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Hydrolysis of caseins and formation of hydrophilic and hydrophobic peptides by wild *Lactococcus lactis* strains isolated from raw ewes' milk cheese

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Aims: To investigate the hydrolysis of α_{S1}-, α_{S0}-, β_B-, β_{A1}- and β_{A2}-caseins by 32 wild lactococci of different randomly amplified polymorphic DNA (RAPD) patterns, isolated from raw ewes' milk cheese, and the production of hydrophilic and hydrophobic peptides from whole casein by those strains.

Methods and Results: Most strains hydrolysed all caseins, and degraded β-caseins to a larger extent than α_S-caseins, when the proteolytic activity of whole cells was determined by capillary electrophoresis. Higher levels of hydrophilic than of hydrophobic peptides were produced from whole casein by all strains, according to reverse-phase high performance liquid chromatography analyses.

Conclusions: Cell envelope proteinases of most lactococci isolated from raw ewes' milk cheese were CEP_{II}, $CEP_{II/III}$ or CEP_{III} (classification of Exterkate *et al.* 1993). A negative correlation was found between degraded α_S- and β-caseins and a highly positive correlation between hydrophilic and hydrophobic peptides.

Significance and Impact of the Study: Fast acid-producing lactococci from raw ewes' milk cheese have considerable and diverse caseinolytic activities. Their peptide production patterns do not reveal serious risks of bitter-flavour defect in cheeses if used as components of dairy starters.

INTRODUCTION

Caseins constitute about 80% of all proteins present in milk. The hydrolysis of caseins by lactococcal cell envelope proteinases (CEP) is the first step in the provision of amino acids essential for starter growth in cheese milk (Pritchard and Coolbear 1993). Peptides produced by lactococcal proteinases are further processed by an array of intracellular peptidases, yielding small peptides and amino acids which can be utilized by the cell. Besides being an important nutritional source for the starter bacteria (Flambard *et al.* 1998), products resulting from the hydrolysis of caseins also play a crucial role in the development of flavour during cheese ripening (Visser 1993).

Lactococcal proteinases, also named lactocepins (Reid and Coolbear 1998), are monomeric serine proteinases with an N-terminal segment of the mature proteinase similar in sequence to the serine proteinases of the subtilisin family. On the basis of the degradation patterns of α_{S1}- and β-casein in aqueous buffers, lactococcal proteinases were classified into two specific groups, the P_I- and P_{III}-type proteinases (Visser *et al.* 1986). Lactococcal proteinases were later classified into seven different groups according to the specificities of their actions on α_{S1}-casein fragment 1–23, which was associated with certain amino acid substitutions in small segments of the proteinase involved in substrate binding (Exterkate *et al.* 1993).

Different proteinases produce a considerable diversity of peptides from the hydrolysis of caseins in simple aqueous buffers (Monnet *et al.* 1986; Monnet *et al.* 1989; Visser *et al.* 1988; Visser *et al.* 1994; Reid *et al.* 1991; Reid *et al.* 1994; Juillard *et al.* 1995), with consequences relevant to cheese flavour. Thus, P_{III}-type proteinase initially cleaves large C-terminal fragments from β-casein, whereas the initial action of P_I-type proteinase on β-casein leads to the formation of a relatively small and very bitter C-terminal fragment (Visser 1993). Proteinase activity primes the entire

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proteolytic system, as differences in the specificities of lactococcal proteinases will have downstream effects on the identities and levels of flavour peptides and peptide precursors of flavour compounds (Reid and Coolbear 1999).

Analysis of the water-soluble fraction of cheeses by reverse-phase high performance liquid chromatography (RP-HPLC) related bitter peptides with late-running material, thought to be hydrophobic peptides, and savoury fractions with early running material, probably hydrophilic peptides and amino acids (Cliffe *et al.* 1993). A good correlation between cheese bitterness scores from a sensory panel and the level of hydrophobic peptides determined by RP-HPLC, or the hydrophobic peptides : hydrophilic peptides ratio, has been reported (Gómez *et al.* 1997).

Information on strain composition within lactococci found in cheeses manufactured from raw milk is scarce (Desmasures *et al.* 1998; Mannu *et al.* 2000). In a study on the physiological and genetic diversity of lactococci found in ewes' raw milk and in cheese made from ewes' raw milk not inoculated with lactic starter cultures, 88 fast acid-producing *Lactococcus lactis* isolates were obtained which exhibited 32 different randomly amplified polymorphic DNA (RAPD) patterns (Gaya *et al.* 1999). The objective of the present work was the characterization of the proteolytic activity of these wild strains, including their specificities towards different caseins and their levels of production of hydrophilic and presumably bitter hydrophobic peptides, in the context of their potential use as components of dairy starters.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Five collection strains of *L. lactis* subsp. *cremoris* and one collection strain of *L. lactis* subsp. *lactis* (Table 1) were chosen according to the specificities of their proteinases for α_{S1}-casein fragment 1–23 and chromogenic substrates (Exterkate *et al.* 1993). Nine wild strains of *L. lactis* subsp. *cremoris* and 23 wild strains of *L. lactis* subsp. *lactis* (Gaya *et al.* 1999) of unknown proteinase type were included in experiments. All lactococcal strains were maintained in M17 broth (Biolife Srl, Milan, Italy) at –80°C.

