Hydrolysis of κ-casein in solution by chymosin, plasmin, trypsin and *Lactobacillus*-proteinases

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The aim of this study was to examine the enzymatic hydrolysis of κ-casein by isolating and identifying the released peptides. The enzymes employed in the study were chymosin, plasmin and trypsin, as well as a cell-free extract from three *Lactobacillus helveticus* and nine *Lactobacillus casei* strains.

The findings showed that the bond most sensitive to the proteolytic activity of chymosin was the Phe 105-Met 106. After 24 hours of hydrolysis a few other bonds in the casein macropeptide were also cleaved. Plasmin was found to have weak proteolytic activity under the conditions of this study. When the enzyme-substrate ratio was raised from 1:200 to 1:50, a few peptides were released from the N-terminal region. Trypsin was found to hydrolyze several κ-casein bonds, and peptides were released from almost all regions of the protein.

The proteases of *Lactobacillus* had less effect than chymosin, plasmin or trypsin. The strains could be divided into three categories. *L. helveticus* strains had activity on bonds in the mid-section and C-terminal region, *L. casei* strains E8, P3, P8 and A1 had activity on bonds in the N- and C-terminal regions, while *L. casei* A5 and M9 had activity only on bonds in the C-terminal region.

Key words: κ-casein, enzymatic hydrolysis, peptides

Introduction

κ-Casein is one of the components of bovine casein, representing around 10% of the total casein. Its primary structure consists of 169 amino acids, one phosphate group and variable amounts of N-acetylgalactosamine, galactose and N-acetylneuraminic acid (SWAISGOOD 1982). κ-Casein is heterogeneous with respect to its carbohydrate moiety, which is exclusively linked to the macropeptide part of κ-casein (MACKINLAY and WAKE 1965).

κ-Casein stabilizes casein micelles (WHEELOCK and KNIGHT 1969) and prevents αs- and β-casein from precipitation in the presence of calcium ions. Hydrolysis of bovine κ-casein by chymosin constitutes the first stage of milk clotting and has been thoroughly investigated. It is known that this proteinase rapidly hydrolyzes one bond, Phe 105-Met 106, of κ-casein, leading to the formation of an N-terminal fragment (para-κ-casein; residues 1-105) and a C-terminal fragment (casein macropeptide, CMP; residues 106-169). In various laboratories, the kinetics of this proteolytic action has been studied by the use of model substrates representing parts of the amino acid sequence around the chymosin-sensitive Phe 105-Met 106 bond of κ-casein

(VISSER et al. 1976, 1977, 1987, RAYMOND and BRICAS 1979).

κ-Casein is totally hydrolyzed during milk coagulation (MELACHOURIS and TUCKEY 1966), whereas para-κ-casein is not hydrolyzed during cheese ripening (GREEN and FOSTER 1974, LEDFORD et al. 1966). This latter result, together with the relatively low concentration and amino acid composition of κ-casein, would suggest that it is not an important source of bitter peptides in cheese.

Biologically active peptides that have been identified as a digestion product of κ-casein include an opioid antagonist peptide (residues 33-38) (CHIBA and YOSHIKAWA 1986) and a peptide (residues 106-116) which inhibits both the aggregation and binding of fibrinogen to platelets (JOLLES et al. 1986).

The aim of this study was to investigate the hydrolysis of κ-casein by proteolytic enzymes involved in the ripening of cheese.

Material and methods

Substrate and enzymes

κ-Casein was isolated from sodium caseinate using ion exchange chromatography on column Mono Q (HR 16/10, Pharmacia LKB, Sweden). The solvents used were (A) 0.02 M Tris-HCl containing 0.1% mercaptoethanol and 4.5 M urea (pH 8.0), and (B) buffer A containing 1 M of NaCl. Solvent was delivered to the column at a flow rate of 4 ml/min as follows: 1% of B for 5 minutes, linear gradient from 1 to 25% of B in 25 minutes and from 25 to 35% of B in 30 minutes. The chromatogram was monitored at 279 nm. The κ-casein fraction was collected, dialyzed against water for 48 hours and lyophilized. κ-Casein was stored at -20°C until used for hydrolysis.

