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Diversity of Cell Envelope Proteinase Specificity among Strains of *Lactococcus lactis* and Its Relationship to Charge Characteristics of the Substrate-Binding Region

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The biochemical and genetical diversity of the subtilisin-like cell envelope proteinase (CEP) among *Lactococcus lactis* strains was investigated. The specificities of the proteinases of 16 strains toward the important cheese peptide α_{s1}-casein fragment 1 to 23 and toward two differently charged chromophoric peptides have been determined. On the basis of the results, these strains could be classified into seven groups. The contribution to the specificity of specific residues in the large C-terminal segment, which differentiates this proteinase from most other members of the subtilisin family, was established with hybrid proteinases, even in the case of the small substrates. These remote residues and the subtilisin-like substrate-binding region are therefore assumed to be spatially close to each other and together constitute most of the binding region of CEP. DNA sequence analysis of fragments of the gene (*prtP*) encoding segments of the proteinase which contain the relevant residues of the substrate-binding region shows that among the strains studied, this binding region is the most negatively charged in the CEP group represented by strain HP and the most positively charged in the CEP group represented by strains AM1 and SK11. Consequently, these two proteinase groups show the most divergent specificities. Each of the proteinases of the other groups shows a different intermediate specificity which in part is the reflection of an intermediate charge in the binding region. However, the results suggest that amino acid residues outside the segments known to be part of the CEP-binding region also contribute to specificity. We propose a new nomenclature of CEPs which is based on their defined specificities toward the α_{s1}-casein fragment 1 to 23 and on their origins.

Lactococcus lactis produces a cell envelope-located serine proteinase (CEP) which, during growth in milk, is pivotal in the process of supplying the cell with essential amino acids and nitrogen (18, 27). During cheese ripening, its action is essential for amino acid production and cheese flavor development (5, 28).

The CEPs of a number of *L. lactis* strains have been characterized biochemically (4, 29) and have been shown to be immunologically related (14). Two major proteinase types have been distinguished on the basis of preliminary biochemical characteristics (4) and on the basis of their cleavage specificities with respect to the caseins (29). Representatives of these major types have been purified and characterized from *L. lactis* subsp. *cremoris* HP (P_I-type proteinase) and AM1 (P_{III}-type proteinase) (11, 12).

Recently, the ability of the P_I-type proteinase, unlike the P_{III} type, to cleave a positively charged chromophoric peptide (methoxysuccinyl-arginyl-prolyl-tyrosyl-*p*-nitroanilide [MS-Arg]) efficiently at low ionic strength was attributed to the presence of a cation-binding site in its substrate-binding region (6). This suggestion was supported when the amino acid sequences of the substrate-binding region of the proteinases of strain Wg2 (showing P_I characteristics) and strain SK11 (P_{III} type) were compared in a three-dimensional model (9). Complete amino acid sequences of these proteinases have been deduced from the nucleotide sequences of their genes, which encode a large preproenzyme of more than 1,900 residues (16, 32). The N-terminal segment (± 500 residues) of the processed, mature proteinase shows significant sequence similarity to the serine proteinases of the subtilisin family (32). This similarity is highest with respect to residues around the catalytic triad and in the substrate-binding region. An important difference from most other members of the subtilisin family is the presence of a large, additional C-terminal extension of more than 1,200 residues which ends in a transmembrane anchor. An amino acid sequence identity of 98% was established between the proteinases of strains Wg2 and SK11. Five of the 44 substitutions are located in parts of the N-terminal segment of 173 amino acid residues of the proteinase which, on the basis of a significant sequence similarity with the subtilisin family, have been predicted to be involved in substrate binding (26, 32). Protein engineering studies employing hybrid Wg2-SK11 proteinases identified this N-terminal segment as one of two segments that significantly affect casein cleavage specificity (31). The second one is the large C-terminal extension in which a small segment (containing residues 747 to 748 and hereafter designated the remote segment) was identified as the relevant part. A model of the catalytic site and substrate-binding region of the lactococcal proteinase, predicted on the basis of its close similarity with subtilisin (9), shows that a negatively charged residue in the binding region of the Wg2 proteinase (Asp-166, the putative cation-binding site) is replaced by the uncharged residue Asn in the SK11 proteinase. Moreover, the replacement of Thr-138 in Wg2 proteinase by Lys in the SK11 proteinase has been suggested to add to the low affinity of the latter proteinase for the above-mentioned, positively charged chromophoric peptide because of charge repulsion (9).

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In a comparative study of the specificities of both proteinase types toward fragment 1 to 23 of α_{s1}-casein [α_{s1}-CN (f1–23)], it has been observed that the charge N terminal to the cleaved bond is a determinant in substrate binding and therefore that long-range electrostatic forces play an important role in the selection of peptide bonds to be cleaved (7, 9). With respect to the bonds cleaved in α_{s1}-CN(f1–23), binding in itself is either mainly electrostatic or (also) hydrophobic. Salt and pH both drastically alter the preference for bonds in α_{s1}-CN(f1–23), which can even result in a totally changed specificity (7).

