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Proteolytic specificity of elastase on bovine α_{s1}-casein

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Abstract

Proteases from polymorphonuclear leukocytes (PMN or neutrophils) and macrophages, the main somatic cells found in milk of healthy cows, may contribute to hydrolysis of caseins at neutral or acid pH in high somatic cell count milks. The objective of this study was to determine the cleavage specificity of elastase, one of the principal PMN proteinases, on α_{s1}-casein. α_{s1}-Casein (5 mg ml^{-1}) was dissolved in phosphate buffer, pH 7.5, and elastase added. Samples were taken over a 24 h period and analyzed by urea polyacrylamide gel electrophoresis and high performance liquid chromatography. Twenty-five cleavage sites were identified showing that elastase had a broad cleavage specificity on α_{s1}-casein.

Keywords: Elastase; α_{s1}-Casein; Specificity; Somatic cells

1. Introduction

There are three main types of somatic cells found in the milk of healthy cows; lymphocytes, polymorphonuclear granulocytes (PMN cells or neutrophils) and macrophages (O'Sullivan, Joyce, Sloan & Shattock, 1992; Sordillo, Sheafer Weaver & Derosa, 1997). Intramammary infections cause elevation of somatic cell count (SCC) and increased proteolytic activity in milk (deRham & Andrews; 1982; Senyk, Barbano & Shipe, 1985; Verdi, Barbano, Devalle & Senky, 1987) thereby leading to hydrolysis of caseins and a reduction in cheese yield (Ali, Andrews & Cheeseman, 1980; Grandison & Ford, 1986). Proteolytic activity may be sufficiently high in mastitic milk that substantial proteolysis occurs in the udder between milk secretion and milking, and a further few hours of incubation at 37°C may be sufficient for complete hydrolysis of the caseins (Ali et al., 1980). Caseins are degraded by leukocyte proteinases in the following order: α_{s1}- > β- ≫ κ-casein (Grieve & Kitchen, 1985). Comparison of low and high SCC milk samples has shown substantial proteolytic breakdown of α_{s1}-casein by proteinases associated with somatic cells (Verdi et al., 1987).

Elevated SCC have also been linked to poor cheesemaking properties of milk and defects in the resulting cheese, including increased losses in the whey, longer rennet clotting times, decreased curd rigidity, poor flavour and increased moisture in the cheese (deRham & Andrews, 1982; Kelly, Sheehan, Tieran, Shattock, Joyce & Foley, 1995). Many cheese varieties have pH of approximately 5.2 and are ripened for several months to develop proper flavour and texture as a result of enzyme-catalysed proteolytic and lipolytic reactions (Fox, 1993). If proteinases of PMN and macrophages are incorporated in the curd and are active under the low pH conditions of cheese ageing, then the flavour and texture could be altered when cheese is made from high SCC milk (Verdi & Barbano, 1991).

PMN contain a number of proteolytic enzymes including neutral and acidic proteases. Proteinases, including elastase and cathepsin G, are the predominant enzymes in somatic cells in milk produced by cows with mastitis (Azzara & Dimick, 1985; Newbould, 1974). Other proteinases found in PMN include the thiol protease cathepsin B and the acid protease cathepsin D (Baggiolini, Bretz & Dewald, 1978). Thus, based on the presence of these proteases in lysosomes of PMN and macrophages, it would be expected that proteases from PMN and macrophages would contribute to hydrolysis of casein in milk at both neutral and acid pH, particularly milk with high SCC (Verdi & Barbano, 1991).

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Elastase, a serine protease with a molecular weight in the region 25.9 kDa (Lewis, Williams & Brink, 1956), digests a wide variety of protein substrates (Lewis et al., 1956), with a preferred specificity for bonds involving uncharged, non-aromatic amino acids (e.g., Ala, Val, Leu, Ile, Gly, Ser) (Naughton & Sanger, 1961). An inhibitor of bovine elastase (EI) has been identified in milk and it has been suggested that EI might function as an inhibitor of PMN elastase present in milk (Christensen, Wiegers, Hermansen & Sottrup-Jensen, 1995).

The proteolytic specificity of elastase on bovine β-casein was determined by Considine, Healy, Kelly and McSweeney (1999), who showed that this enzyme has a broad cleavage specificity on this protein, with some of the elastase cleavage sites being identical to, or near those cleaved by plasmin, chymosin or cell envelope-associated proteinases of several strains of *Lactococcus*. Most of the cleavages sites were located near the N or C-termini of the molecule.

The objective of this study was to investigate further the potential significance of elastase for proteolysis and quality of milk and dairy products, by determining its cleavage specificity towards bovine α_{s1}-casein.

2. Materials and methods

Whole casein was prepared from a sample of bovine skim milk by the method of Mulvihill and Fox (1974). Casein was fractionated by ion-exchange chromatography on diethylaminoethyl cellulose (DE-52, Sigma, St. Louis, MO, USA) using 10 mM imidazole buffer, pH 7, containing 4.5 M urea and 0.15% (v/v) 2-mercaptoethanol (Creamer, 1974); proteins were eluted using a linear NaCl gradient (0–0.5 M). Fractions containing α_{s1}-casein were pooled, dialysed against water and freeze-dried.

