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Investigation of the Substrate Specificity of Glutamyl Endopeptidase Using Purified Bovine β-Casein and Synthetic Peptides

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

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

ABSTRACT: Purified bovine β-casein was digested with glutamyl endopeptidase (GE) at 37 and 50 °C for 4 h. The peptides generated were determined using nano-LC-ESI-qTOF-MS/MS. GE was highly specific and hydrolyzed peptide bonds in β-casein predominantly on the carboxy terminal of Glu and Asp. Pro residues were not preferred, while Met was poorly preferred at the P_1' position. Glu-Met hydrolysis was less preferred in comparison to Asp-Met hydrolysis. Five synthetic peptides corresponding to specific sequences in β-casein were incubated with GE at 37 °C to further characterize the substrate specificity. MS analysis of the digestion products indicated that GE hydrolyzed Glu-Ser in Glu-Glu-Ser. Furthermore, hydrolysis of Glu-Met and Glu-Pro was observed. The presence of multiple-phosphorylated Ser residues upstream from the scissile bond did not appear to affect hydrolysis of Glu-Ser. The results herein are relevant to our understanding of the substrate specificity of GE and the peptides that may be expected during the hydrolysis of β-casein.

KEYWORDS: *glutamyl endopeptidase, substrate specificity, bovine β-casein, synthetic peptides, LC-MS/MS*

INTRODUCTION

Milk proteins are hydrolyzed industrially for various purposes including their subsequent application as ingredients in reduced/hypoallergenic infant formulas and in enteral/sport and parenteral nutrition.[1] Alcalase from *Bacillus licheniformis* is a commercially available proteolytic preparation that is used extensively in the generation of milk protein hydrolysates.[2−8] Alcalase in addition to having a subtilisin-like activity also possesses glutamyl endopeptidase (GE) activity.[7,9] GE appears to play a key role in peptide aggregation and bitterness evolution in whey protein hydrolysates.[6,7] A detailed knowledge of the substrate specificity of GE using specific milk protein substrates may allow the targeted release of specific peptide sequences having different techno- and biofunctional applications. Such information may be relevant to both academic studies and industrial applications of casein/food protein hydrolysates.

GE is reported to be highly specific and cleaves the peptide bond at the carboxy side of acidic (Glu/Asp) amino acid residues.[9−13] Previous studies on GE hydrolysis of α-casein[9] and synthetic peptides[11] indicate that the rate of hydrolysis of Glu-Asp was very slow, whereas Glu and Pro residues at the P_1' position were either not preferred or poorly preferred.

Yokoi et al.[13] 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 h. The hydrolysis generated five peptides, designated 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 kDa, respectively. N-Terminal amino acid sequence analysis showed that 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. It was concluded that Glu-Val (β-CN 91−92), Glu-Ala (β-CN 100−101), and Glu-Ser (β-CN 121−122) were cleaved on incubation with GE. Park and Allen[14] prepared casein phosphopeptides using immobilized GE. A solution of β-casein was recirculated in a fluidized bed bioreactor containing immobilized GE beads at 20 °C for 3 h. The major peptide bands observed in the β-casein GE hydrolysate following SDS-PAGE analysis corresponded to molecular masses of 9.3, 8.2, and 6.2 kDa. Peptides with these masses were attributed to β-casein f12−91, f48−121, and f130−184, respectively. To our knowledge, apart from the above-mentioned studies, no detailed study has been performed on the substrate specificity of GE from *B. licheniformis* using β-casein, a major protein in the casein fraction of bovine milk.

The objective of this study was therefore to investigate the specificity, i.e., the preferred amino acid residues present at the P_1 and P_1' positions, of GE during hydrolysis of bovine β-casein. This was performed by characterizing the peptides released from a purified β-casein preparation following incubation with GE at both 37 and 50 °C for various time intervals. Synthetic peptides corresponding to specific β-casein sequences were also incubated with GE at 37 °C for 120 min to further explore its substrate specificity.

METHODS AND MATERIALS

Acid casein was obtained from Arrabawn Co-op Society Ltd. (Tipperary, Ireland). Syringe filters (0.2 μm, Puradisc) and disc filters (0.2 μm, Supra-200, 47 mm diameter) were from VWR International Ltd. (Dublin, Ireland). Hydrochloric acid, sodium hydroxide, acetonitrile (HPLC grade), water (HPLC grade), and formic acid were obtained from Sigma-Aldrich (Dublin, Ireland). Synthetic peptides corresponding to phosphorylated β-casein f16−26 (LpSpSpSEESITRI), nonphosporylated f16−26 (LSSSEESITRI), f102−112 (MAPKHKEMPFP), f105−119 (KHKEMPFPKYPVEPF), and f115−125 (PVEPFTESQSL) were from GenScript (Piscataway,

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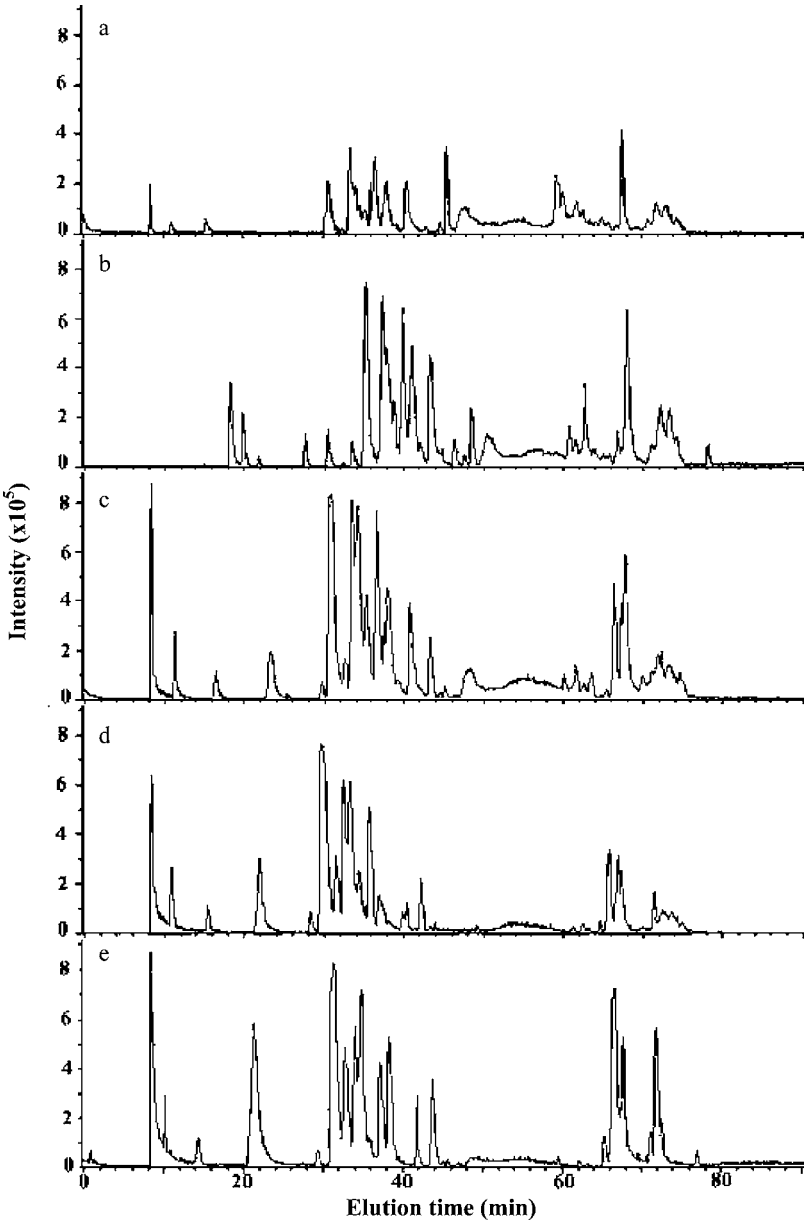

