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Involvement of Enzyme-Substrate Charge Interactions in the Caseinolytic Specificity of Lactococcal Cell Envelope-Associated Proteinases

JULIAN R. REID,[1]* TIM COOLBEAR,[1] CHRISTOPHER H. MOORE,[2]
DAVID R. K. HARDING,[2] AND GRAHAM G. PRITCHARD[2]

New Zealand Dairy Research Institute[1] and Department of Chemistry and Biochemistry, Massey University,[2] Palmerston North, New Zealand

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Three series of oligopeptides were synthesized to investigate the proposal that a major factor in determining the differences in specificity of the lactococcal cell surface-associated proteinases against caseins is the interactions between charged amino acids in the substrate and in the enzyme. The sequences of the oligopeptides were based on two regions of κ-casein (residues 98 to 111 and 153 to 169) which show markedly different susceptibilities to P_I- and P_{III}-type lactococcal proteinases. In each series, one oligopeptide had an identical sequence to that of the κ-casein region, while in the others, one or more charged residues were substituted by an amino acid of opposite charge, i.e., His↔Glu. Generally, substitution of His by Glu in the oligopeptides corresponding to residues 98 to 111 of κ-casein resulted in reduced cleavage of susceptible bonds by the P_I-type proteinase and increased cleavage of susceptible bonds by the P_{III}-type proteinase. In the case of the oligopeptide corresponding to residues 153 to 169 of κ-casein, one major cleavage site was evident, and the bond was hydrolyzed by both types of proteinase (even though this sequence in κ-casein itself is extremely resistant to the P_I-type enzyme). Substitution of Glu by His in this oligopeptide, even in the P_7 position, resulted in increased cleavage of the bond by the P_I-type proteinase and reduced cleavage by the P_{III}-type proteinase. C-terminal truncation of this oligopeptide resulted in a 100-fold decrease in the rate of hydrolysis of the susceptible bond and a change in the pattern of cleavage. While the charge on residues in the immediate vicinity of potentially susceptible bonds has a marked effect on their rate of cleavage by the proteinase, it is clear that other factors are important in determining which bonds in the casein molecule are actually hydrolyzed.

The cell envelope-associated proteinase (CEP) of lactococcal starters plays an important role in the hydrolysis of casein during the manufacture and ripening of cheese and has therefore been the focus of much attention to characterize its properties (see references 6, 9, and 17 for recent reviews). The proteinase has considerable homology to a family of serine proteases designated subtilases (16) because the members of the family have several structural features in common with subtilisin. However, the lactococcal CEP is a much larger enzyme than subtilisin, the molecular mass of the mature in vivo proteinase being about 180 kDa (7).

Different strains of lactococcal starter bacteria may possess different forms of CEP which are distinguished by their specificity of action in hydrolyzing bovine caseins (18). Two different specificity types, designated the HP or P_I-type and the AM1 or P_{III} type, were initially recognized (18), although more recent studies on the specificity of the proteinases have shown that these represent the most divergent specificity types from a range of intermediate forms (4). The proteinase genes from the different strains show a very high degree of homology (98% at the amino acid level), raising the question of the molecular basis for the highly reproducible and selective differences in caseinolytic specificity between such closely related enzymes.

Genetic studies (15, 20) have implicated a small number of amino acid residues in the primary sequence of the lactococcal CEPs as determinants of the specificity differences. Some of these critical residues carry different charges in the P_I- and P_{III}-type enzymes. Structure modeling on the basis of the regions that have close homology to those of the subtilisin-type proteinases (15) has suggested a possible mechanism whereby charge interactions of at least two of these critical residues with particular amino acid residues in the casein substrate might contribute to the differences in specificity of peptide bond hydrolysis. In particular, it has been suggested that the presence of a positively charged substrate residue in the sequence N-terminal to the scissile bond, especially in the P_2 to P_4 substrate positions (by the nomenclature of Schechter and Berger [14]), would favor cleavage by the P_I-type proteinase and inhibit cleavage by a P_{III}-type proteinase, while negative residues at these positions would have the reverse effect (5). The proposed mechanism has received support from the use of synthetic peptidyl *p*-nitroanilide substrates possessing differently charged residues N-terminal to the potential cleavage site (3, 4).

