Prevention of C-terminal autoprocessing of *Lactococcus lactis* SK11 cell-envelope proteinase by engineering of an essential surface loop

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The catalytic domain of the cell-envelope proteinase from *Lactococcus lactis* SK11 has various inserts, situated in external loops of the catalytic domain, compared with the related subtilisins. Protein engineering was employed to analyse the necessity and function of one of these extra loops (residues 205–219), that is predicted to be located in close proximity to the substrate-binding region and is susceptible to autoproteolysis. We constructed a deletion mutant which lacks 14 residues of this surface loop and subsequently introduced various insertion cassettes coding either for the original loop with three mutations (E205S/E218T/M219S: triple-mutant proteinase) or for neutral spacers (1, 4, 7 and 16 serine residues). Engineered proteinases were analysed for activity, (auto)processing, and cleavage specificity. The presence of residues 205–219 is shown to be essential for proteolytic activity, as only triple-mutant proteinase retained activity towards casein substrates. The triple-mutant proteinase was found to be defective in C-terminal autoprocessing, and subsequent release from the lactococcal cell envelope in a calcium-free medium, indicative of either an altered proteolytic specificity or altered accessibility of the processing site. The specificity change appears to be subtle, as only small differences were found between wild-type and triple-mutant proteinase in the breakdown of casein substrates.

INTRODUCTION

Lactococci are Gram-positive bacteria that are used as starter cultures in a variety of dairy fermentation processes. These bacteria have a complex proteolytic system which enables them to grow in milk by degrading caseins, the major milk proteins, into small peptides and free amino acids, some of which are essential for cell growth (Thomas and Pritchard, 1987). A key enzyme in this proteolytic system is an extracellular, cell-envelope-located serine proteinase that is involved in the initial breakdown of the casein substrate. The active proteinase is essential for optimal growth of the lactococcal cells in milk and, indirectly, for flavour development in various dairy products (Thomas and Pritchard, 1987; Visser, 1993).

The structural proteinase gene (*prtP*) of strain *Lactococcus lactis* subsp. *cremoris* SK11 has been isolated and characterized (Vos et al., 1989a). This gene encodes a large pre-proprotein of about 200 kDa, schematically depicted in Figure 1, which is activated by autocatalytic processing at the N-terminus during or after membrane translocation. Furthermore, a second divergently transcribed gene, designated *prtM*, has been identified which encodes a lipoprotein that is also essential for the maturation of the proteinase precursor into an active enzyme (Vos et al., 1989b). The N-terminal, catalytic domain (approx. 500 residues) of the mature lactococcal proteinase shows significant sequence similarity to the serine proteinases of the subtilisin family, also called subtilases (Vos et al., 1989a; Siezen et al., 1991). An important difference with most other subtilases is the presence in the lactococcal proteinase of a large, additional C-terminal extension of over 1200 residues of unknown function. After translocation, the proteinase remains attached to the cell envelope via a C-terminal membrane anchor sequence, which is homologous to those found in a great number of cell-envelope-located proteins from other Gram-positive bacteria (Vos et al., 1989a). Deletion of C-terminal fragments containing this membrane anchor results in secretion of the SK11 proteinase into the growth medium (de Vos et al., 1989; Bruinenberg et al., 1992). Alternatively, C-terminal autodigestion can be induced in a Ca^{2+}-free buffer and leads to release of a truncated, active enzyme of approx. 145 kDa from the lactococcal cells (Vos et al., 1989a,b; de Vos et al., 1991).

Based on known three-dimensional structures of subtilases, a structurally conserved core of the catalytic domain was defined in which the α-helix and β-sheet secondary structures show the highest sequence similarity (Siezen et al., 1991). This sequence similarity is less for the peptide loops, generally located on the surface of the molecule, that connect the core structure elements. In addition, deletions and insertions were generally found in these loops or variable regions (vr) of the subtilases. The SK11 proteinase was found to have 10 inserts of 2–151 residues, all situated in variable regions, with a total of 238 additional residues relative to subtilisin BPN′ (Siezen et al., 1991), as shown schematically in Figure 2.

We have undertaken a protein engineering study to determine the function of one of the largest loop insertions (residues 205–219), which is predicted to be spatially close to the substrate-binding region of the enzyme and which is susceptible to autoproteolysis. The results show that deletion or modification of this extended loop in the SK11 proteinase affects activity and autoprocessing of the proteinase.

MATERIALS AND METHODS

Bacterial strains, media and plasmids

Escherichia coli TG1 (Gibson, 1984) was used to propagate M13 derivatives. *E. coli* strains were grown in L-broth-based medium and handled as described (Sambrook et al., 1989). Strain *L. lactis* MG1363 is a plasmid-free, proteinase-deficient derivative of

Abbreviations used: PrtP, cell-envelope proteinase; TM, triple mutant; 1S, mutant with residues 205–219 replaced by one serine; ΔC190, mutant lacking the 190 C-terminal residues; μ_{max}, maximum specific growth rate; vr, variable region.