Figure 1. Total ion chromatogram of β-casein incubated with glutamyl endopeptidase at 37 °C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min, and (e) 240 min.

NJ, USA). Acetyl-Glu-pNA (ac-Glu-pNA) was from Bachem GmBH (Hergenheimer, Weil am Rhein, Germany).

Purification of Bovine β-Casein from Acid Caseinate. The procedure described by Kalyankar[15] was used. The method employed was a modification of a number of different protocols described in the literature.

Purification of GE. GE was purified from Alcalase using the protocol described by Kalyankar et al.[9]

Digestion of β-Casein with GE. Purified β-casein aqueous solution (2 mL, 1.5% (w/v)) was incubated with GE (256 nmol min^{-1} mL^{-1}, ac-Glu-pNA at 50 °C) at 37 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.

HPLC-ESI-MS and MS/MS Analysis of β-Casein Hydrolysates. The β-casein hydrolysate samples were diluted (25 ng/μL) in dH_2O containing 0.1% (v/v) formic acid and were analyzed using an UltiMate 3000 NanoHPLC (Dionex, Sunnyvale, CA, USA) linked to a MicrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany) as described by Zhu and FitzGerald.[16] Manual detection of short peptides (200−800 Da) was also carried out by the procedure described by Kalyankar et al.[9]

HPLC-ESI-MS and MS/MS Analysis of the Synthetic Peptide Digests. The synthetic peptide hydrolysate samples (1 μM) were also analyzed using an UltiMate 3000 Nano LC instrument linked to a MicrOTOF II mass spectrometer. 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

Table 1. Peptide Sequences Identified upon Incubation of β-Casein with Glutamyl Endopeptidase for Different Time Intervals at 37 °C[a]

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)	1283.621	1283.636	36.77
f1−14	RELEELNVPGEIVE-S	813.4131(2)	1624.812	1624.831	52.61
f1−20	RELEELNVPGEIVESLSSSE-E	889.4104(3)	2665.209	2665.21	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)	1460.553	1460.582	45.63
f32−44	E-KFQS*EEQQQTEDE-L	853.3179(2)	1704.621	1704.615	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	2489.212	63.51
f101−121	E-AM°APKHKEM°PFPKYPVEPFTE-V	836.0634(3)	2505.168	2505.207	35.59
f122−131	E-SQSLTLTDVE-N	1092.5294(1)	1091.522	1091.535	55.35
f185−209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1414.8442(3)	2827.674	2827.629	52.76
Incubation for 30 min					
f1−14	RELEELNVPGEIVE-S	813.41(2)	1624.805	1624.831	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)	3110.365	3110.423	56.02
f6−31	E-LNVPGEIVES*LS*S*S*EESITRINKKIE-K	1064.4503(3)	3190.329	3190.389	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)	1460.548	1460.546	51.02
f32−44	E-KFQSEEQQQTEDE-L	813.3411(2)	1624.668	1624.685	50.79
f32−44	E-KFQS*EEQQQTEDE-L	853.3224(2)	1704.63	1704.615	65.27
f32−47	E-KFQSEEQQQTEDELQD-L	991.4193(2)	1980.824	1980.855	28.64
f32−47	E-KFQS*EEQQQTEDELQD-K	1031.3977(2)	2060.781	2060.821	41.03
f32−52	E-KFQS*EEQQQTEDELQDKIHPF-A	895.3837(3)	2683.129	2683.18	64.65
f92−100	E-VMGVSKVKE-A	488.7705(2)	975.5264	975.5423	40.61
f101−121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4024(3)	2473.185	2473.217	53.21
f101−121	E-AMAPKHKEM°PFPKYPVEPFTE-S	836.0642(3)	2505.171	2505.207	41.94
f122−131	E-SQSLTLTDVE-N	1092.5234(1)	1091.516	1091.535	50.9
f185−209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1414.8406(2)	2827.667	2827.629	51.35
Incubation for 60 min					
f1−11	RELEELNVPGE-I	642.8134(2)	1283.612	1283.636	30.46
f1−14	RELEELNVPGEIVE-S	813.4075(2)	1624.8	1624.831	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)	3110.364	3110.423	30.39
f22−31	E-SITRINKKIE-K	601.3557(2)	1200.697	1200.719	27.92
f32−42	E-KFQS*EEQQQTE-D	731.284(2)	1460.553	1460.582	31.07
f32−44	E-KFQS*EEQQQTEDE-L	853.32(2)	1704.625	1704.652	54.57
f32−47	E-KFQS*EEQQQTEDELQD-K	1031.4032(2)	2060.792	2060.821	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)	2489.172	2489.212	67.56
f122−131	E-SQSLTLTDVE-N	1092.5221(1)	1091.515	1091.535	50.95
β-casein fragment	P_1−peptide sequence−P_1'	ion selected for MSMS (charge)	experimental mass	calculated mass	MASCOT score
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)	1200.707	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)	1726.606	1726.634	73.29
f32−52	E-KFQS*EEQQQTEDELQDKIHPF-A	895.3957(3)	2683.165	2683.18	76.82
f45−91	E-LQDKIHPFAQTQSLVYPFPGPIP NSLPQNIPPLTQTPVVVPPFLQPE-V	1040.7596(5)	5198.762	5198.785	52.57
f92−100	VMGVSKVKE-A	488.7721(2)	975.5296	975.5423	43.22
f101−121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7438(3)	2489.21	2489.212	62.15
f101−121	E-AM°APKHKEM°PFPKYPVEPFTE-S	836.0759(3)	2505.206	2505.207	30.89
f122−131	E-SQSLTLTDVE-N	1092.5396(1)	1091.532	1091.535	53.41