The present study was designed to test the consistency of the proposed model with respect to the specificity differences of two types of lactococcal CEP in hydrolyzing κ-casein (11). The H2 and SK11 proteinases have been shown previously to be representative of the P_I- and P_{III}-type proteinases, respectively (12, 13). The specificity of action of these particular proteinases on α_{s1}-, β-, and κ-casein has been well established (11–13), and they were selected for use in this study because of this. Oligopeptides corresponding to two regions of the κ-casein molecule which show different susceptibility to the two types of proteinase were synthesized. Variants of these oligopeptides in which particular charged residues were substituted by amino

* Corresponding author. Phone: 64 6 350 4649. Fax: 64 6 356 4740.

acid residues of the opposite charge were also synthesized. The rates and specificity of hydrolysis of these synthetic peptides by the two different proteinase types were determined and compared with the action of the proteinases on intact κ-casein.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and harvesting. *Lactococcus lactis* subsp. *cremoris* H2, HP, and SK11 were obtained from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand. Cultures were grown and harvested as described by Coolbear et al. (1).

Purification of the CEP. Proteinase was solubilized by incubating the washed cells in 50 mM sodium phosphate buffer (pH 6.4) at 30°C for two successive 30-min incubations. The released enzyme was purified by ion-exchange and gel-exclusion chromatographies as described by Coolbear et al. (1).

Peptide synthesis, purification, and characterization. Peptide substrates used in this study (see Fig. 1) were synthesized by solid-phase methods on a beaded cellulose resin by use of 9-fluorenylmethylcarbonyl (Fmoc) methodology. The hydroxybenzotriazole active esters were used to couple all Fmoc-amino acids as described by Englebretsen and Harding (2). Oxidation of the methionine residue (which occurred during synthesis of peptide 2A) was reversed by reduction with dithiothreitol as described by Polzhofer and Ney (8). After cleavage from the resin, 50 to 100 μg of the crude peptide material was purified by reverse-phase high-performance liquid chromatography (HPLC). The identity of each of the peptides prepared in this manner was confirmed by determination of the M_r by mass spectrometry as described previously (12).

Hydrolysis of peptides by the proteinase. A freeze-dried sample of each of the purified peptides was dissolved in 10 mM bis-Tris propane buffer (pH 6.4), containing 5 mM $CaCl_2$, immediately prior to digestion. Various quantities of each of the purified peptides (up to 500 nmol) were incubated at 24°C with an appropriate amount of proteinase (standardized by reference to hydrolysis of fluorescein isothiocyanate-labelled β-casein) so that the ratios of enzyme activity to substrate concentration were very similar for each digest. Samples were withdrawn at various times for analysis by HPLC, and the reaction was stopped by the addition of trifluoroacetic acid to a final concentration of 1% (vol/vol).

Identification of the products of peptide hydrolysis. After purification by HPLC, the identity of each of the peptide fragments resulting from digestion of each substrate was confirmed by N-terminal sequence analysis and determination of the M_r by mass spectrometry, as described previously (12).

RESULTS

Digestion of the κ-casein fragment of residues 153 to 169 by P_I- and P_{III}-type proteinases. A major distinction between P_I- and P_{III}-type proteinases in their specificity of hydrolysis of κ-casein lies in the resistance of the C-terminal region to hydrolysis by the P_I-type proteinase as compared with the susceptibility of certain bonds in this region to P_{III}-type proteinase-catalyzed hydrolysis (11, 19). In particular, the Asn-160—Thr-161 bond is hydrolyzed more rapidly than any other bond in κ-casein by the P_{III}-type proteinase but is not detectably hydrolyzed by the P_I-type proteinase (Fig. 1A). The possible involvement of negatively charged residues N-terminal to this bond in determining this difference in cleavage specificity was investigated by studying the hydrolysis of three peptides (designated 1A, 1B, and 1C) based on the κ-casein residue 153-to-169 sequence (Val-169 is the C-terminal residue of κ-casein). The purified CEPs from *L. lactis* subsp. *cremoris* H2 and SK11 were used as sources of P_I- and P_{III}-type proteinases, respectively. Hydrolysis of all three substrates by the P_I-type proteinase produced the complementary fragments of residues 1 to 8 and 9 to 17 (fragment 1-8 and fragment 9-17) only, indicating that cleavage of each substrate resulted exclusively from hydrolysis of the bond Asn-8—Thr-9 (Fig. 1A). Therefore, Asn-8—Thr-9 was the only bond susceptible to hydrolysis within this substrate irrespective of the charge on the P_3 or P_7 residue. Digestion of all three substrates by the P_{III}-type proteinase produced fragment 1-8 and fragment 9-17 as the main hydrolysis products, with only minor quantities of fragment 1-11 and fragment 1-12 (Fig. 1A). Hence, cleavage by the P_{III}-type enzyme resulted primarily from hydrolysis of the peptide bond Asn-8—Thr-9, with only minimal hydrolysis of the bonds Gln-11—Val-12 and Val-12—Thr-13. As found with the P_I-type enzyme, the nature of the charge on the residues at position 2 or 6 had no effect on which bonds were susceptible to hydrolysis by the P_{III}-type proteinase.