Milk-citrate plates (Efstathiou and McKay 1976) were inoculated with 50 μl of an overnight culture in M17 broth and incubated at 30°C for 2–3 d in anaerobic jars (Anaerogen, ANO35A; Oxoid), except for strains AM1 and SK11 which grew rather poorly in anaerobiosis. Cells were harvested from plates with 500 μl 0·02 mol l^{-1} imidazole buffer, pH 6·2. The $O.D._{600}$ of cell suspensions was determined.

Hydrolysis of caseins

Activity on α_S-casein (Sigma-Aldrich Química SA, Alcobendas, Spain) was determined in a 1-g l^{-1} α_S-casein suspension in 0·02 mol l^{-1} imidazol buffer, pH 6·2, incubated with cells at a final concentration of $O.D._{600} = 4$. The activity on β-casein (Sigma) was determined in a 6-g l^{-1} β-casein suspension in the same buffer, with the addition of 0·005 mol l^{-1} $CaCl_2$, incubated with cells at a final concentration of $O.D._{600} = 2$. Aliquots of 150 μl were incubated for 6 h at 30°C in a shaking water-bath and then kept frozen until preparation for analysis. Aliquots were also collected for each strain at zero incubation time.

Residual caseins were determined by capillary electrophoresis (CE), using a Beckman P/ACE System 2100 (Beckman Instruments España SA, Madrid, Spain) controlled by a System Gold Software (Beckman Instruments) data system. The sample buffer was as described by Recio *et al.* (1997). Samples were prepared for CE by mixing 100 μl α_S-casein solution, 400 μl sample buffer and 50 μl 0·33 g l^{-1} α-lactalbumin (internal standard) in 0·02 mol l^{-1} Na citrate or 50 μl β-casein solution, 400 μl sample buffer

Table 1 Strains of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* with different cell envelope proteinases used in this study

Strains	Subspecies	Proteinase type	Origin
AM1	*L. lactis* subsp. *cremoris*	CEP_{III}	NIZO, Ede, The Netherlands
AM2	*L. lactis* subsp. *cremoris*	$CEP_{II/III}$	NIZO
HP	*L. lactis* subsp. *cremoris*	CEP_{I}	NIZO
SK11	*L. lactis* subsp. *cremoris*	CEP_{III}	NIZO
Wg2	*L. lactis* subsp. *cremoris*	$CEP_{I/III}$	NIZO
NCDO 763	*L. lactis* subsp. *lactis*	CEP_{II}	NCDO, Reading, UK
L20, M21, N20, N22, O23, P21, P24, Q25 and R20	*L. lactis* subsp. *cremoris*	Unknown	Gaya *et al.* (1999)
A1, A2, A3, A4, A5, B6, B7, B8, C9, C10, C11, D12, E13, E14, F15, G16, G17, H18, I19, J16, K16, S26 and S27	*L. lactis* subsp. *lactis*	Unknown	Gaya *et al.* (1999)

CEP, Cell envelope proteinase; NIZO, Netherlands Institute for Dairy Research; NCDO, National Collection of Dairy Organisms.

and 50 μl 2 g l^{-1} α-lactalbumin (internal standard) in 0·02 mol l^{-1} citrate. Samples were kept for 1 h at room temperature, filtered (Millex HV 0·45 μm; Millipore, Bedford, MA, USA) and injected in duplicate at the anode using N_2 at 0·5 psi for 15 s. Separation was performed in a hydrophilic coated fused-silica capillary column (CElect P150 (37 cm length (30 cm effective length)); Supelco, Bellefonte, PA, USA) with a final applied voltage of 15 kV. The detection of peaks was at 214 nm. Areas of peaks corresponding to α_{S1}-, α_{S0}-, β_B-, β_{A1}- and β_{A2}-casein were used to calculate the casein degraded. The area of the α_{S2}-casein peak was irrelevant compared with those of the α_{S1}- and α_{S0}-casein peaks and was ignored. Data corresponding to α_S-casein were multiplied by a factor of 0·5 to correct for cell O.D., twice that used for β-casein.

Formation of peptides

Whole casein (Merck Farma, Mollet del Vallés, Spain), 2·5 g l^{-1} in 0·02 mol l^{-1} imidazol buffer, pH 6·2, was incubated with cells at a final concentration of $O.D._{600} = 4$ in a shaking water-bath for 6 h at 30°C. After incubation, 3-ml aliquots were centrifuged and the supernatant fluids frozen, freeze-dried and resuspended in 600 μl distilled water. They were again centrifuged and 500 μl supernatant fluid mixed with 56 μl 10% (v/v) trifluoroacetic acid (TFA), kept for 10 min at room temperature and centrifuged at 5000 rev min^{-1}. Supernatant fluids were filtered (Millex HV 0·45 μm) and subjected to RP-HPLC.