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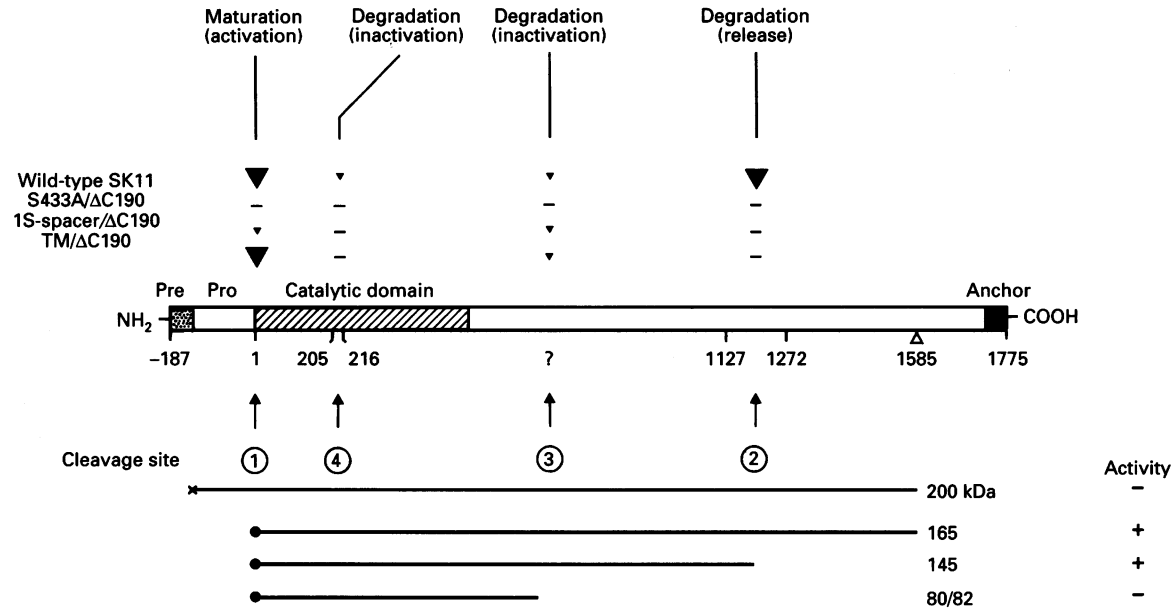

Figure 1 A schematic model depicting the pre-proproteinase, encoded by the *prtP* gene of *L. lactis* SK11, and its autoproteolytic cleavage sites (1–4) and derived products

The various domains in the proteinase are indicated, as are the important cleavage sites (arrows; number in circles). The activity of (mutant) proteinases and autoproteolytic products towards casein substrates is indicated on the right: +, active; −, inactive. At the top of the figure, the sites cleaved autoproteolytically in the different proteinases are indicated as: −, no cleavage; ▼, main cleavage site; ▾, minor cleavage site. Cleavage of wild-type proteinase at site 2 occurs in a Ca^{2+}-free medium and leads to release from the cell; the position of site 2 is as yet unidentified, but presumably lies between residues 1127 and 1272 (P. G. Bruinenberg, R. J. Siezen and W. M. de Vos, unpublished work). Two minor cleavage sites occur: the exact location of site 3 is still unidentified, while site 4 consists of three subsites located between residues 205 and 216. The open triangle at residue 1585 marks the position of deletion of the 190 C-terminal amino acids including the membrane anchor, leading to secretion of the proteinase. The apparent molecular mass of products observed on SDS/polyacrylamide gels, are indicated to the right. Symbols: (●) indicates determined N-termini; (×) indicates blocked N-terminus.

