Food Chemistry 136 (2013) 501–512

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Substrate specificity of glutamyl endopeptidase (GE): Hydrolysis studies with a bovine α-casein preparation

Phanindra Kalyankar, Yishen Zhu, Martina O' Keeffe, Gerard O' Cuinn, Richard J. FitzGerald *

Department of Life Sciences, University of Limerick, Limerick, Ireland

ARTICLE INFO

Article history:
Received 29 June 2012
Received in revised form 18 August 2012
Accepted 20 August 2012
Available online 31 August 2012

Keywords:
Glutamyl endopeptidase
Substrate specificity
Bovine α-casein
LC–MS

ABSTRACT

Glutamyl endopeptidase (GE) from Alcalase™ 2.4 L was purified using hydrophobic interaction (HIC) and ion-exchange (IEX) chromatography. The yield of GE obtained was approximately 42%. Bovine α-casein (containing α_{s1}- and α_{s2}-casein) was digested with GE at 37 and 50 °C for 4 h. Samples were withdrawn at various time intervals and the peptides generated were analysed using mass spectrometry. GE activity was highly specific and hydrolysed the peptide bond predominantly on the carboxy side of Glu residues while hydrolysis on the carboxyl side of Asp residues was also observed. Hydrolysis did not occur when Pro was at the P_1' position. In Glu-Glu-X (X = Arg, Asn, Ile and Ser) and Glu-Glu-Glu-Lys sequences, hydrolysis of Glu-X and Glu-Lys was preferred. The results are relevant to our understanding of the hydrolytic specificity of Alcalase, a food-grade proteolytic preparation containing GE activity which is used in the generation of casein hydrolysates.

1. Introduction

Glutamyl endopeptidase (GE) belongs to a class of serine proteinases and a sub-family of the chymotrypsin-like proteinases mainly found in Bacillus species that specifically cleave after acidic amino acid residues (Rudenskaya, 1998). The molecular mass of GE is dependent on bacterial source and varies from 12 to 31 kDa (Breddam & Svendsen, 1992; Drapeau, Boily, & Houmard, 1972; Kakudo et al., 1992; Kawalec, Potempa, Moon, Travis, & Murray, 2005; Ohara-Nemoto et al., 2002; Yokoi et al., 2001; Yoshida et al., 1988).

Breddam and Meldal (1992), Kakudo et al. (1992) investigated the specificity of GE from *Bacillus licheniformis* employing synthetic peptide substrates. Breddam and Svendsen (1992) also used oxidised ribonuclease and porcine glucagon (Glu deplete) to study the substrate specificity of GE from *B. licheniformis*. Breddam and Meldal (1992) 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 the nature of the amino acid residues present at positions P_4, P_3 and P_2. At positions P_1' and P_2', Val and Phe residues, respectively, were preferred by GE, while Asp or Pro and Pro residues were not preferred at positions P_1' and P_2', respectively. Furthermore, Kakudo et al. (1992) observed that the reactivity of GE towards Asp-paranitroanilide (pNA) was less than 1% of that observed for hydrolysis of Glu-pNA. These authors also observed that GE did not hydrolyse peptide bonds when Ala, Leu, Phe, Tyr and Lys residues were present at the P_1 position. However, Breddam and Svendsen (1992) concluded that GE exhibits non-specific behaviour with respect to the amino acid residue present at the P_1 position when added at high concentration and during extended incubation times.

Alcalase™ is a commercially available food-grade proteinase preparation obtained from *B. licheniformis* that has been shown to contain subtilisin and GE activities (Breddam & Svendsen, 1992; Spellman, Kenny, O'Cuinn, & FitzGerald, 2005). Various researchers have reported the aggregation and gelation properties of peptides obtained on hydrolysis of whey proteins using Alcalase (Creusot & Gruppen, 2007; Creusot, Gruppen, van Koningsveld, de Kruif, & Voragen, 2006; Doucet, Gauthier, Otter, & Foegeding, 2003; Otte et al., 1997; Spellman et al., 2005). The work by Spellman et al. (2005) indicated that the GE activity present in Alcalase was responsible for the enzyme induced aggregation observed during Alcalase hydrolysis of whey protein concentrate. Furthermore, Spellman, O' Cuinn, and FitzGerald (2009) demonstrated that GE was responsible for the release of higher bitterness in Alcalase hydrolysates of whey protein concentrate.

Madsen and Quist (1997) reported that GE (Glu/Asp specific BLP) hydrolysed caseins more effectively than the whey proteins. Given the potential importance of GE from *B. licheniformis* to the sensory and functional properties of milk protein hydrolysates, it is essential to investigate thoroughly the substrate specificity of GE using specific milk protein substrates. To date, no studies appear to have been carried out to investigate the substrate specificity of GE using purified/enriched individual milk proteins. Since caseins are the principal proteins in bovine milk, it was decided to study the substrate specificity of GE using a protein fraction containing α-casein.