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Table 1. continued

β-casein fragment	P_1−peptide sequence−P_1'	ion selected for MSMS (charge)	experimental mass	calculated mass	MASCOT score
Incubation for 120 min					
f189−209	Q-AFLLYQEPVLGPVRGPFPIIV	1163.1602(2)	2324.306	2324.33	50.97
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)	1575.571	1575.573	45.91
f92−100	E-VMGVSKVKE	479.7909(2)	957.5672	957.5859	51.06
f101−121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7328(3)	2489.177	2489.212	76.79
f101−128	E-AM°APKHKEMPFPKYPVEPFTESQSLTLT-D	660.9324(5)	3299.626	3299.565	40.86
f109−121	E-M°PFPKYPVEPFTE-S	799.3684(2)	1596.722	1596.753	23.2
f122−131	E-SQSLTLTDVE-N	546.7653(2)	1091.516	1091.535	26.02
f185−209	D-M°PIQAFLLYQEPVLGPVRGPFPIIV	937.5112(3)	2809.512	2809.561	36.99

[a]The amino acid residues present at P_1 and P_1' are given. S*: phosphorylated serine. M°: oxidized methionine.

precursor ions present in the MS scan using collision-induced dissociation (CID). Electrospray conditions were as follows: capillary temperature, 180 °C; capillary voltage, −1500 V; dry gas (N_2) flow, 4.0 l min^{-1}.

Synthetic phosphorylated β-casein f16−25 at 1 mg mL^{-1} was analyzed by direct infusion in the MS system. The electrospray conditions employed were as follows: capillary voltage, −4500 V; end plate offset voltage, −500 V; nebulizer, 0.6 bar; dry gas, 6.0 l min^{-1}; dry temp, 180 °C. 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.

RESULTS AND DISCUSSION

β-Casein is the major protein in milk, and it accounts for approximately 33% of total caseins.[17] Moreover, in the primary sequence of β-casein the regions corresponding to f1−47 and f90−131 have 14 and 7 theoretical cleavage sites for GE action, respectively. The region corresponding to f132−209 has two theoretical cleavage sites for GE, i.e., Asp (184) and Glu (195). Meanwhile the region of f48−91 has no theoretical cleavage site for GE action. Given the above, it is evident that β-casein is an interesting food protein substrate for investigation of the substrate specificity of GE.

While various temperatures have been employed during protein hydrolysis with Alcalase, it appears that incubation at 50 °C has predominantly been used for milk protein hydrolysis with Alcalase.[3−7] However, Peng et al.[8] hydrolyzed whey protein isolate with Alcalase at 65 °C. Adamson and Reynolds[2] hydrolyzed sodium caseinate with Alcalase at 60 °C. Park and Allen[14] incubated α- and β- caseins with GE at 20 °C, whereas Yokoi et al.[13] incubated α- and β-caseins with GE at 37 °C, and Kakudo et al.[18] used an incubation temperature of 37 °C for characterization of GE activity. It was decided to employ the incubation temperatures used by Kalyankar et al.[9] during hydrolysis of α-casein, i.e., 37 and 50 °C, for the digestion of β-casein with GE.

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 1). It was observed that the intensities of the peaks for the sample incubated for 15 min were lower than those observed in the samples incubated for 30, 60, 120, and 240 min. The greatest number of peaks was 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 was also observed eluting between 60 and 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, and the MASCOT scores following incubation with GE over 240 min are given in Table 1. Peptide fragments corresponding to β-casein were observed only in the β-casein digest. This indicates that the β-casein sample used in the present study was pure. It was also observed that Ser residues at positions 15, 17, 18, 19, and 35 were phosphorylated in β-casein (Table 1).

The β-casein peptide fragments obtained on incubation with GE at 37 °C can be summarized 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 samples 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 samples 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 1).

The GE nonspecific peptide fragments of β-casein detected can be summarized as follows: f32−52 was observed in samples incubated for 30 and 120 min, indicating 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 samples incubated for 240 min, indicating hydrolysis of Thr(128)−Leu(129) (Table 1).

Distinct peaks were also observed in the TIC for the β-casein sample incubated with GE for various time intervals at 50 °C (Figure 2). No overall differences were 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 greater number of peaks was observed between 25 and 40 min, whereas the samples incubated at 50 °C had a greater number