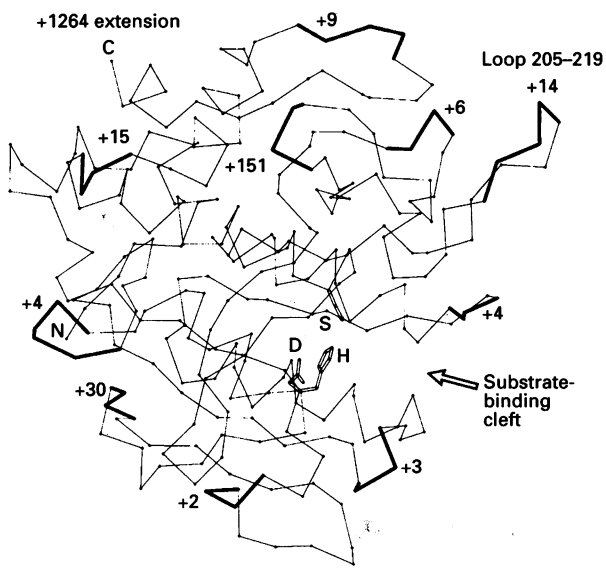

Figure 2 Model of the catalytic domain of *L. lactis* SK11 proteinase

Thin lines represent the Cα-atom backbone of subtilisin BPN′ (Heinz et al., 1991); thick lines indicate the approximate position where extra amino acids (and their number) are predicted to be inserted in the SK11 proteinase relative to subtilisin. Active-site residues D30, H94 and S433 are indicated (double lines), as are the N-terminus (N) and the C-terminus (C) of the catalytic domain and the approximate positions of loop 205–219, the substrate-binding cleft and the C-terminal extension containing other domains, including the membrane anchor.

NCDO 712 (Gasson, 1983) and was used as a host for all plasmid transformations. *L. lactis* strains were generally grown in M17 broth (E. Merck AG, Darmstadt, Germany). For proteinase expression studies *L. lactis* cells were grown in 10% (w/v) pasteurized, reconstituted skimmed milk or in whey permeate medium (de Vos et al., 1989) containing 1.9% (w/v) β-glycerol phosphate and 0.1% (w/v) Casitone (Difco Laboratories, Detroit, MI, U.S.A.). If appropriate the medium contained 0.5% (w/v) glucose and chloramphenicol (10 μg/ml).

Plasmid pNZ521 contains the complete *prtP* gene and a functional *prtM* gene resulting in an active cell-envelope-located proteinase (Vos et al., 1989b). Plasmid pNZ527 [denoted as pNZ521ΔH by Bruinenberg et al. (1992)] encodes a proteinase lacking the 190 most C-terminal amino acids (ΔC190), which results in secretion of the truncated proteinase into the growth medium (de Vos et al., 1991; Bruinenberg et al., 1992). Plasmid pNZ574 is a derivative of pNZ527 containing the S433A active-site mutation (de Vos et al., 1991; Siezen et al., 1993).

Molecular cloning

Isolation of plasmid DNA from *E. coli* and standard recombinant DNA techniques were performed according to Sambrook et al. (1989). Nucleotide sequence analysis of single-stranded M13 DNA was performed by the dideoxy chain-termination method (Sanger et al., 1977). All enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim or New England Biolabs and used according to the manufacturer's instructions. Isolation of plasmid DNA from *L. lactis* and transformation of *L. lactis* was performed as described previously (de Vos et al.,

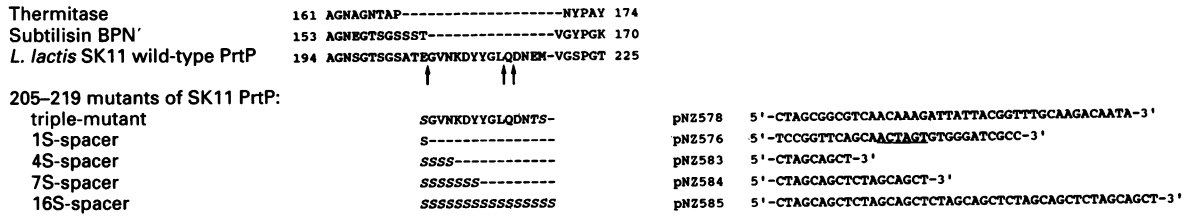

Figure 3 Sequences of wild-type and mutant SK11 proteinases in and around the 205–219 loop region

Relevant amino acid sequences are shown, in the left section, in alignment with those of thermitase (Meloun et al., 1985) and subtilisin BPN′ (Wells et al., 1983; Vasantha et al., 1984). The amino acid sequences are given in one letter code and residue numbering for each sequence is shown. The nomenclature of mutant proteinases is indicated on the left. Amino acid substitutions and insertions are shown in italics; gaps and deletions are indicated with a dashed line. The arrows indicate the mapped autoproteolytic cleavage sites. The oligonucleotides used in mutagenesis and the resulting plasmids are shown in the right-hand section; the underlined segment indicates the unique *Spe*I restriction site used for cassette mutagenesis.

1989; Vos et al., 1989b). Recombinant *L. lactis* plasmids were characterized by restriction enzyme analysis and direct sequencing of double-stranded plasmid DNA (Guo et al., 1983; Yanisch-Perron et al., 1985). Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (Millipore).

