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Processing of the Lactococcal Extracellular Serine Proteinase

ALFRED J. HAANDRIKMAN,[1]* RENE MEESTERS,[1] HARRY LAAN,[2] WIL N. KONINGS,[2] JAN KOK, AND GERARD VENEMA[1]

Departments of Genetics[1] and Microbiology,[2] University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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Activity of the lactococcal cell envelope-located serine proteinase depends on the presence of membrane-associated lipoprotein PrtM. To differentiate between the action of the proteinase and the action of PrtM in the process of proteinase maturation, an inactive form of the lactococcal proteinase was constructed. This was done by mutating one of the three amino acids thought to constitute the active site of the enzyme. The secreted form of this inactivated proteinase was the same size as the inactive secreted form of the proteinase produced in the absence of PrtM. Both inactive proteinases are larger than the active proteinase. Isolation of proteinase by washing lactococcal cells carrying the complete proteinase gene in a Ca^{2+}-free buffer was prevented by the absence of *prtM* or the absence of a functional active site. We propose that PrtM, during or after membrane translocation of the proteinase, effects the autoproteolytic removal of the N-terminal pro region of the proteinase. Subsequent C-terminal autodigestion results in the release of the enzyme from the lactococcal cells.

Lactococcus lactis is used for the production of a wide variety of cheeses. For this application the ability of the bacteria to grow fast in milk is of major importance. Because of the fastidious nature of lactococci, rapid growth in milk is dependent on the presence of a proteolytic system capable of degrading casein into small peptides and amino acids, which serve as a nitrogen source for growth. The key enzyme in this proteolytic system is a large cell-associated serine proteinase (12, 29).

The proteinase gene regions of the following three different lactococcal strains have been cloned and sequenced: *L. lactis* subsp. *cremoris* Wg2 (14) and SK11 (33) and *L. lactis* subsp. *lactis* NCDO 763 (11). All lactococcal proteinase genes (designated *prtP*) reported thus far are plasmid located. They are transcribed from regulatory sequences within a 0.35-kb *Cla*I DNA fragment, as are the divergently transcribed *prtM* genes (11, 14, 33).

In contrast to the differences in immunological properties and specificities of casein breakdown of the proteinases, these enzymes exhibit an extremely high degree of conservation (12). The lactococcal proteinases also exhibit considerable amino acid sequence similarity with subtilisins (14). This similarity especially applies to the regions containing the amino acids of the subtilisin catalytic center, Asp-32, His-64, and Ser-221. On the basis of this similarity, it has been proposed that the residues Asp-30, His-94, and Ser-433 constitute the active site of the lactococcal proteinase. By combining biochemical and genetic data, it was shown that the lactococcal proteinases are, analogous to subtilisins, initially synthesized as precursors that carry a 187-amino acids pre-pro region (11, 19, 34). After translocation across the cytoplasmic membrane, the mature lactococcal proteinases remain associated with the lactococcal cells. A membrane anchorlike structure that is present in the extreme C terminus of the proteinase is responsible for cell association of the enzyme. Consequently, removal of 130 or more C-terminal amino acids leads to the secretion of the proteinases into the culture medium (9, 11, 34).

Deletion of *prtM* leads to the production and (in the case of a C terminally truncated enzyme) secretion of an inactive proteinase (9, 34). Since there is a considerable size difference between the largest forms of the secreted proteinases in the presence and absence of *prtM*, it has been proposed that the PrtM protein is required for maturation of the proteinase precursor (9, 34). The *prtM*-encoded protein, which was isolated after overexpression in *Escherichia coli*, was identified as being a membrane-associated lipoprotein (8a).