Hydrophobic and hydrophilic peptides were determined in duplicate by RP-HPLC, as described by Lau *et al.* (1991). A Beckman System Gold chromatograph, equipped with a programmable solvent module 126, a diode array detector module 168 and an autosampler 502 was used. Detection was at 214 and 280 nm. Peaks with retention times over 14·6 min were considered to correspond to hydrophobic peptides (Gómez *et al.* 1997).

Statistical analysis

Cluster analysis was used to discover the natural grouping of the strains. The statistical program used was SPSS Win 5.0 (SPSS, Chicago, IL, USA). Data obtained from CE electropherograms and RP-HPLC chromatograms were subjected to hierarchical cluster analysis (HCA) using Ward's method, with the Euclidean distance as a measure of the proximity between two strains and variables previously standardized to a normal distribution. Dendrograms obtained with SPSS Win 5.0 show, in a 0–25 scale, the rescaled linkage distance, equivalent to 25 linkage distance/maximal distance.

The degradation of caseins and formation of peptides by groups of strains obtained from HCA using degraded α_{S1}-casein, degraded α_{S0}-casein and degraded α_S-casein: degraded β-casein ratio as variables were subjected to one-way analysis of variance and comparison of means by Tukey's test.

RESULTS

Proteolytic activity towards caseins

Lactococcus lactis HP with CEP_I and *L. lactis* Wg2 with $CEP_{I/III}$ showed extremely low activities towards α_S-casein, as expected (4·5 and 0·7% degradation, respectively). *Lactococcus lactis* NCDO 763 with CEP_{II} and *L. lactis* AM2 with $CEP_{II/III}$ showed intermediate activities on α_S-casein (12·6 and 14·0% degradation, respectively). The highest activities on α_S-casein were recorded for *L. lactis* AM1 and SK11, both with CEP_{III} (24·7 and 32·3% degradation, respectively). In all cases α_{S0}- and α_{S1}-caseins were degraded to similar extents by collection strains (Table 2).

All collection strains were able to hydrolyse β-casein. The degradation shown by the different strains was as follows: *L. lactis* HP and Wg2, 32·4 and 51·9%, respectively; *L. lactis*

Table 2 Degradation* of α_S- and β-caseins by six collection strains and 32 wild strains of *Lactococcus lactis* with different cell envelope proteinases

		Collection strains†			Wild strains		
Casein	0 h concentration (mg l^{-1} casein)	CEP_I, $CEP_{I/III}$ (n = 2)	CEP_{II}, $CEP_{II/III}$ (n = 2)	CEP_{III} (n = 2)	CEP_I, $CEP_{I/III}$ (n = 4)	CEP_{II}, $CEP_{II/II}$ (n = 17)	CEP_{III} (n = 11)
α_{S1}	695	18^a	92^a	199^b	17^a	82^b	132^c
α_{S0}	255	7^a	34^{ab}	71^b	5^a	26^b	52^c
β_B	409	242^a	269^a	147^a	289^b	223^{ab}	154^a
β_{A1}	1126	457^a	542^a	370^a	732^b	483^a	328^a
β_{A2}	1465	566^a	674^a	380^a	850^b	598^{ab}	398^a
α_S : β ratio	0·317	0·024^a	0·085^a	0·301^b	0·012^a	0·102^b	0·218^c

CEP, Cell envelope protein.

*Expressed as mg l^{-1} casein degraded after 6 h at 30 °C. Mean values of collection or wild strains with the same superscript do not differ ($P < 0·05$).

†Collection strains were *L. lactis* HP with CEP_I, Wg2 with $CEP_{I/III}$, NCDO 763 with CEP_{II}, AM2 with $CEP_{II/III}$, AM1 and SK11 with CEP_{III}.

NCDO 763 and AM2, 44·7 and 51·9%, respectively and *L. lactis* AM1 and SK11, 27·0 and 32·8%, respectively. The proteolysis of β-caseins differed, β_B-casein being more susceptible than β_{A1}- or β_{A2}-caseins to degradation by all collection strains. The ratio of degraded α_S-casein : degraded β-casein was found to be particularly useful in the differentiation of collection strains by their caseinolytic activities (Table 2).

