

## Deletion of Various Carboxy-Terminal Domains of *Lactococcus lactis* SK11 Proteinase: Effects on Activity, Specificity, and Stability of the Truncated Enzyme

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**The *Lactococcus lactis* SK11 cell envelope proteinase is an extracellular, multidomain protein of nearly 2,000 residues consisting of an N-terminal serine protease domain, followed by various other domains of largely unknown function. Using a strategy of deletion mutagenesis, we have analyzed the function of several C-terminal domains of the SK11 proteinase which are absent in cell envelope proteinases of other lactic acid bacteria. The various deletion mutants were functionally expressed in *L. lactis* and analyzed for enzyme stability, activity, (auto)processing, and specificity toward several substrates. C-terminal deletions of first the cell envelope W (wall) and AN (anchor) domains and then the H (helix) domain leads to fully active, secreted proteinases of unaltered specificity. Gradually increasing the C-terminal deletion into the so-called B domain leads to increasing instability and autoproteolysis and progressively less proteolytic activity. However, the mutant with the largest deletion (838 residues) from the C terminus and lacking the entire B domain still retains proteolytic activity. All truncated enzymes show unaltered proteolytic specificity toward various substrates. This suggests that the main role played by these domains is providing stability or protection from autoproteolysis (B domain), spacing away from the cell (H domain), and anchoring to the cell envelope (W and AN domains). In addition, this study allowed us to more precisely map the main C-terminal autoprocessing site of the SK11 proteinase and the epitope for binding of group IV monoclonal antibodies.**

Lactococci are gram-positive bacteria used as starters in a variety of dairy fermentation processes. These bacteria have a complex proteolytic system for the degradation of caseins, the major milk proteins, into small peptides and free amino acids that are subsequently used for cell growth, but they can also contribute to flavor development in fermented milk products (29, 32, 38). A single, cell-wall-bound extracellular proteinase (CEP) is generally considered to be responsible for the initial breakdown of caseins (7, 10, 12, 29, 44, 51, 52). Gene deletion and modification studies have demonstrated that *Lactococcus lactis* strains grow very poorly in milk in the absence of a functional CEP (28, 29, 44).

Three distinctly different types of genes encoding CEPs, referred to as *prtB*, *prtH*, and *prtP* (47), have been cloned and sequenced from dairy lactic acid bacteria (20, 25, 27, 30, 43, 54). The *prtP* gene of *L. lactis* SK11 (54) encodes a pre-protein of 1,962 amino acid residues with a calculated molecular mass of >200 kDa. This precursor is autocatalytically processed at the N terminus and thereby activated during or after membrane translocation. A chaperone or maturation protein, PrtM, is required for this activation of PrtP, and the required *prtM* gene is located directly upstream of the *prtP* gene but is oppositely transcribed (22, 55).

A comparative analysis of CEPs from different lactic acid bacteria led to the prediction of a number of different domains, and their homology, characteristics, and putative functions have been described (47). Starting from the N terminus, the

PrtP of *L. lactis* SK11 is predicted to consist of a pre-prodomain (187 residues) for secretion and activation, a serine protease domain (~510 residues, including an internal inserted domain of 151 residues), two large middle domains A (~410 residues) and B (~480 residues) of predicted regulatory and stabilizing function, a helical spacer domain (~210 residues), a hydrophilic cell wall spacer domain (~130 residues), and a cell wall anchor domain (~40 residues). Not all of these domains are present in the other CEPs, which raises the question as to whether and how the various domains of PrtP contribute to protease activity, specificity, or stability.

The catalytic or protease domain is common to all CEPs and belongs to the superfamily of subtilisin-like serine proteases, also referred to as subtilases (48, 49). Using a homology model for its three-dimensional structure, strategies for protein engineering of the PrtP catalytic domain from *L. lactis* SK11 were developed and implemented, strategies aimed at modulating either stability, catalytic activity, or substrate specificity (3, 4, 10, 49, 50). Mutations near the substrate binding site mainly led to changes in activity and specificity (4, 50). Deletion of the insert of 151 residues in the protease domain led to a three-fold-reduced activity and altered the specificity toward caseins (3). The latter result suggests that through deletion of other domains it may be possible to generate novel PrtP variants with altered properties; these could be useful for mechanistic studies to determine the function of various domains, for application in flavor diversification, or for accelerated cheese ripening but also for facilitated isolation, purification, characterization, and perhaps even crystallization. Proteinases of the kexin family of subtilases also consist of an N-terminal protease domain followed by a number of different C-terminal domains (40). Carboxy-terminal deletion analysis in this family has shown that only the highly conserved middle domain of 140 residues directly coupled to the protease domain is required for full proteolytic activity and specificity, while all other C-terminal

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extensions such as Cys-rich or Ser-Thr-rich domains, transmembrane domains, and cytosolic domains can be deleted (1, 18, 24).