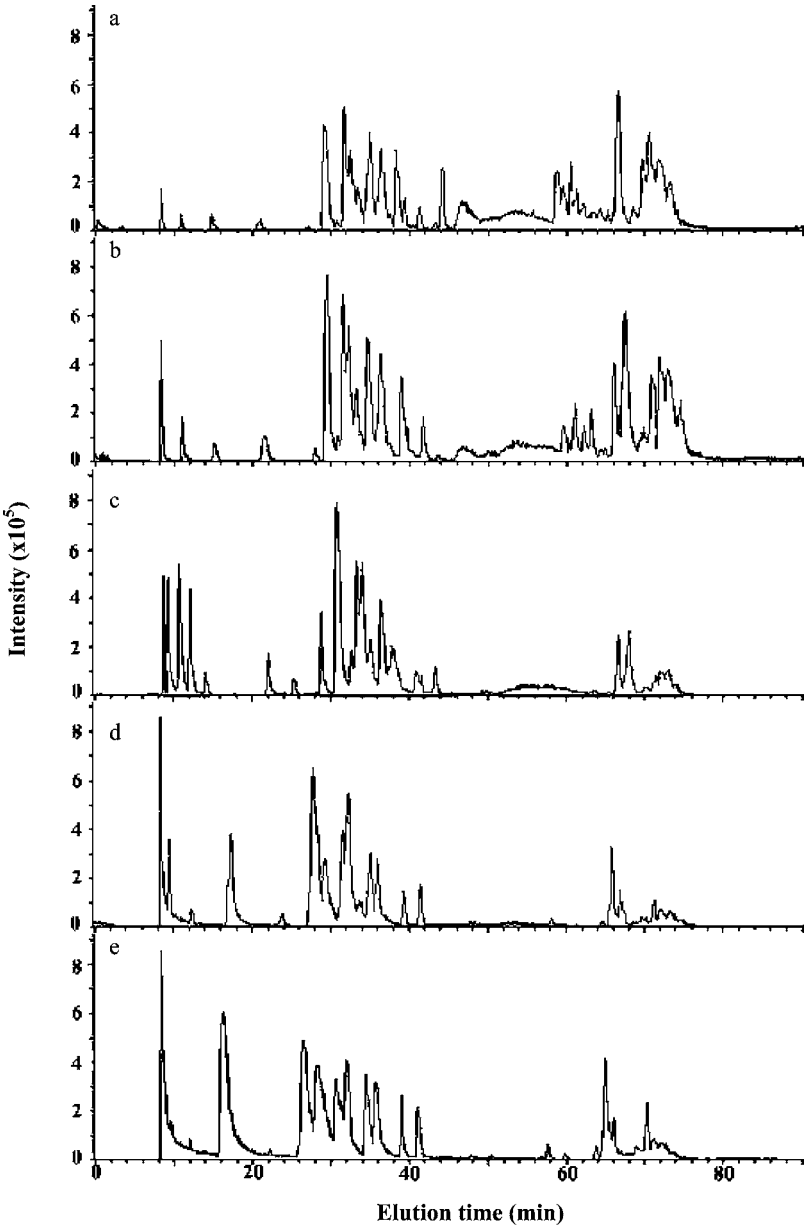

Figure 2. Total ion chromatogram of β-casein incubated with glutamyl endopeptidase at 50 °C for (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min, and (e) 240 min.

of peaks eluting between 25 and 50 min (Figures 1 and 2). In the samples incubated at 50 °C for 15 and 30 min, a cluster of peaks was observed eluting between 60 and 80 min, whereas for incubation at 37 °C these peaks were observed only in the sample incubated for 60 min. The number and intensity of peaks in the samples incubated for 60, 120, and 240 min significantly decreased in the 60−80 min region with increasing incubation time. The peptide sequences, experimental mass, calculated masses of singly or multiply charged ions, and the MASCOT scores following incubation with GE over 240 min at 50 °C are given in Table 2.

The β-casein peptide fragments obtained on incubation at 50 °C with GE can be summarized 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, and 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 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 2).

The GE nonspecific peptide fragments of β-casein detected at 50 °C can be summarized 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 hydrolysis of Leu(192)−Tyr(193) (Table 2).

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Table 2. Peptide Sequences Identified upon Incubation of β-Casein with Glutamyl Endopeptidase for Different Time Intervals at 50 °C[a]

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	642.8135(2)	1283.612	1283.636	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)	2473.207	2473.217	44.78
f122−131	E-SQSLTLTDVE-N	1092.5268(1)	1091.52	1091.535	51.03
Incubation for 30 min					
f1−14	RELEELNVPGEIVE-S	813.4094(2)	1624.804	1624.831	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)	3110.377	3110.423	60.32
f92−100	VMGVSKVKE-A	488.7812(2)	975.5478	975.5423	32
f92−121	E-VMGVSKVKEAMAPKHKEMPFP KYPVEPFTE-S	858.6839(4)	3430.707	3430.749	28.78
f101−121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4003(3)	2473.179	2473.217	51.39
f122−131	E-SQSLTLTDVE-N	1092.5295(1)	1091.522	1091.535	40.13
f185−209	D-M°PIQAFLLYQEPVLGPVRGPFPIIV	1413.8068(3)	2825.599	2825.555	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)	1726.596	1726.621	70.81
f32−47	E-KFQS*EEQQQTEDELQD-K	1031.3985(2)	2060.782	2060.821	30.57
f45−91	E-LQDKIHPFAQTQSLVYPFPGPIHN SLPQNIPPLTQTPVVVPPFLQPE-V	749.393(7)	5238.7	5238.791	22.4
f48−91	D-KIHPFAQTQSLVYPFPGPIHNSLP QNIPPLTQTPVVVPPFLQPE-V	977.5133(5)	4882.53	4882.622	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)	2473.174	2473.217	50.22
f101−121	E-AM°APKHKEMPFPKYPVEPFTE-S	830.7349(3)	2489.183	2489.212	42.66
f101−121	E-AM°APKHKEM°PFPKYPVEPFTE-S	836.0658(3)	2505.176	2505.176	31.45
f122−131	E-SQSLTLTDVE-N	1092.5238(1)	1091.517	1091.535	51.15
f185−209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1414.7571(3)	2827.5	2827.517	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)	1200.698	1200.719	25.02
f32−44	E-KFQS*EEQQQTEDE-L	853.3135(2)	1704.612	1704.615	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	2489.212	71.22
β-casein fragment	P_1−peptide sequence−P_1'	ion selected for MSMS (charge)	experimental mass	calculated mass	MASCOT score
Incubation for 120 min.					
f101−128	E-AM°APKHKEMPFPKYPVEPFTESQSLTLT-D	825.9147(4)	3299.63	3299.565	61.64
f122−131	E-SQSLTLTDVE-N	1092.5239(1)	1091.517	1091.535	51.02
f185−209	D-MPIQAFLLYQEPVLGPVRGPFPIIV	1413.8182(3)	2825.622	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)	1200.713	1200.719	27.05
f32−44	E-KFQS*EEQQQTEDE-L	853.3201(2)	1704.626	1704.615	44.58
f45−91	E-LQDKIHPFAQTQSLVYPFPGPIPNS LPQNIPPLTQTPVVVPPFLQPE-V	1040.7535(5)	5198.731	5198.785	48.71
f48−91	D-KIHPFAQTQSLVYPFPGPIPNSLP QNIPPLTQTPVVVPPFLQPE-V	1211.6489(4)	4842.567	4842.615	40.35
f92−100	E-VMGVSKVKE-A	488.7723(2)	975.53	975.5423	48.18
f101−121	E-AMAPKHKEMPFPKYPVEPFTE-S	825.4064(3)	2473.197	2473.217	41.57
f101−121	E-AM°APKHKEMPFPKYPVEPFTE-S	832.0809(3)	2493.221	2493.221	61.54
f101−121	E-AM°APKHKEM°PFPKYPVEPFTE-S	836.0711(3)	2505.192	2505.207	32.07
f109−121	E-M°PFPKYPVEPFTE-S	799.3791(2)	1596.744	1596.753	43.41