Elastase (E.C. 3.4.21.36; from porcine spleen) containing ~5 units mg^{-1} protein was obtained from the Sigma Chemical Co., St. Louis, MO, USA (1 unit of elastase activity will solubilise 1 mg elastin in 2 min at pH 8.8 and 37°C).

α_{s1}-Casein (5 mg ml^{-1}) was dissolved in 0.1 M phosphate buffer, pH 7.5, containing 0.05% NaN_3. Elastase (1.76×10^{-3} U ml^{-1}) was added and the mixture incubated at 37°C for up to 24 h. Aliquots were taken periodically for analysis by urea-polyacrylamide gel electrophoresis (urea-PAGE) and reverse-phase (RP)-HPLC. Elastase was inactivated in each sample by heating at 100°C for 5 min.

2.1. Urea-PAGE

Samples for urea-PAGE were prepared by the addition of an equal volume of double strength sample buffer (McSweeney, Olson, Fox, Healy & Højrup, 1993a,b). Urea-PAGE was performed according to the method of Andrews (1983) with direct staining using Coomassie Brilliant Blue G250 by the method of Blakesley and Boezi (1977). Peptides which stained on urea-PAGE gels were electroblotted onto polyvinylidenedifluoride membranes (Considine et al., 1999). The electroblots were stored at −18°C until N-terminal sequencing was performed.

2.2. RP-HPLC

RP-HPLC was performed on the 1% trichloroacetic acid (TCA)-soluble fraction of the hydrolyzates. Samples were prepared and RP-HPLC was performed as described by Considine et al. (1999). A Nucleosil $C\hat{8}$, 5 μm, 250×4.6 mm) was used and elution was by means of a gradient formed from solvent A (0.1% trifluoroacetic acid, TFA, in H_2O) and solvent B (0.1% TFA in acetonitrile, Aldrich Chemical Co., Milwaukee, WI, USA) as follows: % B increased from 0 to 30% B at a rate of 1.2% B min^{-1}; from 30 to 50% B at a rate of 0.44% B min^{-1}; 50% B was maintained for 6 minutes and the % B was increased to 95% at a rate of 22.5% B min^{-1}, held at 95% B for 10 min before reduction to 0% B at a rate of 47.5% B min^{-1}. Flow rate was 0.75 ml min^{-1}, detection was at 214 nm and data recorded for 70 min. Peptides were collected manually, freeze-dried and identified by N-terminal sequencing and mass spectrometry.

2.3. Identification of peptides

Peptides were sequenced at the National Food Biotechnology Centre, University College Cork, Ireland and Department of Biochemsitry, Faculty of Medicine and Health Sciences, Queen Medical Centre, University of Nottingham, UK, as described by Considine et al. (1999).

3. Results and discussion

Urea-PAGE electrophoretograms of α_{s1}-casein hydrolysed by elastase are shown in Fig. 1. α_{s1}-Casein was readily degraded by elastase (1.76×10^{-3} units ml^{-1}) with the formation of 3 main peptides with higher electrophoretic mobility. The control α_{s1}-casein, containing no enzyme, showed no degradation after 24 h incubation, indicating the absence of indigenous proteinase activity in the α_{s1}-casein preparation used for this study. Elastase hydrolysed α_{s1}-casein very quickly with degradation products being evident after 5 min of incubation (Fig. 1). The intensity of the peptide bands increased on incubation from 5 min to 12 h and the bands were most intense after 12 h of incubation. The intensity of the bands decreased by 24 h incubation (especially band A) indicating that further hydrolysis of the larger polypep-

tides had taken place. Three peptide bands (A–C, Fig. 1) were isolated by electroblotting and their N-terminal sequence determined, thus allowing identification of some of the primary sites of elastase action on the protein (Table 1). Band B contained 3 peptides and band C contained 2 peptides. The large peptides produced initially by elastase originated from cleavage at Phe_{24}-Val_{25} and Ala_{143}-Tyr_{144} while two peptides, A and B (i), had the same N-terminal sequence as α_{s1}-casein. The C-termini of these large peptides were not determined.

RP-HPLC elution profiles of the 1% TCA-soluble fractions of α_{s1}-casein hydrolysed by elastase for time periods up to 24 h are shown in Fig. 2. The RP-HPLC peptide profiles indicated that 1% TCA-soluble peptides were produced gradually over time during incubation. Fourteen peaks were isolated from the 24 h hydrolysate and identified by sequencing and mass spectrometry (Fig. 3; Table 2). Cleavage sites were identified at Val_{15}-Leu_{16}, Phe_{24}-Val_{25}, Glu_{30}-Val_{31}, Val_{37}-Asn_{38}, Tyr_{104}-Lys_{105}, Pro_{113}-Asn_{114}, Ala_{143}-Tyr_{144}, Glu_{148}-Leu_{149}, Phe_{150}-Arg_{151}, Tyr_{154}-Gln_{155}, Tyr_{159}-Pro_{160}, Ser_{161}-Gly_{162}, Ala_{163}-Trp_{164}, Tyr_{165}-Tyr_{166}, Leu_{169}-Gly_{170}, Thr_{171}-Gln_{172}, Gln_{172}-Tyr_{173}, Tyr_{173}-Thr_{174}, Ala_{176}-Pro_{177}, Phe_{179}-Ser_{180}, Ile_{186}-Gly_{187}, Gly_{187}-Ser_{188}, Thr_{194}-Thr_{195}, Met_{196}-Pro_{197}, Pro_{197}-Leu_{198}. The location of the peptides and elastase cleavage sites on α_{s1}-casein are summarised in Fig. 4.

