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The oligopeptides of sweet and acid cheese whey*

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Summary — The 12% TCA-soluble oligopeptide fraction of sweet whey from Provolone and Grana Padano cheese, and of acid whey from Quarg cheese was fractionated by HPLC and the peptide components identified using a combination of mass spectrometry and Edman degradation. In the sweet whey, in addition to the two differently phosphorylated forms of caseinomacropeptide (CMP), the peptides β–CN(1–29), β-CN(1–28), α_{s1}-CN(f1–22), and α_{s1}-CN(f1–23) were found. The chymosin-derived α_{s1}-CN peptides were absent from the Quarg cheese whey, whereas shortened forms occurred as a consequence of the degradation by starter proteinases. In Quarg cheese whey, 50 peptides were from β–CN, 34 from κ-CN, seven from α_{s1}-CN and none from α_{s2}-CN. Some peptides known to be released during the in vitro action of P_I- and P_{III}- type proteinases on single casein fractions occurred also in Quarg cheese whey.

oligopeptide / whey / RP-HPLC / FAB mass spectrometry

Résumé — Les oligopeptides du lactosérum doux et acide de fromagerie. Les peptides solubles dans le TCA 12 % de lactosérum doux provenant de la fabrication de fromages Provolone et Grana Padano, ainsi que de lactosérum acide provenant de la fabrication de fromage Quarg ont été séparés par CLHP et identifiés en combinant la spectrométrie de masse et la dégradation d'Edman. Tous les lactosérums contenaient les deux formes phosphorylées du caséinomacropeptide (CMP). Les lactosérums doux contenaient les peptides β-CN(1–29), β-CN(1–28), α_{s1}-CN(f1–22) et α_{s1}-CN(f1–23). Ces derniers étaient absents du lactosérum acide, qui présentait néanmoins de nombreux peptides de taille inférieure, signe d'une activité protéasique des bactéries lactiques du levain. La coupure des liaisons peptidiques Phe^{23}-Phe^{24} et Arg^{22}-Phe^{23} a également été observée au cours de l'hydrolyse in vitro de la caséine α_{s1} par la chymosine. Les peptides isolés du lactosérum acide comprenaient 50 peptides issus de la caséine β, 34 de la caséine κ, 7 de la caséine α_{s1} et aucun de la caséine α_{s2}. La caséine α_{s1} était moins hydrolysée que les caséines β ou κ. Plusieurs peptides formés au cours de l'action de protéinases

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de type P_I- et P_{III}- sur les fractions purifiées de caséines ont été retrouvés aussi dans le lactosérum acide de Quarg. Le mécanisme global de formation de ces peptides dans le lactosérum serait une formation de produits primaires résultant de la dégradation des caséines par la chymosine et la plasmine, suivie de la dégradation de ces produits par les protéases associées aux enveloppes cellulaires des bactéries lactiques mésophiles du levain. Les résultats montrent aussi que la composition peptidique du lactosérum de Quarg est plus équilibrée que celle de deux autres lactosérums et pourrait être plus favorable à une croissance rapide des bactéries lors de l'utilisation du lactosérum pour un repiquage de levain lactique.

oligopeptide / lactosérum / CLHP / spectrométrie de masse FAB

INTRODUCTION

The primary function of the addition of a starter culture is the production of lactic acid for cheesemaking. Since lactic acid bacteria can not synthesize many amino acids, they use a complex proteolytic system, consisting of proteases and peptidases, to split most peptide bonds of milk proteins. Thus, the concentration of peptides increases as a consequence of starter bacteria and becomes sufficient to support bacterial growth to the optimal cell density with adequate acid production, both necessary to manufacture a cheese. Therefore, proteolytic activity of starters is very important for bacterial growth in milk and, with other adventitious bacteria, contributes to casein proteolysis during cheese ripening. Identifying peptides produced during fermentation allows us to understand the specificity of the enzymes produced by starter cultures and their further role during cheese ripening. Information is now available on the constitutive enzymes of *Lactococcus*, as well as on the effects of purified enzymes on whole casein and single casein fractions. The specificity of the extracellular P_I- and P_{III}-type proteinase of *Lactococcus lactis* on κ- (Visser et al, 1994), β- (Juillard et al, 1995), and α_{s1}-casein (Reid et al, 1991) has recently been investigated. *Lactococcus* proteinases showed some chymosin-like effects when acting on κ-casein and more than 100 different oligopeptides were produced from the β-casein hydrolysis. About one-fifth of the oligopeptides were small enough, eg, from 4 to 8 amino acid residues, to be taken up by the oligopeptide transport system of *L lactis*. However, neither detailed knowledge of the oligopeptide composition of the whey derived from cheesemaking nor the minimum model for the growth of natural bacterial cultures in casein peptide-media are available at present. The optimization of the preparation of acid lactic bacteria natural whey cultures as a starter for AOC hard cheese remains a sought-after goal. Identifying the oligopeptide-producing enzymes will provide valuable information on the whey quality and may bring us closer to this goal.

In this paper we report the characterization of the 12% TCA soluble oligopeptide fraction of draining whey from Provolone, Grana Padano, and Quarg cheeses. HPLC and mass spectrometry (MS) were the analytical procedures used to fractionate and to identify the oligopeptide components respectively. A comparison of the single whey peptide components to those split in vitro by cell-wall-envelope proteinases allowed the deduction of the different specificity of the proteolytic system associated with the pure starter culture of *Lactococci* for Quarg cheese. No tracer-peptides were found for the endopeptidase action from *Lactobacilli* strains occurring in the natural whey cultures used for Grana Padano and Provolone cheeses.

MATERIALS AND METHODS

Whey samples

Whey was collected from the draining curd in three factories producing Grana Padano, Provolone, and Quarg cheeses. Samples were filtered, freeze-dried, and stored at –20 °C until use. Whole individual casein containing κ-CN A was obtained from a skimmed milk according to the procedure of Aschaffenburg and Drewry (1959).

Isolation of the oligopeptide fraction from whey

Twelve g trichloracetic acid were added to a solution of a whey powder sample in distilled water (10 g/100 mL). The resulting precipitate was discarded by centrifugation at 3000 *g* and the clear supernatant was filtered through a 0.22 μm membrane filter (Waters, Millipore Corp, MA, USA).

