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Specificity of Hydrolysis of Bovine κ-Casein by Cell Envelope-Associated Proteinases from *Lactococcus lactis* Strains

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The cell envelope-associated proteinases from *Lactococcus lactis* subsp. *cremoris* H2 (a P_I-type proteinase-producing strain) and SK11 (a P_{III}-type proteinase-producing strain) both actively hydrolyze the κ-casein component of bovine milk but with significant differences in the specificity of peptide bond hydrolysis. The peptide bonds Ala-23–Lys-24, Leu-32–Ser-33, Ala-71–Gln-72, Leu-79–Ser-80, Met-95–Ala-96, and Met-106–Ala-107 were cleaved by both proteinase types, although the relative rates of hydrolysis at some of these sites were quite different for the two proteinases. Small histidine-rich peptides were formed as early products of the action of the cell envelope-associated proteinases on κ-casein, implicating this casein as a possible significant source of histidine, which is essential for starter growth. The major difference between the two proteinase types in their action on κ-casein was in their ability to cleave bonds near the C-terminal end of the molecule. The bond Asn-160–Thr-161 and, to a lesser extent, the bond Glu-151–Val-152 were very rapidly cleaved by the P_{III}-type proteinase, whereas hydrolysis of these bonds by the P_I-type proteinase was barely detectable (even after 24 h of digestion). Differential hydrolysis of κ-casein at these sites by the two different proteinase types resulted in the formation of distinctive, high-M_r products detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The κ-casein cleavage patterns revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis provide a diagnostic basis for recognizing differences in proteinase specificity and have been used to identify P_I- or P_{III}-type and P_I/P_{III} intermediate-type proteinases from several different lactococcal strains.

Casein hydrolysis by proteolytic enzymes of lactococcal starter bacteria provides the major source of amino acids required for the growth of the starter to high cell densities in milk (12). The initial step in proteolysis is catalyzed by a cell envelope-associated proteinase, which degrades some of the casein components to a series of oligopeptides which are then further degraded to small peptides and amino acids (10, 18, 20, 21, 24).

Two different types of lactococcal cell envelope-associated proteinase have been recognized and distinguished by their specificity of action in hydrolyzing bovine caseins (25). One type (designated P_I) catalyzes the cleavage of β-casein, initially only at peptide bonds located near the C terminus, and has little hydrolytic activity towards α-casein (13, 16, 27). The second type (designated P_{III}) cleaves β-casein, initially at peptide bonds located near both the C-terminal and the N-terminal ends (16, 26), and also hydrolyzes α_{s1}-casein (15, 25).

The respective actions of these two types of proteinase on the κ-casein constituent of bovine milk have been much less thoroughly investigated. Early studies (25) based on gel electrophoretic separation of the hydrolysis products suggested that the P_I-type proteinase was less active than the P_{III}-type proteinase in hydrolyzing κ-casein, but a quantitative comparison was not carried out. However, studies of the growth of a P_I-type proteinase-producing strain in synthetic media (5) indicated that optimal growth on casein as the sole amino acid source required the presence of both κ- and β-caseins. It has been suggested that κ-casein hydrolysis by cell envelope-associated proteinases may specifically supply one or more essential amino acids, such as histidine, required for starter growth (18). Although κ-casein is a quantitatively minor constituent of bovine milk, it is thought to play an important role in stabilizing the structure of the casein micelle (8). Consequently, knowledge of the action of cell envelope-associated proteinases on κ-casein not only provides information on the nature of oligopeptides which are available for starter nutrition and growth or which may contribute to flavor characteristics of dairy products, but is also essential to understanding the action of the starter proteolytic system on micellar casein.

The peptide bonds in κ-casein which are hydrolyzed by the cell envelope-associated proteinase of *Lactococcus lactis* subsp. *lactis* NCDO 763 recently have been identified (14). However, the proteinase from this strain is a P_I/P_{III} intermediate type in that it shows P_I-type specificity for β-casein but hydrolyzes α_{s1}-casein with P_{III}-type specificity (14). The pattern of bond cleavage found for κ-casein by Monnet et al. (14) is therefore not necessarily representative of either of the two main specificity types.