Most elastase cleavage sites identified in α_{s1}-casein were in the regions f1–40 and 141–199, which are two of the most hydrophobic regions of the molecule (Swaisgood, 1992). In addition, the N and C-terminals may be more exposed and, therefore, more readily hydrolysed.

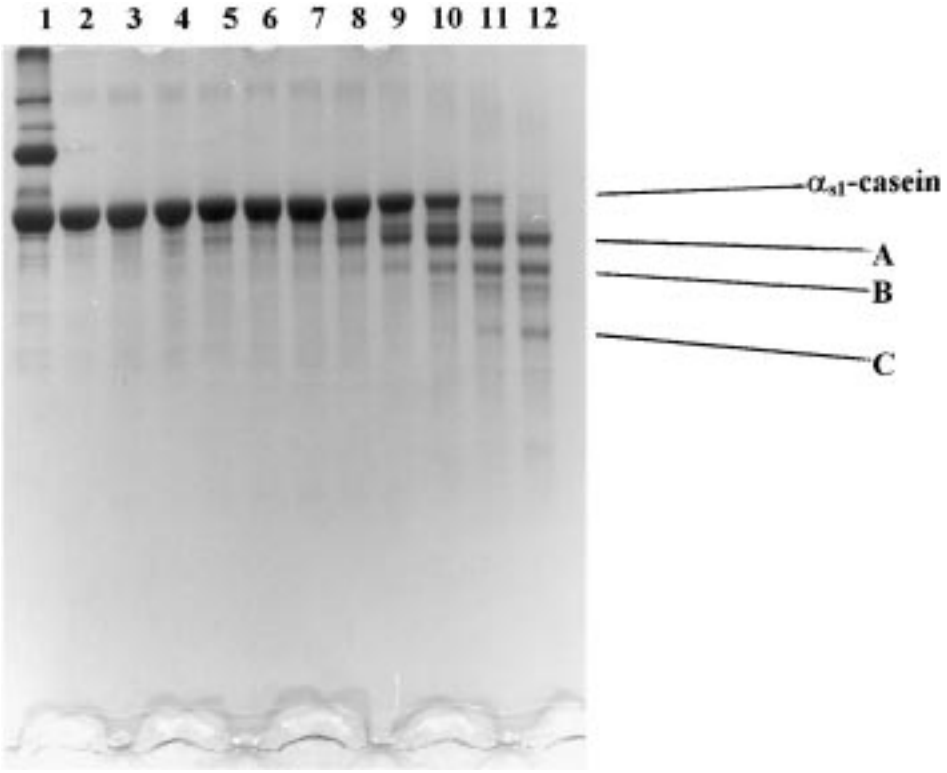

Fig. 1. Urea-polyacrylamide gel electrophoretograms of Na-caseinate (lane 1), and α_{s1}-casein incubated at 37°C in 0.1 M phosphate buffer, pH 7.5, for 0 or 24 h (lanes 2,3) or α_{s1}-casein hydrolysed by elastase (1.76×10^{-3} units ml^{-1}) under the same conditions for 5, 10, 15, 30 min, 1, 3, 6, 12 or 24 h (lanes 4–12).

Table 1
Identity of peptides detected by urea-polyacrylamide gel electrophoresis after hydrolysis of α_{s1}-casein by elastase (see Fig. 1 for location of peptides)

Peptide	N-terminal sequence	Identity	M-terminal cleavage sites
A	Arg-Pro-Lys-His-Pro	α_{s1}-CN fl-[a]	–
B	Val-Ala-Pro-Phe-Pro	α_{s1}-CN f25-[a]	Phe_{24}-Val_{25}
B (i)	Arg-Pro-Lys-His-Pro	α_{s1}-CN fl-[a]	–
C (i)	Tyr-Phe-Tyr-Pro-Glu	α_{s1}-CN fl44-[a]	Ala_{143}-Tyr_{144}
C (ii)	Val-Ala-Pro-Phe-Pro	α_{s1}-CN f25-[a]	Phe_{24}-Val_{25}

[a] Incomplete sequence.