To differentiate between the action of the proteinase and the action of PrtM in proteinase maturation, an active site mutant of the *L. lactis* subsp. *cremoris* Wg2 proteinase was constructed. The inactive proteinase produced by an Asp-30→Asn-30 active-center mutant was the same size as the inactive proteinase produced in the absence of *prtM*. Both inactive forms of the proteinase were larger than the active proteinase made in the presence of *prtM*. We concluded that this size difference results from autoproteolytic activity. Furthermore, we found that the lactococcal proteinase is released from the cells by C-terminal autodigestion.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. *L. lactis* subsp. *lactis* MG1363 (8) was grown in M17 broth supplemented with 0.5% (wt/vol) glucose (28), in whey-based medium (32), or in 10% (wt/vol) reconstituted skim milk supplemented with 2% (wt/vol) β-glycerophosphate and 0.5% (wt/vol) glucose. *Bacillus subtilis* PSL1 (25) was grown in TY broth and was used as a host in the construction of plasmids pGKV1500 and pGKV1552. Erythromycin was added to *B. subtilis* and *L. lactis* cultures to a final concentration of 5 μg/ml. *E. coli* BMH 71-18 and MK 30-3 were grown in TY broth and were the hosts used for M13 phages and their derivatives.

Molecular cloning, site-directed mutagenesis, and DNA sequence analysis. We used the general molecular cloning techniques described by Maniatis et al. (22). Restriction enzymes were purchased from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany. Plasmid DNA was isolated essentially by the method of Birnboim and Doly (1). Protoplast transformation of *B. subtilis* was

* Corresponding author.

TABLE 1. Bacterial strains, phages, and plasmids used

Strain, plasmid, or phage	Relevant phenotype or genotype	Reference or source
E. coli strains		
BMH71-18	Δ(*lac-proAB*) *thi supE F' lacI*^{q} *ZΔM15 proA*$^+$*B*$^+$	16
BMH71-18 mutS	BMH 71-18 *mutS215*::Tn*10*	17
MK 30-3	Δ(*lac-proAB*) *recA galE strA F' lacI*^{q} *ZMΔ15 proA*$^+$*B*$^+$	16
B. subtilis PSL1	*arg leu thi stp recE4*	25
L. lactis MG1363	Prt$^-$, plasmid-free derivative of *L. lactis* NCDO 712	8
Plasmids		
pGKV2	Em^r Cm^r	32
pGKV500	Em^r, containing *prtP* (lacking 130 3′ codons) and *prtM* (lacking three 3′ codons)	15
pGKV507	Em^r, *prtM* deletion derivative of pGKV500	13
pGKV1500	Em^r, pGKV500 specifying Asp-30→Asn-30 proteinase	This work
pGKV552	Em^r, containing the complete *prtP* gene and *prtM* (lacking three 3′ codons)	Laboratory collection
pGKV550	Em^r, containing the complete *prtP* gene but lacking *prtM*	9
pGKV1552	Em^r, pGKV552 specifying Asp-30→Asn-30 proteinase	This work
Phages		
M13mp9		23
M13mp9rev		17

performed as described by Chang and Cohen (3). *L. lactis* was transformed by electroporation, using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), as described by Leenhouts et al. (21). A 645-bp *Hpa*I-*Bam*HI DNA segment from pGKV500 (Fig. 1), which carried the codons for amino acids −41 to 174, was cloned in M13mp9 digested with *Bam*HI and *Sma*I. Single-stranded DNA from the resulting phage was used to form a gapped duplex with heat-denatured replicative-form DNA of phage M13mp9rev digested with *Bam*HI and *Sma*I (23). This gapped duplex and a synthetic 20-mer (CTCGGTTATTΔACAGTGGCA; base substitution is underscored) were used to perform site-directed mutagenesis as described by Kramer et al. (17). A 587-bp *Bam*HI-*Eco*RV fragment from the mutated phage was used to replace the corresponding DNA fragments in pGKV500 and pGKV552, resulting in plasmids pGKV1500 and pGKV1552, respectively (Fig. 1). Plasmids pGKV1500 and pGKV1552 were made single stranded in the region carrying this point mutation by digestion with *Bam*HI and subsequent treatment with *E. coli* exonuclease III (Biolabs Research Laboratories, Gaithersburg, Md.). Nucleotide sequence analysis to confirm the presence of the point mutation was performed by using the dideoxynucleotide sequencing method described by Sanger et al. (26).