* Corresponding author. Tel.: +353 61 2025989.
E-mail address: dick.fitzgerald@ul.ie (R.J. FitzGerald).

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.08.038

2. Materials and methods

Alcalase™ 2.4 L was obtained from Novozyme (Bagsvaerd, Denmark). Acetyl-Glu- pNA (ac-Glu-pNA), succinyl-Ala-Ala-Phe- pNA (s-Ala-Ala-Phe-pNA), succinyl-Ala-Ala-Ala- pNA (s-Ala-Ala-Ala-pNA), benzoyl-arginine- pNA (Bz-Arg-pNA) and succinyl-glycine-proline- pNA (s-Gly-Pro-pNA) were from Bachem, GmbH (Hegenheimer, Germany). Ammonium sulphate was from VWR International Ltd (Dublin, Ireland). Sodium phosphate dibasic, sodium phosphate mono basic, potassium chloride, sodium chloride, sodium hydroxide, dialysis tubing (12,400 Da molecular weight cut-off), calcium hydroxide, calcium chloride, phenyl Sepharose® 6 fast flow (high substitution), HPLC grade water and formic acid were from Sigma–Aldrich (Dublin, Ireland). All the other analytical grade general chemicals were obtained from Sigma Aldrich (Dublin, Ireland).

2.1. Purification of GE from Alcalase

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 matrix packed as per the manufacturer's instructions into a XK 16/10 column (100 × 16 mm 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 the 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 separation, the column was washed using 1 column volume (20 mL) of 1 M 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 analysed for proteolytic activity using synthetic substrates. Fractions containing GE activity were pooled and re-injected onto the HIC column. The active fractions obtained after the 2nd HIC separation step were re-injected onto the HIC column and the gradient volume was extended from 7.5 (150 mL) to 12 column volumes (240 mL) to further separate GE from subtilisin. After the extended gradient third HIC separation, the pooled fractions enriched in GE were concentrated by reapplying onto the HIC column and then eluting the sample in 100% Buffer B. Appropriate amounts of ammonium sulphate and potassium chloride were added to all pooled fractions above to bring the final concentration of both salts to 500 mM prior to injecting onto the HIC column.

The eluted sample was then dialysed overnight (12 h) at 4 °C using a 12,400 Da cut-off dialysis tubing against 50 mM sodium phosphate buffer, pH 6.2. 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). 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 a 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 brought to 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.

2.2. Determination of enzyme activity

The proteolytic activities of different fractions obtained during the purification of GE from Alcalase were assayed using synthetic substrates. The substrates used were s-Ala-Ala-Phe-pNA for quantification of subtilisin activity and ac-Glu-pNA for quantification of GE activity. s-Ala-Ala-Ala-pNA was used for quantification of elastase activity during the initial three HIC separations. 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

