GMIT
GALWAY-MAYO INSTITUTE OF TECHNOLOGY
INSTITIÚID TEICNEOLAÍOCHTA NA GAILLIMHE-MAIGH EO

Investigation of the substrate specificity of glutamyl endopeptidase using casein substrates.

A thesis submitted to the Galway-Mayo Institute of Technology in fulfilment of the academic requirements for the Degree of Doctor of Philosophy.

by

Phanindra Kalyankar M.Sc.

Department of Life Sciences, Galway-Mayo Institute of Technology.

Supervisors

Dr. Gerard O' Cuinn

(Galway-Mayo Institute of Technology)

&

Prof. Richard J. FitzGerald

(University of Limerick)

Submitted to School of Science, Galway-Mayo Institute of Technology.

May 2011.

ABSTRACT

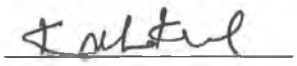
Electrophoretically and chromatographically pure β-casein was prepared from acid caseinate exploiting the differential solubility of different caseins in the presence of Ca^{2+} at 4°C and 30°C. The yield of β-casein was approximately 5%. The β-casein purification process also yielded a co-fraction enriched in α-casein.GE was purified and characterized from Alcalase™ 2.4 L. Various protocols were investigated to purify GE before selecting a protocol employing hydrophobic interaction (HIC) and cation exchange (IEX) chromatography. The yield of GE obtained was approximately 42%.

The purified β-casein and the enriched α-casein fractions were digested with GE at 37°C and 50°C over 4 h. The results show that GE was highly specific and hydrolysed the peptide bond predominantly on the carboxy terminal of Glu residues. GE also appeared to hydrolyze bonds on the carboxy terminal of Asp. The results indicated that Met residues were poorly preferred at the P_1' position. Whereas, Pro residues were not preferred at the P_1' position. It was also observed that the hydrolysis of Glu-X bond in Glu-Glu-X and in Glu-Glu-Glu-X sequences (X= Arg, Asn, Ile and Lys) was preferred in comparison to Glu-Glu, suggesting that at the P_1' position Glu residues were poorly preferred. Non-specific cleavages were observed at the carboxy terminal of Phe (51), Thr (128) and Gln (188) on incubation of β-casein with GE. Non-specific cleavages at the carboxy terminal of Phe (24) and Gln (131) in α_{s1}-casein were observed on incubation of the enriched α-casein with GE only at 50°C. Synthetic peptides corresponding to specific sequences of β-casein were incubated with GE at 37°C for 120 min. The LC-MS analysis on the hydrolysed sample showed that multiple phosphorylated residues near the scissile bond did not influence the substrate specificity of GE. Furthermore, these studies showed that the Glu-Pro and the Glu-Met bonds were hydrolysed.

DECLARATION

This thesis is the original work of the author and due reference and acknowledgement has been made, where necessary to the work of others. The work presented in this thesis has not been submitted to any other university or higher education institution, or for any other academic award in the Galway-Mayo Institute of Technology.

Phanindra Kalyankar — Date



15-09-2011

Author

Department of Life Sciences,
School of Science,
Galway-Mayo Institute of Technology,
Galway- Ireland.

DEDICATION

To my teachers, lectures and grandparents.

ACKNOWLEDGEMENTS

My sincere thanks to Dr. Gerard O' Cuinn and Prof. Dick FitzGerald (University of Limerick) for their invaluable help during the supervision of this thesis.

Thanks to the University of Limerick for hosting my stay at the Life Sciences Department during the duration of this project.Thanks to all the technicians in the Life Sciences Department at the University of Limerick. Thanks also to Ms. Laura McDonagh in the research office at Galway-Mayo Institute of Technology for her support throughout my PhD.

Many thanks to all my friends, at home, in India and in the Life Sciences Department at UL, for their support and words of encouragement that helped me throughout the completion of this thesis.

A very special thanks to my parents, bother, sister-in-law and my wife for their moral and financial support.

A special word of thanks to Dr. Dara O' Sullivan and Dr.Ebenezer Rajaratnam from the Department of Life Sciences, University of Limerick for their support and encouragement.

A special note of gratitude also to Department of Agriculture, Fisheries and Food for funding this research.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 4

Chapter 5

Chapter 6

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 5

Chapter 6

Chapter 7

ABBREVATIONS

ac	acetyl
AC	affinity chromatography
BLP	*Bacillus licheniformis* proteinase
BSA	blood serum albumin
CID	collision induced dissociation
CMP	caseino macro peptide
DEAE	diethylaminoethyl cellulose
DFP	diidopropyl fluorophosphate
EC	Enzyme Commission
FPLC	fast protein liquid chromatography
GE	glutamyl endopeptidase
GPC	gel permation chromatography
HIC	hydrophobic interaction chromatography
IEX	ion-exchange cromatography
Igs	immunoglobulins
IMCU	international milk clotting units
LC-MS	liquid chromatography mass spectrometry
LF	lactoferrin
LP	lactoperoxidase
pI	iso-electric point
PMSF	phenylmethanesulphonyl fluoride
pNA	paranitroanalide

RP-HPLC	reverse phase high performance liquid chromatography
RU	rennet units
s/suc	succinyl
SBTI	soyabean trypsin inhibitor
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TIC	total ion chromatogram
TLCK	tosyl-lysine chloromethyl ketone
TPCK	tosylamido-2-phenylethyl chloromethyl ketone
WPC	whey protein concentate
WPI	whey protein isolate
α-La	α-lactalbumin
β-Lg	β-lactoglobulin

Chapter 1

Introduction

1.1. General introduction

Proteinases are physiologically vital due to their ability to perform highly specific and selective modifications of proteins, e.g., activation of zymogen forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across membranes (Maurer, 2004). Commercially, proteinases are used in the detergent, leather, food and pharmaceutical industries for various purposes.

Food proteins are hydrolysed mostly by bacterial proteinases to modify their functional, bioactivity, sensory and nutritional properties (Sumantha *et al.,* 2006). Alcalase™ is a *Bacillus licheniformis* proteinase (BLP) which was reported to contain subtilisin along with low levels of GE activity (Breddam and Svensdsen, 1992 and Spellman *et al.,* 2005). The aggregation and gelation properties of peptides obtained on hydrolysis of whey proteins using Alcalase was reported by various researchers (Otte *et al.,* 1997; Doucet *et al.,* 2003; Spellman *et al.,* 2005; Creusot *et al.,* 2006 and Creusot and Gruppen, 2007).

The work done by Spellman *et al.,* (2005) indicated that glutamyl endopeptidase (GE) present in Alcalase was responsible for the enzyme induced aggregation in Alcalase hydrolysates of whey protein concentrate. Furthermore, Spellman *et al.,* (2009) implied that GE was also responsible for higher bitterness in Alcalase hydrolysates of whey protein concentrate.

Given the potential importance of GE from *Bacillus licheniformis* to the sensory and functional properties of milk protein hydrolysates, it is essential to thoroughly investigate the substrate specificity of GE using specific milk protein substrates. Caseins are the principal milk proteins which roughly account for 80% of the total proteins in bovine milk. Furthermore, Madsen and Qvist, (1997) reported that GE (Glu/Asp specific BLP) hydrolysed caseins more effectively than the whey proteins. It was therefore decided to study the substrate specificity of GE using purified and enriched fractions of caseins, along with synthetic peptide sequences corresponding to specific regions in the individual caseins.

1.2. Bovine milk proteins

Milk proteins are of major importance for the dairy industry due to their technological and nutritional value. Bovine milk contains 2.3–4.4% (w/w) protein (Walstra *et al.*, 1999). Caseins are the major milk proteins which account for approximately 80% (76-86%) of the total proteins present in milk (Swaisgood, 1992). The remaining 20% is mainly whey or serum proteins, enzymes and other minor proteins and peptides. The most common whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), blood serum albumin (BSA) and immunoglobulins (Igs) (Swaisgood, 1992). Whey also contains proteins such as lactoferrin (LF), lactoperoxidase (LP) and lysozyme as well as more than 60 different enzymes (Andrews, 1992; Steijns and van Hooijdonk, 2000). Several factors affect the composition of proteins and peptides in milk, e.g., breed of cow, animal health, stage of lactation, feeding system, seasonal changes, milking frequency and milking system (Lindmark-Mansson *et al.*, 2005). The concentrations of the major proteins in bovine skim milk are given in Table 1.1.

Table 1.1. Composition of different proteins present in bovine skim milk.

Bovine milk protein	Concentration (g/l)
αs_1-Casein	12-15
αs_2-Casein	3-4
β-Casein	9-11
κ-Casein	2-4
β-lactoglobulin	2-4
α-lactalbumin	0.6-1.7
Serum albumin	0.4
Immunoglobulin G1	0.3-0.6
Immunoglobulin G2	0.05
Immunoglobulin A7	0.01
Immunoglobulin M	0.09
Secretory component	0.02-0.1
Lactoferrin	0.02-0.1

Adopted from Farrell *et al.*, (2004).

1.3. Caseins

Bovine casein contains four distinct gene products designated as α_{s1}-, α_{s2}-, β- and κ-casein occurring at an approximate ratio of 37, 10, 35 and 12%, respectively

(Swaisgood, 1996). All the caseins self-associate to form micelles. Caseins are amphiphilic phosphoproteins. One to 13 residues are phosphorylated depending on the individual protein molecule (FitzGerald, 1997). The number of phosphorylated serine residues present in α_{s1}-, α_{s2}-, β- and κ-casein are 8, 10-13, 5 and 1, respectively (Swaisgood, 1982).

Casein micelles

In milk, casein exists as large colloidal particles 50-60 nm in diameter called casein micelles. Many of the technologically important properties of milk such as white colour, heat stability, ethanol stability and coagulation by rennet are due to the casein micelles (Fox and Brodkorb, 2008). κ-Casein a minor component (12-15%) of the casein system, is soluble in the presence of Ca^{2+} whereas 85% of caseins are precipitated by Ca^{2+}. κ-Casein stabilizes other caseins by forming a micelle (Fox and Brodkorb, 2008). The average characteristics of casein micelles are given in Table 1.2.

Table 1.2. General characteristics of casein micelles.

Characteristic	Value
Diameter	120 nm (range: 50-500 nm)
Surface area	$8x10^{-10}$ cm^2
Volume	$2.1x\ 10^{-15}$ cm^3
Density	1.0632 g cm^3
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O g^{-1} protein
Voluminosity	44 cm^3 g^{-1}
Molecular mass (hydrated)	1.3×10^9 Da
Molecular mass (dehydrated)	5×10^8 Da
No. of peptide chains	5×10^3
No. of particles per ml milk	10^{14}-10^{16}
Surface of micelles per ml milk	5×10^4 cm^3
mean free distance	240 nm

Taken from Fox and Brodkorb (2008)

Genetic variants in caseins

Milk proteins may differ from each other as a result of genetic polymorphism, generally arising from a substitution and/or deletion of amino acids in the primary

structure (Ng-Kwai-Hang and Grosclaude, 1982). All of the caseins exhibit genetic polymorphism (Table 1.3). The genetic variants may be distinguished using a range of techniques including gel electrophoresis. The association of certain genetic variants with unique technological properties can be exploited by the food industry (FitzGerald, 1997).

Table 1.3. Genetic variants of different caseins.

Casein	Genetic variants	Total number of Genetic variants
αs_1-casein	A, B C, D, E, F, G and H	8
αs_2-casein	A, B, C and D	4
β-casein	A^1, A^2, A^3, B, C, D, E F, G, H^1, H^2 and I	12
κ-casein	A, B, C, E, F^1, F^2, G^1, G^2, H, I and J	11

Information from Farrell *et al.*, (2004).

Casein structure

Primary structure of caseins

The primary structures of the individual caseins display several unique properties. The complete amino acid composition of the different caseins along with their molecular mass and hydrophobicity values are given in Table 1.4. The percentage of Pro residues present in caseins is very high and it is approximately 8.5, 5, 17 and 12% per mole for αs_1-, αs_2-, β and κ-casein, respectively. Proline residues are uniformly distributed, giving the caseins a type of poly-proline helix. Thus caseins are very susceptible to proteolysis without prior denaturation by heat or acid treatment. Whereas, in globular whey proteins heat treatment prior to proteolysis is beneficial as these proteins exhibit extensive secondary structure (Cayot and Lorient, 1997).

The percentage of Lys residues present per mole for αs_1-, αs_2-, β and κ-casein is approximately 7, 11.5, 5.3 and 5.3%, respectively. Due to the high content of lysine, caseins and products containing caseins may undergo Maillard reaction (non-enzymatic browning) on heating in the presence of reducing sugars (Fox and McSweeney, 1998).

The percentage of Glu residues present per mole for αs_1-, αs_2-, β and κ-caseins is approximately 12, 12, 9 and 7%, respectively. Caseins contain low amount of sulphur containing amino acid residues in particular Cys residues. Only two Cys residues are present in αs_2-and κ-casein, whereas in αs_1- and β-casein there are no Cys residues.

Caseins are amphiphilic phosphoproteins. In caseins, as already mentioned, 1-13 residues may be phosphorylated. Both αs_1- and β-casein contain hydrophilic N-terminal and hydrophobic C-terminal regions. The primary structure of αs_2-casein shows alternate hydrophilic and hydrophobic segments. αs_2-Casein is more hydrophilic than the other caseins because it has more phosyphorylated sites (10-13). κ-Casein is the only glycosylated casein. The sites of glycosylation can include residues at positions 131, 133, 135, 136 and 142 (Swaisgood, 1992).

Secondary structure of caseins

Caseins were thought to be random coil proteins with little secondary structure based on optical rotary dispersion and circular dichorism studies (Ward, 1998 and references therein). Byler *et al.,* (1988) determined the secondary structures of lyophilised αs_1- and β-caseins using Raman spectroscopy and stated that αs_1- and β-casein had 67 and 49% secondary structures (as α-helix, β-sheet and β-turns), respectively. Raman spectroscopy was not performed on κ-casein. Swaisgood (1992) stated that the caseins cannot be considered as a denatured and random coiled protein and they fall between structured and globular proteins. Caseins have a limited secondary structure due to high content of Pro residues (Kumosinski *et al.,* 1993a, b; Kumosinski and Farrell, 1994).

Tertiary structure of caseins

Using theoretical calculations, Kumosinski *et al.,* (1993a, b) and Kumosinski and Farrell (1994), proposed energy minimized tertiary structures of bovine αs_1-, β- and κ-caseins. Based on theoretical calculations these authors indicated that αs_1-casein has a little α-helix along with some β-sheets and β-turns. It was indicated that the C-terminal of αs_2-casein may possess a globular conformation, while the N-terminal region is thought to contain a randomly structured hydrophobic tail. In β-casein it was suggested that it could have 10% of its residues in α-helix, 17% in β-sheet and 70% in

unordered structures. Whereas κ-casein appears to be the most highly structured of the caseins, it was estimated that 23% of the residues could be in α-helix and 31% in β-sheet structures (Kumosinski et al., 1993a, b; Kumosinski and Farrell, 1994, Figures 1.1, 1.2 and 1.3)

Table 1.4. Amino acid composition, molecular mass and average hydrophobicity of caseins.

Amino acid	αs_1-casein (B*)	αs_s-casein (A*)	β-casein (A2*)	κ-casein (B*)
Asp	7	4	4	3
Asn	8	14	5	8
Thr	5	15	9	14
Ser	8	6	11	12
SerP	8	11	5	1
Glu	25	24	19	12
Gln	14	16	20	14
Pro	17	10	35	20
Gly	9	2	5	2
Ala	9	8	5	15
Cys	0	2	0	2
Val	11	14	19	11
Met	5	4	6	2
Ile	11	11	10	13
Leu	17	13	22	8
Tyr	10	12	4	9
Phe	8	6	9	4
Trp	2	2	1	1
Lys	14	24	11	9
His	5	3	5	3
Arg	6	6	4	5
Total residues	199	207	209	169
Molecular weight	23,623	25,238	23,988	19,006
Hydrophobicity (kJ/residue)	4.89	4.64	5.58	5.12

*: Genetic variant (Taken from Ward, 1998).

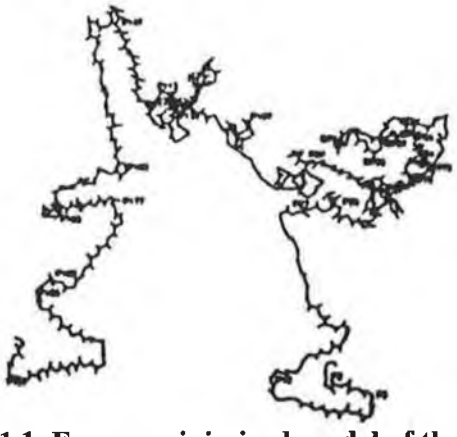


Figure 1.1. Energy minimized model of the tertiary structure of α_{s1}-casein (from Kumosinski and Farrell, 1994)

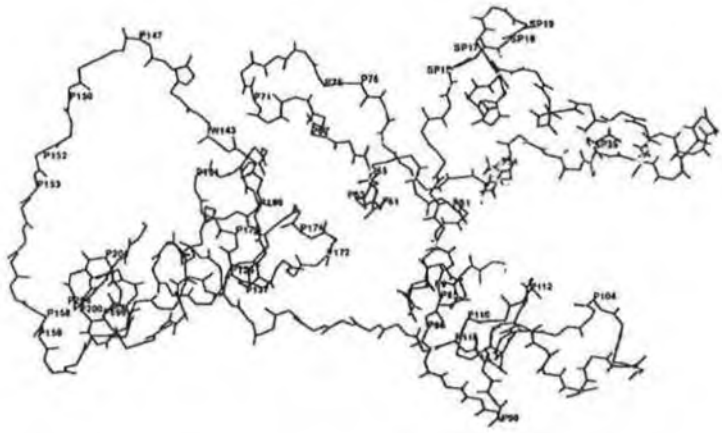


Figure 1.2. Energy minimized model of the tertiary structure of β-casein (from Kumosinski *et al.*, 1993a).

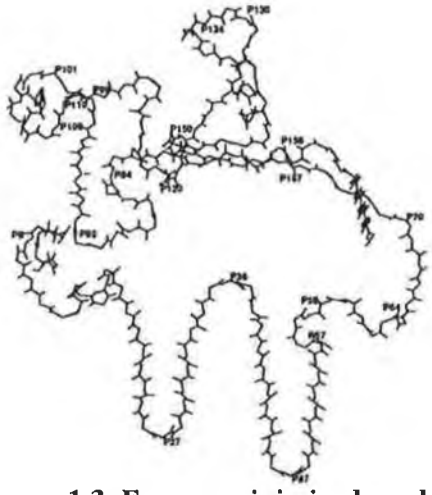


Figure: 1.3. Energy minimized model of the tertiary structure of κ-casein (from Kumosinski *et al.*, 1993b).

Fractionation of caseins from milk

Two common approaches for the fractionation of casein from milk are isoelectric precipitation of the caseins at pH 4.6 and 20°C (acid casein) or by treatment of skimmed milk with chymosin (rennet casein) giving limited proteolysis which yields a curd enriched in the caseins (Swaisgood, 1982). Commercially, caseins are supplied in many formats such as: sodium caseinate, calcium caseinate, acid caseinate and rennet casein. Sodium, calcium and potassium caseinate are prepared by re-dispersing the acid caseinate into aqueous solution by adjusting the pH of the solution to 6.8 either with sodium, calcium or potassium hydroxide, respectively, and then spray drying (Ward, 1998).

The coagulation of milk by rennet is generally divided into two phases. The primary (enzymatic) phase involves the hydrolysis of κ-casein while the secondary (non-enzymatic) phase involves formation of the curd (Brown and Ernstrom, 1988). During the primary phase, κ-casein, which is predominantly present on the outer surface of the casein micelle with its C-terminal hydrophilic tail protruding into the solvent, is cleaved by rennet at the Phe (105) - Met (106) bond releasing fragment (1-105) which is termed para κ-casein and fragment (106-169) termed caseinomacropeptide (CMP). CMP is very hydrophilic and remains in solution whereas para κ-casein along with α_{s1}-, α_{s2}- and β-casein aggregate during the secondary phase of milk coagulation (>20°C) to form a curd.

Fractionation of individual caseins

The common approach to fractionate individual caseins has been based on their differential solubility at 2 or 4°C. Warner (1944) developed a method for fractionation of α- and β-casein based on their differential solubility at 2°C, pH 4.4. Hipp *et al.*, (1952) employed alcohol and urea fractionation at 2°C at various pH values to fractionate different caseins. Later on chromatographic techniques were used for the purification of individual caseins (Thompson, 1966, Andrews *et al.*, 1985, Law and Leaver 2004) Murphy and Fox (1991) developed a method for the fractionation of different caseins from sodium caseinate with ultrafilteration using a 300 kDa cut-off membrane at 4°C.

Enzymatic hydrolysis of caseins

Caseins due to their flexible open structures are generally more susceptible to enzymatic hydrolysis in comparision with the globular whey proteins. This is an important nutritional consideration, especially for neo-natal digestion (Damodaran, 1997). Bio-active peptides can be released from milk proteins, mainly caseins, by various chemical or biological treatments (Rutherfurd and Gill, 2000; Vegarud *et al.*, 2000). Enzymatic hydrolysis of caseins is the most beneficial approach for the release of bio-active peptides (FitzGerald and Meisel, (2003), Korhonen and Pihlanto, 2006). The primary sequence of the caseins act as precursors for many important bio-active peptides which may effect the major bodily systems namely, the cardiovascular, digestive, endocrine, immune and nervous systems (Korhonen, (2009). High amounts (10-60 mg) of bioactive peptides could potentially be produced during the digestion of

the major casein components (Meisel and Olieman, 1998). However, the major drawback of casein hydrolysates is that the peptides generated following proteolytic digestion tend to be bitter due to the high content of hydrophobic peptides (Matoba and Hata, 1972, Cliffe and Law, 1990).

In general, biologically active peptides have short chain lengths. For example, potent angiotensin converting enzyme (ACE)-inhibitory peptides usually contain 2–12 amino acids, although other bioactive peptides with up to 27 amino acids have been identified (Yamamoto *et al.,* 1994; Meisel and Bockelmann, 1999; Pihlanto, 2001; Korhonen and Pihlanto, 2003; Robert *et al.,* 2004; Murray and FitzGerald, (2007); Saito 2008). Commercially, various functional food or ingredients containing casein derived bioactive peptides which claim to have health benefits such as anti-hypertensive, mineral absorption, athletic performance enhancement, etc, are available. Examples of commercially available functional foods or food ingredients containing casein-derived bioactive peptides are listed in Table 1.5.

Table 1.5. Examples of commercially available functional foods or food ingredients containing casein-derived bioactive peptides.

Product name	Manufacturer	Type of food	Heath claim
Calpico (Europe) or CalpisAMEALS(Japan)	Calpis Co., Japan	Fermented milk	Hypotensive
Capolac	Arla Foods, Denmark	Ingredient	Aids mineral absorption
Cardi-04	Chr. Hansen A/S, Denmark	Fermented milk	Hypotensive
Casein DP Peptio Drink	Kanebo, Japan	Soft drink	Hypotensive
CE90CPP	DMV, Netherlands	Ingredient	Aids mineral absorption
C12 Peptide	DMV, Netherlands	Ingredient	Hypotensive
Evolus	Valio, Finland	Fermented milk	calcium enriched Hypotensive
Kotsu Kotsu calcium	Asahi, Japan	Soft drink	Aids mineral absorption
PeptoPro	DSM Food Specialists, Netherlands	Ingredient	Improves athletic performance
ProDiet F200	Ingredia, France	Milk Drink	Reduces stress
Tekkotsu Inryou	Suntory, Japan	Confectionary Soft drink	Aids mineral absorption

(Taken from Phelan *et al.,* 2009).

1.4. Proteinases

Proteinases are enzymes which hydrolyze protein molecules into peptides and amino acids. Proteinases are valuable tools to introduce restricted cleavages into a polypeptide substrate which subsequently help in understanding the primary and higher order structure of proteins (Flannery *et al.*, 1989).

Classification of proteinases

Proteinases belong to Group 3 (hydrolases) sub-group 4 (hydrolyse peptide bonds) as classified by the Enzyme Commission (EC). Based on the source, proteinsases can be divided into three groups, i.e., plant, animal and microbial proteinases (Rao *et al*, 1998). Some examples of different groups of proteinases along with the source and specificity are listed in Table 1.6.

Table 1.6. Examples of some proteinases along with source and specificity.

Proteinase	Source	Preferred amino acid residue at P_1 position	Preferred amino acid residue at P_1' position	Preferred amino acid residue at P_2 position
Trypsin	Animal	Lys/Arg	Non-specific	Non-Specific
Chymotrypsin	Animal	Trp/Tyr/Phe/Leu	Non-Specific	Non-Specific
Pepsin	Animal	Phe/Tyr/Leu-	Non-Specific	Non-Specific
Staphylococuss V8	Microbial	Glu/Asp	Non-Specific	Non-Specific
Thermolysin	Microbial	Non-specific	Leu/Phe	Non-Specific
Subtilisin	Microbial	Trp/Tyr/Phe/Leu	Non-specific	Non-Specific
Papain	Plant	Non-Specific	Non-Specific	Phe/Val/Leu

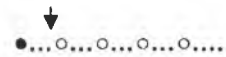
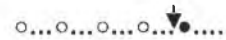
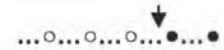
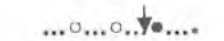
Adopted from Rao *et al.*, (1998).

Due to their broad biochemical diversity and their susceptibility to genetic manipulation microbial proteinsases are excellent source of proteinases. Based on the type of micro-organism, microbial proteinases are classified as bacterial, fungal and viral proteinases (Rao *et al*, 1998).

Based on their ability to cleave peptide bonds, proteinases are classified into two major groups, i.e., endopeptidases and exopeptidases. Exopeptidases are further classified as amino peptidases and carboxy peptidases based on their preferences to cleave on the N- and C-terminal side of peptide bonds, respectively. Endopeptidases

cleave internally located peptide bonds in proteins or peptides from N-and C-terminals. Amino peptidases, carboxyl peptidases and endopeptidases along with their mode of action and EC numbers are given in Table 1.7.

Table 1.7. Classification of proteinases based on their ability to cleave peptide bonds along with their mode of action and Enzyme Commission numbers

Proteinase	Mode of action	Enzyme commission (EC) number
Exopeptidases		3.4.11
Aminopeptidases		3.4.14
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.14
Carboxypeptidases		3.4.13-3.4.18
Serine type protease		3.4.16
Cysteine type protease		3.4.17
Metalloprotease		3.4.18
Peptidyl dipeptidase		3.4.15
Dipeptidases		3.4.13
Oligopeptidases		3.4.19
		3.4.19
Endopeptidases		3.4.21-3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metallo protease		3.4.24
Endopeptidase of unknown catalytic mechanism		3.4.99

(Taken from Rao *et al.*, 1998).

Proteinases are further classified into four mechanistic classes, i.e., serine, cysteine, aspartic and metallo-proteinases. Within these four classes there are six families of proteinases (Neurath, 1989). The six families of proteinases along with an example and corresponding active site residues are given in Table 1.8.

Table 1.8. Families of proteinases along with the active site residues.

Family	Example	Characteristic active site residues.
Serine protease 1 (Mammalian)	Chymotrypsin,	Asp^{102}, Ser^{195}, His^{57}
Serine protease 2 (Bacterial)	Subtilisin	Asp^{32}, Ser^{221}, His^{64}
Cystein proteinases	Papain	Cys^{25}, His^{159}, Asp^{158}
Aspartic proteinases	Penicillopepsin,	Asp^{33}, asp^{213}
Metallo-proteinases 1	Bovine carboxypeptidase A	Zn, Glu^{270}, Try^{248}
Mettalo-proteinases 2	Thermolysin	Zn, Glu^{143}, His^{231}.

(Adopted from Neurath, 1989).

1.5. Serine proteinases

Serine proteinases are the most abundant and functionally diverse group of proteinases (Page and Di Cera, (2008 a, b). They belong to a mechanistic class that contain a catalytic Ser residue. The presence of serine proteinases in viruses, bacteria and eukaryotes suggest that they are vital for all these organisms. The substrate specificity of serine proteinases can be broad (chymotrypsin/subtilisin) or narrow (trypsin) depending on their function. Serine proteinases are classified into 13 clans. Within these 13 clans there are 40 families (Di Cera, 2009). Clan PA is the largest among all serine proteinase clans consisting of 12 families. The mammalian digestive enzymes such as trypsin, chymotrypsin and elastase belong to Clan PA. Subtilisin, an important microbial serine proteinase belongs to Clan SB. The classification of serine proteinases is given in Table 1.9.

Serine proteinase mechanism of action

Serine proteinases commonly contain a catalytic triad consisting of Ser (nucleophile), Asp (electrophile) and His residues (base) (Rao *et al.,* 1998). Serine proteinases usually follow a two step reaction, i.e., 1) acylation and 2) deacylation steps in order to hydrolyze a peptide bond (Fastrez and Fersht, 1973 and Hedstrom, 2002). A generally accepted catalytic model for chymotrypsin-like serine proteinases is given in Figure 1.4.

Table 1.9. Classification of serine proteinases into different clans, corresponding number of families and a representative member of each clan along with the corresponding catalytic residues.

Clan	Number of Families	Representative Member	Catalytic residues
PA	12	Trypsin	His, Asp, Ser
SB	2	Subtilisin	Asp, His, Ser
SC	2	Prolyl oligopeptidase	Ser, Asp, His
SE	6	D-A, D-A carboxypeptidase	Ser, Lys
SF	3	LexA peptidase	Ser, Lys/His
SH	2	Cytomegalovirus assemblin	His, Ser, His
SJ	1	Lon peptidase	Ser, Lys
SK	2	Clp peptidase	Ser, His, Asp
SP	3	Nucleoporin	His, Ser
SQ	1	Aminopeptidase DmpA	Ser
SR	1	Lactoferrin	Lys, Ser
SS	1	L,D-carboxypeptidase	Ser, Glu, His
ST	5	Rhomboid	His, Ser

(Taken from Di Cera, 2009)

1) Acylation step

During the acylation step Ser^{195} attacks the carbonyl of the peptide substrate, assisted by the OH^- of His^{57} of the catalytic triad to yield a tetrahedral intermediate. The His^{57}-H^+ is stabilized by the Asp^{102} residue of the catalytic triad. The oxyanion hole of the tetrahedral intermediate is stabilized by the interactions between main chain amides of the oxyanion hole. The $His^{57}H^+$ acts as a general acid and assist the expulsion of the leaving group leading to the collapse of tetrahedral intermediate which subsequently leads to the formation of an acylenzyme (Figure 1.4)

2) Deacylation step

During the deacylation step, water assisted by His^{57}, attacks the acylenzyme to yield a second tetrahedral intermediate. The tetrahedral intermediate collapses expelling Ser^{195} and a carboxylic acid product (Figure 1.4).

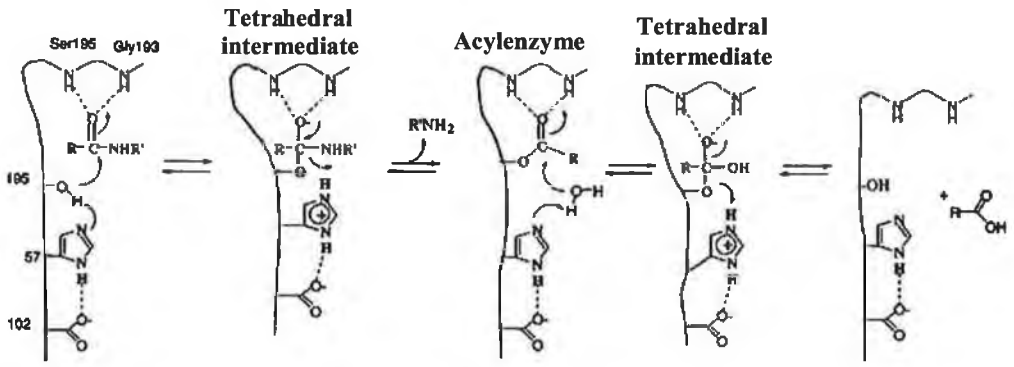


Figure 1.4. The general accepted mechanism of chymotrypsin-like serine proteinases. Taken from Hedstrom (2002).

Serine proteinase inhibitors

Most of the serine proteinase inhibitors are active site specific inhibitors. Mostly these inhibitors irreversibly modify an amino acid residue at the active site of the proteinase (Neurath, 1989). Generally used serine proteinase inhibitors are given in Table 1.10.

DFP, PMSF and chloromethyl ketone derivatives are widely used as serine proteinase inhibitors. They irreversibly modify an amino acid residue at the active site. Whereas, DFP and PMSF react with the active site Ser residue the chloromethyl ketone derivatives react with the His residue of the catalytic traid and irreversibly inhibit proteinase activity (Neurath,1989)

Table 1.10. Commonly used serine proteinase inhibitors.

Inhibitors	Comments
Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)	Limited solubility, limited specificity (inhibits chymotrypsin-like proteinases)
Tosyl-lysine-chloromethyl ketone (TLCK)	Offensive smell, limited specificity (inhibits trypsin-like proteinases)
Phenylmethanesulphonyl flurode (PMSF)	Limited solubility, unusable in aqueous medium, more inhibitor should be added for lengthy preparations, inhibition dependent of enzyme specificity.
Leupeptine , antipain	Reversible inhibitors effective at micromolar level, limited specificity (inhibits trypsin-like proteinases)
Chymostatin	Reversible inhibitor, limited solubility, effective at micromolar concentrations, limited specificity (inhibits chymotrypsin-like proteinases)
Diisopropyl fluorophosphates (DFP)	Highly toxic not recommended for routine use, inhibition dependent of enzyme specificity, also inhibits serine esterases.
Benzamidine, p-amino benzamidine	Reversable inhibitor of trypsin like enzymes.
Elastatinal	Reversible inhibitor, limited specificity, not widely tested.
Soybean trypsin inhibitor (SBTI), Aprotinin (Trasylol)	Most widely used reversible polypeptide inhibitors

(Taken from North 1989)

1.6. Glutamyl endopeptidase (EC 3.4.21.19)

In the literature GE is often referred to as a glutamic acid specific endopeptidase (GSE) and GE from *B. licheniformis* is often referred to as BL-GSE (Breddam and Svensdsen, 1992; Kakudo *et al.,* 1992; Breddam and Meldal 1992; Barbosa *et al.,* 1996; Park and Allen 1998; Stennicke and Breddam, 1998 and Mil'gotina *et al.,* 2003).GE belongs to the class of serine proteinases and sub-family of chymotrypsin proteinases. It exhibits a strong preference for acidic amino acid residues at the P_1 position. A Glu residue is preferred compared to an Asp residue at the P_1 position. GE from bacteria is divided into *Staphylococcal*, *Bacillus* and *Streptomyces* groups based on organism origin and sequence relationship. The *Staphylococcus* and *Bacillus* groups are closer to each other in comparison to the *Streptomyces* group. Therefore, GE from *Staphylococcus* and *Bacillus* groups are termed as GE I (Stennicke and

Breddam, 1998). As stated by Birkfort and Breddam (1994), GE I belongs to clan SA, family S2 and has an EC number 3.4.21.19.

General characteristics of GE

Depending on the bacterial source, i.e., *S. aureus* V8, *S. warneri* M, *B. licheniformis*, *B. intermedius*, *B. licheniformis* ATCC 14580, *Str. thermovulgaris*, *Str. Fradiea*, *Str. griseus* and *E. faecalis* the molecular weight of GE range from 11.4-31.0 kDa, the isoeletric point range from pH 4.5-9.0, pH optimum range from 4-11 and the pH stability range from pH 3.5 to 11.0 (Table 1.11).

Table 1.11. General characteristics of glutamyl endopeptidases.

Glutamyl endopeptidase Source	Molecular Mass (kDa)	Isoelectric point (pI)	pH optimum Z-Glu-pNA	pH stability
B. intermedius	29.0	8.4	8.0	6.5-11.0
Str. thermovulgaris	26.0	6.7	6.5	6.0-10.0
S. aureus V8	11.4-12.0	4.0	4.0* and 7.8*	3.5-9.5
B. licheniformis	23.589	4.8	7.5-8.0	4.0-10.0
B. licheniformis ATCC 14580	25.0	>9.0	8.0	4.0-10.0
Str. griseus	20.0-22.0	8.4	8.8	5.0-8.0
Str. Fradiea	18.702	-	8.2	4.5-9.0
E. faecalis	25.0	-	7.0-7.5[†]	-
S. warneri M	31.0	-	-	-

Adopted from Leshchinskaya *et al.*, (1997) and updated based on the information reported by Yokoi *et al.*, (2001), Kakudo *et al.*, (1992) and Kawalec *et al.*, (2005).
*: pH optimum reported using haemoglobin and casein as substrates. [†]: pH optimum reported using Suc-Ala-Ala-Pro-Glu-pNA.

Methods employed for GE purification from commercially available proteolytic preparations

The purification of GE from two commercially available preparations, Pronase and Alcalase has been reported in the literature (Table 1.12).

Table 1.12. The sequence of chromatographic steps employed during the purification of glutamyl endopeptidase from commercially available proteolytic preparation along with bacterial source, yield and the literature reference.

Proteolytic preparation	Bacterial source	Sequence of chromatographic steps employed (Buffers used).	Yield (%)	Reference
Pronase™	*Str. griseus*	• CM-Toyoperal (IEX) (Buffer A: 10 mM Acetate buffer, 2mM CaCl2, pH 4.6, Buffer B: 0.5M NaCl in buffer A) • Sephadex G-50 (GPC) (Buffer: 50 mM phosphate buffer, pH 6.0). • Hydroxyapatite (IEX) (Buffer A: 10 mM Phosphate buffer, pH 6.5. Buffer B: 50 mM Phosphate buffer, pH 6.5). • Z-Gly-D-Phe-AH-Sepharose 4B (AC) Buffer: 10mM phosphate buffer, pH 6.5. • CM-Toyoperal (IEX) (Buffer-similar to above)	26.0	Yoshida *et al.*, (1988)
Alcalase™	*B. licheniformis*	• CM-Fractogel (IEX) (Buffer A: 10 mM phosphate buffer, pH 6.2. Buffer B: 350 mM NaCl in buffer A). • Bacitracin-Sepharose (AC) (Buffer A: 20 mM Bicine and 2mM CaCl2, pH 8.2). Buffer B: 1 M NaCl in buffer A) • CM-Fractogel (Buffer-similar to above) • Bacitracin-Sepharose (Buffer-similar to above)	50.0	Breddam and Svensdsen (1992)
Alcalase™	*B. licheniformis*	• Phenyl Sepharose HP (HIC) (BufferA: 50 mM phosphate buffer, 500 mM ammonium sulphate and 500 mM potassium chloride, pH 7.0. Buffer B: 50 mM phosphate buffer, pH 7.0). • Phenyl Sepharose HP. (Buffer-similar to above) • Phenyl Sepharose HP. (Buffer-similar to above)	-	Spellman et al., (2005)

AC: affinity chromatography, GPC: gel permeation chromatography, HIC: hydrophobic interaction chromatography and IEX: Ion exchange chromatography.

From Table 1.12, it can be observed that different chromatographic techniques such as affinity, gel permeation, hydrophobic interaction and ion exchange chromatography have been used to purify GE from Pronase and Alcalase. Since the objective of the work of Spellman *et al.*, (2005) was to obtain a fraction enriched in GE activity the yield of the activity was not reported by these authors. However, the hydrophobic interaction chromatography employed by Spellman *et al.*, (2005) was an effective approach to obtain fraction enriched in GE activity

Substrate specificity of GE

Schecter and Berger (1967) introduced a system of nomenclature to describe interaction of proteinases with their substrates. In this system, the amino acid residues present on the amino side of the scissile bond (bond that is cleaved on the substrate) are numbered as P_1, P_2, P_3, etc and the residues on the carboxy terminal of the scissile bond are numbered as P_1', P_2', P_1' etc. Whereas, the amino acids on the proteinase are termed as S_1, S_2, S_3, S_1', S_2', S_3', etc, to compliment the substrate residues that interact with the proteinase (Figure 1.5).

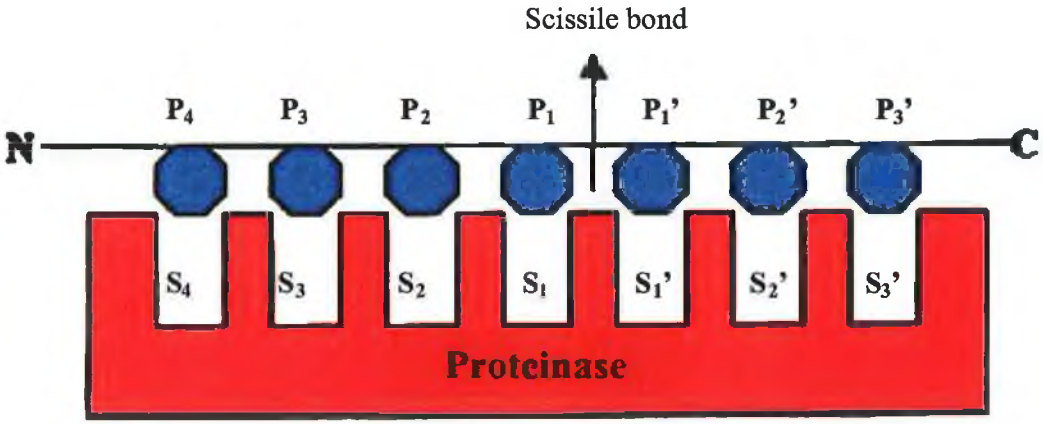


Figure 1.5. The Schecter and Berger nomenclature for binding of a peptide substrate to a proteinase. (Adopted from Rzychon *et al.*, 2004).

Breddam and Meldal, (1992) and Kakudo *et al.*, (1992) investigated the substrate specificity of GE from *B. licheniformis* using synthetic peptides. Whereas, Breddam and Svendsen (1992) investigated substrate specificity using oxidised ribonuclease and porcine glucagons (Glu deplete) as substrates.

Using a variety of synthetic peptide substrates based on intramolecular fluorescence quenching Breddam and Meldal (1992) have investigated the influence of the nature of the amino acid residue present at the P_2, P_3, P_4, P_1' and P_2' positions during incubation with GE at 25°C. Their findings were as follows: GE hydrolysed Glu-Xaa bonds approximately 1000 times more effectively than the hydrolysis of Asp-Xaa (Xaa = any amino acid residue); GE appeared to hydrolyse all types of Glu-Xaa bonds other bonds while the Glu-Asp bond was hydrolysed at a very low rate and Pro residue at P_1' position was not preferred or poorly preferred by GE. Furthermore, these authors indicated that GE also hydrolyses Phe-Xaa and Ala-Xaa bonds at a very low rate. A list of preferred and poorly preferred amino acid residues present at the substrate positions P_4, P_3, P_2, P_1' and P_2' positions for GE are given in Table 1.13.

From Table 1.13, it can be observed that GE prefers negatively charged amino acid residues (Glu/Asp) at the P_1 position, whereas, Phe and Ala residues at P_1 were poorly preferred. It was also observed that GE was non-specific with respect to amino acid residues present at positions P_4, P_3 and P_2. At positions P_1' and P_2', Val and Phe residues were preferred by GE, respectively. Whereas, Asp or Pro and Pro residues were not preferred at positions P_1' and P_2', respectively.

Table 1.13. The amino acid residues that are preferred and poorly preferred by glutamyl endopeptidase at positions P_4, P_3, P_2, P_1, P_1' and P_2'.

Substrate position	Preferred amino acid residue	Poorly preferred amino acid residue
P_4	Asp	None
P_3	Ala	None
P_2	Ala	None
P_1	Glu/Asp	Phe/Ala
P_1'	Val	Asp/Pro
P_2'	Phe	Pro

(Adapted from Breddam and Meldal, 1992).

Kakudo *et al.,* (1992) investigated the substrate specificity of GE purified from *B. licheniformis* ATCC 14580 using human parathyroid hormone (hPTH, f(13-24)) and

synthetic paranitroanalide (pNA) as substrates at 37°C. It was observed that approximately 80% of the glutamyl bonds present in hPTH(13-24) were hydrolysed by GE within a reaction time of 6 min. On the other hand, a 24 h reaction time was required to hydrolyse approximately 75% of the aspartyl bonds in hPTH(13-24). Using synthetic pNA substrates, it was observed that the reactivity of GE towards Asp-pNA was less than 1% that observed for hydrolysis of Glu-pNA. Furthermore, Kakudo *et al.,* (1992) reported that GE did not hydrolyse peptide bonds when Ala, Leu, Phe, Tyr and Lys residues were present at the P_1 position.

As already mentioned, Breddam and Svendsen (1992) investigated the substrate specificity of GE using oxidised ribonuclease and porcine glucagon during incubation with these substrates at 25°C. Oxidised ribonuclease was incubated at two different concentrations of GE, i.e., 0.05 and 1.2 μM. It was found that GE at higher concentration (1.2 μM) hydrolysed at the carboxy terminal of Phe(46) on incubation with oxidised ribonuclease at 25°C. As porcine glucagon has no Glu residues, Breddam and Svendsen (1992) incubated porcine glucagon with GE at a very high concentration (6.4 μM) to further investigate the substrate specificity of GE. Approximately 90% of the observed cleavages corresponded to the hydrolysis of aspartyl bonds. From both these hydrolysis experiments, Breddam and Svendsen (1992) concluded that GE predominantly hydrolysed on the carboxy terminal of Glu residues and to a lesser extent hydrolysed the peptide bonds on the carboxy terminal of Asp residues. Furthermore, it was also concluded that GE exhibits non-specific behaviour at high concentration and during extended incubation times.

To date no studies appear to have been carried out to thoroughly investigate the substrate specificity of GE using purified individual milk proteins and specific synthetic peptides corresponding to regions in bovine β-casein.

1.7. Objectives of thesis

- To generate electrophoretically and chromatographically pure β-casein.

- To purify glutamyl endopeptidase (GE) from Alcalase™ 2.4L and characterize the enzyme activity.

- To investigate the substrate specificity of the purified GE by performing hydrolysis experiments on β-casein and a side fraction (enriched in α-casein) obtained during β-casein purification.

- To hydrolyse synthetic peptides corresponding to specific sequences of β-casein in order to further investigate the specificity of GE.

References

Andrews, A. T. (1992). Indigenous enzymes in milk: General introduction. In: P.F. Fox (Ed.), *Advanced Dairy Chemistry-1: Proteins* (pp. 286-292). Barking: Elsevier Science Publishers Ltd.

Andrews, A. T. Taylor, M. D. and Owen,A. J. (1985). Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *J. Chromat*, **348**, 177-18.

Barbosa, J. A. R. G., Saldanha, J.W. and Garratt, R. C. (1996). Novel features of serine protease active sites and specificity pockets: sequence analysis and modelling studies of glutamate-specific endopeptidases and epidermolytic toxins. *Protein Eng*, **9**, 591–601.

Birktoft, J. J. and Breddam, K. (1994) Glutamyl endopeptidases. *Method Enzymol*, **244**, 114-126.

Breddam, K. and Meldal, M. (1992). Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching. *Eur J Biochem,* **206**, 103-107.

Breddam, K. and Svendsen, I. (1992). Isolation and amino acid sequence of a glutamic acid specific endopeptidase from *Bacillus licheniformis. Eur J Biochem,* **204,** 165-171.

Brown, R. J. and Ernstrom, C. A (1988). Milk clotting enzymes and cheese chemistry.Part I - milk clotting enzymes. In: N. P. Wong, R. Jenness, M. Keeney and E.H. Marth. (Ed.), *Fundamentals of dairy chemistry,* 3rd ed. (pp 609-633. Van Nostrand Reinhold, New York, USA.

Byler, D. M., Farrell Jr., H. M. and Susi, H. (1988). Raman spectroscopic study of casein structure. *J Dairy Res,* **71 (10)**, 2622-2629.

Cayot, P. and Lorient, D. (1997). Structure-function relationships of whey proteins. In S.Damodaran and A. Paraf (Eds.), Food proteons and their applications, (pp 225-256). New York: Marcel Dekker.

Cliffe, A. J and Law, B. A. (1990). Peptide composition of enzyme-treated cheddar cheese slurries, determined by reverse phase high performance liquid chromatography. *Food Chem*, **36 (1)**, 73-80.

Creusot, N., Gruppen, H., van Koningsveld, G.A., de Kruif, C.G. and Voragen, A.G.J. (2006). Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *Int Dairy J,* **16**, 840-849.

Creusot, N. and Gruppen, H. (2007). Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease: Fractionation and identification of aggregating peptides. *J. Agric. Food Chem*, **55,** 9241-9250.

Damodaran, S. (1997). Food Proteins: An overview. In: S.Damodaran and A. Paraf (Eds). *Food proteons and their applications* (pp 1-24). New York: Marcel Dekker.

Di Cera, E. J. (2009). Serine Protease. *Life,* **284**, 36175-36185.

Doucet, D., Gauthier, S. F., Otter, D. E. and Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *J Agric Food Chem,* **51**, 6300-6308.

Drapeau, G. R., Boily, Y. and Houmard, J. Purification and properties of an extracellular protease of Staphylococcus *aureus. J Biol Chem,* **247(20),** 6720-6726.

Fastrez, J., and Fersht, A. R. (1973). Demonstration of the acyl-enzymemechanism for the hydrolysis of peptides and anilides by chymotrypsin. *Biochem,* **12,** 2025–2034.

Farrell, H. M., Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F. and Swaisgood, H.

E. (2004). Nomenclature of the proteins ofcows' milk-sixth revision. *J Dairy Sci*, **87 (6),** 1641-1674.

FitzGerald, R.J. (1997). Exploitation of Casein Variants. In: R.A.S. Welch et al., (Ed.), *Milk Composition, Production and Biotechnology*, Chapter 10 (pp 153-171). New York: CAB International.

FitzGerald, R. J., and Meisel, H. (2003). Milk protein hydrolysates and bioactive peptides. In P. F. Fox, and P. L. H. McSweeney (Eds), *Advanced Dairy Chemistry: Proteins: Volume.1*, Part. A (pp 675–698). New York, NY, USA: Kluwer Academic/Plenum Press.

Flannery, A. V., Beynon, R, J. and Bond, J, S. (1989). Proteolysis of proteins for sequence analysis and peptide mapping. In: Proteolytic enzymes-a practical approach. Ed by Beynon, R. J. and Bond, J. S. (pp145-162). Oxford university press, Oxford, UK.

Fox, P. F. and Brodkorb, A.(2008).The casein micelle: Historical aspects, current concepts and significance. *Int Dairy J,* **18(7),** 677-684.

Fox, P.F. and McSweeney, P.L.H. (1998). Heat induced changes in milk. In: *Dairy Chemistry and Biochemistry*. (pp 347-378). London: Blackie Academic.

Hedstrom, L. (2002). Serine protease mechanism and specificity. *Chem Rev,* **102**, 4501–4524.

Hipp, N. J., Groves, M. L., Custer, J. H., & McMeekin, T. L. (1952). Separation of α, β and γ- casein. *J Dairy Sci,* **35 (3)**, 272-281.

Houmard, J. and Drapeau, G. (1972). Staphylococcal protease: A proteolytic enzyme specific for glutamoyl bonds. *Proc Nat Acad Sc USA,* **69,** 3506-3509.

Kakudo, S., Kikuchi, N., Kitadokoro, K., Fujiwara, T., Nakamura, E., Okamoto, H., Shin, M., Tamaki, M., Teraoka, H., Tsuzuki, H. and Yoshida, N. (1992). Purification,

characterization, cloning, and expression of a glutamic acid-specific protease from Bacillus licheniformis ATCC 14580. *J Biol Chem,* **267(33),** 23782-23788.

Kawalec, M., Potempa, J., Moon, J. L., Travis, J. and Murray, B. E. (2005). Molecular diversity of a putative virulence factor: purification and characterization of isoforms of an extracellular serine glutamyl endopeptidase of Enterococcus *faecalis* with different enzymatic activities. *J Bacteriol,* **187(1),** 266–275

Korhonen, H. (2009). Milk-derived bioactive peptides: from science to applications. *J Functional Foods*, **1**, 177-187.

Korhonen, H. and Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Curr. Pharm. Des*, **9**, 1297-1308.

Korhonen, H. and Pihlanto, A. (2006). Bioactive peptides: production and functionality. *Int Dairy J*, **16**, 945–960.

Kumosinski, T.F., Brown, E.M. and Farrell, H.M. Jr (1993a). Three dimensional molecular modelling of bovine caseins: An energy-minimised β-casein structure. *J Dairy Sci*, **76**, 931-945.

Kumosinski, T.F., Brown, E.M. and Farrell, H.M. Jr (1993b). Three dimensional molecular modelling of bovine caseins: a refined energy-minimised κ-casein structure. *J Dairy Sci*, **76**, 2507-2520.

Kumosinski, T.F. and Farrell, H.M.Jr (1994). Solubility of proteins: salt-water interactions. In N.S.Hittiarachchy and Ziegler, G.R. (eds.), *Protein Functionality in Food Systems (*pp. 33-77). New York: Marcel Dekker.