Proteinase isolation. Secreted proteinase from whey-grown cultures of *L. lactis* MG1363 carrying plasmid pGKV500,

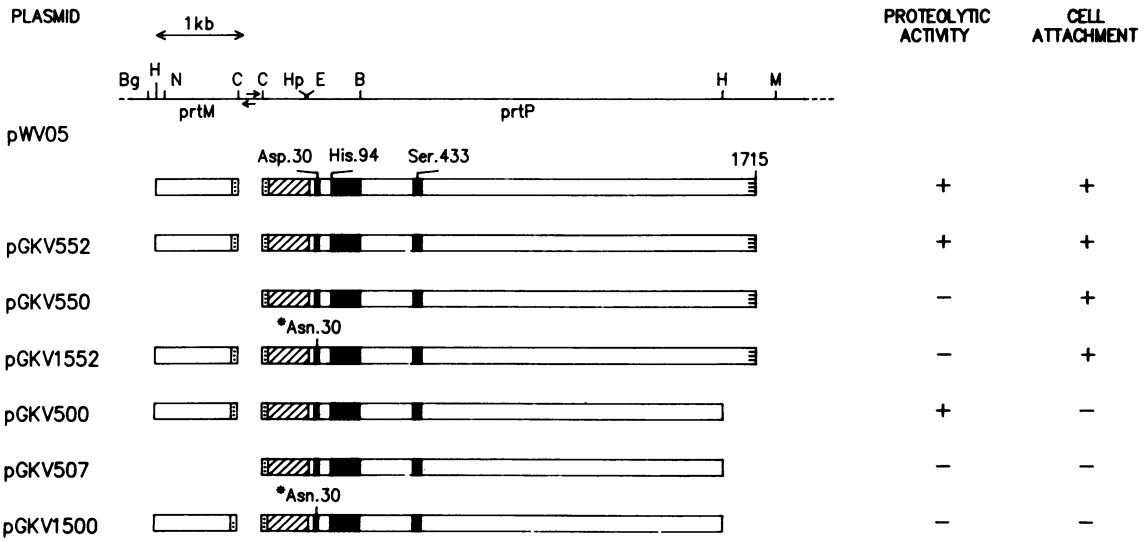

FIG. 1. Schematic representation of the proteinase gene region of *L. lactis* subsp. *cremoris* Wg2 plasmid pWV05. The gene products of proteinase gene *prtP* and maturation gene *prtM* of pWV05 and the plasmids used in this study are indicated as bars. The three regions of homology of the proteinase with subtilisins are indicated by solid boxes. Signal sequences are indicated by stippled boxes. The proteinase membrane anchor is indicated by small horizontally striped regions. The pro region is indicated by cross-hatched boxes. The locations of Asp-30, His-94, and Ser-344 of the active site are shown, as is the location of the Asp-30→Asn-30 mutation. The presence of proteinase activity and the presence of proteinase attachment to lactococcal cells are indicated on the right. The promoter regions of *prtP* and *prtM* are indicated by small arrows. Abbreviations for restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I (not all sites are shown); M, *Mlu*I; N, *Nru*I.

pGKV507, or pGKV1500 was isolated by freeze drying dialyzed culture supernatants, as described by Vos et al. (34). Cell-associated proteinase was released from *L. lactis* MG1363 carrying plasmid pGKV552, pGKV550, or pGKV 1552 by washing the cells from 25-ml portions of overnight whey cultures twice in 0.5-ml portions of 50 mM $NaHPO_4$–acetate (pH 6.5) (34). From the combined release fractions, 10-μl samples were subjected to sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE) (20).

Western blotting and immunodetection. Proteins separated on SDS-polyacrylamide gels were transferred to type BA85 nitrocellulose filters (Schleicher and Schuell, Inc., Keene, N.H.) as described by Towbin et al. (30). Proteinase antigen was detected by using proteinase-specific monoclonal antibodies Wg2-1 and Wg2-9 (19) and alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Promega Biotec, Madison, Wis.) according to the manufacturer's instructions.