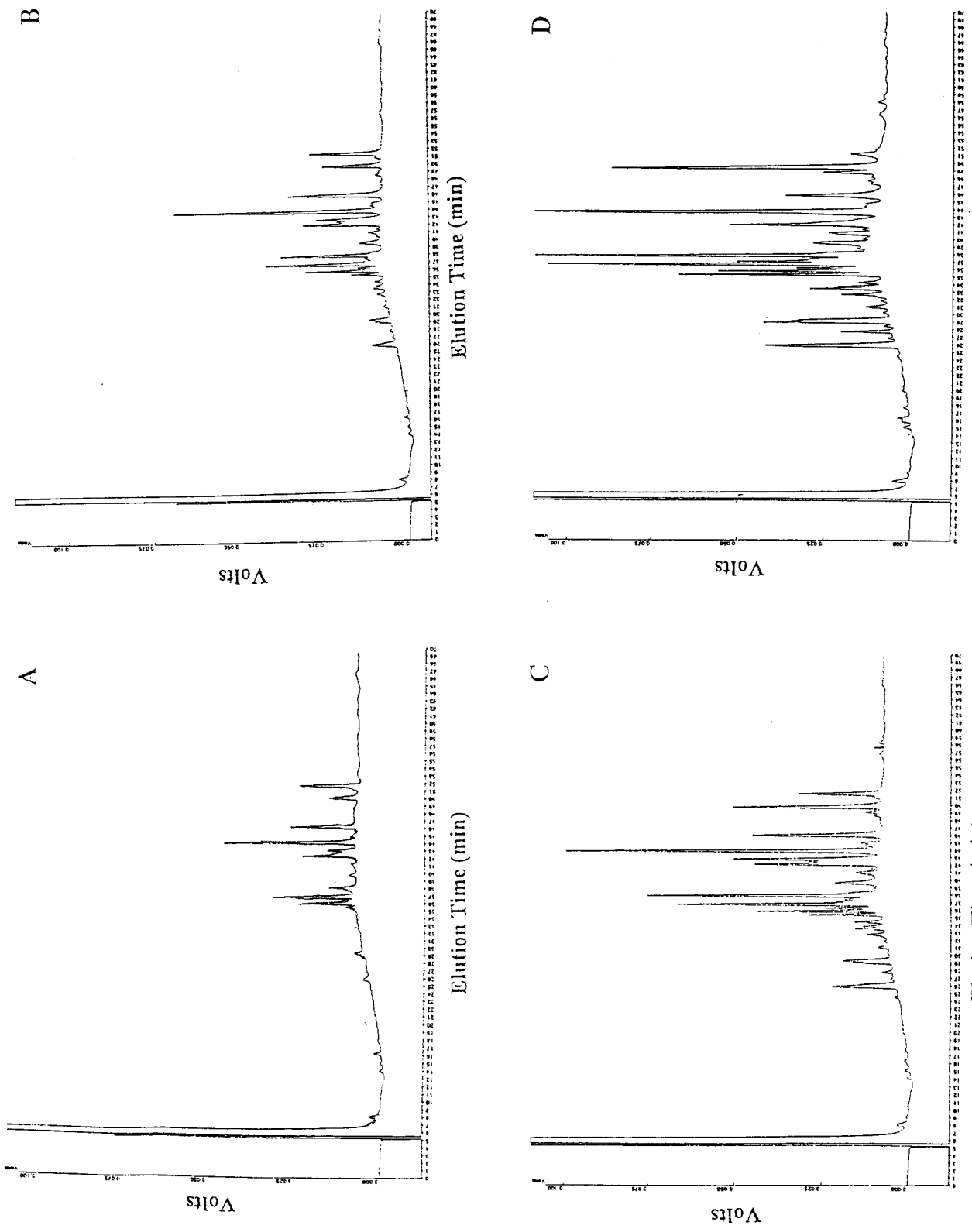

Fig. 2. Reverse-phase HPLC chromatograms of 1% TCA-soluble peptides produced from α_{s1}-casein (5 mg ml^{-1}) by elastase (1.76×10^{-3} units ml^{-1}) incubated in 0.1 M phosphate buffer, pH 7.5 for 3 h (A), 6 h (B), 12 h (C) or 24 h (D).

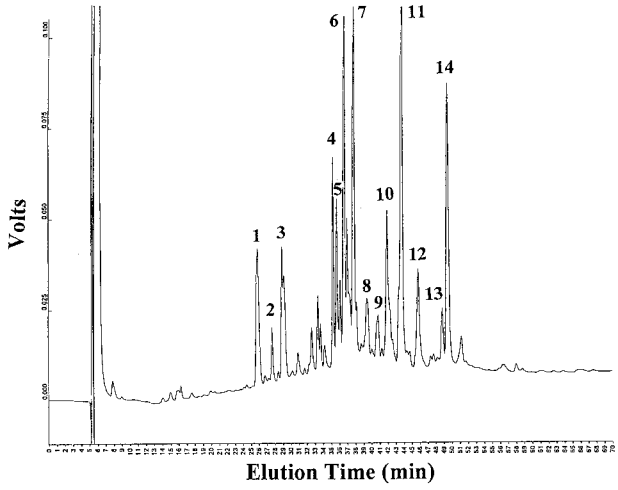

Fig. 3. Reverse-phase HPLC profile of 1% TCA-soluble peptides from bovine α_{s1}-casein (5 mg ml^{-1}) hydrolysed by elastase (1.76×10^{-3} units ml^{-1}) incubated in 0.1 M phosphate buffer, pH 7.5 at 37°C for 24 h. Peptides that were isolated and identified are indicated (1–14).

Considine et al. (1999) showed that elastase also cleaved near the N- and C-terminals of β-casein. Peptides produced from β-casein hydrolysed by elastase (Considine et al., 1999) were much longer than those produced from α_{s1}-casein by elastase at the same concentration. Thus, elastase may be able to cleave α_{s1}-casein more readily, producing peptides which are rapidly cleaved to smaller peptides, or that the structure of α_{s1}-casein contains more potential elastase cleavage sites which would therefore produce a greater number of smaller peptides.

