

Hydrolysis of α_{s2} -casein in solution by chymosin, plasmin, trypsin and *Lactobacillus*-proteinases

ANNE PIHLANTO-LEPPÄLÄ, EERO PAHKALA, MINNA KAHALA and
VEJO ANTILA

PIHLANTO-LEPPÄLÄ, A., PAHKALA, E., KAHALA, M. & ANTILA, V. 1993. **Hydrolysis of α_{s2} -casein in solution by chymosin, plasmin, trypsin and *Lactobacillus*-proteinases.** Agric. Sci. Finl. 2: 133-139. (Agric. Res. Centre of Finland, Food Res. Inst., FIN-31600 Jokioinen, Finland.)

The aim of this study was to examine the enzymic hydrolysis of α_{s2} -casein by isolating and identifying the released peptides. The enzymes applied in the study were chymosin, plasmin and trypsin as well as cell free extracts from three strains of *Lactobacillus helveticus* and nine strains of *L. casei*.

The findings showed that chymosin had weak proteolytic activity on α_{s2} -casein. Plasmin, on the other hand, released numerous peptides under the used conditions. The majority of the identified fragments were released from the C terminal end of the substrate. Plasmin hydrolysed mainly Lys-X bonds. The third enzyme, trypsin, hydrolysed several bonds of α_{s2} -casein. Peptides were released from almost all regions of the protein. Trypsin acted on the carboxyl sides of arginyl and lysyl residues. Cell free extract of lactobacilli had little activity on α_{s2} -casein.

Key words: α_{s2} -casein, enzymatic hydrolysis, peptides

Introduction

α_{s2} -Casein is the most recently discovered casein component of milk, and that is perhaps why it is also the least well known. It constitutes around 10% of the total casein fraction of milk (DAVIES and LAW 1977). α_{s2} -Casein occurs in different forms depending on the extent of phosphorylation. Its primary structure consists of 207 amino acids in a linear chain, with 10-13 phosphoserine residues and two cysteinyl residues. α_{s2} -Casein contains few prolyl residues but numerous lysyl residues. The C terminal of the molecule is hydrophobic and its N terminal is hydrophilic (BRIGNON et al. 1977).

The α_{s2} -casein in milk is hydrolysed rapidly by plasmin and the peptides released have been identi-

fied (LE BARS and GRIBON 1989, VISSER et al. 1989). However, the effect of other proteolytic enzymes on the hydrolysis of α_{s2} -casein has not been studied.

The aim of this research was to examine the hydrolyses of α_{s2} -casein by proteolytic enzymes related to cheese ripening, by isolating and identifying the released peptides.

Material and methods

Substrate and enzymes

The α_{s2} -casein was isolated using the method of VREEMAN and VAN RIEL (1990). 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 autolysed for 48 hours at 42°C. The autolysis suspension was cooled to -20°C, thawed and homogenized in an Ultra-Turrax for 10 min in cold water. The suspension was then centrifuged (20,000 x g, 4°C, 15 min), cell debris were washed with distilled water and finally suspended in 10 ml of distilled water. This suspension was used for the hydrolysis.

Hydrolysis

The enzymes (0.015 - 3% in water) were added into 1.5% (w/v) α_{s2} -casein solution (0.05 M phosphate buffer, pH 6.0). The ratios of enzyme to substrate were:

enzyme	E : S
chymosin	1 : 50
plasmin	1 : 200
trypsin	1 : 10000

Lactobacilli cell extract (200 μ l) was added to 2 ml of 1.5% protein solution. The mixtures were incubated at 40°C. After the reaction period, TFA to 1.1% was added to the mixture. The mixture was filtered (0.45 μ m) and the filtrate was stored at -20°C until analysed.

Separation of peptides in protein hydrolysates

FPLC equipment (Pharmacia LKB, Sweden) was

used in peptide analyses. The column was Pep RPC HR 5/5 (5 μ m, 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 μ l. According to the peptide profile obtained in the first run, the fractions were collected manually from a second run.