In the present study, the oligopeptide products of κ-casein hydrolysis by the cell envelope-associated proteinases from *L. lactis* subsp. *cremoris* H2, a typical P_I-type proteinase-producing strain (16), and from *L. lactis* subsp. *cremoris* SK11, a typical P_{III}-type proteinase-producing strain (15, 25), have been identified. The characteristic patterns of κ-casein cleavage are also shown to be useful for characterizing the types of proteinases produced by other strains of lactococcal starter bacteria.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and harvesting. *L. lactis* subsp. *cremoris* H2, SK11, E8, C5, and 2210 and *L. lactis*

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subsp. *lactis* ML8, ML3, and NCDO 1403 (and its proteinase-negative derivative, NCDO 1403 Prt$^-$, obtained spontaneously by repeated subculturing) were from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand. The particular isolate of strain SK11 used in this study and in previous studies of the P_{III}-type proteinase (15, 16, 29) is that designated SK112 (3), a derivative of *L. lactis* subsp. *cremoris* SK11 containing the proteinase plasmid pSK111. Strain ML3 (or NCDO 763) should probably be reclassified as an *L. lactis* subsp. *cremoris* strain (7), but the older *L. lactis* subsp. *lactis* designation will be retained here to avoid confusion. Culture maintenance, growth, and harvesting were accomplished by procedures described previously (2).

Purification of the cell envelope-associated proteinases. Cells harvested from a 1-liter culture were resuspended in 25 ml of 50 mM phosphate buffer (pH 6.4) and incubated for 1 h to release the proteinase from the cell surface (12). Following centrifugation to remove the cells, the solubilized proteinase in the supernatant was purified by the procedure described previously (2). During purification of the proteinases on a MonoQ HR10/10 ion-exchange column (Pharmacia, Uppsala, Sweden), the P_I-type proteinase from *L. lactis* subsp. *cremoris* H2 was observed reproducibly to elute at a higher ionic strength than the P_{III}-type proteinase from *L. lactis* subsp. *cremoris* SK11. This difference in elution is consistent with the different isoelectric points of the two types of proteinase (6).

Proteinase assay. Proteinase activity during purification was measured by the procedure of Twining (23).

Hydrolysis of κ-casein. κ-Casein was purified from milk obtained from a single cow known to produce the A variant of κ-casein only. The κ-casein fraction was isolated from an isoelectric precipitate of whole casein by the method of Zittle and Custer (31) with the addition of two further purification steps involving gel permeation and ion-exchange chromatography on Sephadex G-150 (superfine) and Q-Sepharose columns, respectively. Mixtures containing 300 μl of purified enzyme (approximately 300 to 400 μg of protein per ml) and 3.0 ml of purified κ-casein (10 mg/ml in 50 mM phosphate buffer [pH 6.4]) were incubated at 24°C. The proteolytic activities of the purified enzymes were first determined with fluorescein isothiocyanate-labelled β-casein as a substrate, and the level of activity of each enzyme solution was adjusted to yield approximately equal activities in the different digest mixtures. While this procedure would not necessarily ensure equivalent rates of digestion of κ-casein, it proved to be an adequate approximation. Samples were withdrawn from the digest mixtures at various times, and the reaction was stopped either by the addition of trifluoroacetic acid (TFA) (final concentration, 1% [wt/vol]) for subsequent high-performance liquid chromatography (HPLC) analysis or by boiling with sodium dodecyl sulfate (SDS)-mercaptoethanol for SDS-polyacrylamide gel electrophoresis (PAGE) analysis by procedures described previously (2).

SDS-PAGE and HPLC analyses. Digest samples were analyzed by SDS-PAGE by the procedure described by Coolbear et al. (2). 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.). HPLC analysis was carried out as described by Reid et al. (15).

Identification of TFA-soluble peptides. TFA-soluble peptides isolated by HPLC analysis of digest samples were identified by N-terminal sequence analysis and molar mass determination by mass spectrometry as described previously (15).