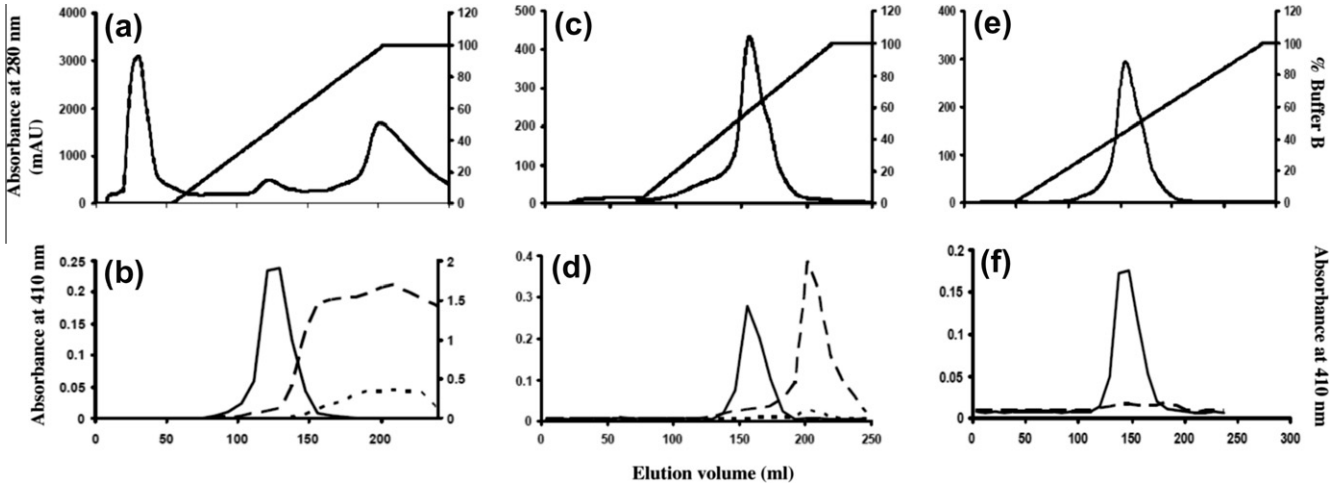

Fig. 1. Chromatograms showing the separation of glutamyl endopeptidase and subtilisin from Alcalase™ 2.4 L using three successive hydrophobic interaction chromatography (HIC) steps (a, c and e). Absorbance at 280 nm and concentration of buffer B are shown. The activity in the respective HIC separations (b, d and f) were expressed as absorbance at 410 nm per mL of fraction for GE activity, ac-Glu-pNA (——), for subtilisin activity, s-Ala-Ala–Phe-pNA (— —) and for elastase, s-Ala-Ala-Ala-pNA(· · ·).

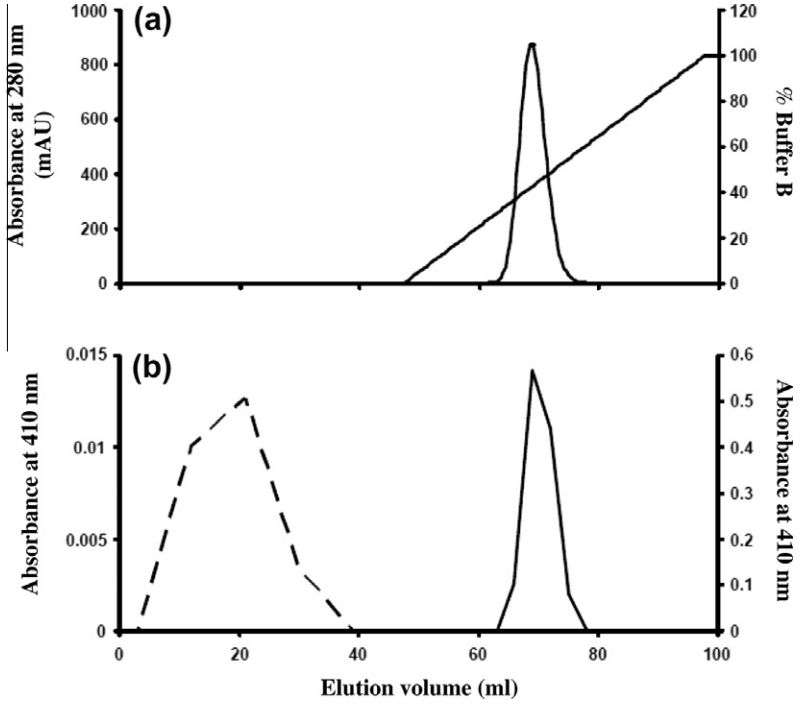

Fig. 2. (a) Cation exchange profile showing separation of the enriched glutamyl endopeptidase active fractions obtained from the third hydrophobic interaction chromatographic separation. Absorbance at 280 nm and concentration of buffer B are shown. (b) Enzyme activity values were expressed as Abs at 410 nm per mL of fraction for ac-Glu-pNA (——, right hand side) and s-Ala-Ala-Phe-pNA (— —, left hand side).

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 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, Yoshida, Noda, & Ito, 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.

2.3. Isolation of α-casein

A protein fraction enriched in α-casein was obtained following the purification procedure described by Kalyankar (2011). Urea-PAGE analysis of the sample showed the presence of α_{s1}- and α_{s2}-casein free of β- or κ-casein contaminant bands (data not shown).

2.4. Digestion of α-casein with GE

An α-casein aqueous solution (2 mL, 1.5% (w/v)) was incubated with GE (256 nmol min^{-1} mL^{-1}) at 37 and 50 °C for 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.

2.5. HPLC-ESI MS and MS/MS analysis of α-casein hydrolysates

The α-casein hydrolysate samples were diluted in HPLC grade water containing 0.1% (v/v) formic acid and were analysed using the LC MS/MS system as described by Zhu and FitzGerald (2010). The search parameters 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 detect low molecular mass peptides manually, theoretical masses were entered on an 'include' list such that they were preferentially fragmented. Masses were searched against theoretical peptide sequences using DataAnalysis, Bio-Tools and Sequence Editor Software packages supplied by Bruker Daltonics GmbH (Bremen, Germany). The peptide mass and fragment mass tolerances were both set to ±0.1 Da.