The 40 most C-terminal residues of PrtP are homologous to A3-type cell wall-membrane anchor sequences identified in a great number of cell envelope proteins from other gram-positive bacteria (37, 41, 54). Initial C-terminal deletion analysis of the *prtP* gene has demonstrated that the absence of this membrane anchor results in secretion of the proteinase into the medium; furthermore, C-terminal segments up to 403 residues can be deleted without loss of PrtP activity or specificity (2, 8, 31). Alternatively, release from the cell envelope of a fully active, truncated form of PrtP can be induced by treating cells with Ca-free buffer (12, 13, 15, 23, 33, 39). This "released form" of PrtP has a molecular mass of about 145 kDa, implying that ca. 500 C-terminal residues have been removed. This release is believed to result from an intramolecular autoproteolytic event at a site that becomes accessible only after the removal of Ca<sup>2+</sup> ions (15, 33); this cleavage site(s) has not been identified yet.

We have undertaken here a more extensive C-terminal truncation analysis by deletion mutagenesis to address the following issues: (i) what is the effect of removal of different domains by progressive C-terminal truncation on the proteolytic activity, specificity and stability of the SK11 proteinase; (ii) what is the minimal size of a stable and active enzyme; and (iii) where is the autocatalytic cleavage site located that leads to "release" of the enzyme in Ca-free medium?

#### MATERIALS AND METHODS

**Bacterial strains and media.** *Escherichia coli* MC1061 (5) was grown in *L* broth-based media and was used as intermediate host for DNA constructions. Strain *L. lactis* subsp. *lactis* MG1363 is a plasmid-free, proteinase-deficient derivative of *L. lactis* subsp. *lactis* NCDO 712 (19) that was used as a host for all proteinase 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% (wt/vol) pasteurized, reconstituted skimmed milk or in whey permeate medium (8) containing 1.9% (wt/vol)  $\beta$ -glycerolphosphate and 0.1% (wt/vol) Casitone (Difco Laboratories, Detroit, Mich.). If appropriate, the media contained 0.5% (wt/vol) glucose and chloramphenicol (10  $\mu$ g/ml).

**Molecular cloning.** Isolation of plasmid DNA from *E. coli* and standard recombinant DNA techniques were performed as described previously (45). All enzymes were purchased from Bethesda Research Laboratories (Breda, The Netherlands), Boehringer Mannheim (Almere, The Netherlands), or New England Biolabs Corp. (Hitchin, Herefordshire, United Kingdom) and were used according to the manufacturer's instructions. Isolation of plasmid DNA from *L. lactis* and transformation of *L. lactis* were performed as described previously (8, 55). Recombinant *L. lactis* strains were analyzed by restriction enzyme analysis and direct sequencing of double-stranded plasmid DNA (21, 46, 57).

**Construction of C-terminal deletions of the proteinase gene.** Plasmid pNZ521 contains the complete *prtP* gene and a functional *prtM* gene of *L. lactis* subsp. *cremoris* strain SK11 resulting in production of an active proteinase located in the cell envelope (8). Plasmid pNZ527 (denoted previously as pNZ521 $\Delta$ H) was constructed by deletion of a 16-bp *Hind*III fragment from pNZ521 (2); frameshift readthrough then reaches a stop codon after four codons. This plasmid encodes a proteinase lacking the 190 most C-terminal residues ( $\Delta$ 190), which results in secretion of the truncated proteinase into the growth medium. Plasmid pNZ596 was constructed by deletion of a 1,671-bp *Kpn*I fragment from pNZ521, filling in the sticky ends with Klenow fragment of DNA polymerase I of *E. coli*, and subsequent ligation of plasmid DNA. Plasmid pPR31 containing the structural *prtP* gene (54) was completely digested with *Bgl*II and *Bst*EII and partially digested with *Nde*I. The sticky ends were filled in with Klenow and ligated. For the production of mutant proteinases in *L. lactis*, correctly mutated *Eco*RI-*Sac*I *prtP* gene fragments were used to construct derivatives of pNZ521 containing a mutant *prtP* gene. The resulting plasmids were designated pNZ522 (*Nde*I), pNZ523 (*Bst*EII), and pNZ524 (*Bgl*II). In this way, all constructed plasmids contained a frameshift mutation (resulting in stop codons within 20 codons) in the C-terminal part of the coding region of the *prtP* gene. Plasmid pNZ574 is a derivative of pNZ527 containing the S433A mutation of the catalytic Ser residue, which leads to an inactive proteinase with the propeptide still attached to the N terminus (9, 50). All constructs were verified by DNA sequence analysis of relevant regions.