Other natural proteinase variants with intermediate P_I and P_{III} type or perhaps different specificities might be expected to occur among lactococci because of substitutions of essential residues of the enzyme which are involved in substrate binding. The above-mentioned shifts in specificity toward α_{s1}-CN(f1–23) induced by pH or salt indicate that this peptide is an ideal substrate to reveal these differences in specificity. We therefore decided to compare proteinases from various strains, most of which were the subject of an earlier comparative study (29), in their action on α_{s1}-CN(f1–23).

Moreover, we selected chromophoric substrates which could be used to obtain additional information about the diversity of lactococcal CEPs. In addition, we assessed the importance of the remote segment for cleavage of these small substrates. In a number of selected proteinases, the differences in specificities are related to amino acid substitutions in the subtilisin-like substrate-binding region and this remote binding segment.

MATERIALS AND METHODS

Origin of the strains. The strains of *L. lactis* subsp. *cremoris* designated C13, E8, and HP were originally obtained from the Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia, and strains Z8, KH, TR, US3, SK11, AM1, and AM2 came from the New Zealand Dairy Research Institute. Strains FD27 and Wg2 were from the Netherlands Institute for Dairy Research (NIZO) collection. Strain SCR 050111 was kindly provided by Christian Hansen's Laboratory, Copenhagen, Denmark, and strain H61 was provided by R. Irie, National Institute of Animal Industry, Tsukuba, Japan. *L. lactis* subsp. *lactis* NCDO 763 was obtained from the National Collection of Dairy Organisms, Reading, England, and strain UC317 was obtained from the University of Cork, Cork, Ireland.

Two recombinant *L. lactis* MG1363 strains containing plasmid-located hybrid proteinase genes (ABcD and abCd) constructed from the SK11 (ABCD) and Wg2 (abcd) wild-type proteinases (32) were from NIZO (W. M. de Vos) and the Department of Genetics, University of Groningen (J. Kok), respectively. These hybrid genes contain an interchanged fragment C (c) (amino acid residues 497 to 1089) with nine substitutions, of which two (i.e., Arg-747 for Leu and Lys-748 for Thr) are associated with the specificity difference between the two wild-type proteinases toward caseins (31).

Isolation of CEP. Crude proteinase fractions were prepared by repeated extraction of milk-grown cells in a Ca^{2+}-free buffer (10). The proteinases from strain HP (P_I type) and strain AM1 (P_{III} type) were further purified as described previously (11, 12). Purified proteinase preparations from all other strains were obtained by gel permeation high-performance liquid chromatography (HPLC) using a precolumn SW (7.5 by 75 mm), a G4000 SW (7.5 by 300 mm) column, and a G3000 SW column (7.5 by 600 mm) (Pharmacia, Brussels, Belgium) in series. A 0.125 M NaH_2PO_4-Na_2HPO_4 buffer (pH 6.65) supplemented with 0.125 M Na_2SO_4 was used as the eluent (elution rate was 0.8 ml · min^{-1}). For details of the HPLC equipment used, see Exterkate et al. (9). The active fractions were dialyzed and concentrated on a YC-30 filter (Amicon, Danvers, Mass.). Proteinase activities in these preparations were determined by measuring the initial rates of hydrolysis of [$^{14}CH_3$]β-casein (10).

TABLE 1. List of primers used

Primer[a]	Target DNA[b]	Sequence (5′ to 3′)
1 (S)	732–756	ATTCACTGATACCGTTAAGCATGGC
2 (A)	1268–1293	TGACATCCGTGTTTTCAGCGGAAGC
3 (S)	2701–2724	AGCATGAAGTATTATCTATTGCGC
4 (A)	2884–2907	AGCCGTCTTGATGTTGCCATCACG

[a] A, antisense sequence; S, sense sequence.
[b] The nucleotide sequence numbering corresponds to that of the *L. lactis* SK11 *prtP* gene (32).

Substrates. α_{s1}-CN(f1–23) was prepared and purified according to the method described previously (7). The chromophoric substrate MS-Arg (S2586) was purchased from Kabi Diagnostica (Stockholm, Sweden). The substrates of the type succinyl-alanyl-X-prolyl-phenylalanyl-*p*-nitroanilide (S-X) were obtained from Bachem AG (Dubendorf, Switzerland). The residue X in the p_3 position (nomenclature according to Schechter and Berger [25]) is Gly, Ala, His, Leu, Phe, Ile, Trp, Gln, Lys, or Glu.