Chymosin action on whole casein and isolation of the derived peptides

The chymosin action was carried out as previously described by Addeo et al (1995) using rennet (Clerici, I-22071 Cadorago). After 30 min the pH was brought to 4.6 by N-HCl dropwise addition and the resultant suspension centrifuged at 3000 *g* for 5 min. The clear supernatant was freeze-dried and the dried sample (5 mg) was dissolved in 200 μL water and injected into the HPLC column equilibrated with 0% solvent B. The mixture of peptides was fractionated by reversed-phase RP-HPLC on a Kontron instrument (Kontron Instruments, Milan, Italy) consisting of two Model 420 pumps, a Rheodyne sample injector (200 μL loop) and a Model 491 solvent programmer. Peptide detection was carried out at 220 nm using a Kontron variable-wavelength detector (Mod 430). The separation was performed on a 214TP54, 5 μm reversed-phase Vydac C18, 250 × 4.6 mm column (Vydac, Hesperia, CA, USA). Solvent A was 1 mL trifluoracetic acid (TFA)/L water. Solvent B was 0.7 mL TFA/L acetonitrile. A linear gradient from 0% to 37% B was applied at a flow rate of 1 mL/min over 60 min. In order to isolate the secondary components of the hydrolysate, a freeze-dried sample of the pH 4.6-soluble fraction (500 mg) was dissolved in distilled water (5 mL) and then diafiltered using a 3-kDa cut-off membrane (Centripep, Amicon Division, Beverly, MA, USA). Each time the retentate was diluted to 5 mL with water and ultrafiltration repeated four times. The permeate containing the oligopeptides with a molecular mass lower than 3 kDa was recovered and freeze dried. Aliquots (5 mg) were dissolved in 200 μL water, injected into the HPLC column equilibrated with 0% solvent B, and fractionated as above. Peptide fractions were collected manually, dried by flushing under nitrogen, and stored at –20 °C.

Fast atom bombardment mass spectrometry (FAB/MS)

FAB/MS spectra were recorded on a VG Analytical ZAB 2SE double focusing mass spectrometer fitted with a cesium gun operating at 25 keV (2 mA). Samples were dissolved in 1 mL/L HCl and loaded onto a glycerol-coated probe tip; thioglycerol was added to the matrix just before introduction of the probe into the ion source. Amplification of the electric signal was decreased during the magnet scan according to the intensity of the mass signals observed on the oscilloscope; mass spectra were recorded on UV-sensitive paper and counted manually.

Peptide identification

Signals recorded in the spectra were associated with the corresponding peptides on the basis of the expected molecular mass deduced from the sequence of the casein fractions (Swaisgood, 1992). In order to confirm these assignments, manual Edman degradation steps were performed either on peptides in mixture or isolated peptides, followed by FAB/MS analysis of the truncated peptides (Addeo et al 1992, 1994).

Electrospray mass spectrometry

ES/MS analysis of peptides was performed with a BIO-Q triple-quadruple mass spectrometer (VG, Manchester, UK). Samples from HPLC separation (10 μL, 50 pmol) were injected into the ion source at a flow rate of 2 μL/min; the spectra were scanned from 1800 to 600 at 10 s/scan. Mass scale calibration was carried out

using the multiple charged ions of a separate introduction of myoglobin.

RESULTS AND DISCUSSION

Three samples of dried whey were examined having a similar total N content accounting for about 12%. The NPN content of Quarg whey was 0.7% whereas that of Provolone and Grana Padano whey samples was about 0.2%. This means a higher content of 12% TCA soluble oligopeptides in Quarg whey with respect to the other two cheese whey samples. Figure 1 shows the HPLC profile of the 12% TCA soluble oligopeptides occurring in the three Grana Padano (fig 1a), Provolone (fig 1b), and Quarg (fig 1c) cheese whey samples. The first two profiles were similar, although the intensity of the individual peaks changed according to the

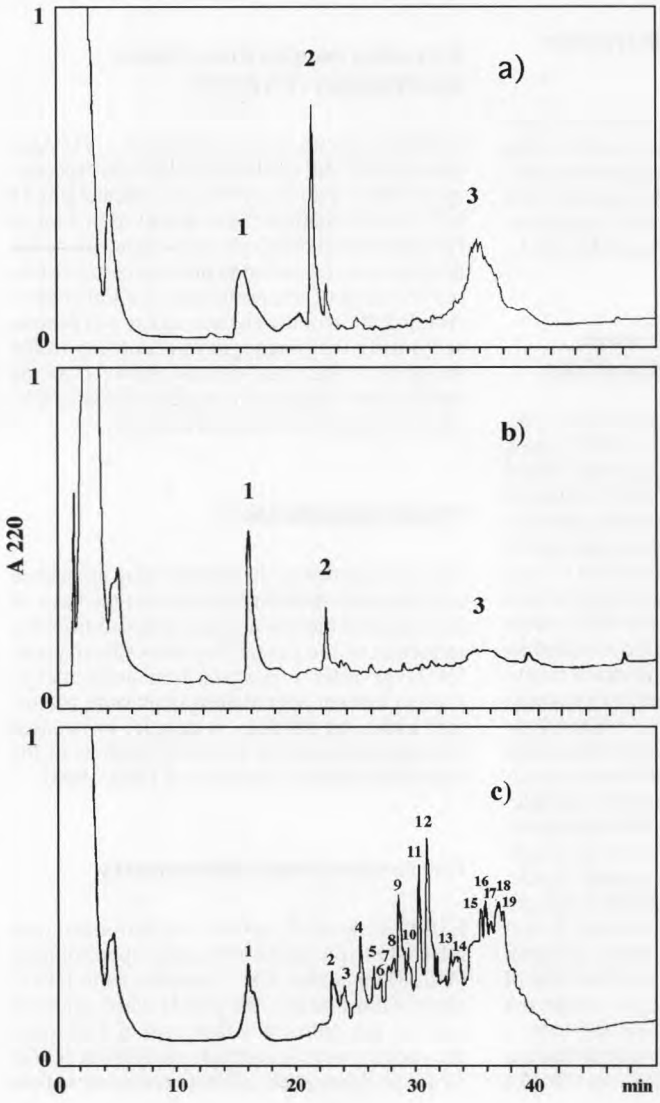

Fig 1. C_{18} reversed-phase HPLC profile of 12% TCA soluble oligopeptides from Grana Padano (**a**), Provolone (**b**), and Quarg cheese whey (**c**) showing the peaks collected and identified as shown in table I.

