING STATEMENT

The functional properties observed for whole whey proteins (WPs) is the sum of the functionality of individual WPs. The use of whole WPs, rather than the individual proteins can account for the high functional variability among commercially available WP products and limits their applications. This shortcoming could be addressed through the development of novel and commercially relevant whey processing technologies that separate enriched fractions, containing mainly individual WPs. This may result in better understanding of the close structure-function relationship of WPs, which is essential for their utilization in food system. The work described in this chapter is the development of a process for separating bovine β-Lg and α-Lac from LW, WPI, and WPC using different chelating agents and addresses the second objective discussed in the "Rationale and Objectives of Study" section of Chapter 1.

Note: This chapter is the text of a paper which has been submitted for publication as follows:

Alomirah, H. F.; Alli, I. Separation and Characterization of β-lactoglobulin and α-lactalbumin from Whey and Whey Protein Preparations. *Int. Dairy J.* (Submitted March, 2002)

Contribution of co-authors: Alli, I. (thesis supervisor).

4.1 ABSTRACT

An improved process for separating bovine β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac) from liquid whey (LW), whey protein isolate (WPI), and whey protein concentrate (WPC) was developed. The method is based on β-Lg solubility at low pH (3.9) and high salt concentration, on the weak calcium binding capacity of α-Lac below pH 3.9, and on the ease of chelating calcium with calcium sequestrates, causing destabilization and precipitation of calcium free α-Lac. The results indicate that the use of sodium citrate (NaC) and sodium hexametaphosphate (SHMP) were more effective in the separation β-Lg and α-Lac than the other tested chelating agents. Yield results indicated that 47-69 % of β-Lg originally present in the whey preparations was recovered, with purities ranging from 84-95%, and protein contents ranging from 40-99%, depending on the source of whey protein preparations and type of chelating agents. The yields of α-Lac in α-Lac enriched fractions obtained without pH adjustment were 23-89 %, with purities ranging from 83-90%, and protein contents ranging from 65-96%; the yields of α-Lac in α-Lac enriched fractions obtained with pH adjustment were 11-43 %, with purities ranging from 68-73%, and protein contents ranging from 44-81%. The method can be investigated further as a means to obtain commercial quantities of β-Lg and α-Lac for food industry application.

4.2 INTRODUCTION

Whey proteins (WP) are well known for their nutritional value and versatile functional properties and are widely utilized in the food industry. The main proteins in whey are β-lactoglobulin (β-Lg, 50%), α-lactalbumin (α-Lac, 21%) and immunoglobulins (10%); bovine serum albumins (BSA, 5%) and proteose-peptone represent minor components (Kinsella and Whitehead, 1989). β-Lg, α-Lac and BSA are primarily responsible for the physicochemical properties of commercial whey proteins. Native β-Lg and α-Lac are compact globular proteins with monomeric molecular weight of about 18300 and 14200 Da, respectively. Each β-Lg molecule has two disulfide bonds and one free thiol group, which exhibit increased reactivity above pH 7, while each α-Lac molecule has four disulfide bonds and one calcium ion which stabilize the molecule against irreversible thermal denaturation (Kinsella and Whitehead, 1989).

The heterogenous nature of WP is typical of many commercial food protein preparations. For these protein preparations, the observed functionality of the complete protein preparation is the sum of the functionality of individual proteins. α-Lac has relatively low solubility and low gelation ability, but good water absorption characteristics after heating, and good emulsifying properties, and it is well suited in bakery products, meats, yogurt and processed cheese, β-Lg and BSA, because of their gelling properties through S-S bond formation, and emulsion and foaming properties, is well suited for soups, gravies, salad dressing, sausages and baked goods. In many food applications, WP are used, rather than the individual protein; this contributes to the functional variability among commercially available WP and can limit their applications (Morr and Ha, 1993; Mate and Krochta, 1994). The use of whey proteins in infant

protein-based foods, is limited by the allergenicity to infants to β-Lg (Gryboski, 1991); this allergenicity can be reduced substantially if the protein is hydrolysed with pepsin, trypsin or using combination of proteolytic enzymes (Ena et al., 1995).

Several techniques have been proposed for laboratory scale separation of WP; these include salting-out (Aschaffenburg and Drewry, 1957), selective precipitation (Amundson et al., 1982; Mailliart and Ribadeau-Dumas 1988; Mate and Krochta, 1994; Bramaud et al., 1995 and 1997), trichloroacetic acid precipitation (Fox et al., 1967), heating at low pH (Pearce, 1983), affinity chromatography (Blackberg and Hernell, 1980), anion exchange chromatography (Skudder, 1985; Manji et al., 1985; Gerberding and Byers, 1998), cation exchange chromatography using conventional resins (Uchida et al., 1996) or cation membranes (Chiu and Etzel, 1997), size exclusion chromatography (Hill et al., 1986), hydrophobic chromatography (Chaplin, 1986) and combination of enzymatic treatment and membrane filtration (Kinekawa and Kitabatake 1996; Pouliot et al., 1999; Sannier et al., 2000). Although these whey fractionation techniques can provide effective protein purification at the laboratory scale, many have not been widely implemented for commercial scale because of their high cost, complexity, low productivity, poor selectivity and/or unacceptable denatured products (Zydney, 1998). On the other hand, some processes have been patented with the capability of being operated on a commercial scale. In some processes (Pearce, 1995; Stack et al., 1995), the mineral content, especially calcium ions, in whey is reduced using electrodialysis and cation exchanger at around pH 3.8, and in other processes (de Wit and Bronts, 1995) whey solution is incubated with strong calcium binding ionic exchange resin to initiate the destabilization of α-Lac.

Our objective was to investigate the use of a combination of techniques reported by Bramaud et al. (1995), Mailliart and Ribadeau-Dumas (1988) and Mate and Krochta (1994) with modification, for the separation of β-Lg and α-Lac enriched fractions from whey and whey protein preparations. Bramaud et al. (1995) purified only α-Lac from only whey protein concentrates (WPC) by increasing the apparent molecular size and destabilization of α-Lac at pH 3.9 using only citric acid as chelating agent while Mailliart and Ribadeau-Dumas (1988) and Mate and Krochta (1994) used high salt concentration and low pH adjustments to purify only β-Lg from liquid whey (LW) and whey protein isolates (WPI), respectively. In the present work, both β-Lg and α-Lac were separated from WPI, WPC and LW using the combinational effects of high salt concentration and chelating agents at low pH.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Liquid whey (LW) obtained from mozzarella cheese preparation, was kept frozen at -20 °C until it was used. Commercial WPC (>75% protein) and WPI (>90% protein) were obtained from Amcan Ingredients (Lachine, Quebec, Canada) and René Rivet Inc. Ingredients (Laval, Quebec, Canada), respectively. Sodium citrate (NaC), sodium hexametaphosphate (SHMP) and citric acid were purchased from Fisher Scientific (Montreal, Canada). Ethylene diamine tetra acetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), β-Lg (containing variant A and B; L-0130), α-Lac (L-6010) and BSA (B-4503) were purchased from the Sigma Chemical Co. (St. Louis, MO). All chemicals were of analytical grade.

4.3.2 Separation of α-Lac and β-Lg

In a preliminary experiment, only LW was used with four chelating agents: sodium-citrate (NaC), sodium hexametaphosphate (SHMP), ethylene diamine tetra acetic acid (EDTA) and ethylene glycol tetra acetic acid (EGTA), each used individually at a concentration of 0.1 M. On the basis of the results from is preliminary experiment, only NaC and SHMP were used in the remainder of the study. The flow diagram used for separation of α-Lac and β-Lg from liquid whey (LW), whey protein isolate (WPI) and whey protein concentrate (WPC) is shown in Figure 4.1. LW was used as is, while WPI and WPC were dispersed in water to provide dispersions of 60 gL^{-1}, based on previous work (Bramaud et al., 1995, 1997; Gésan et al., 1999). LW and the dispersions of WPI and WPC were each treated with NaC and SHMP (150×10^{-3} mol L^{-1}). The mixtures were acidified to pH 3.9 using 6 N citric acid, incubated at 35 °C for 45 min, then centrifuged (5,000 x g, 30 min, 4 °C) (Figure 4.1, step 1). Both the supernatant and the precipitate from step 1 were washed twice with 7% NaCl and centrifuged (10,000 x g, 20 min, 4 °C) (Figure 4.1, step 2B and 2A). The supernatant from step 2B (designated as β-Lg enriched fraction) was dialyzed and lyophilized. The precipitate from step 2A was resolubilized in 0.1 M $CaCl_2$ and centrifuged (10,000 x g, 20 min, 4 °C); the supernatant (designated as α-Lac enriched fraction obtained without pH adjustment) was dialyzed and lyophilized while the precipitate (step 3) was again resolubilized in 0.1 M $CaCl_2$, adjusted to pH 7.5 and centrifuged (10,000 x g, 20 min, 4 °C). The supernatant (designated as α-Lac enriched fraction obtained with pH adjustment) was dialyzed and lyophilized.

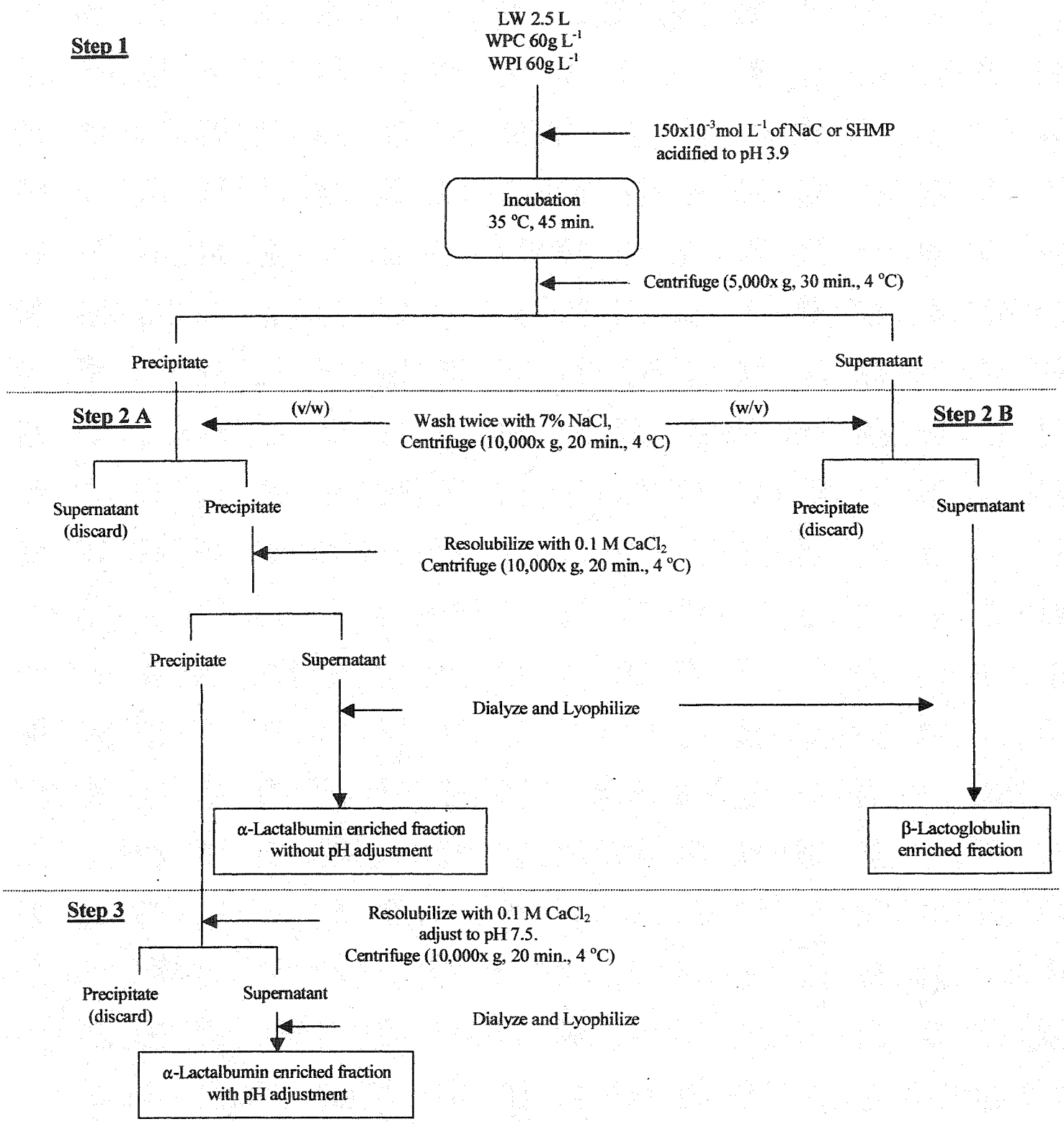


Figure 4.1: Flow diagram showing separation procedure of β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac) from liquid whey (LW), whey protein concentrates (WPC) and whey protein isolates (WPI); NaC, sodium citrate; SHMP, sodium-hexametaphosphate

4.3.3 Electrophoresis

The identification of separated β-Lg and α-Lac proteins in the lyophilized fractions was carried out by polyacrylamide gel electrophoresis (PAGE) both under native conditions (Davis, 1964) and in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970), using a Bio-Rad Mini-Protean® II dual slab cell electrophoresis unit (Richmond, CA); estimation of molecular weight was done using Bio-Rad electrophoresis protein standard (Std-2522). The stacking gel and separation gel were 4% and 10% acrylamide, respectively for native electrophoresis and 4% and 12% acrylamide, respectively for SDS-PAGE. Slab gels (0.75 mm thick) were run at constant current of 6 and 15 mA/gel for native and SDS-PAGE, respectively. After electrophoresis, gels were fixed for 2 h in a mixture of methanol (20% v/v) and acetic acid (10% v/v). Coomassie Brilliant Blue R250 (0.1% w/v in fixing solution) was used to stain protein bands. Destaining was done by storing the gels in the fixing solution until the background color was completely removed. The destained gels were stored in 7% acetic acid until they were photographed.

4.3.4 Glycoprotein Detection

The β-Lg and α-Lac protein fractions which were separated by SDS-PAGE were transferred electrophoretically (90 min at 80 V) using Bio-Rad Mini Trans-Blot® (170-3935, Bio-Rad Laboratories, ON, Canada), to a nitrocellulose membrane (Hybond ECL, RPN-68D, Amersham Pharmacia Biotech, QC, Canada). The transfer buffer (pH 8.3) consisted of 25 mM Tris, 192 mM glycine, 20% v/v methanol.

Glycoproteins on the nitrocellulose membrane were labeled using the ECL™ glycoprotein labelling system (RPN-2190, Amersham Pharmacia Biotech, QC, Canada), then incubated for 1 min in ECL western blotting detection reagents (RPN-2109, Amersham Pharmacia Biotech, QC, Canada). The membrane was wrapped with a saran wrap and placed in a film cassette (FB-XC-810, Fisher Scientific, QC, Canada). In a dark room, a sheet of autoradiography film (Kodak BioMax MS, 111-1681, Kodak, Rochester, New York) was placed on top of the membrane and the film cassette was closed. The file was exposed to the membrane for 1 min, then developed using an automatic film processor (Kodak, M35A-X-OMAT Processor, Rochester, New York).

4.3.5 Determination of Moisture, Protein, and Ash

Dry matter was determined by oven drying at 100 °C for 5h. (AOAC, 1980). Ash content was determined by ashing at 625 °C for 16h (AOAC, 1980) in a furnace chamber(Furnatrol II Furnace, Thermolyne Co, Iowa). Total nitrogen was determined by the Kjeldahl method (AOAC, 1990); nitrogen was converted to protein using the factor of 6.38.

4.3.6 Determination of Purity and Yield of α-Lac and β-Lg

The purity of separated α-Lac and β-Lg enriched fractions was determined as the ratio of their peak areas to the total peak area of peaks separated by RP-HPLC. Reversed-phase HPLC separation of α-Lac and β-Lg enriched fractions was performed with a Waters HPLC (Model 111), C_{18} (0.46 x 25 cm length, Vydac Co., Hesperia, CA) equipped with a diode array, UV visible detector. The following two-buffer gradient system was used to elute the samples at a flow rate of 1 ml/min: buffer A, 0.1%

trifluoroactetic acid (TFA) in water (V/V, pH 2); buffer B, 0.1% TFA in acetonitrile (V/V); solvent B was increased linearly from 0 to 80% over 60 min. The elute was monitored at 210 nm. The yield was calculated based on total β-Lg and α-Lac in initial whey material (68% and 17.5%, respectively), and in the final separated fractions, on dry basis.

4.4 RESULTS AND DISCUSSION

4.4.1 Preliminary Experiments

Figure 4.2 shows the SDS-PAGE patterns of the precipitates obtained by heating LW at 40 °C for 30 min with four chelating agents (NaC, SHMP, EDTA and EGTA), and without chelating agents (untreated). The results indicate that mainly β-Lg and α-Lac proteins were separated in NaC and SHMP treated samples; a minor band corresponding to BSA was also observed. The SDS-PAGE patterns of the EDTA treated LW were similar to those of the untreated LW; both showed a band with MW of 36 kDa, considered to be a dimer of β-Lg. EGTA treated LW showed only faint bands for β-Lg and α-Lac, compared to NaC and SHMP treated LW and untreated LW (Figure 4.2.). Based on the findings of these preliminary experiments, only NaC and SHMP were used as chelating agents in the remainder of the study.

Figure 4.3 shows the SDS-PAGE patterns of the precipitates obtained by heating LW at 25, 35 and 40 °C for 45 min in the absence and presence of NaC and SHMP. The intensity of bands corresponding to precipitated β-Lg and α-Lac in the NaC and SHMP treated LW, increased with increasing heating temperature. The intensity of two other

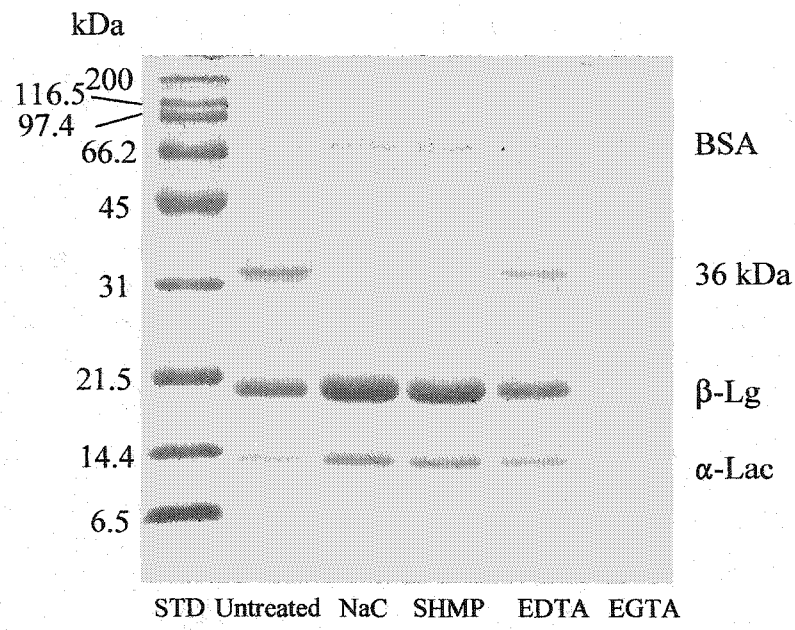


Figure 4.2: SDS-PAGE (12%) patterns of precipitate obtained from liquid whey (LW) by heating at 40 °C for 30 min with different chelating agents (NaC, SHMP, EDTA, EGTA) and without chelating agents (untreated). STD = molecular weight standards, BSA = bovine serum albumin, β-Lg = β-lactoglobulin, α-Lac = α-lactalbumin.

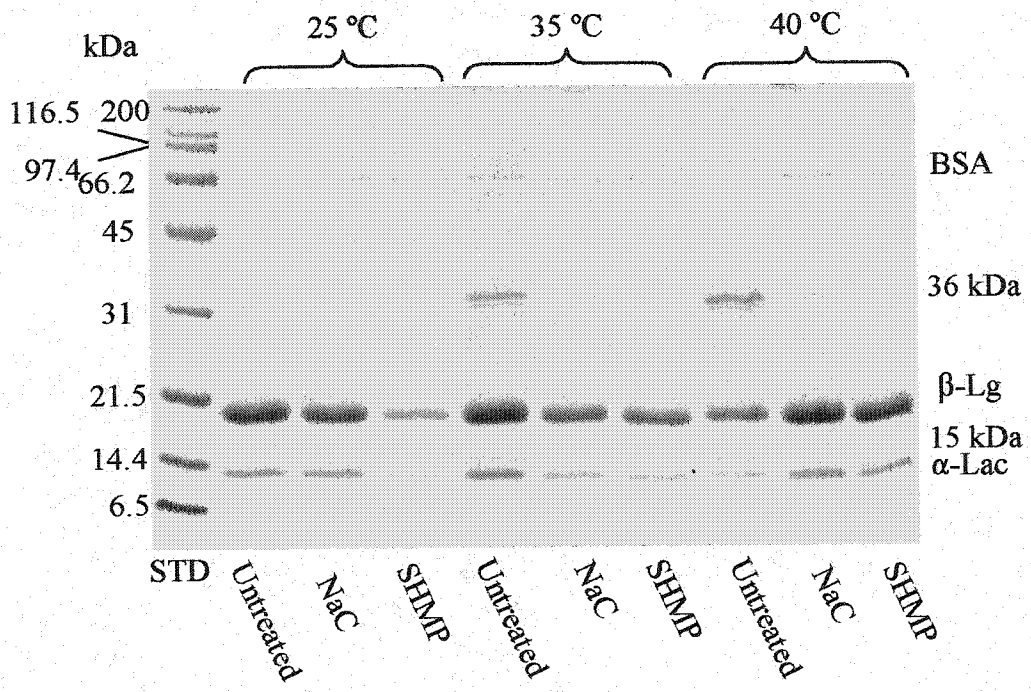


Figure 4.3: SDS-PAGE (12%) patterns of precipitate obtained from liquid whey (LW) by heating at 25, 35 and 40 °C for 30 min with NaC and SHMP and without chelating agents (untreated). STD = molecular weight standards, BSA = bovine serum albumin, β-Lg = β-lactoglobulin, α-Lac = α-lactalbumin.

bands corresponding to MW of 36 and 15 kDa also increased with increasing heating temperature. The former band is considered to be a dimer of β-Lg while the latter band is believed to be composed mainly of polypeptides, such as proteose-peptone, formed by partial proteolysis of β-casein (de Wit, 1998). Heat treatment above 40 °C was not used, since the extent of BSA precipitation can increase when the temperature treatment is > 40 (Bramaud et al., 1997). Consequently, heating at 35 °C was used in the procedure to separate β-Lg and α-Lac enriched fractions from LW, WPI and WPC (Figure 4.1.)

4.4.2 Composition of α-Lac and β-Lg Enriched Factions

Table 4.1 shows the composition of LW, WPI, WPC and the separated β-Lg and α-Lac enriched fractions from these materials using NaC and SHMP. The protein contents of β-Lg enriched fractions (Figure 4.1, step 2B) from WPI, WPC and LW extracted with NaC were 98.6, 69 and 70%, respectively; for β-Lg enriched fraction WPI, WPC and LW extracted with SHMP, the protein contents were 53, 49 and 40%, respectively. The lower protein contents in β-Lg enriched fractions extracted with SHMP, compared those extracted with NaC, is due to their higher ash contents (Table 4.1). Between pH 3.5 and 5.2 β-Lg, is positively charged and associates to form octamers of MW 144 kDa; in solution SHMP is highly negatively charged and can interact electrostatically with β-Lg. Moreover, SHMP is known to promote deflocculation, dispersion or suspension of food constituents (Dziezak, 1990). Therefore, it is likely that there was formation of aggregates containing β-Lg and

residual SHMP resulting in high ash content in these enriched fractions, despite extensive dialysis.

The protein contents of α-Lac enriched fractions obtained without pH adjustment (Figure 4.1, step 2A), from WPI, WPC and LW extracted with NaC were 95.5, 76.2 and 28.8%, respectively; for α-Lac enriched fractions obtained from WPI, WPC and LW extracted with SHMP, the protein contents were 94.1, 71.6 and 65.1%, respectively. These results indicate that protein contents of α-Lac enriched fractions from WPI and WPC, depended on the source of the product, and not on the chelating agents (NaC or SHMP), while the protein contents of α-Lac enriched fractions from LW depended on the chelating agents. For all α-Lac enriched fractions obtained without pH adjustment (Figure 4.1, step 2A), the amounts of ash ranged from 3.7 to 7.5 % except for α-Lac enriched fractions from LW extracted with NaC, which contained 24% ash.

The protein contents of α-Lac enriched fractions obtained with pH adjustment (Figure 4.1, step 3) from WPI, WPC and LW extracted with NaC, were 81.3, 69.9 and 18.7%, respectively; for α-Lac enriched fractions from WPI, WPC and LW extracted with SHMP, the protein contents were 72.1, 75.3 and 43.8%, respectively. As in α-Lac enriched fractions obtained without pH adjustment (Figure 4.1, step 2A), α-Lac enriched fractions from WPI and WPC obtained with pH adjustment (Figure 4.1, step 3), showed different protein contents depending on the source of the protein but not on treatment with different chelating agents, while protein contents of α-Lac enriched fractions from LW depended on the chelating agents. For all α-Lac enriched fractions obtained with pH adjustment (Figure 4.1, step 3), the amounts of ash ranged from 6.1 to 13.7 %.

Table 4.1: Chemical composition of initial whey preparation and separated β-Lg and α-Lac enriched fractions; values calculated as % dry weight basis [a].

Sample		Dry matter %	Ash %	Total protein % (N X 6.38)	Purity[b] %	Yield[c] %
Initial whey preparations						
WPI		95	2.5	90		
WPC		96	3	75		
LW		6.88	7.7	12.35		
β-Lg enriched fractions (Figure 4. 1, step 2 B)						
WPI	NaC	94.03	1.56	98.6	94.8	63.1
	SHMP	94.11	42.60	53.2	ND[d]	ND
WPC	NaC	96.20	31.18	68.9	83.4	46.7
	SHMP	94.16	50.27	48.8	ND	ND
LW	NaC	93.72	5.30	70.0	88.8	68.6
	SHMP	93.53	60.78	40.1	ND	ND
α-Lac enriched fractions without pH adjustment (Figure 4.1, step 2 A)						
WPI	NaC	88.67	7.26	95.5	90	34.8
	SHMP	89.34	3.65	94.1	89.9	88.6
WPC	NaC	91.43	8.65	76.2	83.1	22.9
	SHMP	89.41	7.46	71.6	86.1	43.5
LW	NaC	92.02	24.41	28.8	96.1	7.1
	SHMP	94.07	4.62	65.1	88.3	80.3
α-Lac enriched fractions with pH adjustment (Figure 4.1, step 3)						
WPI	NaC	90.61	6.92	81.3	73.2	24.4
	SHMP	90.83	13.73	72.1	70.1	10.9
WPC	NaC	90.40	10.48	69.9	71.2	16.6
	SHMP	90.39	6.16	75.3	73.2	43.0
LW	NaC	93.10	11.05	18.7	70.0	3.7
	SHMP	93.08	11.53	43.8	68.4	16.3

[a] Means of duplicate determinations

[b] Expressed as % of the ratio of their peak area to the total RP-HPLC peak area

[c] Yield based on total β-Lg and α-Lac in initial material (68% and 17.5%, respectively) and in final products on dry basis

[d] Not determined

WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate

4.4.3 Yield and Purity of α-Lac and β-Lg

Figure 4.4 shows the RP-HPLC chromatogram of WPI; the 5 identified peaks were Ig, BSA, α-Lac and β-Lg (B and A); the RP-HPLC chromatograms of WPC and LW were similar. β-Lg (A and B) (not shown) and α-Lac represent approximately 68% and 17.5 %, respectively, of the total peak area in the WPI, WPC and LW chromatograms. These values are in agreement with values reported by Morr and Foegeding (1990), Mate and Krochta (1994), and Caessens et al. (1997). The yield of the β-Lg enriched fraction from WPI extracted with NaC, was approximately 63% (based on the total protein % and on β-Lg content in the WPI on dry weight basis), with a purity of 95% (Table 4.1). Mate and Krochta (1994) obtained yield of 65% (95% purity), of the β-Lg present in WPI using 7% NaCl at pH 2 and Caessens et al. (1997) reported yield of 60% (95% purity) of purified β-Lg obtained from WPI using 3% trichloroacetic acid at pH 3.5. The yield of β-Lg enriched fraction from WPC and LW extracted with NaC were 46.7 and 68.6%, respectively; this low yield of the β-Lg enriched fractions obtained from LW can be related to its high ash content. The β-Lg enriched fractions obtained by extraction with SHMP were not separated by RP-HPLC, since acidified solution of these fractions failed to pass through the 45 μm syringe membrane filter (Millipore, Beford, MA), prior to HPLC separation. This could be due to the interaction of β-Lg with SHMP resulting in the formation of large protein aggregates in acidic solution (Dziezak, 1990). Although the yield % of β-Lg enriched fractions could not be determined, it can be assumed to be low because of their high ash contents, which ranged from 43 to 61% (Table 4.1). Based on the higher yield and lower

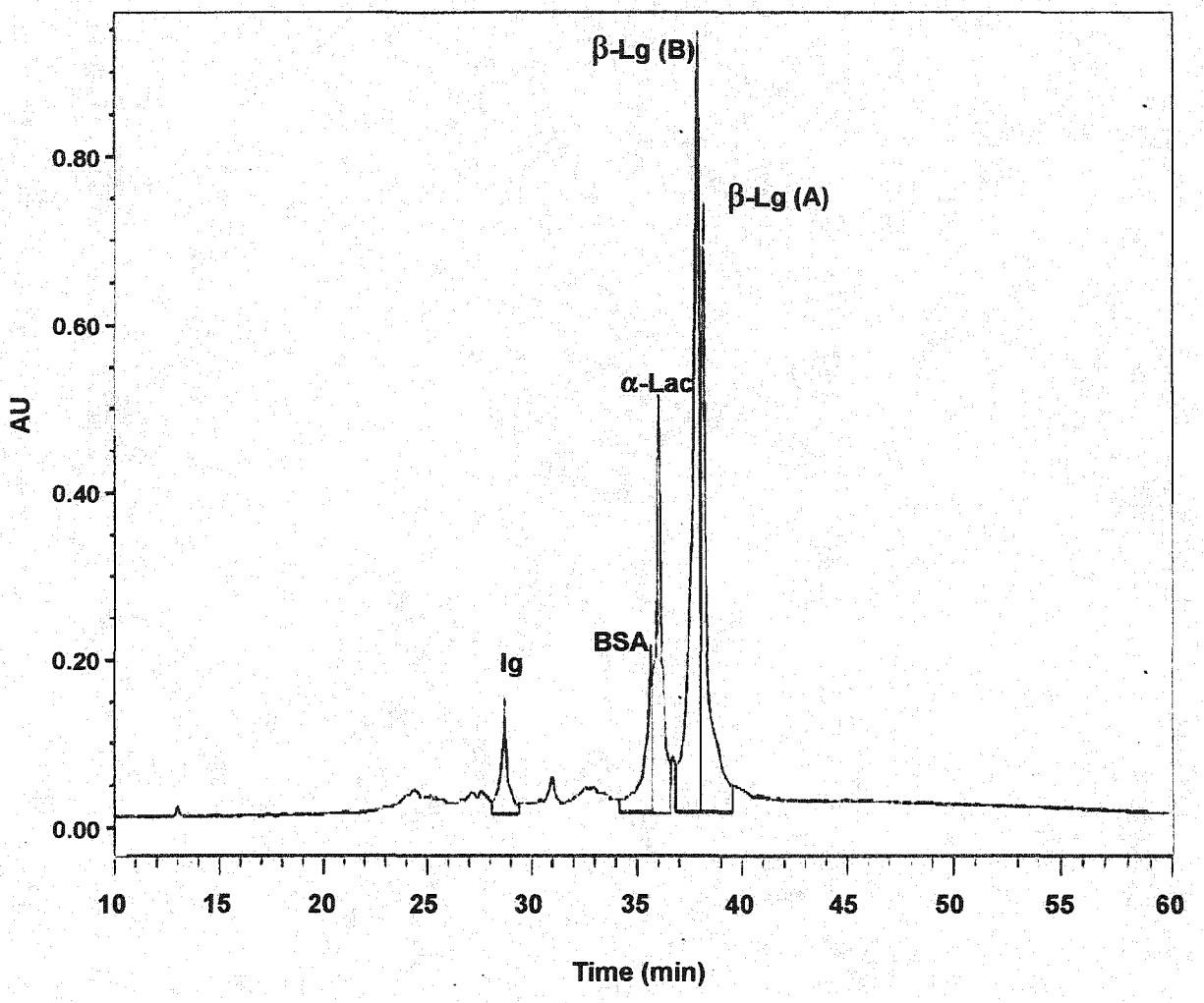


Figure 4.4: Reversed-phase high-performance liquid chromatography of WPI.

ash content of β-Lg enriched fractions extracted with NaC, the use of NaC as a chelating agent for the separation of β-Lg enriched fractions is preferred to the use of SHMP.

The yields of α-Lac in the α-Lac enriched fractions obtained from WPI, WPC and LW extracted with NaC and SHMP without pH adjustment (Figure 4.1, step 2A) are shown in Table 4.1. These yields from WPI, WPC and LW extracted with NaC were 34.8, 22.9 and 7.1%, respectively, with purities of 90, 83 and 96%, respectively; the yields of α-Lac enriched fractions from WPI, WPC and LW extracted with SHMP were 88.6, 43.5 and 80.3, respectively with purities of 90, 86 and 88% respectively. The higher yields of α-Lac enriched fractions extracted with SHMP compared to those of NaC, can be attributed to the greater binding affinity and binding capacity for calcium resulting in greater destabilization and subsequent precipitation of α-Lac. Mailliart and Ribadeau-Dumas (1988) reported that the yield of α-Lac in the α-Lac enriched fraction obtained from LW using 7% NaCl at pH 2 before a desalting step, was approximately 77%, and Gésan et al. (1999) reported yield of 43% (52% purity) of purified α-Lac obtained from WPC. Our recoveries of α-Lac in the α-Lac enriched fractions extracted with SHMP, were higher than reported values, but were lower in the α-Lac enriched fractions extracted with NaC, with purity higher than 83% for both chelating agents. Based on the higher yields and purities and lower ash contents of α-Lac enriched fractions extracted with SHMP, the use of SHMP as a chelating agent for the separation of α-Lac enriched fractions is preferred to the use of NaC.

The yields of α-Lac in the α-Lac enriched fractions from WPI, WPC and LW extracted with NaC and SHMP with pH adjustment to 7.5 and resolubilizing with 0.1 M

$CaCl_2$ (Figure 4.1, step 3) are shown in Table 4.1. The yields of α-Lac enriched fractions from WPI, WPC and LW extracted with NaC were 24.4, 16.6 and 3.7%, respectively, with purities of 73.2, 71.2 and 70%, respectively; the yields of α-Lac enriched fractions from WPI, WPC and LW extracted with SHMP were 10.9, 43.0 and 16.3%, respectively, with purities of 70.1, 73.2 and 68.4%, respectively. The decrease in purities of α-Lac enriched fractions extracted with NaC and SHMP and with pH adjustment compared to those obtained without pH adjustment can be attributed to the effect of higher pH (7.5) on resolubilization of α-Lac and possibly other proteins such as BSA (Bramaud et al., 1997).

4.4.4 Electrophoresis and Glycoprotein Detection

Figure 4.5 shows the electrophoretic patterns for β-Lg enriched fractions obtained from LW, WPC and WPI extracted with NaC and SHMP (Figure 4.1, step 2 B) under native conditions (Figure 4.5 A), in the presence of SDS (Figure 4.5 B) and on nitrocellulose membrane with labelled carbohydrate moieties (Figure 4.5 C). RP-HPLC chromatogram of β-Lg enriched fractions from LW, WPI and WPC extracted with NaC is also shown in Figure 4.5. Individual protein bands were identified from LW, WPI and WPC; similar bands were also identified by Manji et al. (1985). Another band appeared in all β-Lg enriched fractions between the bands of β-Lg (variant B) and BSA (Figure 4.5 A); this band was also visible in the well corresponding to β-Lg standard and could be the dimer of β-Lg. Figure 4.5 (A and B) indicate that β-Lg enriched fractions obtained from LW and WPI extracted with NaC and SHMP (Figure 4.1, step2 B)

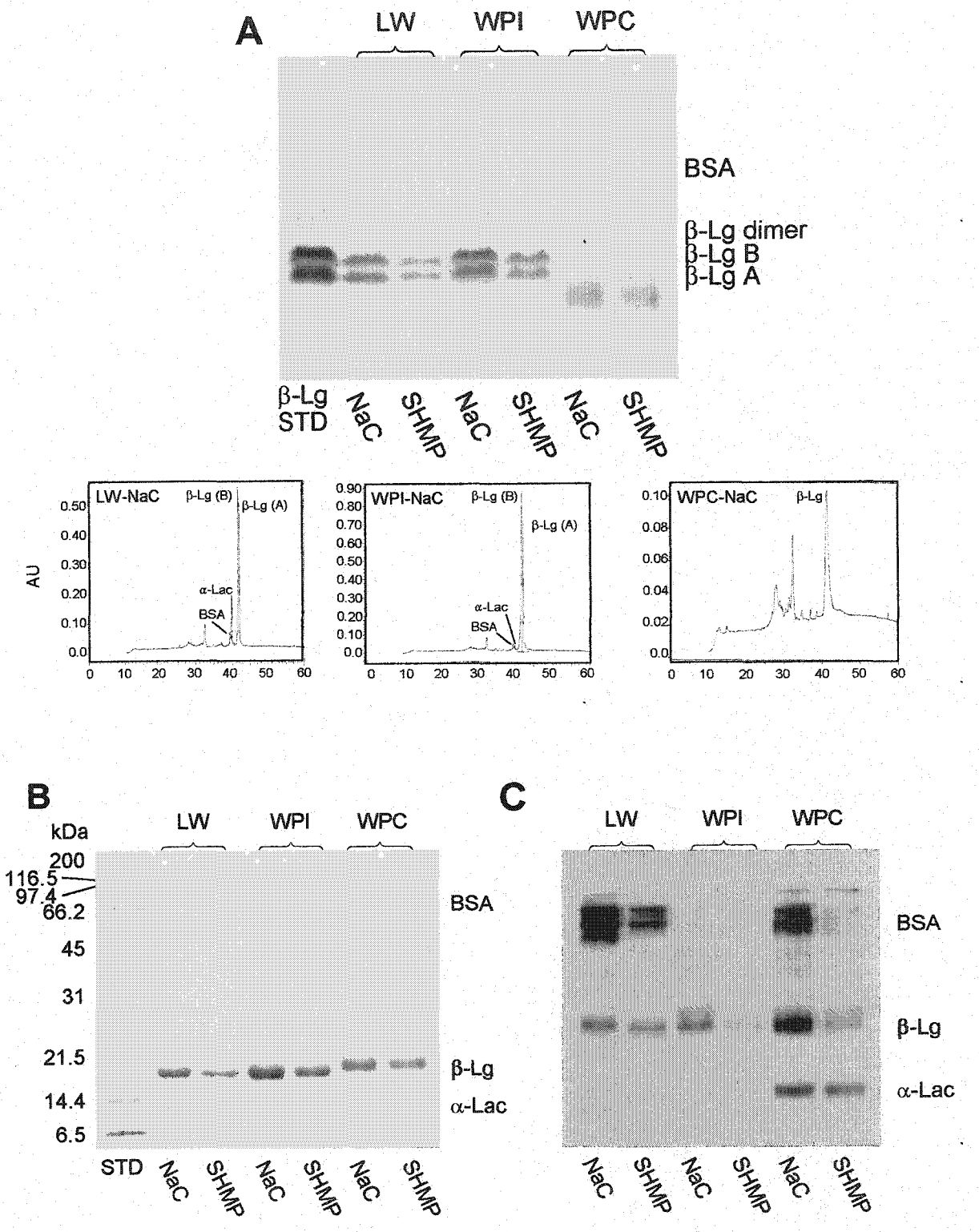


Figure 4.5: Electrophoretic patterns for enriched β-Lg fractions (Figure 4.1, step 2 B) in native conditions (A), in the presence of SDS (B) and in nitrocellulose membrane with labeled carbohydrate moieties (C). See Figure 4.2 for abbreviations.

showed mainly band corresponding to β-Lg; minor bands corresponding to α-Lac and BSA were noted in NaC treated fraction. RP-HPLC (Figure 4.5) confirmed the identity of the bands separated by native electrophoresis. Electrophoretic patterns of β-Lg enriched fraction from WPC under native conditions (Figure 4.5 A) showed greater migration and diffused bands. In the presence of SDS (Figure 4.5 B), the β-Lg enriched fractions obtained from WPC showed band with MW 19,118 Da compared to 18,080 Da for the same fractions from LW and WPI. This could be due to heating and drying treatments (during commercial WPC preparation), which can result in reaction of lactose with available lysine of the β-Lg (Morgan et al., 1998). This was confirmed by carbohydrate specific identification (Figure 4.5 C) which revealed that β-Lg enriched fractions from WPC is glycated with greater glycation in β-Lg enriched fractions obtained with NaC than with SHMP.

Figure 4.6 shows the electrophoretic patterns for α-Lac enriched fractions obtained from LW, WPC and WPI extracted with NaC and SHMP without pH adjustment (Figure 4.1, step 2 A) under native conditions (Figure 4.6 A), in the presence of SDS (Figure 4.6 B) and on nitrocellulose membrane with labeled carbohydrate moieties (Figure 4.6 C). RP-HPLC chromatogram of α-Lac enriched fractions from LW, WPI and WPC extracted with NaC is also shown in Figure 4.6. α-Lac enriched fractions from LW, WPI and WPC extracted with NaC and SHMP without pH adjustment (Figure 4.1, step 2 A), showed a main band corresponding to α-Lac; minor bands corresponding to β-Lg and BSA were also noted. The RP-HPLC chromatograms of α-Lac enriched fraction extracted with NaC without pH adjustment (Figure 4.6)

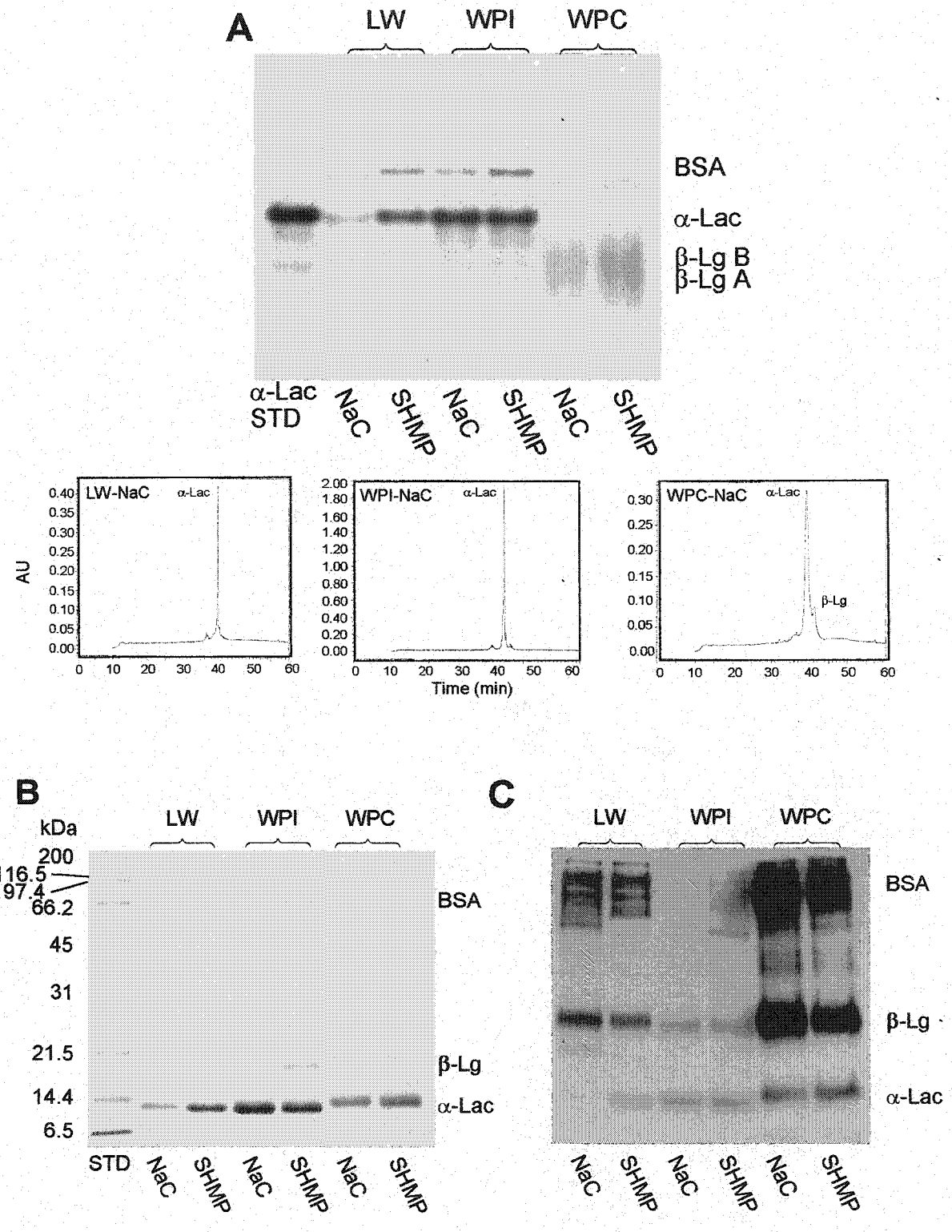


Figure 4.6: Electrophoretic patterns for enriched α-Lac fractions (Figure 4.1, step 2 A) in native conditions (A), in the presence of SDS (B) and in nitrocellulose membrane with labeled carbohydrate moieties (C). See Figure 4.2 for abbreviations.

shows single peaks corresponding to α-Lac. Co-separation of β-Lg and BSA and other protein fractions, represent less than 11% in all α-Lac enriched fractions (Table 4.1). The co-separation of BSA may be explained by the fact that calcium is a stabilizer of BSA (Gumpen et al., 1979) as it is for α-Lac (Bramaud et al., 1995, 1997), and the use of calcium sequestrant (such as NaC and SHMP) would result in a decrease of free calcium ion concentration and thus in both α-Lac and BSA destabilization. Electrophoretic patterns of α-Lac enriched fraction from WPC under native conditions (Figure 4.6 A) showed greater migration and diffused bands. In the presence of SDS (Figure 4.6 B), the α-Lac enriched fractions obtained from WPC showed band with MW 14,770 Da compared to 14,060 Da for the same fractions from LW and WPI. This can be related reaction of lactose with available lysine of the α-Lac. This was confirmed in Figure 4.6 C which indicated that α-Lac enriched fractions from WPC is glycated and co-separated β-Lg and BSA fractions were also glycated.

SDS-PAGE electrophoretograms of α-Lac enriched fractions obtained from LW, WPC and WPI extracted with NaC and SHMP with pH adjustment (Figure 4.1, step 3) are shown in Figure 4.7. RP-HPLC chromatogram of α-Lac enriched fractions from LW, WPI and WPC extracted with NaC with pH adjustment is also shown in Figure 4.7. The results showed that along with the separation of α-Lac, minor bands corresponding to BSA and β-Lg were also observed. Moreover, two faint bands appeared above and below the bands of BSA in all α-Lac enriched fractions and between the bands of β-Lg and α-Lac in α-Lac enriched fractions obtained from WPI.

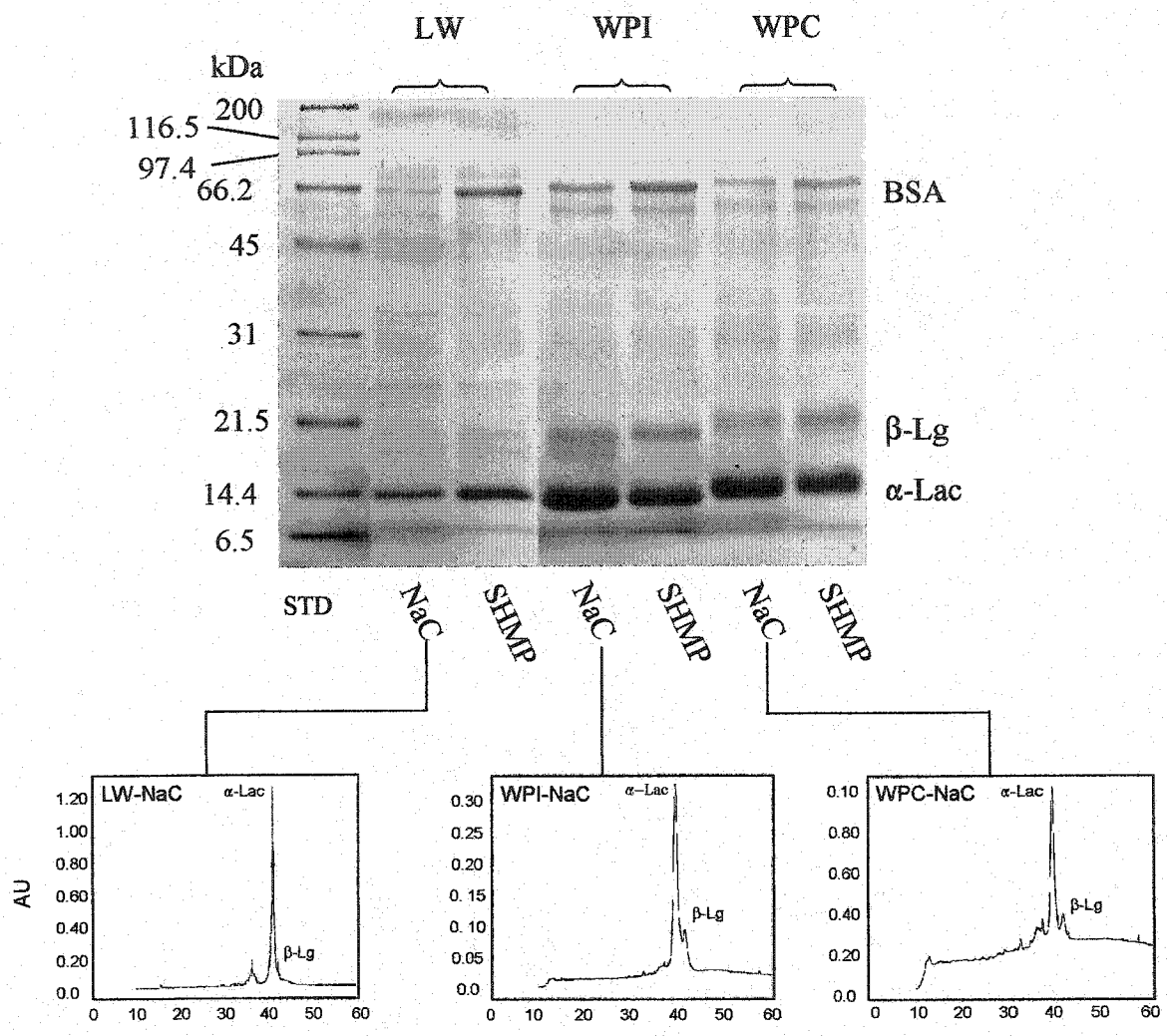


Figure 4.7: SDS-PAGE (12%) patterns for α-Lac enriched fractions after pH adjustment (Figure. 4.1, step 3). See Figure 4.2 for abbreviations.

On average, these co-separated bands represent about 29% of the α-Lac enriched fractions (Table 4.1), indicating that solubilization of precipitated α-Lac with $CaCl_2$ is more selective without pH adjustment.

4.5 CONCLUSIONS

The present work has established the relative performance of NaC and SHMP, in combination with high salt concentration at low pH, to separate β-Lg and α-Lac from LW, WPC and WPI. Results showed that the use of NaC and SHMP were more effective than other chelating agents. The use of NaC as a chelating agent resulted in yield of 47-69% of β-Lg enriched fractions with purities ranging from 83-90% and protein contents ranging from 69-99% while the use of SHMP resulted in yield of 44-89% of α-Lac enriched fractions with purities ranging from 86-90% and protein contents ranging from 65-94% depending on the source of whey protein preparations and type of chelating agents. In addition, results showed that solubilization of precipitated α-Lac with $CaCl_2$ was more selective without pH adjustment. Our process is an improvement over comparable processes for preparation of commercial quantities of β-Lg and α-Lac for food industry applications.

CHAPTER 5

THERMAL DENATURATION AND CONFORMATIONAL STABILITY OF β-LACTOGLOBULIN ISOLATED FROM WHEY AND WHEY PROTEIN PREPARATIONS

5.0 CONNECTING STATEMENT

In Chapter 4, a method was developed to isolate β-Lg with relatively high purity and yields, from liquid whey (LW), whey protein isolate (WPI), and whey protein concentrate (WPC) using different chelating agents. Results showed that the use of NaC and SHMP were more effective than other chelating agents and the use of NaC resulted in high yield of β-Lg isolated fractions. The work described in this chapter is an evaluation of the thermal denaturation and structural stability of β-Lg fractions isolated from whey and whey protein preparations in Chapter 4, and addresses the third objective discussed in the "Rationale and Objectives of Study" section of Chapter 1.

Note: This chapter is the text of a manuscript which is to be submitted for publication as follows:

Alomirah, H. F.; Alli, I.; Ismail, A. A.; Konishi Y. Thermal Denaturation and Conformational Stability of β-lactoglobulin Isolated from Whey and Whey Protein Preparations. *J. Agric. Food Chem.*

Contribution of co-authors: Alli, I. (thesis supervisor); Ismail, A. A. (contributed to interpretation of FTIR spectra); Konishi, Y. (contributed to interpretation of MS data).

5.1 ABSTRACT

The thermal and structural properties of β-lactoglobulin (β-Lg) fractions isolated from fresh liquid whey (LW), whey protein isolates (WPI) and whey protein concentrates (WPC) with sodium-citrate (NaC) or sodium-hexametaphosphate (SHMP) were investigated by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy, respectively. The theromgram of β-Lg isolated fractions showed a single transition with peak temperature (T_d) ranged from 80.7 to 83.5 in deuterated buffer and ranged from 76.5 to 79.2 in aqueous buffer depending on whey source. The β-Lg isolated fractions showed higher thermal stability compared to commercial β-Lg standard. FTIR results showed that although using NaC and SHMP may have partially unfolded certain regions of β-Lg isolated fractions, it may have conferred stability to other regions as evidenced by their higher 1692 cm^{-1} band intensity at 75 °C. The FTIR spectra of β-Lg isolated fractions revealed a shift in the 1677, 1635, 1625 cm^{-1} bands by 2 cm^{-1} compare to β-Lg standard, indicating that their structure was relatively tight and inflexible. Upon heating, β-Lg isolated fractions denatured without the formation of aggregates as indicated by low intensity of the bands at 1684 and 1616 cm^{-1}, suggesting that the addition of NaC and SHMP may have the ability to inhibit aggregate formation.

5.2 INTRODUCTION

The most common functional properties attributed to whey proteins are solubility, viscosity, emulsification, foaming, water absorption and gel formation. These functional properties have been demonstrated to be affected by intrinsic (hydrodynamic and thermodynamic properties) and extrinsic (environmental conditions and processing treatments) factors (Damodaran, 1994). These factors can alter the structure of whey proteins and consequently affect its functionality. Moreover, the variability in whey composition can also affect its functional properties. For example the relative ratios of the proteins present in whey, such as β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), bovine serum albumin (BSA) and immunoglobulins may vary as a function of the bovine breeds, season, and animal feed. In most food applications, whey proteins are used as is, rather than separating the individual protein, this accounts for the high functional variability among commercially available whey proteins products thereby limiting their applications (Morr and Ha, 1993; Mate and Krochta, 1994). The use of whey proteins in infant foods is limited due to the allergenicity of β-Lg of some infants (Gryboski, 1991). Accordingly, selective fractionation of the major whey proteins may be one way to reduce the allergenicity of the whey proteins. Furthermore, large scale separation of the individual whey proteins can result in a better understanding of the structure-function relationship of these proteins and in providing superior control of protein composition.

β-lactoglobulin is approximately 50% of the whey proteins in bovine milk, and is primarily responsible for the physicochemical properties of whey proteins (Schmidt and Morris, 1984; Kinsella and Whitehead, 1989). β-Lg consists of 162 amino acid

residues and contain two intramolecular disulfide bonds and one free buried thiol group; the reactivity of the thiol group increases at pH values above 6.8 (Haque and Kinsella, 1987), upon heating (Kinsella, 1982), or high pressure (Hosseini-nia et al. 1999) and may participate in forming intermolecular disulfide bonds and in thiol-disulfide interchange reaction. The secondary structure of β-Lg consists of an α-helical content of 10-15%, a β-structure content of 50% and 20% turns, and the remaining 15-20% represent amino acid residues in a random coil arrangement (Casal et al., 1988) while crystallographic studies indicated that tertiary structure of β-Lg is made up of nine antiparallel β-strands, eight of which wrap round to form a β-barrel, with an helix at the external face. This configuration has the ability to bind and transport apolar, lipophilic or labile compounds (Papiz et al., 1986). At room temperature and neutral pH, the quaternary structure of β-Lg exist as a dimer. At pH between 3.5 and 5.5, the dimmers associate to form octamers which dissociate to monomers below pH 3.5 and above pH 7.5 (Timasheff et al., 1966; Hambling et al., 1992).

Currently, there is growing interest in studying protein stability-function relations (PSFR) rather than traditional structure-function relations (Apenten and Galani, 1999) since stability is easier to quantify than structure. Although, there is a relative abundance of information in the literature on factors affecting the stability of whey proteins and individual whey protein standards (Boye 1995; Boye et al., 1996b, 1997b, 2000), there is relatively limited information on the structural stability of β-Lg proteins isolated from different whey protein preparations. In this study, differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy; two complementary techniques that have become widely accepted for studying the thermal

and structural behaviour of proteins (Casal et al., 1988); were employed to investigate the thermal and structural changes in β-Lg isolated from fresh liquid whey (LW), and solutions of whey protein isolates (WPI) and whey protein concentrates (WPC) using a chelating agent [sodium-citrate (NaC) or sodium-hexametaphosphate (SHMP)] at pH 3.9 in the presence of 7% NaCl (Chapter 4).

5.3 MATERIALS AND METHODS

5.3.1 Materials

β-Lg standard (containing variant A and B) (L-0130) was obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Deuterium oxide (D_2O) (> 99%) was from Aldrich (Milwaukee, WI).

5.3.2 Samples Preparation

Protein solutions of β-Lg fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4), were extensively dialyzed against excess volumes of water for 3 days. Prior to measurements, all proteins fractions were further desalted 4 times using Centricon-10 filters (Amicon Inc., MA, USA). Desalted samples were then lyophilized and stored at 4 °C. Complete absence of chelating agent attached to the protein was confirmed by electrospray ionization mass spectrometry (ESI-MS) (Chapter 7).

5.3.3 Differential Scanning Calorimetry

Solutions of β-Lg standard and β-Lg isolated fractions were prepared by dispersing the proteins in aqueous (H_2O) and deuterated (D_2O) phosphate buffer solutions (0.01 M, pH 6.8; in this work the relation pD = pH + 0.4 was used) to make 20% (w/v) solutions. The rationale for studying the effect of D_2O and H_2O as a solvent was to determine whether replacement of H_2O with D_2O in FTIR experiments influence the conformational stability of β-Lg. Typically, 25 μL of each solution, were placed in pre-weighed differential scanning calorimetry (DSC) pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC (TA3000, Mettler Instrument Corporation, Greifensee, Switzerland) and scanned from 15 to 100 °C at a programmed heating rate of 5 °C/min. For each run, a sample pan containing the buffer used for dissolving the proteins was used as reference. After heating, the samples were allowed to cool to room temperature and the heating cycle was repeated under the same experimental conditions to determine the degree to which the denaturation was reversible. Cooling of the samples between the two heating cycles was done by the use of a liquid air tank attached to the DSC. Degree of reversibility was determined from the ratio of the areas under the second and first endothermal peaks (Boye and Alli, 2000). The DSC was calibrated by use of indium standards. Peak temperature of denaturation (T_d) and heat of transition or enthalpy (ΔH) (area underneath peak) were computed from each thermal curve. ΔH values are based on the total weight of the protein solutions. Purity percentages samples were determined as the ratio of β-Lg enthalpy (ΔH) divided by total enthalpy (ΔH)

calculated by DSC for each sample. All DSC measurements were done in at duplicate or triplicates.

5.3.4 Fourier Transform Infrared Spectroscopy

Solutions of each protein sample (15% w/v) were prepared using deuterated phosphate buffer solutions (pH 6.8) approximately one hour prior to the start of the infrared measurements. Infrared spectra were recorded on a 8210E FTIR spectrometer (Nicolet, Madison, WI) equipped with a deuterated triglycine sulfate detector. The spectrometer was purged continuously with dry air from a Balston dryer (Balston, Haverhill, MA). 7 μL of the protein solution was placed between two CaF_2 windows separated by 25 μm spacer and held in a temperature-controlled infrared cell. The temperature of the sample was regulated by an Omega temperature controller (Omega Engineering, Laval, QC). The temperature was increased in 5 °C increments and the infrared cell allowed to equilibrate for 5 min prior to data acquisition. The reported temperatures are accurate to within ± 0.1 °C. A total of 512 scans co-added at $4cm^{-1}$ resolution. Deconvolution of the observed spectra was performed using Omnic 5.0 software (Nicolet, Madison, WI). The deconvolution of the infrared spectra was carried out as described by Kauppinen et al. (1981). The signal-to-noise ratio was >20000:1, and the bandwidth used for deconvolution was 13 cm^{-1} with a band narrowing factor of 2.4. The band assignments in the amide I area (1600-1700 cm^{-1}) were based on band assignments from previous FTIR studies (Casal et al., 1988; Susi and Byler, 1988; Boye et al., 1996b; Hosseini-nia et al., 1999).

5.4 RESULTS AND DISCUSSIONS

5.4.1 Thermal Stability of β-Lg

Figure 5.1 shows the DSC thermograms of β-Lg standard and β-Lg fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4); the thermal analysis was done in deuterated (D_2O) phosphate buffer (pH 6.8). Table 5.1 shows the purity % of β-Lg isolated fractions and the thermodynamic parameters. With the expectation of β-Lg isolated from WPI with NaC (β-Lg-WPI-NaC), the purity of the β-Lg isolated fractions ranged from 82 to 95%. These values are in agreement with values obtained by RP-HPLC for the same β-Lg isolated fractions (Chapter 4). RP-HPLC also revealed the presence of small amounts of α-Lac and BSA. A notable exception is the β-Lg-WPI-NaC fraction. RP-HPLC revealed that this fraction had a purity greater than 93% (Chapter 4), whereas a 56% purity was estimated from the DSC data. The source of this discrepancy may be attributed to the presence of small peaks at the low temperature side of the main β-Lg transition peak in the DSC thermogram. Qi et al., 1995, reported that β-Lg solutions at low concentration and at pH 6.75 and pH 8.05 gave complex thermograms with small peaks at the low temperature side of the peak. These peaks were attributed to the dissociation of the dimer before denaturation (Qi et al., 1995).

The theromgram of β-Lg standard in deuterated buffer (Figure 5.1) shows one transition with peak temperature (T_d) at 75 °C and enthalpy (ΔH) of 1.4 J/g (Table 5.1). Similar values have been reported by Boye et al. (1996b) under the same conditions. Cooling of the sample followed by reheating (second heating cycle) resulted in the

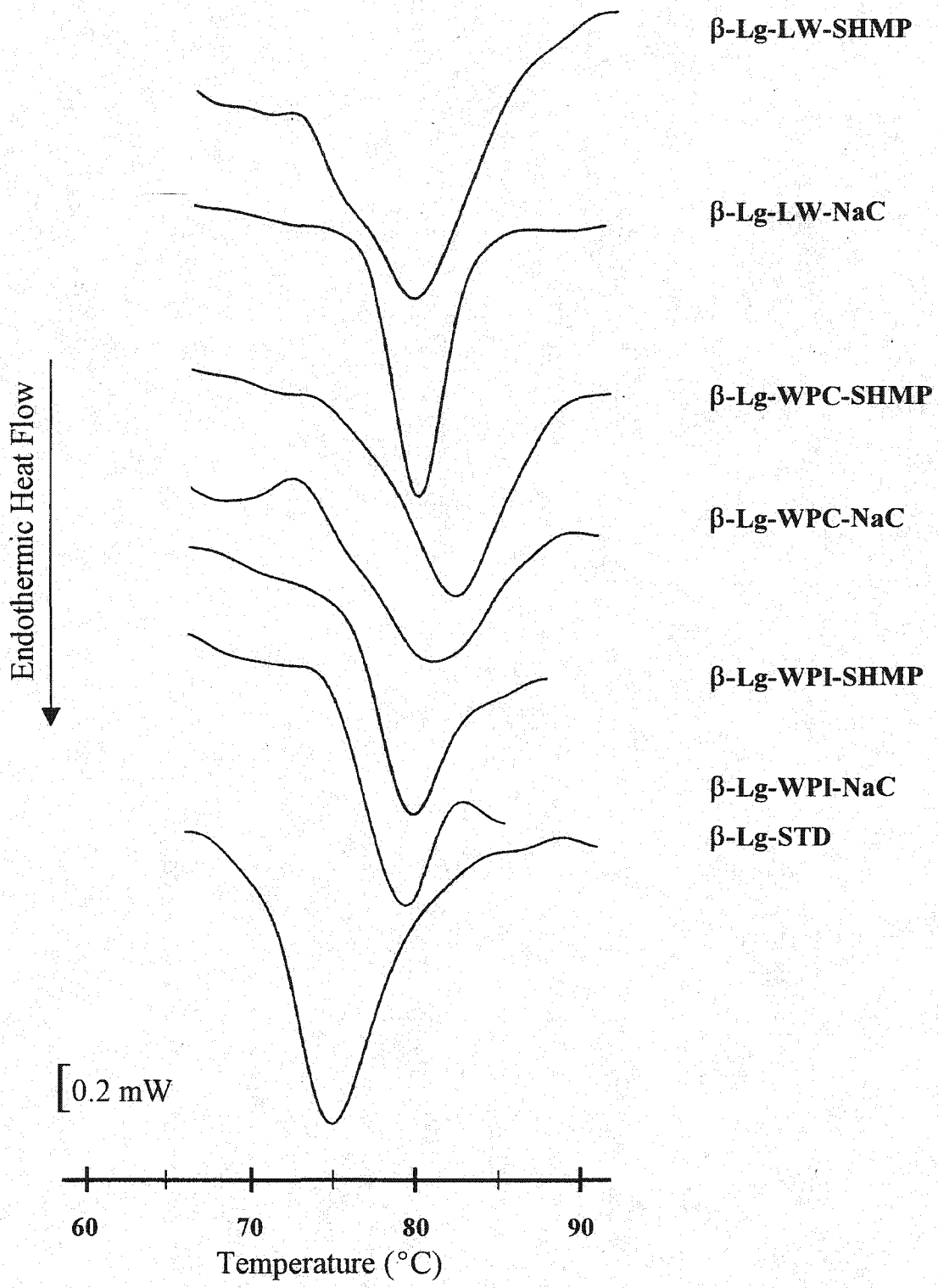


Figure 5.1: DSC thermograms of β-Lg standard and β-Lg fractions isolated from WPI, WPC and LW extracted with NaC or SHMP. Heating at 5 °C/min from 15 to 100 °C in deuterated phosphate buffer (0.01 M, pH 6.8). β-Lg = β-lactoglobulin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

Table 5.1: DSC characteristics of β-lactoglobulin standard and β-lactoglobulin fractions isolated from whey products [a]

		1st heating cycle			2nd heating cycle		
name	solvent	T_d (°C)	ΔH (J/g)	Purity (%)	T_d (°C)	ΔH (J/g)	Reversibility (%)
β-Lg-STD	D_2O	75	1.4	100.0	77.1	0.05	3.3
β-Lg-WPI-NaC	D_2O	80.7	0.6	56.3	81.7	0.05	7.9
β–Lg-WPI-SHMP	D_2O	80.8	0.6	93.9	78.5	0.08	13.3
β-Lg-WPC-NaC	D_2O	82.5	0.6	82.2	77.7	0.04	6.5
β-Lg-WPC-SHMP	D_2O	83.5	0.6	88.7	80.9	0.02	3.8
β-Lg-LW-NaC	D_2O	81.3	0.7	95.3		0.00	0.0
β-Lg-LW-SHMP	D_2O	81.1	0.7	89.0		0.00	0.0
β-Lg-STD	H_2O	72.5	1.5	98.5	70.3	0.02	1.5
β-Lg-WPI-NaC	H_2O	76.5	0.7	50.0	75.9	0.04	5.9
β–Lg-WPI-SHMP	H_2O	78.3	0.8	81.7		0.00	0.0
β-Lg-WPC-NaC	H_2O	79.2	1.5	96.5		0.00	0.0
β-Lg-WPC-SHMP	H_2O	76.5	1.0	98.1		0.00	0.0
β-Lg-LW-NaC	H_2O	77.5	0.6	91.7	75.7	0.01	2.0
β-Lg-LW-SHMP	H_2O	78.1	0.4	75.0		0.00	0.0

[a] T_d, peak temperature of denaturation; ΔH, enthalpy calculated as area underneath β-Lg peak; % purity, enthalpy calculated for β-Lg divided by total enthalpy calculated for each sample × 100; % reversibility, enthalpy calculated from each heating cycle divided by enthalpy of the first heating cycle × 100; β-Lg = β-lactoglobulin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

reappearance of one transition with peak temperature (T_d) at 77.1 °C and low enthalpy (ΔH) of 0.05 J/g, indicating 3.3 % reversibility (Table 5.1). These results demonstrate that β-Lg no longer show endothermal heat effect of unfolding in the second heating cycle. These results agree with statement that β-Lg is irreversible once intermolecular aggregation has began (Wong et al., 1996). The T_d of β-Lg isolated from WPI with NaC and SHMP was 80.7 and 80.8 °C respectively, from WPC were 82.5 and 83.5 °C respectively, and from LW were 81.3 and 81.1 °C, respectively. These results indicate that β-Lg fractions showed different T_d depending on the source of whey (i.e., WPI, WPC and LW), but not on the use of different chelating agents (i.e., NaC and SHMP).

The increase in the thermal stability of all β-Lg isolated fractions compared to that of β-Lg standard cannot be attributed to presence of other proteins in these fractions since previous reports indicate that β-Lg was less thermally stable when heated in the presence of α-Lac (Boye et al., 2000) or κ-casein (Park and Lund, 1984). Also, the thermal stability could not arise from the presence of NaCl since all samples were extensively desalted. Therefore, increased thermal stability of β-Lg isolated fractions may be attributed to conformational change in β-Lg. Table 5.1 shows that ΔH of β-Lg isolated fractions ranged between 0.6 and 0.7 J/g which is substantially lower than ΔH of β-Lg standard (1.4 J/g). It is interesting to note that while β-Lg isolated fractions are more thermally stable, they required less amount of energy to unfold than β-Lg standard. These results suggest that the addition of a chelating agents (NaC and SHMP) may have partially unfolded certain regions of β-Lg resulting in lowering the ΔH and conferred stability in other regions of the protein resulting in higher T_d. Similar effects were reported for β-Lg heated in the presence of SDS (Boye and Alli, 2000).

Furthermore, ESI-MS analysis of β-Lg samples revealed the absence of residual chelating agents, therefore, it is unlikely that the change in the ΔH and T_d are attributed to the interaction between the residual chelating agent and β-Lg.

Table 5.1 shows the T_d of β-Lg isolated from WPC, extracted with NaC and SHMP, increased by 1.8 and 2.7 °C respectively compared to the T_d of β-Lg isolated from WPI, and increased by 1.2 and 2.4 °C compared to the T_d of β-Lg isolated from LW. Cooling of β-Lg fraction isolated from WPC followed by reheating (second heating cycle) was found reversible by 6.5% for NaC-treated WPC and 4% for SHMP-treated WPC samples. These values are higher than the reversibility (3.3%) of β-Lg standard. Similar results were also obtained for WPI, which exhibited a reversibility of 7.9% for NaC-treated and 13.3% for SHMP-treated WPI samples. In contrast, NaC- and SHMP-treated LW samples showed complete lack of reversibility. The above results demonstrate an increase in thermal stability and reversibility of β-Lg fractions obtained from WPC and WPI. The stabilizing effect may be attributed to the presence of lactose. Previous studies employing electrophoresis (Chapter 4) and ESI-MS (Chapter 7) indicated that the β-Lg fraction isolated from WPC and WPI were partially glycated, while β-Lg isolated from LW was nonglycated. Glycation of these fractions could be due to the presence of high concentrations of lactose during the preparation of WPC and the processing of WPI. In the case of LW, the samples were less than one-day old, therefore, minimizing the chance of β-Lg glycation. Earlier studies using DSC (Boye and Alli, 2000) and FTIR (Boye et al., 1996b) have confirmed the stabilizing influence of sugars on β-Lg. Bouhallab et al. (1999) suggest that lactose protects β-Lg from precipitation through steric hindrance and high surface hydrophilicity that prevents the

thiol-disulfide exchange reactions. ESI-MS studies (Chapter 7) also revealed that β-Lg obtained from WPC contained approximately 12-14 sugar residues per protein molecule, while only 2-4 residues of sugar were found in the β-Lg isolated from WPI. It is of interest to note that the percentage reversibility of β-Lg isolated from WPI was greater than that of β-Lg isolated from WPC. It may be possible that the number of sugar residues attached to the protein may play a key role in the reversibility of the protein.

Figure 5.2 shows the DSC thermograms of β-Lg standard and β-Lg fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4); the thermal analysis was done in aqueous (H_2O) phosphate buffer (pH 6.8). Table 5.1 shows that purity of β-Lg isolated fractions, except for β-Lg fractions isolated from WPI and extracted with NaC (β-Lg-WPI-NaC), ranged from 75 to 98%; these values are relatively lower than values obtained by RP-HPLC for the same isolated fractions (Chapter 4). The theromgram of β-Lg standard in aqueous buffer shows single transition with peak temperature (T_d) at 72.5 °C and enthalpy (ΔH) of 1.5 J/g (Table 5.1). Similar values have been reported by Boye and Alli (2000) under similar conditions. Cooling of this sample followed by reheating (second heating cycle) resulted in the reappearance of one transition with peak temperature (T_d) at 70.3 °C and low enthalpy (ΔH) of 0.02 J/g indicating 1.5 % reversibility compared to 3% reversibility in deuterated buffer (Table 5.1). The T_d of β-Lg standard in aqueous buffer decreased by 2.5 °C from T_d of β-Lg standard in deuterated buffer (Table 5.1). Similarly, Verheul et al. (1998) found that β-

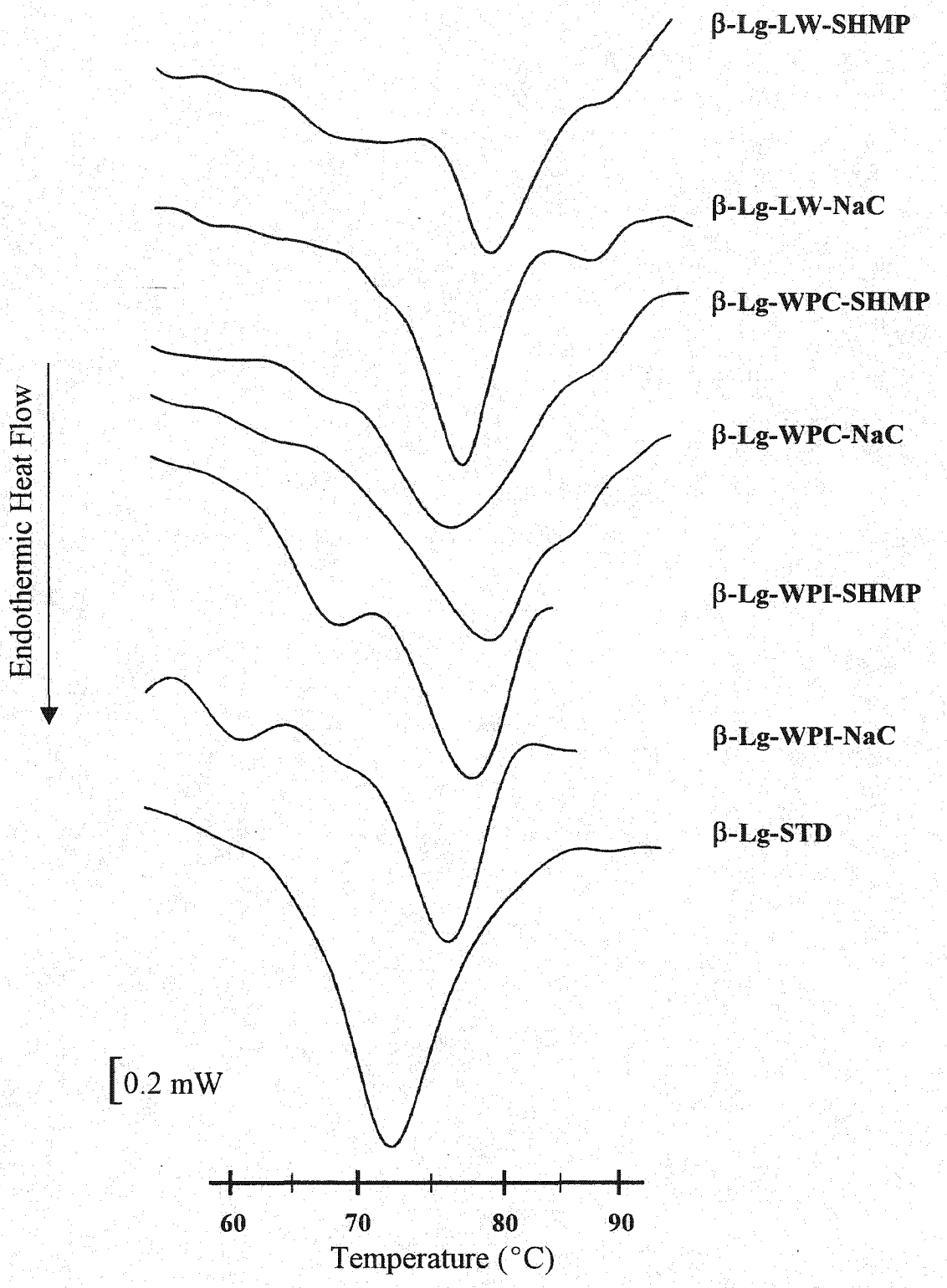


Figure 5.2: DSC thermograms of β-Lg standard and β-Lg fractions isolated from WPI, WPC and LW extracted with NaC or SHMP. Heating at 5 °C/min from 15 to 100 °C in aqueous phosphate buffer (0.01 M, pH 6.8). β-Lg = β-lactoglobulin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

Lg at pH 7.0 is approximately 3 °C higher in D_2O compared to H_2O. The stabilizing effect of D_2O was explained by an increase in hydrophobic interactions and/or an isotope effect on hydrogen bonding (Verheul et al., 1998).

All β-Lg isolated fractions in aqueous buffer showed increased thermal stability compared to β-Lg standard but with lower ΔH. The T_d of all β-Lg isolated fractions in aqueous buffer were lower by 2.5 to 7 °C over that in deuterated buffer, indicating less conformational stability. Table 5.1 indicate that ΔH of β-Lg isolated fraction, except for β-Lg fractions isolated from WPC, were more or less the same as in aqueous buffer (~0.6 J/g). Similar findings have been reported by Verheul et al. (1998) that ΔH of the transition of a β-Lg is not significantly influenced by D_2O.

5.4.2 Structural Changes of β-Lg by FTIR Spectroscopy

In this study changes in the position and relative intensities of the amide I bands in the amide I band absorption region between 1600 and 1700 cm^{-1} in the infrared spectra of the protein was considered. Figure 5.3 (A-D) shows deconvoluted infrared spectra of the β-Lg standard and β-Lg fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4) in deuterated phosphate buffer (pH 6.8) at 25, 55, 75 and 95 °C. The principle bands in the spectra of β-Lg are shown in Table 5.2. The band assignments in Table 5.2 are based on previous infrared studies of β-Lg by Susi and Byler, 1988, Casal et al., 1988; Boye et al., 1996b. FTIR spectroscopy data revealed that at ambient temperatures the secondary structure of the β-Lg isolated fractions were comparable to that of the β-Lg standard. Examination of the infrared spectra of β-Lg fractions isolated from WPC at ambient temperature, revealed the

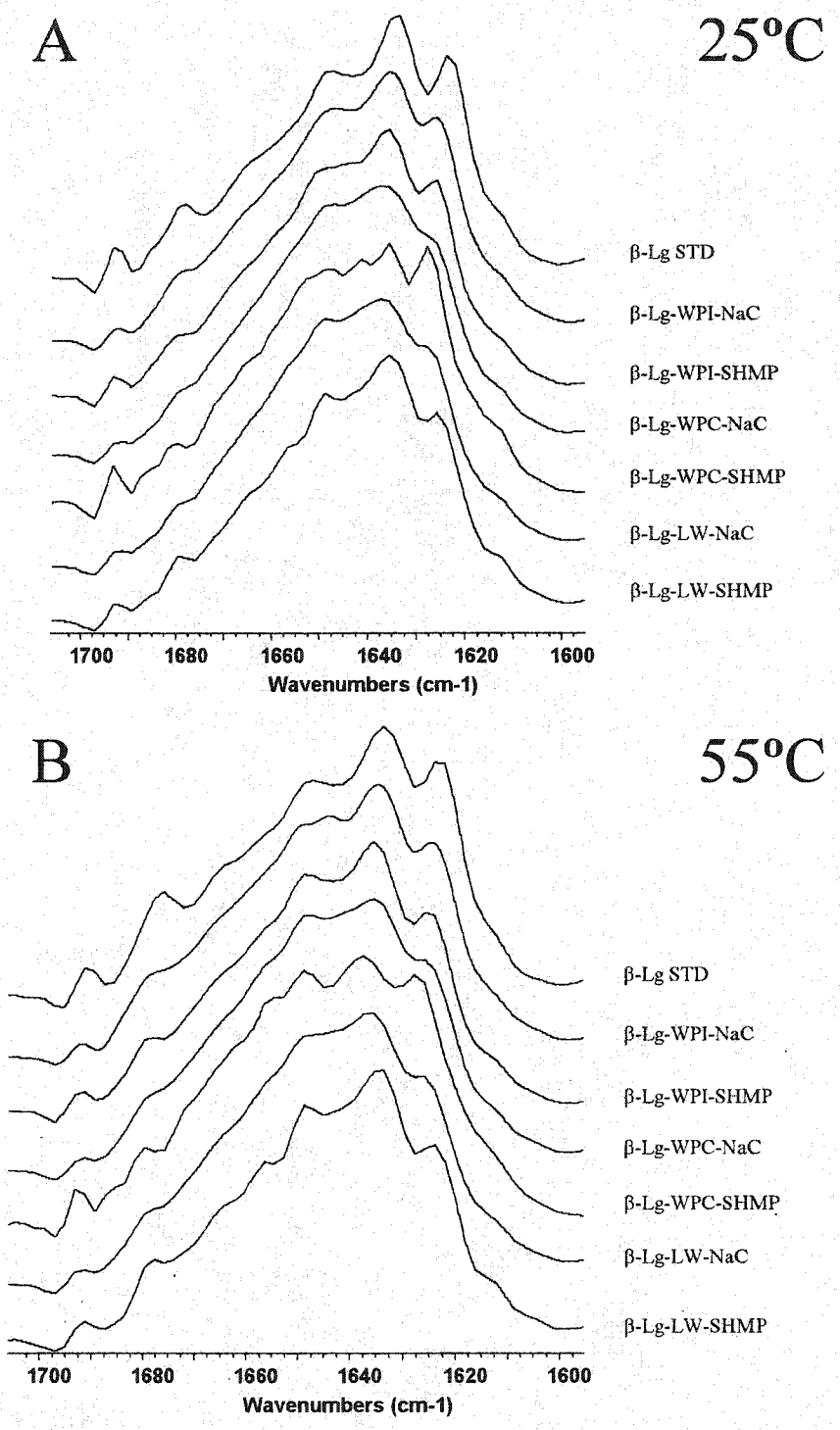


Figure 5.3: Deconvoluted infrared spectra of β-Lg standard and β-Lg isolated fractions (15% w/v) in deuterated phosphate buffer (pH 6.8) at temperature 25 (A), 55 (B), 75 (C) and 95 °C (D). β-Lg = β-lactoglobulin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

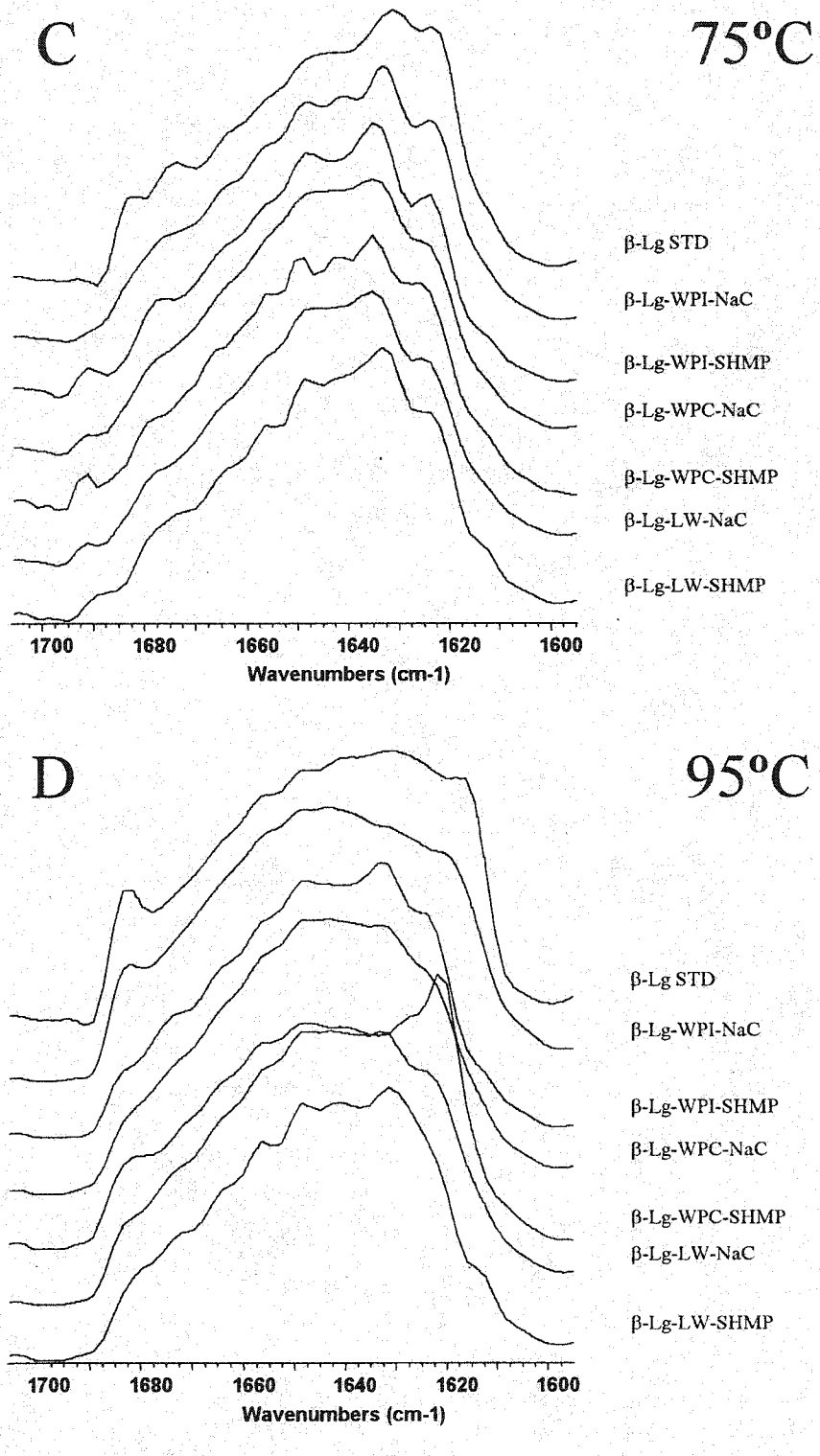


Figure 5.3: (continued).

Table 5.2: Frequencies (cm^{-1}) and band assignments of the amide I regions of β-lactoglobulin [a]

peak position (cm^{-1})	band assignment	peak position (cm^{-1})	band assignment
1690-1693	antiparallel β-turns	1643	random coil
1683	antiparallel β-sheet or β-turns	1637	antiparallel β-sheet
1674-1680	antiparallel β-sheet	1630-1635	antiparallel β-sheet
1656-1658	α-helix	1623-1625	β-sheet
1647-1650	α-helix and random coil	1612	β-sheet aggregated strands

[a] Susi and Byler, 1988, Casal et al., 1988; Boye et al., 1996b.

presence of broad amide I compared to the other protein spectra (Figure 5.3A). The absence of well defined peaks may be attributed to the presence of multiple populations of β-Lg with varying amounts of glycation. ESI-MS data revealed the presence of β-Lg with 2-14 sugar residues (Chapter 7). The infrared spectra of β-Lg at 25 °C (Figure 5.3 A) shows a weak band at 1692 cm^{-1}, attributed to β-type structure (Boye et al., 1996b). Changes in the band intensity of the 1692 cm^{-1} band have been attributed to hydrogen-deuterium exchange in β-type structure and associated with the onset of unfolding of β-Lg upon mild heat treatment of the protein (Boye et al., 1996b) and thus may reflect the tertiary structure of the protein. Accordingly, the 1692 cm^{-1} band can be used as an indicator of the native state of the protein. Results indicate that the relative intensity of this band was dependent on the nature of the chelating agent. At 25 °C, β-Lg extracted with SHMP showed lower intensity of 1692 cm^{-1} to that of the β-Lg standard while β-Lg extracted with NaC show a marked decrease in the intensity of the 1692 cm^{-1} band. The intensity of the 1692 cm^{-1} band gradually decreased upon heating the β-Lg solution. These results are in agreement with those previously reported by Boye et al. (1996) who reported the disappearance of the 1692 cm^{-1} band at 65 °C. However, all β-Lg isolated fractions were more thermally stable than the β-Lg standard. This was reflected in the fact that all β-Lg isolated fractions had to be heated to 85 °C in order to unfold the protein. Heating above 90 °C, resulted in the complete disappearance of this band in all samples. The results suggest that although addition of chelating agents (NaC and SHMP) used for the isolation of β-Lg may have partially unfolded certain regions of β-Lg resulting in an initial decrease in the 1692 cm^{-1} band intensity, it may have conferred stability to other regions of the protein. These findings are consistent with the DSC data,

which showed that the β-Lg isolated fractions have higher thermal stability compared to commercial β-Lg standard.

Above 75 °C, β-Lg standard and β-Lg isolated fractions showed the appearance of two new bands at approximately 1684 and 1618 cm^{-1}; these bands have been assigned previously to intermolecular antiparallel β-sheet stemming from aggregate formation, upon denaturation, with the simultaneous decrease in band intensities 1677 and 1635 cm^{-1} (assigned to intramolecular antiparallel β-sheet) and 1625 cm^{-1} (β-sheet) (Susi and Byler, 1988, Casal et al., 1988; Boye et al., 1996b). The extent of aggregation was significantly variable based on 1684 and 1618 cm^{-1} bands intensity. β-Lg fraction isolated from WPI extracted with NaC denatured to the same extent as the β-Lg standard. All other samples showed minimal aggregate formation. This is supported by the observation that after scanning in the DSC, these samples formed weak gels. Furthermore, β-Lg obtained from WPI extracted with SHMP aggregated the least and upon cooling it was found to retain some of its secondary structure (Figure 5.4). This is further supported by the DSC data that showed a 13.3% reversibility of this fraction.

In order to ascertain effect of temperature on the major secondary structure domains of β-Lg a plot of the percent decrease in the intensity of the 1635 cm^{-1} (antiparallel β-sheet) band in IR spectra of β-Lg standard and β-Lg isolated fractions as a function of increasing temperature was generated (Figure 5.5). The decrease in the intensity of the 1635 cm^{-1} band in IR spectra of β-Lg fractions extracted with NaC and SHMP were similar and accordingly only the plot of the drop in the intensity of 1635 cm^{-1} band in IR spectra of β-Lg fractions extracted with NaC are shown in Figure 5.5. The similarity in thermally-induced unfolding of the antiparallel β-sheets in all β-Lg

fractions extracted with NaC and SHMP revealed that nature of the chelating agent did not affect the denaturation profile of the protein while the source of whey (WPI, WPC and LW) had a more pronounced effect. These findings are consistent with the DSC data, which showed similar thermal stability for β-Lg fractions extracted with NaC and SHMP for the same whey sources.

Heating the β-Lg standard from 25 to 55 °C, resulted in a gradual decrease in the intensity of 1635 cm^{-1}; this drop is attributed to the partial unfolding of the protein without aggregate formation (Boye et al., 1996b). Above 60 °C, the intensity of 1635 cm^{-1} decreased rapidly indicating substantial unfolding of the antiparallel β-sheets in the β-Lg standard (Figure 5.5). The decrease in the intensity of 1635 cm^{-1} for β-Lg fraction isolated from WPC extracted with NaC (β-Lg-WPC-NaC) as a function of increasing temperature was similar to that of the β-Lg standard. However, the rate unfolding of the protein was much slower in the case of WPC (Figure 5.5), will multiple transitions. The presence of multiple transitions could result from the heterogeneous population of glycated β-Lg isolated from the WPC. The theromgram in the DSC of β-Lg from the WPC was also broader indicative of the presence of a heterogeneous population (Figure 5.1).

On the other hand, β-Lg fractions isolated from WPI and LW (β-Lg-WPI-NaC and β-Lg-LW-NaC) showed similar drop in the intensity of 1635 cm^{-1} in the temperature range between 25-55 °C and 55-100 °C; with β-Lg-LW-NaC exhibiting a slower rate of unfolding relative to β-Lg-WPI-NaC. In all cases, the β-Lg isolated fractions unfolded at a slower rate compared to the β-Lg standard. These findings are

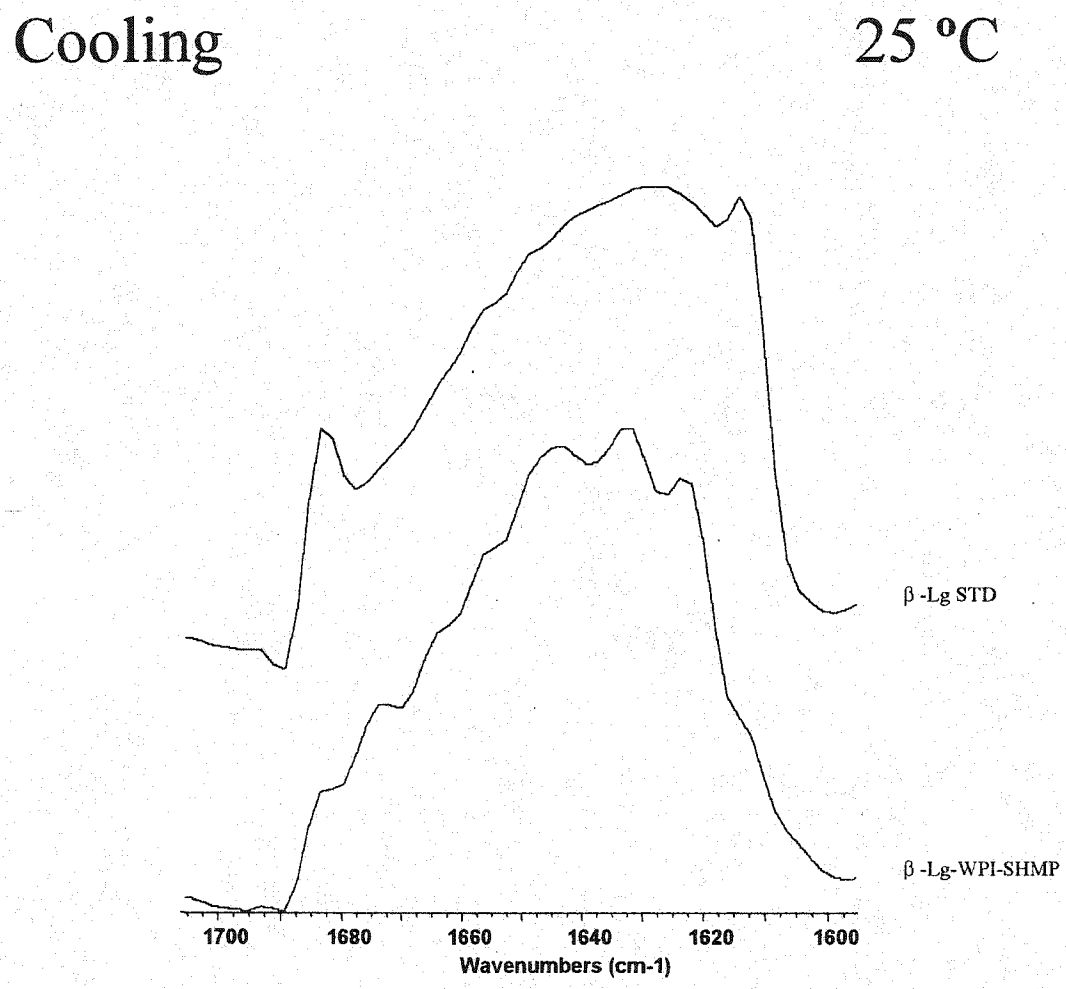


Figure 5.4: Deconvoluted infrared spectra of β-Lg standard and β-Lg-WPI-SHMP fraction (15% w/v) in deuterated phosphate buffer (pH 6.8) at temperature 25 °C after heating to 100 °C. β-Lg = β-lactoglobulin; WPI = whey protein isolate; SHMP = sodium-hexametaphosphate.

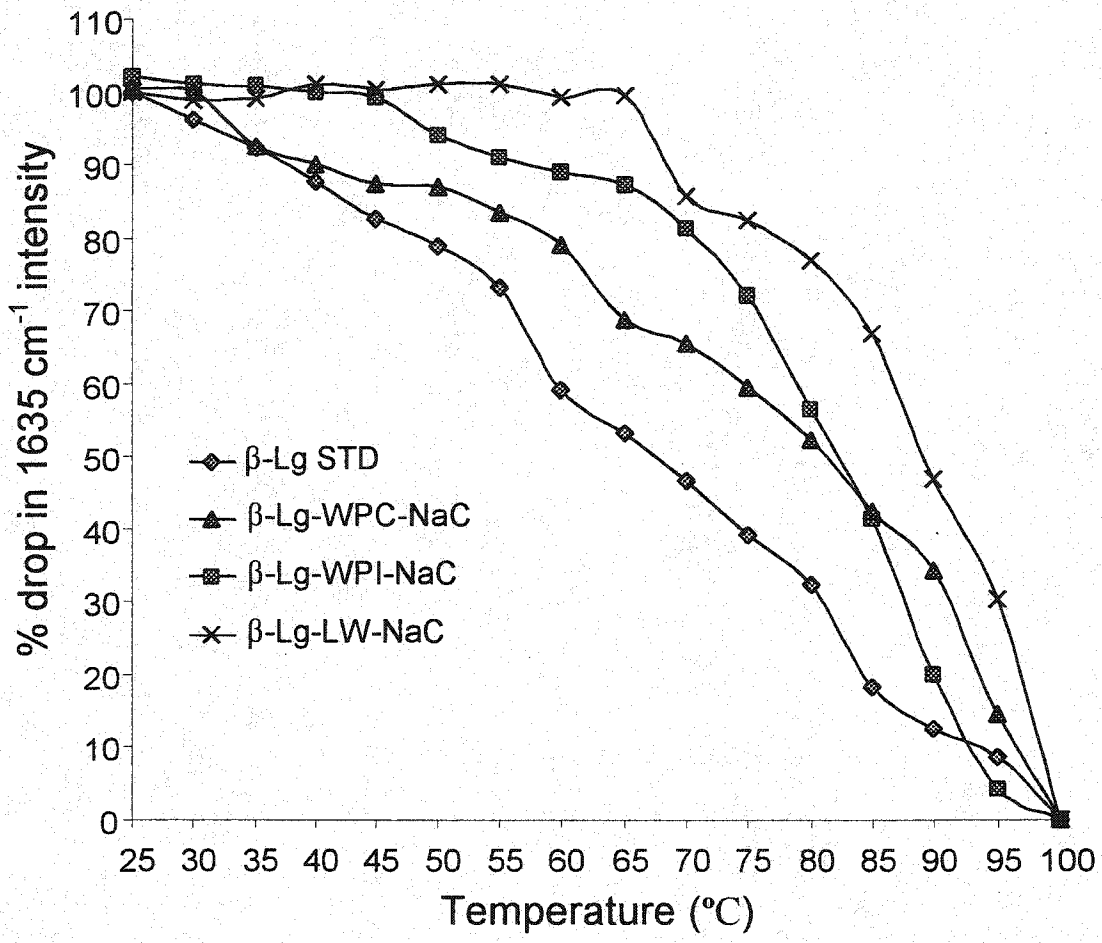


Figure 5.5: Plot of drop % in the intensity of the 1635 cm^{-1} band in the infrared spectra of β-Lg standard and β-Lg fractions isolated from WPI, WPC and LW extracted with NaC as a function of temperature.

consistent with the DSC data, which showed higher peak temperature of denaturation for β-Lg isolated fractions.

Another difference between the infrared spectra of standard β-Lg and β-Lg isolated fractions as a function of increasing temperature, was the shift in the 1677, 1635, 1625 cm^{-1} bands by 2 cm^{-1} to higher wavenumbers in all β-Lg isolated fractions as compared to that of β-Lg standard. This shift in the amide I band to higher wavenumbers can be attributed to increase in the strength of the C=O bond stretching vibration resulting from a decrease in hydrogen bonding (Krimm and Bandekar, 1986). This suggests that the structure of all β-Lg isolated fractions were relatively tight and inflexible, and inhibited solvent accessibility within the protein structure resulting in less hydrogen bonding.

5.5 CONCLUSIONS

In this study, FTIR spectroscopic studies revealed that the secondary structure at ambient temperature of β-Lg isolated from different whey sources (WPC, WPI, LW) was comparable to that of the β-Lg standard. In addition, the use of SHMP as a chelating agent resulted in an increased stability of the native structure of β-Lg. However, the thermally induced unfolding of β-Lg isolated fractions was more dependent on the source of the protein than on the chelating agent employed to isolate the β-Lg. Furthermore, FTIR and DSC results indicated that all β-Lg isolated fractions exhibit increased thermal stability over the β-Lg standard. Both DSC and FTIR studies also revealed that β-Lg fractions isolated from WPC contained a heterogeneous population of glycated β-Lg with varying thermal transitions.

CHAPTER 6

THERMAL DENATURATION AND CONFORMATIONAL STABILITY OF α-LACTALBUMIN ISOLATED FROM WHEY AND WHEY PROTEIN PREPARATIONS

6.0 CONNECTING STATEMENT

In Chapter 4, a method was developed to isolate α-Lac with relatively high purity and yields, from liquid whey (LW), whey protein isolate (WPI), and whey protein concentrate (WPC) using different chelating agents. Results showed that the use of NaC and SHMP were more effective than other chelating agents and the use of SHMP resulted in high yield of α-Lac isolated fractions. The work described in this chapter is an evaluation of the thermal denaturation and structural stability of α-Lac fractions isolated from whey and whey protein preparations in Chapter 4, and satisfies the third objective described in the "Rationale and Objectives of Study" section of Chapter 1.

Note: This chapter is the text of a manuscript which is to be submitted for publication as follows:

Alomirah, H. F.; Alli, I.; Ismail, A. A.; Konishi Y. Thermal Denaturation and Conformational Stability of α-lactalbumin Isolated from Whey and Whey Protein Preparations. *J. Agric. Food Chem.*

Contribution of co-authors: Alli, I. (thesis supervisor); Ismail, A. (contributed to interpretation of FTIR spectra); Konishi, Y. (contributed to interpretation of MS data).

6.1 ABSTRACT

The thermal and structural properties of α-lactalbumin (α-Lac) standard and α-Lac fractions isolated from fresh liquid whey (LW), whey protein isolate (WPI) and whey protein concentrate (WPC) extracted with sodium-citrate (NaC) or sodium-hexametaphosphate (SHMP) at low pH (3.9) were investigated by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy, respectively. The theromgram of α-Lac standard in deuterated buffer shows two reversible transitions with peak temperature (T_d) at 35 °C and 64.5 °C corresponding to the denaturation of the apo-form (Ca^{2+}-free) and holo-form (Ca^{2+} -bound) of α-Lac, respectively. Reversibility percent of holo-form was higher than that of apo-form indicating that removal of Ca^{2+} greatly decreases the thermal stability of α-Lac but the protein retains the folded conformation in the absence of Ca^{2+}. The thermograms of α-Lac isolated fractions in deuterated buffer revealed the presence of a single thermal transition (holo-α-Lac) with T_d lower than that of α-Lac standard but with higher reversibility percent. DSC showed that the use of NaC resulted in a slow interconversion between apo-form and holo-form when α-Lac solutions have concentrations of Ca^{2+} higher than that of protein. FTIR results illustrated that holo-α-Lac have lower relative intensity values of 3_{10}-helix and α-helix than apo-α-Lac indicating slower rate of H/D exchange in the undeuterated 3_{10}-helix (H) component due to increased structural stability. Moreover, results showed that α-Lac fractions extracted with SHMP have lower values of 3_{10}-helix and slower rate of increase in α-helix than in α-Lac extracted with NaC, suggesting less unfolding in the helical structure of SHMP treated samples.

6.2 INTRODUCTION

α-Lactalbumin (α-Lac), is the second major protein component in the whey of milks from various mammalian species, constituting 20% of the proteins in bovine whey, and serves as lactose synthase regulatory protein. α-Lac presents major interest for the food industry because of its functional and nutritional characteristics. For example, α-Lac can be used in infant formulae instead of whey to mimic human milk which result in reduced levels of β-lactoglobulin which is a potential allergen (Gryboski, 1991). Also, α-Lac can be used in nutraceutical because of its high tryptophane content and in meringue-like formulations due to its enhanced whippability (Zydney, 1998). The primary structure of this globular protein consists of 123 amino acid residues, contains four intramolecular disulfide bonds, and contains no free thiol groups. Crystallographic studies reported that the secondary structure of α-Lac consist of an α-helical content of 30%, a 3_{10} helices content of 20% with β-structure content of 6% and the remaining 44% represent amino acid residues in a random coil arrangement while the tertiary structure α-Lac is made up of α-domain which consists of four α-helices and two short 3_{10} helices and of β-domain which consists of a triple-stranded antiparallel β-sheet, a 3_{10} helix and series of loops (Acharya et al., 1989; Pike et al., 1996). This macromolecular structure of α-Lac has a high degree of homology with lysozyme. α-Lac contains a single tightly bound calcium (Ca^{2+}), the binding site being essentially identical in all the known α-Lac crystal structure (Pike et al., 1996). The binding site for the Ca^{2+} is located in a loop between the 3_{10} helix (β-domain) and an α-helix (α-domain).

The binding of Ca^{2+} stabilizes the structure of native α-Lac (Hiraoka et al., 1980). The apo-form (Ca^{2+}-free) and holo-form (Ca^{2+} -bound) of α-Lac has peak temperature (T_d) at 35 and 64 °C corresponding to the denaturation, respectively (Boye et al., 1997a; Boye and Alli, 2000). Although the apo-form is less stable than the holo-form and its refolding is much slower (Hiraoka and Sugai, 1984; Kuwajima et al., 1989; Forge et al., 1999), the folded conformation of the two native forms are similar (Kuwajima et al., 1986; Kuwajima et al., 1989; Forge et al., 1999), based on CD and NMR studies; they concluded that the stability of molten globule (native-like structure in partially folded protein) formed under refolding conditions is enhanced by Ca^{2+}. When α-Lac is subjected to various physicochemical conditions such as low pH values, heated above 50 °C and binding of ions, the native state (N) of α-Lac may undergo various conformational transition to form the molten globule state (A), which is a stable, partially folded state (Kronman, 1989). Under these conditions the protein will aggregate and precipitate. This phenomenon of α-Lac has been applied to the fractionation of individual whey proteins (Bramaud et al., 1997).

Presently, there is growing interest in understanding the relationship between structure stability and protein function to improve the utilization of whey proteins in food systems; this is because stability is easier to quantify than structure (Apenten and Galani, 1999). Although, there is a relative abundance of information in the literature on factors affecting the stability of whey proteins and individual whey protein standards (Boye 1995, Boye et al., 1996, 1997a, b; Boye and Alli, 2000), there is relatively limited information on the structural stability of α-Lac proteins isolated from different whey protein preparations. In this study, differential scanning calorimetry (DSC) and Fourier

transform infrared (FTIR) spectroscopy; two complementary techniques that have become widely accepted for studying the thermal and structural behaviour of proteins (Casal et al., 1988); were employed to investigate the thermal and structural changes in α-Lac isolated from fresh liquid whey (LW), and solutions of whey protein isolates (WPI) and whey protein concentrates (WPC) using a chelating agent [sodium-citrate (NaC) or sodium-hexametaphosphate (SHMP)] at pH 3.9 in the presence of 7% NaCl (Chapter 4).

6.3 MATERIALS AND METHODS

6.3.1 Materials

The α-Lac standard (L-6010, calcium depleted containing less than 0.3 mol. of Ca^{2+}/mol. of α-Lac) was obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Deuterium oxide (D_2O) (> 99%) was from Aldrich (Milwaukee, WI).

6.3.2 Samples Preparation

Protein solutions of α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4), were extensively dialyzed against excess volumes of water for three days. Prior to measurements, all proteins fractions were further desalted 4 times using Centricon-10 filters (Amicon Inc., MA, USA). Desalted samples were then lyophilized and stored at 4 °C. Complete absence of chelating agent attached to the protein was confirmed by electrospray ionization mass spectrometry (ESI-MS) (Chapter 7). The holo-conformer was obtained by adding $CaCl_2$ to α-Lac deuterated solution to a final concentration of 0.5 M (Boye et al., 1997a).

6.3.3 Differential Scanning Calorimetry

Solutions of α-Lac standard and α-Lac isolated fractions were prepared by dispersing the proteins in aqueous (H_2O) and deuterated (D_2O) phosphate buffer solutions (0.01 M, pH 6.8; in this work the relation pD = pH + 0.4 was used) to make 20% (w/v) solutions. The rationale for studying the effect of D_2O and H_2O as a solvent was to determine whether replacement of H_2O with D_2O in FTIR experiments influence the conformational stability of α-Lac. Typically, 25 μL of each solution, were placed in pre-weighed differential scanning calorimetry (DSC) pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC (TA3000, Mettler Instrument Corporation, Greifensee, Switzerland) and scanned from 15 to 100 °C at a programmed heating rate of 5 °C/min. For each run, a sample pan containing the buffer used for dissolving the proteins was used as reference. After heating, the samples were allowed to cool to room temperature and the heating cycle was repeated under the same experimental conditions to determine the degree to which the denaturation was reversible. Cooling of the samples between the two heating cycles was done by the use of a liquid air tank attached to the DSC. Degree of reversibility was determined from the ratio of the areas under the second and first endothermal peaks (Boye and Alli, 2000). The DSC was calibrated by use of indium standards. Peak temperature of denaturation (T_d) and heat of transition or enthalpy (ΔH) (area underneath peak) were computed from each thermal curve. ΔH values are based on the total weight of the protein solutions. Purity percentages of samples were determined as the ratio of apo and holo-α-Lac enthalpies (ΔH) divided by total enthalpy (ΔH) calculated by DSC for each sample. All DSC measurements were done in at least duplicate.

6.3.4 Fourier Transform Infrared Spectroscopy

Solutions of each protein sample (15% w/v) were prepared using deuterated phosphate buffer solutions (pH 6.8) approximately one hour prior to the start of the infrared measurements. Infrared spectra were recorded on a 8210E FTIR spectrometer (Nicolet, Madison, WI) equipped with a deuterated triglycine sulfate detector. The spectrometer was purged continuously with dry air from a Balston dryer (Balston, Haverhill, MA). 7 μL of the protein solution was placed between two CaF_2 windows separated by 25 μm spacer and held in a temperature-controlled infrared cell. The temperature of the sample was regulated by an Omega temperature controller (Omega Engineering, Laval, QC). The temperature was increased in 5 °C increments and the infrared cell allowed to equilibrate for 5 min prior to data acquisition. The reported temperatures are accurate to within ± 0.1 °C. A total of 512 scans co-added at $4cm^{-1}$ resolution. Deconvolution of the observed spectra was performed using Omnic 5.0 software (Nicolet, Madison, WI). The deconvolution of the infrared spectra was carried out as described by Kauppinen et al. (1981). The signal-to-noise ratio was >20000:1, and the bandwidth used for deconvolution was 13 cm^{-1} with a band narrowing factor of 2.4. The band assignments in the amide I area (1600-1700 cm^{-1}) were based on band assignments from previous FTIR studies (Casal et al., 1988; Susi and Byler, 1988; Boye et al., 1997a).

6.4 RESULTS AND DISCUSSIONS

6.4.1 Thermal Stability of α-Lac

Figure 6.1 shows the DSC thermograms of α-Lac standard and α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4); the thermal analysis was done in deuterated (D_2O) phosphate buffer (pH 6.8). Table 6.1 shows the purity % of α-Lac isolated fractions and the thermodynamic parameters. With the expectation of α-Lac fraction isolated from WPC with NaC (α-Lac-WPC-NaC), the purity of the α-Lac isolated fractions ranged from 83 to 95%. These values agree with values obtained by RP-HPLC for the same isolated fractions (Chapter 4). The low purity % in α-Lac-WPC-NaC (73.9%) can be attributed to the presence of additional peak between the two main transitions of α-Lac (transition A and B) (Figure 6.1). This peak has a peak temperature (T_d) at 44.0 °C and enthalpy (ΔH) of 0.22 (data not shown). It is possible that this peak represent the glycated apo-conformer of α-Lac as previous study reported that apo-α-Lac in deuterated buffer containing 50% (w/v) of glucose and sucrose has T_d of 44.6 and 43.7 °C, respectively (Boye et al., 1997a).

The theromgram of α-Lac standard in deuterated buffer shows two transitions with peak temperature (T_d) at 35 °C (transition A) and 64.5 °C (transition B) and with enthalpies (ΔH) of 0.91 and 0.62 J/g, respectively (Table 6.1). Similar values have been reported by Boye et al. (1997a) under the same conditions. These transitions are corresponding to the denaturation of the apo-form (Ca^{2+}-free) and holo-form (Ca^{2+} -bound) of α-Lac (Relkin et al., 1993; Boye et al., 1997a; Hendrix et al., 2000; Boye and Alli, 2000). Results indicate that 60% of α-Lac standard exist as apo-form and holo-

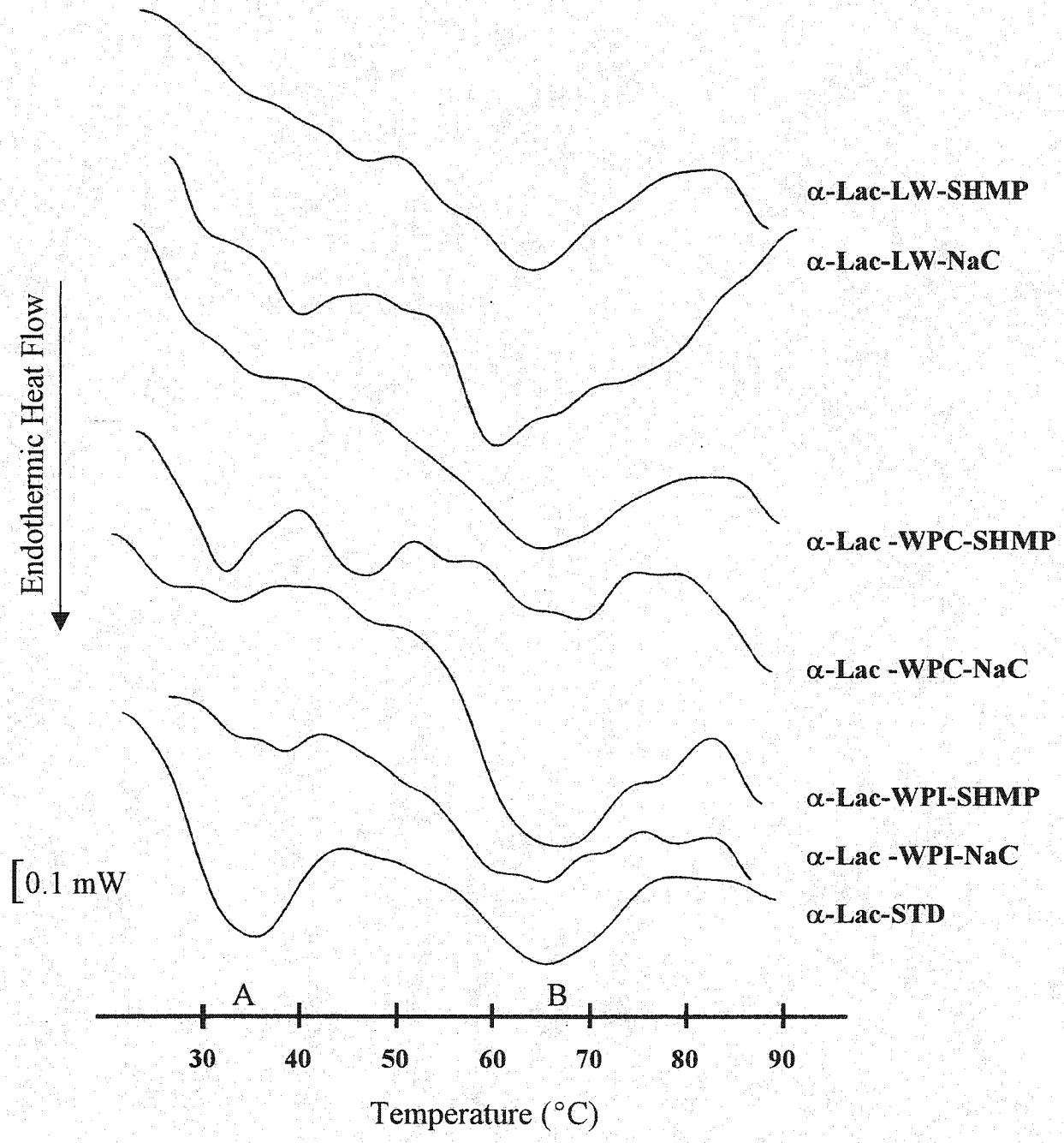


Figure 6.1: DSC thermograms of α-Lac standard and α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP. Heating at 5 °C /min from 15 to 100 °C in deuterated phosphate buffer (0.01 M, pH 6.8). α-Lac = α-lactalbumin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

Table 6.1: DSC characteristics of apo and holo-α-lactalbumin standard and α-lactalbumin fractions isolated from whey products[a]

name	solvent	1st heating cycle					2nd heating cycle		3rd heating cycle	
		A (apo)		B (holo)			A (apo)	B (holo)	A (apo)	B (holo)
		T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)	Purity (%)	Reversibility (%)			
α-Lac STD	D_2O	35.0	0.91	64.5	0.62	96.9	60.2	70.3	37.2	45.5
α-Lac-WPI-NaC	D_2O	37.6	0.12	61.4	0.62	87.4	86.1	89.3	71.1	82.9
α-Lac-WPI-SHMP	D_2O	34.0	0.06	62.7	0.89	92.3	76.0	96.5	43.3	91.8
α-Lac-WPC-NaC	D_2O	33.5	0.24	66.0	0.39	73.9	27.4	82.7	17.0	82.3
α-Lac-WPC-SHMP	D_2O	34.4	0.06	63.0	0.97	91.0	57.4	69.5	47.5	58.2
α-Lac-LW-NaC	D_2O	39.7	0.19	60.0	0.56	95.4	15.8	90.0	14.0	63.6
α-Lac-LW-SHMP	D_2O	34.4	0.04	62.4	0.80	82.6	0.0	68.4	0.0	44.1
α-Lac STD	H_2O	32.5	0.80	62.8	0.79	98.0	81.4	81.5	47.4	73.7
α-Lac-WPI-NaC	H_2O			60.5	0.94	95.1		83.3		61.3
α-Lac-WPI-SHMP	H_2O			61.1	1.19	94.5		65.1		13.6
α-Lac-WPC-NaC	H_2O	37.3	0.04	60.0	0.36	87.9	62.3	72.0	0.0	51.2
α-Lac-WPC-SHMP	H_2O	31.2	0.07	61.7	0.89	93.0	66.7	77.6	0.0	33.0
α-Lac-LW-SHMP	H_2O	31.6	0.10	60.2	0.87	94.0	22.3	70.0	0.0	44.7

[a] A, B first and second transitions observed in the DSC thermogram α-Lac; T_d, peak temperature of denaturation of transitions A and B; ΔH enthalpy of transition A and B calculated as area underneath peaks; % purity, enthalpy calculated for transition A and B divided by total enthalpy calculated for each sample × 100; % reversibility, enthalpy calculated from each heating cycle divided by enthalpy of the first heating cycle × 100; apo = Ca^{2+}-free; holo = Ca^{2+} -bound; α-Lac = α-lactalbumin; WPI = whey protein isolate; WP C = whey protein concentrate; LW = liquid whey; Na-C = sodium-citrate; SHMP = sodium-hexametaphosphate.

form represent only 40% based on their ΔH. Similar findings were reported by Boye et al. (1997a). Cooling of the sample followed by reheating (second heating cycle) resulted in the reappearance of transition A and B with ΔH of 0.64 and 0.37 J/g, respectively indicating 60% and 70% reversibility for transition A and B, respectively (Table 6.1). Third heating cycle resulted in the reappearance of transition A and B with 37.2 and 45.5% reversibility, respectively (Table 6.1). These results demonstrate that although the apo-form and the holo-form of α-Lac standard unfolded at different temperatures, both forms are reversible. However, reversibility percent is higher in the holo-form than in the apo-form indicating that refolding of the apo-form is much slower than the refolding of holo-form. Therefore, removal of Ca^{2+} greatly decreases the thermal stability of α-Lac but the protein retains the folded conformation in the absence of Ca^{2+}. These results are consistent with the findings of previous studies using CD and NMR that folding in the presence of Ca^{2+} is similar to that in its absence, although the rate is increased by more than two orders of magnitude (Kuwajima et al., 1989; Forge et al., 1999).

Figure 6.1 shows the presence of a single thermal transition (transition B) corresponds to holo-α-Lac with the coexistence of small thermal transition (transition A) corresponding to apo-α-Lac in -α-Lac isolated fractions. The predominated presence of holo-form (transition B) in α-Lac isolated fractions may attributed to the use of excess concentration of $CaCl_2$ required to resolubilized α-Lac in the isolation method (Chapter 4). The T_d of holo-α-Lac isolated from WPI, WPC and LW extracted with NaC were 61.4, 66 and 60 °C respectively, and T_d of holo-α-Lac extracted with SHMP were 62.7, 63 and 62.4 °C respectively. On average, these values were lower than that

observed for holo-α-Lac standard (64.5 °C). Lower T_d of holo-α-Lac isolated fractions may be attributed to the broadens of their endothermic peak indicating that nature of transition from native to denatured state of these samples was less cooperative; if denaturation occurs within a narrow range of temperature, the transition is considered highly cooperative (Boye et al., 1997a). The T_d of holo-α-Lac isolated fractions were similar to T_d of holo-α-Lac in the absent of excess Ca^{2+}(~ 64 °C). This indicates the absence of free calcium in α-Lac isolated fractions resulting from extensive desalting, since the addition of calcium (1-10 mM) resulted in transition at ~ 70 °C (Boye et al., 1997a; Hendrix et al., 2000). Table 6.1 shows that percent reversibility of transition B (holo-form) in α-Lac isolated fractions extracted with NaC ranged from 83 to 90 in the second heating cycle and ranged from 64 to 83 in the third heating cycle. These reversibility values are greater than that of transition B in α-Lac standard, suggesting faster refolding rates. Reversibility of transition B (holo-form) in α-Lac fractions isolated from WPC and LW and extracted with SHMP was approximately 69% in the second heating cycle and was 51% in the third heating cycle. These reversibility values were similar to that of transition B in α-Lac standard, suggesting similar refolding rates. However, second and third heating cycle of α-Lac-WPI-SHMP showed 96.5 and 92% reversibility of transition B (holo-form), respectively; these reversibility values are greater than that of transition B in α-Lac standard. These results indicate that α-Lac fractions showed different reversibility percent depending on the source of whey (i.e., WPI, WPC and LW and on the use of different chelating agents (i.e., NaC and SHMP).

The T_d of apo-α-Lac isolated from WPI, WPC and LW extracted with NaC were 37.6, 33.5 and 39.7 °C respectively, and T_d of apo-α-Lac extracted with SHMP were 34,

34.4 and 34.4 °C respectively. On average, the T_d of the apo-α-Lac isolated fractions extracted with NaC (37 °C) were higher than that of α-Lac standard (35 °C) but they showed a marked decrease in their ΔH compared to α-Lac standard; 25% of α-Lac isolated fractions exist as apo-form compared to 60% in α-Lac standard. The average T_d of apo-α-Lac isolated fractions extracted with SHMP (34.3 °C) were similar to that of α-Lac standard (35 °C) but with lower ΔH; 5.6% of α-Lac isolated fractions exist as apo-form compared to 60% in α-Lac standard. It is interesting to notice that T_d and ΔH of apo-α-Lac isolated fractions extracted with NaC were higher than those of apo-α-Lac isolated factions extracted with SHMP (Table 6.1). This indicates that the use of NaC resulted in a slow interconversion between apo-form and holo-form when α-Lac solutions have concentrations of Ca^{2+} higher than that of protein. It is tempting to speculate here that, NaC may have altered partially the structure of the protein, probably at the Ca^{2+} binding site, which may have prevented the complete interconversion of the apo-form to the holo-form in the presence of excess Ca^{2+}. However, this partial modification of α-Lac structure did not alter its refolding properties as shown by its high reversibility percent.

Figure 6.2 shows the DSC thermograms of α-Lac standard and α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP (Chapter 4); the thermal analysis was done in aqueous (H_2O) phosphate buffer (pH 6.8). Table 6.1 shows that purity of α-Lac isolated fractions ranged from 88 to 95%. These values are in agreement with values obtained by RP-HPLC for the same isolated fractions (Chapter 4). The α-Lac-WPC-NaC sample showed the least purity percent (88%) among analyzed isolated factions; this decrease in purity was more pronounced in deuterated

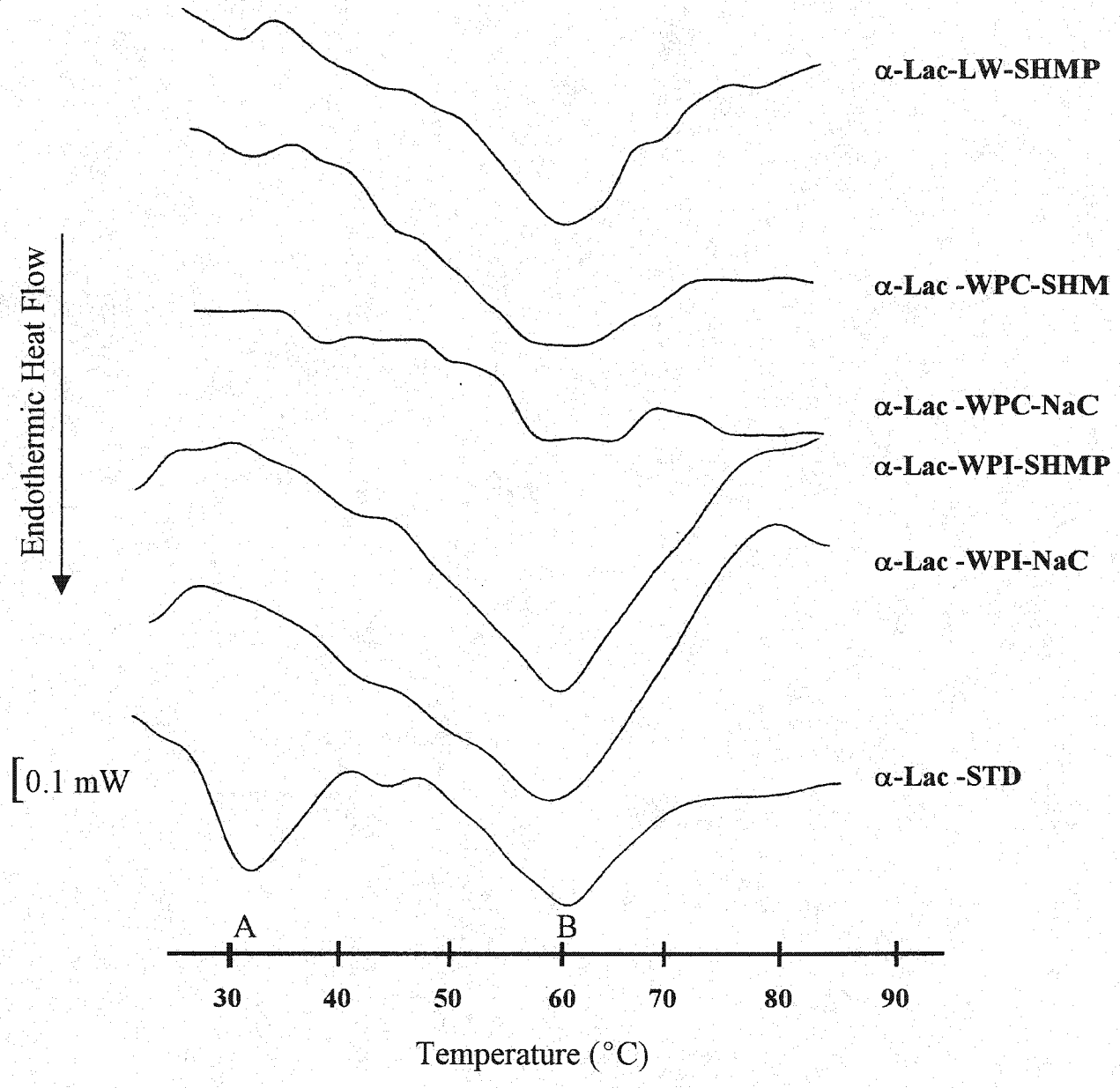


Figure 6.2: DSC thermograms of α-Lac standard and α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP. Heating at 5 °C /min from 15 to 100 °C in aqueous phosphate buffer (0.01 M, pH 6.8). α-Lac = α-lactalbumin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

buffer. The theromgram of α-Lac standard in aqueous buffer shows two transitions with T_d at 32.5 °C (transition A, apo-form) and 62.8 °C (transition B, holo-form) and with enthalpies (ΔH) of 0.80 and 0.79 J/g, respectively (Table 6.1). These values are slightly lower than previously reported Boye and Alli (2000) under similar conditions. Cooling of this sample followed by reheating (second heating cycle) resulted in the reappearance of transition A and B both with ΔH of 0.65 J/g, indicating percent reversibility of 81.5% (Table 6.1). Third heating cycle resulted in different reversibility percent of transition A (47.4%) and B (73.7%). In aqueous buffer, apo- and holo-α-Lac standard exist in equal amounts (50% each) and their T_d decreased by 2.5 and 1.7 °C from T_d of apo- and holo-α-Lac standard in deuterated buffer (Table 6.1). The stabilizing effect of D_2O was explained by an increase in hydrophobic interactions and/or an isotope effect on hydrogen bonding (Verheul et al., 1998).

The results revealed predominance of the holo-form in α-Lac isolated fractions with the complete disappearance of apo-form in α-Lac isolated from WPI extracted with NaC and SHMP. The T_d of holo-α-Lac obtained from WPI, WPC and LW extracted with NaC and SHMP ranged from 60 to 61.7 °C. These values were lower than that observed for holo-α-Lac standard (62.8 °C); this may be attributed to lower cooperative denaturation transitions. Table 6.1 shows that percent reversibility of transition B (holo-form) in α-Lac isolated fractions extracted with NaC and SHMP ranged from 65 to 83% in the second heating cycle and ranged from 14 to 61% in the third heating cycle. Reversibility of α-Lac isolated fractions extracted with NaC were higher than those extracted with SHMP.

6.4.2 Structural Changes of a-Lac by FTIR Spectroscopy

In this study transitions effecting position and intensities of the amide I band in the frequency region between 1600 and 1700 cm^{-1} was considered. Figure 6.3 (A-D) shows deconvoluted infrared spectra of α-Lac standard and α-Lac isolated fractions (15% w/v) in deuterated phosphate buffer (pH 6.8) at temperature 25, 55, 75 and 95 °C. The principle bands in the spectra of α-Lac are shown in Table 6.2. The band assignments in Table 6.2 are based on previous infrared studies of α-Lac by Casal et al., 1988; Susi and Byler, 1988; Boye et al., 1997a; Dzwolak et al., 1999; Troullier et al., 2000 and Dzwolak et al., 2001. To determine the contribution of apo-form and holo-form of α-Lac to the transitions observed in the FTIR and DSC, the FTIR spectra of α-Lac standard dispersed in 0.5 M $CaCl_2$ (holo-α-Lac) were recorded (Boye et al., 1997a). The spectrum at 25 °C shows four main bands at 1656 (α-helix), 1648 (random coil), 1639 (3_{10}-helix), and 1637 cm^{-1} (antiparallel β-sheet) and two shoulders at 1679 (antiparallel β-sheet) and 1612 cm^{-1} (side chain). Increase in temperature from 25 to 35 °C (not shown) resulted in the gradual decrease in intensity of the 1656 cm^{-1} band accompanied by the gain in intensity of 1653 and 1641 cm^{-1} bands indicating partial loss of secondary structure at temperature 35 °C. These changes were more noticeable in the spectra of α-Lac standard (apo and holo-form) than in holo-α-Lac standard and α-Lac isolated fractions. This temperature is close to the T_d of transition A (apo-form, ~ 35 °C) observed in the thermogram of α-Lac heated in deuterated solution (Figure 6.1). Previous infrared studies showed that heat (Boye et al., 1997a; Dzwolak et al., 2001) and pressure (Dzwolak et al., 1999) treatments can induce structural changes in α-Lac at temperature between 35-40 °C. Heating above 55 °C (Figure 6.3 B) resulted in loss of

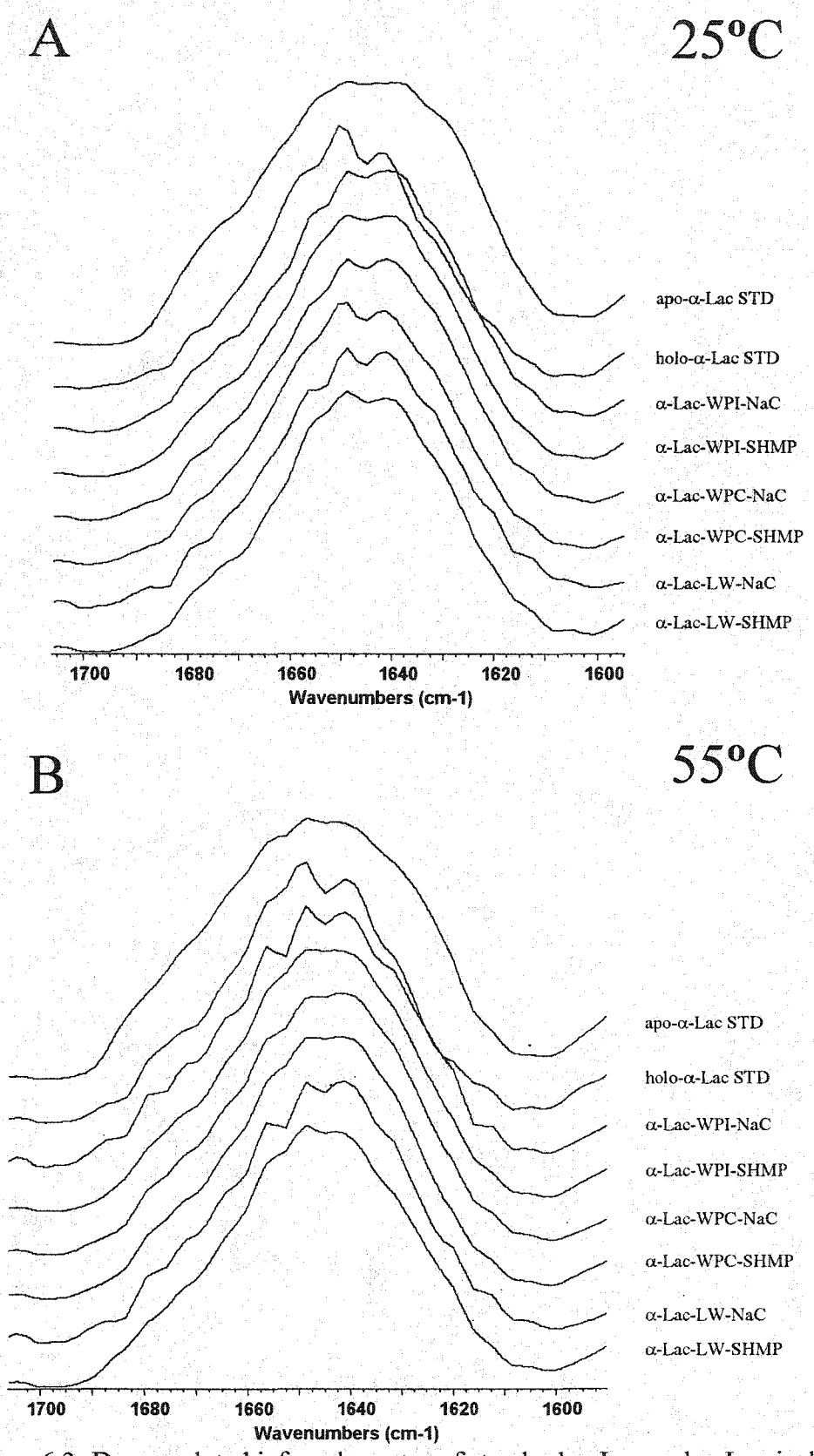


Figure 6.3: Deconvoluted infrared spectra of standard α-Lac and α-Lac isolated fractions (15% w/v) in deuterated phosphate buffer (pH 6.8) at temperature 25 (A), 55 (B), 75 (C) and 95 °C (D). α-Lac = α-lactalbumin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

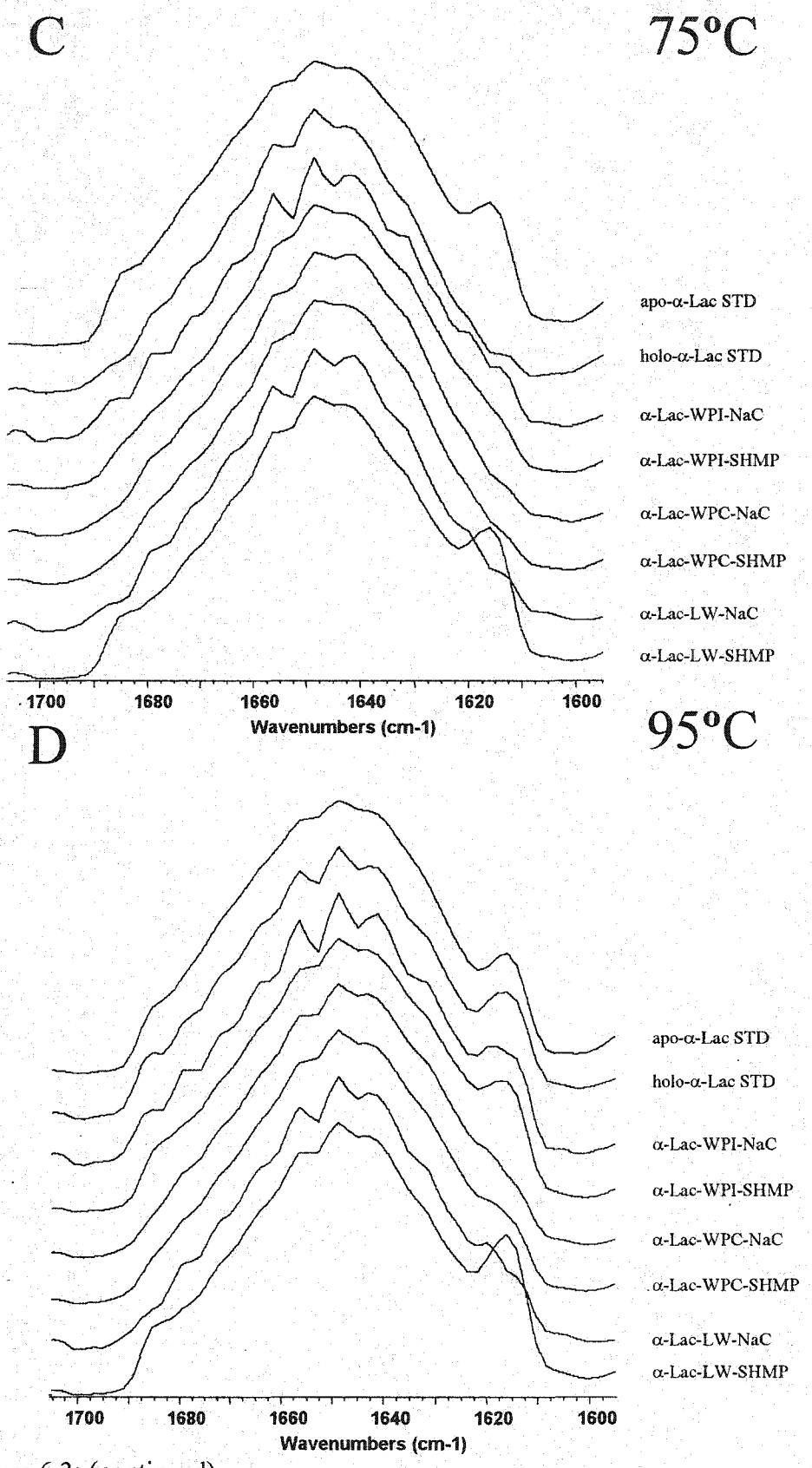


Figure 6.3: (continued).

Table 6.2: Frequencies (cm^{-1}) and band assignments of the amide I regions of α-lactalbumin[a]

peak position (cm^{-1})	band assignment	peak position (cm^{-1})	band assignment
1687-1685	antiparallel β-sheet or β-turns	1639-1641	3_{10}-helix
1672-1679	antiparallel β-sheet	1637	antiparallel β-sheet
1664	turns	1631	β-sheet
1656	α-helix	1619-1616	β-sheet aggregated strands
1648	α-helix and random coil	1612	side chain

[a] Casal et al., 1988; Susi and Byler, 1988; Boye et al., 1997a; Dzwolak et al., 1999; Troullier et al., 2000 and Dzwolak et al., 2001.

1631 cm^{-1} (β-sheet) and 1679 (antiparallel β-sheet) and increase in intensities of the following bands: 1685 (antiparallel β-sheet or turns), 1671 (antiparallel β-sheet), 1664 (turns) and 1648 cm^{-1} (random coil). This temperature (above 55 °C) is close to T_d of transition B (holo-form, ~ 64 °C) observed in DSC. The FTIR results suggest that at high temperature β-sheet structures were transformed to turns and unordered structure indicating protein unfolding. Boye et al. (1997a) and Dzwolak et al. (2001) reported similar findings and indicated that unfolding observed on heating, was reversible on cooling. As the temperature was increased to 95 °C, the intensity of 1613 (side chain), 1617 (β-sheet aggregated strands) and 1685 cm^{-1} (antiparallel β-sheet or turns), bands increased. An increase in the intensity of the latter band accompanied by formation of another band below 1620 cm^{-1} is indicative of the formation of a intermolecular hydrogen-bonded antiparallel β-sheet structure resulting from reassociation of unfolded peptide segments which leads to formation of gel and aggregate structure (Clark et al., 1981).

Figure 6.4 shows the relative intensities of the following infrared main bands; 1656 (α-helix), 1648 (random coil), 1641 (3_{10}-helix). At 25 °C, the intensity of bands at 1656, 1648, 1639 cm^{-1} were higher in holo-form than in apo-form in α-Lac standard and higher in α-Lac isolated fractions extracted with SHMP than in α-Lac isolated fractions extracted with NaC (Figure 6.4). The increase in intensity of these bands can be attributed to the relative stable secondary structure of holo-α-Lac standard and α-Lac isolated fractions extracted with SHMP. These findings supports the results obtained from DSC which showed higher T_d and ΔH of α-Lac isolated fractions

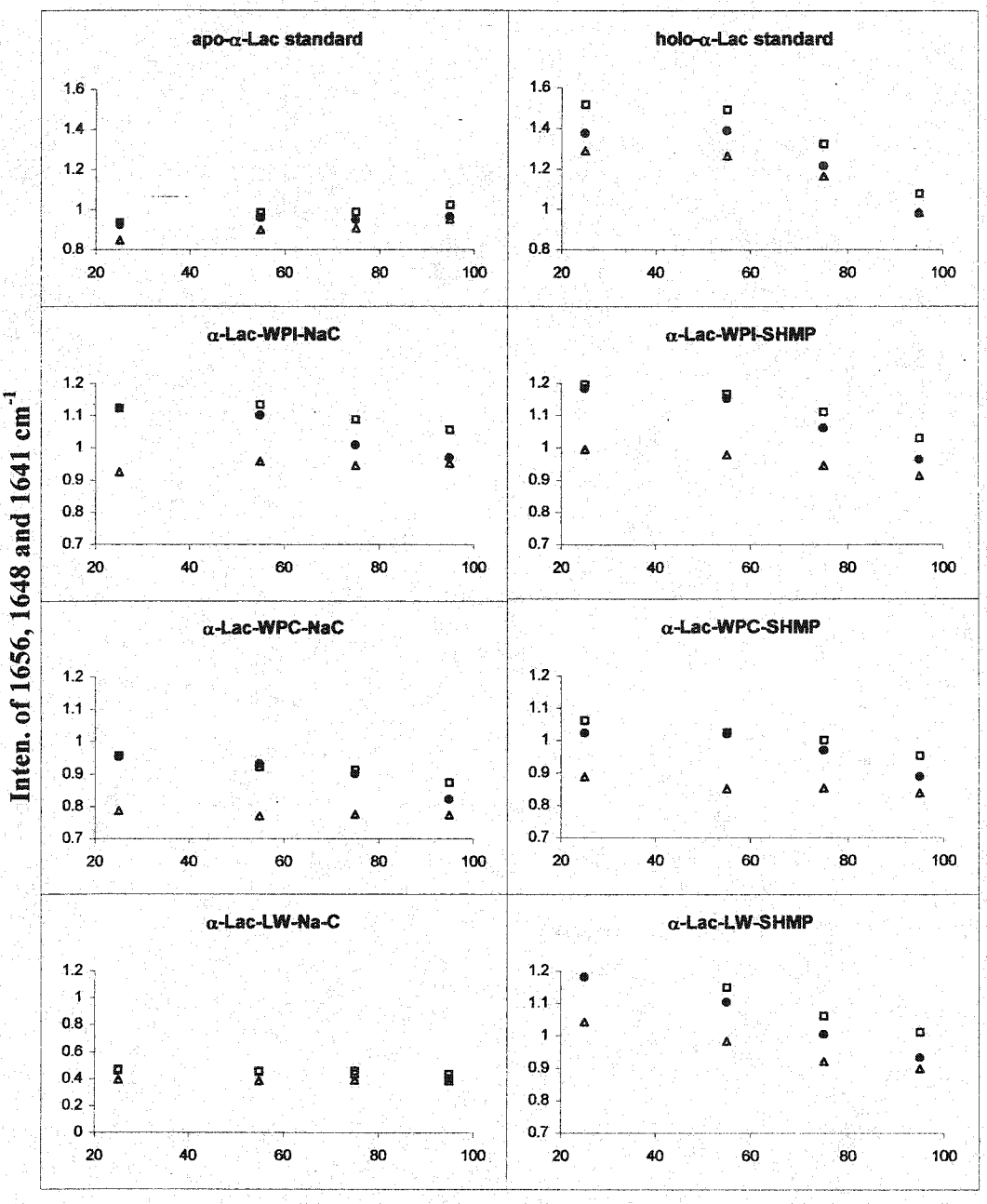


Figure 6.4: Plot of the intensities of the 1656 (▵), 1648 (□), and 1641 cm^{-1} (•) bands in the infrared spectra of α-Lac (apo and holo-form) standard and α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP as a function of temperature.

SHMP and slower interconversion between apo-form and holo-form in excess of Ca^{+2} for samples treated with NaC.

Previous FTIR, studies (Boye et al., 1997a; Dzwolak et al., 1999, 2001) have suggested that structural changes in apo-α-Lac were consisted with gradual hydrogen-deuterium (H/D) exchange process taking place mostly in the helical structure of the protein. In order to better visualize the heat-induced changes in the helical structure region of α-Lac, plots of relative ratio of intensities of the bands at 1657 (α-helix) and 1641 (3_{10}-helix) 7 cm^{-1} to that at 1948.8 cm^{-1} (random coil) as a function of temperature is shown in Figure 6.5. Rationing the intensity of the helical structure of α-Lac to random coil was performed because this band is well resolved and has high intensity. Moreover, this band should not interfere with H/D exchange process, since considerable portion of α-Lac overall random coil is located in the very flexible β-subunit and the random coil within the α-subunit is highly accessible to solvent (Acharya et al., 1989; Pike et al., 1996). Figure 6.5 shows that at all temperatures and in all α-Lac samples, the ratio of band at 1641 cm^{-1} (3_{10}-helix) were constantly higher than ratio of band at 1657 cm^{-1} (α-helix) both relative to band 1647 cm^{-1} (random coil). At temperature 25 °C, apo-α-Lac standard have intensity ratio of approximately 1 and 0.9 for 3_{10}-helix (1641 cm^{-1}) and α-helix (1657 cm^{-1}), respectively. Heating resulted in gradual increase in relative intensity of α-helix (1657 cm^{-1}) and gradual decrease in relative intensity of 3_{10}-helix (1641 cm^{-1}) and at ~95 °C both helices have almost the same relative intensity. At temperature 25 °C, holo-α-Lac standard have intensity ratio of approximately 0.9 and 0.85 for 3_{10}-helix (1641 cm^{-1}) and α- helix (1657 cm^{-1}) respectively, which are lower

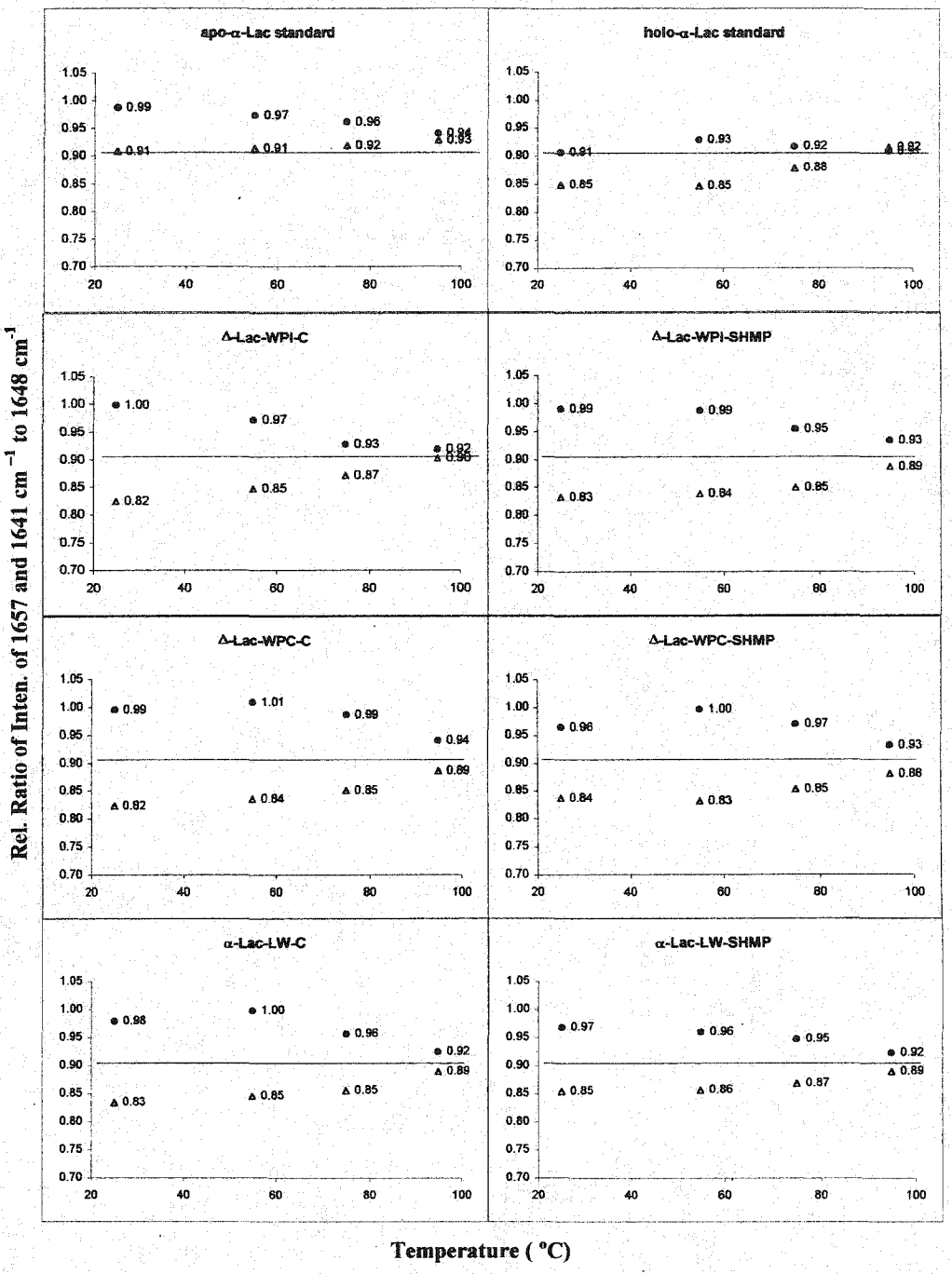


Figure 6.5: Plot of relative ratio of intensities of the bands at 1657 (△) and 1641 (•) cm^{-1} to that at 1648 cm^{-1} in the infrared spectra of α-Lac (apo and holo-form) standard and α-Lac fractions isolated from WPI, WPC and LW extracted with NaC or SHMP as a function of temperature.

than values obtained for apo-α-Lac standard. Similarly, heating resulted in gradual increase in relative intensity of α-helix (1657 cm^{-1}) and gradual decrease in relative intensity of 3_{10}-helix (1641 cm^{-1}) and at ~95 °C both helices have almost the same relative intensity. Dzwolak et al. (1999 and 2001) have proposed that peak centered around 1654 (α-helix) in α-Lac actually contains two spectral components: one originated from the undeuterated 3_{10}-helix (H) and the other one from the deuterated α-helix (D). Crystallographic studies indicated that the structure of α-Lac contains four 3_{10}-helix, two at the termini (residues 12-16 and 115-119) and the another two remain in proximity to calcium-binding loop (residues 76-82 and 101-104) (Acharya et al., 1989; Pike et al., 1996). Therefore one can assume that hydrogens in the two terminal 3_{10}-helix exchange rapidly with deuterons and complete H/D exchange in the buried 3_{10}-helix (close to calcium binding loop) can be achieved only with induced heat or pressure (Dzwolak et al., 1999). Accordingly, we can relate the gradual increase observed in relative intensity of the α-helix to gradual hydrogen-deuterium (H/D) exchange process taking place in the undeuterated 3_{10}-helix (H). Boye et al. (1997) have related the unfolding of apo-α-Lac to the loss of 3_{10}-helix conformation and an increase in β-sheet and related unfolding of holo-α-Lac to the loss of 3_{10}-helix conformation and an increase in the formation of turns. Therefore, gradual decrease in relative intensity of deuterated 3_{10}-helix (D) (1641 cm^{-1}) observed in Figure 6.5 can be related to unfolding of the helical structure probably in the Ca^{2+} binding region. At 25 °C, all α-Lac isolated fractions extracted with NaC and SHMP showed lower values of α-helix (1657 cm^{-1}) relative intensity than that of apo-α-Lac standard (Figure 6.5). This indicates slower rate of H/D exchange in the undeuterated 3_{10}-helix (H) component suggesting that the

structure of all α-Lac isolated fractions were relatively more compact and inaccessible to solvent as compared to apo-α-Lac standard structure. Figure 6.5 shows that α-Lac fractions isolated from WPI, WPC and LW and extracted with SHMP have lower values of 3_{10}-helix (D) (1641 cm^{-1}) compare to α-Lac extracted with NaC suggesting less unfolding in the helical structure of SHMP treated samples. Moreover, the rate of increase in the relative intensity of α-helix (1657 cm^{-1}) was slower in SHMP treated samples than in NaC treated samples suggesting slower rate of H/D exchange in the undeuterated 3_{10}-helix (H) component due to structure stability. These findings support the results obtained from DSC which showed higher T_d and ΔH in α-Lac fractions extracted with SHMP and slower interconversion between apo-form and holo-form in excess of Ca^{+2} for α-Lac fraction extracted with NaC.

6.5 CONCLUSIONS

In this study, the thermal and structural properties of α-Lac isolated fractions were found different from that of α-Lac standard. DSC data, indicate that purity measurement were not influenced by solvent composition (D_2O or H_2O). The thermograms of α-Lac isolated fractions extracted with NaC and SHMP revealed mainly the presence of a single thermal transition (transition B) with reversibility percent greater than α-Lac standard; suggesting faster refolding rates. FTIR shows that transition A (apo-form, ~ 35 °C) observed in DSC is related the gradual decrease in the 1656 cm^{-1} band and accompanied by the gain in intensity of 1653 and 1641 cm^{-1} bands while transition B (holo-form, ~ 64 °C) is associated by breakdown of β-sheet structure and increase in turns and unordered structures. FTIR confirmed DSC findings that the

use of NaC resulted in a slow interconversion between apo-form and holo-form in excess of Ca^{+2}. We have shown that the relative ratio of intensity of α-helix and 3_{10}-helix to that at random coli can be explained exclusively in terms of a complex H/D exchange process. Results illustrated that holo-α-Lac have lower relative intensity values of 3_{10}-helix and α-helix than apo-α-Lac indicating slower rate of H/D exchange in the undeuterated 3_{10}-helix (H) component due to increased structural stability. Moreover, results showed that α-Lac fractions extracted with SHMP have lower values of 3_{10}-helix and slower rate of gradual increase in α-helix than in α-Lac extracted with NaC, suggesting less unfolding in the helical structure of SHMP treated samples.

CHAPTER 7

STUDY OF CONFORMATIONAL STABILITY OF α-LACTALBUMIN AND β-LACTOGLOBULIN ISOLATED FROM WHEY PREPARATIONS BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

7.0 CONNECTING STATEMENT

In Chapters 5 and 6, the thermal and structural properties of β-Lg and α-Lac isolated fractions were found to be different from that of β-Lg and α-Lac standard. By use of differential scanning calorimetry and Fourier transform infrared spectroscopy, a relationship between the thermal transitions occurring during heat treatment and changes in the secondary structure of β-Lg and α-Lac isolated fractions were established. The work described in this chapter addresses the structural stability of α-Lac and β-Lg by monitoring changes in charge state distribution as affected by pH and storage time and by probing hydrogen-deuterium exchange rates using ESI-MS and, satisfies the fourth objective discussed in the "Rationale and Objectives of Study" section of Chapter 1.

Note: This chapter is the text of a manuscript which is to be submitted for publication as follows:

Alomirah, H. F.; Alli, I.; Konishi, Y. Study of Conformational Stability of α-Lactalbumin and β-Lactoglobulin Isolated from Whey Preparations by Electrospray Ionization Mass Spectrometry. *J. Agric. Food Chem.*

Contribution of co-authors: Alli, I. (thesis supervisor); Konishi, Y. (contributed to interpretation of MS data).

7.1 ABSTRACT

Conformational stability of α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) in response to pH (3-8) and storage time (0-10 days) were investigated by monitoring changes in charge state distribution (CSD) in their electrospray ionization (ESI) spectra. Storage of α-Lac at pH 3 resulted in substantial changes in its CSD involving the emergence of new ion species and shifting of its charge state toward higher values indicating less stable conformation. ESI spectra of α-Lac at pH 5 for 4 days showed stable conformation however extending the storage period resulted in substantial changes in its CSD and a decrease in the relative intensity of metal adduct (Ca^{2+}) indicating less stable conformation. The relative intensity of holo-form (Ca^{2+} -bound) of α-Lac increased at pH 6.8 and decreased at pH 8 during storage indicating folded and partially folded conformation, respectively. During storage, β-Lg showed stable CSD at pH 3 with substantial changes at pH 5 and narrow changes at pH 6.8 and 8. Conformational stability of α-Lac and β-Lg fractions isolated from fresh liquid whey (LW), whey protein isolates (WPI) and whey protein concentrates (WPC) extracted with sodium-citrate (NaC) or sodium-hexametaphosphate (SHMP) were studied by measuring hydrogen/deuterium (H/D) exchange rates using ESI-MS. The H/D exchange results demonstrate that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and conformation of β-Lg variant B was more stable than β-Lg variant A. Kinetics of H/D exchange indicated that α-Lac and β-Lg isolated fractions obtained from whey protein preparations have the same or improved conformational stabilities compared to that of α-Lac and β-Lg standard.

7.2 INTRODUCTION

During the past two decades, considerable effort has been devoted to structural characterization of food proteins and peptides. Although much useful information has been obtained through numerous spectroscopic methods, they give relatively little information on specific regions undergoing structural changes and require relatively high concentration and large quantities of purified protein and long analysis time. By contrast, MS can accurately and precisely probe the conformational changes of relatively large protein molecules in relatively short analysis time (10-90 min). The application of mass spectrometry (MS) to large biomolecules has revolutionized in the past decade with the development of electrospray ionization (ESI) (Fenn et al., 1989; Alomirah et al., 2000).

The utility of ESI-MS lies in its ability to generate multiply charged gas phase ions from protein molecules in solution. ESI-MS now plays an important role in studies of primary (covalent and non-covalent) structure of proteins. Its role is more than determination of MW of proteins, since a relationship between the structure of proteins in solution under different environmental conditions and their charge state distributions (CSD) in ESI spectrum has been established (Loo et al., 1990, 1991; Chowdhuray et al., 1990; Mirza et al., 1993). Thus, the changes in CSD of a protein can be interpreted in terms of changes in its conformational stability. CSD is sensitive to tuning conditions in the mass spectrometer, to slight variation in pH and to counter-ion effect (Robinson, 1996). Therefore, more reliable method for detecting protein conformational stability is required. Protein conformational can be studied by ESI-MS using amide hydrogen exchange technique. This technique is based on the mass spectrometric measurement of

the extent of hydrogen/deuterium (H/D) exchange that occurs in different protein conformers over defined periods of time (Katta and Chait, 1991, 1993). Higher order structural (secondary and tertiary) features of proteins can affect amide hydrogen exchange rates drastically, thereby forming the basis for estimating, detecting and locating conformational changes.

Amide hydrogen exchange rates of proteins and peptides is catalyzed by acid and base. Therefore, the isotopic exchange rates are highly pH sensitive and this dictates careful control of pH in all H/D experiments (Smith et al., 1997). Exchange rates of amide hydrogen are also affected by inductive and steric effect of the adjacent amino acid side-chains, but relatively insensitive to more distant side-chains. The effect of neighboring side-chains can decrease the amide hydrogen exchange rates by as much as ten fold, while secondary and tertiary structural features of folded proteins may decrease amide hydrogen exchange rates by as much as 10^8 (Smith et al., 1997). In this context, amide hydrogen exchange is considered as a sensitive probe for detecting and locating conformational changes in proteins. The application of H/D exchange to the study of protein conformational stability in solution was first demonstrated by Katta and Chait (1991) and has since been applied to study protein conformational stability under different environmental conditions. It has been used to study the native state of a protein (Katta and Chait, 1993), exchange rates of α-helices and β-sheets (Wagner et al., 1994), folding pathway of native (Miranker et al., 1993) and reduced (Eyles et al., 1994) lysozyme, to monitor protein/protein (Ehring, 1999), ligand/protein (Wang et al., 1997, 1998) and metal/protein interactions (Johnson and Walsh 1994; Nemirovskiy et al., 1999) and thermal (Zhang and Smith 1996; Maier et al., 1999) and organic solvent (Babu et al., 2001) unfolding of proteins.

Studying protein stability-function relations (PSFR) rather than traditional structure-function relations (Apenten and Galani, 1999) is presently preferred, because stability is easier to quantify than structure. Although, there is a relative abundance of information in the literature on factors affecting structural stability of whey proteins, there is relatively limited information on the structural stability of commercially prepared, individual β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac), isolated from different whey protein preparations. In this study, we have used CSD determinations by ESI-MS to monitor changes in the conformational stability of α-Lac and β-Lg standard induced by change in pH and storage period. In addition, the conformational stability of α-Lac and β-Lg fractions isolated from fresh liquid whey (LW), whey protein concentrates (WPC) and whey protein isolates (WPI) using the combinational effects of high salt concentration (7% NaCl) and chelating agents (sodium citrate (NaC) or sodium hexametaphosphate (SHMP)) at low pH (3.9) (Chapter 4) were probed by H/D exchange ESI-MS.

7.3 MATERIALS AND METHODS

7.3.1 Materials

β-Lg (containing variant A and B; L-0130) and α-Lac standard (L-6010, calcium depleted containing less than 0.3 mol. of Ca^{2+}/mol. of α-Lac) were obtained from Sigma Chemical Co. (St. Louis, MO). β-Lg and α-Lac isolated fractions previously prepared in our laboratory were obtained from whey protein isolates (WPI), whey protein concentrates (WPC) and fresh liquid whey (LW) extracted with NaC or

SHMP (Chapter 4). Deuterium oxide (D_2O) (> 99%) was from Aldrich (Milwaukee, WI). All chemicals were of an analytical grade.

7.3.2 CSD and ESI-MS

Solutions (0.5 mg/ml) of α-Lac and β-Lg standard were prepared by dispersing the proteins in aqueous volatile solution, with pH 3 (1% acetic acid), pH 5.5 (water), pH 6.8 (10 mM ammonium acetate) and pH 8 (10 mM ammonium bicarbonate) and stored for 0, 2, 4, 8, 10 days at refrigerated temperature (4 ± 1 °C). A SCIEX API III- triple-quadrupole mass spectrometer equipped with a standard atmospheric pressure ionization (API) source and a SCIEX ionspray interface (PE-Sciex, Concord, ON, Canada) was used to generate multiply charged protein ions by spraying the sample solution through a stainless steel capillary held at high potential. The voltage on the sprayer was set at 4500 V for positive ion production. The mass scale of the spectrometer was first calibrated with polypropylene glycol and then tuned and recalibrated using peaks from multiply protonated lysozyme. All CSD experiments were done in duplicate.

7.3.3 H/D Exchange and ESI-MS

α-Lac and β-Lg fractions isolated from WPI, WPC and LW (Chapter 4), were dialyzed against excess volumes of water for 3 days. α-Lac and β-Lg standard and α-Lac and β-Lg isolated fractions were further desalted (x 4) using Centricon-10 filters (Amicon Inc., MA, USA). The desalted samples were then lyophilized. Desalting step was essential to obtain a mass spectrum with good sensitivity. For ESI-MS measurements of H/D exchange rates, desalted protein samples were first dissolved (1 mg/100 μL) in 10 mM ammonium acetate aqueous buffer (pH 6.8). After 1 h, the H/D

exchange was initiated by diluting 25 μL of the protein buffered solution in 225 μL of 10 mM deuterated ammonium acetate to produce a final concentration of 1 mg/ml. The final relative amounts of labile deuterium atoms in this mixture is 90%. Initiation of H/D exchange kinetics in this study was done by diluting a concentrated solution of protonated proteins in D_2O, as an alternative for dispersing lyophilized protein in D_2O is based on previous reports that most consistent results and less broad and multiple peaks were achieved with the first technique (Johnson and Walsh, 1994; Szewczuk et al., 2001). This solution was immediately infused into the ionization chamber of the MS from a syringe pump (Model 22, Harvard Apparatus, South Natick, MA, USA) at a rate of 2 μL/min. The enclosed ionization chamber of the MS was kept at room temperature (21 ± 2 °C) and atmospheric pressure. It was constantly flushed with ultra high-purity nitrogen to prevent the back exchange of deuterated atoms by hydrogens in the laboratory air. Ultra high-purity air was used as the nebulizing gas. Under these conditions, exchange of hydrogen by deuterium occurred only in the solution phase, and back exchange of deuterium by hydrogen was negligible during ESI-MS analysis in the gas phase (Wagner et al., 1994). Collection of the initial MS data was started as quickly as possible, usually within 10-20 s of dilution. A small *m/z* range (70 unit) containing a selected charge state of the molecular ions, normally the ion with maximum intensity, was scanned repetitively using a step size of 0.2 Da and dwell time of 10 ms in the first 45 minutes when exchange is rapid. Fewer time points are necessary to define the kinetic curve at later times (60, 2880, 5760, 8640, 11520, and 14400 minutes) (Robinson, 1996). The molecular mass at each time point was calculated from the measured *m/z* (mass-to-charge) ratios and the predetermined charge state. At each time point (*t*), the average number of unexchangeable hydrogens was determined by

subtracting the molecular mass at time *t* from the 90% deuterated molecular mass (Szewczuk et al., 2001). The average numbers of unexchanged hydrogens were plotted against time to give the exchange curves. All H/D exchange experiments were done in duplicate.

7.3.4 Data Analysis

The apo-form (Ca^{2+}-free) of α-Lac contains 123 amino acid residues and a total of 225 hydrogens (Table 7.1), of which 120 are on the amide backbone, 102 are on the side chains, and 3 on the two termini; the total numbers of hydrogens in the holo-form (Ca^{2+} -bound) of α-Lac is 223, due to Ca^{2+} binding to the protein molecule. In our experiment, H/D exchange was initiated in a solution of 90% D_2O, therefore, total labile exchangeable hydrogens for 90% deuterated apo- and holo-α-Lac are 203 and 201, respectively (Table 7.1). β-Lg (variant A and B) contains 162 amino acid residues and a total of 279 (278 for variant B) hydrogens (Table 7.2), of which 153 are on the amide backbone, 123 (122 for variant B) are on the side chains, and 3 on the two termini. The total numbers of labile exchangeable hydrogens for 90% deuterated β-Lg (A) and β-Lg (B) are 251 and 250, respectively (Table 7.2). Free hexose sugar has 5 exchangeable hydrogen. The first hexose sugar that binds to protein molecule will have only 4 exchangeable hydrogens. Additional hexose sugar binds to the first hexose sugar will have 4 exchangeable hydrogens; the first hexose sugar will have 3. Attachment of sugar residues to protein molecule will result in removal of one hydrogen atom from protein molecule. Data (number of unexchanged hydrogens versus the time in minutes after addition of D_2O) were fit to the sum of two exponentials from the following expression (Smith et al., 1997; Szewczuk et al., 2001):

$$Y = A.\exp^{(-k_1 t)} + B.\exp^{(-k_2 t)}$$

Where Y is the number of unexchanged hydrogens remaining; t is the time in minutes after addition of D_2O; A and B are numbers of intermediate and slow exchanged hydrogens class with exchange rate constants of k_1 and k_2, respectively. The number of hydrogens undergoing exchange at very fast rate (C) was calculated by subtracting total exchangeable hydrogens (90%) from A+B.

7.4 RESULTS AND DISCUSSIONS

7.4.1 CSD of α-Lac

Figure 7.1 A to D compares the mass spectra of α-Lac dispersed in solutions at pH 3, 5.5, 6.8 and 8 respectively, and stored for 0, 2, 4, 8, 10. Initially, the spectrum of α-Lac at pH 3 exhibited 4 peaks (+9, +8, +7, +6) with a maximum charge state of +9, 8 charges less than the total number of positive charges based on the number of basic residues (i.e., arginine, lysine, histidine and N-terminal with a primary amine) (Figure 7.1 A). The most intense peak was observed for species +8. Storage for 2 days resulted in the emergence of +10 species with relatively high intensity and substantial increase in the intensity of the +9 species. This indicates increased solvent accessibility to buried basic amino acid residues due to less stable conformation of α-Lac. At day 8 the +11 species was observed. Further storage to day 10 resulted in increase in the relative intensity of charge state +11 indicating decrease in α-Lac conformational stability. At pH 5, the spectrum of α-Lac initially exhibited 3 peaks with a maximum charge state of +8, 9 charges less than the total number of basic residues (Figure 7.1 B). The most

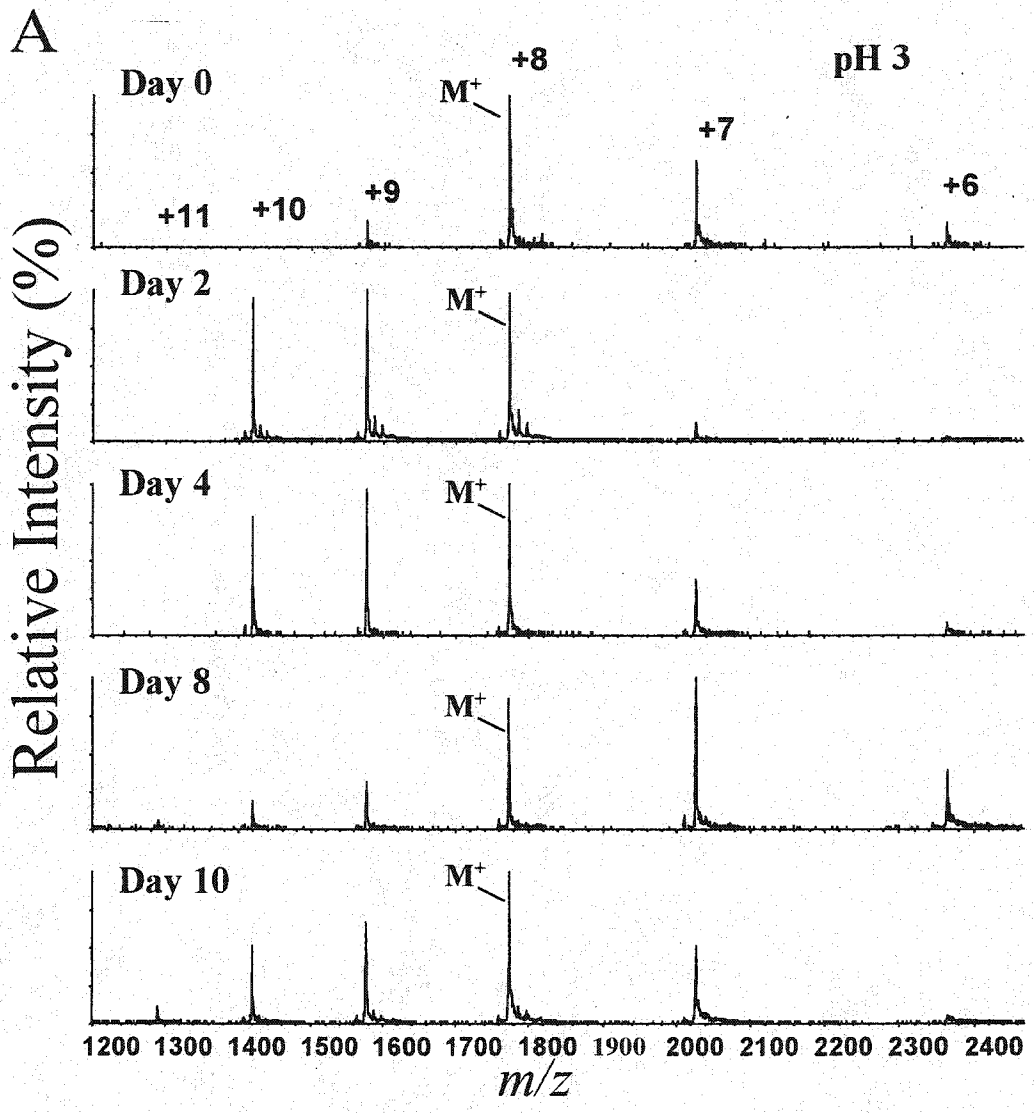
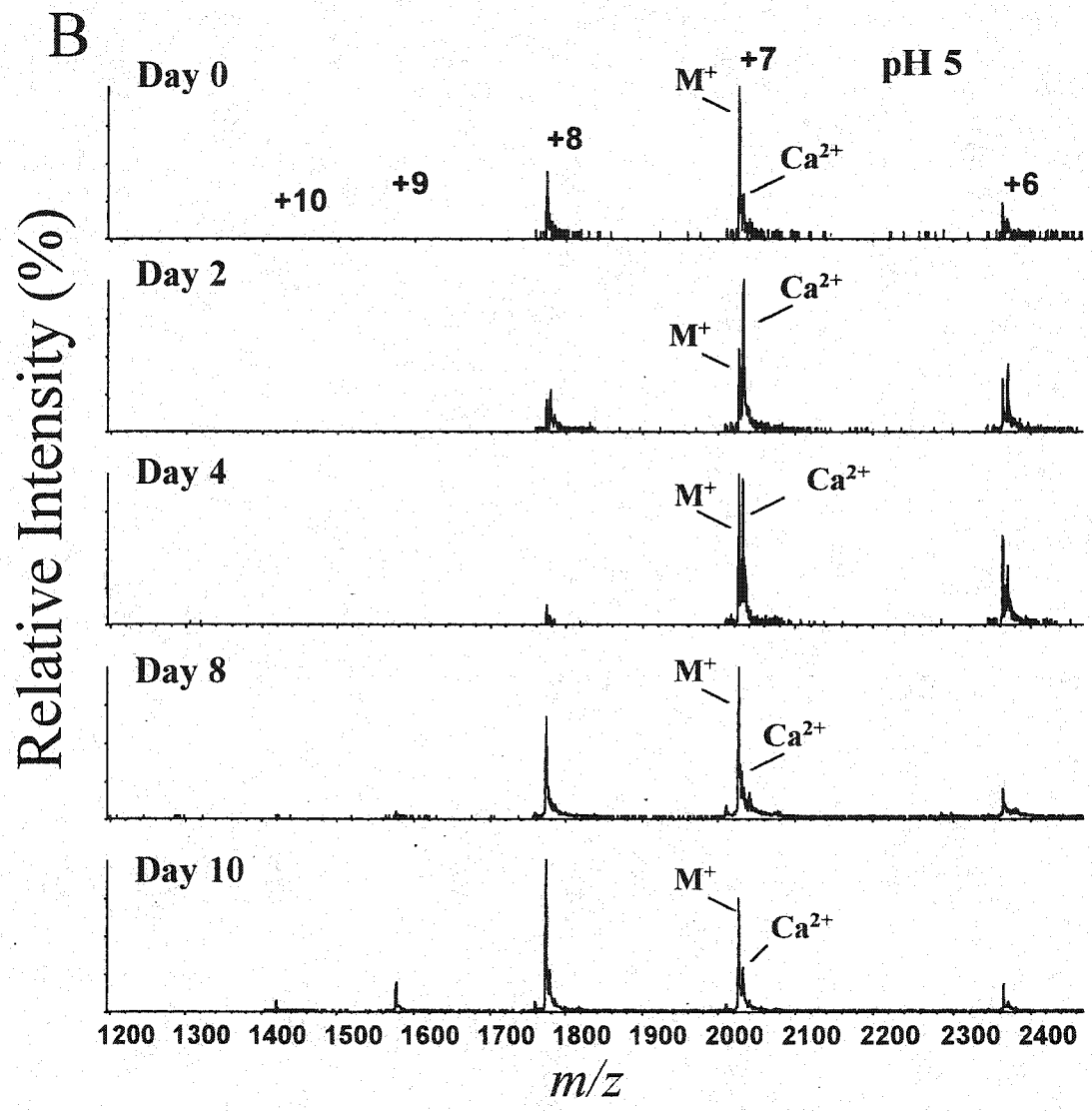


Figure 7.1: ESI mass spectra of α-lactoglobulin at pH 3 (A), 5.5 (B), 6.8 (C) and 8 (D) and stored for 0, 2, 4, 8, 10 days.



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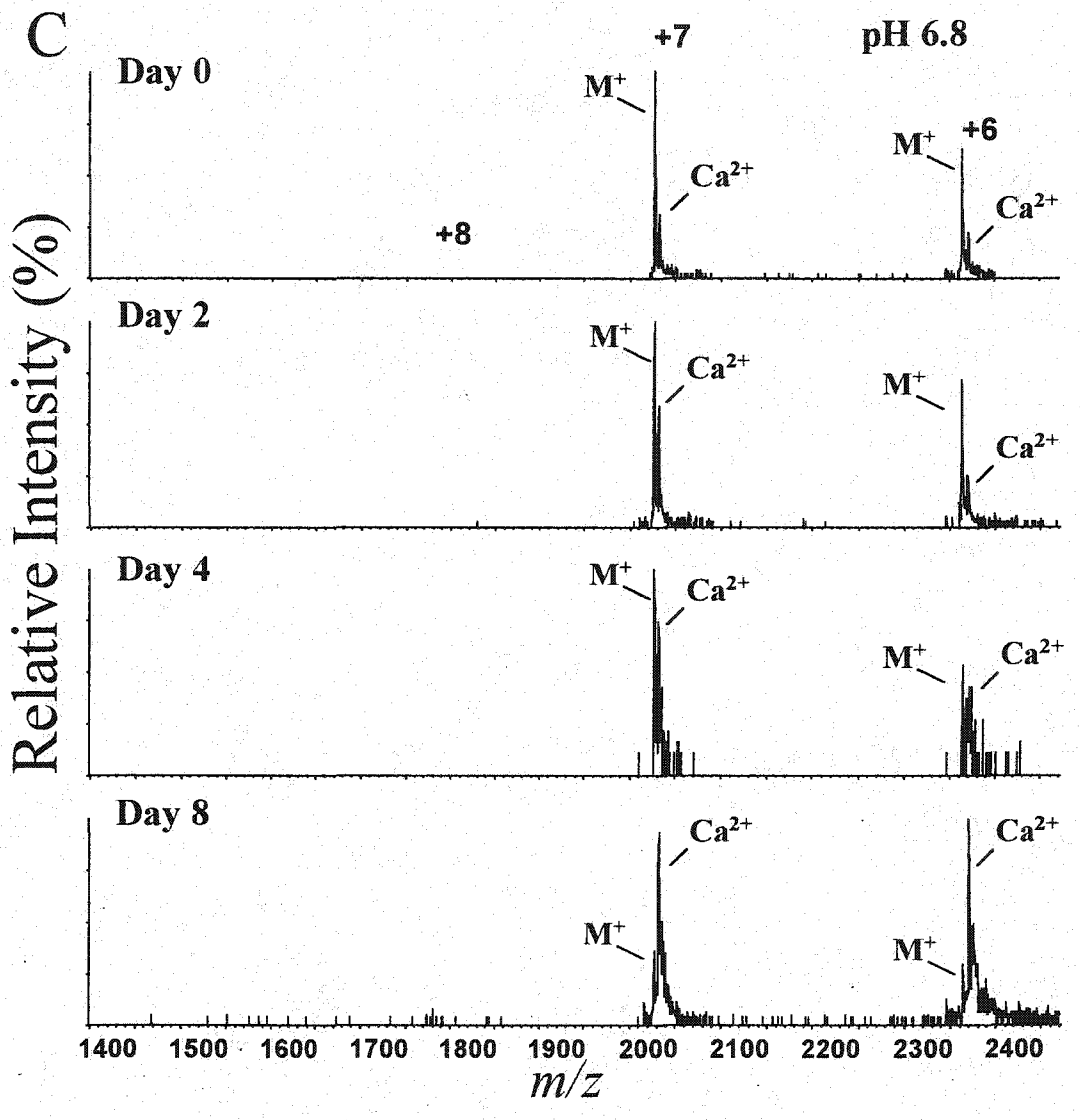

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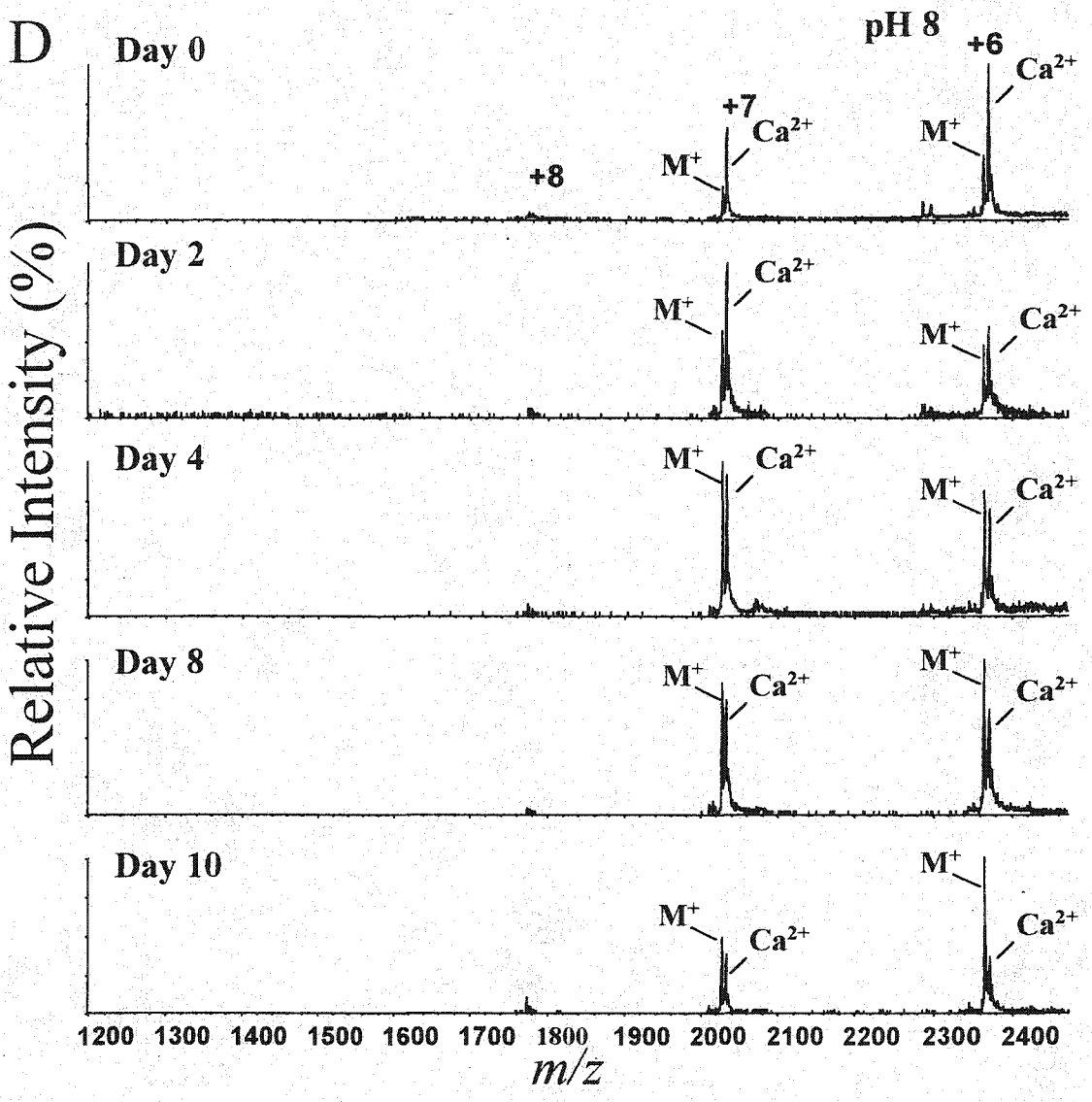


Figure 7.1: (continued).

intense peak was observed for species +7. The molecular mass (M_r) obtained for the molecular ion (M^+) was 14,178 Da and for the metal adduct was 14,216 Da representing the apo-form (Ca^{2+}-free) and holo-form (Ca^{2+} -bound) of α-Lac, respectively. It is known that α-Lac is a Ca^{2+} binding protein and the binding of Ca^{2+} stabilizes the structure of native α-Lac (Hiraoka et al., 1980; Kronman, 1989). Storage for 2 and 4 days resulted in a decrease in the relative signal intensity of α-Lac M^+ (apo-form) and in an increase in the relative signal intensity of metal adduct (holo-form) (Figure 7.1 B). The predominant of the relative M_r of 14,216 Da and the narrow CSD demonstrates that the α-Lac retains its native structure under these conditions for the first 4 days of storage (Robinson et al., 1994; Chung et al., 1997). Extending the storage period to 8 and 10 days resulted in substantial changes in the mass spectrum of α-Lac which involves the emergence of species carrying +9 and +10 charges, shifting of α-Lac charge state toward higher values (i.e., lower m/z values) and a decrease in the relative intensity of metal adduct (holo-form) indicating changes in the α-Lac conformation. The spectrum of α-Lac at pH 6.8 during 8 days of storage exhibited a very narrow CSD in which each spectrum contains only 2 ion peaks, charge states + 6 and +7 (Figure 7.1 C). The relative signal intensity of Ca^{2+} adduct (holo-form) increased with a decrease in the relative signal intensity of M^+ (apo-form) indicating stable conformation of α-Lac during storage at pH 6.8. On the other hand, the quality of ESI mass spectrums obtained at this pH is not optimal since basic groups are not protonated in solution and the use of ammonium acetate causes a shift in charge state of a protein toward lower values (i.e., higher m/z values) (Wang and Cole, 1997). Initially, the spectrum of α-Lac at pH 8 shows a maximum charge state of +8; this CSD was essentially unchanged during

storage (Figure 7.1 D). Unlike the results obtained at pH 6.8, the relative signal intensity of Ca^{2+} adduct (holo-form) decreased with an increase in the relative signal intensity of M^{+} (apo-form) indicating unstable conformation of α-Lac during storage at pH 8. The results revealed that the structure of α-Lac was initially unstable at pH 3 and further storage resulted in substantial loss of stable conformation while the structure of α-Lac initially at pH 5.5 retained its native conformation and further storage resulted in conformation changes. The structure of α-Lac at pH 6.8 was stable and storage at pH 8 resulted in unstable conformation. The above results show the capability of CSD by ESI mass spectrometry to study α-Lac conformational stability as a function of pH and storage time; however the information were only qualitative of relatively low resolution at basic pH solutions (Dobo and Kaltashov, 2001).

7.4.2 CSD of β-Lg

Figure 7.2 A to D compares the mass spectra of β-Lg dispersed in solutions with at pH of 3, 5.5, 6.8 and 8 respectively, and stored for 0, 2, 4, 8, 10. Initially, the spectrum of β-Lg (variant B and A) at pH 3 stored for 12 days exhibited 3 peaks with a maximum charge state of +10, 11 charges less than the total number of basic amino acid residues (Figure 7.2 A); the most intense peak was observed for species +9. This CSD was unchanged during storage for both variants indicating stable conformation of β-Lg at pH 3. These MS findings are consistent with the results from DSC and FTIR which showed that β-Lg has maximum thermal stability at pH 3 (Boye et al., 1996b). At pH 5, the spectrum of β-Lg exhibited 4 peaks with a maximum charge state of +11, 10 charges less than the total number of basic residues (Figure 7.2 B). The most intense peak was observed for species +9. Storage for 2, 4 and 6 days resulted in a gradual

decrease in the relative signal intensity of peaks with charge +9 and +8 and gradual increase in the relative intensity of peaks with higher charge state (i.e., +10 and +11). At day 10, the spectrum of β-Lg shows the emergence of the +12 species. The emergence of new charged species with the shift in an intense peak toward higher CSD, indicate that β-Lg basic amino acid residues are more accessible to solvent under these conditions. The spectrum of β-Lg at pH 6.8 shows only 2 ion peaks with charge states of +8 and 9+ at day 0 (Figure 7.2 C). At day 2, new species carrying +10 was observed. After day 4, the relative intensity of charge +9 and +10 progressively increased. At pH 8, the spectrum of β-Lg shows only 2 ion peaks with charge states of +8 and 9+; this CSD was unchanged during storage (Figure 7.2 D). The relatively minor changes observed in the mass spectra of β-Lg at pH 6.8 and 8 can be attributed to the inability of ESI to produce multiply charged protonated molecule of native β-Lg in basic pH solutions (Figure 7.2 C and D) resulting in a shift of protein CSD toward lower values, in the higher m/z axis. These changes are beyond the capabilities of quadrupole analyzers. The results revealed that initially, the conformation of β-Lg at pH 3 was stable and has less stable conformation at pH 5.5. Storage at pH 6.8 resulted in conformational changes of β-Lg with unchanged CSD at pH 8. These results show the capability of CSD by ESI mass spectrometry to study β-Lg conformational stability at acidic pH but with less effectiveness at basic pH.

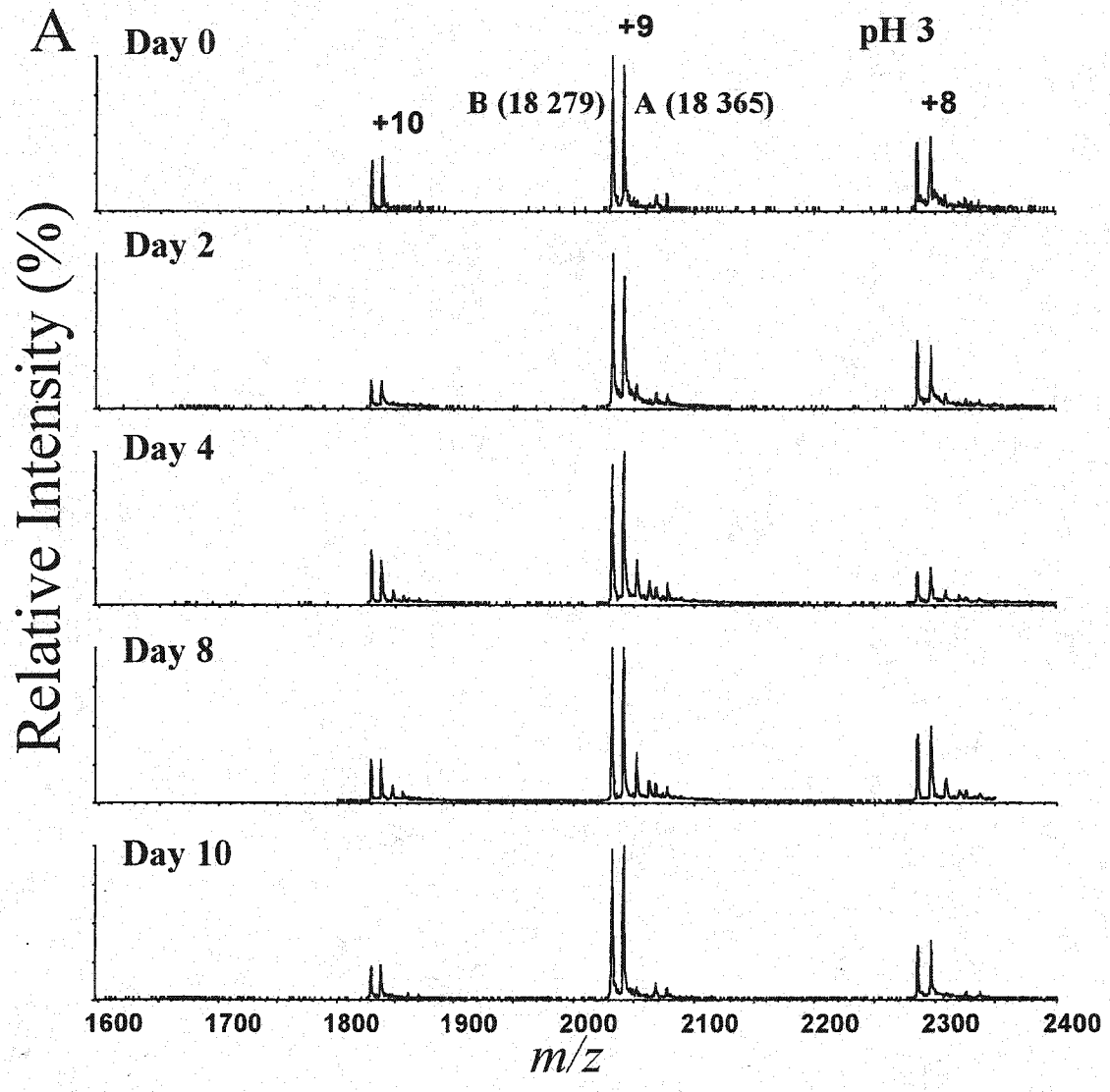
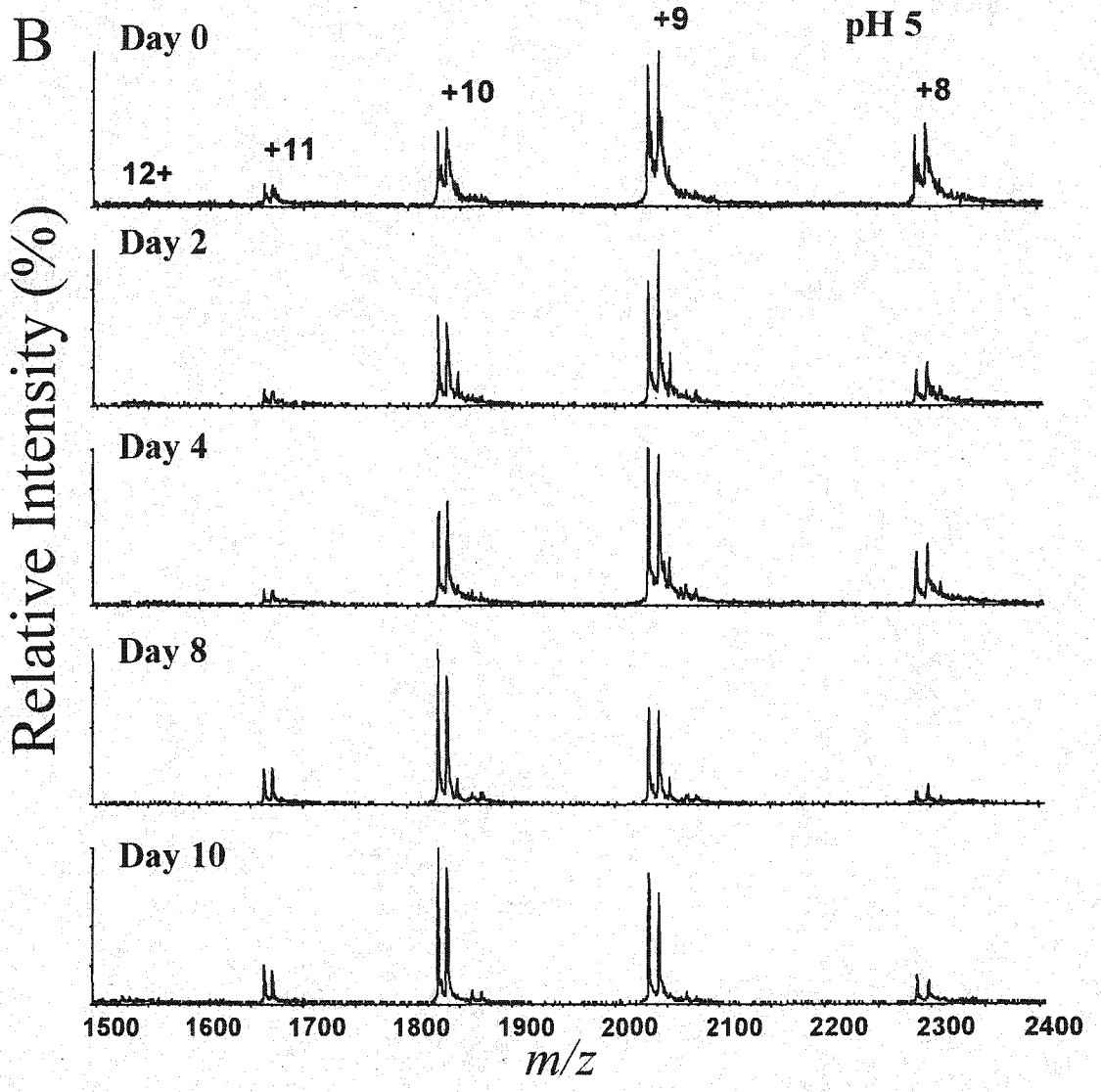


Figure 7.2: ESI mass spectra of β-lactoglobulin at pH 3 (A), 5.5 (B), 6.8 (C) and 8 (D) and stored for 0, 2, 4, 8, 10 days.



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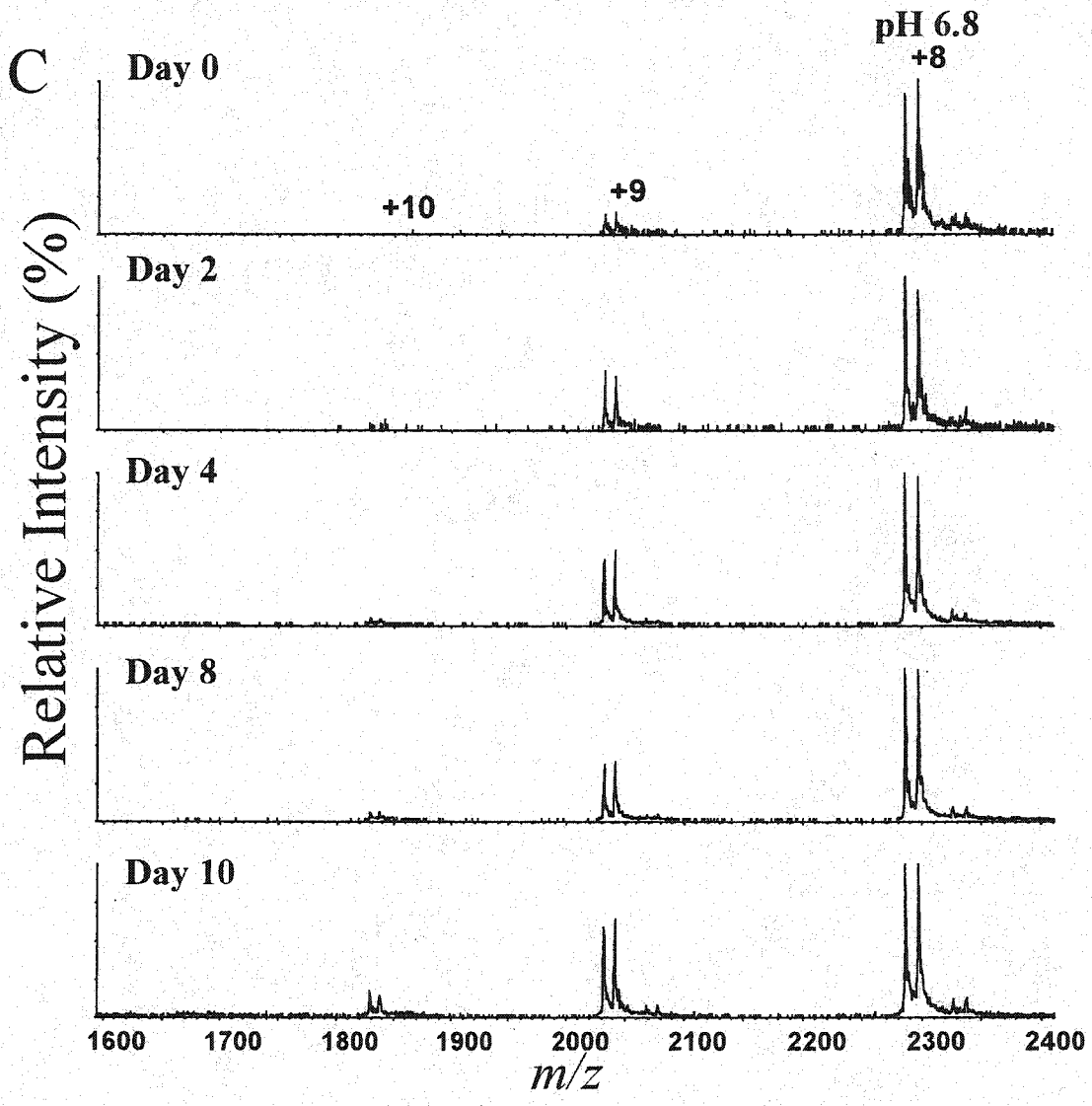

Figure 7.2: (continued).

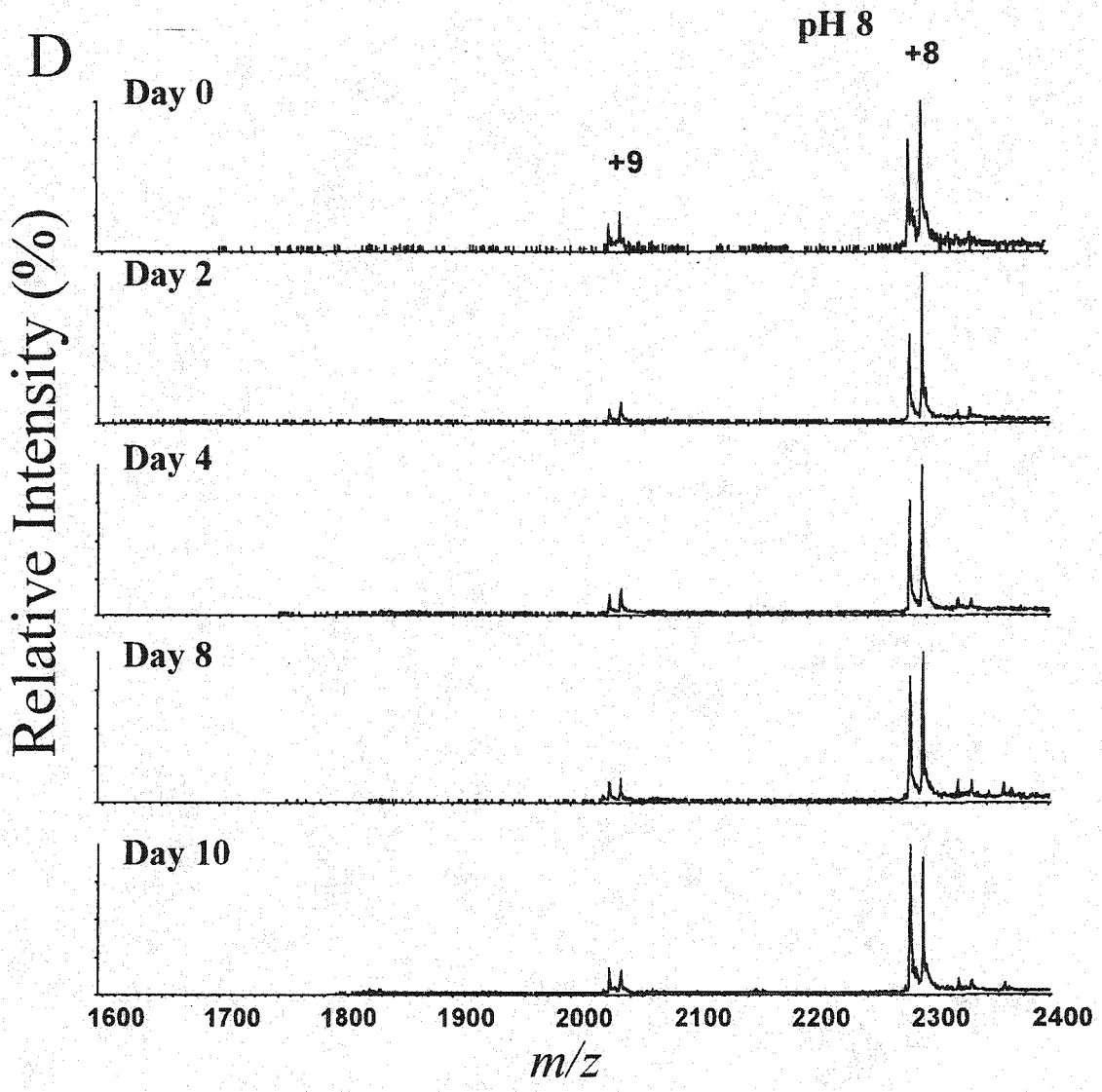

Figure 7.2: (continued).

7.4.3 H/D Exchange of α-Lac

Figure 7.3 shows a representative spectrum of apo- and holo-α-Lac standard incubated in deuterated buffer (pH 6.8) for different time periods. Similar spectra were generated for all α-Lac isolated fractions to obtain the number of unexchanged hydrogens as a function of time (min) after addition of D_2O. Table 7.1 shows the number of hydrogens exchanged in intermediate (A) and slow (B) category, which exchanged with rate constants of k_1 and k_2, respectively. The number of hydrogens in the very fast (C); (exchanged before MS measurements), category was calculated as the total number of exchangeable hydrogens (90%) minus A+B. The most reasonable interpretation of reduced H/D exchange rates in folded proteins are in terms of reduced accessibility of solvent to amide hydrogens (solvent exclusion; Woodward et al., 1982) and/ or increase in amide hydrogen bond stability (local unfolding; Englander and Kallenbach, 1984). It has been shown that, exchange of hydrogen for exposed side chains and unstructured backbone amides can be essentially complete within minutes in D_2O (pH>5) at room temperature (Englander and Mayne, 1992). Therefore, the unexchanged hydrogens in α-Lac remaining after 5 min are expected to be those involved in hydrogen bonding in secondary structure or those on side chain buried inside the hydrophobic core (Table 7.1). Figure 7.4 shows the H/D exchange rates for apo- and holo-α-Lac standard as a function of time (min) at pH 6.8. After 60 s, the total number of unexchanged hydrogens in apo-α-Lac standard were 54 (or 26.6% unexchanged hydrogens) compared to 72 in holo-α-Lac standard (or 35.8% unexchanged hydrogens). The fast exchanged hydrogens are assumed to be on the exposed side chains and on unstructured backbone amides, while the remaining

exchangeable hydrogens are assumed to be either buried, or involved in intramolecular hydrogen bonding (Smith et al., 1997). The difference in extents of unexchanged hydrogens between apo- and holo-α-Lac standard remained constant for the first 20 minutes (difference of 9.7% unexchanged hydrogens at 5 min and 9% at 20 min, Figure 7.4 and Table 7.1). At 45 and 60 min, the percentage of unexchanged hydrogens was 5.8% and 4.0%, respectively. After which the difference between the two curves remained about the same for period of 10 days (data not shown). Table 7.1 shows that (i) 22% unexchanged hydrogens of apo-α-Lac standard belong to the intermediate (A) exchanging category corresponding to 37% of the backbone amide hydrogens, (ii) 7% belong to the slow (B) exchanging category corresponding to 11% of the backbone amide hydrogens and (iii) 71% belongs to the very fast (C) exchanging category. The results for holo-α-Lac, which contains Ca^{2+}, show 62% of the very fast (C), 17% of the intermediate (A), and 21% of the slow (B) exchangeable hydrogens. These results demonstrate the effect of Ca^{2+} on the stability of the structure of holo-α-Lac. These results are consistent with H/D-MS data of Chung et al. (1997) who reported that in the absence of Ca^{2+}, protection against hydrogen exchange is decreased. The accelerating H/D exchange rates in apo-α-Lac compared to holo-α-Lac can suggest that the absence of Ca^{2+} can causes exposure of many amide groups to the solvent or possibly the breakage of many hydrogen bonds. Recently, X- ray (Chrysina et al., 2000) and NMR (Wijesinha et al., 2001) studies have shown that removal of Ca^{2+} from α-Lac effect mainly the Ca^{2+}-binding region of the protein. The NMR study showed that the amide hydrogens in holo-α-Lac are more protected against exchange compared to apo-α-Lac;

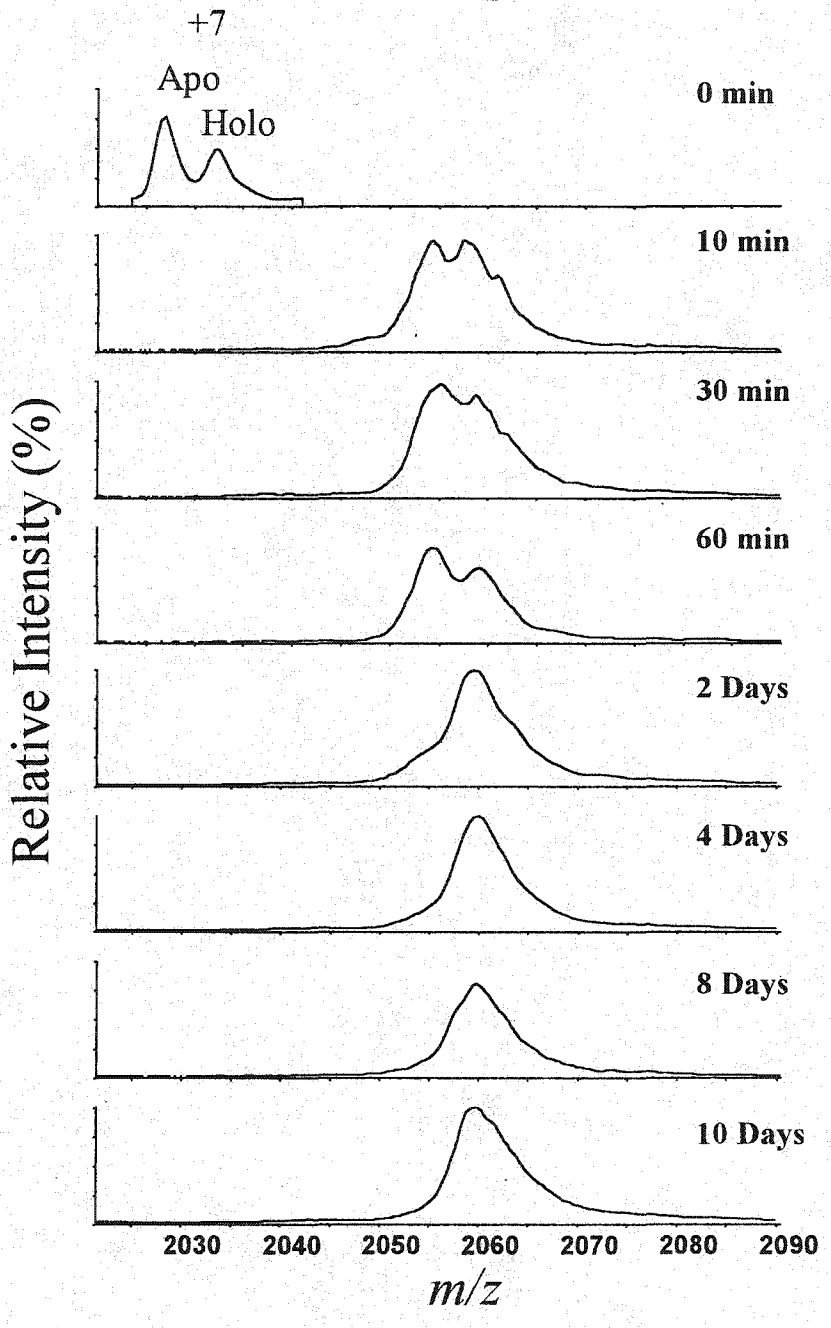

Figure 7.3: ESI mass spectrum of apo- and holo-α-lactoglobulin standard incubated in deuterated buffer (pH 6.8) for different time periods.

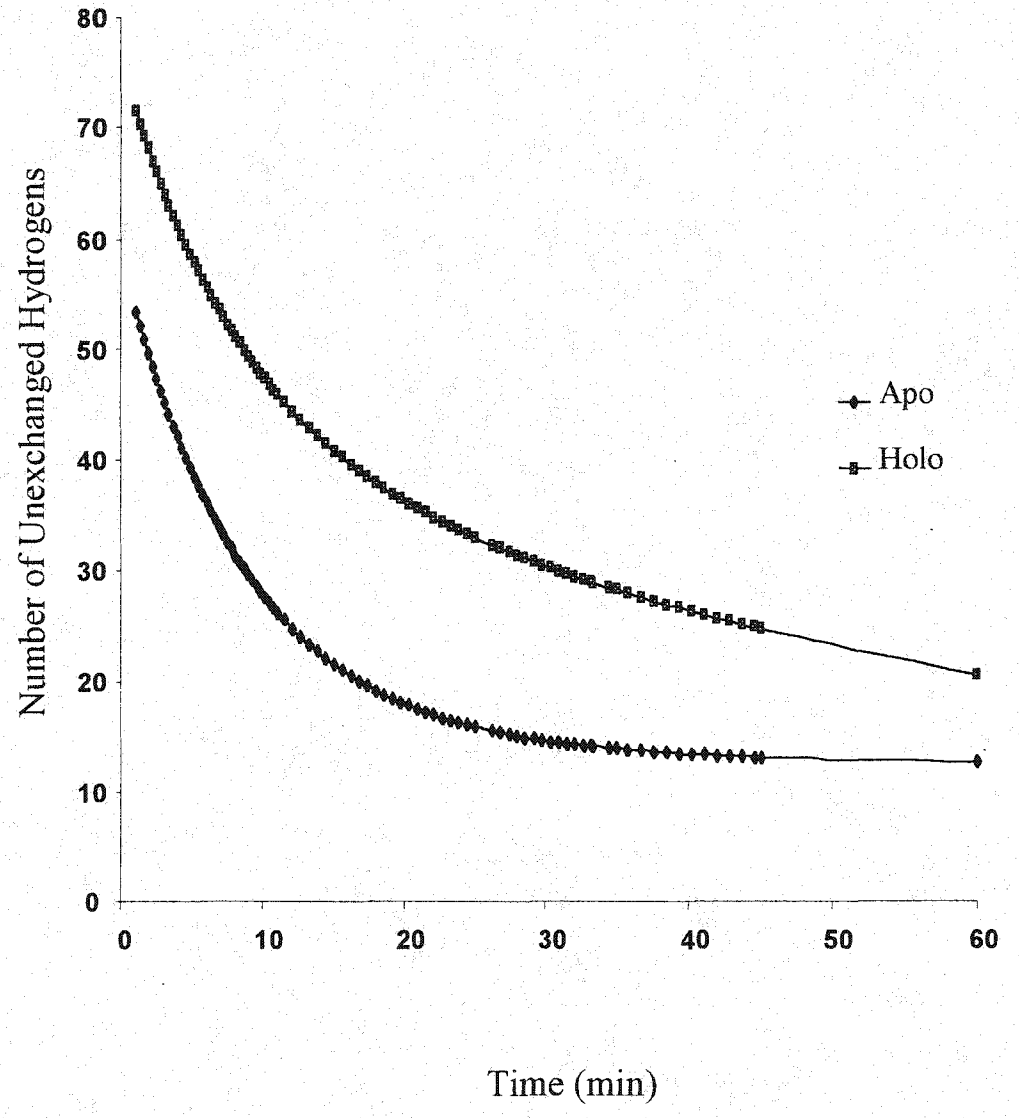

Figure 7.4: H/D exchange rates of apo- and holo-α-Lac standard as a function of time (min) at pH 6.8.

Table 7.1: Hydrogen/Deuterium exchange characteristics of α-lactalbumin standard and α-lactalbumin isolated from whey preparations

Origin of α-Lac	Total Hydrogens (100%)	Exchangeable Hydrogens (90%)	Unexchanged Hydrogens After			k_1 (min^{-1})	k_2 (min^{-1})	Intermediate Exchanged Hydrogens (A)	(A) (%)	Slow Exchanged Hydrogens (B)	(B) (%)	Very Fast Exchanged Hydrogens (C)	(C) (%)	(A) Amide (%)	(B) Amide (%)	Total Amide (%)
			5 min	20 min	45 min											
Apo-STD	225	203	38	18	13	0.113	0.001	45	22	14	7	144	71	37	11	49
Holo-STD	223	201	58	36	25	0.122	0.012	33	17	42	21	125	62	28	35	63
WPI-NaC	223	201	77	38	23	0.095	0.017	65	32	40	20	96	48	54	33	87
WPI-SHMP	223	201	77	49	42	0.138	0.003	59	29	49	24	93	46	49	41	90
LW-NaC	223	201	63	45	33	0.072	0.008	28	14	45	23	128	64	23	38	61
LW-SHMP	223	201	72	47	25	0.370	0.026	14	7	80	40	107	53	12	66	78
WPC-NaC+4Hex	235	212	74	44	24	0.054	0.014	55	26	35	16	122	58	46	29	75
WPC-NaC+5Hex	238	215	82	55	33	0.042	0.012	53	25	41	19	120	56	44	35	79
WPC-NaC+6Hex	242	217	94	68	45	0.038	0.005	65	30	42	19	110	51	54	35	89
WPC-SHMP+3Hex	232	209	68	40	18	0.037	0.033	22	11	58	28	128	61	19	49	67
WPC-SHMP+4Hex	235	212	73	45	25	0.051	0.012	53	25	34	16	125	59	45	28	73
WPC-SHMP+5Hex	238	215	83	57	36	0.041	0.007	58	27	37	17	120	56	49	31	79

these most highly protected amide groups are clustered around the Ca^{2+}-binding region (Ca^{2+}-binding loop and the C-helix) and residues 53 to 62, which are adjacent to this region (Wijesinha et al., 2001). This could explain the increased involvement of the total amide % in holo-α-Lac standard (63.3%) compared to those of apo-α-Lac standard (48.6%) measured in our experiment (Table 7.1).

Figure 7.5 shows ESI spectra of α-Lac fractions isolated from LW, WPI, WPC extracted with NaC or SHMP. The spectrum of α-Lac standard was similar to that of α-Lac isolated from LW. The α-Lac isolated from WPI contained 2 glycated species with 2 and 4 hexose sugar units, while α-Lac isolated from WPC contained glycated species with 2 to 10 hexose sugar units and extend of glycation has more than 10 hexose sugar units. For all α-Lac fractions isolated from LW, WPI and WPC, Table 7.1, shows the H/D exchange for only the holo-form of the α-Lac, since the relative intensity of its mass-to-charge ratio (*m/z*) peak is more abundant compared to *m/z* of apo-form. α-Lac fraction isolated from WPI extracted with NaC (α-Lac-WPI-NaC) showed 77 (or 38%) unexchanged hydrogens after 5 min which is higher than that of holo-α-Lac standard (58 or 29%). After 5 min, unexchanged hydrogens in α-Lac-WPI-NaC and holo-α-Lac standard were similar. For α-Lac-WPI-NaC, the % of very fast (C) exchanging hydrogens (48%) was less than that for holo-α-Lac standard (62%), the % of intermediate (A) exchanging hydrogens (32%) was greater than that of holo-α-Lac standard (17%) samples and the % of slow (B) exchanging hydrogens was similar. These results, along with increased involvement of the total amide % in α-Lac-WPI-NaC sample (87%) compared to those of holo-α-Lac standard (63%), indicate that α-

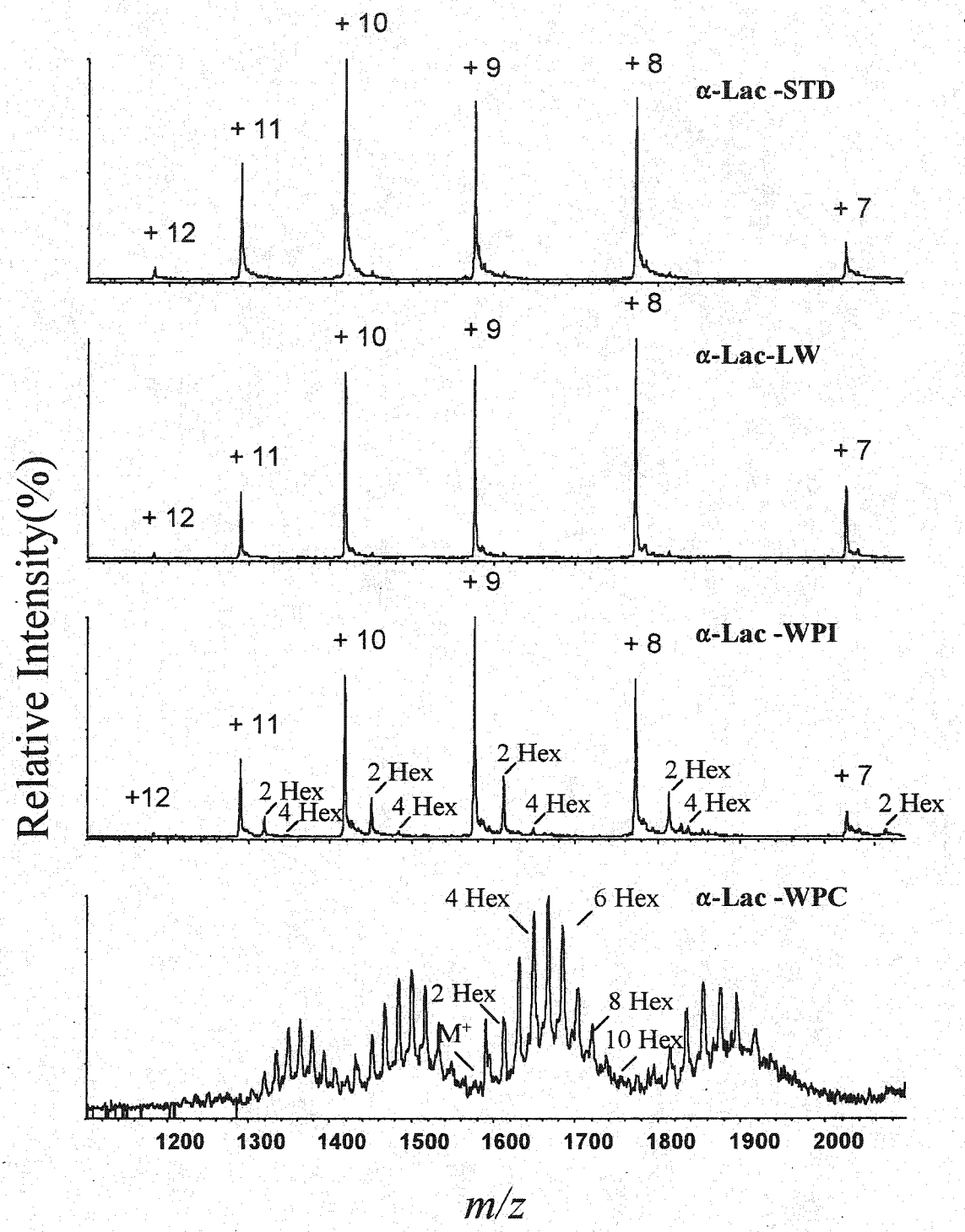

Figure 7.5: ESI mass spectrum of α-Lac fractions isolated from WPI, WPC, LW extracted with NaC or SHMP. α-Lac = α-lactalbumin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate; SHMP = sodium-hexametaphosphate.

Lac-WPI-NaC is more protected against hydrogen exchange than holo-α-Lac standard due to increase in structured hydrogen bonding in its secondary structure. Table 7.1 shows the number of unexchanged hydrogens after 5, 20 and 45 min and the rates of H/D exchange of α-Lac fraction isolated from WPI extracted with SHMP (α-Lac-WPI-SHMP). The α-Lac-WPI-SHMP sample showed higher unexchanged hydrogens than the α-Lac-WPI-NaC sample. The % of slow (B) exchanging hydrogens in the α-Lac-WPI-SHMP sample (24%) was higher than that of α-Lac-WPI-NaC sample (20%) with little decrease in the very fast (46%) and intermediate (29%) exchanging hydrogens and with an increase in total amide hydrogen % in α-Lac-WPI-SHMP samples (90%) compared to that in α-Lac-WPI-NaC (87%). These results indicate that α-Lac fractions isolated from WPI extracted with SHMP were more protected against hydrogen exchange than α-Lac fractions isolated from WPI extracted with NaC, and therefore more than holo-α-Lac standard, due to increase in structured hydrogen bonds in its secondary structure. Our DSC study (Chapter 6) showed higher T_d and ΔH for the α-Lac-WPI-SHMP that those for the α-Lac-WPI-NaC, whereas our FTIR study showed less unfolding in the helical structure of α-Lac-WPI-SHMP sample than in the α-Lac-WPI-NaC sample (Chapter 6).

Table 7.1 shows the number of unexchanged hydrogens after 5, 20 and 45 min and the rates of H/D exchange of α-Lac-LW-NaC and α-Lac-LW-SHMP. The % of slow (B) exchanging hydrogens in α-Lac-LW-SHMP sample (40%) was greater than that of α-Lac-LW-NaC sample (23%) and the % of the very fast (C) exchanging hydrogens in α-Lac-LW-SHMP sample (53%) was less than that for α-Lac-LW-NaC sample (64%) with an increase in total amide hydrogen % in α-Lac-LW-SHMP sample

(78%) compared to that in α-Lac-LW-NaC (61%). These results are similar to those obtained with α-Lac-WPI fractions, which indicate that α-Lac fractions obtained with SHMP extraction was more protected against hydrogen exchange than α-Lac fractions obtained with NaC extraction.

Table 7.1 shows that 14% of unexchanged hydrogens in α-Lac-LW-NaC belongs to the intermediate (A) exchanging category, 22.5% to the slow (B) exchanging class and 64% belong to the very fast (C) exchanging category with involvement of 61% of total number of amide backbone hydrogens. These values are comparable to those obtained for holo-α-Lac standard; however the rates of exchange (k_1 and k_2) of α-Lac LW-NaC (0.072 and 0.008 min^{-1}) was much slower, than that of holo-α-Lac standard (0.122 and 0.012 min^{-1}). Similarity, the rate of exchange in α-Lac-LW-NaC was slower than that of α-Lac-WPI-NaC (0.095 and 0.017 min^{-1}). This explains the higher number of unexchanged hydrogens after 20 and 45 min in α-Lac-LW-C (45 and 33) compared to those of holo-α-Lac standard (36 and 25) and α-Lac-WPI-C (38 and 23). The increased protection against H/D exchange in α-Lac-LW-NaC compared to those of holo-α-Lac standard or α-Lac-WPI-NaC, can be attributed to less structural modifications induced by preparation conditions of α-Lac from LW when compared to preparation conditions of α-Lac from the other sources.

Table 7.1 shows the number of unexchanged hydrogens after 5, 20 and 45 min and the rates of H/D exchange in glycated α-Lac isolated from WPC (4 to 6 hexose residues) extracted with NaC (α-Lac-WPC-NaC) and SHMP (3 to 5 hexose residues) (α-Lac-WPC-SHMP). It should be noted that based on molecular mass measurement, the discrimination between the addition of lactose unit and multiple hexose residues

cannot be achieved. The results show that the numbers of unexchanged hydrogens in α-Lac-WPC-NaC and α-Lac-WPC-SHMP increased as the number of hexose residues attached to them increased. Also, the rates of exchange (k_1 and k_2) of α-Lac-WPC-NaC and α-Lac-WPC-SHMP were generally slower, as the numbers of attached sugar moieties increased (Table 7.1). Two possible explanations for the observed decrease in the H/D exchange rate in glycated α-Lac-WPC-NaC and α-Lac-WPC-SHMP, are (i) the hexose residues cover a region of α-Lac protein surface, protecting it from solvent and delaying its exchange (this is evident from the decrease in % of very fast (C) exchange hydrogens with the increase in numbers of sugar moieties attached to the protein) and (ii) attachment of 3 or more hexose residues to α-Lac protein could result in more stable conformational structure, making local unfolding, and therefore H/D exchange slower (this is evident from the increase in the % of total number of amide backbone hydrogens involved in α-Lac with the increase in numbers of attached sugar moieties). Moreover, both explanations could be contributing to some extent to cause the observed change. The number of unexchanged hydrogens after 5, 20 and 45 min and the rates of H/D exchange in α-Lac-WPC-NaC and α-Lac-WPC-SHMP glycated with same number of sugar residues was essentially the same. This indicates that the increased protection against hydrogen exchange in α-Lac fractions extracted with SHMP compared to α-Lac fractions extracted with NaC, was removed by glycation.

7.4.4 H/D Exchange of β-Lg

There are two major variants of β-Lg, A (18,363.3 Da) and B (18,277.2 Da) which have mass difference of 86 Da accounted for the two amino acid substitutions occurring in the B variant (Asp_{64} → Gly and Val_{118} →Ala). Figure 7.6 shows the ESI

spectrum of β-Lg standard (variant A and B) incubated in deuterated buffer (pH 6.8) for different time periods. Similar spectra were generated for β-Lg fractions isolated from LW, WPI and WPC extracted with NaC to obtain the number of unexchanged hydrogens as a function of time (min) after addition of D_2O. β-Lg fractions isolated from WPI, WPC and LW extracted with SHMP were not measured by MS, since aqueous liquid solutions of these fractions failed to pass through the 45μm syringe membrane filter (Millipore, Beford, MA), prior to MS measurement. This could probably be due to the interaction of β-Lg with SHMP resulting in the formation of large protein aggregates. Figure 7.7 shows the ESI spectrum of β-Lg (variant B and A) fractions isolated from LW, WPI and WPC extracted with NaC. The results show a similarity between spectrum of β-Lg standard and that of β-Lg fraction isolated from LW. β-Lg fraction isolated from WPI was glycated with 2 and 4 hexose sugar units, while β-Lg fraction isolated from WPC was highly glycated with the extent of glycation greater than 12 hexose units. The high level of glycation in β-Lg fraction isolated from WPC resulted in complicated mass spectra which made the H/D exchange kinetic data difficult to interpret. Therefore, H/D exchange experiments were carried only for β-Lg standard and β-Lg fractions isolated from LW and WPI extracted with NaC.

Table 7.2 shows the number of unexchanged hydrogens after 5, 20 and 45 min and the rates of H/D exchange of β-Lg (variant B and A) standard and β-Lg (variant B and A) fractions isolated from LW and WPI extracted with NaC. The results show that (i) 39% unexchanged hydrogens of β-Lg (variant B) standard belong to the intermediate (A) exchanging category, corresponding to 64% of the backbone amide hydrogens, (ii)

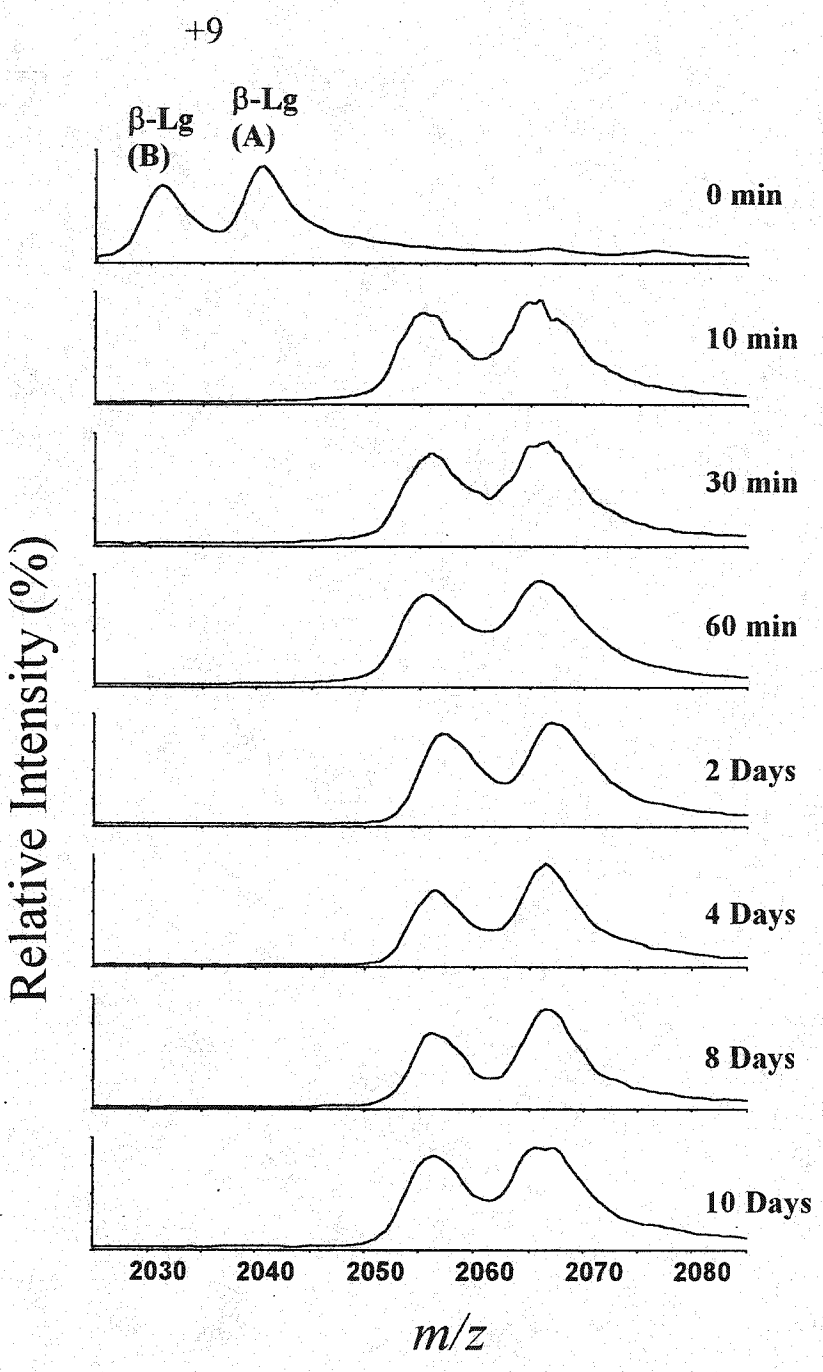

Figure 7.6: ESI mass spectrum of β-lactoglobulin (variant B and A) standard incubated in deuterated buffer (pH 6.8) for different time periods.

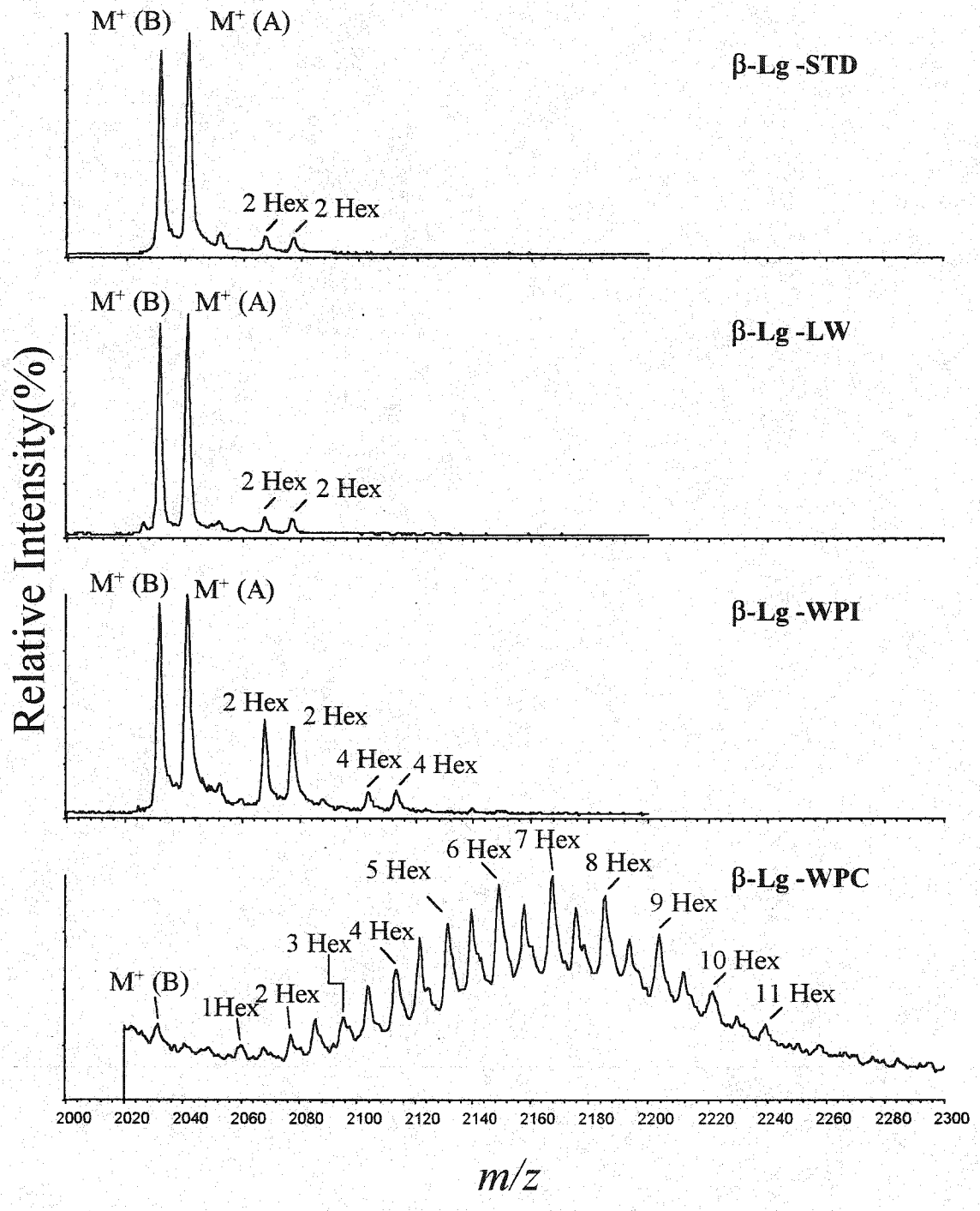

Figure 7.7: ESI mass spectrum of β-Lg (variant B and A) fractions isolated from WPI, WPC, LW extracted with NaC. β-Lg = β-lactoglobulin; WPI = whey protein isolate; WPC = whey protein concentrate; LW = liquid whey; NaC = sodium-citrate.

Table 7.2: Hydrogen/Deuterium exchange characteristics of β-lactoglobulin standard and β-lactoglobulin isolated from whey preparations

Origin of β-Lg	Total Hydrogens (100%)	Exchangeable Hydrogens (90%)	Unexchanged Hydrogens After			k_1 (min^{-1})	k_2 (min^{-1})	Intermediate Exchanged Hydrogens (A)	A (%)	Slow Exchanged Hydrogens (B)	B (%)	Very Fast Exchanged Hydrogens (C)	C (%)	(A) Amide (%)	(B) Amide (%)	Total Amide (%)
			5 min	20 min	45 min											
STD (B)	278	250	77	46	38	0.244	0.007	98	39	51	21	101	40	64	34	97
STD (B)+Na^+	277	249	78	46	39	0.241	0.007	95	38	52	21	102	41	62	34	96
STD (A)	279	251	74	43	37	0.264	0.005	108	43	47	19	95	38	71	31	102
STD (A)+Na^+	278	250	74	44	38	0.267	0.005	109	44	48	19	93	37	71	31	102
WPI (B)	278	250	79	46	40	0.238	0.005	105	42	50	20	95	38	68	33	101
WPI (B)+Na^+	277	249	79	47	40	0.243	0.005	104	42	51	20	94	38	68	33	101
WPI (B)+2Hex	284	255	75	42	35	0.211	0.006	91	36	46	18	118	46	59	30	90
WPI (A)	279	251	74	43	36	0.277	0.007	113	45	49	20	89	36	74	32	106
WPI (A)+Na^+	278	250	74	44	37	0.284	0.007	113	45	50	20	87	35	74	33	106
WPI (A)+2Hex	285	256	72	39	32	0.218	0.006	93	36	43	17	119	47	61	28	89
LW (B)	278	250	80	46	40	0.243	0.005	110	44	50	20	90	36	72	33	104
LW (B)+Na^+	277	249	80	47	42	0.248	0.004	110	44	51	20	89	36	72	33	105
LW (A)	279	251	74	43	36	0.271	0.006	114	45	48	19	89	35	74	31	106
LW (A)+Na^+	278	250	75	43	37	0.269	0.006	115	46	48	19	87	35	75	31	106

21% belong to the slow (B) exchanging category, corresponding to 34% of the backbone amide hydrogens and (iii) 40% belong to the very fast (C) exchanging category. Table 7.2 shows that (i) 43% unexchanged hydrogens of β-Lg (variant A) standard belong to the intermediate (A) exchanging category, corresponding to 71% of the backbone amide hydrogens, (ii) 47% belong to the slow (B) exchanging category, corresponding to 31% of the backbone amide hydrogens and (iii) 38% belong to the very fast (C) exchanging category. It is noteworthy that the high values of total amide % in all samples (Table 7.2) are not only originating from exchangeable bonded amide hydrogens but also from side-chain hydrogens that are assumed to be buried or involved in intramolecular hydrogen bonding. Figure 7.8 shows the H/D exchange rates for β-Lg (variant B and A) standard as a function of time (min) at pH 6.8. After 60 s, the total number of unexchanged hydrogens in β-Lg variant B and A was 140 (or 56% unexchanged hydrogens). The number of unexchanged hydrogens in variant B and A after 5-min was 77 (31% unexchanged hydrogens) and 74 (29% unexchanged hydrogens), respectively. After 20 min, the extents of unexchanged hydrogens between variant B and A of β-Lg standard was similar. The rate of exchange in the intermediate category (k_1) of β-Lg (variant A) standard (0.264 min^{-1}) was faster than that of β-Lg (variant B) standard (0.244 min^{-1}). These results indicate that β-Lg (variant B) standard is more protected against hydrogen exchange than that of β-Lg (variant A) standard. These results are consistent with results reported by various methods. DSC showed that β-Lg variant B seems to be thermally more stable than β-Lg variant A (Imafidon et al., 1991; Huang et al., 1994a; Boye et al., 1997b). Also, it has been reported that gels made with β-Lg B have a higher gelation point (Huang et al., 1994b), display a higher elasticity (Allmere et al., 1998) and the gel matrix is formed by larger aggregate

structures (Boye et al., 1997b) than those made with β-Lg A. Proteolytic susceptibility of β-Lg (variant A) was greater than that of β-Lg (variant B); indicating increased structural flexibility of β-Lg (variant A) (Huang et al., 1994a). Under pressure, FTIR (Hosseini-nia et al., 1999) and NMR (Belloque et al., 2000) studies found that the structure of β-Lg (variant A) was more sensitive and unfold faster than that of β-Lg (variant B), respectively. Belloque et al. (2000) suggest that the relative structural flexibility of β-Lg (variant A) arises from the structural difference existing between the variants, caused by the change of the residue Val_{118} in β-Lg variant A to Ala_{118} in β-Lg variant B. On the other hand, crystallographic study suggest that creation of a cavity by the loss of two methyl groups (Val_{118} →Ala) with no movement of the protein main chain, make the B variant less stable than the A variant (Qin et al., 1999).

Figure 7.9 shows the rates of H/D exchange of β-Lg (variant B and A) standard and β-Lg (variant B and A) fractions isolated from nonglycated WPI, glycated WPI (2 hexose sugar units) and LW as a function of time at pH 6.8. The results indicate that β-Lg variant A, isolated from WPI (glycated and nonglycated) and LW, has less numbers of unexchanged hydrogens after 5, 20 and 45 min and has faster rate of exchange in the intermediate category (k_1) than that of variant B; indicating more flexible structure (Table 7.2). The presences of Na^+ adduct ion in all β-Lg (variant B and A) isolated fractions did not alter their protection against hydrogen exchange. This is evident from the similar values obtained for (i) unexchanged hydrogen after 5, 20 and 45 min, (ii) rates of exchange (k_1 and k_2) and (iii) distribution of exchanged hydrogens in intermediate (A), slow (B) and very fast (C) category in each β-Lg isolated fraction. However, binding of two hexose sugar residues to β-Lg (variant B and A) isolated from

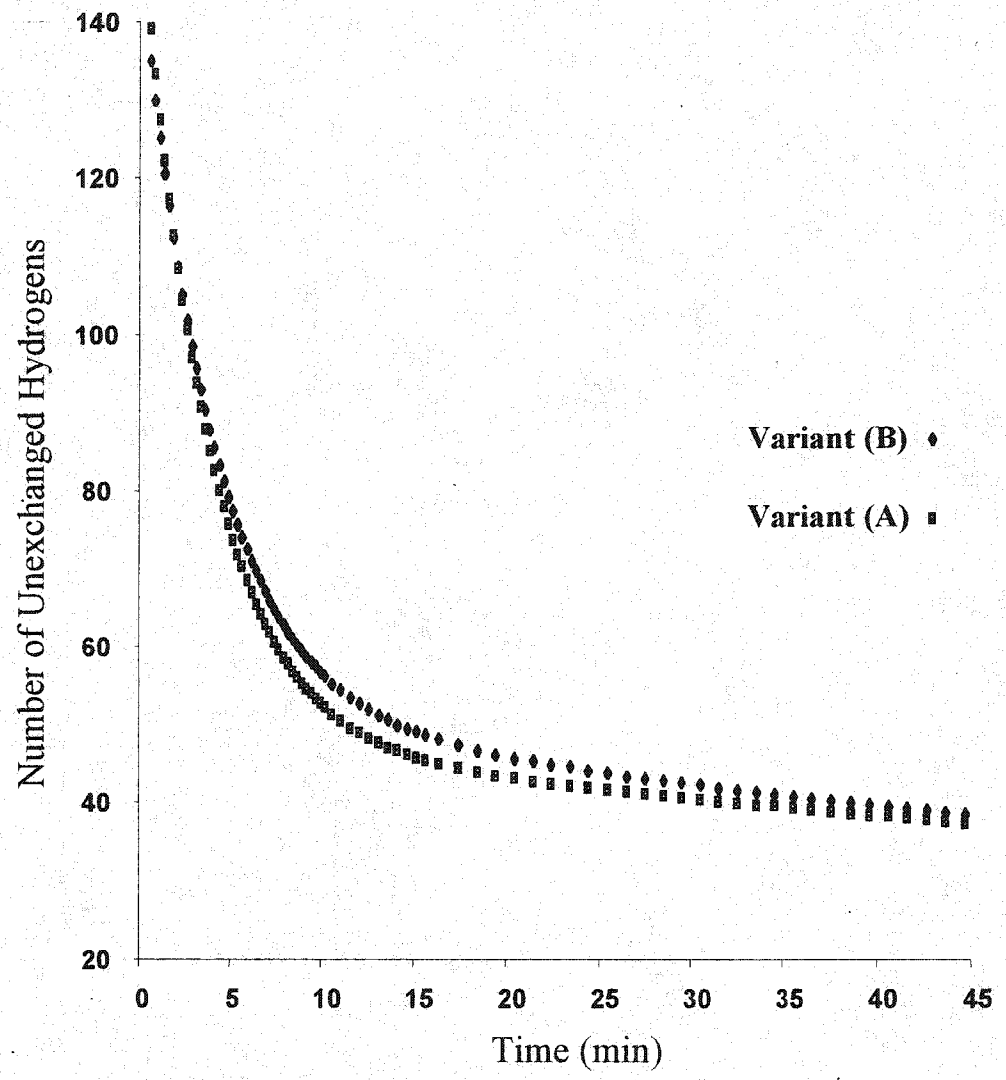

Figure 7.8: H/D exchange rates of β-Lg (variant B and A) standard as a function of time (min) at pH 6.8.

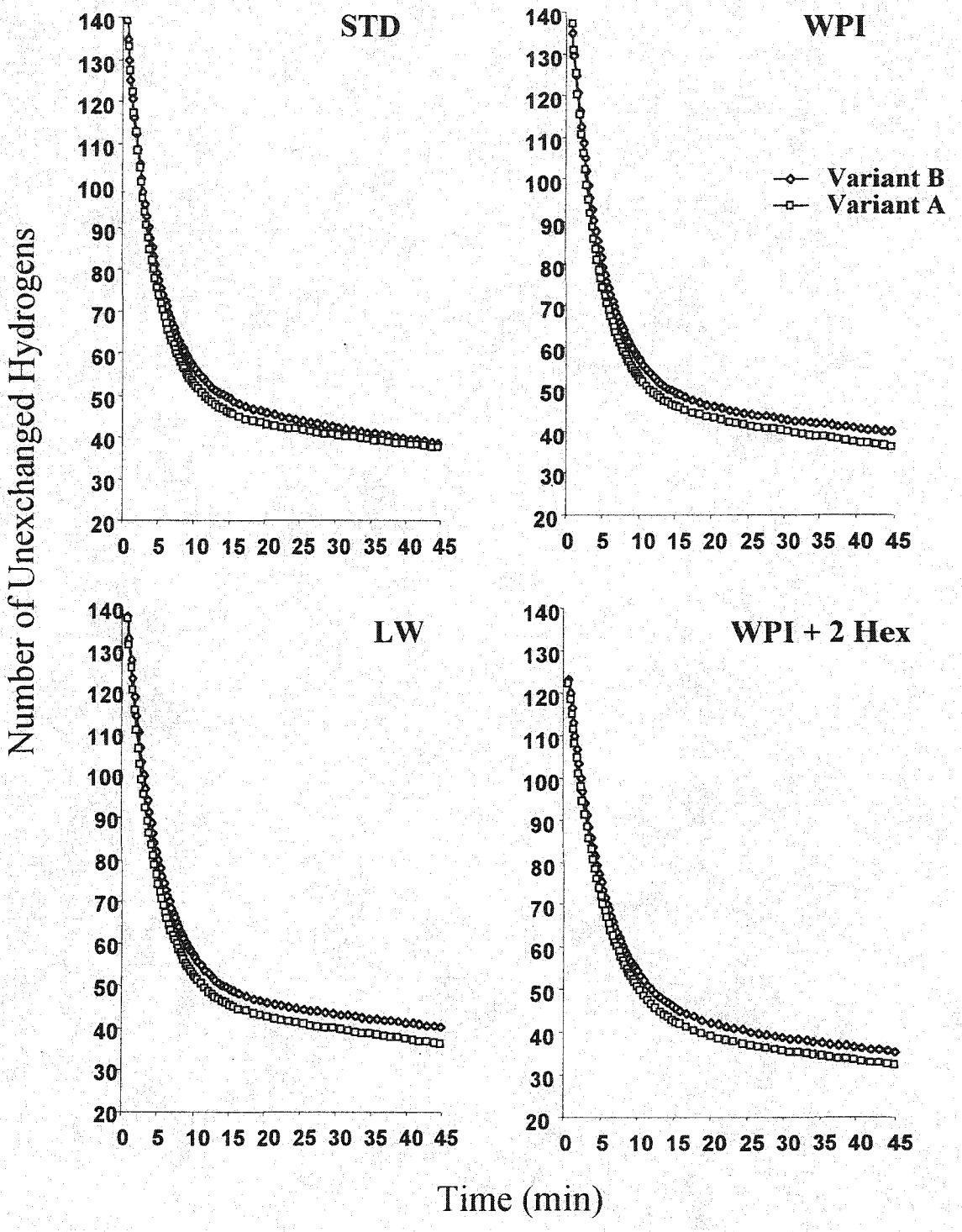

Figure 7.9: H/D exchange rates of β-Lg (variant B and A) standard and β-Lg (variant B and A) fractions isolated from nonglycated WPI, glycated WPI (2 hexose sugar units) and LW as a function of time (min) at pH 6.8.

WPI decreased their protection against hydrogen exchange. The number of unexchanged hydrogens after 5, 20 and 45 min in glycated β-Lg (variant B) (two hexose residues) was less than that for nonglycated β-Lg (variant B) sample (Table 7.2). Glycation of β-Lg variant B resulted in a decrease in the % of intermediate (A) and slow (B) (36 and 18%) exchanging hydrogen compared to nonglycated β-Lg variant B (42 and 20%), respectively and increase in the % of very fast exchanging hydrogens (C) in glycated β-Lg variant B (46%) compared to nonglycated β-Lg variant B (38%). Similarly, the binding of two hexose sugar residues to β-Lg variant A isolated from WPI resulted in less protein stable structure. One possible explanation for the observed change in exchanging behavior is that large conformational change in β-Lg occurred upon attachment of two hexose residues and this could result in accelerated H/D exchange rate. This is evident from the increase in the % of very fast exchanging hydrogens (C) in glycated β-Lg variant B (46%) compare to in nonglycated β-Lg variant B (38%); this increase was more pronounced in glycated β-Lg variant A (Table 7.2). FTIR study revealed that sugars promote the unfolding of β-Lg via multiple transition pathways leading to a transition state resisting aggregation (Boye et al., 1996b). Moreover, Morgan et al. (1999) showed that glycation led to important conformational changes of β-Lg as probed by fluorescence, susceptibility to pepsin and immunochemical characterization.

7.5 CONCLUSIONS

The results showed the capability of CSD of ESI mass spectra to investigate α-Lac and β-Lg conformational stability as a function of pH and storage time; however

the information obtained was only qualitative and was of relatively low resolution at basic pH. H/D exchange rates measured by MS, provided a sensitive measure of conformational stability of the whey proteins. The H/D exchange results demonstrate that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and conformation of β-Lg variant B was more stable than β-Lg variant A. Kinetics of H/D exchange indicated that α-Lac and β-Lg fractions isolated from whey protein preparations, using chelating agents, have the same or improved conformational stabilities compared to that of α-Lac and β-Lg standard. Generally, α-Lac and β-Lg fractions extracted with SHMP were more stable than those extracted with NaC. The covalent binding of 3 or more hexose residues to α-Lac enhanced its conformational stability but covalent binding of two hexose residues to β-Lg resulted in less stable conformation.

GENERAL CONCLUSIONS

Selective fractionation of whey major proteins may allow exploitation of their different functional, nutritional and therapeutic properties and may result in better understanding of the close structure-function relationship of these proteins which is essential to utilize whey protein products completely in food system. In this study the structural and thermal properties of α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) fractions isolated from different whey protein sources were investigated.

The results obtained showed that the use of high salt concentration and chelating agents at low pH was effective in isolating β-Lg and α-Lac, with relatively high purity and yield, from different whey protein sources. Results showed that the use of NaC and SHMP were more effective than other chelating agents. The use of NaC as a chelating agent resulted in high yield % of β-Lg isolated fractions while the use of SHMP resulted in high yield % of α-Lac isolated fractions and solubilization of precipitated α-Lac with $CaCl_2$ was more selective without pH adjustment.

Comparison of the β-Lg and α-Lac isolated fractions from different whey protein sources showed that all β-Lg isolated fractions exhibits an increase thermal stability over β-Lg standard and difference in thermal properties depend in protein source (WPI, WPC and LW) but not by chelating agent type (NaC or SHMP). Results demonstrated a stabilizing effect of SHMP in β-Lg isolated fractions over NaC and both may have the ability of inhibiting aggregate formation upon heating. All α-Lac isolated fractions showed mainly the presence of a single thermal transition (Ca^{2+} -bound form, holo-α-Lac) with reversibility percent greater than α-Lac standard, suggesting faster refolding rates. Denaturation of apo-α-Lac was related the gradual decrease in the α-

helix band and accompanied by the gain in intensity of 1653 and 1641 cm^{-1} bands while denaturation of holo-α-Lac was associated by breakdown of β-sheet structure and increase in turns and unordered structures. Results illustrated that holo-α-Lac has lower relative intensity values of 3_{10}-helix and α-helix than apo-α-Lac indicating slower rates of hydrogen/deuterium (H/D) exchange in the undeuterated 3_{10}-helix (H) component due to increased structural stability.

Storage of α-Lac at pH 3 resulted in substantial changes in its charge state distribution (CSD), involving the emergence of new ion species and shifting of its charge state toward higher values indicating less stable conformation. Spectra of α-Lac at pH 5 for 4 days showed stabile conformation, however, extending the storage period resulted in substantial changes in its CSD and a decrease in the relative intensity of metal adduct (Ca^{2+}) indicating less stabile conformation. During storage, β-Lg showed stable CSD at pH 3 with substantial changes at pH 5 and narrow changes at pH 6.8 and 8. The changes in CSD were only qualitative and were of relatively low resolution at basic pH. H/D exchange rates measured by electrospray ionization mass spectrometry (ESI-MS), provided a sensitive measure of conformational stability of whey proteins. The H/D exchange results demonstrate that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and conformation of β-Lg variant B was more stable than β-Lg variant A. Kinetics of H/D exchange indicated that α-Lac and β-Lg fractions isolated from different whey protein sources have the same or improved conformational stabilities compared to that of α-Lac and β-Lg standard.

REFERENCES

Acharya, K. R.; Stuart, D. I.; Walker, N. P. C.; Lewis, M.; Phillips, D. C. Refined structure of baboon α-lactalbumin at 1.7 Å resolution. Comparison with c-type lysozyme. *J. Mol. Biol.* **1989**, *208*, 99-127.

Adamson, N. J.; Reynolds, E. C. Relationship between degree of casein hydrolysis and phosphopeptide release. *J. Dairy Res.* **1997**, *64*: 505-514.

Aleksandrov, M .L.; Gall, L. N.; Krasnov, N. V.; Nikolaev, V. I.; Pavlenko, V. A.; Shkurov, V. A.; Baram, G. I.; Grachv, M.A.; Knorre, V. D.; Kusner, Y. S. Direct coupling of microcolumn liquid chromotogrophy and a mass spectometer. *Bioorg. Khim.* **1984b,** *10*, 710-712.

Aleksandrov, M. L.; Gall, L. N.; Krasnov, N. V.; Nikolaev, V. I.; Pavlenko, V. A.; Shkurov, V. A. Formation of the distribution of cluster ions in a molecular beam. *Dokl. Akad. Nauk. SSSR.* **1984a**, *277*, 379-383.

Alli, I.; Boye, J. I; Gibbs, B.F. and Konishi, Y. 1999. Amass spectrometric analysis of whey proteins. *Intern. Dairy J.* 1999. In preparation.

Alli, I.; Gibbs, B.F.; Okoniewska, M. K.; Konishi, Y.; Dumas, F. Identification of phaseolin polypeptide subunits in a crystalline food protein isolate from large lima bean (*Phaseolus lunatus*). *J. Agric. Food Chem.* **1994,** *42*, 2679-2683.

Alli, I.; Gibbs, B.F.; Okoniewska, M. K.; Konishi, Y.; Dumas, F. Identification and characterization of phaseolin polypeptides in a crystalline protein isolate from white kidney beans (*Phaseolus vulgaris*). *J. Agric. Food Chem.* **1993,** *41,* 1830-1834.

Alli, I.; Okoniewska, M. K; Gibbs, B. F.; Konishi, Y. Identification of peptides in cheddar cheese by electrospray ionization mass spectrometry. *Int. Dairy J.* **1998**, *8*, 643-649.

Allmere, T.; Andren, A.; Lindersson, M.; Bjorck, L. Studies on rheological properties of stirred milk gels made from milk defined genetic variants of κ-casein and β-lactoglobulin. *Int. Dairy J.* **1998**, *8*, 899-905.

Alomirah, H. F. Proteolytic degradation products as indicators for quality assessment in meat and fish. M.Sc. Thesis, McGill University, Montreal, Canada. 1996.

Alomirah, H. F.; Alli, I.; Gibbs, B. F.; Konishi, Y. Identification of proteolytic products as indicators of quality in ground and whole meat. *J. Food Quality* **1998**, *21*, 99-316.

Alomirah, H. F.; Alli, I.; Ismail, A. A.; Konishi, Y. Thermal denaturation and structural stability of α-lactalbumin separated from whey and whey protein preparations. (*J. Agric. Food Chem.* submitted. 2002.

Alomirah, H. F.; Alli, I.; Konishi, Y. Applications of mass spectrometry to food proteins and peptides. *J. Chromatogr. A*, **2000**, *893*, 1-21.

Alomirah, H. F.; Alli, I. Proteolytic degradation products as indicators for quality assessment in fish. I.F.T. Conference. New Orleans, Louisiana. 1996.

Alomirah, H. F.; Alli, I. Separation and characterization of β-lactoglobulin and α-lactalbumin from whey and whey protein preparations. *Int. Dairy J.* submitted. 2002.

Amano, M.; Ogawa, H.; Kojima, K.; Kamidaira, T.; Suetsugu, S.; Yoshihama, M.; Satoh, T.; Samejima, T.; Matsumoto, I. Identification of the major allergens in wheat flour responsible for bakers asthma. *Biochem. J.* **1998**, *330*, 1229-1234.

Amundson, C. H.; Watanawanichakorn, S.; Hill, C. G. Production of enriched protein fractions of β-Lactoglobulin and α-Lactalbumin from cheese whey. *J. Food Process. Preser.* **1982**, *6*, 55-71.

Anfinsen, C. B. Principles that govern the folding of protein chains. *Science* **1973**, *181*, 223-230.

Angeletti, R.; Gioacchini, A.M.; Seraglia, R.; Piro, R.; Traldi, R. The potential of matrix-assisted laser desorption/ ionization mass spectrometry in the quality control of water buffalo mozzarella cheese. *J. Mass Spectrom.* **1998**, *33*, 525-531.

AOAC. Official Methods of Analysis, Association of Official Analytical Chemists, **1980**, Washington, DC.

AOAC. Official Methods of Analysis, Association of Official Analytical Chemists international, **1990**, Washington, DC.

Apenten, R. K. O.; Galani, D. Is the rate of sulfur-disulfide exchange between the native β-lactoglobulin and PDS related to protein conformational stability?. *Int. J. Food Sci. Technol.* **1999**, *34*, 483-486.

Arnold, R. J.; Karty, J. A.; Ellington, A. D.; Reilly, J. P. Monitoring the growth of a bacteria culture by MALDI-MS of whole cells. *Anal. Chem.* **1999**, *71*, 1990-1996.

Arnold, R. J.; Reilly, J. P. Fingerprint matching of E. *coli* strains with MALDI-TOF mass spectrometry of whole cells using a modified correlation approach. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 630-636.

Aschaffenburg, R.; Drewry, J. Improved method for the preparation of crystalline β-LG and α-LA from cow's milk. *Biochemistry* **1957**, *65*, 273-277.

Aubagnac, J. L.; Salesse, A.; Jauregui_Adell J. Fast atom bombardment mass spectra of various peptides from sarcoplasmic calcium-binding protein. Biomed. Environ. Mass Spectrom. **1988**, *16*, 469-472.

Babu, K. R.; Moradian, A.; Douglas, D. J. The methanol-induced conformational transition of β-lactoglobulin, cytochrome c, and ubiquitin at low pH: a study by electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 317-328.

Bai, Y.; Milne, J. S.; Mayne, L.; Englander. S. W. Primary structure effects on peptide group hydrogen exchange. *Proteins* **1993**, *17*, 75-86.

Bailey, A. G. In *Electrostatic Spraying of liquids; Electrostatics and Electrostatic Applications Series*. Wiley: New York, 1988.

Barber, M.; Bordoli, R. S; Elliott, G. J.; Sedgwick, R. D.; Tyler, A. N. Fast atom bombardment mass spectrometry. *Anal. Chem.* **1982**, *54*, 645A-657A.

Beckey, H. D. In: *Principles of Field ionization and Field Desorption Mass Spectrometry*; (International Series in Analytical Chemistry, Vol. 61), Pergamon Press, Oxford, 1977.

Belitz, H. D.; Grosch, W. Milk and dairy products. In *Food Chemistry - 2nd ed.;* Springer, Berlin, 1999; pp 470-512.

Belloque, J.; López-Fandiño, R.; Smith, G. M. A ^{1}H-NMR study on the effect of high pressures on β-lactoglobulin. *J. Agric. Food Chem.* **2000**, *48*, 3906-3912.

Bembers, M. B.; Satterlee, L. D. Physicochemical characterization of normal and PSE porcine muscle myoglobins. *J Food Sci.* **1975**, *40*, 40-43.

Bergeron, D.; Nielsen, S. S. Partial characterization of trypsin inhibitors and N-terminal sequence of five isoinhibitors of great northern beans (*Phaseolus vulgaris*). *J. Agric. Food Chem.* **1993**, *41*, 1544-1549.

Beuhler, R. J.; Flanigan, E.; Green, L. J.; Friedman, L. Proton transfer mass spectrometry of peptides. Rapid heating techniques for underivatized peptides containing arginine. *J. Am. Chem. Soc.* **1974**, *96*, 3990-3999.

Biemann, K. Contribution of mass spectrometry to peptide and protein structure. *Biomed Environ. Mass Spectrom.* **1988**, *16*, 99-111.

Biemann, K.; Vetter, W. Separation of peptide derivatives by gas chromatography combined with mass spectrometry determination of the amino acid sequence. *Biochem. Biophys. Res. Commun.* **1960**, *3*; 578-584.

Birmingham, J.; Demirev, P.; Ho, Y. P.; Thomas, J.; Bryden, W.; Fenselau, C. Corona plasma discharge for rapid analysis of microorganisms by mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 604-606.

Blackberg, L.; Hernell, O. Isolation of lactoferrin from human whey by a single chromatographic step. *FEBS Letters* **1980**, *109*, 180-183.

Blakley, C. R.; Vestal, M. L. Thermospray interface for liquid chromatography/ mass spectrometry. *Anal. Chem.* **1983**, *55*, 750-754.

Bouhallab, S.; Morgan, F.; Henry, G.; Mollé, D.; Léonil, J. Formation of stable covalent dimer explains the high solubility at pH 4.6 of lactose-β-lactoglobulin conjugates heated near neutral pH. *J. Agric. Food Chem.* **1999**, *47*, 1489-1494.

Boye, J. I. Thermodynamic and structural properties related to the gelation of whey proteins. Ph.D. Thesis. McGill University. Montreal, Canada. 1995.

Boye, J. I.; Alli, I. Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study. *Food Res. Int.* **2000**, *33*, 673-682.

Boye, J. I.; Alli, I.; Ismail, A. A. Interactions involved in the gelation of bovine serum albumin. *J. Agric. Food Chem.* **1996a**, *44*, 996-1004.

Boye, J. I.; Alli, I.; Ismail, A. A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. *J. Agric. Food Chem.* **1997a**, *45*, 1116-1125.

Boye, J. I.; Ismail, A. A.; Alli, I. Effect of Physicochemical factors on the secondary structure of β-Lactoglobulin. *J. Dairy Res.* **1996b**, *63*, 97-109.

Boye, J. I.; Ma, C. Y.; Ismail, A.; Harwalkar, V. R.; Kalab, M. Molecular and microstructural studies of the thermal denaturation and gelation of β-lactoglobulins A and B. *J. Agric. Food Chem.* **1997b**, *45*, 1608-1618.

Bramaud, C.; Aimar, P.; Daufin, G. Thermal isoelectric precipitation of α-lactalbumin from a whey protein concentrate: influence of protein-calcium complexation. *Biotech. Bioeng.* **1995**, *47*, 121-130.

Bramaud, C.; Aimar, P.; Daufin, G. Whey protein fractionation: isoelectric precipitation of α-lactalbumin under gentle heat treatment. *Biotech. Bioeng.* **1997**, *56*, 391-397.

Brown, E. M.; Farrell, H. M. Jr. Interaction of β-lactoglobulin and cytochrome *c*: Complex formation and iron reduction. *Arch. Biochem. Biophys.* **1978**, *185*, 156-164.

Bryant, C. M.; McClements, D. J. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci. Technol.* **1998**, *9*, 143-151.

Burr, R.; Moore, C. H.; Hill, J. P. Evidence of multiple glycosylation of bovine β-lactoglobulin by electrospray ionization mass spectrometry. *Milchwissenschaft* **1996**, *51*, 488-492.

Caessens, P. W. J. R.; Visser, S.; Gruppen, H. Method for the isolation of bovine β-lactoglobulin from a cheese whey protein fraction and physicochemical characterization of purified product. *Int. Dairy J.* **1997**, *7*, 229-235.

Camafeita, E.; Alfonso, P.; Acevedo, B.; Mendez, E. Sample preparation optimization for the analysis of gliadins in food by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **1997a**, *32*, 444-449.

Camafeita, E.; Alfonso, P.; Mothes, T.; Mendez, E. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric micro-analysis: The first non-immunological alternative attempt to quantify gluten gliadins if food samples. *J. Mass Spectrom.* **1997b**, *32*, 940-947

Camafeita, E.; Mendez, E. Screening of gluten avenins in foods by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **1998**, *33*, 1023-1028.

Camafeita, E.; Solis, J.; Alfonso, P.; Lopez, J. A.; Sorell, L.; Mendez, E. Selective identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of different types of gluten in foods made with cereal mixtures. *J. Chromatogr A.* **1998**, *823*, 299-306.

Casal, H. L.; Kohler, U.; Mantsch, H. H. Structural and conformational changes of β-lactoglobulin B: An infrared spectroscopic study of the effect of pH and temperature. *Biochim. Biophys. Acta* **1988**, *957*, 11-20.

Catinella, S.; Traldi, P.; Pinelli, C.; Dallaturca, E. Matrix-assisted laser desorption ionization mass spectrometry: A valid analytical tool in the dairy industry. *Rapid Commun. Mass Spectrom.* **1996a**, *10*, 1123-1127.

Catinella, S.; Traldi, P.; Pinelli, C.; Dallaturca, E.; Marsilio, R. Matrix-assisted laser desorption mass spectrometry in milk science. *Rapid Commun. Mass Spectrom.* **1996b**, *10*, 1629-1637.

Catinella, S; Seraglia, R; Marsilio, R. Letters to the editor - evaluation of protein profile of human milk by matrix-assisted laser desorptiontionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1546-1549.

Cayot, P.; Lorient, D. Structure-function relationships of whey proteins. In *Food Proteins and Their Applications* Damodaran, S.; Paraf, A.; Eds.; Marcel Dekker, New York, 1997; pp 225-256.

Chait, B. T.; Kent, S. B. H. Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins. *Science* **1992**, *257*, 1885-1894.

Chaplin, L. C. Hydrophobic interaction fast protein liquid chromatography of milk proteins. *J. Chromatogr.* **1986**, *363*, 329-335.

Chiu, C. K.; Etzel, M. R. Fractionation of lactoperoxidase and lactoferrin from bovine whey using a cation exchange membrane. *J. Food Sci.* **1997**, *62*, 996-1000.

Chong, B. E.; Wall, D. B.; Lubman, D. M.; Flynn, S. J. Rapid profiling of E-coli proteins up to 500 kDa from whole cell lysates using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1900-1908.

Chowdhuray, S. K.; Katta, V.; Chait, B. T. Probing conformational changes in proteins by mass spectrometry. *J. Am. Chem. Soc.* **1990**, *112*, 9012-9013.

Chrysina, E. D.; Brew, K.; Acharya, K. R. Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2 Å resolution reveal an effect of calcium on inter-lobe interactions. *J. Biol. Chem.* **2000**, *275*,37021-37029.

Chung, E. W.; Nettleton, E. J.; Morgan, C. J.; Groß, M.; Miranker, A.; Radford, S. E.; Dobson, C. M.; Robinson, C. V. Hydrogen exchange properties of proteins in native and denatured states monitored by mass spectrometry and NMR. *Protein Sci.* **1997**, *6*, 1316-1324.

Clark, A. H.; Saunderson, D. H .P.; Suggett, A. Infrared and laser raman spectroscopic studies of thermally-induced globular protein gels. *Int. J. Pept. Protein Res.* **1981**, *17*, 353-364.

Covey, T. R.; Bonner, R. F.; Shushan, B. I.; Henion, J. The determination of protein, oligonucleotide and peptide molecular weights by ion-spray mass spectrometry. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249-256.

Creamer, L. K.; Parry, D. A. D.; Malcolm, G. N. Secondary structure of bovine β-lactoglobulin B. *Arch. Biochem. Biophys.* **1983**, *227*, 98-105.

Cruz, L.; Garden, R. W.; Kaiser, H. J.; Sweedler, J. V. Studies of the degradation products of nisin, a peptide antibiotic, using capillary electrophoresis with off-line mass spectrometry. *J. Chromatogr. A* **1996**, *735*, 375-385.

Dai, Y.; Li.; L.; Roser, D. C.; Long, S. R. Detection and identification of low-mass peptides and proteins from solvent suspensions of escherichia coli by HPLC fractionation and MALDI mass spectrometry, *Rapid Commun. Mass Spectrom.* **1999**, 13, 73-78.

Damodaran, S. Food proteins: An overview. In *Food Proteins and Their Applications* Damodaran, S., Paraf, A., Eds.; Marcel Dekker, New York, 1997; pp 1-24.

Damodaran, S. Structure-function relationship of food proteins. In *Protein Functionality in Food Systems.* Hettiarachchy, N. S., Ziegler, G. R., Eds.; Marcel Dekker Inc: New York, 1994; pp 1-37.

Davis, B. J. Disc electrophoresis. II. Method and application to human serum protein. *Ann. New York Acad. Sci.* **1964**, *121*, 404-427.

de Wit, J. N. Marschall Rhone-Poulenc award lecture. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.* **1998**, *81*, 597-608.

de Wit, J. N.; Bronts, H. Process for the recovery of α-lactalbumin and β-lactoglobulin from a whey protein product. *United State Patent.* **1995,** Patent number: US 5, 420,249.

deFrutos, M.; Cifuentes, A.; DiezMasa, J. C.; Camafeita, E.; Mendez, E. Multiple peaks in HPLC of proteins: Bovine serum albumin eluted in a reversed-phase system. *J. High Res. Chrom.* **1998**, *21*, 18-24.

Deighton, N.; Glidewell, S. M.; Goodman, B. A. Potential use of electrospray ionisation mass spectrometry for the detection of irradiated egg white. *Food Sci. Technol. Today* **1995**, *9*, 144-145.

Demirev, P. A.; Ho, Y. P.; Ryzhov, V.; Fenselau, C. Microorganism identification by mass spectrometry and protein database searches. *Anal. Chem.* **1999**, *71*, 2732-2738.

Dill, K. A. Dominant forces in protein folding. *Biochemistry* **1990**, *29*, 7133-7155.

Dobo, A.; Kaltashov, I. A. Detection of multiple protein conformational ensembles in solution via deconvolution of charge-state distribution in ESI MS. *Anal. Chem.* **2001**, *73*, 4763-4773.

Domin, M. A .; Welham, K. J.; Ashton, D. S. The effect of solvent and matrix combinations on the analysis of bacteria by matrix assisted laser desorption/ionisation Time-of-flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 222-226.

Dworschak, R. G.; Ens, W.; Standing, K. G.; Preston, K. R.; Marchylo, B. A.; Nightingate, M. J.; Stevenson, S. G.; Hatcher, D. W. Analysis of wheat gluten proteins by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **1998**, *33*, 429-435.

Dziezak, J.D. Phosphates improve many foods. *Food Technol.* **1990**, *44*, 80-82, 85-86, 90, 92.

Dzwolak, W.; Kato, M.; Shimizu, A.; Taniguchi, Y. Fourier-transform infrared spectroscopy study of the changes in the structure of the bovine α-lactalbumin: the stabilizing role of the calcium ion. *Biochim. Biophys. Acta* **1999**, *1433*, 45-55.

Dzwolak, W.; Kato, M.; Shimizu, A.; Taniguchi, Y. FTIR study on heat-induced and pressure-assisted cold-induced changes in structure of bovine α-lactalbumin: stabilizing role of calcium ion. *Biospectroscopy* **2001**, *62*, 29-39.

Edelhoch, H.; Osborne, J. C. The thermodynamic basis of the stability of proteins, nucleicacid and membranes. *Adv. Prot. Chem.* **1976**, *30*,183-250.

Ehring, H. Hydrogen exchange/electrospray ionization mass spectrometry studies of structural features of proteins and protein/protein interactions. *Anal. Biochem.* **1999**, *267*, 252-259.

Ena, J. M.; van Beresteijn, E. C. H.; Robben, A. J. P. M.; Schmidt, D. G. Whey protein antigenicity reduction by fungal proteinases and a pepsin/pancreatin combination. *J. Food Sci.* **1995**, *60*, 104-110.

Englander, S. W.; Kallenbach, N. R. Structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys.* **1984**, *16*, 521-655.

Englander, S. W.; Mayne, L. Protein folding studies using hydrogen exchange labeling and two-dimensional NMR. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 243-265.

Eyles, S. J.; Robinson, C. V.; Radford, S. E.; Dobson, C. M. Kinetic consequence of the removal of a disulfide bond on the folding of hen lysozyme. *Biochemistry* **1994**, *33*, 1534-1538.

Fanton, C.; Delogu, G.; Maccioni, E.; Podda, G.; Seraglia, R.; Traldi, P. Matrix-assisted laser desorption/ionization mass spectrometry in the dairy industry 2. The protein

fingerprint of ewe cheese and its application to detection of adulteration by bovine milk. Rapid Commun. *Mass Spectrom.* **1998**, *12*, 1569-1573

Fedele, L; Seraglia, R; Battistotti, B; Pinelli, C; Traldi, P. Matrix-assisted laser desorption/ionization mass spectrometry for monitoring bacterial protein digestion in yogurt. *J. Mass Spectrom.* **1999a**, *34*, 1338-1345.

Fedele, L; Seraglia, R; Battistotti, B; Pinelli, C; Traldi, P. Qualitative characterization of bacterial strains employed in the production of yogurt by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **1999b**, *34*, 1385-1388.

Fenn, J. B.; Mann, M.; Mengs, C. K.; Wong, S.F.; Whitehouse, C. M. Electrospray ionization for mass spectrometry of large biomolecules. *Science* **1989**, *246*, 64-71.

Ferranti, P.; Addeo, F.; Malorni, A.; Chianese, L.; Leroux, C. Martin, P. Differential splicing of pre-messenger RNA produces multiple forms of mature caprine alpha (s1)-casein. *Eur. J. Biochem.* **1997a**, *249*, 1-7.