Elastase can attack a wide variety of peptide bonds involving neutral amino acids having aliphatic side chains (Naughton & Sanger, 1961). Considine et al. (in press), who determined the proteolytic specificity of elastase on bovine β-casein, showed that peptide bonds involving non-aromatic amino acids were preferentially cleaved by elastase. The specificity of purified porcine pancreatic elastase on the oxidized B chain of insulin (Narayanan & Anwar 1969; Naughton & Sanger, 1961)

Table 2
Identity of 1% TCA-soluble peptides produced from bovine α_{s1}-casein (5 mg ml^{-1}) by elastase (1.76×10^{-3} units ml^{-1})

HPLC peak No.	Sequence identity	Experimental mass (DA)	Theoretical mass (Da)	Probable peptide identity	N-terminal cleavage site	C-terminal cleavage site
1(i)	H_2N-Gln-Leu-Asp-Ala-Tyr-COOH	611.9	608.65	α_{s1}-CN f155-159	Tyr_{154}-Gln_{155}	Tyr_{159}-Pro_{160}
1 (ii)	H_2N-Tyr-Val-Pro-Leu-Gly-Thr-COOH	611.9	648.76	α_{s1}-CN f166-171	Tyr_{165}-Tyr_{166}	Thr_{171}-Gln_{172}
2	H_2Tyr-Phe-Tyr-Pro-Glu-COOH	666	717.77	α_{s1}-CN f144-148	Ala_{143}-Tyr_{144}	Glu_{148}-Leu_{149}
3	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-COOH	1765.3	1764.06	α_{s1}-CN f1-15	–	Val_{15}-Leu_{16}
4 (i)	H_2N-Thr-Asp-Ala-Pro-Ser-Phe-COOH	635.4	636.66	α_{s1}-CN f1174-179	Tyr_{173}-Thr_{174}	Phe_{179}-Ser_{180}
4 (ii)	H_2N-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-Ser-COOH	1387.2	1387.51	α_{s1}-CN f151-161	Phe_{150}-Arg_{151}	Ser_{161}-Gly_{162}
5	H_2N-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pri-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-COOH	2518.4	2535.71	α_{s1}-CN f174-197	Tyr_{173}-Thr_{174}	Pro_{197}-Leu_{198}
6 (i)	H_2N-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-COOH	1477.3	1479.62	α_{s1}-CN f187-199	Ile_{186}-Gly_{187}	–
6 (ii)	H_2N-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-COOH	1726.3	1721.84	α_{s1}-CN f172-187	Thr_{171}-Gln_{172}	Gly_{187}-Ser_{188}
6 (iii)	H_2N-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-COOH	2882.5	2888.06	α_{s1}-CN f170-196	Leu_{169}-Gly_{170}	Met_{196}-Pro_{197}
7 (i)	H_2N-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-COOH	1445.5	1446.71	α_{s1}-CN f25-37	Phe_{25}-Val_{25}	Val_{37}-Asn_{38}
7 (ii)	H_2N-Tyr-Val-Pro-Leu-Gly-Thr-COOH	645.6	648.76	α_{s1}-CN f166-171	Tyr_{165}-Try_{166}	Thr_{171}-Gln_{172}
7 (iii)	H_2N-Thr-Met-Pro-Leu-Trp-COOH	645.6	561.69	α_{s1}-CN f195-199	Thr_{194}-Thr_{195}	–
8 (i)	H_2N-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-COOH	997.3	1041	α_{s1}-CN f164-172	Ala_{163}-Trp_{164}	Gln_{172}-Tyr_{173}
8 (ii)	H_2N-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-COOH	997.3	1022.25	α_{s1}-CN f105-113	Tyr_{104}-Lys_{105}	Pro_{113}-Asn_{114}
9	H_2N-Val-Ala-Pro-Phe-Pro-Glu-COOH	667.2	658.72	α_{s1}-CN f25-30	Phe_{24}-Val_{25}	Glu_{30}-Val_{31}
10	H_2N-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-COOH	2762.3	2749.98	α_{s1}-CN f174-199	Tyr_{173}-Thr_{174}	–
11	H_2N-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-COOH	978.4	978.11	α_{s1}-CN f144-150	Ala_{143}-Tyr_{144}	Phe_{150}-Arg_{151}
12 (i)	H_2N-Leu-Asn-Glu-Asn-Leu-Leu-Arg-Phe-Phe-COOH	1200.4	1165	α_{s1}-CN f16-24	Val_{15}-Leu_{16}	Phe_{24}-Val_{25}
12 (ii)	H_2N-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-COOH	1200.4	1227.33	α_{s1}-CN f166-176	Tyr_{165}-Tyr_{166}	Ala_{176}-Pro_{177}
13	H_2N-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-COOH	1610.9	1572.78	α_{s1}-CN f144-154	Ala_{143}-Try_{144}	Tyr_{154}-Gln_{155}
14	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-Leu-Arg-Phe-Phe-COOH	2910.2	2911.4	α_{s1}-CN f1-24	–	Phe_{24}-Val_{25}