Analyse par CLHP (colonne C_{18}) des peptides solubles dans le TCA 12% isolés à partir du lactosérum issu de la fabrication des fromages Grana Padano (a), Provolone (b), et Quarg (c). L'identification des pics est présentée dans le tableau I.

cheese whey. They were different from the Quarg cheese whey sample profile (fig 1c), which contained some additional peaks between 20 and 40 min retention time (RT) as a result of more intense proteolysis. The main HPLC peaks of profiles 1a, 1b, and 1c were collected and analyzed by ES/MS and/or FAB/MS. The signals occurring in the spectra were as signed to the corresponding peptides along the casein sequence on the basis of their molecular mass. All the assignments were confirmed by mass analysis of the truncated peptides after two Edman degradation steps respectively (Addeo et al, 1992). Thereafter, following two consecutive Edman degradation steps, the mass spectrum was again determined on the truncated peptides. This allowed the identification of the peptides from the N-terminal sequence and the molecular mass. In table I the identity of the main low-molecular mass peptides is shown with the indication of their origin.

Table I. Main peptides isolated by HPLC analysis in figure 1 from 12% trichloroacetic acid-soluble fraction from Grana Padano, Provolone and Quarg cheese whey and identified by FAB/MS and ES/MS.

Oligopeptides isolés par séparation CLHP de la figure 1 à partir de la fraction soluble dans le TCA 12 % du lactosérum provenant de la fabrication des fromages Grana Padano, Provolone et Quarg et identifiés par spectrométrie de masse FAB et spectrométrie de masse électrospray.

Peak	*MH⁺*	*N-terminal sequence*	*Peptide*
1[a*]	3476+3604	Arg-Glu	β(f1-28)4P+β(f1-29)4P
2[a*]	2617+2764	Arg-Pro	α_{s1}(f1-22)+α_{s1}(f1-23)
3[a*]	6787	Met-Ala	κ(f106-169)1P
1[b*]	3476+3604	Arg-Glu	β(f1-28)4P+β(f1-29)4P
2[b*]	2617+2764	Arg-Pro	α_{s1}(f1-22)+α_{s1}(f1-23)
3[b*]	6787	Met-Ala	κ(f106-169)1P
1[c]	1126	Glu-Val	κ(151-160)
	1057	Pro-Pro	β(85-93)
	997	Val-Ile	κ(152-160)
	905	Asn-Glu	α_{s1}(17-23)
2[c*]	3476+3604	Arg-Glu	β(f1-28)4P+β(f1-29)4P
3[c]	1551	Thr-Gln	β(78-91)
	1392	Gln-Glu	β(194-206)
	1282	Leu-Ser	β(164-175)
	1057	Pro-Pro	**β(85-93)**
	997	Val-Ile	**κ(152-160)**
	905	Asn-Glu	**α_{s1}(17-23)**
4[c]	817	Gly-Val	**β(94-101)**
	791	Glu-Asn	α_{s1}(18-23)
	634	Arg-Pro	α_{s1}(1-5)
	577	Val-Lys	**β(98-102)**
5[c]	934	Ser-Gln	κ(132-140,T^{136})
	735	Thr-Val	**κ(161-167)**

Peak	*MH*$^+$	*N-terminal sequence*	*Peptide*
	997	Val-Ile	κ(152-160)
	634	Val-Gln	κ(162-167)
6[c]	1207	Gln-Asn	β(72-82)
	1195	Leu-Ser	β(165-175)
	1126	Glu-Val	κ(151-160)
	1118	Gln-Gln	β(38-46)
	1082	Ala-Val	κ(66-75)
	1040	Val-Ala	κ(143-151,A^{148}S^{149})
	1021	Ser-Thr	κ(132-141,T^{136})
	997	Val-Ile	κ(152-160)
	941	Ala-Thr	κ(144-151, A^{148}S^{149})
	934	Ser-Thr	**κ(132-140,T^{136})**
	905	Asn-Glu	α_{s1}(17-23)
	791	Glu-Asn	α_{s1}(18-23)
	763	Asp-Ile	α_{s1}(56-61)
	703	Ser-Ile	**β(22-27)**
7[c]	1422	Thr-Gln	β(78-90)
	1221	Thr-Val	κ(142-151,S^{149},T^{145},A^{148})
	1141	Thr-Val	κ(142-151, A^{148}, S^{149})
	1122	Ser-Thr	**κ(132-142)**
	1040	Val-Ala	κ(143-151)
	996	Val-Ala	κ(143-151,A^{148},S^{149})
	785	Glu-Ser	κ(154-160)
	703	Ser-Ile	**β(22-27)**
	674	Asp-Met	β(184-188)
	656	Ser-Pro	κ(155-160)
	603	Val-Pro	β(178-182)
8[c]	1228	Ser-Thr	**κ(141-151, A^{148}, S^{149})**
	1184	Ser-Thr	κ(141-151, D^{148}, S^{149})
	1141	Thr-Val	κ(142-151, A^{148}, S^{149})
	905	Thr-Val	**κ(161-169)**
	830	Arg-Asp	β(183-189)
	802	Lys-Ala	β(176-182)
	756	Asp-Lys	β(47-52)
	603	Val-Pro	β(178-182)
	569	Pro-Pro	κ(156-160)
	559	Met-Pro	β(185-189)
9[c]	1527	Ala-Val	κ(138-151, A^{148}, S^{149})
	1184	Ser-Thr	κ(141-151, D^{148}, S^{149})
	997	Val-Ile	κ(152-160)
	979	Thr-Glu	β(120-128)
	946	Ser-Thr	κ(132-140)T^{136}
	898	Leu-Glu	**κ(153-160)**
	856	Asn-Val	**β(7-14)**
	788	Arg-Glu	**β(1-6)**

