Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* SK11

Graham G. Pritchard,¹ Andrea D. Freebairn¹ and Tim Coolbear²

Author for correspondence: Graham G. Pritchard. Tel: +64 6 356 9099. Fax: +64 6 350 5682.

¹ Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand

² New Zealand Dairy Research Institute, Palmerston North, New Zealand

An endopeptidase has been purified from *Lactococcus lactis* subsp. *cremoris* SK11. The enzyme is a 70 kDa monomer, strongly inhibited by the metalloproteinase inhibitors 1,10-phenanthroline and phosphoramidon but relatively insensitive to EDTA. It is not significantly inhibited by the thiol enzyme inhibitor *p*-chloromercuribenzoate nor by the serine protease inhibitor phenylmethylsulphonyl fluoride. The action of the endopeptidase in catalysing the hydrolysis of several peptide hormones has been studied and the hydrolysis products identified by sequence analysis. The enzyme catalyses hydrolysis of peptide bonds in which a hydrophobic amino acid (most commonly a Phe or Leu) residue occupies the position immediately C-terminal to the hydrolysed bond. It thus has a specificity very similar to that of thermolysin. Two of the oligopeptides produced during the early stages of β -casein digestion by the lactococcal cell-wall proteinases were hydrolysed by the endopeptidase, the others were resistant to hydrolysis. Cell fractionation studies have shown that the distribution of endopeptidase activity between the different cell fractions is the same as that of the intracellular marker enzyme fructose biphosphate aldolase, and thus indicate a cytoplasmic location for the enzyme. These observations argue against a role for this enzyme in the early stages of casein breakdown by the lactococcal proteolytic system.

Keywords: *Lactococcus lactis* subsp. *cremoris*, endopeptidase, proteases, dairy microbiology

INTRODUCTION

The hydrolysis of milk proteins by dairy starter lactic acid bacteria involves the action of a cell-envelope-associated proteinase, various peptidases and transport systems for peptides and amino acids (Kok, 1990; Smid *et al.*, 1991; Pritchard & Coolbear, 1993; Tan *et al.*, 1993). The cell-envelope-associated proteinase catalyses the partial hydrolysis of one or more of the casein components of bovine milk to a series of oligopeptide products, the nature of which has been well characterized (Monnet *et al.*, 1989, 1992; Visser *et al.*, 1988, 1991; Reid *et al.*, 1991a, b). The subsequent degradation of these oligopeptides to provide the free amino acids required for starter growth is less clearly defined. Several peptidases have now been isolated from lactococci (Kok, 1990; Smid *et al.*, 1991; Tan *et al.*, 1993). Most of these are aminopeptidases which could potentially degrade the

large casein oligopeptides by systematic cleavage of amino acids or dipeptides from the N-terminus. This would require some of the aminopeptidases to be located external to the cell membrane since large oligopeptides are probably not readily transported across the membrane (Law, 1978; Rice *et al.*, 1978). However, current evidence on the cellular location of the aminopeptidases indicates that they are mostly located intracellularly (Tan *et al.*, 1992; Exterkate *et al.*, 1992; Nardi *et al.*, 1991; Booth *et al.*, 1990).

Compared to the well-defined characterization and classification of the aminopeptidase enzymes from lactococci and other lactic acid bacteria (Kok, 1990; Tan *et al.*, 1993), the relationship of the various enzymes with endopeptidase activity which have been reported from lactic acid bacteria, under a variety of names and designations, is less clearly understood. Two different endopeptidases, designated LEP-I and LEP-II, were isolated from *Lactococcus lactis* subsp. *cremoris* H61 (Yan *et al.*, 1987a, b). LEP-I was shown to be a 98 kDa monomer, unable to

Abbreviations: BTP, Bis-tris propane; *p*CMB, *p*-chloromercuribenzoate.