Enzymic hydrolysis. Initial activities toward the chromophoric substrates (1 mM) were measured in 50 mM NaH_2PO_4 buffer (pH 6.8) with or without 1.2 M NaCl at 25°C by monitoring the release of *p*-nitroaniline at 410 nm with a Uvikon 810 spectrophotometer with a thermostatically controlled cell compartment (Kontron, Zürich, Switzerland) (6). Enzymic hydrolysis of the substrate α_{s1}-CN(f1–23) (final concentration, 4 mg · ml^{-1}) with each of the proteinases was performed in 25 mM imidazole-HCl buffer (pH 6.5) at 30°C. The incubation mixtures contained levels of proteinase activity at which the conversion of the substrate could be monitored conveniently. In the case of the E8 proteinase, significantly less activity was used, since the yield of proteinase from this strain was very low (10). At several time intervals, aliquots (20 μl) were withdrawn from the incubation mixture, heated in small, sealed tubes at 80°C for 5 min, cooled on ice, and then diluted fourfold with solvent A (acetonitrile-water-trifluoroacetic acid [100:900:1 by volume]). A 20-μl sample was injected for analytical reverse-phase HPLC. For further details, see Exterkate et al. (9).

PCR amplification and nucleotide sequence analysis. The *L. lactis* strains were grown in lactose-M17 broth (E. Merck AG, Darmstadt, Germany). Plasmid DNA was isolated as described previously (3). Primers used for polymerase chain reaction (PCR) amplification of segments of the coding region of the *prtP* gene are listed in Table 1. PCR amplifications were performed as described previously (17) with a Thermocycler 60 (BioMed, Amstelstad, Holland). The amplification was done in 30 cycles by melting the DNA at 93°C for 1 min, annealing at 50°C for 1.5 min, and elongation at 72°C for 2.5 min. The DNA fragments were purified by Tris-acetate-EDTA–agarose gel electrophoresis (23) and recovered with the GeneClean kit (Bio 101, La Jolla, Calif.) according to the instructions of the manufacturer. Nucleotide sequence analysis of double-stranded DNA was per-

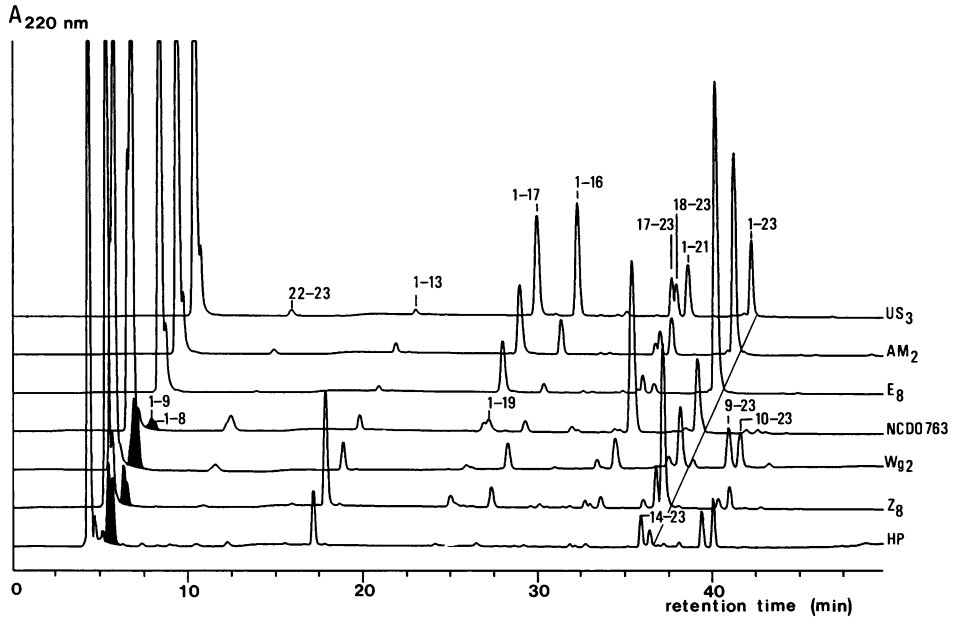

FIG. 1. Analytical reverse-phase HPLC patterns of the products of α_{s1}-CN(f1-23) degradation by the CEP of representative strains of *L. lactis* of the groups a (US3), b (AM2), c (E8), d (NCDO 763), e (Wg2), f (Z8), and g (HP). The extent of hydrolysis of the substrate is no measure for the relative activities of these enzymes.

formed by the dideoxy chain termination method (24) with the PCR primers.