Peak	*MH*$^+$	*N-terminal sequence*	*Peptide*
	780	Lys-Val	β(169-175)
	756	Asp	β(47-52)
	674	Asp-Met	β(184-188)
	632	Glu-Leu	β(2-6)
10[c]	2037	Met-Ala	κ(106-123)
	1162	Ala-Phe	α_{s1}(31-40)
	997	Val-Ile	κ(152-160)
	898	Ile-Glu	κ(153-160)
	1082	Ala-Val	κ(66-75)
11[c]	2037	Met-Ala	κ(106-123)
	1876	Arg-Pro	α_{s1}(1-16)
	1554	Glu-Leu	**β(44-56)**
	1425	Leu-Gln	β(45-56)
	1325	Glu-Leu	β(44-54)
	1282	Ser-Leu	**β(164-175)**
	1082	Ala-Val	κ(66-75)
	1198	Ala-Arg	κ(96-105)
	1126	Glu-Val	κ(151-160)
	997	Val-Ile	κ(152-160)
12[c]	1883	Val-Ile	κ(152-169)
	1563	Lys-Val	β(170-182)
	1197	Val-Ile	κ(152-162)
	1126	Glu-Val	**κ(151-160)**
	1020	His-Gln	α_{s1}(8-16)
	969	Leu-Asn	β(6-14)
	997	Val-Ile	κ(152-160)
	978	Ser-Leu	β(69-77)
	954	Ala-Val	κ(66-74)
	941	Ala-Thr	κ(144-151, A^{148}, $\underline{S}^{149}$)
	898	Ile-Glu	κ(153-160)
	548	Leu-Leu	α_{s1}(20-23)
13[c]	2793	Met-Ala	**κ(106-131)**
14[c]	1542	Ser-Pro	**κ(155-169)**
	1405	Ser-Leu	**β(69-81)**
	978	Ser-Leu	**β(69-77)**
	905	Asn-Glu	α_{s1}(17-23)
	791	Gln-Asn	α_{s1}(18-23)
	891	Leu-Pro	β(70-77)
	603	Val-Pro	β(178-182)
15[c]	1340	Ser-Leu	**β(57-68,H^{67})**
	1392	Gln-Glu	**β(194-206)**
	1264	Glu-Pro	β(195-206)
	1038	Val-Leu	**β(197-206)**
	1001	Tyr-Pro	**β(60-68,H^{67})**

Peak	*MH*$^+$	*N-terminal sequence*	*Peptide*
	689	His-Leu	β(134-139)
	978	Ser-Leu	β(69-77)
	1092	Asn-Ser	β(68-97)
16[c]	1555	Tyr-Gln	**β(193-206)**
	1392	Gln-Glu	**β(194-206)**
	1264	Glu-Pro	β(195-206)
17[c]	2193	Ala-Gln	**β(53-72,H^{67})**
	1765	Ser-Leu	**β(57-72,H^{67})**
	1678	Leu-Val	**β(58-72,H^{67})**
	1563	Asp-Met	**β(184-196)**
	1300	Ser-Leu	**β(57-68,H^{67}L^{58})**
	1213	Leu-Val	**β(58-68,P^{67})**
	1151	Gly-Pro	β(199-209)
	1124	Val-Val	β(82-91)
	997	Val-Ile	κ(152-160)
	898	Ile-Glu	κ(153-160)
18[c]	1671	Glu-Ser	κ(154-169)
	1551	Thr-Gln	**β(78-91)**
	1542	Ser-Pro	κ(155-169)
	1455	Pro-Pro	κ(156-169)
	1375	Gln-Gln	**β (194-206, Pyr^{194} , G^{195})**
	1505	Gln-Glu	β(194-207)
	1300	Ser-Leu	**β(57-68,P^{67})**
	1200	Thr-Thr	κ(141-151,T^{141},D^{148}, S^{149})
	1213	Leu-Val	β(58-68,P^{67})
	1322	Thr-Pro	β(80-91)
	1422	Thr-Gln	β(78-90)
19[c]	1781	Thr-Gln	**β (78-93)**
	1717	Gln-Glu	**β (194-209)**
	1700	Pyr-Glu-Glu	**β (194-209)**
	1200	Thr-Thr	κ(141-151, S^{149}A^{148})

[a]Grana Padano; [b]Provolone; [c]Quarg cheese. S, SerP, T, ThrP.
The most represented peptide, in peak mixtures are indicated in bold.
* Molecular mass determined by electrospray mass spectrometry.

Les peptides les plus abondants dans chaque pic chromatographique sont indiqués en gras.
**Masse moléculaire déterminée par spectrométrie de masse électrospray.*

Peptide composition of cheese whey samples

Both β-CN(1–29) and β-CN(1–28) (ie, the proteose-peptone-8 fast) peptides occurred in the first HPLC peak relative to the three cheese whey samples likely as result of the Lys^{28}-Lys^{29} and Lys^{29}-Ile^{30} bond splitting by plasmin. This enzyme is therefore able to cleave also the Lys^{29}-Ile^{30} bond which may explain the presence of a β-CN(1–29) peptide in the whey. Taking into account that

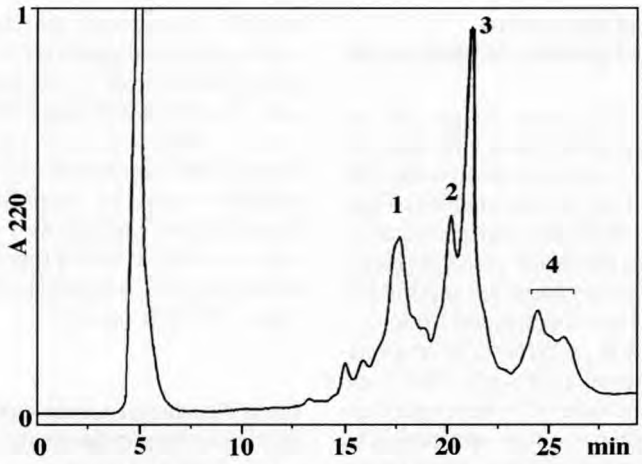

Fig 2. C_{18} reversed-phase HPLC profile of the pH 4.6 soluble peptides obtained from a rennet-hydrolysate of whole casein.