Law, A. J. R & Leaver, J. (2004). Method for extracting casein fractions from milk and caseinates and production of novel products. *United States Patent Application,* Publication No. US2004/0234666A1.

Leshchinskaya, I. B., Shakirov, E. V., Itskovitch, E. L., Balaban, N. P., Mardanova, A. M., Sharipova, M. R., Viryasov, M. B., Rudenskaya, G. N. and Stepanov, V. M.(1997) Glutamyl endopeptidase of Bacillus intermedius, strain 3-19. *FEBS Lett*, **404**, 241-244.

Lindmark-Månsson, H., Timgren, A., Aldén, G. and Paulsson. M. (2005). Two-dimensional gel electrophoresis of proteins and peptides in bovine milk, *Int Dairy J,* **15,** 111–121

Matoba, T. and Hata, T. (1972). Relationship between bitterness of peptides and their chemical structures. *Agr Biol Chemi*, **36**, 1423–1431.

Madsen, J. S. and Qvist, K. B. (1997). Hydrolysis of milk protein by a *Bacillus licheniformis* protease specific for acidic aminoacid residues. *J Food Sci,* **62,** 579–582.

Maurer, K.H. (2004). Detergent proteases. *Curr. Opin. Biotechno*, **15**, 330-334.

Meisel, H., & Bockelmann, W. (1999). Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Ant. van Leeuwen.*, **76**, 207-215.

Meisel, H., and Olieman, C. (1998). Estimation of calcium-binding constants of casein phosphopeptides by capillary zone electrophoresis. *Anal. Chim. Acta,* **372,** 291–297.

Mil'gotina, E. I., Voyushina, T. L. and Chestukhina, G. G. (2003). Glutamyl endopeptidases: structure, function and practical application. *Bioorg Khim*,**29(6),** 563-76.

Murphy, J. M. & Fox, P. F. (1991). Fractionation of sodium caseinate by ultrafiltration. *Food Chem*, **39 (1)**, 27-38.

Murray, B.A. and FitzGerald, R.J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Curr Pharm Des,* **13**, 773-791.

Nagase, H. and Salvesen, G. (1989). Inhibition of proteolytic enzymes. In: R. J. Beynon and R.J Bond (Ed.), *Proteolytic enzymes-a practical approach* (pp 83-104). Oxford University press, Oxford, England.

Neurath, H. (1989). The diversity of proteolytic enzyme. In: R. J. Beynon and R.J Bond (Ed.), *Proteolytic enzymes-a practical approach* (pp 83-104). Oxford University press, Oxford, England.

Ng-Kwai-Hang, K.F. and Grosclaude, F. (1982). Genetic polymorphism of milk proteins. In P.F. Fox (Ed.), *Advanced Dairy Chemistry*, Vol. 1 (pp. 405-455). London: Elsevier Science.

North, M. J. (1989). Prevention of unwanted proteolysis. In: R. J. Beynon and R.J Bond (Ed.), *Proteolytic enzymes-a practical approach* (pp 105-124). Oxford University press, Oxford, England.

Ohara-Nemoto Y., Ikeda, Y., Kobayashi, M., Sasaki, M., Tajika, S. and Kimura, S. (2002). Characterization and molecular cloning of a glutamyl endopeptidase from Staphylococcus *epidermidis. Microb. Pathog*, **33,** 33-41.

Otte, J., Lomholt, S. B., Ipsen, R., Stapelfeldt, H., Bukrinski, J. T. and Qvist, K. B. (1997). Aggregate formation during hydrolysis of β-lactoglobulin with a Glu and Asp specific protease for *Bacillus licheniformis. J Agr Food Chem*, **45**, 4889–4896.

Page, M. J. and Di Cera, E. (2008a). Serine peptidases: classification, structure and function. *Cellular and Molecular Life Sci*, **65**, 1220-1236.

Page, M.J. and Di Cera, E. (2008b). Evolution of peptidase diversity. *J Biol Chem*, **283**, 30010-30014.

Phelan, M., Aherne, A., FitzGerald, R.J. and O'Brien, N.M. (2009). Review: Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *International Dairy Journal*, **19**, 643-654.

Park, O. and Allen, J. C. (1998). Preparation of phosphopeptides derived from α_s-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. *J. Dairy Sci*, **81(11),** 2858-2865.

Pihlanto, A. (2001). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends Food Sci Technol*, **11**, 347-356.

Rao, M.B., Tanksale, A. M., Ghatge. And M. S, Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev* , **62**, 597–635.

Robert, M.C., Razaname, A., Mutter, M. and Juillerat, M.A. (2004). Identification of angiotensin-I-converting enzyme inhibitory peptides derived from sodium caseinate hydrolysates produced by *Lactobacillus helveticus* NCC 2765. *J Agric Food Chem,* **52**, 6923–6931.

Rudenskaya, G. N. (1998) Glutamylendopeptidases from microorganisms, a new subfamily of chymotrypsin proteases. *Russ J Bioorg Chem,* **24,** 256–261.

Rutherfurd, K. J., & Gill, H. S. (2000). Peptides affecting coagulation. *Br J Nutr*, **84**, 99-102.

Rzychon, M., Chmiel, D. and Stec-Niemczyk, J. (2004). Modes of inhibition of cysteine proteases. *Acta Biochim Pol,* **51**, 861–873.

Saito, T. (2008). Antihypertensive peptides derived from bovine casein and whey proteins. *Adv Exp Med Biol*, **606**, 295-317.

Schechter, I. and Berger, A. (1967). On the size of the active site in proteases. I. Papain, *Bichem Biophys Res Commun*, **27**, 157-167.

Spellman, D., Kenny, P., O'Cuinn, G. & FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with Bacillus licheniformis proteinase activities. *J Agric Food Chem,* **5(4)**, 1258-1265.

Spellman, D., O'Cuinn, G. & FitzGerald, R. J. (2009). Bitterness in Bacillus proteinase hydrolysates of whey proteins. *J Agric Food Chem,* **114 (2)**, 440-446.

Steijns, J. M., & van Hooijdonk, A. C. M. (2000). Ocurrence, structure, biochemical properties technological characteristics of lactoferrin. *Br J Nutr*, **84**, S11-S17.

Stennicke, H.R. and Breddam, K. (1998). Glutamyl endopeptidase I. In: A.J. Barrett, N.D. Rawlings and J.F. Woessner (Ed.). *Handbook of Proteolytic Enzymes* (pp. 243-246). Academic Press, San Diego, CA, USA.

Sumantha, A., Larroche, C. and Pandey, A. (2006) Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technol Biotech,* **44 (2)**, 211–220.

Swaisgood, H.E. (1982). Chemistry of milk proteins. In: P. F. Fox (Ed.), *Developments in Dairy Chemistry*, Vol.1 (pp. 1-59). London: Applied Science.

Swaisgood, H. E. (1992). Chemistry of the caseins. In: P. F. Fox (Ed.), *Advanced Dairy Chemistry-1: Proteins.* (pp. 63–110). Barking: Elsevier Science Publishers Ltd..

Swaisgood, H.E. (1996). Characteristics of Milk. In: O.R. Fennema (Ed.), *Food Chemistry*, 3rd ed. (pp. 841-878). New York: Marcel Dekker Inc.

Thompson, M. P. (1966). DEAE-cellulose-urea chromatography of casein in the presence of 2-mercaptoethanol. *J Dairy Sci*, **49 (7)**, 792-795.

Vegarud, G. E., Langsrud, T., & Svenning, C. (2000). Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *Br J Nutr,* **84**, 91-98.

Walstra, P., T. J. Geurts, A. Noomen, A. Jellema, and M. A. J. S. von Boekel. 1999. Composition, structure, and properties, (pp 3-26). In: *Dairy technology: principles of milk properties and processes.* Marcel Dekker, New York, N.Y

Ward, L. S. (1998). Isolation of Caseins fractions by manipulating inherent solubility and association properties of caseins. Ph.D. dissertation, University of Minnesota, St. Paul, USA.

Warner, R. C. (1944). Separation of α and β-casein. *J Am Chem Soc,* **66,** 1725-1731.

Yamangata, A., Yoshida, N., Noda, K. and Ito, A. (1995). Purification and characterization of a new serine proteinase from Bacillus *subtili* with specificity for amino acids at P_1 and P_2 positions. *Biochim Biophys Acta,* **1253,** 224-228.

Yamamoto, N., Akino, A. and Takano, T. (1994). Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Biosci Biotechnol Biochem*, **58**, 776-778.

Yokoi, K., Kakikawa, M., Kimoto, H., Watanabe, K., Yasukawa, H., Yamakawa, A., Taketo, A. and Kodaira, K. (2001). Genetic and biochemical characterization of glutamyl endopeptidase of Staphylococcus *warneri* M. *Gene,* **281,** 115–122.

Yoshida, N., Tsuruyama, S., Nagata, K., Hirayama, K., Noda, K. and Makisumi, S. (1988). Purification and characterization of an acidic amino acid specific endopeptidase of Streptomyces *griseus* obtained from a commercial preparation (Pronase). *J Biochem,* **104,** 451-456.

Chapter 2

Purification of bovine β-casein.

2.1 ABSTRACT

A protocol was adopted for obtaining electrophoretically and chromatographically pure β-casein from acid caseinate. Calcium caseinate was prepared from acid caseinate (3% (w/v)) by adjusting to pH 6.8 using 1M calcium hydroxide. Calcium chloride was added to the solution to make the final concentration to 10 mM. The solution was subsequently rennet coagulated at 31°C for 30 min prior to heat inactivation of rennet at 70°C for 30 min. The whey was discarded and the curd was re-suspended in distilled water, cooled and held at 4°C for 24 h. The pH was then adjusted to 4.6 to dissociate β-casein from the caseinate gel and the sample was centrifuged at 5000 x g at 4°C for 15 min. The supernatant obtained was warmed (30°C), precipitating a fraction enriched in β-casein. The enriched β-casein sample was then further purified using ion exchange chromatography. Chromatographic (RP-HPLC and GPC) and urea-PAGE analysis showed that the β-casein sample was homogeneous. The yield of β-casein was approximately 5%. Using this method a fraction enriched in α-casein (both α_{s1}- and α_{s2}-casein) was also obtained with a yield of approximately 31%.

2.2 Introduction

β-Casein is the major protein in bovine milk which accounts for approximately 33% of total caseins (Farrell, *et al.*, 2004). It is composed of 209 amino acid residues with a molecular weight varying from 23,946 to 24,097 Da depending on the genetic variant (Liveny *et al.*, 2004). It is highly amphiphilic containing hydrophilic N-terminal and hydrophobic C-terminal regions. The N-terminal region of the molecule contains 5 phosphorylated serine residues at positions 15, 17, 18, 19 and 35 and the C-terminal is mainly composed of apolar amino acid residues with no phosphorylated sites. The complete amino-acid composition of β-casein is given in Table 2.1.

Table 2.1. The complete amino acid composition of β-casein-A2.

Amino Acid	Number present in β-casein	Amino Acid	Number present in β-casein
Asp	04	Cys	00
Asn	05	Val	19
Thr	09	Met	06
Ser	11	Ile	10
SerP	05	Leu	22
Glu	19	Tyr	04
Gln	20	Phe	09
Pro	35	Trp	01
Gly	05	Lys	11
His	05	Arg	04
Ala	05		

(Taken from: Swaisgood (1982))

Due to its amphipathic nature, β-casein self aggregates into a micellar structure composed of 15-60 molecules (Schmidt and Payens, 1972). Removal of the 20 residue C-terminal hydrophobic portion of the molecule eliminates the self-association properties of β-casein (Creamer and Berry, 1975). The size of the self associated polymers also varies depending on the temperature and the protein concentration (Buchheim and Schmidt, 1979; Evans *et al*, 1979). Furthermore, if calcium is added, β-casein polymerizes and precipitates out of solution. The precipitation of β-casein in the presence of calcium is attributed to the presence of the

five phosphorylated sites at its N-terminus. The amount of bound calcium decreases with decreasing pH (< pH 6) or as the ionic strength increases (Dickson and Perkins, 1971). The temperature dependence of β-casein was reported as early as 1944 by Warner. β-Casein exists as a monomer at 4°C and it was observed that even in the presence of calcium up to 400 mM, it does not precipitate (Thompson *et al.*, 1969). The concentration of calcium required to induce precipitation in β-casein at 20, 30 and 40°C (pH 7.0) was reported to be 20, 8.6 and 4.25 mM, respectively (Parker and Dalgleish, 1981). As the caseins are refractive to crystallization, it is not possible to determine their secondary structure. However the secondary structure prediction as determined by Fourier transform infrared or circular dichroism spectroscopies at 25°C indicate that β-casein may contain 29 or 20% α-helix, 34 or 32% β-structure, 32 or 28% turns and 4 or 22% irregular structure (Farrell, *et al.*, 2001).

In the literature, different approaches have been reported for the purification of β-casein. Warner (1944) was probably the first to report a method for isolation of β-casein from skim milk. This technique involved precipitation of skim milk at various pH values at 2°C. In 1952, Hipp *et al.*, reported on two methods for the purification of β-casein. The first method involved fractionation of acid casein in 50% alcohol in the presence of ammonium sulphate at various pH values. The crude β-casein fraction obtained was cooled to 2°C and was adjusted to pH 4.5. The sample was then filtered and the filtrate obtained was warmed to 32°C to precipitate fractions enriched in β-casein. The second method involved urea fractionation of whole caseins. In this method whole casein was dissolved in 6.6 M Urea and it was observed that at 1.7 M urea β-casein selectively precipitated from the solution. Using this method, the authors claimed to obtain a yield of 40% of β-casein. Groves *et al.*, (1962) reported that the β-casein samples prepared by Warner (1944) and Hipp *et al.*, (1952) were not pure following analysis by acid and alkaline starch gel electrophoresis. The second method of Hipp *et al.*, (1952) was later modified by Aschaffenburg in 1963. Whereas Hipp *et al.*, (1952) used 6.6 M urea for reconstitution of acid precipitated caseins, Aschaffenburg (1963) used 3.3 M urea. This method was reported to yield approximate 30% of β-casein with no major contamination with α-casein and κ-casein on analysis by paper electrophoresis.

Groves *et al.,* (1962) used both DEAE and CM-cellulose chromatography to purify β-casein and claimed to obtain a homogeneous preparation of β-casein following analysis using both acid and alkaline starch gel electrophoresis. Thompson and Pepper (1964) chromatographed enriched β-casein fractions obtained by the urea fractionation method described by Hipp *et al.,* (1952) using the DEAE-cellulose-urea protocol and obtained a pure preparation of β-casein. This method was further modified by Thompson (1966) for removing the κ-casein contaminant by including 2-mercaptoethanol during chromatography. Murphy and Fox (1991) developed a method for the fractionation of sodium caseinate using ultrafiltration through 300,000 Da cut-off membranes at 4°C. The β-casein preparation obtained, however, was only 80% pure. Ward and Bastian (1996) reported a method for obtaining pure β-casein from calcium caseinate. In this procedure, calcium caseinate was reconstituted, renneted to form a gel, cooled to and held at 4°C to allow β-casein dissociation from the caseinate gel and then centrifuged. The supernatant was warmed to 45°C, precipitating pure β-casein from solution. However, when using the Ward and Bastian (1996) method, extensive hydrolysis of β-casein by chymosin was observed by Huppertz *et al.,* (2006).

In 1999, Bouchier developed a method for isolating β-casein from bovine milk using the urea fractionation method described by Aschaffenburg (1963) and the modified ion exchange chromatography method described by Thompson and Pepper (1964). Whereas, Thomson and Peppper used 3.3 M urea in the elution buffers, that method was modified by Bouchier (1999) by excluding urea from the elution buffers. Bouchier reported a yield of approximately 7% of β-casein. More recently, Huppertz *et al.*, (2006) claimed to purify β-casein from bovine skim milk. The method involved rennet coagulation of skim milk at 30°C and re-suspending the curd in distilled water, cooling to and holding at 4°C for 24 h. After 24 h the sample was filtered using Whatman No. 113 filter paper. The filtrate which was enriched in β-casein was lyophilized. The authors reported a yield of approximately 20%.

The objective of this study was to develop a protocol for the purification of β-casein from acid-caseinate or skim milk which yields an electrophoretically and chromatographically pure substrate (β-casein) for subsequent targeted enzymatic hydrolysis studies.

2.3. Materials and Methods

Dawn™ skim milk, (Kerry Group, Ireland), acid casein (89% (w/w) protein content, Kerry Ingredients, Ireland), rennet a- (Maxiren® 180 DSM Foods Specialties, Delft, Netherlands), rennet b- (Chy-Max® plus, 190 IMCU/ml, Chr. Hansen, Cork, Ireland), 0.2 μm syringe filters (Puradisc, VWR International Ltd, Ireland), 0.2 μm disc filters (Supra-200, 47 mm diameter, VWR International Ltd, Ireland), calcium chloride, calcium hydroxide, urea, dialysis tubing (high retention seamless cellulose tubing, molecular weight cut-off 12400 Da), 1 M hydrochloric acid, 1 M sodium hydroxide, acetonitrile (HPLC grade), water (HPLC grade), trifluoroacetic acid (TFA), bovine pancreatic trypsin and chymotrypsin were from Sigma-Aldrich, Dublin, Ireland.
(Note: All other chemicals were obtained from Sigma-Aldrich, Dublin, Ireland.)

2.3.1. Isolation of β-casein

Two different approaches were used starting with either skim milk or acid casein as source material.

Isolation of β-casein from bovine milk (Protocol 1)

The isolation of β-casein from bovine skim milk was initially carried out using a modification of the procedure described by Huppertz *et al.,* (2006). The following main modifications were made:

- The starting volume of skim milk was 1 liter.
- The sample was gently stirred during storage at $4^{o}C$.

The procedure was further modified by re-suspending the curd in 10 mM $CaCl_2$ during cold storage at $4^{0}C$ and subsequent warming of the supernatant to $45^{0}C$ in order to collect the precipitate enriched in β-casein as described by Ward and Bastian (1996). The procedure makes use of the differential solubility of caseins in the presence of low concentrations of calcium at different temperatures. The flow chart of the modified procedure is given in Figure 2.1.

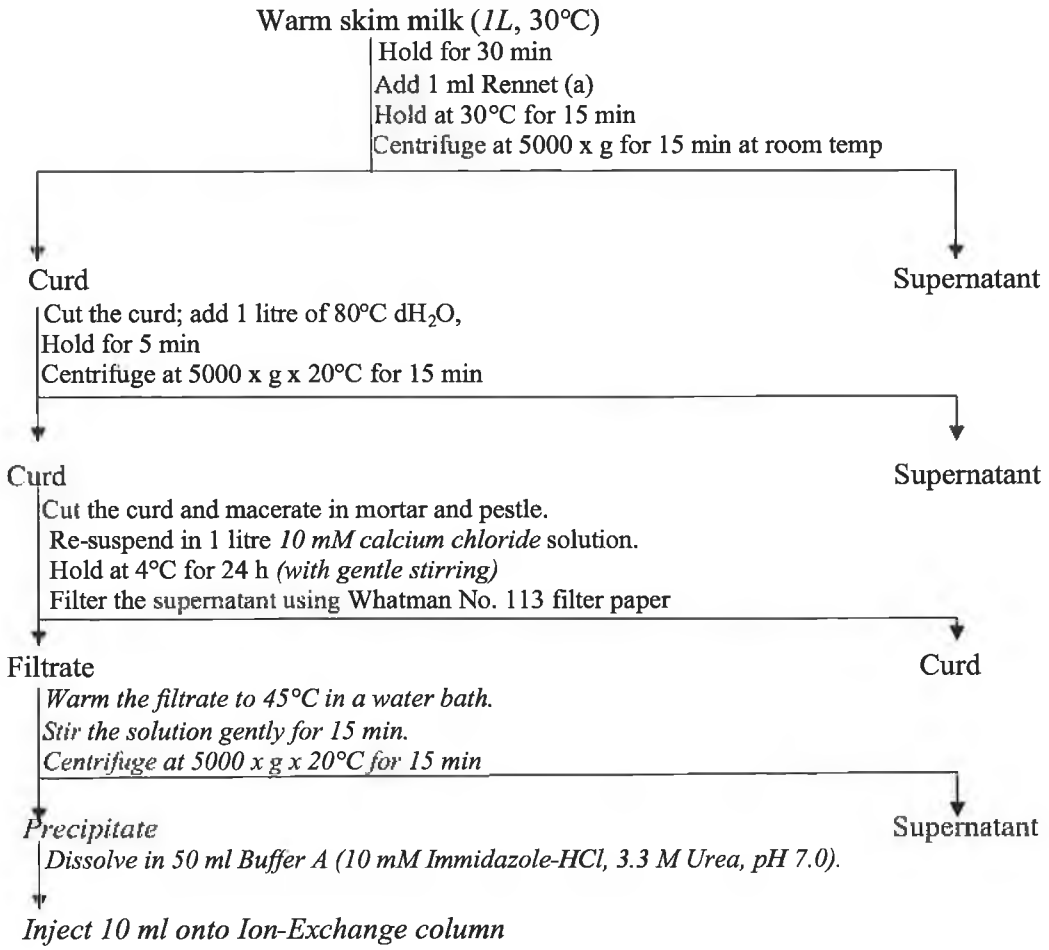


Figure 2.1. Flow chart showing the modified procedure of Huppertz *et al.*, (2006) for the isolation of β-casein from bovine milk. The modifications made presented are in italics.

The ion exchange (IEX) chromatography procedure as described by Thompson and Pepper (1964) was used in the further purification of the enriched β-casein fraction. Whereas Thompson and Pepper (1964) performed IEX chromatography using a step gradient with urea in the buffers that method was modified herein where elution was via a continuous gradient of buffer B containing 3.3 M urea, 10 mM imidazole-HCl, 0.3 M NaCl, pH 7.0 and buffer A contained 3.3 M Urea, 10 mM imidazole-HCl, pH 7.0.

The IEX column was prepared by packing DEAE 52 (Whatman GmbH, Germany) into a XK16/10 column (100 mm x 16 mm I.D, column volume 20 ml, Pharmacia Biotech, Cambridge, England). The column was attached to a Pharmacia fast protein

liquid chromatography (FPLC) system. The FPLC system consisted of two P-500 pumps, a LCC-501 controller and a FRAC-100 fraction collector. Conductivity and absorbance at 280nm were monitored throughout the separation procedure using a Pharmacia conductivity monitor and a UV-MII detector. The system was interfaced with FPLC Director Software (Pharmacia Biotech, Cambridge, England).

The IEX column was first equilibrated using two column volumes of buffer A. The enriched β-casein sample was dissolved in 50 ml buffer A, of which a 10 ml aliquot was injected with the aid of a 10 ml super-loop onto the column for chromatographic separation of the different casein components. Separation was by an increasing linear gradient of buffer B. The gradient was generated over 10 column volumes (200 ml). The flow rate was 1 ml min^{-1}. Fractions (5 ml) were collected. Pooled fractions from the column were dialysed using 12,400 Da molecular weight cut-off dialysis tubing (Sigma-Aldrich, Ireland) against distilled water at 4^0C for 24 h. The dialysed fractions corresponding to individual peaks were lyophilized using a Free Zone, 4.5 freeze-dryer system (Labconco, Kansas City, MO) and the dry samples were stored at -20^0C until further use.

Isolation of β-casein from acid casein (Protocol 2)

The process described by Ward and Bastian (1996) was followed to isolate β-casein from calcium caseinate. Given the lack of direct access to calcium caseinate, acid casein was used in the preparation of calcium caseinate. The procedure followed is outlined below:

A 500 ml suspension of calcium casein was prepared by adjusting the pH of a 3% (w/v) aqueous solution of acid casein to pH 6.8 using 1 M $Ca(OH)_2$. Calcium chloride was then added to bring the final concentration of Ca^{2+} in the solution to 10 mM. The temperature was raised to 31°C by holding the solution in a water bath at 31°C for 1 h. Rennet b (23.8 IMCU/L) was added and the solution was held at 31°C for 30 min. The curd formed was disrupted by stirring for 3 min using a magnetic stirrer at room temperature and the sample was then held at 4°C for 24 h. After 24 h the sample was centrifuged at 5000 x g (Sorvall® RC 5C plus, Fisher Scientific, Dublin, Ireland) at

4°C for 30 min. The supernatant was then filtered using Whatman No.4 filter paper. The filtrate was then warmed in a water bath to 45°C for 30 min. The sample was centrifuged at 20°C and 5000 x g (Sorvall® RC 5C plus, Fisher Scientific, Dublin, Ireland) for 30 min. The precipitate obtained was resuspended in 300 ml distilled water and lyophilised using a Free Zone, 4.5 freeze-dryer system (Labconco, Kansas City, MO). The freeze-dried sample was stored at -20°C until further use.

Isolation of β-casein from acid casein (Protocol 3)

- Enrichment of β-casein

The process described in Protocol 2 was modified to obtain a sample enriched in β-casein. The following modifications were made to Protocol 2:

- Deactivation of rennet was at 80°C for 30 min. The sample was initially warmed in a microwave until the temperature reached 80°C and it was then held in a water bath at 80°C for 30 min.
- Warming the filtrate obtained after filtration at 4°C to 30°C.

The enriched β-casein sample obtained was further purified by ion exchange chromatography as already outlined. The flow chart of the process used is given in Figure 2.2.

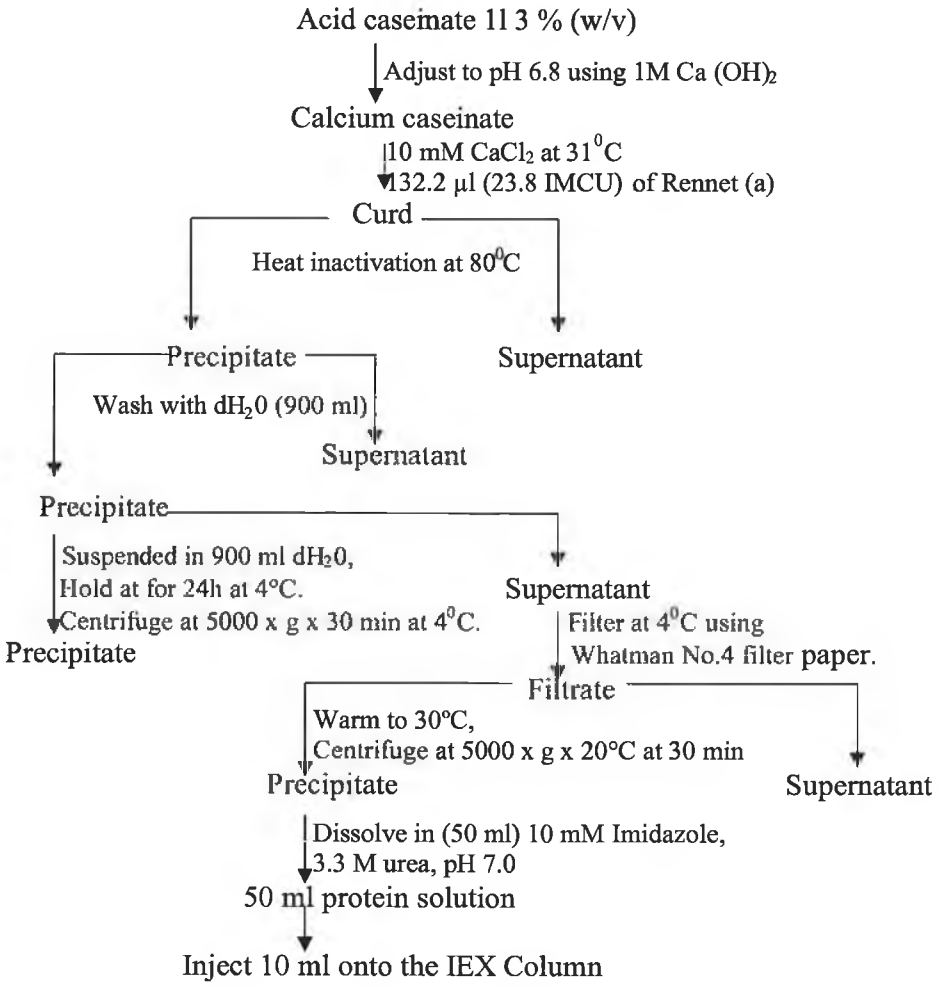


Figure 2.2. Schematic showing the method used for the purification of β-casein from acid caseinate (Protocol 3).

Isolation of β-casein from acid casein (Protocol 4)

- Preparation of enriched β-casein:

Calcium casein was prepared as described earlier. The following modifications were made to the process described in Protocol 3 for the extraction of enriched β-casein.

(A) the rennet deactivation temperature was reduced from 80°C to 70°C (Huppertz *et al.* , 2006). Sample was initially warmed in a microwave until the temperature reached 70°C and it was then held in a water bath at 70°C for 30 min.

(B) adjusting the pH of the sample stored at 4°C after 24 h to pH 4.6 (Law and Leaver, 2004)

The flow chart of the enrichment protocol is given in Figure 2.3.

- Ion exchange (IEX) chromatographic isolation of β-casein:

The IEX chromatographic procedure described by Thomson and Pepper (1964) as outlined earlier was followed. In this instance, IEX chromatography was carried out using a HiPrep™ DEAE FF 16/10 column (GE Healthcare, Bucks, UK) connected to an AKTA™ Purifier (GE Healthcare, Bucks, UK). The IEX column was first equilibrated using two column volumes of buffer A (40 ml of 10 mM imidazole-HCl, pH 7.0 containing 3.3 M urea). The precipitate after warming the solution at 30°C (enriched β-casein sample, see Figure 2.3) was dissolved in 150 ml buffer A and this was loaded onto the column via pump inlet A2. Chromatographic separation was brought about using a linear gradient up to 30% buffer B (3.3 M urea, 10 mM imidazole-HCl, 1 M NaCl, pH 7.0). The gradient was generated over 10 column volumes (200 ml). After the proteins were separated the column was then washed with 1 column volume of 30% buffer B and then with 100 ml (5 column volumes) of 100% buffer B before re-equilibrating in buffer A. The flow rate was 2 ml min^{-1}. Fractions (6 ml) were collected and pooled, fractions corresponding to individual protein peaks were dialysed against distilled water at 4^0C for 24 h.

After dialysis, the pH of the pooled fractions was adjusted to pH 4.6 using 1M HCl allowed to settle and then centrifuged at 5000 x g (Sorvall® RC 5C plus, Fisher Scientific, Dublin, Ireland) for 15 min at room temperature (20°C). The precipitate was resuspended in 500 ml distilled water and the pH was adjusted to pH 7.5 using 1M NaOH. The precipitation and resuspension of the sample was repeated twice and the final protein solution (500 ml) was then lyophilized and the dry sample was stored at -20^0C until further use.

- Chromatographic separation of α-casein:

A small amount (600 mg) of the freeze dried crude α-casein fraction (see Figure 2.3) was dissolved in 100 ml of buffer A (10 mM imidazole-HCl, pH 7.0 containing 3.3 M urea). The IEX chromatography was carried out using a HiPrep™ DEAE FF 16/10

column (GE Healthcare, Bucks, UK) connected to an AKTA™ Purifier (GE Healthcare, Bucks, UK). The column was first equilibrated using two column volumes of buffer A. The crude α-casein sample (600 mg) dissolved in 100 ml buffer A was injected onto the column via pump inlet A2. Chromatographic separation was carried out by modifying the procedure described by Thomson and Kiddy (1964). Whereas Thompson and Kiddy (1964) performed IEX chromatography with a step gradient with urea and different concentrations of NaCl in the elution buffers, that method was modified herein as follows, elution was achieved using a linear gradient from 0-20% B in 2 column volumes (CV) and then from 20-45%B in 5 CV. The flow rate was 2 ml min^{-1}. Fractions (6 ml) were collected; fractions corresponding to individual protein peaks were pooled and dialysed against distilled water at 4^0C for 24 h. The dialysed fractions were lyophilized and dry samples were stored at -20^0C until further use.

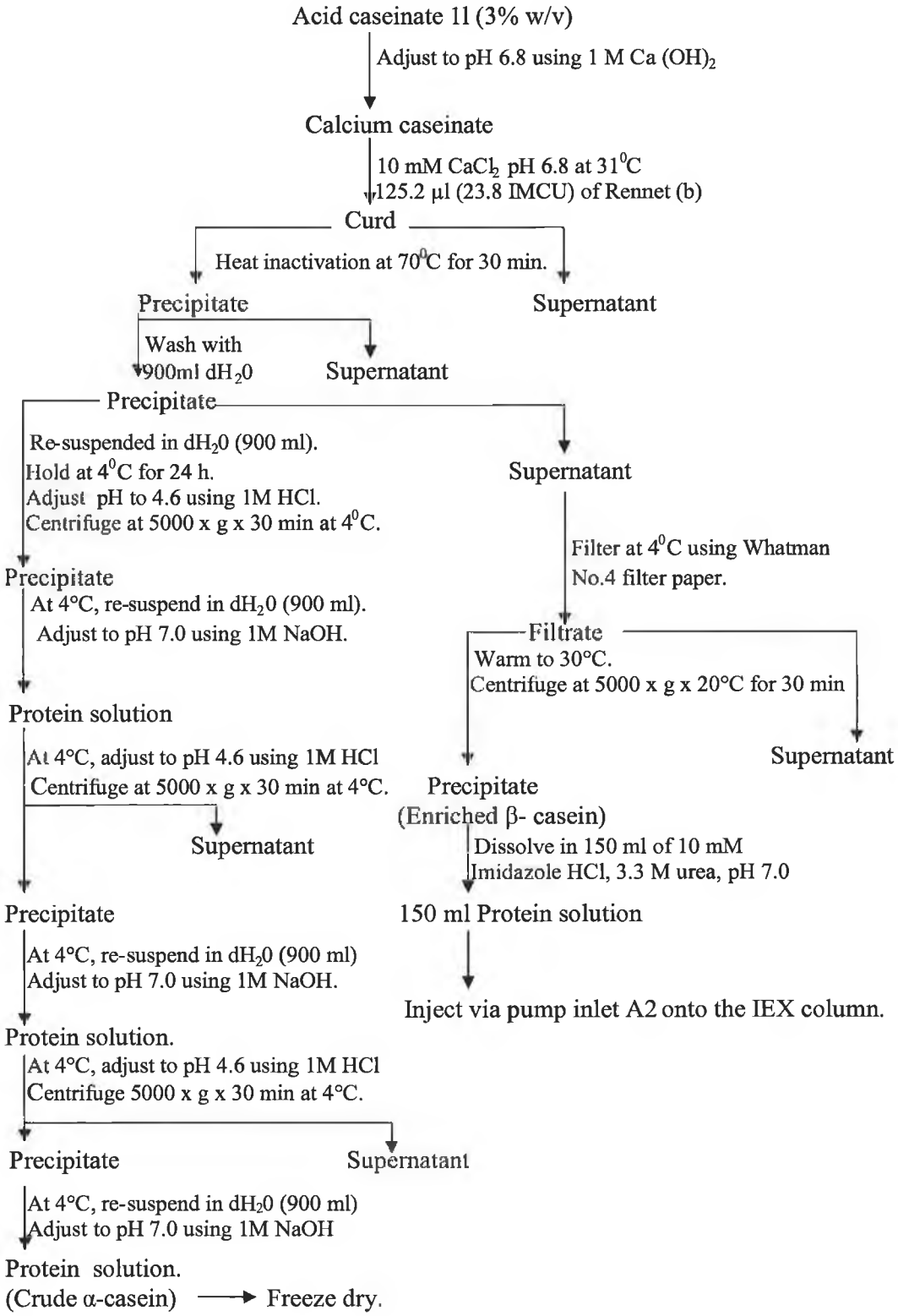


Figure 2.3. Schematic showing the method used for the purification of β-casein from acid caseinate (Protocol 4).

2.3.2. Urea-polyacrylamide gel electrophoresis (Urea-PAGE)

Urea-polyacrylamide gel electrophoresis (Urea-PAGE) was used to monitor the progress of the purification processes. A Protean®ii xi cell (Bio-Rad, Hertfordshire, UK) supplied with a power pack 1000 (Bio-Rad, Hertfordshire, UK) was used to run the gel. For Urea-PAGE, a modified procedure described by Poulik (1957) and Thompson *et al.,* (1964) was followed. A 12.5% resolving gel and a 4.5% stacking gel was prepared and run at a constant voltage of 200 volts. The lyophilised test samples were dissolved in aqueous sample buffer (1.2% (v/v) of 0.5 M Tris HCl, pH 6.8, 1% (v/v) of 8M urea, 1% (v/v) glycerol, 0.4% (v/v) 2-mercaptoethanol and 0.4% (v/v) of 1% Bromophenol blue) at a concentration of 10 mg ml^{-1}. The gels were stained for 1 h using 0.25% (w/v) Comassie Blue dissolved in 40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) distilled water and destained overnight in destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) distilled water).

2.3.3. Reversed-phase (RP) HPLC

RP HPLC was performed using a Waters HPLC system comprising a model 1525 binary pump, a model 717 Plus auto-sampler and a model 2487 dual λ absorbance detector interfaced with a Breeze™ data-handling package (Milford, MA, USA). The column used was a Phenomenex Jupiter (C18, 250 mm x 4.6 mm I.D., 5 μm particle size, 300 Å pore size) with a Security Guard system containing a C18 (ODS) wide pore cartridge (4 mm x 3 mm I.D, Phenomenex, Cheshire, U.K.). The column was equilibrated with solvent A (0.1% TFA) at a flow rate of 1.0 ml min^{-1} and protein was eluted with an increasing gradient of solvent B (0.1% TFA, 80% acetonitrile) as follows: 0-4 min 0 % B, 4-54 min 0-60% B, 54-55 min 60-100% B, 55-65 min 100 % B, 65-70 min 100-0% B, 70-85 min 0% B. Detector response was monitored at 214 nm (Spellman *et al.*, 2009). Protein samples (0.5% w/v) dissolved in 0.1% TFA were filtered through 0.2 μm syringe filters (Puradisc, VWR International Ltd, Ireland) and 20 μl was applied to the column.

2.3.4. Gel-permeation chromatography (GPC)

GPC was performed using a Waters HPLC system comprising a model 1525 binary pump, a model 717 Plus auto-sampler and a model 2487 dual λ absorbance detector interfaced with a Breeze^{TM} data-handling package (Milford, MA). Protein samples 0.25% (w/v) diluted in 0.1% TFA in 30% acetonitrile were filtered through 0.2 μm syringe filters and 20 μl applied to a TSK gel G2000 SW separating column (600 mm x 7.5 mm I.D.) connected to a TSKGEL SW guard column (75 mm x 7.5 mm I.D.). Separation was by isocratic elution with a mobile phase of 0.1% TFA in 30% acetonitrile at a flow rate of 1.0 ml min^{-1} (Smyth and FitzGerald, 1998, Spellman *et al.*, 2005). Detector response was monitored at 214 nm.

2.3.5. Digestion of purified β-casein with trypsin and chymotrypsin

β-Casein (20 mg), purified using protocol 4 was dissolved in 1 ml of 50 mM ammonium bicarbonate solution, pH 8.0. Trypsin (Bovine pancreas, 10 mg, 9.1 mmol min^{-1} mg^{-1}) and chymotrypsin (Bovine pancreas, 10 mg, 55 μmol min^{-1} mg^{-1}) were dissolved separately in 0.5 ml of 50 mM ammonium bicarbonate solution, pH 8.0. β-Casein solution (1 ml) was held at 37°C for 5 min. Trypsin and chymotrypsin, (20 μl) were added to the β-casein solution (E:S=1:50 (w/w)) and this was incubated at 37°C for 120 min after which the enzymes were inactivated by holding in a water bath at 90°C for 10 min. The sample was then stored at -20°C until further use.

2.3.6. HPLC-ESI MS and MSMS analysis of β-casein

The β-casein hydrolysate samples were dissolved in HPLC grade water (with 0.1 % (v/v) formic acid and analyzed using a UltiMate® 3000 NanoHPLC instrument (Dionex, Sunnyvale, USA) connected to a MicrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany). The β-casein hydrolysate sample (1μl) was loaded onto a C18 PepMap 100 precolumn cartridge (Dionex, Sunnyvale, USA) with the μl Pickup mode (Dionex) at a flow rate of 25 μL min^{-1} for 3 min. Being eluted from the precolumn, sample was then separated on a C18 PepMap 100 column (Dionex, 75 μm × 150 mm, 3 μm, Sunnyvale, USA) at a flow rate of 300 nl min^{-1}. Mobile phase A

was water with 0.1% v/v formic acid, and mobile phase B was acetonitrile with 0.1% v/v formic acid. A linear gradient was employed from 2.0 to 40% B in 80 min. Column temperature was maintained at 25°C. The MS and tandem MS experiments were controlled by MicrOTOF control software (version 2.3, Bruker Daltonics). Full scans were performed between a m/z range of 150 and 3000. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan using collision induced dissociation (CID). Electrospray conditions were as follows: capillary temperature, 150°C; capillary voltage, -1700V; dry gas (N_2) flow, 6.0 L min-1. Peak list files were searched by the MASCOT search engine (v. 2.3) against SwissProt (v. 57.15). The peptide mass tolerance and fragment mass tolerance were set to ±0.1 Da and ±0.1 Da, respectively. The search included enzyme TrypChymo, taxonomy other mammalia, max missed cleavages 3, variable modifications for oxidation of methionine and phosphorylation of serine, threonine and error tolerant search of all significant protein hits. MASCOT ions score cut-off was set to 30. The significance threshold was set to 0.05.

2.4. Results

2.4.1. Isolation of β-casein from skim milk (Protocol 1)

The IEX chromatogram obtained during the purification of β-casein from bovine milk is shown in the Figure 2.4. During the IEX run, fractions (5 ml) were collected, fractions corresponding to the individual peaks were pooled and dialysed using a 12,400 Da molecular weight cut-off membrane against distilled water at 4°C. Three peaks were obtained after the IEX run. The first peak eluted during the injection and eluted in fractions 1-7. The second peak eluted into fractions 22-24 and the third peak eluted into fractions 25-31. The weight of sample corresponding to individual peaks obtained after dialysis and subsequent lyophilization is given in Table 2.2.

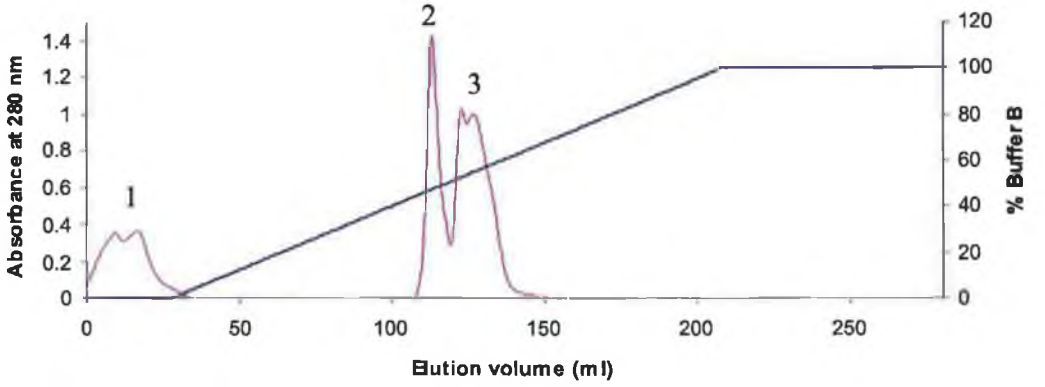


Figure 2.4. Chromatogram showing the purification of β-casein from bovine milk using an ion exchange (DE 52 matrix) column. Absorbance at 280 nm and concentration of buffer B are given.

Table 2.2. Peak numbers, corresponding pooled fractions, total volume of the pooled fractions and the weight recovered after dialysis and lyophilization of the corresponding peaks obtained following DEAE ion exchange chromatography of enriched β-casein using Protocol 1.

Peak Number	Corresponding pooled Fractions	Total volume (ml)	Weight after lyophilization (mg)
1	1-7	35	71.0 (355.0)*
2	22-24	15	12.2 (61.0)*
3	25-31	35	32.5 (162.5)*

*Since a 10 ml aliquot of 50 ml sample was injected onto the ion exchange column, the weight of the sample recovered was multiplied by 5 (value in brackets) to estimate the amount in the individual peaks corresponding to the total amount in 1l of skim milk.

The samples corresponding to the individual peaks were analysed using Urea-PAGE (Figure 2.5). The first peak was observed to be κ-casein along with some low molecular weight peptides (Figure 2.5, lane 8). The 2nd and 3rd peaks (Figure 2.5, lane 5, 6 and 7) were observed to contain both α- and β-caseins along with κ-casein and some low molecular weight peptides. Since both the 2nd and 3rd peaks were observed to contain significant levels of β-casein, the total yield of β-casein was estimated by combining the yields of both the 2nd and 3rd peaks. This yielded 223.5 mg l^{-1} (Table 2.2) or 2.23% of total β-casein present in skim milk, assuming that skim milk contains 10 g l^{-1} of β-casein. Since the purity and the yield of the β-casein was not satisfactory, it was decided to employ an alternative method for purification of β-casein.

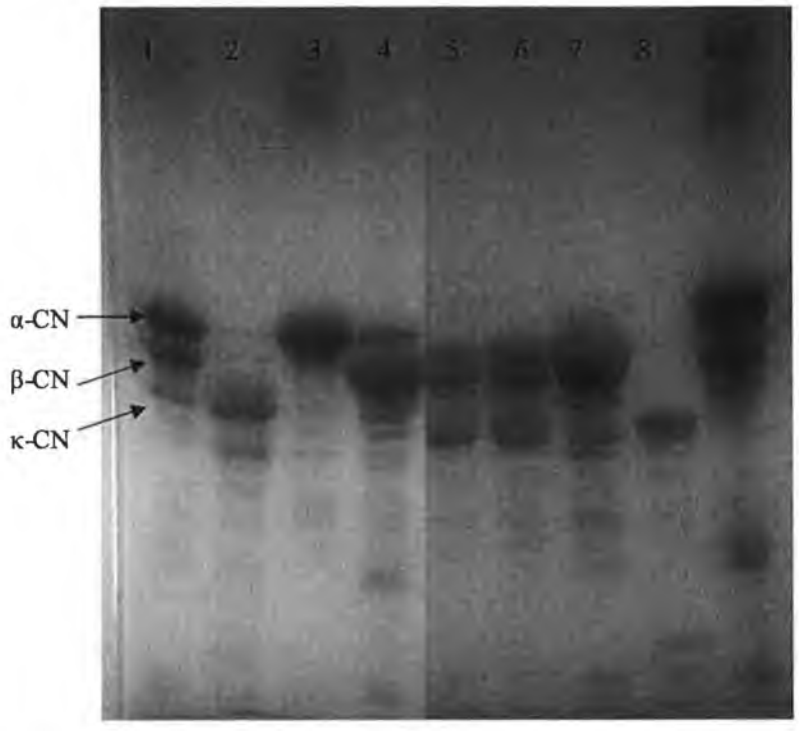


Figure 2.5. The Urea-PAGE profiles of the samples obtained following the purification of β-casein from skim milk using Protocol 1.

Lane No.	Sample	Amount
1.	Na-CN	20μg
2.	κ-Casein standard	20μg
3.	α-Casein standard	20μg
4.	β-Casein standard	20μg
5.	2nd peak from IEX	20μg
6.	2nd peak from IEX	30μg
7.	3rd peak from IEX	20μg
8.	1st peak from IEX	20μg
9.	Acid-CN	20μg

2.4.2. Isolation of enriched β-casein from acid casein (Protocol 2)

Since the primary objective was to isolate large quantities of pure β-casein irrespective of the starting material, the process described by Ward and Bastian (1996) using calcium caseinate was then adopted for β-casein purification.

The amount of freeze dried sample obtained following adoption of Protocol 2 was 702 mg/ 500 ml or 16% of the theoretical yield of β-casein from 500 ml of 3% acid casein solution. The yield (16%) was significantly lower than the yield (55%) reported by Ward and Bastian (1996). This may be due to the fact that the authors dried the precipitated β-casein fraction in a desiccator, and as a result the moisture present in the sample may not have been completely removed. Therefore, the yield (55%) reported by Ward and Bastian (1996) may be higher than the actual value due to the presence of moisture in the sample. This observation was also made by Huppertz *et al.,* (2006). Moreover, from urea-PAGE analysis in the present study (Figure 2.6, lane 5 6 and 7), it was observed that the β-casein sample was contaminated with α-casein, κ-casein and some low molecular weight peptides. In agreement with these results Huppertz *et al.*, (2006) also reported extensive hydrolysis of β-casein by chymosin when using the Ward and Bastian (1996) isolation method.

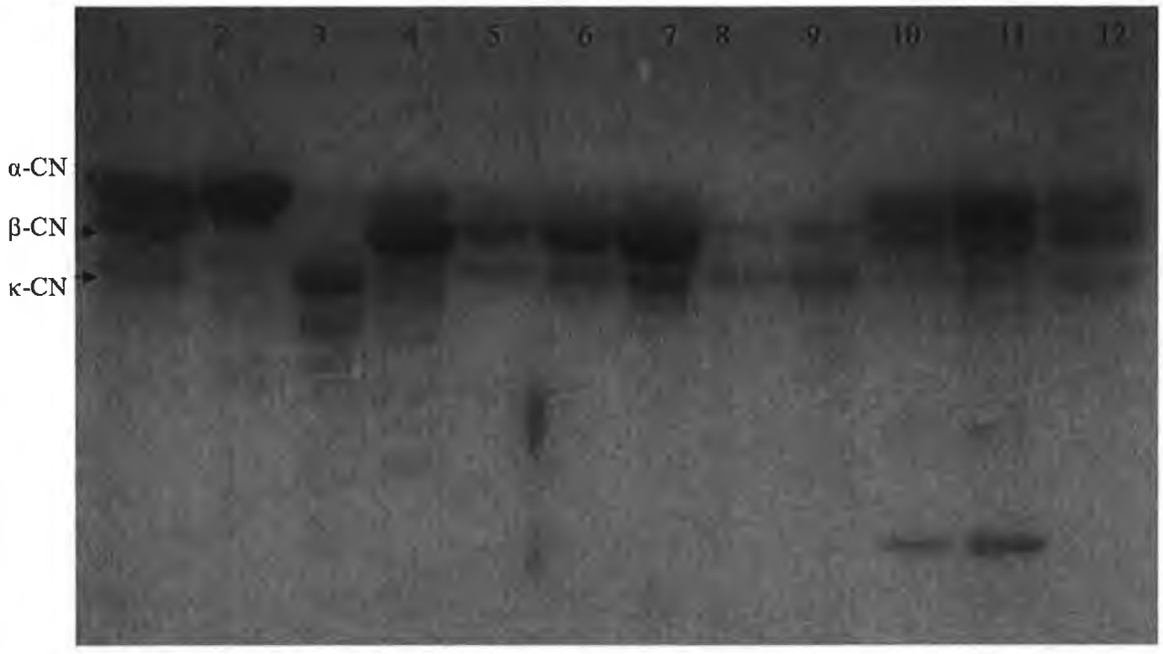


Figure 2.6. The urea-PAGE profile of the samples obtained following the purification of β-casein from acid casein using Protocol 2.

Lane No.	Sample	Amount
1.	Na-Cn	20 μg
2.	α-Casein standard	20 μg
3.	κ-Casein standard	20 μg
4.	β-Casein standard	20 μg
5.	Precipitate at 45°C (enriched β-casein)	5 μg
6.	Precipitate at 45°C (enriched β-casein)	10 μg
7.	Precipitate at 45°C (enriched β-casein)	20 μg
8.	Supernatant at 45°C	10 μg
9.	Supernatant at 45°C	20 μg
10.	Precipitate at 4°C , 24 h (enriched α-casein)	10 μg
11.	Precipitate at 4°C , 24 h (enriched α-casein)	20 μg
12.	Acid-Cn	20 μg

2.4.3. Isolation of β-casein from acid casein (Protocol 3)

The β-casein fraction isolated using Protocol 2 was observed to be contaminated with α-casein, κ-casein and what appeared to be some low molecular weight peptides as previously described. Therefore, it was decided to modify Protocol 2 as described in section 2.3.1 (Protocol 3), to obtain an enriched β-casein fraction which was subsequently purified using IEX. The IEX chromatogram obtained during the purification of β-casein from acid casein is shown in the Figure 2.7.