Proteinase activity measurements. Proteinase activity in overnight milk cultures of *L. lactis* was assayed by using the *O*-phthaldialdehyde spectrophotometric assay (4). Proteinase activity in cultures grown overnight in whey-based medium was assayed with the synthetic substrate methoxy-succinyl-arginyl-prolyl-tyrosyl-*p*-nitroanilide (MeOSuc-Arg-Pro-Tyr-pNA) (Kabi Diagnostica, Stockholm, Sweden) (5). To 200 μl of a *L. lactis* culture (if necessary diluted with fresh culture medium) 25 μl of 10 mM MeOSuc-Arg-Pro-Tyr-pNA and 25 μl of 100 mM $NaHPO_4$ (pH 6.5) were added. Following 15 min of incubation at 30°C, 50 μl of 80% (vol/vol) acetic acid was added. When incubation times longer than 15 min were necessary, 5 μg of chloramphenicol per ml was added to prevent further growth of the *L. lactis* cells. After centrifugation, 250 μl of each supernatant was transferred to a microtiter plate. The A_{405} was measured by using a Titertek Multiskan model MCC/340 P instrument (Flow Laboratories, Rickmansworth, United Kingdom).

RESULTS

Effect of the Asp-30→Asn-30 proteinase mutation or the absence of PrtM on proteinase activity. A lactococcal proteinase active-site mutant was made on the basis of the amino acid sequence similarity between this proteinase and subtilisin (14). We replaced the Asp-30 GAC codon with an Asn AAC codon by performing site-directed mutagenesis as described in Materials and Methods. To investigate whether the Asp-30→Asn-30 mutation introduced into the lactococcal proteinase resulted in the formation of a proteolytically inactive enzyme, pGKV1500 and pGKV1552 were transferred to plasmid-free and proteinase-deficient *L. lactis* strain MG1363. The main difference between pGKV1500 and pGKV1552 is the absence in the former of the nucleotide sequence encoding the C-terminal 130 amino acids of the proteinase. This region is responsible for the attachment of the proteinase to cells (9). Whereas plasmids pGKV500 (14) and pGKV552 complemented the proteinase deficiency, MG1363, pGKV1500, and pGKV1552 did not. As shown in Table 2, *L. lactis* carrying either pGKV1500 or pGKV1552 was unable to grow to a high cell density in milk. Hydrolysis of milk proteins could not be detected in milk-grown overnight cultures. The synthetic substrate MeOSuc-Arg-Pro-Tyr-pNA (5) was used to determine proteinase activity in overnight cultures of *L. lactis*. No proteinase activity was detected in cultures of *L. lactis* that produced the Asp-30→Asn-30 mutated proteinase specified by plasmids pGKV1500 and pGKV1552 (Table 2). Even after incubation for up to 7 h no substantial hydrolysis of the substrate was detected. From these results we concluded that the activity of the lactococcal proteinase was reduced to less than 0.1% of its original level when it carried the Asp-30→Asn-30 mutation. *L. lactis* carrying an intact proteinase gene but lacking the maturation gene *prtM* produced a caseinolytically inactive proteinase (9, 34). As shown in Table 2, the proteinases produced in the absence of *prtM* from pGKV507 and pGKV550 were also incapable of hydrolyzing the substrate MeOSuc-Arg-Pro-Tyr-pNA.

TABLE 2. Proteolysis of milk proteins, as measured by the *O*-phthaldialdehyde spectrophotometric assay (A_{340}), hydrolysis of the synthetic substrate MeOSuc-Arg-Pro-Tyr-pNA by *L. lactis* MG1363 carrying various plasmids, and cell densities of strains grown in milk

Strain(plasmid)	*O*-phthaldialdehyde assay (A_{340})	Density (CFU/ml)[a]	Activity[b]
Milk blank	0.153	ND[c]	ND
MG1363	0.113	2.0×10^8	≤0.0005
MG1363(pGKV500)	0.702	3.0×10^9	0.659
MG1363(pGKV507)	0.113	2.1×10^8	≤0.0005
MG1363(pGKV1500)	0.115	1.8×10^8	≤0.0005
MG1363(pGKV552)	0.718	2.8×10^9	0.436
MG1363(pGKV550)	0.117	2.1×10^8	≤0.0005
MG1363(pGKV1552)	0.111	3.2×10^8	≤0.0005

[a] Determined in cultures grown overnight at 30°C in 10% (wt/vol) reconstituted skim milk.
[b] Proteolytic activity is expressed as ΔA_{405} per minute per milliliter of whey-grown overnight culture with an optical density at 600 nm of 1.
[c] ND, not determined.