hydrolyse α_{s1} -, β - or κ -caseins or insulin B-chain but catalysing hydrolysis of smaller peptides such as bradykinin, angiotensin, neurotensin and the N-terminal 23-residue fragment from α_{s1} -casein. LEP-II was reported to be a dimer of two identical 40 kDa subunits catalysing hydrolysis of a wider range of oligopeptides including insulin B-chain, glucagon and two α_{s1} -casein oligopeptides, although as with LEP-I, whole α_{s1} -, β - and κ -caseins were not hydrolysed. More recently an endopeptidase was isolated from *L. lactis* subsp. *cremoris* Wg2 (Tan *et al.*, 1991) which, like LEP-II, could catalyse hydrolysis of insulin B-chain and glucagon although the particular bonds hydrolysed were not identified. However, it differed from LEP-II in its molecular mass (70 kDa for both the native and subunit molecular mass). This enzyme was shown, by immunoreactivity to a polyclonal antibody raised to the purified enzyme, to be present in several strains of lactococci (Tan *et al.*, 1992). An apparently similar enzyme, designated a neutral oligo-endopeptidase (NOP), has been described from *L. lactis* subsp. *cremoris* C13 (Baankreis, 1992). The nucleotide sequence of the gene (designated *pepO*) encoding an endopeptidase in *L. lactis* subsp. *cremoris* P8-2-47 has been determined (Mierau *et al.*, 1993) and shown to have sequence homology to mammalian enkephalinase.

Other lactococcal enzymes with endopeptidase activity (apart from the cell-envelope-associated proteinase) include enzymes designated as intracellular proteinases (Desmazeaud & Zevaco, 1976; Muset *et al.*, 1989; Zevaco & Desmazeaud, 1980). These enzymes differ from the endopeptidases described above in their ability to hydrolyse β -casein.

The present study describes the purification and properties of an endopeptidase from *L. lactis* subsp. *cremoris* SK11. A comparison of the specificity and other properties of this enzyme with those of previously studied lactococcal endopeptidases and 'proteinases' suggests that many of these enzymes have a very similar specificity of action in hydrolysing peptides and may therefore be related enzymes.

METHODS

Bacterial strain, growth conditions and harvest. *Lactococcus lactis* subsp. *cremoris* SK11 was obtained from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand (isolate number 5221). Bacterial cultures for enzyme purification were grown in a 40 litre fermenter in a lactose/peptone/yeast extract medium following the procedure described by Lloyd & Pritchard (1991). Bacterial cells were harvested by centrifugation at the end of the exponential phase (approximately 6 h after inoculation), washed twice with cold 50 mM phosphate buffer (pH 6.4), and the washed pellets were stored frozen at -15°C until required.

Enzyme assays. Endopeptidase activity was determined using the peptide hormone bradykinin as substrate. The rate of hydrolysis was followed by measuring the decrease in bradykinin concentration after separating substrate and products by reverse-phase HPLC. The standard assay mixture contained 80 μl bradykinin (0.5 mg ml^{-1} in deionized water) and 20 μl appropriately diluted enzyme solution in either 20 mM phosphate buffer (pH 6.4) or 20 mM-Bis-tris propane (BTP) buffer

(pH 6.4). The reaction mixture was incubated at room temperature and, at appropriate times, 50 μl samples were removed and added to 25 μl 3% (v/v) trifluoroacetic acid (TFA) to stop further reaction. The samples were centrifuged at 10000 *g* in a microcentrifuge (MSE, MicroCentaur) and then 50 μl of supernatant was injected onto a Vydac 218 TP C18 reverse-phase column (250 mm $\times$ 4.6 mm; Alltech Associates) linked to a Philips PU4100 HPLC system. Residual bradykinin was separated from the peptide products using the following elution conditions. Solution A contained 0.1% TFA in glass-distilled deionized water; solution B contained 0.08% TFA in acetonitrile. Elution was by a linear gradient of 5–40% B at 1 ml min^{-1} over 15 min. Peptides were detected by their absorbance at 220 nm. The system was calibrated using known concentrations of bradykinin. One unit of endopeptidase activity is defined as that amount of enzyme catalysing the hydrolysis of 1 μmol bradykinin min^{-1} .

Lysine aminopeptidase activity was measured using lysyl *p*-nitroanilide as substrate and following the rate of appearance of *p*-nitroaniline spectrophotometrically at 405 nm. The reaction mixture contained: 780 μl 100 mM MES buffer (pH 6.8); 200 μl lysine *p*-nitroanilide (5 mM) and 20 μl appropriately diluted enzyme. One unit of enzyme is defined as that amount catalysing the formation of 1 μmol *p*-nitroaniline min^{-1} .

Fructose-1,6-bisphosphate aldolase activity was measured essentially according to the procedure of Crow & Thomas (1982).