RESULTS

Comparison of proteinase specificities toward α_{s1}-CN(f1-23). Figure 1 shows HPLC patterns of the products of α_{s1}-CN(f1-23) degradation by the CEPs of representative strains of *L. lactis*. Identical patterns were obtained in replicate experiments with the same proteinase. During the course of the reactions—until complete conversion of the peptide—the relative amounts of the cleavage products do not change significantly, indicating that secondary conversion involving initial products does not occur at an important rate. The strains are divided into seven groups (groups a to g) according to the extent of similarity of these patterns (Fig. 2). The groups show striking differences with respect to the cleavage products formed and/or with respect to the ratio of these products. Apart from the two extreme proteinases, P_I (HP) proteinase (group g) and P_{III} (US3) proteinase (group a), three deviating P_{III}-type proteinases (i.e., those of strain AM2 [group b], strain E8 [group c], and strain NCDO 763 [group d]) and two intermediate proteinases represented by those of strain Wg2 (group e) and strain Z8 (group f) can be distinguished.

The AM2 proteinase (group b) showed a reversed preference concerning peptide bonds 16—17 and 17—18 compared with the proteinase of group a. The E8 proteinase (group c) exhibits a narrow specificity as seen from the clear preference for bond 17—18. The Wg2 and Z8 proteinases both showed a P_I-type specificity, with additional affinity for bonds 16—17 and 21—22, while Z8 proteinase demonstrated a more dominant preference for bond 13—14. Both showed in addition cleavage of bond 19—20. The proteinases of strains NCDO 763 and UC317 (group d) clearly preferred bond 21—22; apart from this preference, they typically resembled the mixed-type specificity of the Wg2 and Z8 proteinases but with the higher relative cleavage rate of bond 19—20.

Previously, we found that cleavage of bond 21—22 by the free P_{III} (AM1) type proteinase is completely suppressed at high ionic strength and when this enzyme is in the cell-bound form (7). Therefore, we determined whether these two conditions also affected the cleavage of this bond by the NCDO 763 enzyme. Indeed, cleavage of bond 21—22 was completely suppressed in both cases. In contrast to the HP

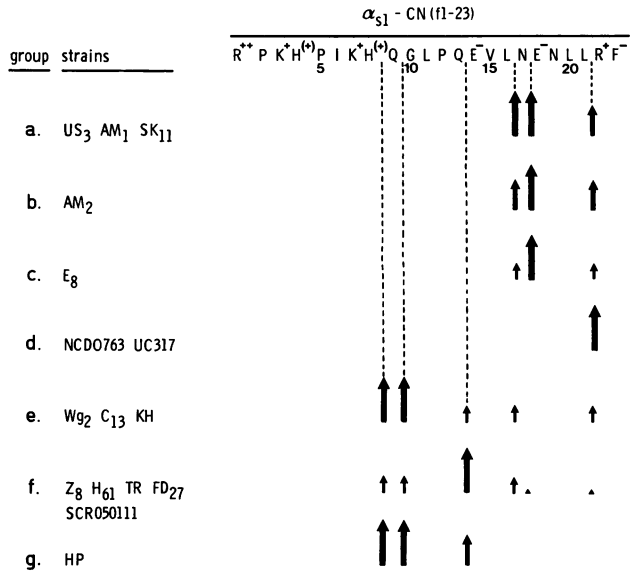

FIG. 2. Classification of strains of *L. lactis* according to the specificities of their CEPs toward α_{s1}-CN(f1-23). The main cleavage sites are indicated by arrows; the sizes of the arrows are related to the relative cleavage rates.

TABLE 2. Relative initial activities of the P_I (HP) and P_{III} (AM1) proteinases toward the substrates MS-Arg and S-X[a]

Substrate	HQ[b]	Vol[c]	Proteinase activity (nmol · 10^2 pNA · min^{-1} · ml^{-1})[d]	
			P_I (HP)	P_{III} (AM1)
MS-Arg			451	45
S-X				
Glu^-	−9.9	89	<1	574
Lys^+	−4.2	105	47.5	73
Gln	−0.3	95	13.0	255
Gly	0	0	3.5	98
Ala	0.5	26	7.5	129
$His^{(+)}$	0.5	101	120.0	306
Leu	1.8	102	13.0	158
Phe	2.5	137	44.0	431
Ile	2.5	103	21.5	618
Trp	3.4	172	134.0	940

[a] Relative initial activities at pH 6.8 (50 mM NaH_2PO_4-NaOH) and 25°C. X represents the different amino acid residues indicated in the table. The substrates were at a concentration of 1 mM.
[b] HQ, hydrophobicity of the amino acid side chain in kilocalories (1 kcal = 4.184 kJ) · mol^{-1}.
[c] Vol, mean volume of amino acid side chain buried in proteins (in cubic angstroms [1 Å = 0.1 nm]) obtained by subtracting the volume of a glycine residue (1).
[d] pNA, *p*-nitroaniline.

proteinase (7), the presence of salt stimulated the cleavage of bonds 8—9 and 9—10. Bonds 16—17, 13—14, 9—10, and 8—9 were preferred by the bound NCDO 763 enzyme. Similar results were obtained with the UC317 proteinase (data not shown).