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Table 2. continued

β-casein fragment	P_1−peptide sequence−P_1'	ion selected for MSMS (charge)	experimental mass	calculated mass	MASCOT score
Incubation for 240 min					
f122−129	E-SQSLTLTD-V	864.4234(1)	863.4161	863.4236	33.95
f122−131	E-SQSLTLTDVE-N	1092.5281(1)	1091.521	1091.535	51.22
f193−209	L-YQEPVLGPVRGPFPIIV	941.0256(2)	1880.037	1880.056	45.01

[a]The amino acid residues present at P_1 and P_1' are given. S*: phosphorylated serine. M°: oxidised methionine.

Interestingly, from Table 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 different peptides obtained upon incubation of β-casein with GE at 50 °C (Table 2).

Theoretically, 23 different GE specific cleavage sites may exist in β-casein. It was observed that GE was highly specific at both 37 and 50 °C, predominantly hydrolyzing peptides on the carboxyl terminal of Glu and Asp residues (Table 3 and Figure 3). However, some of the theoretically expected cleavage sites were not hydrolyzed. Hydrolysis post Glu residues at positions 2, 21, 36, 117, and 195 along with the Asp residue at position 43 did not appear to occur on incubation at 37 °C. Furthermore, on incubation at 50 °C, hydrolysis post Glu residues at 2, 20, 21, 36, 117, and 195 along with the Asp residue at position 43 did not appear to occur. The Glu(2)−Leu(3) bond may have been cleaved; however, due to instrumental setup limitations, it was not detected. Interestingly, the Glu(20)−Glu(21) bond appears to be hydrolyzed only in the sample incubated at 37 °C for 15 min. However, no evidence was found of Glu(20)−Glu(21) hydrolysis on incubation at 50 °C. In addition, no evidence was found for Glu(36)−Glu(37) hydrolysis on incubation at either 37 or 50 °C. Interestingly, Glu(4)−Glu(5) appeared to be hydrolyzed after 60, 120, and 240 min on incubation at both 37 and 50 °C (Tables 1−3 and Figure 3).

Table 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[a]

theoretical cleavage sites (P_1 position)	amino acid residues at P_1−P_1'	cleavage observed at 37 °C	cleavage 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

[a]The amino acid residues present at P_1−P_1' are also given.

Figure 3. 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 at both 37 and 50 °C. Arrows represent the theoretical cleavage sites, residues in bold represent the cleavages observed at both 37 and 50 °C, the italic residues represent the cleavages observed only at 37 °C, and the underlined residues represent the cleavage observed only at 50 °C.

Park and Allen[14] reported that Glu residues present at positions 11, 14, 20, 91, and 121 were cleaved upon recirculation of β-casein over beads containing immobilized GE at 20 °C for 3 h. The results presented herein are in agreement with those reported by Park and Allen.[14] In addition to the cleavages observed by Park and Allen,[14] it was also found that Glu residues present at positions 4, 5, 31, 42, 44, 100, and 131 were hydrolyzed in samples incubated at 37 and 50 °C in the present study. However, the Glu residue present at position 20 did not appear to be hydrolyzed in any of the samples incubated at 50 °C, whereas surprisingly the Glu(20)−Glu(21) bond appeared to be hydrolyzed only in the sample incubated for 15 min at 37 °C. Yokoi et al.[13] reported that cleavage occurred post Glu residues present at positions 91, 100, and 121 upon digestion of β-casein with GE at 37 °C for 1 and 3 h. Park and Allen[14] also reported that cleavage post Asp residues present at positions 47, 129, and 184 occurred upon recirculation of β-casein on immobilized GE beads at 20 °C for 3 h. In the present study, it was observed that hydrolysis post Asp residues present at positions 43, 47, and 184 occurred 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 cleavages post Asp residues by GE was reported by Yokoi et al.[13]

As previously mentioned, Breddam and Meldal[11] reported that the rate of hydrolysis of Glu−Asp was slow and the Glu−Pro bond was either not hydrolyzed or hydrolyzed to a low extent. The results herein indicate that the Glu−Pro bond (117−118 and 195−196 in β-casein) was not hydrolyzed. It was also observed that the Glu(108)−Met(109) bond was only hydrolyzed 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 Asp−Met (184−185) was readily hydrolyzed 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 1 and 2). These results indicate that Glu−Met was hydrolyzed to a lower extent in comparison to Asp−Met.

There were also some nonspecific cleavages observed at the carboxyl terminal of Phe (52), Thr(128), and Gln(188) on incubation at 37 °C, and nonspecific cleavages at the carboxyl terminal of Thr(128) and Leu(192) were observed on incubation at 50 °C. Breddam and Svensden[12] and Kalyankar et al.[9] have previously reported nonspecific cleavage at the carboxyl terminal of Phe. The hydrolysis of Leu (192)−Tyr (193) may be due to low levels of residual chymosin activity. Chymosin primarily cleaves the Phe−Met (105−106) bond in κ-casein; however, it may also hydrolyze 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 released following the action of chymosin on β-casein.[19]

Hydrolysis of β-casein with GE should yield a number of short peptide sequences. In the present study only one 6-mer fragment of β-casein (f6−11) was detected on incubation at both 37 and 50 °C (Tables 1 and 2). However, f15−20, which contains the tetraphosphorylated peptide pSLpSpSpSE was not detected in either the 37 or 50 °C incubated samples. Park and Allen[14] observed release of 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 dipeptides (f1−2 (RE) and f3−4 (LE)), one tripeptide (f12−14, (IVE)), one tetrapeptide (f118−121 (PFTE)), and two pentapeptides (f32−36 (KFQSE) and f38−42 (QQQTE), Table 3 and Figure 3). Under the main detection method (MASCOT search engine) employed in this study none of these peptides were observed. However, since the Glu−Pro bond (195−196 and 117−118) was not hydrolyzed, the tetrapeptide f118−121 (PFTE) was unlikely to be present. A manual search of the MS spectra was carried out in order to detect these low molecular mass peptides. During this manual detection, RELEE, f(1−5), was observed only in the sample incubated for 120 min at 37 °C. Meanwhile, this peptide was observed in the 60 and 120 min samples incubated at 50 °C.

