APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1992, p. 3263–3270
0099-2240/92/103263-08$02.00/0

Vol. 58, No. 10

Stability and Specificity of the Cell Wall-Associated Proteinase from *Lactococcus lactis* subsp. *cremoris* H2 Released by Treatment with Lysozyme in the Presence of Calcium Ions

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Received 24 March 1992/Accepted 16 July 1992

The cell wall-associated proteinase from *Lactococcus lactis* subsp. *cremoris* H2 (isolate number 4409) was released from the cells by treatment with lysozyme, even in the presence of 50 mM calcium chloride. Cell lysis during lysozyme treatment was minimal. The proteinase activity released by lysozyme treatment fractionated on ion-exchange chromatography as three main forms, the molecular masses of which were determined by gel exclusion chromatography and polyacrylamide gel electrophoresis. Two of the enzyme forms released, 137 and 145 kDa, were the same as those released by incubation of cells in calcium-free phosphate buffer. In the presence of calcium, lysozyme treatment also resulted in the release of a 180-kDa enzyme molecule. The total proteinase activity released by lysozyme treatment (in the presence or absence of calcium) was not only greater than that released by phosphate buffer but was also greater than that initially detectable on the surface of whole cells, suggesting an unmasking of enzyme on the cell surface. The presence of calcium during release treatment resulted in increased stability of the crude enzyme preparations. For the proteinase preparation released by using lysozyme with 50 mM $CaCl_2$, the half-life of proteinase activity at 37°C was 39 h, compared with 0.22 h for the calcium-free phosphate buffer-released preparation. In all cases, maximum stability was observed at pH 5.5. Comparison of β-casein hydrolysis by the three forms of the enzyme showed that the products of short-term (5- to 30-min) digestions were very similar, although subtle differences were detected with the 180-kDa form.

Proteinases of starter lactococci catalyze the initial steps in the hydrolysis of milk proteins by the starter proteolytic system, thereby initiating the process which provides the cell with the amino acids that are essential to or promote growth (16, 23, 25). Rapid growth of starter lactococci is industrially important, since it ensures an appropriate rate of acid production in milk fermentations. The proteinases also contribute to the overall process of milk protein breakdown during cheese maturation and may therefore influence texture and flavor (20, 25).

Previous studies (22, 27, 28) have indicated the existence of two main classes of lactococcal proteinases, designated P_I and P_{III} types, which differ in their specificities of action on the various caseins. The genes encoding the two different types of proteinase have been sequenced (8, 11, 30) and are approximately 98% homologous. A high degree of homology to the subtilisin class of protease is revealed by the amino acid sequence, particularly in regions surrounding the active site residues, although the lactococcal proteinases are generally much larger proteins than most members of the subtilisin family (24).

The method routinely used to isolate the enzyme is to release it from the lactococcal cell surface by washing the cell in calcium-free buffer, although alternative methods, including treatment with lysozyme, have been used in earlier studies (see reference 25). Two mechanisms for this release in the absence of calcium have been proposed. The first involves an autoproteolytic cleavage from the C-terminal membrane anchor sequence which is a consequence of a conformational change induced by the removal of calcium. This proposal is supported by recent work in which the release of the enzyme, detected by a specific monoclonal antibody assay, was shown to be enhanced by calcium-specific chelators and prevented by serine protease inhibitors or inactivation of the proteolytic activity by site-specific mutagenesis of an active-site residue (10, 12, 13). The second model (4, 5) proposed the direct involvement of calcium ions in the binding of the active moiety of the enzyme to the anchor sequence. With either of these proposed mechanisms, the moiety released in a calcium-free environment is an active fragment rather than an intact molecule. The released activity is unstable, although calcium ions have been shown to have a stabilizing effect (12, 18). These findings pose the question as to whether the properties of the enzyme released with calcium-free buffer are comparable with those of the enzyme in situ, i.e., in close association with cell surface components.

In this study, we show that the proteinase can be released from the lactococcal cell surface by using lysozyme even in the presence of high concentrations of calcium ions. The properties of the enzyme preparations obtained in this manner are compared with those obtained by using calcium-free methods.

MATERIALS AND METHODS

Bacterial strain, growth conditions, and harvest. *Lactococcus lactis* subsp. *cremoris* H2 (isolate number 4409) was from the culture collection of the New Zealand Dairy Re-

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search Institute. The strain was stored as a frozen culture at −70°C in 10% (wt/vol) reconstituted skim milk (sterilized by autoclaving at 121°C for 15 min) and subcultured twice in the same medium at 30°C before being used to inoculate 1-liter batch cultures (1% inoculum) of sterilized 10% reconstituted skim milk buffered with 70 mM β-glycerophosphate (autoclaved separately). Batch cultures were grown at 30°C until the pH had dropped to 5.0 to 5.2. The cultures were then strained through muslin, 25% sodium citrate was added (60 ml/liter), and the pH was adjusted to 7.0 with 10 M NaOH. The cells were harvested by centrifugation (13,000 × *g*, 10 min, 4°C) and then washed three times by suspending them in 50 mM sodium acetate-phosphate buffer (pH 6.4, 4°C, 250 ml/liter of culture) and centrifuging them each wash.

Release of cell wall-bound proteinase. Harvested washed cells were suspended in one of the following buffers (25 ml/liter of original culture): 50 mM sodium phosphate buffer (pH 7.0), 50 mM Tris-HCl buffer (pH 7.5) containing 24% sucrose, or Tris-sucrose buffer containing $MgCl_2$ (20 mM) or $CaCl_2$ (2, 20, or 50 mM). Samples (1 ml) of each cell suspension were retained for assay of in situ proteinase activity. Lysozyme (Sigma Chemical Co., St. Louis, Mo.; 51,500 U/mg of solid) was added to cell suspensions in Tris-sucrose buffer (with or without $MgCl_2$ or $CaCl_2$) to give a final concentration of 1 mg/ml. The various cell suspensions were then incubated for three successive 30-min periods at either 30°C (cells in phosphate buffer) or 37°C (all other cell suspensions). After each incubation, the solubilized material was removed by centrifugation (13,000 × *g*, 10 min, 20°C) and retained for proteinase assay, the pelleted cells being resuspended in the original volume of the appropriate buffer (plus lysozyme where required) each time, with 1-ml samples retained for proteinase assay in each case. Cell lysis was monitored by determination of the levels of fructose 1,6-bisphosphate aldolase released into the solubilized material by using the assay method of Crow and Thomas (2).

Proteinase and protein assay. Proteinase activity was measured by using fluorescein isothiocyanate-labeled β-casein as substrate (26), generally in 50 mM sodium acetate-phosphate buffer (pH 6.4). This procedure was modified in thermostability experiments by increasing the strength of the buffer to 0.5 M. Protein was quantified by the method of Bradford (1).

Assessment of proteinase stability. The stabilities of the unpurified lactococcal proteinase preparations were assessed by determining the half-life of enzyme activity at 37°C. Samples of enzyme preparation were incubated at 37°C in the presence or absence of 10 mM $CaCl_2$ in an equal volume of constant-ionic-strength sodium citrate-phosphate buffers in the pH range of 4.5 to 7.5 (all at 0.125 M) for up to 2.5 h. Samples were removed at 30-min intervals and assayed for remaining proteinase activity. Half-lives of the enzyme preparations at each pH were determined from the initial linear portion of the curves obtained by plotting $\log_{10}$% activity remaining against time of incubation.

Purification of lactococcal cell wall-bound proteinase. Both phosphate buffer-released and lysozyme-released enzyme preparations were purified by using anion-exchange and gel exclusion chromatography (MonoQ HR10/10 and Superose 6 HR10/30, respectively; Pharmacia, Uppsala, Sweden) under identical conditions. However, those preparations containing phosphate buffer were first subjected to fast desalting column chromatography (FDS HR10/10; Pharmacia) with 20 mM bis-Tris-propane (pH 6.4) as running buffer. Ion-exchange chromatography on MonoQ was performed at a flow rate of 2.0 ml/min with 20 mM bis-Tris-propane (pH 6.4) containing 10 mM $CaCl_2$ as buffer A and the same buffer but containing 1 M NaCl as buffer B. Samples of enzyme (2.0 ml) were loaded onto the column equilibrated in buffer A, and after 10 min under isocratic conditions, a linear 30-min gradient to 40% buffer B was applied. Fractions (1.0 ml) were collected throughout, and A_{280} of the column eluent was monitored (UV-M monitor; Pharmacia). Each fraction was assayed for proteinase activity, and those peak fractions containing maximum levels of activity were further purified on the Superose 6 column with 20 mM bis-Tris-propane (pH 6.4) containing 10 mM $CaCl_2$ and 0.15 M NaCl as running buffer. A flow rate of 0.5 ml/min was used, and fractions (0.5 ml) were collected. Each fraction was assayed for proteinase activity, and the molecular masses of the active protein peaks were assessed by reference to known standards used to calibrate the column (thyroglobulin, 669 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa [all from Sigma]). Peak fractions containing maximum proteinase activity were also assessed for purity and molecular mass by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Hydrolysis of casein. Activities of each of the purified proteinase solutions were determined by using fluorescein isothiocyanate–β-casein as substrate to ensure that the ratio of proteinase activity to β-casein was equivalent in all the different digest mixtures. Mixtures containing an appropriate amount of purified enzyme, 1.0 ml of purified β-casein (10 mg/ml), and 0.25 ml of 50 mM phosphate buffer (pH 6.4) were incubated at 24°C, and samples were withdrawn at 5, 10, 30, and 90 min and at 4 and 24 h. The reaction was stopped either by addition of trifluoroacetic acid (TFA) to give a final concentration of 1% (for subsequent high-pressure liquid chromatography [HPLC] analysis) or by boiling with SDS-mercaptoethanol as described below (for SDS-PAGE analysis). Samples for HPLC analysis were centrifuged to remove TFA-insoluble peptides, and the supernatants were filtered with a Centricon 10 microconcentrator (10-kDa cutoff; Amicon, Danvers, Mass.).

SDS-PAGE. Samples for analysis by SDS-PAGE were prepared by being boiled with an equal volume of 0.125 M Tris-HCl (pH 6.8) containing 4% (wt/vol) SDS, 20% (vol/vol) glycerol, and 10% (vol/vol) β-mercaptoethanol. For electrophoresis of the proteinase, the running gel contained 8% (wt/vol) acrylamide; for analysis of β-casein digests, a running gel containing 15% (wt/vol) acrylamide was used. The procedure for preparation, running, and staining followed the general method of Laemmli (15) with details as specified in Reid et al. (21).

HPLC analysis. Analysis of the low-molecular-mass products of β-casein hydrolysis by HPLC was carried out according to the procedures described by Reid et al. (21).

RESULTS

Comparison of different methods of proteinase release. Proteinase activities in the cell suspensions, solubilized cell wall material, and pelleted fractions prepared as described in Materials and Methods were compared. The results (Table 1) show that the levels of proteinase activity were similar in cell suspensions in phosphate and Tris-sucrose buffers, with higher levels in the presence of 20 mM $MgCl_2$ and 2 mM $CaCl_2$ but lower levels at the two higher $CaCl_2$ concentrations, presumably because the inhibitory effects of the higher calcium concentrations carried over into the proteinase assay (6; unpublished data). In phosphate buffer, the major proportion of the detected proteinase was released during

TABLE 1. Proteinase activity in cell suspensions and soluble fractions during enzyme release treatments

Sample[a]	Activity after treatment with[b]:					
	PO_4^{2-}	TS	TSM	TSC2	TSC20	TSC50
Susp	157	147	175	185	132	122
S1	162	256	325	229	107	104
S2	28	53	109	208	76	56
S3	5	38	41	180	58	38
P1	43	185	273	381	345	162
P2	15	89	221	340	409	201
P3	5	38	132	183	272	162

[a] Abbreviations: Susp, whole-cell suspension; S1 to S3, first, second, and third soluble fractions, respectively, from release incubations; P1 to P3, first, second, and third particulate fractions, respectively, from release incubations.
[b] Abbreviations: PO_4^{2-}, phosphate buffer; TS, Tris-sucrose plus lysozyme; TSM, Tris-sucrose-lysozyme containing 20 mM $MgCl_2$; TSC2, TSC20, and TSC50, Tris-sucrose-lysozyme containing 2, 20, and 50 mM $CaCl_2$, respectively. See Materials and Methods for further details. Activity is given in arbitrary fluorescence units (measured at 525-nm emission and 490-nm excitation).

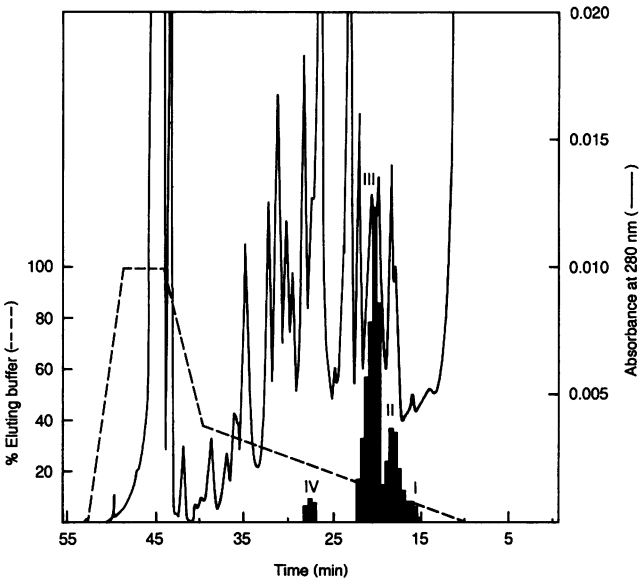

FIG. 1. MonoQ anion-exchange column chromatography of soluble material released from the cell surface of *L. lactis* subsp. *cremoris* H2 by treatment with lysozyme in the presence of 20 mM $MgCl_2$. Histograms denote fractions in which proteinase activity (in arbitrary fluorescence units: 525-nm emission, 490-nm excitation) was detected, with peaks of activity numbered I to IV. Note that the time axis runs from right to left.

the first 30-min incubation. Successive treatments released diminishing amounts of proteinase, but the total activity recovered was greater (by about 27%) than that determined to be present initially with the whole-cell suspensions. For lysozyme-treated cells, the total proteinase activity released by the three washes (plus that remaining in the final pellet) exceeded the initially determined activity by a considerably larger factor up to a maximum recovery, obtained in the presence of 2 mM $CaCl_2$, of over 400%. This apparent unmasking of "latent" activity resulted in the release of a significant quantity of proteinase activity in the second and third treatments with lysozyme. At 50 mM $CaCl_2$, the inhibitory effect of calcium on both activity and release is evident, but even at this high calcium concentration, 55% of the total recovered activity (and 162% of the initial, "whole-cell" activity) was solubilized by the three successive washes. Cell lysis during treatments was minimal; at no stage was the level of aldolase recovered in the proteinase preparations greater than 1.5% of the total aldolase activity measured in cell extracts, with approximately 0.5% of the total aldolase being detected in each of the three sequential release treatments.

Purification of proteinase activity. In general, up to three defined peaks of activity were eluted from the MonoQ column. A fourth, incompletely resolved area of activity was eluted very early in the gradient when lysozyme-released material was purified (Fig. 1). These active forms were designated I to IV in order of elution. The proportions of the total eluted activity found in each peak for the different samples are given in Table 2, the unresolved activities in peaks I and II being considered together. While the actual proportions of peaks I and II varied between preparations, the general trend was that increasing levels of calcium resulted in increasing amounts of peak I material.

With phosphate-buffer-released enzyme, only two peaks of activity, II and III, were found (this designation was used because their positions on the elution profile corresponded to those of peaks II and III obtained when lysozyme was used to release the proteinase), with only 5% of the activity in peak I/II. With samples obtained using lysozyme to release proteinase, the proportion of enzyme activity present in peak II was much greater (22 to 44%, depending on calcium concentration) than that with the phosphate-released proteinase. With samples released in the presence of 50 mM $CaCl_2$, a significant proportion of the proteinase activity did not bind to the column because of the relatively high ionic strength of the original sample. This could be avoided by subjecting these samples to fast-desalting column chromatography prior to MonoQ separation. The proteinase activity in the unbound fraction was found on rechromatography to comprise peak I and peak II forms of the enzyme. In samples obtained using lysozyme release treatment in the absence of calcium (or in the presence of $MgCl_2$), activity was observed eluting at relatively high ionic strength (peak IV), but this accounted for only a very small proportion of total activity. Further minor areas of activity were detectable eluting from the MonoQ column at high salt concentration, but the amounts of these varied between preparations using the same conditions. Study of these minor peaks was not pursued further at this stage.

TABLE 2. Relative proportions of proteinase activity associated with active fractions separated by MonoQ column chromatography

Sample[a]	% Activity in peaks			
	Unbound	Peaks I and II[b]	Peak III	Peak IV
PO_4^{2-}	0	4.9	96.1	0
TS	0	22.9	74.0	3.1
TSM	0	21.5	76.8	1.6
TSC2	0	37.8	62.2	0
TSC20	0	44.5	55.5	0
TSC50	41.8[c]	3.0	55.2	0

[a] See Table 1, footnote *b*, for explanations of abbreviations.
[b] Peak I and II material were assessed together. No peak I material was detected when phosphate buffer was used to release enzyme activity.
[c] This activity represents peak I and peak II material which did not bind because of the high concentration of $CaCl_2$ in the sample.

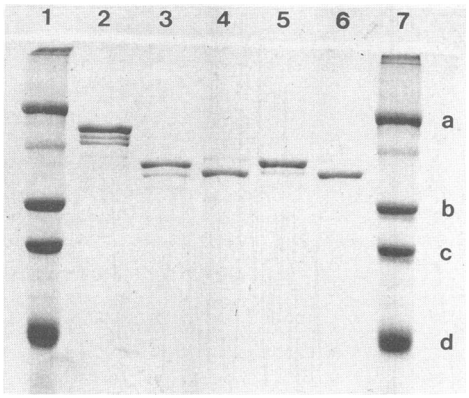

FIG. 2. SDS-PAGE analysis of the different forms of cell wall proteinase from *L. lactis* subsp. *cremoris* H2. The different forms were obtained by anion-exchange chromatography (MonoQ) and gel filtration (Superose 6) of the soluble material obtained following incubation of cells in Tris-sucrose buffer containing lysozyme and 2 mM $CaCl_2$ (Lyso I, II, and III forms) or in calcium-free phosphate buffer (Phos II and III forms). Lanes 1 and 7, standard proteins; lanes 2 through 4, Lyso I, II, and III forms, respectively; lanes 5 and 6, Phos II and III forms, respectively. Standard proteins were myosin (205 kDa) (a), β-galactosidase (116 kDa) (b), phosphorylase *b* (97.4 kDa) (c), and bovine serum albumin (66 kDa) (d).

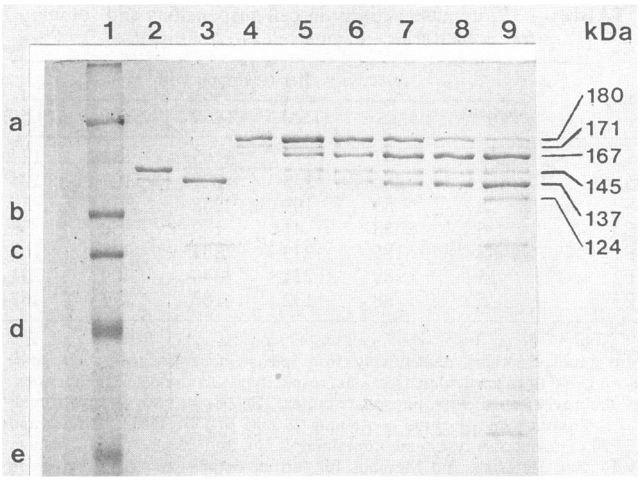

FIG. 3. SDS-PAGE analysis of Lyso I form of the proteinase from *L. lactis* subsp. *cremoris* H2, showing the effect of storage at 24°C for various periods up to 96 h. Lane 1, standard proteins; lane 2, Phos II (mainly 145 kDa) form of the proteinase, freshly purified; lane 3, Phos III (mainly 137 kDa) form, freshly purified; lanes 4 through 9, Lyso I form of the proteinase, freshly purified (lane 4) and after 5, 24, 48, 72, and 96 h of storage at 24°C, respectively. Purification procedures were as described in Materials and Methods. All preparations contained 10 mM calcium chloride. Standard proteins were as in Fig. 2 with the addition of egg albumin (45 kDa) (e).

Peaks II and III from the phosphate buffer-released samples (designated Phos II and Phos III, respectively) each eluted from a Superose 6 gel filtration column as two protein peaks, one major and one minor. In each case, only the major peak was proteolytically active, and comparison with the standard proteins gave molecular masses of 280 kDa for Phos II and 270 kDa for Phos III. This difference, although small, was nevertheless completely reproducible. The minor, proteolytically inactive peak in the two preparations gave a molecular mass of 63 to 65 kDa. The protein from MonoQ peaks II and III of the lysozyme-released samples (Lyso II and Lyso III, respectively) both eluted from the Superose 6 column in three peaks, only one of which was proteolytically active. The Lyso II and Lyso III peaks of proteinase activity eluted in positions identical to those for the Phos II and Phos III material, respectively. However, the proteinase activity from MonoQ peak I, which was found only in lysozyme-released samples, eluted from the Superose column much earlier in a position corresponding to a considerably higher molecular mass, suggesting that the enzyme was in an aggregated or multimeric form. The molecular mass could not be accurately estimated, since the activity eluted in a position outside the linear limits of resolution, i.e., between apoferritin (443 kDa) and thyroglobulin (669 kDa).

Characterization of different proteinase types by SDS-PAGE. SDS-PAGE of each of the three forms revealed a major protein band with one or more minor bands (Fig. 2). The proteinases from Phos II and Lyso II each had a major protein band with a molecular mass of 145 kDa, with minor bands at 137 and 124 kDa, while the proteinases from Phos III and Lyso III each ran as a major band at 137 kDa. The major protein band in Lyso I corresponded to a molecular mass of 180 kDa, with minor bands at 171 and 167 kDa. When the Lyso I form was stored for 96 h at 24°C and periodically assessed by SDS-PAGE, the band corresponding to the 180-kDa moiety progressively decreased in intensity while that corresponding to a molecular mass of 167 kDa increased and 145- and 137-kDa bands appeared (Fig. 3). After 96 h, other bands corresponding to lower molecular masses (124, 98, 50, and 46 kDa) appeared. These may have some relationship to the minor activities observed with MonoQ column chromatography. Similarly, a significant proportion of the 145-kDa major component of peak II material was converted into the 137-kDa form after storage for 48 h at 4°C (data not shown). It therefore seems justifiable to conclude that the major protein-staining band present in each of the three forms was the main proteolytically active form originally present in each case, while the minor lower-molecular-mass bands were autoproteolytic products. In addition, since identical levels of activity were loaded in each lane, assignment of a minor band (rather than the major band) as the main proteolytic activity would imply an extraordinarily high specific activity to the protein in the minor band, which would be unprecedented.

Actions of different proteinase types on β-casein. The high-molecular-weight digestion products of β-casein given by the different proteinase preparations and analyzed by SDS-PAGE (Fig. 4) are virtually identical over the first 90 min of hydrolysis, indicating that the initial sites of cleavage are the same for the different-molecular-weight forms of the enzyme. However, after 4 h and, even more markedly, after 24 h, the pattern of digestion products resulting from incubation with the Lyso I form of the proteinase shows some differences.

HPLC analysis of the low-molecular-weight β-casein digestion products was used to further define the specificities of the enzyme preparations. The profiles obtained with Phos II and Lyso I forms of the proteinase are shown in Fig. 5. The profiles obtained with Phos III and Lyso II forms were identical with that obtained using the Phos II form of the enzyme even at a fine level of detail for up to 24 h of digestion and are therefore not shown. The profile obtained

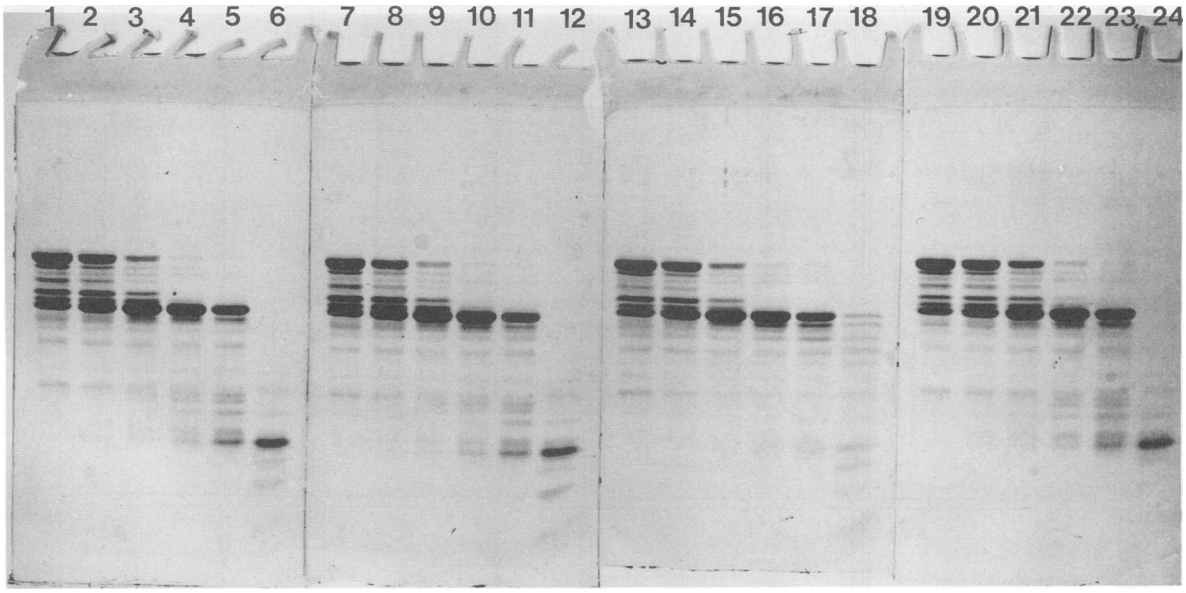

FIG. 4. SDS-PAGE analysis showing hydrolysis of β-casein by the different forms of cell wall proteinase released from *L. lactis* subsp. *cremoris* H2 by incubation in the presence of lysozyme plus 2 mM $CaCl_2$ or in calcium-free phosphate buffer. β-casein was incubated with each form of proteinase, and samples were taken for analysis at (from left to right for each set of 6 lanes) 5, 10, 30, and 90 min and at 4 and 24 h. Lanes show β-casein digestion by Phos II form (lanes 1 through 6), Phos III form (lanes 7 through 12), Lyso I form (lanes 13 through 18), and Lyso II and Lyso III forms combined (lanes 19 through 24).

with the Lyso I form is similar (but not identical) to that for the other two forms over the first 30 min of digestion. However, as the time of digestion of β-casein increased, the Lyso I digestion profiles showed an increasing number of differences from those obtained with Phos II.

One subtle difference is evident in the HPLC profile of the Lyso I digestion products even after only 5 min of hydrolysis. The relative heights of peaks 15 and 16 are the same in this profile, whereas in that for Phos II (and also for Phos III and Lyso II [data not shown]), peak 15 is consistently higher than peak 16. Analysis of HPLC peak 16 from the Lyso I digestion profile by amino acid sequence determination revealed the presence of two peptides from the C-terminal region of β-casein, one comprising residues 183 to 193 and the other being a significant quantity of the overlapping peptide, residues 193 to 209.

Thermostability of proteinase preparations. Thermostability studies (Table 3) showed that the phosphate buffer-released preparations and those obtained using lysozyme in the absence of $CaCl_2$ or in the presence of $MgCl_2$ were extremely unstable at 37°C, but the inclusion of 10 mM $CaCl_2$ conferred a significant stabilizing effect. For lysozyme-released enzyme preparations, the concentrations of $CaCl_2$ in the enzyme release incubation were double those in the thermostability incubations (due to dilution with various incubation buffers; see Materials and Methods). Thus, for enzyme released by lysozyme in the presence of 20 mM $CaCl_2$, the resulting concentration in the thermostability incubation corresponds to 10 mM $CaCl_2$. Therefore, the stabilization observed is not due solely to carryover of calcium into the incubation but is a consequence of calcium being present in the release treatment, and there is a correlation between the concentration of $CaCl_2$ present during release and the subsequent stability of the enzyme. In all cases, the enzyme proved to be most stable at pH 5.5.

DISCUSSION

When the proteolytic enzymes of lactic acid bacteria are studied with a view to the future control of proteolysis in cheese manufacture and ripening, it is necessary that the correlation between in vitro enzyme activity and the proteolytic process in situ be established. Since the use of calcium-free buffers to release the proteinase activity results in modification of the enzyme structure, alternative methods were used to obtain soluble proteinase preparations from the cell walls of lactococci in an attempt to release enzyme molecules retaining a greater part of the in vivo structure predicted by genetic information (see reference 9).

In this study, we have shown that the proteinase can be released from the cell walls of lactococci in the presence of relatively high levels of calcium by partial digestion of the cell wall with lysozyme. Although this procedure released proteinase forms which were apparently the same as those released by calcium-free phosphate buffer, a significantly larger form of the enzyme was also obtained. The concentration of calcium present during the release process appears to be a major factor in determining the relative proportions of the various enzyme forms obtained.

The largest form of the enzyme was found to have a molecular mass of 180 kDa, which is considerably larger than previously reported values for the released proteinase and virtually equal to the proposed value of the mature, in situ form of the proteinase (14). Laan and Konings (13), who used monoclonal antibodies to investigate autoproteolytic degradation of the proteinase from *L. lactis* subsp. *cremoris* Wg2, reported the presence of a 165-kDa enzyme as the highest-M_r form released in calcium-free buffer. This form was unstable and was converted to lower-M_r forms on continued incubation in calcium-free buffer but was more stable in the presence of calcium. The 165-kDa form de-

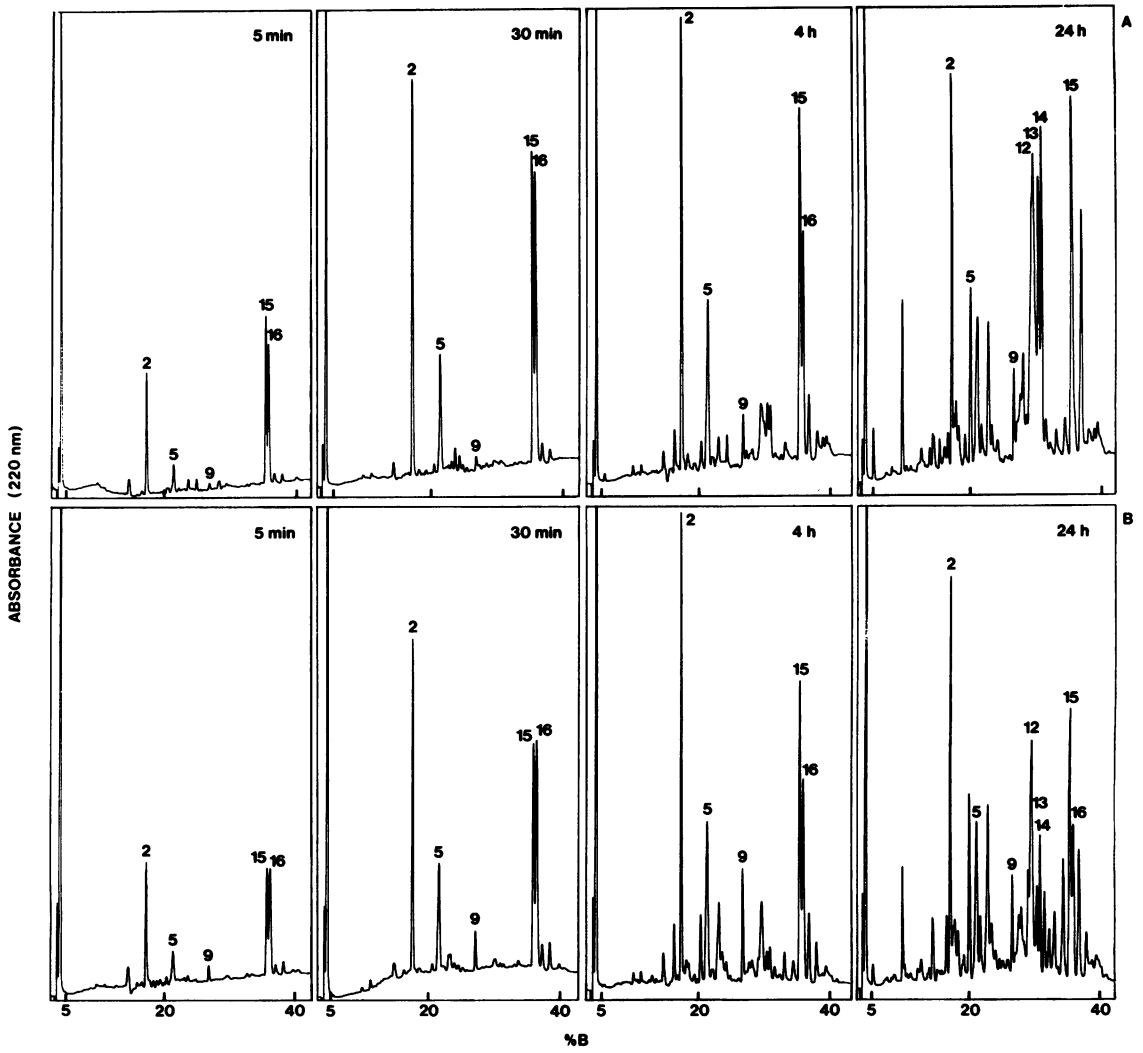

FIG. 5. Reversed-phase HPLC profiles of 1.0% TFA-soluble peptides from β-casein digested by the different forms of the cell wall proteinase from *L. lactis* subsp. *cremoris* H2. β-Casein was digested with the Phos II form (A) or Lyso I form (B). The HPLC profiles obtained using the Phos III form and the Lyso II and III forms combined were identical at all sampling times with those obtained using the Phos II form (data not shown). Peaks are numbered according to a previous study (22) in which the identities of the peptides in each HPLC peak were established. Solvent A was 0.1% TFA in water, and solvent B was 0.08% TFA in acetonitrile. Peptides eluted with a linear gradient of 0 to 50% solvent B over 50 min. Absorbance unit full scale = 2.0.

scribed by Laan and Konings (13) may correspond to the 167-kDa protein found in the present study as a minor component of freshly purified Lyso I and shown to be derivable (along with other lower-M_r forms) from the 180-kDa form after storage of Lyso I at room temperature for 3 or 4 days. The lower-molecular-mass forms found in the present study may correspond to the two different proteolytically active forms (133 and 126 kDa) detected by Exterkate and de Veer (4).

The 145- and 137-kDa forms of the enzyme isolated in this study exist as dimers in the native form as determined by Superose 6 column chromatography, whereas the 180-kDa form is possibly a larger aggregate in the native form (eluting in a position between the 449- and 660-kDa markers). Nissen-Meyer and Sletten (19) described the dimeric nature of a free form of the proteinase released from the surface of *L. lactis* subsp. *cremoris* NCDO1201 which had a subunit size of 150 kDa. This free dimer is presumably related to the dimeric forms of the enzyme described here.

The stabilities of the proteinases in crude extracts obtained by lysozyme release were greater in the presence of calcium. Magnesium also conferred some stability but was not as effective as calcium. The stabilizing influence of calcium on proteinase activity following release has been observed on several previous occasions (12, 18), although there is very little quantitative information on this effect or on its pH dependence.

It has been clearly established that the release of proteinase in calcium-free buffer involves an autoproteolytic event (13). The fact that high levels of proteinase activity were released by lysozyme treatment even in the presence of 50 mM $CaCl_2$ suggests that the release of the enzyme from the cell wall is not necessarily simply a consequence of the absence of calcium per se but may be a result of changes in

TABLE 3. Thermostability at 37°C of proteinase released from the lactococcal cell surface

Sample[a]	Half-life (h) of prepn at pH:				
	4.5	5.5	6.0	6.5	7.5
PO_4^{2-}	0.12	0.22	0.12	<0.07	<0.07
PO_4^{2-} + 10 mM $CaCl_2$	0.25	6.10	1.95	0.50	<0.07
TS	0.25	0.22	0.28	0.16	0.15
TSM	0.37	0.64	0.36	0.1	0.14
TSM + 10 mM $CaCl_2$	2.50	7.50	4.00	1.92	0.15
TSC2	1.60	3.70	1.60	0.46	0.17
TSC20	2.20	15.00	12.00	4.95	0.17
TSC50	4.76	39.00	18.50	7.50	1.00

[a] See Table 1, footnote *b*, for explanations of abbreviations.

the interaction of the enzyme with those cell wall components in close association with it. This interaction may itself be calcium mediated and therefore disrupted by the chelating action of phosphate when cells are incubated in calcium-free phosphate buffer. On the other hand, lysozyme treatment, through partial digestion of the cell wall, could lead to relaxation of the enzyme-cell wall interaction, allowing autoproteolytic release even in the presence of calcium, with cleavage occurring at several possible sites. This provides a mechanism for release which incorporates elements of both models previously proposed to account for the release of the enzyme in a calcium-free buffer (5, 12), i.e., autoproteolytic events which are induced by alterations in the integrity of the enzyme-cell wall interactions (by whatever means) rather than solely by the absence of calcium itself. Release of the proteinase as a result of alteration in the degree of interaction of the enzyme with cell wall components may explain the reported secretion of the proteinase by *L. lactis* subsp. *cremoris* ML1 (3, 7).

It seems more probable that the 180-kDa form of the enzyme is released as a consequence of autoproteolysis at a site very close to the putative membrane anchor sequence instead of being the entire proteinase molecule. Release of the entire proteinase molecule would involve disruption of the membrane structure with which the anchor sequence interacts. This would be expected to be accompanied by a high level of leakage of intracellular components if not by complete lysis. Since the levels of lysis found following lysozyme treatment were minimal, it seems likely that the integrity of the membrane has largely been retained. While the exact nature of the 180-kDa form of the enzyme remains to be determined, considerable loss of structural integrity of the cell wall is apparently necessary for its release. The apparent unmasking of latent proteinase activity by lysozyme treatment can also be accounted for if it is accepted that a proportion of the proteinase molecules may be partially enclosed by the cell wall matrix (and therefore unable to bind and subsequently hydrolyze substrate). The action of lysozyme could lead to release of these "protected" proteinase molecules by hydrolysis of the interfering cell wall structure, whereas phosphate buffer would not be expected to alter the cell surface in such a manner.

Since release of the proteinase from the cell may alter its conformation and, hence, its specificity of action, it was of interest to investigate the specificities of the different forms of the enzyme in digesting β-casein. It has been shown previously that the P_I-type proteinases isolated from lactococci to date have a highly specific action on β-casein that results in initial cleavage at a small number of sites close to the C terminus, although with longer periods of digestion, slower cleavage at other sites towards the N terminus occurs (17, 22, 29). For the various enzyme forms described here, it is clear that the products obtained by digestion of β-casein with the 145-kDa form (the predominant species in Phos II and Lyso II) and the 137-kDa form (the predominant species in Phos III and Lyso III) are identical and are not influenced either by the release mechanism or by their differences in size. The initial digestion pattern obtained with both of these forms is highly characteristic of the P_I-type proteinase (17, 22, 29) and results from progressive C-terminal shortening of β-casein at five specific sites and accumulation of the large N-terminal residual fragment (residues 1 to 153). With the Lyso I form (containing predominantly the 180-kDa species), both SDS-PAGE and HPLC analyses of the high- and low-molecular-mass cleavage products, respectively (Fig. 4 and 5), suggested that while the initial cleavage pattern was very similar to that obtained with the other forms, the slower cleavage (i.e., after 4 h) at sites more distant from the β-casein C terminus was, at least quantitatively, different.

In the early digest samples obtained with the Lyso I form, the β-casein peptide comprising residues 193 to 209 (resulting from cleavage of the Leu-192–Tyr-193 bond) was present as a significant product. This peptide is present normally as only a very minor early constituent of P_I-type proteinase action (22). The presence of a significant quantity of the peptide 193-209 in the Lyso I digest of β-casein is of interest, since early cleavage of the Leu-192–Tyr-193 bond is characteristic of the P_{III}-type proteinase from *L. lactis* subsp. *cremoris* SK11 (22) and AM1 (28). Thus, while the specificities of action of all three proteinase forms in cleaving β-casein are similar, subtle differences detectable between the 180-kDa form and the two lower-M_r forms may indicate that cleavage of β-casein by the proteinase in situ differs in certain respects from that obtained in vitro.

ACKNOWLEDGMENT

This research was supported in part by the Foundation for Research, Science and Technology, New Zealand (contract DRI104).

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