Construction of loop 205–219 mutant SK11 proteinases

A 1.3 kb *Bam*HI–*Eco*RI fragment from pNZ521 containing part of the coding region of the *prtP* gene (Vos et al., 1989b) was subcloned into M13 (Yanisch-Perron et al., 1985). Site-directed mutagenesis was performed essentially as described by Sayers et al. (1988) using a 29-nucleotide mutagenic primer: 5′-TCCGGTTCAGCAACTAGTGTGGGATCGCC-3′. This resulted in a deletion of 42 nucleotides coding for amino acids 206–219 and introduction of the mutation E205S. In addition, in this way a unique *Spe*I restriction site was introduced in the coding sequence at this position, which was used to introduce various insertion cassettes. An overview of the mutagenic oligonucleotides used and the resulting mutations in the amino acid sequence 205–219 of the proteinase are given in Figure 3. For the production of mutant proteinases in *L. lactis*, mutated gene fragments produced in *E. coli* and verified by sequence analysis were used to construct derivatives of pNZ521 containing an intact mutant *prtP* gene. Proteinase mutants lacking the C-terminal 190 amino acids were constructed by replacing the 4.9 kb *Bgl*II–*Sst*I DNA fragment (Vos et al., 1989a) for the corresponding fragment from pNZ527. All constructs were verified by DNA sequence analysis of relevant regions.

Proteinase expression studies

Lactococcal cells were grown in whey-based medium to the mid-log growth phase (A_{600} 0.9) and proteinase was released from the cell envelope by incubation in Ca^{2+}-free buffer (cell-envelope release fraction) as described previously (Exterkate and de Veer, 1985). Secreted proteinase was isolated from the culture medium by freeze-drying of dialysed samples (de Vos et al., 1989). Proteinase samples isolated from equal amounts of lactococcal cells (as determined by measuring A_{600}) were analysed on SDS/10% polyacrylamide gels (Laemmli, 1970), that were stained with Coomassie Brilliant Blue.

Proteinase activity assays

L. lactis cells were harvested at the mid-log growth phase (A_{600} 0.9) and washed in 50 mM potassium phosphate, pH 6.5, supplemented with 5 mM $CaCl_2$. The proteolytic activity of lactococcal cells towards α_{S1}- and β-casein was assayed as described previously (Vos et al., 1991). Enzymic hydrolysis of the substrate α_{S1}-casein(1–23) was performed according to Exterkate et al. (1991).

The ability of *L. lactis* cells to produce a functional proteinase was assayed by growth of these cells in 10% (w/v) pasteurized reconstituted skimmed milk. The maximum specific growth rate ($\mu_{max.}$) of lactococcal strains in milk was determined by measuring the absorbance at 600 nm of cultures clarified by a modified EDTA–borate treatment (Otto, 1981).

N-terminal amino acid analysis

Proteins were separated by SDS/PAGE, blotted on Protoblot membrane (Applied Biosystems) and visualized by staining with Coomassie Brilliant Blue according to the manufacturer's instructions. Small pieces of Protoblot membrane containing proteinase bands were directly analysed in a gas-phase Sequenator (Applied Biosystems) (Vandekerckhove et al., 1985).

RESULTS

Construction of loop 205–219 mutant proteinases

The amino acid sequence of the *L. lactis* SK11 proteinase has an insert at position 205–219 which is 14 and 18 residues longer than the corresponding loops of subtilisin BPN′ and thermitase respectively (Figure 3). The inserted sequence has high predictions for β-turn secondary structure (Garnier et al., 1978) and surface probability (Emini et al., 1985)(results not shown). This 205–219 insert is located in loop vr11 (Siezen et al., 1991) and is predicted to be in close proximity to the substrate-binding region of the catalytic domain of the lactococcal proteinase, as is evident from the model in Figure 2. Furthermore, loop 205–219 was found to be susceptible to autoproteolysis after residue Leu-214 in the proteinase from *L. lactis* strains Wg2 (Laan and Konings, 1991) and NCDO 1201 (Nissen-Meyer and Sletten, 1991); our N-terminal sequencing of degradation products confirmed this cleavage site in the SK11 proteinase and revealed additional autoproteolysis after residues Glu-205 and Gln-215 (results not shown).

To determine the necessity and function of loop 205–219, we first constructed a deletion mutant lacking 14 residues (i.e. residues 205–219 replaced by a single Ser) to obtain the 1S proteinase, and at the same time introduced a unique *Spe*I restriction site. This deletion was designed to reduce loop vr11 to the same length and very similar sequence as in subtilisin BPN′ (Figure 3). Secondly, we reinserted the wild-type amino acid sequence from position 206 to 217, which due to the cassette

Table 1 Maximum specific growth rates ($\mu_{max.}$) in milk of *L. lactis* strain MG1363 producing wild-type and mutant SK11 proteinases

$\mu_{max.}$ values are the average of three experiments (S.D. ± 0.03). *L. lactis* MG1363 is a proteinase-deficient strain which is unable to grow in milk (Gasson, 1983; Bruinenberg et al., 1992).