Effect of the Asp-30→Asn-30 mutation on the size of the secreted proteinase. The proteinases produced by *L. lactis* containing pGKV500, pGKV1500, or pGKV507 were compared by using SDS-PAGE. The proteinases encoded by *prtP* on plasmids pGKV500 and pGKV507 are secreted into the culture medium (9). As shown in Fig. 2, the same applied to the inactive C terminally truncated proteinase specified by pGKV1500. When the proteinase from the culture supernatant of *L. lactis* containing pGKV1500 was subjected to SDS-PAGE, major protein bands at 185, 170, and 58 kDa were visible in a Coomassie-stained gel (Fig. 2A, lane 4). The presence of multiple proteinase bands in all samples may well have resulted from other proteolytic activities that may have been present in the culture medium. In addition, we cannot exclude the possibility that a possible low residual level of proteolytic activity of the proteinases produced by *L. lactis* containing pGKV1500 or *L. lactis* containing pGKV507 resulted in autodigestion. Residual proteolytic activity has also been observed in an Asp-32→Ala-32 subtilisin mutant (2). In the Western blot analysis with lactococcal proteinase-specific monoclonal antibodies, all of the protein bands except the 58-kDa band reacted with the antibodies (Fig. 2B, lane 4). Only in the culture supernatant of *L. lactis* containing pGKV500 (thus, in the presence of active proteinase) was the 58-kDa protein absent. This 58-kDa secreted protein, most probably Usp45 (31), is degraded by the proteinase (unpublished data). The largest forms of the inactive proteinases produced by *L. lactis* containing pGKV507 and *L. lactis* containing pGKV1500 appeared to be the same size, approximately 185 kDa (Fig. 2, lanes 3 and 4). This is approximately 20 kDa larger than the largest form of the active proteinase produced by *L. lactis* containing pGKV500 (Fig. 2, lanes 2). From these results we