**Growth studies.** The ability of *L. lactis* cells to produce a functional proteinase was assayed by growth of these cells in 10% (wt/vol) pasteurized, reconstituted skimmed milk. The maximum specific growth rate ( $\mu_{\max}$ ) values of lactococcal strains in milk were determined by measuring the optical density at 600 nm (OD<sub>600</sub>) of cultures clarified by using a modified EDTA-borate treatment (26, 42).

**Proteinase expression studies.** Lactococcal cells were grown in whey-based medium to the mid-log growth phase (OD<sub>600</sub> = 0.9), and wild-type proteinase was released from the cell envelope by incubation in Ca<sup>2+</sup>-free buffer (cell-envelope "release fraction") as described previously (13, 39). Secreted proteinase was isolated from the culture medium by freeze-drying dialyzed samples (8). Proteinase samples isolated from equal amounts of lactococcal cells (as determined by measuring the OD<sub>600</sub>) were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (36) that were stained with Coomassie brilliant blue. Proteinases were detected by Western blotting using polyclonal antibodies raised against SK11 proteinase (8) and monoclonal antibodies (MAbs) of groups I and IV raised against the cell envelope proteinase PrtP of *L. lactis* strain Wg2 which cross-react with those of strain SK11 (34, 35).

**Proteinase activity assays.** The proteolytic activity of secreted SK11 proteinases was measured at pH 6.5 and at 30°C toward  $\alpha$ - and  $\beta$ -casein (56) and the cheese peptide  $\alpha$ -s<sub>1</sub>-casein-(1-23) fragment (14, 16). Initial activities toward the chromophoric substrate Suc-Ala-Glu-Pro-Phe-pNA (Bachem AG) were measured at pH 6.8 and 25°C (11).

#### RESULTS

**Analysis of proteinase C-terminal truncation mutants.** Lactococcal plasmids carrying mutant *prtP* genes encoding wild-type or truncated SK11 proteinases were introduced into *L. lactis* MG1363 and functionally expressed. An overview of the resulting C-terminally truncated proteinases is given in Table 1 and Fig. 1.

Wild-type and recombinant strains were assayed for their ability to grow in milk (Table 2). Only strain MG1363(pNZ527), which specifies a proteinase lacking the C-terminal 190 residues, showed a  $\mu_{\max}$  in milk identical to that of strain MG1363(pNZ521) expressing wild-type proteinase (Table 2). The  $\mu_{\max}$  of other mutant strains gradually declines as the C-terminal truncation of the proteinase increases. While strain MG1363(pNZ522) encoding PrtP lacking 503 C-terminal residues grew only slightly more slowly than wild type, the MG1363(pNZ524) strain expressing PrtP with the largest C-terminal truncation (838 residues) showed a fourfold reduced  $\mu_{\max}$  in milk. In contrast, strains without proteinase (MG1363) or an inactive proteinase (MG1363 harboring pNZ574) grew extremely slowly and did not reach high cell densities in milk (Table 2).

Since all PrtP mutants lack the C-terminally located membrane anchor, the truncated proteinases are secreted into the growth medium. SDS-polyacrylamide gel electrophoresis (PAGE) of supernatant fractions shows that strain MG1363(pNZ527) produces a proteinase with an apparent molecular mass of about 145 kDa (Fig. 2A, lane 1), despite the fact that a truncated proteinase of 168 kDa is encoded (Table 1). It has been shown previously that MG1363(pNZ521) produces wild-type PrtP that remains anchored to the cell but can be released as a 145-kDa autodigestion product upon incubation in Ca-free medium (2, 4, 50). Therefore, it appears that the PrtP(1-1585) encoded by strain MG1363(pNZ527) is further autoprocessed C-terminally to generate the same 145-kDa product as the "released" wild-type PrtP. As a control, the strain with plasmid pNZ574, specifying the inactive S433A-PrtP lacking the last 190 residues (8, 50), secretes a main component with an apparent molecular mass of about 190 kDa (Fig. 2A, lane 6), as expected in the absence of both C-terminal autoprocessing and N-terminal autoprocessing of the propeptide of 154 residues (Table 1).