Identification of peptides in fractions

Amino acid analyses using HPLC

After collection, peptide fractions were evaporated on a Waters PICO TAG Work Station and hydrolysed using 6 M HCl (1% phenol) in the gas phase for 24 h at 110°C. Amino acids were analysed as phenylthiocarbamate (PTC) derivatives. Derivatization and HPLC runs were performed according to instructions issued by Millipore Corporation (1987). The HPLC equipment consisted following components: 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).

Sequence analysis

N terminal amino acid sequence analysis from some of the peptides was performed using a manual method (TARR 1986).

Results

Hydrolysis by proteolytic enzymes

The peptide profiles obtained from chymosin hydrolysates of α_{s2} -casein are shown in Figure 1. The results show that the proteolytic activity of chy-

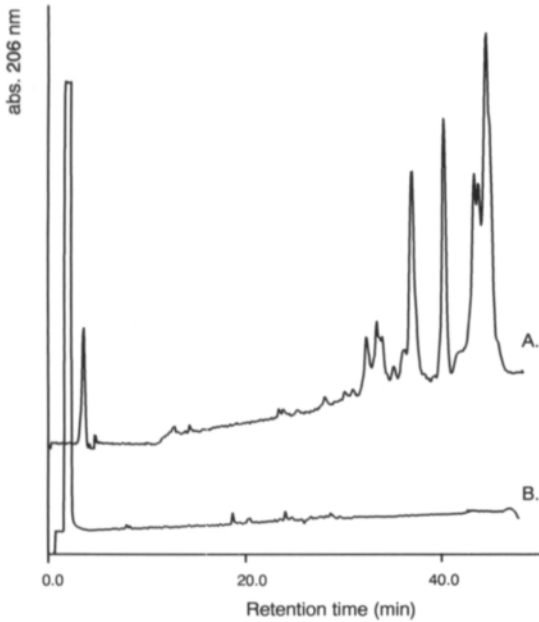


Fig. 1. Peptide profile of a 24 h hydrolysate of α_{s2} -casein by chymosin (E:S = 1:50), without TFA (A) and with TFA (B). Detection at 206 nm, injection volume of 100 μ l.

mosin on α_{s2} -casein was weak. At the enzyme/substrate ratio 1:50, no substantial amounts of peptides were released even after incubation for 24 hours (Figure 1).

In order to study the possibility that the peptides produced might precipitate on addition of TFA, the reaction was stopped by rapidly freezing the samples. Hydrophobic peptides were observed in the chromatogram, but it was not possible to identify them by the applied methods. These peptides were detectable after hydrolysis for 2 hours.

The hydrolysis of α_{s2} -casein by plasmin under the given conditions released numerous peptides (Figure 2). The majority of the peptides were released from the C terminal end of the protein. Only one peptide from the N terminal end (α_{s2} -CN 1-24) was identified. The peptides released after 2 and 4 hour were approximately the same, and their concentration increased with continued incubation.

Figure 3 shows that several bonds in α_{s2} -casein were hydrolysed by trypsin. Peptides were released almost all along the length of the amino acid chain. Altogether, 17 cleaved sites were identified. The N