Proteinase	$\mu_{max.}$ (h^{-1})
Wild-type	0.63
Wild-type/ΔC190	0.63
TM	0.63
TM/ΔC190	0.41
1S-spacer	< 0.05
1S/ΔC190	< 0.05
4S-spacer	< 0.05
7S-spacer	< 0.05
16S-spacer	< 0.05
S433A/ΔC190	< 0.05
None	< 0.05

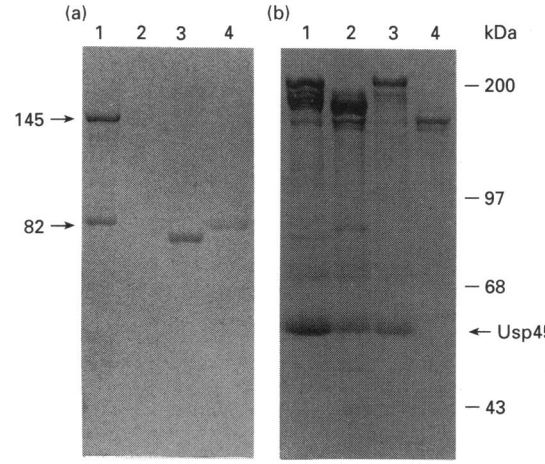

Figure 4 SDS/PAGE of cell-envelope proteinases released in Ca^{2+}-free buffer (a) or proteins secreted into the growth medium (b)

(**a**) Proteinase released from *L. lactis* MG1363 producing wild-type SK11 proteinase (lane 1), wild-type SK11/ΔC190 proteinase (lane 2), 1S proteinase (lane 3), and TM proteinase (lane 4). (**b**) Proteins secreted by *L. lactis* MG1363 producing 1S/ΔC190 proteinase (lane 1), TM/ΔC190 proteinase (lane 2), S433/ΔC190 proteinase (lane 3), and wild-type SK11/ΔC190 proteinase (lane 4). Molecular-mass markers (in kDa) and the position of the 60 kDa Usp45 protein are indicated to the right. The positions of the 145 kDa, truncated form of SK11 proteinase and of the 82 kDa autodegradation product are indicated to the left.

mutagenesis approach was modified at three connecting positions (i.e. mutations E205S/E218T/M219S) to obtain the TM proteinase (Figure 3). Thirdly, to verify whether the spacing between the core-structure elements is important, we reinserted at this position various neutral spacers of different lengths, resulting in the 4S, 7S and 16S proteinases with 4, 7 and 16 inserted serine residues respectively.

In a few cases the loop mutations were combined with a deletion of the 190 C-terminal residues (ΔC190) of the SK11 proteinase. This deletion of the membrane anchor sequence results in secretion of the truncated (mutant) proteinase into the growth medium and allows isolation and further analysis of the purified enzyme.

Growth in milk

The wild-type and mutant proteinases were expressed in *L. lactis* and recombinant strains were assayed for their ability to grow in milk (Table 1). Only the strain containing the cell-bound TM proteinase showed a $\mu_{max.}$ identical to cells expressing wild-type proteinase. The growth rate of cells expressing wild-type proteinase is not affected by deletion of the membrane anchor; in contrast, cells secreting the TM/ΔC190 proteinase into the growth medium (see below) showed a considerably (35%) reduced $\mu_{max.}$ in milk when compared with cells with cell-bound TM proteinase (Table 1). All other mutant strains were unable to grow in milk, irrespective of the location of their proteinases, indicating that these mutant proteinases were inactive.

Isolation and size characterization of mutant proteinases

The released wild-type proteinase, isolated from the cell envelope in a Ca^{2+}-free buffer (cell-envelope release fraction), showed a predominant band of approx. 145 kDa in SDS/PAGE (Figure 4a, lane 1) and a minor band of 82 kDa, which is an inactive autodegradation product of the 145 kDa proteinase (Siezen et al., 1993). In contrast, the 145 kDa component was not present in the release fraction of cells producing the 1S and TM proteinases (Figure 4a, lanes 3 and 4) or the other loop 205–219 mutant proteinases (results not shown). Various factors could account for the absence of the 145 kDa proteinase band in the cell-envelope release fractions of these mutant strains, e.g. altered synthesis, secretion, processing, release, or degradation. To distinguish between these possibilities, the truncated forms (ΔC190) of the 1S and TM proteinases were constructed, which should lead to their secretion into the growth medium (Vos et al., 1989b; Bruinenberg et al., 1992). Wild-type/ΔC190 proteinase is fully secreted (Figure 4b, lane 4), and none is left attached to the cells since no release is observed upon washing in a Ca^{2+}-free medium (Figure 4a, lane 2).