Enzyme purification. All steps, except where specified otherwise, were carried out at a temperature of $0\text{--}4^{\circ}\text{C}$. Approximately 100 g frozen cells (collected from about 7.5 l of culture) were thawed and resuspended in 170 ml 20 mM phosphate buffer (pH 6.4). Bacterial cells were disrupted by a single passage through a French pressure cell operated at 55 MPa. The resulting homogenate was centrifuged at 27000 *g* for 15 min and the supernatant collected. The supernatant was loaded onto a column (3.5 cm $\times$ 17 cm) of DEAE-cellulose (Whatman DE23) equilibrated with 10 mM sodium phosphate buffer (pH 6.4). The column was washed with 100 ml 10 mM sodium phosphate buffer (pH 6.4) containing 0.1 M NaCl and then bound protein was eluted with a linear 0.1–0.6 M NaCl gradient using a total elution volume of 500 ml and a flow rate of 1.2 ml min^{-1} . Fractions containing high endopeptidase (bradykinin-hydrolysing) activity (eluting between 0.4 and 0.6 M NaCl) were pooled and concentrated to a volume of 5–10 ml by ultrafiltration through a PM 30 Diaflo membrane (Amicon). The concentrated sample was loaded onto a column (2.6 cm $\times$ 90 cm) of Sephacryl S300 equilibrated with 20 mM BTP buffer (pH 6.0) containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 0.2 ml min^{-1} . Fractions with high endopeptidase activity (eluting at 280–330 ml) were pooled and concentrated to less than 10 ml by ultrafiltration. The concentrated fractions were then loaded onto a MonoQ HR 10/10 FPLC column (Pharmacia) equilibrated with 20 mM BTP buffer (pH 6.5) and operated at room temperature. The column was washed with approximately 10 ml equilibration buffer and bound protein was then eluted with a linear 0–1 M NaCl gradient over 40 min at a flow rate of 2 ml min^{-1} . Fractions containing high endopeptidase activity were pooled and dialysed for 3 h against 20 mM-BTP buffer (pH 6.5) containing 3 M NaCl. The dialysed solution was loaded onto a 0.7 cm $\times$ 6.5 cm column of phenyl-Sepharose equilibrated with the 20 mM BTP (pH 6.5) containing 3 M NaCl, the column was washed with approximately 50 ml of equilibration buffer and enzyme eluted with a linear 3–0 M NaCl gradient using a total elution volume of 240 ml and a flow rate of 0.5 ml min^{-1} . Contaminating proteins were eluted in the wash buffer, while the endopeptidase activity bound to the phenyl-Sepharose

and was eluted as a homogeneous protein between 1.75 and 1 M NaCl. Active fractions were pooled and concentrated by ultrafiltration using a Centricon 10 (Amicon) micro-concentrator.

Protein determination. Protein concentration was measured using the bicinchoninic acid method of Smith *et al.* (1985) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis (PAGE). Samples for analysis by SDS-PAGE were prepared by boiling with an equal volume of 0.125 M Tris/HCl (pH 6.8) containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) β -mercaptoethanol. SDS-PAGE was carried out according to the procedure of Laemmli (1970).

Determination of molecular mass. The molecular mass of the denatured monomer was determined using SDS-PAGE with the following proteins as standards: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The molecular mass of the native purified protein was determined from its elution volume from a Superose 12 gel permeation column (Pharmacia) eluted with 20 mM BTP (pH 6.5) containing 0.15 M NaCl at a flow rate of 0.5 ml min⁻¹. The following proteins were used as standards: β -amylase (200 kDa); alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa).

N-terminal sequence analysis. The N-terminal amino acid sequence of the purified enzyme was determined by the automated Edman method using a gas-phase protein sequencer (Applied Biosystems 470A, with a 120A PTH analyser).

Isolation and identification of peptide products of hydrolysis. The products of peptide hydrolysis by the purified endopeptidase were separated by HPLC using the apparatus and procedure described above for bradykinin (see **Enzyme assays**). Peptide peaks were collected and dried under vacuum. Peptides were identified by sequence analysis.

Cell fractionation. Bacteria were grown in reconstituted skim milk and harvested following the procedures described by Coolbear *et al.* (1992a). The harvested cells were separated into 'cell-wall lysate', 'cytoplasmic' and 'particulate' fractions using lysozyme plus mutanolysin to solubilize cell wall material and sucrose as osmotic stabilizer following the procedures described by Crow *et al.* (1993).

Materials. Bradykinin, neurotensin, insulin B-chain, met-enkephalin and other small peptides were obtained from Sigma, as were other enzyme assay substrates. Oligopeptides produced by digestion of β -casein by purified cell-envelope-associated proteinases from *L. lactis* subsp. *cremoris* strains H2 and SK11 were isolated and identified as described by Reid *et al.* (1991b). Glycerol-3-phosphate dehydrogenase/triosephosphate isomerase was from Boehringer. Angiotensin was synthesised by Dr D. Engelbrechtsen of the Massey University Separation Science Unit. DEAE-cellulose DE23 was from Whatman Biochemicals, and all other column matrices and pre-packed columns were from Pharmacia. Acetonitrile and 2-propanol were HPLC-grade solvents from either BDH or Mallinckrodt. Yeast extract was from Gibco and Trypsin peptone from BBL.