2.6. Determination of protein content

The protein content of the enzyme preparations was determined by the Bensadoun and Weinstein (1976) modification of the Lowry, Rosebrough, Farr, and Randal (1951) method.

3. Results and discussion

3.1. Purification of GE from Alcalase

GE activity was purified from Alcalase using a combination of HIC and IEX chromatographic steps. The chromatograms obtained during the chromatographic separations are given in Figs. 1 and 2. From Fig. 1a, three distinct peaks can be observed following the first HIC separation. Enzyme activity analyses (Fig. 1b) showed that the initial peak eluting between 10 and 70 mL had no proteolytic activity, the peak eluting between 100 and 150 mL contained GE activity while subtilisin and elastase activities were observed from 160 mL until the completion of the separation. These results are in agreement with those reported by Spellman et al. (2005) where good separation could be obtained, but where the GE activity was contaminated with a co-eluting subtilisin activity, especially in the later eluting fractions of the second protein peak. It can also be observed that subtilisin and elastase activities co-eluted indicating that subtilisin may exhibit low levels of elastase-like activity.

The fractions enriched in GE activity were pooled and then re-chromatographed twice more using the HIC column for further

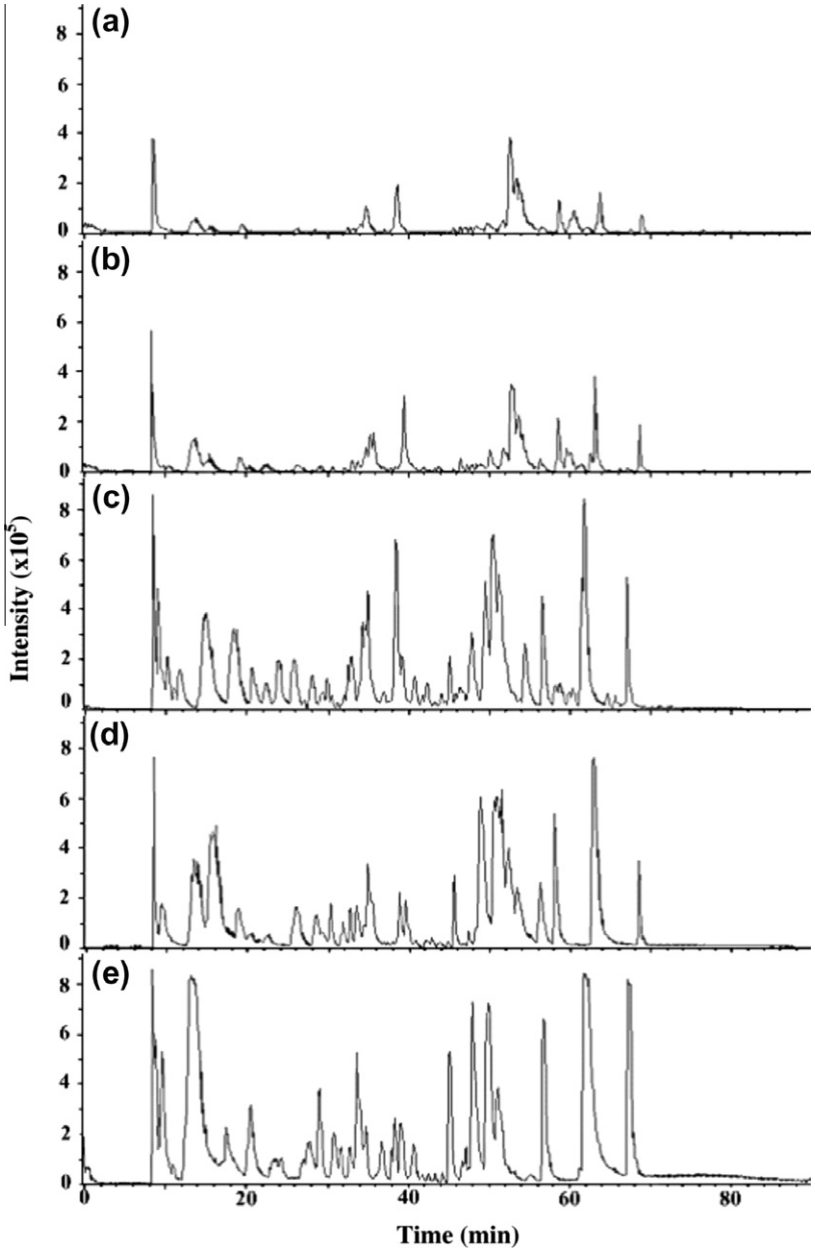

Fig. 3. Total intensity profiles of the enriched α-casein preparation following incubation with glutamyl endopeptidase at 37 °C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min and (e) 240 min.