Comparison of proteinase specificities toward small chromophoric substrates. The activities of the P_I (HP) and P_{III} (AM1) proteinases toward the substrates S-X, which differ in the amino acid residue X in the p_3 position, are listed in Table 2 and compared with the activities of both proteinases measured with the substrate MS-Arg. In all cases, the activity of P_I proteinase is lower and that of P_{III} proteinase is higher toward the S-X substrates than the respective activities toward MS-Arg. At a P_I/P_{III} activity ratio of approximately 10 in the case of MS-Arg as the substrate, the ratios obtained with each of these S-X substrates vary from 0.64 with S-Lys to less than 2×10^{-3} with S-Glu. In general, P_{III} proteinase activity increases with the hydrophobicity of the p_3 side chain, with the exception of S-Leu, S-Gln, and S-Glu; the lowest activity was measured toward S-Lys, which, like MS-Arg, has a positive charge on p_3. P_I has the lowest activity toward S-Glu. As has been shown previously (6), one can distinguish between P_I-type (HP) and P_{III}-type (AM1) proteinases by a differential effect exerted by a high ionic strength on their activities toward MS-Arg (i.e., an inhibition and activation, respectively). On the basis of these results, the peptides MS-Arg and S-Glu were selected to characterize the proteinases of different strains by their activities toward these substrates at low and high ionic strength (Table 3). According to these activities, the proteinase can be identified as either a P_{III} type (group a), a P_I type (groups e, f, and g), or an intermediate type (groups b, c, and d).

Comparison of hybrid proteinases with the wild-type variants. It has been shown that a so-called remote segment contributes to the difference between the SK11 and Wg2 type proteinases with respect to initial degradation of caseins (31). This small segment (with essential residues 747 and 748) is in the C-terminal extension of the proteinase which has no counterpart in the subtilisins. Consequently, it is not part of the subtilisin-like-binding region.

TABLE 3. Relative initial activities of the proteinases from different strains of *L. lactis* toward MS-Arg and S-Glu in the presence (+) or absence (−) of 1.2 M NaCl

Group	Strain(s)	Initial activities (%) with the following substrates[a]:			
		MS-Arg		S-Glu	
		−NaCl	+NaCl	−NaCl	+NaCl
1					
a	US3	10.5	100	73	195
	AM1	7.2	100	91	191
	SK11 (ABCD)	6.6	100	79	196
	Hybrid ABcD[b]	14.0	100	35	137
2					
b	AM2	52	100	2.1	17.6
c	E8	73	100	4.1	21.7
d	NCDO 763	64	100	3.0	18.5
	UC317	54	100	4.8	21.2
	Hybrid abCd[b]	85	100	2.9	17.6
3					
e	Wg2 (abcd)	130	100	0.9	9.1
	KH	125	100	0.9	5.7
	C13	122	100	0.8	9.3
f	Z8	147	100	1.9	10.3
	H61	144	100	1.3	10.9
	SCR 050111	144	100	1.2	10.4
	TR	169	100	<0.1	18.9
	FD27	163	100	5.2	ND[c]
g	HP	146	100	0.8	10.5

[a] Activities are expressed as percentages of the activity toward MS-Arg at 1.2 M NaCl.
[b] Values for the hybrid proteinases ABcD and abCd, constructed from the wild-type SK11 (ABCD) and Wg2 (abcd) proteinases, are given for comparison. These hybrids were not classified as belonging to one of the groups a to g.
[c] ND, not determined.

To assess whether this remote segment also affects the activity and specificity toward substrates smaller than casein, we compared the wild-type proteinases of strains SK11 (ABCD) and Wg2 (abcd) with hybrid proteinases in which fragments C and c containing this segment were exchanged (viz., ABcD and abCd) in their actions toward α_{s1}-CN(f1–23) (Fig. 3) and the chromophoric substrates (Table 3). The results presented are representative of replicate trials.

The most striking differences with respect to the hybrid abCd proteinase compared with the wild-type abcd proteinase are the decreased relative rates of cleavage of bonds 8—9, 9—10, 13—14, and 16—17 in favor of bonds 21—22 and 17—18 and the occurrence of a new product (Fig. 3, arrow). This hybrid most resembles the group d proteinases. This resemblance is also suggested by its relative cleavage rates of the chromophoric peptides (Table 3). Hybrid proteinase ABcD showed an increased preference for bond 16—17 and therefore is distinct from the wild-type ABCD and all other proteinases. Also with respect to its relative activity toward the chromophoric peptides, this hybrid cannot be classified in one of the groups a to g. It showed a decreased relative activity toward S-Glu and a slightly increased relative activity toward MS-Arg compared with those of wild-type ABCD proteinase.