Plasmid DNA isolation and probing. Plasmid DNA was isolated from late-log-phase cells grown in M17 broth as described by Anderson and McKay (1). The DNA was purified by phenol and chloroform extraction and ethanol precipitation. A 4.6-kb *Hin*dIII-*Bgl*II fragment spanning 70% of the cloned proteinase gene from *L. lactis* subsp. *cremoris* H2 (30) was used as a probe. After separation from residual DNA by gel electrophoresis on 0.7% SeaKem agarose (FMC Bioproducts, Rockland, Maine), the fragment was excised, purified by a freeze-squeeze procedure similar to that described by Thuring et al. (22), and labelled with ^{32}P by the random primer method (Megaprime; Amersham International, Buckinghamshire, United Kingdom). The plasmid DNA was electrophoresed on a 0.7% agarose gel and transferred to Hybond-N nylon membranes (Amersham) by the method of Southern (19). Hybridization was carried out at 65°C under established conditions (9). Following hybridization, the membranes were washed twice for 15 min each time at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then once for 15 min at 65°C in 0.5× SSC. The washed membranes were wrapped in plastic film, and autoradiograms were made with Agfa Curix RP1 X-ray film and exposure for 1 to 5 days.

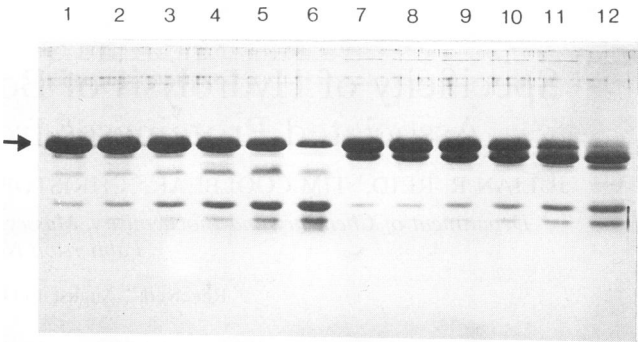

FIG. 1. SDS-PAGE analysis of samples taken at various times during the digestion of κ-casein by purified cell envelope-associated proteinases from *L. lactis* subsp. *cremoris*. Lanes: 1 to 6, samples taken at 5, 10, 30, and 90 min and at 5 and 24 h, respectively, during digestion by P_I-type proteinase from strain H2; 7 to 12, samples taken at 5, 10, 30, and 90 min and at 5 and 24 h, respectively, during digestion by P_{III}-type proteinase from strain SK11. The position of the κ-casein band is indicated by the arrow on the left-hand side of the gel.

RESULTS

Comparative specificity of κ-casein hydrolysis by P_I- and P_{III}-type proteinases. The P_I- and P_{III}-type proteinases purified from *L. lactis* subsp. *cremoris* H2 and SK11, respectively, were used to digest κ-casein. Samples taken at 5, 10, 30, and 90 min and at 5 and 24 h from each digest were analyzed by SDS-PAGE (Fig. 1). Although some of the products of κ-casein hydrolysis observed on gel staining appeared to be similar for the two proteinase types, distinct differences were also evident. One conspicuous difference between the actions of the two proteinases was the formation by the P_{III}-type proteinase of a major hydrolysis product which can be seen (Fig. 1) as a heavily stained band just below undigested κ-casein in SDS-PAGE. Only a minute trace of this product was evident in the digestion of κ-casein by the P_I-type proteinase. This difference was apparent from the earliest digest sample (5 min).