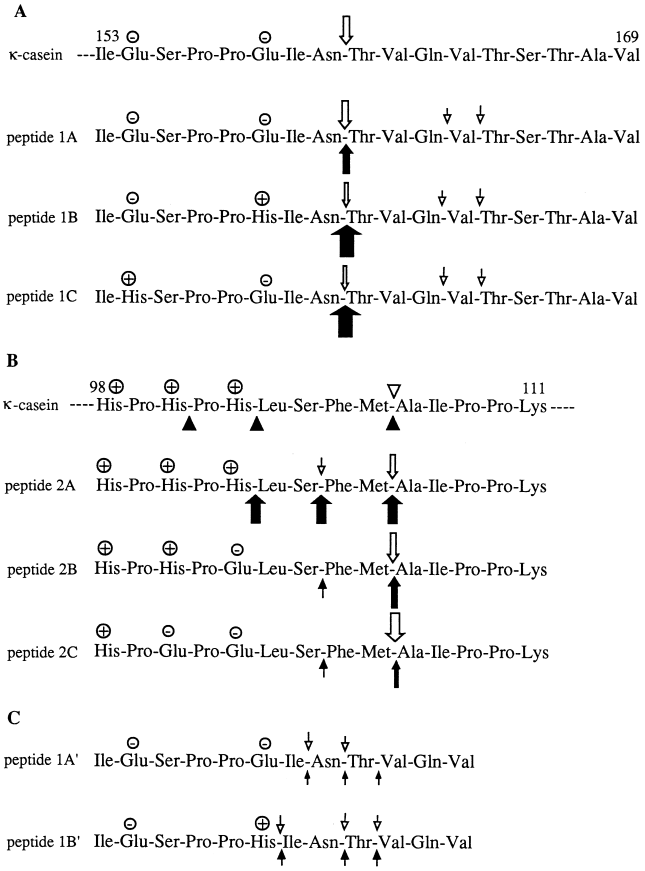

FIG. 1. Specificity of cleavage of the synthetic peptides 1A, 1B, and 1C (after 1 h of digestion) and the residue 153-to-169 region of κ-casein upon which the sequences of the peptides were based (A), the synthetic peptides 2A, 2B, and 2C (after 1.5 h of digestion) and the residue 98-to-111 region of κ-casein upon which the sequences of the peptides were based (B), and the synthetic peptides 1A′ and 1B′ (after 24 h of digestion) based on the residue 153-to-164 region of κ-casein (C). Peptide bonds hydrolyzed by the P_I-type (⬆ or ▲) and P_{III}-type (⇩ or ▽) proteinases are shown. Arrow size gives an indication of the relative rates of hydrolysis of the scissile bonds, whereas triangles indicate scissile bonds for which no rate comparison is possible. Residues located N-terminally to the cleavage sites and which possess charged side chains are indicated.

A comparison of the various rates of substrate hydrolysis revealed that peptide 1A, which had a sequence identical to that of the region of κ-casein consisting of residues 153 to 169, was cleaved by the two proteinases at similar rates (Fig. 2). The rates at which the P_I-type proteinase cleaved peptides 1B and 1C (each of which had one positively charged residue substituted in their N-terminal regions) were almost identical and were significantly higher than the rate at which this proteinase hydrolyzed peptide 1A. In contrast, the P_{III}-type proteinase cleaved both peptides 1B and 1C at somewhat lower rates than it did peptide 1A (Fig. 2).