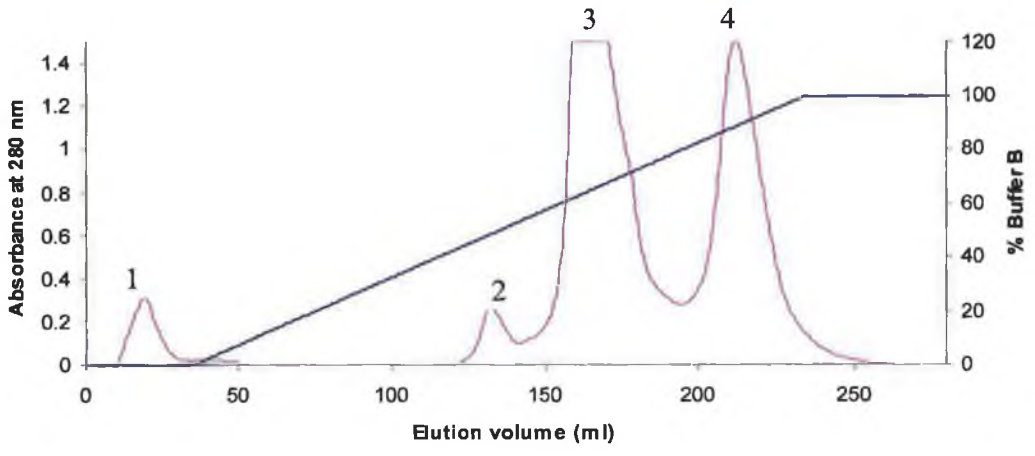


Figure 2.7. Chromatogram showing the purification of β-casein from acid casein using ion exchange (DE 52 matrix) chromatography. Absorbance at 280 nm and concentration of buffer B are given.

During the IEX run, fractions (6 ml) were collected, fractions corresponding to the individual peaks were pooled and dialysed using a 12,400 Da molecular weight cut-off membrane against distilled water at 4°C. Four peaks were obtained during the IEX run, the first peak eluted into fractions 3-8, the second peak eluted into fractions 25-29, the third peak eluted into fractions 31-38 and the fourth peak eluted into fractions 41-52 (Figure 2.7). The weight of fractions corresponding to individual peaks obtained after dialysis and subsequent lyophilization is given in Table 2.3. The third peak obtained during the above chromatographic run (Figure 2.7) was observed by urea-PAGE (Figure 2.8, lane 5 and 6) analysis to be highly enriched in β-casein. The percentage protein in the original acid caseinate sample was 89% of which approximately 33% is β-casein and therefore the estimated total yield of β-casein obtained by Protocol 3 was approximately 7.61%. However, urea-PAGE (Figure 2.8,

lane 5 and 6) analysis, indicated that the sample still contained low levels of α-casein and either κ-casein or some peptides (potentially fragments of β-casein) which eluted just below the β-casein band

Table 2.3. Peak numbers, corresponding pooled fractions, total volume of the pooled fractions and the weight recovered after dialysis and lyophilization of the corresponding peaks obtained following DEAE ion exchange chromatography of enriched β-casein using Protocol 3.

Peak Number	Corresponding pooled Fractions	Total volume (ml)	Weight after lyophilization (mg)
1	3-8	30	3.8 (19.0)*
2	25-29	25	1.4 (7.0)*
3	31-38	40	134.2 (671)*
4	41-52	60	17.4 (87.0)*

*Since a 10 ml aliquot of 50 ml sample was injected onto the ion exchange column, the weight of the sample recovered was multiplied by 5 (values in brackets) to estimate the amount in the individual peaks corresponding to the total amount in 1l of 3% (w/v) of acid caseinate.

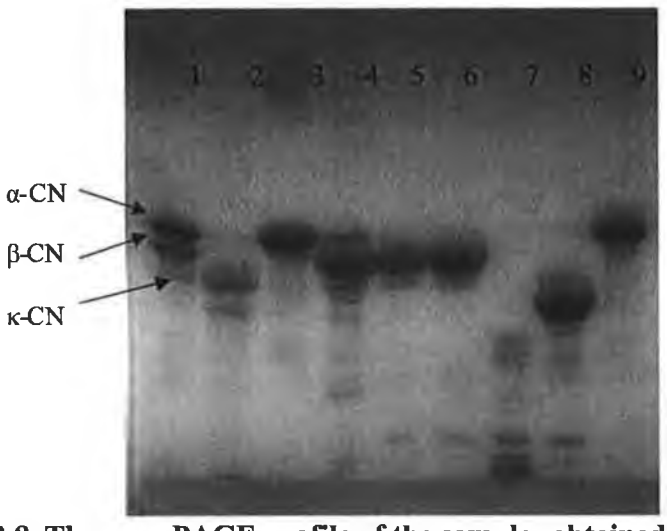


Figure 2.8. The urea-PAGE profile of the samples obtained following the purification of β-casein from acid casein using Protocol 3.

Lane No.	Sample	Amount
1.	Na-CN	20μg
2.	κ-Casein standard	20μg
3.	α-Casein standard	20μg
4.	β-Casein standard	20μg
5.	3rd peak after IEX	20μg
6.	3rd peak after IEX	30μg
7.	1st peak after IEX	20μg
8.	2nd peak after IEX	20μg
9.	4th peak after IEX	20μg

In all the above mentioned protocols i.e., Protocol 1, 2 and 3 it was observed that the β-casein preparation obtained was contaminated with other proteins or peptides. Given that Protocol 3 yielded a more pure β-casein sample than Protocol 1 and 2, it was decided to further modify Protocol 3 for the purification of β-casein.

2.4.4. Isolation of β-casein from acid casein (Protocol 4)

As mentioned earlier the objective of the study was to purify β-casein to homogeneity it was therefore decided to further modify the method described in Protocol 3 utilising different steps employed in the protocols described by Ward (1998), Law and Leaver (2004) and Huppertz *et al.*, (2006). The full sequence of steps used is outlined in Figure 2.3. The IEX chromatogram obtained during the purification of the enriched β-casein from acid casein (Protocol 4) is shown in the Figure 2.9.

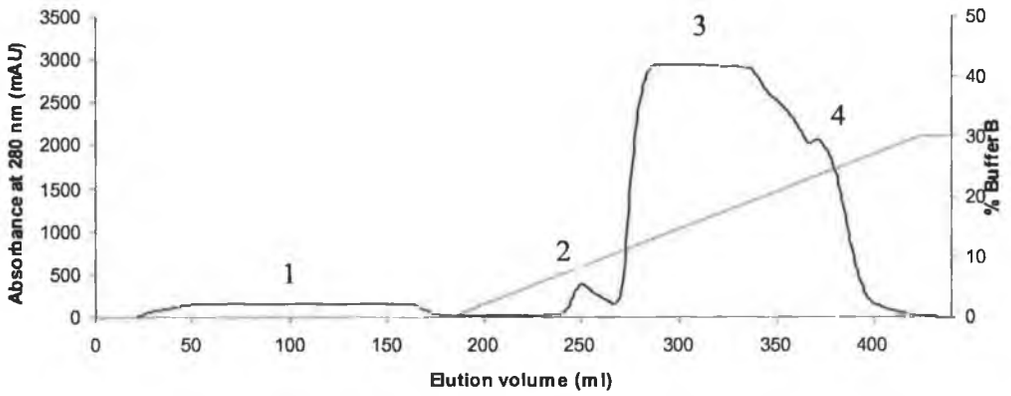


Figure 2.9. Chromatogram showing the purification of the enriched β-casein from acid casein (Protocol 4) using an ion exchange (HiPrep DEAE FF 16/10) column. Absorbance at 280 nm and concentration of buffer B are given.

During the IEX run, fractions (6 ml) were collected, fractions corresponding to the individual peaks were pooled and dialysed using a 12,400 Da molecular weight cut-off membrane against distilled water at 4°C. Four peaks were obtained during the IEX run, the first peak eluted in fractions 5-31, the second peak eluted in fractions 41-45, the third peak eluted in fractions 47-61 and the fourth peak eluted in fractions 62-72 (Figure 2.9). For peak three after dialysis, the sample was precipitated at pH 4.6 and re-suspended at pH 7.5. Precipitation and re-suspension steps were carried out three times prior to lyophilization. The weight of fractions corresponding to individual peaks obtained after dialysis and subsequent lyophilization is given in Table 2.4.

Table 2.4. Peak numbers, corresponding pooled fractions, total volume of the pooled fractions and the weight recovered after dialysis and lyophilization of the corresponding peaks obtained following DEAE ion exchange chromatography of enriched β-casein using Protocol 4.

Peak Number	Corresponding pooled Fractions	Total volume (ml)	Weight after lyophilization (mg)
1	5-31	162	60
2	41-45	30	29
3	47-61	90	434
4	62-72	66	109

Table 2.5. Purification table showing the theoretical amount and percentage yield of β-casein retained during different stages of purification using Protocol 4.

Sample	Total protein (g)	Amount of β-casein (g)	Yield (g/g) (%)
Acid Caseinate	26.700	8.81	100.00
Enriched β-casein	0.980	0.980	11.12
Purified β-casein	0.434	0.434	4.92

The purification table for β-casein is given Table 2.5. The amount of β-casein present in acid caseinate was approximately 33% (w/w) of total protein. It was observed 11% (w/w) of β-casein was obtained prior to the IEX step and after IEX step approximately 5% β-casein was obtained.

Figure 2.10. The urea-PAGE profiles of the samples obtained following the purification of β-casein from acid caseinate using Protocol 4.

Lane No.	Sample	Amount
1.	Na-CN	20μg
2.	κ-CN standard	20μg
3.	α-CN standard	20μg
4.	β-CN standard	20μg
5.	Supernatant after heat inactivation of rennet	20μg
6.	Precipitate after storage at 4°C after 24 h, pH 4.6	20μg
7.	Supernatant after storage at 4°C after 24 h, pH 4.6	20μg
8.	Supernatant at 30°C	20μg
9.	Precipitate at 30°C (enriched β-CN)	20μg
10.	Purified β-casein	20μg
11.	Precipitate at 30°C (enriched β-CN)	30μg
13.	Purified β-CN	30μg
14.	Enriched α-CN	20μg
15.	Enriched α-CN	30μg

From urea-PAGE analysis, Figure 2.10 (lane 9 and 11), it can be observed that in the enriched β-casein fraction obtained from Protocol 4 there was no α- or κ-casein contaminant. Whereas, the enriched β-casein fraction obtained from Protocol 2 was observed to be contaminated with α- and κ-casein along with some low molecular weight peptides (see Figure 2.6, lane 5, 6 and 7). Therefore, from Figures 2.6 and 2.10 it was concluded that adjusting the pH of the cold (4°C) stored sample to pH 4.6 after 24 h, prior to centrifugation yields a purer fraction of β-casein than the sample obtained without adjusting the pH to 4.6. This step was adopted from the procedure outlined by Law and Leaver (2004). Furthermore, it was observed that when 20 μg of the enriched β-casein sample was applied onto urea-PAGE (Figure 2.10, lane 9) it appeared to be pure, however, when 30 μg of enriched β-casein was applied (Figure 2.10, lane 11) it was observed that the sample was contaminated with what appeared to be some low molecular weight peptides.

The contaminating band observed in the enriched β-casein preparation obtained using Protocol 4 may be due to degradation products of β-casein by the action of plasmin or chymosin. β-Casein is the preferred substrate for plasmin and it generally cleaves polypeptide chains predominantly after lysine and to a lesser extent after arginine residues. β-Casein plasmin hydrolysis products are know as γ-casein and proteose-peptones. There are three different γ-caseins reported which are termed as γ_1-, γ_2- and γ_3-casein which correspond to 29-209, 106-209 and 108-209 of the β-casein molecule, respectively (Andrews, 1983). Chymosin primarily cleaves the Phe-Met (105-106) bond of κ-casein but if the enzyme is not properly inactivated, it will also hydrolyse specific bonds in other caseins, preferentially in β-casein. Fragments βI, βII and βIII corresponding to β-casein fragment 1-189/192, 1-163 and 1-139, respectively, were reported following the action of chymosin on β-casein. (Fox *et al.,* 1993). The temperature for inactivation of chymosin used in Protocol 4 was 70°C, which as reported by Huppertz *et al.,* (2006) was sufficient to inactivate chymosin activity. The contaminants observed in the enriched β-casein preparation (Figure 2.10, lanes 9 and 11), which may be the hydrolysis products of β-casein by the above mentioned enzymes, were removed using IEX chromatography (Figure 2.9). After this IEX step, the β-casein sample obtained was pure as observed using urea- PAGE (Figure 2.10, lane 10 and 13) RP-HPLC (Figure 2.11) and GPC (Figure 2.12) analysis. On urea-PAGE only a single band corresponding to β-casein was observed (Figure 2.10, lane

10 and 13). Both RP-HPLC and GPC chromatograms showed that purified β-casein had only a single peak. From Figure 2.14, it can be observed that a combination of trypsin and chymotrypsin extensively hydrolysed β-casein. The sequences of the 30 peptides with major intensity peaks in the total intensity chromatogram (TIC, Figure 2.13) of β-casein incubated with trypsin and chymotrypsin at 37°C for 120 min were summarised in Table 2.6. The molecular mass information of singly or multiply charged ions corresponding to different peaks in the TIC spectrum (Figure 2.13) along with calculated and experimental mass are also given in Table 2.6. Theoretically, in β-casein there are five phosphorylated Ser residues at positions 15, 17, 18, 19 and 35 (Swaisgood, 1982). From the peptides obtained following the incubation of β-casein with trypsin and chymotrypsin, no evidence of phosphorylation at positions 15, 17, 18 and 19 was found (Table 2.6). However, in fragments f(30-48), f(32-48),f(33-48) and f(33-52) it was observed that Ser at positions 35 was phosphorylated (Table 2.6). The sequence coverage of β-casein was 78%. From the HPLC-ESI MS and MSMS analysis it is evident that the β-casein purified using Protocol 4 was pure given that no non β-casein peptides were identified in the digest .

The results obtained show that using Protocol 4 electrophoretically and chromatographically pure β-casein can be obtained with a yield of approximately 5% (see Tables 2.4 and 2.5). The yield was less than that reported by Bouchier (1999), who obtained electrophoretically pure β-casein with a yield of approximately 7%. Bouchier purified β-casein from 6 L of skim milk using urea fractionation and ion-exchange chromatography. In that method the crude β-casein sample was exposed to a high concentration of urea for extended periods of time. Whereas, in the procedure described herein, Section 2.3.1 (Protocol 4), the sample was not exposed to high concentrations of urea for long time periods. The amount of crude β-casein dissociated from the casein curd after 24 h extraction using the method described in Section 2.3.1 (Protocol 4) was approximately 11% (Table 2.5). This was also lower than the values reported in the literature which vary from 20-55% (Hipp *et al.*, 1952; Aschaffenburg., 1963; Ward and Bastian, 1996, Bouchier 1999 and Huppertz *et al.*, 2006). The lower yield in the present study may be related to losses on adjustment of the pH to 4.6 after cold (4°C) storage during which β-casein may be trapped in the precipitated α-casein. It is evident from urea-PAGE analysis (Figure 2.10, lane 6), that this precipitate obtained at 4°C (pH 4.6) had a significant amount of β-casein.

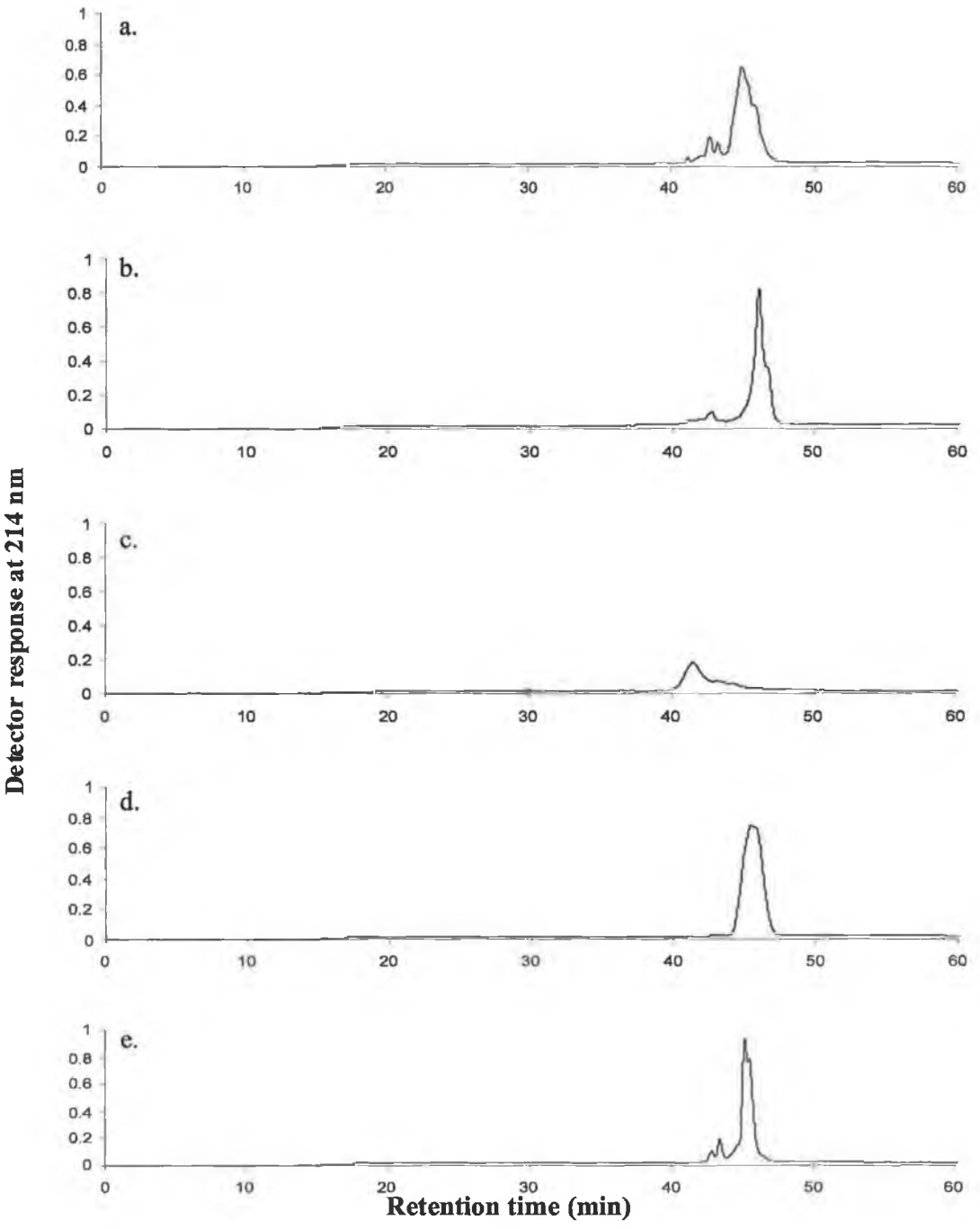


Figure 2.11. Reversed-phase-HPLC chromatograms of a) α-casein standard, b) β-casein standard, c) κ-casein standard, d) purified β-casein and e) enriched α-casein.

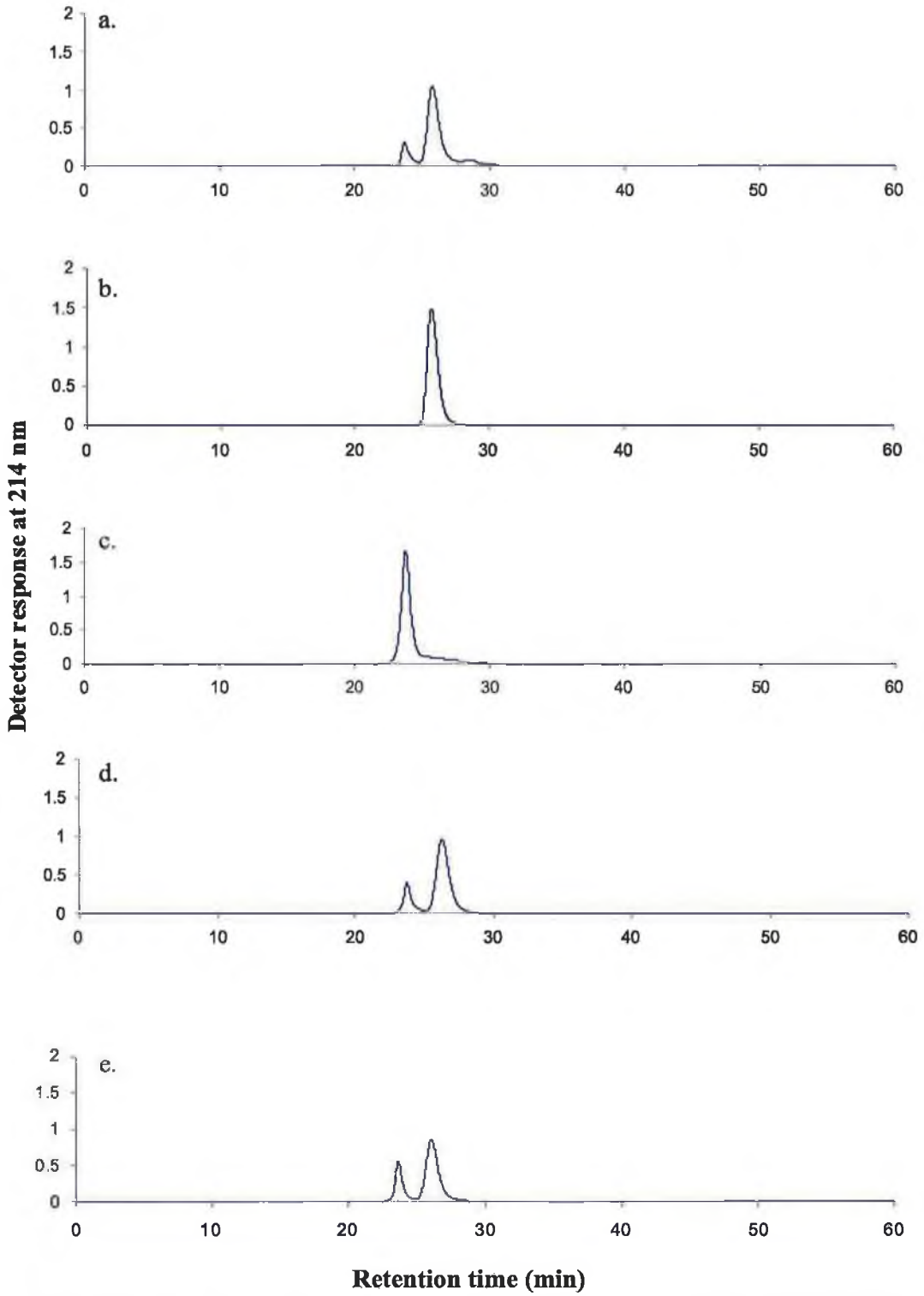


Figure 2.12. Gel permeation chromatograms of a) β-casein standard, b) purified β-casein, c) κ-casein d) α-casein standard and e) enriched α-casein.

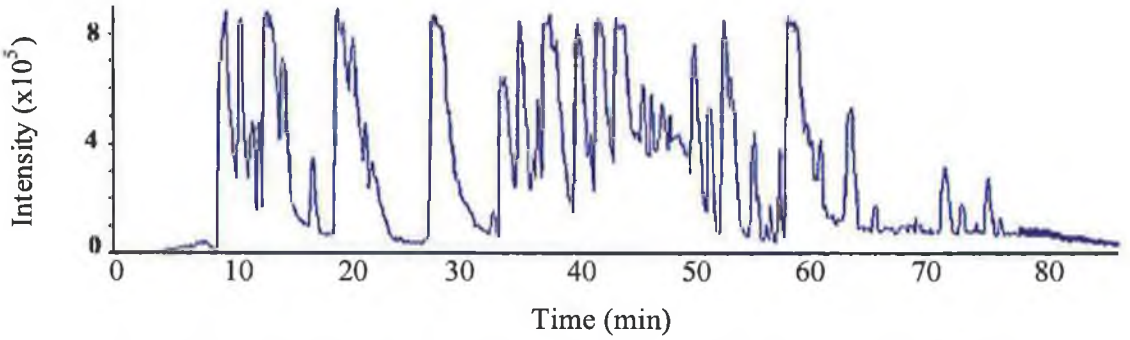


Figure 2.13. Total ion chromatogram of β-casein incubated with trypsin and chymotrypsin at 37°C for 2 h.

Table 2.6. Peptide sequences of β-casein identified upon incubation of β-casein with trypsin and chymotrypsin for 2 h at 37°C.

S.No	β-Casein fragment	Peptide sequence	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
1	f1-16	RELEELNVPGEIVESL	913.4817(2)	1824.9488	1824.9469	60.85
2	f30-48	IEKFQS*EEQQQTEDELQDK	811.3562(3)	2431.0468	2431.0428	60.91
3	f32-48	KFQS*EEQQQTEDELQDK	730.6486(3)	2188.924	2188.9161	49.87
4	f33-48	FQS*EEQQQTEDELQDK	1031.4204(2)	2060.8262	2060.8212	137.78
5	f33-52	FQS*EEQQQTEDELQDKIHPF	852.706(3)	2555.0962	2555.0853	95.68
6	f34-48	QSEEQQQTEDELQDK	917.9003(2)	1833.786	1833.7864	116.77
7	f34-52	QSEEQQQTEDELQDKIHPF	777.0275(3)	2328.0607	2328.0506	78.96
8	f69-97	SLPQNIPPLTQTPVVVPPFLQPEVM°GVSK	1043.5794(3)	3127.7164	3127.6992	51.96
9	f78-93	TQTPVVVPPFLQPEVM	891.4822(2)	1780.9498	1780.9434	42.97
10	f78-97	TQTPVVVPPFLQPEVM°GVSK	1085.0835(2)	2168.1524	2168.1552	29.07
11	f83-97	VVPPFLQPEVM°GVSK	821.9484(2)	1641.8822	1641.88	53.49
12	f88-97	LQPEVM°GVSK	552.2935(2)	1102.5724	1102.5692	42.61
13	f100-105	EAM°APK	662.3198(1)	661.3125	661.3105	39.04
14	f106-113	HKEM°PFPK	515.2684(2)	1028.5222	1028.5113	32.98
15	f108-113	EMPFPK	748.3735(1)	747.3662	747.3625	39.16
16	f114-119	YPVEPF	751.37(1)	750.3627	750.3588	38.28
17	f114-125	YPVEPFTESQSL	709.8284(2)	1417.6422	1417.6377	60.33
18	f120-133	TESQSLTLTDVENL	837.4211(2)	1672.8276	1672.8204	51.82
19	f126-133	TLTDVENL	908.4904(1)	907.4831	907.4634	39.71
20	f134-139	HLPLPL	689.4383(1)	688.431	688.4272	35.81
21	f144-156	MHQPHQPLPPTVM	756.8749(2)	1511.7352	1511.7377	47.31
22	f144-163	MHQPHQPLPPTVM°FPPQSVL	771.7243(3)	2312.1511	2312.1446	72.71
23	f145-163	HQPHQPLPPTVM°FPPQSVL	722.7184(3)	2165.1334	2165.1092	81.31
24	f164-169	SLSQSK	649.3536(1)	648.3463	648.3442	30.85
25	f170-175	VLPVPQ	652.412(1)	651.4047	651.3956	48.58
26	f177-182	AVPYPQ	674.3551(1)	673.3478	673.3435	52.05
27	f184-191	DM°PIQAFL	950.4676(1)	949.4603	949.4579	22.26
28	f191-202	LLYQEPVLGPVR	692.8964(2)	1383.7782	1383.7762	53.41

Table 2.6: Continued.

S.No	β-Casein fragment	Peptide sequence	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
29	f192-202	LYQEPVLGPVR	635.8653(2)	1269.716	1269.7081	68.63
30	f192-202	LYQEPVLGPVR	635.8653(2)	1269.716	1269.7081	68.63

S*: Phosphorylated serine. M°: Oxidised methionine.

- Ion exchange chromatography of α-casein enriched fraction.

During the IEX run, Fractions (6 ml) were collected, fractions corresponding to the individual peaks were pooled and dialysed using a 12,400 Da molecular weight cut-off membrane against distilled water at 4°C.The first peak eluted in fractions 4-25 , the second peak eluted in fractions 31-35 and the third peak eluted in fractions 37-51. From the urea-PAGE (Figure 2.10, lane 14 and 15), RP-HPLC (Figure 2.11) and GPC (Figure 2.12) analysis it was observed that the 3rd peak obtained after the IEX chromatographic separation of the α-casein enriched fraction (Figure 2.14) was enriched in both α_{s1}- and α_{s2}-caseins and the sample was free of β-casein. The weight of sample obtained was 254 mg, which corresponds to approximately 31% combined yield of α_{s1}- and α_{s2}-casein from the original starting material (1l of 3% (w/v) acid caseinate solution), assuming that 45 and 11.7% of α_{s1}- and α_{s2}-caseins, respectively, are present in acid caseinate.

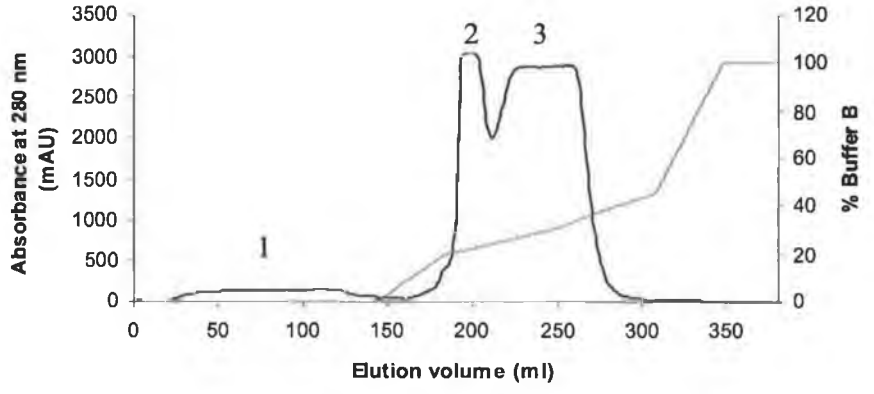


Figure 2.14. Chromatogram showing the purification of α-casein from acid casein (Protocol 4) using an ion exchange column. Absorbance at 280 nm and concentration of buffer B are given.

2.5. Conclusion

Based on the results presented in this Chapter it can be proposed that, for the large scale isolation of enriched β-casein, the procedure described in Section 2.3.1 (Protocol 4) can be followed without including a chromatographic step to obtain an enriched β-casein with an approximate yield of 11%. From a research prospective the method described in section 2.3.1 (Protocol 4) is an effective protocol for obtaining electrophoretically and chromatographically pure β-casein free of any other protein or peptide contaminant with a yield of approximately 5%. Furthermore, by following Protocol 4, fractions enriched in α_{s1}- and α_{s2}-caseins free from β-casein contamination can be obtained with a yield of approximately 31%.

For future studies, it is proposed after adjusting the pH of the curd sample following holding at 4°C for 24 h to pH 4.6 that the sample could be held at this pH for a further 24 h at 4°C with gentle stirring. This additional step may increase the yield of enriched β-casein by facilitating its further dissociation from the α-casein precipitate.

References

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on the caseins. *J. Dairy Res*, **50**, 45-55.

Aschaffenburg, R. (1963). Preparation of β-casein by a modified urea fractionation method. *J. Dairy Res*, **30**, 259-260.

Bouchier, P.J. (1999). Hydrolytic and peptide debittering properties of purified lactococcal aminopeptidases. Ph.D. dissertation, *National University of Ireland, Galway.*

Brown, R. J. & Ernstrom, C. A. (1988). Milk clotting enzymes and cheese chemistry. In: Noble P. Wong (Ed.), *Fundamentals of Dairy Chemistry,* 3rd ed. (p 619).Van Nostrand Reinhold, New York, U.S.A.

Buchheim, W. & Schmidt, D. G. (1979). On the size of monomers and polymers of β-casein. *J. Dairy Sci*, **46 (2)**,277-280.

Creamer, L. K. & Berry, G. P (1975). A study of properties of dissociated bovine casein micelles. *J. Dairy Res,* **42**, 169-183.

Dickson, I. R. & Perkins, D. J. (1971). Studies on the interactions between purified bovine caseins and alkaline-earth metal ions. *Biochem. J.*, **124 (1)**, 235-240.

Evans, M. T. A., Phillips, M. & Jones, M. N. (1979). The conformation and aggregation of bovine β-casein A. II. Thermodynamics of thermal association and the effects of changes in polar and apolar interactions on micellization. *Biopolymers,* **18 (5)**, 1123-1140.

Farrell, H. M., Jr., Jimenez-Flores, R.,Bleck, G. T., Brown, E. M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K. F. & Swaisgood, H.E.

(2004). Nomenclature of the proteins of cow's milk-sixth revision. *J. Dairy Sci.***87(6)**, 1641-1674.

Farrell, H. M., Jr., Wickham, E. D., Unruh, J. J., Qi, P. X. & Hoagland, P. D. (2001). Secondary structural studies of bovine caseins: Temperature dependence of β-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization. *Food Hydrocoll.* **15**, 341-354.

Fox, P. F., Law, J., McSweeney. P. L. H. & Wallace, J. (1993). Cheese: Chemistry, Physics and Microbiology. In: P. F. Fox (Ed.), *Biochemistry of Cheese Ripening*, 2nd ed. (p 399). Chapman and Hall, London, England.

Groves, M., McMeekin, T., Hipp, N. & Gordon, W. (1962). Preparation of β- and γ-caseins by Column chromatography. *Biochim. Biophys Acta*, **57**, 197-203.

Hipp, N. J., Groves, M. L., Custer, J. H., & McMeekin, T. L. (1952). Separation of α, β and γ- casein. *J.Dairy Sci,* **35 (3)**, 272-281.

Huppertz, T., Hennebel, J.B., Considine, T., Shakeel Ur, R., Kelly, A. L. & Fox, P. F. (2006). A method for the large-scale isolation of β-casein. *Food Chem*, ***99*(1)**, 45-50.

Law, A. J. R & Leaver, J. (2004). Method for extracting casein fractions from milk and caseinates and production of novel products. *United States Patent Application,* Publication No. US2004/0234666A1.

Livney, Y. D., Schwan, A. L. & Dalgleish D. G. (2004). A study of β-casein tertiary structure by intramolecular crosslinking and mass spectrometry. *J. Dairy Sci,* **87 (11)**, 3638-3647.

Murphy, J. M. & Fox, P. F. (1991). Fractionation of sodium caseinate by ultrafiltration. *Food Chem*, **39 (1)**, 27-38.

Parker,T.G. & Dalgleish, D.G. (1981). Binding of calcium ions to bovine β-casein. *J. Dairy Sci,* **48 (1)**, 71-76.

Poulik, M. D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. *Nature*, **180**, 1477-1479.

Schmidt, D. G. & Payens, T. A. J., (1972). Evaluation of positive and negative contributions to the second virial coefficient of some milk proteins. *J. Colloid Interface Sci*, **39 (3)**, 655-662.

Smyth, M. & FitzGerald, R. J. (1998). Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int. Dairy J.*, **8 (9)**, 819-827.

Spellman, D., Kenny, P., O'Cuinn, G. & FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with Bacillus licheniformis proteinase activities. *J Agric Food Chem*, **5(4)**,1258-1265.

Spellman, D., O'Cuinn, G. & FitzGerald, R. J. (2009). Bitterness in Bacillus proteinase hydrolysates of whey proteins. *Food Chem*, **114 (2)**, 440-446.

Swaisgood, H. E. (1982). Chemistry of caseins. In: P.F. Fox (Ed.), *Advanced Dairy Chemistry*, Vol:3 (pp 1-59). Applied Science, London.

Thompson, M. P. (1966). DEAE-cellulose-urea chromatography of casein in the presence of 2-mercaptoethanol. *J Dairy Sci*, **49 (7)**, 792-795.

Thompson, M. P., Gordan, W. G. & Boswell, T.T. Jr. (1969). Solubility, solvation and stabilization of α_{s1} and β-casein. *J. Dairy Sci*, **52 (8)**, 1166-1173.

Thompson, M. P & Kiddy, C. A. (1964). Genetic polymorphism is cow's milk iii. Isolation and properties of α_{s1}-caseins A, B and C. *J. Dairy Sci*, **47 (6)**, 626-632.

Thompson, M. P., Kiddy, C. A., Johnston, J.O. & Weinburg, R.M. (1964). Genetic polymorphism in caseins of cow's milk. II. Conformation of the genetic control of β-casein variation. *J. Dairy Sci*, **47(4)**, 378-381

Thompson, M. P. & Pepper, L. (1964). Genetic polymorphism in casines of cow's milk.iv. Isolation and properties of β-Casines A, B and C. *J. Dairy Sci,* **47 (6),** 633-637.

Ward, L. S. & Bastian, E. D. (1996). A method for isolating β-casein. *J. Dairy Sci*, **79 (8),** 1332-1339.

Ward, L. S. (1998). Isolation of caseins fractions by manipulating inherent solubility and association properties of caseins. Ph.D. Dissertation, *University of Minnesota,* St. Paul, USA, pp 2-49.

Warner, R. C. (1944). Separation of α and β-casein. *J. Am. Chem. Soc.*, **66**, 1725-1731.

Chapter 3

Purification and characterization of glutamyl endopeptidase (GE).

3.1. ABSTRACT

A protocol for the purification of glutamyl endopeptidase (GE) from Alcalase™ 2.4 L was developed using hydrophobic interaction chromatography (HIC) and ion-exchange (IEX) chromatography. The yield of GE obtained was approximately 42%. The pH optimum of GE was found to be around pH 7.5-8.0. The molecular weight was estimated using SDS-PAGE and observed to be approximately 24 kDa. Addition of Ca^{2+} ions had no effect on the stability of GE when stored at three different temperatures (4°C, 20°C and -20°C) for 2 months. GE activity was stable on heating at 50°C for 4 h, however, at 90°C a complete loss in the GE activity was observed after 5 min heating. The Michaelis constant (K_m) was estimated to be 3.33 and 10.80 mM at 37°C and 50°C, respectively, using acetyl-glutamic acid-paranitroanalide. GE was partially inhibited by 1 and 10 mM ethylenediaminetetraacetic acid (EDTA) and no significant loss in activity was observed in the presence of 1 and 10 mM phenylmethylsulphonylfluoride (PMSF).

3.2. Introduction

Serine proteinase are divided into three groups based on the cleavage preferences at the carboxy terminal of positively charged, large apolar and small apolar amino acid residues. These are termed as trypsin, subtilisin/chymotrypsin and elastase-like proteinases, respectively (Stennicke *et al.*, (1996). Glutamyl endopeptidase (GE) belongs to a class of serine proteinases and a sub-family of chymotrypsin-like proteinases that specifically cleave after acidic amino acid residues (Rudenskaya, 1998). Bacterial GE can be divided into three groups based on organism origin and primary sequence relationships, i.e., into Staphylococcal, Bacillus and Streptomyces groupings (Stennicke and Breddam, 1998). Drapeau *et al.*, (1972) and Houmard and Drapeau (1972) were the first to describe proteinases that cleave specifically on the carboxy-terminal of acidic amino acids (Glu and Asp). The molecular weight of GE varies from 12 to 31 kDa and is dependent on bacterial source (Drapeau *et al.*, (1972), Yoshida *et al.*, (1988), Kakudo *et al.*, (1992), Svendsen and Breddam (1992), Yokoi *et al.*, (2001), Ohara-Nemato *et al.*, (2002), Kawalec *et al.*, (2005)).

Drapeau *et al.*, (1972) purified a proteinase that cleaved specifically on the carboxy-terminal of acidic amino acids (Glu and Asp) from *Staphylococcus aureus* strain V8 by a combination of procedures. These included precipitation of the culture filtrate with ammonium sulphate followed by acetone fractionation, an ion chromatography step on DEAE-cellulose and preparative electrolysis using polyacrylamide. The authors reported that the proteinase obtained was active in the pH range of pH 3.5 to 9.5 with a maximum activity at pH 4.0 and 7.8. The molecular weight was estimated to be approximately 12 kDa using SDS-PAGE. The authors reported a purification yield of 25.1%. The isolated proteinase was inhibited by diisopropylfluorophosphate (DFP) suggesting that it was a serine endopeptidase.

Svendsen and Breddam (1992) purified GE from Alcalase™, a commercially available proteolytic preparation from *Bacillus licheniformis*, by ion-exchange (IEX) and affinity chromatography (AC) with a yield of approximately 50%. The purified GE was observed to preferentially cleave peptide bonds on the carboxy side of acidic amino acid residues, predominantly at glutamic acid and to lesser extent at aspartic acid. However, the authors observed non-specific cleavage at the carboxy terminal of

phenylalanine during the hydrolysis of ribonuclease at high GE concentration (1.2 μM). The isolated GE consisted of a single peptide chain of 222 amino acid residues with a molecular mass of 23,589 Da. The pH optimum was observed to be between pH 7.5-8.0. It was completely inhibited by DFP suggesting that GE from *B. lichenformis* was also a serine endopeptidase. Kakudo *et al.,* (1992) purified GE from *Bacillus lichenformis* ATCC 14580 by IEX, hydrophobic interaction (HIC) and AC with a yield of 30.3%. The authors reported that GE was completely inhibited by DFP and Boc-Leu-Glu-chloromethyl ketone thereby confirmed that GE was a serine proteinase.

Yokoi *et al.,* (2001) purified GE from *Staphylococcus warnier* strain M using ion-exchange (IEX) and size exclusion chromatography (SEC) and reported a yield of 50.4%. Kawalec *et al.*, (2005) purified GE from *Enterococcus faecalis* using a combination of procedures which included acetone precipitation followed by IEX and SEC. Spellman *et al.,* (2005) purified GE from Alcalase™ 2.4 L using HIC on phenyl Sepharose.

The work carried out by Spellman *et al.,* (2009) indicated that GE activity contributed to high bitterness levels in whey protein hydrolysates. The authors generated whey protein hydrolysates using two *B. licheniformis* preparations, i.e., Alcalase 2.4 L and Prolyve 1000. The difference between those two proteinase preparations was the presence of GE activity in Alcalase 2.4 L, which was not detected in Prolyve 1000. GE was also observed to play a role in peptide aggregation in whey protein hydrolysates (Spellman *et al.,* 2005).

The objective of this study was to purify GE from *Bacillus licheniformis* (Alcalase™ 2.4 L) and to characterize the purified enzyme activity.

3.3. Materials and methods

Alcalase™ 2.4 L was obtained from Novozymes (Bagsvaerd, Denmark). Acetyl-Glu-paranitroanilide (ac-Glu-pNA), succinyl-Ala-Ala-Phe-paranitroanilide (s-AAF-pNA), succinyl-Ala-Ala-Ala-paranitroanilide (s-AAA-pNA), Benzoyl-Arginine-paranitroanilide (Bz-Arg-pNA) and succinyl-Gly-Pro-paranitroanilide (s-Gly-Pro-pNA) were from Bachem, GmbH (Hegenheimer, Germany). Ethanol (96%w/v) and ammonium sulphate were from VWR International Ltd (Dublin, Ireland). N-ethylmaleimide (NEM), phenylmethylsulphonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), 1,10 phenanthroline, sodium phosphate dibasic, sodium phosphate mono basic, potassium chloride, sodium chloride, methanol, acetic acid, glycerol, sodium dodecyl sulphate (SDS), dialysis tubing (12,400 Da molecular weight cut-off membrane), boric acid, sodium hydroxide, calcium chloride, phenyl Sepharose® 6 fast flow (high substitution) were from Sigma-Aldrich (Dublin, Ireland).
(All the other general chemicals were obtained from Sigma Aldrich, Dublin, Ireland).

3.3.1. Purification of glutamyl endopeptidase (GE) from Alcalase

Three different protocols were used for the purification of GE from Alcalase.

Purification of glutamyl endopeptidase from Alcalase (Protocol 1)

GE was purified from Alcalase™ 2.4 L using a combination of HIC and IEX. HIC was carried out using a phenyl Sepharose® 6 fast flow (high substitution) matrix packed as per the manufacturers instructions into a XK 16/10 column (100mm x 16mm I.D, column volume 20 ml, Pharmacia Biotech, Cambridge, England). The HIC column was connected to an AKTA™ Purifier (GE Healthcare, Bucks, UK). The column was first equilibrated with 5 column volumes (100 ml) of buffer A (50 mM sodium phosphate buffer, pH 7.0, containing 500 mM ammonium sulphate and 500 mM KCl). All buffers were filtered through 0.2 µm filters (Puradisc, VWR International Ltd, Dublin, Ireland) prior to use. The Alcalase™ 2.4 L preparation was diluted 1:4 in buffer A and filtered through a 0.2 µm syringe filter (Puradisc, VWR International Ltd, Dublin, Ireland) prior to injection.

Sample (10 ml of diluted Alcalase preparation) was loaded onto the HIC column using a 10 ml super-loop (GE Healthcare, Bucks, UK). Chromatographic separation was by a linear gradient of buffer B (50 mM sodium phosphate buffer, pH 7.0). The gradient was generated over 7.5 column volumes (150 ml). After completion of the run, the column was washed using 1 column volume (20 ml) of 1M NaOH followed by 2 column volumes (40 ml) of distilled water. The flow rate was 1 ml min^{-1}. Fractions (3 ml) were collected and every third fraction was immediately analyzed for proteolytic activity using synthetic substrates. The substrates used were s-AAF-pNA for quantification of subtilisin activity and ac-Glu-pNA for quantification of GE activity. The substrate s-AAA-pNA for quantification of elastase activity was used for initial three HIC runs. The substrates s-Gly-Pro-pNA and Bz-Arg-pNA were used for the quantification of prolyl-dipeptidylpeptidase and plasmin activity, respectively, in the purified GE fraction. Assays were performed by the addition of 50 µl of enzyme solution to 450 µl of 1.11 mM substrate in 50 mM sodium phosphate buffer, pH 7.0. The assay mixture was incubated at 50°C for 15 min and the reaction was terminated by the addition of 1 ml of 1.5 M acetic acid (Spellman *et al.*, 2005). The quantity of paranitroanilide (pNA) released was determined by measuring the absorbance at 410 nm using an extinction coefficient of 8800 M^{-1} cm^{-1} for pNA at 410 nm (Yamangata *et al.*, 1995). Buffer instead of enzyme was used as control. Activity was expressed on the basis of the quantity of pNA released per min per mg of enzyme. Fractions containing GE activity were pooled and re-injected twice onto the HIC column. The active fractions obtained after the 3rd HIC step were dialysed using 12,400 Da molecular weight cut-off dialysis tubing against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h.

The dialysed fraction (45 ml) was injected using a 10 ml super-loop (GE Healthcare, Bucks, UK) onto an anion exchange column (Hiprep™ DEAE FF 16/10, GE Healthcare, Bucks, UK). Five injections were made (4 x 10 ml and 1 x 5 ml). The column was connected to an AKTA™ Purifier (GE Healthcare, Bucks, UK). Prior to injection the column was equilibrated with 5 column volumes (100 ml) of buffer A (50 mM sodium phosphate buffer, pH 7.0). Elution was by linear gradient of buffer B (50 mM sodium phosphate buffer, 400 mM NaCl, pH 7.0). The gradient was generated in 5 column volumes (100 ml). The flow rate was 1 ml min^{-1}. Fractions (3 ml) were collected and every third fraction was immediately analyzed for proteolytic

activity using the synthetic substrates as described earlier. Fractions enriched in GE did not bind to the IEX column and eluted during the injection step.

The GE enriched samples obtained after the IEX run were pooled and an appropriate amount of ammonium sulphate and KCl were added to bring the final concentration of both salts to 500 mM. The sample (51 ml) was injected onto the HIC column using a 50 ml super-loop (GE Healthcare, Bucks, UK). The HIC run was carried out as described earlier and fractions enriched in GE activity were pooled and dialysed using a 12,400 Da molecular weight cut-off dialysis membrane against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h. The sample after dialysis was stored in 30% (v/v) glycerol in the presence of 5 mM $CaCl_2$ at 4°C until further use.

Purification of glutamyl endopeptidase from Alcalase (Protocol 2)

Since the GE activity obtained using Protocol 1 was observed to be contaminated with low levels of subtilisin activity it was decided to further modify Protocol 1 to try to obtain pure GE activity. Therefore, it was decided to employ a GPC step in place of the final HIC run for the purification of GE. The method employed was as follows:

The initial three HIC runs followed by an ion exchange run as described in Protocol 1 were followed and GPC was employed as a final purification step. After the IEX run fractions enriched in GE activity were pooled and freeze dried using a Free Zone, 4.5 freeze-dryer system (Labconco, Kansas City, MO). The dried sample was re-suspended in 2 ml of 50 mM sodium phosphate buffer, pH 7.0, filtered using a 0.2 µm syringe filter and injected onto a HiLoad™ Superdex™ 75 pg column (column volume 120 ml, GE Healthcare, Bucks, UK) connected to an AKTA™ Purifier. The column was equilibrated with 2 column volumes (240 ml) of 50 mM sodium phosphate buffer, pH 7.0 prior to injection. Chromatographic separation was by isocratic elution with 50 mM sodium phosphate buffer, pH 7.0. The flow rate was 0.5 ml min^{-1}. Fractions (3 ml) were collected. Fractions enriched in GE activity were diluted in 30% (v/v) glycerol and $CaCl_2$ was added to bring the final concentration to 5 mM. These samples were then stored at 4°C until further use.

As described earlier, throughout all the chromatographic runs, every third fraction was analysed for the presence of GE and subtilisin activity using the synthetic substrates,

every third fraction for the initial three HIC steps was analysed for the presence of elastase activity. The final purified GE fraction obtained was analysed for the presence of prolyl-dipeptidylpeptidase and plasmin activity.

Purification of glutamyl endopeptidase from Alcalase (Protocol 3)

The GE sample obtained from Protocol 2 was also found to be contaminated with low levels of subtilisin activity. The subtilisin activity co eluted with the GE activity.

Since an enriched GE preparation was obtained using HIC, it was decided to follow the initial two HIC runs as described in Protocol 1 without change but for the third HIC run it was decided to extend the gradient volume from 7.5 column volumes (150 ml) to 12 column volumes (240 ml). After the extended third HIC run, the enriched GE fractions obtained were concentrated by reapplying the active fractions onto the HIC column and then eluting the sample in 100% buffer B. The eluted sample was then dialysed using a 12,400 Da cut-off dialysis tubing against 50 mM sodium phosphate buffer, pH 6.2 overnight (12 h) at 4°C. The dialysed sample (63 ml) was injected using a 50 ml super-loop (GE Healthcare, Bucks, UK) onto a HiTrap™-CM FF cation exchange column (column volume 5 ml, GE Healthcare, Bucks, UK). Two injections were made (1 x 50 ml and 1 x 13 ml). Prior to sample injection, the column was equilibrated using 5 column volumes (25 ml) of buffer A (50 mM sodium phosphate buffer, pH 6.2). Chromatographic separation was by a linear gradient to 100% buffer B (50 mM sodium phosphate buffer 0.5 M NaCl, pH 6.2) in 10 column volumes (50 ml). The flow rate was 1 ml min^{-1}. Fractions (3 ml) were collected. Fractions enriched in GE activity were dialysed using 12,400 Da molecular weight cut-off membrane against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h. The dialysed sample was diluted in 30% (v/v) glycerol and $CaCl_2$ was added to bring the final concentration to 5 mM. These samples were then stored at 4°C until further use.

As described earlier, throughout all the chromatographic runs, every third fraction was analysed for the presence of GE and subtilisin activity using the synthetic substrates, every third fraction for the initial three HIC steps was analysed for the presence of elastase activity and the purified GE fraction obtained was analysed for the presence of prolyl-dipeptidylpeptidase and plasmin.

3.3.2. Characterization of glutamyl endopeptidase

The GE sample obtained using Protocol 2 was used for these characterization studies.

Determination of Michaelis constant (K_m)

The K_m of GE was determined at both 50°C and 37°C using ac-Glu-pNA. A stock substrate solution (4 mM) was made in 50 mM sodium phosphate buffer, pH 7.0 and solutions of 3, 2, 0.8, 0.6 and 0.4 mM were prepared from the 4 mM stock solution by making necessary dilutions in 50 mM sodium phosphate buffer, pH 7.0. A stock solution of 1.11 mM of ac-Glu-pNA was also used.

Assays were performed by the addition of 50 µl of appropriately diluted enzyme solution to 450 µl of substrate solution at the above mentioned concentrations. Therefore, the working concentration of the ac-Glu-pNA solutions was 3.6, 2.7, 1.8, 1, 0.72, 0.54 and 0.36 mM. The assay mixture was incubated at 50°C/37°C for 30 min before termination by the addition of 1 ml of 1.5 M acetic acid. The quantity of paranitroanilide (pNA) released was determined by measuring the absorbance at 410 nm using an Ultraspec 2000 (Pharmacia Biotech, Cambridge, England) at an extinction coefficient of 8800 M^{-1} cm^{-1} for pNA at 410 nm (Yamangata *et al.*, 1995). Reaction rate was expressed on the basis of the quantity of pNA released per sec. A Michaelis–Menten curve was plotted by plotting initial rate (v), against substrate concentration [s] and Lineweaver-Burk plots were generated by plotting 1/v sec $nmol^{-1}$ against 1/[s] mM^{-1}. In Lineweaver-Burk plots the slope represents K_m/Vmax (maximal rate) and the y-intercept equals 1/Vmax. The equation of the line obtained was used to calculate Vmax and K_m (Stryer, 1995).