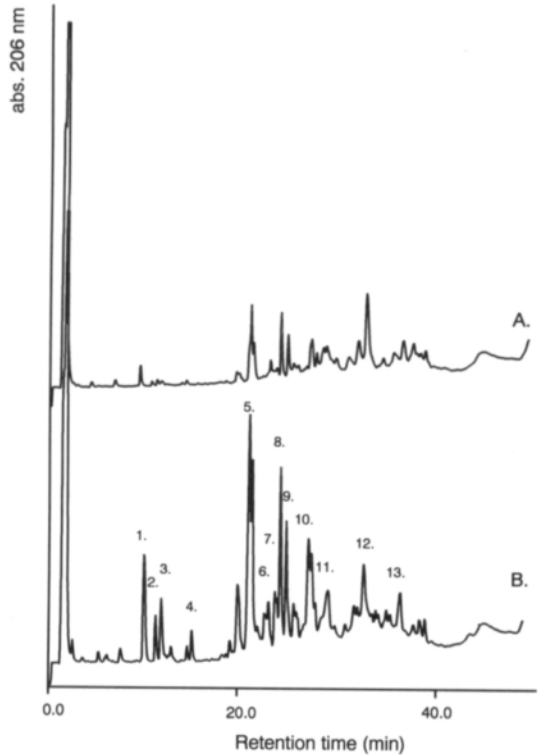


Fig. 2. Peptide profile of α_{s2} -casein after hydrolysis by plasmin (E:S = 1:200) for 4 (A) and 24 (B) hours. Detection at 206 nm, injection volume 100 μ l. Identified fragments: 1. 182-188; 2. 167-173; 3. 166-173; 4. 71-80; 5. 1-24; 6. 150-165; 7. 151-165; 8. 115-150; 9. 115149; 10. 174-181; 11. 198-207; 12. 153-170 + 182-197; 13. 153-207.

terminal amino acid sequence was determined from the fragments No 5, 9, 10, 13 and 14.

Hydrolysis by lactobacilli-proteases

The proteolytic effect of lactobacilli, namely *L. helveticus* LH5, *L. casei* E8 and *L. casei* subsp. *rhamnosus* M1, on α_{s2} -casein is presented in Figure 4. It can be seen that these lactobacilli had limited ability to hydrolyse this protein. The hydrolysis of α_{s2} -casein effected by the other *Lactobacillus*-strains was closely similar to that shown in Figure 4.

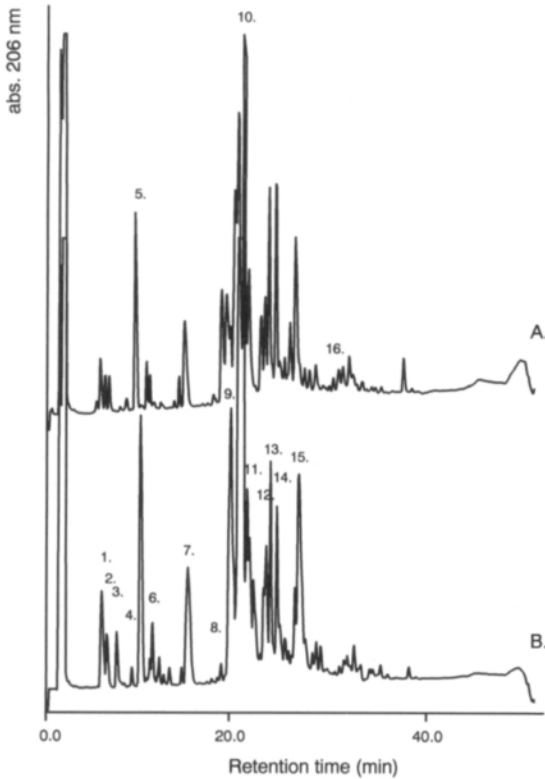


Fig. 3. Peptide profile of α_{s2} -casein after hydrolysis by trypsin (E:S = 1:10000) for 2 (A) and 4 (B) hours. Detection at 206 nm, injection volume 100 μ l. Identified fragments: 1. 171-173; 2. 167-170; 3. 166-170; 4. 42-45; 5. 182-188; 6. 167-173; 7. 205-207; 8. 192-197; 9. 198205; 10. 1-24; 11. 138-149; 12. 153-165; 13. 115-150; 14. 115-149; 15. 171-181; 16. 174-181.