From Table 4, it can be observed that the mean peptide sequence coverage values for 37 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 and 50 °C was observed. This may be due to the fact that the MASCOT scorecut 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 1). Both f45−91 and f48−91 were observed only in the sample incubated at 50 °C for 60 and 240 min (Table 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 that are generally considered to be hydrophobic.[20] Therefore, the low mean percentage peptide sequence coverage values obtained need to be considered with these facts in mind.

Table 4. 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

In order to obtain further insight into the substrate specificity of GE, five synthetic peptides corresponding to specific sequences of β-casein were incubated with GE. The synthetic phosphorylated 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 4a) during LC-MS analysis. A direct infusion was also performed to try to detect this peptide. 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 occurs only following direct infusion at high concentration. The fragmentation information following direct infusion of phosphorylated β-casein f16−26 is given in Figure 5a. Using this LC-MS injection approach, a GE digestion fragment, SITRI (f22−26), was detected in the phosphorylated peptide sample incubated with GE (Figure 4b). The fragmentation information of f22−26 is given in Figure 5b.

The detection of β-casein f22−26 indicates that GE hydrolyzed Glu(21)−Ser(22). Furthermore, this result also indicates that the Glu(20)−Glu(21) bond was not hydrolyzed 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 nonphosphorylated peptide corresponding to f16−26 was incubated with GE at 37 °C. The synthetic nonphosphorylated peptide LSSSEESITRI corresponding to β-casein f16−26 was detected in the control sample, i.e., without GE following LC-MS analysis (Figure 4c). The fragment information of this peptide is given in Figure 5c. In the sample incubated with GE at 37 °C for 120 min, fragments SITRI and LSSSEE were detected (Figure 4d). The fragment information for LSSSEE is given in Figure 5d. The detection of SITRI (f22−26) and LSSEE (f16−21) again indicates that GE hydrolyzed Glu−Ser. Therefore, it can be stated that in Glu−Glu−Ser GE hydrolyses Glu−Ser. It can also be concluded that the presence of multiple phosphorylated Ser residues upstream (toward the N-terminus) of the peptide did not alter the ability of GE to hydrolyze Glu−Ser. Furthermore,

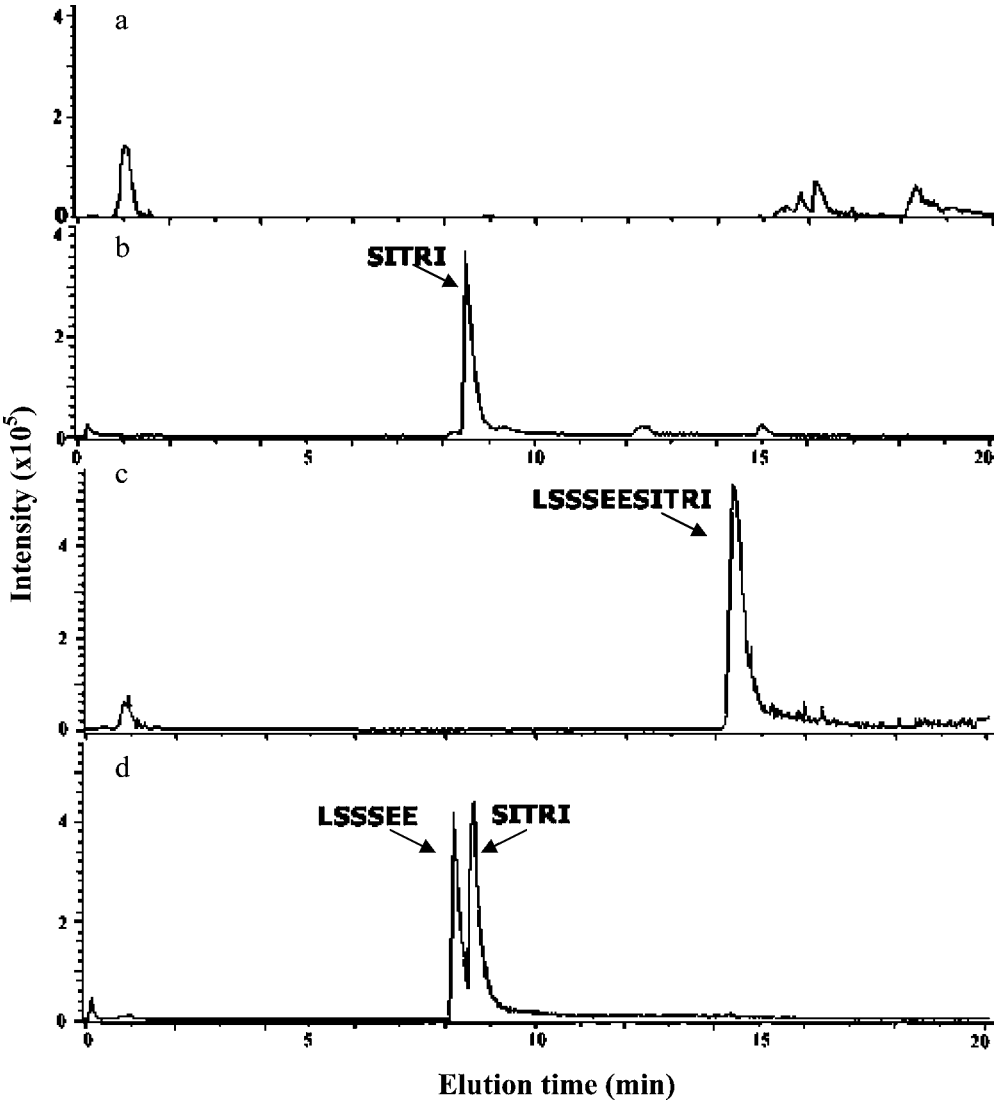

Figure 4. Total ion chromatogram of peptide (a) LS*S*S*EESITRI (control), (b) LS*S*S*EESITRI, (c) LSSSEESITRI (control), and (d) LSSSEESITRI incubated with glutamyl endopeptidase at 37 °C for 120 min. The peptide sequences present in the respective peaks are shown. *: phosphorylated serine residue.

the LC-MS results obtained for both phosphorylated and nonphosphorylated β-casein f16−26 indicate that neither the phosphorylated β-casein f16−26 nor the phosphorylated cluster sequence, i.e., β-casein f16−20/21, was detected with the LC-MS method used.