Inhibitory studies

Commonly used proteolytic inhibitors such as N-ethylmaleimide (NEM), phenyl methyl sulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and 1,10 phenanthroline which mostly inhibit proteinases belonging to cysteine, serine and metallo proteinase classes (Nagase and Salvesen, 1989 and North, 1989) were used to determine the effect of inhibitors on GE. Stock solutions of 10 mM and 100 mM

NEM, PMSF and 1,10 phenanthroline were prepared in 96% (v/v) ethanol, whereas EDTA was prepared in 50 mM sodium phosphate buffer, pH 7.0. A final working concentration of 1 and 10 mM inhibitors were maintained in the reaction mixture and the inhibitory studies were performed by the addition of 50 µl of enzyme solution to 450 µl of substrate solution (400 µl of 1.11 mM ac-Glu-pNA and 50 µl of inhibitor solution). The enzyme assay was performed using ac-Glu-pNA as described earlier. For the control samples, 50 mM sodium phosphate buffers, pH 7.0 and 96% (v/v) ethanol were used. The percentage inhibition was reported as the difference in initial rates of the test versus control samples.

pH optimum studies

The pH optimum of GE was estimated over the pH range 6 to 10. For pH 6.0 to 8.0, 50 mM sodium phosphate buffer was used, whereas, for pH 8.5 to 10.0, 50 mM Clark and Lubs solution (KCl, H_3BO_3 and NaOH) was used (Bower and Bates, 1952). The initial rate (v) was plotted against pH to determine the optimum pH of GE (Plummer, 1978). The assay was performed using ac-Glu-pNA as described earlier. Rate was expressed on the basis of the quantity (n moles) of pNA released per sec.

Effect of Ca^{2+} at different storage temperatures

The substantially purified enzyme obtained using Protocol 2 was stored in the presence of 30% (v/v) glycerol, 0.02% (w/v) sodium azide, with and without 5 mM calcium chloride. Three different storage temperatures, i.e., -20°C, room temperature (20°C) and 4°C were studied. GE activity was monitored over a two month storage period.

Temperature stability studies

The temperature stability of GE at four different temperatures, i.e., 50°C, 60°C, 70°C and 90°C was monitored by maintaining GE (1 ml, 96.24 µmol min^{-1} mg^{-1}) at the above temperatures in a water bath for different time intervals. Enzyme assays were performed using ac-Glu-pNA as described earlier. The loss in activity is reported as the percentage drop in the initial rate (v) as a function of holding time.

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to estimate the molecular weight of GE. A Mini-Protean®ii electrophoresis system (Bio-Rad, Hertfordshire, UK) supplied with a power pack 1000 (Bio-Rad, Hertfordshire, UK) was used to run the gel. The procedure described by Laemmli (1970) was followed. Lyophilised test samples were dissolved in sample buffer at a concentration of 10 mg ml^{-1}.

Determination of protein content

The protein content of the enzyme preparations was determined by the modified Lowry *et al.,* (1951) method (Bensadoun and Weinstein (1976)). Bensadoun and Weinstein (1976) included a protein precipitation step using trichloroacetic acid, and sodium deoxycholate to the method described by Lowry *et al.,* (1951).

3.4. Results and Discussion

3.4.1. Purification of glutamyl endopeptidase from Alcalase (Protocol 1)

As outlined in section 2.1. (Protocol 1) the purification of GE was carried out by injecting Alcalase™ 2.4 L diluted 1:4 in buffer A onto the phenyl Sepharose HIC column. The active fractions were reapplied after a further two HIC runs (Figures 3.1-3.3), IEX (Figure 3.4) and HIC (Figure 3.5). GE and subtilisin activity was assayed throughout with the aid of ac-Glu-pNA and s-AAF-pNA, respectively. Whereas, elastase activity was assayed for the initial three HIC runs using s-AAA-pNA.

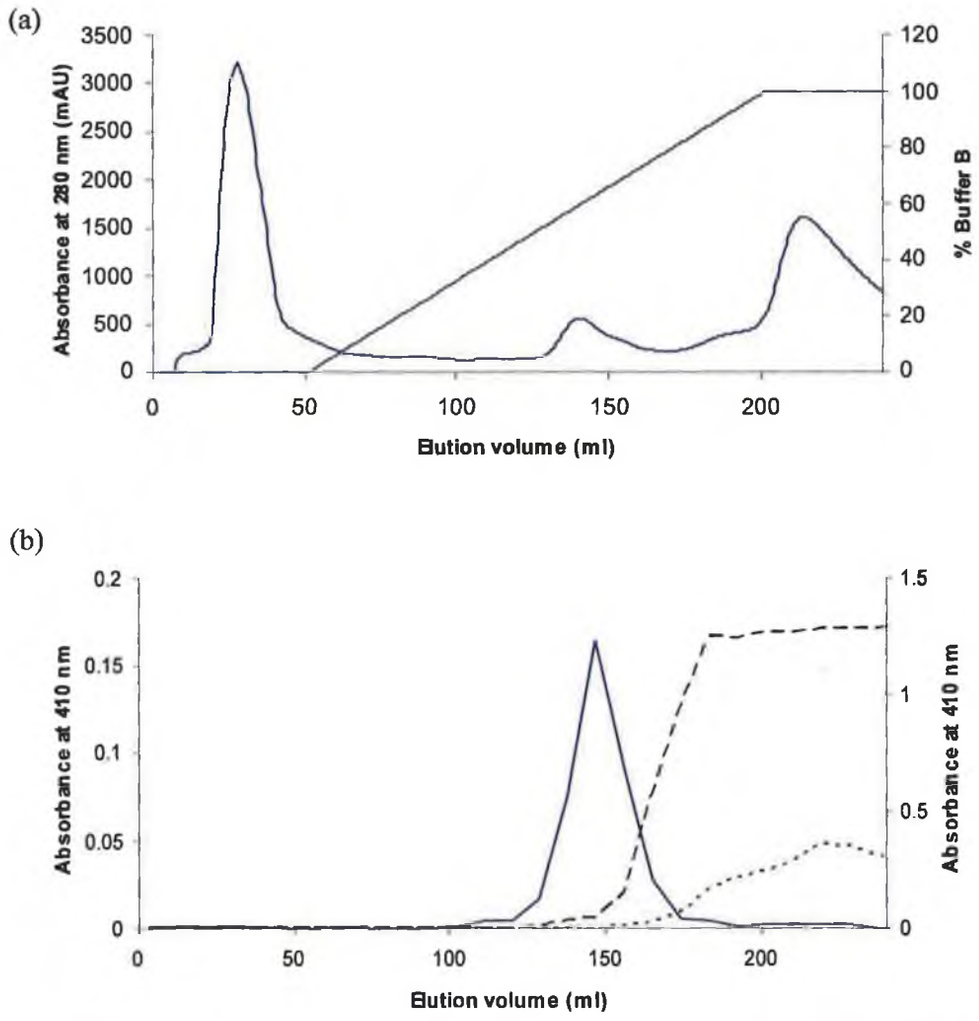


Figure 3.1. (a) Chromatogram showing the separation of Alcalase™ 2.4 L by hydrophobic interaction chromatography (HIC). Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values were expressed as Abs at 410 nm per ml of fraction for ac-Glu-pNA (___, left hand side), s-AAF-pNA (---, right hand side) and s-AAA-pNA(....., right side).

From Figure 3.1a, three distinct peaks can be observed. Enzyme activity analyses (Figure 3.1b) showed that the initial peak eluting between 10 and 60 ml had no proteolytic activity, the peak eluting between 130 and 170 ml contained GE activity while subtilisin and elastase activity was observed from 150 ml until the completion of the run. These results are in agreement with those reported by Spellman *et al.,* (2005) where good separation was obtained but where the GE activity was contaminated with the coeluting subtilisin activity especially in the later eluting fractions containing GE activity. It can be observed that subtilisin and elastase activities were coeluting, indicating that subtilisin may also exhibit low levels of

elastase like activity. The peak enriched in GE activity (fraction 44 to 57, Figure 3.1) was pooled giving a total volume of 42 ml after the 1st HIC run. Appropriate amounts of ammonium sulphate and potassium chloride were added to the pooled fractions to achieve a final concentration of both salts in solution of 500 mM.

The pooled fractions (42 ml) were re-injected onto the HIC column for further purification of GE. The elution profile and the proteolytic activity of the different fractions obtained during the second HIC run are given in Figure 3.2. These results again show that fractions containing GE also had a subtilsin contaminant activity. Elastase activity was observed to be significantly lower in fractions containing GE activity.

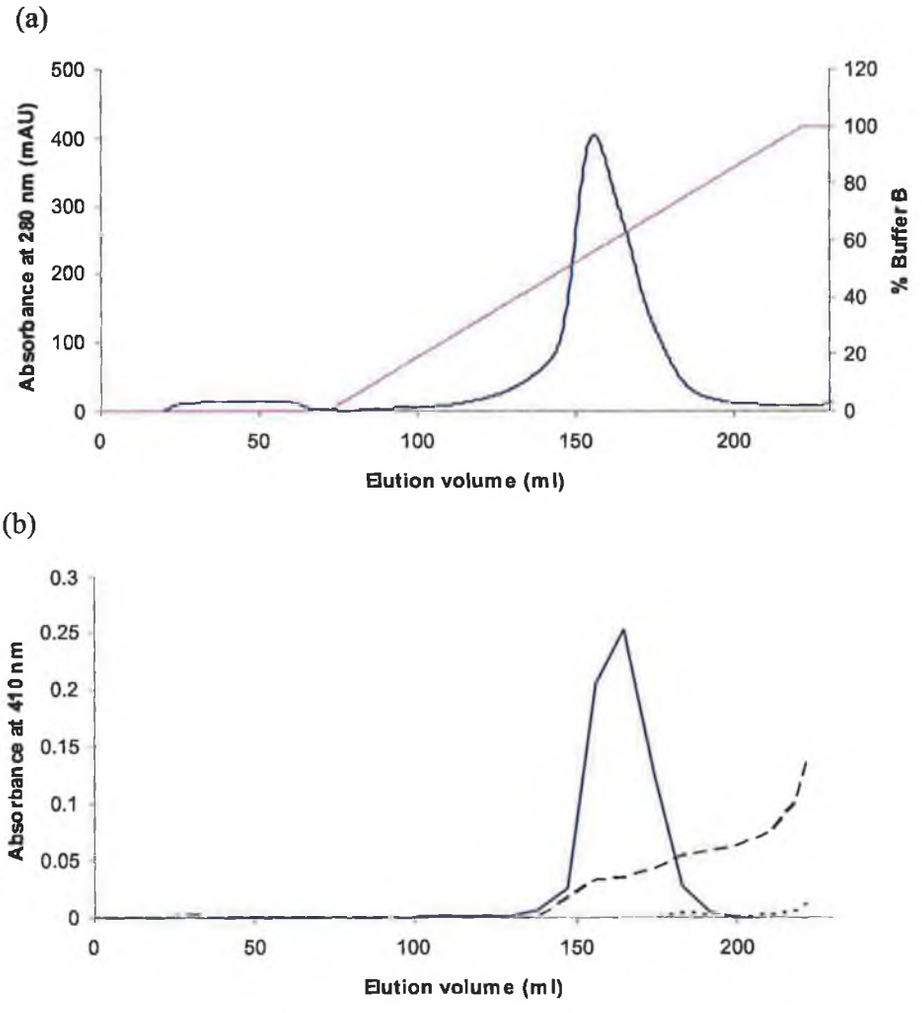


Figure 3.2. (a) Hydrophobic interaction chromatography (HIC) chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from the first HIC run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___), s-AAF-pNA (----) and s-AAA-pNA(.....).

The peak enriched in GE activity (fraction no. 47 to 61, Figure 3.2) was pooled. Appropriate amounts of ammonium sulphate and potassium chloride were added to the pooled fractions (45 ml) to bring the final concentration of both salts in solution to 500 mM. The pooled fractions (45 ml) were rechromatographed for the third time on the HIC column in an attempt to separate the subtilisin contaminant from GE. The HIC chromatogram and the proteolytic activity of the different fractions obtained during third run are given in Figure 3.3.

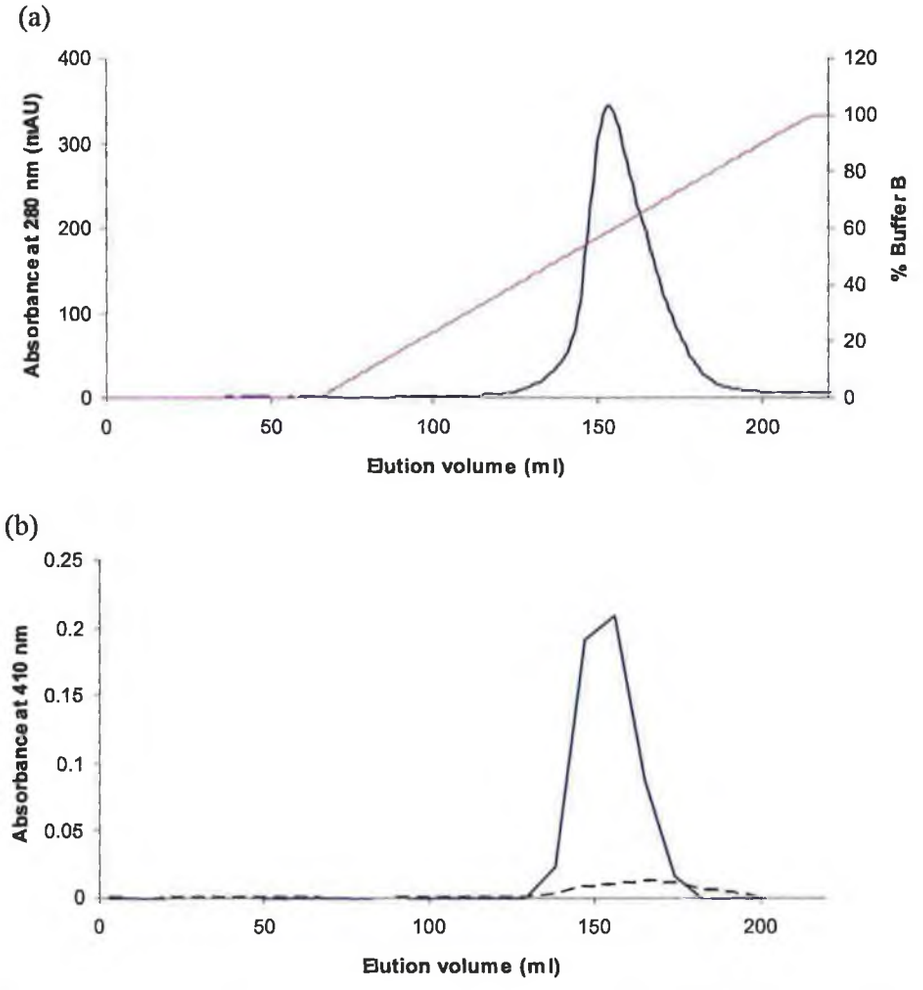


Figure 3.3. (a) Hydrophobic interaction chromatography (HIC) chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from the second HIC run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___) and s-AAF-pNA (----).

Again it was observed that the third HIC step was not capable of fully separating the subtilisin contaminat from GE activity. No elastase activity was observed in fractions enriched in GE activity. The fractions containing GE activity (fraction 47 to 60, Figure 3.3) were pooled and a total volume of 45 ml was obtained. The pooled fractions were dialysed against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h. The dialysed pooled fractions were then injected onto a Hiprep™ DEAE FF 16/10 column. The IEX chromatogram and the proteolytic activity of the different fractions obtained during the IEX run are given in Figure 3.4.

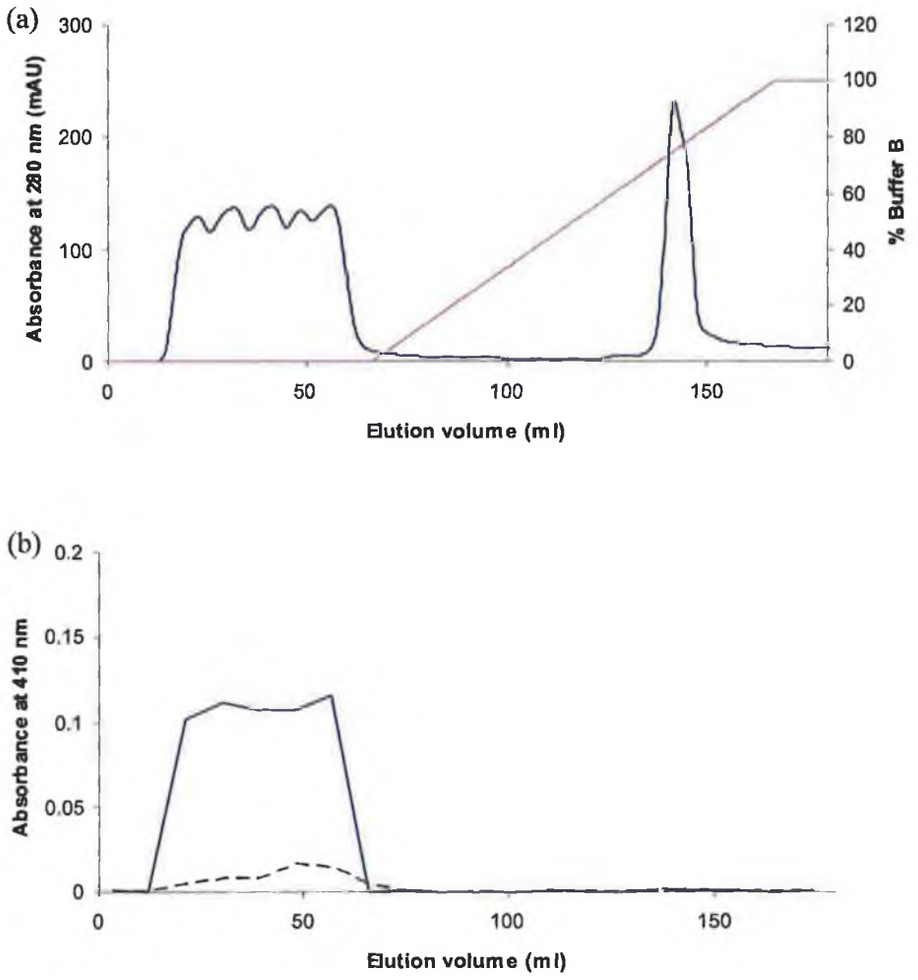


Figure 3.4. (a) Anion exchange chromatogram showing the separation of dialysed enriched glutamyl endopeptidase activity fractions obtained from the third hydrophobic interaction chromatographic run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___) and s-AAF-pNA (----).

From Figure 3.4 it can be observed that under the chromatographic conditions the pooled fractions enriched in GE activity as well as the subtilisin contaminant did not bind to the IEX column and eluted during sample injection. The fractions enriched in GE activity (fraction 6 to 22, Figure 4) yielded a total volume of 51 ml. It is also observed from Figure 4a, that the peak eluting between 130 and 150 ml had no activity against ac-Glu-pNA or s-AAF-pNA. This indicates that, while the activities did not bind to this column, some purification had been achieved by using this IEX step.

Appropriate amounts of ammonium sulphate and potassium chloride were added to the pooled fractions (51 ml) to bring the final concentration of both salts in solution of 500 mM. The pooled fractions (51 ml) were again injected onto the HIC column in an attempt to further purify the enriched GE activity. The HIC chromatogram and the proteolytic activity in the different fractions obtained during this run are given in Figure 3.5.

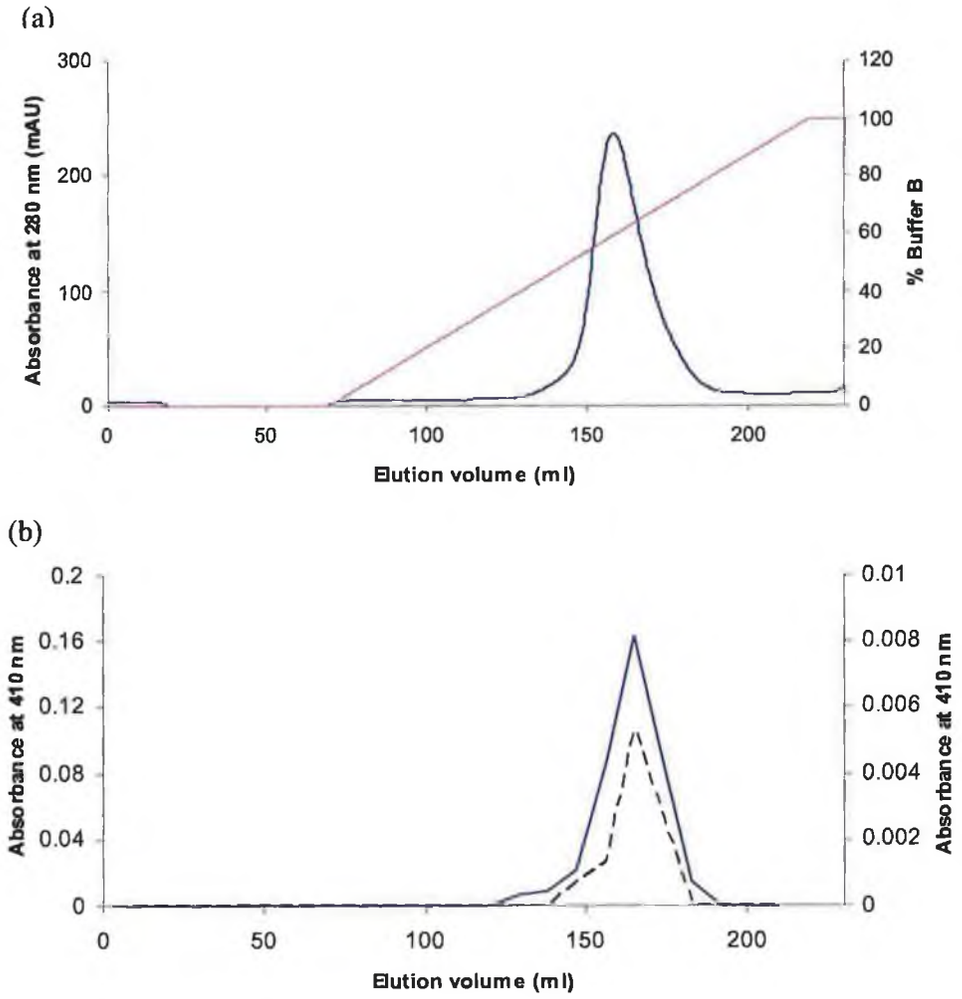


Figure 3.5. (a) Hydrophobic interaction chromatography (HIC) chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from the ion exchange chromatographic run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___, left hand side) and s-AAF-pNA (----, right hand side).

From the Figure 3.5b, it can be seen that the GE as well as subtilisin activity co-eluted during this 4th HIC run. The fractions enriched in GE activity were pooled (fractions

50 to 61, Figure 3.5). The pooled fractions (39 ml) were dialysed using a 12,400 Da cut-off dialysis membrane against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h. The dialysed sample was diluted in 30% (v/v) glycerol and an appropriate amount of $CaCl_2$ was added to bring to a final concentration of 5 mM. The sample was then stored at 4°C until further use. The purified GE did not have any activity towards Bz-Arg-pNA and S-Gly-Pro-pNA. The purification tables for GE and subtilisin obtained using Protocol 1 are given in Tables 3.1 and 3.2.

Table 3.1. Glutamyl endopeptidase (GE) purification table obtained during the purification of GE from Alcalase™ 2.4 L using Protocol 1. The units (U) for activity are given as µmol min^{-1}.

Sample	Volume (ml)	Activity (U/ml)	Total activity	Total Protein (mg)	Specific activity (U/mg)	Yield
Alcalase	10	107.10	1071.00	200	5.35	100.00
1st HIC	41	19.14	803.88	8.36	96.15	75.05
2nd HIC	45	14.36	646.20	7.25	89.13	60.33
3rd HIC	42	14.69	616.98	5.07	121.69	57.60
IEX	51	12.04	614.04	4.91	125.05	57.33
4th HIC	39	10.45	407.55	3.69	110.44	38.05

HIC: Hydrophobic interaction chromatography and IEX: Ion-exchange chromatography.

Table 3.2. The subtilisin purification table obtained during the purification of glutamyl endopeptidase from Alcalase™ 2.4 L using Protocol 1. The units (U) for activity are given as µmol min^{-1}.

Sample	Volume (ml)	Activity U/ml	Total activity	Total Protein (mg)	Specific activity (U/mg)	Yield
Alcalase	10	879.50	8795.00	200	43.10	100.00
1st HIC	41	40.60	1664.50	8.36	199.10	18.93
2nd HIC	45	3.88	174.60	7.25	24.08	1.10
3rd HIC	42	1.28	53.76	5.07	10.60	0.61
IEX	51	1.46	74.46	4.91	15.16	0.84
4th HIC	39	0.50	19.50	3.69	5.30	0.22

HIC: Hydrophobic interaction chromatography and IEX: Ion-exchange chromatography.

From the purification tables (Tables 3.1 and 3.2) it was observed that approximately 38% of GE activity was recovered, however, this contained low levels (5.30 µ mol min^{-1} mg^{-1}) of a subtilisin contaminant. From Tables 3.1 and 3.2, it was also observed after the first HIC run that 25% of GE activity was lost, however, a significant reduction (81%) in subtilisin was also achieved. During the second HIC run a further

15% and 18% of GE and subtilisin, respectively, were lost. After the third HIC step no significant reduction in subtilisin contaminant was observed, whereas the GE yield decreased from 57 to 38%. This implied that the chromatographic steps after the third HIC were not effective. Furthermore, it was observed that low levels of subtilisin co-eluted with GE throughout all the chromatographic runs (Figures 3.1-3.5). The specific activity of GE as well as subtilisin increased significantly after the first HIC step. However, a small decrease in GE specific activity and a significant decrease in subtilisin activity was observed after the second HIC step. After the third HIC and IEX steps a gradual increase in GE specific activity was observed. However, after the final HIC step a decrease in specific activity of GE was observed. Therefore, it can be concluded that the chromatographic steps after the third HIC were not effective.

3.4.2. Purification of glutamyl endopeptidase from Alcalase (Protocol 2)

The chromatograms obtained during the chromatographic runs associated with Protocol 2 are given in Figures 3.6-3.10.

After the first HIC run the fractions enriched in GE activity (fraction 38 to 53, Figure 3.6) were pooled and a total volume 48 ml was obtained after the 1st HIC run. Appropriate amounts of ammonium sulphate and potassium chloride was added to the pooled fractions (48 ml) to bring final concentration of both salts in solution of 500 mM.

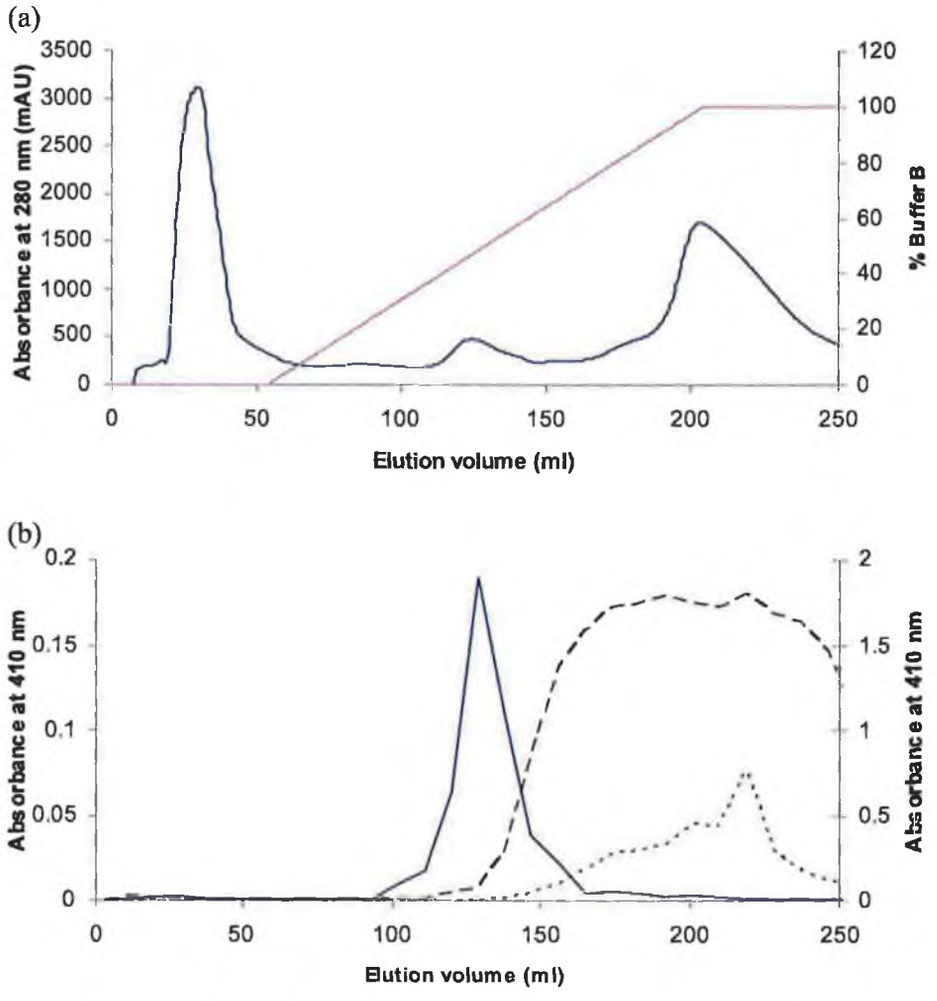


Figure 3.6. (a) Chromatogram showing the separation of Alcalase™ 2.4 L by hydrophobic interaction chromatography. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values were expressed as Abs at 410 nm per ml of fraction for ac-Glu-pNA (___, left hand side), s-AAF-pNA (---, right hand side) and s-AAA-pNA(....., right side).

The elution profiles observed after the first HIC step were similar to that observed in Protocol 1 (Figure 3.1). The enriched GE fractions (48 ml) were re-injected onto the HIC column for the further enrichment of GE activity. The HIC chromatogram and the proteolytic activity of different fractions obtained during the second run are given in Figure 3.7.

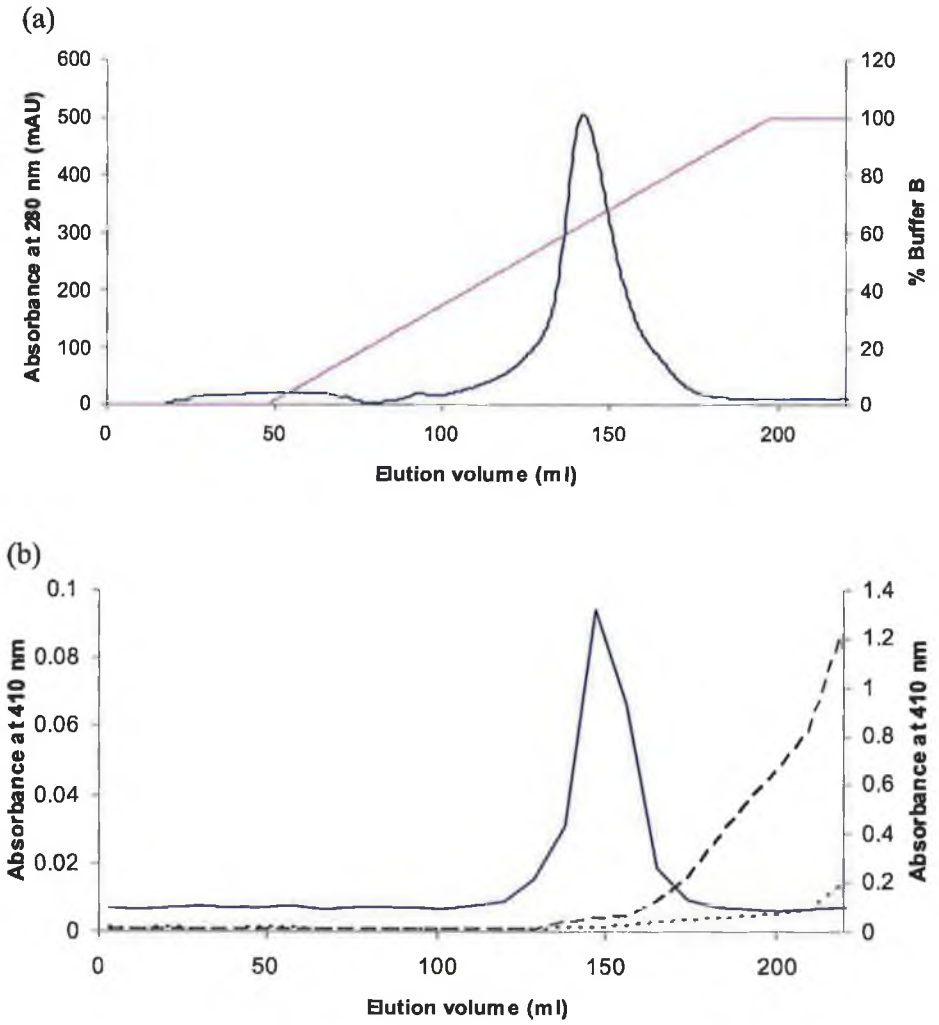


Figure 3.7. (a) Hydrophobic interaction chromatography chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from the first HIC run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___, left hand side) and s-AAF-pNA (----, right hand side) and s-AAA-pNA(….., right side).

The fractions enriched in GE activity, (fraction 42 to 54, Figure 3.7) were pooled and a total volume of 39 ml was obtained. An appropriate amount of ammonium sulphate and potassium chloride was added to the pooled fractions to bring final concentration of both salts in solution of 500 mM. The pooled fractions (39 ml) were re-injected for the 3rd time onto the HIC column in an attempt to separate the subtilisin contaminant from the enriched GE activity. The HIC chromatogram and the proteolytic activity of

the different fractions obtained during the third run are given in Figure 3.8. It was observed that subtilisin again co-eluted with GE.

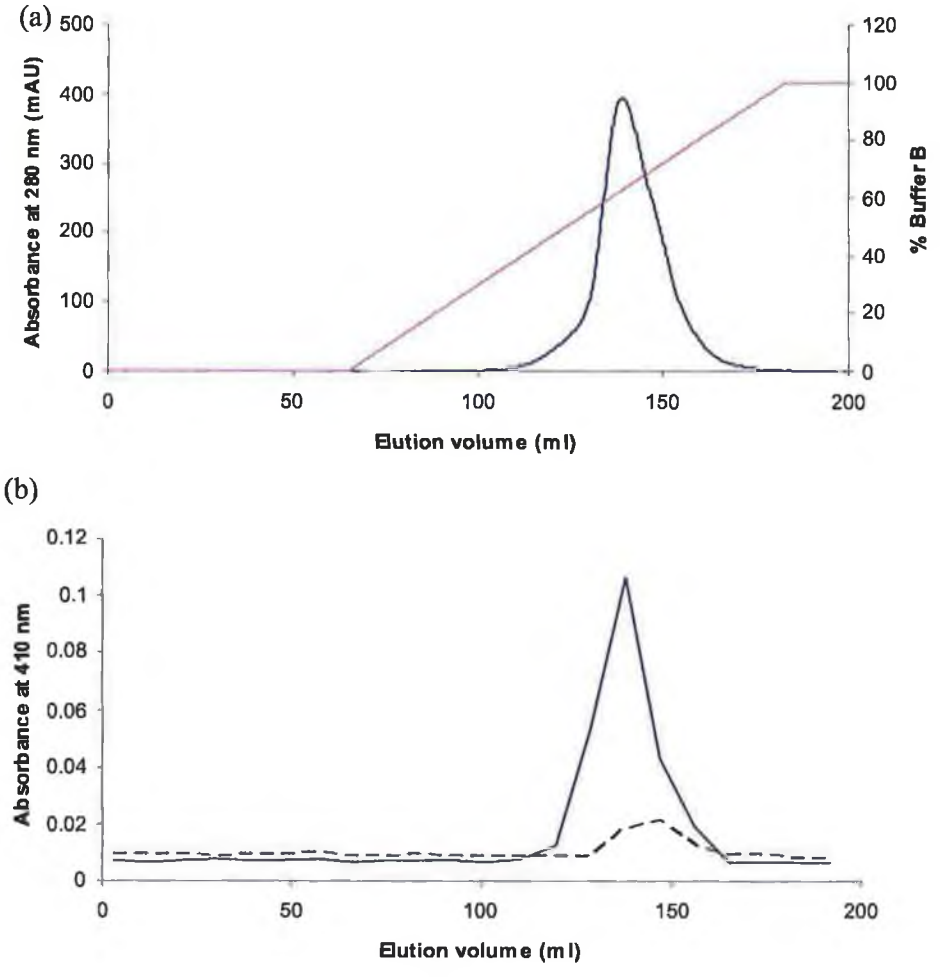


Figure 3.8. (a) Hydrophobic interaction chromatography (HIC) chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from second HIC run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___) and s-AAF-pNA (----).

The fractions enriched in GE activity (fraction 41 to 54, Figure 3.8) were pooled and a total volume of 42 ml was obtained. The pooled fractions were dialysed against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h. The dialysed pooled fractions were then injected onto a Hiprep™ DEAE FF 16/10 column. The IEX chromatogram and the proteolytic activity of the different fractions obtained during the IEX run are given in Figure 3.9.

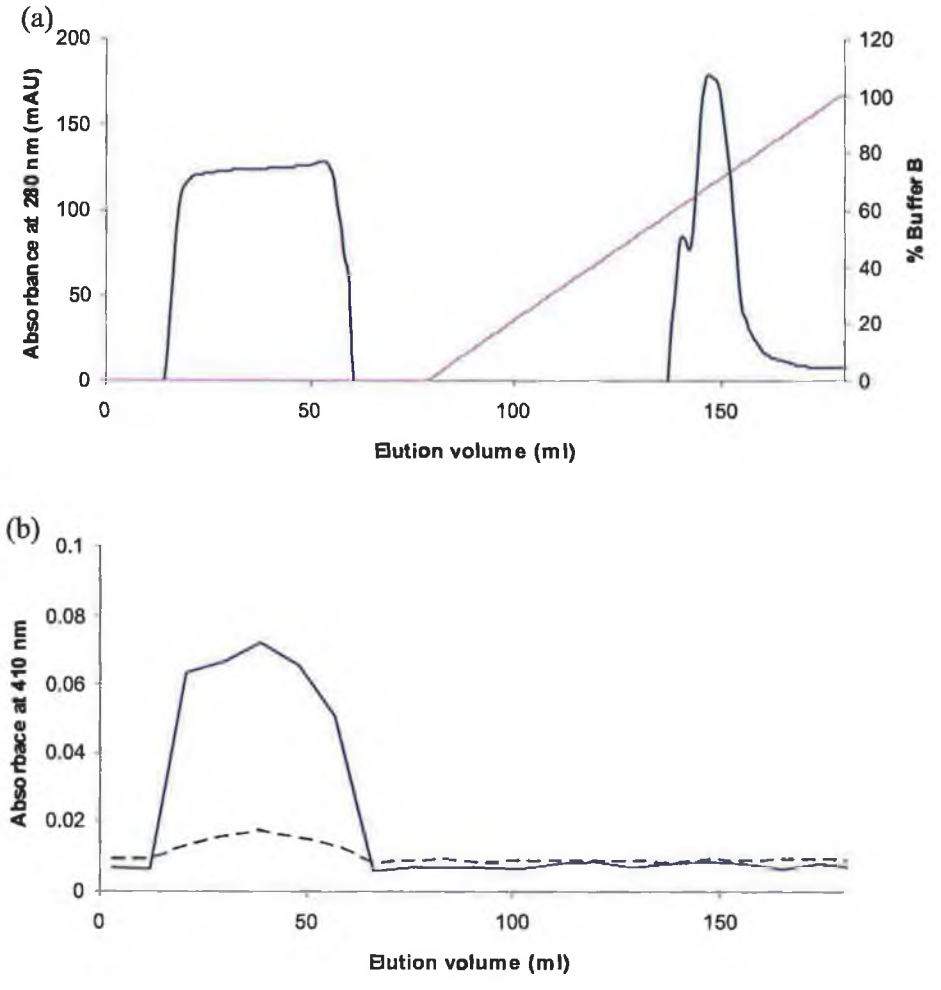


Figure 3.9. (a) Anion exchange chromatogram showing the separation of dialysed enriched glutamyl endopeptidase activity fractions obtained from third hydrophobic interaction chromatographic run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___) and s-AAF-pNA (----).

From Figure 3.9, it can be seen that under the chromatographic conditions used the pooled fractions enriched in GE activity as well as the subtilisin contaminant activity did not bind to the IEX column and eluted during sample injection step. The behaviour of the sample on the IEX column was the same as observed during Protocol 1 (Figure 3.4). The fractions enriched in GE activity (fraction 6 to 21, Figure 3.9) were pooled (48 ml) and then freeze-dried. It is also observed from Figure 3.9a, that the peak eluting between approximately 140 and 160 ml had no activity against ac-Glu-pNA or s-AAF-pNA. This observation was also made in Protocol 1 after the IEX step. Low levels of subtilisin activity co-eluted with GE.

The dried sample was dissolved in 2 ml of 50 mM sodium phosphate buffer and injected onto the GPC column in an attempt to separate GE and subtilisin activities based on differences in their molecular weights. The GPC chromatogram and the proteolytic activity in the different fractions obtained are given in Figure 3.10.

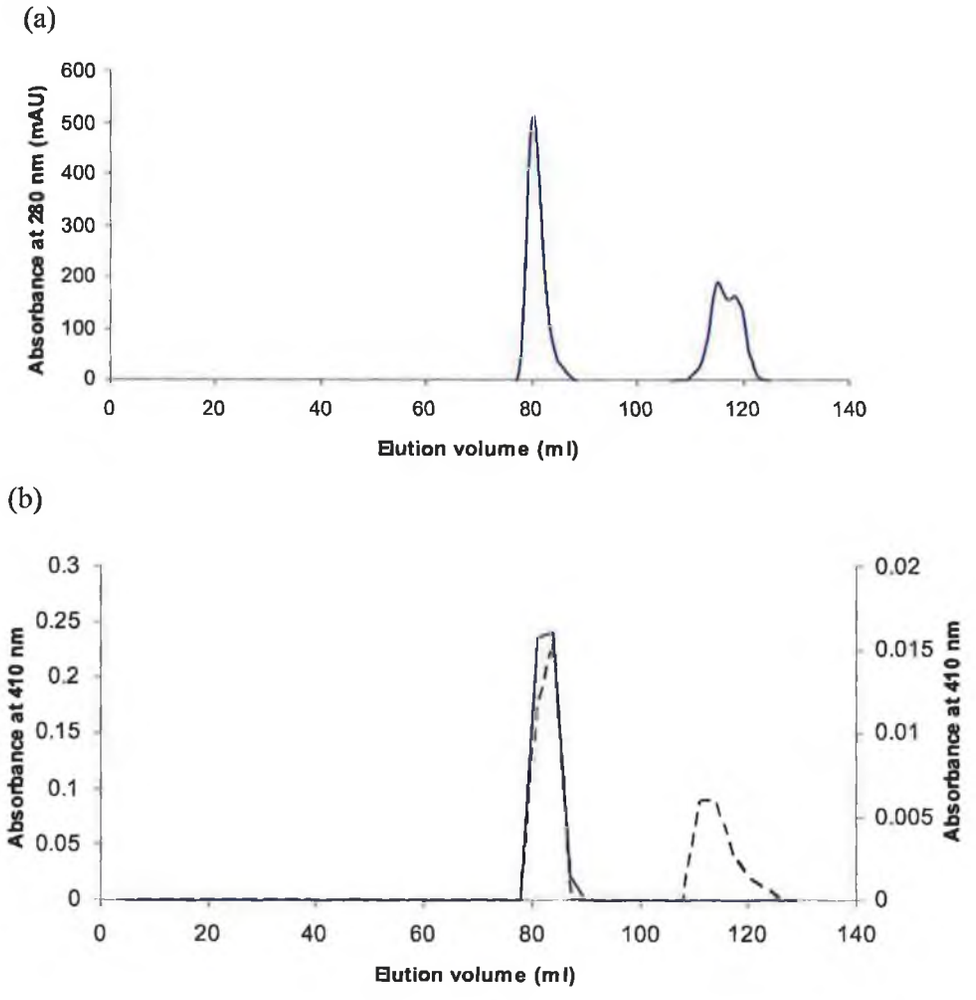


Figure 3.10. (a) Gel permeation chromatogram showing the separation of dialysed enriched glutamyl endopeptidase activity fractions obtained from the ion exchange chromatographic run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values were expressed as Abs at 410 nm per ml of fraction for ac-Glu-pNA (___, left hand side) and s-AAF-pNA (---, right hand side).

From the Figure 3.10b, it can be observed that GE as well as subtilisin activity co-eluted and in addition a low level of subtilisin activity eluted near the total column volume, suggesting non-specific (maybe weak ionic) interaction of the subtilisin contaminant with the column matrix due to the absence of salt (i.e., NaCl or KCl) in

the elution buffer. The fractions enriched in GE activity were pooled (fraction 27 to 30, Figure 3.10). The sample was diluted in 30% (v/v) glycerol and an appropriate amount of $CaCl_2$ to bring the final concentration to 5 mM and was stored at 4°C until further use. Again purified GE did not have any activity towards Bz-Arg-pNA and S-Gly-Pro-pNA. The purification tables for GE as well as subtilisin activity obtained using Protocol 2 are given in Tables 3.3 and 3.4.

Table 3.3 Glutamyl endopeptidase (GE) purification table obtained during the purification of GE from Alcalase™ 2.4L using Protocol 2. The units (U) for activity are given as µmol min^{-1}.

Sample	Volume (ml)	Activity U/ml	Total activity	Total Protein (mg)	Specific activity (U/mg)	Yield
Alcalase	10	107.10	1071.00	200	5.35	100.00
1st HIC	48	19.50	936.00	9.11	102.74	87.40
2nd HIC	39	15.68	611.52	6.68	91.54	57.10
3rd HIC	42	13.46	565.32	5.97	94.69	52.78
IEX	48	11.28	541.44	5.11	105.95	50.55
GPC	12	17.42	209.04	2.17	96.24	19.51

HIC: Hydrophobic interaction chromatography, IEX: ion-exchange chromatography and GPC: gel permeation chromatography.

Table 3.4. The subtilisin purification table obtained during the purification of glutamyl endopeptidase from Alcalase™ 2.4 L using Protocol 2. The units for activity (U) are given as µmol min^{-1}.

Sample	Volume (ml)	Activity (U/ml)	Total activity	Total Protein (mg)	Specific activity (U/mg)	Yield
Alcalase	10	879.50	8795.00	200	43.98	100.00
1st HIC	48	94.64	4542.72	9.11	498.65	51.65
2nd HIC	39	11.50	448.50	6.68	67.14	5.10
3rd HIC	42	2.17	91.14	5.97	15.27	1.04
IEX	48	1.54	73.92	5.11	14.47	0.84
GPC	12	0.53	6.36	2.17	2.93	0.07

HIC: Hydrophobic interaction chromatography, IEX: ion-exchange chromatography and GPC: gel permeation chromatography.

From the Tables 3.3 and 3.4, it was observed that approximately 19.5% of GE activity was obtained with low levels (2.93 µ mol min^{-1} mg^{-1}) of subtilisin contaminant. This yield was approximately 50% less than that obtained by Protocol 1, however, the extent of subtilisin contamination (2.93 µmol min^{-1} mg^{-1}) was significantly less than that obtained using the Protocol 1 (5.30 µ mol min^{-1} mg^{-1}).

From Tables 3.3 and 3.4, it was also observed that after the first HIC run only 12.5 % of GE was lost which is less than that lost in Protocol 1 (25%). However, a significant amount (52%) of subtilisin was observed in the GE active peak after the first HIC run. This was significantly higher than that observed (19%) in Protocol 1. During the second HIC run 87% and 5% of GE and subtilisin, respectively, were obtained. After the third HIC run no significant reduction in subtilisin contaminant was observed, whereas a significant decrease in the yield of GE was observed, i.e., from 53 to 19.5%. Since during the IEX step, GE and subtilisin did not bind to the column this IEX step was not considered to be effective in the purification of GE. The GPC step included in this Protocol was also observed to be ineffective for GE purification during which a significant decrease in GE yield was observed. Therefore, the results imply that the chromatographic steps (IEX and GPC) after the third HIC run were not effective in the further purification of GE. Furthermore, it also was observed that low levels of a subtilisin contaminant activity co-eluted with GE throughout all the chromatographic runs (Figures 3.6-3.10).

3.4.3. Purification of glutamyl endopeptidase from Alcalase (Protocol 3)

Since the GE sample obtained following the procedure described in Protocol 2 was also observed to contain a subtilisin contaminant it was decided to further modify Protocol 2. Therefore, in Protocol 3 the third HIC run was modified by increasing the elution gradient with buffer B from 7.5 column volumes to 12 column volumes. In addition, a cation exchange chromatographic step was included and both the GPC and the anion exchange chromatographic steps followed in Protocol 2 were excluded.

The chromatograms obtained during the chromatographic runs associated with Protocol 3 are given in Figures 3.11-3.14.

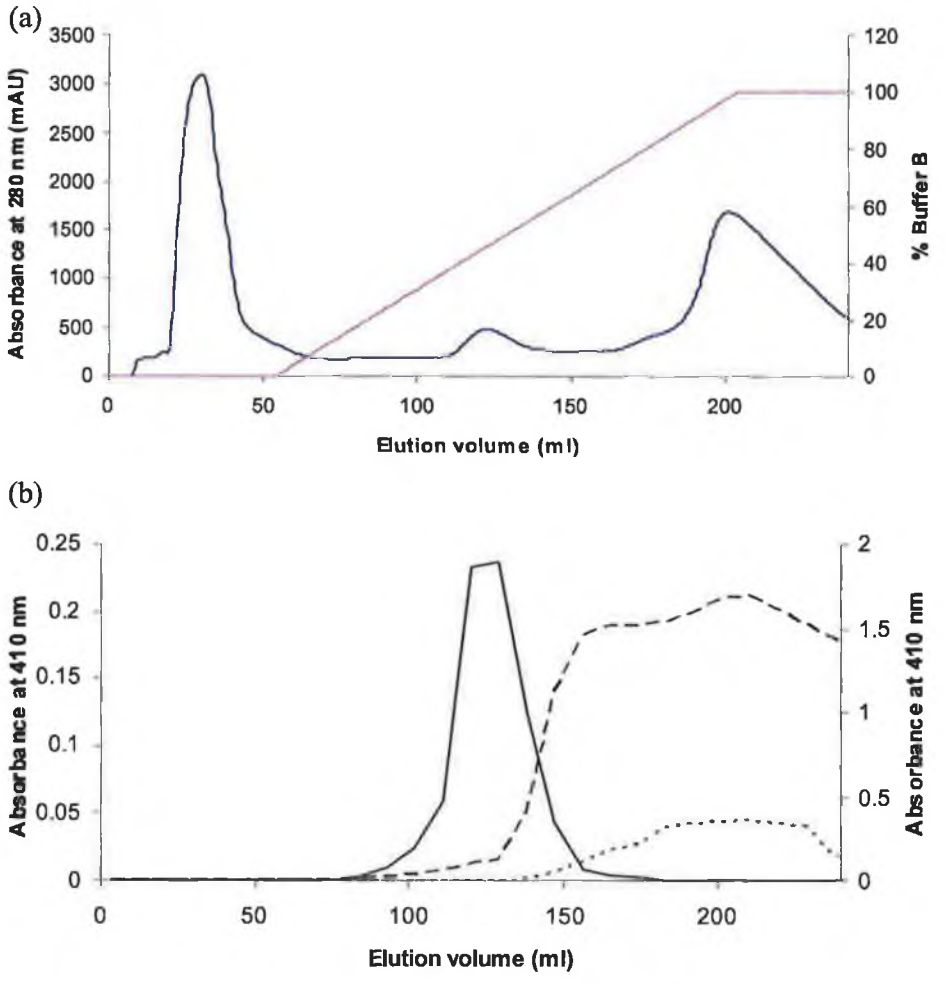


Figure 3.11. (a) Chromatogram showing the separation of Alcalase™ 2.4 L by hydrophobic interaction chromatography. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values were expressed as Abs at 410 nm per ml of fraction for ac-Glu-pNA (___, left hand side), s-AAF-pNA (---, right hand side) and s-AAA-pNA(....., right side).

The elution profiles observed after the first HIC step were similar to that observed in Protocol 1 and 2. The fractions enriched in GE activity (fraction 34 to 50, Figure 3.6) were pooled and a total volume 51 ml was obtained after the 1st HIC run. Appropriate amounts of ammonium sulphate and potassium chloride was added to pooled fractions (51 ml) to bring the final concentration of both salts in solution of 500 mM.

The GE enriched fractions (51 ml) were rechromatographed onto the HIC column for further enrichment of GE activity. The elution profile and the proteolytic activity of the different fractions obtained during the second HIC run are given in Figure 3.12.