Figure 1 shows the results of the application of HCA to all strains, with degraded α_{S1}-casein, degraded α_{S0}-casein and degraded α_S-casein : degraded β-casein ratio as variables. *Lactococcus lactis* AM1 and SK11 formed a cluster with three wild strains which, together with another cluster of eight wild strains, were in one of the main branches of the dendrogram and distant from the rest. The proteinases of the lactococci in this main branch were considered to be CEP_{III}. *Lactococcus lactis* HP and Wg2 were in the second main branch in a cluster with four wild strains, of proteinases considered to be CEP_I or $CEP_{I/III}$. Finally, *L. lactis* NCDO 763 and AM2 formed a cluster with six wild strains, and two other clusters close to it included five and six wild strains, respectively. The proteinases of the strains

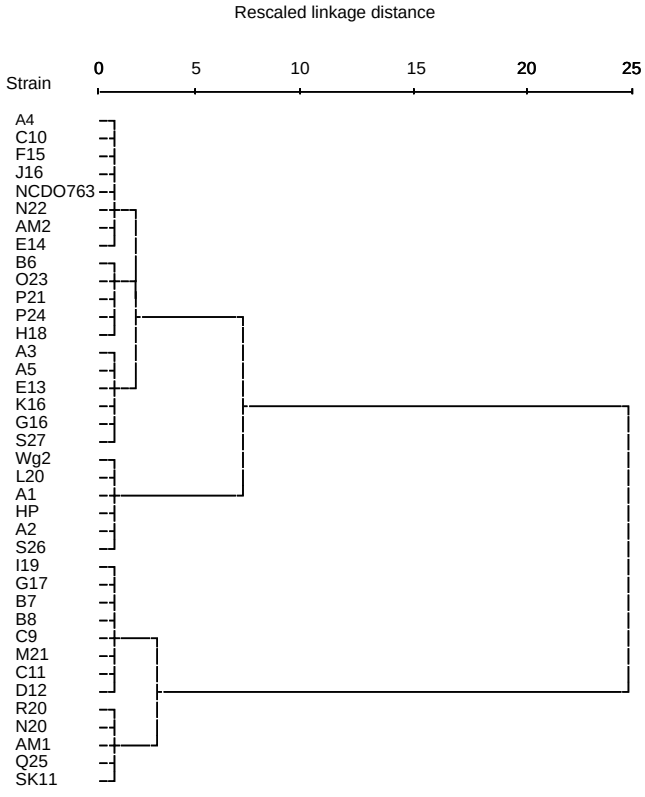

Fig. 1 Dendrogram from hierarchical cluster analysis of collection and wild strains of *Lactococcus lactis*, with degraded α_{S1}-casein, degraded α_{S0}-casein and degraded α_S-casein : degraded β-casein ratio as variables

in these three clusters were considered to be CEP_{II} or $CEP_{II/III}$. Although other HCA strategies were assayed, *L. lactis* HP and Wg2 remained, in all cases, together in the same cluster as did *L. lactis* NCDO 763 and AM2, according to their activities on α_S- and β-caseins.

Wild strains in the CEP_{III} group included four *L. lactis* subsp. *cremoris* strains and seven *L. lactis* subsp. *lactis* strains, those in the CEP_I or $CEP_{I/III}$ group included one *L. lactis* subsp. *cremoris* strain and three *L. lactis* subsp. *lactis* strains and wild strains in the CEP_{II} or $CEP_{II/III}$ group included four *L. lactis* subsp. *cremoris* strains and 13 *L. lactis* subsp. *lactis* strains.

Casein degradation by the three groups of *L. lactis* wild strains defined by the dendrogram in Fig. 1 is shown in Table 2. The degradation of α_S-casein by strains with CEP_I or $CEP_{I/III}$ ranged from 0·1% for A1 to 4·3% for A2, degradation by strains with CEP_{II} or $CEP_{II/III}$ from 7·7% for A3 to 17·1% for E14 and degradation by strains with CEP_{III} from 15·3% for D12 to 25·1% for Q25. Significant differences ($P < 0·05$) between the three groups of wild strains were found for both α_{S0}- and α_{S1}-caseins.

With respect to β-casein, degradation by *L. lactis* wild strains with CEP_I or $CEP_{I/III}$ ranged from 60·9% for A1 to 63·9% for L20, by strains with CEP_{II} or $CEP_{II/III}$ from 18·9% for S27 to 77·1% for J16 and by strains with CEP_{III} from 14·9% for D12 to 38·8% for C11. Wild strains with CEP_I or $CEP_{I/III}$ achieved significantly higher ($P < 0·05$) extents of degradation of β_B-, β_{A1}- and β_{A2}-caseins than wild strains with CEP_{III}.

Lactococcus lactis wild strains hydrolysed α_{S0}- and α_{S1}-casein at the same or similar extents. However, β_B-casein was hydrolysed to a greater extent than β_{A1}- and β_{A2}-caseins by all lactococcal wild strains expect A2. A negative correlation between degraded α_S-casein and degraded β-casein ($r = -0·371$; $P < 0·05$) was obtained for all 32 lactococcal wild strains investigated.