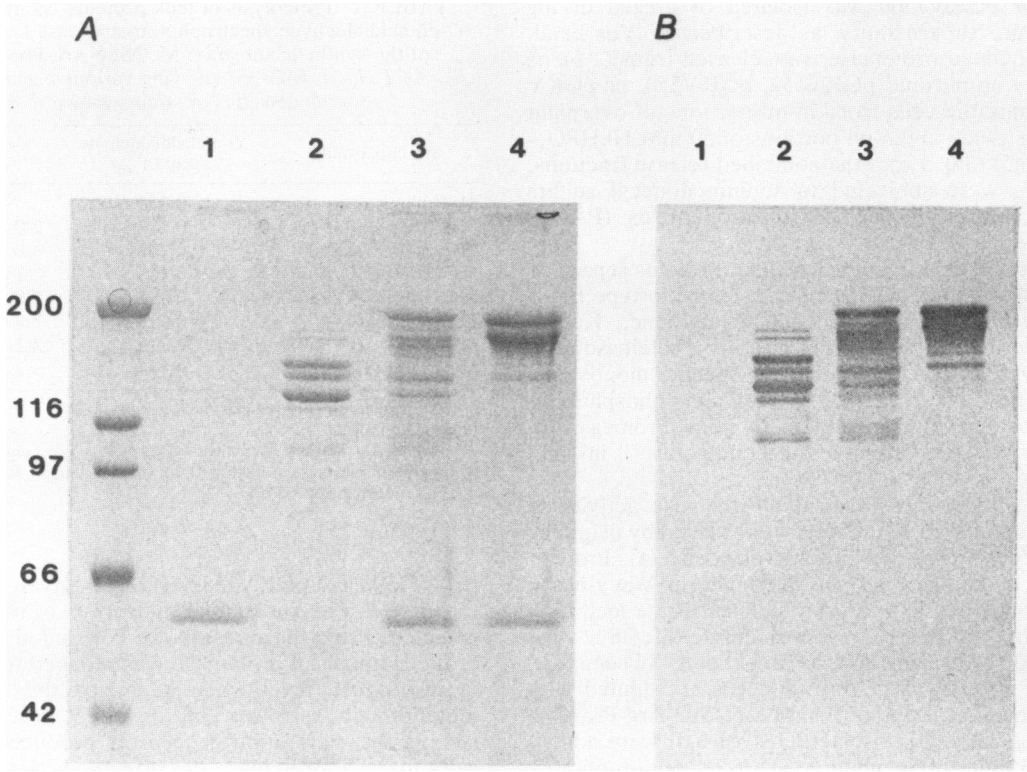

FIG. 2. SDS–7.5% PAGE (A) and Western blot analysis in which we used proteinase-directed monoclonal antibodies (B) of proteins isolated from the culture media of *L. lactis* MG1363 carrying plasmids pGKV2 (lanes 1), pGKV500 (lanes 2), pGKV507 (lanes 3), and pGKV1500 (lanes 4). The following high-molecular-weight standards were used: myosin (molecular weight, 200,000), β-galactosidase (116,250), phosphorylase *b* (97,400), bovine serum albumin (66,200), and ovalbumin (42,900). The molecular masses (in kilodaltons) are indicated on the left.

concluded that the size difference between the active and inactive proteinases is the result of autodigestion and that PrtM itself has no proteolytic activity.

Effect of the Asp-30→Asn-30 mutation on proteinase release from lactococcal cells. The cell-associated proteinase of *L. lactis* can be isolated by washing the cells in a Ca^{2+}-free buffer (29). To establish whether this proteinase release resulted from C-terminal autodigestion or from the action of another proteinase or PrtM, we incubated the various *L. lactis* strains in a Ca^{2+}-free buffer. The release fractions were subjected to Western blot analysis. Proteinase release was observed neither from *L. lactis* lacking PrtM (pGKV 550) (Fig. 3, lane 2) nor from *L. lactis* cells producing PrtM but synthesizing an inactive proteinase (pKKV1552) (Fig. 3, lane 3). Proteinase with an apparent molecular mass of 145 kDa was isolated only from *L. lactis* carrying a gene encoding an active proteinase, as is the case in pGKV552 (Fig. 3, lane 1). Even the presence of 10 mM EDTA, which enhances proteinase release from *L. lactis* cells (18), did not result in release of proteinase antigen from *L. lactis* carrying either pGKV550 or pGKV1552 (data not shown). From these results we concluded that activity of the lactococcal proteinase, for which the presence of PrtM is a prerequisite, is essential for release of the proteinase from the lactococcal cells.

DISCUSSION

In this paper we describe the construction of an Asp-30→Asn-30 lactococcal proteinase mutant by site-directed mutagenesis. As expected from the sequence similarity between the *L. lactis* subsp. *cremoris* Wg2 proteinase and subtilisin (14), the Asp-30→Asn-30 substitution resulted in the complete loss of proteolytic activity. In addition to the absence of caseinolytic activity, no hydrolysis of the synthetic substrate MeOSuc-Arg-Pro-Tyr-pNA by the active-site mutant was detectable. Similar results were obtained when we assayed the proteolytic activity of *L. lactis* cells carrying a functional proteinase gene but lacking *prtM* (Table 2). These results suggest that the presence of PrtM directly influences the catalytic center of the proteinase and that PrtM is not involved in the interaction between the proteinase and the preferred substrate β-casein.

Removal of the C-terminal 130 amino acids results in secretion of both the active and the inactive proteinases (9). The apparent size of the largest form of the secreted inactive proteinase carrying the active-site mutation coincided with the size of the largest form of the inactive proteinase secreted in the absence of *prtM* (approximately 185 kDa) (Fig. 2, lanes 3 and 4). The molecular mass of largest form of the secreted active proteinase is about 165 kDa. Apparently, the absence of *prtM* blocks specific autocatalytic degradation, resulting in the active 165-kDa form of the enzyme. Since in the presence of *prtM* the inactive Asp-30→Asn-30 substituted proteinase is not subject to size reduction, we concluded that PrtM itself has no proteolytic activity.