Analyse par CLHP (colonne C_{18}) de la fraction soluble à pH 4,6 d'un hydrolysat de caséine entière par la présure.

Table II. Average molecular mass of the main peptides occurring in the HPLC profile of the chymosin-hydrolysate of whole casein determined by electrospray mass spectrometry.

Masse moléculaire moyenne, établie par spectrométrie de masse électrospray, des peptides isolés par séparation CLHP d'un hydrolysat de caséine entière obtenu par action de la présure.

HPLC peak	*Measured molecular mass (Da)*	*Peptide*	*Calculated molecular mass* [a] *(Da)*
1	2 616.7 ± 0.3	α_{s1}-CN(f1-22)	2 617.0
2	2 763.8 ± 0.9	α_{s1}-CN(f1-23)	2 764.2
3	6 787.7 ± 0.3	κ-CN(f106-169) 1P	6 787.4
	6 867.1 ± 0.9	κ-CN(f106-169) 2P	6 867.4
4	19 038.9 ± 1.9	κ-CN A 1P	19 039.4

[a] Calculation based on the κ-, and α_{s1}-casein sequence determined by Mercier et al (1973) and Mercier et al (1971) respectively and relative corrections reported by Swaisgood (1992).

Calculs basés sur la séquence des caséines κ et α_{s1} publiée par Mercier et al (1973) et Mercier et al (1971) avec les corrections de Swaisgood (1992).

peptides having a similar sequence present a reduced competition effect during the ionization process in FAB/MS (Naylor et al, 1986), a rough evaluation of the relative amount can be tentatively made by a comparison of the relative quasi-molecular ion intensity. Accordingly, peptides (1–29) and (1–28) accounted for about 50% each. Peak *n* 2 was also found to be a mixture of two α_{s1}-CN-derived components, a_{s1}-CN(f1–22) and α_{s1}-CN(f1–23). This latter has been reported to be produced very early during chymosin action on the Phe^{23}-Phe^{24} bond of α_{s1}-CN. The occurrence of α_{s1} CN(f1–22) is also new in whey or cheese. Neither α_{s1}-CN(f1–22) nor α_{s1}-CN(f1–23) occurred in the Quarg whey.

Identification of the in vitro rennet-mediated products of whole casein

In order to identify the origin of the α_{s1}-CN(f1–22) peptide, an in vitro assay of rennet on whole casein was carried out. The HPLC profile of the pH 4,6 soluble fraction relative to this hydrolysate is shown in figure 2. The components identified in the chymosin digest of the whole casein are reported in table II. Peaks 1 and 2 corresponded to α_{s1}-CN(f1–22) and α_{s1}-CN(f1–23), respectively. This indicates that Arg^{22}-Phe^{23} and Phe^{23}-Phe^{24} bond of α_{s1}-CN were both cleaved by chymosin (or rennet enzymes). The relative proportions of these components were roughly 60 and 40%, although a detailed evaluation was hindered by the uncompleted separation of α_{s1}-CN(f1–23) and CMP peaks. The occurrence of α_{s1}-CN(f1–22) means that the Arg^{22}-Phe^{23} bond is as susceptible as the Phe^{23}-Phe^{24}, and that cleavage of these two peptide bonds of α^{s1}-CN occurs simultaneously after the addition of rennet to milk. The main chymosin-mediated products of κ-CN were κ-CN (f106–169)1P and κ-CN(f106–169)2P corresponding to CMP species at two degrees of phosphorylation. The broad peak eluted between RT 22 and 25 min corresponded to κ-CN A, indicating that the α_{s1}-CN chymosin-mediated products were formed in the chymosin hydrolysate before the entire conversion of κ-CN into CMP and para-κ-casein. Therefore κ-CN and α_{s1}-CN was found to be simultaneously hydrolyzed to different extents by chymosin. Since mass signals of low intensity were detected in the mass spectra, it was supposed that other minor peptide components occurred in the digest of whole casein.

Table III. Identification by FAB mapping of the peptides occurring in the chymosin-hydrolysate of whole casein after permeation through a 3-kDa cut-off membrane.

Identification par le couplage de la dégradation d'Edman à la spectrométrie de masse FAB des peptides présents dans un hydrolysat de caséine entière obtenu par action de la présure après perméation à travers une membrane de porosité de 3 kDa.

MH⁺	*N-terminal*	*Peptide*
1593	Phe-Val	α_{s1}(f24–37)
1430	Thr-Met	κ(f94–105)
1251	Thr-Lys	α_{s2}(f198–207)
1237	Phe-Val	α_{s1}(f24–34)
1022	Val-Ile	α_{s2}(f200–207)
979	Phe-Ala	α_{s2}(f174–181)

Identification of minor components in the in vitro hydrolysate of whole casein by rennet

In order to concentrate these components in a single fraction, the whole digest was further fractionated by diafiltration through a 3-kDa cut-off membrane. The permeate containing oligopeptides having a molecular mass lower than 3 kDa were directly analyzed by FAB/MS and the identified components shown in table III. Two peptides were from α_{s1}-, three from α_{s2}-, one from κ-CN, and no component from β-CN. Para-κ-CN was also affected by chymosin with the formation of the κ-CN(f94–105) peptide. These results confirm that there were minor components in the chymosin-hydrolysate of whole casein, whereas the main components were CMP, α_{s1}-CN(f1–22), and α_{s1}-CN(f1–23).