(a)

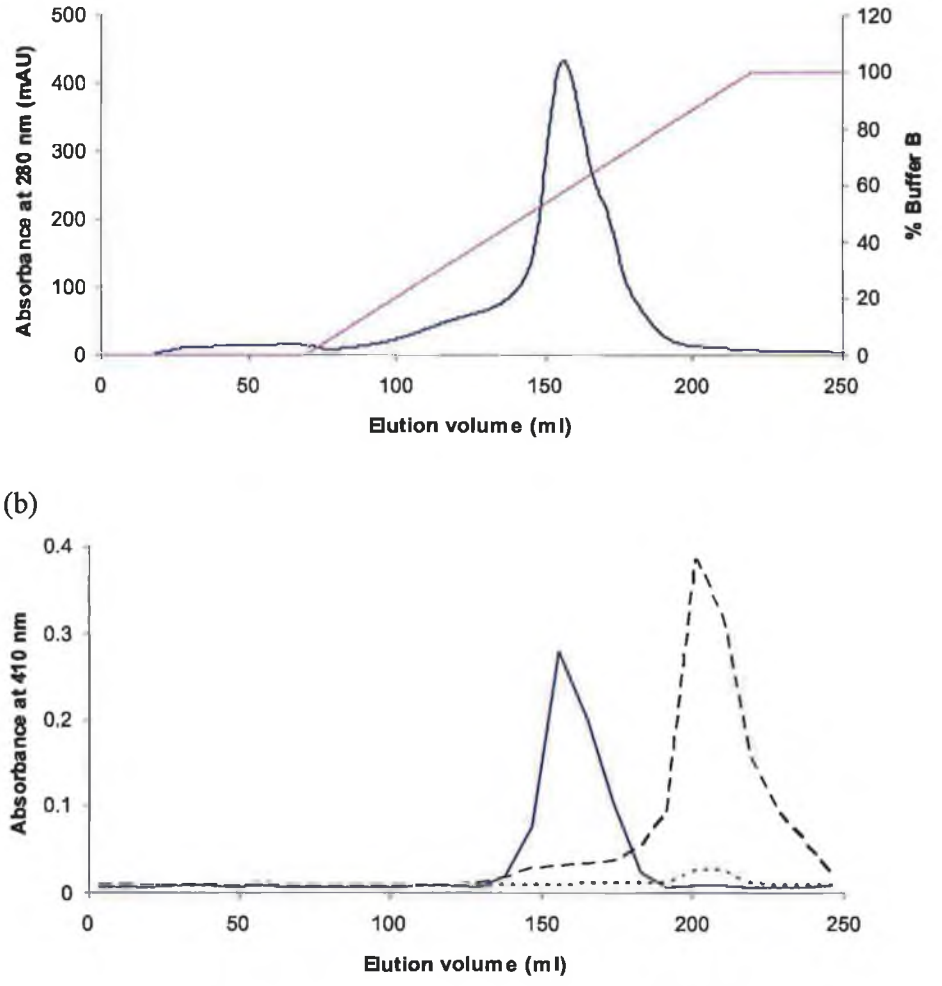


Figure 3.12. (a) Hydrophobic interaction chromatography chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from the first HIC run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___), s-AAF-pNA (----) and s-AAA-pNA(.....).

The peak enriched in GE activity (fraction 46 to 54, 135 to 183 ml, Figure 3.12) was pooled and a total volume of 48 ml was obtained. Appropriate amount of ammonium sulphate and potassium chloride was added to the pooled fractions to bring the final concentration of both salts in solution of 500 mM. The pooled fractions (48 ml) were injected for the 3rd time onto the HIC column in an attempt to separate the subtilisin contaminant present from the enriched GE activity. The HIC chromatogram and the proteolytic activity of the different fractions during the 3rd HIC run are given in Figure 3.13.

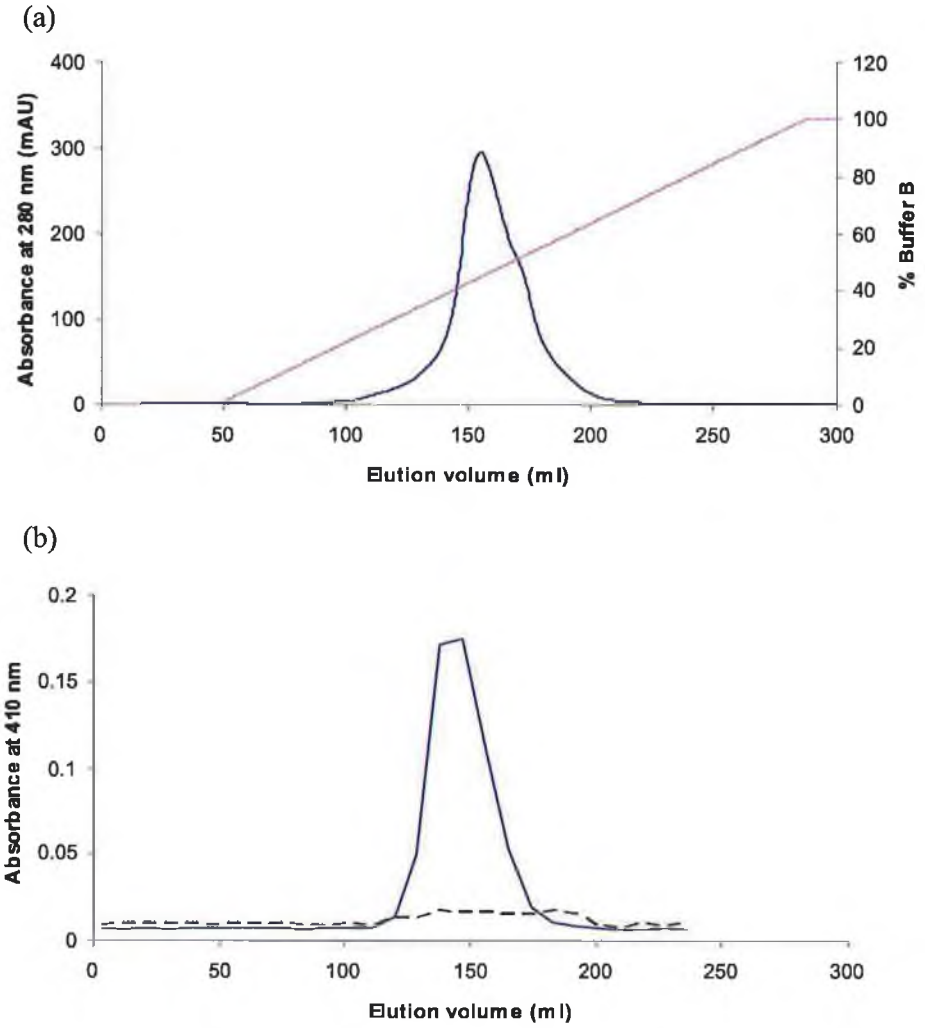


Figure 3.13. (a) Hydrophobic interaction chromatography chromatogram showing the separation of enriched glutamyl endopeptidase activity fractions obtained from the second HIC run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___) and s-AAF-pNA (----).

It was observed that the third HIC step was not capable of fully separating the subtilisin contaminat from GE activity. The fractions containing GE activity (fraction 40 to 60, Figure 3.13) were pooled and a total volume of 63 ml was obtained. The pooled fractions were concentrated by injecting the fractions onto the HIC column and eluting with 100 % B. The concentrated fractions obtained were dialysed using a 12,400 Da molecular weight cut-off dialysis membrane against 50 mM sodium phosphate buffer, pH 6.2 at 4°C for 12 h. The dialysed sample was then injected onto

a HiTrap™-CM FF cation exchange column. The elution profile and the proteolytic activity of the different fractions obtained during the IEX run are given in Figure 3.14.

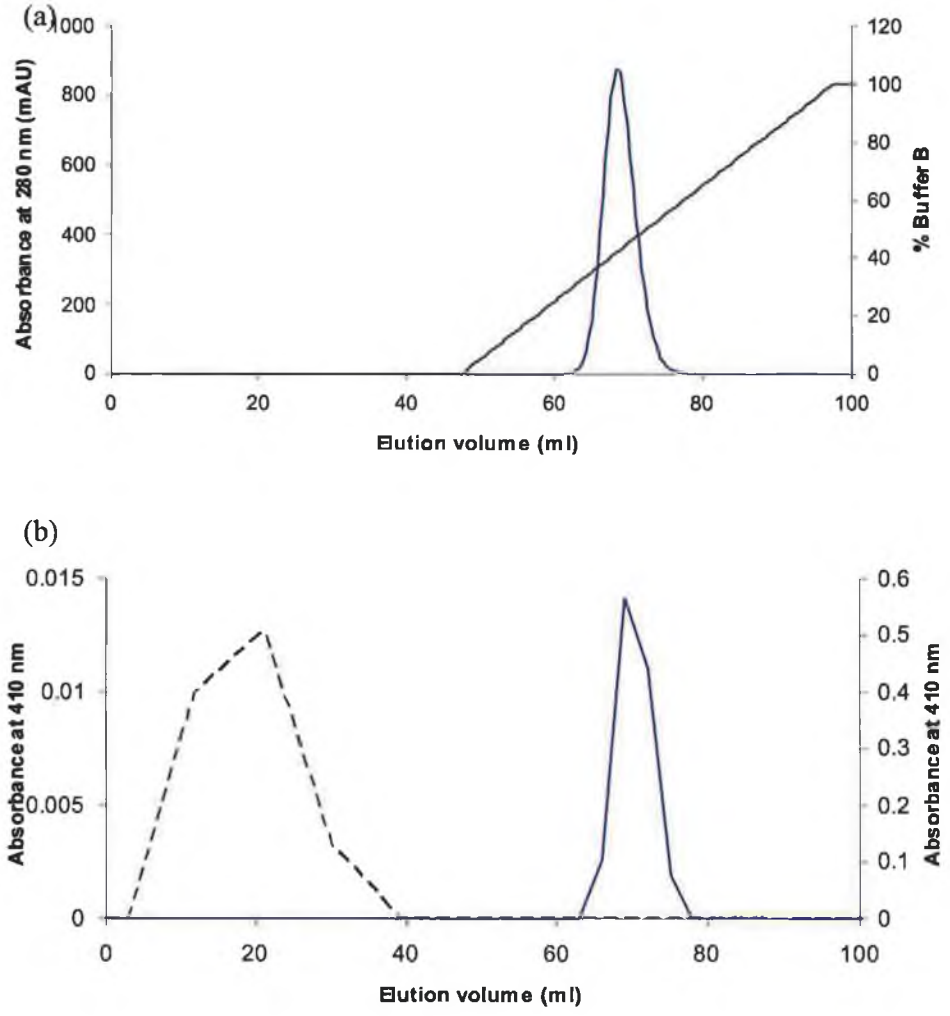


Figure 3.14. (a) The cation exchange chromatogram showing the separation of dialysed enriched glutamyl endopeptidase activity fractions obtained from third hydrophobic interaction chromatographic run. Absorbance at 280 nm and concentration of buffer B are shown. (b) The activity values are expressed as Abs at 410 nm per ml of fractions for ac-Glu-pNA (___, right hand side) and s-AAF-pNA (----, left hand side).

From Figure 3.14 it can be observed that the subtilisin contaminant did not bind to the IEX column whereas the GE activity bound and eluted during the gradient. The GE active fractions (fraction 22 to 25, Figure 14) were pooled. The pooled fractions (12 ml) were dialysed using a 12,400 Da cut-off dialysis membrane against 50 mM sodium phosphate buffer, pH 7.0 at 4°C for 12 h. After 12 h dialysis the sample was diluted in 30% (v/v) glycerol and an appropriate amount of $CaCl_2$ was added to bring

it to a final concentration of 5 mM. The sample was then stored at 4°C until further use. Purified GE did not have any activity towards Bz-Arg-pNA and s-Gly-Pro-pNA. The purification Tables for GE as well as subtilisin activity obtained using Protocol 3 are given in Tables 3.5 and 3.6.

Table 3.5. Glutamyl endopeptidase (GE) purification table obtained during the purification of GE from Alcalase™ 2.4L using Protocol 3. The units (U) for activity are given as µmol min^{-1}.

Sample	Volume (ml)	Activity U/ml	Total activity	Total Protein (mg)	Specific activity (U/mg)	% Yield
Alcalase	10	107.10	1071.0	213.60	5.35	100.00
1st HIC	51	11.84	603.84	10.32	58.51	56.38
2nd HIC	48	12.20	585.6	8.63	67.86	54.68
3rd HIC	63	7.20	453.6	6.57	69.04	42.35
IEX	12	37.2	446.4	4.35	102.62	41.68

HIC: Hydrophobic interaction chromatography and IEX: ion-exchange chromatography.

Table 3.6. The subtilisin purification table obtained during the purification of glutamyl endopeptidase from Alcalase™ 2.4 L using Protocol 3. The units (U) for activity are given as µmol min^{-1}.

Sample	Volume (ml)	Activity U/ml	Total activity	Total Protein (mg)	Specific activity (U/mg)	% Yield
Alcalase	10	879.50	1071.0	213.60	41.18	100.00
1st HIC	51	36.80	603.84	10.32	192.56	22.59
2nd HIC	48	2.68	585.6	8.63	14.91	1.46
3rd HIC	63	0.73	453.6	6.57	7.00	0.52
IEX	12	0.025	0.30	4.35	0.07	0.003

HIC: Hydrophobic interaction chromatography and IEX: ion-exchange chromatography.

From the Tables 3.5 and 3.6, it was observed that approximately 41.7% of GE activity was obtained with no significant level (0.07 µ mol min^{-1} mg^{-1}) of subtilisin contamination. This yield was higher than that obtained by Protocol 1 and 2. Therefore, from the results it can be concluded that for the isolation of GE from Alcalase™ 2.4 L the method described in Protocol 3 should be followed.

From the purification tables (Tables 3.5 and 3.6), the trends in specific activity for the three HIC steps were similar to those observed in Protocols 1 and 2. However, after the final IEX chromatographic step, the sample was shown to be highly enriched in GE with no significant subtilisin contaminant. The yield (41.68%) was less than the

yield (50%) reported by Svensden and Breddam (1992) and more than the yield (30.3%) reported by Kakudo *et al.*, (1992). From the results presented herein, it can be said that the cation IEX step employed by Svensden and Breddam (1992) was a very useful approach for GE separation from subtilisin. The lower yield in comparison with that reported by Svensden and Breddam (1992) may be due to the fact that a high level of GE activity (44%) was lost during the first HIC step. The sequence of chromatographic steps used by Svensden and Breddam (1992) were different and also a higher volume (750 ml) of Alcalase was used in the purification of GE.

3.4.4. Characterization of enriched glutamyl endopeptidase activity

As mentioned in section 3.3.2, characterization studies with GE were carried out with the sample enriched using Protocol 2.

Determination of Michaelis constant (K_m) at 37°C

A Michaelis–Menten curve was plotted by plotting initial rate (v), against the substrate concentration [s] (Figure 3.15a) and a Lineweaver-Burk plot (Figure 3.15b) was generated by plotting 1/v against 1/[s].

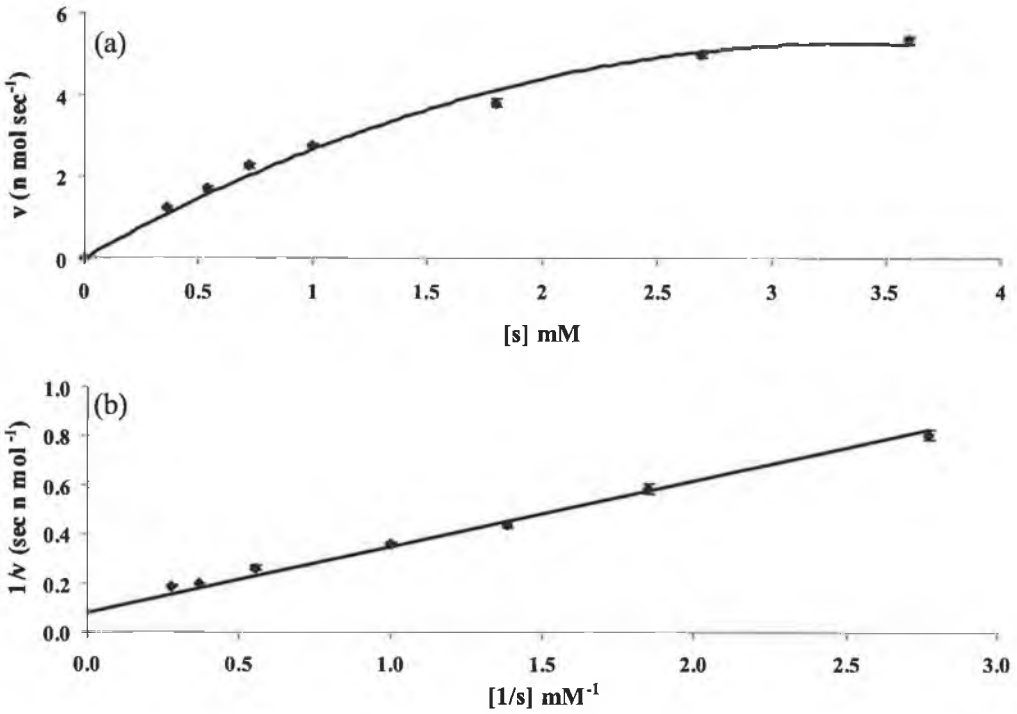


Figure 3.15. (a) Initial rate (v) (nmol sec-1) of pNA released at 37°C versus substrate concentration [s]. (b) The Lineweaver-Burk plot (1/v versus 1/[s]) at 37°C for enriched glutamyl endopeptidase activity using acetyl-Glu-pNA as substrate. The values plotted are means of ± SD of independent triplicate analysis.

From the linear equation Figure 3.15b, i.e., y = 0.2677x + 0.0804, it is known that the slope represents K_m/Vmax which equals to 0.2677 and the y-intercept is equal to 1/Vmax which was found to be 0.0804. By using these results Vmax and K_m values were calculated and were found to be 12.44 n mol sec^{-1} and 3.33 mM, respectively.

Determination of Michaelis constant (K_m) at 50°C

A Michaelis–Menten curve was plotted by plotting initial rate (v), against the substrate concentration [s] (Figure 3.16a) and a Lineweaver-Burk plot (Figure 3.16b was generated by plotting 1/v against 1/[s].

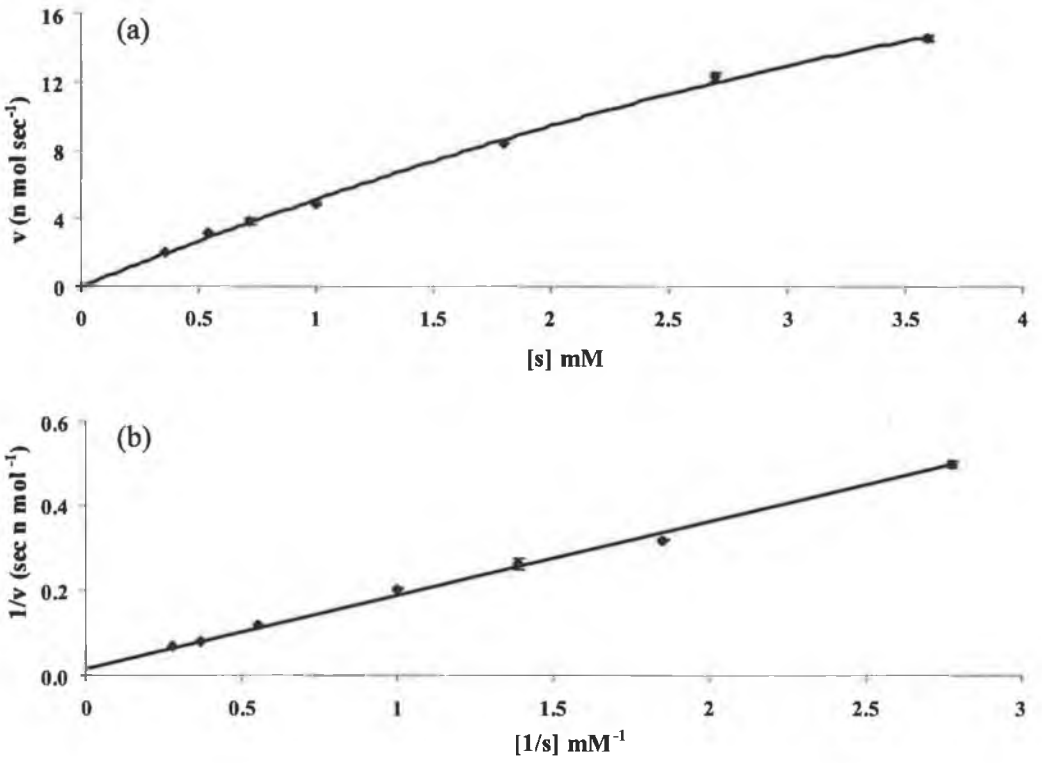


Figure 3.16. (a) Initial rate (v) (nmol sec-1) of pNA released at 50°C versus substrate concentration [s]. (b) The Linewaver-Burk plot (1/v versus 1/[s]) at 37°C for enriched glutamyl endopeptidase activity using acetyl-Glu-pNA as substrate. The values plotted are means of ± SD of independent triplicate analysis.

From the linear equation Figure 3.16b, i.e., y = 0.1739x + 0.0161, it is known that the slope represents K_m/Vmax which equals to 0.1739 and the y-intercept is equal to 1/Vmax which was found to be 0.0161. Using these results Vmax and K_m values are calculated and were found to be 62.11 n mol sec^{-1} and 10.80 mM, respectively

The K_m value for GE at 37°C was observed to be 3.33 mM which is in the range of the K_m values reported by Kakudo *et al.,* (1992). Kakudo *et al.,* (1992) reported the K_m values for GE purified from *B. lichenformis* ATCC 14580, at 37°C using eight different substrates with Glu at the P_1 position. K_m values were observed to be in the range of 1.5 to 5.5 mM depending on the substrate used. However, a K_m value for GE using ac-Glu-pNA was not reported by Kakudo *et al.,* (1992) or by any other researchers. A K_m of 10.80 mM at 50°C using ac-Glu-pNA, implies that this is not the

preferred temperature for GE activity in comparison with the K_m value obtained at 37°C.

Inhibitory studies

The inhibition of GE was monitored employing commonly used proteolytic inhibitors such as N-ethylmaleimide (NEM), phenyl methyl sulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and 1,10 phenanthroline. Since 96% (v/v) ethanol was used to make up stock solutions of most of these reagents, the potential inhibition of GE with ethanol was also monitored. Two different concentrations of inhibitor were used, i.e., 1 and 10 mM and the percentage inhibition values obtained on incubation at these concentrations are given in Table 3.7.

Table 3.7. The percentage inhibition of glutamyl endopeptidase activity for 1 and 10 mM final concentrations of N-ethylmaleimide (NEM), phenylmethylsulphonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and 1, 10 phenanthroline and 96% v/v ethanol (control).

Sample	Inhibition of activity at 1mM (%)	Inhibition of activity at 10 mM (%)
Control	0.00	0.00
Ethanol	2.99 ± 1.22	3.55 ± 1.18
EDTA	68.29 ± 2.44	64.50 ± 0.59
PMSF	7.69 ± 1.78	16.46 ± 0.61
NEM	1.83 ± 0.61	2.37 ± 1.18
1,10 phenanthroline	1.22 ± 1.22	2.37 ± 1.19

The values are means of ± SD of independent triplicate analysis.

From Table 3.7 it is evident that, with the exception of EDTA, the GE sample was not significantly inhibited by most of the inhibitors used. The partial inhibition of GE with EDTA was previously reported by Svendsen and Breddam (1992) and Kakudo *et al*., (1992).The results herein re-confirm the observation made by Svendsen and Breddam (1992) that metal ions (Ca^{2+}) are important but not essential for the GE activity isolated from *B.lichenformis.* The partial inhibition of GE activity with EDTA was stated to be a reversible inhibition (Kakudo *et al.,* 1992). In the literature, it was shown that, the GE isolated from *B. licheniformis* was inhibited by DFP (Kakudo *et*

al., (1992), Svendsen and Breddam (1992) and Park and Allen (1998)). It was also inhibited by Boc-Leu-Glu-CH_2Cl (Kakudo *et al.*, (1992)). Both the above mentioned inhibitors were not used in the present study because of the toxicity associated with DFP and due to financial limitations in acquiring Boc-Leu-Glu-CH_2Cl (€7000 per 50 mg).

From the results, it is seen that PMSF, which is classified as a serine endo proteinase inhibitor does not significantly inhibit GE activity. Using NEM or 1, 10 phenanthroline no inhibition of GE was also observed. To our knowledge no information appears in the literature as to whether or not PMSF or NEM or 1, 10 phenanthroline inhibit GE activity purified form Alcalase or purified from *B. licheniformis*.

pH optimum studies

The pH optimum of GE was estimated by monitoring the initial rate of hydrolysis of Ac-Glu-pNA by GE at various pH values (pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10) during incubation at 50°C.

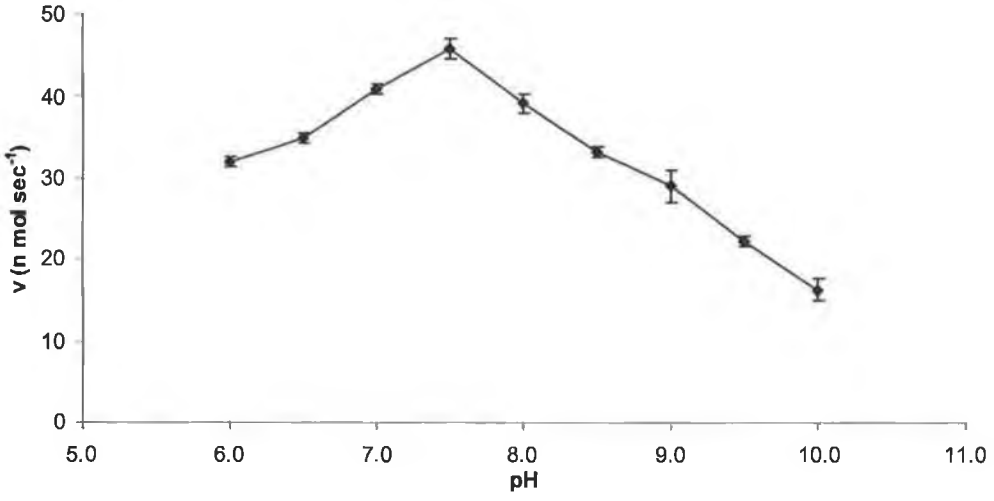


Figure 3.17. Initial rate (v, nmol sec-1) versus pH for glutamyl endopeptidase at 50°C using ac-Glu-pNA as substrate. The values plotted are mean ± SD of independent triplicate analyses.

From Figure 3.17, it is evident that the pH optimum of GE at 50°C was in the range of pH 7.5-8.0, which is in agreement with the values previously reported by Svendsen and Breddam (1992).

Effect of Ca^{2+} at different storage temperatures

Approximately 10% of the activity was lost over the 2 month storage period in sample with and without added calcium at the three different storage temperatures (Figure 3.18). Therefore the activity was very stable at all the storage temperatures with and without added calcium for 2 months. Spellmen *et al.*, (2005) reported that GE was stable at 4°C in the presence of 5 mM $CaCl_2$ for 3 months. The results obtained herein indicate that addition of Ca^{2+} and storage temperature had little effect on GE stability over a 2 month storage period.

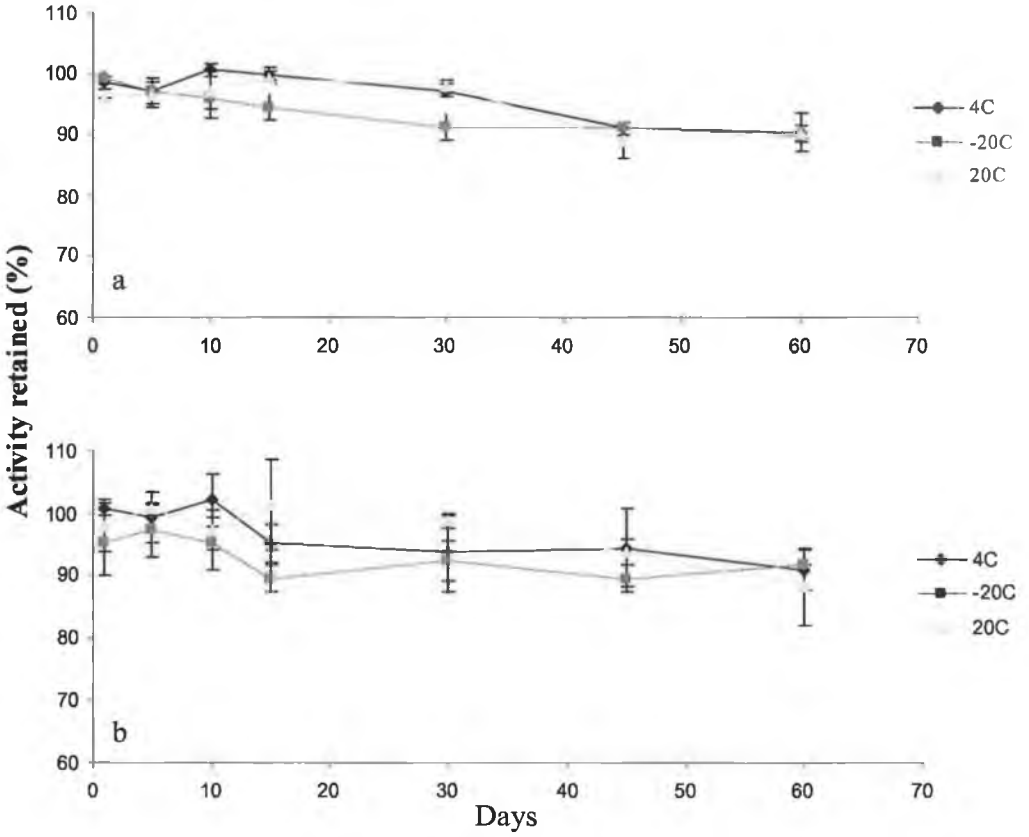


Figure 3.18. Glutamyl endopeptidase stability measured over a 2 month period at 4°C, -20°C and 20°C (a) without added Ca+2 (b) with added Ca+2 (5 mM). The values plotted are means ± SD of independent triplicate analyses.

Temperature stability studies

It was observed that GE was stable up to 4 h when held at 50°C, at 60°C it lost 69% activity after 4 h, at 70°C it lost 97% activity in 15 min and completely lost the activity in less than 30 min. At 90°C a complete loss in activity was observed in less than 5 min (Figure 3.19). To our knowledge no information appears in the literature on the temperature stability of GE.

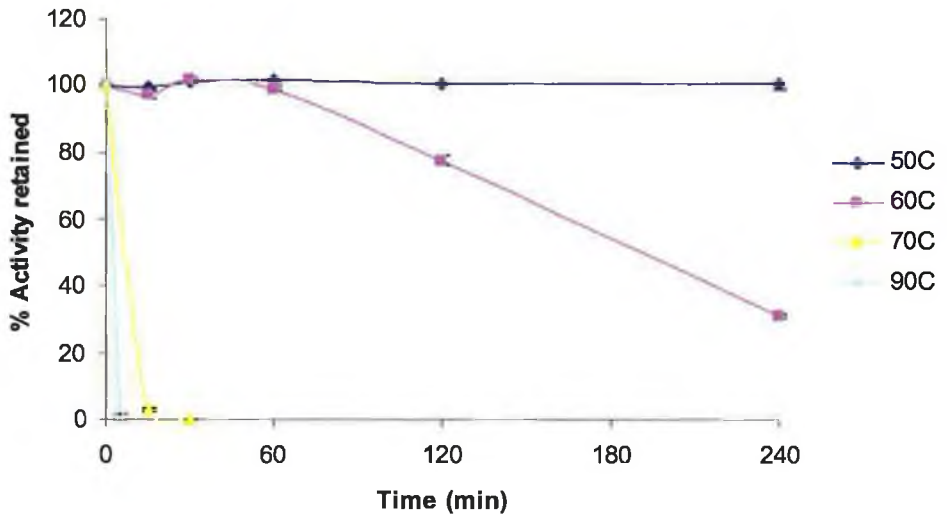


Figure 3.19. Temperature stability of glutamyl endopeptidase showing the percentage activity retained versus time for four different temperatures (50°C, 60°C, 70°C and 90°C). The values plotted are means ± SD of independent duplicate analyses.

Gel electrophoresis

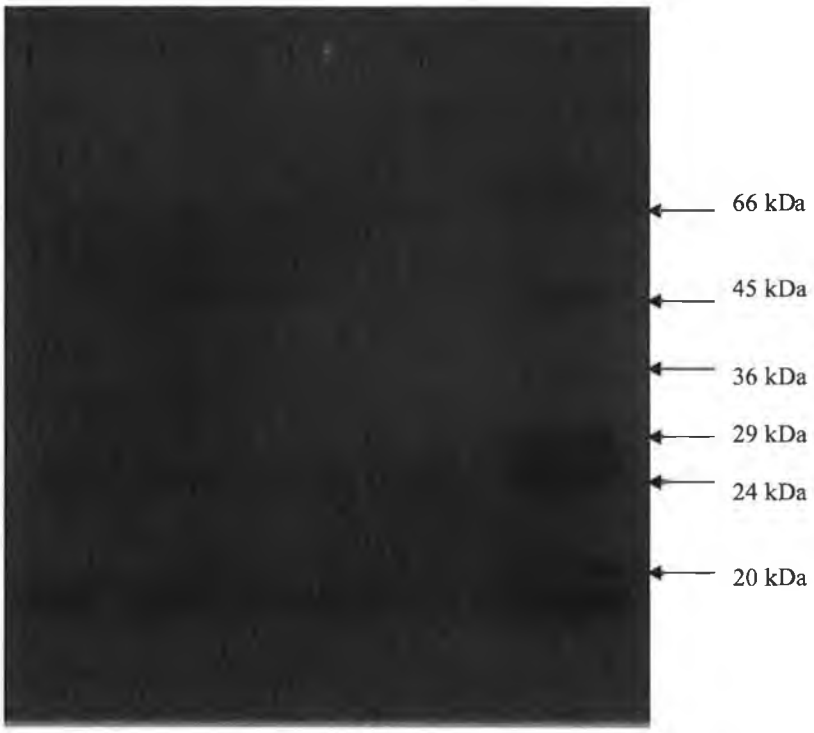


Figure 3.20. The SDS-PAGE profile obtained for glutamyl endopeptidase obtained following purification using Protocol 2.

Well No.	Sample	Amount of Sample
1.	Purified GE Sample (Protocol 2)	10µg
2.	Purified GE Sample (Protocol 2)	8µg
3.	Purified GE Sample (Protocol 2)	4µg
4.	Purified GE Sample (Protocol 2)	2µg
5.	Low molecular weight standards	20µl

From Figure 3.20, it was observed that the purified GE sample had a single band (lanes 1, 2, 3 and 4) with a molecular weight of approximately 24 kDa which is in agreement with the molecular weight (23,589 Da) reported by Svendsen and Breddam (1992) and the molecular weight (25 kDa) reported by Kakudo *et al.,* (1992).

3.5. Conclusion

Based on the results presented in this Chapter it can be concluded for the isolation of GE activity from Alcalase™ 2.4 L that Protocol 3 is an effective method for obtaining pure GE, i.e., free of subtilisin and elastase contamination, with a yield of approximately 42%. The molecular weight of GE was approximately 24 kDa and it had a pH optimum in the range of pH 7.5-8.0. The Michaelis constant (K_m) of GE using ac-Glu-pNA at 37°C and 50°C was estimated to be 3.33 and 10.80 mM, respectively. The activity was stable at different storage temperatures (-20°C, 4°C and 20°C) with or without the addition of Ca^{2+} ions for up to 2 months. GE was partially inhibited by EDTA. The method described in Protocol 3 could be modified further by reducing the number of HIC steps and by also using an IEX step as the first and last chromatographic steps for GE purification.

References

Bensadoun, A. and Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70(1),** 241-250.

Bower, V. E. and Bates, R. G. (1955). The pH values of the Clark and Lubs buffer solutions at 25°C. *J. Res. Nat. Bur. Stand.* **55**, 197–200.

Breddam, K. and Svendsen, I. (1992). Isolation and amino acid sequence of a glutamic acid specific endopeptidase from Bacillus *licheniformis. Eur. J. Biochem.* **204,** 165-171.

Drapeau, G. R., Boily, Y. and Houmard, J. (1972). Purification and properties of an extracellular protease of Staphylococcus *aureus. J. Biol. Chem.* **247(20),** 6720-6726.

Houmard, J. and Drapeau, G. (1972). Staphylococcal protease: A proteolytic enzyme specific for glutamoyl bonds. *Proc. Nat. Acad. Sci. USA.* **69,** 3506-3509.

Kakudo, S., Kikuchi, N., Kitadokoro, K., Fujiwara, T., Nakamura, E., Okamoto, H., Shin, M., Tamaki, M., Teraoka, H., Tsuzuki, H. and Yoshida, N. (1992). Purification, characterization, cloning, and expression of a glutamic acid-specific protease from Bacillus licheniformis ATCC 14580. *J. Biol. Chem,* **267(33),** 23782-23788.

Kawalec, M., Potempa, J., Moon, J. L., Travis, J. and Murray, B. E. (2005). Molecular diversity of a putative virulence factor: purification and characterization of isoforms of an extracellular serine glutamyl endopeptidase of Enterococcus *faecalis* with different enzymatic activities. *J Bacteriol*, **187(1),** 266–275

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227,** 680-685.

Lowry, O.H., Rosebrough, N. J., Farr, AL., and Randal, RJ. (1951). Protein measurement with the Folin phenol reagent. *J Biol. Chem.* **193 (1),** 265-275.

Nagase, H. and Salvesen, G. (1989). Inhibition of proteolytic enzymes. In: R. J. Beynon and R.J Bond (Ed.), *Proteolytic enzymes-a practical approach* (pp 83-104). Oxford University press, Oxford, England.

North, M. J. (1989). Prevention of unwanted proteolysis. In: R. J. Beynon and R.J Bond (Ed.), *Proteolytic enzymes-a practical approach* (pp 105-124). Oxford University press, Oxford, England.

Ohara-Nemoto Y., Ikeda, Y., Kobayashi, M., Sasaki, M., Tajika, S. and Kimura, S. (2002). Characterization and molecular cloning of a glutamyl endopeptidase from Staphylococcus *epidermidis. Microb Pathog*, **33,** 33-41.

Park, O. and Allen, J. C. (1998). Preparation of phosphopeptides derived from α_s-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. *J. Dairy Sci*, **81(11),** 2858-2865.

Plummer, D. T. (1978). In: *Enzymes: An introduction to practical biochemistry.* 2nd ed. (pp 243-287). McGraw-Hill book company (UK) limited, Maidenhead, England.

Rudenskaya, G. N. (1998) Glutamyl endopeptidases from microorganisms, a new subfamily of chymotrypsin proteases. *Bioorg. Khimiya, Moscow,* **24,** 256–261.

Spellman, D., Kenny, P., O'Cuinn, G. & FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with Bacillus licheniformis proteinase activities. *J Agric Food Chem*, **5(4)**, 1258-1265.

Spellman, D., O'Cuinn, G. and FitzGerald, R.J. (2009). Bitterness in *Bacillus* proteinase hydrolysates of whey proteins. *Food Chemistry*, **114**, 440-446.

Stennicke, H.R., Birktoft, J.J. and Breddam, K. (1996). Characterisation of the S1 binding site of glutamic acid-specific protease from Streptomyces *griseus*. *Protein Sci.* **5,** 2266–2275.

Stennicke, H.R. and Breddam, K. (1998). Glutamyl endopeptidase I. In: A.J. Barrett, N.D. Rawlings and J.F. Woessner (Ed.). *Handbook of Proteolytic Enzymes* (pp. 243-246). Academic Press, San Diego, CA, USA,

Stryer, L. (1995). Enzymes: Basic concepts and kinetics. In: *Biochemistry*. 4th ed. (pp 192-194). W.H Freeman and Company, New York, USA,

Yamangata, A., Yoshida, N., Noda, K. and Ito, A. (1995). Purification and characterization of a new serine proteinase from Bacillus *subtili* with specificity for amino acids at P_1 and P_2 positions. *Biochim. Biophys. Acta,* **1253,** 224-228.

Yokoi, K., Kakikawa, M., Kimoto, H., Watanabe, K., Yasukawa, H., Yamakawa, A., Taketo, A. and Kodaira, K. (2001). Genetic and biochemical characterization of glutamyl endopeptidase of Staphylococcus *warneri* M. *Gene,* **281,** 115–122.

Yoshida, N., Tsuruyama, S., Nagata, K., Hirayama, K., Noda, K. and Makisumi, S. (1988). Purification and characterization of an acidic amino acid specific endopeptidase of Streptomyces *griseus* obtained from a commercial preparation (Pronase). *J. Biochem,* **104,** 451-456.

Chapter 4

Substrate specificity of glutamyl endopeptidase (GE): hydrolysis studies with β-casein.

4.1. Abstract

Purified β-casein (2 ml, 1.5% (w/v) aqueous solution) was digested with glutamyl endopeptidase (256 nmol min^{-1} ml^{-1}) at 37°C and 50°C for 4 h. Samples (250 µl) were withdrawn at various time intervals (15, 30, 60, 120 and 240 min) and the peptides generated were analysed using mass spectrometry. It was observed that the GE activity was highly specific and hydrolysed the peptide bond predominantly on the carboxy terminal of Glu and Asp. It was observed that Pro residues were not preferred at the P_1' position. Whereas, Met was poorly preferred at the P_1' position. It was also observed that Glu-Met was hydrolysed to a lower extent when compared to Asp-Met. Non- specific cleavages were observed at the carboxy terminal of Phe (51), Thr (128) and Gln (188) on incubation at 37°C, and at Thr (128) during incubation at 50°C. Mass spectrometry analysis also showed that Ser residues in β-casein were phosphorylated to different extents (tri, tetra and penta) at positions 15, 17, 18, 19 and 35. The mean peptide sequence coverage was 53.4 and 49.8% for samples incubated with GE at 37 and 50°C, respectively.

4.2 Introduction

Milk proteins are hydrolysed industrially for various purposes, e.g., for use as ingredients for reduced/hypoallergenic infant formulae, and for enteral/sport and parenteral nutrition (FitzGerald and Meisel, 2003). Alcalase™ is a commercially available proteolytic preparation which is used extensively in the generation of milk protein hydrolysates (Adamson and Reynolds, 1996, McDonagh and FitzGerald, 1998, van der Ven *et al.,* 2001, Severin and Xia, 2006, Spellman *et al.,* 2005, Spellman *et al,* 2009 and Peng *et al.,* 2009). Alcalase from *B.licheniformis* in addition to having a subtilisin-like activity also possesses glutamyl endopeptidase (GE) activity (Spellman *et al.,* (2005)). GE appears to play a key role in peptide aggregation and bitterness in whey protein hydrolystaes (Spellman *et al.,* 2005 and Spellman *et al,* 2009). A detailed knowledge of the substrate specificity of GE may allow the targeted release of specific peptide sequences having different techno- and biofunctional applications.

As described in Chapter 2, GE is reported to be highly specific and cleaves the peptide bond at the carboxy side of acidic amino acid residues (Glu/Asp). However, Breddam and Svensden (1992) also observed some non-specific cleavage at the carboxy terminal of Phe during the hydrolysis of ribonuclease at high GE concentrations (1.2 μM). It was therefore proposed that GE was somewhat non-specific with respect to the nature of the amino acid residue present at the P_1' position. Breddam and Meldal (1992) also reported that the rate of hydrolysis of Glu-Asp was very slow and that Pro residues at the P_1' position was not preferred or poorly preferred by GE.

Yokoi *et al.,* (2001) purified GE from Staphylococcus *warneri* strain M. They digested β-casein (20 μg) with GE (0.03 μg) in the presence of 50 mM Tris-HCl buffer pH 7.5 at 37°C for 3 hours. The hydrolysis generated five peptides, represented as b_1, b_2, b_3, b_4 and b_5 which corresponded in molecular mass to approximately 12.0, 10.5, 8.6, 7.8 and 6.0 k Da, respectively. The seven N-terminal amino acid residues corresponding to b_1, b_3, b_4 and b_5 were, RELEELN (β-CN 1-7), VMGVSKV (β-CN 92-98), AMAPKHK (β-CN 101-106) and SQSLTLT (β-CN 122-128), respectively. The authors therefore concluded that Glu-Val (β-CN 91-92), Glu-Ala (β-CN 100-101) and Glu-Ser (β-CN 121-122) were cleaved upon incubation with

GE. Park and Allen (1998) prepared casein phosphopeptides using immobilized GE. Solutions of α- and β-casein (1% (w/v), 20 ml) in 50 mM Tris-HCl buffer, pH 8.0, containing 0.02% (w/v) NaN_3) were recirculated in a fluidized bed bioreactor containing 2 ml of immobilised GE beads at 20°C for 3 h. The major peptide bands observed following SDS-PAGE corresponded to molecular weight of 9.3, 8.2 and 6.2 k Da, which were thought to correspond to β-casein f12-91, f48-121, and f130-184, respectively.

The objective of this study was to investigate GE specificity with respect to the preferred amino acid residues present at the P_1, P_1' and P_2 positions. This was carried out while characterizing the peptides released from a purified β-casein preparation following incubation at both 37°C and 50°C for various time intervals.

4.3. Methods and Materials

HPLC grade water and formic acid were from Sigma-Aldrich, Dublin, Ireland. β-Casein and glutamyl endopeptidase (GE) were purified by the methods described in Chapter 1 (Protocol 4) and Chapter 2 (Protocol 3), respectively.

4.3.1. Digestion of β-casein with glutamyl endopeptidase (GE)

Purified β-casein aqueous solution (2 ml, 1.5% (w/v)) was incubated with GE (256 nmol min^{-1} ml^{-1}) at 37°C and 50°C over 240 min. Samples (250 µl) were withdrawn at 15, 30, 60, 120 and 240 min and were immediately diluted with 450 µl of 0.1% (v/v) formic acid in HPLC grade water to inactivate the enzyme.

4.3.2. HPLC-ESI MS and MSMS analysis of β -casein hydrolysate

The β-casein hydrolysate samples were further dissolved (25 ng/µl) in ddH_2O with 0.1 % (v/v) formic acid and analyzed using an UltiMate® 3000 NanoHPLC (Dionex, Sunnyvale, CA, USA) linked to a MicrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany). The β-casein hydrolysate sample (1 µl) was loaded onto a C18 PepMap 100 precolumn cartridge (Dionex) using the µl Pickup mode (Dionex) at a flow rate of 25 µl min^{-1} for 3 min. After the precolumn elution, the sample was then

separated on a C18 PepMap 100 column (Dionex, 75 μm × 150 mm, 3 μm) at a flow rate of 300 nL min^{-1}. Mobile phase A was water with 0.1% v/v formic acid and mobile phase B was acetonitrile with 0.1% v/v formic acid. A linear gradient was employed from 2.0 to 40% B in 80 min. Column temperature was maintained at 25°C.

The MS and tandem MS experiments were controlled using MicrOTOF control software (version 2.3.0, Bruker Daltonics). Full scans were performed between an m/z range of 50 and 3000. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan using collision induced dissociation (CID). Electrospray conditions were as follows: capillary temperature, 150°C; capillary voltage, -1700V; dry gas (N_2) flow, 6.0 L min^{-1}.

Peak list files were searched by the MASCOT search engine (v. 2.3.0) against NCBInr (20101008). The peptide mass tolerance and fragment mass tolerance were set to ±20 ppm and ±0.05 Da, respectively. The search included enzyme V8-DE (cleavage at D and E), taxonomy- other mammalia, max missed cleavages-5, variable modifications for oxidation of methionine, phosphorylation of serine and threonine, and error tolerant search of all significant protein hits. MASCOT ion score cut-off was set to 20. The significance threshold was set to 0.05.

To manually detect low molecular mass peptides peak list files were searched in the molecular mass range of 200-800 Da using DataAnalysis, Bio-Tools and Sequence Editor software packages supplied by Bruker Daltonics GmbH (Bremen, Germany).The peptide mass tolerance and fragment mass tolerance were set to ±20 ppm and ±0.05 Da, respectively.

4.4. Results and Discussion

Various temperatures have been used for protein hydrolysis with Alcalase. From reports in the literature it appears that an incubation temperature of 50°C has predominantly been used for hydrolysis of milk proteins with Alcalase (McDonagh and FitzGerald, 1998, van der Ven *et al.,* 2001, Severin and Xia, 2006, Spellman *et al.,* 2005 and Spellman *et al,* 2009). However, Peng *et al.*, (2009) hydrolysed whey protein isolate with Alcalase at 65°C. Adamson and Reynolds (1995) hydrolysed sodium caseinate with Alcalase at 60°C. While Park and Allen (1998) incubated α- and β- caseins with GE at 20°C. Whereas Yokoi *et al.,* (2001) incubated α- and β-caseins with GE at 37°C. Moreover, Kakudo *et al.*, (1992) used an incubation temperature of 37°C for characterization of GE activity. Therefore it was decided to incubate β-casein with GE both at 37 and 50°C.

Distinct peaks were observed in the total ion chromatograms (TIC) for the β-casein sample incubated with GE at 37°C for various time intervals (Figure 4.1). From Figure 4.1, it was observed that for the sample incubated for 15 min the intensities of the peaks were lower than those observed in the samples incubated for 30, 60, 120 and 240 min. The maximum number of peaks were observed in the 25-50 min elution time range in all the samples. In the samples incubated for 15, 30 and 60 min a cluster of peaks were also observed between 60-80 min. However, in the samples incubated for 120 and 240 min the number of peaks decreased in the 60-80 min elution time range in comparison to those observed eluting at 15, 30 and 60 min. The peptide sequences, experimental mass, calculated masses of singly or multiply charged ions along with the MASCOT scores following incubation with GE over 240 min are given in Table 4.1.

As only peptide fragments corresponding to β-casein were obtained upon digestion of β-casein with GE this indicates that the β-casein sample used in the present study was pure (Table 4.1). It was also observed that Ser residues in β-casein at positions 15, 17, 18, 19 and 35 were phosphorylated (Table 4.1).

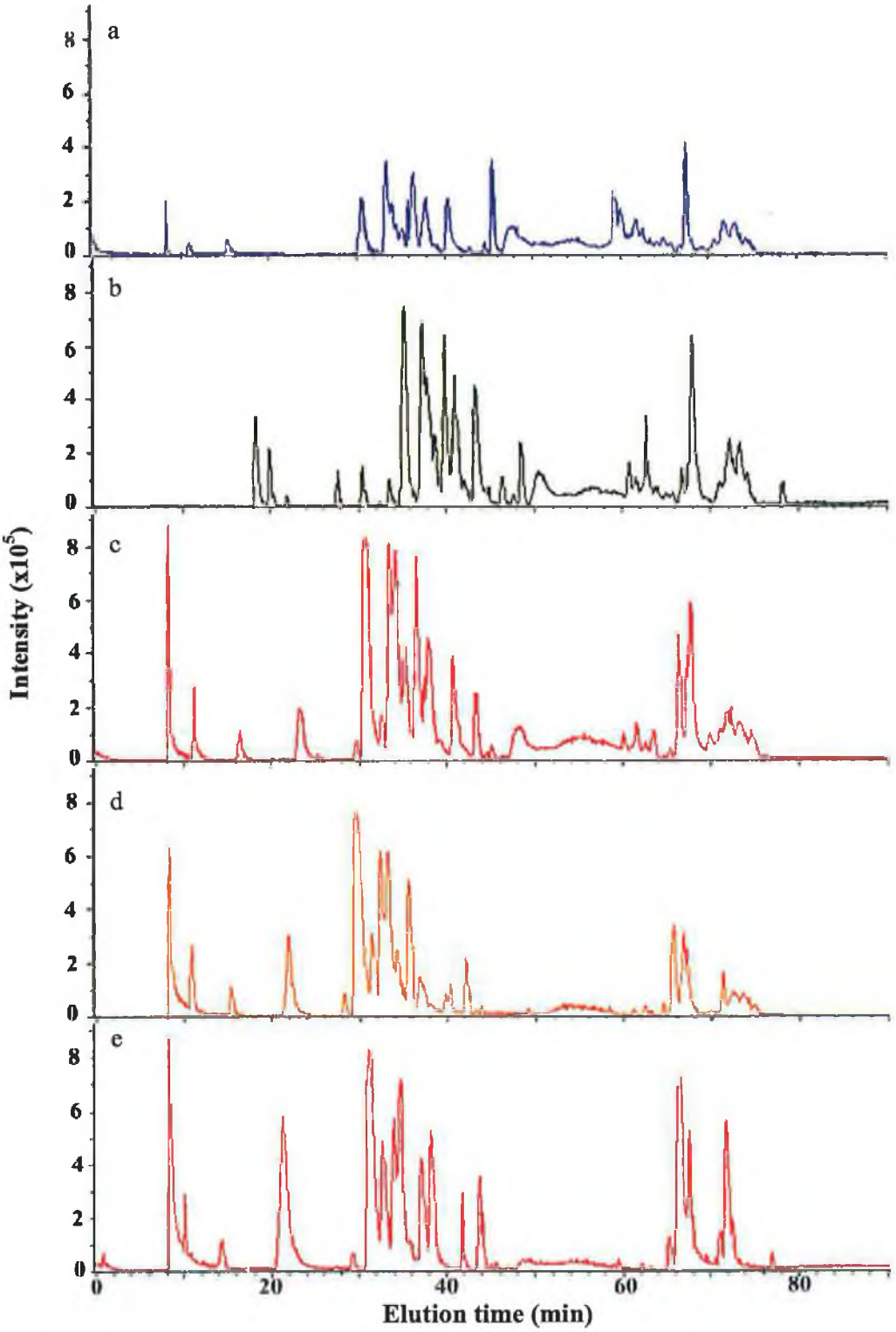


Figure 4.1. The total ion chromatogram of β-casein incubated with glutamyl endopeptidase at 37°C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min and (e) 240 min.

The peptide fragments of β-casein obtained due to GE activity can be summarised as follows: f6-11, f92-100, f101-121 and f122-131 were observed following incubation for 15, 30, 60, 120 and 240 min, f32-42 and f32-44 were observed following incubation for 15, 30, 60 and 120 min, f1-14 was observed in sample incubated for 15, 30 and 60 min, f1-11 was observed in samples incubated for 15 and 60 min, f185-209 was observed in samples incubated for 15, 30 and 240 min, f6-14 was observed in samples incubated for 15, 120 and 240 min, f6-31 and f32-47 were observed following incubation for 30 and 60 min, f32-37 was observed in samples incubated for 30 and 120 min, f5-11 and f22-31 were observed following incubation for 60, 120 and 240 min, f1-20 was observed only in sample incubated for 15 min, f32-43 and f45-91 were observed following incubation for 120 min and f109-121 was observed in the sample incubated for 240 min (Table 4.1).

The non-specific peptide fragments of β-casein obtained by GE can be summarised as follows: f32-52 was observed in samples incubated for 30 and 120 min indicating the hydrolysis of Phe(52)-Ala(53), f189-209 was observed in sample incubated for 120 min indicating hydrolysis of Gln(188)-Ala(189) and f101-128 was observed in sample incubated for 240 min indicating hydrolysis of Thr(128)-Leu(129) (Table 4.1).

Table 4.1. Peptide sequences identified upon incubation of β-casein with glutamyl endopeptidase for different time intervals at 37°C. The amino acid residues present at P_1 and P_1' are given.

β-Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 15 min.					
f1-11	RELEELNVPGE-I	642.818(2)	1283.6214	1283.6357	36.77
f1-14	RELEELNVPGEIVE-S	813.4131(2)	1624.8116	1624.8308	52.61
f1-20	RELEELNVPGEIVESLSSSE-E	889.4104(3)	2665.2094	2665.2103	41.62
f6-11	E-LNVPGE-I	628.3178(1)	627.3105	627.3228	20.58
f6-14	E-LNVPGEIVE-S	969.5095(1)	968.5022	968.5179	26.8
f32-42	E-KFQS*EEQQQTE-D	731.2839(2)	1460.5532	1460.582	45.63
f32-44	E-KFQS*EEQQQTEDE-L	853.3179(2)	1704.6212	1704.6152	68.21
f92-100	E-VMGVSKVKE-A	488.7704(2)	975.5262	975.5423	48.07
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-V	830.7336(3)	2489.179	2489.2123	63.51
f101-121	E-AM°APKHKEM°PFPKYPVEPFTE-V	836.0634(3)	2505.1684	2505.2072	35.59
f122-131	E-SQSLTLTDVE-N	1092.5294(1)	1091.5221	1091.5346	55.35
f185-209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1414.8442(3)	2827.6738	2827.6287	52.76

Table 4.1. Continued.

β-Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 30 min.					
f1-14	RELEELNVPGEIVE-S	813.41(2)	1624.8054	1624.8308	55.99
f6-11	E-LNVPGE-I	628.3192(1)	627.3119	627.3228	20.58
f6-31	E-LNVPGEIVESLS*S*S*EESITRINKKIE-K	1037.7957(3)	3110.3653	3110.4226	56.02
f6-31	E-LNVPGEIVES*LS*S*S*EESITRINKKIE-K	1064.4503(3)	3190.3291	3190.3889	48.64
f32-37	E-KFQS*EE-Q	847.3092(1)	846.3019	846.316	35.91
f32-42	E-KFQS*EEQQQTE-D	731.2814(2)	1460.5482	1460.5457	51.02
f32-44	E-KFQSEEQQQTEDE-L	813.3411(2)	1624.6676	1624.6853	50.79
f32-44	E-KFQS*EEQQQTEDE-L	853.3224(2)	1704.6302	1704.6152	65.27
f32-47	E-KFQSEEQQQTEDELQD-L	991.4193(2)	1980.824	1980.8548	28.64
f32-47	E-KFQS*EEQQQTEDELQD-K	1031.3977(2)	2060.7808	2060.8212	41.03
f32-52	E-KFQS*EEQQQTEDELQDKIHPF-A	895.3837(3)	2683.1293	2683.1803	64.65
f92-100	E-VMGVSKVKE-A	488.7705(2)	975.5264	975.5423	40.61
f101-121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4024(3)	2473.1854	2473.2174	53.21
f101-121	E-AMAPKHKEM°PFPKYPVEPFTE-S	836.0642(3)	2505.1708	2505.2072	41.94
f122-131	E-SQSLTLTDVE-N	1092.5234(1)	1091.5161	1091.5346	50.9
f185-209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1414.8406(2)	2827.6666	2827.6287	51.35
Incubation for 60 min.					
f1-11	RELEELNVPGE-I	642.8134(2)	1283.6122	1283.6357	30.46
f1-14	RELEELNVPGEIVE-S	813.4075(2)	1624.8004	1624.8308	41.62
f5-11	E-ELNVPGE-I	757.3597(1)	756.3524	756.3654	35.37
f6-11	E-LNVPGE-I	628.3206 (1)	627.3133	627.3228	21.31
f6-31	E-LNVPGEIVESLS*S*S*EESITRINKKIE-K	1037.7951(3)	3110.3635	3110.4226	30.39
f22-31	E-SITRINKKIE-K	601.3557(2)	1200.6968	1200.719	27.92
f32-42	E-KFQS*EEQQQTE-D	731.284(2)	1460.5534	1460.582	31.07
f32-44	E-KFQS*EEQQQTEDE-L	853.32(2)	1704.6254	1704.6516	54.57
f32-47	E-KFQS*EEQQQTEDELQD-K	1031.4032(2)	2060.7918	2060.8212	30.41
f92-100	E-VMGVSKVKE-A	488.7727(2)	975.5308	975.5423	49.01
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7311(3)	2489.1715	2489.2123	67.56
f122-131	E-SQSLTLTDVE-N	1092.5221(1)	1091.5148	1091.5346	50.95
Incubation for 120 min.					
f5-11	E-ELNVPGE-I	757.3687(1)	756.3614	756.3654	35.3
f6-11	E-LNVPGE-I	628.3317(1)	627.3244	627.3228	28.91
f6-14	E-LNVPGEIVE-S	969.5158(1)	968.5085	968.5179	26.94
f22-31	E-SITRINKKIE-K	601.3607(2)	1200.7068	1200.719	26.67
f32-37	E-KFQS*EE-Q	847.3119(1)	846.3046	846.316	27.9
f32-42	E-KFQS*EEQQQTE-D	742.2753(2)	1482.536	1482.564	42.19
f32-44	E-KFQS*EEQQQTEDE-L	864.3101(2)	1726.6056	1726.6335	73.29
f32-52	E-KFQS*EEQQQTEDELQDKIHPF-A	895.3957(3)	2683.1653	2683.1803	76.82
f45-91	E-LQDKIHPFAQTQSLVYPFPGPIP NSLPQNIPPLTQTPVVVPPFLQPE-V	1040.7596(5)	5198.7616	5198.7849	52.57
f92-100	VMGVSKVKE-A	488.7721(2)	975.5296	975.5423	43.22
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7438(3)	2489.2096	2489.2123	62.15
f101-121	E-AM°APKHKEM°PFPKYPVEPFTE-S	836.0759(3)	2505.2059	2505.2072	30.89
f122-131	E-SQSLTLTDVE-N	1092.5396(1)	1091.5323	1091.5346	53.41
f189-209	Q-AFLLYQEPVLGPVRGPFPIIV	1163.1602(2)	2324.3058	2324.3297	50.97

Table 4.1: Continued.

β-Casein fragment	Peptide sequence	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 240 min.					
f5-11	E-ELNVPGE-I	757.3643(1)	756.357	756.3654	26.24
f6-11	E-LNVPGE-I	628.3183(1)	627.311	627.3228	22.1
f6-14	E-LNVPGEIVE-S	969.5079(1)	968.5006	968.5179	55.97
f22-31	E-SITRINKKIE-K	601.3573(2)	1200.7	1200.719	26.1
f32-43	E-KFQS*EEQQQTED-E	788.7927(2)	1575.5708	1575.5726	45.91
f92-100	E-VMGVSKVKE	479.7909(2)	957.5672	957.5859	51.06
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7328(3)	2489.1766	2489.2123	76.79
f101-128	E-AM°APKHKEMPFPKYPVEPFTESQSLTLT-D	660.9324(5)	3299.6256	3299.5647	40.86
f109-121	E-M°PFPKYPVEPFTE-S	799.3684(2)	1596.7222	1596.7534	23.2
f122-131	E-SQSLTLTDVE-N	546.7653(2)	1091.516	1091.5346	26.02
f185-209	D-M°PIQAFLLYQEPVLGPVRGPFPIIV	937.5112(3)	2809.5118	2809.5605	36.99

S*: phosphorylated serine. M°: oxidised methionine.

Again distinct peaks were observed in the TIC for the β-casein sample incubated with GE for various time intervals at 50°C (Figure 4.2). No significant difference was observed in the TIC profiles of the samples incubated at 37 and 50°C. However, the intensities of the peaks observed on incubation at 50°C were higher than the intensities observed at 37°C. At 37°C, a maximum number of the peaks were observed between 25-40 min, whereas, the samples incubated at 50°C had a maximum number of peaks eluting between 25-50 min. In the samples incubated at 50°C for 15 and 30 min, a cluster of peaks were observed eluting between 60-80 min, whereas, for incubation at 37°C these peaks were only observed in the 60 min incubated sample. In the samples incubated for 60, 120 and 240 min the number of peaks significantly decreased in the 60-80 min elution time range. The peptide sequences, experimental mass, calculated masses of singly or multiply charged ions along with the MASCOT scores following incubation with GE over 240 min are given Table 4.2.

The peptide fragments of β-casein obtained due to GE activity can be summarised as follows:f92-100, f101-121 and f122-131 were observed in the sample incubated for 15, 30, 60, 120 and 240 min, f6-11 was observed in the sample incubated for 15, 60, 120 and 240 min, f6-14 was observed in the sample incubated for 15 and 30 min, f185-209 was observed in the sample incubated for 30, 60, 120 min, f5-11 and f32-44 were observed in the sample incubated for 60, 120 and 240 min, f45-91 and f48-91 were observed in the sample incubated for 60 and 240 min, f22-31 was observed in

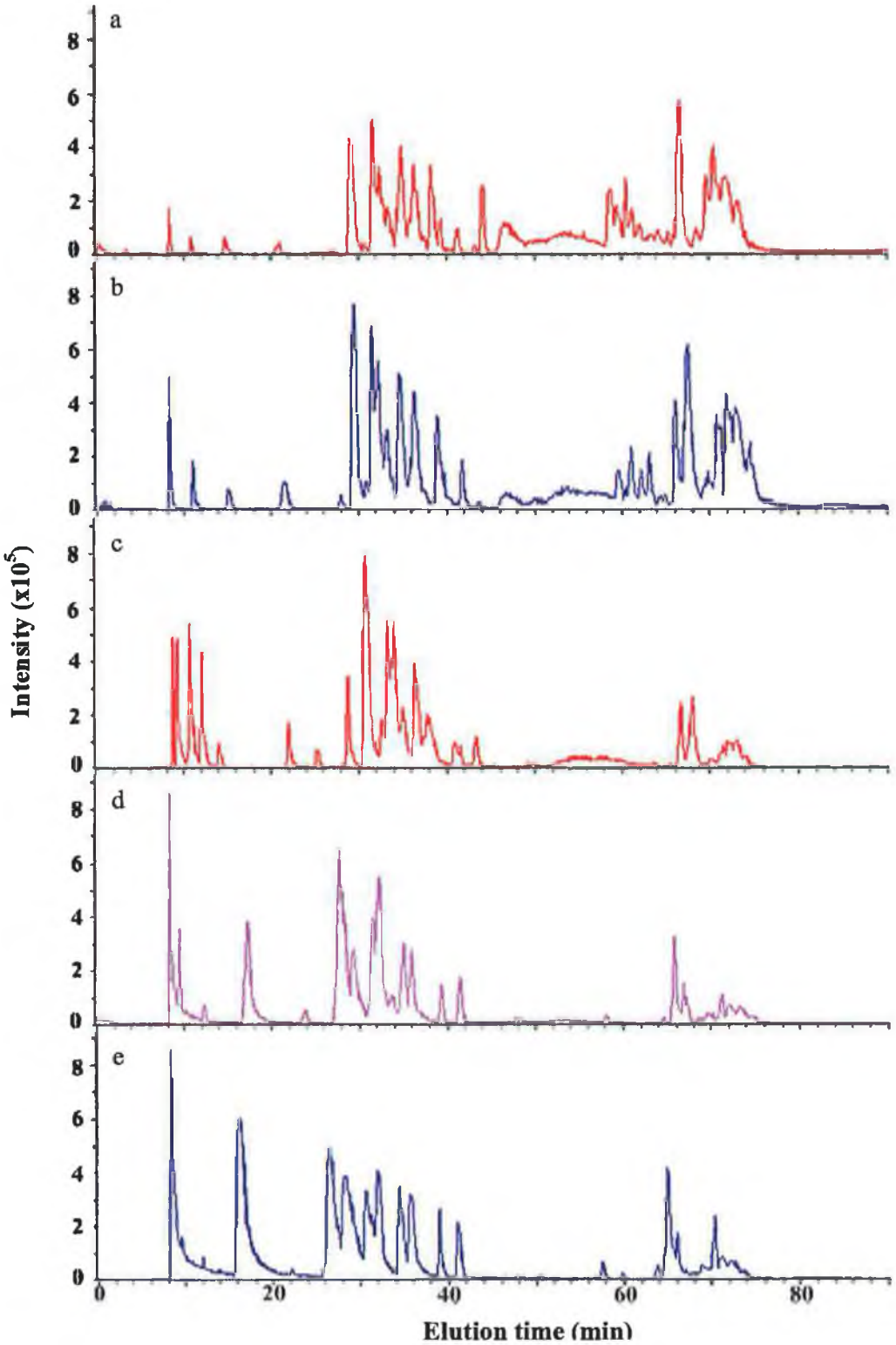


Figure 4.2. The total intensity chromatogram of β-casein incubated with glutamyl endopeptidase at 50C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min and (e) 240 min.

the sample incubated for 120 and 240 min, f1-11 was observed in the sample incubated for 15 min, f1-14, f6-31 and f92-121 were observed in the sample incubated for 30 min, f32-42 and f32-47 were observed in the sample incubated for 60 min and f109-121 and 122-129 were observed in the sample incubated for 240 min (Table 4.2).

The peptide fragments of β-casein obtained by non-specific GE activity can be summarised as follows: f101-128 was observed in the sample incubated for 120 min, indicating hydrolysis of Thr(128)-Leu(129) and f193-209 was observed in the sample incubated for 240 min indicating the hydrolysis of Leu(192)-Tyr(193) (Table 4.2).

Interestingly, from Table 4.2, it was observed that the Ser residue at the 15th position was dephosphorylated, whereas, Ser residues at positions 17, 18, 19 and 35 were phosphorylated for the peptides obtained upon incubation of β-casein with GE at 50°C (Table 4.2).

Table 4.2. Peptide sequences identified upon incubation of β-casein with glutamyl endopeptidase for different time intervals at 50°C. The amino acid residues present at P_1 and P_1' are given.

β-Casein fragment	Peptide sequence	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 15 min.					
f1-11	RELEELNVPGE-I	642.8135(2)	1283.6124	1283.6357	36.46
f6-11	E-LNVPGE-I	628.32(1)	627.3127	627.3228	20.21
f6-14	E-LNVPGEIVE-S	969.5077(1)	968.5004	968.5179	24.42
f92-100	E-VMGVSKVKE-A	488.7704(2)	975.5262	975.5423	47.18
f101-121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4097(3)	2473.2073	2473.2174	44.78
f122-131	E-SQSLTLTDVE-N	1092.5268(1)	1091.5195	1091.5346	51.03
Incubation for 30 min.					
f1-14	RELEELNVPGEIVE-S	813.4094(2)	1624.8042	1624.8308	51.85
f6-14	E-LNVPGEIVE-S	969.512(1)	968.5047	968.5179	22.44
f6-31	E-LNVPGEIVESLS*S*S*EESITRINKKIE-K	1037.7997(3)	3110.3773	3110.4226	60.32
f92-100	VMGVSKVKE-A	488.7812(2)	975.5478	975.5423	32
f92-121	E-VMGVSKVKEAMAPKHKEMPFP KYPVEPFTE-S	858.6839(4)	3430.7065	3430.7491	28.78
f101-121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4003(3)	2473.1791	2473.2174	51.39
f122-131	E-SQSLTLTDVE-N	1092.5295(1)	1091.5222	1091.5346	40.13
f185-209	D-M°PIQAFLLYQEPVLGPVRGPFPIIV	1413.8068(3)	2825.599	2825.5554	52.38
Incubation for 60 min.					
f5-11	E-ELNVPGE-I	757.3639(1)	756.3566	756.3654	20.68
f6-11	E-LNVPGE-I	628.3242(1)	627.3169	627.3228	22.07
f32-42	E-KFQS*EEQQQTE-D	731.2848(2)	1460.555	1460.582	43.77
f32-44	E-KFQS*EEQQQTEDE-L	864.3055(2)	1726.5964	1726.621	70.81
f32-47	E-KFQS*EEQQQTEDELQD-K	1031.3985(2)	2060.7824	2060.8212	30.57
f45-91	E-LQDKIHPFAQTQSLVYPFPGPIHN SLPQNIPPLTQTPVVVPPFLQPE-V	749.393(7)	5238.7001	5238.791	22.4

Table 4.1: Continued.

β-Casein fragment	Peptide sequence	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 60 min.					
f48-91	D-KIHPFAQTQSLVYPFPGPIHNSLP QNIPPLTQTPVVVPPFLQPE-V	977.5133(5)	4882.5301	4882.6215	26.94
f92-100	E-VM°GVSKVKE-A	496.7673(2)	991.52	991.5372	41.4
f101-121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.3985(3)	2473.1737	2473.2174	50.22
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7349(3)	2489.1829	2489.2123	42.66
f101-121	E-AM°APKHKEM°PFPKYPVEPFTE-S	836.0658(3)	2505.1756	2505.1756	31.45
f122-131	E-SQSLTLTDVE-N	1092.5238(1)	1091.5165	1091.5346	51.15
f185-209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1414.7571(3)	2827.4996	2827.5169	42.13
Incubation for 120 min.					
f5-11	E-ELNVPGE-I	757.3602(1)	756.3529	756.3654	35.04
f6-11	E-LNVPGE-I	628.3189(1)	627.3116	627.3228	21.42
f22-31	E-SITRINKKIE-K	601.3562(2)	1200.6978	1200.719	25.02
f32-44	E-KFQS*EEQQQTEDE-L	853.3135(2)	1704.6124	1704.6152	53.42
f92-100	E-VMGVSKVKE-A	488.7664(2)	975.5182	975.5059	54.62
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7316(3)	2489.173	2489.2123	71.22
f101-128	E-AM°APKHKEMPFPKYPVEPFTESQSLTLT-D	825.9147(4)	3299.6297	3299.5647	61.64
f122-131	E-SQSLTLTDVE-N	1092.5239(1)	1091.5166	1091.5346	51.02
f185-209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1413.8182(3)	2825.6218	2825.622	62.06
Incubation for 240 min.					
f5-11	E-ELNVPGE-I	757.3683(1)	756.361	756.3654	39.61
f6-11	E-LNVPGE-I	628.3275(1)	627.3202	627.3228	33.59
f22-31	E-SITRINKKIE-K	601.3636(2)	1200.7126	1200.719	27.05
f32-44	E-KFQS*EEQQQTEDE-L	853.3201(2)	1704.6256	1704.6152	44.58
f45-91	E-LQDKIHPFAQTQSLVYPFPGPIPNS LPQNIPPLTQTPVVVPPFLQPE-V	1040.7535(5)	5198.7311	5198.7849	48.71
f48-91	D-KIHPFAQTQSLVYPFPGPIPNSLP QNIPPLTQTPVVVPPFLQPE-V	1211.6489(4)	4842.5665	4842.6153	40.35
f92-100	E-VMGVSKVKE-A	488.7723(2)	975.53	975.5423	48.18
f101-121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4064(3)	2473.1974	2473.2174	41.57
f101-121	E-AM°APKHKEMPFPKYPVEPFTE-S	832.0809(3)	2493.2209	2493.2208	61.54
f101-121	E-AM°APKHKEM°PFPKYPVEPFTE-S	836.0711(3)	2505.1915	2505.2072	32.07
f109-121	E-M°PFPKYPVEPFTE-S	799.3791(2)	1596.7436	1596.7534	43.41
f122-129	E-SQSLTLTD-V	864.4234(1)	863.4161	863.4236	33.95
f122-131	E-SQSLTLTDVE-N	1092.5281(1)	1091.5208	1091.5346	51.22
f193-209	L-YQEPVLGPVRGPFPIIV	941.0256(2)	1880.0366	1880.056	45.01

S*: phosphorylated serine. M°: oxidised methionine.

It could be estimated that 23 different GE specific cleavage sites may exist in β-casein (Table 4.3). From Table 4.3, it can be observed that GE was highly specific both at 37 and 50°C. It can be concluded that GE predominantly hydrolysed peptides on the carboxy terminal of Glu and Asp residues. However, some of the theoretically expected cleavage sites were not hydrolysed. Glu residues present at positions 2, 21, 36, 117 and 195 along with the Asp residue present at 129 appeared not to be hydrolysed on incubation at 37°C. On incubation at 50°C, Glu residues at 2, 20, 21, 36, 117 and 195 along with Asp residue at position 43 did not appear to be hydrolysed.

The Glu(2)-Leu(3) bond may have been cleaved but due to instrumental set-up limitations it was not detected. Interestingly, the Glu(20)-Glu(21) bond, appears to be hydrolysed in the sample incubated at 37°C for 15 min. However, no evidence was found of the Glu(20)-Glu(21) bond hydrolysis on incubation at 50°C. In addition, no evidence was found of Glu(36)-Glu(37) hydrolysis on incubation either at 37 or 50°C. Interestingly, Glu(4)-Glu(5) appeared to be hydrolysed after 60, 120 and 240 min for incubation both at 37°C and 50°C (Tables 4.1, 4.2 and 4.3).

Table 4.3. Theoretically expected cleavage site for glutamyl endopeptidase action on β-casein along with the actual cleavages observed upon incubation of purified β-casein with GE at 37 and 50°C. The amino acid residues present at P_1-P_1' are also given.

Theoretical cleavage sites (P_1 position)	Amino acid residues at P_1-P_1'	Cleavages observed at 37°C	Cleavages observed at 50°C
02	E-L	No	No
04	E-E	Yes	Yes
05	E-L	Yes	Yes
11	E-I	Yes	Yes
14	E-S	Yes	Yes
20	E-E	Yes	No
21	E-S	No	No
31	E-K	Yes	Yes
36	E-E	No	No
37	E-Q	Yes	Yes
42	E-D	Yes	Yes
43	D-E	Yes	Yes
44	E-L	Yes	Yes
47	D-K	Yes	Yes
91	E-V	Yes	Yes
100	E-A	Yes	Yes
108	E-M	Yes	Yes
117	E-P	No	No
121	E-S	Yes	Yes
129	D-V	No	Yes
131	E-N	Yes	Yes
184	D-M	Yes	Yes
195	E-P	No	No

Park and Allen (1998) reported that Glu residues present at positions 11, 14, 20, 91 and 121 were cleaved upon recirculation of β-casein on immobilised GE beads at 20°C for 3 h. The results presented in this Chapter are in agreement with those Park and Allen (1998). In addition to the cleavages observed by Park and Allen (1998), it was also found that Glu residues present in positions 4, 5, 31, 42, 44, 100 and 131 were hydrolysed in samples incubated at 37°C and 50°C in the present study. However, the Glu residue present at position 20 did not appear to be hydrolysed in

any of the samples incubated at 50°C. Whereas, surprisingly the Glu(20)-Glu(21) bond appeared to be hydrolysed in the sample incubated for 15 min at 37°C. Yokoi *et al.,* (2001) reported that Glu residues present at positions 91, 100 and 121 were cleaved upon digestion of β-casein with GE at 37°C for 1 and 3 h. Park and Allen (1998) also reported that Asp residues present at positions 47, 129 and 184 were cleaved upon recirculation of β-casein on immobilised GE beads at 20°C for 3 h. In the present study, it was observed that Asp residues present at positions 43, 47 and 184 were cleaved in samples incubated at 37°C. While the Asp residue present at positions 47, 129 and 184 was cleaved in samples incubated at 50°C. No evidence of Asp residues being cleaved by GE was reported by Yokoi *et al.,* (2001).

As previously mentioned, Breddam and Meldal (1992) reported that the rate of hydrolysis of Glu-Asp was slow and the Glu-Pro bond was either not hydrolysed or hydrolysed to a low extent. The results presented in this Chapter indicate that the Glu-Pro (117-118 and 195-196 in β-casein) bond was not hydrolysed. It was also observed that the Glu(108)-Met(109) bond was only hydrolysed in the sample incubated for 240 min at both 37 and 50°C, suggesting that Met residues at the P_1' position were poorly preferred by GE. No previous literature reports appear to exist about the hydrolysis of peptide bonds with Glu and Met at the P_1 and P_1' positions, respectively. Interestingly, it was observed in the present study that the Asp-Met (184-185) was readily hydrolysed in the samples incubated at 37°C for 15, 30 and 240 min and in the samples incubated for 30, 60 and 120 min at 50°C (Tables 4.1 and 4.2). Based on these results it can be concluded that Glu-Met is hydrolysed to a lower extent in comparision to Asp-Met.

There were also some non-specific cleavages observed at the carboxy terminal of Phe (52), Thr(128), Gln(188) on incubation at 37°C and non-specific cleavages at the carboxy terminal of Thr(128) and Leu(192) were observed on incubation at 50°C. Breddam and Svensden (1992) have previously reported non-specific cleavage at the carboxy terminal of Phe during the hydrolysis of ribonuclease at a high GE concentration (1.2 μM). The hydrolysis of Leu (192)-Tyr(193) may be due to low levels of residual chymosin activity. Primarily chymosin cleaves the Phe-Met (105-106) bond of κ-casein but if the enzyme is not properly inactivated, it will also hydrolyse specific bonds in other caseins, preferentially in β-casein. Fragments βI, βII

and βIII corresponding to β-casein fragment 1-189/192, 1-163 and 1-139 respectively are reported by the action of chymosin on β-casein (Fox *et. al.,* 1993).

Hydrolysis of β-casein with GE should yield a number of short peptide sequences. In the present study only a 6 amino acid (aa) residue fragment of β-casein (f6-11) was detected on incubation at both 37°C and 50°C (Table 4.1 and 4.2). However, f15-20 (6 aa residues), which contain the tetra phosphorylated peptide, pSLpSpSpSE was not detected in either the 37 or 50°C incubated samples. Park and Allen (1998) observed the phosphorylated peptide (f15-20) upon incubation of β-casein with GE at 20°C. Theoretically, the action of GE on β-casein should release two di-peptides (f1-2 (RE) and f3-4 (LE)), one tri-peptide (f12-14, (IVE)), one tetra peptide (f118-121(PFTE)) and two penta-peptides (f32-36 (KFQSE) and f38-42 (QQQTE), Table 4.3). Under the main detection method (MASCOT search engine) employed in this study none of these peptides were observed. However, since the peptide bond Glu-Pro (195-196 and 117-118) was not hydrolysed, the tetra peptide f118-121 (PFTE) was unlikely to be present. In order to detect these low molecular mass peptides a manual search was carried out as described in Section 4.3.2. During this manual detection only peptide RELEE (1-5) was observed in the sample incubated for 120 min at 37°C and on incubation at 50°C, peptide RELEE was observed in the 60 and 120 min samples.

Table 4.4. The percentage peptide sequence coverage for various incubation times upon incubation of β-casein with glutamyl endopeptidase at 37 and 50°C.

Incubation time (min)	Peptide sequence coverage at 37°C (%)	Peptide sequence coverage at 50°C (%)
15	46.9	25.8
30	56.0	45.9
60	41.6	63.2
120	72.7	45.5
240	49.8	68.4
Mean	53.4	49.8

From Table 4.4, it can be observed that the mean peptide sequence coverage values for 37°C and 50°C incubations were 53.4% and 49.8%, respectively. A marked difference in the peptide sequence coverage values for 15 min incubated samples at 37°C and 50°C was observed. This may be due to the fact that the MASCOT score cut off was set at 20, this may account for some of the peptides present for the 50°C incubated sample not being observed. The generally low percentage peptide sequence coverage values observed may be due to the fact that the C-terminal peptide f132-184 was not detected in any of samples incubated for the various time intervals at either 37

or 50°C. The amino acid residues present (53) in f132-184 account for 25.4% of total amino acid residues present in β-casein (209). Moreover, f48-91, containing 44 amino acid residues corresponding to 21.05% of the total amino acid residues present in β-casein was only detected in the sample incubated at 37°C for 120 min (Table 4.1). Both f45-91 and f48-91 were only observed in the sample incubated at 50°C for 60 and 240 min (Table 4.2). There are no potential cleavage sites for GE within either f48-91 or f132-184. Furthermore, f48-91 and f132-184 occur in regions which are generally considered to be hydrophobic (Ward, 1998). Therefore the low mean percentage peptide sequence coverage values obtained need to be considered with these facts in mind.

4.5. Conclusion

As GE appeares to play a key role in peptide aggregation and bitterness in whey protein hydrolystaes (Spellman *et al.,* 2005 and Spellman *et al,* 2009), it was decided to investigate the substrate specificity of GE using an actual milk protein substrate. As caseins are the principal milk proteins which account for 76-86% of protein present in bovine milk (Swaisgood, 1982), it was decided to use casein as a substrate for investigating the substrate specificity of GE. β-Casein is the major protein in milk which accounts for approximately 33% of total casein (Farrell *et al.,*2004). Moreover, in β-casein f1-45/47 and f90-131 have 14 and 7 theoretical cleavage sites for GE action, respectively. It was expected that f132-209 would have two theoretical cleavage sites for GE action, i.e., Asp (184) and Glu (195). While f48-91 has no theoretical cleavage sites for GE action. Due to the above reasons β-casein was therefore expected to be an interesting substrate to investigate the substrate specificity of GE. In addition Glu residues present in β-casein have unique amino acid residues at the P_2, P_3, P_4, etc and P_1', P_2', P_3', etc, positions.

The results herein show that since only peptide fragments corresponding to the primary sequence of β-casein were observed upon incubation of β-casein with GE it can be concluded that the β-casein sample used in the present study was pure. The phosphorylated peptides obtained (f6-31 and f32-n (n=37, 42, 44, 47 and 52), Table 4.1 and 4. 2) showed that there were 5 phosphorylated serine residues present in the purified β-casein at positions 15,17,18,19 and 35, which is in agreement with the information present in the literature (Swaisgood, 1982).

GE was observed to hydrolyze predominantly on the carboxy terminal of Glu residues present in β-casein on incubation at 37 and 50°C. This implies that the GE preparation used in the present study was significantly pure. However, GE also hydrolysed peptide bonds at the carboxy terminal of Asp residues in β-casein on incubation at 37 and 50°C. It was observed that GE was non-specific with respect to the nature of the amino acid residue present at the P_1' position. However, the hydrolysis of the Glu-Pro bond was not observed and therefore can be concluded that GE does not prefer a Pro residue at the P_1' position. Breddam and Meldal (1992) reported that Pro residues at P_1' position were not preferred or poorly preferred by GE. Breddam and Meldal (1992) also reported that the hydrolysis of the Glu-Asp bond was slow. However, in the present study it was observed that the Glu-Asp bond appeared to be hydrolysed in samples incubated at 37°C after 15, 30, 60 and 120 min (Table 4.1). However, for incubation at 50°C, surprisingly the hydrolysis of the Glu-Asp bond was observed only in the sample incubated for 60 min.

The results herein suggest that Glu-Met was hydrolysed to a lower extent when compared to Asp-Met. Hydrolysis of Asp(184)-Met(185) was observed after 15, 30 and 240 min incubation at 37°C, and on incubation at 50°C hydrolysis of Asp(184)-Met(185) was observed after 30, 60 and 120 min. Whereas, hydrolysis of Glu(108)-Met(109) was only observed after 240 min incubation at both 37°C and 50°C indicative of a less favoured cleavage site. To the best of our knowledge, no previous work appears to have reported on GE hydrolysis of peptide bonds between Glu or Asp and Met at the P_1 and P_1' positions, respectively.

Breddam and Svensden (1992) reported non-specific cleavage at the carboxy terminal of Phe during the hydrolysis of ribonuclease at high GE concentration (1.2 μM). The results obtained in the present study showed that GE hydrolysed the peptide bond at the carboxy terminal of Phe (51), Thr (128) and Gln (188) upon incubation of β-casein at 37°C. It was also observed that GE hydrolysed the peptide bond at the carboxy terminal of Thr (128) upon incubation of β-casein at 50°C. However, the hydrolysis of Leu(192)-Tyr(193) in the samples incubated at 50°C for 240 min is indicative of the presence of low levels of residual chymosin activity. The mean peptide sequence coverage was 53.4 and 49.8% for samples incubated at 37 and 50°C, respectively.

References

Adamson, N. J. and Reynolds, E. C. (1996) Characterisation of casein phosphopeptides prepared using alcalse: Determination of enzyme specificity. *Enzyme microb tech,* **45**, 196-204.

Breddam, K. and Svendsen, I. (1992). Isolation and amino acid sequence of a glutamic acid specific endopeptidase from *Bacillus licheniformis*. *Eur. J. Biochem.* **204,** 165-171.

Breddam, K. and Meldal, M. (1992). Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching *Eur. J. Biochem.* **206**, 103-107.

Farrell, H. M., Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K. F. & Swaisgood, H.E. (2004). Nomenclature of the proteins of cow's milk-sixth revision. *J. Dairy Sci.* **87(6),** 1641-1674.

Fox, P. F., Law, J., McSweeney. P. L. H., & Wallace, J., (1993). Cheese: Chemistry, Physics and Microbiology. In: P. F. Fox (Ed.), *Biochemistry of Cheese Ripening*, 2nd ed. (p 399). Chapman and Hall, London, England.

Kakudo, S., Kikuchi, N., Kitadokoro, K., Fujiwara, T., Nakamura, E., Okamoto, H., Shin, M., Tamaki, M., Teraoka, H., Tsuzuki, H. and Yoshida, N. (1992). Purification, characterization, cloning, and expression of a glutamic acid-specific protease from *Bacillus licheniformis* ATCC 14580. *J. Biol. Chem,* **267(33),** 23782-23788.

McDonagh, D. and FitzGerald, R. J. (1998). Production of caseinophosphopeptides (CPPs) from sodium caseinate using a range of protease preparations. *Int Dairy J*, **8**, 39-45

FitzGerald, R. J. and Meisel, H. (2003). Milk protein hydrolysates and bioactive peptides. In: P. F. Fox and P. L. H. McSweeney (Ed.), *Advanced Dairy Chemistry- 1 Proteins.*3rd ed. (pp 675–698). New York, NY, USA.

Park, O. and Allen, J. C. (1998). Preparation of phosphopeptides derived from α_s-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. *J. Dairy Sci*, **81(11),** 2858-2865.

Peng, X., Xiong, Y. L. and Kong, B. (2009). Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. *Food Chem*, **113 (1)**, 196-201.

Spellman, D., Kenny, P., O'Cuinn, G. & FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with *Bacillus licheniformis* proteinase activities. *J Agric Food Chem*, **5(4)**, 1258-1265.

Spellman, D., O'Cuinn, G. & FitzGerald, R. J. (2009). Bitterness in Bacillus proteinase hydrolysates of whey proteins *J. Food Chem,* **114 (2)**, 440-446.

Severin, S. and Xia, W.S. (2006). Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *J Food Bioche*, **30**, 77–97.

Swaisgood, H. E. (1982). Chemistry of caseins. In: P.F. Fox (Ed.), *Advanced Dairy Chemistry,* Vol: 1(pp 1-59). Applied Science, London.

van der Ven, C., Gruppen, H., Bont, D.B. and Voragen, A.G., 2001. van der de Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics. *J Agric Food Chem,* **49,** 5005-5012.

Ward, L. S. (1998). Isolation of caseins fractions by manipulating inherent solubility and association properties of caseins. Ph.D. Dissertation, *University of Minnesota,* St. Paul, USA, p 6.

Yokoi, K., Kakikawa, M., Kimoto, H., Watanabe, K., Yasukawa, H., Yamakawa, A., Taketo, A. and Kodaira, K. (2001). Genetic and biochemical characterization of glutamyl endopeptidase of Staphylococcus *warneri* M. *Gene,* **281,** 115–122.

Chapter 5

Substrate specificity of glutamyl endopeptidase (GE): hydrolysis studies with an enriched α-casein preparation.

5.1. ABSTRACT

A protein preparation enriched in bovine α-casein (2 ml, 1.5% (w/v) aqueous solution) was digested with glutamyl endopeptidase (GE, 256 μmol min^{-1} ml^{-1}) at 37°C and 50°C for 4 h. Samples (250 μl) were withdrawn at various time intervals (15, 30, 60, 120 and 240 min) and the peptides generated were analysed using mass spectrometry. GE activity was highly specific and hydrolysed the peptide bond predominantly on the carboxy terminal of Glu residues. GE was also observed to hydrolyse peptide bonds on the carboxy terminal of Asp residues. It was observed that hydrolysis did not occur when Pro was at the P_1' position. In Glu-Glu-X and Glu-Glu-Glu-X sequences (X= Arg, Asn, Ile, and Lys), the Glu-X bond hydrolysis was preferred by GE in comparison with Glu-Glu bond hydrolysis. Non specific cleavages at the carboxy terminal of Phe (24) and Gln (131) were observed upon incubation of the enriched α-casein preparation for various time intervals at 50°C. The mean peptide sequence coverage values corresponding to α_{s1}-casein were 51.8 and 81.8%, respectively, for the samples incubated with GE at 37°C and 50°C.

5.2. Introduction

Park and Allen (1998) prepared casein phosphopeptides using immobilized GE. Solutions of α- and β-casein (1% (w/v), 20 ml) in 50 mM Tris-HCl buffer pH 8.0 containing 0.02% (w/v) NaN_3 were recirculated in a fluidized bed bioreactor containing 2 ml of immobilised GE beads at 20°C for 3 h. It was reported that hydrolysis of α_{s1}-casein with GE occurred rapidly, i.e., after 5 min incubation. The electrophoretic profile showed four well defined bands in the molecular mass range of 3.6 to 10.8 kDa. Based on the Ca^{2+} binding isotherms Park and Allen (1998) stated that the four fragment of α_{s1}-casein observed on SDS-PAGE were f52-141, f44-117, f86-141 and f90-118 corresponding to molecular masses of 10.8, 9.0, 6.6 and 3.6 kDa, respectively. Yokoi *et al.,* (2001) incubated α-casein (20 μg) with GE (0.03 μg) in the presence of 50 mM Tris-HCl buffer (pH 7.5) at 37°C for 3 h. It was reported that that GE cleaved α–casein into several fragments based on the SDS-PAGE profiles obtained. However, no specific peptide sequence details were reported by Yokoi *et al.,* (2001).

The results presented in Chapter 4 indicate that GE was highly specific and predominantly hydrolysed the peptide bond on the carboxy terminal of Glu and Asp residues. However, some non-specific cleavages on the carboxy terminal of Phe, Gln, and Thr were observed upon incubation of β-casein with GE. It was also observed that the Glu-Pro bond was not hydrolysed by GE, whereas, Glu-Met was hydrolysed to a lower extent when compared to hydrolysis of Asp-Met. The primary structure of alpha (α_{s1}- and α_{s2}) casein has some interesting sequences, e.g., Glu(69)-Glu(70)-Ile(71), Glu(117)-Glu(118)-Arg(119) and Glu(133)-Pro(134) in α_{s1}-casein and Glu(11)-Glu(12)-Ser(13), Glu(59)-Glu(60)-Ser(61), Glu(67)-Glu(68)-Val(69), Glu(132)-Glu(133)-Asn(134), Asp(74)-Asp(75)-Lys(76), Glu(49)-Glu(50)-Glu(51)-Tyr(52) and Glu(155)-Glu(156)-Glu(157)-Lys(52) in α_{s2}-casein which may be susceptible to hydrolysis with GE. Therefore, it was decided to further investigate the substrate specificity of GE by incubating a preparation enriched in alpha (α_{s1}- and α_{s2}) casein with GE for various time intervals at 37 and 50°C.

The objective of this study was to further investigate the substrate specificity of GE regarding the amino acid residues present at the P_1 and P_1' positions using a preparation enriched in bovine α_{s1}- and α_{s2}-casein.

5.3. Methods and Materials

HPLC grade water and formic acid were from Sigma-Aldrich, Dublin, Ireland. The enriched α-casein preparation and GE were obtained by the methods described in Chapter 1 (Protocol 4) and Chapter 2 (Protocol 3), respectively.

5.3.1. Digestion of α-casein with GE

Enriched α-casein aqueous solution (2 ml, 1.5% (w/v)) was incubated with GE (256 μmol min^{-1} ml^{-1}) at 37°C and 50°C over 240 min. Samples (250 μl) were withdrawn at 15, 30, 60, 120 and 240 min and were diluted with 450 μl of 0.1% (v/v) formic acid in HPLC grade water to immediately inactivate the enzyme

5.3.2. HPLC-ESI MS and MSMS analysis of α-casein hydrolysates

The hydrolysate samples of enriched α-casein were dissolved (25 ng/μl) in ddH_2O with 0.1 % (v/v) formic acid and analyzed using an UltiMate® 3000 NanoHPLC (Dionex, Sunnyvale, CA, USA) and ESI- MicrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany). The enriched α-casein hydrolysate samples (1 μl) were loaded onto a C18 PepMap 100 precolumn cartridge (Dionex) with the μl Pickup mode (Dionex) at a flow rate of 25 μl min^{-1} for 3 min. Being eluted from the precolumn, sample was then separated on a C18 PepMap 100 column (Dionex, 75 μm × 150 mm, 3 μm) at a flow rate of 300 nL min^{-1}. Mobile phase A was water with 0.1% (v/v) formic acid, and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. A linear gradient was employed from 2.0 to 40% B in 80 min. Column temperature was maintained at 25°C.

The MS and tandem MS experiments were controlled using MicrOTOF control software (version 2.3.0, Bruker Daltonics GmbH, Bremen, Germany). Full scans were performed between an m/z range of 50 and 3000. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan using collision induced dissociation (CID). Electrospray conditions were as follows:

capillary temperature, 150°C; capillary voltage, -1700V; dry gas (N_2) flow, 6.0 L min^{-1}.

Peak list files were searched by the MASCOT search engine (v. 2.3.0) against NCBInr (20101008). The peptide mass tolerance and fragment mass tolerance were set to ±20 ppm and ±0.05 Da, respectively. The search included enzyme V8-DE (cleavage at D and E), taxonomy- other mammalia, max missed cleavages-5, variable modifications for oxidation of methionine, phosphorylation of serine and threonine, and error tolerant search of all significant protein hits. MASCOT ion score cut-off was set to 20. The significance threshold was set to 0.05.

To manually detect low molecular mass peptides peak list files were searched in the molecular mass range of 200-800 Da using DataAnalysis, Bio-Tools and Sequence Editor software packages supplied by Bruker Daltonics GmbH (Bremen, Germany).The peptide mass tolerance and fragment mass tolerance were set to ±20 ppm and ±0.05 Da, respectively.

5.4. Results and Discussion

Distinct peaks were observed in the total ion chromatogram (TIC, Figure 5.1) in the enriched α-casein preparation following incubation at 37°C for various time intervals. However, the intensity of the peaks for the samples incubated for 15 and 30 min were significantly lower than that observed in the samples incubated for 60, 120 and 240 min. It was observed that the peaks were evenly distributed between 10-70 min. However, the intensities of peaks observed between 10-20 min and 45-60 min in all TICs were significantly higher than the other peptides. In particular, the intensity of the peaks eluted at approximately 14 and 62 min increased with an increase in incubation time. The peptide sequences, experimental mass, calculated masses of singly or multiply charged ions along with the MASCOT scores following incubation with GE over 240 min are given in Table 5.1.

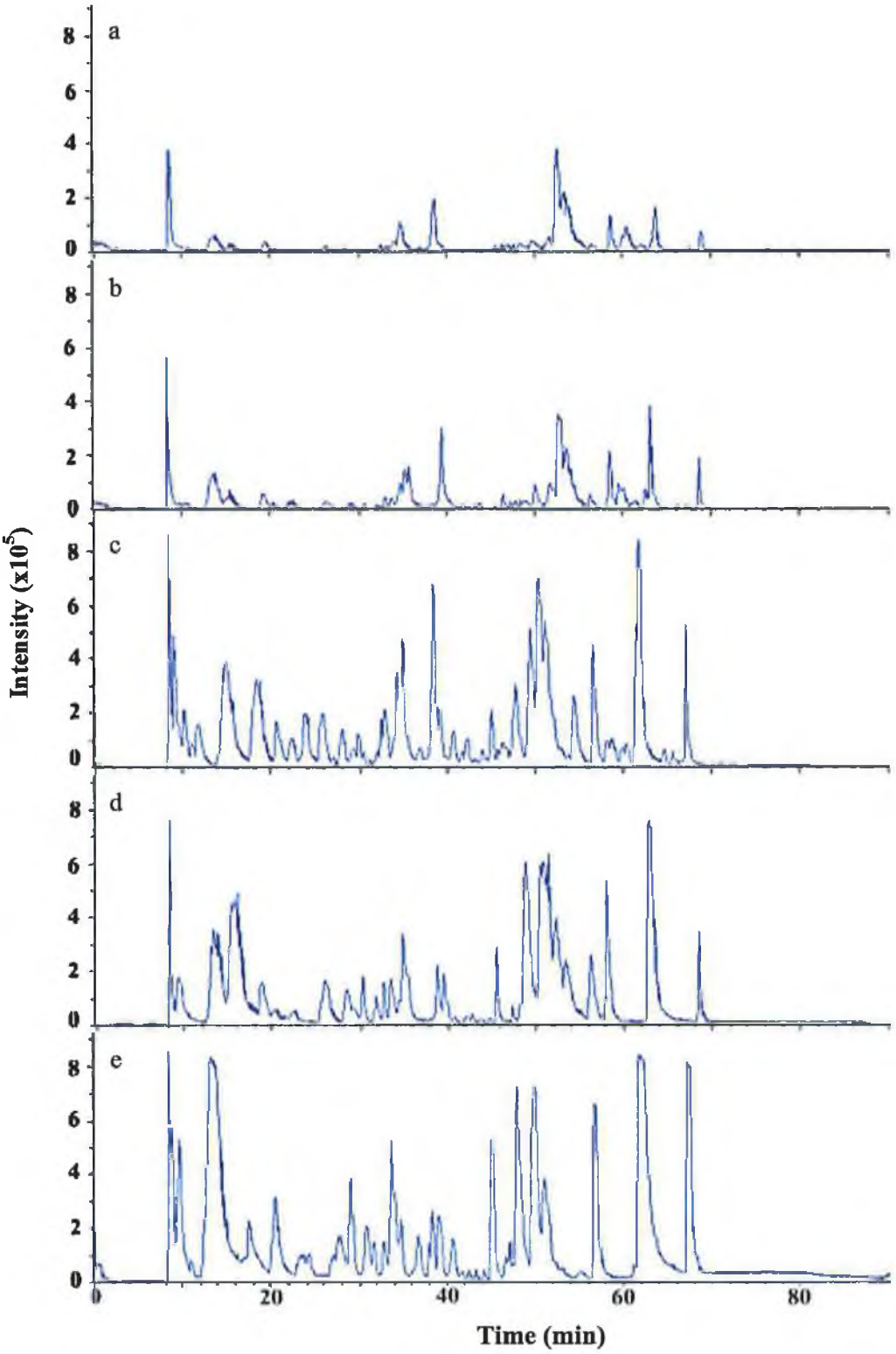


Figure 5.1. The total intensity chromatogram of enriched α-casein incubated with glutamyl endopeptidase at 37°C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min and (e) 240 min.

From Table 5.1, it was observed that α_{s1}- and α_{s2}-casein were phosphorylated to different extents. Ser residues present at positions 46, 48, 75 and 115 in α_{s1}-casein and Ser residues present at positions 16, and 143 in α_{s2}-casein were phosphorylated. Theoretically, the Ser residues present at positions 46, 48, 64, 66, 67, 68, 75 and 115 in α_{s1}- casein and positions 8, 9, 10, 16, 56, 57, 58, 61, 129, 131 and 143 in α_{s2}-caseins are phosphorylated (Swaisgood, 2003). However, in κ-casein there is only one phosphorylated Ser residue at position 149 and in the present study it was observed to be phosphorylated (Swaisgood, 2003). Some of the Ser residues both in α_{s1}- and α_{s2}-casein may either be de-phosphorylated during the purification of enriched α-casein preparation or de-phosphorelated α_{s1}- and α_{s2}-casein might have been present in the starting material (acid caseinate) used for purification of β-casein.