In contrast, for *L. lactis* MG1363 harboring pNZ522, pNZ596, pNZ523, and pNZ524 the slowest-migrating proteinase band corresponded to an apparent molecular mass of ca. 145, 125, 110, and 100 kDa, respectively (Fig. 2A, lanes 2 to 5),

TABLE 1. Characteristics of wild-type and truncated *L. lactis* PrtP

Plasmid	Truncation at restriction site	Residual N-terminal residues (range)	Deleted C-terminal residues (total no.)	Apparent mass (kDa)		Residual activity <sup>a</sup>	Binding of MAb IV	Comments (reference)
				Calculated	SDS-PAGE <sup>b</sup>			
pNZ521		1-1775	0	186	145	++++	+	Wild type (8)
pNZ527	<i>Hind</i> III	1-1585	190	168	145	++++	+	This work (2)
pNZ511	<i>Sal</i> I	1-1372	403	147	145	++++	ND	Reference 8
pNZ522	<i>Nde</i> I	1-1272	503	137	145	+++	+	This work
pNZ596	<i>Kpn</i> I	1-1129	646	121	125	++	+	This work
pNZ523	<i>Bst</i> EII	1-1091	684	116	110	+	-	This work
pNZ524	<i>Bgl</i> II	1-937	838	102	100	+	-	This work
pNZ574	<i>Hind</i> III	(-154)-1585	190	184	190	-	+	Active-site mutant S433A (9)

<sup>a</sup> Approximate average of activities against various substrates (see text): +++++, 100%; +++, 50 to 100%; ++, 10 to 50%; +, 1 to 10%; and -, <1%.

<sup>b</sup> Apparent mass of the largest component observed.

which is in good agreement with the theoretical calculated mass (Table 1). In addition, the supernatant from strains MG1363(pNZ596) and MG1363(pNZ523) (Fig. 2A, lanes 3 and 4, respectively) show several PrtP autodegradation products, including two major proteolytic fragments of approximately 90 kDa (from pNZ596) and 74 kDa (from pNZ523). These results indicate that the truncated proteinases PrtP(1-1129) and PrtP(1-1091) are more sensitive to autoproteolysis than PrtP(1-1272) or longer variants. The enzyme with the largest C-terminal truncation, PrtP(1-937), is extremely sensitive to autoproteolysis since very little undegraded protein is detected on the gel (Fig. 2A, lane 5).

Usp45, the secreted 60-kDa protein of *L. lactis* of unknown

function (53), is a natural substrate for the cell envelope proteinase, and it is completely degraded in the supernatant of strains expressing PrtP with a wild-type level of activity, viz., MG1363 harboring pNZ521 (2, 4), pNZ527 (Fig. 2A, lane 1), and pNZ522 (Fig. 2A, lane 2). Analysis of extracellular proteins of strains expressing PrtP with larger C-terminal deletions (Fig. 2A, lanes 3 to 5) or expressing an inactive PrtP (Fig. 2A, lane 6) show significant amounts of undigested Usp45 protein, suggesting that these mutants show reduced proteolytic activity toward this natural substrate.

**Identification of (autodegradation) products and mapping of epitope.** Polyclonal antibodies raised against SK11 proteinase and MABs of groups I and IV raised against Wg2 protein-