The enzymes used in the study were chymosin (Chr. Hansen, from calf), plasmin (Sigma, bovine plasma) and TPCK trypsin (Sigma). The selection of *Lactobacilli* was based on their differing peptidase and caseinolytic activity (PAHKALA and ANTILA 1987). The following *Lactobacilli* were included:

Lactobacillus helveticus LH1, LH5 and LH7
Lactobacillus casei G2, S9, E8, P3, P8, A1 and A5
Lactobacillus casei subsp. *rhamnosus* M1 and M9

The bacteria were grown, isolated and disintegrated according to the method used by PAHKALA et al. (1986). After overnight cultivation (200 ml), the cells were centrifuged and washed twice with distilled water. The cells were then suspended in distilled water (25 ml) and autolyzed for 48 hours at 42°C. The autolysis suspension was cooled to -20°C, thawed and homogenized with an Ultra-Turrax for 10 minutes in cold water. The suspension was then centrifuged (20,000 x g, 4°C, 15 min), and the cell debris was washed with distilled water and finally suspended in 10 ml of distilled water. This suspension was used for the hydrolysis of κ-casein.

Hydrolysis

The enzymes (0.015 - 3% in water) were added into 1.5% (w/v) solutions (0.05 M phosphate buffer, pH 6.0) of κ-casein. The ratios of enzyme to substrate were:

Enzyme	E:S
chymosin	1:100
plasmin	1:200, 1:100, 1:50
trypsin	1:10000

The cell-free extract of *Lactobacillus* was added to the protein solution at a ratio of 1:10.

The mixtures were incubated at 40°C. After the reaction period, 2, 4 and 24 hours for chymosin, plasmin and trypsin, and 24 and 48 hours for *Lactobacillus*-proteases, TFA to 1.1% was added to the mixture. The mixture was filtered (0.45 µm) and the filtrate stored at -20°C until analyzed.

Separation of peptides in protein hydrolysates

FPLC equipment (Pharmacia LKB, Sweden) was used in the peptide analyzes. The column was Pep RPC HR 5/5 (5 µm, 100 Å). The runs were conducted at room temperature at a flow rate of

1.0 ml/min. Solvents and gradient were prepared as described by PAHKALA et al. (1989a). Peak detection was at 206 nm and the injection volume was 100 µl. Fractions were collected manually from a second run according to the peptide profile obtained in the first run.

Identification of peptides in fractions

After collection, peptide fractions were evaporated on a Waters PICO TAG Work Station and hydrolyzed using 6 M HCl (1% phenol) in the gas phase for 24 hours at 110°C. Amino acids were analyzed as phenylthiocarbamate (PTC) derivatives. Derivatization and HPLC runs were performed according to instructions issued by Millipore Corporation (1987). The HPLC equipment consisted of the following parts: Waters Model 510 pumps, Waters automatic sample feeder (Wisp Model 710), Pharmacia LKB VWM 2141 spectrophotometer, and data processing equipment Nec APV IV (program Baseline 810). The column was PICO-TAG (3.9 mm x 15 cm) and its temperature was held at 40°C (Waters Column Heater/Temperature Control Module).

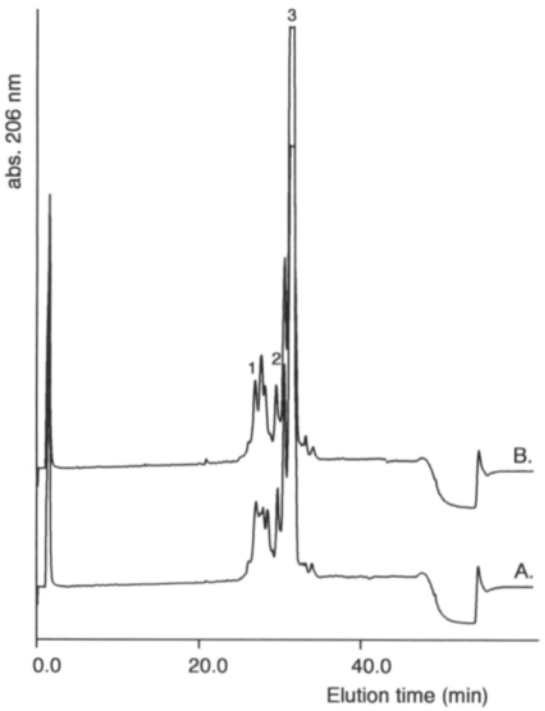

Fig. 1. Peptide profile of κ-casein after 4 (A) and 24 (B) hours of hydrolysis by chymosin (E:S = 1:100). Detection at 206 nm, injection volume 100 µl. Identified fragments: 1. 120-124 & 148-152; 2. 130-139; 3. 106-169.

Results

Hydrolysis by proteolytic enzymes

Figure 1 shows the chromatogram for the chymosin hydrolysis of κ-casein and the location of the peptides identified on the basis of their amino acid composition. It was shown that under these conditions, the bond most sensitive to the activity of chymosin was Phe 105-Met 106. The release of the casein macropeptide was observed after 2 hours of hydrolysis. After 24 hours, a few other bonds in the casein macropeptide were also cleaved. Peptides from the para-κ-casein part were not identified.

Figure 2 gives the results for the plasmin hydrolysis of κ-casein when the E:S ratio was 1:50 after 2, 4 and 24 hours of hydrolysis. Plasmin was found to act on bonds in N-terminal region of κ-casein. N-terminal fragments 1-16 and 1-24 were observed already after 2 hours of hydrolysis. Plasmin cleaved specifically at the carboxyl side of lysine. The Phe 105-Met 106 bond was found to hydrolyze slowly, since the release of the casein macropeptide was observed (Peak No. 10 fig. 2) only after 24 hours of hydrolysis.

Figure 3 shows the chromatograms of 2 and 4 hours of trypsin hydrolysates of κ-casein. The results indicate that under the conditions used, bonds were cleaved by trypsin all along the amino acid chain of κ-casein.

Hydrolysis by *Lactobacillus*-proteases

Figures 4 and 5 present the proteolytic effect of cell-free extracts of *L. helveticus* LH7 and *L. casei* subsp. rhamnosus M9 on κ-casein. The identified fragments released from κ-casein by the different

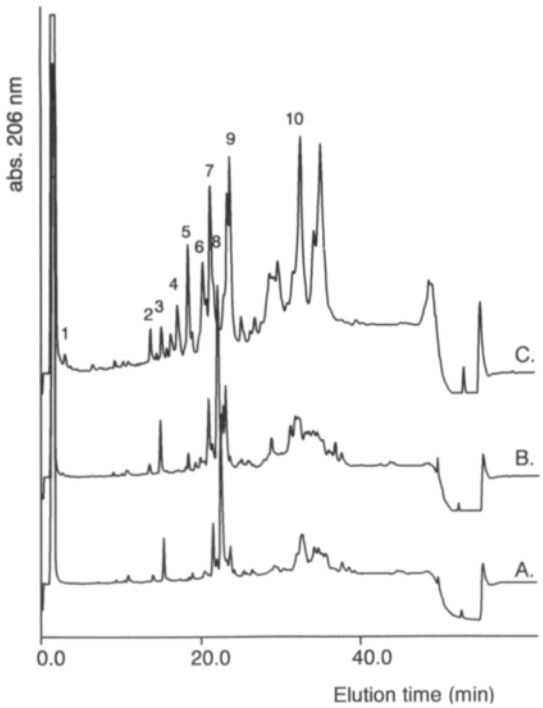

Fig. 2. Peptide profile of κ-casein after 2 (A), 4 (B) and 24 (C) hours of hydrolysis by plasmin (E:S = 1:50). Detection at 206 nm, injection volume 100 µl. Identified fragments: 1. 22-24; 2. 17-21; 3. 1-16; 4. 11-24; 5. 17-24; 6. 1-16; 7. 1-21; 8. 1-24; 9. 1-24; 10. 106-169.

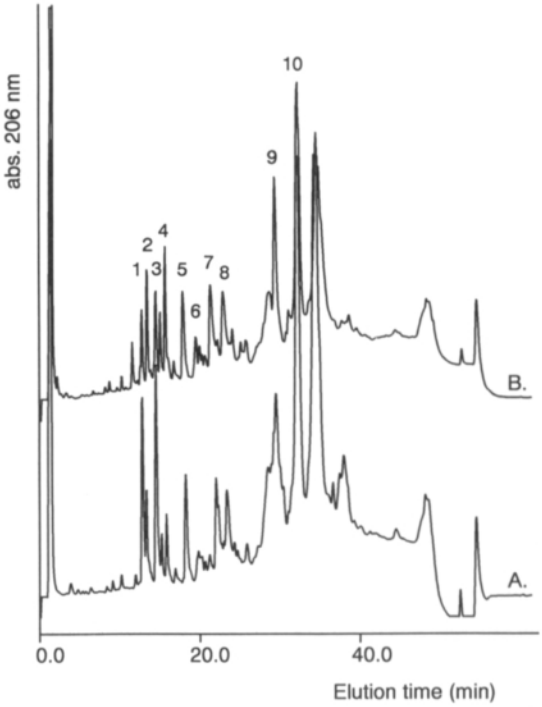

Fig. 3. Peptide profile of κ-casein after 2 (A) and 4 (B) hours of hydrolysis by trypsin (E:S = 1:10000). Detection at 206 nm, injection volume 100 µl. Identified fragments: 1. 1-16; 2. 1-10; 3. 1-21; 4. 87-99; 5. 17-24; 6. 98-105; 7. 98-112; 8. 98-111; 9. 69-86; 10. 106-169.

Lactobacillus-proteases are shown in Table 1. *L. helveticus* strains released fragments from the mid-section and the C-terminal region of κ-casein. N-terminal and C-terminal peptides were identified in hydrolysates by *L. casei* strains E8, P3, P8 and A1. In hydrolysates by *L. casei* strains A5, M9, G2 and S9 only C-terminal peptides were identified. No peptides could be identified in hydrolysates by *L.casei* M1. Free amino acids were released by cell-free extracts of all *L. casei* and *L. helveticus* strains. The strongest proteolysis, in terms of number of released peptides, was observed in *L. casei* strains G2, S9 and A5.

Discussion

The rapid and specific enzymatic cleavage of the sensitive Phe 105-Met 106 peptide bond of κ-casein by chymosin has been the subject of many investigations (LAWRENCE and CREAMER 1969, VISSER et al. 1976, 1977, 1987). Also in this study, cleavage of the 105-106 bond was observed after 2 hours of incubation. After 24 hours of hydrolysis, other bonds in the casein macropeptide were also split by chymosin. These results are consistent with those of SHAMMET et al. (1992), who observed an increase in the number of peaks in the chromatogram after 30 minutes of hydrolysis by chymosin. This indicated that the enzyme started to cleave other peptide bonds in the substrate.

EIGEL (1977) reported that κ-casein is resistant to proteolysis by plasmin: even after 60 minutes of incubation with plasmin no changes could be detected in the electrophoretic pattern of κ-casein. The present study showed κ-casein is hydrolyzed slowly by plasmin, when the enzyme - substrate

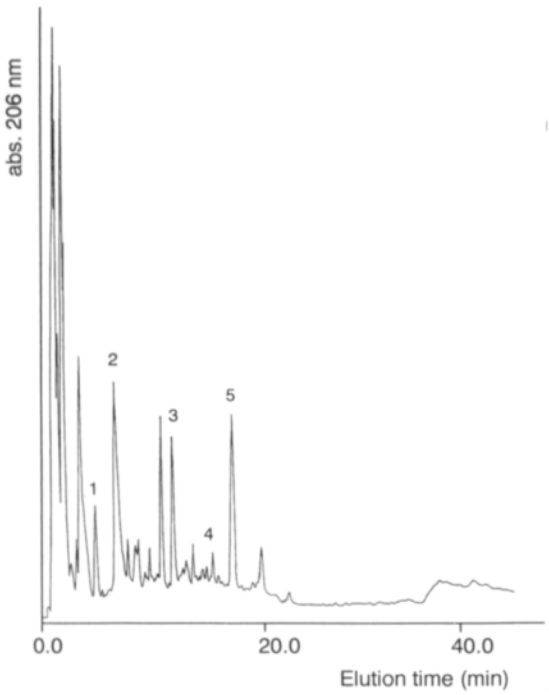

Fig. 4. Peptide profile of κ-casein after 48 hours of hydrolysis by cell-free extracts of *L. helveticus* LH7. Detection at 206 nm, injection volume 100 µl. Identified fragments: 1. Tyr (free amino acid); 2. Phe (free amino acid); 3. 86-96; 4. 132-152; 5. 132-141.

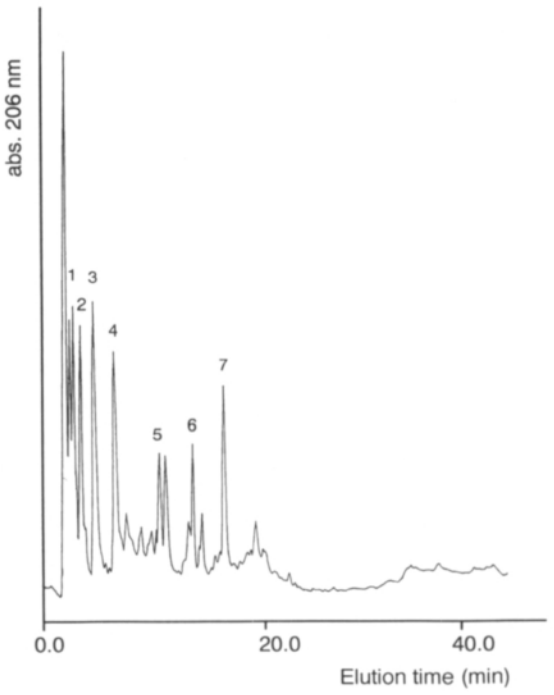

Fig. 5. Peptide profile of κ-casein after 48 hours of hydrolysis by cell-free extracts of *L. casei* subsp. *rhamnosus* M9. Detection at 206 nm, injection volume 100 µl. Identified fragments: 1. Met (free amino acid); 2. Ile (free amino acid); 3. Tyr (free amino acid); 4. Phe (free amino acid); 5. 145-161; 6. 147-163; 7. 128-148.

ratio was raised from 1:200 to 1:50. Under the conditions of this study the N-terminal Lys-X bonds were found to be sensitive to the proteolytic activity of plasmin. The resistance of κ-casein to proteolysis by plasmin is fortunate considering the importance of κ-casein in stabilizing other major caseins against precipitation by calcium (ZITTLE et al. 1959, ZITTLE and WALTER 1963).

Trypsin mainly hydrolyzed the carboxyl side of arginine and lysine residues. This supports previous findings concerning the specificity of trypsin (ADLER-NISSEN 1986). On the basis of the hydrolysis products that were identified, it could be seen that 9 of the 13 lysyl and argynyl containing bonds of κ-casein were split. Five of the cleaved bonds were Lys-X and four were Arg-X bonds. In this study, trypsin mainly hydrolyzed bonds in the N-terminal and mid-section. It is presumed that sequence 25-68 contains three trypsin-sensitive bonds; however, no peptides were detected from this region. Sequence determinations have shown that sequence 28-79 is resistant to enzymatic degradation (JOLLES et al. 1970). In this sequence there are three stretches of extended structure and an α-helix, which may form a hydrophobic core and render the bonds resistant to enzymatic degradation (RAAP et al. 1983). LEONIL and MOLLE (1990) have investigated the effect of trypsin on the casein macropeptide. They observed three trypsin-sensitive bonds, namely 111-112, 112-113 and 116-117; bonds 111-112 and 112-113 were split much faster than 116-117. In this study, the bond Lys 116-Thr 117 was not split, while the other two trypsin-sensitive bonds (111-112, 112-113) were hydrolyzed under the conditions used.