The intensity of the synthetic β-casein f115−125 (PVEPF-TESQSL) was low in the LC-MS detection method used (Figure 6a). However, GE digestion fragments SQSL, PFTE, and PVEPFTE were observed in the sample incubated at 37 °C for 120 min (Figure 6b). The detection of these peptides indicates that GE hydrolyzed the Glu(117)−Pro(118) and Glu(121)−Ser(122) bonds. However, hydrolysis of Glu−Pro was not observed during incubation of β-casein with GE. The detection method used herein did not allow performance of 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 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[11] and Kalyankar et al.[9]

The intensity of synthetic β-casein f102−112 (MAPKH-KEMPFP) was also very low under the detection method employed (Figure 6c). However, in the sample incubated with GE at 37 °C two peptide sequences, MAPKHKEMPFP and MPFP, were detected (Figure 6d). However, again the intensity of MPFP was very low. These results indicate that Glu−Met was hydrolyzed by GE. This observation was also made during analysis of the results from the β-casein hydrolysis experiments. 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.

Synthetic β-casein KHKEMPFPKYPVEPF was not detected either in the 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 6e and f). A very low intensity peptide corresponding to KHKEMPFPKYPVE was detected in the

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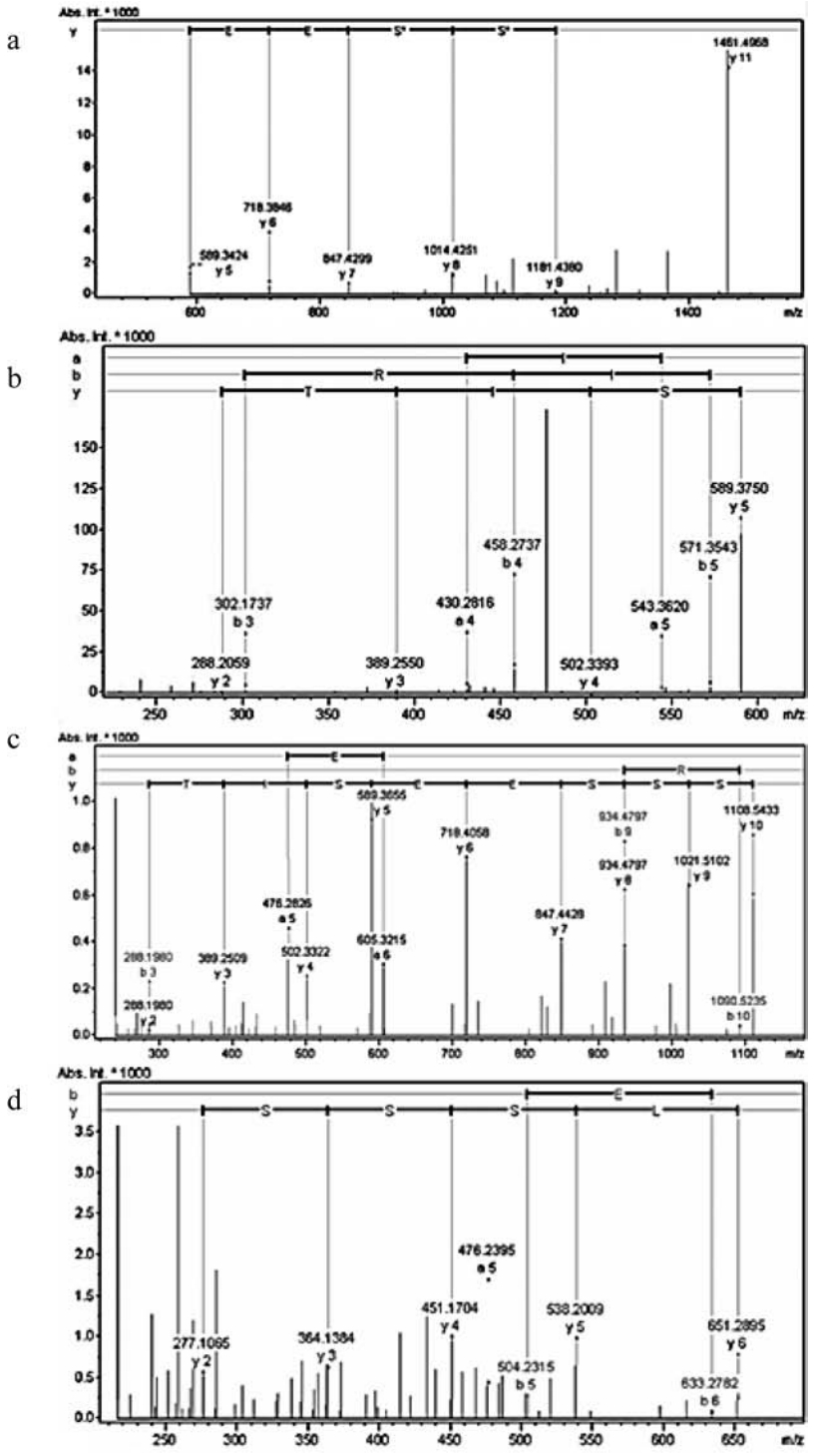

Figure 5. Fragmentation information of β-casein (a) f16−26 (LS*S*S*EESITRI), (b) f22−26 (SITRI), (c) f16−26 (LSSSEESITRI), and (d) f16−21 (LSSSEE). *: phosphorylated serine residue.

sample incubated with GE at 37 °C (Figure 6f). The detection of KHKEMPFPKYPVE again indicates that Glu−Pro was hydrolyzed. These results also suggest that Glu−Met was not hydrolyzed. 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 hydrolyzed sequences of synthetic peptides of β-casein analyzed using MS are summarized in Table 5.