RESULTS AND DISCUSSION

Purification

A typical purification is summarized in Table 1. The use of hydrophobic interaction chromatography on phenyl-Sepharose was particularly effective in removing protein

contaminants at the final stage, yielding a virtually homogeneous protein as determined by SDS-PAGE (Fig. 1).

Molecular mass

The subunit molecular mass, estimated from three separate SDS-polyacrylamide gels, was 69.5 ± 1.2 kDa. This is in agreement with the value of 70 kDa reported by Tan *et al.* (1991) for the molecular mass of the endopeptidase from *L. lactis* subsp. *cremoris* Wg2 and with the value of 71.5 kDa calculated from the gene sequence for the endopeptidase from *L. lactis* subsp. *cremoris* P8-2-47 (Mierau *et al.*, 1993). The native molecular mass determined by gel permeation was 93 kDa (two separate determinations agreeing within ± 2 kDa). Thus this method results in an overestimation of the molecular mass. This is of interest since the mass of the intracellular proteinase purified from *L. lactis* subsp. *lactis* NCDO 763 (Muset *et al.*, 1989) was also found to be 93 kDa by gel permeation. A value of 80 kDa (also determined by gel permeation) was reported for the LEP-II endopeptidase from *L. lactis* subsp. *cremoris* H61 by Yan *et al.* (1987a) although the subunit molecular mass of this enzyme, determined by SDS-PAGE, was only 40 kDa, indicating a dimeric native enzyme.

N-terminal sequence

The sequence of the first 20 amino acid residues of the purified endopeptidase was determined (Table 2) except for the initial N-terminal residue which could not be unambiguously identified. This N-terminal sequence is identical to that predicted from the nucleotide sequence for the endopeptidase from *L. lactis* subsp. *cremoris* P8-2-47 (Mierau *et al.*, 1993). However, it shows almost no homology with the N-terminal sequence determined for LEP-II from *L. lactis* subsp. *cremoris* H61 by Yan *et al.* (1987a) except for the three residues which are the same in both sequences (see Table 2).

pH dependence

The pH dependence of the endopeptidase using the peptide met-enkephalin as substrate (as used in the work of Tan *et al.*, 1991) showed a simple pH profile with an optimum value of around 6.0. This is in agreement with values reported for other lactococcal endopeptidases (Desmazeaud & Zevaco, 1976; Yan *et al.*, 1987a; Tan *et al.*, 1991). However, when the pH dependence was studied using bradykinin as the peptide substrate, high enzyme activity was maintained over the range from pH 6.0 to 9.5. No difference in the bradykinin hydrolysis products was observed over this pH range. The reason for this difference in pH profiles using the two different substrates is unknown.

Effect of proteinase inhibitors

The sensitivity of the endopeptidase purified in the present study to various proteinase inhibitors (Table 3) was similar in most respects to previously reported findings on

Table 1. Purification of an endopeptidase from *L. lactis* subsp. *cremoris* SK11

The results presented are for a purification from 75 g wet wt of cells.

Purification step	Protein concn (mg ml ⁻¹)	Total protein (mg)	Activity (units ml ⁻¹)	Total activity (units)	Specific activity (units mg ⁻¹)	Purification (-fold)	Yield (%)
Crude extract	37.5	3700	3.4	256.7	0.085	1	100
DEAE-cellulose	50.6	359	14.5	97.3	0.271	3.15	37
Sephacryl S300	12.2	73	2.4	27.1	0.371	4.3	10
MonoQ	6.8	9.8	4.9	13.0	1.32	16.0	5
Phenyl-Sepharose	0.52	0.2	6.1	2.5	12.5	147	1

Fig. 1. SDS-PAGE showing purification of an endopeptidase from *L. lactis* subsp. *cremoris* SK11. Lanes 1 and 7, molecular mass standards; lane 2, crude cell extract; lane 3, pooled fractions from DEAE-cellulose column; lane 4, pooled fractions from Sephacryl S300 column; lane 5, active fractions from MonoQ column; lane 6, active fractions from phenyl-Sepharose column.

the inhibition of lactococcal endopeptidases. However, a significant difference was the inability of EDTA (even at 10 mM) to inhibit the enzyme to less than 60% of the control activity, although activity was completely inhibited by two other metalloproteinase inhibitors, phosphoramidon (0.1 mM) and 1,10-phenanthroline (1 mM). Previous studies on lactococcal endopeptidases

Table 3. Effect of protease inhibitors on activity of an endopeptidase from *L. lactis* subsp. *cremoris* SK11

The enzyme was preincubated for 30 min in the presence of inhibitor at the concentration shown and then the reaction was started by addition of substrate. Final inhibitor concentrations after addition of substrate were 60% of the concentrations shown.