Representative HPLC profiles showing the TFA-soluble low-M_r peptides produced by the action of the two different proteinases on κ-casein are shown in Fig. 2. As anticipated from the SDS-PAGE analysis of these same digest samples, the HPLC profiles showed that a distinctive set of low-M_r peptide

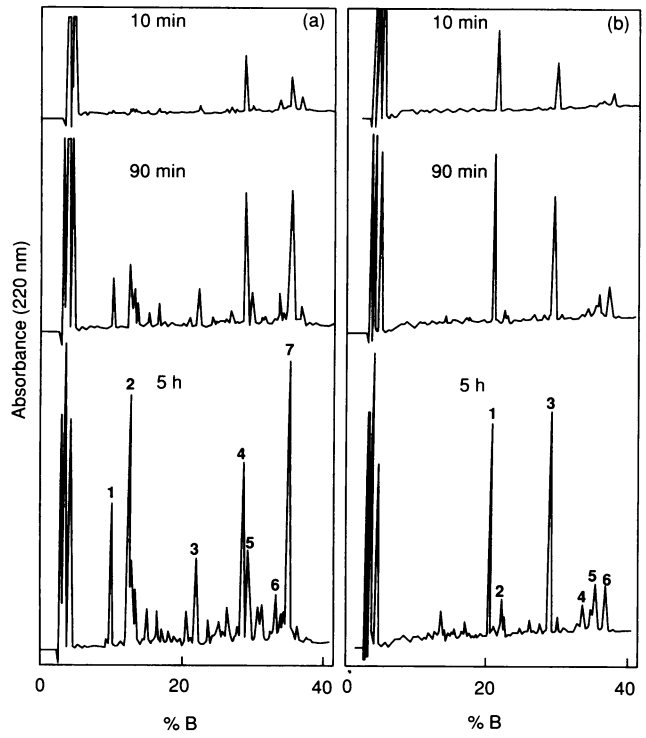

FIG. 2. Reverse-phase HPLC profiles of 1.0% TFA-soluble peptides from κ-casein digested with purified cell envelope-associated proteinases from *L. lactis* subsp. *cremoris* H2 (a) and SK11 (b). Samples were taken from the digest mixture at the times indicated. Solvent A was 0.1% TFA in water; solvent B was 0.08% TFA in acetonitrile. Peptides were eluted with a linear gradient of 0 to 50% solvent B over 50 min.

products was generated by the action of the two different proteinases.

Peptides collected from the major HPLC peaks were identified by sequence analysis, and the identifications were confirmed by determination of the M_rs by mass spectrometry. Alignment of the low-M_r peptide products resulting from κ-casein hydrolysis by the P_I- and P_{III}-type proteinases with the primary structure of κ-casein (11) is shown in Fig. 3.

The initial TFA-soluble low-M_r peptides produced from κ-casein by the P_I-type proteinase from *L. lactis* subsp. *cremoris* H2 (i.e., those clearly evident after 5 and 10 min of digestion) were the peptides comprising residues 72 to 79 (in HPLC peak 7), 96 to 106 (present in peak 4), and 103 to 106 (also present in peak 4). Thus, the initial sites of cleavage by the P_I-type proteinase within the κ-casein molecule resided close to the center of its primary structure. After longer periods of digestion (i.e., longer than 90 min), several additional TFA-soluble peptides, namely, the peptides comprising residues 24 to 32 (peak 6), 33 to 41 (peak 3), 96 to 100 (peak 1), 96 to 102 (peak 2), and 101 to 106 (peak 5), were detected in the digests.

Hydrolysis of κ-casein by the P_{III}-type proteinase from *L. lactis* subsp. *cremoris* SK11 resulted in the very early production of three TFA-soluble peptides comprising residues 66 to 79 (peak 6), 96 to 106 (peak 3), and 161 to 169 (peak 1), of which only the peptide comprising residues 96 to 106 was also an early product of P_I-type proteinase action. Of these early products, the peptide comprising residues 161 to 169, which includes the C terminus of the κ-casein molecule, appeared to be produced first. It therefore seems likely that the large N-terminal complement to this peptide (i.e., residues 1 to 160) was the very early hydrolysis product which ran just below the undigested κ-casein band in SDS-PAGE (Fig. 1). The C-terminal peptide comprising residues 161 to 169 was only present in trace amounts among the products of the P_I-type proteinase from strain H2. Continued hydrolysis of κ-casein by the P_{III}-type SK11 proteinase resulted in the slow release of peptides comprising residues 24 to 32 (peak 4), 72 to 79 (peak 5), and 152 to 160 (peak 2). Of these "late" P_{III}-type proteinase products, the peptide comprising residues 24 to 32 was also a late product of P_I-type proteinase action and the peptide