Discussion

Chymosin was found to have weak proteolytic activity on α_{s2} -casein. Under the conditions used and with a relatively high proportion of enzyme (E:S = 1:50), peptides were precipitated when TFA was added. It may be presumed that the peptides released were long and hydrophobic. Unhydrolysed casein and high molecular weight peptides are known to precipitate at pH 4.6. Precipitation of peptides by trichloroacetic acid (TCA) is influenced by hydrophobicity, in particular, and also to a certain extent by length (YVON et al. 1989). The addition of TFA (1.1%) reduces the pH below 4.6,

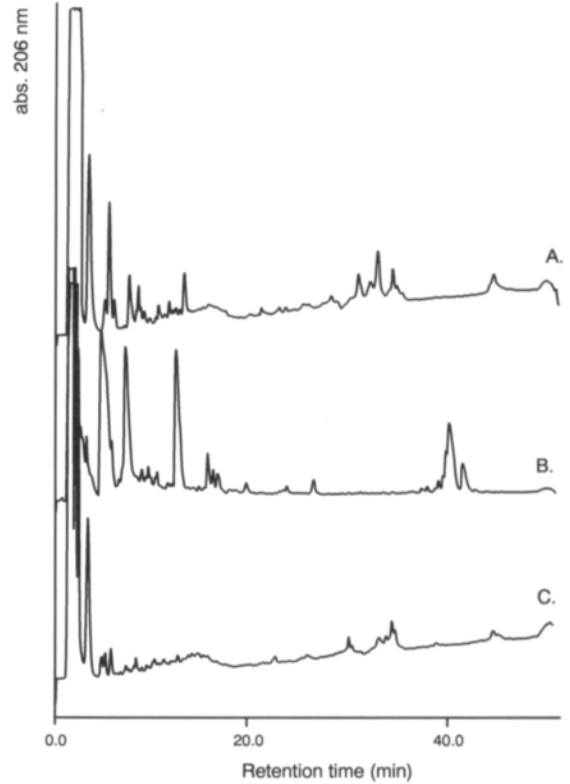


Fig. 4. Peptide profile of 48 h hydrolysates of α_{s2} -casein by *L. helveticus* LH5 (A), *L. casei* E8 (B) and *L. casei* subsp. *rhamnosus* M1 (C) strains. Detection at 206 nm, injection volume 100 μ l.

causing the large peptides to precipitate. The hydrophobicity of peptides may also affect their solubility on addition of TFA.

In previous studies, the proteolytic activity of chymosin on β -casein was found to be weak. In the case of β -casein, peptides are released primarily from its hydrophobic C terminal region (VISSER and SLAGEN 1977, PAHKALA et al. 1989b).

Plasmin readily hydrolyses α_{s2} - and β -caseins (SNOEREN and VAN RIEL 1979). The sensitivity of α_{s2} -casein to plasmin is most likely due to the relatively high lysine concentration in its sequence, which meets the primary specificity requirements of the enzyme. At least 14 peptide bonds were

hydrolysed, 12 of which were Lys-X bonds and 2 were Arg-X. This result supports previous data concerning the specificity of plasmin, since plasmin has been found to favor Lys-X but it is also capable of hydrolysing Arg-X bonds slowly (WEINSTEIN and DOOLITTLE 1972). The cleaved sites identified in this study are consistent with the findings of previous studies (LE BARS and GRIPON 1989, VISSER et al. 1989).

Only one peptide (α_{s2} -CN 1-24) from the N terminal was identified in the plasmin hydrolysates of α_{s2} -casein. This peptide was contaminated with peptide which could not be identified by the methods used. Previous studies (LE BARS and GRIPON 1989, VISSER et al. 1989, PAHKALA et al. 1989a) showed that fragments 1-24 and 1-21 occur one after another and thus it is possible that the unidentified fragment was 1-21.