Inhibitor	Activity (percentage of uninhibited control)
EDTA (10 mM)	62
<i>o</i> -Phenanthroline (1 mM)	0
Phosphoramidon (0.1 mM)	0
<i>p</i> CMB (1 mM)	89
PMSF (1 mM)	83

(Tan *et al.*, 1991; Yan *et al.*, 1987a, b) have reported strong inhibition of endopeptidase activity by 1 mM EDTA. Muset *et al.* (1989) found that the phosphoramidon-sensitive intracellular proteinase from *L. lactis* subsp. *lactis* NCDO 763 was inhibited to only 58% of the control level by 1 mM EDTA at pH 6, although inhibition increased at lower pH values. The endopeptidase from *L. lactis* subsp. *cremoris* C13 was found to be only partially inhibited by 1 mM EDTA and to require 10 mM EDTA to achieve complete inhibition (Baankreis, 1992). In the present study, prolonged incubation of the enzyme with EDTA (10 mM), prior dialysis of the enzyme to remove or reduce the content of bound metal ions, variation of

Table 2. N-terminal amino acid sequences of lactococcal endopeptidases

Endopeptidase*	N-terminal sequence
(a)	?-Arg-Ile-Gln-Asp-Asp-Leu-Phe-Ala-Thr-Val-Asn-Ala-Glu-Trp-Leu-Glu-Asn-Ala-Glu-Ile...
(b)	Thr-Arg-Ile-Gln-Asp-Asp-Leu-Phe-Ala-Thr-Val-Asn-Ala-Glu-Trp-Leu-Glu-Asn-Ala-Glu-Ile
(c)	?-Phe-Thr-Leu-Pro-Glu-Leu-Pro-Tyr-Ala-Pro-Asn-Ala-Leu-Glu-Phe-Phe-Cys-Met...

* (a) Endopeptidase purified from *L. lactis* subsp. *cremoris* SK11 in the present study; (b) endopeptidase PepO from *L. lactis* subsp. *cremoris* P8-2-47 (Mierau *et al.*, 1993); (c) endopeptidase LEP II from *L. lactis* subsp. *cremoris* H61 (Yan *et al.*, 1987a).

Table 4. Peptide bonds hydrolysed in hormone peptides by the endopeptidase from *L. lactis* subsp. *cremoris* SK11

Peptides (150–160 µg) were incubated with purified endopeptidase (1–4 µg protein in 20 mM BTP buffer, pH 6.0) from *L. lactis* subsp. *cremoris* SK11 for periods up to 4 h. For insulin B-chain, 240 µg peptide was incubated with 9 µg endopeptidase for up to 1 h. Hydrolysis products were separated by HPLC and identified by amino acid sequence determination as described in Methods. Hydrolysed bonds are indicated by ▼. For comparison the bonds cleaved by the endopeptidase LEP-II from *L. lactis* subsp. *cremoris* H61 (Yan *et al.*, 1987a) are shown by △.

Peptide	Sequence and peptide bond(s) hydrolysed
Metenkephalin	Tyr-Gly-Gly-▼Phe-Met △
Bradykinin	Arg-Pro-Pro-Gly-▼Phe-Ser-Pro-▼Phe-Arg △
Angiotensin	Asp-Arg-Val-Tyr-Ile-His-Pro-▼Phe-His-Leu △
Insulin B-chain	Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-▼Leu-Val-Glu-Ala-▼Leu-Tyr-▼Leu- △ Val-Cys-Gly-Glu-Arg-Gly-▼Phe-▼Phe-Tyr-Thr-Pro-Lys-Ala △
Neurotensin	pGlu-Leu-▼Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-▼Ile-Leu △