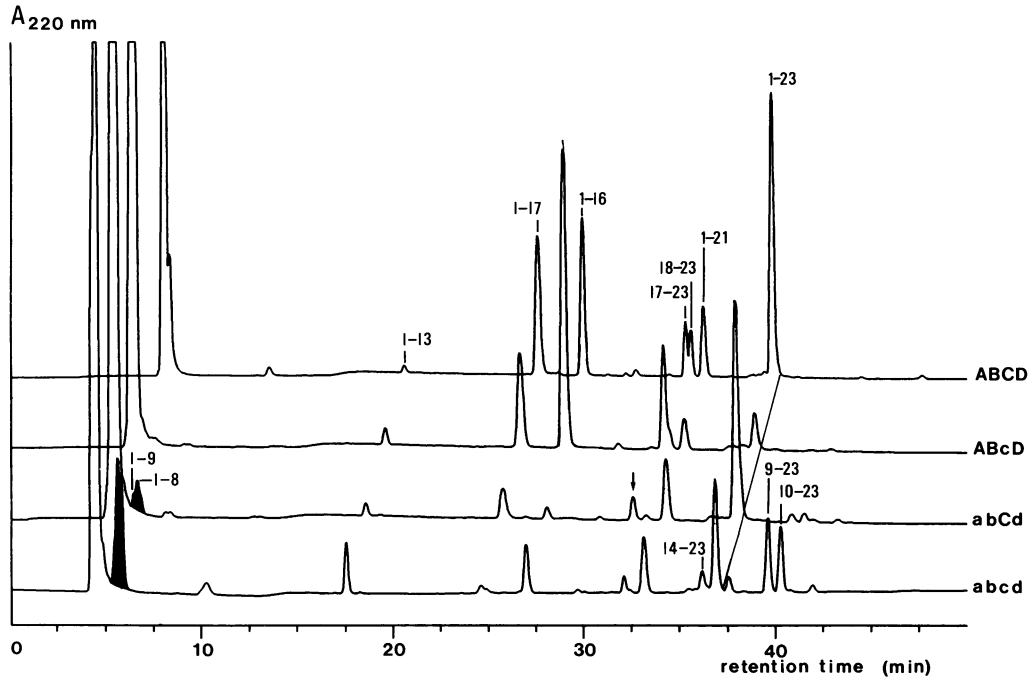

FIG. 3. Analytical reverse-phase HPLC patterns of the products of α_{s1}-CN(f1–23) degradation by the wild-type SK11 (ABCD) and Wg2 (abcd) CEPs and by the derived hybrid proteinases ABcD and abCd. The arrow indicates an unidentified product in the pattern obtained with abCd.

Comparison of amino acid sequences in the substrate-binding region. The *prtP* genes of *L. lactis* subsp. *cremoris* AM2, E8, FD27, and HP were subjected to PCR amplification with primers based on the nucleotide sequence of the SK11 *prtP* gene. Two DNA fragments of 561 and 207 bp were isolated and subjected to sequence analysis. The amino acid sequences 113 to 195 and 726 to 773 (numbering according to the SK11 proteinase sequence) were derived; they contain parts of the subtilisin-like-binding region of the proteinase and the region containing the remote segment that have now been shown to contribute to the cleavage specificity toward small substrates as well as to caseins. These amino acid sequences were compared with those of published sequences of proteinases of lactococcal strains SK11, WG2, and NCDO 763, and the differences are listed in Table 4. Each of these strains represents one of the seven groups as presented in Fig. 2. Interestingly, each of the representative proteinases has its own characteristic substitutions. The most striking feature of this comparison is the presence of a positive charge on residue 138 in the subtilisin-like region of the SK11 proteinase exclusively and the presence of a negative charge on residue 142 in the HP proteinase exclusively. Furthermore, in contrast to the SK11 enzyme, the HP proteinase and all other proteinases show a negative charge on residue 166. Additional conservative amino acid substitutions are found at positions 131 (Ser for Thr), 144 (Val for Leu), and 177 (Leu for Ile).

As far as the C terminally located remote segment is concerned, the proteinases of SK11, AM2, E8, and NCDO 763 show two successive residues (747 and 748), each with a

TABLE 4. Amino acid substitutions in CEPs of strains of *L. lactis* at positions (possibly) relevant for substrate binding[a]