Table 5.1. Peptide sequences identified upon incubation of enriched α-casein with glutamyl endopeptidase for different time intervals at 37°C. The amino acid residues present at the P_1 and P_1' positions.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 15 min.					
α_{s1}-casein					
f1-18	RPKHPIKHQGLPQEVLNE-N	707.3848(3)	2119.1326	2119.165	42.48
f1-30	RPKHPIKHQGLPQEVLNENLLRFFVAPFPE-V	888.4758(4)	3549.8741	3549.9361	47.43
f3-30	P-KHPIKHQGLPQEVLNENLLRFFVAPFPE-V	825.1885(4)	3296.7249	3296.7822	43.14
f15-39	E-VLNENLLRFFVAPFPEVFGKEKVNE-L	979.1802(3)	2934.5188	2934.5643	55.69
f19-30	E-NLLRFFVAPFPE-V	725.389(2)	1448.7634	1448.7816	30.43
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	827.4393(3)	2479.2961	2479.3264	53.86
f31-39	E-VFGKEKVNE-L	525.2754(2)	1048.5362	1048.5553	28.28
f40-55	E-LSKDIGSES*TEDQAME-D	950.3399(2)	1898.6652	1898.6798	43.82
f111-118	E-IVPNSAEE-R	858.404(1)	857.3967	857.413	28.74
f111-125	E-IVPNS*AEERLHSMKE-G	607.2744(3)	1818.8014	1818.8335	20.12
f119-141	E-RLHSMKEGIHAQQKEPMIGVNQE-L	665.8317(4)	2659.2977	2659.3322	24.72
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	892.7662(3)	2675.2768	2675.3271	28.43
f193-199	E-KTTMPLW	876.4529(1)	875.4456	875.4575	32.74
f193-199	E-KTTM°PLW	446.7247(2)	891.4348	891.4524	21.16
α_{s2}-casein					
f13-18	E-SIIS*QE-T	756.3044(1)	755.2971	755.3102	26.52
f13-23	E-SIIS*QETYKQE-K	703.3076(2)	1404.6006	1404.6174	34.35
Caseinomacropeptide					
f36-64	E-STVATLEDS*PEVIESPPEINTVQVTSTAV	1547.2113(2)	3092.408	3092.469	30.14
f47-64	E-VIESPPEINTVQVTSTAV	942.483(2)	1882.9514	1882.9888	55.05
Incubation for 30 min.					
α_{s1}-casein					
f1-18	RPKHPIKHQGLPQEVLNE-N	707.3824(3)	2119.1254	2119.165	42.63
f19-30	E-NLLRFFVAPFPE-V	725.3864(2)	1448.7582	1448.7816	43.96

Table 5.1: Continued.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
α_{s1}-casein					
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	827.4363(3)	2479.2871	2479.3264	53.31
f31-39	E-VFGKEKVNE-L	525.275(2)	1048.5354	1048.5553	20.91
f31-55	E-VFGKEKVNELSKDIGSES*TEDQAME-D	977.4048(3)	2929.1926	2929.2246	46.17
f31-55	E-VFGKEKVNELSKDIGSESTEDQAM°E-D	982.7325(3)	2945.1757	2945.2195	63.45
f40-55	E-LSKDIGSES*TEDQAME-D	950.3349(2)	1898.6552	1898.6798	51.09
f40-55	E-LSKDIGSESTEDQAM°E-D	958.3322(2)	1914.6498	1914.6748	55.14
f97-118	E-QLLRLKKYKVPQLEIVPNS*AEE-R	892.4726(3)	2674.396	2674.4458	37.18
f111-118	E-IVPNS*AEE-R	938.3703(1)	937.363	937.3794	24.33
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9504(3)	1793.8294	1793.8366	47.33
α_{s2}-casein					
f13-18	E-SIIS*QE-T	756.3029(1)	755.2956	755.3102	26.15
f13-23	E-SIIS*QETYKQE-K	703.3031(2)	1404.5916	1404.6174	36.68
Incubation for 60 min.					
α_{s1}-casein					
f31-39	E-VFGKEKVNE-L	525.2803(2)	1048.546	1048.5553	24.57
f31-55	E-VFGKEKVNELSKDIGS*ESTEDQAME-D	977.4012(3)	2929.1818	2929.2246	45.66
f31-55	E-VFGKEKVNELSKDIGSES*TEDQAM°E-D	982.7313(3)	2945.1721	2945.2195	44.03
f40-55	E-LSKDIGSES*TEDQAM°E-D	918.3527(2)	1834.6908	1834.7179	25.04
f40-55	E-LSKDIGS*ESTEDQAM°E-D	958.3322(2)	1914.6498	1914.6748	50.82
f40-55	E-LSKDIGSES*TEDQAME-D	910.3534(2)	1818.6922	1818.723	36.94
f71-77	E-IVPNS*VE-Q	837.3604(1)	836.3531	836.3681	22.13
111-118	E-IVPNS*AEE-R	938.37(1)	937.3627	937.3794	28.82
f111-125	E-IVPNS*AEERLHSMKE-G	607.2766(3)	1818.808	1818.8335	44.74
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9492(3)	1793.8258	1793.8366	50.13
f149-157	E-LFRQFYQLD-A	615.3076(2)	1228.6006	1228.6241	22.64
f193-199	E-KTTM°PLW-	446.7255(2)	891.4364	891.4524	21.49
α_{s2}-casein					
f13-23	E-SIIS*QETYKQE-K	703.3036(2)	1404.5926	1404.6174	36.06
f13-33	E-SIIS*QETYKQEKNM°AINPSKE-N	845.3829(3)	2533.1269	2533.1771	22.44
f64-84	E-VATEEVKITVDDKHYQKALNE-I	810.7394(3)	2429.1964	2429.2438	60.61
f134-145	E-NSKKTVDMES*TE-V	724.7872(2)	1447.5598	1447.5538	44.11
Incubation for 120 min.					
α_{s1}-casein					
f1-18	RPKHPIKHQGLPQEVLNE-N	707.3927(3)	2119.1563	2119.165	50.49
f3-30	P-KHPIKHQGLPQEVLNENLLRFFVAPFPE-V	825.1972(4)	3296.7597	3296.7822	49.36
f15-30	E-VLNENLLRFFVAPFPE-V	953.0052(2)	1903.9958	1904.0196	24.48
f15-35	E-VLNENLLRFFVAPFPEVFGKE-K	822.4397(3)	2464.2973	2464.3155	43.1
f19-30	E-NLLRFFVAPFPE-V	725.3963(2)	1448.778	1448.7816	52.04
f19-35	E-NLLRFFVAPFPEVFGKE-K	670.6957(3)	2009.0653	2009.0775	36.79
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	827.4446(3)	2479.312	2479.3264	40.73
f31-39	E-VFGKEKVNE-L	525.2818(2)	1048.549	1048.5553	21.68
f31-55	E-VFGKEKVNELSKDIGSESTEDQAM°E-D	982.7523(3)	2945.2351	2945.2195	58.5
f31-55	E-VFGKEKVNELSKDIGS*ESTEDQAME-D	977.4172(3)	2929.2298	2929.2246	43.42
f40-55	E-LSKDIGSESTEDQAM°E-D	918.3683(2)	1834.722	1834.7179	55.55
f40-55	E-LSKDIGS*ESTEDQAME-D	950.3551(2)	1898.6956	1898.6798	55.38
f40-55	E-LSKDIGS*ES*TEDQAME-D	950.3527(2)	1898.6908	1898.6893	30.16
f71-77	E-IVPNS*VE-Q	837.3765(1)	836.3692	836.3681	20.27
f90-96	E-RYLGYLE-Q	457.242(2)	912.4694	912.4705	22.36

Table 5.1: Continued.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
α_{s1}-casein					
f90-102	E-RYLGYLEQLLRLK-K	555.6617(3)	1663.9633	1663.9773	41.82
f90-110	E-RYLGYLEQLLRLKKYKVPQLE-I	663.3869(4)	2649.5185	2649.537	35.37
f97-110	E-QLLRLKKYKVPQLE-I	439.7782(4)	1755.0837	1755.077	21.53
f97-118	E-QLLRLKKYKVPQLEIVPNS*AEE-R	892.4842(3)	2674.4308	2674.4458	30.72
f111-118	E-IVPNS*AEE-R	938.3875(1)	937.3802	937.3794	24.63
f111-125	E-IVPNS*AEERLHSMKE-G	607.2888(3)	1818.8446	1818.8335	39.89
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	665.843(4)	2659.3429	2659.3322	29.65
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	892.7794(3)	2675.3164	2675.3271	25.56
f126-141	E-GIHAQQKEPMIGVNQE-L	598.9615(3)	1793.8627	1793.873	47.52
f126-141	E-GIHAQQKEPMIGVNQE-L	593.6341(3)	1777.8805	1777.8781	28.97
f126-148	E-GIHAQQKEPM°IGVNQELAYFYPE-L	893.4334(3)	2677.2784	2677.2846	21.98
f149-157	E-LFRQFYQLD-A	615.3177(2)	1228.6208	1228.6241	25.58
f149-192	E-LFRQFYQLDAYPSGAWYYVPL GTQYTDAPSFSDIPNPIGSENSE-K	1251.8276(4)	5003.2813	5003.3242	31.42
f190-199	E-NSEKTTMPLW	603.7944(2)	1205.5742	1205.575	23.4
f193-199	E-KTTM°PLW	892.4598(1)	891.4525	891.4524	28.61
α_{s2}-casein					
f13-18	E-SIIS*QE-T	756.3174(1)	755.3101	755.3102	26.36
f13-23	E-SIIS*QETYKQE-K	703.3209(2)	1404.6272	1404.6174	46.85
f13-33	E-SIIS*QETYKQEKNMAINPSKE-N	840.069(3)	2517.1852	2517.1821	20.28
f64-84	E-VATEEVKITVDDKHYQKALNE-I	810.7583(3)	2429.2531	2429.2438	60.56
f69-84	E-VKITVDDKHYQKALNE-I	634.3432(3)	1900.0078	1900.0054	35.31
f134-145	E-NSKKTVDMES*TE-V	724.7961(2)	1447.5776	1447.5902	45.45
Incubation for 240 min.					
α_{s1}-casein					
f19-30	E-NLLRFFVAPFPE-V	725.3852(2)	1448.7558	1448.7816	21.57
f40-50	E-LSKDIGS*ES*TE-D	663.2359(2)	1324.4572	1324.4836	21.58
f40-55	E-LSKDIGSES*TEDQAME-D	910.3516(2)	1818.6886	1818.723	22.04
f111-118	E-IVPNS*AEE-R	938.3708(1)	937.3635	937.3794	25.72
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9539(3)	1793.8399	1793.873	31.79
α_{s2}-casein					
f134-145	E-NSKKTVDMES*TE-V	724.7935(2)	1447.5724	1447.5902	24.27

S*: phosphorylated serine. M°: oxidised methionine.

The peptide fragments of α_{s1}-casein released by GE can be summarised as follows: f40-55 and f111-118 were observed in samples incubated for 15, 30, 60, 120 and 240 min, f31-39 was observed in samples incubated for 15, 30, 60 and 120 min, f126-141 was observed in samples incubated for 30, 60, 120 and 240 min, f 1-18, f19-30 and f19-39 were observed in samples incubated for 15, 30 and 120 and 240 min, f111-125 and f193-199 were observed in samples incubated for 15, 60 and 120 min, f31-55 was observed in samples incubated for 30, 60 and 120 min, f15-39 and 119-141 were observed in samples incubated for 15 and 120 min, f97-118 was observed in samples

incubated for 30 and 120 min, f71-77 and f149-157 were observed in samples incubated for 60 and 120 min, f1-30 was observed in samples incubated for 15 min, f15-30, f15-35, f90-96, f90-110, f97-110, f126-148, f149-192 and f190-199 were observed in samples incubated for 120 min and f40-50 was observed in samples incubated for 240 min (Table 5.1).

The peptide fragments of α_{s1}-casein obtained by non-specific GE activity can be summarised as follows: f3-30 was observed in samples incubated for 15 and 120 min and f90-102 was observed in samples incubated for 120 min. These results indicate that Pro(2)-Lys(3) and Lys(102)-Lys(103) were hydrolysed. Le Bars and Gripon (1993) reported that bovine plasmin hydrolysed the peptide bonds in α_{s1}-casein at 11 different positions. The authors reported that the following peptide bonds in α_{s1}-casein were hydrolysed by bovine plasmin: Arg(22)-Phe(23), Lys(34)-Glu(35), Lys(79)-His(80), Arg(90)-Tyr(91), Lys(100)-Leu(101), Lys(102)-Lys(103), Lys(103)-Tyr(104), Lys(105)-Val(106), Lys(124)-Glu(125), Arg(151)-Gln(152) and Lys(193)-Thr(194). Therefore, the cleavage of Lys(102)-Lys(103) may be due to low levels of residual plasmin activity present in the enriched α-casein preparation. To the best of our knowledge no evidence of GE hydrolysing on the carboxy terminal of Pro has been reported in literature. Therefore, the hydrolysis Pro(2)-Lys(3) may not be due to the non-specific action of GE but protein or peptides without two amino acid residues at the N-terminal would have been present in the starting material (acid-casein or the enriched α-casein). Since the detailed procedure used in the preparation of acid caseinate (starting material for β-casein purification) was not supplied by the manufacture it is difficult to interpret the origin of peptide f3-X (X > 30). Furthermore, to the best of our knowledge hydrolysis of Pro(2)-Lys(3) is not due to any of the proteinases which may be present in bovine milk.

The peptide fragments of α_{s2}-casein released by GE can be summarised as follows: f13-18 and f13-23 were observed in samples incubated for 15, 30 and 120 min, f134-145 was observed in samples incubated for 60, 120 and 240 min, f64-84 was observed in samples incubated for 60 and 120 min and f13-33 and f69-84 were observed in samples incubated for 120 min. From these results it was observed that the Glu-Glu bond was did not appear to be hydrolysed (i.e., Glu(11)-Glu(12), Glu(132)-Glu(133)).

No non-specific cleavages were observed corresponding to α_{s2}-casein in the samples on incubation at 37°C (Table 5.1).

Two of CMP fragments f36-64 and f47-64 were also observed upon incubation of the enriched α-casein preparation with GE at 37°C for 15 min (Table 5.1)

The results indicated that the α-casein fraction used in this experiment was enriched in α_{s1}-casein and contained α_{s2}-casein and κ-casein/fragments at lower levels. This is as expected since the approximate ratio of α_{s1}- to α_{s2}-casein in bovine milk is 4:1 (Swaisgood, 1996).

The TIC profiles on incubation at 50°C for various time intervals are shown in Figure 5.2. The intensities of peaks were higher than that observed for incubation at 37°C. The TIC profiles for samples incubated at 50°C were generally similar to those observed at 37°C (Figure 5.1). The intensity of the peak eluted approximately after 14 min increased with an increase in incubation time. The intensity of the peak eluted around 20 min increased with an increase in incubation time up to 60 min and interestingly this peak was not observed in samples incubated for 120 and 240 min. A peak eluted at approximately 66 min was observed in all the samples. The peak eluted at approximately 70 min was not detected in the sample incubated for 240 min, however, it was observed in the samples incubated for 15, 30, 60 and 120 min. The peptide sequences, experimental mass, calculated masses of singly or multiply charged ions along with the MASCOT scores following incubation with GE over 240 min are given in Table 5.2.

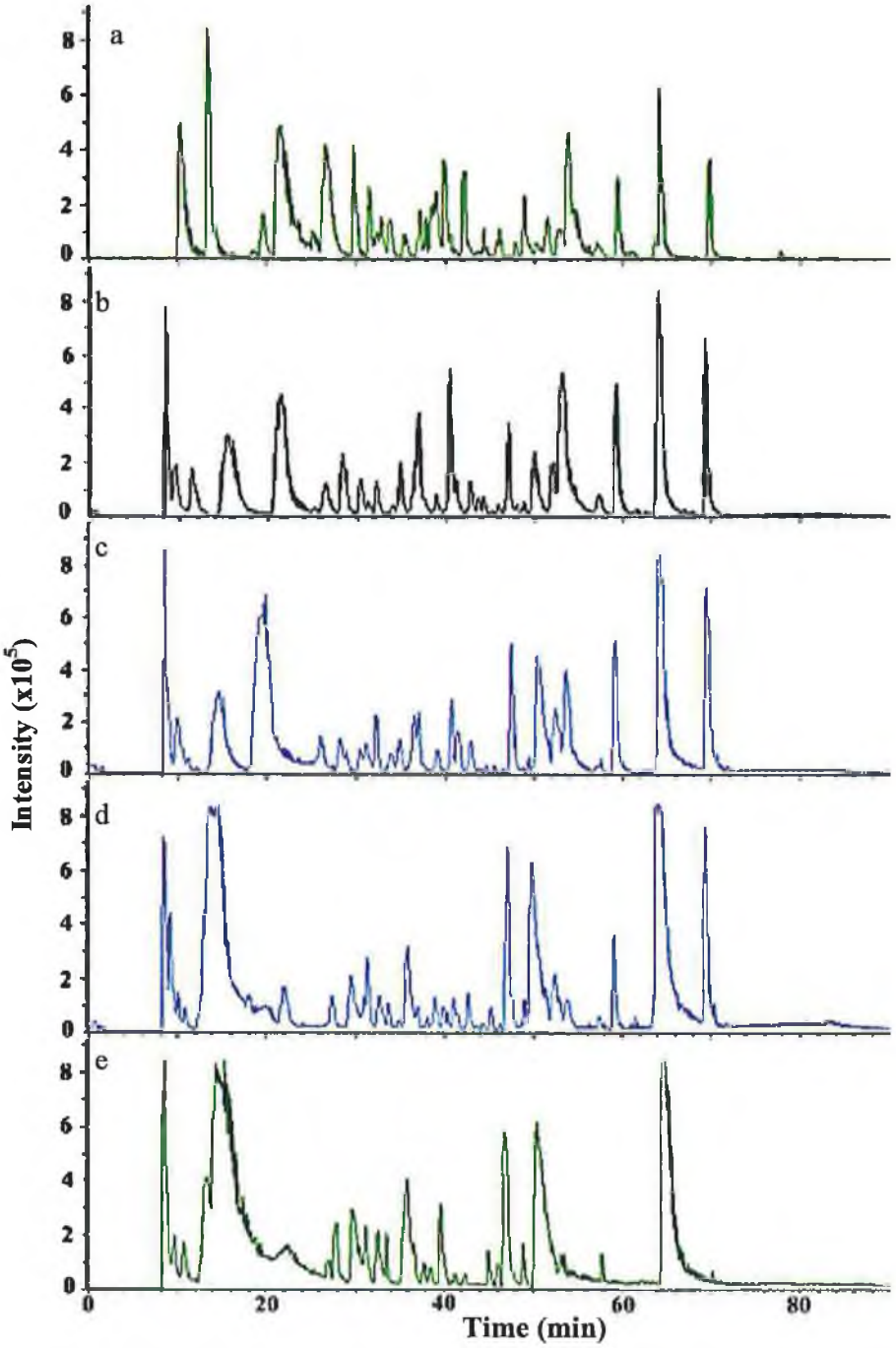


Figure 5.2. The total intensity chromatogram of enriched α-casein incubated with glutamyl endopeptidase at 50°C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min and (e) 240 min.

Table 5.2. Peptide sequences identified upon incubation of enriched α-casein with glutamyl endopeptidase for different time intervals at 50°C. The amino acid residues present at the P_1 and P_1' positions are given.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 15 min.					
α_{s1}-casein					
f1-18	RPKHPIKHQGLPQEVLNE-N	707.3815(3)	2119.1227	2119.165	43.86
f31-39	E-VFGKEKVNE-L	1049.5404(1)	1048.5331	1048.5189	44.95
f31-55	E-VFGKEKVNELSKDIGSESTEDQAM°E-D	982.7294(3)	2945.1664	2945.2195	64.83
f40-55	E-LSKDIGSESTEDQAM°E-D	958.3293(2)	1914.644	1914.6748	58.42
f40-55	E-LSKDIGSES*TEDQAME-D	950.3307(2)	1898.6468	1898.6798	51.83
f56-61	E-DIKQME-A	763.3508(1)	762.3435	762.3582	29
f97-110	E-QLLRLKKYKVPQLE-I	878.5291(2)	1755.0436	1755.077	27.42
f119-141	E-RLHSM°KEGIHAQQKEPMIGVNQE-L	887.4296(3)	2659.267	2659.2959	47.08
f119-141	E-RLHSM°KEGIHAQQKEPM°IGVNQE-L	892.7601(3)	2675.2585	2675.2908	42.89
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9504(3)	1793.8294	1793.8366	56.71
α_{s2}-casein					
f13-23	E-SIIS*QETYKQE-K	703.3028(2)	1404.591	1404.6174	32.98
f19-33	E-TYKQEKNMAINPSKE-N	890.9297(2)	1779.8448	1779.8461	53.95
f134-145	E-NSKKTVDMES*TE-V	724.7888(2)	1447.563	1447.5902	56.41
f146-157	E-VFTKKTKLTEEE-K	726.8862(2)	1451.7578	1451.7508	51.27
Incubation for 30 min.					
α_{s1}-casein					
f1-18	E-RPKHPIKHQGLPQEVLNE-N	707.3989(3)	2119.1749	2119.165	49.12
f1-30	E-RPKHPIKHQGLPQEVLNENLLRFFVAPFPE-V	710.9962(5)	3549.9446	3549.9361	40.35
f3-30	P-KHPIKHQGLPQEVLNENLLRFFVAPFPE-V	825.2065(4)	3296.7969	3296.7822	49.1
f15-30	E-VLNENLLRFFVAPFPE-V	953.01(2)	1904.0054	1904.0196	56.25
f15-35	E-VLNENLLRFFVAPFPEVFGKE-K	822.442(3)	2464.3042	2464.3155	49.84
f15-39	E-VLNENLLRFFVAPFPEVFGKEKVNE-L	734.6386(4)	2934.5253	2934.5643	24.95
f19-30	E-NLLRFFVAPFPE-V	725.3935(2)	1448.7724	1448.7816	57.73
f19-35	E-NLLRFFVAPFPEVFGKE-K	670.696(3)	2009.0662	2009.0775	49.07
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	827.4537(3)	2479.3393	2479.3264	58.55
f31-39	E-VFGKEKVNE-L	525.2921(2)	1048.5696	1048.5553	28.08
f31-55	E-VFGKEKVNELSKDIGSESTEDQAM°E-D	1009.4105(3)	3025.2097	3025.1763	63.85
f31-55	E-VFGKEKVNELSKDIGSES*TEDQAME-D	977.4263(3)	2929.2571	2929.2246	53.07
f31-55	E-VFGKEKVNELS*KDIGSES*TEDQAME-D	1004.0792(3)	3009.2158	3009.1909	44.81
f40-55	E-LSKDIGSES*TEDQAM°E-D	950.3571(2)	1898.6996	1898.6798	84.2
f40-55	E-LSKDIGS*ESTEDQAM°E-D	958.3544(2)	1914.6942	1914.6748	67.1
f40-55	E-LSKDIGSES*TEDQAME-D	910.3753(2)	1818.736	1818.723	62.07
f40-55	E-LSKDIGS*ES*TEDQAME-D	646.5567(3)	1936.6483	1936.6452	44.87
f40-61	E-LSKDIGSESTEDQAM°EDIKQME-A	887.3535(3)	2659.0387	2659.0224	45.91
f71-77	E-IVPNS*VE-Q	837.376(1)	836.3687	836.3681	22.09
f71-84	E-IVPNS*VEQKHIQKE-D	576.9711(3)	1727.8915	1727.8607	32.4
f71-89	E-IVPNS*VEQKHIQKEDVPSE-R	752.7039(3)	2255.0899	2255.0835	21.46
f71-90	E-IVPNS*VEQKHIQKEDVPSER-Y	603.8065(4)	2411.1969	2411.1846	52.2
f90-102	E-RYLGYLEQLLRLK-K	555.6696(3)	1663.987	1663.9773	40.17
f90-110	E-RYLGYLEQLLRLKKYKVPQLE-I	663.3977(4)	2649.5617	2649.537	33.84
f97-118	E-QLLRLKKYKVPQLEIVPNS*AEE-R	892.4941(3)	2674.4605	2674.4458	47.11
f111-118	E-IVPNS*AEE-R	938.3875(1)	937.3802	937.3794	27.58
f111-125	E-IVPNS*AEERLHSMKE-G	607.2893(3)	1818.8461	1818.8335	47.28
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	892.7866(3)	2675.338	2675.3271	56.63

Table 5.2: Continued.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 30 min.					
α_{s1}-casein					
f119-141	E-RLHSMKEGIHAQQKEPMIGVNQE-L	887.4549(3)	2659.3429	2659.3322	40.3
f119-141	E-RLHSM°VKEGIHAQQKEPM°IGVNQE-L	898.1202(3)	2691.3388	2691.3221	34.23
f119-148	E-RLHSMKEGIHAQQKEPM°IGVNQELAYFYPE-L	712.7567(5)	3558.7471	3558.7387	26.41
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9674(3)	1793.8804	1793.873	52.53
f126-141	E-GIHAQQKEPMIGVNQE-L	593.6353(3)	1777.8841	1777.8781	41.98
f126-148	E-GIHAQQKEPMIGVNQELAYFYPE-L	888.1066(3)	2661.298	2661.2897	28.84
f149-157	E-LFRQFYQLD-A	615.3244(2)	1228.6342	1228.6241	23.63
f149-192	E-LFRQFYQLDAYPSGAWYYVPLGTQ YTDAPSFSDIPNPIGSENSE-K	1251.8237(4)	5003.2657	5003.3242	53.57
f149-199	E-LFRQFYQLDAYPSGAWYYVPLGTQYTDAPS FSDIPNPIGSENSEKTTM°PLW-	1470.1786(4)	5876.6853	5876.7661	49.4
f149-199	E-LFRQFYQLDAYPSGAWYYVPLGTQYTDAP SFSDIPNPIGSENSEKTTMPLW-	1466.1872(4)	5860.7197	5860.7712	33.03
f190-199	E-NSEKTTMPLW-	603.7992(2)	1205.5838	1205.575	29.48
f193-199	E-KTTM°PLW-	892.4624(1)	891.4551	891.4524	27.8
α_{s2}-casein					
f13-23	E-SIIS*QETYKQE-K	703.3208(2)	1404.627	1404.6174	37.71
f64-84	E-VATEEVKITVDDKHYQKALNE-I	810.7601(3)	2429.2585	2429.2438	49.52
f69-84	E-VKITVDDKHYQKALNE-I	634.3461(3)	1900.0165	1900.0054	37.63
f85-126	E-INQFYQKFPQYLQYLYQGPIVLNPWDQ VKRNAVPITPTLNRE-Q	1019.3335(5)	5091.6311	5091.6763	39.55
f85-133	E-INQFYQKFPQYLQYLYQGPIVLNPWDQVKRNA VPITPTLNREQLS*TS*EE-N	1206.1856(5)	6025.8916	6025.9485	25.38
f134-145	E-NSKKTVDMES*TE-V	724.806(2)	1447.5974	1447.5902	45.91
Caseinomacropeptide					
f47-64	E-VIESPPEINTVQVTSTAV	942.5064(2)	1882.9982	1882.9888	61.78
Incubation for 60 min.					
α_{s1}-casein					
f1-18	RPKHPIKHQGLPQEVLNE-N	707.3899(3)	2119.1479	2119.165	47.57
f1-30	RPKHPIKHQGLPQEVLNENLLRFFVAPFPE-V	888.4775(4)	3549.8809	3549.9361	48.98
f15-30	E-VLNENLLRFFVAPFPE-V	953.0001(2)	1903.9856	1904.0196	57.85
f15-35	E-VLNENLLRFFVAPFPEVFGKE-K	822.4295(3)	2464.2667	2464.3155	34.44
f15-39	E-VLNENLLRFFVAPFPEVFGKEKVNE-L	979.1813(3)	2934.5221	2934.5643	66.33
f15-47	E-VLNENLLRFFVAPFPEVFGK EKVNELSKDIGSE-S	1270.6624(3)	3808.9654	3809.0119	41.72
f19-30	E-NLLRFFVAPFPE-V	725.3838(2)	1448.753	1448.7816	54.25
f19-35	E-NLLRFFVAPFPEVFGKE-K	670.6876(3)	2009.041	2009.0775	46.18
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	620.8311(4)	2479.2953	2479.3264	46.27
f24-30	E-FVAPFPE-V	806.4013(1)	805.394	805.401	55.36
f31-39	E-VFGKEKVNE-L	525.2861(2)	1048.5576	1048.5553	29.86
f31-50	E-VFGKEKVNELSKDIGS*ES*TE-D	786.0119(3)	2355.0139	2355.0284	27.82
f31-55	E-VFGKEKVNELSKDIGSES*TEDQAME-D	982.7449(3)	2945.2129	2945.2195	71.71
f31-55	E-VFGKEKVNELSKDIGSES*TEDQAME-D	977.4133(3)	2929.2181	2929.2246	70
f31-55	E-VFGKEKVNELS*KDIGSES*TEDQAME-D	1004.0696(3)	3009.187	3009.1909	62.81
f40-50	E-LSKDIGS*ES*TE-D	663.242(2)	1324.4694	1324.4836	24.22
f40-55	E-LSKDIGS*ESTEDQAME-D	910.3635(2)	1818.7124	1818.723	60.05
f40-55	E-LSKDIGS*ESTEDQAM°E-D	958.3428(2)	1914.671	1914.6748	56.12
f40-55	E-LSKDIGS*ES*TEDQAME-D	950.3451(2)	1898.6756	1898.6893	21.92
f71-77	E-IVPNS*VE-Q	837.3684(1)	836.3611	836.3681	26.11
f71-84	E-IVPNS*VEQKHIQKE-D	576.9598(3)	1727.8576	1727.8607	46.6
f71-89	E-IVPNS*VEQKHIQKEDVPSE-R	752.699(3)	2255.0752	2255.0835	21.14

Table 5.2: Continued.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 60 min.					
α_{s1}-casein					
f90-100	E-RYLGYLEQLLR-L	712.3966(2)	1422.7786	1422.7983	47.87
f90-102	E-RYLGYLEQLLRLK-K	555.6594(3)	1663.9564	1663.9773	42.32
f90-110	E-RYLGYLEQLLRLKKYKVPQLE-I	663.3813(4)	2649.4961	2649.537	43.32
f97-110	E-QLLRLKKYKVPQLE-I	586.0325(3)	1755.0757	1755.077	27.8
f97-118	E-QLLRLKKYKVPQLEIVPNS*AEE-R	892.4824(3)	2674.4254	2674.4458	40.85
f111-118	E-IVPNS*AEE-R	938.3815(1)	937.3742	937.3794	27.41
f111-125	E-IVPNS*AEERLHSMKE-G	607.2807(3)	1818.8203	1818.8335	27.56
f119-141	E-RLHSMKEGIHAQQKEPMIGVNQE-L	665.8354(4)	2659.3125	2659.3322	58.86
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	892.7748(3)	2675.3026	2675.3271	56.65
f119-141	E-RLHSM°KEGIHAQQKEPM°IGVNQE-L	673.8315(4)	2691.2969	2691.3221	29.68
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9582(3)	1793.8528	1793.873	46.12
f126-141	E-GIHAQQKEPMIGVNQE-L	593.6286(3)	1777.864	1777.8781	40.42
f126-148	E-GIHAQQKEPM°IGVNQELAYFYPE-L	893.4257(3)	2677.2553	2677.2846	47.07
f149-157	E-LFRQFYQLD-A	615.316(2)	1228.6174	1228.6241	24.33
f149-192	E-LFRQFYQLDAYPSGAWYYVPLGTQYT DAPSFSDIPNPIGSENSE-K	1251.8135(4)	5003.2249	5003.3242	76.84
f149-192	E-LFRQFYQLDAYPSGAWYYVPLGTQYTDAPS*F SDIPNPIGSENSE-K	1256.8028(4)	5023.1821	5023.2542	42.72
f149-199	E-LFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIP NPIGSENSEKTTMPLW-	1466.1731(4)	5860.6633	5860.7712	34.54
f190-199	E-NSEKTTMPLW-	603.7896(2)	1205.5646	1205.575	36.5
f193-199	E-KTTM°PLW-	446.729(2)	891.4434	891.4524	23.33
α_{s2}-casein					
f13-23	E-SIIS*QETYKQE-K	703.3112(2)	1404.6078	1404.6174	37.03
f13-33	E-SIISQETYKQEKNM°AINPSKE-N	845.3962(3)	2533.1668	2533.1771	24.65
f64-84	E-VATEEVKITVDDKHYQKALNE-I	810.7513(3)	2429.2321	2429.2438	72.63
f68-84	E-EVKITVDDKHYQKALNE-I	677.3492(3)	2029.0258	2029.048	29.87
f69-84	E-VKITVDDKHYQKALNE-I	634.3395(3)	1899.9967	1900.0054	41.93
f85-110	E-INQFYQKFPQYLQYLYQGPIVLNPWD-Q	947.4812(4)	3785.8957	3785.8392	48.74
f85-126	E-INQFYQKFPQYLQYLYQGPIVLNPWDQV KRNAVPITPTLNRE-Q	1273.9012(4)	5091.5757	5091.6763	26.95
Caseinomacropeptide					
f47-64	E-VIESPPEINTVQVTSTAV	953.4824(2)	1904.9502	1904.9707	43.5
Incubation for 120 min.					
α_{s1}-casein					
f1-18	RPKHPIKHQGLPQEVLNE-N	707.3944(3)	2119.1614	2119.165	50.95
f1-30	RPKHPIKHQGLPQEVLNENLLRFFVAPFPE-V	888.4885(4)	3549.9249	3549.9361	60.19
f15-22	E-VLNENLLR-F	485.7849(2)	969.5552	969.5607	52.49
f15-30	E-VLNENLLRFFVAPFPE-V	953.0078(2)	1904.001	1904.0196	57.46
f15-35	E-VLNENLLRFFVAPFPEVFGKE-K	822.438(3)	2464.2922	2464.3155	64.09
f15-39	E-VLNENLLRFFVAPFPEVFGKEKVNE-L	979.1927(3)	2934.5563	2934.5643	41.53
f15-47	E-VLNENLLRFFVAPFPEVFG KEKVNELSKDIGSE-S	1270.6798(3)	3809.0176	3809.0119	46.9
f19-30	E-NLLRFFVAPFPE-V	725.3912(2)	1448.7678	1448.7816	57.92
f19-35	E-NLLRFFVAPFPEVFGKE-K	670.6939(3)	2009.0599	2009.0775	41.06
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	827.4455(3)	2479.3147	2479.3264	48.02
f31-39	E-VFGKEKVNE-L	525.2864(2)	1048.5582	1048.5553	27.7

Table 5.2: Continued.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 120 min.					
α_{s1}-casein					
f31-55	E-VFGKEKVNELSKDIGSESTEDQAM°E-D	1009.4027(3)	3025.1863	3025.1763	66.57
f31-55	E-VFGKEKVNELS*KDIGSESTEDQAME-D	1004.0726(3)	3009.196	3009.1814	65.92
f31-55	E-VFGKEKVNELS*KDIGSES*TEDQAME-D	1004.0732(3)	3009.1978	3009.1909	60.41
f40-50	E-LSKDIGS*ES*TE-D	663.2463(2)	1324.478	1324.4836	37.63
f40-55	E-LSKDIGSESTEDQAM°E-D	958.3507(2)	1914.6868	1914.6748	59.86
f40-55	E-LSKDIGSES*TEDQAME-D	950.3469(2)	1898.6792	1898.6798	43.67
f71-77	E-IVPNS*VE-Q	837.3726(1)	836.3653	836.3681	25.54
f71-84	E-IVPNS*VEQKHIQKE-D	576.9627(3)	1727.8663	1727.8607	40.06
f71-89	E-IVPNSVEQKHIQKEDVPSE-R	726.042(3)	2175.1042	2175.1171	29.31
f71-89	E-IVPNS*VEQKHIQKEDVPSE-R	1128.5539(2)	2255.0932	2255.0835	24.02
f90-100	E-RYLGYLEQLLR-L	712.4017(2)	1422.7888	1422.7983	67.61
f90-110	E-RYLGYLEQLLRLKKYKVPQLE-I	663.3874(4)	2649.5205	2649.537	50.73
f111-118	E-IVPNS*AEE-R	938.3796(1)	937.3723	937.3794	29.27
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	892.7838(3)	2675.3296	2675.3271	47.09
f119-141	E-RLHSMKEGIHAQQKEPMIGVNQE-L	665.8354(4)	2659.3125	2659.3322	44.85
f126-141	E-GIHAQQKEPMIGVNQE-L	889.944(2)	1777.8734	1777.8781	69.48
f126-141	E-GIHAQQKEPM°IGVNQE-L	598.9632(3)	1793.8678	1793.873	40.96
f132-141	Q-KEPM°IGVNQE-L	580.7812(2)	1159.5478	1159.5543	56.84
f132-141	Q-KEPMIGVNQE-L	572.7872(2)	1143.5598	1143.5594	48.88
f142-192	E-LAYFYPELFRQFYQLDAYPSGAWYYVPLG TQYTDAPSFSDIPNPIGSENSE-K	1472.6691(4)	5886.6473	5886.7358	42.93
f149-157	E-LFRQFYQLD-A	615.3148(2)	1228.615	1228.6241	23.93
f149-192	E-LFRQFYQLDAYPSGAWYYVPLGTQYTD APSFSDIPNPIGSENSE-K	1251.8261(4)	5003.2753	5003.3242	67.87
f158-192	D-AYPSGAWYYVPLGTQYTDAPS FSDIPNPIGSENSE-K	1265.2252(3)	3792.6538	3792.7108	25.72
f190-199	E-NSEKTTM°PLW-	611.7912(2)	1221.5678	1221.57	29.11
f193-199	E-KTTMPLW-	876.4603(1)	875.453	875.4575	28.94
f193-199	E-KTTM°PLW-	892.4503(1)	891.443	891.4524	26.92
α_{s2}-casein					
f13-23	E-SIIS*QETYKQE-K	703.3139(2)	1404.6132	1404.6174	52.72
f64-84	E-VATEEVKITVDDKHYQKALNE-I	810.7551(3)	2429.2435	2429.2438	73.99
f69-84	E-VKITVDDKHYQKALNE-I	634.3401(3)	1899.9985	1900.0054	47.06
f85-126	E-INQFYQKFPQYLQYLYQGPIVLNPWD QVKRNAVPITPTLNRE-Q	1273.918(4)	5091.6429	5091.6763	33.35
f85-133	E-INQFYQKFPQYLQYLYQGPIVLNPWDQVKRN AVPITPTLNREQLS*TS*EE-N	1206.1821(5)	6025.8741	6025.9485	30.42
f134-145	E-NSKKTVDMES*TE-V	724.8032(2)	1447.5918	1447.5902	37.78
Caseinomacropeptide					
f36-46	E-STVATLEDS*PE-V	625.7404(2)	1249.4662	1249.4727	58.57
f47-64	E-VIESPPEINTVQVTSTAV	953.487(2)	1904.9594	1904.9707	47.47

Table 5.2: Continued.

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MSMS (charge)	Experimental mass	Calculated mass	MASCOT score
Incubation for 240 min.					
α_{s1}-casein					
f15-22	E-VLNENLLR-F	485.7817(2)	969.5488	969.5607	40.98
f15-23	E-VLNENLLRF-F	559.3173(2)	1116.62	1116.6291	43.96
f15-30	E-VLNENLLRFFVAPFPE-V	953.0118(2)	1904.009	1904.0196	51.27
f19-30	E-NLLRFFVAPFPE-V	725.3942(2)	1448.7738	1448.7816	60.04
f19-39	E-NLLRFFVAPFPEVFGKEKVNE-L	827.4473(3)	2479.3201	2479.3264	42.86
f25-30	F-VAPFPE-V	659.3375(1)	658.3302	658.3326	39.79
f31-39	E-VFGKEKVNE-L	525.2822(2)	1048.5498	1048.5553	29.74
f31-50	E-VFGKEKVNELS*KDIGS*ES*TE-D	1218.5027(2)	2434.9908	2434.9947	24.74
f31-55	E-VFGKEKVNELS*KDIGSESTEDQAME-D	1004.0728(3)	3009.1966	3009.1814	65.72
f40-50	E-LSKDIGSES*TE-D	1325.4714(1)	1324.4641	1324.4836	48.12
f40-50	E-LSKDIGSES*TE-D	625.2713(2)	1248.528	1248.5258	45.65
f40-55	E-LSKDIGS*ESTEDQAME-D	950.3541(2)	1898.6936	1898.6798	53.83
f40-55	E-LSKDIGS*ESTEDQAM°E-D	958.3483(2)	1914.682	1914.6748	51.88
f71-77	E-IVPNS*VE-Q	837.3705(1)	836.3632	836.3681	22.68
f71-84	E-IVPNS*VEQKHIQKE-D	576.9577(3)	1727.8513	1727.8607	40.67
f71-89	E-IVPNS*VEQKHIQKEDVPSE-R	1128.5497(2)	2255.0848	2255.0835	22.74
f90-96	E-RYLGYLE-Q	913.476(1)	912.4687	912.4705	23.61
f90-99	E-RYLGYLEQLL-R	634.3483(2)	1266.682	1266.6972	42.88
f90-100	E-RYLGYLEQLLR-L	712.4029(2)	1422.7912	1422.7983	63.38
f90-110	E-RYLGYLEQLLRLKKYKVPQLE-I	663.3885(4)	2649.5249	2649.537	40.61
f111-118	E-IVPNS*AEE-R	938.3767(1)	937.3694	937.3794	23.02
f119-141	E-RLHSMKEGIHAQQKEPM°IGVNQE-L	892.7768(3)	2675.3086	2675.3271	50.11
f119-141	E-RLHSMKEGIHAQQKEPMIGVNQE-L	665.8352(4)	2659.3117	2659.3322	37.87
f119-141	E-RLHSM°KEGIHAQQKEPM°IGVNQE-L	673.8306(4)	2691.2933	2691.3221	25.56
f126-141	E-GIHAQQKEPM°IGVNQE-L	897.9355(2)	1793.8564	1793.873	73.89
f132-141	Q-KEPM°IGVNQE-L	580.7799(2)	1159.5452	1159.5543	44.7
f149-157	E-LFRQFYQLD-A	615.3139(2)	1228.6132	1228.6241	20.16
f149-192	E-LFRQFYQLDAYPSGAWYYVPLGTQYT DAPSFSDIPNPIGSENSE-K	1251.8257(4)	5003.2737	5003.3242	41.47
f190-199	E-NSEKTTM°PLW	611.7927(2)	1221.5708	1221.57	33.69
α_{s2}-casein					
f64-84	E-VATEEVKITVDDKHYQKALNE-I	810.7596(3)	2429.257	2429.2438	64.38
f69-84	E-VKITVDDKHYQKALNE-I	951.0024(2)	1899.9902	1900.0054	48.31
f85-126	E-INQFYQKFPQYLQYLYQGPIVLNPWD QVKRNAVPITPTLNRE-Q	1019.3309(5)	5091.6181	5091.6763	40.78
Caseinomacropeptide					
f43-64	E-DSPEVIESPPEINTVQVTSTAV	1174.5574(2)	2347.1002	2347.1196	26.07
f47-53	E-VIESPPE-I	770.3822(1)	769.3749	769.3858	20.19
f47-64	E-VIESPPEINTVQVTSTAV	942.5006(2)	1882.9866	1882.9888	73.13

S*: phosphorylated serine. M°: oxidised methionine.

From Table 5.2, it can be observed that six Ser residues present at positions 41, 46, 48, 75, 88 and 115 in α_{s1}-casein and four Ser residues present at positions 16, 129, 131 and 143 in α_{s2}-casein were phosphorylated in the sample incubated at 50°C. However, surprisingly in the samples incubated at 37°C only four and two phosphorylated Ser

residues were observed in α_{s1}- and α_{s2}-casein, respectively (Table 5.1). However, a phosphorylated Ser residue at position 149 was observed in κ-casein on incubation at both 37 and 50°C.

The peptide fragments of α_{s1}-casein specifically released by GE can be summarised as follows: f31-39, f31-55, f40-55, f119-141 and f126-141 were observed in samples incubated for 15, 30, 60, 120 and 240 min, f1-18 was observed in samples incubated for 15, 30, 60 and 120 min, f15-30, f19-30, f19-39, f71-77, f71-84, f71-89, f90-110, f111-118, f149-157, f149-192 and f190-199 were observed in samples incubated for 30, 60, 120 and 240 min, f1-30, f15-35, f15-39, f19-35 and f193-199 were observed in samples incubated for 30, 60 and 120 min, f40-50 was observed in samples incubated for 60, 120 and 240 min, f111-125 was observed in samples incubated for 30 and 60 min, f97-110 was observed in samples incubated for 15 and 60 min, f97-118, f126-148 and 149-199 were observed in samples incubated for 30 and 60 min, f3-30 was observed in samples incubated for 30 and 240 min, f15-47 was observed in samples incubated for 60 and 120 min, f90-96 was observed in sample incubated for 240 min f56-61 was observed in samples incubated for 15 min, f40-61, f71-90 and f119-148 were observed in samples incubated for 30 min, f24-30 observed in samples incubated for 60 min and f142-192 and f158-192 were observed in samples incubated for 120 min.

The peptide fragments of α_{s1}-casein obtained by non-specific GE activity can be summarised as follows: f90-100 was observed in samples incubated for 60, 120 and 240 min indicating hydrolysis of Arg(100)-Leu(101), f90-102 was observed in samples incubated for 30 and 60 min indicating hydrolysis of Lys(102)-Lys(103) , f15-22 and f132-141 indicating hydrolysis of Arg(22)-Phe(23) and Gln(131)-Lys(132) respectively, were observed in samples incubated for 120 and 240 min, f3-30 was observed in samples incubated for 30 min indicating hydrolysis of Pro(2)-Lys(3) and f15-23 and f25-30 indicating hydrolysis of Phe(23)-Phe(24), Phe(24)-Val(25) respectively, were observed in samples incubated for 240 min. The hydrolysis of Pro(2)-Lys(3) and Lys(102)-Lys(103) was also observed in samples incubated at 37°C. As described earlier the hydrolysis of Pro(2)-Lys(3), Arg(22)-Phe(23), Arg(100)-Leu(101), Lys(102)-Lys(103) may not be due to the non-specific behaviour of GE. As mentioned earlier, the hydrolysis of Arg(22)-Phe(23), Arg(100)-Leu(101), Lys(102)-

Lys(103) may be due to residual plasmin activity. The hydrolysis of Phe(23)-Phe(24) may be due to the action of a residual chymosin activity on α_{s1}-casein. Coker *et al.*, (1999) and McSweeney *et al.*, (1993) reported that Phe(23)-Phe(24) was hydrolysed in α_{s1}-casein due to the action of chymosin. Therefore, the results indicate that the hydrolysis of Phe(24)-Val(25) and Gln(131)-Lys(132) may be due to the non-specific action of GE (Table 5.2). The results also indicate that the above mentioned non-specific cleavages occur at longer incubation times (120 or 240 min) on incubation at 50°C. Interestingly, no evidence of hydrolysis of Phe(24)-Val(25) and Gln(131)-Lys(132) was observed on incubation at 37°C (Table 5.1). These results suggest that GE is more highly specific during incubation at 37°C in comparison to incubation at 50°C.

The peptide fragments of α_{s2}-casein (Table 5.2) specifically released by GE can be summarised as follows: f13-23 was observed in samples incubated for 15, 30, 60, 120 and 240 min, f64-84, f69-84 and f85-126 were observed in samples incubated for 30, 60, 120 and 240 min, f134-145 was observed in samples incubated for 15, 30 and 120 min, f85-133 was observed in samples incubated for 30 and 120 min, f19-33 and f146-157 were observed in samples incubated for 15 min, f13-33 and f68-84 were observed in samples incubated for 60 min and f85-110 was observed in samples incubated for 120 min (Table 5.2).

The peptide fragments of CMP (Table 5.2) specifically released by GE can be summarised as follows: f47-64 was observed in samples incubated for 30, 60, 120 and 240 min, f36-64 was observed in samples incubated for 120 min and f43-64 and f47-53 were observed in samples incubated for 240 min. However, due to the low peptide sequence coverage obtained during incubation at 37°C only two fragments i.e., f36-64 and f47-64 of CMP were observed in the samples incubated for 15 min.

Table 5.3. Theoretically expected cleavage sites for glutamyl endopeptidase (GE) action in α_{s1}-casein along with the actual cleavages observed upon incubation of enriched α_s-casein with GE at 37 and 50°C. The amino acid residues present at P_1-P_1' are also given.

Theoretical expected (P_1 residue)	Amino acid residues at P_1-P_1'	Cleavages observed at 37°C	Cleavages observed at 50°C
14	E-V	Yes	Yes
18	E-N	Yes	Yes
30	E-V	Yes	Yes
35	E-K	Yes	Yes
39	E-L	Yes	Yes
43	D-I	No	No
47	E-S	No	Yes
50	E-D	Yes	Yes
51	D-Q	No	No
55	E-D	Yes	Yes
56	D-I	No	No
61	E-A	No	Yes
63	E-S	No	No
69	E-E	No	No
70	E-I	Yes	Yes
77	E-Q	Yes	Yes
84	E-D	No	Yes
85	D-V	No	No
89	E-R	Yes	Yes
96	E-Q	Yes	Yes
110	E-I	Yes	Yes
117	E-E	No	No
118	E-R	Yes	Yes
125	E-G	Yes	Yes
133	E-P	No	No
141	E-L	Yes	Yes
148	E-L	Yes	Yes
157	D-A	Yes	Yes
175	D-A	No	No
181	D-I	No	No
189	E-N	Yes	Yes
192	E-K	Yes	Yes

It can be estimated that 32 different GE specific cleavage sites may exist in α_{s1}-casein (Table 5.3). From Table 5.3, it is shown that no evidence was found for the hydrolysis of the following peptide bonds in samples incubated at 37°C for various time intervals: Glu(47)-Ser(48), Glu(61)-Ala(62) and Glu(84)-Asp(85). However, the above mentioned peptide bonds were hydrolysed in samples incubated at 50°C the reasons for not observing these cleavages at 37°C may be related in some way to the lower mean peptide coverage value for samples incubated at 37°C (51.8%) than for

the samples incubated at 50°C (81.8%). From Table 5.3, it can be conclude that Glu-Pro was not hydrolysed, an observation also made in Chapter 4 and previously by Breddam and Meldal (1992). It was also observed that Glu-Glu was not hydrolysed. Apart from Glu-Glu and Glu-Pro all the other theoretically expected peptide bonds were hydrolysed including Glu-Asp, an observation also made in Chapter 4. From Table 5.3 it was observed that GE hydrolysed on the carboxy terminal of only Asp(157) on incubation at 37 and 50°C.

Theoretically, the action of GE on α_{s1}-casein should release one di-peptide (AE (f166-117)), two tri-peptides (STE (f48-50) and NSE (f190-192)), five tetra-peptides (VLNE (f15-18), KVNE (f36-39), LSKD (f40-43), IGSE (f44-47) and VPSE (f86-89)) and 4 penta-peptides IKQME (f57-61), DVPSE (f85-89) and VFGKE (f31-35)). However, under the main detection method (MASCOT search engine) employed in this study none of these peptides were observed. In order to detect these low molecular mass peptides a manual search was carried out as described in Section 4.3.2. However, during this manual detection only VLNE (f15-18) was observed in the sample incubated for 240 min at 50°C.

From Table 5.4, it was observed that theoretically there are 28 different GE specific cleavage sites exist in α_{s2}-casein. However, the number of cleavages observed in α_{s2}-casein were lower than that observed in α_{s1}-casein. This may be due to the fact that the ratio of α_{s1}- and α_{s2}-casein in bovine milk is approximately 4:1. From Table 5.4, it was observed that the Glu-Glu bond was hydrolysed in α_{s2}-casein, i.e., Glu(11)-Glu(12), Glu(49)-Glu(50), Glu(50)-Glu(51), Glu(132)-Glu(133), Glu(155)-Glu(156), Glu(156)-Glu-(157). However, interestingly it was observed that Glu(67)-Glu(68) was hydrolysed only in the sample incubated for 60 min at 50°C (Table 5.2 and 5.4). From the results obtained it can be concluded that in Glu-Glu-X and Glu-Glu-Glu-X (X=Arg, Asn, Ile, and Lys), Glu-X hydrolysis was preferred by GE in comparison with Glu-Glu hydrolysis.

Theoretically, the action of GE on α_{s2}-casein should release one di-peptide (ME (f141-142)), two tri-peptides (SAE (f61-63) and STE (f143-145)), one tetra-peptides (VATE (f64-67)) and 3 penta-peptides (KNTME (f1-5), TYKQE (f19-23) and VATEE (f64-

68)). None of these peptides were observed using both the automated and the manual detection methods.

Table 5.4. Theoretically expected cleavage sites for glutamyl endopeptidase (GE) action in α_{s2}-casein along with the actual cleavages observed upon incubation of enriched α-casein with GE at 37 and 50°C. The amino acid residues present at P_1-P_1' are also given.

Theoretical expected P_1 residue.	Amino acid residues at P_1-P_1'	Cleavages observed at 37°C	Cleavages observed at 50°C
5	E-H	No	No
11	E-E	No	No
12	E-S	Yes	Yes
18	E-T	Yes	Yes
23	E-K	Yes	Yes
33	E-N	Yes	Yes
42	E-V	No	No
49	E-E	No	No
50	E-E	No	No
51	E-Y	No	No
59	E-E	No	No
60	E-S	No	No
63	E-V	Yes	Yes
67	E-E	No	Yes
68	E-V	No	Yes
74	D-D	No	No
75	D-K	No	No
84	E-I	Yes	Yes
110	D-Q	No	Yes
126	E-Q	No	Yes
132	E-E	No	No
133	E-N	Yes	Yes
140	D-M	No	No
142	E-S	No	No
145	E-V	Yes	Yes
155	E-E	No	No
156	E-E	No	No
157	E-K	No	Yes

Surprisingly, fragments of κ-casein specifically that of CMP were also found in the enriched α-casein preparation (Table 5.1, 5.2 and 5.5). Since chymosin was used during the initial purification stages to obtain fractions enriched in α-casein, it was expected that κ-casein would be cleaved but it was surprising to observe peptide fragments corresponding to CMP in the enriched α-casein sample. CMP which is hydrophilic was not expected to be present in the enriched α-casein fraction. However, it may have been trapped in the enriched α-casein precipitate during the purification procedure. From Table 5.5 it can be theoretically observed that there are 10 different

GE specific cleavage sites exist in CMP. Again, the results obtained indicated that that Glu(24)-Pro(25) bond present in CMP was not hydrolysed.

Table 5.5. Theoretically expected cleavage sites for glutamyl endopeptidase (GE) action in caseinomacropeptide along with the actual cleavages observed upon incubation of enriched α-casein with GE at 37 and 50°C. The amino acid residues present at P_1-P_1' are also given.

Theoretical expected (P_1 residue)	Amino acid residues at P_1-P_1'	Cleavages observed at 37°C	Cleavages observed at 50°C
10	D-K	No	No
13	E-I	No	No
24	E-P	No	No
32	E-A	No	No
35	E-S	Yes	Yes
42	E-D	No	Yes
43	D-S	No	No
46	E-V	Yes	Yes
49	E-S	No	No
53	E-I	No	Yes

Theoretically, the action of GE on caseinomacropeptide (CMP) should release four tri-peptides and 2 penta-peptides. The sequences of the expected four tri-peptides are KTE (f11-13), AVE (f33-35), SPE (f44-46) and VIE (f47-49) and the sequence of expected two tetra-peptides are DSPE(f43-46), SPPE(f50-53). Again these peptides were not detected either using the automated or manual detection methods.