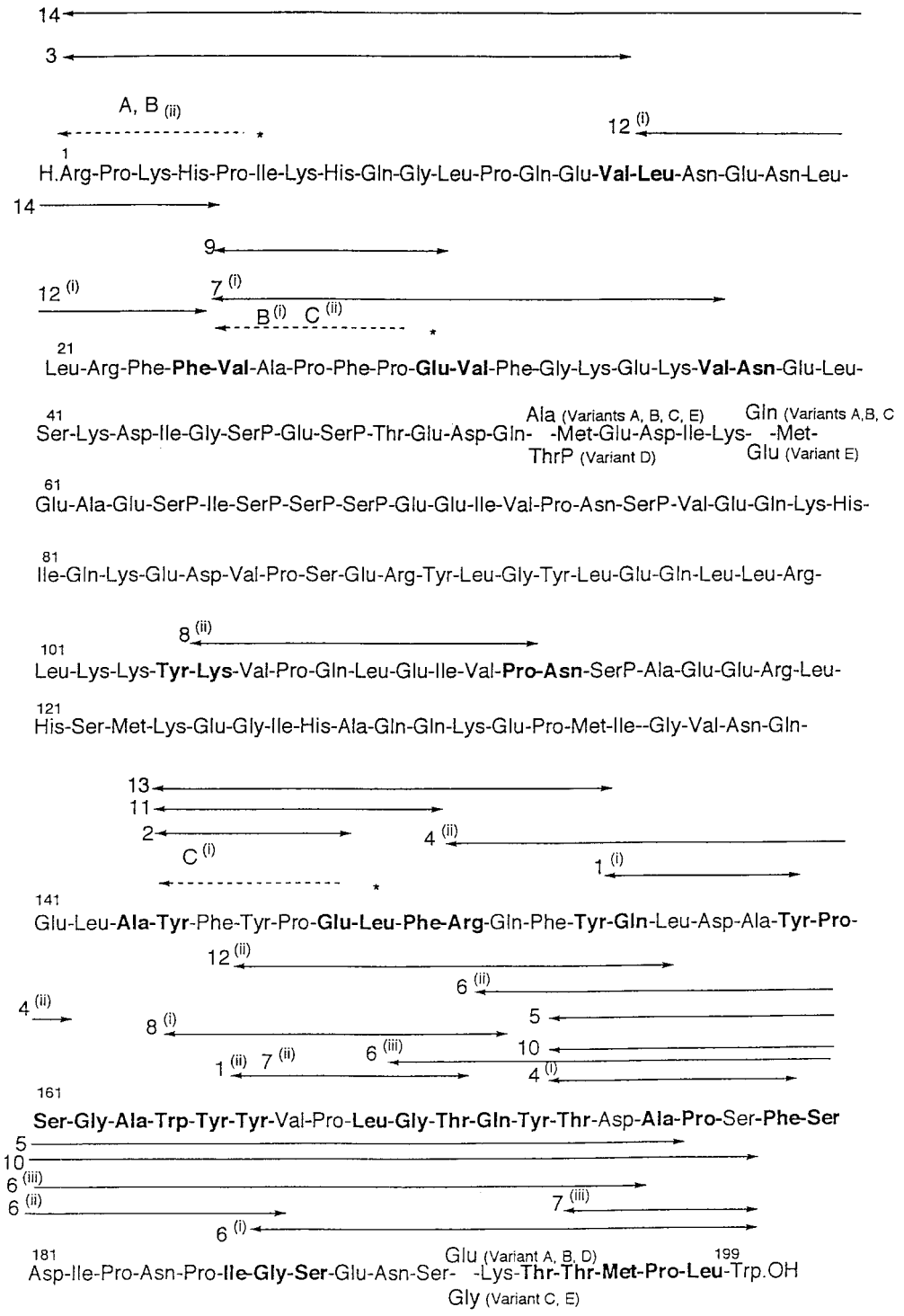

Fig. 4. Primary structure of bovine α_{s1}-casein (Swaisgood, 1992) showing the position of the peptides produced on hydrolysis with elastase at pH 7.5. Cleavage sites are indicated in bold. Peptides identified by urea-polyacrylamide gel electrophoresis (Table 1) are indicated as — — — — and peptides isolated by reverse-phase-HPLC (Table 2) as ———— (* Incomplete sequence).

and the oxidized A-chain of insulin (Naughton & Sanger, 1961) have been determined. These authors found that the peptide bonds, containing Tyr, Ala, Leu, Val, Cys, Gly, Phe, Ser and His, were most suceptible to hydrolysis. Similar amino acid residues were very prevalent among the peptide bonds cleaved by elastase in α_{s1}-casein. In this study these amino acids were also present in the bonds cleaved rapidly by elastase. Elastase from human neutrophils (Blow, 1977), porcine pancreas (Narayanan & Anwar, 1969) and horse (Dubin, Potempa & Travis, 1994) preferentially attack peptide bonds with small aliphatic amino acids (Val and Ala). Bonds containing

Ala were cleaved more frequently by elastase than Val in β-casein (Considine et al., 1999), whereas, in α_{s1}-casein bonds containing Val were cleaved more frequently.

Elastase cleaved α_{s1}-casein at several sites, including Tyr_{159}-Pro_{160}, Phe_{179}-Ser_{180}, Phe_{24}-Val_{25}, Glu_{148}-Leu_{149}, Phe_{150}-Arg_{151}, Tyr_{154}-Gln_{155}, Tyr_{165}-Tyr_{166}, Ala_{163}-Trp_{164}, Tyr_{166}-Val_{167}, Val_{15}-Leu_{16}, Glu_{30}-Val_{31}, Tyr_{104}-Lys_{105} and Ala_{143}-Tyr_{144}, which are close to or identical to those of chymosin on α_{s1}-casein [Tyr_{159}-Pro_{160}, Phe_{23}-Phe_{24}, Leu_{149}-Phe_{150}, Trp_{164}-Tyr_{165}, Leu_{11}-Pro_{12}, Phe_{32}-Gly_{33}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143}, (McSweeney et al. 1993a,b)].