pH and the use of angiotensin or metenkephalin as alternative substrates to bradykinin all yielded the same result, i.e. no more than 40% inhibition of enzyme activity. The complete inhibition by 1,10-phenanthroline and phosphoramidon suggests that the enzyme is indeed, like other lactococcal endopeptidases, a metalloenzyme, but the failure to achieve marked inhibition by EDTA suggests some conformational protection of the metal ion from this inhibitor. With respect to its insensitivity to inhibition by the serine proteinase inhibitor PMSF, and the thiol proteinase inhibitor pCMB, the endopeptidase from *L. lactis* subsp. *cremoris* SK11 is similar to that of other lactococcal endopeptidases and intracellular proteinases (Yan *et al.*, 1987a; Muset *et al.*, 1989; Desmazeaud & Zevaco, 1976). The endopeptidase from *L. lactis* subsp. *cremoris* Wg2, on the other hand, was reported to be inhibited by 1 mM pCMB (Tan *et al.*, 1991) although the percentage inhibition was not stated.

Substrate specificity

The bonds hydrolysed in various hormone peptides are shown in Table 4, which also indicates the sites of hydrolysis found by Yan *et al.* (1987a) for LEP-II from *L. lactis* subsp. *cremoris* H61. It is evident that the specificity of action of endopeptidase from *L. lactis* subsp. *cremoris* SK11 in cleaving these five peptides is almost identical to that found for LEP-II (except for an additional three sites found only in the present study). Most of the bonds identified as cleavage sites in insulin B-chain in the present study were also found for the intracellular proteinase from *L. lactis* subsp. *lactis* NCDO 763 by Muset *et al.* (1989) and for an endopeptidase from *L. lactis* subsp. *lactis* bv.

diacetylactis by Desmazeaud & Zevaco (1976), although these two studies also reported a few additional cleavage sites. However, the cleavage patterns found in the present study are very different from those found for the endopeptidase LEP-I from the H61 strain (Yan *et al.*, 1987b), which does not hydrolyse insulin B-chain and which hydrolyses neurotensin and bradykinin in a different manner. The bond specificity of the endopeptidase purified from *L. lactis* subsp. *cremoris* Wg2 (Tan *et al.*, 1991) was not reported but that enzyme catalysed hydrolysis of neurotensin, metenkephalin, bradykinin and insulin B-chain (angiotensin was not investigated) and thus showed a similar spectrum of peptide susceptibility to hydrolysis to that of the enzyme purified in the present study. Inspection of the cleaved bonds (Table 4) reveals that the enzyme shows a strong preference for bonds in which a Leu or Phe residue occupies the P1' site (i.e. in the position immediately C-terminal to the cleaved bond). It thus has a specificity very similar to that of thermolysin. In fact, the particular bonds hydrolysed in insulin B-chain are virtually the same as those cleaved by thermolysin and various other neutral metalloproteases from a variety of microbial sources (Moriyama, 1974). The only thermolysin-sensitive bond in B-insulin not identified as a cleavage site in the present study is the His₅-Leu₆ peptide bond.

Hydrolysis of casein oligopeptides

As found for other lactococcal endopeptidases (Yan *et al.*, 1987a, b; Tan *et al.*, 1991), the endopeptidase from *L. lactis* subsp. *cremoris* SK11 was not able to hydrolyse intact α-, β- or κ-caseins. However, it did catalyse hydrolysis of

Table 5. Peptide bonds hydrolysed in casein oligopeptides by the endopeptidase from *L. lactis* subsp. *cremoris* SK11

Casein oligopeptides were generated by digesting β - and κ -casein with cell envelope proteinases from *L. lactis* subsp. *cremoris* strains H2 and SK11 as described by Reid *et al.* (1991a, b). The peptides were isolated from the digest by reverse-phase HPLC and identified by the methods described previously (Reid *et al.*, 1991a). The casein peptides were incubated with purified endopeptidase (approx. 10 μ g) for periods up to 24 h. Samples were taken after various times and the peptide products were isolated by reverse-phase HPLC and identified by amino acid sequence analysis. Numbers in parentheses refer to the residue numbers in the amino acid sequence of the respective caseins. Peptides bonds hydrolysed are indicated by ▼.