Relative hydrolysis rates of peptide 1A and κ-casein. The sequence of peptide 1A was identical to that of the region consisting of residues 153 to 169 of κ-casein. To compare the rates of hydrolysis of the equivalent peptide bonds Asn-8—Thr-9 within peptide 1A and Asn-160—Thr-161 within κ-casein, each of these substrates was digested with the P_I- and P_{III}-type proteinases. The molar concentration of each sub-

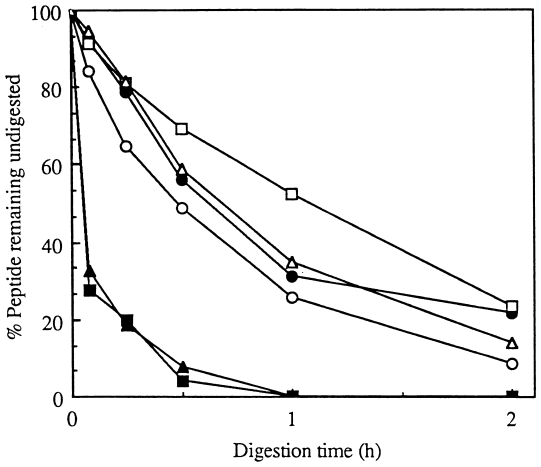

FIG. 2. Hydrolysis of peptides 1A (●, ○), 1B (■, □), and 1C (▲, △) by the P_I-type (closed symbols) and P_{III}-type (open symbols) proteinases. Samples taken at various times during the digestions were analyzed by reverse-phase HPLC, and hydrolysis was monitored by measuring the amount of substrate remaining undigested at each sampling time.

strate was adjusted to the same value, and hydrolysis of the equivalent Asn—Thr bond within each substrate was monitored by determining the appearance of either the peptide 1A-derived fragment 9-17 or the identical κ-casein-derived peptide consisting of residues 161 to 169 (Fig. 3). As expected, the peptide consisting of residues 161 to 169 of κ-casein was not detected in the H2 P_I-type proteinase digest of κ-casein since this proteinase has been shown previously not to hydrolyze the Asn-160—Thr-161 bond (11). However, in the P_{III}-type proteinase digest of κ-casein, the fragment consisting of residues 161 to 169 was produced at a little under half the rate at which the corresponding peptide 1A-derived fragment 9-17 appeared. This is not surprising since the proteinase must bind to several regions of the κ-casein molecule in addition to that involved during hydrolysis of the Asn-160—Thr-161 bond (11, 19), and thus, a lower overall rate of hydrolysis of this bond in κ-casein relative to that in peptide 1A would be expected.

Digestion of the κ-casein fragment consisting of residues 98 to 111 and variants by P_I- and P_{III}-type proteinases. Digestion of κ-casein by the P_I- and P_{III}-type proteinases has been shown previously to involve hydrolysis of bonds within the region consisting of residues 98 to 111 (11, 19). The P_I-type proteinase cleaves three bonds within this region, whereas just one bond is susceptible to hydrolysis by the P_{III}-type proteinase (Fig. 1B). To study the influence of neighboring charged residues upon the differential susceptibility to hydrolysis of the scissile bonds within this region, hydrolysis of the three synthetic peptides based on the amino acid sequence of the residue 98-to-111 region of κ-casein (designated peptides 2A, 2B, and 2C) was studied. Hydrolysis of peptide 2A by the P_I-type proteinase produced the fragments 1-5, 1-7, 1-9, 10-14, and 6-14, indicating that the bonds (His-5—Leu-6, Ser-7—Phe-8, and Met-9—Ala-10) were hydrolyzed (Fig. 1B). Two of these bonds (His-5—Leu-6 and Met-9—Ala-10) were equivalent to those hydrolyzed in κ-casein by the P_I-type proteinase (11). Substitution of the positively charged His residue at position 5 by a negatively charged Glu residue markedly affected hydrolysis since the main products resulting from hydrolysis of peptide 2B were fragments 1-9 and 10-14 with only a small quantity of fragment 1-7 produced, indicating that the bond Met-9—Ala-10 was the main hydrolysis site and the bond Ser-7—Phe-8 was only a minor cleavage site. When the His residues at positions 3 and 5 were both substituted by Glu residues (peptide 2C), the fragments produced following hydrolysis by the P_I-type proteinase were identical to those produced by hydrolysis of peptide 1B. Thus, the presence of a second negative charge did not result in any further change to the peptide bonds susceptible to hydrolysis. Hydrolysis of peptide 2A by the P_{III}-type proteinase produced fragment 1-9 and fragment 10-14 as the main products, with only small amounts of fragment 1-7, indicating that the bonds Met-9—Ala-10 and Ser-7—Phe-8 were major and minor hydrolysis sites, respectively (Fig. 1B). Introduction of negative charges in the N-terminal region, as in peptides 2B and 2C, suppressed cleavage of the Ser-7—Phe-8 bond by the P_{III}-type proteinase, with only the Met-9—Ala-10 bond being hydrolyzed.