VISSER et al. (1989) noted the reduction in the concentration of fragments 182-207, 189-207, 115-207/150-207 and 151-207 during incubation for 1 to 4 hours. These peptides were not observed after hydrolysis periods of 4 and 24 hours, and it is likely that they had been hydrolysed to shorter fragments. New sites sensitive to plasmin were found within the sequence 150-207 (Lys 153-Leu 154, Lys 155-Lys 156, Lys 156-Ile 157, Arg 170-Tyr 171, Lys 173-Phe 174). Previous research failed to show the formation of peptides from the segment 25-114, and it was presumed that these peptides were together with some intact α_{s2} -casein (VISSER et al. 1989). In the present study, however, fragment 71-80, was found (Figure 2, peak No. 4). The fragment was clearly detectable only after hydrolysis for 24 hours which indicates that the release of peptides from the central part of the protein requires a relatively long hydrolysis time.

Trypsin hydrolysed mainly bonds on the carboxyl side of arginine and lysine residues. This is consistent with the previous findings concerning the specificity of trypsin (ADLER-NISSEN 1986). On

the basis of the 16 hydrolysis products that were characterized from the trypsin hydrolysates, it was established that 19 of the 30 trypsin-sensitive bonds of α_{s2} -casein were hydrolysed. Under the conditions of this study, the activity of trypsin hydrolysed at the N and C terminals. Peptides in the sequence 25-41, which contains the cysteine residues of the protein, were not identified.

Previous findings have shown differences in the proteolytic activity of lactobacilli (PAHKALA et al. 1986), but none was observed in this research. Other studies have reported weak proteolytic activity of lactobacilli on α_{s1} - and β -caseins. *L. helveticus* strains were shown to be more proteolytic on α_{s1} - and β -caseins than *L. casei* strains (PAHKALA et al. 1989a, b). However, the findings of the present study indicated no differences in activity between the lactobacilli, and not even the *L. helveticus* strains differed from other *Lactobacillus*-strains in terms of stronger and more extensive proteolysis.

The amino acid sequence of fragment 174-181, isolated from the plasmin and trypsin hydrolysates, corresponds to that of the bitter peptide isolated by MATOBA et al. (1970) from the trypsin hydrolysates of total casein. This means that bitter peptides can also be formed from α_{s2} -casein. The identified N terminal peptide fragment (α_{s2} -CN 1-24), which contains a cluster of 4 phosphoserine residues, could be of special interest for studies of its functional properties. Although the concentrations of substrate and enzymes used in this study differed from the circumstances in milk, it may be assumed that at least some of the detected hydrolysis products are constituents of the very heterogeneous proteoseptone fraction of milk (ANDREWS 1983).

Acknowledgements. The writers wish to express their thanks to the Ministry of Agriculture and Forestry, which has supported the study through its biotechnical research fund.