enrichment of GE activity (Fig. 1c and e). However, it was observed that both the second and third HIC steps were not capable of fully separating the subtilisin contaminant from GE activity (Fig. 1d and f). An IEX step was therefore employed to further purify GE. The dialysed pooled fractions obtained from the 3rd HIC separation were injected onto a HiTrap™-CM FF cation exchange column. From Fig. 2, it can be observed that the subtilisin contaminant did not bind to the IEX column, while GE bound and eluted during the gradient. The GE active fractions were pooled, dialysed using a 12,400 Da cut-off dialysis as described earlier and stored at 4 °C in 30% (v/v) glycerol and 5 mM $CaCl_2$. The purified GE preparation did not have any activity on incubation with s-Ala-Ala-Phe-pNA, Bz-Arg-pNA and s-Gly-Pro-pNA.

From the purification table (data not shown), it was observed that approximately 42% of GE activity was obtained following the HIC and IEX steps. It was also observed after the first HIC separation that approximately 45% of GE activity was lost, while no significant reduction in the yield of GE was observed after the second HIC separation. However, during the third HIC separation approximately 12% of GE activity was lost. The specific activity of GE increased significantly after the first HIC step, while a gradual increase in GE specific activity was observed after the second and third HIC steps. However, after the IEX step a marked increase in GE specific activity was observed. The yield (41.68%) was less than the yield (50%) reported by Breddam and Svensden (1992) and was higher than the yield (30.3%) reported by Kakudo et al. (1992). From the results presented herein, it can be seen that the cation IEX step, as originally employed by Breddam and Svensden (1992), was a very useful approach for separating GE from subtilisin. The lower yield obtained herein in comparison with that reported by Breddam and Svensden (1992) may be due to the fact that a high level of GE activity (45%) was lost during the first HIC step. Furthermore, the sequence of

Table 1
Peptide sequences identified upon incubation of the α-casein preparation with glutamyl endopeptidase for different time intervals at 37 °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 MS/MS (charge)	Experimental mass	Calculated mass	MASCOT score
15 min Incubation					
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-KTTM°PLW	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
κ-Casein (caseinomacropeptide)					
f141–169	E-STVATLEDS*PEVIESPPEINTVQVTSTAV	1547.2113(2)	3092.408	3092.469	30.14
f152–169	E-VIESPPEINTVQVTSTAV	942.483(2)	1882.9514	1882.9888	55.05
30 min Incubation					
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
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
60 min Incubation					
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
60 min Incubation					
α_{s1}-Casein					
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
120 min Incubation					
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

(*continued on next page*)

Table 1 (*continued*)

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MS/MS (charge)	Experimental mass	Calculated mass	MASCOT score
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
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
240 min Incubation					
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.

chromatographic steps used by Breddam and Svensden (1992) were different to those carried out in this work.

3.2. Digestion of α-casein with GE

From the total intensity chromatogram (TIC) profiles obtained upon incubation of the enriched α-casein preparation with GE at 37 °C (Fig. 3) and 50 °C (data not shown) for various time intervals, it was observed that the intensities of the peaks at 50 °C were higher than that on incubation at 37 °C. However, the overall TIC profiles for samples incubated at 50 °C were generally similar to those observed at 37 °C. The peptide sequences, experimental mass, calculated mass of single and multiple charged ions along with the MASCOT scores of the samples incubated with GE over a period of 240 min both at 37 and 50 °C are given in Tables 1 and 2, respectively, where peptides whose MASCOT scores were above 20 are reported.

The cleavages observed in α_{s1}-casein both at 37 and 50 °C are represented in Fig. 4.

From Fig. 4, it was observed that GE was highly specific and hydrolysed peptide bonds on the carboxy terminal of Glu residues and to a lesser extent on the carboxyl side of Asp residues.

It can be shown that 32 different GE specific cleavage sites may exist in α_{s1}-casein (Fig. 4a). No evidence was found for the hydrolysis of Glu(47)-Ser(48), Glu(61)-Ala(62), Glu(84)-Asp(85) peptide bonds in samples incubated at 37 °C for various time intervals. However, the above peptide bonds were hydrolysed in samples incubated at 50 °C. While this may be an incubation temperature effect, the reason(s) for not observing these cleavages at 37 °C may be related in some way to the lower mean peptide coverage value obtained for samples incubated at 37 °C (51.8%) compared with samples incubated at 50 °C (81.8%). Spellman et al. (2005) reported that GE was responsible for aggregation of whey protein hydrolysates. The differences in the sequence coverage values obtained at 37 °C versus 50 °C may be due to different peptide aggregation behaviours during digestion.