The specificity of whey peptides in Quarg cheese

By using the FAB-mapping procedure, the HPLC peaks within RT 20 and 32 min were shown to contain a number of peptides already shown in table I. They arise from κ-, β- and α_{s1}-CN, whereas no peptide was from α_{s2}-CN. Without taking into account the multi-phosphorylated CMP forms 64 residues long, the maximal length of α_{s1}- and β-CN peptides was of 16 and 20 resi-

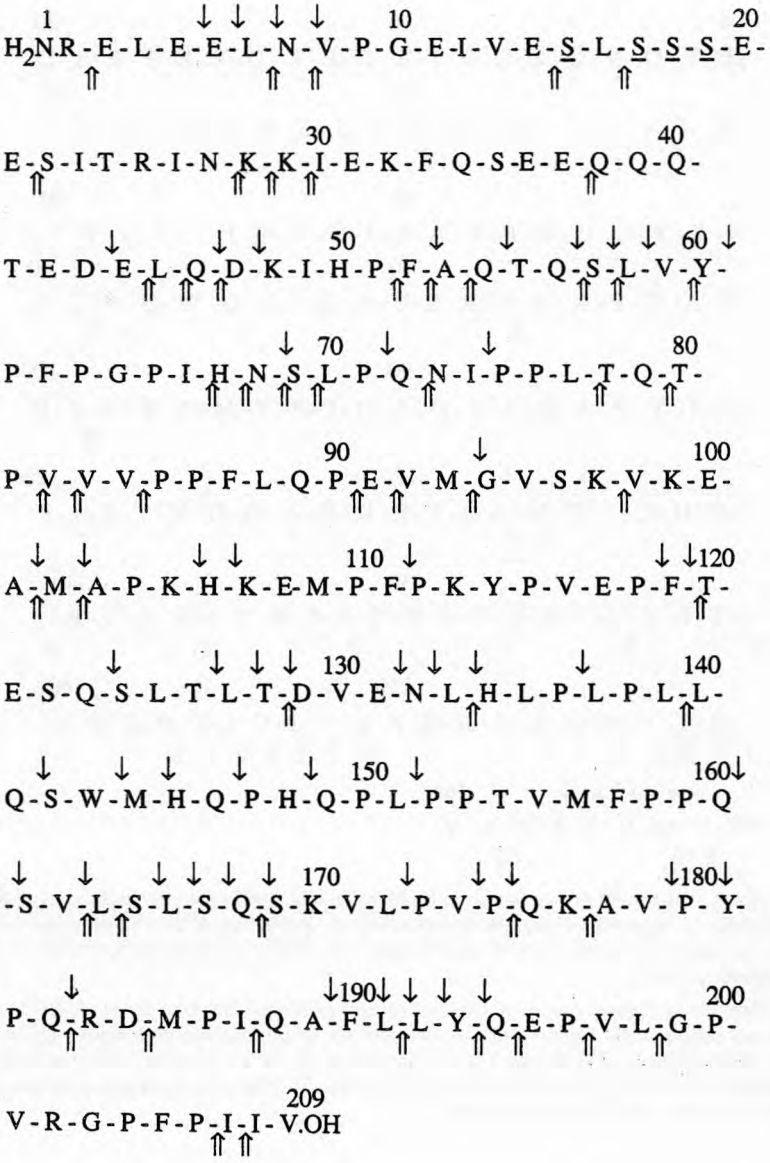

Fig 3. Primary structure of bovine β-casein A^2 (Ribadeau Dumas et al, 1972) with the localization of the 12% TCA-soluble peptides occurring in the whey of a Quarg cheese. Cleavage sites identified in this work (⇑) and those derived by in vitro action of the cell-envelope P_I- and P_{III}- type proteinase (↓)(Tan et al, 1993).

Localisation sur la séquence de la caséine β (variant génétique A^2) (Ribadeau Dumas et al, 1972) des sites de coupures et des peptides libérés dans la fraction soluble dans le TCA 12 % d'un échantillon de lactosérum de fromage Quarg. Sites de coupure identifiés dans ce travail (⇑) et ceux libérés par action in vitro des protéases de paroi des bactéries de types P_I et P_{III} (↓) (Tan et al, 1993).

Fig 4. Primary structure of bovine κ-casein B (Mercier et al, 1973) with the indication of the cleavage sites identified in this work (⇑) and those derived by in vitro action of the cell-envelope proteinase of *Lactococcus lactis* ssp *cremoris* AM[1] (↓) (Visser et al, 1994); (⇓), peptide bond split by chymosin; pE, pyroglutamic acid.

Localisation sur la séquence de la caséine κ (variant génétique B) (Mercier et al, 1973) des sites de coupures et des peptides libérés dans un échantillon de lactosérum de fromage Quarg. Peptides identifiés dans ce travail (⇑) et ceux libérés par action in vitro de protéase de paroi de la souche de Lactococcus lactis *subsp* cremoris *AM[1] (↓) (Visser et al, 1994); (⇓), liaison peptidique coupée par la chymosine; pE = acide pyroglutamique.*

dues respectively. In all, 50 peptides came from β-CN, 34 from κ-CN, and seven from α_{s1}-CN. In order to identify the enzyme involved in the casein proteolysis, peptide components arising from a common casein fraction have been grouped together in figures 3, 4, 5 according to the casein fraction and are discussed separately.

β-CN-derived oligopeptides

The β-casein chain was thoroughly affected by hydrolysis indicating that no region was intrinsically resistant to starter endopeptidase(s) (fig 3). A couple of peptides differing only for the amino acid substitution His^{67} –> Pro^{67} was detected, which indicated that cheese milk consisted of a mixture

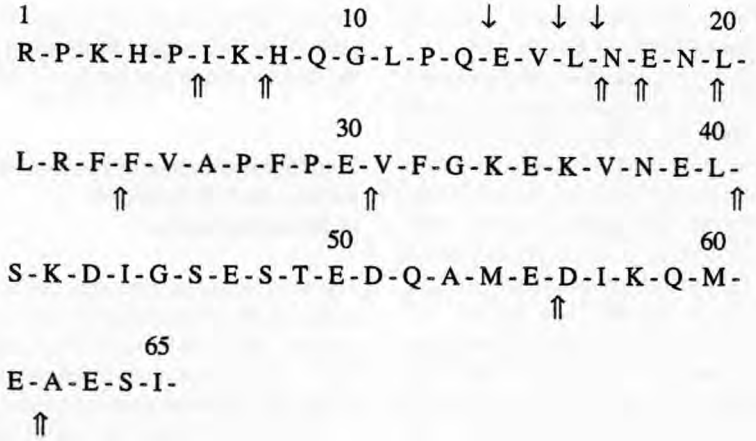

Fig 5. N-terminal primary structure of bovine α_{s1}-casein B (Mercier et al, 1971) with the indication of the cleavage sites identified in this work (⇑) and those derived by in vitro action of the cell-envelope proteinase of some lactococcus strains (↓) (Exterkate and Alting, 1993; Exterkate et al, 1993).