Hydrophilic and hydrophobic peptides

Collection strains showed three patterns of RP-HPLC chromatograms, the first of which corresponded to *L. lactis* HP and Wg2, the second to *L. lactis* NCDO 763 and AM2 and the third to *L. lactis* AM1 and SK11 (Fig. 2). A retention time of 14·6 min was considered to separate early-running hydrophilic peptides from late-running hydrophobic peptides (Gómez *et al.* 1997). The retention times of hydrophilic peptides remained fairly constant in duplicate analyses, whereas a certain variation between duplicate analyses was observed for the retention times of most hydrophobic peptides.

Cellular material from lactococci accounted for a large amount of the peptides with retention times under 8·0 min,

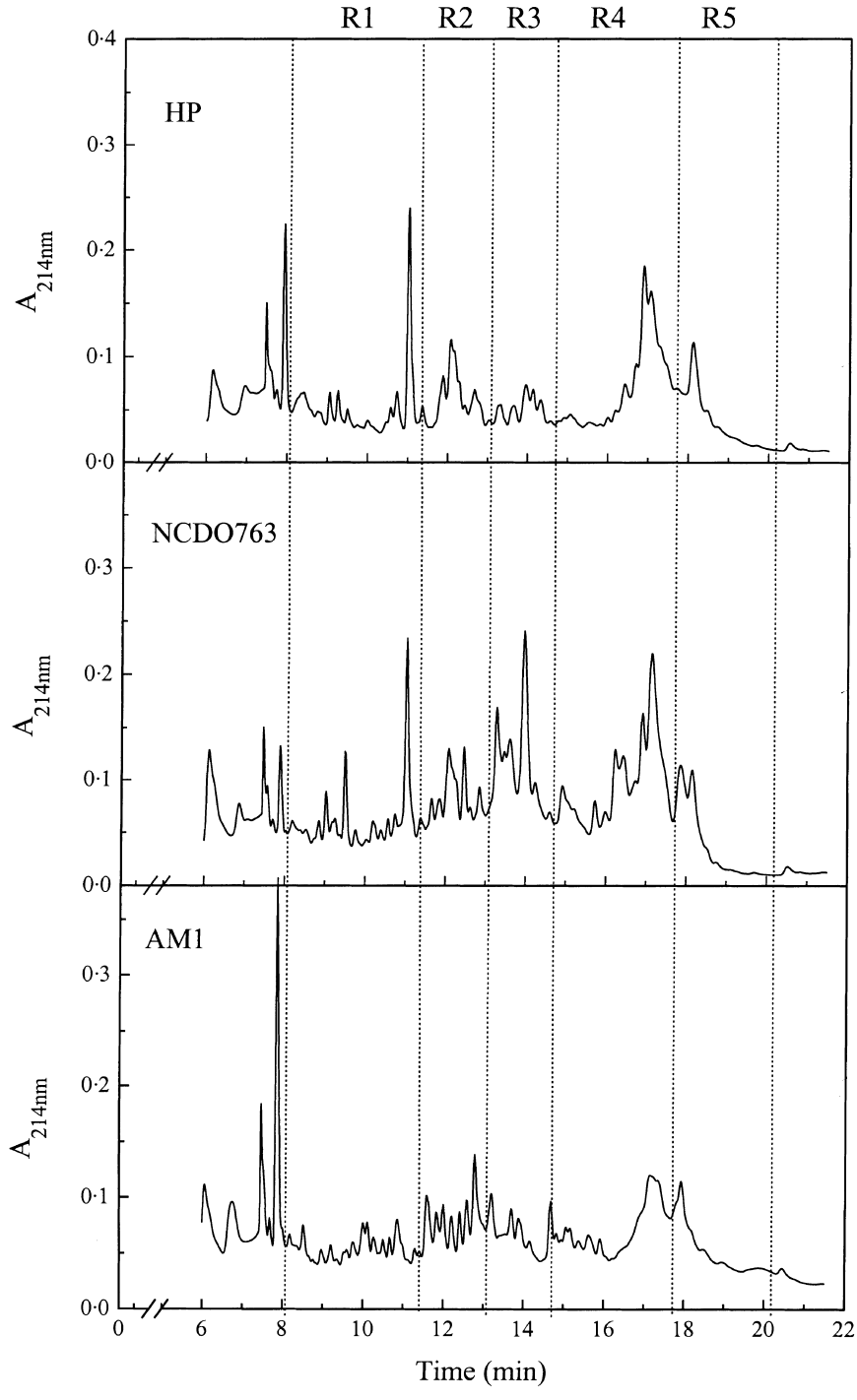

Fig. 2 Reverse-phase high performance liquid chromatograms ($A_{214\ nm}$) of *Lactococcus lactis* subsp. *cremoris* AM1 and HP and *L. lactis* subsp. *lactis* NCDO 763 with three regions of hydrophilic peptides (R1, R2 and R3) and two regions of hydrophobic peptides (R4 and R5)