Substrate-binding region	Residue	Amino acid substitution for CEPs of the following strains (groups):						
		SK11 (a) (α_{s1}, β [P_{III}])[b]	AM2 (b)	E8 (c) (α_{s1}, β [P_{III}/P_I])[b]	NCDO 763 (d) (α_{s1}, β [P_I])[b]	Wg2 (e) (β [P_I])[b]	FD27 (f) (α_{s1}, β [P_I/P_{III}])[b]	HP (g) (β [P_I])[b]
Subtilisin-like-binding region	131	Ser	Thr	Thr	Thr	Thr	Thr	Thr
	138	Lys^+	Thr	Thr	Thr	Thr	Thr	Thr
	142	Ala	Ala	Ala	Ala	Ser	Ala	Asp^-
	144	Val	Leu	Leu	Leu	Leu	Leu	Leu
	166	Asn	Asp^-	Asp^-	Asp^-	Asp^-	Asp^-	Asp^-
	(177)	Leu	Leu	Ile	Leu	Leu	Leu	Ile
Remote segment in fragment C (c) (31)	747	Arg^+	Arg^+	Arg^+	Arg^+	Leu	Leu	Leu
	748	Lys^+	Lys^+	Lys^+	Lys^+	Thr	Thr	Thr
	(763)	Asn	Asn	Asn	$His^{(+)}$	Asn	$His^{(+)}$	$His^{(+)}$

[a] Numbering is according to the sequence of the SK11 proteinase (32). The data for the other strains are taken from reference 15 (NCDO 763) or 16 (Wg2) or are the results of the present work (E8, FD27, AM2, and HP). Strain UC317 (19) is identical to NCDO 763 with respect to the residues listed here.

[b] Casein substrate specificity; the data in brackets represent degradation of β-casein in a P_I, P_{III}, or mixed but mainly P_I [P_I/P_{III}] or mainly P_{III} [P_{III}/P_I] manner (19, 20, 29).

positive charge. Remarkably, Arg-747 and Lys-748 are both found to be present, or both are replaced by Leu and Thr, respectively. Finally, residue Asn-763 is replaced by His in NCDO 763, FD27, and HP proteinases. It is not known whether this residue contributes to substrate binding.

DISCUSSION

The results of this comparative study show that CEPs with specificities deviating from those of the P_I-type (HP) and the P_{III}-type (AM1) proteinases occur among members of the species *L. lactis*. The amino acid sequences of the whole polypeptide chain (15, 16, 32) or of relevant parts thereof (19 and the present work) have now been established for the proteinases from different lactococcal strains. These proteinases differ in amino acid residues in small segments which have been predicted, on the basis of a close sequence similarity with the subtilisins, to be involved in substrate binding (32), or they differ in a small remote segment in which two simultaneous mutations, both involving a change in charge, viz., Arg-747 for Leu and Lys-748 for Thr, have been shown to significantly affect casein degradation (31).

The present results demonstrate that these last two substitutions influence the binding not only of large substrates like the caseins but also of α_{s1}-CN(f1–23) and even of small chromophoric peptides. This is clearly demonstrated by the differences in cleavage of these peptides between the wild-type Wg2 (abcd) proteinase and its derived hybrid abCd and between the wild-type SK11 (ABCD) proteinase and its derived hybrid ABcD. Therefore, we conclude that the remote segment is oriented toward the substrate-binding region in such a way that residues 747 and 748 are close enough to exert, if positively charged and possibly together with Lys-138, at least a long-range electrostatic force on (parts of) the substrate and thus contribute to the specificity demonstrated by the group a proteinases. This specificity is characterized by cleavages only in the C-terminal, negatively charged half of α_{s1}-CN(f1–23).

The absence of a positive charge on residues 747 and 748 (hybrid proteinase ABcD) may have drastically decreased the overall positively charged character of the binding region present in the SK11 (ABCD) proteinase. Introduction in the Wg2 proteinase of positively charged residues in positions 747 and 748 (hybrid proteinase abCd) may have neutralized the influence of the negatively charged Asp-166. In both cases, charge neutralization of the substrate-binding region is apparently responsible for the changed relative cleavage rates of the charged chromophoric peptides and peptide bonds in α_{s1}-CN(f1–23). Neutralization of Asp-166 thus contributes to the differences in relative cleavage rates observed with the AM2, E8, and NCDO 763 proteinases compared with that of the Wg2 proteinase. However, the differences in specificity among the AM2, E8, and NCDO 763 proteinases suggest that an additional amino acid substitution(s) outside the remote segment and outside the catalytic, subtilisin-like-binding region must contribute to specificity. Substrate binding could be affected directly by involvement of this residue(s) or indirectly by having an influence on enzyme conformation. The presence in the P_I (HP) proteinase (group g) of a negative charge on residue 142 (Asp) at the surface of the enzyme may be an example of such a residue; it is located just outside the region known to largely determine specificity by interactions with substrate residues p_1 to p_4 (9). Together with Asp-166, it may be responsible for the unique specificity of this proteinase toward α_{s1}-CN(f1–23). This specificity, in clear contrast to that of group a proteinases, is characterized by the distinct preference for the positively charged N-terminal part of the molecule. A positive charge on substrate residue p_6 may interact with Asp-142 on the surface of the enzyme molecule.