Localisation sur la séquence de la caséine α_{s1} (variant génétique B) (Mercier et al, 1971) des sites de coupures et des peptides libérés dans un échantillon de lactosérum du fromage Quarg. Peptides identifiés dans ce travail (⇑) et ceux liberés par action in vitro des protéases de paroi de plusieurs souches de lactocoques (↓) (Exterkate and Alting, 1993; Exterkate et al, 1993).

of β-CN variants such as A^1, B, and C and A^2 and A^3, having His^{67} and Pro^{67} as marker-residues, respectively. The peptides identified in the present work were compared to those released from β-casein by the P_I- and P_{III}- type proteinases (Tan et al, 1993). The following peptides released in the whey by the starter proteinases, 1–6, 47–52, 57–68, 58–68, 120–128, 164–175, 169–182, 176–182, and 194–209, were also detected in the in vitro hydrolysate of β-casein by P_I- and P_{III}- type proteinases (Tan et al, 1993). The cleavage of the peptide bonds such as 192–193, 43–44, 44–45, 46–47, 52–53, and 193–194, considered as typical for the P_I- and P_{III}-type proteinases respectively, were found to be hydrolyzed also by the starter proteinase of the Quarg cheese. Although our sample was an industrial whey resulting from a 16 h-long acidified milk, no significant difference was recognized in the hydrolytic pattern between both P_I- and P_{III}- type proteinase and the starter enzymes. This means that the low quantity of clotting enzyme added for the cheesemaking did not produce any significant hydrolysis of β-casein, which is known among the casein fractions to be the most resistant to chymosin.

κ-CN-derived oligopeptides

In addition to the CMP, a number of κ-CN peptides were formed taking origin by the action of bacterial enzymes on both the intact protein and CMP. Examining the list of peptides occurring in the cheese whey, CMP was found to be more sensitive than para-κ-casein to enzymes since a high number of peptides came from the C-terminal part of κ-casein. In figure 4 the primary structure of bovine κ-casein B is reported with the positions of cleavage sites as derived from the identification of main low-molecular mass peptides in comparison with those derived by the cell-envelope proteinase of *Lactococcus lactis* ssp *cremoris* AM^1. Of the 24 κ-casein cleavage sites iden-

tified in the Quarg cheese whey, five corresponding to peptide bonds 151–152, 160–161, 162–163, and 167–168, were common with the in vitro hydrolysate of κ-casein by cell-envelope proteinase of *Lactococcus lactis* ssp *cremoris* AM[1] (Visser et al, 1994). The most represented peptides in cheese whey were κ-CN(f152–160) and κ-CN(f161–169), also shared with the in vitro hydrolysate of κ-CN by cell-envelope proteinase (CEP). This indicates that the splitting of the 160–161 and 151–152 peptide bonds previously indicated by Visser et al (1994) to be characteristic for the CEP_{III} action also occurs in the lactococci containing commercial starter used for cheesemaking. In cheese whey, additional cleavage products were detected originating from the 140–143, 150–156, and 161–169 κ-CN regions. This allowed the formation of some peptides, differing by one or more residues at the N- and/or C-end, co-eluting in single HPLC peaks. The FAB mapping procedure, a very sensitive analytical procedure of identification, allowed us to detect these complex mixtures of peptides and to identify single components in mixtures. Monnet et al (1992), studying the specificity of CEP_{II} from *L lactis* ssp *lactis* NCDO 763 towards κ-casein, found more positions of cleavage than did Visser et al (1994). This difference was attributed by Visser et al (1994) to the mixed-type specificity of the enzyme used by Monnet et al (1992). The case of the commercial starter culture growth in cheese milk where a variety of enzymes acts simultaneously on the four casein fractions is more complicated than that studied by Visser et al (1994), Monnet et al (1992), and Reid et al (1991) whose studies considered only model systems with a single casein fraction and an active CEP preparation obtained from a single strain. This might explain the high number of cleavage positions of κ-casein found in the whey in our study, in comparison with the fewer positions observed in the model systems. Our results are hardly consistent with the outcome of Monnet et al (1992) and Visser et al (1994) on the susceptibility of κ-casein to the cell-envelope proteinases of lactococci.

Peptides indicative of the κ-casein variant and of its degree of phosphorylation

The two series of peptides containing the amino acid substitutions of the two κ-casein variants, Ile^{136} (B) –> Thr^{136} (A) and Ala^{148} (B) –> Asp^{148} (A), were also isolated indicating that both the parent proteins occurred in cheese milk (table I). Ser^{149} was found phosphorylated in all the peptides isolated except one κ-CN(f143–151) where no phosphate was found. One extra-phosphorylated peptide was the κ-CN(f142–151) component containing both Ser^{149} and Thr^{145} phosphorylated. These results confirm those of Vreeman et al (1977) on the presence of a κ-CN 2P. The occurrence of a κ-CN devoid of phosphate is new. The analysis of the CMP by ES/MS allows us to conclude that there are at least three species of κ-casein differing in the degree of phosphorylation, 0, 1, and 2P. The presence of a silent κ-CN variant in cheese milk might be deduced from the isolation of the peptide κ-CN(f141–151, $SerP^{149}$, Ala^{148}, Thr^{141}) containing the Ser^{141} –> Thr^{141}. However this needs confirmation by analysis of native milk, possibly individual.