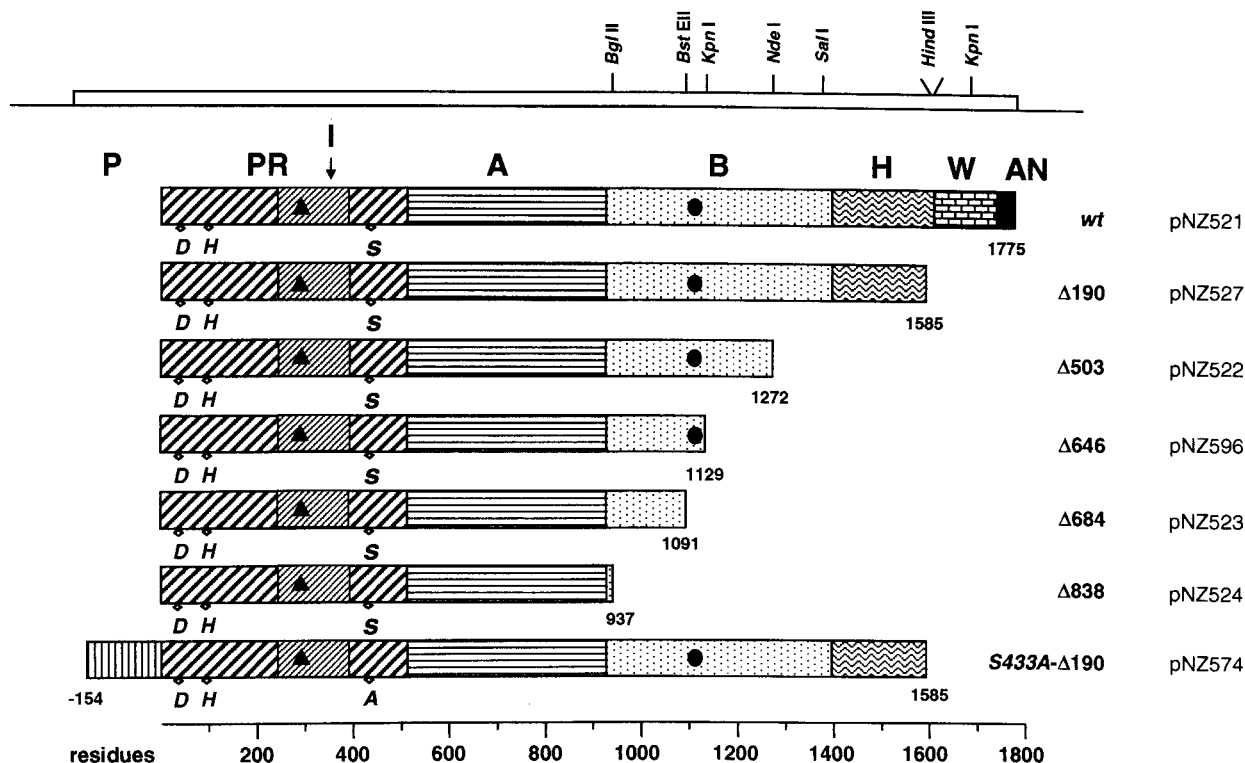


FIG. 1. Schematic representation of wild-type and C-terminally truncated SK11 proteinases. Domains: propeptide, P; protease, PR; insert, I; A; B; helix, H; wall, W; anchor, AN (47). Residue numbering begins at the N terminus of the mature enzyme. The number of C terminally deleted residues is indicated at the right. The approximate position of catalytic residues D, H, and S in the PR domain are indicated. Putative MAB I (▲) and IV (●) binding sites are indicated. The positions of relevant restriction sites used in cloning experiments are indicated in the coding region of the *prtP* gene at the top, and plasmids encoding the proteinases are shown at the far right.

TABLE 2. Maximum specific growth rates ( $\mu_{\max}$ ) in milk of *L. lactis* MG1363 harboring different proteinase plasmids

Plasmid	$\mu_{\max}$ ( $\text{h}^{-1}$ ) <sup>a</sup>
pNZ521 (wild-type PrtP) .....	0.63
pNZ527.....	0.63
pNZ522.....	0.58
pNZ596.....	0.34
pNZ523.....	0.23
pNZ524.....	0.15
pNZ574.....	<0.05
None <sup>b</sup> .....	<0.05

<sup>a</sup>  $\mu_{\max}$  values are the average of three experiments (standard deviation,  $\pm 0.03$ ).

<sup>b</sup> Strain *L. lactis* MG1363 is a plasmid-free, proteinase-deficient strain which is unable to grow in milk (19).

ase, which cross-react with SK11 proteinase (34, 35), were used to identify autodegradation products of the C-terminally truncated SK11 proteinases. Group I MAbs are directed against an epitope of SK11 proteinase between residues 238 and 388, consisting of an external loop of the proteolytic domain (3), also referred to as the I (insert) domain (47). These group I MAbs were found to react with the major 145-kDa protein band of SK11 proteinase secreted by MG1363(pNZ527) and MG1363(pNZ522) (Fig. 2B, lane 1 and 2) and with the major 125-, 110-, and 100-kDa protein bands produced by strains with plasmids pNZ596, pNZ523, and pNZ524, respectively (Fig. 2B, lanes 3, 4, and 5).

Group IV MAbs are directed against an epitope located between Thr816 and Leu1219 of the SK11 and Wg2 proteinases (35). These group IV antibodies reacted with the 145-kDa band from strains MG1363(pNZ527) and MG1363(pNZ522) and the 125-kDa band from MG1363(pNZ596) (Fig. 2C, lanes 1, 2, and 3, respectively) but also with several other PrtP fragments present in these supernatants, particularly from MG1363(pNZ596). However, these group IV MAbs do not bind to any protein bands from strains MG1363(pNZ523) and MG1363(pNZ524) secreting the truncated enzymes PrtP(1-1091) and PrtP(1-937), respectively (Fig. 2C, lanes 4 and 5). These results indicate that the epitope of group IV MAbs is located in a region between Ala1092 and Thr1129 of the SK11 proteinase. The 90- and 74-kDa protein bands found in supernatants of cells with pNZ596 and pNZ523 both react with polyclonal antibodies raised against SK11 proteinase (not shown), identifying these bands as proteolytic fragments derived from SK11 proteinase. Furthermore, the 90-kDa protein reacts with group IV MAbs (Fig. 2C, lane 3) but not with group I MAbs (Fig. 2B, lane 3), while the 74-kDa band binds neither group I MAbs (Fig. 2B, lane 4) nor group IV MAbs (Fig. 2C, lane 4). These results indicate that both the 90-kDa and the 74-kDa fragments represent N-terminal autodegradation products lacking the group I epitope between residues 238 and 388.