It was also observed for α_{s2}-casein that GE predominantly hydrolysed on the carboxyl side of Glu residues and to a lesser extent on the carboxyl side of Asp residues on incubation at 37 and 50 °C (Fig. 4b). Theoretically, it is estimated that there are 28 different GE specific cleavage sites in α_{s2}-casein (Fig. 4b). It was observed that the Glu-Glu bond was not 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 2). The mean peptide sequence coverage values of α_{s2}-casein for the 37 and 50 °C incubations were 11.7% and 37.2%, respectively.

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). The action of GE on α_{s2}-casein should theoretically release one di-pep-

Table 2
Peptide sequences identified upon incubation of the α-casein preparation 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 MS/MS (charge)	Experimental mass	Calculated mass	MASCOT score
15 min Incubation					
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
30 min Incubation					
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
30 min Incubation					
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	1206.1856(5)	6025.8916	6025.9485	25.38

(*continued on next page*)

Table 2 (*continued*)

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MS/MS (charge)	Experimental mass	Calculated mass	MASCOT score
	VPITPTLNREQLS*TS*EE-N				
f134–145	E-NSKKTVDMES*TE-V	724.806(2)	1447.5974	1447.5902	45.91
κ-Casein (caseinomacropeptide)					
f152–169	E-VIESPPEINTVQVTSTAV	942.5064(2)	1882.9982	1882.9888	61.78
60 min Incubation					
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
60 min Incubation					
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
κ-Casein (caseinomacropeptide)					
f152–169	E-VIESPPEINTVQVTSTAV	953.4824(2)	1904.9502	1904.9707	43.5
120 min Incubation					
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

Table 2 (*continued*)

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MS/MS (charge)	Experimental mass	Calculated mass	MASCOT score
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
120 min Incubation					
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
κ-Casein (caseinomacropeptide)					
f141–151	E-STVATLEDS*PE-V	625.7404(2)	1249.4662	1249.4727	58.57
f151–169	E-VIESPPEINTVQVTSTAV	953.487(2)	1904.9594	1904.9707	47.47
240 min Incubation					
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
240 min Incubation					
α_{s1}-Casein					
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

(*continued on next page*)

Table 2 (*continued*)

Casein fragment	P_1-Peptide sequence-P_1'	Ion selected for MS/MS (charge)	Experimental mass	Calculated mass	MASCOT score
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
κ-Casein (caseinomacropeptide)					
f148–169	E-DSPEVIESPPEINTVQVTSTAV	1174.5574(2)	2347.1002	2347.1196	26.07
f152–158	E-VIESPPE-I	770.3822(1)	769.3749	769.3858	20.19
f152–169	E-VIESPPEINTVQVTSTAV	942.5006(2)	1882.9866	1882.9888	73.13

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

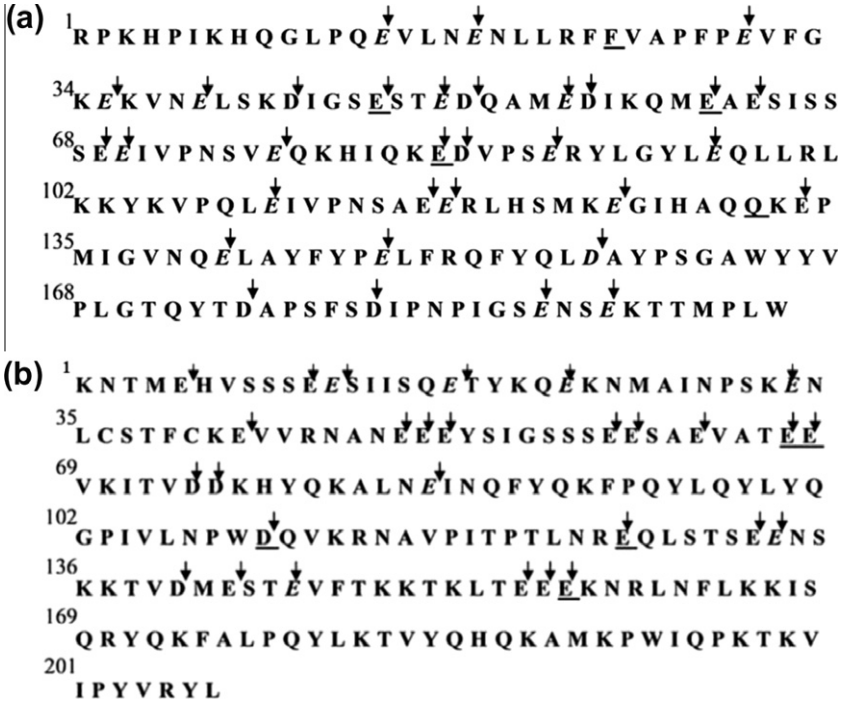

Fig. 4. Theoretically expected cleavage sites for glutamyl endopeptidase (GE) on (a) α_{s1}-casein and (b) α_{s2}-casein along with the actual cleavages observed on incubation of the α-casein preparation with GE both at 37 and 50 °C. Arrows represent the theoretical cleavage sites, residues in italics represent the cleavages observed both at 37 and 50 °C and the underlined residues represent cleavages observed only at 50 °C.