References

- ADLER-NISSEN, J. 1986. Some fundamental aspects of food proteins hydrolysis. In: *Enzymatic Hydrolysis of Food Proteins*. I. Elsevier Applied Science Publishers, London, p. 20-21.
- ANDREWS, A. T. 1983. Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* 50: 45-55.
- BRIGNON, G., RIBADEAU DUMAS, B., MERCIER, J.-C., PELISSIER, J.-P. & DAS, B.C. 1977. Complete amino acid sequence of bovine α_{s2} -casein. *FEBS Lett.* 76: 274-279.
- DAVIES, D. T. & LAW, A. J. R. 1977. An improved method for the quantitative fractionation of casein mixtures using ion-exchange chromatography. *J. Dairy Res.* 44: 213-221.
- LE BARS, D. & GRIBON, J.-C. 1989. Specificity of plasmin towards bovine α_{s2} -casein. *J. Dairy Res.* 56: 817-821.
- MATOKA, T., HAYASHI, R. & HATA, T. 1970. Isolation of bitter peptides from tryptic hydrolysate of casein and their chemical structure. *Agr. Biol. Chem.* 34: 1235-1243.
- Millipore Corporation 1987. Liquid chromatographic analysis of amino acids in feeds and foods using a modification of the PICO-TAG method. Revision from the PICO-TAG amino acid analysis system, Waters 1984.
- PAHKALA, E. & ANTILA, V. 1987. Proteolysis in cheese. *Meijeritiet. Aikak.* 45, 1: 33-42.
- , ANTILA, V. & PITKÄRANTA, T. 1986. Accelerating the ripening of cheese by the addition of proteolytic enzymes. III. The proteolytic characteristics of lactobacilli. *Meijeritiet. Aikak.* 44, 2: 74-85.
- , PIHLANTO-LEPPÄLÄ, A., LAUKKANEN, M. & ANTILA, V. 1989a. Decomposition of milk proteins during the ripening of cheese. I. Enzymatic hydrolysis of α_s -casein. *Meijeritiet. Aikak.* 47, 1: 39-7.
- , PIHLANTO-LEPPÄLÄ, A., LAUKKANEN, M. & ANTILA, V. 1989b. Decomposition of milk proteins during the ripening of cheese. II. Enzymatic hydrolysis of β -casein. *Meijeritiet. Aikak.* 47, 1: 6370.
- SNOEREN, T. H. M. & VAN RIEL, J. A. M. 1979. Milk proteinase, its isolation and action on α_{s2} - and β -casein. *Milchwissenschaft* 34: 528-1.
- TARR, G. E. 1986. Manual Edman sequencing system. In: Shively, J. E. (ed.). *Methods of Protein Microcharacterization, A Practical Handbook*. Humana Press, Clifton, New Jersey, p. 155-193.
- WEINSTEIN, M. J. & DOOLITTLE, R. F. 1972. Different specificities of thrombin, plasmin, and trypsin with regard to synthetic and natural substrates and inhibitors. *Biochim. Biophys. Acta* 258: 577-590.
- VISSER, S. & SLANGEN, K. J. 1977. On the specificity of chymosin (rennin) in its action on bovine β -casein. *Neth. Milk Dairy J.* 31: 1630.
- , SLANGEN, K. J., ALTING, A. C. & VREEMAN, H. J. 1989. Specificity of bovine plasmin in its action on bovine α_{s2} -casein. *Milchwissenschaft* 44: 335-9.
- VREEMAN, H. J. & VAN RIEL, A. M. 1990. The largescale isolation of α_{s2} -casein from bovine casein. *Neth. Milk Dairy J.* 44: 43-8.
- YVON, M., CHABANET, C. & PELISSIER, J.-P. 1989. Solubility of peptides in trichloroacetic acid (TCA) solutions. *Int. J. Peptide Protein Res.* 34: 166-176.

Manuscript received August 1992

Anne Pihlanto-Leppälä
Eero Pakkala
Minna Kahala
Veijo Antila
Agricultural Research Centre of Finland
Food Research Institute
FIN-31600 Jokioinen, Finland

SELOSTUS

**α_2 -kaseiinin pilkkoutuminen kymosiinilla, plasmiinilla, trypsiinillä ja
Lactobacillus-proteinaaseilla**

ANNE PIHLANTO-LEPPÄLÄ, EERO PAHKALA, MINNA KAHALA ja
VEIJO ANTILA

Maatalouden tutkimuskeskus

Tässä tutkimuksessa selvitettiin α_2 -kaseiinin entsyymaattista hydrolyysiä eristämällä ja identifioimalla vapautuvia peptidejä. Entsyymeinä käytettiin kymosiinia, plasmia, trypsiiniä sekä kolmesta *Lactobacillus helveticus*- ja yhdeksästä *Lactobacillus casei*-kannasta eristettyä preparaattia.

Kymosiinilla oli heikko proteolyttinen aktiivisuus hydrolysoida käytettyä substraattia. Plasmia vapautti käytetyissä

olosuhteissa lukuisia peptidejä. Suurin osa identifioituista fragmenteista vapautui C-terminaalista. Trypsiini hydrolysoi useita α_2 -kaseiinin sidoksia. Peptidejä vapautui lähes koko aminohappoketjun matkalta. Trypsiinin vaikutus kohdistui arginiiniin ja lysiniin karboksyylipeihin. Laktobasillien proteolyttinen vaikutus oli vähäistä.