The presence of a pro region is a common feature among proteinases secreted by gram-positive bacteria (35). As established for the alkaline and neutral proteinases produced

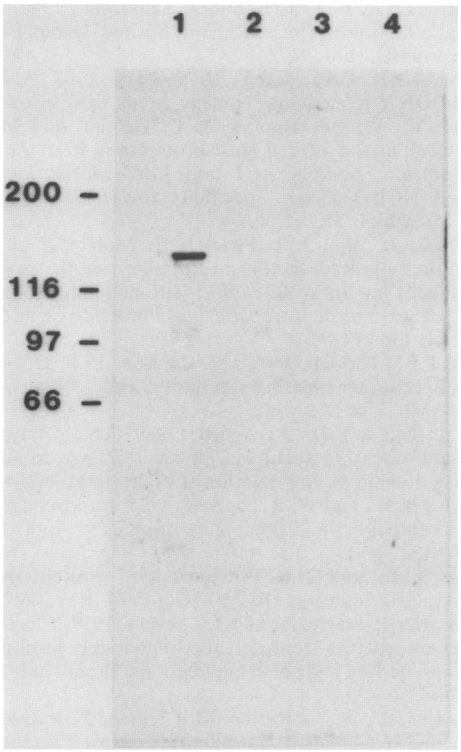

FIG. 3. Western blot analysis, using proteinase-directed monoclonal antibodies, of proteins released from cells of *L. lactis* MG1363 carrying plasmids pGKV552 (lane 1), pGKV550 (lane 2), pGKV1552 (lane 3), and pGKV2 (lane 4). The molecular weight markers were the same as those described in the legend to Fig. 2. Molecular masses (in kilodaltons) are indicated on the left.

by bacilli and the α-lytic proteinase produced by the gram-negative bacterium *Lysobacter enzymogenes*, the pro region acts as a template to promote the folding of the proteinase into an active conformation (27, 35). In subtilisins, removal of the pro region during proteinase maturation has been shown to be caused by an intramolecular self-digestion step (10). Maturation by autodigestion has also been proposed for a number of other bacterial proteinases, based on the fact that no maturation was observed in active-site mutants (35). Removal of the pro region of the lactococcal proteinase by N-terminal self-digestion, analogous to other bacterial proteinases, may well explain at least part of the observed size difference between the largest form of the proteinase produced by the active-site mutant and the wild-type proteinase. On the basis of the results of this study and our previous finding that PrtM is a membrane-associated lipoprotein, maturation of the lactococcal proteinase may be envisaged as follows: during or directly following membrane translocation of the proteinase precursor, PrtM induces the proproteinase to remove its N-terminal pro region by a self-digestion step. Although the exact nature of the PrtP-PrtM interaction remains to be elucidated, it is tempting to assume that PrtM, perhaps in association with the pro region of the proteinase precursor, guides the enzyme to adopt an active conformation. This hypothesis is supported by the results of this study; the largest forms of the inactive proteinases encoded by pGKV1500 and pGKV507 are the same size (Fig. 2). This hypothesis could be tested if the N-terminal amino acid sequences of the proteinases produced by *L. lactis* carrying pGKV1500 and *L. lactis* carrying pGKV507 were available. However, as was the case for the N-terminal amino acid sequence of the proteinase produced in the absence of PrtM (9, 34), we were repeatedly unable to determine the N-terminal amino acid sequence of the Asp-30→Asn-30 mutated proteinase.

Two different models have been proposed to explain the release of the lactococcal proteinase when cells are washed in a Ca^{2+}-free buffer (29). Kok et al. (13) have proposed a model for proteinase degradation. In this model proteinase release from *L. lactis* cells is envisaged to occur by a C-terminal autodigestion step. A number of possible self-digestion sites in the proteinase C terminus were proposed on the basis of the digestion sites of lactococcal proteinases in β-casein (14, 24). Exterkate and de Veer presented an alternative model, because the kinetics of proteinase release were thought to be incompatible with an enzymatic reaction (6, 7). These authors proposed that the release occurs by diffusion of the proteinase. In this model the proteinase is originally associated by Ca^{2+}-mediated interactions with a membrane-bound anchor protein unit originating from the proteinase C terminus. Recently, Laan and Konings (18) showed that proteinase release is mediated by a serine proteinase activity. Proteinase release from proteolytically active lactococcal cells was inhibited in the presence of phenylmethylsulfonyl fluoride but enhanced in the presence of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. In that study, however, the possible involvement of another serine proteinase for proteinase release could not be excluded. These results, together with the results of this study which prove that proteinase release depends only on the action of the enzyme itself, conclusively show that C-terminal self-digestion is required for proteinase release and that this self-digestion step is initiated when cells are washed in a Ca^{2+}-free buffer.

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