The NCDO 763 and UC317 proteinases demonstrate a distinct preference for cleavage of bond 21—22. Complete inhibition of the cleavage of this peptide bond was obtained at high ionic strength, confirming the dominating electrostatic character of substrate binding in this case, presumably arising from a strong interaction between p_1' (Arg) and these enzymes (7). Cleavage of bond 21—22 does not take place when these enzymes are in the cell-bound form; instead, other bonds are preferred. As suggested previously (7), this indicates that catalytic features of these enzymes are also determined by their in situ structural organization. This is in contrast with the findings of Coolbear et al. (2), which show similar casein hydrolysis patterns by both the free and the bound forms of the proteinase of *L. lactis* subsp. *cremoris* H2. However, long-term digestions may be more revealing with respect to a difference in specificity between these two forms. We cannot confirm the conclusion of Monnet et al. (21) that the NCDO 763 proteinase cleaves α_{s1}-CN(f1–23) with a specificity similar to that of the SK11 proteinase. Our results suggest that, apart from the highly specific cleavage of bond 21—22, the NCDO 763 enzyme has a mixed specificity similar to those of the Wg2- and Z8-type proteinases. At high ionic strength, this enzyme, unlike the P_{III} (AM1)-type proteinase, even showed a typical P_I (HP)-type specificity. The difference in specificity between the NCDO 763 proteinase and the HP proteinase observed with α_{s1}-CN(f1–23) at low ionic strength was not established with respect to the initial degradation of β-casein (20, 30). A remarkably consistent pattern was found for the initial sites of cleavage, which were all in the C-terminal hydrophobic, low-charged part of the molecule.

This suggests that differences in specificity can be observed only in the case of peptide bonds in more-charged parts of the molecule where charged residues might be expected to become involved in substrate binding. This has been established by comparing the actions of the P_I (H2)-type and P_{III} (SK112)-type proteinases on β-casein (22) and might also be established if long-term digests of β-casein by the NCDO 763 and HP and other proteinases are compared. In this respect, it is important to mention that preliminary experiments with the H2 proteinase and α_{s1}-CN(f1–23) have clearly shown that cleavage at bond 17—18 is as important as cleavage at bonds 8—9, 9—10, and 13—14; additionally, bonds 16—17 and 21—22 are cleaved. This means that this proteinase has a mixed specificity which is different from that of group e (Wg2) and group f (Z8) proteinases (8).

Our results confirm the involvement of positively charged residues in P_{III} (SK_{11})-type proteinase (viz., Lys-138, Arg-747, and Lys-748) with binding of substrates having charged p_3 residues. However, a hydrophobic side chain on p_3 was found to promote binding even more efficiently, possibly owing to interaction with the Lys-138 backbone. The present results also substantiate the involvement of a negatively charged residue in the binding region (Asp-166) of the Wg2 and HP proteinases (6, 9); they suggest the contribution of an additional negatively charged residue (Asp-142) to substrate binding by the HP proteinase. The relatively low activity of the latter proteinase with the S-X substrates is most probably due to repulsive forces directed toward the negatively charged succinyl moiety of the S-X substrates.

The diversity of proteinases among the strains tested and

the possible occurrence of other proteinase variants with distinct specificities emphasize the need for a simple but clear nomenclature. It is proposed to use the name CEP followed by the current subscript in roman numerals to indicate proteinases which mutually show a clearly different, characteristic specificity toward α_{s1}-CN(f1–23). The designations CEP_I, CEP_{II}, and CEP_{III} should be reserved exclusively for the group g-type, group d-type, and group a-type proteinases, respectively. $CEP_{I/III}$ or $CEP_{II/III}$ should be used to name the remaining intermediate or mixed-type proteinases, viz., those of groups e and f and groups b and c, respectively. Preferably, a strain reference should also be given. For obvious reasons, this is important for the mixed-type proteinases but also for the other three types, because amino acid substitutions may have occurred without implications for specificity toward α_{s1}-CN(f1–23). Moreover, in order to make a distinction between the proteinases of *Lactococcus* species and closely related proteinases of *Lactobacillus* species (13), the prefix Lc or Lb could be used, e.g., Lc-CEP_I, etc.

In conclusion, the present results indicate that the observed differences in specificity among naturally occurring CEPs of lactococci can be largely but not exclusively traced back to amino acid substitutions which are associated with changes in charge in the predicted subtilisin-like-binding region and in a small remote segment. This suggests that the CEP-binding region is even more complex than previously suggested. In general, it could be that, apart from the assumed vicinity of this remote segment, the proximity to the subtilisin-like-binding region of other residues, particularly in domains which have no counterpart in subtilisin, may very well influence the substrate-binding behavior of CEP. Alternatively, these domains could have some impact on the conformation of the enzyme and consequently on substrate cleavage specificity. Engineering experiments involving such domains are currently being performed and may elucidate the importance of (segments in) these domains for specificity.

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