To examine the effects of the substitutions within peptides 2A, 2B, and 2C on the overall rate of hydrolysis, the rate of decrease in substrate concentration in each digest was measured (Fig. 4). The P_I-type proteinase hydrolyzed peptide 2A (which was identical to the region of residues 98 to 111 of κ-casein) at a significantly higher rate than did the P_{III}-type proteinase. Of the three substrates, peptide 2A was hydrolyzed by the P_I-type proteinase at a higher rate than that of peptide 2B, which in turn was hydrolyzed at a higher rate than that of peptide 2C. When digested with the P_{III}-type proteinase, the relative rates of hydrolysis were in the opposite order, with peptide 2C being the most rapidly hydrolyzed and peptide 2A being the least rapidly hydrolyzed.

Digestion of the κ-casein fragment consisting of residues 153 to 164 and variant. To study the importance of the residues C-terminal to the cleaved bond, shorter versions of peptides 1A and 1B (corresponding to the region within κ-casein consisting of residues 153 to 164) were synthesized. These shorter peptides, designated 1A′ and 1B′ (Fig. 1C), had N-terminal sequences identical to those of peptides 1A and 1B, respectively, but were C-terminally truncated by five residues. By comparing both the rate of cleavage and the hydrolysis products resulting from digestion of peptides 1A′ and 1B′ with those obtained from peptides 1A and 1B, the effect of C-terminal truncation on hydrolysis was assessed.

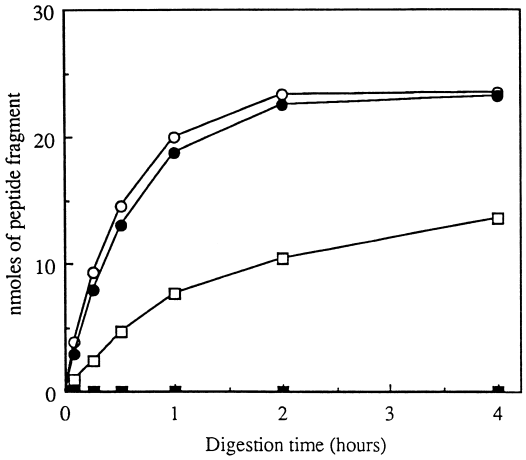

FIG. 3. A comparison of the rates of hydrolysis of the equivalent peptide bonds Asn-160—Thr-161 in κ-casein and Asn-8—Thr-9 in peptide 1A. Equal quantities (27 nmol) of peptide 1A and κ-casein were hydrolyzed by the P_I-type (closed symbols) or P_{III}-type (open symbols) proteinases, and samples were taken at various times for analysis by HPLC. Hydrolysis of the two equivalent peptide bonds was monitored by measuring the amounts of the identical peptide products consisting of residues 161 to 169 of κ-casein (■, □) and residues 9 to 17 of peptide 1A (●, ○) in the respective samples.

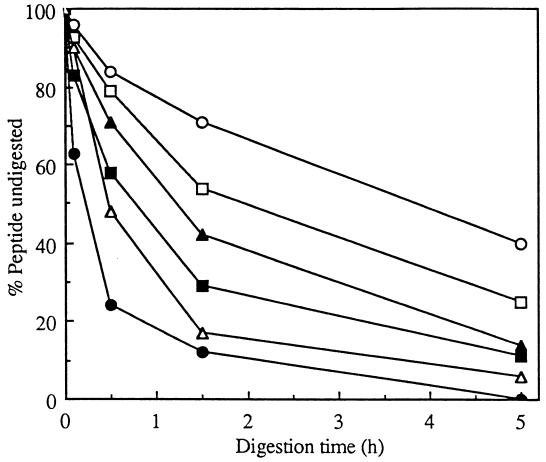

FIG. 4. Hydrolysis of peptides 2A (●, ○), 2B (■, □), and 2C (▲, △) by the P_I-type (closed symbols) and P_{III}-type (open symbols) proteinases. Samples taken at various times during the digestions were analyzed by reverse-phase HPLC, and hydrolysis was monitored by measuring the amount of substrate remaining undigested at each sampling time.

