

Peptides in fermented Finnish milk products

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This study was conducted to investigate the rate of proteolysis and peptide profiles of different Finnish fermented milk products. The highest rate of proteolysis was observed in Biokefir, while the greatest change in the rate of proteolysis was observed in Gefilus®. Differences in starters and manufacturing processes reflected on the peptide profiles of the products. Most of the identified peptides originated from either the N- or C-terminal region of β -casein or from the N-terminal region of α_{s1} -casein.

Key words: fermented milk products, proteolysis, peptides

Introduction

The enzymatic degradation of milk proteins yields amino acids and peptides of varying size. The quality and quantity of the products of degradation depend on the components of the milk protein fraction and the enzymes involved, i.e. bacterial proteases and plasmin. The temperature and pH of the processes and products also have their effect on the liberation of amino acids and peptides.

The starters of fermented milk products contain proteolytic enzymes, which cause proteolysis during manufacture and storage. The products formed by proteolysis have a great effect on the final product. They can contribute to its texture, nutritional properties and flavour of the product. Peptides and amino acids are not solely responsible for the flavour, but they can act as precursors of the flavour-producing enzymatic reactions (TAMINE and ROBINSON 1989). Certain peptides contribute to a bitter taste in the product. The formation of bitter peptides in the manufacture of yoghurts is favoured by a temperature below 38°C as well as by the

activity of the enzymes during cold storage (ROBINSON and TAMINE 1981).

Only a few reports have been published on the peptide content of fermented milk products. TANEV and ZIVKOVA (1977) investigated the peptide content of milk and Bulgarian yoghurt during storage using an electrophoretic method. Free amino acids have been the subject of several studies (MILLER et al. 1964, ALM 1982). Also, the degradation of casein components with different lactic acid bacteria has been studied by PAHKALA et al. (1989a, 1989b)

The aim of this study was to investigate the peptide profiles of various fermented milk products with high performance liquid chromatography (HPLC) methods.

Material and methods

The investigated fermented milk products were Gefilus®, plain yoghurt, Bulgarian yoghurt, viili, soured skimmed milk, acidophilus milk and Biokefir. The samples were obtained from two dairies in

Table 1. Micro-organisms used in the fermented milk products.

| Product | Starter type | Micro-organisms |
|------------------------|----------------------------|--|
| 1. Bulgarian yoghurt | thermophilic | <i>Streptococcus salivarius</i> ssp. <i>thermophilus</i> , <i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> |
| 2. Plain yoghurt | thermophilic | <i>Streptococcus salivarius</i> ssp. <i>thermophilus</i> , <i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> |
| 3. Gefilus | mixed mesophilic + special | <i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>L. lactis</i> ssp. <i>cremoris</i> , <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> , <i>Lactobacillus</i> GG |
| 4. Viili | mixed mesophilic + special | <i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>L. lactis</i> ssp. <i>cremoris</i> , <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> , <i>Geotrichum candidum</i> |
| 5. Biokefir | mixed mesophilic + special | <i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>L. lactis</i> ssp. <i>cremoris</i> , <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> , Kefir grains |
| 6. Acidophilus milk | mixed mesophilic + special | <i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>L. lactis</i> ssp. <i>cremoris</i> , <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> , <i>Lactobacillus acidophilus</i> |
| 7. Soured skimmed milk | mixed mesophilic | <i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>L. lactis</i> ssp. <i>cremoris</i> , <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> |

southern Finland and stored at +4°C during the study period. The micro-organisms used in the manufacture of these fermented milk products are listed in Table 1.

Sample preparation

The proteins and larger peptides were precipitated with 6% trichloroacetic acid (TCA). The supernatant was centrifuged at +7°C for 15 minutes and 6000 G and filtered through a 0.45 µm filter.

The samples were precipitated on the second and eighth day after manufacture as well as on the

sell-by date. The proteolysis was monitored by determining the total nitrogen in the product and in the filtrate with the Kjeldahl method.

Separation of peptides with HPLC

The HPLC equipment consisted of two pumps (Waters 510), an automatic sample processor (Waters Wisp 710), a UV detector (Pharmacia LKB VWM 2141) and a personal computer (NEC APV IV) with an analysis programme (Waters Baseline 810). The column was SuperPac Pep-S (4.0 x 250 mm, 5 µm; Pharmacia LKB) equipped with precol-

umn Pep-S (4.0 x 10 mm, 5 µm; Pharmacia LKB). The column temperature was 30°C and flow rate 1.0 ml/min. The eluants were: A 0.05% trifluoroacetic acid (TFA) in water and B 0.05% TFA, 90% acetonitrile (CH₃CN) in water. The gradient grew linearly from 2% to 60% B in 45 minutes.

Before HPLC analysis the samples were filtered once again through a 0.45 µm filter. The injection volume was 100 µl and the peptides were detected at wavelengths of 214 and 280 nm. The peptides were collected manually after the detector.

Peptide identification and amino acid analysis

After collection the peptides were dried under vacuum. The total amino acid content and the N-terminal sequence of three amino acid residues were determined for each peptide. Free amino acids were analyzed in the TCA soluble fraction. The peptides were hydrolyzed for the total amino acid analysis in gas phase (6 N HCl + 1% phenol) at 110°C for 24 hours. The amino acids were analyzed as phenylthiocarbamate (PTC) derivatives using the Waters Pico Tag method. The preparation of the derivatives and the HPLC analysis were performed according to the instructions of Millipore Corporation (1987). The equipment was the same as in the peptide analysis, except that the column was Waters Pico Tag (3.9 x 150 mm) and the temperature 40°C.

The manual N-terminal sequence analysis was performed according to the method of TARR (1986). Phenylthiohydantoin (PTH) amino acids were analyzed with the above-mentioned HPLC equipment on a Waters Pico Tag column. The running conditions were according to LEMIEUX and AMIOT (1990).

Results and discussion

Table 2 shows the change in the rate of proteolysis of the different fermented milk products between the first day and the sell-by date. In each product the rate of proteolysis increased during the storage period. The greatest change after one week of storage was observed in Gefilus (+2.3%), while there was

Table 2. Rate of proteolysis (%) in fermented milk products during storage. (6% TCA soluble N/total N × 100).

| Product | age | | | |
|------------------------|-------|--------|--------------|--------|
| | 1 day | 7 days | sell-by date | (days) |
| 1. Bulgarian yoghurt | 6.3 | 7.8 | 8.5 | (15) |
| 2. Plain yoghurt | 6.8 | 7.7 | 8.1 | (14) |
| 3. Gefilus | 6.2 | 8.3 | 8.5 | (18) |
| 4. Viili | 8.2 | 9.4 | 9.6 | (13) |
| 5. Biokefir | 9.1 | 9.5 | 10.1 | (13) |
| 6. Acidophilus milk | 8.0 | - | 9.6 | (11) |
| 7. Soured skimmed milk | 7.8 | - | 8.1 | (10) |

only a slight change in soured skimmed milk after 10 days of storage. The highest rate of proteolysis was found in Biokefir both at the age of one day and on the sell-by date.

Figures 1-7 show the peptide profiles of the different fermented milk products at the age of one day and on the sell-by date. The suggested sequence of the identified peptides is also indicated. Figure 8 shows the peptide profile of a pasteurized milk sample on the day of manufacture and after four days' cold storage.

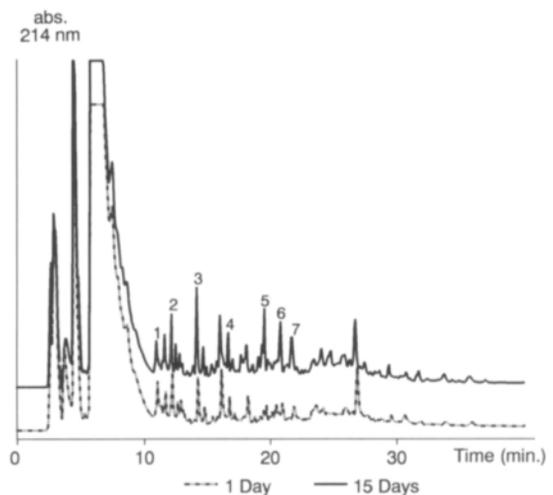


Fig. 1. Peptide profile of Bulgarian yoghurt at the age of 1 and 15 days. Identified fractions: 1. Leu, 2. Tyr, 3. Phe, 4. α_1 -CN 1-14, 5. β -CN 47-57, 6. β -CN 166-175, 7. β -CN 176-188.

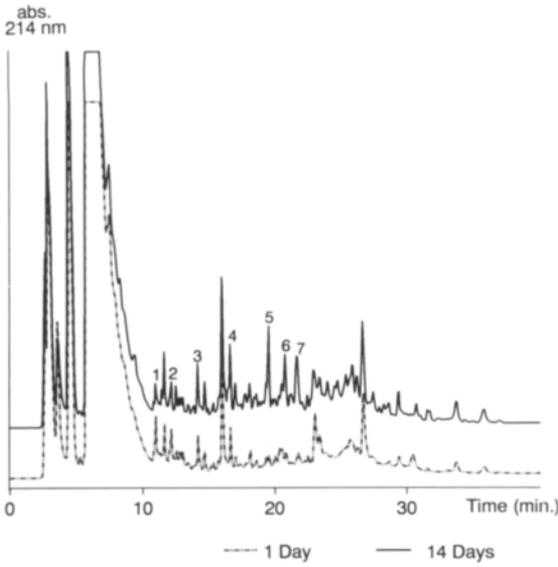


Fig. 2. Peptide profile of plain yoghurt at the age of 1 and 14 days. Identified fractions: 1. Leu, 2. Tyr, 3. Phe, 4. α_{s1} -CN 1-14, 5. β -CN 47-57, 6. β -CN 166-175, 7. β -CN 176-188.

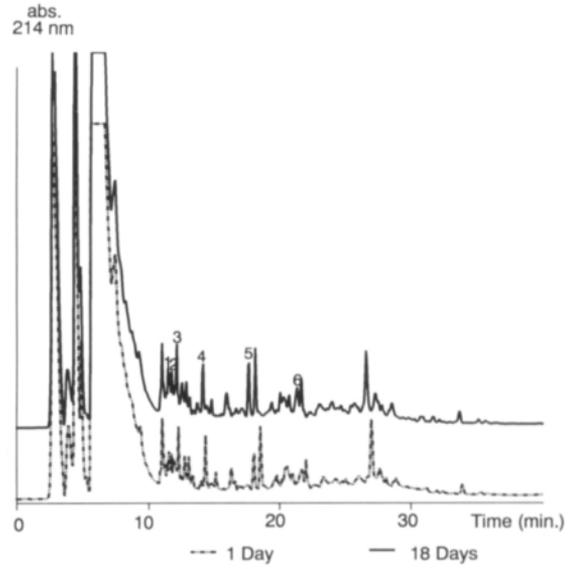


Fig. 3. Peptide profile of Gefilus at the age of 1 and 18 days. Identified fractions: 1. Ile, 2. Leu, 3. Tyr, 4. Phe, 5. β -CN 176-182, 6. β -CN 7-15.

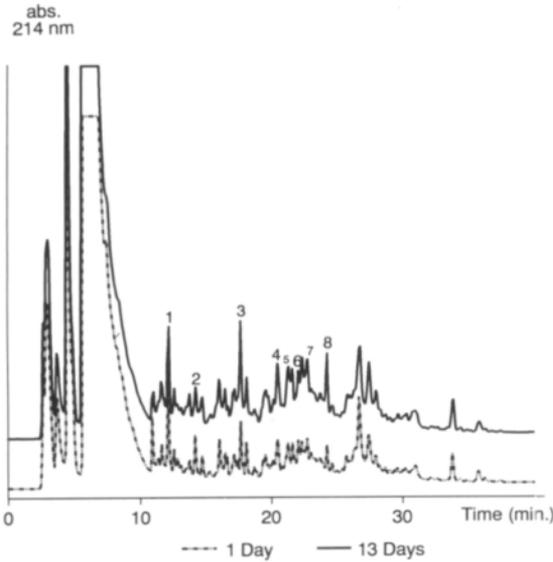


Fig. 4. Peptide profile of viili at the age of 1 and 13 days. Identified fractions: 1. Tyr, 2. Phe, 3. β -CN 176-182, 4. β -CN 169-175, 5. β -CN 7-15, 6. β -CN 1-9, 7. β -CN 44-52.

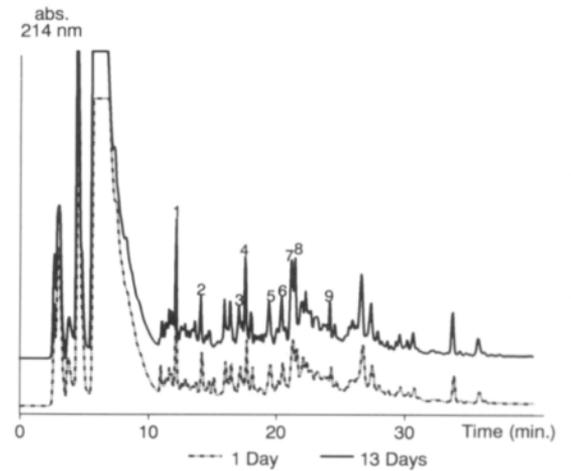


Fig. 5. Peptide profile of biokefir at the age of 1 and 13 days. Identified fractions: 1. Tyr, 2. Phe, 3. β -CN 22-28, 4. β -CN 176-182, 5. β -CN 47-57, 6. β -CN 169-175, 7. β -CN 7-15, 8. β -CN 1-9, 9. β -CN 44-52.

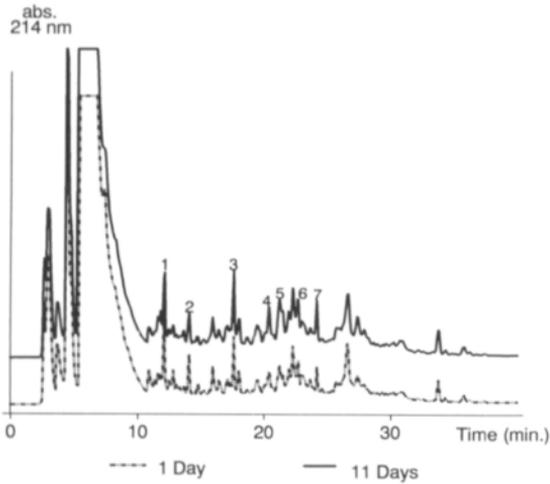


Fig. 6. Peptide profile of acidophilus milk at the age of 1 and 11 days. Identified fractions: 1. Tyr, 2. Phe, 3. β -CN 176-182, 4. β -CN 169-175, 5. β -CN 7-16, 6. β -CN 164-175, 7. β -CN 44-52.

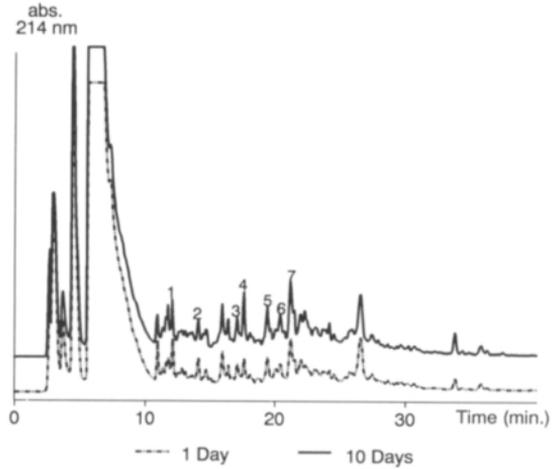


Fig. 7. Peptide profile of soured skimmed milk at the age of 1 and 10 days. Identified fractions: 1. Tyr, 2. Phe, 3. β -CN 22-28, 4. β -CN 176-182, 5. κ -CN 161-169, 6. β -CN 169-175, 7. β -CN 7-15.

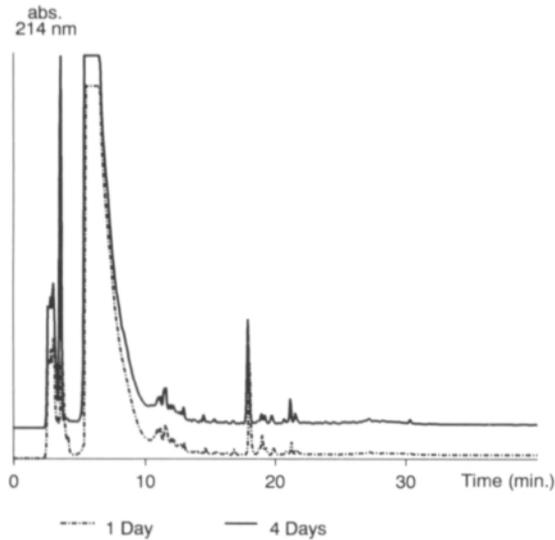


Fig. 8. Peptide profile of pasteurized milk at the age of 1 day and after 4 days' storage.

A wide peak caused by TCA can be seen at the beginning of each chromatogram. This is followed by the peaks of free amino acids and peptides. Only a few peaks are found on the chromatogram of pasteurized milk (Figure 8). A comparison between

fermented milk products and pasteurized milk shows the great effect of the fermentation process on the peptide content of the product. Also, the increase in both the height and area of the peptide peaks after storage gives information on the intensity of proteolysis.

In the free amino acid determinations, a high content of proline compared to other amino acids was observed, showing the highest content of all amino acids in all the fermented milks except in Gefilus (Figures 9 and 10). Glutamine content was also high in the products. During the manufacture and storage the content of all amino acids and peptides increased. ALM (1982) also found great variations in the amino acid content of different products. In yoghurt the proline content was found to be higher than average. Kefir was rich in lysine and proline at that study.

Differences caused by starters during the fermentation process become obvious when the peptide profile and content of the products are compared. A clear difference was between yoghurts and other fermented milk products. Yoghurts contained peptides which could not be found in the other products. Most of the identified peptides in all products originated from β -casein. However, opioid

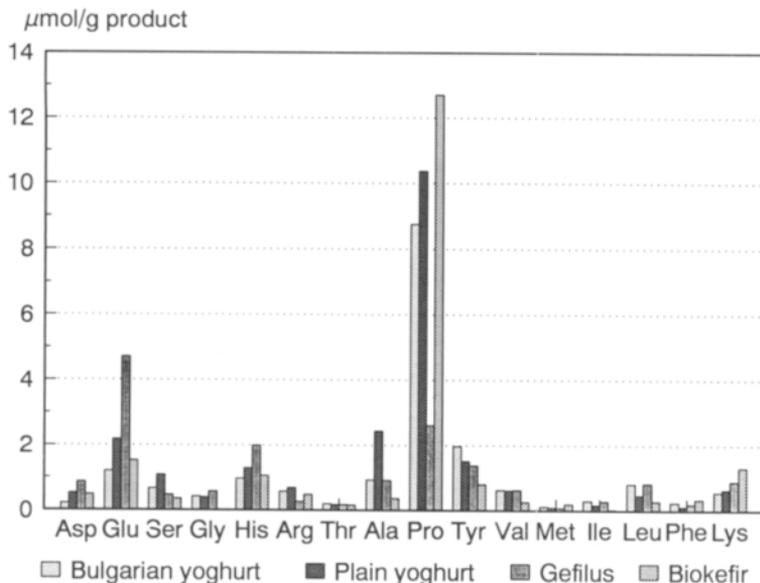


Fig. 9. Free amino acid content ($\mu\text{mol/g}$ product) of Bulgarian yoghurt, Plain yoghurt, Gefilus and Biokefir. The three letters amino acid codes used.

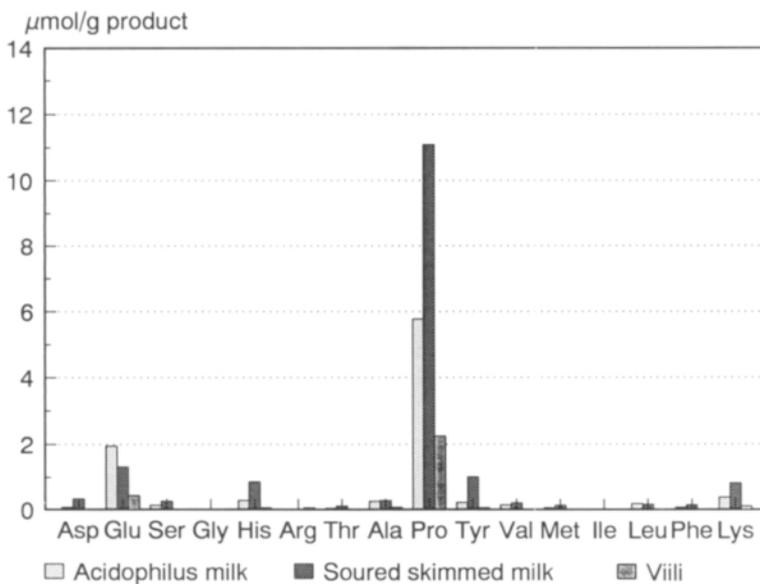


Fig. 10. Free amino acid content ($\mu\text{mol/g}$ product) of acidophilus milk, soured skimmed milk and viili. The three letters amino acid codes used.

β -casomorphines (β -CN f 60-70) were not observed.

Table 3 summarizes the peptides which were identified during the study. Peptides typical for yoghurts include fragments 1-14 from α_{s1} -casein and

47-57, 166-175 and 176-188 from β -casein. Peptide β -CN 176-188 contains a fragment which, according to MARUYAMA et al. (1985), possesses antihypertensive activity (β -CN f 177-183). Despite the apparent similarity of the peptide profiles of Bul-

Table 3. Identified peptide sequences in fermented milk products. 1. Bulgarian yoghurt, 2. Plain yoghurt, 3. Gefilus, 4. Viili, 5. Biokefir, 6. Acidophilus milk, 7. Soured skimmed milk.

| Sequence | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---------------------------|----|----|----|----|----|----|----|
| α_{11} -CN 1-14 | x | x | | | | | |
| β -CN 1-9 | | | | x | x | | |
| 7-16 | | | x | x | x | x | x |
| 22-28 | | | | | x | | x |
| 44-52 | | | | x | x | x | |
| 47-57 | x | x | | | x | | |
| 164-175 | | | | x | | x | |
| 166-175 | x | x | | | | | |
| 169-175 | | | | x | x | x | x |
| 176-182 | | | x | x | x | x | x |
| 176-188 | x | x | | | | | |
| κ -CN 161-169 | | | | | | | x |

garian and plain yoghurt, slight differences exist which are presumably caused by differences in the combination of starter and the manufacturing processes. The peptide profile of Gefilus differs very much from that of both yoghurts. The identified peptides are different and the free amino acid content is also higher than in yoghurts.

There is a certain similarity between the peptide profiles of viili, soured skimmed milk and Gefilus (Figures 3-7). Identical fragments of β -casein were found in these products. The most common peptides were fragments 7-15, 44-52, 169-175 and 176-182 of β -casein. Fragment 161-169 from the C-terminal region of κ -casein appears in soured skimmed milk. This peptide was not identified in any other product. Fragment 22-28 from β -casein appears only in kefir and soured skimmed milk. One of the peptides identified in viili and acidophilus milk was β -CN f 164-175.

The effect of plasmin on the peptide profile of the studied fermented milk products seems to be negligible. Its high specificity towards α_{s2} -casein should be easily recognizable (VISSER et al. 1989, PAHKALA et al. 1989a). The activity is obviously weakened by the high heat treatment of milk and low pH. An additional cause might be the relatively short storage period which may have led to an overlapping of the proteolytic activity of starters with the activity of plasmin. The high heat treatment of milk during the manufacture of all the fermented milk products denatures the whey proteins and causes the association of κ -casein and β -lactoglobulin (DALGLEISH 1990). The effect of these phenomena on the proteolytic end products was, however, not distinguishable in this study.

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SELOSTUS

Hapanmaitotuotteiden peptideistä

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Maatalouden tutkimuskeskus

Suomalaisten hapanmaitotuotteiden valkuaisaineiden pilkkoutumista tutkittiin varastoinnin aikana. Tuotteista määritettiin proteolyysiaste sekä eristettiin ja identifioitiin peptidejä peptidikartalta. Suurin proteolyysiaste todettiin biokefiirissä, kun taas suurin proteolyysiasteen muutos todettiin gefilukses-

sa. Erot käytetyissä hapatteissa ja valmistusmenetelmissä kuvastuivat tuotteiden peptidikartoissa. