EFFECTS OF ENZYMATIC DEPHOSPHORYLATION ON PROPERTIES OF BOVINE CASEIN

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ABSTRACT

Milk proteins represent an important source of protein ingredients due to their distinctive physico-chemical, nutritional, technological and functional properties. Casein content of milk represents about 80% of milk proteins. The distinguishing property of phosphorylation provides important properties to caseins. The objectives of this research were to investigate enzymatic dephosphorylation of caseins, to characterize products of dephosphorylation and to examine the effects of dephosphorylation on biological properties of caseins.

Bovine whole casein, α-casein and β-casein were dephosphorylated with potato acid phosphatase; optimum dephosphorylation conditions were 37 °C, pH 5.8 for 6 h. The extents of dephosphorylation accounted for 71.6, 89.2 and 73.7% for whole casein, α-casein and β-casein, respectively. The apparent V_{max} and apparent K_m for dephosphorylation of whole casein were 0.283 μmol P/mg casein min and 9.951 mg casein/l, respectively. SDS-PAGE, urea-PAGE, RP-HPLC and ESI-MS demonstrated effects of dephosphorylation on the caseins. Urea-PAGE and ESI-MS confirmed the identities of the individual fractions. ESI-MS established (a) the molecular weight for α-casein and β-casein as 23, 612 and 24, 017 Da, respectively; (b) random removal of 1, 2, 4, 6, 7 and 8 phosphate groups from α-casein and 1, 2, 3, 4 and 5 phosphate groups from β-casein and (c) effects of incubation conditions. The effects of dephosphorylation of α-casein and β-casein on the action of pepsin and trypsin were evaluated. Peptide mapping by RP-HPLC indicated that both proteases generated a complex mixture of peptides, with dephosphorylated peptides showing an increase in retention time. LC-ESI-MS and

MS/MS in conjunction with the use of advanced bioinformatics software resulted in the identification of the peptides generated. Dephosphorylated α-casein and β-casein showed the presence of peptides in which phosphate groups were removed, and were not observed in peptides from the corresponding native protein. Several of the peptides identified contained sequences that have been reported to be biologically active. Residual allergenicity of dephosphorylated whole casein, α-casein and β-casein as well as peptic and tryptic products of these caseins was determined by an ELISA technique. The results demonstrated that removal of phosphate groups from whole casein, α-casein and β-casein reduced allergenicity by 33, 31.2 and 24.4%, respectively. Proteolysis and dephosphorylation resulted in a significantly ($p < 0.05$) higher reduction in the antigen-antibody binding capacity compared to non-hydrolyzed and non-dephosphorylated caseins, particularly in the highly allergenic α-casein.

RÉSUMÉ

Les protéines du lait constituent une source importante d'ingrédients protéiniques grâce à leurs propriétés caractéristiques physico-chimiques, nutritionnelles, technologiques et fonctionnelles. La teneur en caséine du lait représente environ 80% des protéines du lait. La propriété distinctive de la phosphorylation dote les caséines d'importantes propriétés. Les objectifs de cette recherche étaient d'étudier la déphosphorylation enzymatique des caséines afin de caractériser les produits de déphosphorylation et d'examiner les effets de la déphosphorylation sur les propriétés biologiques des caséines.

La caséine bovine entière, la α-caséine et la β-caséine ont été déphosphorylées avec la phosphatase acide de pomme de terre; les conditions optimales de déphosphorylation étaient 37 °C à pH 5.8 pendant 6 h. L'ampleur de la déphosphorylation atteignit 71.6, 89.2 et 73.7% pour la caséine entière, la α-caséine et la β-caséine, respectivement. La V_{max} et K_m apparente pour la déphosphorylation de la caséine entière étaient de 0.283 μmol P/mg caséine min et 9.951 mg caséine/l, respectivement. Les méthodes SDS-PAGE, urea-PAGE, RP-HPLC et ESI-MS ont démontré des effets de déphosphorylation sur les caséines. Les méthodes urea-PAGE et ESI-MS ont confirmé les identités des fractions individuelles. La méthode ESI-MS a établi (a) le poids moléculaire pour la α-caséine et la β-caséine à 23, 612 et 24, 017 Da, respectivement ; (b) l'élimination aléatoire des groupes de phosphate de α-caséine 1, 2, 4, 6, 7 et 8 et des groupes de phosphate de β-caséine 1, 2, 3, 4 et 5 et (c) les effets des conditions d'incubation. L'influence de la déphosphorylation de α-caséine et de β-caséine sur l'action de la pepsine et la trypsine a été évaluée. Le traçage peptidique par la

méthode RP-HPLC a indiqué que les deux protéases ont produit un mélange de peptides complexe, les peptides déphosphorylés affichant une augmentation du temps de rétention. Les méthodes LC-ESI-MS et MS/MS couplées à l'utilisation d'un logiciel avancé en bioinformatique on permit l'identification des peptides obtenus. La α-caséine et la β-caséine déphosphorylées ont révélé la présence de peptides là où des groupes phosphates avaient été enlevés, et n'ont pas été observés dans les peptides de la protéine originale correspondante. Plusieurs des peptides identifiés, contenait des séquences ayant été rapportée comme étant biologiquement active. Le facteur allergénique résiduel de la caséine entière, la α-caséine et la β-caséine déphosphorylées de même que les produits de la pepsine et trypsine de ces caséines a été déterminé à l'aide d'une technique ELISA. Les résultats ont démontré que l'élimination des groupes phosphate de la caséine entière, la α-caséine et la β-caséine a réduit le facteur allergénique de 33, 31.2 et 24.4%, respectivement. La dégradation enzymatique et la déphosphorylation ont donné une plus grande réduction significative ($p < 0.05$) de la capacité de liaison antigène-anticorps comparativement aux caséines non-hydrolysées et non-déphosphorylées, particulièrement pour la hautement allergéniqe α-caséine.

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CLAIMS TO ORIGINAL RESEARCH

1. This is the first study to investigate the effects of enzymatic dephosphorylation of whole casein and to report the apparent kinetic parameters for enzymatic dephosphorylation of whole casein using an acid phosphatase enzyme.

2. This is the first study to investigate the extents of enzymatic dephosphorylation of α-casein and β-casein by determining casein molecular changes and dephosphorylated casein species which occur during dephosphorylation using electrospray ionization mass spectrometry (ESI-MS).

3. This is the first study to evaluate the effects of dephosphorylation of α-casein and β-casein on peptic, tryptic and combined peptic-tryptic hydrolysis and to characterize all the proteolysis products by using ESI coupled with food proteomics tools, including analysis by specialized protein database software and search engines.

4. This is the first study to investigate the effects of dephosphorylation and proteolysis on reduction in antigen-antibody binding of a mixture of dephosphorylated and peptic, tryptic and combined peptic-tryptic hydrolysis products of α-casein and β-casein.

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ABBREVIATIONS

ACE	Angiotensin-I-Converting Enzyme
Dalton	Da
ELISA	Enzyme-Linked Immunosorbent Assay
ESI-MS	Electrospray Ionization-Mass Spectrometry
IgE	Immunoglobulin E
kDa	KiloDalton
K_m	Michaelis-Menten Constant
LC-ESI-MS	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
LC-ESI-MS/MS	Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Time of Flight
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MW	Molecular Weight
m/z	Mass-to-Charge Ratio
PAGE	Polyacrylamide Gel Electrophoresis
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TCA	Trichloro Acetic Acid
V_{max}	Maximum Reaction Velocity
Z	Charge

LIST OF AMINOACIDS, THEIR ABBREVIATION AND LINEAR STRUCTURE

Amino Acid	Three Letter Code	Single Letter Code	Linear Structure
Alanine	ala	A	CH3-CH(NH2)-COOH
Arginine	arg	R	HN=C(NH2)-NH-(CH2)3-CH(NH2)-COOH
Asparagine	asn	N	H2N-CO-CH2-CH(NH2)-COOH
Aspartic Acid	asp	D	HOOC-CH2-CH(NH2)-COOH
Cysteine	cys	C	HS-CH2-CH(NH2)-COOH
Glutamic Acid	glu	E	HOOC-(CH2)2-CH(NH2)-COOH
Glutamine	gln	Q	H2N-CO-(CH2)2-CH(NH2)-COOH
Glycine	gly	G	NH2-CH2-COOH
Histidine	his	H	NH-CH=N-CH=C-CH2-CH(NH2)-COOH
Isoleucine	ile	I	CH3-CH2-CH(CH3)-CH(NH2)-COOH
Leucine	leu	L	(CH3)2-CH-CH2-CH(NH2)-COOH
Lysine	lys	K	H2N-(CH2)4-CH(NH2)-COOH
Methionine	met	M	CH3-S-(CH2)2-CH(NH2)-COOH
Phenylalanine	phe	F	Ph-CH2-CH(NH2)-COOH
Proline	pro	P	NH-(CH2)3-CH-COOH
Serine	ser	S	HO-CH2-CH(NH2)-COOH
Threonine	thr	T	CH3-CH(OH)-CH(NH2)-COOH
Tryptophan	trp	W	Ph-NH-CH=C-CH2-CH(NH2)-COOH
Tyrosine	tyr	Y	HO-Ph-CH2-CH(NH2)-COOH
Valine	val	V	(CH3)2-CH-CH(NH2)-COOH

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Milk consists of approximately 13% solids and 87% water; however, the solids content determines the value of milk (Jenness, 1988). Technology allows processors to disassemble milk into its various component parts; these can be combined in different ways to make new products or to be used as specific ingredients in other foods (Fox, 2001). Milk composition is also of extreme importance to the consumer from a nutritional point of view; it contains a mixture of high value proteins, necessary amino acids and significant quantities of inorganic salts (Murphy & Allen, 2003).

In general, milk proteins are trusted by consumers as wholesome, natural and nutritious food components. In the food industry, milk proteins comprising of caseins and whey have been recognized by possessing essential functional and physicochemical properties as foods ingredients. The protein system in milk, especially in bovine milk, has been studied for nearly 200 years and very significant progress has been made during the past years (Fox & McSweeney, 1998). Although, the principal milk-specific proteins have been very well characterized already, the rapidly expanding tools of biotechnology are enabling a new perception of food proteins. Proteomics and their analyses are revealing the molecular details of the remarkable structural complexity and design of milk proteins (Chalkley et al., 2005).

Milk caseins precipitate by acidification of milk; the whey proteins remain soluble (Wong et al., 1996). Casein has been produced commercially for at least 80 years. Initially casein was used for industrial purposes because of its good binding properties

(e.g., adhesives, paper coating, paints and cosmetics). However, in the 1960's commercial casein preparations gained importance as food proteins, due mainly to pioneering work carried out in Australia and New Zealand. Presently, casein produced by acid or rennet coagulation, is one of the principal functional food proteins with an annual world production of approximately 250, 000 tonnes; casein is considered to posses unique properties and cannot be replaced by other proteins in certain food applications (Fox, 2001). α_s-casein and β-casein are the major protein components of milk.

A characteristic of caseins is their microheterogeneity with varying extents of phosphorylation and glycosylation. Caseins contain phosphate groups bound to serine and threonine. The phosphate groups of caseins are known to affect many characteristics of the protein including casein digestion and bioavailability of divalent cations. Additionally, caseins may influence the immune system causing allergenic reactions, especially as the phosphoseryl residues are considered to be immunoreactive and resistant to digestion. In most food applications there is growing interest in studying protein modifications that can provide new benefits and safety to the consumers.

Modification of caseins without compromising their functional properties is desirable for many food applications. One approach to minimizing the undesirable biological effects of phosphorylated caseins is to remove the phosphate groups; this dephosphorylation can be achieved both chemically and enzymatically.

1.2 Rationale and Objectives of Study

Knowledge on milk proteins generally advances as new and more sophisticated analytical techniques are developed; milk proteins continue to be characterized in every

increasing detail. Even though, there is relative abundance of information in the literature on dephosphorylation of casein fractions, understanding of the effects on the properties of casein after post-translational modification of the phosphate groups and the characterization of dephosphorylation products are required.

The overall objectives of this research were to investigate enzymatic dephosphorylation of caseins and to examine the effects of dephosphorylation on properties of caseins. The specific objectives of this study were:

1. To enzymatically dephosphorylate whole casein, α-casein and β-casein, to investigate the kinetics and extent of dephosphorylation and to establish optimum dephosphorylation conditions of caseins.

2. To investigate molecular characteristics of products of dephosphorylated whole casein, α-casein and β-casein using a combination of polyacrylamide gel electrophoresis (SDS and urea-PAGE), reversed phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS).

3. To evaluate the effects of dephosphorylation on biological properties of caseins, including *in vitro* enzymatic hydrolysis simulating human gastric conditions and peptide mass mapping based on proteomic techniques, including liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (MS/MS), followed by database search.

4. To study the effects of dephosphorylation and *in vitro* enzymatic hydrolysis of caseins on allergy reduction evaluated by enzyme-linked immunosorbent assay (ELISA).

CHAPTER 2

LITERATURE REVIEW

2.1 Milk Proteins

Bovine milk represents an important and valuable source of protein ingredients due to their recognized superior nutritional, organoleptic and functional properties of milk proteins (Singh, 2003). Milk is a polyphasic secretion of the mammalian gland and also a source of fat and lactose as food ingredients, contains 3.9% fat, 3.3% protein, 5% lactose and 0.7% minerals (Jenness, 1988). The milk proteins include several caseins, several whey proteins, fat globule membrane proteins and enzymes. The caseins are phosphoproteins and compromise approximately 80% of the total protein content in milk. Whey proteins represent 20% of the total milk proteins and consist mainly of α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobins (Farrell et al., 2004). Both the caseins and whey protein fractions are quite heterogeneous and proteins within each group have very different molecular and physico-chemical properties. Some of the more important properties of the milk proteins are summarized in Table 2.1. A review of the information on properties of the caseins follows.

2.2 Physicochemical Characteristics of Caseins

Unlike many typical globular proteins, caseins are not present in milk as individual molecular structures, but rather as large protein complexes which also incorporate milk salts, particularly calcium phosphate. The native structure is a protein complex resulting from the interaction of all the individual caseins. Nevertheless,

Table 2.1: Characteristics of the principal proteins in bovine milk (Ng-Kwai-Hang, 2003)

Protein	Molecular Mass (Da)	Amino acid Residues			PO_4 residues	Genetic variants detected
		Total	Pro	Cys		
α_{s1}-casein	23 614	199	17	0	8	A, B*, C, D, E, F,G, H
α_{s2}-casein	25 388	207	10	2	10-13	A*, B, C, D
β-casein	23 983	209	35	0	5	A^1, A^{2}*, A^3, B, C, D, E, F, G
κ-casein	19 038	169	20	2	1	A*, B, C, E, F^S, F^I, G^S, G^E, H, I, J
β-lactoglobulin	18 277	162	8	5	0	A, B*, C, D, E, F, H, I, J
α-lactalbumin	14 175	123	2	8	0	A, B*, C
Serum albumin	66 267	582	28	35	0	

* Molecular mass for the genetic variant

knowledge of the physicochemical behavior of the individual caseins provides for an understanding of their individual structures as well as how these structures may interact to form the casein micelle.

2.2.1 Casein Structure

Casein micelles are the colloidal aggregates of caseins and mineral calcium phosphate. Destabilization of the casein micelles results in coagulation or gelation, which is exploited in the production of a range of dairy products (Horne, 2003). In the most widely accepted casein micelle model, the casein micelle is composed of sub-micelles (Schmidt, 1982). The sub-micelles are held together by nano-crystals of calcium phosphate and by hydrophobic and hydrogen bonds. This rearrangement results in the whole micelle having a hairy appearance (Figures 2.1 and 2.2).

Caseins have low levels of secondary and tertiary structure (Farrell, 1988). They are neither globular proteins nor truly random coil polymers. They are rheomorphic proteins, adopting open and flexible structures in solution dictated by the environmental circumstances in which they find themselves (Swaisgood, 1982). Caseins have little α-helical structure, no denaturation temperature and hydrodynamic volumes (Sawyer & Holt, 1993). Their open conformation allows relatively easy access to proteolytic enzymes, facilitating rapid and extensive proteolysis. Caseins are stable to denaturing agents, e.g. urea, and they possess high surface activity (Horne, 2003).

The three-dimensional structure of the major caseins cannot be determined by X-ray crystallography (caseins cannot be crystallized) or by nuclear magnetic resonance (NMR) spectroscopy (Swaisgood, 2003), this is because it is not possible to dissolve the caseins at a sufficient high concentration at which the native-like monomeric structure is

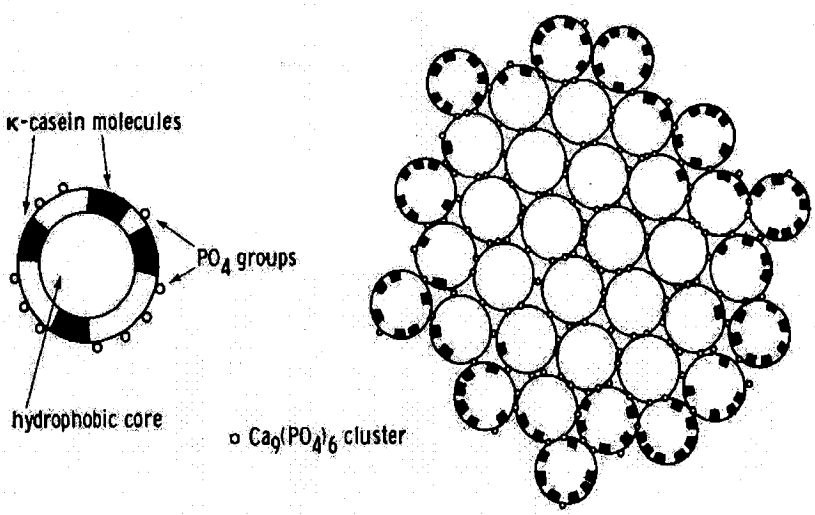


Figure 2.1: Schematic representations of a submicelle and a casein micelle composed of submicelles (Schmidt, 1982)

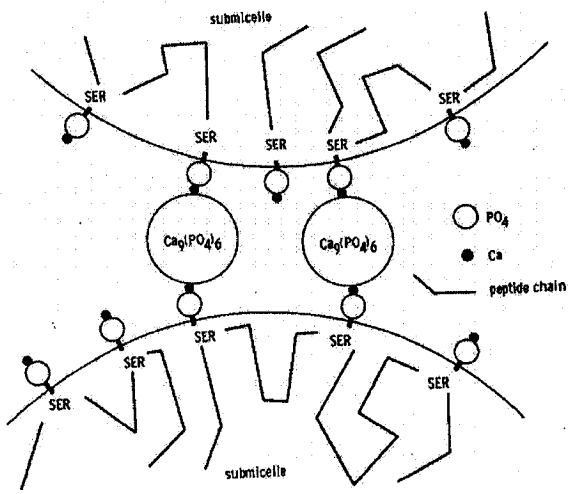


Figure 2.2: Schematic diagram of the binding of two submicelles via $Ca_9(PO_4)_6$ clusters (Schmidt, 1982)

retained. Estimates of structural features have been made by using prediction methods, from peptide structures determined by NMR spectroscopy and from physicochemical studies (Kumosinski et al., 1994).

2.2.2 Heterogeneity of Caseins

Whole casein is isolated from milk by destabilizing the micelle suspension and can be fractionated to give several individual caseins. Bovine casein consists of the following four caseins: α_{s1}-, α_{s2}-, β- and κ-casein. These represent approximately 37, 10, 35 and 12% respectively, of whole casein (Fox & McSweeney, 1998). The molecular weight of caseins varies from 20 to 25 kDa. All caseins contain high levels of proline which prevents the formation of α-helices, β-sheets and β-turns (Ng-Kwai-Hang, 2003).

Caseins are very heterogeneous with few structural features in common. This heterogeneity is complicated by their genetic polymorphism, resulting in several variants for each protein (Farrell et al., 2004). To date, 32 variants of the bovine caseins have been identified; genetic polymorphism is normally detected either by electrophoresis, when substitutions cause a change in charge or when substitutions involving uncharged residues (silent substitution) can be detected by mass spectrometry (Fox & Kelly, 2003). Caseins contain phosphate bound to the amino acid serine and occasionally threonine. The caseins are relatively hydrophobic and have a high surface hydrophobicity due to their open structure. The hydrophobic, polar and charged residues are not uniformly distributed through the sequences but occur as hydrophobic or hydrophilic patches, giving the caseins strongly amphiphatic structures, which make them highly surface active (O'Connell et al., 2003).

α_{s1}-casein contains eight phosphate residues located in the hydrophilic region between residues 45 and 89 (Swaisgood, 2003). α_{s1}-casein has the highest negative charge (22 at pH 6.5); it contains more acidic amino acids than basic ones (Wong et al., 1996). Both prediction and spectral methods indicate 24% helical structure in α_{s1}-casein (Holt & Sawyer, 1988); this little discernible secondary structure may be due to the 8.5% proline content distributed in the polypeptide chain. Three hydrophobic regions are identified in the residues: 1 to 44, 90 to 113 and 132 to 199. Variant A differs by a deletion of 13 residues (14 to 26) in the N-terminal sequence; the sequence 41-80 is very polar due to the presence of seven seryl phosphate residues, eight glutamates and three aspartates (Ng-Kwai-Hang, 2003). The C and D variants consist of Gly, Thr-P and Glu, and Ala at positions 192 and 53, respectively; the five genetic variants A, B, C, D and E show a decreasing electrophoretic mobility in urea alkaline gels; these variants constitute the major components of α_{s1}-casein (Wong et al., 1996). Variants F, G and H have been recently reported (Farrell et al., 2004). α_{s0}-casein is a minor component identified (Eigel et al., 1984); it has the same amino acid sequence as α_{s1}-casein B-8P but contains one additional phosphoseryl residue at position 41.

α_{s2}-casein is phosphorylated at various extents, containing 10, 11, 12 or 13 phosphate residues; the reference casein for this family is α_{s2}-casein A-11P (Farrell et al., 2004). α_{s2}-casein shows similar characteristics to α_{s1}-casein except that there is a minimum association at a NaCl concentration of about 0.2 mol/l (Creamer, 2003). α_{s2}-contains 2 cysteine residues at position 36 and 40 and it can occur as disulphide-linked dimers of α_{s2}-casein A-12P and α_{s2}-casein A-11P (Rasmussen et al., 1994). Four variants are known, A, B, C and D. The complete amino acid sequence of α_{s2}-casein A-11P

consists of 207 residues (Swaisgood, 1992). The polypeptide chain consists of a large number of positively charged side chains, especially in the C-terminal segment in the regions: 5 to 18, 49 to 68 and 126 to 145; it possesses a large hydrophilic region 90 to 120 (Creamer, 2003). α_{s2}-casein contains a low proline content and among caseins is the least hydrophobic (Farrell et al., 2004). The segments 50 to 123 and 130 to 207 contain 38% identical residues, indicating high internal homology a possible result of a duplicate primitive gene (Wong et al., 1996).

β-casein contains five phosphate residues; some β-casein molecules contain four phosphate residues (Fox & Kelly, 2003). The reference casein for this family is β-casein A1-5P which is a single polypeptide chain of 209 residues (Ribadeau Dumas et al., 1972). Seven genetic variants have been identified, A1, A2, A3, B, C, D and E (Eigel et al., 1984); recently variants F, G and H have been reported (Farrell et al., 2004). The absence of cysteine from α_{s1}- and β-casein increases their flexibility (Wong et al., 1996). β-casein has a high negative charge located in the N-terminal segment (1 to 43) containing all the phosphoseryl residues. Half of the C-terminal (residues 136-209) is highly hydrophobic (Cassiano & Areas, 2003); this concentration of hydrophilic and hydrophobic residues in the terminal ends makes β-casein the most surfactant casein among the others. Plasmin (a protease in milk) can cause a proteolytic effect of β-casein generating γ-caseins by cleaving 28-29 and 105-106/107-108 bonds (Swaisgood, 2003). β-casein contains very low contents of periodic structure, approximately 13% of predicted helical content (Holt & Sawyer, 1988).

κ-casein contains one or two phosphate groups. The only glycosylated casein is κ-casein. It contains galactose, galactosamine and N-acetylneuraminic acid which occur

either as trisaccharides or tetrasaccharides attached to threonine residues in the C-terminal region (Fox & Kelly, 2003); the presence of oligosaccharides increases κ-casein hydrophilicity. The N-terminal 2/3 of k-casein is hydrophobic while the C-terminal 1/3 is strongly hydrophilic; this structural feature is significant for the properties and stability of casein micelles (Sood et al., 2003). The primary structure of κ-casein contains 169 amino acid residues. The amphiphatic character of κ-casein enhances the formation of micelles in solution (Swaisgood, 2003); unlike the other casein, κ-casein does not bind calcium extensively and it is not sensitive to calcium precipitation. The genetic variants of κ-casein are A, B, C, E F^S, F^1, G^S, G^E, H, I and J (Ng-Kwai-Hang, 2003). κ-casein occurs in the form of a mixture of disulfide-bonded polymers (Groves et al., 1992). κ-casein structure can be affected by chymosin (rennin), the bond 105-106 is the cleavage site that generates para-κ-casein (residues 1-105) and the macropeptide (residues 106-169) (Bingham, 1975). The stability of the casein micelle depends on the presence of k-casein on the surface of the micelle where it functions as an interface between the hydrophobic caseins (Creamer et al., 1998).

2.2.3 Phosphorylated Caseins

Milk contains about 900 mg phosphorus/l, which occurs in five types of phosphate-containing compounds including: inorganic phosphorus, insoluble and colloidal phosphates; organic phosphorus in phospholipids, casein and sugars phosphates and nucleotides. Whole casein contains about 0.85% phosphorous, α_{s1}-, β- and κ-caseins contain 1.1, 0.6 and 0.16% P, respectively (Fox & McSweeney, 1998). The phosphate groups are esterified as monoesters of serine or, to a minor extent of threonine.

Adenosine triphosphate (ATP) provides the phosphate and phosphorylation is catalyzed by casein kinase. A specific sequence, Ser.X.A (where X is any amino acid and A is an anionic residue, i.e. Glu, Asp or SerP) is required for phosphorylation. Most of the phosphoserine residues in casein are present in clusters (Fox & Kelly, 2003). A phosphate cluster occurs when the phosphoseryl residues are concentrated within a short length of the polypeptide chain as shown in Figure 2.3. The phosphorus is covalently bound to the protein and is removed only by severe heat treatment, high pH or some phosphatases. Phosphate groups are important in many aspects: nutritionally, because they bind large amounts of Ca^{2+} (Lopez-Huertas et al., 2006). However, they also bind Zn^{2+} and other polyvalent cations strongly (Bouhallab & Bougle, 2004). They increase the solubility of caseins; and they probably contribute to the high heat stability of casein (Besler et al., 2001).

2.3 Modification of Caseins

Modification of proteins is used in protein chemistry to study the relationship between structure and biological functions. It is also used extensively to improve the functional properties of food proteins with potential applications in the food industry.

2.3.1 Chemical Modification

The chemical process of modifying caseins usually involves acetylation, phosphorylation, dephosphorylation, succylation, deamidation, decarboxylation, esterification, glycosylation, reductive alkylation, sulfitolysis and thiolation of isolated and purified proteins (Means & Feeney, 1990). Processes for modification of caseins to

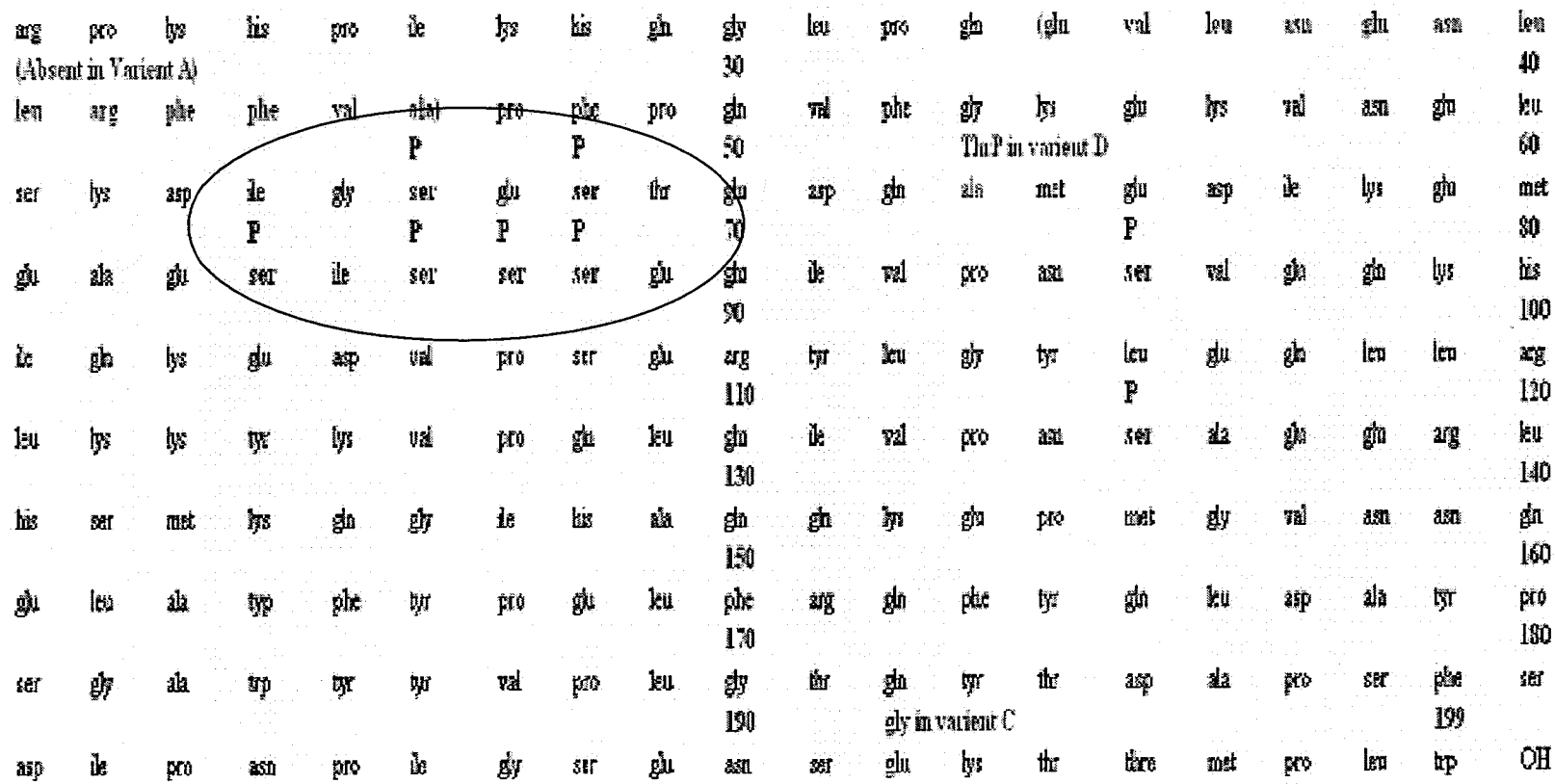


Figure 2.3: Primary structure of α_{s1}-casein, indicating the presence of phosphate clusters (Mercier et al., 1971)

meet the requirements of food products and pharmaceutical industries have been developed (Arai et al., 1986). Certain functional groups of amino acid residues (amino, carboxyl, disulfide, guanidine, hydroxyl, indole, imidazole, phenolic, sulfhydryl and thioether) are the target for physicochemical manipulation that lead to the formation of products with enhanced functionalities compared to the native proteins (Feeney, 1987). Caseins serve as functional food additives, contributing adhesive, emulsion, coagulation, or viscoelastic properties to foods by means of hydrophobic associations and electrostatic interactions (Kinsella, 1984). However caseins have a minimal number of sulfhydryl groups. Disulfide-sulfhydryl interactions are important forces in the conformation and activity of the protein. Sulfhydryl groups were introduced to α_{s1}-casein by the process of thiolation resulting in a very stable foam of this casein (Okumura et al., 1990); foam stability is affected by the tertiary structure of proteins, which was stabilized by disulfide bonds. β-casein was esterified to different extents by using different alcohols; after this modification the emulsifying activity and stability of esterified β-casein in the acidic pH range of 3-5 were generally higher compared to the corresponding native proteins (Sitohy et al., 2001). The effects of chemical glycosylation and increase of the ionic strength of α_{s1}- and β-casein were evaluated (Cayot et al., 1991); it was demonstrated that after chemical modification there was an improvement in the solubility of these caseins and glycosylation had an improvement on the emulsifying properties of a mixture of α_{s1}-casein, β-casein and galactosylated β-casein.

2.3.2 Enzymatic Modification

The use of enzymes to modify the structure of caseins provides a more systematic approach since enzymes are specific and enzymatic reactions can be controlled more than chemical reaction. The principles and methods for casein enzymatic modification include the use of rennet, bacteria proteinases and peptidase during cheese production (Fox, 1988). The classic example of enzyme modification of milk proteins is the action of chymosin, which cleaves the sensitive bond Phe_{105}-Met_{106} of κ-casein (Fox et al., 1990); this triggers a sequence of events that lead to the aggregation of casein micelles that encapsulate some fats to form cheese curd. Proteolysis in cheese is affected by many factors including activity of residual rennet and indigenous milk proteases (Grappin et al., 1985); this type of protein modification affects both the texture and flavor of the cheese in which occurs.

Bioactive peptides formation is another approach to modify caseins, when caseins are digested by proteolytic enzymes such as pepsin, trypsin and chymotrypsin (Torres-Llanez Mde et al., 2005). Other approaches to modify caseins include transglutamination, deamination, dephosphorylation and, succinylation (Imafidon et al., 1997). For example, α_{s1}-casein and β-casein can be modified with more amphiphilic structure and higher surfactancy than the native casein by succinylating (Schwenke et al., 1981).

2.3.3 Dephosphorylation

2.3.3.1 Chemical Dephosphorylation

Dephosphorylation of casein occurs as a result of alkaline hydrolysis, releasing phosphate and producing serine. Dephosphorylation can also proceed through a β-

elimination reaction producing dehydroalanine residues capable of intermolecular or intramolecular reaction with lysine to form lysinoalanine. The optimum pH for the reaction is 12 (Ward & Bastian, 1998). The use of hydrofluoric acid has been also applicable to remove phosphate moieties from phosphoproteins and peptides, including α-casein, without any cleavage of other bonds. The phosphate ester linkage of serine, threonine or tyrosine was successfully cleaved despite the high acidity of hydrofluoric acid (Kuyama et al., 2003). Some of the chemical reactions available for intentional protein modification can be replaced by enzymatic reactions, which have the advantage of milder reaction conditions.

2.3.3.2 Enzymatic Dephosphorylation

Enzymatic dephosphorylation of milk proteins is more acceptable to the food processors than those from chemical alterations (Rolle, 1998). Dephosphorylation of caseins by enzymes results in release of the phosphate group and the serine residue is preserved. Milk contains both alkaline and acid phosphatases, which are phosphomonoesterases capable of dephosphorylating phosphoserine residues. A variety of phosphatases have been used to remove the phosphate groups from casein (Li-Chan & Nakai, 1989; Lorient & Linden, 1976). However, acid phosphatase from potato shows several advantages; it does not require activators, is active at pH 7, can be obtained commercially in a highly purified form and hydrolyzes only monoesters of phosphate. Acid phosphatase has a molecular weight of approximately 42, 000 and has a pH optimum of 4.9 using p-nitrophenyl phosphate as substrate (Bingham et al., 1976). Alkaline phosphatase is a dimer with a molecular weight of approximately 160 000-190

000 Da and contains four to five Zn atoms per molecule (Andrews, 1992).The pH optimum for dephosphorylation of isolated CN in buffered solutions is between 6 and 7 (Lorient & Linden, 1976). Studies on dephosphorylation of individual caseins by alkaline phosphatase have been conducted. Lorient & Linden (1976) reported a more efficient dephosphorylation of individual α-casein, β-casein and κ-casein rather than mixtures of the same caseins; however at alkaline pH required for this phosphatase the presence of lactose, whey proteins and phosphate ions has an inhibitory effect on alkaline phosphatase. (Li-Chan & Nakai (1989) investigated the dephosphorylation of caseins with calf intestinal alkaline phosphatase and potato acid phosphatase. Incubation with the first enzyme produced a lower percentage in dephosphorylation when compared with the acid phosphatase results. Dephosphorylation of whole casein was less efficient than dephosphorylation of individual casein fractions. Dephosphorylation of bovine skim milk with acid phosphatase indicated a resistance of the casein micelles to the enzyme. β-casein and a derived phosphopeptide were dephosphorylated by *Escherichia coli* alkaline phosphatase and bovine spleen phosphoprotein phosphatase (West & Towers, 1976); the first enzyme was able to remove phosphate groups in two stages, since casein molecules retaining three of the original phosphoseryl residues were resistant to further dephosphorylation at low enzyme concentrations. In contrast, complete dephosphorylation of these caseins was achieved by bovine spleen phosphoprotein phosphatase without any inhibition. Pearse et al. (1986) studied the dephosphorylation of β-casein and κ-casein with alkaline phosphatase in order to evaluate the effect of altering the casein composition of an artificial milk micelle; incorporation of partial or complete dephosphorylated β-casein had an adverse result on coagulation and syneresis.

There is relatively little information with regards to kinetic studies of dephosphorylation of casein. Bingham et al. (1976) assessed the effect of varying α_{s1}-casein concentration on potato acid phosphatase activity; results indicated that phosphate groups are competitive inhibitors of this enzyme. Additionally, kinetic constants were determined for the dephosphorylation of α_{s1}-casein at pH 7 and *p*-nitrophenyl phosphate as substrates. The V_{max} value for α_{s1}-casein was seven-fold lower than that of V_{max} for *p*-nitrophenyl phosphate, while the K_m values for both substrates were similar. West & Towers (1976) studied a phosphate transfer reaction through kinetics of dephosphorylation of a phosphopeptide derived from β-casein, containing a cluster of phosphate groups, by bovine spleen phosphoprotein phosphatase; results indicated that the phosphate groups were removed in a sequential manner; the rate constants were calculated for each dephosphorylation stage indicating an enhancement of the activity of the phosphate cluster in transferring phosphate.

2.4 Biological Properties of Caseins

The understanding of the biological role of the caseins is required beyond their nutritional functions, especially since caseins are widely used in numerous food ingredients. The biological effects of caseins related to casein proteolysis and allergenicity play important roles in the development of new food ingredients that decrease or mitigate adverse consequences. Modification of caseins without compromising their functional properties is considered important to reduce undesirable biological effects.

2.4.1 Proteolysis of Caseins

Caseins are relatively easily digested in the intestine compared with many other food proteins (Bos et al., 2000); successive *in vitro* digestion of casein with pepsin and trypsin yields peptides that contain physiological and biological functions (Pihlanto-Leppälä et al., 1994). During the complete digestion process, before proteins reach the large intestine, a less specific digestion is required in the small intestine, carried out by pepsin, which is an aspartate protease occurring in the stomach with an optimum pH 2–7; further digestion is accomplished by trypsin and chymotrypsin, which are serine proteases from the intestine with an optimum pH 7–9 (Schmelzer et al., 2004); these endopeptidases are not specific to a certain substrate, but are capable of cleaving almost any protein inside the peptide chain.

Increasing attention has been placed on the extent of proteolysis, the type of enzymes used and the conditions for proteolysis. Several researchers have studied peptides, non-dephosphorylated and dephosphorylated products derived from hydrolysis with specific gastric proteases such as trypsin and chymosin (Visser et al., 1995; Lemieux & Amiot, 1989) using β-casein as model. Tryptic digestion of β-casein yields several important nutraceutic and nutritious peptides. However, a final peptide (1-105) product causes enzyme inhibition; Chobert et al. (1998) described a mutation replacement of Lys_{188} for aromatic amino acid residues that altered the specificity of trypsin for cleaving β-casein in many places, hydrolyzing as well the fragment 1-105. Guillou et al. (1991) investigated the hydrolysis of βA2-casein by bovine chymosin and pepsin A to compare the hydrolysis of the two enzymes on this protein; several peptides were identified as well as the preferred cleavage site for both proteases. The peptides produced from native and

dephosphorylated α-casein need also to be studied since α-casein is the major fraction present in bovine milk (Blanc, 1981).

2.4.1.1 Proteolysis of Dephosphorylated Casein

Phosphate removed enzymatically from casein serine residues produce proteins that possess unique and specific biological properties. Removal of some or most of the negatively charged phosphate groups from caseins cause a modification in the net casein charge and consequently, the flexibility and properties of the new modified casein structure are altered (Cassiano & Areas, 2003). The conformational change achieved by dephosphorylation may influence the effect of proteinases, altering the biological significance of the caseins. The high degree of casein phosphorylation may cause inhibition of certain digestive enzymes, leading to the formation and accumulation of longer peptides, which may negatively affect final organoleptic characteristics of cheese, and influence its final quality (Alli et al., 1998). Dephosphorylated casein hydrolysates have been used recently as nutritional food ingredients as they have shown to exert favorable digestive and metabolic effects on people suffering from severe gastrointestinal diseases (Maubois & Leonil, 1989). Additionally, curd or clot formation can influence digestion of casein, especially in infants; *in vitro* digestibility of modified caseins by gastrointestinal enzymes, demonstrated that dephosphorylated casein improved digestibility properties and acid clotting of bovine casein for infant feeding (Li-Chan & Nakai, 1989). Dephosphorylated caseins can find applications in the formulation of infant formulae in which rapid digestion by gastric enzymes is highly desirable (West, 1986).

2.4.1.2 Products of Casein Proteolysis

Proteolysis of milk proteins to generate peptides has been practiced for a long time. Enzymatic modification of casein produces peptides and in some cases free amino acid residues (Kilara & Panyam, 2003). The accumulation of hydrophobic peptides causes bitterness which is a common occurrence and problem in many cheese varieties (Singh et al., 2005). Some of the peptides in milk proteins are capable of affecting biological functions of an organism (Meisel et al., 2001); these biological effects may play an important role in the development of medical foods that treat or mitigate the effects of diseases and increase the nutraceutical aspects of foods containing casein peptides. Additionally, casein-derived bioactive peptides represent a source of health enhancing components that may be incorporated in functional foods and in pharmaceutical preparations.

2.4.1.3 Bioactive Peptides

A variety of food protein peptides has been demonstrated to elicit biological effects in various *in vitro* or *in vivo* test systems. These compounds from food proteins are known as biologically active peptides and are identified as digestion products of several food proteins. Bioactive peptides are absorbed intact and modulate digestion, appetite and endocrine metabolism by binding to specific receptors (Yamamoto et al., 2003).

Biologically active peptides derived from milk proteins are inactive within the sequence of the precursor proteins but can be released by enzymatic proteolysis. Bioactive peptides of milk protein have been classified (Meisel, 2005) into four groups:

1) opiod agonist, including casomorphins, exorphins and α- and β-lactorphins, 2) opiod antagonist (casoxins), 3) immunostimulants (immunopeptides) and 4) mineral carriers or caseinophosphopeptides. The term opiod refers to morphine-like effects which include signs of sedation, tolerance, sleep induction and depression. Opiod receptors (μ-, δ- and κ-type) are located in the nervous, endocrine, and immune system as well as in the gastrointestinal tract of the mammalian organism (Teschemacher et al., 1997). The common structural feature among exogenous and endogenous opiod peptides is the presence of a tyrosine residue at the amino terminal end (except in α-casein opiods) and the presence of another aromatic residue such as phenylalanine or tyrosine in the third or fourth position (Chang et al., 1981); this structural motif fits into the binding site of opiod receptors. A negative potential formed close to the phenolic hydroxyl group of tyrosine is essential for opiod activity. Even though bioactive peptides have already found interesting applications as dietary supplements and as pharmaceutical preparations; Garvey (2002) stated that peptides with opiod activity may cause or trigger autism. Autism being a syndrome with multiple nongenetic and genetic causes has been reported to increase 556% in pediatric prevalence between 1991-1997 (Muhle et al., 2004). Peptides with opiod activity are highly resistant to further hydrolysis by intestinal enzymes; once adsorbed into the blood, these peptides can pass to the brain and various other organs to elicit an opiod or morphine-like effect (Teschemacher et al., 1997).

Food bioactive peptides from milk proteins are also claimed to be health enhancing components for functional foods that are used to reduce the risk of disease or to enhance a certain physiological function, even insignificant amounts of liberated peptides maybe sufficient to exert biological effects (Meisel et al., 2001). Many different

roles can be attributed to these peptides. Milk-derived casomorphins contribute to the regulation of nutrient assimilation by decreasing intestinal motility and improving digestion and absorption, particularly β-casomorphins (Froetschel, 1996); when released into the small intestine during the digestion of β-casein, these bioactive peptides are absorbed intact and inhibit gastrointestinal motility and the emptying rate of the stomach (Daniel et al., 1990). Moreover, β-casomorphins possess antidiarrheal effects which results from the enhancement of water and electrolyte absorption stimulated by the casomorphins. Several studies (Miguel et al., 2006; FitzGerald & Meisel, 2000) have demonstrated that casokinins and lactokinins sequences can inhibit the activity of Angiotensin-I-converting enzyme (ACE), which is involved in vasoconstriction of blood vessels, a part of blood flow regulation, this action has antihypertensive effects.

Bioactive peptides are also related to bactericidal activity, especially lactoferricin derived from the whey iron-binding glycoprotein lactoferrin (Tomita et al., 1994). This peptide fragment (17-41) from lactoferrin is generated *in vitro* upon enzymatic digestion of lactoferrin with pepsin. The mode of action against micro-organisms is by increasing the cell membrane permeability (Strom et al., 2000). A casein derived peptide from κ-casein glycopeptide has the ability to protect against caries, since it inhibits viral of bacterial adhesion to oral surfaces (Brody, 2000). Several peptide fractions can affect immune functions by simulate phagocyte activity of macrophages that cause biochemical changes in the cell; when the phagocytic activity of immune cells increases, it confers protection against bacterial infections (Faith et al., 1984).

Phosphopeptides can exert an influence on absorption in the intestine of calcium or other minerals including iron, magnesium and zinc; casophosphopeptides can form

soluble organophosphate salts that work as mineral carries (Sato et al., 1986). It was found that the high number of phosphate residues present in α_{s1}-casein, α_{s2}-casein and β-casein represent the binding site for these minerals (West, 1986). Yeung et al. (2001) reported that iron bound to casein phosphoserine clusters becomes less available for digestion unless successive digestion of casein with pepsin and trypsin yields peptides that can be dephosphorylated by intestinal alkaline phosphatase; hence the divalent metals bound can be accessible for absorption.

2.4.1.4 Techniques for Identification of Products of Casein Proteolysis

Three methods used to analyze casein peptides are: (i) electrophoretic techniques using polyacrylamide gels with urea (Pardo & Natalucci, 2002) or sodium dodecylsulphate (Janer et al., 2005), isoelectric focusing (Gauthier & Pouliot, 2003) and more recently two-dimensional gel electrophoresis (Mamone et al., 2003), (ii) high-performance liquid chromatography (HPLC) by ion-exchange (Juillard et al., 1998) and especially reversed-phase modes (Lemieux & Amiot, 1989; Janssen et al., 1984) and (iii) immunological methods (Lv et al., 2003). Each method has its own advantages; however liquid chromatography has become a powerful tool, especially when coupled with mass spectrometry (Weber et al., 2006; Miquel et al., 2005). This technique has made possible to obtain major advances in the identification of peptides derived from milk proteins.

2.4.1.4.1 Food Proteomics

Proteomics is an area of molecular biology that is concerned with the systematic analysis of proteins. The proteome is a complex and dynamic entity that can be defined in

terms of the sequence, structure, abundance localization, modification, interaction and biochemical function of its components, providing a rich and varied source of data and information (Twyman, 2004). Post-translational modification either by cleavage of the polypeptide backbone, chemical or enzymatical modification of specific amino acids side chains increases the complexity of the proteome; therefore there is a need for techniques that allow the separation of protein mixtures into their individual components followed by characterization of the individual proteins or peptides (Kwon et al., 2006).

Several bioanalytical techniques are currently used to solve emerging challenges associated with proteomics; they include two-dimensional polyacrylamide gel electrophoresis 2D-PAGE, electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionization mass spectrometry time of flight (MALDI-TOF MS), tandem mass spectrometry (MS/MS) and liquid chromatography LC-MS/MS. Combination of these techniques can result in powerful tools to characterize post-translational modification of proteins (Figure 2.4). MS is complementary to other protein and peptide analysis methods such as protein sequencing, amino acid analysis and high performance liquid chromatography peptide mapping. Additionally, it is particularly useful for identifying post-translational modifications which are quite difficult to distinguish by other methods; MS also can replace alternative methods for protein sequencing, such as Edman degradation (Jensen, 2004).

2.4.1.4.2 Mass Spectrometry for Identification of Proteolysis Products

Milk and derived peptides have been the subject of numerous MS investigations; these include identification of milk protein variants and glycoforms, fingerprinting,

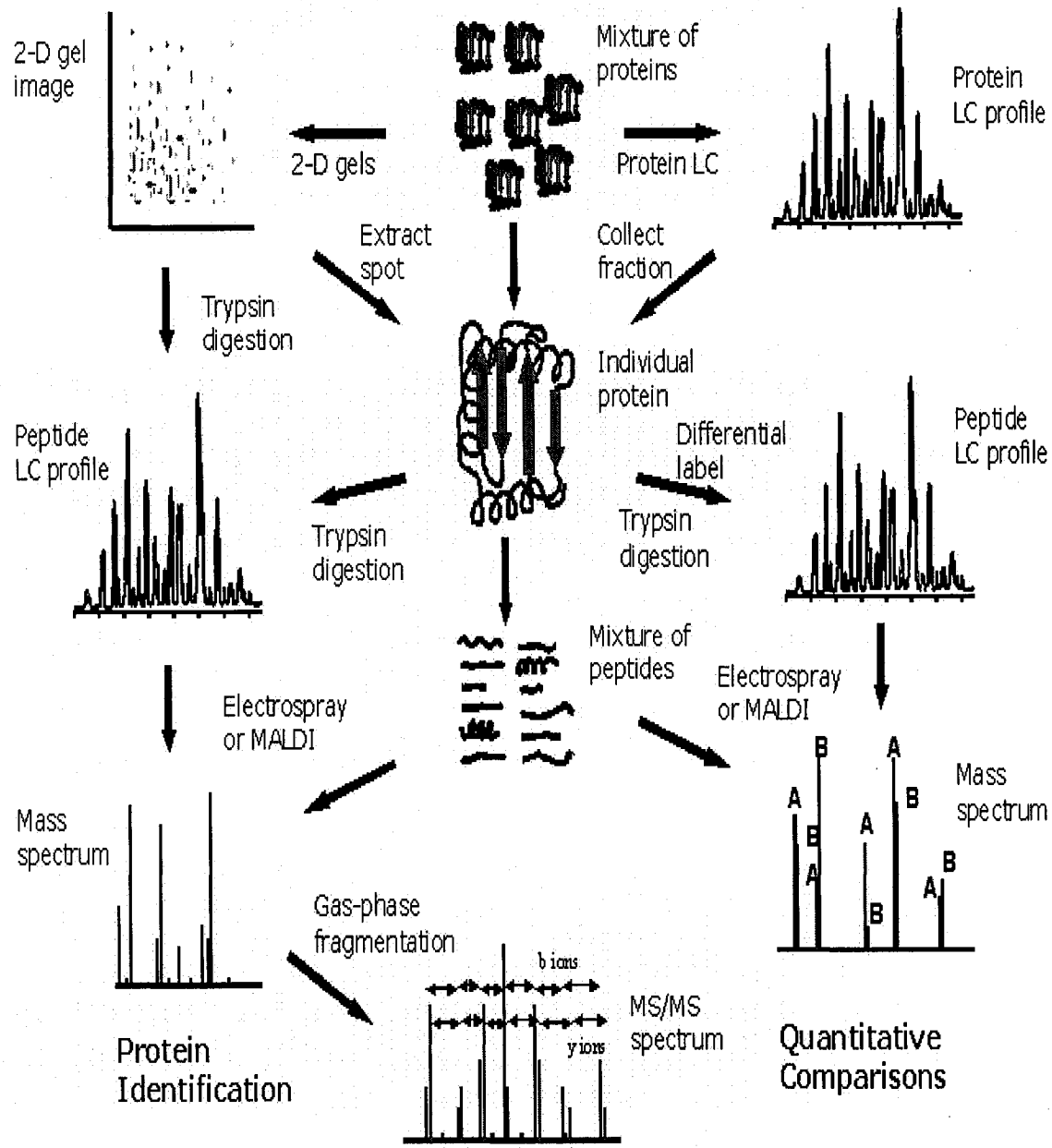


Figure 2.4: Schematic representation of bioanalytical techniques combined in proteomic studies (Proteomics research and development center, Indiana University 2006)

degree of glycoforms and post-translational modification such as phosphorylation (Alomirah et al., 2000). Mass spectrometers are used to measure the mass-to-charge ratios (m/z) of biological molecules, and then the m/z values can be used to calculate molecular weight as well as determine primary sequence information when tandem mass spectrometry experiments are performed. MS can provide accurate molecular weights for peptides and proteins with masses up to 500 KDa using only few picomoles of sample (Calbiani et al., 2006); the accuracy achieved is better than 0.1% of the calculated mass. Using MS/MS, complete or partial sequence information may be obtained at the femtomole level for peptides containing up to 25 amino acid residues (Speicher, 1996). In MS/MS two consecutive stages of mass analysis are used to detect secondary fragment ions that are formed from a particular precursor ion. Peptide fragment ions are produced in the mass spectrometer primary by cleavage of the amide bonds that join pairs of amino acid residues (Koomen et al., 2005); two types of ions are formed: y (C-terminal charge retention) and b (N-terminal charge retention) as illustrated in Figure 2.5. Using a full series of one type of ion, the sequence of the entire peptide as well as post-translational modifications can by determined by the mass difference between fragments (Kuster & Mann, 1998).

MS/MS can provide sequencing information even if the peptides are present in complex mixtures, and more importantly, even if they are modified (Carr et al., 1991). Moreover, it plays an important role in mapping dephosphorylation. Dephosphorylation of milk proteins produce a shift of 80 Da in the molecular mass of casein, which corresponds to the removal of HPO_3^- from the –OH group of a serine or threonine residue (Lund & Ardo, 2004). The importance of phosphorylation and dephosphorylation has

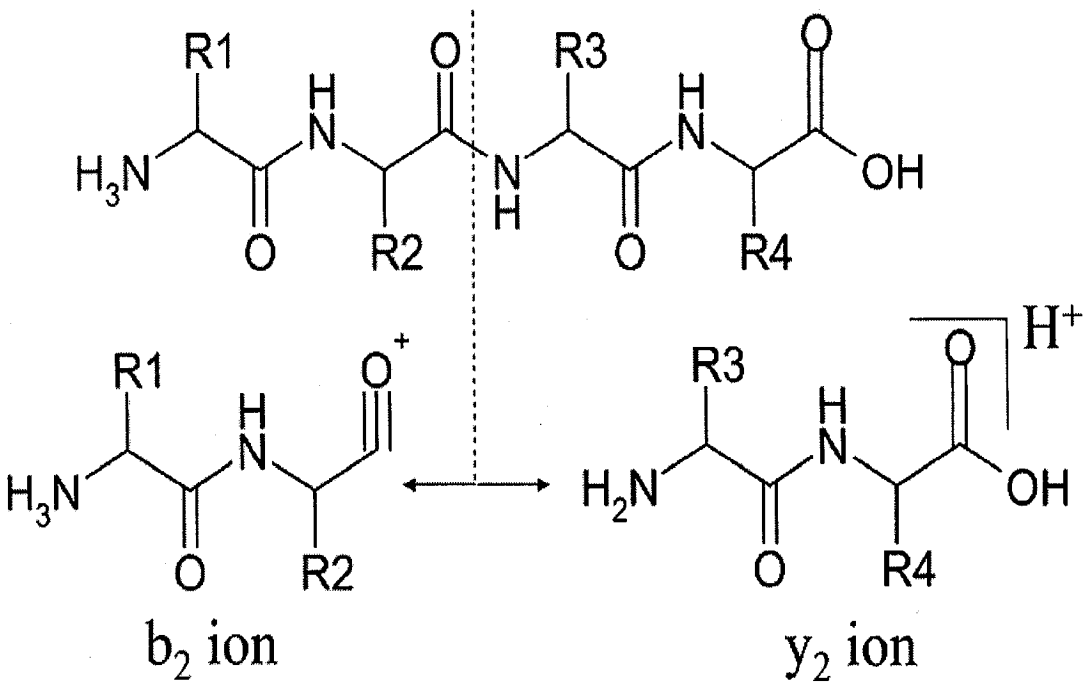


Figure 2.5: Fragmented ions generated by the cleavage of the amide bond by collision-induced dissociation during MS/MS analysis. R represents the variable amino acid side chain (Koomen et al., 2005)

lead to the development of phosphoproteomics, which can de defined as the study of the phosphorylation state of proteins (McCormick et al., 2005). Recently, several methods have been developed and used to locate phosphorylation sites of peptides and proteins. Many of them employ the strategy of degrading the phosphoprotein either chemically (Jaffe et al., 1998) or enzymatically (Liao et al., 1994) into small peptides, followed by analysis of the composition and mapping of each fragment. Many examples of the application of mass spectrometry to identify phosphorylation sites have been published. Hunter & Games (1994) studied the use of LC-MS/MS for the identification of phosphorylation by neutral ion loss of phosphoric acid (H_3PO_4) via gas-phase β-elimination of this ion (-98 Da) from phosphoserine and phosphothreonine residues of a tryptic digests of α_{s1}-casein. Carr et al. (1996) characterized the lost of a phosphate group (-79 Da) and sequencing of serine, threonine and tyrosine phosphopetides by using LC-MS/MS under alkaline conditions in a negative-ion mode at femtomole level of protein digests. Yip & Hutchens (1992) investigated the use of MALDI-TOF mass analysis to monitor protein and peptide phosphorylation and dephosphorylation reaction rates, even in complex unfractionated mixtures.

The type of ion source used determines the types of ions that can be obtained during a mass spectrometry analysis. The most common ionization methods MALDI and ESI are widely used (Alomirah et al., 2000). In MALDI a solution containing the analyte is mixed with another organic solution containing the matrix, upon drying of the matrix a laser irradiates the matrix deposits and desorbs the analyte molecules, producing ions that correspond to the protonated molecules of the analyte (Tanaka et al., 1988). In ESI-MS a solution containing the analyte is sprayed from a capillary by using a high voltage,

generating small droplets in the gas phase which evaporate leaving multiply charged ions, in proteins and peptides these ions are multiprotonated (Whitehouse et al., 1985).

2.4.1.4.3 Proteomics Database Analysis and Search Engines

The availability of bioinformatics solutions is crucial for proteomics technologies. One of the most used strategies in proteomics is correlative database searching; this involves searching the sequence databases for proteins containing peptides that match experimental data obtained by mass spectrometry resulting in definitive protein or peptide identification (Chalkley et al., 2005). Computer algorithms have been developed to use two different types of data; one approach involves collection of the molecular weight generated by protein digestibility after mass spectrometry analysis; thus the mass map fingerprint can be generated (Henzel et al., 1993). The second approach is based on the use of data created by tandem mass spectrometers; MS/MS spectra contain highly specific information in the fragmentation pattern as well as sequence information (Yates, 1998). Several web sites offer free access to web-based database-searching programs for peptide mass fingerprinting and the identification of sequence tags, all of which provide other tools such as prediction of enzyme digests. Examples of databases include UniProtKB/Swiss-Prot provided by the Swiss Institute of Bioinformatics (http://us.expasy.org/tools/) and UniProtKB/TrEMBL provided by the European Bioinformatics institute (http://www.ebi.ac.uk/trembl/); which are available for protein sequences annotated for function, subcellular location and known potential post-translational modifications.

Mascot is a search engine developed by the Imperial Cancer Research Fund, London, UK and Matrix Science Ltd., London, UK researchers, that incorporates probability-based scoring (Perkins et al., 1999). It supports three types of search: i) peptide mass fingerprint, ii) sequence query and iii) MS/MS ion search. Mascot can be freely accessible across the World Wide Web at Uniform Resource Locator (URL) http://www.matrixscience.com. The fundamental approach of Mascot is to calculate the probability that the observed match between the experimental data seat and each sequence database entry is a chance event (Weber et al., 2006). In the case of MS/MS fragment ion data are matched to calculated values for user-selected ion series. The advantages of this search engine are based on probability and include a simple rule to decide whether a result is significant or not and Mascot scores can be compared with those from other types of search like sequence homology (Perkins et al., 1999).

Recently, a study to detect milk allergens in different food matrixes, involved the analysis of tryptic digest of casein by using LC-MS/MS and database analysis (Weber et al., 2006); MS/MS data from the mass spectrometry system were processed with specialized MS/MS software and submitted for database search using Mascot for protein identification. This study demonstrated the capability of the MS/MS database search to provide reliable confirmation of the presence or absence of milk allergenic proteins.

2.4.2 Casein Allergenicity

Bovine milk is recognized as one on the main food allergens. Milk allergy incidence ranges from 0.3 to 7.5% of newborn or young children (Kajosaari & Saarinen, 1983) and less than 2% in adults (Stoger & Wuthrich, 1993). The multiplicity and diversity of proteins that are involved in milk allergy, includes polysensitization to

several proteins (Sharma et al., 2001). Caseins appear to be the major allergens responsible for bovine milk allergy; among caseins, α_{s1}-casein is a major allergen in milk (Docena et al., 1996). Most patients allergic to casein are sensitized to the four casein fractions; this cosensitization to the different caseins likely occurs after disruption of the casein micelle during the digestive process; polysensitization also appeared to be due to cross-sensitization mechanisms and involved the regions that contain the major sites of phosphorylation (Wal, 2004).

In healthy subjects, orally ingested milk proteins, induce antigen-specific systemic responsiveness, referred as oral tolerance (Juvonen et al., 1999). This phenomenon is well described in animal models; however, the real pathogenic mechanisms of allergenic reactions have been intensively studied over the last five years in order to increase the understanding of food-induced allergenic reactions (Sampson, 2004). Food initially contact the gut mucosa and the allergenic symptoms spread outside the gastrointestinal tract, although Hsieh et al. (2003) demonstrated that food allergy can be also induced by allergen exposure through the skin. Milk proteins tend to stay intact as milk is converted to dairy products of all types (Besler et al., 2001), when caseins are ingested they may be absorbed into the blood fully undigested provoking an immune response and malabsorption, especially pronounced in infants (Savilahti, 2000).

The above immune response occurs in two phases, an initial sensitization to an allergen followed by elicitation of an allergic reaction on subsequent exposure to the same allergen as shown in Figure 2.6 (US Food and Drug Administration; Threshold Working Group, 2006). Sensitization occurs when a susceptible individual produces IgE antibodies against specific proteins found in food; re-exposure to the same food allergen

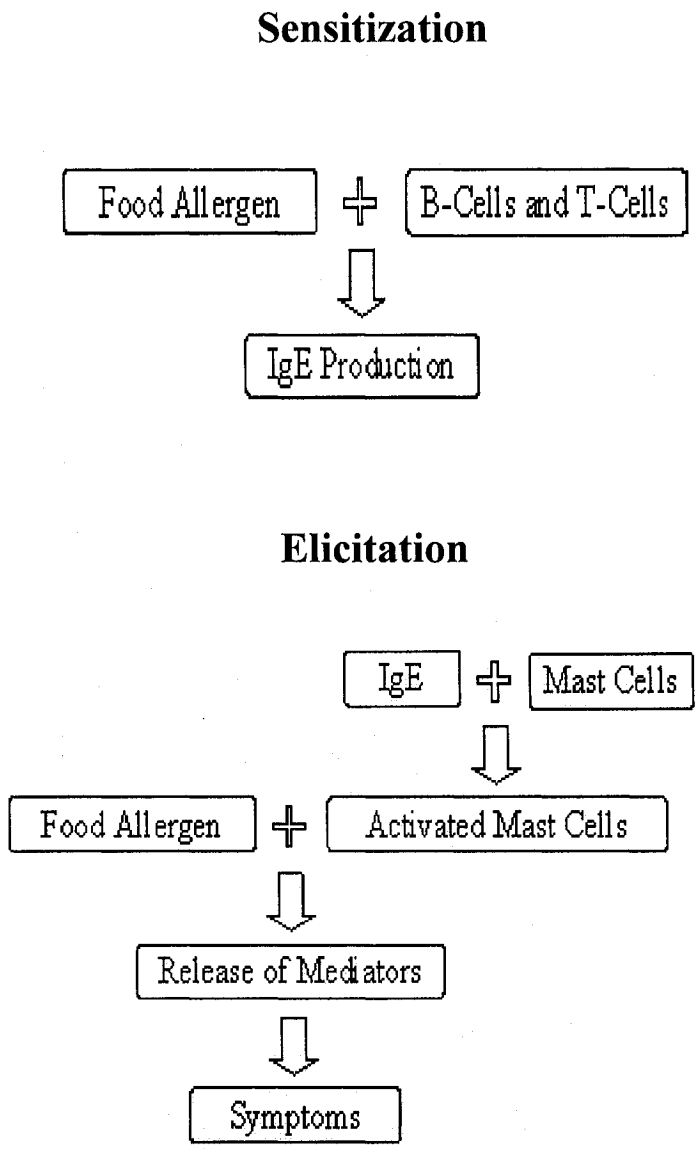


Figure 2.6: General mechanism of IgE mediated allergenic reactions (US Food and Drug Administration, Threshold Working Group, 2006)

initiates binding of the allergenic proteins to IgE molecules on immune mediator cells (basophiles and mast cells), leading to activation of these mediator cells (Goldsby et al., 2003); this elicitation causes the release of inflammatory molecules like leukotrienes and histamine. The specific symptoms and severity of an allergic reaction are affected by the concentration of allergen, route of exposure, and the organ systems involved, including skin, gastro intestinal tract, respiratory tract, and blood (Ebo & Stevens, 2001).

2.4.2.1 Molecular Characteristics of Allergenic Structures of Casein

Advances in technology have lead to allergenic maps sites for many food proteins. Immunoglobulin E (IgE) binding sites or epitopes on the allergenic protein may consist of segments of consecutive amino acids referred as sequential epitopes (e.g., caseins), or amino acids from different parts of the protein sequence (e.g., whey proteins), brought together by protein folding named conformational epitopes (Nowak-Wegrzyn, 2003); they are represented in Figure 2.7. In milk proteins both types of epitopes might be responsible for allergenic reactions; they are both conformational and linear epitopes, widely spread all along the protein molecules (Wal, 2004).

The phosphate groups of caseins bound to serine are present in the major epitopes regions that affect the IgE immunoreactivity of caseins, especially a homologous sequence present in α_{s1}-, α_{s2}- and β-casein, SerP-SerP-SerP-Glu-Glu, which is a highly conserved major site of phosphorylation (Wal, 2002). This sequence plays an important part in cross-reactivity, since it has already been described as immunoreactive (Bernard et al., 1998) and resistant to digestive degradation (Hynek et al., 2002). Bernard et al. (2000) determined that modification of such well-characterized site in casein affects their

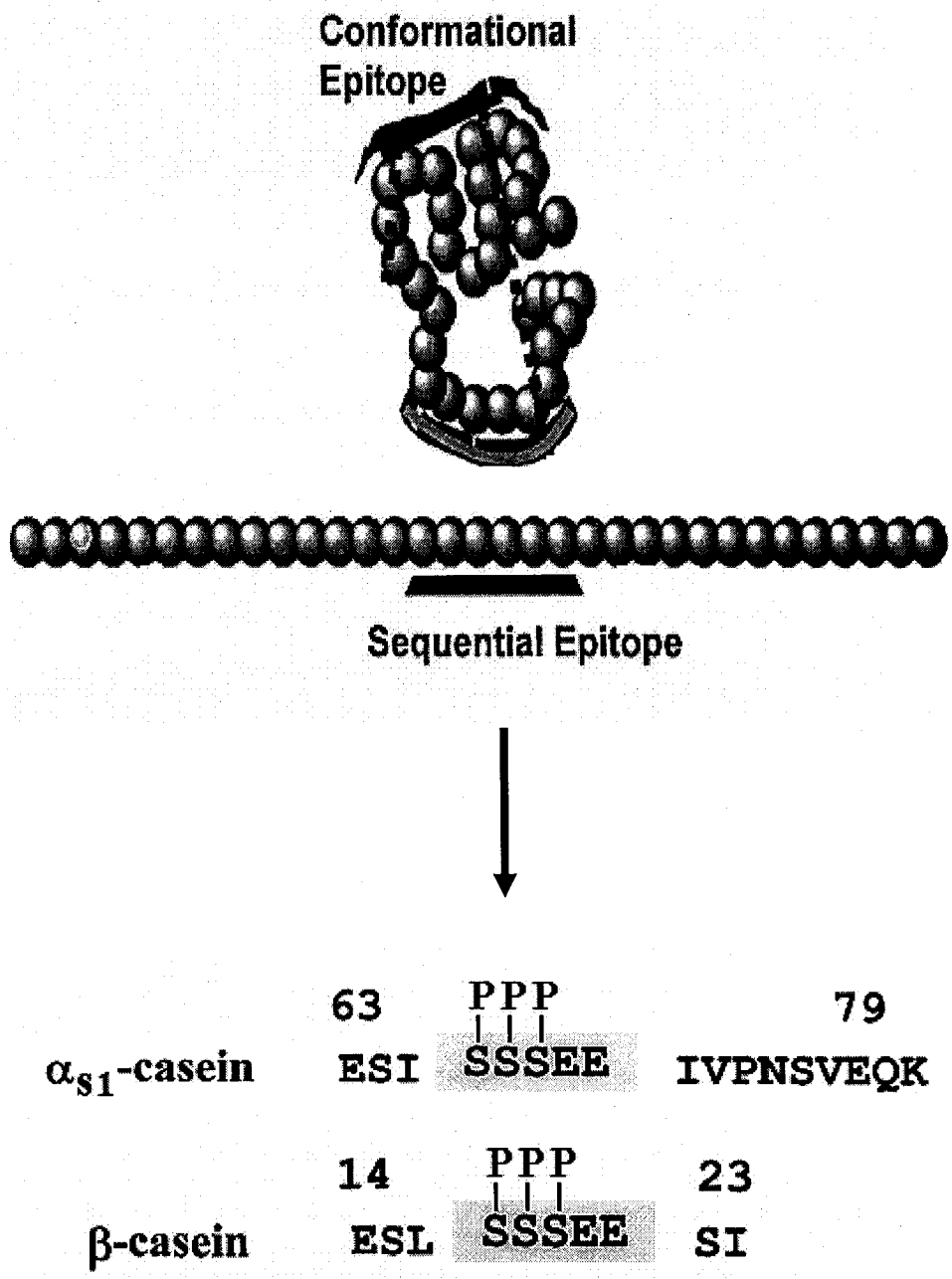


Figure 2.7: Sequential and conformational allergen epitopes (modified from (Sampson, 2004 and Wal, 2004))

immunoreactivity. Otani et al. (1987) evaluated that anti-native-casein antibody had a higher affinity to native caseins than to dephosphorylated caseins, suggesting that dephosphorylation affected the antigen-antibody reaction.

2.4.2.2 Effect of Proteolysis on the Allergenicity of Caseins

In the last few years, sensitivity to casein during the digestive process has increased in terms of both frequency and intensity of IgE response (Stoger & Wuthrich, 1993); the potentially allergenicity of casein phosphopeptides should be taken into account when considering their biological interest and possible functional use in foods. After ingestion most of casein structure the potential antigens may be modified by gastric conditions, action of digestive enzymes and uptake through the intestinal mucosa (Otani et al., 1987). The major epitopes on α_{s1}-caseins are continuous epitopes located in the hydrophobic regions of the molecule, where they are not accessible to antibodies unless the casein is hydrolyzed during digestion (Chatchatee et al., 2001). Milk protein hydrolysates, although frequently used as substitutes for milk proteins because of their allergenicity, show reduced but never complete absence of allergenicity (Caffarelli et al., 2002). Hypoallergenic hydrolysates are widely used on the market for the treatment of children cow's milk allergy. However, some anaphylactic reactions have been experienced (Walker-Smith, 2003). The degree of hydrolysis necessary in infant hydrolysates formulae to obtain the desired reduction of allergenicity and what peptide fractions are responsible for this residual allergenicity in the hydrolysates are not yet known (Niggemann et al., 1999). The importance of peptide chain length on allergenicity has been reviewed. Otani et al. (1986) studied the allergenicity of α_{s1}-casein and some

peptides derived form this protein, by using a combination of a chemical agent different fragments were obtained. Fragments 1-54, 61-123, 124-135 and 136-196 showed 19.4%, 31.4%, 10% and 20.3% of antibody-binding activity, respectively. This indicates that one site for high antigenicity is found in the phosphorylated 61-123 fragment; these researchers also found that polar amino acids such as lysine, histidine and glutamic acid were important components of some antigenic reactive regions of α_{s1}-casein and β-casein.

2.4.2.3 Techniques for Determining Casein Allergenicity

Information about the epitopes of a protein can be obtained by several methods, including: i) evaluation of the antigenic properties of peptides resulting from enzymatic or chemical fragmentation of the protein, ii) epitope mapping carried out by immunological testing of synthetic of peptides according to the protein entire sequence and iii) epitope mapping based on a set of overlapping sequential peptides shifted by one amino acid (Spuergin et al., 1996). *In vitro* immunoenzymatic techniques like immunoblotting and Enzyme-Linked Immunosorbent Assay (ELISA) are widely used to detect the presence of residual allergenicity in food products.

2.4.2.3.1 Immunoblotting

The immunoblotting technique provides information about presence, molecular weight, and quantity of an antigen by combining protein separation via gel electrophoresis with specific recognition of antigens by antibodies (Goldsby et al., 2003); immunoblotting is useful when the antigen of interest is insoluble or readily degraded.

Since most gel electrophoresis procedures result in denaturation of the antigen, only polyclonal and monoclonal antibodies that recognize the denatured form of an antigen can be utilized in immunoblotting (Restani et al., 1996).

2.4.2.3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA detects the presence of an antibody or an antigen in the sample to be analyzed. It utilizes two antibodies, one of which is specific to the antigen and the other which is coupled to an enzyme (horseradish peroxidase or alkaline phosphatase). This second antibody causes a chromogenic or fluorogenic substrate to produce a signal (Rabin et al., 1992). ELISA can be a quantitative, rapid and dependable assay since many samples can be tested in parallel. In the most common format an antigen is immobilized by adsorption to the wells of a microtitre plate, and then reacted with the analyte. Three types of ELISA assays can be found: 1) Sandwich ELISA, 2) Direct ELISA and 3) Indirect ELISA (Figure 2.8). The most commonly used ELISA is the sandwich assay. The analyte to be measured is bound between two antibodies– the capture antibody and the detection antibody. The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate. The antigen does not need to be purified prior to use, and the assay is very specific. However, one disadvantage is that not all antibodies can be used (Rabin et al., 1992). The direct detection method labeled antibodies with a fluorescent tag to mark tissue antigens. In this technique, a labeled primary antibody reacts directly with the antigen. Direct detection is not widely used in ELISA but is quite common for immunohistochemical staining of tissues and cells (Coons & Kaplan, 1950). The indirect method uses a labeled secondary

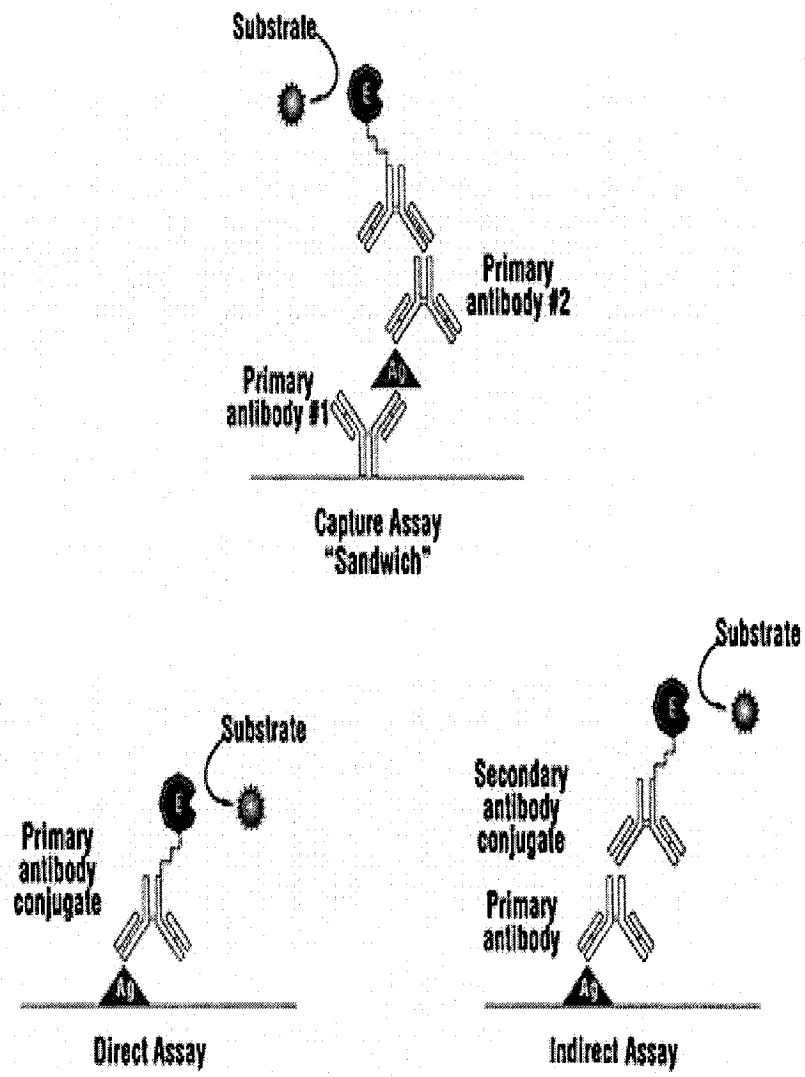


Figure 2.8: ELISA immunoassay formats, sandwich, direct and indirect assay (Pierce, 2006)

antibody for detection. First, a primary antibody is incubated with the antigen. This is followed by incubation with a labeled secondary antibody that recognizes the primary antibody (Weller & Coons, 1954). For ELISA it is important that the antibody enzyme conjugate is of high specific activity, this can be achieved when the enzyme conjugation hemistry preserves antibody specificity as well as enzyme activity (Goldsby et al., 2003).

CHAPTER 3

ENZYMATIC DEPHOSPHORYLATION OF BOVINE CASEIN

3.1 Justification

Enzymatic dephosphorylation of casein proteins is preferred over chemical dephosphorylation for many food applications. Enzymatic dephosphorylation results in release of phosphate groups leaving the dephosphorylated serine residue intact on the protein. Although there have been several studies on enzymatic dephosphorylation of caseins (Li-Chan & Nakai, 1989; Bingham et al., 1976), there is relatively little information on the kinetics of dephosphorylation of caseins. It is considered important to establish optimum dephosphorylation conditions of caseins before a detailed approach on products of dephosphorylation is performed. This chapter addresses the first research objective to enzymatically dephosphorylate whole casein and its α-casein and β-casein fractions and to investigate the kinetics and extents of dephosphorylation.

3.2 Materials and Methods

3.2.1 Materials

Commercial dry skim milk powder was purchased and reconstituted with distilled water (370 g/l). Whole casein was precipitated with 1M ammonium acetate buffer at 20 °C and pH 4.6. The casein was washed with acetone, air dried and stored in a desiccator until further analysis (Veloso et al., 2002). Commercial bovine α-casein and β-casein standards and potato acid phosphatase (EC 3.1.3.2; activity: 1.2 units/mg solid) were purchased from Sigma Chemicals Co. (St.-Louis, MO); the α-casein was not separated further into its α_{s1}- and α_{s2}-casein fractions.

3.2.2 Protein and Phosphorus Contents

Total phosphorus was determined by dry-ashing (600 °C, 5 h; Furnatrol II Furnace, Thermolyne Co, Iowa) followed by colorimetric analysis (823 nm, Ultrospec 100 UV/Vis; Biochrom Ltd., Cambridge, UK) using the method of Pulliainen & Wallin (1994). For measurement of free phosphate, the dry-ashing step was excluded; only the colorimetric step was necessary. Protein content of whole casein and dephosphorylated whole casein was determined using the method of Lowry et al. as modified by Hartree (1972); bovine serum albumin was used as standard.

3.2.3 Caseins Dephosphorylation

3.2.3.1 Effect of Enzyme Concentration

Dephosphorylation of whole casein with potato acid phosphatase was carried out using the method of Bingham et al. (1976) with modification (citrate buffer 0.1 M, pH

5.8, 37 °C). Controls were prepared using whole casein but without the addition of the enzyme. The enzyme reaction was performed in duplicate using enzyme concentrations of 0.05, 0.10, 0.15, 0.20 and 0.25 mg/ml; the total reaction mixture was 10 ml and contained 25 mg casein. The reaction was carried out at time intervals of 1 h for 4 h and stopped by the addition of 5 ml 15% trichloroacetic acid (TCA). The precipitated protein was recovered by centrifugation (3,000 x g, 5 min) and analyzed for phosphorus content as described before in Section 3.2.2.

3.2.3.2 Effect of Reaction Time

Based on results from the preliminary experiment described in Section 3.2.3.1, an enzyme concentration of 0.2 mg solid/ml was selected to investigate the effect of reaction time. The dephosphorylation reaction mixture was 10 ml and contained 25 mg whole casein. It was carried out at time intervals of 1 h for 8 h and stopped by the addition of 5 ml 15% TCA. The precipitated protein was recovered by centrifugation (3,000 x g, 5 min) and analyzed for phosphorus content (Section 3.2.2). Optimum reaction time of 6 h for whole casein was determined and these final optimum conditions were used to dephosphorylate α-casein and β-casein.

3.2.3.3 Effect of Substrate Concentration

The effect of varying whole casein concentration (1.25, 2.5, 3.5, 5, 6.25, and 7.5 mg/ml) on dephosphorylation was determined. The reaction was carried out at intervals of 30 min for 2 h at 37 °C and stopped by the addition of TCA (5 ml, 15%). The dephosphorylated whole casein was recovered by centrifugation (3,000 x g, 5 min,) and analyzed for

phosphorus content (Section 3.2.2). The activity of the phosphatase (μg P/ml released after dephosphorylation) vs. the reaction time for each casein concentration were plotted in order to determine the initial velocity of dephosphorylation (V) before 30% of bioconversion was obtained; it was expressed in μmol P/ml min. The double reciprocal plot of these data was used to calculate the apparent kinetic parameters (V_{max} and K_m) using the Lineweaver-Burk linear equation (1934).

3.3 Results and Discussion

Protein and total phosphate content of whole casein were 94.6% and 0.8%, respectively and 92.3% protein for dephosphorylated whole casein. The free phosphate before enzymatic dephosphorylation represented 7.7% of the total phosphate; this free phosphate may be due to the presence of indigenous phosphatases in milk. These results agree with the values reported by Fox and McSweeney (1998).

3.3.1 Extent of Dephosphorylation

Figure 3.1 shows the phosphate released from whole casein by potato acid phosphatase as a function of incubation time. As enzyme concentration increased, the extent of dephosphorylation increased as the incubation time is extended. Highest dephosphorylation (65.4%) was obtained at an incubation time of 4 h at enzyme concentration of 0.2 mg/ml. For enzyme concentration of 0.25 mg/ml relatively low dephosphorylation of 34.8% could be related to low enzyme solubility.

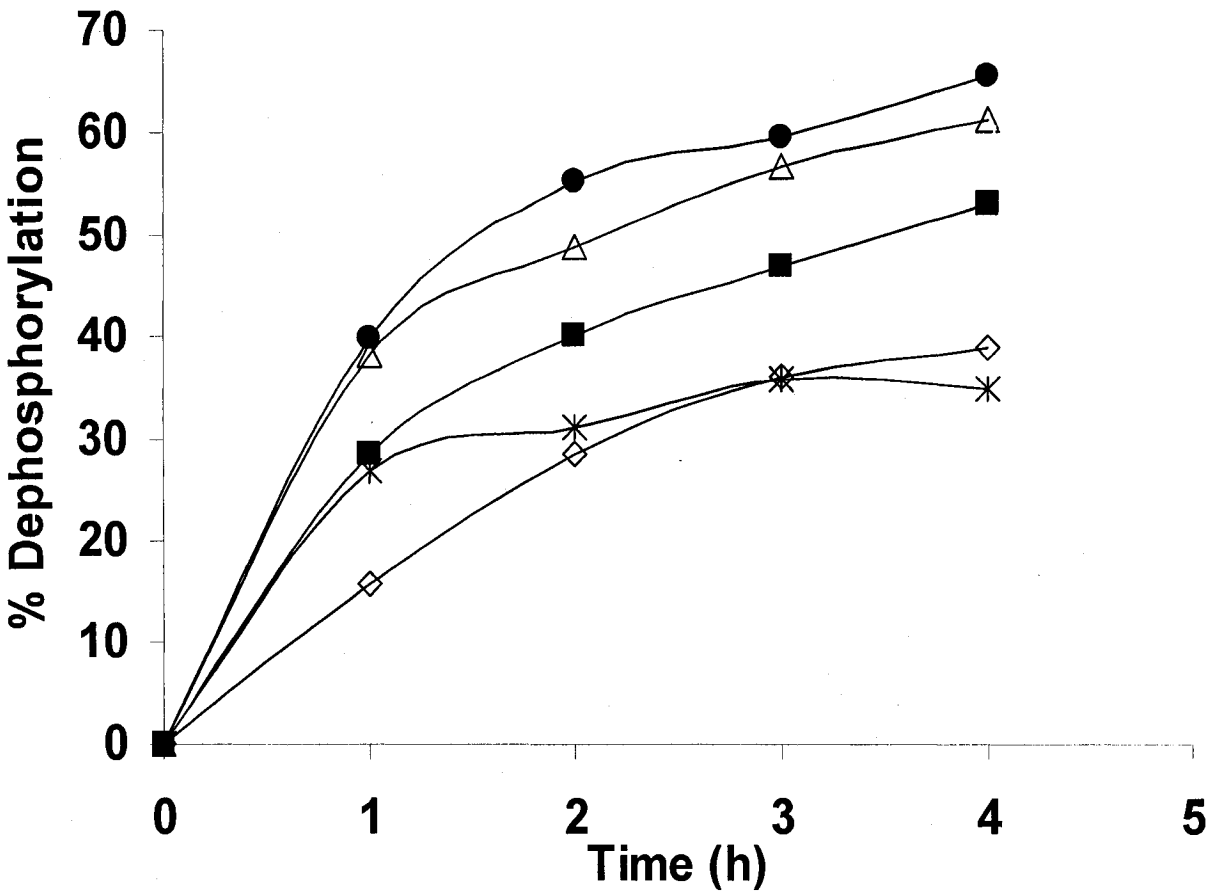


Figure 3.1: Phosphate released from whole casein by potato acid phosphatase as a function of incubation time. Enzyme concentration: ◇ 0.05 mg/ml, ■ 0.10 mg/ml, △ 0.15 mg/ml, ● 0.20 mg/ml and X 0.25 mg/ml

Figure 3.2 shows the phosphate released from whole casein (2.5 mg/ml) as a function of incubation time with a selected enzyme concentration of 0.2 mg/ml. At 6 h and 8 h, 71.6% and 74% of dephosphorylation occurred, respectively. Optimum dephosphorylation conditions can be considered at 6 h; an increase of approximate 2.4% of dephosphorylation of whole casein was observed between 6 and 8 h. Figure 3.3 demonstrates that α-casein (2.5 mg/ml) was dephosphorylated at 89.2% and β-casein (2.5 mg/ml) was 73.7% dephosphorylated. Previous work by Bingham et al. (1976) showed a maximum dephosphorylation of approximately 70% for α_{s1}-casein at pH 7 and 38 °C. Li-Chan et al. (1989) reported 97%, 70% and 99% of dephosphorylation for whole casein, α_{s1}-casein and β-casein, respectively. Other researchers (van Hekken & Strange, 1993) obtained 40% partially dephosphorylated whole casein at pH 6.5, 37 °C. The different extents of dephosphorylation reported in other studies could be related to phosphate end-product inhibition; Bingham et al. (1976) suggested that phosphate is a competitive inhibitor of potato acid phosphatase activity. Additionally, β-casein with three phosphoseryl residues can resist the action of phosphatase; this resistance was attributed to intermolecular interactions involving the phosphate groups (West & Towers, 1976).

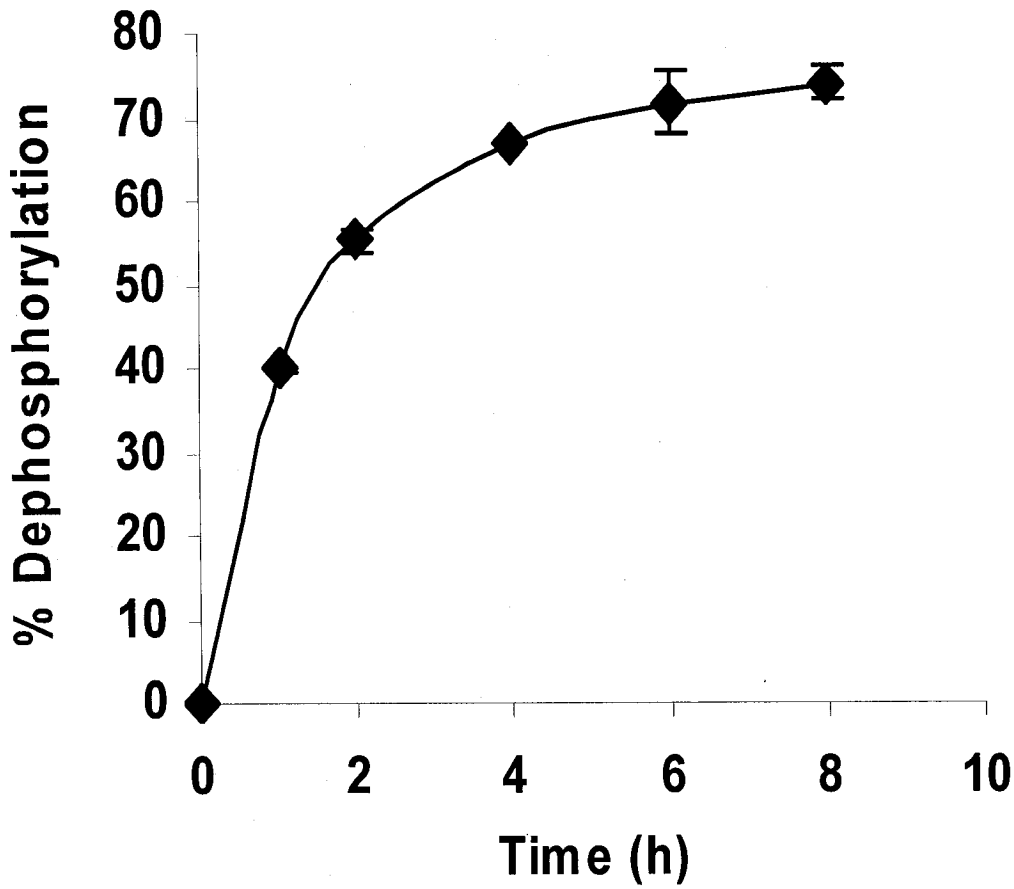


Figure 3.2: Phosphate released from whole casein by potato acid phosphatase as a function of incubation time

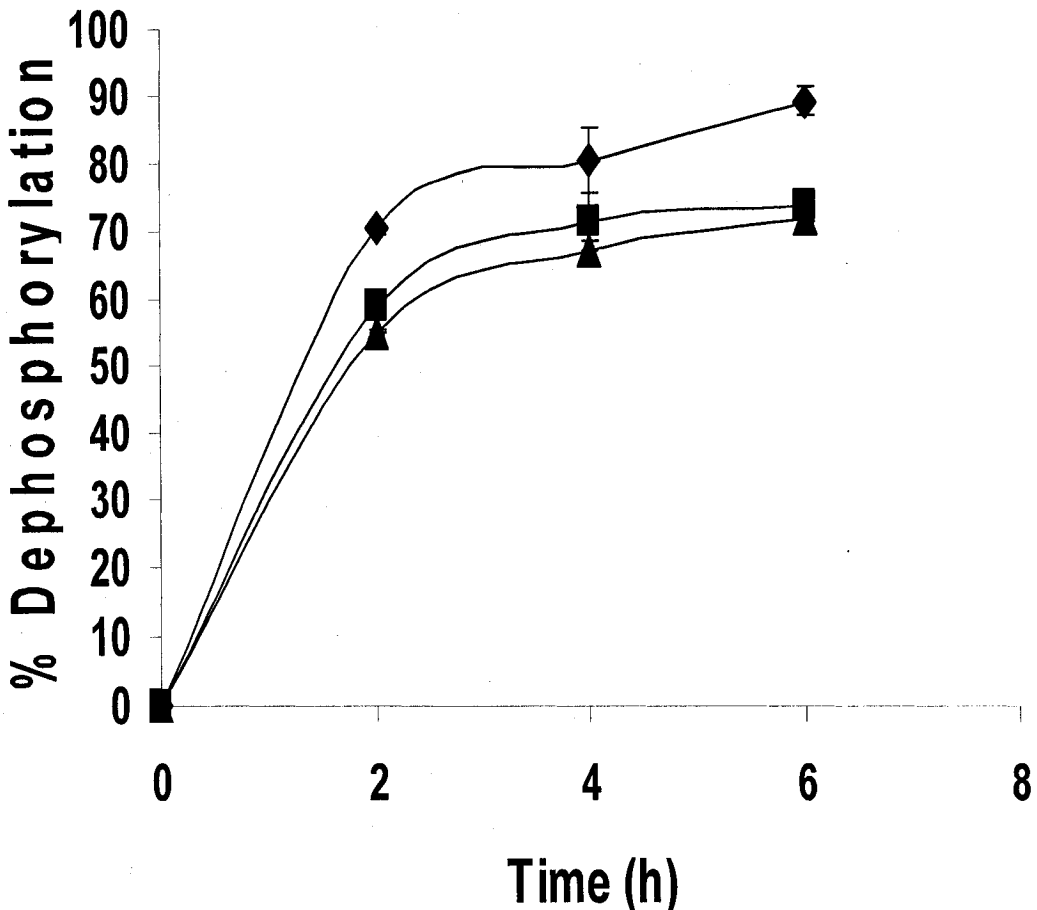


Figure 3.3: Phosphate released from ◆ α-casein, ■ β-casein and ▲ whole casein by acid phosphatase as a function of incubation time

3.3.2 Kinetics of Dephosphorylation

Figure 3.4 A shows the effect of varying whole casein concentration on dephosphorylation. The results indicate a linear relationship between casein concentration and dephosphorylase activity over the range of substrate concentration studied. The double reciprocal plot (Figure 3.4 B) was used to calculate the apparent kinetic parameters for whole casein; the values for the apparent V_{max} and apparent K_m were 0.283 μmol P/mg casein min and 9.951 mg casein/l, respectively. Bingham et al. (1976) reported kinetics parameters only for α_{s1}-casein which cannot be compared with those obtained for whole casein; these researchers did not report inhibition data for whole casein because of the difficulty of measuring phosphate released from whole casein in the presence of KH_2PO_4 as inhibitor.

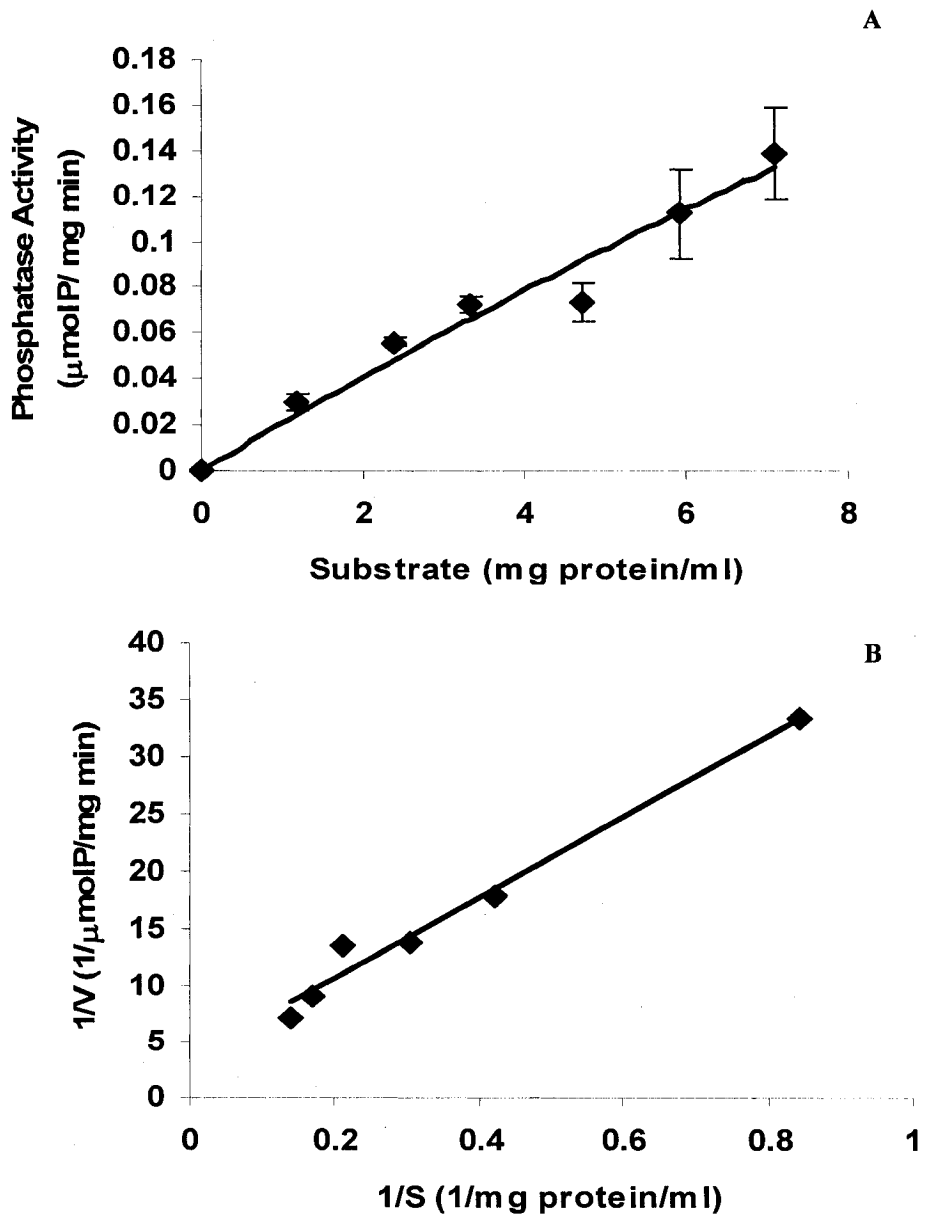


Figure 3.4: (A) Effect of the substrate (whole casein) concentration on phosphatase activity, (B) Double reciprocal plot of linear data of the effect of whole casein concentration on phosphatase activity

CHAPTER 4

MOLECULAR CHARACTERISTICS OF PRODUCTS FROM DEPHOSPHORYLATION OF BOVINE CASEINS

4.1 Justification

Relatively little information on the identification of the dephosphorylated casein species can be found; hence there is a need to study in detail the products of the enzymatic dephosphorylation reaction of caseins, in order to clearly identify the molecular changes occurring in the caseins molecule during dephosphorylation. The work described in this chapter addresses the second research objective related to the investigation of molecular characteristics of products of dephosphorylation using a combination of PAGE (urea and SDS), RP-HPLC and ESI-MS.

4.2 Materials and methods

4.2.1 Materials

Whole casein was obtained as described previously in Section 3.2.1. Commercial bovine α-casein and β-casein standards and potato acid phosphatase (EC 3.1.3.2; activity: 1.2 units/mg) were purchased from Sigma Chemicals Co. (St. Louis, MO). The α-casein was not separated further into its α_{s1}- and α_{s2}-casein fractions. Chemicals of electrophoretic and HPLC grade were used. Dephosphorylation of caseins was conducted as described in Section 3.2.3.

4.2.2 Electrophoresis

Whole casein, α-casein and β-caseins and their dephosphorylated products were subjected to slab gel polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970), using a Bio-Rad Miniprotean® II dual slab cell electrophoresis unit (Richmond, CA). The conditions used were: staking and separation gel 4% and 12% acrylamide, respectively and sample injection volume of 20 μl in the gels. Estimation of molecular weight was done by SDS-PAGE broad range molecular weight standards (Bio-Rad Hercules; CA). Slab gel at constant current, 15 mA/gel; run time approximately 2 h. After electrophoresis, gels were fixed for 1 h in a mixture of methanol (20% v/v) and acetic acid (10% v/v). Coomassie Brilliant Blue R-350 (0.1% w/v in fixing solution) was used to stain protein bands. Destaining was done using the fixing solution. Urea-PAGE was performed to achieve complete separation of whole casein, α-casein and β-casein (Coker, 1991). The conditions used were: 6% and 20% acrylamide, respectively. Sample injection volume was 20 μl in the gels. Slab gel at

constant voltage, 100 volts/gel; run time approximately 1.5 h. Protein bands were stained with Coomassie Brilliant Blue R-350 (0.1% w/v in fixing solution). Destaining was done using the fixing solution.

4.2.3 Reverse-Phase High Performance Liquid Chromatography

RP-HPLC was used to separate caseins and their dephosphorylated products with a liquid chromatograph (Beckman; CA, US) equipped with a Programmable Solvent Module (model 126) for high pressure solvent delivery, a manual injector (20 µl loop), a Programmable Detector Module (model 166) and an Ultra C4 reverse phase column 5µm, 250 X 4.6 mm column (Restek Co., US). Chromatographic data were analyzed by the Gold System (version V810), translated into PRN format for Microsoft Excel© manipulation. Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoracetic acid (TFA) in water and solvent B was 0.04% acidified water (TFA) in acetonitrile. Elution was performed at a flow rate of 1 ml/min with a 50 min linear gradient starting at 25% of solvent B and increasing up to 45% of solvent B over 40 min, then the linear conditions were re-established over 10 min (Bernard et al., 2000); detection was at 220 nm. Fractions eluting from the column were collected by Water Fraction Collector (NE, US), then pooled and concentrated, freeze-dried and stored at room temperature for further analysis.

4.2.4 Electrospray Ionization Mass Spectrometry

α-casein and β-casein standards were dissolved in 10% acetic acid and subjected to ESI-MS directly. For the controls (incubated without phosphatase), dephosphorylated

α-casein and dephosphorylated β-casein, dialysis against distilled water (4 °C) was used to remove buffer salts that can suppress the analyte signal during the electrospray ionization. A Waters Micromass QTOF Ultima Global (Micromass; Manchester, UK) hybrid mass spectrometer equipped with a nanoflow electrospray source was used. It was operated in positive ionization mode (+ESI) at 3.80 kV; with source temperature of 80 °C and desolvation temperature of 150 °C. The TOF was operated at an acceleration voltage of 9.1 kV, a cone voltage of 100 V and collision energy of 10 eV (for MS survey). For the MS survey mass range, m/z was 400-1990, scanned continuously over the chromatographic run. The mass spectrometer was tuned and calibrated with [Glu]-Fibrinopeptide B (Sigma Chemicals Co.). Instrument control and data analysis were carried out by MassLynx V4.0 software (Waters Corporation, 2005).

4.3 Results and Discussion

4.3.1 Effects of Dephosphorylation on Molecular Characteristics

Figure 4.1 shows the SDS-PAGE patterns for whole casein, α-casein and β-casein standards, controls (whole casein, α-casein and β-casein incubated without phosphatase), and dephosphorylation products of the caseins; the controls allowed us to determine if experimental incubation conditions affected the casein fractions in the absence of the phosphatase enzyme. No attempt was made to estimate molecular weights for the caseins because of the known anomalous behavior of caseins in SDS-PAGE (Creamer & Richardson, 1984). The identification of the casein fractions was done by comparison of migration distance with those of the known standards. The high intensity band of whole casein standard (lane 2) indicates that it was not separated into its individual α-casein

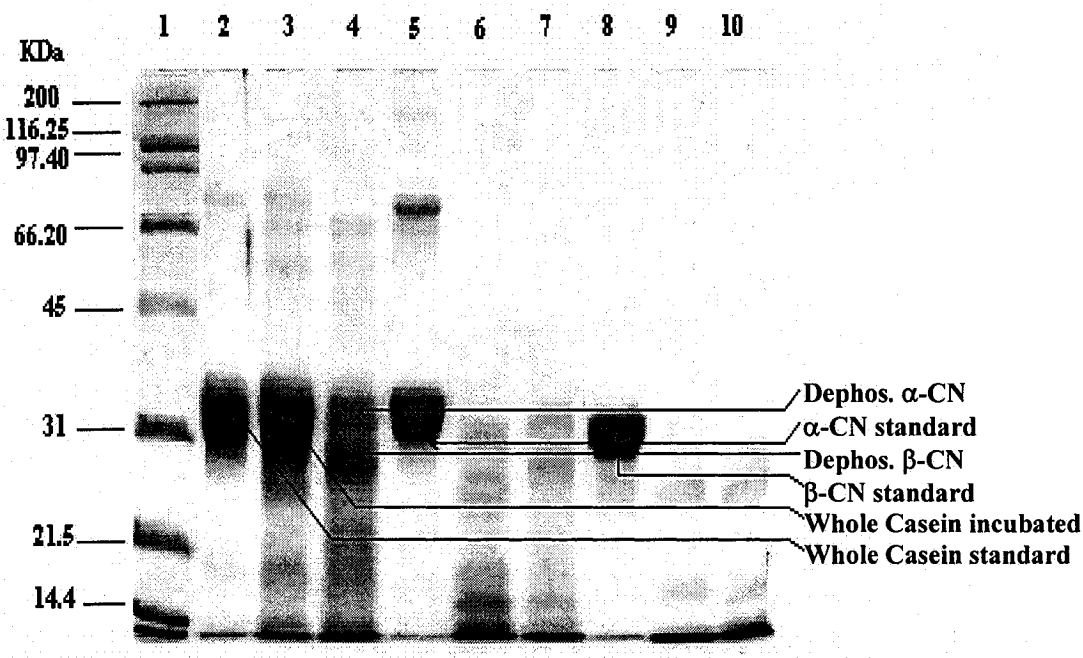


Figure 4.1: SDS-PAGE electrophoretic patterns of caseins. (1) Standards, (2) whole casein standard, (3) control whole casein, (4) dephosphorylated whole casein, (5) α-casein standard, (6) control α-casein, (7) dephosphorylated α-casein, (8) β-casein standard, (9) control β-casein and (10) dephosphorylated β-casein. (Controls: incubated without phosphatase)

and β-casein fractions as their theoretical individual molecular weights are very similar (23.6 KDa, α_{s1}-casein, 24 KDa β-casein; Farrell et al., 2004). Lane 4 shows dephosphorylated whole casein with two bands which are likely the separated dephosphorylated α-casein and β-casein fractions (comparison with whole casein, lanes 2 and 3). The differences between the control and the phosphatase incubated α-casein (lanes 6 and 7) and β-casein (lanes 9 and 10) reflect the effect of dephosphorylation of these fractions. The SDS-PAGE results suggest changes in structures of α-casein (lanes 5 and 6) and β-casein (lanes 8 and 9) in the absence of the phosphatase (control); however, there was no change in the structure of whole casein in the absence of the phosphatase (lanes 2 and 3).

Figure 4.2 shows the urea-PAGE patterns for whole casein, α-casein and β-casein standards, controls (whole casein, α-casein and β-casein incubated without phosphatase), and dephosphorylation products of the caseins. For whole casein standard (lane 1) and whole casein control (no phosphatase, lane 2), two intense bands corresponding to α-casein and β-casein were observed. Urea-PAGE separated the casein fractions present in whole casein that was not obtained with SDS-PAGE. For dephosphorylated whole casein (lane 3), the two main bands were identified as dephosphorylated α-casein and dephosphorylated β-casein; the migration of the dephosphorylated casein fraction is reduced as a result of the removal of the negative charge of the phosphate group (Li-Chan & Nakai, 1989). The presence of several bands with α-casein (lane 6) and β-casein (lane 9) suggests that proteolysis also occurred during dephosphorylation in the presence of phosphatase. The differences between the control and the phosphatase incubated α-casein

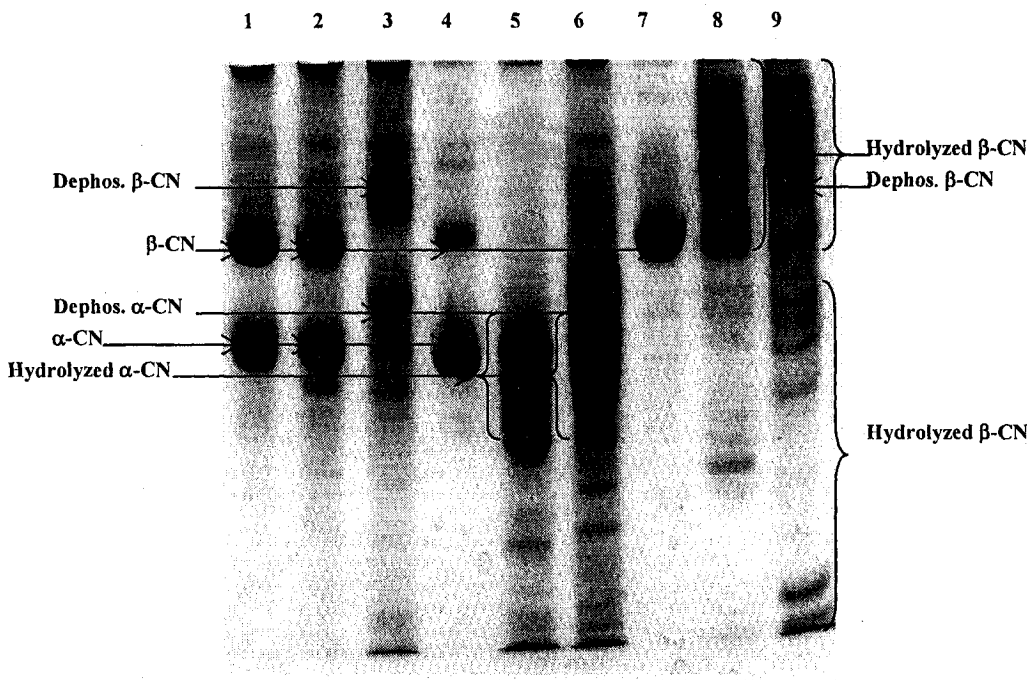


Figure 4.2: Urea-PAGE electrophoretic patterns of caseins. (1) Whole casein standard, (2) control whole casein, (3) dephosphorylated whole casein, (4) α-casein standard, (5) control α-casein, (6) dephosphorylated α-casein, (7) β-casein standard, (8) control β-casein and (9) dephosphorylated β-casein. (Controls: incubated without phosphatase)

(lanes 5 and 6) and β-casein (lanes 8 and 9) reflect the actual effect of dephosphorylation of these fractions; these effects were also evident with SDS-PAGE (Figure 4.1). Urea-PAGE also suggested that proteolysis occurred in the control experiments (incubation without phosphatase) with α-casein (lane 5) and β-casein (lane 8). Li-Chan & Nakai (1989) also reported that both proteolysis and dephosphorylation of α_{s1}-casein occurred in the presence of an alkaline phosphatase. It is likely that the observed proteolysis in the α–casein and β-casein controls (no phosphatase) was the effect of the incubation conditions on these proteins. Swaisgood (1982) reported that α-casein and β-casein showed greater susceptibility to proteolysis when they were separated from the casein micelle. Additionally, α-casein and β-casein exhibit more chain flexibility than typical globular proteins, which increases susceptibility to hydrolysis (Church et al., 1981).

Figure 4.3A shows the RP-HPLC chromatograms for whole casein standard (I) and dephosphorylated whole casein (II). From the chromatogram of whole casein, the 3 peaks were assigned as the κ-casein, α-casein and β-casein fractions; the identities of these fractions were confirmed by SDS-PAGE (Figure 4.3B) and urea-PAGE (Figure 4.3C). Bobe et al. (1998) reported similar elution pattern for the three casein fractions from whole casein. The RP-HPLC results showed slight changes in the retention times of the α-casein and β-casein as a result of the dephosphorylation. Removal of the phosphate groups is expected to increase hydrophobicity of the casein fractions, compared with the fully phosphorylated caseins (Strange et al., 1991; Bernard et al., 2000).

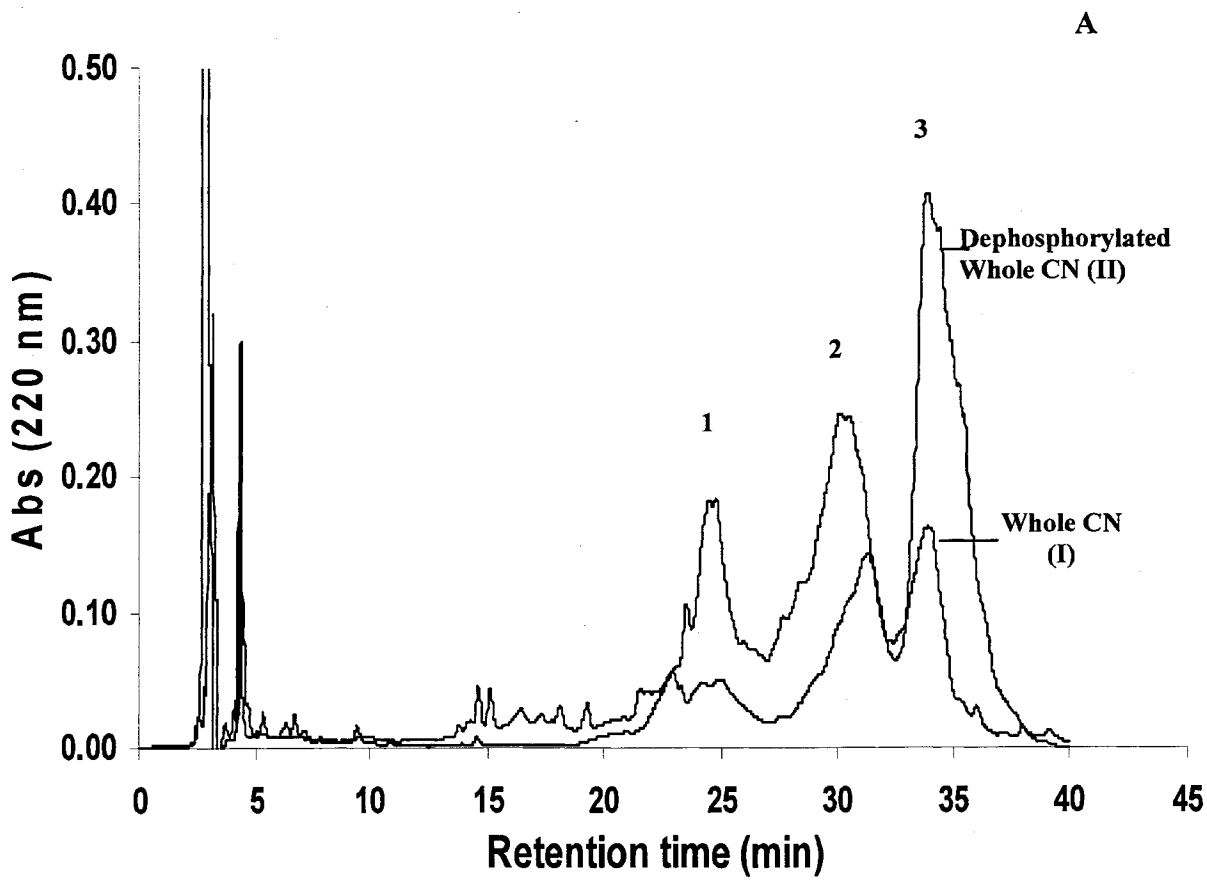


Figure 4.3: (A) RP-HPLC chromatograms of (I) standard whole casein and (II) dephosphorylated whole casein. Peak identity: (1) k-casein, (2) α-casein and (3) β-casein

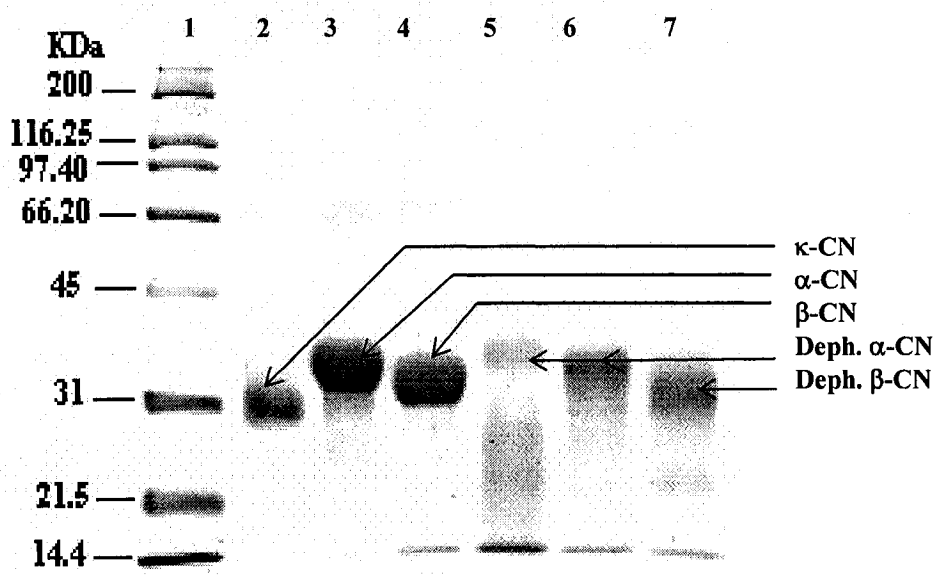


Figure 4.3: (B) Casein profiles obtained by SDS-PAGE. Fractions collected from RP-HPLC separation. (1) Standards, (2) k-casein, (3) α-casein, (4) β-casein, (5) dephosphorylated k-casein, (6) dephosphorylated α-casein and (7) dephosphorylated β-casein.

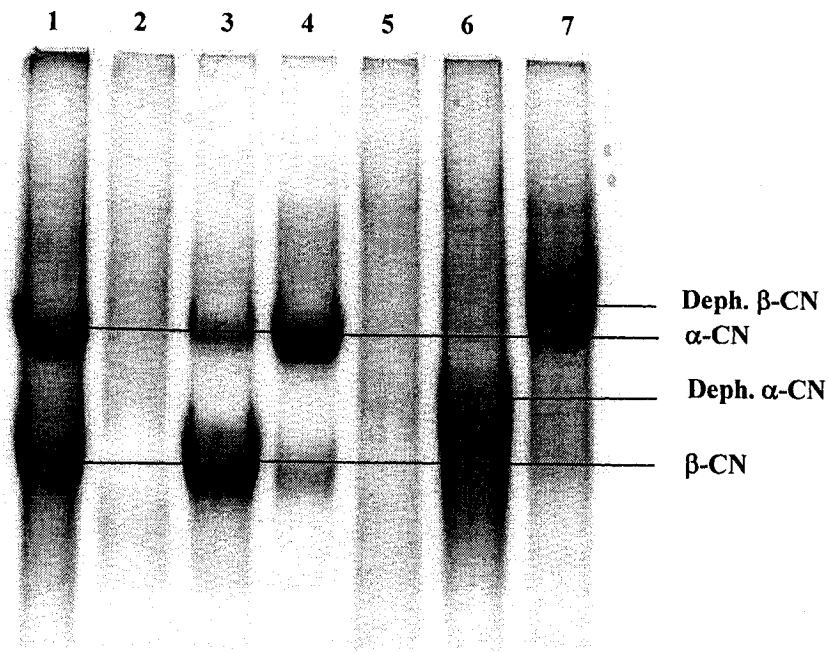


Figure 4.3: (C) Casein profiles obtained by urea-PAGE. Fractions collected from RP-HPLC separation. (1) Whole casein standard (2) k-casein, (3) α-casein, (4) β-casein, (5) dephosphorylated k-casein, (6) dephosphorylated α-casein and (7) dephosphorylated β-casein

Figure 4.4A shows the chromatograms for α-casein standard (I), control α-casein (II) and dephosphorylated α-casein (III). α-casein was observed as a major peak with retention time of 31.9 min. The chromatogram of the control α-casein (no phosphatase, Figure. 4.4A, II) also gave the α-casein peak at 31.9 min, as well as several peaks which could be hydrolysis products of the α-casein; these results support our findings from electrophoretic analysis, which suggested that incubation conditions in the absence of the phosphatase resulted in some proteolysis of α-casein and β-casein. Dephosphorylated α-casein (Figure 4.4A, III) gave a major peak at 32.3 min; it is likely that this shift in retention time from 31.6 min is due to the increased hydrophobicity of the dephosphorylated α-casein (Bernard et al., 2000). Figure 4.4B shows the chromatogram for β-casein standard (I), control β-casein (II) and dephosphorylated β-casein (III). The β-casein standard was observed as a doublet peak likely due to self-association of this casein (Bobe et al., 1998) with retention time 34.6 min. Control β-casein (no phosphatase, Figure 4.4B, II) shows the β-casein peak at 34.6 min as well as several peaks which could be hydrolysis products of the β-casein; similar observations were made from the previous in electrophoretic analysis. Dephosphorylated β-casein showed several overlapping peaks in the retention time range of 34.8 and 36.7 min. It is likely that these peaks represent dephosphorylated β-casein and dephosphorylated hydrolysis products of β-casein; similar results were observed with dephosphorylated α-casein.

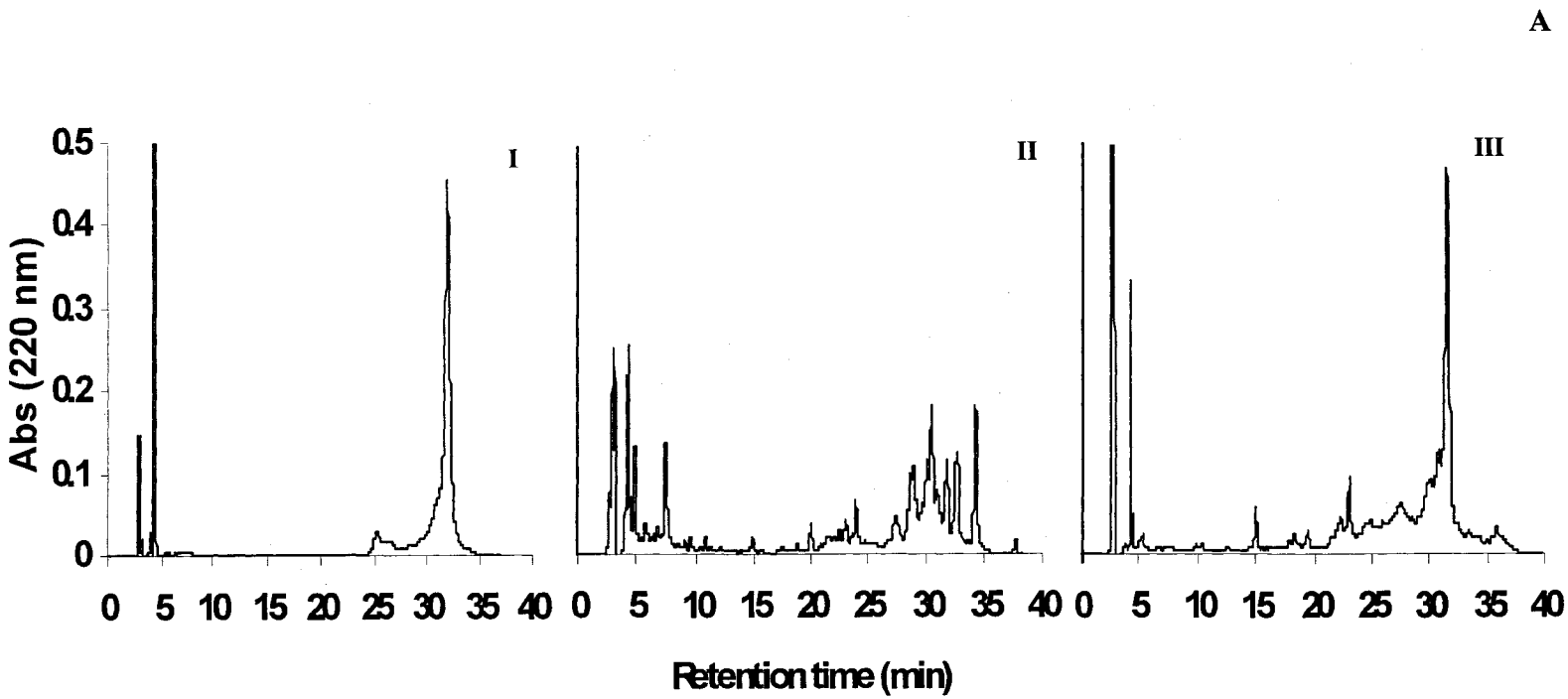


Figure 4.4: (A) Chromatographic profiles of (I) α-casein standard, (II) control α-casein and (III) dephosphorylated α-casein obtained by RP-HPLC. (Controls: incubated without phosphatase)

B

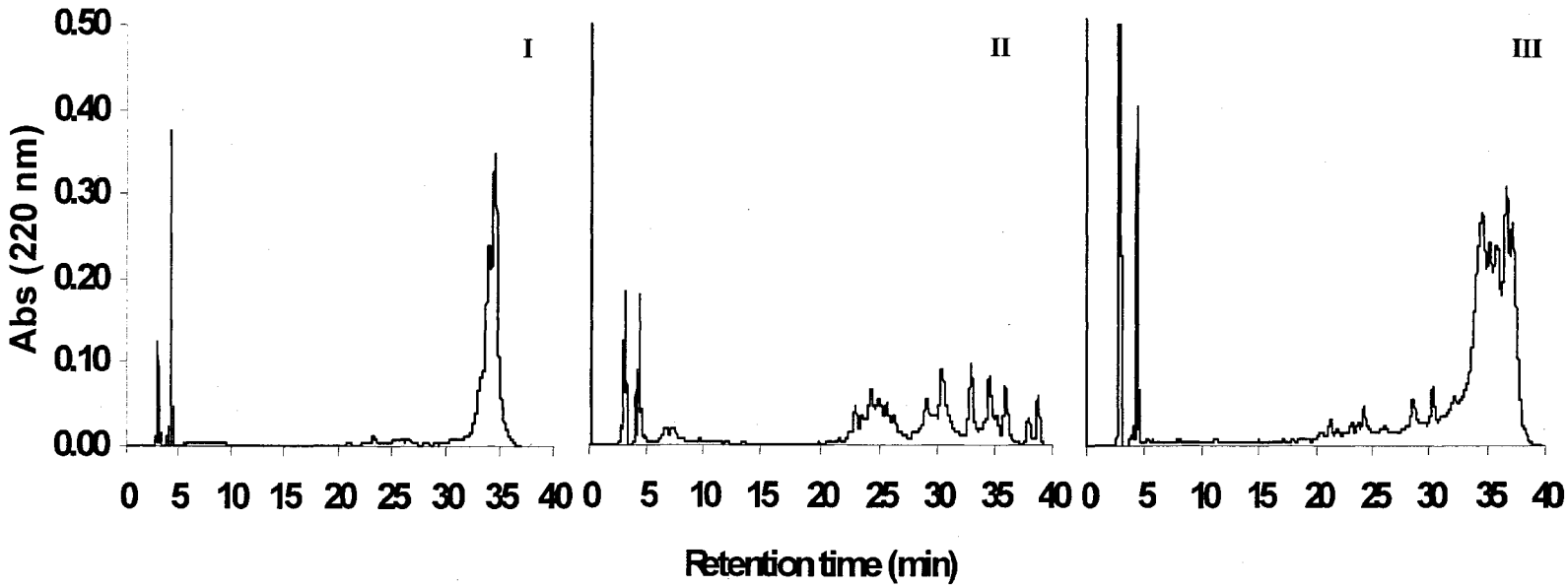


Figure 4.4: (B) Chromatographic profiles of (I) β-casein standard, (II) β-casein control and (III) dephosphorylated β-casein obtained by RP-HPLC. (Controls: incubated without phosphatase)

Figure 4.5 shows the mass spectra and deconvoluted spectra of standard α-casein (A, B), incubated (no phosphatase) α-casein (C, D) and dephosphorylated α-casein (E, F). Using ESI-MS data to identify dephosphorylation in proteins, it can be expected that a mass decrease of 80 Da will be observed for removal of each phosphate group (Hirschberg et al., 2004); with ESI tandem MS of phosphopeptides, loss of a phosphate group results in a decrease of 98 Da from a phosphoserine residue (Lund & Ardo, 2004). However, in this particular work a difference of 80 Da is considered since dephosphorylation with acid phosphatase leaves intact the serine residue and only HPO_3^- is removed. MS techniques have been used to identify the presence of phosphate groups in phosphoproteins and in phosphopeptides resulting from enzymatic or chemical degradation of the phosphoproteins (Lee et al., 2001; Zhong et al., 2004). MW species corresponding to removal of phosphate groups form α-casein are identified in Table 4.1; in addition MW species not assigned to removal of phosphate but present both in the incubated (no phosphatase) and dephosphorylated α-caseins are identified as products of hydrolysis. Standard α-casein gave a MW of 23 612 Da; this compares well with both the theoretical MW of α_{s1}-casein B variant (23 618 Da) calculated from the cDNA sequence as reported by Stewart et al. (1984) and the observed MW (23 614 Da) reported by Léonil et al. (1995).

On the basis of comparison of expected MW from theoretical dephosphorylation of α-casein and observed MW from the dephosphorylated α-casein (Table 4.1), six dephosphorylated species (MW 23 536, 23 458, 23 287, 23 140, 23 050 and 22 975 Da) were identified, corresponding to loss of 1, 2, 4, 6, 7 and 8 phosphate groups during dephosphorylation. Four of the phosphoseryl residues occur at the Ser_{64}– Ser_{68} region of

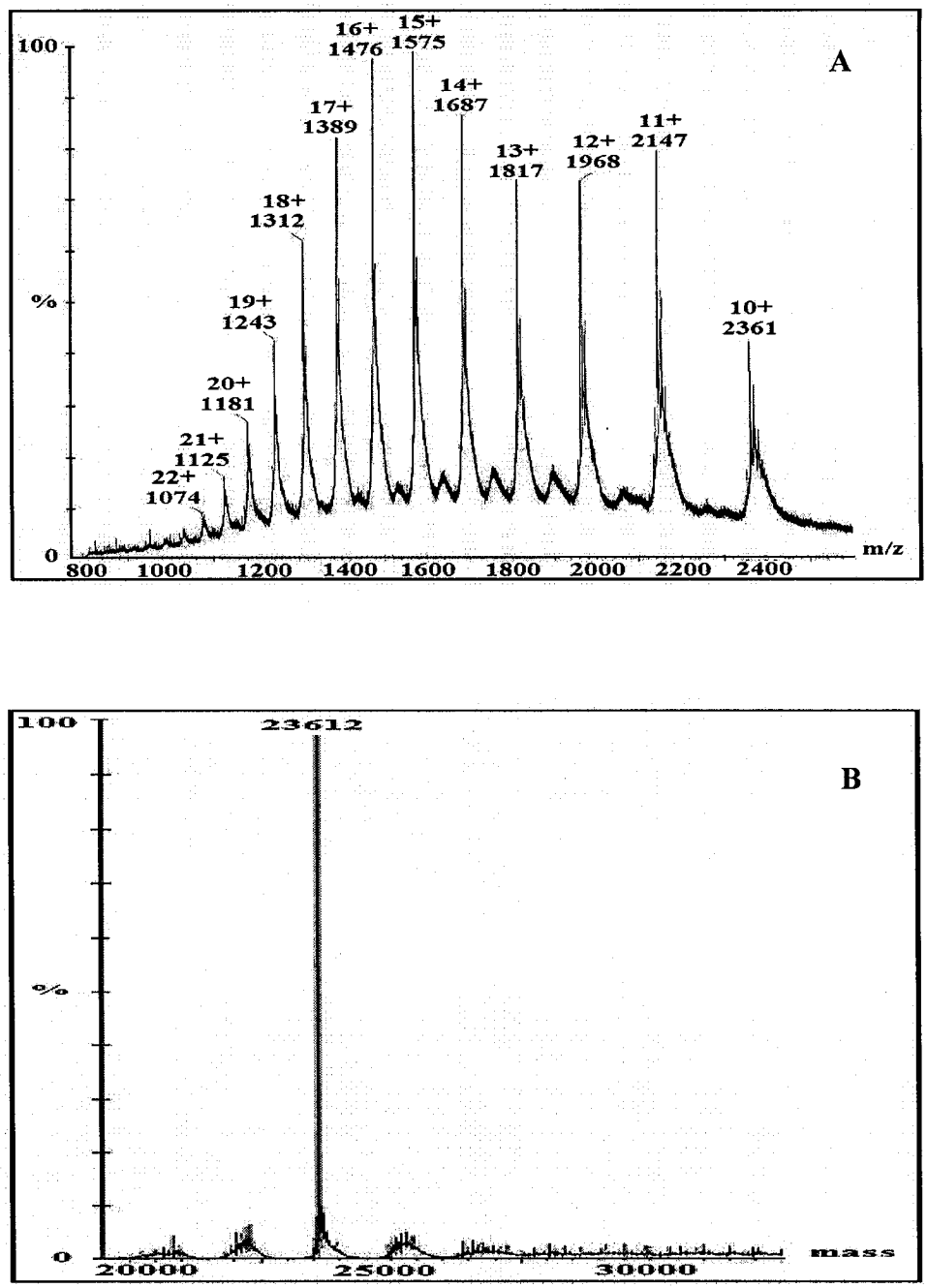


Figure 4.5: (A) ESI-MS spectra of α-casein standard indicating the net charge of the multiprotanated ions and (B) deconvoluted electrospray mass spectra of α-casein standard

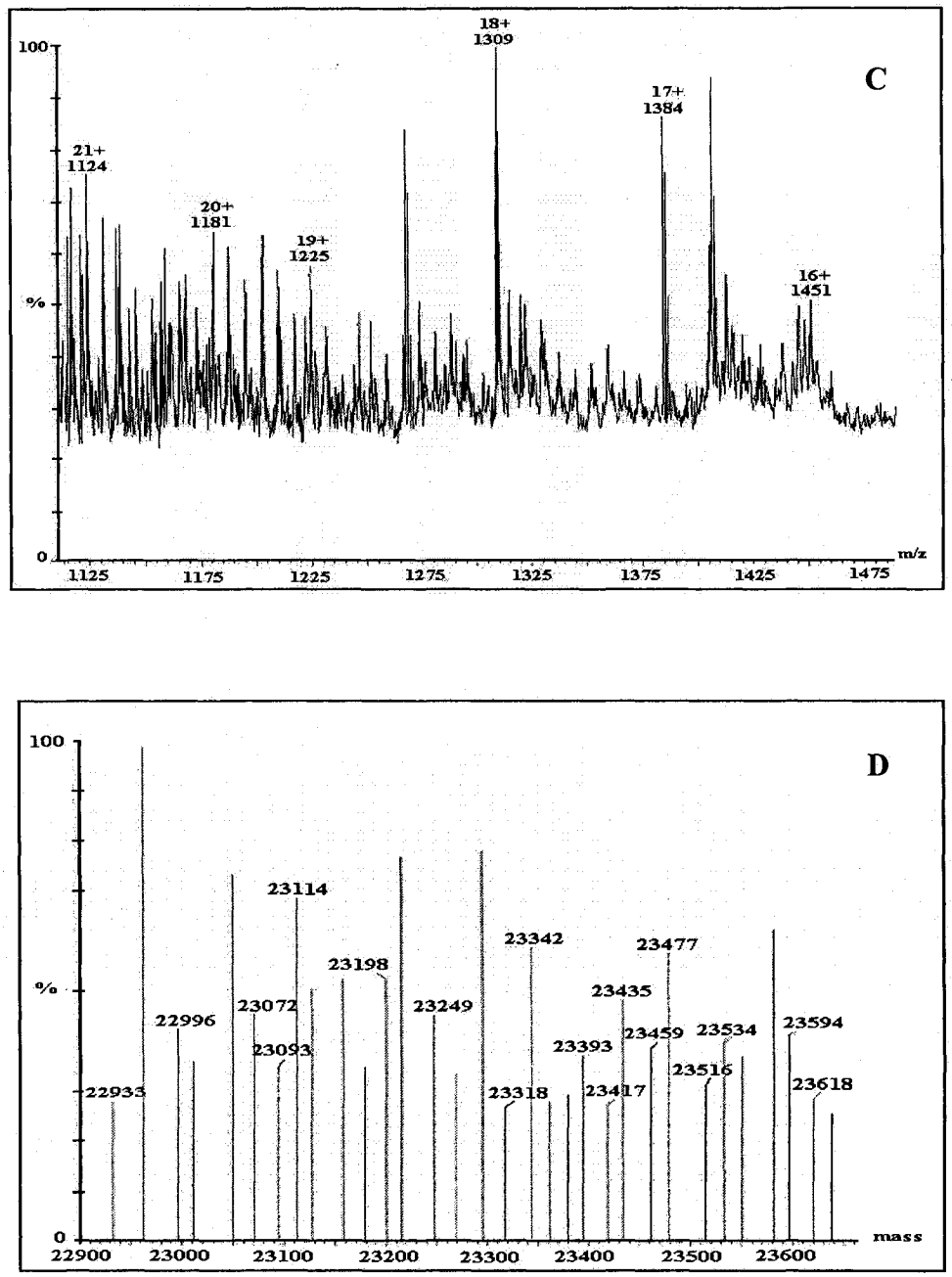


Figure 4.5: (C) ESI-MS spectra of α-casein incubated indicating the net charge of the multiprotanated ions and (D) deconvoluted electrospray mass spectra of α-casein incubated

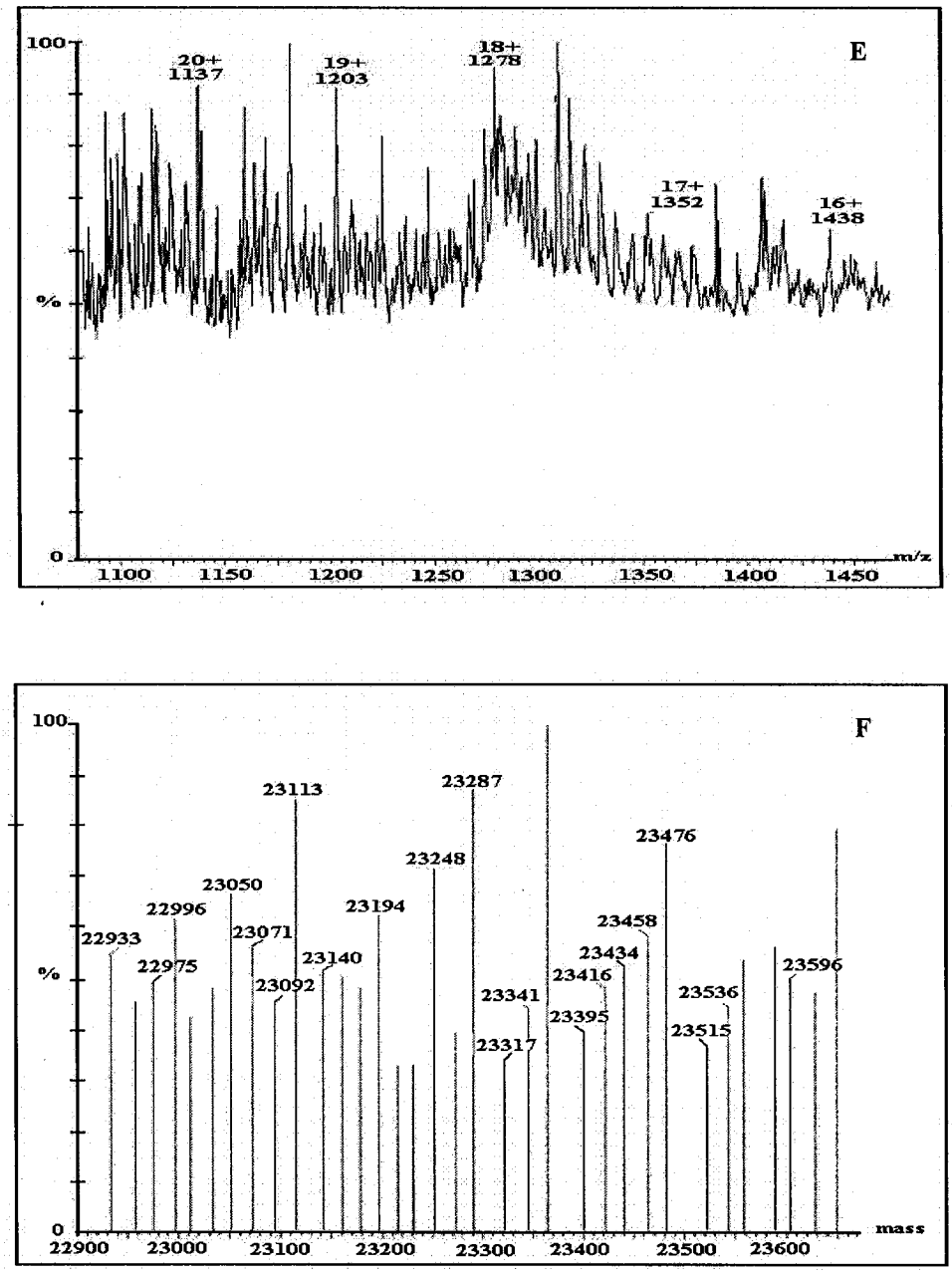


Figure 4.5: (E) ESI-MS spectra of dephosphorylated α-casein indicating the net charge of the multiprotanated ions and (F) deconvoluted electrospray mass spectra dephosphorylated α-casein

Table 4.1: Molecular species of α-casein, and dephosphorylated α-casein after deconvolution of mass spectra

Observed in α-casein standard (Da)	Theoretical MW in α-casein after dephosphorylation (Da)	Observed MW in α-casein incubated (Da)	Observed MW in α-casein dephosphorylated (Da)	Reason
23 612*		23 618 (6)		
		23 594 (-18)	23 596 (-16)	Hydrolysis
	23 532 (-1 Phosphate)	23 534 (-78) Léonil et al., (1995)	23 536 (-76)	Dephosphorylation (- 1 Phosphate)
		23 516 (-96)	23 515 (-97)	Hydrolysis
		23 477 (-135)	23 476 (-136)	Hydrolysis
	23 452 (-2 Phosphates)	23 459 (-153)	23 458 (-154)	Dephosphorylation (-2 Phosphates)
		23 435 (-177)	23 434 (-178)	Hydrolysis
		23 417 (-195)	23 416 (-196)	Hydrolysis
		23 393 (-219)	23 395 (-217)	Hydrolysis
		23 342 (-270)	23 341 (-271)	Hydrolysis
		23 318 (-294)	23 317 (-295)	Hydrolysis
	23 292 (-4 Phosphates)		23 287 (-325)	Dephosphorylation (-4 Phosphates)
		23 249 (-363)	23 248 (-364)	Hydrolysis
		23 198 (-414)	23 194 (-418)	Hydrolysis
	23 132 (-6 Phosphates)		23 140 (-472)	Dephosphorylation (-6 Phosphates)
		23 114 (-498)	23 113 (-499)	Hydrolysis
		23 093 (-519)	23 092 (-520)	Hydrolysis
		23 072 (-540)	23 071 (-541)	Hydrolysis
	23 052 (-7 Phosphates)		23 050 (-562)	Dephosphorylation (-7 Phosphates)
		22 996 (-616)	22 996 (-616)	Hydrolysis
	22 972 (-8 Phosphates)		22 975 (-637)	Dephosphorylation (-8 Phosphates)
		22 933 (-679)	22 933 (-679)	Hydrolysis

* Theoretical molecular weight of α_{s1}-casein B 23 618 Da (Stewart et al., 1984)

the α_{s1}-casein molecule, with three of the phosphates on adjacent serine residues but separated from a fourth by a single Ile_{65} residue; the other four serilphosphates are distributed in the remainder of the molecule (Ng-Kwai-Hang, 2003). The control α-casein (no phosphatase) showed the presence of the original α-casein (MW 23 618), as well as MW species (MW 23 534 and 23 459 Da) corresponding to loss of 1 and 2 phosphate groups respectively; this suggests partial dephosphorylation of α-casein in the absence of added phosphatase; Léonil et al. (1995) also observed with ESI-MS, the occurrence of partially dephosphorylated casein with the loss of 1 phosphate group in the absence of added enzyme. Figure 4.6 shows the mass spectra and deconvoluted spectra of standard β-casein (A, B), incubated (no phosphatase) β-casein (C, D) and dephosphorylated β-casein (E, F). Table 4.2 shows the MW species identified from these spectra. MW species corresponding to removal of phosphate groups from β-casein are summarized in Table 4.2; in addition MW species not assigned to removal of phosphate but present both in the incubated (no phosphatase) and dephosphorylated β-caseins are identified as products of hydrolysis. Standard β-casein gave a MW of 24 017 Da, which compares well with the values of 24 024 Da and 24 028 Da reported for variant A^1-5P (Jimenez-Flores et al., 1987; Swaisgood, 1992). On the basis of comparison of expected MW from theoretical dephosphorylation of β-casein and observed MW from the dephosphorylated β-casein (Table 4.1), five dephosphorylated species (MW 23 942, 23 855, 23 774, 23 687 and 23 615 Da) were identified, corresponding to loss of 1, 2, 3, 4 and 5 phosphate groups during dephosphorylation. West & Towers (1976) reported that dephosphorylation of β-casein was non-random with an alkaline phosphatase from *E.*

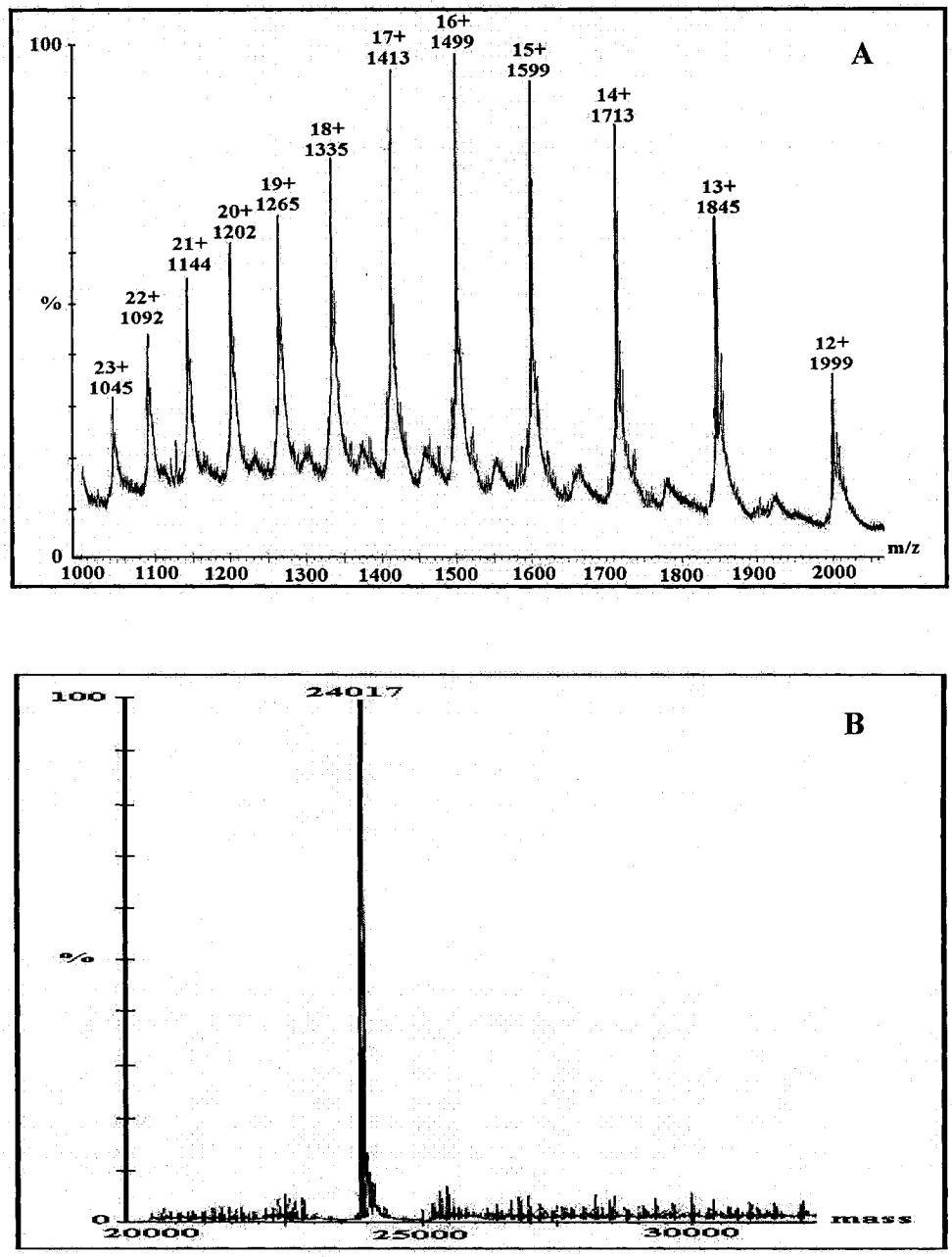


Figure 4.6: (A) ESI-MS spectra of β-casein standard indicating the net charge of the multiprotanated ions and (B) deconvoluted electrospray mass spectra of β-casein standard

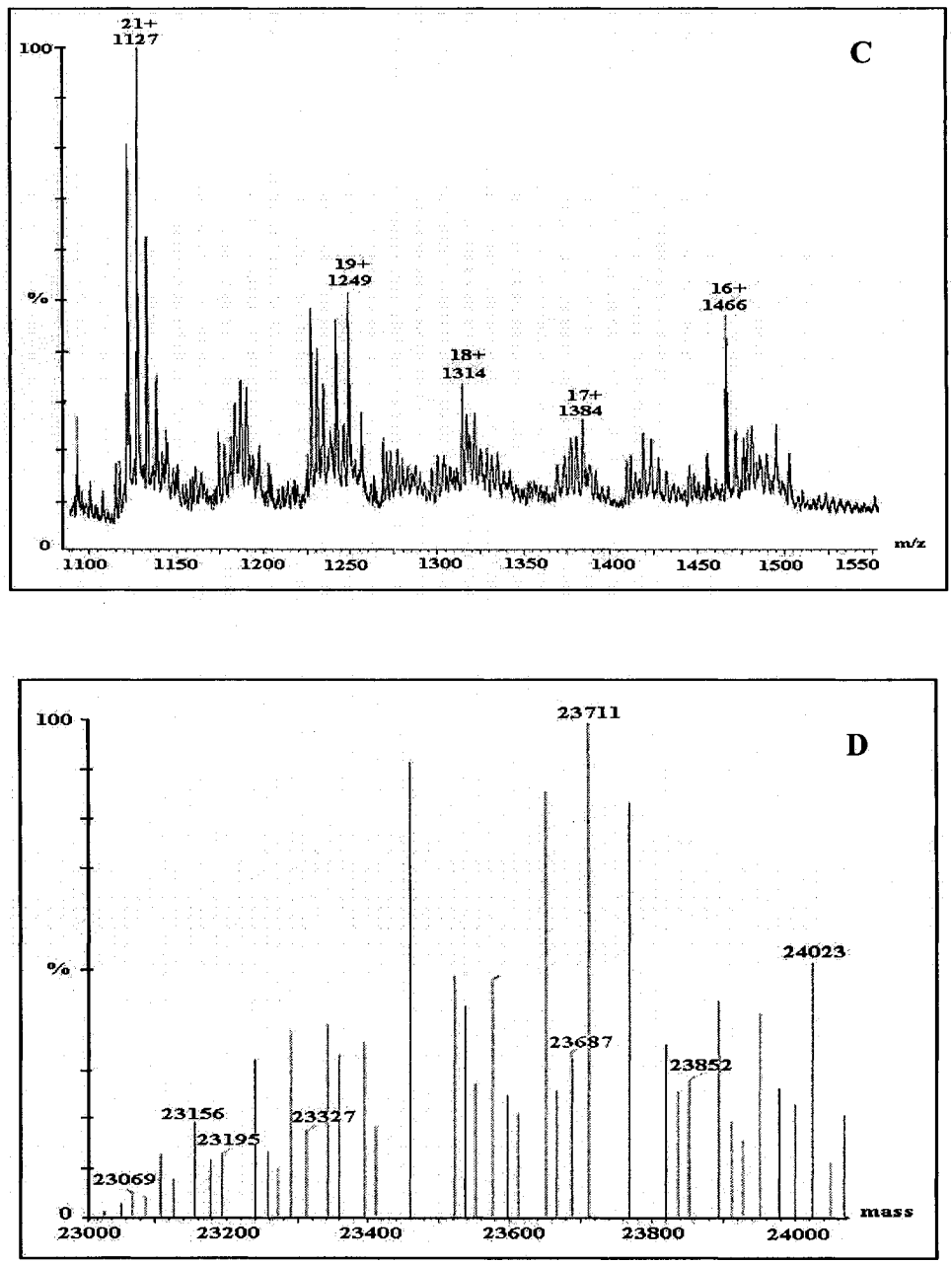


Figure 4.6: (C) ESI-MS spectra of β-casein incubated indicating the net charge of the multiprotanated ions and (D) deconvoluted electrospray mass spectra of β-casein incubated

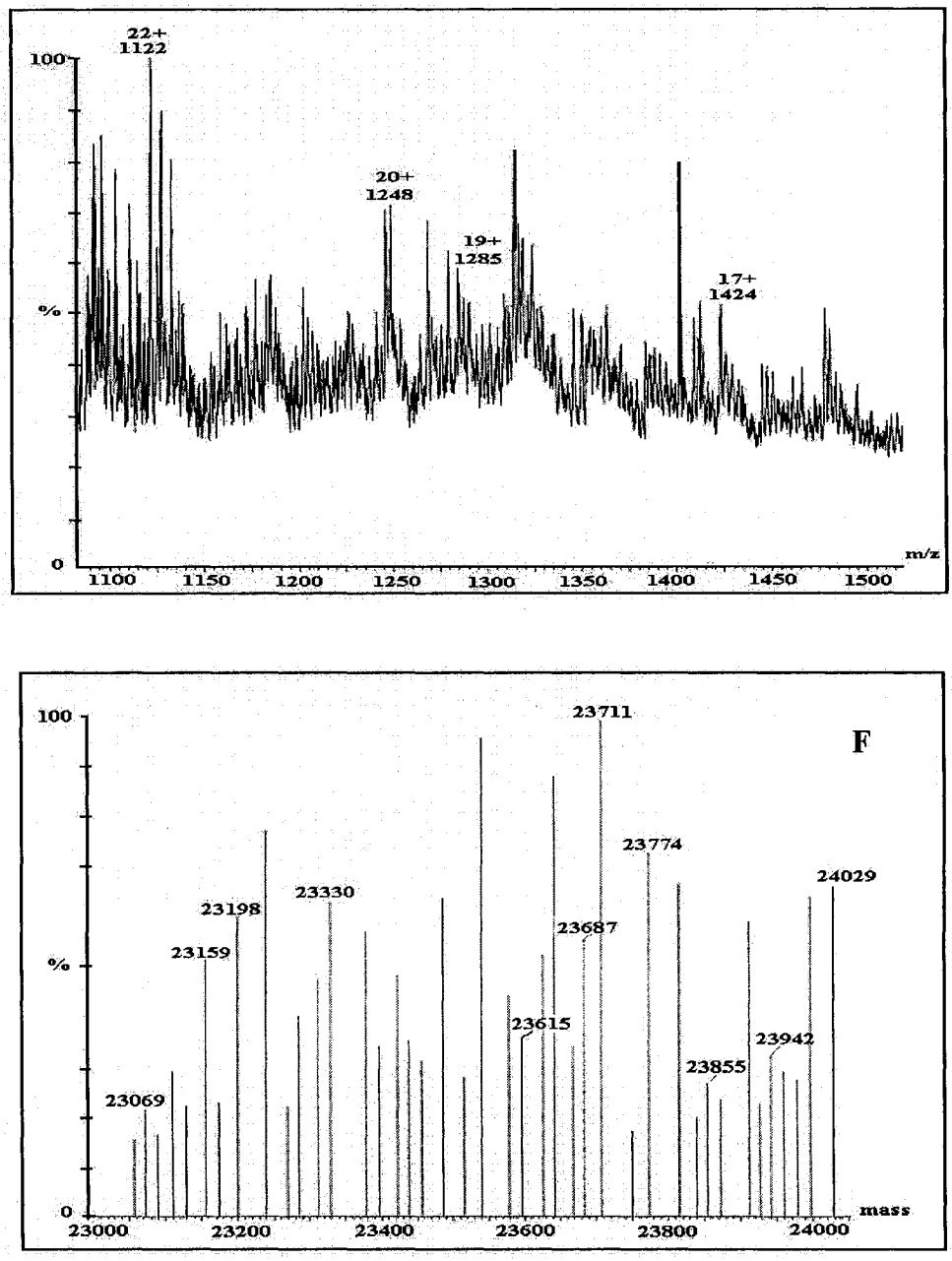


Figure 4.6: (E) ESI-MS spectra of dephosphorylated β-casein incubated indicating the net charge of the multiprotanated ions and (F) deconvoluted electrospray mass spectra of dephosphorylated β-casein

Table 4.2: Molecular species of β-, and dephosphorylated β-casein after deconvolution of mass spectra

Observed in β-casein standard (Da)	Theoretical MW in β-casein after dephosphorylation (Da)	Observed MW in β-casein incubated (Da)	Observed MW in β-casein dephosphorylated (Da)	Reason
24 017*		24 023 (-6)	24 029 (-12)	
	23 937 (-1 Phosphate)		23 942 (75)	Dephosphorylation (-1 Phosphate)
	23 857 (-2 Phosphates)	23 852 (-165)	23 855 (-162)	Dephosphorylation (-2 Phosphates)
	23 777 (-3 Phosphates)		23 774 (-243)	Dephosphorylation (-3 Phosphates)
	-	23 711(-306)	23 711 (-306)	Hydrolysis
	23 697 (-4 Phosphates)	23 687 (-330)	23 687 (-330)	Dephosphorylation (- 4 Phosphates)
	23 617 (-5 Phosphates)		23 615 (-402)	Dephosphorylation (-5 Phosphates)
		23 327 (-690)	23 330 (-687)	Hydrolysis
		23 195 (-822)	23 198 (-819)	Hydrolysis
		23 156 (-861)	23 159 (-858)	Hydrolysis
		23 069 (-948)	23 069 (-948)	Hydrolysis

* Theoretical molecular weight of β-casein 24 024 Da (Jimenez-Flores et al., 1987)

coli, but was different from the action observed with a bovine spleen phosphatase. The control β-casein showed the presence of the original β-casein (MW 23 023 Da), as well as a MW species of 23 852 Da corresponding to loss of 2 phosphate groups; this suggests partial dephosphorylation of β-casein in the absence of added phosphatase.

CHAPTER 5

EFFECTS OF DEPHOSPHORYLATION ON PROTEOLYSIS OF CASEINS: PEPTIDE IDENTIFICATION AS PRODUCTS OF PROTEOLYSIS

5.1 Justification

The products of enzymatic dephosphorylation of caseins were identified in chapter four; dephosphorylated caseins were identified by ESI-MS. The effects of dephosphorylation on proteolysis of the caseins was the next step of this study. Food proteomics is an emerging field in biological sciences. Most proteins, like caseins, undergo post-translational modifications such as phosphorylation. Knowledge of these modifications is important because they may alter casein properties, including stability, activity, and consequently, function of the casein. Proteomics has become a useful tool to analyze biological samples; it uses mass spectrometry extensively to identify and characterize proteins (Chalkley et al., 2005). Caseins are relatively simple molecules to manipulate; protein digestibility and enzymatic modification methods were used to determine the effect of phosphate groups on biological properties. In addition, novel mass spectrometric peptide sequencing coupled with database analysis are emerging technologies for investigating casein modifications.

This part of the study addresses the third research objective related to the effect of dephosphorylation on the hydrolysis of α- and β-caseins. The identification and characterization of LC-ESI-MS/MS peptide mass mapping for the *in vitro* enzymatic hydrolysis simulating human gastric conditions is also described.

5.2 Material and Methods

5.2.1 Materials

Whole casein was obtained as described previously in Section 3.2.1. Commercial α-casein and β-casein standards, potato acid phosphatase (EC 3.1.3.2; activity: 1.2 units/mg), pepsin from porcine stomach mucosa (E.C. 3.4.23.1; activity 3, 600 units/mg protein) and trypsin Type IX-S from porcine pancreas (E.C. 3.4.21.4; activity 16, 100 units/mg protein) were purchased from Sigma Chemicals Co. (St. Louis, MO). MS reagents were HPLC grade.

5.2.2 Enzymatic Dephosphorylation

Dephosphorylation of caseins was conducted as described in Section 3.2.3 with a modification; in order to obtain adequate quantities of dephosphorylated caseins, the enzyme reaction was performed in 100 ml of citrate buffer containing 250 mg of different caseins and phosphatase concentration of 0.20 mg/ml. The reaction time was 6 h and it was stopped by lowering the temperature to 0 °C. Caseins were dialyzed overnight against distilled water at 4 °C to remove salts from the buffer. Desalted samples were freeze-dried for further analysis. Controls (no phosphatase enzyme) were prepared under the same experimental conditions.

5.2.3 Enzymatic Digestibility of Dephosphorylated Caseins

In vitro proteolysis simulating human gastrointestinal conditions of native and dephosphorylated caseins was performed using the method of (Pihlanto-Leppälä et al., 1994) with an enzyme/substrate ratio of 1:100. 0.3% (w/v) solution of freeze-dried

caseins in (i) 10 mM HCl at pH 2 were prepared for peptic hydrolysis, and (ii) 10 mM phosphate buffer at pH 8 for tryptic hydrolysis. 0.1% (w/v) solutions of pepsin or trypsin in distilled water were prepared; the enzyme-substrate mixture was incubated for 3 h at 37 °C and the reaction was stopped by reducing the temperature to 0 °C. For combined proteolysis with pepsin and trypsin, initial conditions were set at pH 2; after 1.5 h the reaction was adjusted to pH 8 and then trypsin solution was added to the reaction mixture. Substrate and enzyme solutions were prepared immediately before the hydrolysis experiments. Controls (native and dephosphorylated α- and β-casein) were performed without the addition of pepsin and trypsin enzymes.

5.2.4 Peptide Analysis

5.2.4.1 Reverse-Phase High Performance Liquid Chromatography

The products form proteolytic hydrolysis of caseins and the dephosphorylated caseins were subjected to RP-HPLC. The analysis was carried out as described in Section 4.2.3. An octadecyl (C18) reverse phase 5μm, 250 X 4.6 mm column (Mallinckrodt Baker, Inc.; New Jersey, USA) was used. Solvent A consisted of 0.1% TFA in water and solvent B was 60% acetonitile/40%water/0.1% TFA acidified water (TFA) in acetonitrile (Pihlanto-Leppälä et al., 1994); elution was performed at a flow rate of 0.8 ml/min with a 60 min linear gradient starting at 0% of solvent B and increasing up to 80% over 50 min, then the linear conditions were re-established over 10 min. Detection was carried out at 215 nm.

5.2.4.2 Liquid Chromatography Electrospray Ionization Mass Spectrometry and Tandem Mass Spectrometry

Hydrolyzed α-casein and β-casein and their dephosphorylated hydrolyzed products were dialyzed, lyophilized and dissolved in 0.1 % formic acid. Controls (no proteolytic enzymes) were subjected to the same procedure. Samples were analyzed by as described in Section 4.2.4. For LC-MS/MS analysis, the mass spectrometer was coupled to a Waters CapLC system, operated at a flow rate of 6 µl/min. A splitter placed before the column delivered a final flow rate of 0.3 µl/min. The guard column consisted of Waters Symmetry 300 NanoEase C18, 5 µm. The separation was achieved using a Waters Atlantis dC18, 3 µm, 75 µm x 50 mm column. A binary gradient of solvent B (acetonitrile: 0.1% formic acid) and solvent A (water: 0.1% formic acid) was increased from 5 to 50% in 25 min. Injection volume was 5 µl. For the MS survey mass range, m/z, was 400-1990 and for MS/MS was 50-1990, scanned continuously over the chromatographic run (Waters Corporation Manual, 2005). The mass spectrometer was tuned and calibrated with [Glu]-Fibrinopeptide B (Sigma Chemicals Co; St. Louis, MO). Instrument control and data analysis software were carried out by MassLynx V4.0 and ProteinLynx Global Server 2.1 (Waters Corporation, 2005). MS data analysis was carried out by MassLynx V4.0 software.

5.2.4.3 Database Analysis

Evaluation of the effect of dephosphorylation on proteolysis and peptide sequences characterization was achieved using the survey MS/MS data; two softwares were used: ProteinLynx Global Server v2.1 (Waters Corporation, 2005) and Mascot

(Perkins et al., 1999). ProteinLynx is an integrated software used for protein and peptide identification and includes a database search engine. The survey data acquired were processed by ProteinLynx to create a peak list (Appendix 1), which contained the MS/MS data to be searched against a FASTA-formatted protein databank (SwissProt protein database, Swiss Institute of Bioinformatics, 2006); the factors contributing to the database search scores in ProteinLynx were the number of entries in the database, the mass accuracy, and the peak intensity. For the MS/MS scoring, every precursor mass in the query was compared to the mass of each theoretically determined peptide in the database, taking into account the tolerance of the precursor mass and the fragmentation characteristics. The probability for each identified peptide was then calculated and given a score, which were reported as natural logs. For instance, in a database with 100000 entries, the maximum score would be 11.5 and the 95% significance threshold would be 11.4. The created peak list (Appendix 1) from ProteinLynx was transformed into PKL format and analyzed using the software search algorithm Mascot in the mode MS/MS Ion Search. The searches were not taxonomically restricted. The enzyme entry was set as pepsin, trypsin or none, according to each raw data set analyzed. The scoring of Mascot incorporated a probability-based implementation of the Mowse algorithm with the formula: Mowse score = - 10 X log (p); this score is based on the probability (p) that a peptide identified from the experimental fragment matched a peptide in a protein database. A random match will have a high probability (thus, a low Mowse score), whereas a valid match will have a low probability and consequently a high Mowse score. Mascot ranks the quality of the peptide matches and sums the scores of detected peptides to calculate a total protein score (Appendix 2). In order to eliminate ambiguous peptide

mass results, the results obtained from Mascot, with a score indicating peptide identity, were correlated by comparison of the fragmentation spectra obtained by ProteinLynx. All peptides masses were obtained as monoisotopic masses. The softwares (Mascot and ProteinLynx) used to process the MS/MS raw data were able to detect signal peaks within a speed time of approximately 10 sec. Additionally, these mechanisms were capable of using information hidden in low peaks that otherwise could not been distinguished from noise, allowing to considerably improve the identification rate and sequence coverage of the caseins.

5.3 Results and Discussion

5.3.1 Peptide Mapping by RP-HPLC

Figure 5.1 shows the RP-HPLC chromatographic patterns of α-casein and dephosphorylated α-casein casein obtained after treatment with (A) pepsin, (B) trypsin and (C) combined proteolysis. Comparison of the chromatograms in Figure 5.1 for native and dephosphorylated α-casein indicates that many peptides are common to every enzymatic digests since they eluted at the same retention time (8-45 min), only slightly differences were noted in some peaks for dephosphorylated α-casein indicated an increase in retention time due to change in hydrophobicity obtained by the removal of phosphate groups. These results agree with those reported by other researchers (Lemieux & Amiot, 1990; Carles & Ribadeau Dumas, 1986). The pepsin treatment produced peptides mainly in a retention time range of 18-43 min whereas trypsin treatment generated diverse peptides at different retention times (8-48 min) compared to the peptic hydrolysis. The effects of both proteases produced a mixture of peptides over a wider

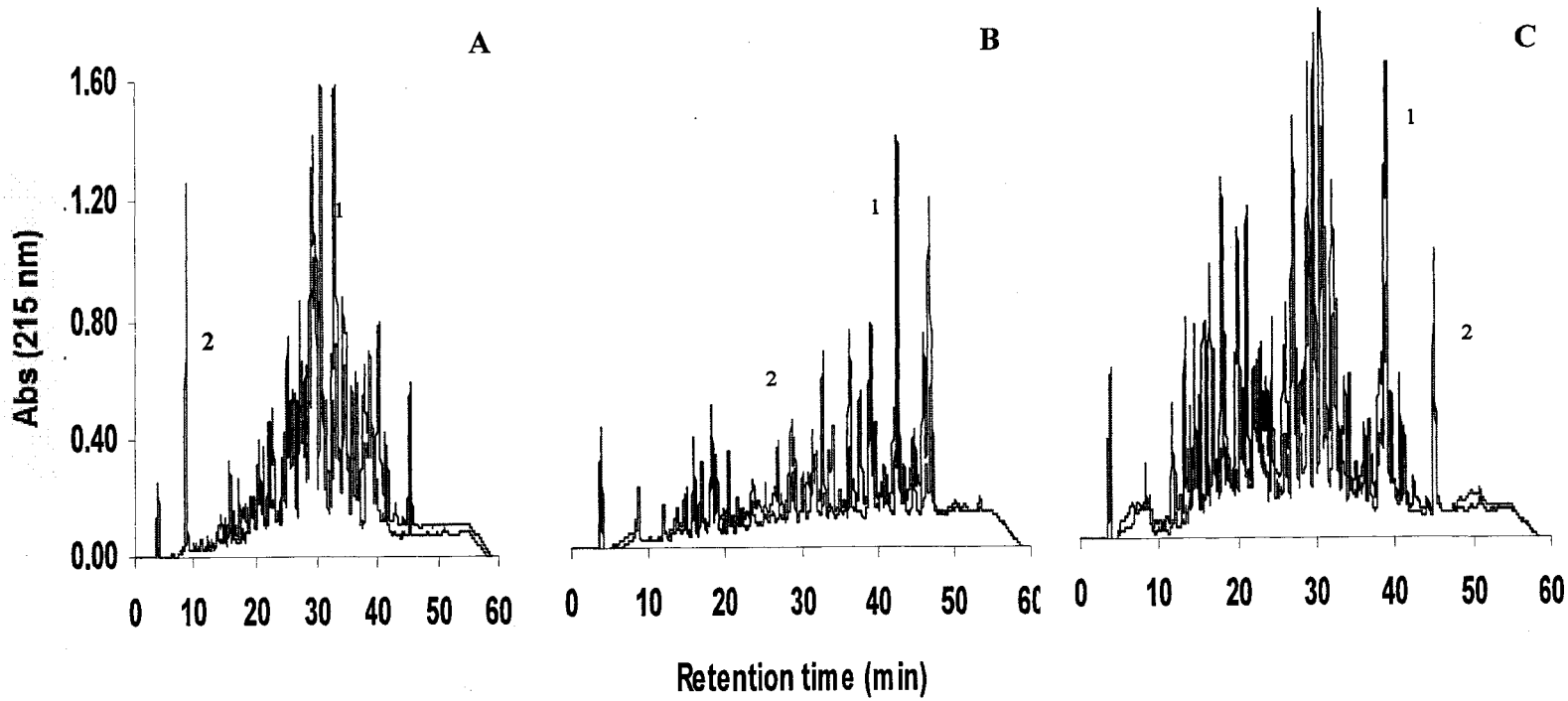


Figure 5.1: Chromatographic peptide mapping of (1) α-casein and (2) dephosphorylated α-casein after: (A) pepsin, (B) trypsin and (C) combined pepsin-trypsin proteolysis

retention time range (5-48 min). Peptides that eluted at the first part of the gradient are likely more polar and less strongly adsorbed; short hydrophilic peptides with basic character are poorly absorbed and have minor interaction with the column support (Janssen et al., 1984). Retention time of peptides on a reversed-phase column has been shown to depend on their amino acid composition, ionization state, the localization of charges and the hydrophobicity rather than in the amino acid sequence (Lemieux & Amiot, 1989).

Figure 5.2 represents the elution patterns of β- and dephosphorylated β-casein after treatment with (A) pepsin, (B) trypsin and (C) combined proteolysis. Pepsin hydrolysis produced peptides within a retention time interval of 18-48 min, trypsin generated peptides within 5-58 min and an extensive number of peaks was observed in the peptic/tryptic combined proteolysis occurring during 5-55 min; for this treatment a combined chromatographic profile included peaks obtained individually for pepsin and trypsin. In general, same peaks were detected in β-casein and dephosphorylated β-casein. A previous study (Grego et al., 1984) indicated that the retention times of phosphorylated peptides, were less than those of the corresponding dephosphorylated peptides; a similar finding was observed for some peptides corresponding to dephosphorylated β-casein in our study. It is expected that a peptide containing less or no phosphoserine residues is more strongly bound to the stationary phase than a peptide containing phosphoserine residues. The RP-HPLC chromatogram for each enzyme treatment studied could not be used to identify the peptides produced; characterization of the peptides obtained for the native and dephosphorylated caseins was performed using LC-ESI-MS and MS/MS because of their ability to deal with complex peptide mixtures observed in the chromatograms obtained.

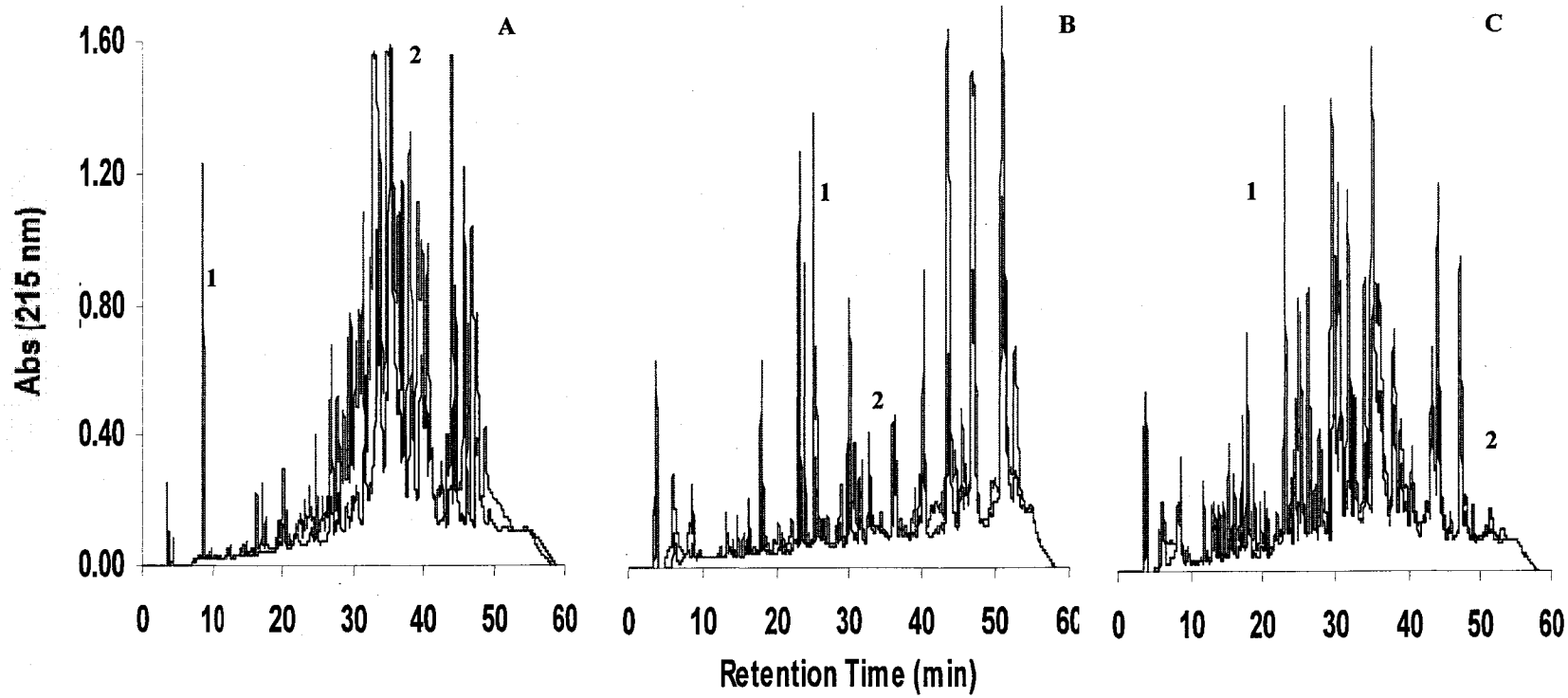


Figure 5.2: Chromatographic peptide mapping of (1) β-casein and (2) dephosphorylated β-casein after: (A) pepsin, (B) trypsin and (C) combined pepsin-trypsin proteolysis

5.3.2 Identification of Peptides

5.3.2.1 LC-ESI-MS of Native and Dephosphorylated α-casein and β-casein

Figure 5.3 shows a representative LC-ESI-MS spectra for α-casein and dephosphorylated α-casein after combined proteolysis with pepsin and trypsin. The signals (m/z) 506.3 and 769.3 were identified only in Figure 5.3 (B) for dephosphorylated α-casein, indicating effects of dephosphorylation on α-casein proteolysis. From complete analysis of the LC-ESI-MS spectra a total of 25, 28 and 49 molecular species were identified for α-casein treatment with pepsin, trypsin and successive proteolysis (Appendix 3), respectively. For dephosphorylated α-casein 26, 25 and 52 peptides were identified (Appendix 3) only by molecular weights, after pepsin, trypsin and combined proteolysis. These signals were further analyzed by MS/MS (Section 5.3.2.2), in order to more fully identify all the peptides generated.

Figure 5.4 shows a representative LC-ESI-MS spectra for β-casein and dephosphorylated β-casein after combined proteolysis with pepsin and trypsin. The signals (m/z) 497.2 and 553.8 were identified only in Figure 5.4 (B) for dephosphorylated β-casein, likely indicating effects of dephosphorylation on β-casein proteolysis. For β-casein 44, 23 and 49 and for dephosphorylated β-casein 48, 27 and 43 peptides were noted after pepsin, trypsin and combined proteolysis, respectively in both caseins (Appendix 4). These signals were further analyzed by MS/MS (Section 5.3.2.3), in order to clearly identify all the peptides generated. Our LC-ESI-MS results suggest that mass suppression did not occurred in the peptides derived from the caseins before and after dephosphorylation; thus contrary to the results obtained by Larsen et al. (2001), where

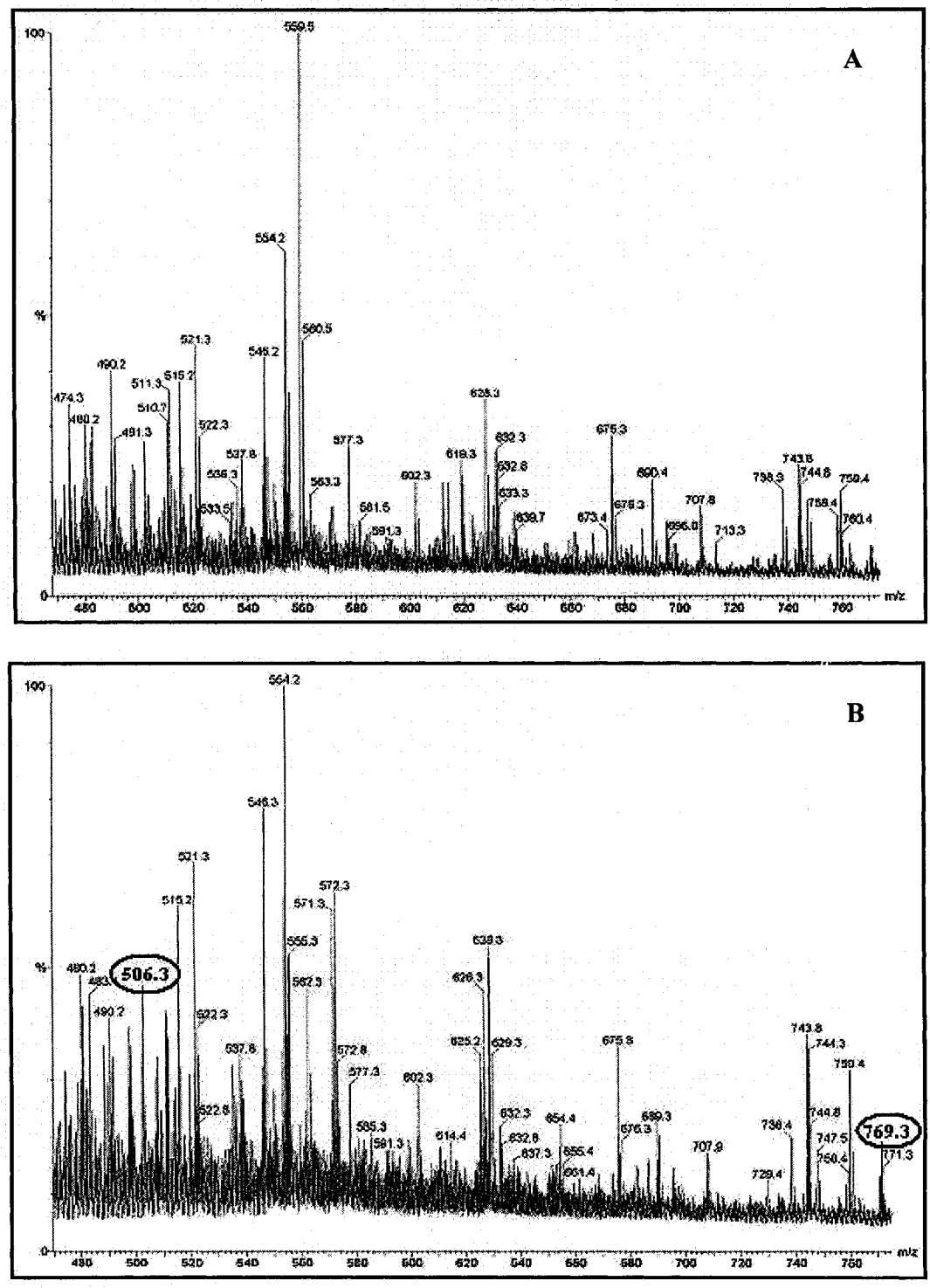


Figure 5.3: (A) LC-ESI-MS spectra of α-casein and (B) LC-ESI-MS spectra of dephosphorylated α-casein hydrolyzed by pepsin-trypsin. Signals in bold type/circled indicate dephosphorylated peptides

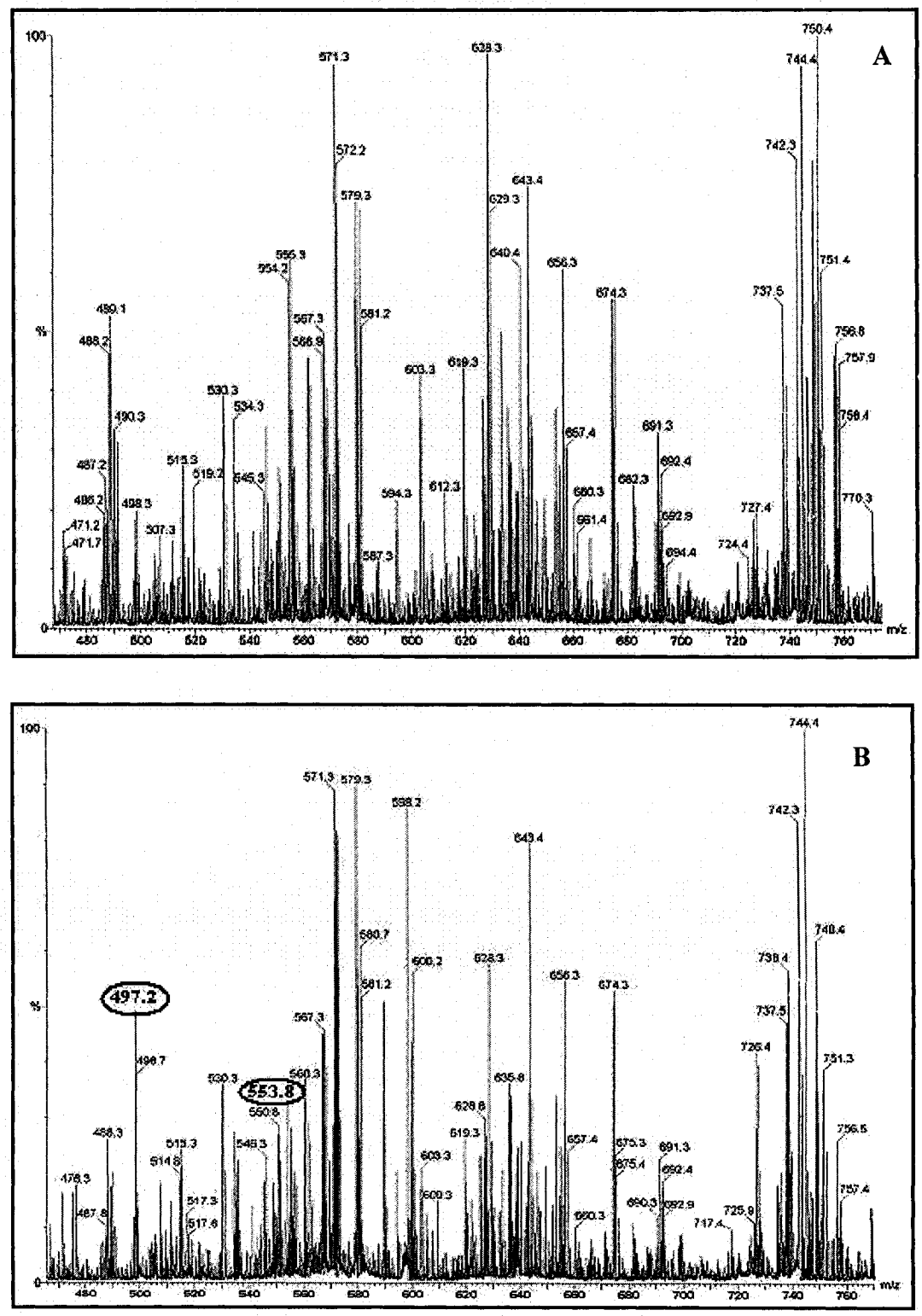


Figure 5.4: (A) LC-ESI-MS spectra of β-casein and (B) LC-ESI-MS spectra of dephosphorylated β-casein hydrolyzed by pepsin-trypsin. Signals in bold type/circled indicate dephosphorylated peptides

the intensity signals retention time range for phosphorylated peptides were very low compared to other peptide signals in the spectrum, due to a combination of specific suppression of the signals for the phosphopeptides and metastable fragmentation of the phosphate groups. In the molecular weight range from 5 to 50 kDa analysis by LC-ESI-MS exhibits better mass resolution than MALDI (Carr & Annan, 1996), which permitted the detection of molecular weight that varies slightly in mass. It was observed (Figure 5.5) that the maximum number of charges for the peptides correlated reasonably well with the number of amino acids side chains (arginine, lysine and histidine) than can accept a proton at the low pH used in the analyte stream. In the case of peptides produced by the three proteases treatments, generally double charged species were present with a dominant $[M+2H]^{2+}$ ion (Figure 5.5 A). Few smaller peptides with a single charged $[M+H]^{+}$ ion were identified (Figure 5.5 B). The evidence that these ions carried a single charge was obtained from the spacing of the isotope peaks, which were one m/z unit apart; doubly charged form of the parent ions was observed when the peaks from the isotope cluster of the daughter ions were spaced 0.5 m/z units apart (Yeboah et al., 1999).

5.3.2.2 LC-ESI-MS/MS ProteinLynx and Mascot Search Engine for Identification of Peptides

LC-ESI-MS/MS spectral data were analyzed using ProteinLynx and Mascot search engine (Section 5.2.4.3). The results presented are the MS and the MS/MS spectra of selected peptides from the spectral analysis; the peptides sequences were identified as well as the specific parts of the parent molecule from which the peptides were originated.

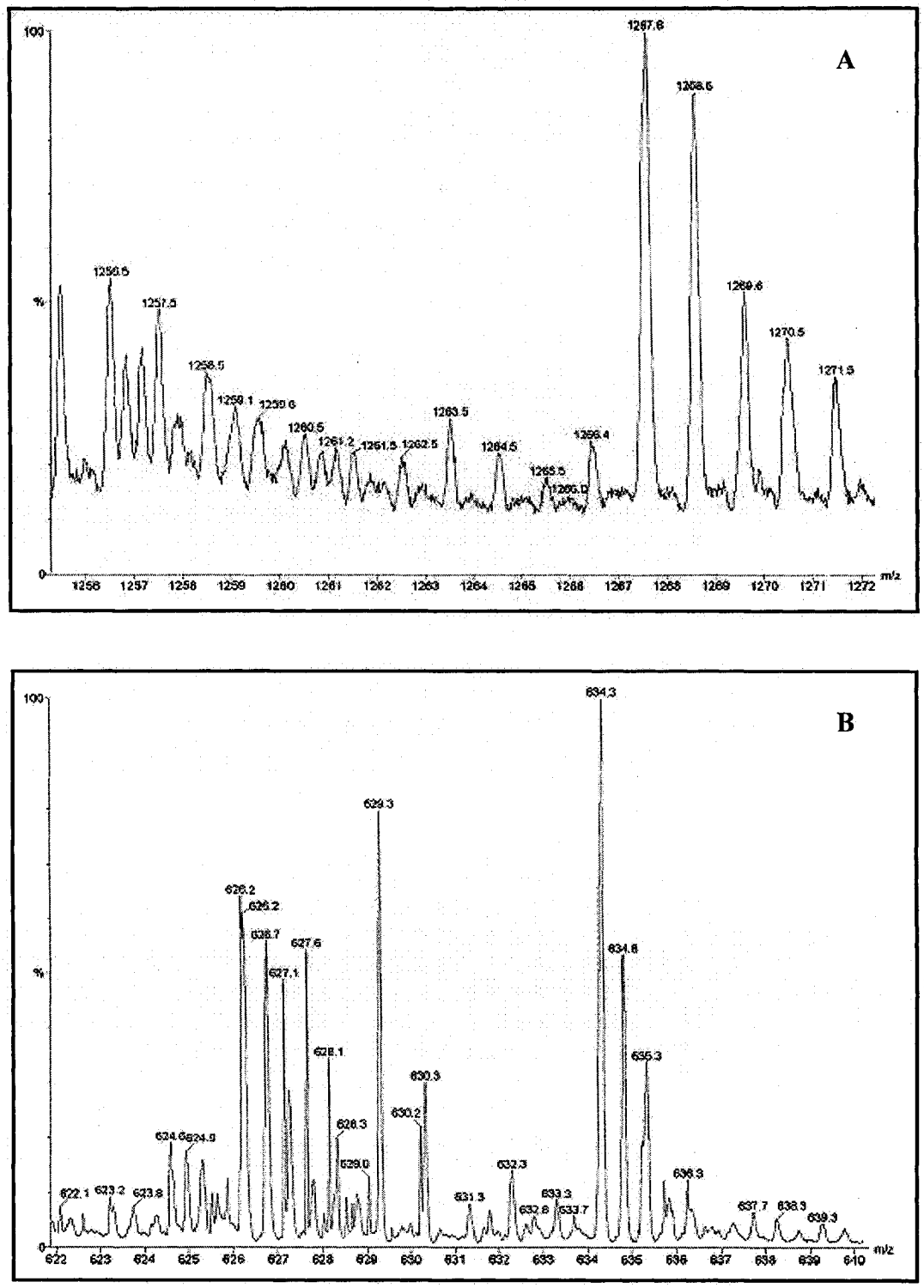


Figure 5.5 (A) LC-ESI-MS of dephosphorylated α-casein hydrolyzed by trypsin representing a dominant $[M+H]^+$ ion and (B) LC-ESI-MS spectra of dephosphorylated β-casein hydrolyzed by trypsin indicating a dominant $[M+H]^{2+}$ ion

5.3.2.2.1 Identification of Peptides from α-casein and Dephosphorylated α-casein

Figure 5.6 (A) shows the LC-ESI-MS spectra used to generate the LC-ESI-MS/MS spectra (Figure 5.6 B) of the triple charged peptide R_{100}-L_{109} (m/z 423.9) from dephosphorylated α-casein after pepsin hydrolysis; from these spectra the identified peptide was RLKKYKVPQL (MW 1271.7 Da). All identified peptides are listed in Table 5.1 and the origin of these peptides is shown in Figure 5.7. Three similar peptides were obtained by the action of pepsin in α-casein and dephosphorylated α-casein, respectively. Native and dephosphorylated α-casein were cleaved by pepsin at the same five peptide bonds F_{32}-G_{33}, L_{40}-S_{41}, L_{99}-R_{100}, L_{101}-K_{102} and L_{109}-E_{110}, this enzyme did not hydrolyze the dephosphorylated α-casein where the clusters of phosphoserine residue were removed. Pepsin showed a preferential splitting at the L-X and F-X bonds for both native α-casein and dephosphorylated α-casein. It has been reported that pepsin cleaves C-terminal to aromatic amino acids and leucine (Athaudaa & Takahashia, 2002).

Figure 5.8 (A) shows the LC-ESI-MS spectra used to generate the LC-ESI-MS/MS spectra (Figure 5.8 B) of the triple charged peptide Q_{59}-K_{79} (m/z 773.3) from dephosphorylated α-casein tryptic hydrolysis; the molecular weight indicates the loss of 5 phosphate groups. Six and eight peptides were obtained by the action of trypsin in α-casein and dephosphorylated α-casein, respectively (Table 5.1). The following ten peptide bonds were cleaved in both the native and the dephosphorylated α-casein: K_7-H_8, R_{22}-F_{23}, K_{34}-E_{35}, K_{42}-D_{43}, K_{58}-E_{59}, K_{83}-E_{84}, R_{90}-Y_{91}, R_{100}-L_{101}, K_{132}-E_{133} and R_{151}-Q_{152}; some of these cleavage bonds have been reported (Lemieux & Amiot, 1990; Lemieux & Amiot, 1989). Trypsin gave preferential cleavage at the K-X and R-X bonds for both

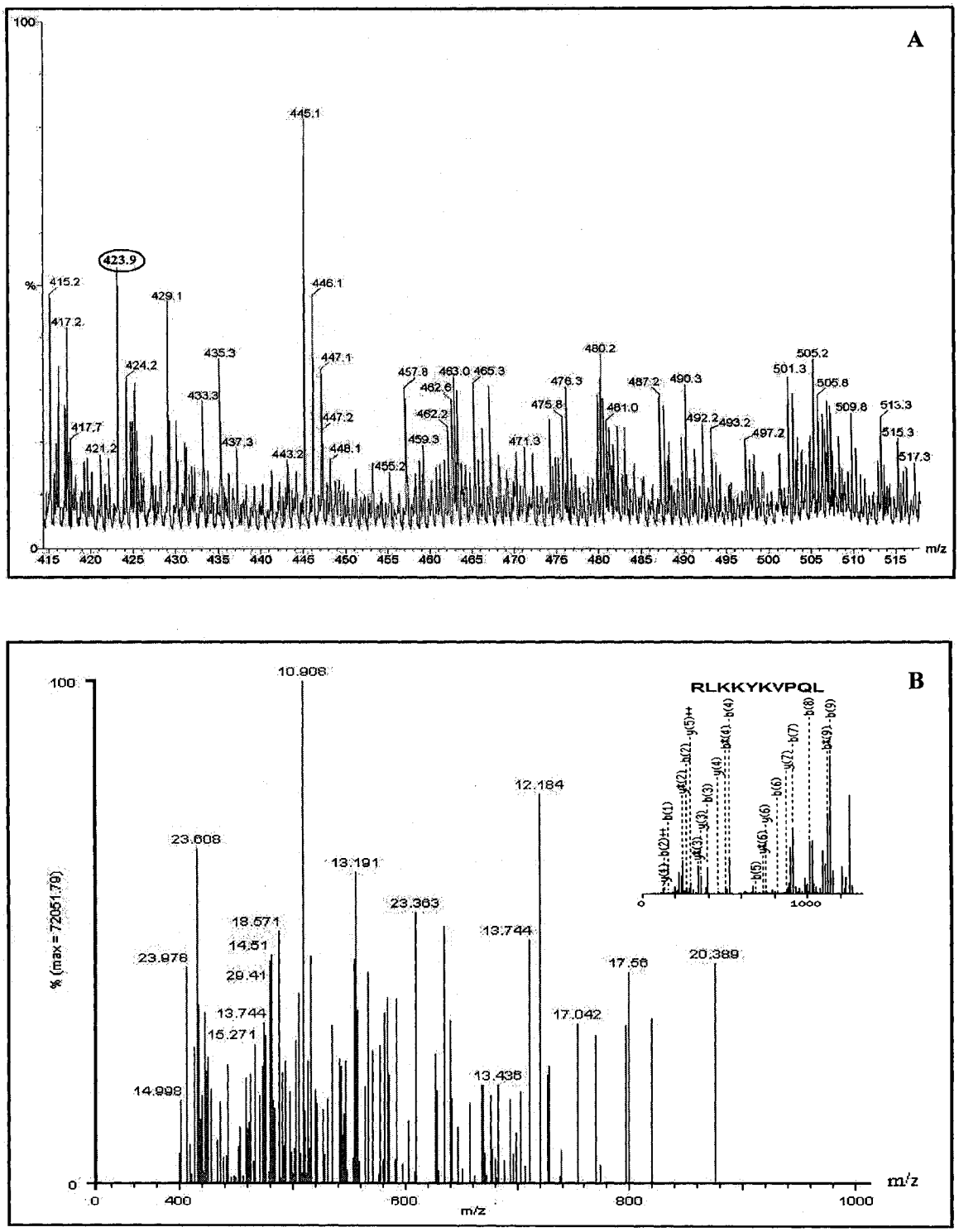


Figure 5.6: (A) LC-ESI-MS spectra (m/z region 415 to 515), m/z 423.9 signal in bold type/circled and (B) LC-ESI-MS/MS spectra (m/z region 320 to 1000) of dephosphorylated α-casein peptic hydrolysis. Inset represents the fragmentation spectra of the peptide RLKKYKVPQL, experimental MW 1271.7 Da

Table 5.1: Peptides identified in enzyme hydrolysis products of α- and dephosphorylated α-casein by LC-ESI-MS/MS

Peptide: Theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN, Reference	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN
570, 1 EIVPN 70-74								570.2
807.4, 2 HQGLPQE 8-14								807.4
830.4, 2 EDVPSER 84-90, (Lemieux & Amiot, 1990; 1989)	830.4				830.3	830.4	830.3	830.3
915.5, 2 GKEKVNEL 33-40			915.4	915.5				
945.5, 2 EKVNELSK 35-42							945.5	
958.5, 2 KEDVPSER 83-90							958.4	958.5
964.4, 2 (-2 Phosphate) KDIGSESTE 42-50								964.4

Table 5.1 (continued)

Peptide: Theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN, Reference	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN
1002.6, 2 KKYKVPQL 102-109			1002.5	1002.6				
1012.5, 2 (-1 Phosphate) IVPNSVEQK 71-79								1012.5
1019.4, 2 HQGLPQEVL 8-16							1019.5	1019.5
1089.6, 2 VAPFPEVFGK 25-34, (Lemieux & Amiot, 1990; 1989)							1089.6	1089.6
1133.6, 2 HQGLPEVLN 8-17								1133.6
1142.6, 2 EIVPNSAEER 110-119							1142.6	1140.5
1236.6, 2 FVAPFPEVFGK 24-34							1237.7	
1262.6, 2 HQGLPQEVLNE 8-18							1262.5	1262.6

Table 5.1 (continued)

Peptide: Theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN, Reference	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN
1266.7, 2 YLGYLEQLLR 91-100	1266.5	1266.7			1266.6	1266.7		
1270.8, 2 (-1 Phosphate) EEIVPNSVEQK 69-79								1270.6
1271.7, 3 RLKKYKVPQL 100-109			1271.8	1271.7				
1283.6, 2 IPNPIGSENSEK 182-193							1283.7	
1336.7, 2 HIQKEDVPSER 80-90, (Lemieux & Amiot, 1989)	1336.6	1336.6						
1383.7, 2 FFVAPFPEVFGK 23-34	1383.5	1383.7			1383.6	1383.7	1383.7	
1384.9, 3 LRLKKYKVPQL 99-109							1384.9	
1485.5, 2 SDIPNPIGSENSEK 180-193							1485.7	1485.6

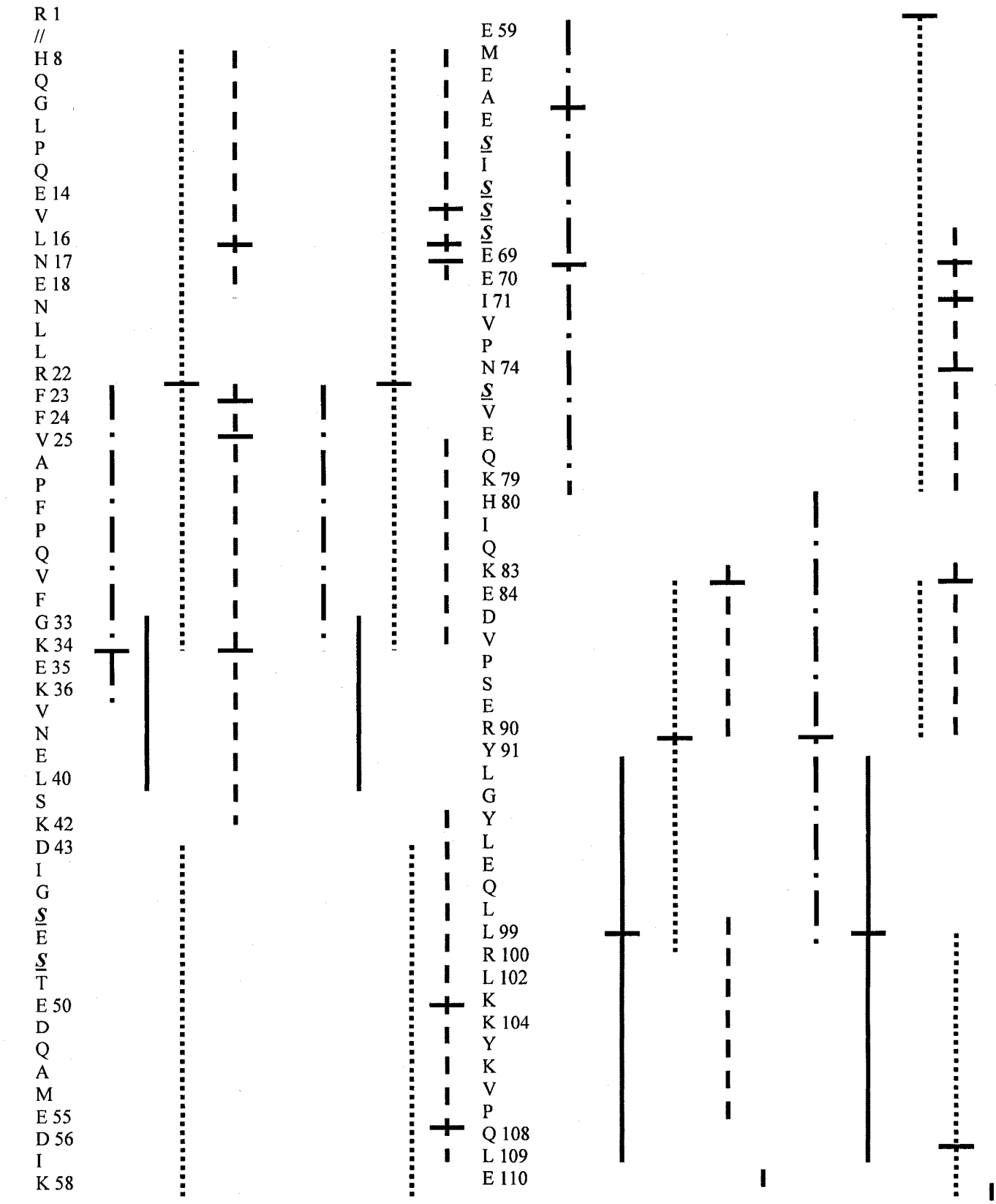
Table 5.1 (continued)

Peptide: Theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN, Reference	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN
1538.7, 2 (-2 Phosphate) KDIGSESTEDQAME 42-55								1538.6
1640.8, 3 FFVAPFPEVFGKEK 23-36	1640.7							
1653.74, 2 (-2 Phosphate) KDIGSESTEDQAMED 42-56								1653.7
1759, 3 HQGLPQEVLNENLLR 8-22, (Lemieux & Amiot, 1990; 1989)					1758.9	1759		
1766.7, 2 (-1Phosphate) DIGSESTEDQAMEDIK 43-58						1766.7		
1846.7, 2 DIGSESTEDQAMEDIK 43-58					1846.6			

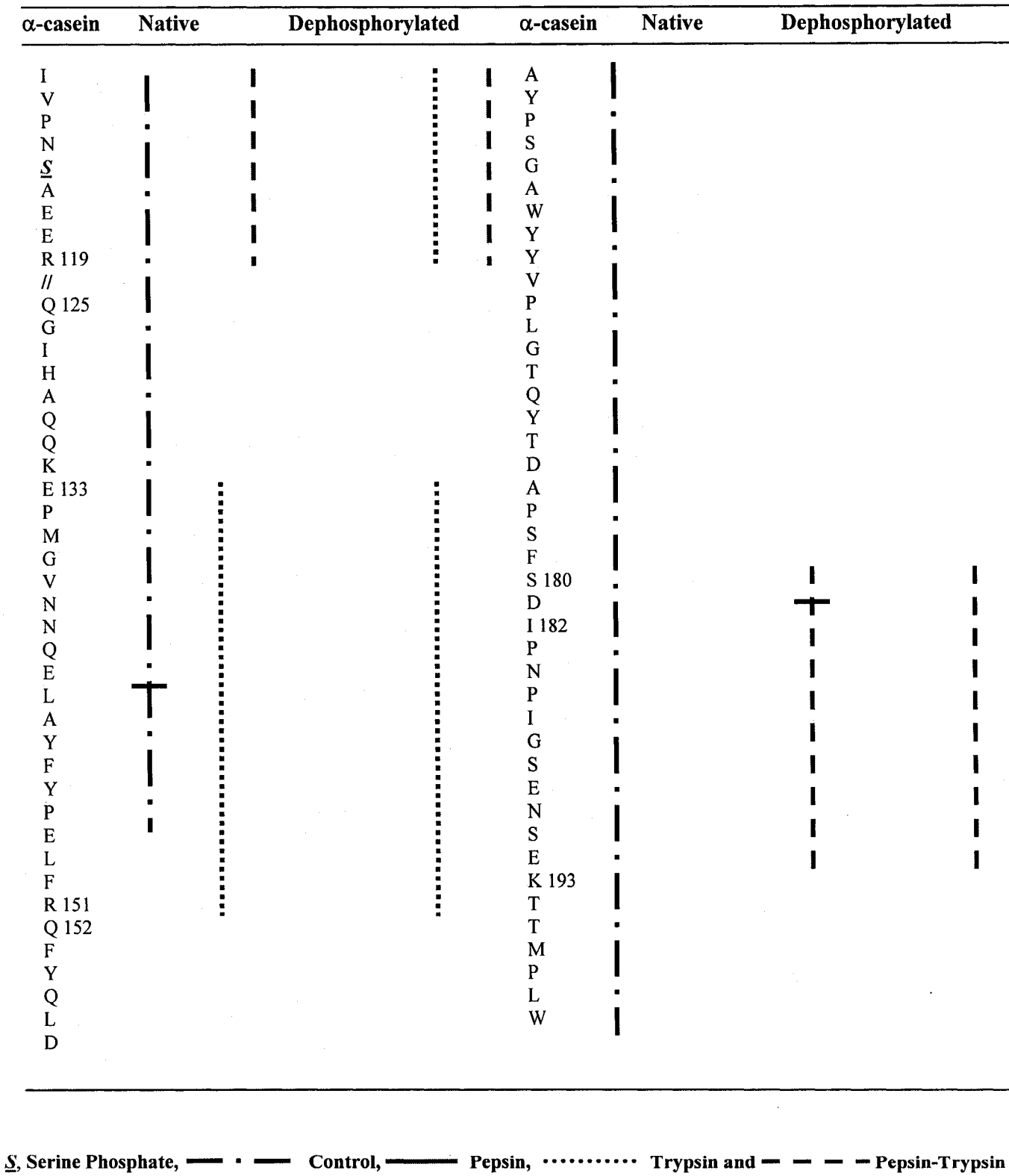
Table 5.1 (continued)

Peptide: Theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN, Reference	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN	α-CN	Dep. α-CN
1871, 3 (-1Phosphate) YKVPQLEIVPNSAEER 104-119						1871		
2079, 2 EDVPSERYLGYLEQLLR 84-100	2078.9							
2315, 2 EPMIGVNQELAYFYPELFR 133-151					2315.1	2315		
2320, 3 (-5 Phosphate) QMEAESISSSEEIVPNSVEQK 59-79						2320		
3206.6, 4 EGIHAQQKEPMIGVNQELAYFYP ELFR 125-151	3206.5							
5444.4, 4 QFYQLDAYPSGAWYYVPLGTQY TDAPSFSDIPNPIGSENSEKTTMPL W 152-199	5444.5							

Figure 5.7: Identified peptides by LC-ESI-MS/MS from α- and dephosphorylated α-casein after proteolysis



(Continued in page 98); see footnote at end of Figure



S, Serine Phosphate, — · — Control, ——— Pepsin, ····· Trypsin and — — — Pepsin-Trypsin

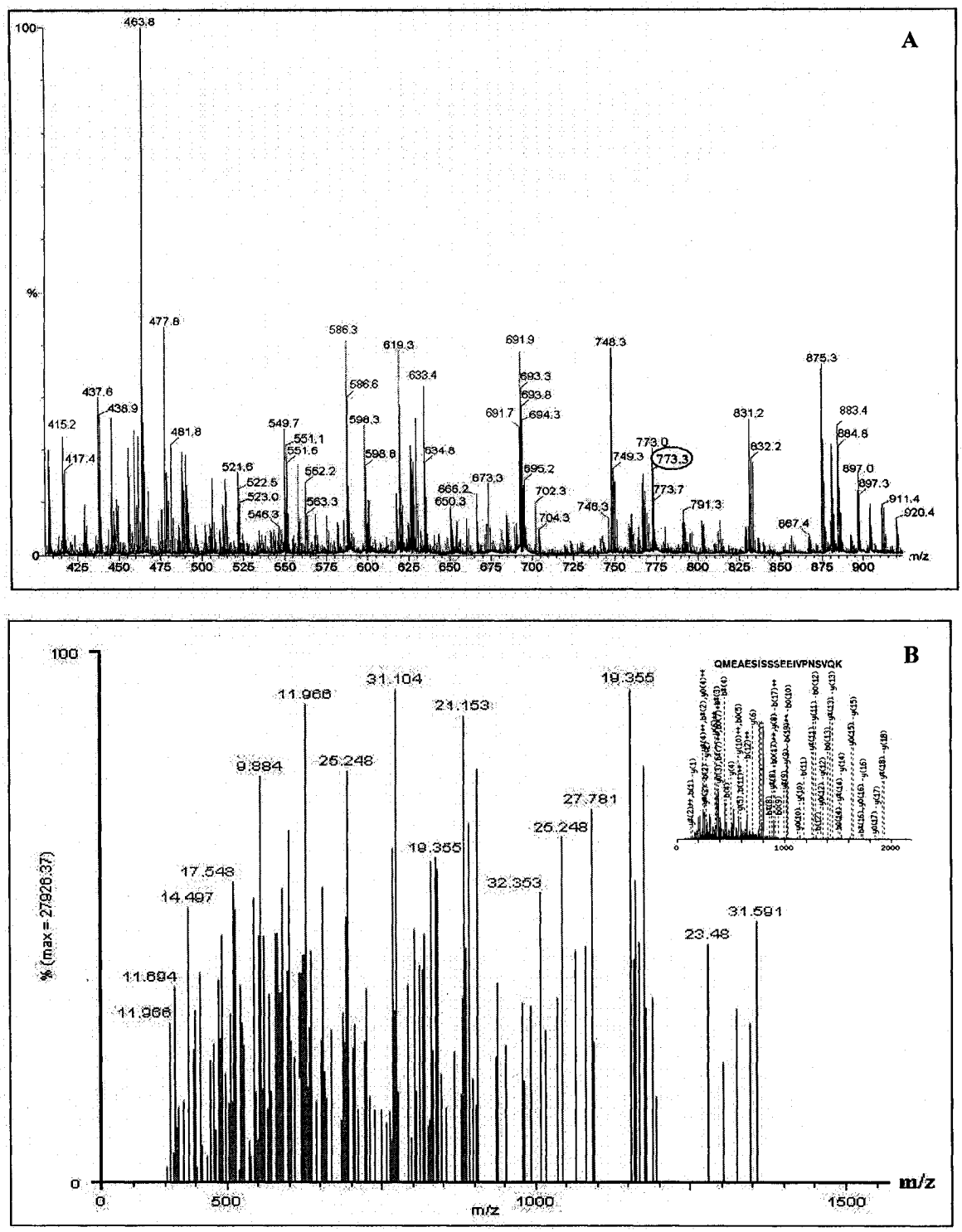


Figure 5.8: (A) LC-ESI-MS spectra (m/z region 420 to 900), m/z 773.3 signal in bold type/circled and (B) LC-ESI-MS/MS spectra (m/z region 300 to 1000) of dephosphorylated α-casein tryptic hydrolysis. Inset represents the fragmentation spectra of the peptide QMEAESISSSEEIVPNSVEQK, experimental MW 2320.3 Da, indicating the loss of 5 phosphate groups

native casein and dephosphorylated α-casein. It is known that trypsin has affinity for peptide bonds which have arginine or lysine at the carbonyl side of the peptide bond (Trevor, 1991). Dephosphorylated α-casein treatment with trypsin hydrolyzed the peptide bonds K_{79}-H_{80} (Lemieux & Amiot, 1989), K_{103}-Y_{104} and R_{119}-L_{120}; removal of the phosphate groups was determined from the m/z 623.6 and m/z 773.3 signals. Trypsin also hydrolyzed the peptides bonds K_{42}-D_{43} and K_{58}-E_{59} where two phosphoseryl residues are present in α-casein. A difference in molecular weight of 80 Da for the same doubly charged peptide (D_{43}-K_{58}, m/z 923.4 vs. m/z 883.4) in dephosphorylated α-casein was noted; confirming the loss of 1 phosphate group from dephosphorylation. Previous work (Adamson & Reynolds, 1997) reported the recovery of a peptide corresponding to α_{s1}-casein (D_{43}-K_{79}) rather than the expected α_{s1}-casein (D_{43}-K_{58}) and α_{s1}-casein (Q_{59}-K_{79}); this suggests that the cleavage site at K_{58}-Q_{59} of α_{s1}-casein was more resistant to tryptic hydrolysis than those at K_{42}-D_{43} and K_{79}-H_{80}. Dephosphorylation produced two peptides (D_{43}-K_{58} and Q_{59}-K_{79}; Table 5.1), indicating that the loss of phosphate made the K_{58} more susceptible to trypsin cleavage.

Figure 5.9 (A) shows the LC-ESI-MS spectra used to generate the LC-ESI-MS/MS (Figure 5.9 B) spectra of dephosphorylated α-casein peptic-tryptic hydrolysis for the double charged peptide K_{42}-D_{56} (m/z 826.9) which experimental molecular weight determined (1653.7 Da); the data indicate the loss of two phosphate groups when compared with the theoretical molecular weight expected (1814.6 Da). Combined pepsin/trypsin proteolysis of α-casein and dephosphorylated α-casein resulted in twelve and fifteen peptides, respectively (Table 5). Three different peptide cleavage sites were identified in α-casein F_{23}-F_{24}, L_{98}-L_{99} and D_{181}-I_{182} (Figure 5.7), in addition to L_{109}-E_{110}

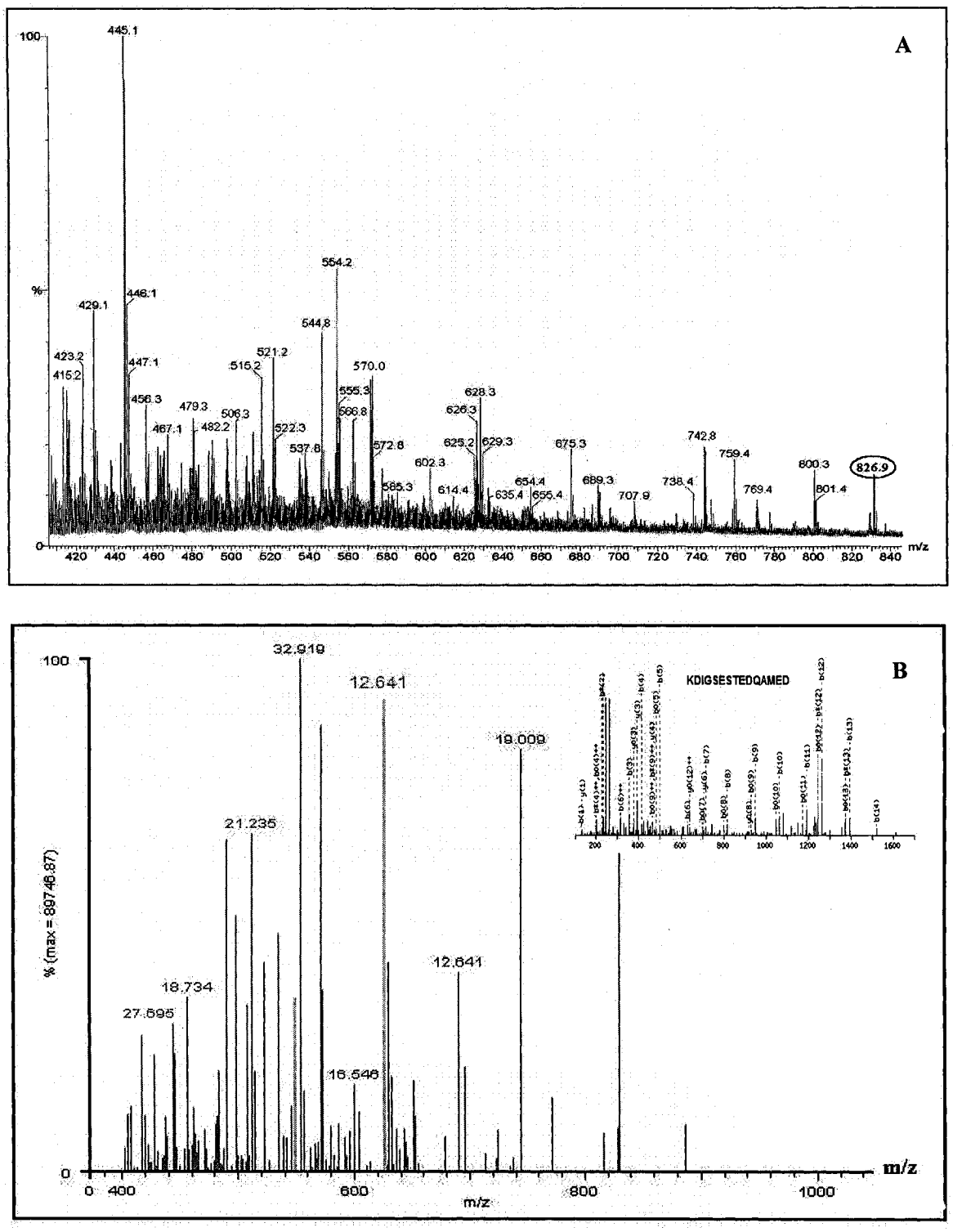


Figure 5.9: (A) LC-ESI-MS spectra (m/z region 410 to 840), m/z 826.9 signal in bold type/circled and (B) LC-ESI-MS/MS spectra (m/z region 360 to 1040) of dephosphorylated α-casein combined (peptic-tryptic) hydrolysis. Inset represents the fragmentation spectra of the peptide KDIGSESTEDQAMED, experimental MW 1653.7 Da, indicating the loss of 2 phosphate groups

produced by pepsin and R_{22}-F_{23} and K_{34}-E_{35} (Lemieux & Amiot, 1990) formed by trypsin, which were established earlier. For dephosphorylated α-casein the following twelve peptide cleavage sites were observed after trypsin hydrolysis: E_{14}-V_{15}, N_{17}-E_{18}, K_{34}-E_{35}, S_{41}-K_{42}, E_{50}-D_{51}, E_{55}-D_{56}, D_{56}-I_{57}, S_{68}-E_{69}, E_{69}-E_{70}, E_{70}-I_{71}, N_{74}-S_{75}, E_{109}-E_{110}, K_{79}-H_{80}, some of these cleavage bonds have been reported (Lemieux & Amiot, 1989). Both native and dephosphorylated α-casein showed in common ten cleavaged sites K_7-H_8, E_{18}-N_{19}, F_{24}-V_{25}, Q_{82}-K_{83}, K_{83}-E_{84}, R_{90}-Y_{91}, R_{119}-L_{120}, F_{179}-S_{180} and K_{193}-T_{194}, some of these cleavage bonds have been reported (Lemieux & Amiot, 1990; Lemieux & Amiot, 1989). In addition to the known preferred cleavage sites for pepsin and trypsin enzymes (Athaudaa & Takahashia, 2002; Trevor, 1991); our results suggest that E-X, D-X, S-X and N-X are also cleavage sites (Figure 5.7).

The higher number of peptide cleavaged bonds determined in both the native and dephosphorylated α-casein for the successive proteolysis may be due to the ability of pepsin to facilitate the tryptic proteolysis of casein (Ono et al., 1998). The presence of neighboring peptide bonds cleaved (Figure 5.7) indicates that degradation of some peptides occurred; for example dephosphorylated α-casein peptides H_8-E_{14} and H_8-L_{16} (m/z 403.7 and m/z 509.7) are likely from degradation of H_8-N_{17} (m/z 566.8). Identification of the peptides I_{71}-K_{79} (m/z 506.3) and presumably the degradation peptides K_{42}-E_{50} (m/z 482.2), K_{42}-E_{55} (m/z 769.4) and E_{69}-K_{79} (m/z 635.4) were only observed in dephosphorylated α-casein subjected to combined pepsin/trypsin proteolysis after the removal of one or two phosphate groups, respectively (Figure 5.7). Dephosphorylation favored the action of the proteases since the presence of phosphate may cause a steric effect on the enzymes by blocking the access to a close cleavage site

where a phosphoserine residue is present (Ono et al., 1998); caseins are known to have a strong negative charge at the phosphoryl groups on the surface; it may be difficult for the protease enzymes to reach the cleavage sites of the caseins in the proximity of the negative charge.

5.3.2.2.2 Identification of Peptides from β-casein and Dephosphorylated β-casein

Figure 5.10 (A) shows the LC-ESI-MS spectra used to generate the LC-ESI-MS/MS spectra (Figure 10 B) of the peptide S_{17}-L_{45} (m/z 859.8) obtained after cleavage of the bond L_{16}-S_{17} in dephosphorylated β-casein; the molecular mass of this peptide (3439.5 Da) indicates the efficient removal of 4 phosphate groups. All identified peptides are listed in Table 5.2 and the origin of these peptides is shown in Figure 5.11.

Four and five peptides were obtained for β- and dephosphorylated β-casein after peptic hydrolysis, respectively. β- and dephosphorylated β-casein were cleaved by pepsin at the same following six peptide bonds: L_{45}-Q_{46}, L_{58}-V_{59}, L_{70}-P_{71}, L_{192}-Y_{193}, L_{198}-G_{199} and F_{205}-P_{206} (Table 5.2); some of these findings have been confirmed previously (Guillou et al., 1991; Lemieux & Amiot, 1990). Pepsin showed a favored splitting at the L-X and F-X bonds for both β- and dephosphorylated β-casein (Athaudaa & Takahashia, 2002). Additionally, pepsin cleaved a quadruple charged large peptide once dephosphorylation occurred (Figure 5.11).

Figure 5.12 (A) shows the LC-ESI-MS spectra used to generate the LC-ESI-MS/MS LC-ESI-MS spectra (Figure 5.12 B) of the peptide E_2-R_{25} (m/z 881.7) obtained after tryptic hydrolysis of dephosphorylated β-casein, indicating the loss of 4 phosphate

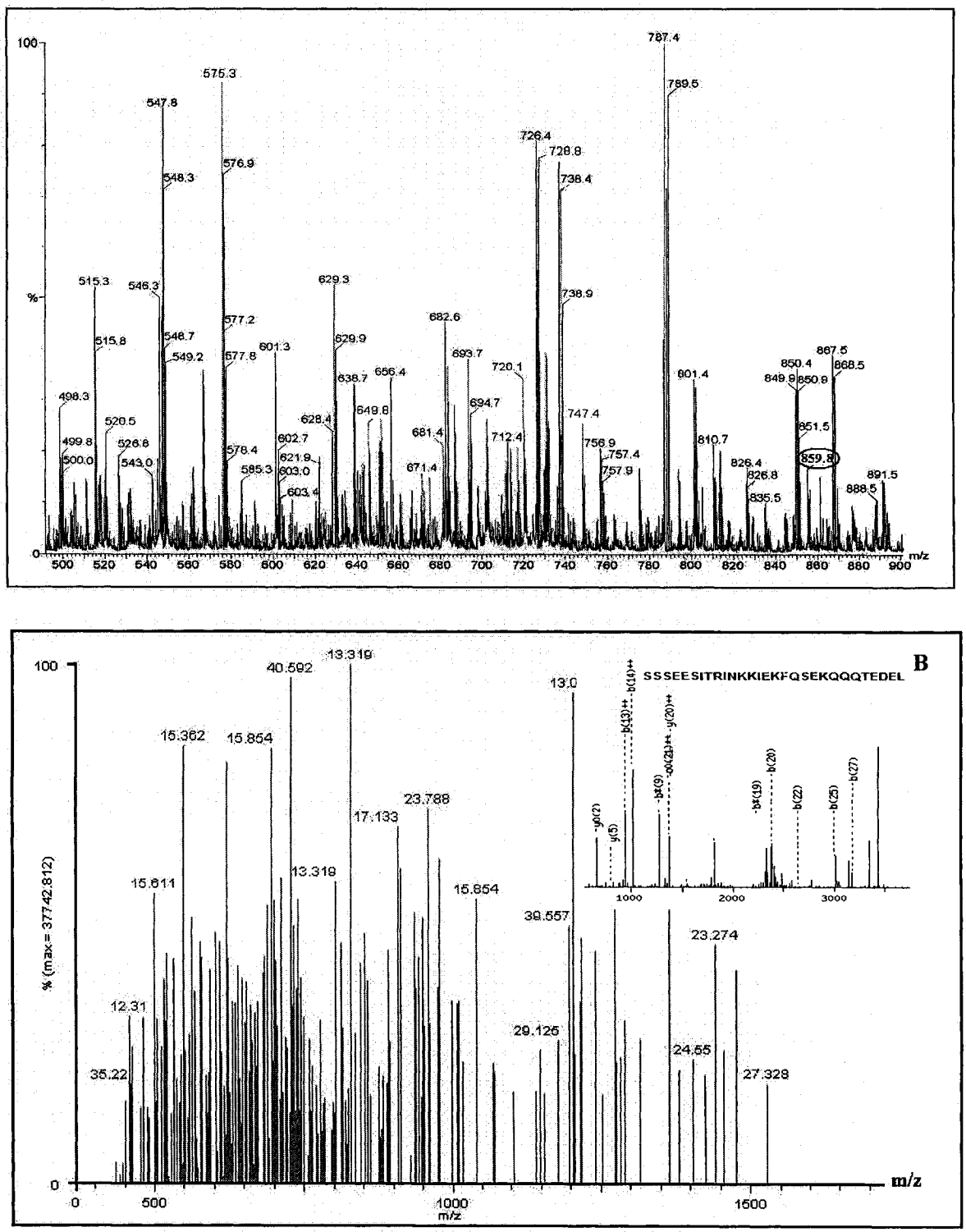


Figure 5.10: (A) LC-ESI-MS spectra (m/z region 480 to 900), m/z 859.8 signal in bold type/circled and (B) LC-ESI-MS/MS spectra (m/z region 320 to 1040) of dephosphorylated β-casein peptic hydrolysis. Inset represents the fragmentation spectra of the peptide SSSEESITRINKKIEKFQSEKQQQTEDEL, experimental MW 3439.5 Da, indicating the loss of 4 phosphate groups

Table 5.2: Peptides identified in enzyme hydrolysis products of β-casein by LC-ESI-MS/MS

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
741.4, 1 GPFPIIV 203-209, (Visser et al., 1995; Lemieux & Amiot, 1989, 1990; Miralles et al., 2003)					741.3			
747.3, 1 YQEPVL 193-198, (Lemieux & Amiot, 1989, 1990; Schmelzer et al., 2004)				747.4				
829.4, 2 AVPYPQR 177-183, (Visser et al., 1995; Lemieux & Amiot, 1989, 1990; Carles & Ribadeau Dumas, 1986)					829.4	829.4		829.4
877.4, 2 PFPGPIHN 61-68							877.4	877.4
940.4, 2 IHPFAQTQ 49-56	940.5						940.4	940.4

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
993.5, 2 QEPVLGPVR 194-202, (Lemieux & Amiot, 1989, 1990)							993.6	
994.3, 2 SSSEESITR (-3 Phosphates) 17-25								994.3
1027.3, 2 IHPFAQTQS 49-57								1027.3
1028.6, 2 NLHLPLPLL 132-140	1028.6							1028.5
1107.5, 2 LSSSEESITR (- 3 Phosphate) 16-25								1107.5
1129.6, 2 LHLPLPLLQS 133-142	1129.7							1129.7

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1139.5, 2 VYPFPGPIHN 59-68, (Lemieux & Amiot, 1989)							1139.6	1139.5
1140.4, 2 IHPFAQTQSL 49-58	1140.5						1140.5	1140.5
1150.6, 2 GPVRGPFPIIV 199-209	1150.7		1150.5	1150.6				
1156.5, 2 YQEPVLGPVR 193-202, (Lemieux & Amiot, 1990)	1156.6						1156.5	1156.5
1196.3 SLSSSEESITR (-3 Phosphates) 15-25								1196.3
1252.5, 2 LVYPFPGPIHN 58-68							1252.5	1252.6

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1256.7, 2 VENLHLPLPLL 130-140	1256.6							1256.7
1269.6, 2 LYQEPVLGPVR 192-202, (Lemieux & Amiot, 1989, 1990)							1269.7	1269.6
1282.5, 2 YPVEPFTESQS 114-124	1283.6							
1299.6, 2 VYPFPGPIPNSL 59-70			1299.6	1299.7				
1307.6, 2 SLPQNIPPLTQT 69-80								1307.7
1319.6, 2 PVVVPPFLQPEV 81-92								1319.6
1339.6, 2 VYPFPGPIHNSL 59-70								1339.7

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1360.6, 2 QQQTEDELQDK 38-48								1360.6
1367.5, 2 FQSEEQQQTED (- 1 Phosphate) 33-43								1367.5
1371.7, 2 DVENLHLPLPLL 127-140	1371.7							1371.8
1380.5, 2 HQPHQPLPPTVM 145-156							1380.7	1380.6
1382.6, 2 LLYQEPVLGPVR 191-202 (Carles & Ribadeau Dumas, 1986; Visser et al., 1995)							1382.6	1382.6
1395.6, 2 YPVEPFTESQSL 114-125							1395.7	1395.7
1432.8, 2 IQAFLLYQEPVL 187-198								1432.8

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1446.7, 2 PVEPFTESQSLTL 115-127								1446.7
1450.7, 2 PVVVPPFLQPEVM 81-93, (Schmelzer et al., 2004)	1450.7						1450.8	1450.7
1452.7, 2 LVYPFPGPIHNSL 58-70							1452.7	
1457.6, 2 YQEPVLGPVRGPF 193-205			1457.7	1457.8				
1459.9, 2 PVLGPVRGPFPIIV 196-209, (Schmelzer et al.,2004)							1459.9	
1468.7, 2 ELEELNVPGEIVE 2-14								1468.7
1471.7, 2 VENLHLPLPLLQS 130-142								1471.7
1472.7, 2 TDVENLHLPLPLL 128-140, (Schmelzer et al.,2004)	1472.6						1472.8	1472.7

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1489.6, 2 EQQQTEDELQDK 37-48							1489.7	1489.7
1507.8, 2 PVVVPPFLQPEVMG 81-97							1507.8	
1510.7, 2 MHQPHQPLPPTVM 144-156							1510.7	1510.7
1511.7, 2 QDKIHPFAQTQSL 46-58	1511.6		1511.7					
1535.7, 2 IVESLSSSEESITR (-4 Phosphates) 12-25								1535.7
1564.7, 2 VYPFPGPIHNSLPQ 59-72								1564.7
1585.8, 2 LTDVENLHLPLPLL 127-140								1585.9

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1600.8, 2 TDVENLHLPLPLLQ 128-141	1600.9							1600.9
1609.7, 2 YPVEPFTESQSLTL 114-127								1609.7
1624.7, 2 RELEELNVPGEIVE 1-14	1624.8							1624.6
1677.8, 2 LVYPFPGPIHNSLPQ 58-72							1677.9	
1687.7, 2 TDVENLHLPLPLLQS 128-142, (Schmelzer et al., 2004)							1687.9	1687.9
1697.6, 2 WMHQPHQPLPPTVM 143-156, (Schmelzer et al., 2004)	1697.6						1697.6	1697.6
1800.9, 2 LTDVENLHLPLPLLQS 127-142							1800.9	

Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
1833.7, 2 QSEEQQQTEDELQDK (- 1 Phosphate) 34-48								1833.7
1879.9, 2 YQEPVLGPVRGPFPIIV 193-209, (Schmelzer et al., 2004)							1880	
1902, 2 TLTDVENLHLPLPLLQS 126-142								1902.2
1912.7, 2 QSWMHQPHQPLPPTVM 141-156	1912.7						1912.7	1912.7
1980.8, 2 (-1 Phosphate) FQSEEQQQTEDELQDK 33-48, (Miralles et al., 2003)					1980.7	1980.9		1980.8
2145, 2 LNVPGEIVESLSSSEESITR (-4 Phosphates) 6-25								2145.2

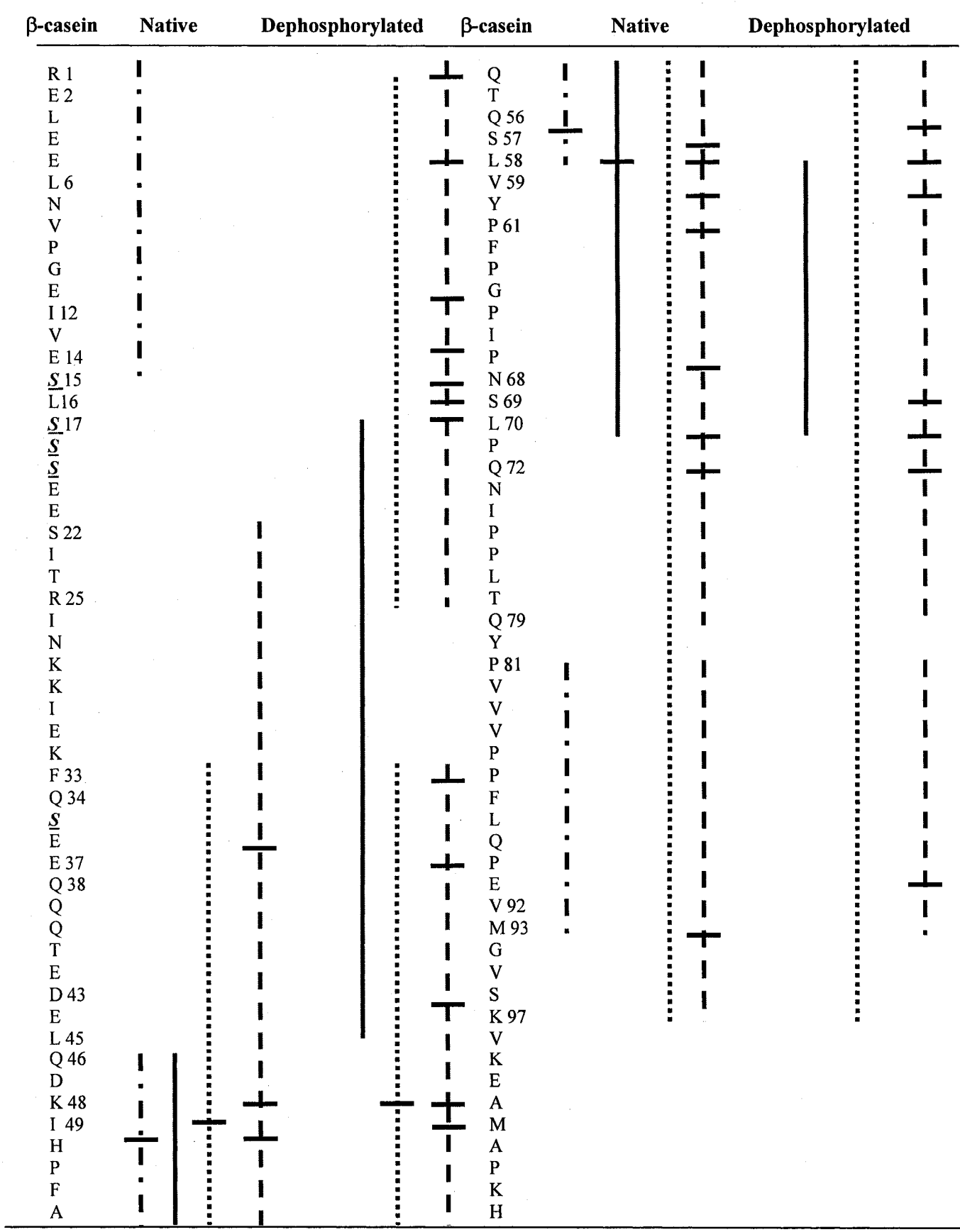
Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
2185.1, 3 DMPIQAFLLYQEPVLGPVR 184-202, (Visser et al., 1995; Miralles et al., 2003)					2185	2185.1		
2280.1, 3 MHQPHQPLPPTVMFPPQSVL 144-161							2280.1	
2429.1, 2 VYPFPGPIHNSLPQNIPPLTQT 59-80, (Schmelzer et al., 2004)							2429.1	2429.1
2466.1, 2 WMHQPHQPLPPTVMFPPQSVL 143-161	2466.1						2466.1	2466.1
2542.2, 2 LVYPFPGPIHNSLPQNIPPLTQT 58-80							2542.2	2542.2
2645.2, 3 (- 4 Phosphates) ELEELNVPGEIVESLSSSEESITR 2-25						2645.3		

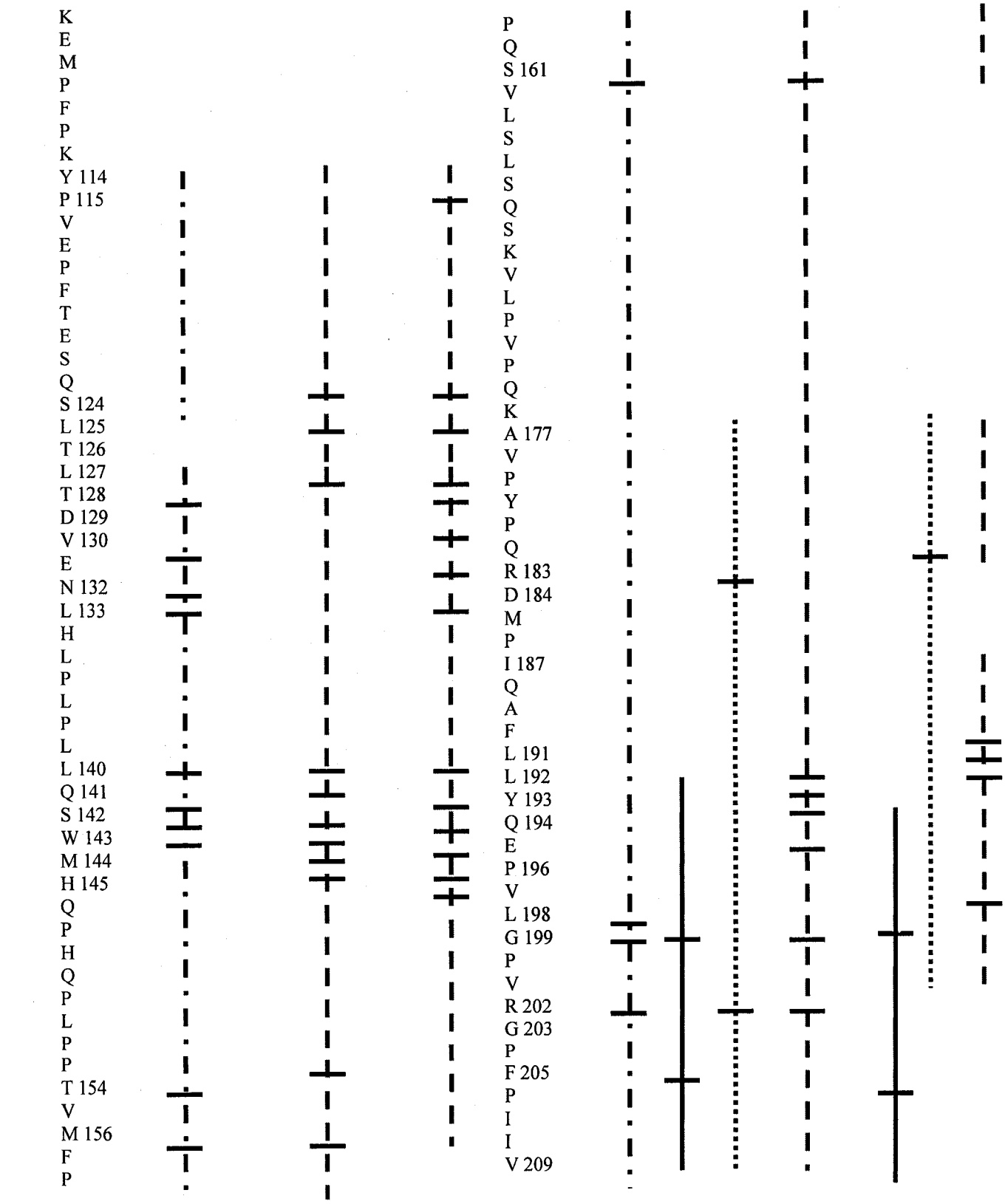
Table 5.2 (continued)

Peptide: theoretical MW (Da), charge (Z) amino acid sequence amino acid sequence in α-CN	Control Exp MW (Da)		Pepsin Exp MW (Da)		Trypsin Exp MW (Da)		Pepsin-Trypsin Exp MW (Da)	
	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN	β-CN	Dep. β-CN
2805.5, 4 SLSQSKVLPVPQKAVPYPQRDM PIQ 154-198	2804.5						2805.5	
2876.5, 4 SLSQSKVLPVPQKAVPYPQRDM PIQA 154-199	2876.5						2876.6	
2920.5, 4 SITRINKKIEKFQSEEQQQTEDEL 22-45							2920.5	
3439.5, 4 (-4 Phosphate) SSSEESITRINKKIEKFQSEKQQQ TEDEL 17-45				3439.7				
5355.7, 4 IHPFAQTQSLVYPFPGPIHNSLPQ NIPPLTQTPVVVPPFLQPEVMGV SK 49-97, (Visser et al., 1995; Miralles et al., 2003)					5355.7	5355.9		

Figure 5.11: Identified peptides by LC-ESI-MS-MS from β- and dephosphorylated β-casein after proteolysis



(Continued in page 117); see footnote at end of Figure



S, Serine Phosphate, — · — Control, ——— Pepsin, ····· Trypsin and — — — Pepsin-Trypsin

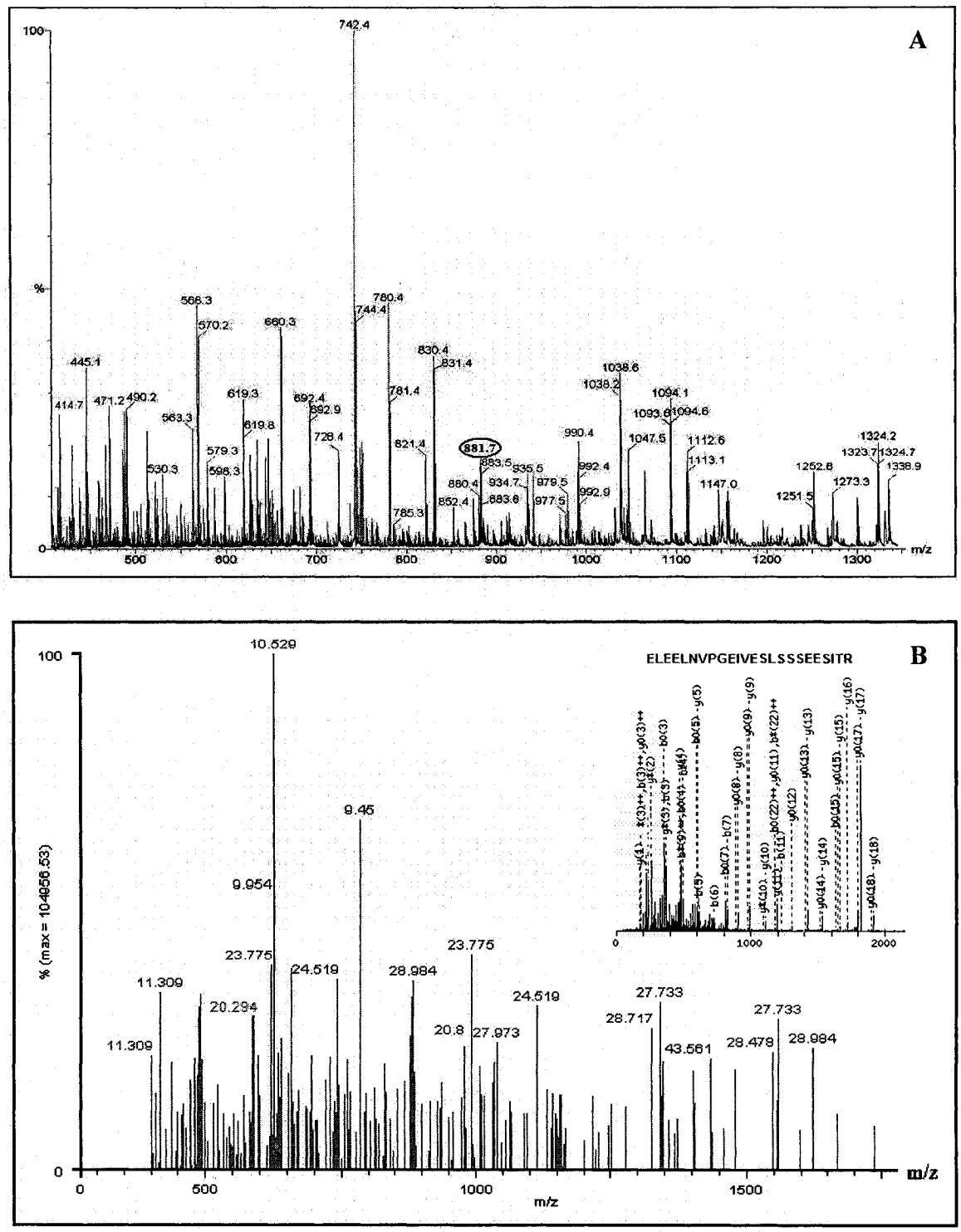


Figure 5.12: (A) LC-ESI-MS spectra (m/z region 400 to 1350), m/z 881.7 signal in bold type/circled and (B) LC-ESI-MS/MS spectra (m/z region 400 to 1600) of dephosphorylated β-casein tryptic hydrolysis. Inset represents the fragmentation spectra of the peptide ELEELNVPGEIVESLSSSEESITR, experimental MW 2645.2 Da, indicating the loss of 4 phosphate groups

groups. Trypsin cleaved the same six peptide bonds for β- and dephosphorylated β-casein K_{32}-F_{33}, K_{48}-I_{49}, K_{97}-V_{98}, K_{176}-A_{177}, R_{183}-D_{184} and R_{202}-G_{203} (Table 5.2); some of these cleavage bonds have been reported (Miralles et al., 2003; Visser et al., 1995; Carles & Ribadeau Dumas, 1986); only the cleaved bonds R_1-E_2 and R_{25}-I_{26} were found in dephosphorylated β-casein. The peptide F_{33}-K_{48} also reported previously (Miralles et al., 2003) occurred in both the native and dephosphorylated β-caseins; it was determined from its signal (m/z 990.4) that one phosphate group was lost before the action of acid phosphatase, this phosphate likely was lost during the incubation conditions required for dephosphorylation. The preferred cleaved sites R-X and K-X for trypsin (Trevor, 1991) were also confirmed. β-casein has been reported (Swaisgood, 1992) as a flexible molecule in the region of the N-terminal domain, resulting in susceptibility of the lysine residues to proteolysis by trypsin. Hynek et al. (2002) demonstrated with a model phosphopeptide of β-casein that tryptic hydrolysis of the peptide bond K-S was easily cleaved when phosphate was removed, making evident that phosphorylation inhibit proteolytic breakdown, supporting the results obtained.

Figure 5.13 shows the (A) LC-ESI-MS used to generate the LC-ESI-MS/MS spectra (Figure 5.13 B) of the peptide S_{15}-R_{25} (m/z 598.2) obtained from dephosphorylated β-casein after combined pepsin/trypsin hydrolysis, indicating the loss of 3 phosphate groups. Successive proteolysis (pepsin/trypsin) of β- and dephosphorylated β-casein resulted in thirty one and forty eight peptides, respectively (Table 5.2). Nine different peptide bonds were cleaved in β-casein as follows: E_{21}-S_{22}, L_{45}-Q_{46}, Q_{56}-S_{57}, K_{58}-I_{59}, G_{97}-V_{98}, L_{153}-S_{154}, Y_{193}-Q_{194}, E_{195}-P_{196}, G_{199}-F_{200}, in addition to Q_{198}-G_{199} produced by pepsin hydrolysis (Figure 5.11); some of these cleavage bonds

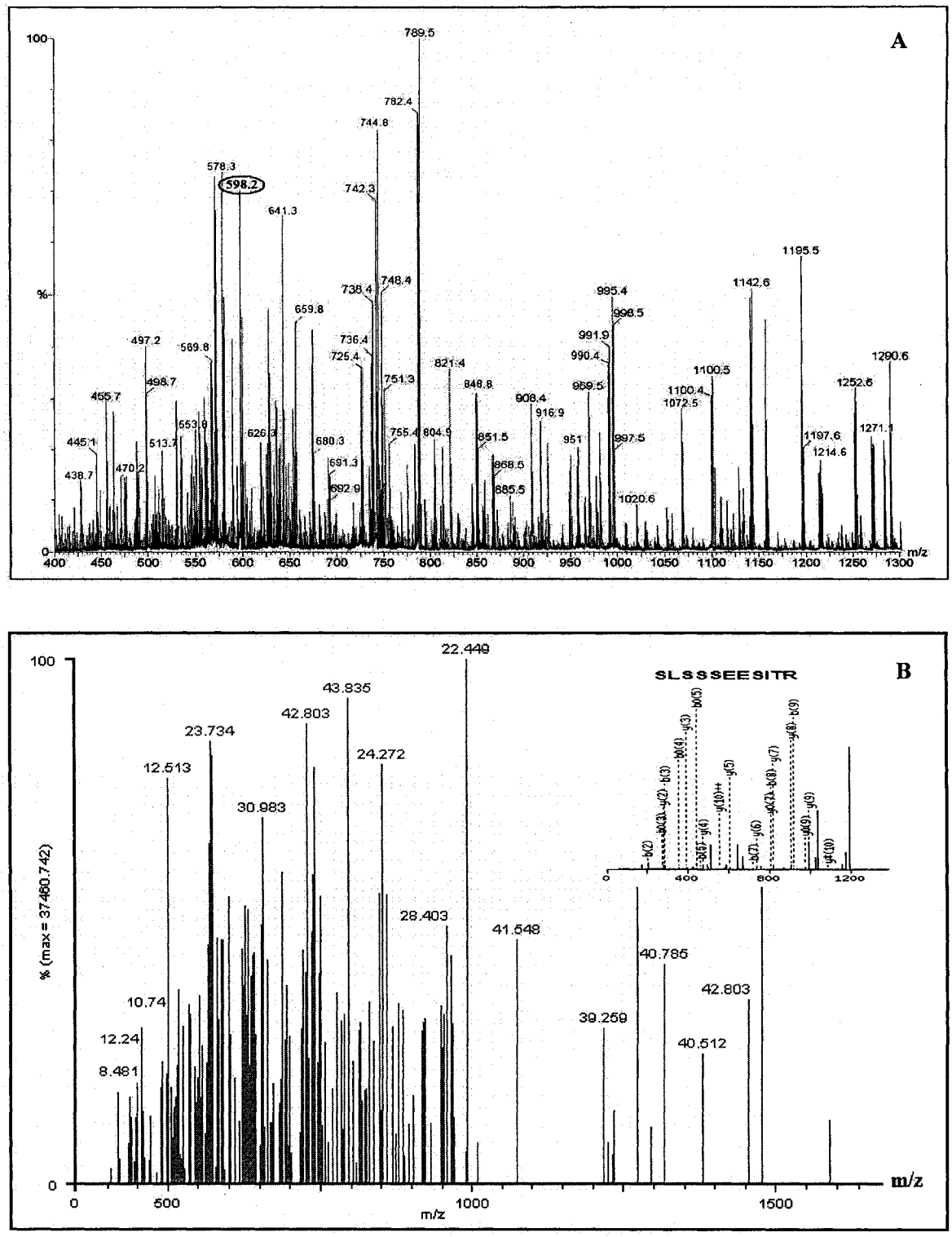


Figure 5.13: (A) LC-ESI-MS spectra (m/z region 400 to 1300), m/z 598.2 signal in bold type/circled and (B) LC-ESI-MS/MS spectra (m/z region 4400 to 1580) of dephosphorylated β-casein combined (peptic-tryptic) hydrolysis. Inset represents the fragmentation spectra of the peptide SLSSSEESITR, experimental MW 1196.3 Da, indicating the loss of 3 phosphate groups

have been reported (Schmelzer et al., 2004; Visser et al., 1995; Lemieux & Amiot, 1989). For dephosphorylated β-casein nineteen different peptide bonds were cleaved as follows: E_{131}-N_{132}, S_{15}-L_{16}, N_{132}-L_{133}, E_{14}-S_{15}, D_{129}-V_{130}, E_{37}-Q_{38}, P_{186}-I_{187}, Y_{114}-P_{115}, E_{11}-I_{12}, T_{128}-L_{129}, L_{129}-T_{130}, F_{33}-Q_{34}, E_5-L_6, Q_{56}-S_{57}, V_{92}-M_{93}, D_{43}-E_{44}, Q_{72}-N_{73}, L_{145}-Q_{146} and Q_{142}-S_{143}; in addition to L_{16}-S_{17} and L_{198}-G_{199} that were cleaved by pepsin and K_{176}-A_{177}, K_{48}-I_{49}, K_{32}-F_{33}, R_1-E_2, R_{183}-D_{184}, R_{25}-I_{26} cleaved by trypsin hydrolysis (Figure 5.11); some of these cleavage bonds have been reported (Schmelzer et al., 2004; Visser et al., 1995; Lemieux & Amiot, 1989). Additionally to the reported cleavage sites for both proteases (pepsin and trypsin); the cleavage of N-X, M-X, T-X, E-X, Q-X, G-X, D-X, P-X, W-X, and V-X was noted; this supports some of the found cleaved sites determined for peptic hydrolysis of β-casein (Schmelzer et al., 2004). Dephosphorylation of β-casein treatment with both pepsin and trypsin enzymes also produced the signals: m/z 497.2 and 553.8, as degradation products of the peptide S_{15}-R_{25} with 3 phosphate groups removed. Four phosphate groups were eliminated from the peptide E_2-R_{25} (m/z 881.7), degradation peptides L_6-R_{25} (m/z 1072.5) and I_{12}-R_{25} (m/z 767.9) and S_{17}-L_{45} (m/z 859.9) were observed (Figure 11). Dephosphorylation of β-casein enhanced the action of both proteases (pepsin and trypsin) where the presence of phosphate prevents cleavage. Moreover, the presence of a higher number of peptides found in β-casein and dephosphorylated β-casein than those found in α-casein and dephosphorylated α-casein may be due to the high content of proline residues (35/209) in β-casein and the non compact structure of this casein; resulting in β-casein and dephosphorylated β-casein more susceptible to be cleavage by proteases (Farrell et al., 2004).

5.3.3 Bioactive Peptides

Several of the identified peptides from both native and dephosphorylated α-casein and β-casein can be considered as bioactive peptides precursors based on published information. Table 5.3 and 5.4 summarize the identified peptides containing a sequence with possible biological or physiological functions. Sequences peptides: YKVPQL, VAP, FVAPFPEVFGK, LAYFYP, DAYPSGAWY, TTMPLW, YPFPG, IPP, VPP and LLY have been reported to have antithrombotic or antihypertensive activity (Meisel & FitzGerald, 2000; Philanto-Leppala et al., 1998; Maeno et al., 1996; Maruyama et al., 1987); RYLGYLE and YPFPGPI have been reported to have opioid activity (Meisel & FitzGerald, 2000); LLY has been reported to have immunomodulating (Migliore-Samour et al., 1989) and KIEKFQSEEQQQ has been reported to have mineral utilization properties (Chabance et al., 1998).

Table 5.3: Bioactive peptides precursors found in α-casein hydrolysis products

Peptide: MW (Da), Z amino acid sequence amino acid sequence in α-CN	Bioactivity	Origin	Reference
1271.7, 3 RLKKYKVPQL 100-109	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN pepsin, Dep α-CN pepsin	Maeno et al., 1996
1384.9, 3 LRLKKYKVPQL 99-109	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN pepsin-trypsin	Maeno et al., 1996
*1871, 3 (-1Phosphate) YKVPQLEIVPNSAEER 104-119	Angiotensin-l-converting enzyme (ACE) inhibitory	Dep α-CN trypsin	Maeno et al., 1996
1089.6, 2 VAPFPEVFGK 25-34	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN pepsin-trypsin, Dep α-CN pepsin-trypsin	Maruyama et al., 1987
1236.6, 2 FVAPFPEVFGK 24-34	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN pepsin-trypsin	Maruyama et al., 1987
1383.7, 2 FFVAPFPEVFGK 23-34	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN & Dep α-CN control, α-CN & Dep α-CN trypsin, α-CN pepsin-trypsin	Maruyama et al., 1987
1640.8, 3 FFVAPFPEVFGKEK 23-36	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN control	Maruyama et al., 1987
1846.7, 2 DIGSESTEDQAMEDIK 43-58	Mineral binding	α-CN trypsin	Schlimme & Meisel, 1995
2079, 2 EDVPSERYLGYLEQLLR 84-100	Opioid	α-CN control	Maruyama et al., 1985
2315,2 EPMIGVNQELAYFYPELFR 133-151	Angiotensin-l-converting enzyme (ACE) inhibitory	α-CN Trypsin, Dep α-CN trypsin	Philanto-Leppala et al., 1998
5444.4, 4 QFYQLDAYPSGAWYYV PLGTQYTDAPSFSDIPNP IGSENSEKTTMPLW 152-199	Angiotensin-l-converting enzyme (ACE) inhibitory Immunomodulatory	α-CN control	Philanto-Leppala et al., 1998

Underlined sequences reported as bioactive peptides

Table 5.4: Bioactive peptides precursors found in β-casein hydrolysis products

Peptide: MW (Da), Z amino acid sequence amino acid sequence in β-CN	Bioactivity	Origin	Reference
1139.5, 2 VYPFPGPIHN 59-68	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Meisel & FitzGeald, 2000
1252.5, 2 LVYPFPGPIHN 58-68	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Meisel & FitzGeald, 2000
1299.6, 2 VYPFPGPIPNSL 59-70	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin, Dep β-CN pepsin	Meisel & FitzGeald, 2000
1299.6, 2 VYPFPGPIPNSL 59-70	Immunomodulatory	β-CN pepsin, Dep β-CN pepsin	Migliore-Samour et al., 1989
1299.6, 2 VYPFPGPIPNSL 59-70	Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin, Dep β-CN pepsin	FitzGerald & Meisel, 2000
1307.6, 2 SLPQNIPPLTQT 69-79	Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Philanto-Leppala et al., 1998; FitzGerald & Meisel, 2000
1319.6, 2 PVVVPPFLQPEV 81-92	Angiotensin-l-converting enzyme (ACE) inhibitory	Dep β-CN pepsin-trypsin	Philanto-Leppala et al., 1998; FitzGerald & Meisel, 2000
1339.6, 2 VYPFPGPIHNSL 59-70	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	Dep β-CN pepsin-trypsin	Chabance et al., 1998
1382.6, 2 LLYQEPVLGPVR 191-202	Immunomodulatory	β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Migliore-Samour et al., 1989

Table 5.4 (continued)

Peptide: MW (Da), Z amino acid sequence amino acid sequence in β-CN	Bioactivity	Origin	Reference
1432.8, 2 IQAFLLYQEPVL 187-198	Immunomodulatory	Dep β-CN pepsin-trypsin	Migliore-Samour et al., 1989
1450.7, 2 PVVVPPFLQPEVM 81-93	Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN control, β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Philanto-Leppala et al., 1998; FitzGerald & Meisel, 2000
1452.7, 2 LVYPFPGPIHNSL 58-70	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin	Chabance et al., 1998
1507.8, 2 PVVVPPFLQPEVMG 81-97	Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin	Philanto-Leppala et al., 1998; FitzGerald & Meisel, 2000
1564.7, 2 VYPFPGPIHNSLPQ 59-72	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	Dep β-CN pepsin-trypsin	Chabance et al., 1998
1677.8, 2 LVYPFPGPIHNSLPQ 58-72	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin	Chabance et al., 1998
2185.1, 3 DMPIQAFLLYQEPVLGPV R 184-202	Immunomodulatory	β-CN trypsin, Dep β-CN trypsin	Migliore-Samour et al., 1989
2429.1, 2 VYPFPGPIHNSLPQNIPPL TQT 59-79	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Chabance et al., 1998
2542.2, 2 LVYPFPGPIHNSLPQNIPP LTQT 58-79	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN pepsin-trypsin, Dep β-CN pepsin-trypsin	Chabance et al., 1998

Table 5.4 (continued)

Peptide: MW (Da), Z amino acid sequence amino acid sequence in β-CN	Bioactivity	Origin	Reference
2805.5, 4 SLSQSKVLPVPQKAVPYP QRD MPIQ, 154-198	Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN control, β-CN pepsin-trypsin	Murayama et al., 1987; Maeno et al., 1996
2876.5, 4 SLSQSKVLPVPQKAVPYP QRD MPIQA 154-199	Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN control, β-CN pepsin-trypsin	Muyarama et al., 1987b; Maeno et al., 1996
2920.5, 4 SITRINKKIEKFQSEEQQQ TEDEL 22-45	Mineral binding	β-CN trypsin	Chabance et al., 1998
5355.7, 4 IHPFAQTQSLVYPFPGPIH NSL PQNIPPLTQTPVVVPPFLQ PEV MGVSK 49-97	Opioid Angiotensin-l-converting enzyme (ACE) inhibitory	β-CN Trypsin, Dep β-CN trypsin	Chabance et al., 1998; Philanto-Leppala et al., 1998; FitzGeald & Misel, 2000

Underlined sequences reported as bioactive peptides

CHAPTER 6

EFFECTS OF DEPHOSPHORYLATION AND PROTEOLYSIS ON ALLERGENICITY OF CASEINS

6.1 Justification

Previous chapters described experiments on enzymatic dephosphorylation (Chapter 3), characterization of products of dephosphorylation (Chapter 4) and the effects of dephosphorylation on proteolysis of caseins (Chapter 5). In this chapter the effects of dephosphorylation on casein allergenicity is described. Milk allergy is common among infants, young children and some adults and is an abnormal response of some individuals to certain proteins present in bovine milk. An important component of milk allergy investigation is the evaluation of the allergenic response after dephosphorylation of caseins, since the phosphate groups are present in the epitope regions of casein and are immunoreactive (Bernard et al., 2000). A significant reduction of casein allergenicity is highly desirable; it is worthy to investigate whether this reduction can be achieved by proteolytic activity and whether hydrolysis of dephosphorylated caseins can generate molecules not recognized by the immune system as antigens. The fourth research objective of this study was to investigate the effects of dephosphorylation and *in vitro* hydrolysis of caseins on allergy reduction evaluated by enzyme-linked immunosorbent assay (ELISA); this Chapter addresses this objective.

6.2 Material and Methods

6.2.1 Materials

Whole casein was obtained as described previously in Section 3.2.1. Commercial α-casein and β-casein standards, potato acid phosphatase (EC 3.1.3.2; activity: 1.2 units/mg), pepsin from porcine stomach mucosa (E.C. 3.4.23.1; activity 3, 600 units/mg protein) and trypsin Type IX-S from porcine pancreas (E.C. 3.4.21.4; activity 16, 100 units/mg protein) were purchased from Sigma Chemicals Co. (St. Louis, MO).Immunological test, ELISA, was obtained from ELISA Systems Pty Ltd. (Queensland, Australia).

6.2.2 Dephosphorylation of Caseins

Dephosphorylation of whole casein, α-casein and β-casein was conducted as described in Section 5.2.2. Controls were prepared under the same experimental conditions using the caseins without the addition of acid phosphatase.

6.2.3 Preparation of Casein Hydrolysates

Proteolysis of native and dephosphorylated α-casein and β-casein was performed according to Section 5.2.3. Controls (native whole casein, α-casein and β-casein) were treated similarly without the addition of proteolytic enzymes. All hydrolyzed caseins were dialyzed overnight against distilled water at 4 °C to remove salts from the buffer.

6.2.4 Allergenicity Evaluation

6.2.4.1 Antigens Preparation

Native and dephosphorylated whole, α-casein and β-casein as well as the products of proteolysis of native and dephosphorylated α-casein and β-casein by pepsin, trypsin and combined proteolysis were analyzed. 2 mg of each casein were dissolved in 50 ml of extraction buffer, mixed and placed in a water bath (60 °C, 15 min) with shaking for 1 min every 5 min (ELISA Systems Pty Ltd. Protocol, 2005). The casein solutions were diluted to obtain a final protein concentration of 5 mg/l.

6.2.4.2 Enzyme-Linked Immunosorbent Assay

ELISA Systems Pty Ltd (Queensland, Australia) assay was used to detect casein residues after dephosphorylation and hydrolysis by a double antibody (sandwich) ELISA coated with specific anti-Bovine casein antibodies, 72 microwells were used as follows (ELISA Systems Pty Ltd. Protocol, 2005); 100 µl casein solution was added to each well of the flexible round-bottom microtitre plate, followed by mixing for 10 sec and incubation for 15 min. A thorough washing step (x5) was used to remove excess of free caseins. 100 µl peroxidase conjugated anti-bovine casein polyclonal antibodies was added to each well, mixed for 10 sec and incubated for 15 min, followed by additional washing (x5). 100 µl stabilized tetramethylbenzidine (TMB) as substrate was added to each well, mixed for 10 sec and incubated for 10 min. Finally, 100 µl 1M phosphoric acid was added to stop the reaction. The results were read in a microplate reader (BioRad laboratories; Hercules, CA) set at 450 nm and 620 nm; the raw data were processed by a Microplate Manager V5.2 (ELISA Systems Pty Ltd. Protocol, 2005); absorbance

readings are obtained as the subtraction of Absorbance 450 nm – Absorbance 620 nm. A negative control (buffered base) and positives controls (milk powder in buffer, at concentrations of 1, 2.5, 5 and 10 mg/l) were included. Results were expressed as the % reduction of allergenicity caseins after dephosphorylation and proteolysis by using the following equation:

$$\%\ \text{Reduction Allergenicity} = 100 - \frac{\text{Abs (Sample 450-620 nm)} \times 100}{\text{Abs (Control 450-620 nm)}}$$

6.2.5 Statistical Analysis

Student's *t* test to compare two sets of replicate measurements: i) native vs. dephosphorylated casein and ii) native vs. dephosphorylated casein after each different protease treatment, was performed using the $Abs_{(450\text{-}620nm)}$ data and analyzed with Microsoft Excel©; 95 % confidence level was used in order to establish whether casein modification (dephosphorylation and proteolysis) had a significant effect on reducing allergenicity.

6.3 Results and Discussion

6.3.1 Effect of Dephosphorylation of Caseins on Allergenicity Reduction

Figure 6.1 shows the antigenicity binding capacity of whole casein, α-casein and β-casein and dephosphorylated whole casein, α-casein and β-casein. The highest binding capacity ($Abs_{450\text{-}620nm}$ 2.72) was obtained for the α-casein fraction, followed by whole

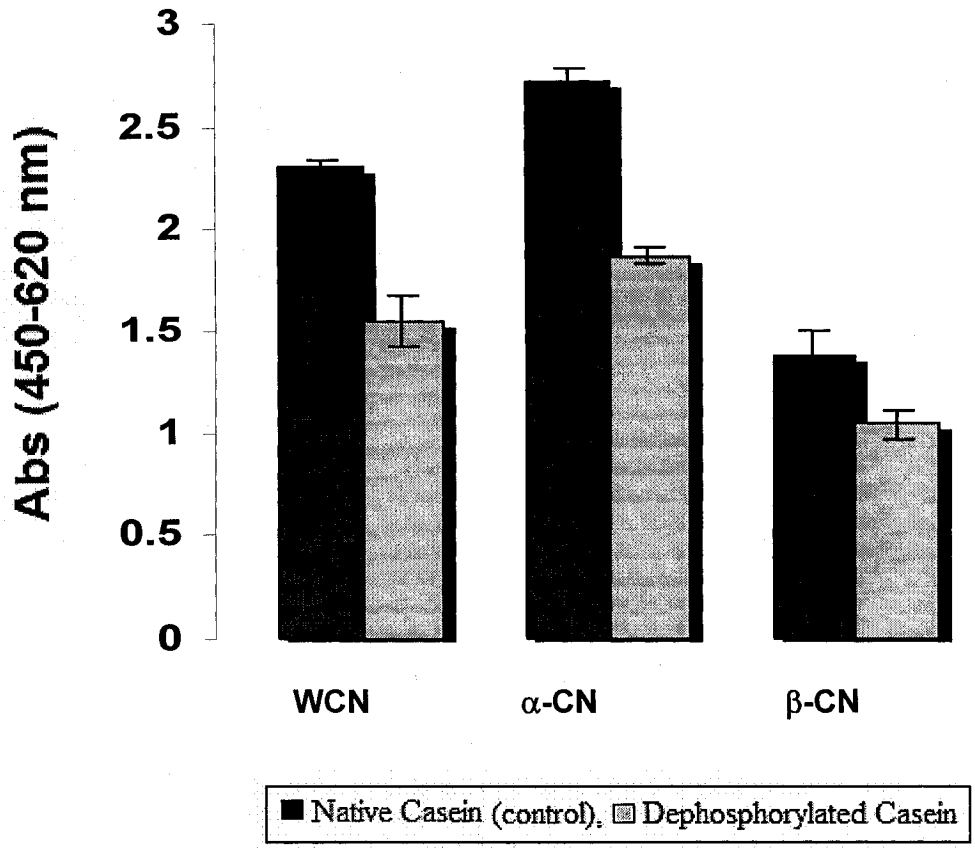


Figure 6.1: Antigen-antibody binding of whole, α-casein and β-casein (controls) compared to dephosphorylated whole casein, α-casein and β-casein

casein ($Abs_{450\text{-}620nm}$ 2.32) and β-casein ($Abs_{450\text{-}620nm}$ 1.37). Dephosphorylation of the caseins resulted in a decrease in the antigenicity binding capacity; results show $Abs_{450\text{-}620nm}$ of 1.87, 1.55 and 1.04 for dephosphorylated α-casein, whole casein and β-casein, respectively; these values are similar to those reported by Nentwich et al. (2004) for native caseins and dephosphorylated α-casein. There was a significant difference ($p < 0.05$) in the binding capacity of dephosphorylated whole casein and dephosphorylated α-casein when compared to the respective control caseins (non-dephosphorylated whole casein and α-casein); however, no significant difference ($p < 0.05$) was observed between dephosphorylated β-casein and β-casein control. Table 6.1 shows the calculated reduction in allergenicity for each casein; removal of phosphate groups from whole casein, α-casein and β-caseins resulted in relatively low reduction of allergenicity of 33%, 31.2% and 24.4%, respectively. The lack of defined tertiary structure of caseins due to the high content of proline and hydrophobic amino acids (Fox & McSweeney, 1998) likely contributed to this relatively low reduction in allergenicity; the major epitopes containing the phosphate groups were probably not completely dephosphorylated. Otani et al. (1987) reported that modification of caseins by dephosphorylation changed their immunological properties; this agrees with our results which decreased IgE binding of dephosphorylated whole casein, α-casein and β-casein confirms a reduction of the antigen bonding. The highest binding obtained for the control α-casein is consistent with current knowledge that α-casein is the major milk allergen (Spuergin et al., 1996). Dephosphorylation of α-casein is important since α-casein plays a crucial role in allergenicity, especially as the phosphoseryl residues in α_s-caseins have been described as being immunoreactive and resistant to digestive degradation (Bernard et al., 2000). In a review by Wal (2004), it has

Table 6.1: % Reduction* of allergenicity after dephosphorylation and dephosphorylation/proteolysis of whole casein, α-casein and β-casein

Type of Proteolysis	Protein	% Reduction allergenicity
None	Dephosphorylated Whole Casein	33
None	Dephosphorylated α-casein	31.2
None	Dephosphorylated β-casein	24.4
Peptic	α-casein	61.9
	Dephosphorylated α-casein	74.7
Tryptic	α-casein	61.9
	Dephosphorylated α-casein	75.4
Peptic-Tryptic	α-casein	58.5
	Dephosphorylated α-casein	71.3
Peptic	β-casein	4
	Dephosphorylated β-casein	5.6
Tryptic	β-casein	0.6
	Dephosphorylated β-casein	69.5
Peptic-Tryptic	β-casein	70.3
	Dephosphorylated β-casein	74.9

* Based on average absorbance data (Abs $_{420\text{-}620nm}$) in Figures 6.1, 6.2 and 6.3

been suggested that there is no specific structure or function that can be associated with allergenicity of milk proteins due to the high variability and heterogeneity of the IgE response; it was stated that no single allergen or particular structure can be responsible for milk allergy. On the other hand, Spuergin et al. (1996) demonstrated that sera from allergic subjects to milk showed reactions with three regions of α_{S1}-casein, corresponding to the amino acids residues 19-30, 93-98, and 141-150 which contain phosphoserine residues.

6.3.2 Effect of Proteolysis and Dephosphorylation/Proteolysis on Allergenicity Reduction of Caseins

Figure 6.2 shows the antigenicity binding capacity of α-casein and dephosphorylated α-casein obtained after peptic, tryptic and combined peptic-tryptic proteolysis. The highest binding capacity ($Abs_{450\text{-}620nm}$ 0.62) was obtained for the α-casein peptic treatment, followed by α-casein combined proteolysis treatment ($Abs_{450\text{-}620nm}$ 0.45) and α-casein tryptic treatment ($Abs_{450\text{-}620nm}$ 0.43). The dephosphorylated α-casein showed a decrease in the antigenicity binding capacity after proteolysis; results show an $Abs_{450\text{-}620nm}$ 0.47, 0.41 and 0.51 for peptic, tryptic and combined proteolysis, respectively; there was no significant difference ($p < 0.05$) in allergenicity reduction between the α-casein and dephosphorylated α-casein hydrolysates obtained after trypsin and combined pepsin-trypsin proteolysis; however, pepsin treatment showed a significant difference ($p < 0.05$) between the α-casein and dephosphorylated α-casein hydrolysates. Additionally, a significant difference ($p < 0.05$) in the reduction of allergenicity was noted between dephosphorylated α-casein as control (non-hydrolyzed) and the

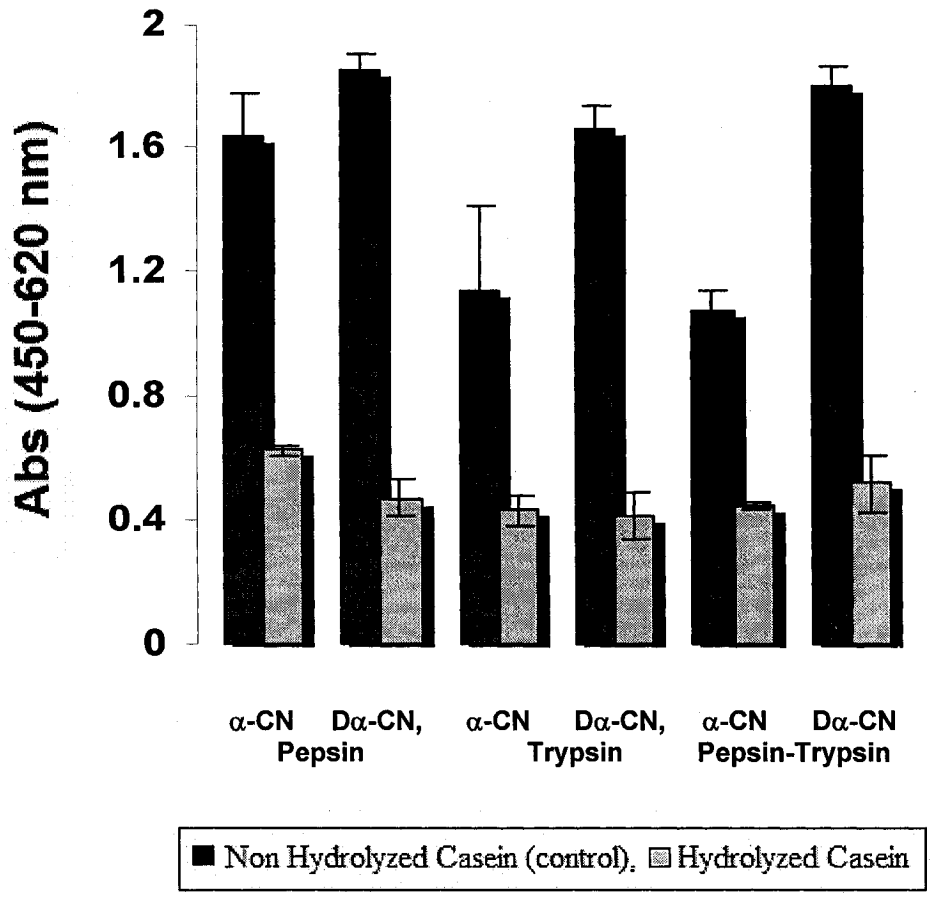


Figure 6.2: Antigen-antibody binding of α-casein and dephosphorylated α-casein after pepsin, trypsin or combined proteolysis

proteolysis products of the same dephosphorylated α-casein obtained after the three proteolytic enzyme treatments. Table 6.1 shows the calculated reduction in allergenicity for α-casein and dephosphorylated α-casein after each proteolytic treatment; a reduction in allergenicity of 61.9%, 61.9% and 58.5% was obtained for the pepsin, trypsin and combined pepsin-trypsin proteolysis of α-casein, respectively; slightly higher allergenicity reduction of 74.7%, 75.5% and 71.33% was obtained for dephosphorylated α-casein after pepsin, trypsin and combined proteolysis, respectively.

Figure 6.3 shows the antigenicity binding capacity of β-casein and dephosphorylated β-casein obtained after peptic, tryptic and combined peptic-tryptic proteolysis. The highest binding capacity ($Abs_{450\text{-}620nm}$ 0.60) was obtained for the β-casein peptic treatment, followed by β-casein tryptic treatment ($Abs_{450\text{-}620nm}$ 0.44) and β-casein combined peptic-tryptic proteolysis treatment ($Abs_{450\text{-}620nm}$ 0.28). Dephosphorylated β-casein showed a decrease in the antigenicity binding capacity after proteolysis; binding capacities were $Abs_{450\text{-}620nm}$ 0.48, 0.22 and 0.19 for peptic, tryptic and combined proteolysis, respectively; there was a significant difference ($p < 0.05$) in allergenicity reduction between β-casein and dephosphorylated β-casein hydrolysates after all proteolysis treatments; additionally, a significant difference ($p < 0.05$) in allergenicity reduction was noted between dephosphorylated β-casein as control (non-hydrolyzed) and dephosphorylated β-casein after trypsin and combined pepsin-trypsin proteolysis. Peptic treatment for β-casein control (non- hydrolyzed), β-casein hydrolyzed and dephosphorylated showed a non significant difference ($p < 0.05$) in the binding capacity; this was also the case for β-casein control (non-hydrolyzed) and β-casein after

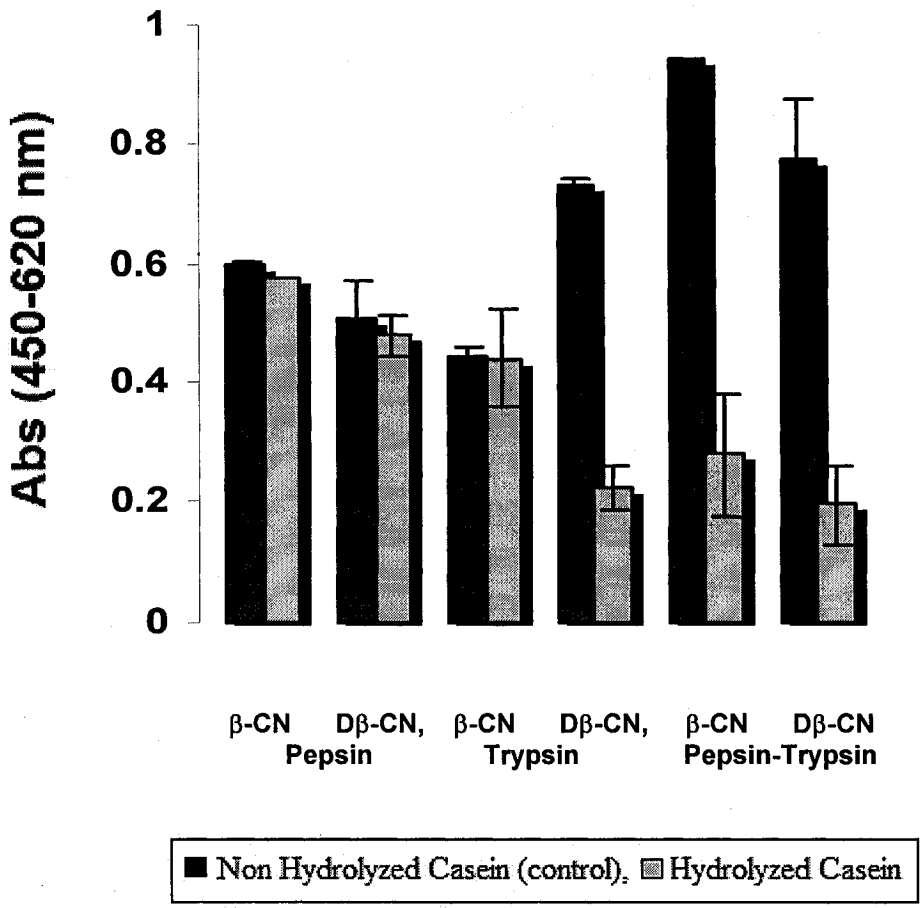


Figure 6.3: Antigen-antibody binding of β- and dephosphorylated β-casein after pepsin, trypsin or combined pepsin-trypsin proteolysis

trypsin proteolysis. Table 6.1 shows the calculated reduction in allergenicity for β-casein and dephosphorylated β-casein after each proteolytic treatment; a reduction in allergenicity of 4%, 0.6% and 70.3% was obtained for the pepsin, trypsin and combined proteolysis of β-casein, respectively; at this time no explanation can be given for the relatively low decrease in allergenicity by pepsin hydrolysis. Higher results were obtained for dephosphorylated β-casein 4%, 69.5% and 74.9% after pepsin, trypsin and combined proteolysis, respectively.

The results demonstrated that dephosphorylation favored the proteolytic cleavage of regions originally containing the major phosphorylated epitopes; there is a higher reduction in allergenicity (more than 70%) when compared to the non-hydrolyzed native and dephosphorylated caseins. α-casein showed the strongest antigenic binding when compared to the β-casein; an evaluation of reduction in allergenicity of a particular casein fraction may indicate the extent of hydrolysis necessary for efficient allergenicity reduction. Although, there is evidence on the reduced allergenicity of caseins hydrolyzate used in infant formulas (Miquel et al., 2005), the adequate degree of hydrolysis necessary to obtain the desired reduction in allergenicity and the particular polypeptides that are responsible for residual allergenicity have not been established. Commercial hydrolyzed formulas were evaluated for allergenic responses in children with IgE mediated bovine milk allergy (Caffarelli et al., 2002); it was observed that most patients tolerated extensively hydrolyzed casein formulas; although, highly sensitized children experienced immediate clinical reactions.

CHAPTER 7

GENERAL CONCLUSIONS

7.1 Conclusions

Milk proteins are considered an active subject of research and development of new and improved food products and ingredients. The modern food-processing industry is placing more and more emphasis on the utilization of protein ingredients to provide specific functional properties to a wide range of formulated foods. Phosphate groups are responsible for many important characteristics of caseins. Modification of caseins can be achieved by a number of chemical and enzymatic means; this research studied the effects of enzymatic dephosphorylation on whole casein, α-casein and β-casein on biological properties of whole casein, α-casein and β-casein.

The present work has established optimum dephosphorylation conditions for whole casein, α-casein and β-casein. Results demonstrated that whole casein and its individual α-casein and β-casein fractions showed substantial (>70%) dephosphorylation by an acid phosphatase. Apparent kinetic parameters V_{max} (0.283 μmol P/mg casein min) and K_m (9.951 mg casein/l) for dephosphorylation of whole casein were obtained. Knowledge of these parameters is important to characterize the reaction system; the V_{max} value varied with the total concentration of the phosphatase present but the K_m value was independent of the enzyme concentration and it is considered characteristic of the reaction investigated.

This work established that dephosphorylation of the whole casein did not show the non-specific proteolysis which was observed with α-casein and β-casein. Random

dephosphorylation was observed with both α-casein and β-casein. Limited dephosphorylation was observed with both α-casein and β-casein in the absence of the phosphatase. Using ESI-MS would allow us to identify dephosphorylated species.

Dephosphorylation of the casein fractions facilitated the proteolytic effects of pepsin and trypsin. The information obtained using mass spectrometry techniques about protein structure in conjunction with advanced bioinformatics interfaced with protein database search engines, resulted in the identification of peptides liberated by proteolysis and dephosphorylation effects on the peptides released by proteolysis.

Evaluation of allergenicity by Enzyme-Linked Immunosorbent Assay after dephosphorylation of whole casein, α-casein and β-casein and proteolysis, indicated that there was a significant reduction of the antigen binding capacity of dephosphorylated casein. However, proteolysis reduced considerably more the antigen binding capacity of caseins compared to the entire caseins molecules. Proteolysis and dephosphorylation results showed a higher reduction in allergenicity (approximately 70%); especially in the highly allergenic α-casein after dephosphorylation and trypsin treatment.

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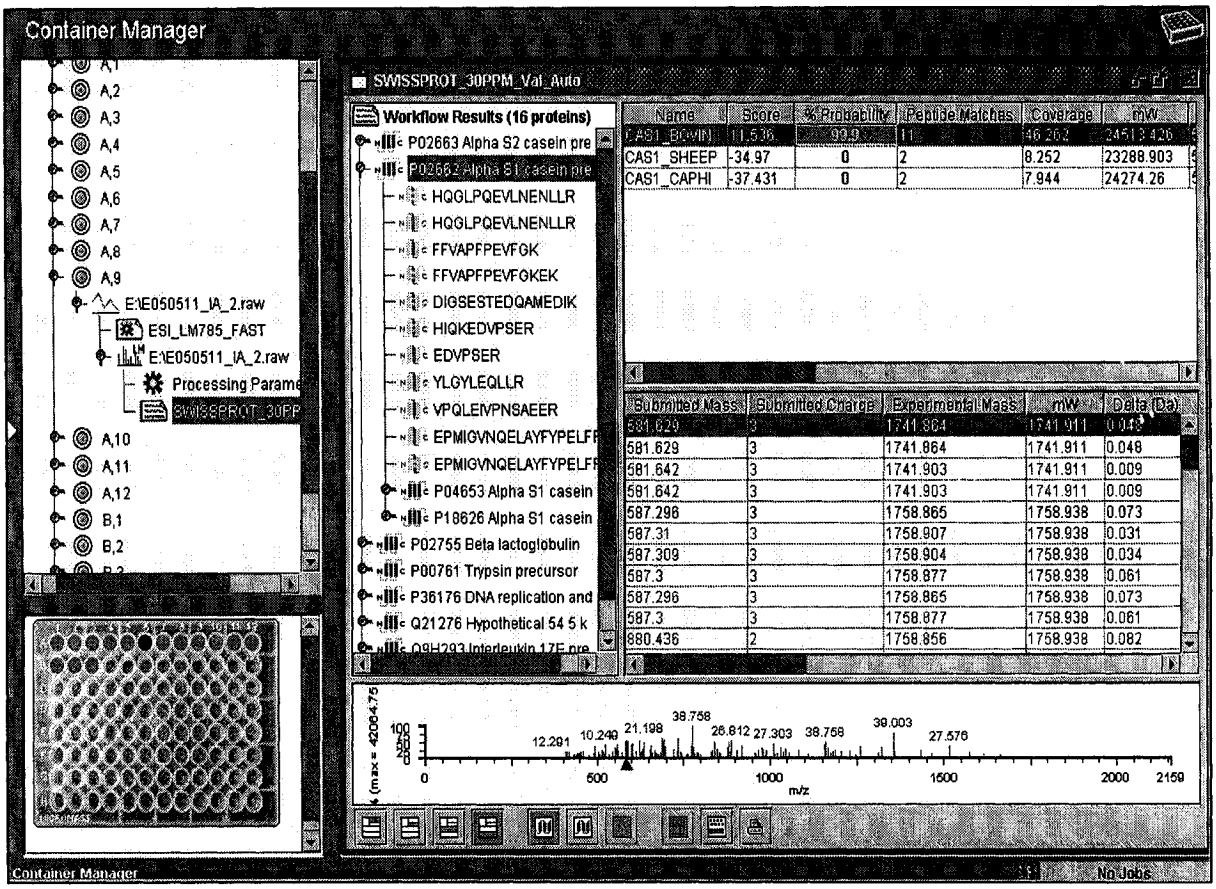
APPENDIX 1

Screen capture of portions of a typical LC-ESI-MS/MS data analysis search report of α-casein tryptic hydrolysis using ProteinLynx showing (a) list of significant hits and information on individually identified proteins, with the sequence of the matched peptide, (b) probability-based score (99.9%) illustrated by the bar chart, (c) information on individually identified proteins including mass, charge, experimental mass and error, and (d) the MS/MS spectrum (ProteinLynx, Global Server 2005).



APPENDIX 2

Screen capture of portions of a typical LC-ESI-MS/MS data search report of α-casein trypsin hydrolysis using Mascot (Matrix Science, 2005), showing (a) top list of significant hits, (b) probability-based Mowse Score (bar chart), (c) information on individually identified proteins including mass, error, and (d) sequence of matched peptide (Matrix Science, Mascot, 2005)

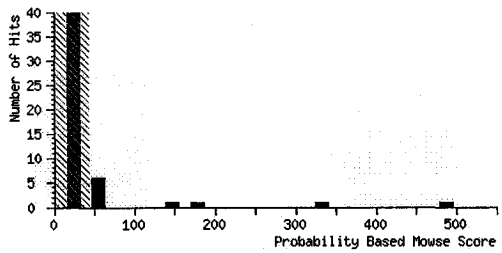
Mascot Search Results

User : aline
Email : aline.tezcucano@mail.mcgill.ca
Search title : a-casein trypsin
MS data file : E050511_IA_2.pkl
Database : SwissProt 47.6 (243668 sequences; 110671679 residues)
Timestamp : 21 Aug 2005 at 02:01:19 GMT
Warning : Too many peptide masses in your data file. Mascot has a limit of 1000000
Significant hits:

P02662-00-00-00	(CAS1_BOVIN) Splice isoform Displayed; Variant Displayed
P02663	(CAS2_BOVIN) Alpha-S2 casein precursor [Contains: Casocidin-I] Alpha-S2 casein
P02754-00-04-00	(LACB_BOVIN) Splice isoform Displayed; Variant variant A
P67975	(LACB_OVIMU) Beta-lactoglobulin (Beta-LG) Beta-lactoglobulin (Beta-LG)
P02666-00-05-00	(CASB_BOVIN) Splice isoform Displayed; Variant variant B
P02668-00-00-00	(CASK_BOVIN) Splice isoform Displayed; Variant Displayed
Q8B9D5	(CATV_NPVRO) Viral cathepsin (EC 3.4.22.50) (V-cath) (Cysteine proteinase)

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 44 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. P02662-00-00-00 Mass: 24513 Score: 487 Queries matched: 65
(CAS1_BOVIN) Splice isoform Displayed; Variant Displayed; Conflict Displayed; from P02662 Alpha-S1 casein

☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	27	416.16	830.31	830.38	-0.07	0	(32)	1.8	1	K.EDVPSER.Y
☑	28	416.17	830.33	830.38	-0.05	0	(31)	2.2	1	K.EDVPSER.Y
☑	29	416.20	830.38	830.38	0.00	0	33	1.5	1	K.EDVPSER.Y
	43	455.74	909.46	909.47	-0.01	0	(5)	4.7e+02	10	K.EGIHAQQK.E
	44	455.74	909.46	909.47	-0.01	0	9	1.9e+02	3	K.EGIHAQQK.E
☑	113	634.29	1266.57	1266.70	-0.13	0	64	0.00057	1	R.YLGYLEQLLR.L
☑	114	634.29	1266.57	1266.70	-0.13	0	(61)	0.0014	1	R.YLGYLEQLLR.L

APPENDIX 3

Peptides Identified by MS from α-casein and Dephosphorylated α-casein in Section 5.3.2.1 after Enzyme Proteolysis

Control (MW, Da)		Pepsin (MW, Da)		Trypsin (MW, Da)		Pepsin-Trypsin (MW, Da)	
α-casein	Deph α-casein	α-casein	Deph α-casein	α-casein	Deph α-casein	α-casein	Deph α-casein
807.4	810.4	818.4	812.4	816.4	830.4	830.4	570.3
827.6	826.6	826	827	830.4	911.4	856.2	809.6
849.6	930.6	835.4	829.4	877.4	969.6	870.4	830.4
861.6	968.6	843.4	834	897.4	983.6	930.6	840.6
928.6	1000.6	845.4	842.4	910.4	1011.6	944.4	863.6
947.6	1050.6	867.6	937.4	956.4	1027.4	945.5	872.6
974.6	1090.6	915.5	952	980.4	1048.6	956.4	925.4
1008.6	1137.6	922.4	960.4	1002.4	1066.6	958.5	946.6
1029.6	1266.7	929.4	969.4	1025.4	1145.6	976.6	958.5
1100.6	1336.7	945.4	976	1044.4	1197.6	982.6	960.4
1336.7	1383.7	952	983.6	1067.6	1207.6	994.4	964.4
1350.9	1496.8	966.4	1002.6	1083.6	1237.6	1001.6	976.6
	1834.8	977.6	1020.6	1117.6	1266.7	1006.6	1004.6
	1964.4	981.4	1071.6	1146.6	1268.6	1019.4	1012.5
		994.4	1087.6	1198.6	1294.6	1024.6	1013.6
		1002.6	1102.6	1226.6	1338.6	1034.6	1019.4
		1011	1123.6	1239.6	1347.6	1041.6	1020.6
		1128.6	1138.6	1268.6	1362.6	1079.6	1030.6
		1142.6	1156.6	1298.6	1383.7	1089.6	1042.6
		1271.7	1168.6	1324.6	1743.9	1093.6	1060.6
		1297.5	1183.6	1366.6	1759	1116.4	1075.6
		1305.6	1194.6	1383.7	1766.7	1123.6	1089.6

Control (MW, Da)		Pepsin (MW, Da)		Trypsin (MW, Da)		Pepsin-Trypsin (MW, Da)	
α-casein	Deph α-casein	α-casein	Deph α-casein	α-casein	Deph α-casein	α-casein	Deph α-casein
		1378.5	1271.7	1385.6	1871	1142.6	1093.6
		1563.6	1296.9	1412.4	2315	1163.6	1102.6
		1748.1	1410.6	1542.4	2320	1181.6	1115.6
			1422.9	1585.8		1200.6	1123.6
				1846.7		1236.6	1133.6
				2315		1236.6	1142.6
						1247.8	1142.6
						1255.8	1154.6
						1264.6	1164.6
						1284.6	1181.6
						1289.7	1200.6
						1290.6	1234.6
						1302.6	1251.6
						1319.5	1262.6
						1350.6	1262.6
						1364.6	1270.8
						1388.6	1278.6
						1406.6	1289.6
						1449.6	1303.4
						1487.6	1324.6
						1503.4	1372.6
						1509.9	1400.6
						1532.4	1407.6
						1540.6	1410.6
						1558.4	1450.6
						1593.6	1485.5

Control (MW, Da)		Pepsin (MW, Da)		Trypsin (MW, Da)		Pepsin-Trypsin (MW, Da)	
α-casein	Deph α-casein	α-casein	Deph α-casein	α-casein	Deph α-casein	α-casein	Deph α-casein
						1600.6	1490.8
							1540.6
							1583.4
							1653.7

APPENDIX 4

Peptides Identified by MS from β-casein and Dephosphorylated β-casein in Section 5.3.2.1 after Enzyme Proteolysis

Control (MW, Da)		Pepsin (MW, Da)		Trypsin (MW, Da)		Pepsin-Trypsin (MW, Da)	
β-casein	Deph β-casein	β-casein	Deph β-casein	β-casein	Deph β-casein	β-casein	Deph β-casein
867.6		827.6	747.4	741.4	816.6	813.4	813.4
926.6		849.6	845.6	816.4	829.4	817.6	822.4
976.6		882.6	882.6	985.6	852.4	844.6	828.6
1028.6		901.6	899.6	1002.6	893.4	867.6	829.4
1051.6		940.6	912.6	1029.6	936.6	880.6	848.6
1088.6		958.6	940.6	1088.6	1001.6	921.6	866.6
1129.6		970.6	952.6	1107.6	1028.6	927.6	882.6
1140.4		975.6	966.6	1195.6	1060.6	934.6	921.6
1150.6		986.6	978.6	1202.6	1088.6	972.4	940.4
1196.6		996.6	996.6	1233.6	1110.6	980.6	942.6
1264.8		1004.6	1012.6	1253.6	1136.6	992.6	975.6
1282.5		1020.6	1109.6	1270.6	1158.6	993.5	993.5
1371.7		1030.6	1118.8	1408.8	1197.6	1002.6	1011.6
1400.8		1041.6	1193.8	1414.6	1230.6	1010.6	1026.6
1472.7		1057.6	1199.8	1460.8	1289.6	1024.6	1028.6
1511.7		1062.6	1203.2	1540.6	1342.6	1030.6	1064.6
		1080.6	1208.8	1565.6	1362.8	1040.6	1102.6
		1100.6	1245.8	1569.6	1385.8	1057.6	1107.5
		1135.6	1256.8	1643.6	1424.8	1070.6	1120.6
		1167.6	1282.8	1734	1448.8	1095.8	1129.6

Control (MW, Da)		Pepsin (MW, Da)		Trypsin (MW, Da)		Pepsin-Trypsin (MW, Da)	
β-casein	Deph β-casein	β-casein	Deph β-casein	β-casein	Deph β-casein	β-casein	Deph β-casein
		1210.8	1310.8	1980.8	1605	1122.6	1194.6
		1185.6	1291.8	1747	1490.8	1101.6	1140.4
		1204.8	1304.8	1783	1586	1110.6	1140.6
		1225.6	1312.8		1643.6	1138.6	1214.8
		1231.8	1321.8		1707.8	1158.6	1239.8
		1260.6	1336.8		1980.4	1187.6	1251.6
		1276.8	1348.8			1202.8	1269.6
		1278.9	1365.8			1215.8	1273.6
		1281.8	1373.8			1224.6	1286.8
		1296.8	1404.8			1267.8	1303.8
		1314.8	1412.8			1298.8	1307.6
		1336.8	1422.8			1307.6	1328.8
		1373.8	1433.8			1342.8	1339.6
		1381.8	1448.8			1386.8	1341.8
		1384.8	1452.8			1395.6	1367.5
		1404.8	1457.6			1399.8	1371.7
		1437.8	1474.8			1406.8	1374.8
		1454.8	1496.8			1432.8	1383.8
		1457.6	1528.8			1444.8	1398.8
		1474.8	1550.8			1452.7	1432.8
		1488.8	1588			1487.8	1448.8
		1513.8	1625			1507.8	1468.8
		1539	1698			1510.7	1902
		1550.8	1715			1512.8	

Control (MW, Da)		Pepsin (MW, Da)		Trypsin (MW, Da)		Pepsin-Trypsin (MW, Da)	
β-casein	Deph β-casein	β-casein	Deph β-casein	β-casein	Deph β-casein	β-casein	Deph β-casein
			1737			1697.6	
			1754			1731	
			1757.7			1769	
			1773			1800.9	
						1879.9	