Analysis of the digestion products revealed that the P_I-type proteinase cleaved peptide 1A′ to give the fragments 1-7, 1-8, 1-9, and 9-12, indicating that the three adjacent bonds, Ile-7—Asn-8, Asn-8 — Thr-9, and Thr-9—Val-10 were hydrolyzed (Fig. 1C). Replacement of a negatively charged Glu residue with a positively charged His residue at position 6 (in peptide 1B′) enhanced the overall rate of hydrolysis by the P_I-type enzyme by two- to threefold (data not shown). The cleavage pattern was also altered, with fragments 1-6, 1-8, 1-9, and 7-12 (Fig. 1C) produced as a result of hydrolysis of the His-6—Ile-7, Asn-8—Thr-9, and Thr-9—Val-10 bonds. Hydrolysis of peptide 1A′ by the P_{III}-type proteinase gave fragments 1-8 and 9-12 as the main products and smaller quantities of fragment 1-7, indicating that cleavage of this substrate resulted primarily from hydrolysis of the Asn-8—Thr-9 bond with limited hydrolysis of the Ile-7—Asn-8 bond (Fig. 1C). Cleavage of peptide 1B′ by the P_{III}-type proteinase was also influenced by the change of charge at position 6 since the fragments 1-6, 1-8, 1-9, and 7-12 were produced. Thus, both proteinase types cleaved the same bonds within this substrate.

To provide an indication of the relative rates of hydrolysis of peptides 1A and 1A′, each substrate was incubated separately with the P_I- and P_{III}-type proteinases and hydrolysis was monitored by measuring the formation of fragment 1-8. This provided a reasonable measure of the hydrolysis rate for peptide 1A by both proteinases and of peptide 1A′ by the P_{III}-type proteinase, since in these cases the major site of hydrolysis was the bond Asn-8—Thr-9. However, hydrolysis of peptide 1A′ by the P_I-type proteinase was underestimated by this method since this proteinase cleaved several other bonds within this substrate at similar rates. Nevertheless, the rate of hydrolysis of the Asn-8—Thr-9 bond in peptide 1A was 100-fold greater than that of the Asn-8—Thr-9 bond in peptide 1A′ (results not shown).

Comparison of the specificities of the HP and H2 proteinases. The proteinase from *L. lactis* subsp. *cremoris* H2 has been used in the present study (and in previous studies [11–13]) as representative of a P_I-type proteinase. In a recent comparison of the specificity of peptide bond hydrolysis by proteinases from several lactococcal strains (4), the proteinase of the H2 strain was reported to show some differences in specificity from that of the proteinase from the strain HP, which, on the basis of its hydrolytic action on the α_{s1}-casein fragment of residues 1 to 23, was shown to be typical of the P_I (or CEP_I) type of lactococcal CEP. In view of this, the specificities of the HP and H2 proteinases in hydrolyzing two of the small peptides used in the present study (peptides 1A and 2A) and κ-casein were compared.

The HPLC profiles obtained upon analysis of the low-M_r peptide products resulting from hydrolysis of κ-casein by each proteinase were highly similar, with just a few minor differences apparent in peak heights (data not shown). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the complementary high-M_r products appeared to be identical for each proteinase (data not shown). Identical specificities were also observed with peptide 1A, and both enzymes produced the same fragments from hydrolysis of peptide 2A, although the Ser-7—Phe-8 bond in this substrate was a relatively minor hydrolysis site for the HP proteinase and it was a major site for the H2 proteinase (Fig. 5). Therefore, with respect to the substrates used in our studies, the specificities of the two proteinases proved to be very similar. Of the three substrates used, only peptide 2A was cleaved in a manner that allowed the activity of the two proteinases to be differentiated, and this was

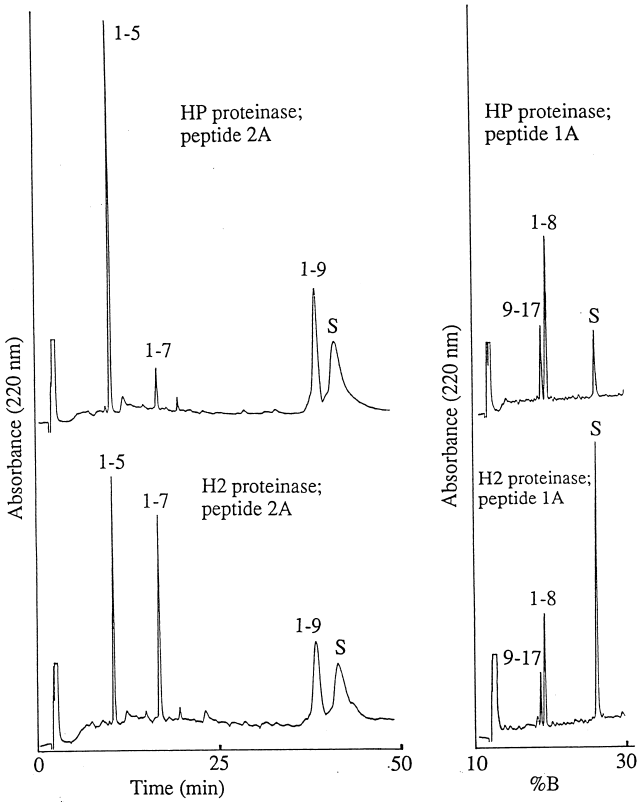

FIG. 5. HPLC profiles of the products recovered after incubating peptides 1A and 2A for 1 h with the proteinases from *L. lactis* subsp. *cremoris* HP and H2. The peptide and proteinase used are indicated on each profile. Numbers identify the peptide fragments detected within each peak; the substrate peak is labelled S. The gradient used to separate the products resulting from hydrolysis of peptide 1A was 19 to 30% solvent B in 20 min. The gradient used to separate the products resulting from hydrolysis of peptide 2A was as follows: 0 to 17.9% solvent B in 17.9 min, isocratic at 17.9% solvent B for 16 min, 17.9 to 22.5% solvent B in 1 min, isocratic at 22.5% solvent B for 15 min. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.08% trifluoroacetic acid acetonitrile. The flow rate was 1 ml/min.

based on a quantitative rather than a qualitative difference in cleavage specificity.

DISCUSSION

The main objective of the present study was to test the proposal that charge interactions make a significant contribution to the differences in specificity among the proteinases from different lactococcal strains. Previous evidence for the proposal is based on correlations between the substrate specificity and the occurrence of positively or negatively charged residues in the amino acid sequence of specificity-determining regions of the enzyme (3–5, 15, 20). The present study provides a complementary approach by varying the nature of charged residues in peptides possessing amino acid sequences corresponding to the sequences of particular regions of the casein substrate.

The relative rate of cleavage of the 17-residue peptide 1A was of the same order as that of the corresponding region in κ-casein, indicating that the oligopeptides do provide satisfactory model systems for studying interaction of the proteinase with particular regions of the casein molecule. However, when a C-terminally shortened version of peptide 1A (peptide 1A′) with only four residues C-terminal to the scissile bond was used, the rate of hydrolysis was markedly reduced and the pattern of cleavage was altered. Thus, enzyme-substrate interaction during hydrolysis may involve a more extended binding site than the current models suggest (5, 15). Alternatively, the low hydrolysis rate may be a consequence of the proximity of the negative charge on the α-carboxyl group of the C-terminal residue of the shortened peptide. This charge may lead to electrostatic repulsion by a negatively charged group in the proteinase. The major cleavage sites within the small peptides used in the present study (except for peptides 1A′ and 1B′) were mostly the same as those in κ-casein, i.e., the actual peptide bonds cleaved are determined mainly by the sequence of amino acid residues in the close vicinity of the scissile bonds rather than by gross conformational features of the whole molecule. However, the accessibility of particular regions of the casein molecule to hydrolysis may be determined largely by its general conformation.

Substitution of charged residues located in the N-terminal regions of the various peptides by residues possessing the opposite charge had a major influence on the rate at which hydrolysis occurred. In particular, the substitution of negatively charged Glu residues by positively charged His residues (in peptides 1A, 1B, and 1C) enhanced the rate of substrate cleavage by the P_I-type H2 proteinase and reduced the rate of cleavage by the P_{III}-type SK11 proteinase. When His residues were substituted by Glu residues (peptides 2A, 2B, and 2C), the opposite effect was observed. These findings provide further support for the suggestion of Exterkate et al. (5) that the P_I-type proteinase, which possesses a negatively charged binding region, prefers positively charged substrate residues located N-terminally to the scissile bond, whereas the P_{III}-type proteinase, which possesses a positively charged binding region, prefers negatively charged residues located in this region of the substrate. However, the actual peptide bonds cleaved within the larger substrates for which the rate of hydrolysis was relatively high (peptides 1A, 1B, 1C, 2A, 2B, and 2C) were, with the exception of peptide 2A, largely unaffected by the changes in charged residues. This indicates that although the side chain charge on the particular residues involved is important in the general binding process, other interactions are more important in determining which particular bonds are susceptible to hydrolysis. The substitution of a Glu residue in peptide 1A′ by a His residue in peptide 1B′ did affect the pattern of peptide bond cleavage. However, the significance of this observation is difficult to assess given the very low rates of hydrolysis obtained with each of these substrates.

Current models of the active site of the proteinase show interaction between the P_4 to P_1 (5) or P_6 to P'_1 (15) substrate residues and amino acid residues of the active-site cleft. However, in the present study, hydrolysis of peptide 2C revealed that the charge on the P_7 residue had a marked effect on the rate of cleavage. Moreover, with peptides 1B and 1C, the introduction of a positive charge in the P_7 position had the same marked effect on the rate of substrate cleavage as the introduction of a positive charge in the P_3 position. These results suggest that proteinase-substrate charge interactions involving residues more distant from the scissile bond than those identified in current models can influence hydrolysis. Indeed, Exterkate et al. (4) suggested that the negatively charged Asp-142 residue of the P_I-type proteinase from *L. lactis* subsp. *cremoris* HP may be involved in electrostatic interactions with substrate residues that are remote from the scissile bond. These authors suggest that this residue, which is predicted to be located at the surface of the proteinase just beyond the region known to interact with the P_4 and P_1 substrate residues, may be able to interact with charged P_6 residues. It is possible that Asp-142 might interact with a more distal substrate residue such as that in the P_7 position. The identity of the amino acid residue at position 142 of the H2 proteinase is unknown, and so its possible role in electrostatic interaction of this proteinase with substrate residues cannot be assessed. However, the SK11 proteinase possesses an Ala residue at position 142 (21) which is not capable of participating in electrostatic interactions. Thus, the proteinase residue(s) responsible for interacting with the P_7 substrate residue may be additional to those that are shown in the current models of the substrate binding site.

The one major exception to the general similarity between the action of the proteinases on the peptides and that on the corresponding regions within κ-casein was the susceptibility to hydrolysis of the Asn-8—Thr-9 bond in peptide 1A and the corresponding Asn-160—Thr-161 bond in κ-casein. The P_I-type H2 proteinase readily hydrolyzed this bond within peptide 1A but is unable to hydrolyze the corresponding bond within κ-casein (11). Therefore, failure of the P_I-type proteinase to hydrolyze the κ-casein Asn-160—Thr-161 bond cannot be due to the amino acid sequence immediately surrounding the bond and may be related to features of the amino acid sequence in regions of the κ-casein molecule lying outside the residue 153-to-169, C-terminal region. Rapp et al. (10) used a combination of methods to predict secondary structural features of κ-casein and showed that the sequence Pro-156—Pro-157 is likely to be at the center of a β-turn. This reversal of chain direction may cause the C-terminal segment of κ-casein to fold back towards the region consisting of residues 147 to 151, which possesses a high negative charge density (residues 147, 148, and 151 possess negatively charged side chains, and Ser-149 is phosphorylated). If the Asn-160—Thr-161 bond is positioned close to this cluster of negatively charged residues in the three-dimensional structure of κ-casein, then electrostatic repulsion between the negatively charged substrate binding region of the P_I-type proteinase and the region consisting of residues 147 to 151 may prevent hydrolysis of this bond. Conversely, the P_{III}-type proteinase, which possesses a positively charged binding region, should bind strongly to such a negatively charged region of the substrate.

Thus, the results of the present study, while largely consistent with the original model proposed to account for specificity

differences between lactococcal CEPs (3, 5), indicate that the nature of the interactions which may occur between the CEPs and caseins may be much more complex than the simple, subtilisin-based model predicts.

ACKNOWLEDGMENTS

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