as observed by incubating resting cells alone and, therefore, this early-eluting fraction was not considered further. The remaining peptides were subdivided into three regions (R1, 8·0–11·2 min, R2, 11·2–13·0 min and R3, 13·0–14·6 min) of hydrophilic peptides and two regions (R4, 14·6–17·6 min and R5, 17·6–20·0 min) of hydrophobic peptides (Fig. 2). The formation of the peptides included in each of these five regions by *L. lactis* collection strains and wild strains is shown in Table 3. The levels of hydrophilic peptides ranged from 141 for L20 to 296 for A2 for wild strains with CEP_I or $CEP_{I/III}$, from 195 for P21 to 482 for N22 for wild strains with CEP_{II} or $CEP_{II/III}$ and from 281 for C11 to 474 for M21 for wild strains with CEP_{III}. With respect to hydrophobic peptides, their levels ranged from 84

Table 3 Hydrophilic and hydrophobic peptides* formed from whole casein by six collection strains and 32 wild strains of *Lactococcus lactis* with different cell envelope proteinases

		Collection strains†			Wild strains		
Peptides	Region	CEP_I, $CEP_{I/III}$ (n = 2)	CEP_{II}, $CEP_{II/III}$ (n = 2)	CEP_{III} (n = 2)	CEP_I, $CEP_{I/III}$ (n = 4)	CEP_{II}, $CEP_{II/III}$ (n = 17)	CEP_{III} (n = 11)
Hydrophilic	R1	164[a]	173[a]	161[a]	106[a]	137[b]	154[b]
Hydrophilic	R2	99[a]	130[ab]	137[b]	42[a]	82[b]	98[b]
Hydrophilic	R3	85[a]	167[b]	95[a]	40[a]	84[b]	94[b]
Hydrophobic	R4	197[a]	269[b]	204[a]	87[a]	147[ab]	171[b]
Hydrophobic	R5	115[a]	113[a]	114[a]	45[a]	60[a]	83[b]
Total peptides		659[a]	852[b]	711[a]	319[a]	511[b]	600[b]
Hydrophobic : hydrophilic ratio		0·81[a]	0·82[a]	0·90[a]	0·69[a]	0·67[a]	0·72[a]

CEP, Cell envelope proteinase.
*Expressed as chromatogram area units per sample. Mean values of collection or wild strains with the same superscript do not differ ($P < 0·05$).
†As in Table 2.

for L20 to 221 for A2 for wild strains with CEP_I or $CEP_{I/III}$, from 110 for K16 to 383 for N22 for wild strains with CEP_{II} or $CEP_{II/III}$ and from 144 for C11 to 407 for M21 for wild strains with CEP_{III}.

Within each proteinase group, the strains responsible for the maximum levels of hydrophilic and hydrophobic peptides were generally coincident. A highly significant correlation ($r = 0·916$; $P < 0·001$) was obtained between hydrophilic and hydrophobic peptides for all 32 wild lactococcal strains. Wild strains with CEP_{III} achieved the highest levels of peptides included in each of the five regions of the chromatogram (Table 3). However, there were no significant differences between the ratios of hydrophobic : hydrophilic peptides of wild strains belonging to the three groups defined by the dendrogram in Fig. 1.

The dendrogram obtained by HCA of collection strains and wild strains with the areas of the five regions of peptides defined in Fig. 2 as variables did not separate *L. lactis* HP and Wg2, respectively, with CEP_I or $CEP_{I/III}$, from *L. lactis* AM1 and SK11, with CEP_{III} (Fig. 3).

From the RP-HPLC chromatograms of collection strains (Fig. 2), 14 distinct peaks corresponding to hydrophilic peptides were selected for further separation of strains. When the areas of these 14 peaks in the chromatograms of collection strains and wild strains were submitted to analysis of variance, a significant effect of the proteinase group was found for only six peaks, with retention times of 9·0, 12·2, 12·4, 12·8, 13·2 and 13·9 min, which were used to construct the dendrogram in Fig. 4. A main branch included *L. lactis* HP and Wg2 together with four wild strains with CEP_{II} or $CEP_{II/III}$ and three wild strains with CEP_I or $CEP_{I/III}$. *Lactococcus lactis* NCDO 763 and AM2 were in a division of the second main branch, together with four wild strains with CEP_{II} or $CEP_{II/III}$ and seven wild strains with CEP_{III}. In another division of the second main branch were *L. lactis* AM1 and SK11, together with one wild strain with CEP_I or $CEP_{I/III}$, nine wild strains with CEP_{II} or $CEP_{II/III}$ and four wild strains with CEP_{III}.

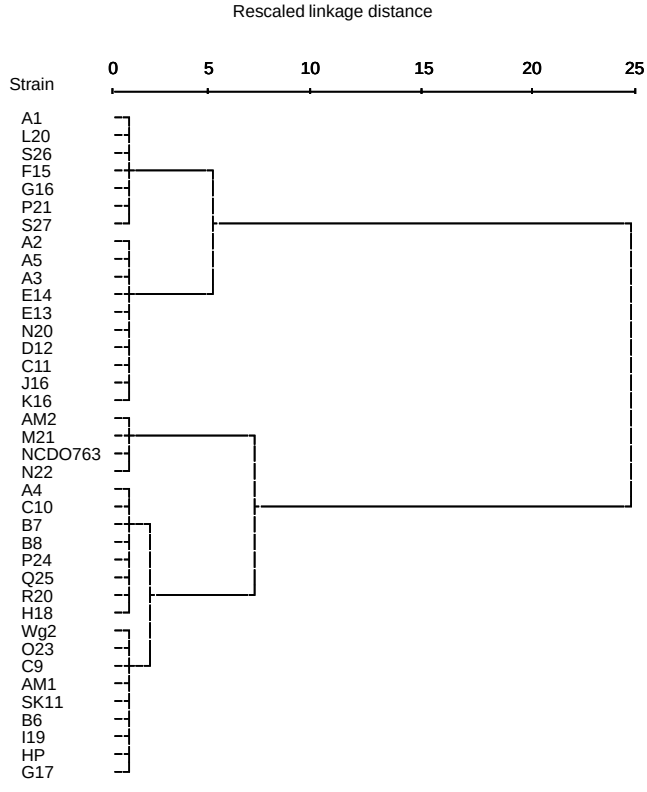

Fig. 3 Dendrogram from hierarchical cluster analysis of collection and wild strains of *Lactococcus lactis*, with hydrophilic peptides (three regions) and hydrophobic peptides (two regions) as variables

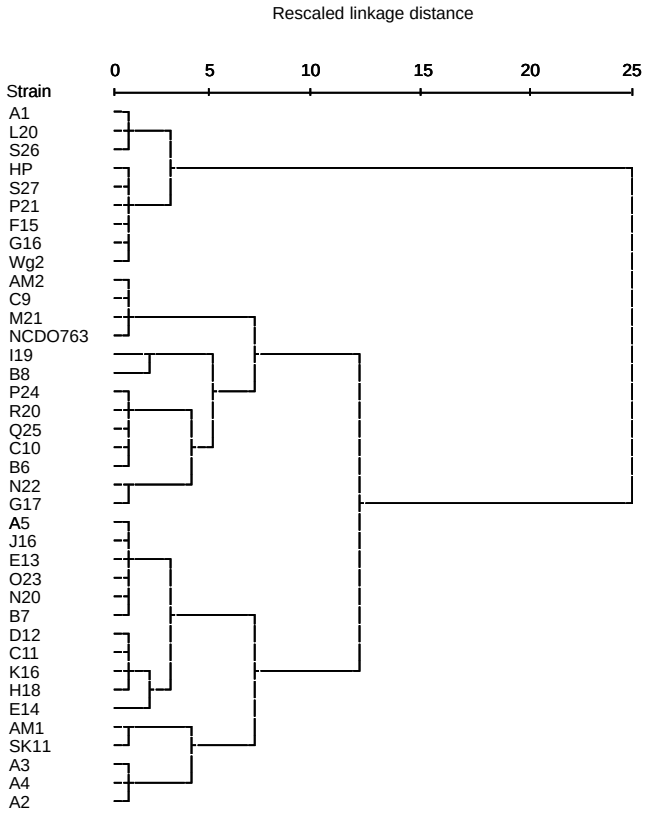

Fig. 4 Dendrogram from hierarchical cluster analysis of collection and wild strains of *Lactococcus lactis*, with the areas of six selected hydrophilic peptides as variables

DISCUSSION

The proteolytic system of lactococci consists of a cell envelope proteinase, transport systems for oligopeptides generated and a multitude of intracellular peptidases (Kunji *et al.* 1996). According to this widely accepted model, cell envelope proteinases would be mostly responsible for the effects observed in our experiments during the incubation of caseins with lactococci for 6 h. Transport of peptides into the cell cytoplasm was expected to be of minor importance under those conditions.

The extents of α_{S0}- and α_{S1}-casein degradation by each of the collection strains were very similar under our experimental conditions, and this conclusion was also valid for wild strains (Table 2). The presence in α_{S0}-casein of one more phosphoseryl residue than occurs in α_{S1}-casein (Manson *et al.* 1977) had no apparent effect on their susceptibilities to degradation by lactococcal proteinases. On the contrary, the extent of β_B-casein degradation was higher than those of β_{A1}- and β_{A2}-casein, independently of the proteinase group, for collection and wild strains. Genetic variants of β-casein are due to mutations resulting in amino acid substitutions in the polypeptide chain (Ng-Kwai-Hang and Grosclaude 1992), which might influence the substrate affinity of lactococcal proteinases.

Lactococcus lactis subsp. *cremoris* proteinases were classified by Visser *et al.* (1986) on the basis of α_{S1}-, β- and κ-casein degradation patterns. The activities of lactococcal proteinases on α_{S1}- and β-casein are relevant to cheese ripening, but their activities on κ-casein seem to be of minor practical interest as κ-casein is mainly degraded by rennet during cheese manufacture (Smid *et al.* 1991; Visser 1993). Four wild strains which lacked a clear activity on α_S-casein, *L. lactis* A1, A2, L20 and S26, were clustered (Fig. 1) with *L. lactis* HP and Wg2, strains considered to possess P_I-type proteinases (Visser *et al.* 1986). Amino acids essential for growth in milk must, therefore, be obtained by *L. lactis* A1, A2, L20 and S26 mainly from β_B-, β_{A1}- and β_{A2}-caseins, substrates on which they exhibited the highest activities recorded among wild strains (Table 2). *Lactococcus lactis* NCDO 763 and AM2, respectively, with CEP_{II} and $CEP_{II/III}$ (Exterkate *et al.* 1993) and intermediate activities on α_{S1}- and α_{S0}-caseins, were close in the dendrogram to 17 wild strains of intermediate activities on α_{S1}- and α_{S0}-caseins (Fig. 1). Distant from them were *L. lactis* AM1 and SK11, with CEP_{III}, in the same cluster as *L. lactis* N20, R20 and Q25, the three wild strains with the highest activities on α_{S1}- and α_{S0}-caseins and, in the other cluster of the same main branch, were eight wild strains also with considerable activities on α_{S1}- and α_{S0}-caseins.

Information on the incidence of the different cell envelope proteinases in *L. lactis* strains is scarce. Exterkate *et al.* (1993) reported one strain with CEP_I, eight with $CEP_{I/III}$, two with CEP_{II}, two with $CEP_{II/III}$ and three with CEP_{III}, out of 16 lactococcal strains assayed. In the present work, a lower proportion of lactococci with CEP_I or $CEP_{I/III}$ and a higher proportion of lactococci with CEP_{II} or $CEP_{II/III}$ were found. Similar incidences of the different proteinase groups were found for wild strains belonging to *L. lactis* subsp. *cremoris* or *L. lactis* subsp. *lactis*.

The RP-HPLC chromatograms revealed the large diversity of peptides produced by lactococcal proteinases from whole casein. The formation of more than 100 different oligopeptides from β-casein by P_I-type proteinase has been reported (Juillard *et al.* 1995) and the broader specificity of P_{III}-type proteinase compared with that of P_I-type proteinase in their action on β-casein is also well known (Visser *et al.* 1986, 1988). In the present work, more complex RP-HPLC patterns were found for *L. lactis* NCDO 763 and AM1, with CEP_{II} and CEP_{III}, respectively, than for *L. lactis* HP, with CEP_I (Fig. 2), and complex RP-HPLC patterns were also frequently observed for wild strains with CEP_{II}, $CEP_{II/III}$ and CEP_{III}.

Hierarchical cluster analysis of the areas of the five regions of hydrophilic and hydrophobic peptides (Fig. 3) resulted in

a classification of strains quite different from that obtained with caseinolysis data (Fig. 1), probably due to the fact that most of the peptides taken into account for the construction of Fig. 3 originated from β-casein, which had a lower weight than α_S-casein used in the construction of Fig. 1. In spite of this, collection strains of the same proteinase group remained together, i.e. *L. lactis* NCDO 763 with AM2, *L. lactis* HP with Wg2 and *L. lactis* AM1 with SK11, these four last strains in the same cluster.

The dendrogram obtained with the peak areas of six selected hydrophilic peptides (Fig. 4) succeeded in the separation of *L. lactis* HP and Wg2 from *L. lactis* AM1 and SK11 and from *L. lactis* NCDO 763 and AM2. Separation of *L. lactis* HP from Wg2, and of *L. lactis* NCDO 763 from AM2, can be accomplished by working with purified proteinases and α_{S1}-casein fragment 1–23 as substrate (Exterkate *et al.* 1993). The separation of *L. lactis* AM1 from SK11 seems more difficult as they share CEP_{III} and separation could only rely on quantitative aspects of peptide production. When RP-HPLC profiles of miniature Cheddar-type cheeses manufactured with different lactococcal strains were compared, *L. lactis* AM1 and SK11 remained together in dendrograms independently of cheese ages of 2 or 4 months (Pripp *et al.* 1999). However, the separation of *L. lactis* HP and Wg2 in dendrograms increased as the cheeses aged. Changes in the incubation conditions of whole lactococcal cells with casein might also be useful in the strain differentiation on the basis of RP-HPLC patterns.

Lactococci isolated from raw ewes' milk cheese showed considerable and diverse caseinolytic activities. An interesting feature of our wild strains, from a technological point of view, is their low hydrophobic : hydrophilic peptide ratios, independently of their proteinase group, compared with those of collection strains. No serious risk of bitterness seems thus to derive from the inclusion of these wild strains in dairy starters, in the light of their reduced formation of hydrophobic peptides.

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