**Activity and specificity of mutant proteinases.** We analyzed the effect of increasing C-terminal deletions on the activity and specificity of the SK11 enzyme toward several substrates. All truncated proteinases were still able to degrade  $\beta$ -casein (Fig. 3) and  $\alpha_{s1}$ -casein (data not shown), but less casein degradation was found as C-terminal truncation of PrtP increased (Table 1). Similar results were obtained using the  $\alpha_{s1}$ -casein-(1-23) substrate (Fig. 4) and the chromophoric substrate suc-Ala-Glu-Pro-Phe-pNA (data not shown). The specific activity of the PrtP mutants toward these substrates cannot be determined accurately, since the truncated proteinases significantly differ in their stability (see Fig. 2). Based on the incubation times needed for a comparable amount of degradation of various

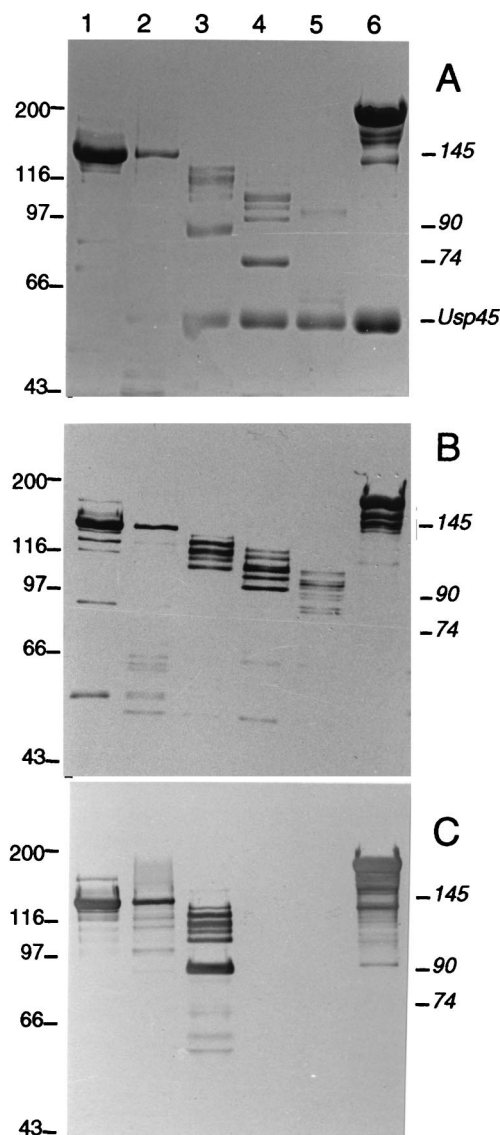


FIG. 2. SDS-PAGE and Western blot analysis of proteins secreted into the growth medium of lactococcal cells harboring pNZ527 (lane 1), pNZ522 (lane 2), pNZ596 (lane 3), pNZ523 (lane 4), pNZ524 (lane 5), and pNZ574 (lane 6). (A) Coomassie brilliant blue staining. (B) Western blot with MAbs of group I. (C) Western blot with MAbs of group IV. Molecular mass markers (in kilodaltons) are indicated to the left. Positions of Usp45 and the 145-, 90-, and 74-kDa products of SK11 proteinase are indicated on the right.

substrates, the supernatant containing SK11 proteinase with the largest truncation (838 residues) has approximately 5% residual activity compared to that of the wild-type enzyme (see, for example, Fig. 4). While the various truncated PrtP species differed considerably in residual activity, no clear differences were found in specificity toward the tested substrates, e.g., the bonds cleaved in  $\alpha_{s1}$ -casein-(1-23) were always 16-17, 17-18, and 21-22 (Fig. 4), the same as those cleaved by wild-type PrtP (14, 50).

## DISCUSSION

Using a strategy of deletion mutagenesis we have analyzed the function of several C-terminal domains of the SK11 pro-

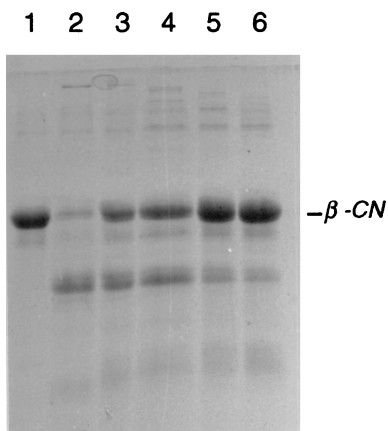


FIG. 3. Caseinolytic activity of C terminally truncated SK11 proteinase mutants toward  $\beta$ -casein. Lane 1, no proteinase added; lanes 2 to 6, supernatant fractions added containing truncated proteinases specified by plasmids pNZ527 (lane 2), pNZ522 (lane 3), pNZ596 (lane 4), pNZ523 (lane 5), and pNZ524 (lane 6).

teinase which are absent in other CEPs of lactic acid bacteria (47). The truncations led first to loss of the cell envelope W (wall) and AN (anchor) domains (pNZ527), then to further loss of the H (helix) domain (pNZ511), and then to partial deletions (pNZ522, pNZ596, and pNZ523) or entire deletion of the B domain (pNZ524), as schematically depicted in Fig. 1. The various deletion mutants were functionally expressed in *L.*

*lactis* and analyzed for enzyme activity, (auto)processing, and specificity toward several substrates. All of the C-terminally truncated enzymes were secreted and exhibited the expected size of normally N-terminally-autoactivated proteinases, except the enzyme specified by pNZ527 which was also C-terminally autoprocessed (Fig. 2), presumably in the same way as wild-type lactococcal proteinase in Ca-free medium (2, 23).

We previously found that the activity and caseinolytic specificity of two secreted SK11 proteinases specified by plasmids pNZ527 (lacking 190 3' codons) and pNZ511 (lacking 402 3' codons) is identical to that of the cell-envelope-bound, wild-type enzyme (2, 8). Therefore, we can now conclude that the three most C-terminal domains, H, W, and AN, are not required for obtaining wild-type activity, specificity, or stability. Their function appears to be tethering to the cell envelope (W and AN domains) and acting as spacers (H and W domains) to position the other N-terminal domains away from the cell (47). Our present study indicates that larger C-terminal deletions ranging from 503 up to 838 codons, corresponding to progressive deletion of the B domain, significantly affect the stability and residual activity of the proteinase toward various tested substrates such as Usp45 (Fig. 2A),  $\beta$ -casein (Fig. 3),  $\alpha_{s1}$ -casein-(1-23) (Fig. 4), and total milk protein (Table 2). We have shown earlier that the thermal stability and residual activity of the wild-type SK11 proteinase is directly related to autoproteolysis (50). Therefore, the observed lower stability of C-terminally truncated enzymes presumably makes them increasingly sensitive to autoproteolysis (Fig. 2), leading to loss of active enzyme. Domain B may therefore play a role in the stabilization of the PR and/or A domains, at least in the se-

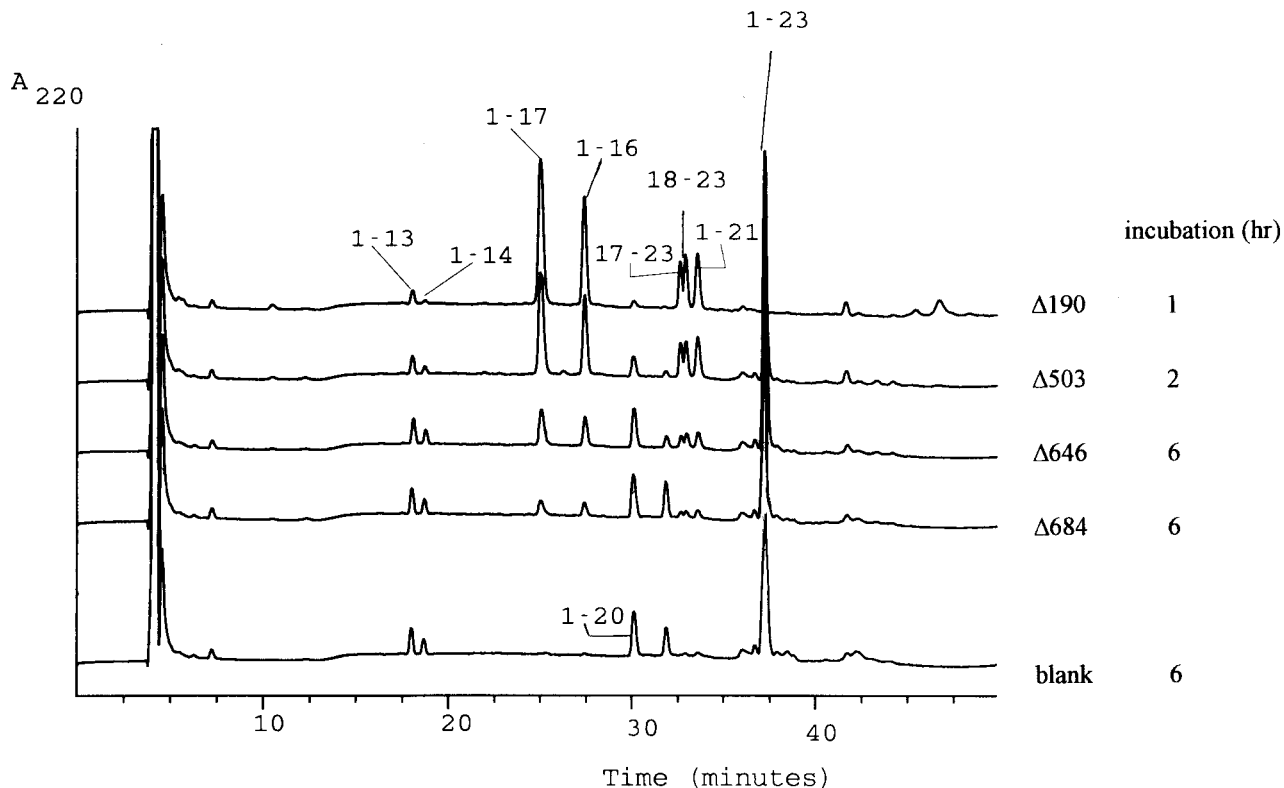


FIG. 4. Caseinolytic activity and specificity of C terminally truncated SK11 proteinase mutants toward  $\alpha_{s1}$ -casein-(1-23). Analytical reversed-phase high-pressure liquid chromatography patterns of the products of  $\alpha_{s1}$ -casein-(1-23) degradation. Note that incubation times were varied between 1 and 6 h to allow for the large differences in proteinase activity. The "blank" data refer to S433A/ $\Delta 190$  (pNZ527), which has no PrP activity but shows a low background activity toward the substrate due to released intracellular PepO activity (12, 15).

creted enzyme. Although the smallest construct PrtP(1–937), consisting only of the PR and A domains (called the PR-A form), still appears to be active, its specificity and specific activity cannot be determined due to its great instability. Suggestions for stabilization could come from studies of the homologous streptococcal proteinases ScpA from *Streptococcus pyogenes* (6), Csp from *S. agalactiae* (T. O. Harris and C. E. Rubens, personal communication), and PrtS from *S. thermophilus* (V. Monnet, personal communication), which are active and stable in the absence of a B domain (47).

While having a profound effect on stability, none of the C-terminal deletions of PrtP altered the specificity of the truncated proteinases. Therefore, the B, H, W, and AN domains do not play a role in determining substrate specificity, and they are presumably not in the vicinity of the substrate binding region of the PR domain. This is clearly in contrast to deletion of the I domain that led to altered specificity in addition to lower activity (3).

We have identified main 90- and 74-kDa components present in the supernatant of MG1363(pNZ596) and MG1363(pNZ523) cells, respectively, as N-terminal autodegradation products of the SK11 proteinase (Fig. 2). Both the 90- and 74-kDa components lack the MAb group I epitope, and their size suggests that both lack ca. 300 N-terminal residues, so autocleavage presumably occurs in the I domain after the group I MAb binding site. As a consequence, these 74- and 90-kDa forms are likely to be inactive since they lack the catalytic Asp and His residues (Fig. 1). If these inactive fragments can be purified they could prove to be useful in the elucidation of the structure and possibly the function of the A domain, but only if correct folding is retained. The epitope of group IV MAbs, previously mapped between residues 816 and 1219 of the Wg2 and SK11 proteinases (35), has now been more precisely mapped between residues Ala1092 and Thr1129 of the SK11 proteinase sequence (Fig. 1 and 2). Therefore, group IV MAb can be used as a specific probe for the B domain of PrtP (and in particular its central part).

Washing of the lactococcal cells in a  $Ca^{2+}$ -free buffer results in release of the active 145-kDa proteinase from the lactococcal cell envelope due to autoproteolysis. This deletion mutagenesis study has allowed us to locate this important C-terminal autoprocessing site(s) in the B domain somewhere near residue 1272, since the truncated enzyme PrtP(1–1272) also has an apparent molecular mass of 145 kDa on SDS-PAGE (Fig. 1, Table 1).

In summary, we have answered many of the questions initially posed at the outset but, unfortunately, we were not yet able to generate stable, C terminally truncated proteinases with altered specificity. An alternative approach to elucidate functions and/or necessity of domains could be to construct genes encoding CEPs that lack one or more "internal" domains, for instance, by deleting the A, B, and/or H domains while retaining the W and/or AN domains. Such natural CEPs have already been found in other lactic acid bacteria (47). Novel hybrid enzymes could also be made by recombination of the domains of CEPs from different species of lactic acid bacteria. Previously, such hybrid enzymes were made between two highly homologous PrtP variants and provided insight into residues involved in determining substrate specificity (56). Hybrids formed from more distantly related CEPs could broaden the scope of innovation and application of extracellular proteinases, not only by modulating known functions such as stability, proteolytic activity/specificity, or cell wall attachment but also to introduce entirely new functions, such as adhesion, antibody binding, receptor binding, etc.

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