Elastase also cleaved α_{s1}-casein at Phe_{24}-Val_{25}, Tyr_{104}-Lys_{105}, Phe_{150}-Arg_{151}, Tyr_{154}-Gln_{155} which are close to plasmin cleavage sites [Arg_{22}-Phe_{23}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Arg_{151}-Gln_{152}; (McSweeney et al., 1993b)]. Since the cleavage sites of elastase on α_{s1}-casein are very similar to those of plasmin it is possible that elastase may produce "plasmin-like" peptides by hydrolysis of the caseins, especially in milks with very high somatic cell count.

Elastase also cleaved α_{s1}-casein at Val_{15}-Leu_{16}, Phe_{24}-Val_{25}, Glu_{148}-Leu_{149}, Ala_{163}-Trp_{164}, Thr_{171}-Gln_{172}, Pro_{197}-Leu_{198}, which are close to sites hydrolysed by cell-envelope-associated proteinases (CEP) of *Lactococus lactis* subsp *lactis* NCDO 763 (Glu_{148}-Leu_{149}, Leu_{156}-Asp_{157}, Ser_{161}-Gly_{162}, Leu_{169}-Gly_{170}, Ser_{191}-Glu_{192}; Monnet, Ley & Gonzàlez, 1992), *L. lactis* subsp. *cremoris* AM_2 (Leu_{21}-Arg_{22}; Exterkate, Alting & Bruinenberg, 1993) and *L. lactis* subsp. *cremoris* FD_{27} [Gln_{16}-Glu_{14} and Leu_{16}-Asn_{17} (Exterkate et al., 1993)].

The order in which the 1% TCA-soluble peptides were produced from α_{s1}-casein is shown in Fig. 5. The peptide α_{s1}-CN f144-150 was produced most rapidly, suggesting the bonds Ala_{143}-Tyr_{144} and Phe_{150}-Arg_{151} were very susceptible to hydrolysis. The peptides α_{s1}-CN f187-199, α_{s1}-CN f172-187, α_{s1}-CN f170-196 were liberated next, suggesting that bonds Leu_{169}-Gly_{170}, Thr_{171}-Gln_{172}, Ile_{186}-Gly_{187}, Gly_{187}-Ser_{188} and Met_{196}-Pro_{197} were also hydrolysed rapidly.

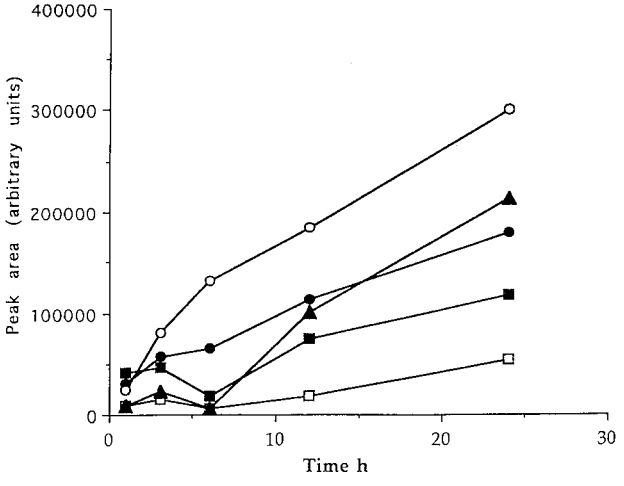

Fig. 5. Time course of changes in the area of the HPLC peaks representing the major peptides produced on hydrolysis of bovine α_{s1}-casein by elastase at 37°C in 0.1 M phosphate buffer, pH 7.5: ■, f155–159, f166–171; □, f1–15; ●, f170–196, f172–187, f187–199; ○, f144–150; ▲, f1–23. Experimental details are given in the text.

These cleavage sites are located towards the C-terminus of α_{s1}-casein, suggesting that this portion of the molecule was easily degraded by elastase. The peptide α_{s1}-CN f1-24 was not produced until after 6 h of incubation, indicating that the bond Phe_{24}-Val_{25} was cleaved more slowly. The area of peak 1 increased, decreased and then began to increase after 6 h incubation, perhaps because this peak contained 2 peptides, α_{s1}-CN f155-159 and α_{s1}-CN f166-171, which may be produced at different rates. Peptide α_{s1}-CN f1-15 was produced most slowly and did not reach as high a concentration as the other peptides.

Elastase had a broad cleavage specificity towards α_{s1}-casein, as indicated by the range of cleavage sites that have been identified. Elastase cleaves α_{s1}-casein at at least 25 sites, principally towards the C- and N-terminus of the molecule. Indigenous elastase in milk may thus be of significance to the proteolysis of milk proteins in dairy products.

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References

Ali, A. E., Andrews, A. T., & Cheeseman, G. C. (1980). Influence of elevated somatic cell count on casein distribution and cheese-making. *Journal of Dairy Research, 47*, 393–400.

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research, 50*, 45–55.

Azzara, C. D., & Dimick, P. S. (1985). Lipoprotein lipase activity of milk from cows with prolonged subclinical mastitis. *Journal of Dairy Science, 68*, 3171–3175.

Baggiolini, M., Bretz, U., & Dewald, B. (1978). Subcellular localization of granulocyte enzymes. In K. Havemann, & A. Janoff, *Neutral proteases of human polymorphonuclear leucocytes* (pp. 3–17). Baltimore, MD: Urban and Schwarzenburg Inc.

Blakesley, R. W., & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Blue G250. *Analytical Biochemistry, 82*, 580–585.

Blow, A. M. J. (1977). Action of human lysosomal elastase on the oxidised B chain of insulin. *Biochemical Journal, 161*, 13–16.

Christensen, S., Wiegers, T., Hermansen, J., & Sottrup-Jensen, L. (1995). Plasma-derived proteinase inhibitors in bovine milk. *International Dairy Journal, 5*, 439–449.

Considine, T., Healy, A., Kelly, A. L., & McSweeney, P. L. H. (1999). Proteolytic specificity of elastase on bovine β-casein. *Food Chemistry, 66*, 463–470.

Creamer, L. K. (1974). Preparation of α_{s1}-casein A. *Journal of Dairy Science, 57*, 341–344.

deRham, O., & Andrews, A. T. (1982). Qualitative and quantitative determination of proteolysis in mastitic milk. *Journal of Dairy Research, 49*, 587–596.

Dubin, A., Potempa, J., & Travis, J. (1994). Structural and functional characterisation of elastase from horse neutrophils. *Biochemical Journal, 300*, 401–406.

Exterkate, F. A., Alting, A. C., & Bruinenberg, P. G. (1993). Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. *Applied and Environmental Microbiology, 59*, 3640–3647.

Fox, P. F. (1993). Cheese: an overview. In P. F. Fox, *Cheese: chemistry, physics and microbiology* (Vol. 1, 2nd ed. pp. 1–36). London: Chapman and Hall.

Grandison, A. S., & Ford, G. D. (1986). Effects of variations in somatic cell count on the rennet coagulation properties of milk and on the yield, composition and quality of Cheddar cheese. *Journal of Dairy Research, 53*, 645–655.

Grieve, A., & Kitchen, B. J. (1985). Proteolysis in milk: the significance of proteinases from milk leucocytes and a comparison of the action of leucocytes, bacterial and natural milk proteinases on caseins. *Journal of Dairy Research, 52*, 101–112.

Kelly, A. L., Sheehan, J., Tiernan, D., Shattock, A., Joyce, P., & Foley, J. (1995). Bovine milk PMN content and its effects in cheese quality. Proceedings of the 3rd IDF Mastitis Symposium, Tel Aviv. 29 May–1 June.

Lewis, U. J., Williams, D. E., & Brink, N. G. (1956). Pancreatic elastase: purification, properties and function. *Journal of Biological Chemistry, 222*, 705–720.

McSweeney, P. L. H., Olson, N. F., Fox, P. F., Healy, A., & Højrup, P. (1993a). Proteolytic specificity of chymosin on bovine α_{s1}-casein. *Journal of Dairy Research, 60*, 401–412.

McSweeney, P. L. H., Olson, N. F., Fox, P. F., Healy, A., & Højrup, P. (1993b). Proteolytic specificity of plasmin on bovine α_{s1}-casein. *Food Biotechnology, 7*, 143–158.

Monnet, V., Ley, J. P., & Gonzàlez, S. (1992). Substrate specificity of the cell envelope-located proteinase of *Lactococcus lactis* subsp. *lactis* NCDO 763. *International Journal of Biochemistry, 24*, 707–718.

Mulvilhill, D., & Fox, P. F. (1974). Proteolysis of α_{s1}-casein by chymosin: influence of pH and urea. *Journal of Dairy Research, 44*, 533–540.

Narayanan, A. S., & Anwar, R. A. (1969). The specificity of purified porcine pancreatic elastase. *Biochemical Journal, 114*, 11–17.

Naughton, M. A., & Sanger, F. (1961). Purification and specificity of pancreatic elastase. *Biochemical Journal, 78*, 156–163.

Newbould, F. H. S. (1974). Microbial diseases of the mammary gland. In B. L. Larson, & V. R. Smith, *Lactation, a comprehensive treatise* (Vol. 2, pp. 269–316). New York: Academic Press.

O'Sullivan, C. A., Joyce, P. J., Sloan, T., & Shattock, A. G. (1992). Capture immunoassay for the diagnosis of bovine mastitis using a monoclonal antibody too polymorphonuclear granulocytes. *Journal of Dairy Research, 59*, 123–131.

Senyk, G. F., Barbano, D. J., & Shipe, W. F. (1985). Proteolysis in milk associated with increasing somatic cell counts. *Journal of Dairy Science, 68*, 2189–2194.

Sordillo, L. M., Shaefer-Weaver, K., & Derosa, D. (1997). Bovine immunology of the mammary gland. *Journal of Dairy Science, 80*, 1851–1865.

Swaisgood, H. E. (1992). Chemistry of the caseins. In P. F. Fox, *Advanced dairy chemistry, Volume 1. Proteins* (pp. 63–110). London: Elsevier Applied Science.

Verdi, R. J., & Barbano, D. M. (1991). Properties of proteases from milk somatic cells and blood leukocytes. *Journal Dairy Science, 74*, 2077–2081.

Verdi, R. J., Barbano, D. M., Devalle, M. E., & Senky, G. F. (1987). Variability in true protein, casein, nonprotein nitrogen and proteolysis in high and low somatic cell milks. *Journal of Dairy Science, 70*, 230–242.