The bond 105-106 seems to be sensitive to the

Table 1. Peptide sequences identified in hydrolysates of κ-casein produced by cell-free extracts of *Lactobacillus*-strains.

Strain	N-terminal peptides	Mid-section peptides	C-terminal peptides
L. helveticus LH1		87-94	126-140
L. helveticus LH5		87-95	154-168
L. helveticus LH7		86-96	132-141, 132-152
L. casei E8	1-8		126-151, 147-161
L. casei P3	1-8		147-161
L. casei P8	1-8		147-152
L. casei A1	1-8		112-138, 147-161
L. casei G2			112-118, 114-118
L. casei S9			114-120
L. casei A5			114-120
L. casei (rhamnosus) M9			128-148, 145-161, 147-163

action of proteinases other than chymosin, since the casein macropeptide was released also by plasmin and trypsin.

κ-Casein was observed to be more susceptible to the proteolytic action of the different *Lactobacillus*-cultures used in this study than the other caseins studied earlier (PAHKALA et al. 1989a, 1989b, PIHLANTO-LEPPÄLÄ et al. 1993). The differences in activity observed earlier between different *Lactobacillus*-strains (PAHKALA et al. 1986) were not apparent in this study. Instead, the *Lactobacillus*-strains could be divided into three categories according to the identified fragments; those that hydrolyzed bonds in the C-terminal region and mid-section, only in the C-terminal region, or in the N- and C-terminal regions of κ-casein. No clear substrate specificity was observed among the peptides identified. The results of this study would indicate that para-κ-casein is hydrolyzed during cheese ripening. HARPER et al. (1971) have obtained similar results which showed extensive degradation of para-κ-casein during the ripening of Cheddar cheese slurries. However, other previous studies (GREEN and FOSTER 1974, LEDFORD et al. 1966) have reported no hydrolysis of para-κ-casein during cheese ripening.

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SELOSTUS

κ-kaseiinin hydrolysoituminen kymosiinilla, plasmiinilla, trypsiinillä ja Lactobacillus-proteinaaseilla

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Maatalouden tutkimuskeskus

Tässä tutkimuksessa selvitettiin κ-kaseiinin entsymaattista hydrolyysiä eristämällä ja identifioimalla vapautuvia peptidejä. Entsyymeinä käytettiin kymosiinia, plasmiinia, trypsiiniä sekä kolmesta *Lactobacillus helveticus*- ja yhdeksästä *Lactobacillus casei* -kannasta eristettyä preparaattia.

κ-kaseiinin Phe 105-Met 106 sidos oli herkkä kymosiinin proteolyyttiselle aktiivisuudelle. Havaittiin, että 24 tunnin hydrolyysin jälkeen myös muutamia muita kaseiinimakropeptidin sidoksia oli hydrolysoitunut. Plasmiinin proteolyyttinen aktiivisuus hydrolysoida käytettyä substraattia oli heikko, sillä muutamia N-terminaalisia peptidejä vapautui kun entsyymi-proteiini suhde nostettiin 1:200:sta 1:50:neen. Trypsiini hydrolysoi useita κ-kaseiinin sidoksia, ja peptidejä vapautui lähes koko aminohappoketjun matkalta.

Lactobacillus-proteinaasien vaikutus oli vähäisempi kuin käytetyillä proteolyyttisillä entsyymeillä. κ-kaseiinin hydrolysoituminen laktobasilleilla oli voimakkaampaa kuin muilla aikaisemmin tutkituilla kaseiini-komponenteilla. Tunnistettujen peptidien perusteella *Lactobacillus*-kannat voitiin jakaa kolmeen luokkaan: *L. helveticus* kannat hydrolysoivat sidoksia aminohappoketjun keskialueelta ja C-terminaalista, *L. casei* E8, P3, P8 ja A1 kannat hydrolysoivat sidoksia N- ja C-terminaalista ja *L. casei* A5 ja M9 hydrolysoivat sidoksia C-terminaalista.