Ferranti, P.; Barone, F.; Chianese, L.; Addeo, F.; Scaloni, A.; Pellegrino, L.; Resmini, P. Phosphopeptides from Grana Padano cheese: nature, origin and changes during ripening. *J. Dairy Res.* **1997b**, *64*, 601-615.

Ferranti, P.; Malorni, A.; Nitti, G.; Laezza, P.; Pizzano, R.; Chianese, L.; Addeo, F. Primary structure of ovine alpha s1-caseins: localization of phosphorylation sites and characterization of genetic variants A, C and D. *J. Dairy Res.* **1995**, *62*, 281-296.

Forge, V.; Wijesinha, R. T.; Balbach, J.; Brew, K.; Robinson, C. V.; Redfield, C.; Dobson, C .M. Rapid collapse and slow structural reorganisation during the refolding of bovine α-lactalbumin. *J. Mol. Biol.* **1999**, *288*, 673-688.

Fox, K. K.; Holsinger, V. H.; Posati, L. P.; Pallansch, M. J. Separation of β-lactoglobulin from other milk serum proteins by trichloroacetic acid. *J. Dairy Sci.* **1967**, *50*, 1363-1367.

Fox, P. F.; McSweeney, P. L. H. Milk proteins. In *Dairy chemistry and biochemistry*; Blackie Academic and Professional, London, UK, 1998; pp 146-238.

Gagnaire, V; Pierre, A.; Molle, D.; Leonil, J. Phosphopeptides interaction with colloidal calcium phosphate isolated by tryptic hydrolysis of bovine casein micelles. *J. Dairy Res.* **1996**, *63*,405-422.

Gaskell, S. J. Electrospary: principles and practice. *J. Mass Spectrom.* **1997**, *32*, 677-688.

Gerberding, S. J.; Byers, C. H. Preparative ion-exchange chromatography of proteins from dairy whey. *J. Chromatogr. A* **1998**, *808*, 141-151.

Gésan, G.; Daufin, G.; Timmer, M.; Allersma, D.; Van der Horst, C. Process steps for the preparation of purified fractions of α-lactalbumin and β-lactoglobulin from whey protein concentrates. *J. Dairy Res.* **1999**, *66*, 225-236.

Gezimati, J; Creamer, L. K.; Singh, H. Heat-induced interactions and gelation of mixtures of β-lactoglobulin and α-Lactalbumin. *J. Agric. Food Chem.* **1997**, *45*, 1130-1136.

Gibbs, B. F.; Alli, I. Characterization of a purified alpha-amylase inhibitor from white kidney beans (Phaseolus vulgaris). *Food Res. Int.* **1998**, *31*, 217-225.

Godovac-Zimmermann, J.; Conti, A.; James, L.; Napolitano, L. Microanalysis of the amino-acid sequence of monomeric β-lactoglobulin from Donkey (Equus asinus) milk. Biol. Chem. Hoppe-Seyler **1988**, *369*, 171-179.

Godovac-Zimmermann, J.; Shaw, D. The primary structure, binding site and possible function of β-lactoglobulin from Eastern Grey Kanguroo (Macropus giganteus). *Biol. Chem. Hoppe-Seyler* **1987**, *368*, 879-886.

Gouldsworthy, A. M.; Leaver, J.; Banks, J. M. Application of a mass spectrometry sequencing technique for identifying peptides present in cheddar cheese. *Int. Dairy J.* **1996**, *6*, 781-790.

Griko, Y. V; Remeta, D. P. Energetic of solvent and ligand-induced conformational changes in α-lactalbumin. *Protein Sci.* **1999**, *8*, 554-561.

Gross, J.; Strupta, K. Matrix-assisted laser desorption/ ionization-mass spectrometry applied to biological macromolecules. *Trends. Anal. Chem.* **1998**, *17*, 470-484.

Gryboski, J. D. Gastrointestinal aspects of cow's milk protein intolerance and allergy. *Immunol. Allergy Clin. N. Am.* **1991**, *11*,773 –797.

Gumpen, S.; Hegg, P. O.; Martens, M. Thermal stability of fatty acid serum albumin complexes studied by differential scanning calorimetry. *Biochem. Biophys. Res. Commun.* **1979**, *95*, 1098-1104.

Haag, A. M; Taylor, S. N; Johnston, K. H; Cole, R. B. Rapid identification and speciation of haemophilus bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **1998**, *33*, 750-756.

Hall, G. M. In *Methods of Testing Protein Functionality*. Hall, G. M., Ed.; Blackie Academic and Professional, London, 1996, pp. 1-10.

Hambling, S. G.; McAlpine, A. S.; Sawyer, L. β-lactoglobulin. *In Advanced Dairy Chemistry -1. Proteins*; Fox, F. P., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 141-190.

Haque, Z.; Kinsella, J. E. Interaction between κ-casein and β-lactoglobulin. Effect of calcium. *Agr. Biol. Chem.* **1987**, *51*, 1997.

Harwalkar, V. R.; Kalab, M. Thermal denaturation and aggregation of β-lactoglobulin at pH 2.5. Effect of ionic strength and protein concentration. *Milchwissenschaft*, **1985**, *40*, 31-34.

Harwalkar, V. R.; Ma, C. Y. Studies of thermal properties of oat globulin by differential scanning calorimetry *J. Food Sci.* **1987**, *52*, 394-398.

Havel, H. A. In *Spectroscopic Methods for Determining Proteins in Solution.* Havel, H. A., Ed.; VCH. Publisher, 1996, pp.1-4.

Hayakawa, S.; Nakai, S. Relationship of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.* **1985**, *50*, 486-491.

Hendrix, T.; Griko, Y. V.; Privalov, P. L. A calorimetric study of the influence of calcium on the stability of bovine α-lactalbumin. *Biophys. Chem.* **2000**, *84*, 27-34.

Hermansson, A. M. Methods of studying functional characteristics of vegetable proteins. J. *Am. Oil Chem. Soc.* **1979**, *56*, 272-279.

Hickman, D. R.; Roepstorfft, P.; Shewry, P. R.; Tatham, A. S. Molecular weights of high molecular weight subunits of glutenin determined by mass spectrometry. *J. Cereal Sci.* **1995**, *22*, 99-103.

Hill, A. R.; Irvine, D. M.; Kakuda, Y.; Manji, B. Separation and quantification of whey proteins by size exclusion chromatography. *Can. Inst. Food Sci. Technol. J.* **1986**, *19*, 227-230.

Hiraoka, Y.; Segawa, T.; Kuwajima, K.; Sugai, S.; Murai, N. α-lactalbumin: a calcium metalloprotein. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1098-1104.

Hiraoka, Y.; Sugai, S. Equilibrium and kinetic study of sodium and potassium-induced conformational changes of apo-α-lactalbumin. *Int. J. Pept. Protein Res.* **1985**, *26*, 252-261.

Hiraoka, Y.; Sugai, S. Thermodynamics of thermal unfolding of bovine apo-α-lactalbumin. *Int. J. Pept. Protein Res.* **1984**, *23*, 535-542.

Holland, R. D.; Wilkes, J. G.; Rafii, F.; Sutherland, J. B.; Persons, C. C.; Voorhees, K. J.; Lay Jr, J. O. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996**,*10*, 1227-1232.

Horning, E. C.; Carroll, D. I.; Haegele, K. D.; Horning, M. D.; Stillwell, R. N. Atmospheric pressure ionization (API) MS. Solvent-Mediated ionization of samples introduced in solution and in a liquid chromatograph. Effluent stream. *J. Chromatogr. Sci.* **1974**, *12*, 725-729.

Hosseini-nia, T.; Ismail, A. A.; Kubow, S. Pressure-induced conformational changes of β-lactoglobulin by variable-pressure fourier transform infrared spectroscopy. *J. Agric. Food Chem.* **1999**, *47*, 4537-4542.

Hu, P.; Ye, Q. Z; Loo, J. A. Calcium stoichiometry determination for calcium binding proteins by electrospray ionization mass spectrometry. *Anal. Chem.* **1994**, *66*, 4190-4194.

Huang, X. L.; Catignani, G. L.; Foegeding, E. A.; Swaisgood, H. E. Comparison of the gelation properties of β-lactoglobulin genetic variants A and B. *J. Agric. Food Chem.* **1994b**, *42*, 1064-1067.

Huang, X. L.; Catignani, G. L.; Swaisgood, H. E. Relative structural stabilities of β-lactoglobulins A and B as determined by proteolytic susceptibility and differential scanning calorimetry. *J. Agric. Food Chem.* **1994a**, *42*, 1276-1280.

Huyghues-Despointes, B. M. P.; Pace, C. N.; Englander, S. W.; Scholtz, J. M. Measuring the conformational stability of a protein by hydrogen exchanged. In *Methods in Molecular Biology: Protein Structure, Stability, and Folding*. Murphy, K. P., Ed.; Humana Press, 2001, pp 69-92.

Imafidon, G. I.; Ng-Kwai-Hang, K.; Harwalkar, V. R.; Ma, C.-Y. Effect of genetic polymorphism on the thermal stability of β-lactoglobulin and κ-casein mixture. *J. Dairy Sci.* **1991**, *74*, 1791-1802.

Ismoyo, F. Biochemical changes associated with Rhizopus fermentation of soybean. M.Sc. Thesis, McGill University, Montreal, Canada. 1995.

Johnson, R. S.; Walsh, K. A. Mass spectrometric measurement of protein amide hydrogen exchange rates of apo- and holo-myoglobin. *Protein Sci.* **1994**, *3*, 2411-2418.

Jones, A. D.; Tier, C. M.; Wilkins, J. P. G. Analysis of the Maillard reaction products of β-lactoglobulin and lactose in skimmed milk powder by capillary electrophoresis and electrospray mass spectrometry. *J. Chromatogr. A* **1998**, *822*, 147-154.

Kabuki, T.; Saito; T.; Kawai, Y.; Uemura, J.; Itoh, T. Production, purification and characterization of reutericin 6, a bacteriocin with lytic activity produced by lactobacillus reuteri LA6. *Int. J. Food Microbiol.* **1997**, *34*: 145-156.

Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. Influence of the wavelength in high-irradiance ultraviolet laser desorption mass spectrometry of organic molecules. *Anal. Chem.* **1985**, *57*, 2935-2939

Karas, M.; Hillenkamp, F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* **1988**, *60*, 2299-2301.

Kato, A.; Tsutsui, N.; Matsudomi, N.; Kobayashi, K.; Nakai, S. Effects of partial denaturation on surface properties of ovalbumin and lysozyme. *Agric. Biol. Chem.* **1981**, *45*, 2755-2760.

Katta, V.; Chait, B. T. Conformational changes in proteins probed by hydrogen-exchange electrospray-ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 241-217.

Katta, V.; Chait, B. T. Hydrogen/deuterium exchange electrospary ionization mass spectrometry: A method for probing protein conformational changes in solution. *J. Am. Chem. Soc.* **1993**, *155*, 6317-6321.

Kaufmann, R. Matrix-assisted laser desorption (MALDI) mass spectrometry: a novel analytical tool in molecular biology and biotechnology. *J. Biotechnol.* **1995**, *41*, 155-175

Kauppinen, J. K.; Moffatt, D. J.; Mantsch, H. H.; Cameron, D. G. Fourier transforms in the computation of self deconvoluted and first-order derivative spectra of overlapped band contours. *Anal. Chem.* **1981**, *53*, 1454-1457.

Kella, N. K. D.; Kinsella, J. E. Enhanced thermodynamic stability of β-lactoglobulin at low pH - A possible mechanism. *Biochem. J.* **1988a**, *255*; 113-118.

Kella, N. K. D.; Kinsella, J. E. Structural stability of β-lactoglobulin in the presence of kosmotropic salts. A kinetic and thermodynamic study. *Int. J. Pept. Prot. Res.* **1988b**, *32*, 396-405.

Kilara, A.; Harwalkar, V. R. In *Food Proteins Properties and Characterization.* Nakai, S., Modler, H. W., Eds.; VCH Publ., New York, 1996, pp 71-148.

Kim, H. J.; Leszyk, J.; Taub, I. A. Direct observation of protein glycosylation by matrix-assisted laser desorption/ionization time-of-light mass spectrometry. *J. Agric. Food. Chem.* **1997**, *45*, 2158-2165.

Kinekawa, Y.-I.; Kitabatake, N. Purification of β-lactoglobulin from whey protein concentrate by pepsin treatment. *J. Dairy Res.* **1996**, *79*, 350-356.

Kinsella, J. E. In *Food Proteins*. Fox, P., London, J. J., Eds.; Elsevier Applied Science Publ, London, 1982, pp. 51-103.

Kinsella, J. E. Milk proteins: Physicochemical and functional properties. *CRC Crit. Rev. Food Sci. Nutr.* **1984**, *21*, 197-262.

Kinsella, J. E.; Whitehead, D. M.. Proteins in whey: Chemical, physical, and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343-438.

Konermann, L.; Douglas, D. L. Unfolding of proteins monitored by electrospray ionization mass spectrometry: A comparison of positive and negative ion modes. *J. Am. Soc. Spectrom.* **1998**, *9*, 1248-1254.

Krimm, S.; Bandekar, J. Vibrational spectroscopy and conformation of peptides, polypeptides and proteins. *Adv. Protein Chem.* **1986**, *38*, 181-354.

Krishnamurthy, T.; Davis, M. T.; Stahl, D. C.; Lee, T. D. Liquid chromatography/microspray mass spectrometry for bacterial investigations. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 39-49.

Krishnamurthy, T.; Ross, P. L. Rapid identification of bacteria by direct assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1992-1996.

Krishnamurthy, T.; Ross, P. L.; Rajamani, U. Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 883-888.

Kronman, M. J. Metal-ion binding and the molecular conformational properties of α-lactalbumin. *Crit. Rev. Biochem. Mol. Biol.* **1989**, *24*, 565-667.

Kuwajima, K.; Harushima, Y.; Sugai, S. Influence of Ca^{2+} binding on the structure and stability of bovine α-lactalbumin studied by circular dichroism and nuclear magnetic resonance spectra. *Int. J. Pept. Protein Res.* **1986**, *27*, 18-27.

Kuwajima, K; Mitani, M; Sugai, S. Characterization of the critical state in protein folding. Effects of guanidine hydrochloride and specific Ca^{2+} binding on the folding kinetics of alpha-lactalbumin. *J Mol Biol* **1989**, *206*: 547- 561.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680-685.

Lavanant, H.; Derrick, P. J.; Heck, A. J. R.; Mellon, F. A. Analysis of nisin A and some of its variants using Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Biochem.* **1998**, *255*, 74-89.

Le Blanc, J. C. Y.; Beauchemin, D.; Siu, K. W.; Guevremont, R.; Berman, S. S. Thermal denaturation of some proteins and its effect on their eectrospray mass spectra. *Org. Mass Spectrom.* **1991**, *26*, 831-839.

Lefèvre, T.; Subirade, M. Structural and interaction properties of β-lactoglobulin as studied by FTIR spectroscopy. *Int. J. Biol. Macromol.* **1999**, *26*, 337-344.

Léonil, J.; Mollé, D.; Fauquant, J.; Maubois, J. L.; Pearce, R. J.; Bouhallab, S. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* **1997**, *80*, 2270-2281.

Liao, P. C.; Leykam, J.; Andrews, P. G.; Gage, D.A.; Allison, J. An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* **1994**, *219*, 9-20.

Lin, L.; Pinker, R. J.; Forde, K.; Rose, G. D.; Kallenbach, N. R. Molten globular characteristics of the native state of apomyoglobin. *Nature Struct. Biol.* **1994**, *1*, 447-452.

Loo, J. A.; Edmonds, C. G.; Udseth, H. R.; Smith, R. D. Effect of reducing disulfide-containing proteins on electrospray ionization mass spectra. *Anal. Chem.* **1990**, *62*, 693-698.

Loo, J. A.; Edmonds, C. G; Smith, R. D. Tandem mass spectrometry of very large moleculs: serum albumins sequence information from multiply charged ions formed by electrospray ionization. *Anal. Chem.* **1991**, *63*, 2488-2499.

Loo, J. A.; Ogorzalek Loo, R. R.; Light, K. J; Edmonds, C. G; Smith, R. D. Multiply charged negative ions by electrospray ionization of polypeptides and proteins. *Anal. Chem.* **1992**, *64*, 81-88.

Loo, J. A.; Ogorzalek Loo, R. R. Electrospray ionization mass spectrometry of peptides and proteins. In *Electrospray Intonation Mass Spectrometry: fundamentals, instrumentation, and applications*. Cole, R. B., Ed; John Wiley and Sons: New York, 1997; pp 385-419.

Loo, J. A.; Orgorzalek Loo, R. R.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. Solvent-induced conformational changes of polypeptides probed by electrospray-ionization mass spectrometry. *Rapid Commun Mass Spectrom.* **1991**, *5*, 101-105.

Loo, J. A.; Udseth, H .R.; Smith, R. D. Solvent effects on the charge distribution observed with electrospray ionization-mass spectrometry. *Biomed. Environ. Mass Spectrom.* **1988**, *17*, 411-414.

Loo, J. A.; Udseth, H. R.; Smith, R. D. Peptide and protein analysis by electrospray ionization-mass spectrometry and capillary electrophoresis-mass spectrometry. *Anal. Biochem.* **1989**, *179*, 404-412.

Lynn, E C; Chung, M. C; Tsai, W. C; Han, C.C. Identification of enterobacteriaceae bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2022-2027.

Ma, C. Y.; Harwalkar, V. R. Study of thermal denaturation of oat globulin by ultraviolet and fluorescence spectrophotometry. *J. Agric. Food Chem.* **1988**, *36*, 155-160.

Maier, C. S.; Schimerlik, M. I.; Deinzer, M. L. Thermal denaturation of Escherichia coli thioredoxin studied by hydrogen/deuterium exchange and electrospray ionization mass spectrometry: monitoring a two-state protein unfolding transition. *Biochemistry* **1999**, *38*, 1136-1143.

Mailliart, P.; Ribadeau-Dumas, B. Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. *J. Food Sci.* **1988**, *53*, 743-745, 752.

Mandal, D. K.; Nieves, E.; Bhattacharyya, L.; Orr, G. A.; Roboz, J.; Yu, Q. T.; Brewer, C. F. Purification and characterization of three isolectins of soybean agglutinin: evidence for C-terminal truncation by electrospray ionization mass spectrometry. *Eur. J. Biochem.* **1994**, *221*, 547-553.

Manji, B.; Hill, A. R.; Kakuda, Y.; Irvine, D. M. Rapid separation of milk whey proteins by anion exchange chromatography. *J. Dairy Sci.* **1985**, *68*, 3176-3179.

Mann, M.; Meng, C. K.; Fenn, J. B.; Interpreting mass spectra of multiply charged ions. *Anal. Chem.* **1989**,*61*, 1702-1708.

Mann, M; Wilm, M. Electrospray mass spectrometry for protein characterization. *Trends Biochem. Sci.* **1995**, *20*, 219-224.

Marmorino, J. L.; Auld, D. S.; Bets, S. F.; Doyle, D. F.; Young, G. B.; Pielak, G. J. Amide proton exchange rates of oxidized and reduced Saccharomyces cerevisiae iso-1-cytochrome c. *Protein Sci.* **1993**, *2*, 1966-1974.

Mate, J. I.; Krochta, J. M. β-lactoglobulin separation from whey protein isolates on a large scale. *J. Food Sci.* **1994**, *59*, 1111-1114.

Matsuda, T.; Watanabe, K.; Sato, Y. Secondary structure of reduced ovomucoid and renaturation of reduced ovomucoid and its reduced fragments A (1-130) and B (131-186). *FEBS Letters* **1981a**, *124*, 185-188.

Matsuda, T.; Watanabe, K.; Sato, Y. Temperature-induced structural changes in chicken egg white ovomucoid. *Agric. Biol. Chem.* **1981b**, *45*, 1609-1614.

Mattarella, N. L.; Creamer, L. K.; Richardson, T. Amidation or esterification of bovine β-lactoglobulin to form positively change proteins. *J. Agric. Food Chem.* **1983**, *31*, 968-972.

Mauri, P L; Pietta, P G; Maggioni, A.; Cerquetti, M.; Sebastianelli, A.; Mastrantonio, P. Characterization of surface layer proteins from clostridium difficile by liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 695-703.

Mayne, L.; Paterson, Y.; Cerasoli, D.; Englander, S. W. Effect of antibody binding on protein motions studied by hydrogen exchange labeling and two-dimensional NMR. *Biochemistry* **1992**, *31*, 10678-10685.

Miller, D. W.; Dill, K. A. A statistical mechanical model for hydrogen exchange in globular proteins. *Protein Sci.* **1995**, *4*, 1860-1873.

Miranker, A.; Robinson, C. V.; Radford, S. E.; Aplin, R. T.; Dobson, C. M. Detection of transient protein folding populations by mass spectrometry. *Science*. **1993**, 262, 896-900.

Mirza, U. A.; Cohen, S. L.; Chait, B. T. Heat-induced conformational changes in proteins studied by electrospray ionization mass spectrometry. *Anal. Chem.* **1993**, *65*, 1-6.

Mollé, D.; Léonil, J. Heterogeneity of the bovine κ-casein caseinomacropeptide resolved by liquid chromatography on-line with electrospray ionization mass spectrometry. *J. Chromatogr. A* **1995**, *708*, 223-230.

Morgan, F.; Bouhallab, S.; Molle, D.; Henry, G.; Maubois, J. L.; Leonil, J. Lactolation of β-lactoglobulin monitored by electrospray ionization mass spectrometry. *Int. Dairy J.* **1998**, *8*, 98-98.

Morgan, F.; Leonil, J.; Molle, D.; Bouhallab, S. Nonenzymatic Lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413-417.

Morgan, F.; Molle, D.; Henry, G.; Venien, A.; Leonil, J.; Peltre, G.; Levieux, D.; Maubois, J. L.; Bouhallab, S. Glycation of bovine β-lactoglobulin: Effect on the protein structure. *Int. J. Food Sci. Tech.* **1999**, *34*, 429-435.

Morr, C. V.; Foegeding, E. A. Composition and functionality of commercial whey and milk protein concentrates and isolates: A statutes report. *Food Technol.* **1990**, *44*, 100, 102-104, 106, 108, 110, 112.

Morr, C. V.; Ha, E. Y. W. Whey protein concentrates and isolates: Processing and functional properties. *Crit. Rev. Food Sci. Nutr.* **1993**, *33*, 431-476.

Munson, M. S. B.; Field, F. H. Chemical ionization mass spectrometry I. General introduction. *J. Am. Chem. Soc.* **1966**, *88*, 2621-2630.

Murphy, K. P. Stabilization of protein structure. In *Methods in Molecular Biology: Protein Structure, Stability, and Folding*. Murphy, K. P., Ed.; Humana Press, 2001, pp 1-16.

Nemirovskiy, O.; Giblin, D. E.; Gross, M. L. Electrospray ionization mass spectrometry and hydrogen/deuterium exchange for probing the interaction of calmodulin with calcium. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 711-718.

Ng-Kwai-Hang, K. G.; Grosclaude, F. Genetic polymorphism of milk proteins. *In Advanced Dairy Chemistry -1. Proteins*; Fox, F. P., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 405-455.

Nguyen, D. N.; Becker, G. W.; Riggin, R. M.. Protein mass spectrometry: Applications to analytical biotechnology. *J. Chromatogr. A* **1995**, *705*, 21-45.

Nilsson, C. L.; Fingerprinting of helicobacter pylori strains by matrix-assisted laser desorption/ionization mass spectrometric analysis. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1067-1071.

Nordhoff, E.; Kirpekar, F.; Roepstroff, P. Mass spectrometry of nucleic acids. *Mass Spectrom. Rev.* **1996**, *15*, 67-138.

Osborne, H. B.; Nabedryk-Viala, E. Infrared measurement of peptide hydrogen exchange in rhodopsin. *Methods Enzymol.* **1982**, *88*, 676-680.

Pace, C. N.; Tanford, C. Thermodynamics of the unfolding of β-lactoglobulin A in aqueous urea solutions between 5 and 5.5. *Biochemistry* **1968**, *1*, 198-208.

Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of

β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383-385.

Park, K. H.; Lund, D. B. Calorimetric study of thermal denaturation of β-lactoglobulin. *J. Dairy Sci.* **1984**, *67*, 1699-1706.

Pasdar, N. A. Functional and structural characteristics of acid-hydrolyzed whey protein concentrate. M.Sc. Thesis, McGill University, Montreal, Canada. 1995.

Patel, P. D.; Fry, J. C. In *Developments in Food Proteins*. Hudson, B. J. T., Ed.; Elsevier Applied Science Publ., 1982, pp 299-333.

Paulsson, M.; Hegg, P. O.; Castberg, H. B. Thermal stability of whey proteins studied by differential scanning calorimetry. *Thermochimica Acta.* **1985**, *95*, 435-440.

Pearce, R. J. Enriched whey protein fractions and method for the production thereof. *United State Patent.* **1995**, Patent number: US 5, 455,331.

Pearce, R. J. Thermal separation of β-lactoglobulin and α-lactalbumin in bovine cheddar cheese whey. *Aust. J. Dairy Technol.* **1983**, *38*, 144-149.

Permyakov, E. A.; Berliner, L. J. α-Lactalbumin: structure and function. *FEBS Letters* **2000**, *473*, 269-274.

Pessen, H.; Purcell, J. M.; Farrell, H. M. Jr. Proton relaxation rates of water in dilute-solutions of β-lactoglobulin: Determination of cross relaxation and correlation with structural changes by the use of 2 genetic-variants of a self associating globular protein. *Biochim. Biophys. Acta.* **1985**, *828*,1-12

Phillips, L. G. The relationship between the structure, interfacial and foaming properties of β-lactoglobulin. Ph.D. Thesis. Cornell University, Ithaca, New York. 1992.

Phillips, L. G.; Whitehead, D. M.; Kinsella, J. In *Structure-Function Properties of Food Proteins*. Phillips, L. G., Whitehead, D. M, Eds.; Academic Press, San Diego, Calif., U.S.A. 1994, pp 75-106.

Pike, A. C. W.; Brew, K.; Acharya, K. R. Crystal structures of guinea-pig, goat and bovine α-lactalbumins highlight the enhanced conformational flexibility of regions that are significant for its action in the lactose synthase. *Structure* **1996**, *4*, 691-703.

Pouliot, Y.; Wijers, M. C.; Gauthier, S. F.; Nadeau, L. Fractionation of whey protein hydrolysates using charged UF/NF membranes. *J. Membrane Sci.* **1999**, *158*, 105-114.

Pour-El, A. In *Protein Functionality in Foods.* Cherry, J. P., Ed.; American Chemical Society Symposium Series 147, Washington D.C., 1981, pp 1-20.

Qi, X. L.; Brownlow, S.; Holt, C.; Sellers, P. Thermal denaturation of β-lactoglobulin: Effect of protein concentration at pH and pH 8.05. *Biochem. Biophys. Acta* **1995**, *1248*, 43-49.

Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, E. N.; Jameson, G. B. Functional implications of structural differences between variants A and B of bovine β-lactoglobulin. *Protein Sci.* **1999**, *8*, 75-83.

Rahmelow, K.; Hübner, W.; Ackermann, T. Infrared absorbances of protein side chains. *Anal. Biochem.* **1998**, *257*, 1-11.

Reid, J. R.; Coolbear, T.; Ayers, J. S.; Coolbear, K. P. The action of chymosin on κ-casein and its macropeptide: Effect of pH and analysis of products of secondary hydrolysis. *Int. Dairy J.* **1997**, *7*, 559-569.

Relkin, P.; Launay, B.; Eynard, L. Effect of sodium and calcium addition on thermal denaturation of apo-α-lactalbumin: A differential scanning calorimetric study. *J. Dairy Sci.* **1993**, *76*, 36-47.

Relkin, P. Thermal Unfolding of β-lactoglobulin, a-lactalbumin, and bovine serum albumin. A thermodynamic approach. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 565-601.

Resing, K. A.; Ahn, N. G. Deuterium exchange mass spectrometry as a probe of protein kinase activation. Analysis of wild-type and constitutively active mutants of MAP kinase Kinase-1. *Biochemistry* **1998**, *37*, 463-475.

Robinson, C. V. Protein secondary structure investigation by electrospray ionization. In *Methods in Molecular Biology, Vol. 61: Proteins and peptides analysis by mass spectrometry*; Chapman, J. R., Ed.; Humana Press Inc., Totowa, NJ, 1996, pp 129-139.

Robinson, C. V.; Groß, M.; Eyles, S. J.; Ewbank, J. J.; Mayhew, M.; Hartl, F. U.; Dobson, C. M.; Radford, S. E. Conformation of GroEl-bound α-lactalbumin probed by mass spectrometry. *Nature* **1994**, *372*, 646-65.

Rohl, C. A.; Scholtz, J. M.; York, E. J.; Stewart, J. M.; Baldwin, R. L. Kinetics of amide proton exchange in helical peptides of varying chain lengths. Interpretation by the Lifson-Roig equation. *Biochemistry* **1992**, *31*,1263-1269.

Rose, N. L.; Sporns, P.; McMullen, L. M. Detection of bacteriocins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Appl. Environ. Microbiol.* **1999a**, *65*, 2238-2242.

Rose, N. L.; Sporns, P.; Stiles, M. E.; McMullen, L. M. Inactivation of Nisin by Glutathione in fresh meat. *J. Food Sci.* **1999b**, *64*, 759-762.

Sabbadin, S.; Seraglia, R.; Allegri, G.; Bertazzo, A.; Traldi, P. Matrix-assisted laser desorption/ionization mass spectrometry in evaluation of protein profiles of infant formulae. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1438-1443.

Saenz, A. J.; Petersen, C. E.; Valentine, N. B.; Gantt, S. L.; Jarman, K. H.; Kingsley, M. T.; Wahl, K. L. Reproducibility of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for replicate bacterial culture analysis. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1580-1585.

Sannier, F.; Bordenave, S.; Piot, J. M. Purification of goat β-lactoglobulin from whey by an ultrafiltration membrane enzymatic reactor. *J. Dairy Res.* **2000**, *67*, 43-51.

Sawyer, L. One fold among many. *Nature* **1987**, *327*, 659-659.

Sawyer, L.; Kontopidis, G.; Wu, S.-Y. β-lactoglobulin-a three-dimensional perspective. *Int. J. Food Sci. Tech.* **1999**, *34*, 409-418.

Sawyer, W. H.; Norton, R. S.; Nichol, L. W.; McKenzie, G. H. Thermodenaturation of bovine β-lactoglobulin. Kinetics and the introduction of β-structure. *Biochim. Biophys. Acta* **1971**, *243*, 19-30.

Sawyer, W. H.; Puckridge, J. The dissociation of proteins by chaotropic salts. *J.Biol. Chem.* **1973**, *248*, 8429-8433.

Schmid, F. X. In *Protein Structure*. Creighton, T. E., Ed.; IRL Press, Oxford, 1997, pp 253-297.

Schmidt R. H.; Morris, H. A. Gelation properties of milk proteins, soy proteins, and blended protein systems. *Food Technol.* **1984**, *38*, 85-96.

Singh, T. K.; Fox, P. F.; Healy, A. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *J. Dairy Res.* **1997**, *64*, 433-443.

Siuzdak, G. The emergence of mass spectrometry in biochemical research. *Proc. Natl. Acad Sci.* USA. **1994**, *91*, 11290-11297.

Sivaraman, T.; Robertson, A. D. Kinetics of conformational fluctuations by EX1 hydrogen exchange in native proteins. In *Methods in Molecular Biology: Protein Structure, Stability, and Folding*. Murphy, K. P., Ed.; Humana Press, 2001, pp 193-214.

Skudder, P. J. Evaluation of a porous silica based ion-exchange medium for the production of protein fractions from rennet-and acid-whey. *J. Dairy Res.* **1985**, *52*, 167-181.

Slangen, C. J.; Visser, S. Use of mass spectrometry to rapidly characterize the heterogeneity of bovine α-lactalbumin. *J. Agric. Food Chem.* **1999**, *47*, 4549-4556.

Smith, D. L.; Deng, Y.; Zhang, Z. Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. *J. Mass Spectrom.* **1997**, *32*, 135-146.

Smith, R. D.; Loo, J. A.; Barinaga, C. J.; Edmonds, C. G.; Udseth, H. R. Capillary zone electrophoresis and isotachophoresis-mass spectrometry of polypeptides and proteins based upon an electrospray ionization interface. *J. Chromatogr.* **1989**, *480*, 211-232.

Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. New development in biochemical mass spectrometry: Electrospray ionization. *Anal. Chem.* **1990**, *62*, 882-899.

Smithers, G. W.; Ballard, F. J.; Copeland, A. D.; De Silva, K. J.; Dionysius, D. A.; Francis, G. L.; Goddard, C.; Grieve, P. A.; McIntosh, G. H.; Mitchell, I. R.; Pearce, R. J.; Regester, G. O. New opportunities from the isolation and utilization of whey proteins. *J. Dairy Sci.* **1996**, *79*, 1454-1459.

Sporns P.; Abell, D. C. MALDI mass spectrometry for food analysis. *Trends Food Sci. Technol.* **1996**, *7*, 187-190.

Stack, F. M.; Hennessy, M.; Mulvihill, D.; O'Kennedy, B. T. Process for the fractionation of whey constituents. *World Intellectual Property Organization.* **1995,** Patent number: WO 9, 534,216.

Sumual, M. F. Fractionation and characterization of proteins from coconut milk. M.Sc. Thesis, McGill University, Montreal, Canada. 1994.

Surewicz, W. K. Mantsch, H. H. In *Spectroscopic Methods for Determining Proteins in Solution.* Havel, H. A., Ed.; VCH. Publisher, 1996, pp 135-162.

Susi, H.; Byler, D. M. Fourier transform infrared spectroscopy in protein conformation studies. In *Methods for Protein Analysis*; Cherry, J. P., Barford, R. A., Eds.; American Oil Chemists Society: Champaign, IL, 1988; pp 235-250.

Swaisgood, H. E. In *Developments in Dairy Chemistry-1.* Fox, P. F., Ed.; Elsevier Applied Science, London, 1982, pp 1-35.

Szewczuk, Z.; Konishi, Y.; Goto, Y. A two-process model describes the hydrogen exchange behavior of the cytochrome *c* in the molten globule state with various extents of acetylation. *Biochemistry* **2001**, *40*, 9623-9630.

Tang, X. J.; Brewer, C. F.; Saha, S.; Chernushevich, I.; Ens, W.; Standing, K. G. Investigation of protein-protein noncovalent interactions in soybean agglutinin by electrospray ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1994**; *8*, 750-754.

Timasheff, S. N.; Townend, R.; Mescanti, L. The optical rotatory dispersion of the β-lactoglobulins. *J. Biol. Chem.* **1966**, *241*, 1863-1870.

Torgerson, D. F.; Skowronski, R. P.; Macfarlane, R. D. New approach to the mass spectrometry of non-volatile compounds. *Biochem. Biophys. Res. Commun.* **1974**, *60*, 616-621.

Troullier, A.; Reinstädler, D.; Dupont, Y.; Naumann, D.; Forge, V. Transient non-native secondary structures during the refolding of α-lactalbumin detected by infrared spectroscopy. *Nature Struct. Biol.* **2000**, *7*, 78-86.

Uchida, T.; Sato, K.; Kawasaki, Y.; Dosako, S. Separation of lactoperoxidase, secretory component and lactoferrin from milk or whey with a cation exchange resin. *United State Patent.* **1996,** Patent number. US 5, 516,675.

Verheul, M.; Roefs, S. P. F. M.; de Kruif, K. G. Aggregation of β-lactoglobulin and influence of D_2O. *FEBS Letters* **1998**, *421*, 273-276.

Wagner, D. S.; Anderegg, R. J.; Conformation of cytochrome *c* studied by denterium exchange-electrospray ionization mass spectrometry. *Anal. Chem.* **1994**, *66*, 706-711

Wagner, D. S.; Melton, L. G.; Yan, Y.; Erickson, B. W.; Anderegg, R. J. Deuterium exchange of α-helices and β-sheets as monitored by electrospray ionization mass spectrometry. *Protein Sci.* **1994,** *3*, 1305-1314.

Waissbluth, M. D.; Grieger, R. A. Alkaline denaturation of β-lactoglobulins. Activation parameters and effect on dye binding site. Biochemistry **1974**, *13*, 1285-1288.

Wang, F.; Blanchard, J. S.; Tang, X. J. Hydrogen exchange/electrospray ionization mass spectrometry studies of substrate and inhibitor binding and conformational changes of Escherichia coli dihydrodipicolinate reductase. *Biochemistry* **1997**, *36*, 3755-3759.

Wang, F.; Scapin, G.; Blanchard, J. S.; Angeletti, R. H. Substrate binding and conformational changes of clostridium glutamicum diaminopimelate dehydrogenase

revealed by hydrogen/deuterium exchange and electrospray mass spectrometry. *Protein Sci.* **1998**, *7*, 293-299.

Wang, G.; Cole, R. B. Solution, gas-phase, and instrumental parameter influences on charge state distributions in electrospray ionization mass spectrometry. In *Electrospray ionization mass spectrometry: fundamentals, instrumentation, and applications*. Cole R. B Ed.; John Wiley and Sons Inc: New York, 1997: pp 137-174.

Wang, Z.; Russon, L.; Li, L.; Roser, D.C.; Long, S. R. Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 456-464.

Welham, K. J.; Domin, M. A.; Scannel, D. E; Cohen E.; Ashtomn, D. S. The characterization of micro-organisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 176-180.

Wijesinha, R.; Dobson, C. M.; Redfield, C. Comparison of the structural and dynamical properties of holo and apo bovine α-lactalbumin by NMR spectroscopy. *J. Mol. Biol.* **2001**, *307*, 885-898.

Wong, D. W. S.; Camirand, W. M.; Pavlath, A. E. Structure and functionalities of milk proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 807-844.

Woodward, C. K.; Simon, I.; Tuchsen, E. Hydrogen exchange and the dynamic structure of proteins. *Mol Cell Biochem.* **1982**, *48*, 135-160.

Wu, S. Y.; Pérez, M. D.; Puyol, P.; Sawyer, L. β-lactoglobulins binds palmitate within its central cavity. *J. Biol. Chem.* **1999**, *274*, 170-174.

Yamagishi, T.; Ebina, F.; Yamauchi, F. Analysis of the state of aromatic amino acid residues in heated soybean 7S globulin by absorption derivative spectrophotometry and specrtofluorimetry. *Agric. Biol. Chem.* **1982**, *46*, 2441-2448.

Yamashita, M.; Fenn, J. B. Electrospray ion source: Another variation on the free-jet theme. *J. Phys. Chem.* **1984a**, *88*, 4551-4459.

Yamashita, M.; Fenn, J. B. Negative ion production with the electrospray ion source. *J. Phys. Chem.* **1984b**, *88*, 4671-4675.

Yeboah, F. K.; Alli, I.; Simpson, B. K.; Konishi, Y.; Gibbs, B. F. Tryptic fragments of phaseolin from protein isolates of Phaseolus beans. *Food Chem.* **1999**, *67*, 105-112.

Zaluzec, E. J.; Gage, D. A.; Watson, T. J. Quantitative assessment of cysteine and cystine in peptides and proteins following organomercurial derivatization and analysis by matrix-assisted laser desorption ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 359-366.

Zappacosta, F.; Diluccia, A.; Ledda, L.; Addeo, F. Identification of C-terminally truncated forms of beta-lactoglobulin in whey from Romagnola cows' milk by two dimensional electrophoresis coupled to mass spectrometry. *J. Dairy Res.* **1998**, *65*, 243-252.

Zayas, J. F. In *Functionality of Proteins in Food.* Zayas, J. F., Ed.; Springer-Verlag, Berlin, 1997, pp 1-5.

Zhang Z; Post C. B.; Smith D. L.; Amide hydrogen exchange determined by mass spectrometry: Application to rabbit muscle aldolase. *Biochemistry* **1996**, *35*, 779-791.

Zhang, Z.; Smith D. L. Thermal-induced unfolding domains in aldolase identified by amide hydrogen exchange and mass spectrometry. *Protein Sci.* **1996**, *5*, 1282-1289.

Zydney, A. L. Protein separation using membrane filtration: new opportunities for whey fractionation. *Int. Dairy J.* **1998**, *8*, 243-250.