Peptide	Sequence and peptide bond(s) hydrolysed
κ -Casein (161–169)	Thr-Val-Gln - Val-Thr-Ser-Thr-Ala-Val ▼
β -Casein (1–209; total sequence)	Not hydrolysed
(47–52)	Asp-Lys-Ile-His-Pro-Phe; not hydrolysed
(166–175)	Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln; not hydrolysed
(176–182)	Lys-Ala-Val-Pro-Tyr-Pro-Gln; not hydrolysed
(183–193)	Arg-Asp-Met-Pro - Ile-Gln-Ala - Phe - Leu - Leu-Tyr ▼ ▼ ▼
(194–209)	Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro - Ile-Ile-Val ▼

Fig. 2. Time course of hydrolysis by the endopeptidase of two oligopeptides derived from β -casein by action of the cell envelope proteinase from *L. lactis* subsp. *cremoris* H2. 1, The peptide Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr (Arg₁₈₃-Tyr₁₉₃ in β -casein). 1a–1c, Hydrolysis products of peptide 1: 1a, Phe₁₉₀-Tyr₁₉₃; 1b, Arg₁₈₃-Ala₁₈₉; 1c, the tetrapeptide Arg₁₈₃-Pro₁₈₆ plus the dipeptide Leu₁₉₂-Tyr₁₉₃ (these two peptides have the same retention time in the HPLC system used). 2, The peptide Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (Gln₁₉₄-Val₂₀₉ in β -casein). 2a, Hydrolysis product of peptide 2: Gln₁₉₄-Pro₂₀₆.

some oligopeptides derived from these caseins (Table 5). The peptides shown in Table 5 were isolated from digests obtained by incubating the intact β - and κ -caseins with

purified cell-envelope-associated proteinases from either *L. lactis* subsp. *cremoris* SK11 (a P_{III}-type proteinase-producing strain) or *L. lactis* subsp. *cremoris* H2 (a P_I-type proteinase-producing strain). The κ -casein (161–169) peptide, a major product of κ -casein digestion by the P_{III}-type proteinase at the earliest digestion times (J. R. Reid, T. Coolbear & G. G. Pritchard, unpublished), is readily hydrolysed by the endopeptidase at the Gln₁₆₃-Val₁₆₄ bond. Of the four major peptides generated during the initial stage of hydrolysis of β -casein by the P_I-type lactococcal cell envelope-associated proteinase (Reid *et al.*, 1991b), the peptides Arg₁₈₃-Tyr₁₉₃ and Gln₁₉₄-Val₂₀₉ were hydrolysed by the endopeptidase at one or more of the peptide bonds with hydrophobic residues in the P1' position (Table 5). There was, however, a significant difference in the rate of hydrolysis of these two peptides. At identical enzyme concentrations, the peptide Arg₁₈₃-Tyr₁₉₃ was almost completely degraded after 1 h whereas the C-terminal β -casein peptide Gln₁₉₄-Val₂₀₉ was more slowly hydrolysed over a period of 6 h (Fig. 2). Hydrolysis of the former peptide (Arg₁₈₃-Tyr₁₉₃) occurred initially at the Ala₁₈₉-Phe₁₉₀ bond, leading to the transient accumulation of the heptapeptide Arg-Asp-Met-Pro-Ile-Gln-Ala and the tetrapeptide Phe-Leu-Leu-Tyr (Fig. 2). Both of these initial peptide products were subsequently degraded to smaller peptides at the bonds indicated in Table 5. The C-terminal β -casein peptide (Gln₁₉₄-Val₂₀₉) was hydrolysed only at the Pro₂₀₆-Ile₂₀₇ bond. The large N-terminal product of this hydrolysis (Gln₁₉₄-Pro₂₀₆) accumulated and was still present as a major product after 24 h digestion. The relative resistance of this peptide to hydrolysis is surprising in view of the presence of hydrophobic residues in the sequence which

Table 6. Subcellular distribution of endopeptidase activity in *L. lactis* subsp. *cremoris* SK11

Percentage distribution of total cellular activity of endopeptidase, lysine aminopeptidase and fructose-1,6-bisphosphate aldolase activity between different cellular fractions obtained using the procedure of Crow *et al.* (1993).

Enzyme	Cell wall lysate (%)	Cytoplasmic fraction (%)	Particulate fraction (%)
Lysine aminopeptidase	0.2	99.1	0.7
Fructose bisphosphate aldolase	0.3	99.0	0.7
Endopeptidase	1.4	98.0	0.6

could indicate likely cleavage sites, suggesting that conformational factors as well as the primary sequence determine the susceptibility to hydrolysis. The peptides from this region of β -casein have been shown to be extremely bitter (Shinoda *et al.*, 1985) and have been implicated as contributing to the bitter flavour defect of cheese (Visser *et al.*, 1983). Other early products of P₁-type cell-envelope-associated proteinase action on β -casein, namely the peptides Lys₁₇₆-Gln₁₈₂, a series of three overlapping, not easily separated, peptides from the sequence Ser₁₆₆-Gln₁₇₅ and also the hexapeptide Asp₄₇-Phe₅₂, an early product of P₁₁₁-type proteinase action (Reid *et al.*, 1991b), were not hydrolysed by the endopeptidase even after 24 h incubation.