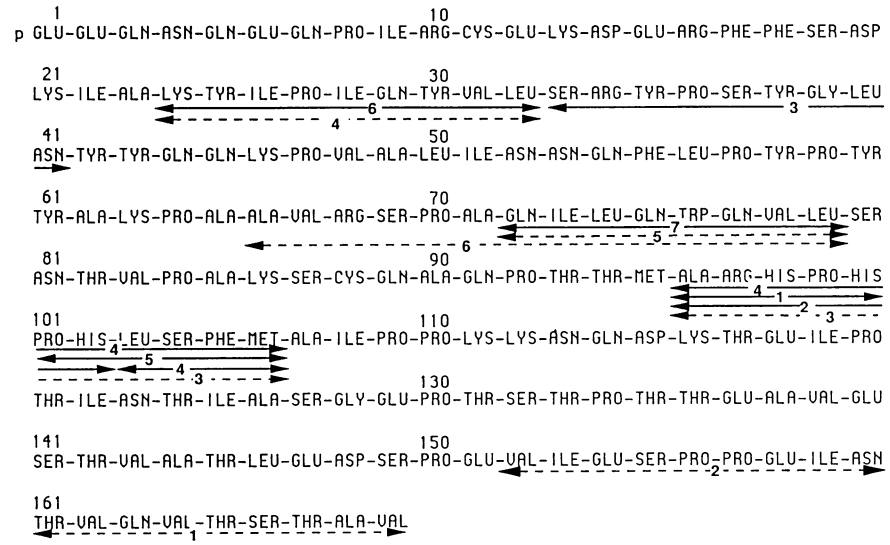

FIG. 3. Amino acid sequence of κ-casein showing the positions of TFA-soluble peptides produced by digestion with the P_I-type cell envelope-associated proteinase from *L. lactis* subsp. *cremoris* H2 (solid lines) and by the P_{III}-type proteinase from *L. lactis* subsp. *cremoris* SK11 (broken lines). Numbers refer to the HPLC peaks in Fig. 2.

comprising residues 72 to 79 was a very early P_I-type proteinase product, while the peptide comprising residues 152 to 160 was a distinctive P_{III}-type proteinase product not detectable among the products of P_I-type proteinase action for up to 24 h.

Hydrolysis of κ-casein by cell envelope-associated proteinases from other lactococcal strains. The relatively simple but distinctive nature of the κ-casein hydrolysis patterns obtained by digestion with the P_I- and P_{III}-type proteinases from H2 and SK11 (Fig. 1 and 2) suggested that digestion of this substrate might prove useful as a simple diagnostic test for identifying P_I- and P_{III}-type proteolytic activities from other lactococcal strains. The proteinases from *L. lactis* subsp. *lactis* ML3, ML8, and NCDO 1403 and from *L. lactis* subsp. *cremoris* E8, C5, 2210, H2, and SK11 were partially purified by anion-exchange chromatography. From each of these strains, except for *L. lactis* subsp. *lactis* NCDO 1403, a single major proteinase activity peak was obtained on anion-exchange chromatography. In the case of strain NCDO 1403, two distinct, well-separated peaks of proteolytic activity eluted from the anion-exchange column. To eliminate the possibility that the culture of this strain used was a mixture of two different strains, eight single-colony isolates were taken from M17 agar plates streaked with the original NCDO 1403 culture. The isolates were grown in milk, and the cell envelope-associated proteinase activity was released from harvested cells of each isolate in the usual way. Anion-exchange chromatography was performed on each of these crude proteinase preparations and, in all eight cases, two distinct peaks of proteolytic activity were detected eluting at the same salt concentrations and in about the same proportions as those previously found. The proteinases from the two anion-exchange peaks from *L. lactis* subsp. *lactis* NCDO 1403 were used separately in subsequent digestion studies, the early-eluting species being referred to as 1403A and the late-eluting species being referred to as 1403B.