tide, ME (f141–142), two tri-peptides, SAE (f61–63) and STE (f143–145), one tetra-peptide VATE (f64–67) and 3 penta-peptides, KNTME (f1–5), TYKQE (f19–23) and VATEE (f64–68). However, under the main detection method (MASCOT search engine) employed in this study none of these peptides was observed. In order to detect the above mentioned low molecular mass peptides, a manual search was carried out as described in Section 2.5. An additional six peptides were detected, four from α_{s1}-casein (NSE f(190–192), VLNE f(15–18), KVNE f(36–39) and VFGKE f(31–35) and 2 from α_{s2}-casein (TYKQE f(19–23) and VATEE f(64–68) using the manual search approach.

There were some non-specific cleavages observed both at 37 and 50 °C (Tables 1 and 2). The results indicate that Pro(2)-Lys(3), Arg(22)-Phe(23), Phe(23)-Phe(24), Phe(24)-Val(25), Arg(90)-Tyr(91), Arg(100)-Leu(101), Lys(102)-Lys(103) and Gln(131)-Lys(132) were hydrolysed. However, most of these cleavages may be attributed to the action of residual chymosin and plasmin activities associated with the α-casein preparation (see Coker, Creamer, Burr, Hill, 1999; Le Bars Gripon, 1993; McSweeney, Olson, Fox, Healy, Hjrup, 1993). Furthermore, Breddam and Svensden (1992) also reported non-specific cleavage by GE (at higher concentrations, 1.2 μM) on the carboxyl side of Phe residues upon incubation with ribonuclease.

Fragments of κ-casein specifically arising from caseino-macropeptide (CMP f(105–169)) were also found in the α-casein preparation (Tables 1 and 2). 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 α-casein fraction. However, it may have been trapped in the α-casein precipitate during the purification procedure. The peptide fragments of κ-casein specifically released by GE can be summarised as follows: f152–169 was observed in samples incubated for 30, 60, 120 and 240 min, f141–151 was observed in samples incubated for 120 min and f120–126, f127–148, f131–137 and f131–148 were observed in samples incubated for 240 min. During incubation at 37 °C only two CMP fragments, i.e., f152–169 and f141–169, were observed in the samples incubated for 15 min (Tables 1 and 2).

Overall, from the results obtained, it was observed that Glu-Pro was not hydrolysed by GE, an observation also made by Breddam and Meldal (1992) using synthetic peptides. It was also observed that in Glu-Glu-X sequences present in α_{s1}-casein, the hydrolysis of Glu-X (X = Ile (71) and Arg (119)) was preferred over hydrolysis of Glu-Glu (69–70 and 117–118) (see Table 1 and Fig. 4). Similar observations were made in α_{s2}-casein, where Glu-X (X = Ser (13) and Asn (134) hydrolysis was preferred over Glu-Glu (11–12 and 132–133) hydrolysis. In α_{s2}-casein, it was also observed in Glu-Glu-Glu-Lys (f155–158) that Glu-Lys (157–158) hydrolysis was preferred over Glu-Glu (155–156 and 156–157) hydrolysis (Table 2). The hydrolysis of Glu-Glu (67–68) was observed (corresponding to α_{s2}-casein) only once in the present study in a sample incubated at 50 °C for 60 min (Table 2) implying that a Glu residue at the P_1' position was less preferred by GE. Apart from Glu-Glu and Glu-Pro, all the other theoretically expected peptide bonds were hydrolysed, including Glu-Asp. It was also observed that, with the exception of Pro and Glu residues at the P_1' position, GE is non-specific with respect to the nature of the amino acid residues present up stream (P_2, P_3, P_4, P_5, etc.) and down stream (P_1', P_2', P_3', P_4', etc.) of the scissile bond.

4. Conclusion

The results presented herein indicate that highly pure GE could be obtained using a combination of HIC and IEX. GE was observed to hydrolyse predominantly on the carboxy terminal of Glu residues present in α_{s1}-, α_{s2}-and κ-casein (CMP f(105–169)) on incubation at 37 and 50 °C. GE also hydrolysed (to a lesser extent) the peptide bond on the carboxy terminal of Asp residues present in α_{s1}- and α_{s2}-caseins.

From the results obtained it was also concluded that GE does not hydrolyse Glu-Pro in α-casein. In Glu-Glu-X (X = Arg, Asn, Ile and Ser), Glu-X hydrolysis was preferred by GE in comparison with hydrolysis of Glu-Glu. Whereas, in Glu-Glu-Glu-Lys, Glu-Lys hydrolysis was preferred over Glu-Glu hydrolysis. 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 large difference in the mean peptide coverage values for the samples incubated at 37 and 50 °C suggests that peptide aggregation was higher on incubation at 37 °C in comparison to incubation at 50 °C. Further work needs to be carried out to confirm the above observation. It may be that in the presence of α-casein that GE is more active at 50 °C than at 37 °C.

In conclusion, the work presented herein gives an insight into the hydrolytic specificity of GE, an important side-activity present in Alcalase, a widely used food grade proteolytic preparation. Moreover, to our knowledge, this is the first demonstration of the substrate specificity of GE on an actual food protein substrate, i.e., α-casein.

Acknowledgement

This work was funded under the National Development Plan (2006-2010), through the Food Institutional Research Measure, administered by the Irish Department of Agriculture and Food.

References

Bensadoun, A., & Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. *Analytical Biochemistry, 70*(1), 241–250.

Breddam, K., & Meldal, M. (1992). Substrate preferences of glutamic-acid-specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching. *European Journal of Biochemistry, 206*, 103–107.

Breddam, K., & Svendsen, I. (1992). Isolation and amino acid sequence of a glutamic acid specific endopeptidase from *Bacillus licheniformis*. *European Journal of Biochemistry, 204*, 165–171.

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

Creusot, N., & Gruppen, H. (2007). Hydrolysis of whey protein isolate with *Bacillus licheniformis* protease: Fractionation and identification of aggregating peptides. *Journal of Agricultural and Food Chemistry, 55*, 9241–9250.

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

Doucet, D., Gauthier, S. F., Otter, D. E., & Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: Peptide identification and determination of enzyme specificity. *Journal of Agricultural and Food Chemistry, 51*, 6300–6308.

Drapeau, G. R., Boily, Y., & Houmard, J. (1972). Purification and properties of an extracellular protease of *Staphylococcus aureus*. *Journal of Biological Chemistry, 247*(20), 6720–6726.

Kakudo, S., Kikuchi, N., Kitadokoro, K., Fujiwara, T., Nakamura, E., Okamoto, H., et al. (1992). Purification, characterization, cloning, and expression of a glutamic acid-specific protease from *Bacillus licheniformis* ATCC 14580. *Journal of Biological Chemistry, 267*(33), 23782–23788.

Kalyankar, P. (2011). *Investigation of the substrate specificity of glutamyl endopeptidase using casein substrates. Ph.D dissertation*, Galway-Mayo Institute of Technology, Galway, Ireland, 32–65.

Kawalec, M., Potempa, J., Moon, J. L., Travis, J., & 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. *Journal of Bacteriology, 187*(1), 266–275.

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

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randal, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

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

McSweeney, P. L., Olson, N. F., Fox, P. F., Healy, A., & Højrup, P. (1993). Proteolytic specificity of chymosin on bovine alpha s_1-casein. *Journal of Dairy Science, 60*(3), 401–412.

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

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

Rudenskaya, G. N. (1998). Glutamyl endopeptidases from microorganisms, a new subfamily of chymotrypsin proteases. *Russian Journal of Bioorganic Chemistry, 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. *Journal of Agricultural and Food Chemistry, 5*(4), 1258–1265.

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

Yamangata, A., Yoshida, N., Noda, K., & Ito, A. (1995). Purification and characterization of a new serine proteinase from *Bacillus subtilis* with specificity for amino acids at P_1 and P_2 positions. *Biochimica et Biophysica Acta, 1253*, 224–228.

Yokoi, K., Kakikawa, M., Kimoto, H., Watanabe, K., Yasukawa, H., Yamakawa, A., et al. (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., & Makisumi, S. (1988). Purification and characterization of an acidic amino acid specific endopeptidase of *Streptomyces griseus* obtained from a commercial preparation (Pronase). *Journal of Biochemistry, 104*, 451–456.

Zhu, Y.-S., & FitzGerald, R. J. (2010). Direct nanoHPLC-ESI-QTOF MS/MS analysis of tryptic caseinophosphopeptides. *Food Chemistry, 123*, 753–759.