Cellular location of endopeptidase activity

Harvested cells were separated into three subcellular fractions designated 'cell-wall lysate', 'cytoplasmic' and 'particulate' fractions using the procedure described by Crow *et al.* (1993). The cell-wall lysate fraction contains proteins, such as the cell-envelope-associated proteinase, released from the cell wall by treatment with the lytic enzymes, lysozyme and mutanolysin. The cytoplasmic fraction contains soluble (and possibly loosely membrane-bound) proteins released into the supernatant when spheroplasts are lysed and centrifuged, while the particulate fraction contains proteins and other materials tightly bound to the cell wall and membrane (Coolbear *et al.*, 1992b; Crow *et al.*, 1993). The distribution of endopeptidase activity between the three fractions was compared with that of the cytoplasmic marker enzyme, fructose-1,6-bisphosphate aldolase, and of the enzyme lysine aminopeptidase which, on the basis of current evidence, also has a cytoplasmic location (Tan *et al.*, 1992; Exterkate *et al.*, 1992). The results of the present study (Table 6) clearly indicate that endopeptidase (bradykinin-degrading) activity is located in the cytoplasmic fraction. The endopeptidase isolated from *L. lactis* subsp. *cremoris* Wg2 was also shown, by immunogold labelling and antibody binding to subcellular fractions, to be located predominantly in the cytoplasm (Tan *et al.*, 1992). In the recently determined nucleotide sequence of the endo-

peptidase gene, *pepO*, from *L. lactis* subsp. *cremoris* P8-2-47 (Mierau *et al.*, 1993), the translation start site corresponds to the codon specifying the N-terminus of the mature protein, showing that no signal sequence characteristic of membrane-translocated proteins is present; this provides further confirmation of the intracellular location.

Concluding remarks

The endopeptidase purified in the present study is probably closely related to, or identical with, the 70 kDa enzymes from the *L. lactis* subsp. *cremoris* strains Wg2 (Tan *et al.*, 1991), C13 (Baankreis, 1992) and P8-2-47 (Mierau *et al.*, 1993). It also resembles the endopeptidase LEP-II from *L. lactis* subsp. *cremoris* H61 (Yan *et al.*, 1987a) in its specificity of hydrolysis of five different oligopeptides (see Table 4) but its molecular mass and N-terminal amino acid sequence clearly distinguish it from the latter enzyme. This raises the question of the relationship of the endopeptidase LEP-II from *L. lactis* subsp. *cremoris* H61 to the other recently studied lactococcal endopeptidases. It would seem rather improbable that a totally different enzyme, with identical specificity to that of the 70 kDa enzyme purified in the present study, exists in *L. lactis* subsp. *cremoris* H61, especially since Tan *et al.* (1991) showed, using an antibody raised to the purified endopeptidase from the Wg2 strain, that a 70 kDa endopeptidase was present in the H61 strain.

The specificity properties of the endopeptidase from *L. lactis* subsp. *cremoris* SK11 (Table 4) suggest that it could potentially contribute to the degradation of β -casein oligopeptides with a high content of hydrophobic residues. The hydrophobic C-terminal peptide of β -casein (Pro₁₉₆-Val₂₀₇) has been shown to be very bitter (Shinoda *et al.*, 1985). This peptide was slowly hydrolysed by the endopeptidase but only at one peptide bond, leaving a relatively large hydrophobic fragment undegraded. The endopeptidase NOP from *L. lactis* subsp. *cremoris* C13 was also found to hydrolyse this peptide (Baankreis, 1992). However, the cytoplasmic location of the endopeptidase would suggest that it plays only a very limited, if any, role in the casein degradation pathway in the intact cell, unless the recently described oligopeptide transport system (Kunji *et al.*, 1993) is capable of transporting peptides larger than the previously suggested upper size limit (6 to 7 amino acid residues) for transport across the cell membrane (Rice *et al.*, 1978; Law, 1978). Indeed, Mierau *et al.* (1993), using gene disruption to obtain a *pepO* mutant lacking this endopeptidase, showed that the growth and rate of acid production of this mutant were identical to the parent *pepO*⁺ strain, demonstrating clearly that this endopeptidase is not essential for lactococcal growth in milk. It may, however, play a significant role in the ripening phase of cheese manufacture if it is liberated by starter lactococci into the cheese curd.

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