The partially purified proteinases from *L. lactis* subsp. *lactis* ML3, ML8, and NCDO 1403 and from *L. lactis* subsp. *cremoris* E8, C5, 2210, H2, and SK11 were used to hydrolyze κ-casein, and the digests were analyzed by SDS-PAGE (Fig. 4). A comparison of the SDS-PAGE profiles revealed that, along with the SK11 enzyme, the E8, C5, and 1403A proteinases could be classified as possessing P_{III}-type proteinase activity since, in each case, the slowly running band just below undigested κ-casein was clearly present. The κ-casein digest produced by the proteinase from strain 2210 had only a trace amount of this fragment, so this proteinase could therefore be classified with the H2 proteinase as possessing P_I-type proteinase activity. In the samples digested with the ML3, ML8, and 1403B proteinases, the characteristic P_{III}-type proteinase hydrolysis product running just below κ-casein was clearly visible, although in relatively small quantities compared with those present in the typical P_{III}-type proteinase digests. Other than this product, these three enzymes appeared to produce the same hydrolysis products as the P_I-type proteinases from strains H2 and 2210. On this basis, the ML3, ML8, and 1403B proteinases can be classified as having mixed P_I/P_{III} intermediate-type proteinase specificity. Analysis of the low-M_r peptide products of hydrolysis by HPLC supports this classification (data not shown). The peptides generated by the SK11, E8, C5, and 1403A proteinases were very similar and, in particular, the C-terminal κ-casein peptide (residues 161 to 169) was present as the major product in the digests obtained with all four proteinases. On the other hand, the HPLC profiles for the 2210 and ML3 proteinase digests were similar to that for the H2 P_I-type proteinase digest, and the HPLC profiles for the ML8 and 1403B proteinase digests were somewhat different from either of the typical P_I- or P_{III}-type proteinase patterns.

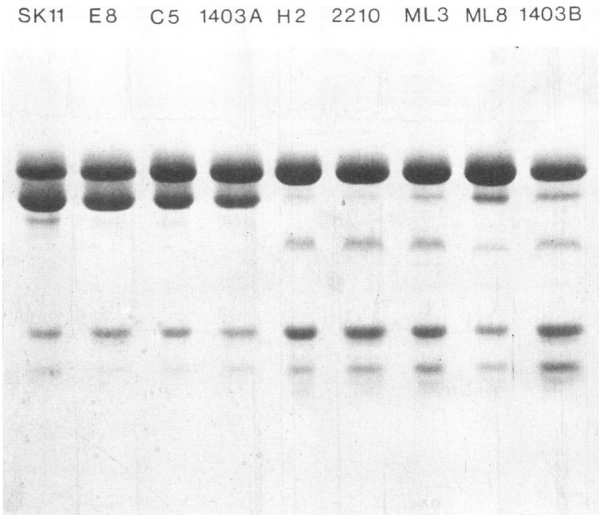

FIG. 4. SDS-PAGE analysis of samples of κ-casein digested for 4 h by cell envelope-associated proteinases from various strains of *L. lactis*. The strains are indicated at the top of each lane. Strains SK11, E8, C5, H2, and 2210 are *L. lactis* subsp. *cremoris* strains; strains ML8 and NCDO 1403 are *L. lactis* subsp. *lactis* strains; and strain ML3 is usually classified as an *L. lactis* subsp. *lactis* strain but may, on the basis of recent findings, be more correctly designated an *L. lactis* subsp. *cremoris* strain (7). The lanes labelled 1403A and 1403B represent digests of κ-casein with the two different proteinases isolated from strain NCDO 1403 (see the text).