From the results, it can be said that GE predominantly hydrolyzed peptide bonds on the carboxyl terminal of Glu residues present in β-casein and five synthetic peptides corresponding to specific sequences within β-casein. Furthermore, GE also hydrolyzed peptide bonds at the carboxyl terminal of Asp residues in β-casein. It was also observed that GE was nonspecific with respect to the nature of the amino acid residue present at the P_1' position. However, hydrolysis of the Glu−Pro bond was not observed upon incubation of β-casein

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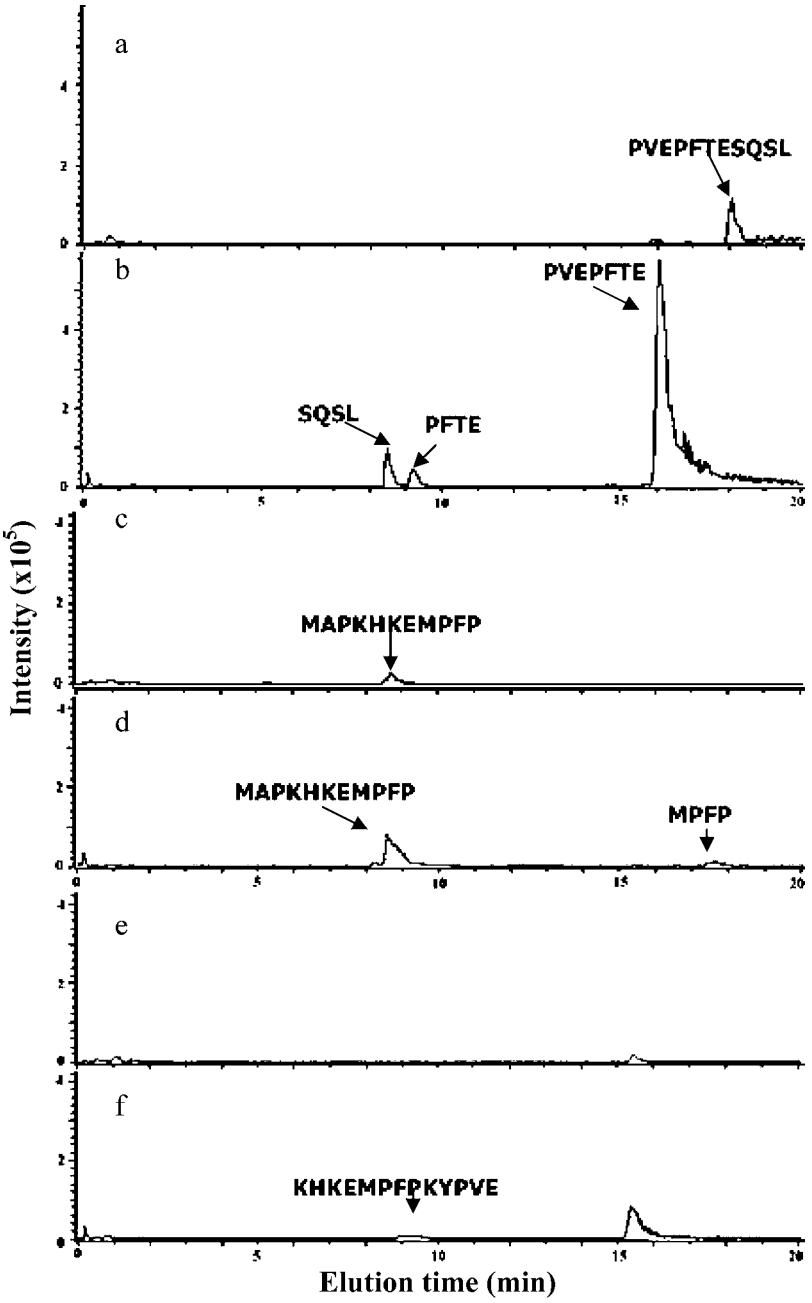

Figure 6. Total ion chromatogram of peptide (a) PVEPFTESQSL (control) and (b) PVEPFTESQSL, (c) MAPKHKEMPFP (control), (d) MAPKHKEMPFP, (e) KHKEMPFPKYPVEPF (control), and (f) KHKEMPFPKYPVEPF incubated with glutamyl endopeptidase at 37 °C for 120 min. The peptide sequences present in the respective peaks are shown.

with GE, suggesting that a Pro residue at the P_1' position was not preferred by GE. Interestingly, the results from synthetic peptide studies suggest that the Glu−Pro bond was hydrolyzed by GE. On the basis of the above observations, it can be stated that a Pro residue at the P_1' position was not preferred or poorly preferred by GE.

Breddam and Meldal[11] reported that hydrolysis of the Glu−Asp bond was slow. However, during the hydrolysis of β-casein with GE, it was observed that the Glu−Asp bond appeared to be hydrolyzed in samples incubated at 37 °C after 15, 30, 60, and 120 min (Table 1). Surprisingly however, on incubation at 50 °C, hydrolysis of the Glu−Asp bond was observed only in the sample incubated for 60 min (Table 2).

The results from the hydrolysis of β-casein with GE also suggest that Glu−Met was hydrolyzed to a lesser 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 observed only after 240 min incubation at both 37 and 50 °C, indicative of a less favored cleavage site (Tables 1 and 2). The results obtained during the incubation of MAPKHKEMPFP, β-casein f(102−112), indicate that the Glu−Met bond was hydrolyzed. To 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.

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Table 5. Summary Details of the Synthetic Peptide Sequences (of β-Casein Primary Sequence) along with Their Measured and Calculated Masses Detected Using Mass Spectrometry[a]

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

[a]p: phosphorylated. nd: not detected.

The results obtained for the incubation of the synthetic nonphosphorylated and phoshorylated β-casein f(16−26) confirm that in Glu−Glu−Ser-containing sequences, GE hydrolyzes the Glu−Ser bond, suggesting that Glu at the P_1' position was not preferred by GE. However, the results from hydrolysis of β-casein with GE suggest that a Glu residue at the P_1' position was poorly preferred by GE. This observation was also made by Kalyankar et al.[9] when studying GE hydrolysates of α-casein. The results with synthetic nonphosphorylated and phoshorylated β-casein f(16−26) confirm that multiple phosphorylated residues near the scissile bond do not appear to affect the cleavage capability of GE.

In conclusion, detailed novel information in relation to the hydrolysis of β-casein with GE has been elucidated. It is evident that a quantitative LC-MS method is required in order to fully study the extent of hydrolysis of peptide bonds by GE when Pro, Met, and Glu residues are present at the P_1' position. The results herein provide information on GE hydrolysis of β-casein that may be relevant in the generation of casein hydrolysates with different techno- and biofunctional properties.

AUTHOR INFORMATION

Corresponding Author

*E-mail: dick.fitzgerald@ul.ie. Tel: +353 61 2025989.

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Notes

The authors declare no competing financial interest.

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