The mean peptide sequence coverage values of α_{s1}-casein for the 37°C and 50°C incubations were 51.8 and 81.8%, respectively (Table 5.6) and mean peptide sequence coverage values of α_{s2}-casein for 37°C and 50°C incubations were 5.8 and 37.2%, respectively (Table 5.7). Spellman *et al.,* (2005) reported that GE was responsible for aggregation of whey protein hydrolysates. The differences in the peptide coverage values obtained at 37 versus 50 °C may be due to peptide aggregation. Since only 250 µl of hydrolysate sample was withdrawn at various time intervals it was difficult to obtain a representative sample due to peptide aggregation. These results may indicate that peptide aggregation was higher on incubation at 37°C in comparison to incubation at 50 °C. In order to confirm the observations made herein, further work could be carried out by performing particle size analysis on the peptide aggregates formed on hydrolysis at different temperatures.

Table 5.6. The percentage peptide coverage of α_{s1}-casein observed on incubation of enriched α-casein sample with glutamyl endopeptidase both at 37°C and 50°C for various times.

Incubation time (min)	Peptide sequence coverage at 37°C (%)	Peptide sequence coverage at 50°C
15	47.2	43.2
30	46.7	95.5
60	48.7	92.5
120	86.4	92.5
240	29.6	85.4
Mean	51.1	81.8

Table 5.7. The percentage peptide coverage of α_{s2}-casein observed on incubation of enriched α-casein sample with glutamyl endopeptidase both at 37°C and 50°C for various times.

Incubation time (min)	Peptide sequence coverage at 37°C (%)	Peptide sequence coverage at 50°C
15	5.3	21.7
30	5.3	44.9
60	15.9	40.6
120	26.1	44.9
240	5.8	33.8
Mean	11.7	37.2

5.6. Conclusion

The results presented in this Chapter indicate that GE purified using Protocol 3 (Chapter 3) was highly pure. GE was observed to hydrolyze predominantly on the carboxy terminal of Glu residues present in α_{s1}- and α_{s2}- and κ-casein on incubation at 37 and 50°C. GE also hydrolysed the peptide bond on the carboxy terminal of Asp residues present in α_{s1}- and α_{s2}-caseins. However, some non-specific cleavages were observed at the carboxy terminal of Phe (24) and Gln (131) corresponding to sequences within α_{s1}-casein upon incubation of the enriched α-casein preparation at 50°C. Breddam and Svensden (1992) reported non-specific cleavage at the carboxy terminal of Phe during the hydrolysis of ribonuclease at high GE concentration (1.2 μM). However, no non-specific cleavages were observed in the present experiment for the enriched α-casein fraction when incubated with GE for various time intervals at 37°C.

It was also observed that the enriched α-casein sample used was significantly enriched in α_{s1}-casein. This was expected as the ratio of α_{s1}- and α_{s2}-casein in bovine milk is approximately 4:1. The results herein also showed that the Ser residues present at positions 41, 46, 48, 75, 88 and 115 in α_{s1}-casein, the Ser residues present at positions 16, 129, 131 and 143 in α_{s2}-casein and the Ser residue present at position 149 in κ-casein were phosphorylated.

From the results it was concluded that GE does not hydrolyse the Glu-Pro peptide bond. In Glu-Glu-X sequences (X=Asn, Ile and Lys), the Glu-X bond was strongly preferred over Glu-Glu and also in Glu-Glu-Glu-Lys, Glu-Lys hydrolysis was preferred over Glu-Glu. It can be concluded that Glu residues at the P_1' position were poorly preferred, whereas, Pro residues at the P_1' position were not preferred.

The Phe(23)-Phe(24) bond was hydrolysed in α_{s1}-casein upon incubation of the enriched α-casein at 50°C suggesting the presence of a low level of residual chymosin activity. This is not unexpected as chymosin was used in the initial stages of β-casein purification. The Lys(102)-Lys(103) and Arg(22)-Phe(23), Arg(90)-Tyr(91), Arg(100)-Leu(101), Lys(102)-Lys(103) bonds were hydrolysed in α_{s1}-casein upon incubation of enriched α-casein at 37 and 50°C, respectively suggesting the presence of a low level of residual bovine plasmin activity.

The large difference in the mean peptide recovery values for the samples incubated at 37°C and 50°C suggest that peptide aggregation was higher at 37°C than 50°C. Further work needs to be carried out to confirm the above observation. It might just be of course that in the presence of α-casein that GE is more active at 50 than at 37°C. Finally, to our knowledge this is the first demonstration of the substrate specificity of GE on an actual food protein substrate, i.e., α-casein.

References

Breddam, K. and Svendsen, I. (1992). Isolation and amino acid sequence of a glutamic acid specific endopeptidase from Bacillus *licheniformis*. *Eur. J. Biochem.* **204,** 165-171.

Breddam, K. and Meldal, M. (1992). Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching *Eur. J. Biochem.* **206**, 103-107.

Coker, J. C., Creamer, L. K., Burr, R. G. and Hill, J. P. (1999). The hydrolysis of the α_{s1}-casein A, B and C variants by plasmin and chymosin. *Int. Dairy. J,* **9**, 371-372

Le Bars, D., & Gripon, J.-C. (1993). Hydrolysis of α_{s1}-casein by bovine plasmin. *Lait*, **73**, 337-344.

McSweeney, P. L, Olson, N.F., Fox, P.F., Healy. A. and Højrup, P. (1993). Proteolytic specificity of chymosin on bovine alpha s1-casein. *J. Dairy Sci*, **60(3)**, pp 401-412.

Park, O. and Allen, J. C. (1998). Preparation of phosphopeptides derived from α_s-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. *J. Dairy Sci*, **81(11),** 2858-2865.

Spellman, D., Kenny, P., O'Cuinn, G. and FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with Bacillus licheniformis proteinase activities. *J Agric Food Chem*, **5(4)**, 1258-1265.

Spellman, D., O'Cuinn, G. and FitzGerald, R. J. (2009). Bitterness in Bacillus proteinase hydrolysates of whey proteins *J. Food Chem,* **114 (2)**, 440-446.

Swaisgood, H. E. (1996). Characteristics of milk. In: O. R. Fennema (Ed.). Food Chemistry, 3rd ed. (pp 841-878). Marcel Dekker, Inc.: New York.

Swaisgood, H. E. (2003). Chemistry of the caseins. In P.F.Fox and P.L.H. McSweeney (Ed.) Advanced Dairy Chemistry. Volume 1 Proteins, 3rd edn (pp 139–201), Kluwer Academic/Plenum Publishers, New York.

Yokoi, K., Kakikawa, M., Kimoto, H., Watanabe, K., Yasukawa, H., Yamakawa, A., Taketo, A. and Kodaira, K. (2001). Genetic and biochemical characterization of glutamyl endopeptidase of Staphylococcus *warneri* M. *Gene*, **281,** 115–122.

Chapter 6

Substrate specificity of glutamyl endopeptidase (GE): hydrolysis studies using synthetic peptides.

6.1. ABSTRACT

Synthetic peptides corresponding to f16-26 (LpSpSpSEESITRI), non-phosporylated f16-26 (LSSSEESITRI), f115-125 (PVEPFTESQSL), f102-112 (MAPKHKEMPFP) and f105-119 (KHKEMPFPKYPVEPF) of β-casein (1 ml, 0.01 mM) were incubated with glutamyl endopeptidase (GE, 256 nmol min^{-1} ml^{-1}) at 37°C for 120 min. The hydrolysed samples were subsequently analysed using liquid chromatography mass spectrometry. The results obtained for non-phosphorylated and phosphorylated peptides indicated that in Glu-Glu-Ser, GE hydrolysed Glu-Ser. It was observed that multiple phosphorylated Ser residues present close to the scissile bond did not affect the substrate specificity of GE. The results obtained with PVEPFTESQSL, MAPKHKEMPFP and KHKEMPFPKYPVEPF indicated that GE hydrolyses the Glu-Met and the Glu-Pro bonds.

6.2. Introduction

In the literature, it was reported that GE from *Bacillus licheniformis* hydrolysed peptide bonds at the carboxy terminal of Glu and Asp residues (Breddam and Svensdsen, 1992; Kakudo *et al.,* 1992; Breddam and Meldal 1992; Barbosa *et al.,* 1996; Park and Allen 1998; Stennicke and Breddam, 1998 and Mil'gotina *et al.,* 2003). The results obtained in Chapters 4 and 5, also showed that GE purified from Alcalase™ hydrolysed peptide bonds at the carboxy terminal of Glu and Asp residues. The results obtained during GE hydrolysis of α- and β-casein indicated that Glu-Pro was not hydrolysed, Glu-Met was hydrolysed to a low extent and in Glu-Glu-X and Glu-Glu-Glu-X hydrolysis of Glu-X was preferred in comparison to hydrolysis of Glu-Glu. Breddam and Meldal (1992) previously reported that a Pro residue at the P_1' position was not preferred or poorly preferred by GE. These authors used a fluorogenic substrate Abz-Ala-Ala-Glu-Pro-TyrNO_2-Asp-OH and an incubation temperature of 25°C for their specificity studies However, no results seem to be available on the ability of GE to hydrolyse Glu-Pro in peptide sequences corresponding to actual food protein substrates.

The number of Glu-Met, Glu-Pro, Glu-Glu-X and Glu-Glu-Glu-X sequences (X= amino acid residue) found in the primary sequences of α_{s1}-, α_{s2}- and β-casein, and caseinomacropeptide are given in Table 6.1. From Table 6.1, it can be observed that apart from Glu-Glu-Glu-X sequences all the other sequences, i.e., Glu-Met, Glu-Pro and Glu-Glu-X sequences occur in β-casein. Therefore, to get a more detailed insight about the specificity of GE with respect to the amino acid residues present at the P_1' position, it was decided to incubate β-casein f16-26 , f102-112, f105-119 and f115-125 which contain Glu-Glu-X, Glu-Met and Glu-Pro sequences with GE at 37°C.

Table 6.1. The number of Glu-Met, Glu-Pro, Glu-Glu-X and Glu-Glu-Glu-X sequences present in α_{s1}-, α_{s2}- and β-casein and caseinomacropeptide.

Peptide Sequence	α_{s1}-casein	α_{s2}- casein	β-casein	caseinomacropeptide
Glu-Met	0	0	1	0
Glu-Pro	1	0	2	1
Glu-Glu-X	2	0	3	0
Glu-Glu-Glu-X	0	2	0	0

X: amino acid residue.

The results obtained in Chapter 4 during GE hydrolysis of β-casein also indicated that Glu(20)-Glu(21) and Glu(4)-Glu(5) bonds were hydrolysed. Whereas, the results obtained during GE hydrolysis of the enriched α-casein fraction (Chapter 5) indicated that in Glu-Glu-X and Glu-Glu-Glu-X sequences (X= Arg, Asn, Ile and Lys) hydrolysis of Glu-X was preferred in comparison to Glu-Glu. Therefore, both phosporylated (p) β-casein f16-26 (LpSpSpSEESITRI) and non-phosphorylated f16-26 (LSSSEESITRI) were chosen for incubation with GE to characterise the specificity of GE when Glu was present at the P_1' position and also to investigate the affect of multiple phosphorylated Ser residues at the N-terminus of potential scissile bonds.

6.3. Materials and Method

GE was purified as described in Chapter 3 using Protocol 3. Synthetic peptides corresponding to β-casein f16-26 (LpSpSpSEESITRI), non-phosporylated f16-26 (LSSSEESITRI), f102-112 (MAPKHKEMPFP), f105-119 (KHKEMPFPKYPVEPF) and f115-125 (PVEPFTESQSL) were from GenScript (Piscataway, NJ, USA). HPLC grade water and formic acid were from Sigma-Aldrich (Dublin, Ireland).

6.3.1. Digestion of synthetic peptides with GE

A stock solution of 10 mg ml^{-1} of the synthetic peptides was prepared using HPLC grade water. A working concentration of 0.01 mM was prepared by appropriate dilution of the stock solution with HPLC grade water. Synthetic peptide solutions (2 ml, 0.01 mM) were then incubated with GE (256 nmol min^{-1} ml^{-1}) at 37°C over 120 min. Samples (200 µl) were withdrawn at 15, 30, 60 and 120 min and were diluted with 200 µl of 0.1% (v/v) formic acid in HPLC grade water. The samples incubated for 120 min were analysed using mass spectrometry.

6.3.2. HPLC-ESI MS and MSMS analysis of peptide hydrolysates

The synthetic peptide hydrolysate samples (1 µM) were analyzed using an UltiMate® 3000 Nano LC instrument (Dionex, Sunnyvale, CA, USA) linked to a MicrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany). The hydrolysate

sample (1 µl) was loaded onto a C18 PepMap 100 precolumn cartridge (Dionex) in the µl Pickup mode (Dionex) at a flow rate of 25 µl min^{-1} for 1.5 min. On elution from the precolumn, the sample was then separated on a C18 PepMap 100 column (Dionex, 75 µm × 150 mm, 3 µm) at a flow rate of 300 nl min^{-1}. Mobile phase A was HPLC grade water with 0.1% (v/v) formic acid and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. The sample was eluted with an increasing gradient of solvent B as follows: 0-1.5 min 1% B; 1.5-16 min 1-60% B; 16.1-19 min 95% B; 19.1-20 min 1% B. The column temperature was maintained at 25°C.

The MS and tandem MS experiments were controlled using MicrOTOF control software (version 2.3, Bruker Daltonics). Full scans were performed between an m/z range of 50 and 2000. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan using collision induced dissociation (CID). Electrospray conditions were as follows: capillary temperature, 180°C; capillary voltage, -1500V; dry gas (N2) flow, 4.0 l min^{-1}.

6.3.3. Direct infusion

A concentration of 1 mg ml^{-1} of synthetic phosphorylated β-casein f16-25 was analysed by direct infusion in the MS system. Electrospray conditions employed are as follows: capillary voltage -4500V; end plate offset voltage: -500V; nebulizer 0.6 bar; dry gas: 6.0 l min^{-1}; dry temp: 180C; Full scans were performed between an m/z range of 50 and 3000. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan using CID.

6.4. Results and Discussion

The synthetic phosphorylated (p) peptide LpSpSpSEESITRI corresponding to β-casein f16-26 was not detected under the LC MS detection method used, however, peaks with unidentified sequences were observed in the TIC for this sample (Figure 6.1a) during LC MS analysis. In order to detect this fragment, direct infusion was performed as described in section 6.3.3. During direct infusion phosphorylated β-casein f16-26 was detected with a measured mass of 1460.529 Da. The theoretically

expected mass of this peptide is 1460.524 Da. It seems that detection of this phosphorylated peptide sequence only occurs following direct infusion at high concentration. The fragmentation information following direct infusion of phosphorylated β-casein f16-26 is given in Figure 6.2. The TIC for phosphorylated β-casein f16-26 when incubated with GE at 37°C for 120 min is given in Figure 6.1b. Using the LC MS detection approach, a GE digestion fragment, SITRI (f22-26), was detected in the phosphorylated peptide sample incubated with GE (Figure 6.1b). The fragmentation information of f22-26 is given in Figure 6.3.

The detection of β-casein f22-26 indicates that GE hydrolysed Glu(21)-Ser(22). Furthermore, this result also indicates that the Glu(20)-Glu(21) bond was not hydrolysed since ESITRI (f21-26) was not detected after incubation with GE. These results, however, are not in agreement with the results obtained during GE hydrolysis of β-casein where hydrolysis of Glu(4)-Glu(5) and Glu(20)-Glu(21) was observed. In order to further investigate the affect of multi phosphorylated Ser residues present at the N-terminus on the hydrolysis of Glu(20)-Glu(21), a non-phosphorylated peptide corresponding to f16-26 was incubated with GE at 37°C.

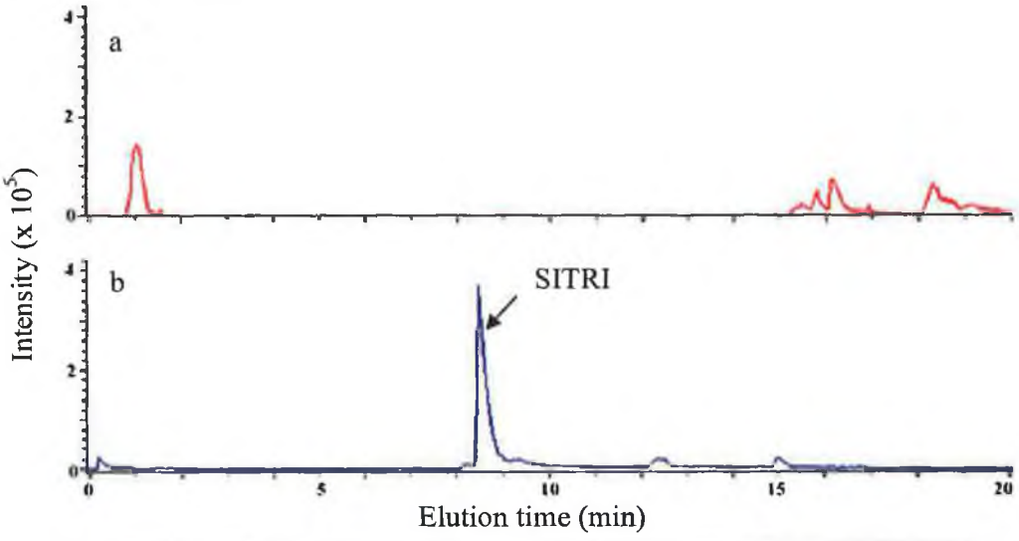


Figure 6.1. Total ion chromatogram of peptide (a) LS*S*S*EESITRI (control) and (b) LS*S*S*EESITRI incubated with glutamyl endopeptidase 37°C for 120 min. The peptide sequence present in the main peak is shown. *: phosphorylated serine residue.

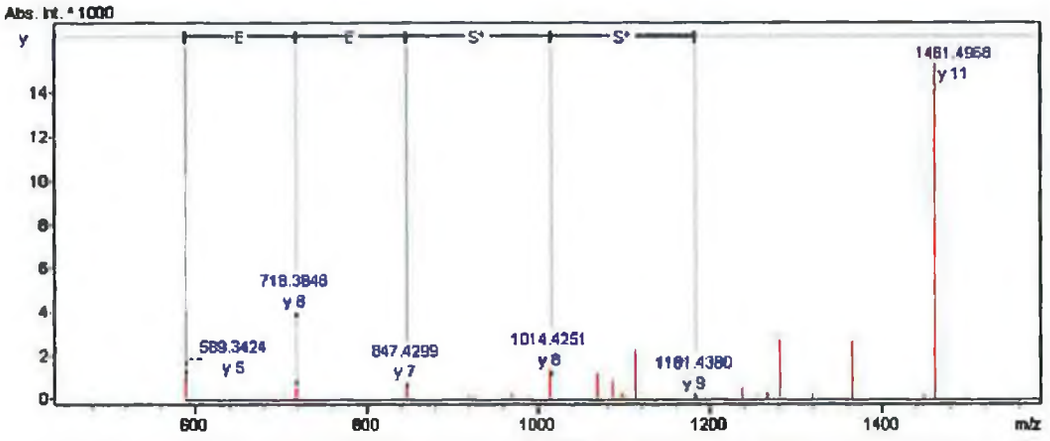


Figure 6.2. The fragmentation information of β-casein f16-26 (LS*S*S*EESITRI). *: phosphorylated.

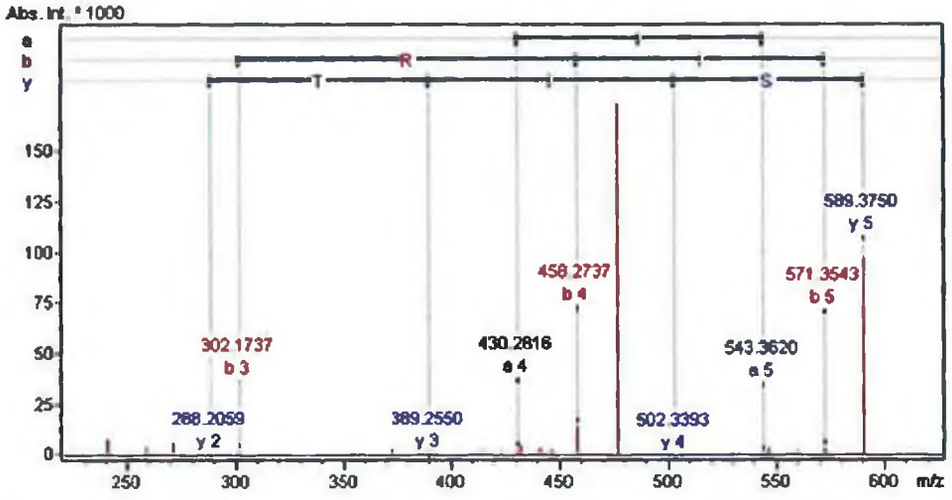


Figure 6.3. The fragmentation information of β-casein f22-26 (SITRI).

The synthetic non-phosphorylated peptide LSSSEESITRI corresponding to β-casein f16-26 was detected in the control sample, i.e., without GE following LC MS analysis (Figure 6.4a). The fragment information of this peptide is given in Figure 6.5. In the sample incubated with GE at 37°C for 120 min, fragments SITRI and LSSSEE were detected (Figure 6.3b). The fragment information for SITRI and LSSSEE are given in Figures 6.6 and 6.7, respectively. The detection of SITRI (f22-26) and LSSEE (f16-21) again indicates that GE hydrolysed Glu-Ser. Therefore, from Figures (6.1-6.7) it can be concluded that in Glu-Glu-Ser, GE hydrolyses Glu-Ser. It can also be concluded that the presence of multiple phosphorylated Ser residues upstream (towards the N-terminus) of the peptide did not alter the ability of GE to hydrolyse Glu-Ser.

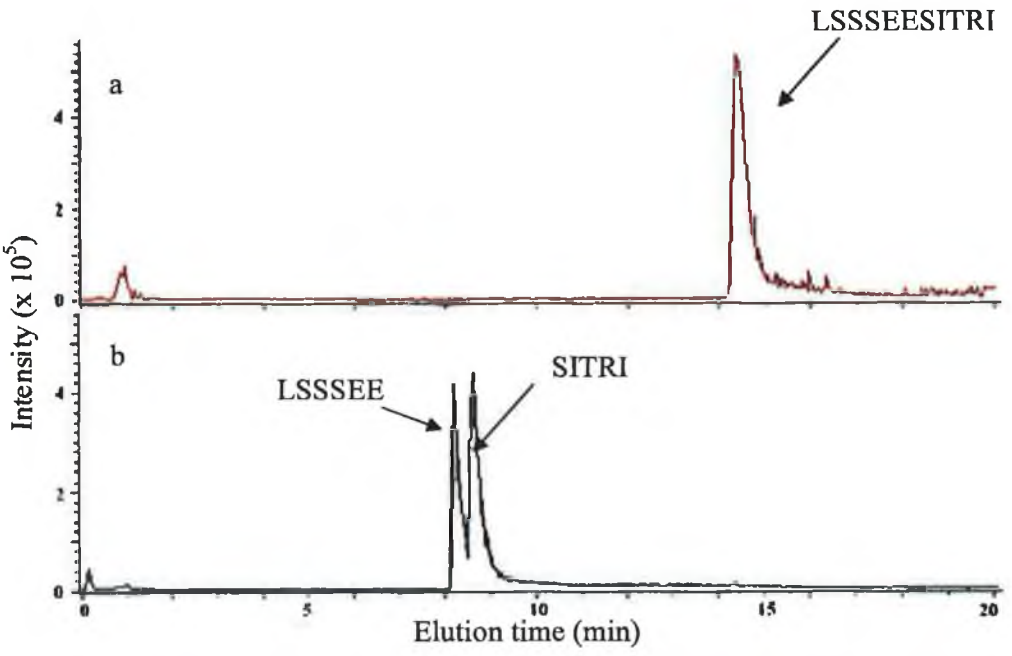


Figure 6.4. Total ion chromatogram of peptide (a) LSSSEESITRI (control) and (b) LSSSEESITRI incubated with glutamyl endopeptidase at 37°C for 120 min. The peptide sequences present in the respective peaks are shown.

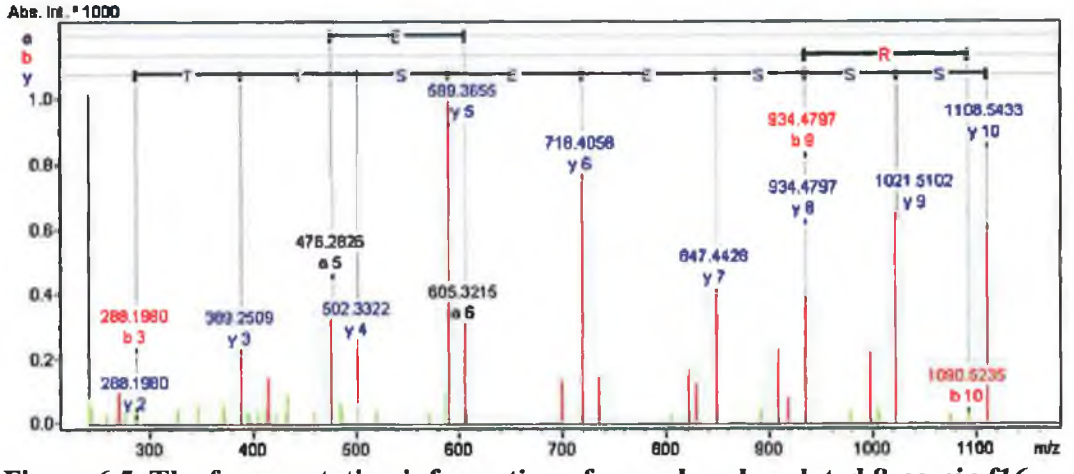


Figure 6.5. The fragmentation information of non-phosphorylated β-casein f16-26 (LSSSEESITRI).

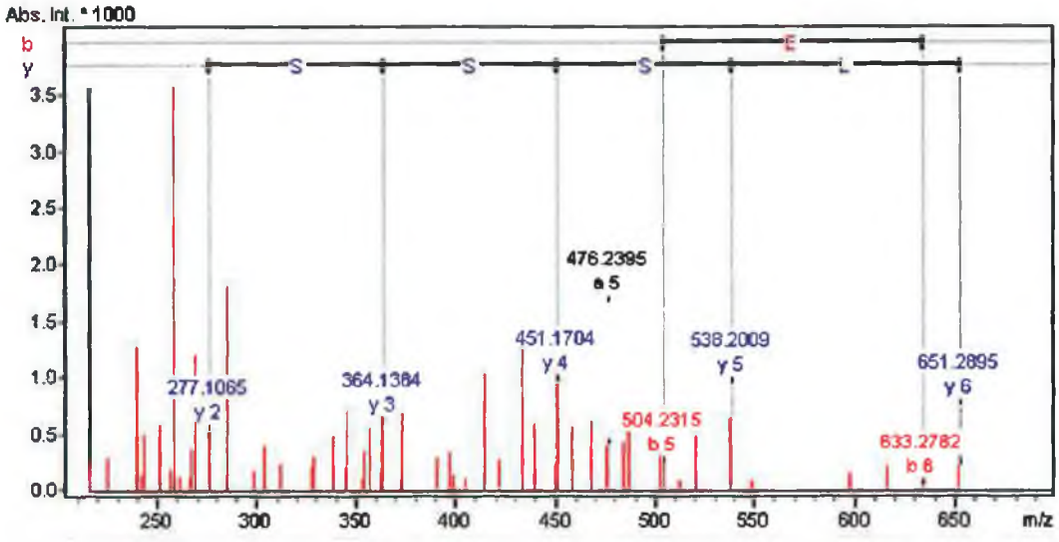


Figure 6.6.The fragmentation information of β-casein f16-21 (LSSSEE)

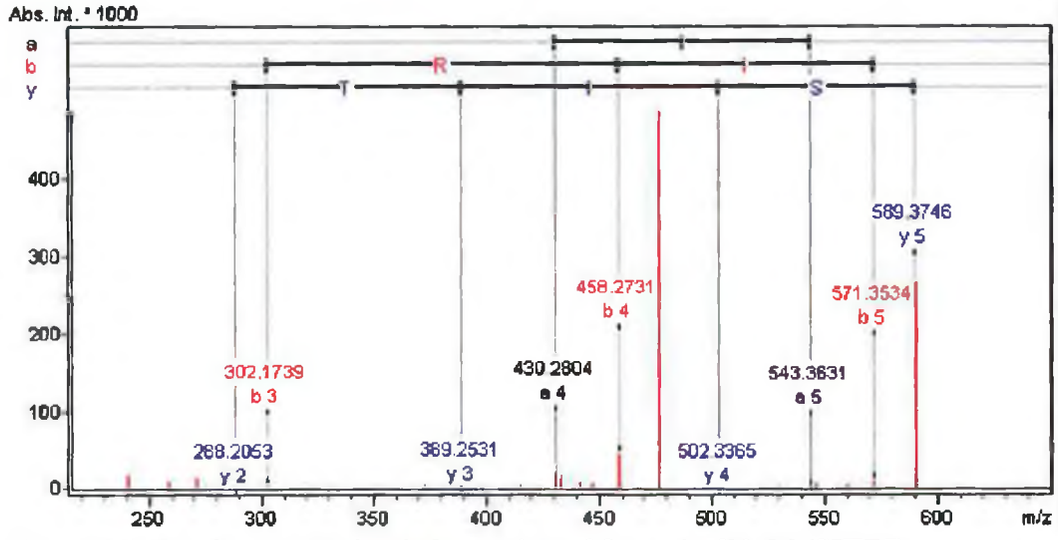


Figure 6.7. The fragmentation information of β-casein f22-26 (SITRI).

The intensity of the synthetic β-casein f115-125 (PVEPFTESQSL) was low in the LC MS detection method used (Figure 6.8a). However, GE digestion fragments SQSL, PFTE and PVEPFTE were observed in the sample incubated at 37°C for 120 min (Figure 6.8b). The detection of these peptides indicate that GE hydrolysed the Glu(117)-Pro(118) and Glu(121)-Ser(122) bonds. However, hydrolysis of Glu-Pro was not observed during incubation of α- and β-casein with GE. The detection method used herein did not allow us to perform a quantitative comparison of the different fragments obtained on incubation of the synthetic peptides with GE. Therefore, the extent of hydrolysis of Glu-Pro cannot be ascertained. However, the results from

hydrolysis of α- and β-casein and β-casein f115-125 suggest that Pro residues at the P_1' position are either not preferred or poorly preferred by GE. A similar observation was previously made by Breddam and Meldal (1992) when working with a synthetic fluorogenic Glu-Pro containing peptide.

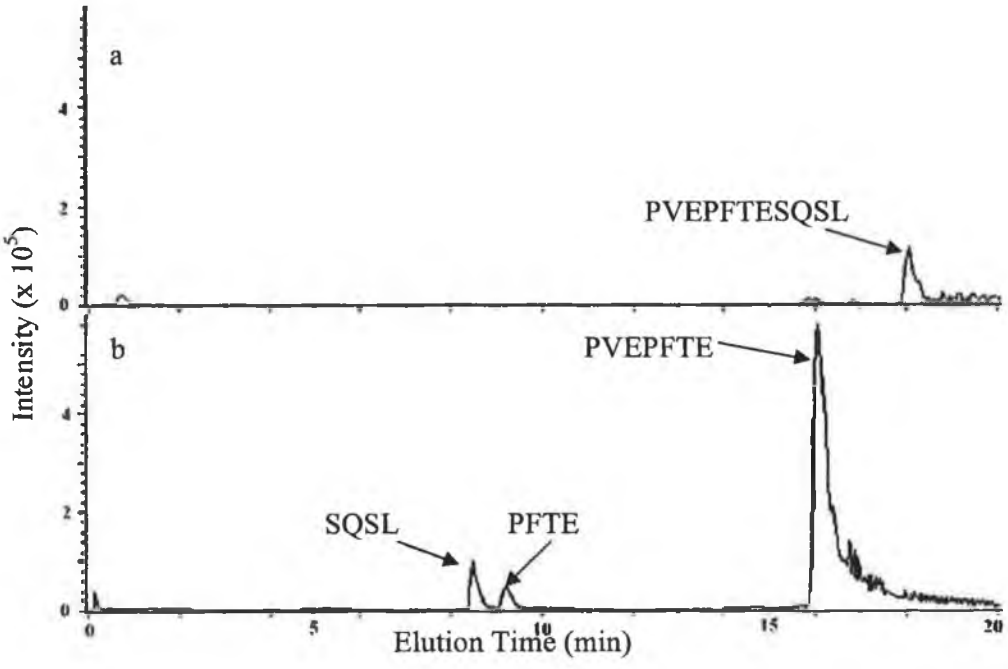


Figure 6.8. Total ion chromatogram of peptide (a) PVEPFTESQSL (control) and (b) PVEPFTESQSL incubated with glutamyl endopptidase 37°C for 120 min. The peptide sequences present in the respective peaks are shown.

The intensity of synthetic β-casein f102-112 (MAPKHKEMPFP) was also very low under the detection method employed (Figure 6.9a). However, in the sample incubated with GE at 37°C two peptide sequences MAPKHKEMPFP and MPFP were detected (Figure 6.9b). However, again the intensity of MPFP was very low. These results indicate that Glu-Met was hydrolysed by GE. This observation was also made during analysis of the results from the β-casein hydrolysis experiments (Chapter 4). However, it must be again stressed that the extent of hydrolysis of this bond could not be determined as the analytical procedure employed during MS analysis did not allow quantification of the amount of MPFP produced.

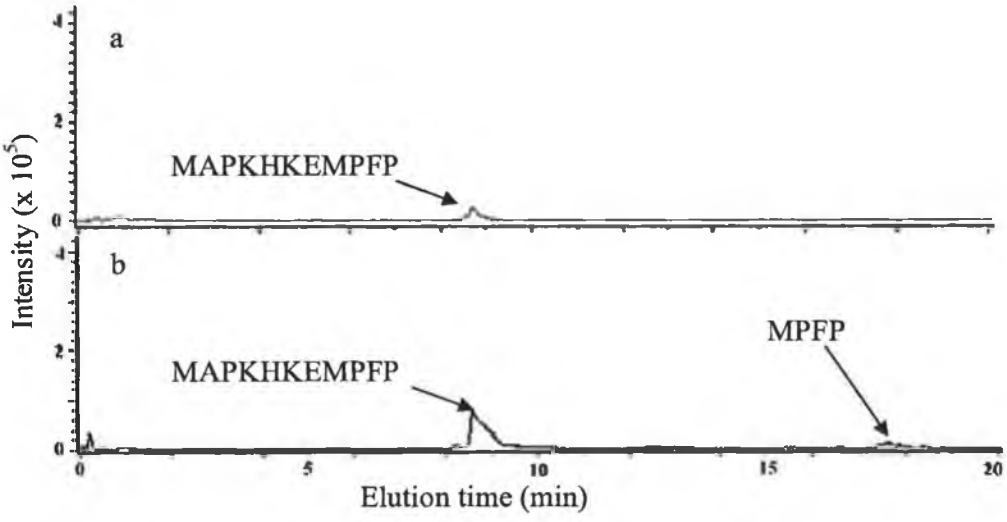


Figure 6.9. Total ion chromatogram of peptide (a) MAPKHKEMPFP (control) and (b) MAPKHKEMPFP incubated with GE at 37°C for 120 min. The peptide sequences present in respective peaks are shown

Synthetic β-casein KHKEMPFPKYPVEPF was not detected either in control or in the sample when incubated with GE using both LC-MS and direct infusion methods. However, a low intensity unidentified peak was observed in the TICs of both control and test sample at around 15.5 min following LC MS analysis (Figure 6.10). A very low intensity peptide corresponding to KHKEMPFPKYPVE was detected in the sample incubated with GE at 37°C (Figure 6.10). The detection of KHKEMPFPKYPVE again indicates that Glu-Pro was hydrolysed. These results also suggest that Glu-Met was not hydrolysed. However, as the intensities obtained were very low, it is proposed that further experiments be carried out in order to quantitatively substantiate the observations made herein.

The measured and calculated masses of both control and hydrolysed sequences of synthetic peptides of β-casein analysed using mass spectrometry are summarised in Table 6.2.

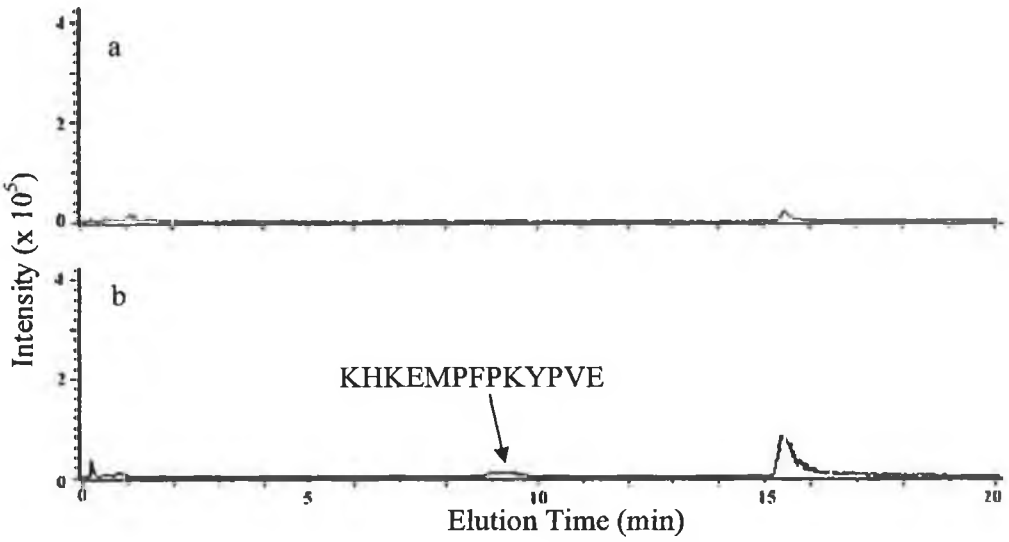


Figure 6.10. Total ion chromatogram of peptide (a) KHKEMPFPKYPVEPF (control) (b) KHKEMPFPKYPVEPF incubated with glutamyl endopeptidase 37°C for 120 min. The peptide sequence present in the main peak is shown.

Table 6.2: Summary details of the sequences of the synthetic peptides along with their measured and calculated masses when analysed using mass spectrometry.

Sequence	Measured mass (Da)	Calculated mass (Da)
LpSpSpSEESITRI (f16-26)	1460.529	1460.524
SITRI (f22-26)	588.371	588.3595
LSSSEESITRI (f16-26)	1220.644	1220.625
LSSSEE (f16-21)	650.285	650.2759
SITRI (22-26)	588.368	588.3595
PVEPFTESQSL(f115-125)	1232.606	1232.592
PVEPFTE (f115-121)	817.398	817.3858
PFTE (f118-121)	492.228	492.222
SQSL (f122-125)	433.221	433.2173
MAPKHKEMPFP (f102-112)	1311.656	1311.647
MPFP (f109-112)	490.227	490.225
KHKEMPFPKYPVEPF (f105-119)	nd	1872.96
KHKEMPFPKYPVE (f105-117)	1628.853	1628.839

p: phosphorylated. nd: not detected. In the brackets the corresponding β-casein fragment numbering is given. nd: not determind.

6.5. Conclusion

The results obtained for the incubation of the synthetic non-phosphorylated and phoshorylated β-casein f(16-26) confirm that in Glu-Glu-Ser containing sequences, GE hydrolyses the Glu-Ser bond. The results obtained also confirmed that multiple phosphorylated residues near the scissile bond do not affect the cleavage capability of GE. The results obtained during the incubation of PVEPFTESQSL and MAPKHKEMPFP corresponding to β-casein f(115-125) and f(102-112), respectively, indicate that the Glu-Pro and the Glu-Met bonds were hydrolysed. The results obtained from the enriched α- and β-casein hydrolysis experiments (Chapter 4 and 5) indicated that the Glu-Pro bond was not hydrolysed. To our knowledge, no information appears in the literature regarding the GE mediated hydrolysis of Glu-Met or Glu-Glu bonds.

For future studies, it is proposed to develop a quantitative liquid chromatography mass spectroscopy method to study the extent of hydrolysis of peptide bonds when Pro, Met and Glu residues are present at the P_1' position.

References

Barbosa, J. A. R. G., Saldanha, J.W. and Garratt, R. C. (1996). Novel features of serine protease active sites and specificity pockets: sequence analysis and modelling studies of glutamate-specific endopeptidases and epidermolytic toxins. *Protein Eng*, **9**, 591–601.

Breddam, K. and Meldal, M. (1992). Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching. *Eur J Biochem,* **206**, 103-107.

Breddam, K. and Svendsen, I. (1992). Isolation and amino acid sequence of a glutamic acid specific endopeptidase from *Bacillus licheniformis. Eur J Biochem,* **204,** 165-171.

Kakudo, S., Kikuchi, N., Kitadokoro, K., Fujiwara, T., Nakamura, E., Okamoto, H., Shin, M., Tamaki, M., Teraoka, H., Tsuzuki, H. and Yoshida, N. (1992). Purification, characterization, cloning, and expression of a glutamic acid-specific protease from *Bacillus licheniformis* ATCC 14580. *J Biol Chem,* **267(33),** 23782-23788.

Mil'gotina, E. I., Voyushina, T. L. and Chestukhina, G. G. (2003). Glutamyl endopeptidases: structure, function and practical application. *Bioorg Khim*,**29(6),** 563-76.

Park, O. and Allen, J. C. (1998). Preparation of phosphopeptides derived from α_s-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. *J. Dairy Sci*, **81(11),** 2858-2865.

Stennicke, H.R. and Breddam, K. (1998). Glutamyl endopeptidase I. In: A.J. Barrett, N.D. Rawlings and J.F. Woessner (Ed.). *Handbook of Proteolytic Enzymes* (pp. 243-246). Academic Press, San Diego, CA, USA.

Chapter 7

Overall summary of major findings and recommendations for future work

7.1. Overall summary of major findings

Glutamyl endopeptidase (GE) has been reported to induce peptide aggregation and bitterness on digestion of bovine whey protein concentrate. To date no detailed study of the specificity of GE using actual food protein substrates appears to have been carried out. Due to the potential importance of GE to the sensory and functional properties of milk protein hydrolysates it was decided to investigate the substrate specificity of GE using isolated/enriched bovine casein substrates.

The objectives of the present study were therefore to prepare purified/enriched casein substrates for subsequent substrate specificity studies with purified GE. A purified β-casein preparation and a fraction enriched in α-casein were incubated with GE for various time intervals at 37°C and 50°C. The peptides generated on hydrolysis of these substrates were analysed using liquid chromatography mass spectrometry. Based on the results obtained by mass spectrometry analysis, it was aimed to elucidate and confirm the substrate specificity of GE using actual food protein substrates. To further investigate the substrate specificity of GE, synthetic peptides corresponding to specific sequences of β-casein were also incubated with GE and the resultant fragments obtained were analysed by LC-MS analysis.

Chapter 2 reports on how the differential solubility of the caseins in the presence of Ca^{2+} at 4°C and 30°C was exploited to obtain fractions enriched in both α- and β-casein from acid caseinate. An ion-exchange (IEX) chromatographic step was employed in order to further purify the enriched casein fractions. Electrophoretically and chromatographically pure β-casein was obtained with an approximate yield of 5% and a fraction enriched in α-casein (both α_{s1}- and α_{s2}-casein) without β-casein was also obtained with a yield of approximately 31%.

Chapter 3 outlines how GE was purified from Alcalase™ 2.4L by employing hydrophobic interaction (HIC) and IEX chromatography with an approximate yield of 42%. The purified GE fraction was free from subtilisin, elastase, prolyl-dipeptidylpeptidase and plasmin activities. The molecular mass of GE was found to be approximately 24 kDa. The pH optimum was between pH 7.5- 8.0. The Michaelis constant (K_m) was estimated to be 3.33 and 10.80 mM at 37°C and 50°C, respectively.

It was observed that addition of Ca^{2+} ions had no effect on the stability of GE when stored at three different temperatures (4°C, 20°C and -20°C) for 2 months. GE was partially inhibited by 1 and 10 mM ethylenediaminetetraacetic acid.

Chapter 4 outlines the results obtained when purified β-casein was digested with GE at 37°C and 50°C for various time intervals. It was observed that the GE activity was highly specific and hydrolysed the peptide bond predominantly on the carboxy terminal of Glu and Asp. It was observed that the Glu-Pro bond was not hydrolysed on incubation at both 37 and 50°C. It was also observed that the Glu-Met bond was hydrolysed only at longer incubation times (240 min) both at 37 and 50°C. This suggested that Met residues were poorly preferred at the P_1' position. Interestingly, hydrolysis of Asp-Met was observed after 15, 30 and 240 min incubation at 37°C and for incubation at 50°C hydrolysis of Asp-Met was observed after 30, 60 and 120 min. These results indicate that the Glu-Met bond was hydrolysed by GE to a lower extent when compared to the hydrolysis of Asp-Met. GE hydrolysed the Glu-Asp bond in β-casein in samples incubated for 15, 30, 60 and 120 min at 37°C. However, during incubation at 50°C hydrolysis of Glu-Asp was only observed in the sample incubated for 60 min. The results obtained also indicated that GE hydrolysed the peptide bond at the carboxy terminal of Phe (51), Thr (128) and Gln (188) upon incubation of β-casein at 37°C. It was also observed that GE hydrolysed the peptide bond at the carboxy terminal of Thr (128) on incubation of β-casein at 50°C. The mean peptide sequence coverage was 53.4 and 49.8% for samples incubated with GE at 37 and 50°C, respectively.

Chapter 5 outlines the results obtained when an enriched α-casein fraction was digested with GE at 37°C and 50°C for various time intervals. It was again observed that GE was highly specific and hydrolysed the peptide bond predominantly on the carboxy terminal of Glu. It was also observed that GE hydrolysed bonds on the carboxy terminal of Asp residues. The peptides obtained corresponding to α_{s1}-casein and caseinomacropeptide (CMP) indicated that Glu-Pro was not hydrolysed. The result obtained also indicated that in Glu-Glu-X, hydrolysis of Glu-X was preferred by GE in comparison to Glu-Glu hydrolysis (X=Asn and Ile, Lys). This suggested that Glu residues at the P_1' position were poorly preferred. Non-specific cleavages at the

carboxy terminal of Phe (24) and Gln (131) corresponding to α_{s1}-casein were observed on incubation of the enriched α-casein for various time intervals at 50°C. However, non-specific cleavages were not observed on incubation at 37°C. The peptides obtained on α_{s2}-casein hydrolysis also indicated that in Glu-Glu-Glu-Lys sequences, hydrolysis of Glu-Lys is preferred by GE in comparison to Glu-Glu hydrolysis, which again indicates that Glu residues at the P_1' position were poorly preferred. GE hydrolysed the Glu-Asp bond in α_{s1}-casein in samples incubated for 15, 30, 60, 120 and 240 min at both 37°C and 50°C. The mean peptide sequence coverage values for α_{s1}-casein were 51.8 and 81.8%, respectively, for the samples incubated at 37°C and 50°C. The mean percentage peptide sequence coverage for α_{s2}-casein were 11.7 and 37.2%, respectively, for the samples incubated at 37°C and 50°C

Chapter 6 reports on the hydrolysis of various synthetic peptides corresponding to β-casein on incubation with GE at 37°C. The results obtained confirmed that in Glu-Glu-Ser, GE preferentially hydrolysed the Glu-Ser bond in comparison to Glu-Glu. Met and Pro residues at the P_1' position was also hydrolysed. However, the methodology used did not allow a quantitative comparison of the different fragments obtained on incubation of the synthetic peptides with GE. The results also confirmed that multiple phosphorylated residues near the scissile bond do not affect the substrate specificity of GE.

Overall, the results obtained indicate that the method employed for purification of β-casein was effective and yielded electrophoretically and chromatographically pure β-casein. It was observed that GE purified from Alcalase, was highly specific and hydrolysed peptide bonds predominantly on the carboxy terminal of Glu residues. GE was also observed to hydrolyse peptide bonds on the carboxy terminal of Asp residues. In general, GE appeared to be non-specific with respect to the nature of amino acid residue present at the P_1' position. However, the specificity studies carried out using casein substrates suggested that Pro residues at the P_1' position were not preferred. Whereas, Glu and Met residues at the P_1' position were poorly preferred. The poor preference of GE for Met and Glu residue at the P_1' position has not been reported in the literature. In the literature it was reported that Asp was not preferred at the P_1' position, however, in this present study it was observed that GE hydrolysed the Glu-Asp bond in both the purified β-casein and the enriched α-casein fractions even after

short incubation times (15-60 min). It was also observed that the Glu-Met bond was hydrolysed to a lesser extent by GE in comparison with hydrolysis of Asp-Met. GE also appeared to hydrolyse bonds on the carboxy terminal of Phe, Gln, and Thr residues. The hydrolysis of the Phe-X bond (X= amino acid residue) was previously reported in the literature. However, no evidence was found in the literature regarding the hydrolysis of peptide bonds on the carboxy side of Gln and Thr residues. In Figures 7.1-7.4 all the cleavages observed on incubation of purified β-casein and the enriched α-casein (α_{s1}-and α_{s2}-casein and CMP) at 37°C and 50°C are summarised.

Finally, to date no work has been carried out prior to this study to thoroughly investigate the substrate specificity of GE using real food substrates (caseins) or on the synthetic peptides corresponding to specific sequences of a real food substrate (caseins) or on the synthetic phosphorylated and non-phosphorylated peptides corresponding to a real food substrate (caseins).

Figure 7.1. Theoretical cleavage sites for glutamyl endopeptidase (GE) action present in β-casein along with the actual cleavages observed on incubation of purified β-casein with GE both at 37 and 50°C. Arrows represent the theoretical cleavage sites, residues in blue, orange and green represent the cleavages observed both at 37 and 50°C, only at 37°C and only at 50°C, respectively.

Figure 7.2. Theoretical cleavage sites for glutamyl endopeptidase (GE) action present in α_{s1}-casein along with the actual cleavages observed on incubation of the enriched α-casein fraction with GE both at 37 and 50°C. Arrows represent the theoretical cleavage sites, residues in blue and green represent the cleavages observed both at 37°C and 50°C and only at 50°C, respectively.

Figure 7.3. Theoretical cleavage sites for glutamyl endopeptidase (GE) action present in α_{s2}-casein along with the actual cleavages observed on incubation of the enriched α-casein fraction with GE both at 37 and 50°C. Arrows represent the theoretical cleavage sites, residues in blue and green represent the cleavages observed both at 37°C and 50°C and only at 50°C, respectively.

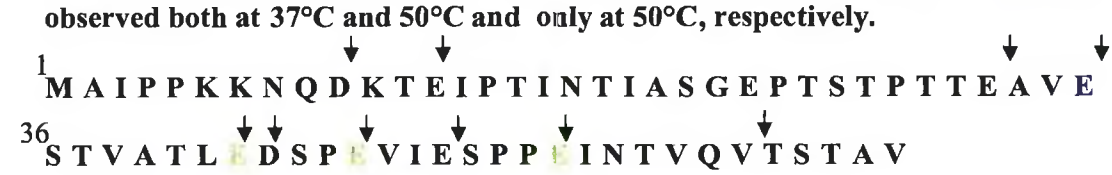


Figure 7.4. Theoretical cleavage sites for glutamyl endopeptidase (GE) action present in observed in caseinomacropeptide along with the actual cleavages observed on incubation of the enriched α-casein fraction with GE both at 37 and 50°C. Arrows represent the theoretical cleavage sites, residues in blue and green represent the cleavages observed both at 37°C and 50°C and only at 50°C, respectively.

7.2. Recommendations for future work.

- The method described for the purification of β-casein in Chapter 2 (Protocol 4), could be optimised in order to obtain a higher yield of pure β-casein. It is suggested that, after adjusting the pH of the curd sample following holding at 4°C for 24 h to pH 4.6 the sample could be held at this pH for a further 24 h at 4°C with gentle stirring. This additional step may increase the yield of enriched β-casein by facilitating its further dissociation from the α-casein precipitate. Alternatively, after adjusting the pH of the curd sample following holding at 4°C for 24 h to pH 4.6 multiple extractions of β-casein from the precipitate could be carried out by re-suspension and precipitation steps at 4°C in the presence of 10 mM $CaCl_2$. This approach may also increase the yield of enriched β-casein.
- Protocol 3 in Chapter 3 could be modified further for the purification of GE by reducing the number of HIC steps and by also using an IEX step as the first and last chromatographic steps.
- The substrate specificity of GE could be studied in more detail with respect to the number of amino acid residues required both upstream and downstream of the scissile bond in order for efficient hydrolysis to take place. This analysis could be carried out either using real food substrates or specific synthetic peptides.
- A quantitative liquid chromatography mass spectrometry protocol should be developed to study the rate and extent of hydrolysis of specific peptide bonds under different experimental conditions.
- The bioactivity, physiochemical and sensory properties of the peptides generated on hydrolysis of β-casein and the enriched α-casein fraction with GE could be determined.

Finally, hydrolysis of β-casein and the enriched α-casein fraction could be carried out using GE in combination of other enzyme activities for the targeted release of specific bioactive sequences.