SDS/PAGE of supernatant fractions from these strains showed that the 1S/ΔC190 mutant proteinase was completely secreted (Figure 4b, lane 1) as a major component of approx. 200 kDa, while the TM/ΔC190 proteinase was secreted as a major 165 kDa component (Figure 4b, lane 2); in both cases various minor bands were also found. As controls we included the secreted form of wild-type proteinase (wild-type/ΔC190), which appeared as a major 145 kDa protein band (Figure 4b, lane 4) equivalent to the released wild-type proteinase (Figure 4a, lane 1), and the secreted form of the active-site mutant proteinase (S433A/ΔC190; de Vos et al., 1991), which appeared as a major band of approx. 200 kDa (Figure 4b, lane 3).

A natural substrate for the cell-envelope proteinase is Usp45, the secreted 60 kDa protein of *L. lactis* (van Asseldonk et al., 1990), that is completely degraded by strains expressing proteinase with wild-type level of activity, as in Figure 4(b) (lane 4) (de Vos et al., 1991). Analysis of extracellular proteins of the mutant strains shows the persistence of the Usp45 protein (Figure 4b, lanes 1–3), suggesting that their mutant proteinases show lower or no activity towards this natural substrate.

These results imply that the TM and 1S proteinases are mainly present as large precursors (> 145 kDa) and that for these mutant *L. lactis* strains the absence of the active 145 kDa enzyme in cell-envelope release fractions is probably a result of inhibition of autoproteolytic release of the proteinase from the cell envelope in a Ca^{2+}-free buffer. To verify this hypothesis, whole cells expressing either wild-type, TM or 1S proteinase were examined for the presence of proteinase before and after the release procedure, using antibodies against the SK11 proteinase. As expected, after performing the release procedure, the proteinase

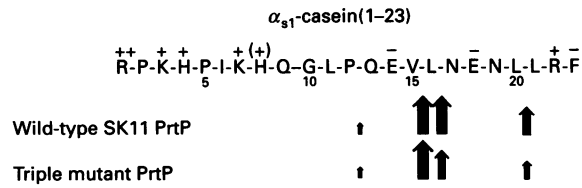

Figure 5 Proteolytic specificity of wild-type SK11 proteinase and TM/ΔC190 proteinase towards α_{s1}-casein(1–23)

Only the main cleavage sites are indicated; the size of the arrows is related to the cleavage preference.

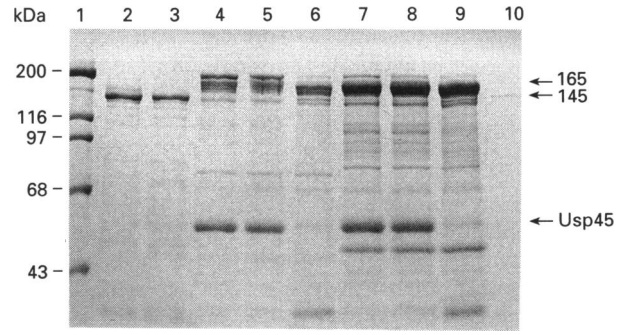

Figure 6 SDS/PAGE analysis of proteinase stability of wild-type SK11 proteinase (lanes 2,3 and 10), 1S proteinase (lanes 4–6) and TM proteinase (lanes 7–9)

Proteinases were incubated for 5 h at either 0 °C (lanes 2,4 and 7) or 30 °C (lanes 3,5 and 8). In mixing experiments a small amount of wild-type proteinase (lane 10) was incubated for 5 h at 30 °C together with 1S proteinase (lane 6) or TM proteinase (lane 9). Molecular-mass markers (in kDa) are in lane 1. The positions and apparent mass (in kDa) of the main forms of the proteinase are indicated to the right, as is the position of the 60 kDa Usp45 protein.

was still present in whole cells expressing TM and 1S proteinase (results not shown). This result confirms that the TM and 1S proteinases, unlike the wild-type enzyme, cannot be released from the cell envelope in a Ca^{2+}-free buffer.

Activity and specificity of loop 205–219 mutant proteinases

Since the release of the proteinase from the cell envelope is proposed to be an autocatalytic event (Laan and Konings, 1989; Haandrikman et al., 1991; de Vos et al., 1991), we determined the effect of alterations in loop 205–219 on the activity and specificity of the enzyme. The cell-bound wild-type and TM proteinases showed only small differences in activity and specificity towards α_{s1}- and β-casein; in contrast, secreted TM/ΔC190 proteinase showed an approx. 3-fold reduced activity towards α_{s1}- and β-casein when compared with cell-bound TM proteinase (results not shown). These activities towards whole caseins are in general agreement with the cell growth experiments in milk (Table 1). The specificity of the secreted TM/ΔC190 proteinase towards the peptide substrate α_{s1}-casein(1–23) was found to differ somewhat from that of the wild-type enzyme (Figure 5). Secreted S433A/ΔC190 and 1S/ΔC190 proteinases, as well as the released 80–82 kDa degradation products were unable to degrade these casein substrates, indicating that these mutant proteinases and fragments were inactive (results not shown).