The distinctive nature of both the SDS-PAGE and the HPLC patterns of the digestion products resulting from the action of the 1403A and 1403B proteinases suggests that these are two distinct enzymes rather than that one is an autoproteolytic product of the other. The presence of two different proteinases in strain NCDO 1403 was further confirmed by hybridization of plasmid DNA with a probe derived from the proteinase gene of *L. lactis* subsp. *cremoris* H2 (30). In the plasmid profile of this strain (Fig. 5), two separate plasmids were found to hybridize to this probe (Fig. 5b), while for a wide range of other lactococcal strains tested with this probe, only a single hybridization signal was found (data not shown). In the plasmid profile of a Prt^- derivative of NCDO 1403, no hybridization signal was obtained (Fig. 5b), although agarose gel analysis of this derivative showed that only one of the two plasmids which hybridized in the Prt^+ parent was missing (Fig. 5a). It is possible that the band corresponding to the position of the other hybridizing plasmid actually contained two plasmids with similar M_rs, only one of which was lost in the Prt^- derivative.

DISCUSSION

Both P_I- and P_{III}-type lactococcal cell envelope-associated proteinases hydrolyze κ-casein but, as with β-casein (16), there are several significant differences in the specificity of peptide bond cleavage between the two proteinase types. A comparison of the peptides produced from κ-casein by the action of the P_I- and P_{III}-type proteinases reveals that just three peptides, namely, those corresponding to residues 24 to 32, 72 to 79, and 96 to 106 in the κ-casein sequence, are common products of both proteinase types. The very early cleavage of the Met-106–Ala-107 bond by both types of proteinase is of interest, since this bond is immediately adjacent to the chymosin-sensitive Phe-105–Met-106 bond. Thus, the action of the starter cell

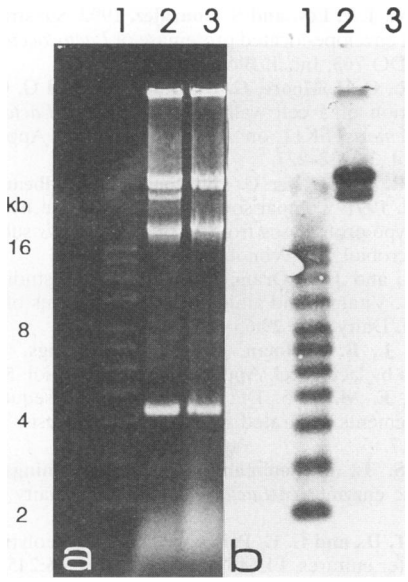

FIG. 5. (a) Agarose gel analysis of plasmids isolated from *L. lactis* subsp. *lactis* NCDO 1403. Lanes: 1, size standards (BRL supercoiled ladder); 2, *L. lactis* subsp. *lactis* NCDO 1403 Prt^+; 3, *L. lactis* subsp. *lactis* NCDO 1403 Prt^-. (b) Southern blot of plasmid gels shown in panel a hybridized to a ^{32}P-labelled proteinase gene probe isolated from *L. lactis* subsp. *cremoris* H2. Lanes are as in panel a.

envelope-associated proteinases of both types on micellar casein would be expected to be similar to that of chymosin in initiating the clotting of milk.

The molecular basis for the differences in peptide bond specificity between the two proteinase types is still subject to speculation, although manipulation of the proteinase genes has identified certain amino acid sequence differences between the proteinases which appear to be responsible for the specificity differences (28). An examination of the amino acid residues surrounding both the P_I- and the P_{III}-type proteinase cleavage sites identified within κ-casein reveals no obvious consensus sequence. Exterkate et al. (4) recently showed that digestion of the peptide comprising residues 1 to 23 of α_{s1}-casein by a P_I-type proteinase resulted in cleavage within the positively charged N-terminal region of the substrate, whereas digestion by a P_{III}-type proteinase resulted in cleavage at three sites within the negatively charged C-terminal region. Similarly, Visser et al. (26) found that the P_{III}-type proteinase from *L. lactis* subsp. *cremoris* AM1 acting on β-casein also displayed a preference for bonds at which negatively charged residues occupied the P_2-P_3 and/or P'_2-P'_3 regions of the substrate. This preference for cleavage of bonds at which a negative charge occurs in the vicinity has been attributed to the more positively charged nature of the substrate-binding region of the P_{III}-type proteinase (4). This suggestion is largely consistent with the results of the present study, which show that negatively charged residues are located close to two distinctive P_{III}-type proteinase cleavage sites located near the C terminus of κ-casein (i.e., Glu-151–Val-152 and Asn-160–Thr-161). Indeed, the Asn-160–Thr-161 bond, which has a negatively charged neighbor at position P_3, appeared to be the bond most susceptible to cleavage within κ-casein by the P_{III}-type proteinase from strain SK11. Neither of these two bonds is hydrolyzed to any significant extent by the P_I-type proteinase from strain H2. However, the location of the Ala-65–Ala-66 bond, which is specifically cleaved by the P_{III}-type proteinase, is not consistent with the proposed preference for negatively charged residues, since Lys and Arg residues are present in the P_3 and P'_3 positions, respectively.

In the only previous study of the lactococcal proteinase cleavage sites within κ-casein, Monnet et al. (14) identified the peptides produced by hydrolysis of a range of substrates, including κ-casein, by the cell envelope-associated proteinase from *L. lactis* subsp. *lactis* NCDO 763. They found that the proteinase from this strain exhibited a mixed P_I/P_{III} intermediate-type proteinase specificity with respect to its action on α_{s1}- and β-caseins. Of the 13 peptide bonds found to be cleaved in κ-casein by the *L. lactis* subsp. *lactis* NCDO 763 proteinase, 5 are the same as those cleaved by both the P_I- and the P_{III}-type proteinases from *L. lactis* subsp. *cremoris* H2 and SK11 (i.e., the bonds Leu-32–Ser-33, Ala-71–Gln-72, Leu-79–Ser-80, Met-95–Ala-96, and Met-106–Ala-107), 1 is the same as the unique H2 P_I-type proteinase cleavage site (Asn-41–Tyr-42), and 2 are the same as the unique SK11 P_{III}-type proteinase cleavage sites (Ala-65–Ala-66 and Asn-160–Thr-161). Five other bonds found to be cleaved by the NCDO 763 proteinase were not identified as cleavage sites in the present study, although examination of the κ-casein hydrolysate HPLC peptide profile published by Monnet et al. (14) indicates that most of these are probably minor cleavage sites.

The apparent presence of two different plasmid-linked cell envelope-associated proteinases in one strain (*L. lactis* subsp. *lactis* NCDO 1403) is unusual and does not appear to have been reported previously for any other lactococcal strain. The demonstration that eight single-colony isolates from this culture all showed the same two proteinases in the same relative proportions argues against the possibility that the culture used was a mixture of two strains.

One finding from the present study that is of particular physiological significance is the early release of the peptide comprising residues 96 to 106 from κ-casein by both P_I- and P_{III}-type proteinase action. This peptide is rich in histidine, which is known to be an essential amino acid for the nutrition of *L. lactis* (17). The peptides previously shown to be released from the C-terminal region of β-casein in the early stages of digestion by P_I-type proteinase action (13, 16, 27) contain all of the amino acids essential for lactococcal growth, with the exception of histidine. Since the P_I-type proteinase does not significantly digest α-casein, this situation poses the problem of the source of histidine for strains with this type of proteinase; κ-casein has been postulated as a possible source (18). The results of the present study showing the early release of the histidine-rich peptides comprising residues 96 to 106, 96 to 102, and 96 to 100 from κ-casein by the P_I-type proteinase from strain H2 suggest that these peptides may indeed supply the cells with their histidine requirement. These results also provide an explanation for the observation of Exterkate and de Veer (5) that the optimal growth of *L. lactis* subsp. *cremoris* HP, a P_I-type proteinase-producing strain, was obtained with β-casein in combination with a low concentration of κ-casein.

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ADDENDUM IN PROOF

An independent investigation of the hydrolysis of κ-casein by the P_{III}-type, cell envelope-associated proteinase from *L.*

lactis subsp. *cremoris* AM1 (S. Visser, C. J. Slangen, A. J. P. M. Robben, W. D. van Dongen, W. Heerma, and J. Haverkamp, Appl. Microbiol. Biotechnol., in press) has revealed a cleavage specificity almost identical to that found for the P_{III}-type proteinases in the present study.

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