α_{s1}-CN-derived oligopeptides

The cleavage sites of the α_{s1}-casein chain giving rise to peptides in Quarg cheese whey (table I) are indicated with arrows in figure 5. The 16–17 and 23–24 bonds were the most susceptible to enzyme attack as shown from the higher level of the relative peptides 1–16 and 17–23 in comparison with other peptides in secondary amount. Peptides 1–16, 17–23, and 18–23 of the Quarg cheese whey are also typical of the different CEP from

several *Lactococcus* strains already identified in previous studies (Exterkate and Alting, 1993; Exterkate et al, 1993). This may indicate that the CEPs active in the cheese milk degrade α_{s1}-casein with a pattern similar to that described for CEP from *Lactococcus lactis* ssp *cremoris* SK11 (Reid *et al*, 1991). However, this latter produced, in addition to peptides from the N-terminal (1–17, 17–23, and 34–37) and central region of α_{s1}-casein, 11 components from the C-terminal region which were not detected in the Quarg-like cheese whey. Some peptides are too large to be soluble in 12% TCA and might be lost in the insoluble fraction. There were some peptides such as 1–5, 8–16, and 20–23 indicative of the degradation of α_{s1}–CN(f1-23) peptide which were not detected intact in the Quarg cheese whey. In a model experiment, the peptides 1–9, 1–13, 1–14, 1–7, 1–18, 1–20, and 14–23 were derived from the in vitro digestion of the α_{s1}-CN(f1–23) peptide with a cell free extract from *L lactis* ssp *cremoris* H61 (Kaminogawa et al 1986). P_I- and P_{III}- type proteinases were shown to have different modes of action (Exterkate et al, 1991) on the α_{s1}-CN(f1–23) peptide, but some overlap of specificity occurred. The absence of the α_{s1}-CN(f1–23) peptide from the Quarg-like cheese whey can be justified by both the low level of chymosin added to milk and also by its quick conversion into shorter peptides by starter proteinases. This mechanism was demonstrated in Gouda cheese, where in absence of CEP, α_{s1}-CN(f1–23) accumulated, whereas in the presence of CEP, characteristic for the different lactococcal strains, the typical degradation pattern of α_{s1}-CN(f1–23) was recognized (Exterkate and Alting, 1995). These authors showed also that using a four-fold amount of rennet for cheesemaking produced a higher rate of amino acid nitrogen than a normal amount of rennet and α_{s1}-CN (f1–23) peptide accumulated to some extent in absence of CEP, whereas using mixed-strain mesophilic starter cultures or single stains CEP positive, α_{s1}-CN (f1–23) was degraded into Phe, 1–9, 1–13, 1–16, 1–17, and 1–14 peptides. Thus, the presence of intact 1–23 peptide as well as of other soluble casein fragments in the whey seems to be governed by the relative activity of both chymosin (or other endoproteases) and the cell-envelope proteinase proteolysis. Both Pélissier et al (1974) and McSweeney et al (1993) failed to detect Arg^{22}-Phe^{23} as a scissile bond of α_{s1}-CN by chymosin. Additional bonds of α_{s1}-CN such as 34–35 and 37-38 were also broken during in vitro action of chymosin and 24–34 and 24–37 peptides were formed confirming the higher susceptibility to chymosin of the N-terminal α_{s1}-CN area (Pélissier et al 1974; McSweeney et al 1993).

α_{s2}-CN-derived oligopeptides

Although during the in vitro experiments, the C-terminal part of the α_{s2}-CN was hydrolyzed by chymosin giving rise to three peptides with a main length of nine amino acid residues, no α_{s2}-CN-derived oligopeptides were found in the Quarg whey cheese, thus confirming that the reduced level of chymosin added for cheesemaking had a limited effect on the extent of the α_{s2}-CN hydrolysis.

CONCLUSIONS

During the first stages of cheesemaking, peptides of different size were released in the whey from the casein by the indigenous milk enzymes such as plasmin, and by rennet. With few exceptions such as those components within the peaks 4c–7c, 9c, and 12c, the whey peptides are too large to be taken up by the growing starter cells, the upper size limit for the oligopeptide transport system being usually thought to be 6–7 aminoacyl residues (Law, 1978; Rice et al, 1978). Peptidases released from lactococcal cells possibly by leakage or lysis (Pritchard

and Colbear, 1993) seem to act cooperatively in reducing casein oligopeptides to a proper size (Pritchard and Colbear, 1993) and in rendering them transportable across the cytoplasmic membrane (Smid et al, 1991).

The mixture of κ-CN-mediated peptides, eg, κ-CN(f106–169), at different degrees of phosphorylation, was found to be in common with both the sweet and acid whey, regardless of the cheese variety. A detailed structural study on the glycosylated CMPs occurring in bovine casein has been recently reported (Mollé and Léonil, 1995). Signals of low intensity corresponding to some glycosylated CMP A and B in mixture with the corresponding unglycosylated CMP forms were also detected. Since our main objective was monitoring the casein degradation, no attempts were made to identify the glycosylated CMP species. The main difference between 12% TCA-soluble oligopeptides of the sweet whey derived from Provolone and Grana Padano cheese, and of the acid one from the fresh Quarg cheese consisted in the level of the primary peptides. In the first two whey samples there was a considerable level of β-CN(f1–28) (ie, the proteose-peptone-8 fast), β-CN(f1–29), α_{s1}-CN (f1–23), and α_{s1}-CN (f1–22), and no derived degradation products owing to the relatively short time between the addition of the starter culture and the draining of the whey sample. In the Quarg cheese whey, the level of primary peptides was low owing to the prolonged contact with casein, 16 h vs about 1 h for Grana Padano and Provolone cheese. The major source of essential and growth-stimulating peptides was β- and κ-CN which, judging from the number of resulting peptides in whey, were both more susceptible than α_{s1}-CN to the cell-envelope proteinases of starter lactococci. The present study focused on the composition of casein-derived peptides in whey cheese gave the specific indication that the acid Quarg cheese whey appears more balanced for a rapid growth of the starter cultures than that of the sweet whey recovered from the making of a hard cheese. It is noteworthy to underline that some peptides recognized as bioactive themselves or precursors of bioactive peptides such as the β-casomorphin-7 containing sequence, eg, β-CN (f60-66) (Brantl et al, 1979), phosphopeptides and κ-CN(f106–123) occurred in Quarg cheese whey. This latter is a precursor of the κ-CN(f106–116) recognized as a potent inhibitor of blood clotting through the formation of platelet-fibrinogen bonds (Jollès et al, 1986). Other peptides can represent the key growth-stimulating factors especially for non-proteolytic bacterial strains. Whey and tryptic digest of either whole casein and κ-CN promoted the growth of most species of the genus *Bifidobacterium* (Poch and Bezkorovainy, 1988, 1991), although the growth-promoting activity peptide has not been definitely identified. Therefore, whey peptides can represent a source of bioactive compounds and growth factor for starter cultures. In the future, we aim to establish just which peptides the starter uses for its growth and to obtain information on the size restriction and substrate specificity for its natural substrates.

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