Stability of mutant proteinases (ΔC190 forms)

Incubation at 30 °C or higher leads to autoproteolysis and inactivation of the 145 kDa wild-type proteinase, mainly through progressive C-terminal shortening (Exterkate and de Veer, 1989; Laan and Konings, 1991; Nissen-Meyer and Sletten, 1991). To test the stability of mutant proteinases we incubated their ΔC190 forms for 5 h at 30 °C. The wild-type proteinase showed some loss of the 145 kDa component (Figure 6, lanes 2 and 3), whereas no change was seen for the 1S proteinase (Figure 6, lanes 4 and 5) or the TM proteinase (Figure 6, lanes 7 and 8). Hence, the mutant enzymes are at least as stable as the wild-type proteinase.

Determination of autoproteolytic processing sites

In order to identify autodegradation products, N-terminal sequencing was performed of several of the protein bands obtained from SDS/PAGE gels. The first ten amino acids of the 165 kDa (TM/ΔC190 proteinase) and 80 kDa (1S proteinase) fragments were Xaa-Ala-Lys-Ala-Asn-Xaa-Met-Xaa-Asn-Val and Asp-Ala-Lys-Ala-Asn-Ser-Met-Ala-Asn-Val respectively, which is the same N-terminus as that of the released, active wild-type enzyme of 145 kDa (Vos et al., 1989a). Several attempts to determine the N-terminal sequence of the 200 kDa form of either the S433A/ΔC190 proteinase or the 1S/ΔC190 proteinase were not successful, probably because these N-termini were blocked. These results suggest that the N-terminal propeptide has not been removed in the 1S/ΔC190 proteinase.

Incubation of mutant proteinases (ΔC190 forms) with wild-type SK11 proteinase

In mixing experiments we investigated whether the 145 kDa wild-type SK11 proteinase could further process the truncated 1S and TM proteinases. The added amount of wild-type proteinase was low enough to be barely detectable by SDS/PAGE (Figure 6, lane 10), but high enough for complete conversion of the Usp45 protein (Figure 6, lanes 6 and 9). Indeed, we found that the 1S proteinase was converted from the 200 kDa into a 165 kDa form (Figure 6, lane 6), equivalent to the main form of the TM proteinase (lane 7), suggesting that the propeptide could be removed intermolecularly. However, this 165 kDa processed form of 1S proteinase was still inactive. No further processing of the 165 kDa form to a 145 kDa component was seen for either the 1S or TM proteinase (Figure 6, lanes 6 and 9). The TM proteinase did not become more active after incubation with wild-type proteinase.

DISCUSSION

Mutagenesis of the loop 205–219 region of the predicted catalytic domain of *L. lactis* SK11 proteinase had a large impact on enzyme activity and autoprocessing. Our results indicate that the original activity and specificity of the enzyme depend on the presence of surface loop 205–219 with the wild-type amino acid sequence. Deletion of loop residues 206–219 resulted in inactivation of the enzyme; the strain that produces the resulting mutant (1S) proteinase was unable to grow in milk-based media (Table 1). Full recovery of enzyme activity of the proteinase, deduced from the growth rate of the mutant strain in milk, was established

by reinserting the wild-type sequence 206–217 with only three amino acid substitutions (i.e. TM proteinase: E205S/E218T/M219S) at the connecting ends of this loop. In contrast, activity could not be restored by insertion at position 205–219 of loops with length varying from 1 to 16 serine residues. The combined results suggest that the spacing between the conserved structural core elements is less important for activity than the appropriate sequence and/or conformation.

The effects of loop mutation on maturation and autoprocessing are summarized in Figure 1. In the wild-type lactococcal proteinase, N-terminal processing at site 1 leads to removal of the propeptide and concomitant activation. In addition, in a Ca^{2+}-free buffer, C-terminal autodigestion at site 2 results in the release of the active 145 kDa enzyme from the lactococcal cells. The position of cleavage site 2 is as yet unidentified, but presumably lies between residues 1127 and 1272 (P. G. Bruinenberg, R. J. Siezen, W. M. de Vos, unpublished work). In contrast, proteinases with mutations in loop 205–219 cannot be released from the lactococcal cells in a Ca^{2+}-free buffer; cell-envelope release fractions of these cells lacked the major proteinase of 145 kDa (Figure 4a). These results demonstrate that C-terminal autoprocessing is inhibited in these mutant enzymes.

Although the TM proteinase is inhibited in C-terminal processing at site 2 it is still active, which suggests that its specificity is altered when compared with wild-type SK11 proteinase. This altered specificity may only be subtle, however, since only small differences were found between wild-type and TM proteinase in the breakdown of casein substrates. Together, these findings support the hypothesis that C-terminal processing of lactococcal proteinases is an autocatalytic event and therefore depends on the specificity of the enzyme. In previous studies, substitutions involving charged residues in the subtilisin-like substrate-binding region and the more distant residues Arg-747 and Lys-748 were found to contribute significantly to the cleavage specificity of the lactococcal proteinases, especially in the case of electrostatic interactions with charged substrates (Vos et al., 1991; Exterkate et al., 1991; Siezen et al., 1993). In the TM proteinase, two of the substitutions in loop 205–219 involve mutation of a negatively charged residue to an uncharged polar residue, i.e. E205S and E218T (Figure 3). Therefore, it is likely that one or both of these charged residues also contributes to the caseinolytic specificity of the enzyme.

The secreted wild-type proteinase (ΔC190) also appeared as a major 145 kDa protein band (Figure 4b), since this secreted form is also processed at site 2 near the C-terminus. In contrast, secreted forms of the mutant proteinases had molecular masses much larger than 145 kDa, which indicates that N- and/or C-terminal processing of these mutant proteinases had not occurred. The truncated, active-site mutant proteinase (S433A/ΔC190) appears predominantly in a 200 kDa form, representing inactive unprocessed proteinase that is not cleaved at any of the sites 1–4 (de Vos et al., 1991); the main form of the 1S/ΔC190 proteinase is also 200 kDa, again suggesting a complete lack of processing. The secreted TM/ΔC190 proteinase is found mainly as a 165 kDa component; the size difference with the S433A/ΔC190 and 1S/ΔC190 proteinases must be explained by removal of the propeptide (approx. 17 kDa), since the determined N-terminus of TM/ΔC190 proteinase corresponds to that of wild-type proteinase. We were unable to determine the N-termini of the 200 kDa S433A/ΔC190 and 1S/ΔC190 proteinases, which supports the hypothesis that both proteins are unprocessed at the N-terminus (see Figure 1). Removal of the propeptide of the 1S/ΔC190 proteinase, by cleavage at site 1, could be induced intermolecularly by addition of wild-type proteinase. In contrast, cleavage at site 2 could not be induced in this fashion, neither in TM/ΔC190 nor in 1S/ΔC190 proteinase. Apparently, cleavage site 2 is no longer accessible in these secreted, mutant proteinases.

Quite surprisingly, when truncated TM proteinase was secreted into the medium a lowered growth rate in milk was observed (Table 1), and also a 3-fold reduction in its activity *in vitro* towards α_{s1}- and β-casein. This phenomenon was not observed for the wild-type SK11 enzyme. We hypothesize that the lowered activity may be due to inhibition of the secreted TM proteinase by its C-terminal extension of over 300 residues, which extends from the position of cleavage site 2 up to residue 1585, possibly through folding back towards the substrate-binding region. This extension is absent in secreted wild-type proteinase, since it is autoprocessed at site 2 (see Figure 1).

Loop 205–219 is located in close proximity to the substrate-binding region (Figure 2), but homology modelling of this insert was not possible since there is no equivalent in the structure of subtilisin (Siezen et al., 1991,1993). However, it is conceivable that this loop is folded back towards the substrate-binding cleft and contributes to substrate binding and to the specificity of the enzyme, as previously also predicted for the more distant residues Arg-747 and Lys-748 (Vos et al., 1991). Alternatively, the 205–219 loop may be involved in binding of the proteinase to another factor, e.g. the maturation protein PrtM that enhances or is required for autocatalytic processing (Vos et al., 1989b) and probably is an extracellular chaperone (Kok and de Vos, 1993). In another engineering study, we recently demonstrated that deletion of residues 137–139 in the predicted substrate-binding region of SK11 proteinase, and subsequent insertions of various size and sequence are tolerated much better and do not lead to inactivation of the mutant enzyme (Siezen et al., 1993).

In summary, the presence of residues 205–219 in loop vr11 is essential for proteolytic activity of the SK11 proteinase. Moreover, we have identified loop 205–219 as a third segment involved in substrate-cleavage specificity. The wild-type amino acid sequence of this loop is required for autoproteolytic processing at site 2 near the C-terminus of the SK11 proteinase. Apparently, processing at site 2 can be prevented by the three mutations in TM proteinase, i.e. E205S, E218T and M219S.

Currently, further deletion studies are being conducted to determine the function of other segments in the SK11 proteinase, located either in the catalytic domain or in the large C-terminal domain, which do not have a counterpart in subtilisin. Deletion of the 151-residue insert (see Figure 2) alters the caseinolytic specificity substantially, but does not affect the N- and C-terminal autoprocessing (Bruinenberg et al., 1994).

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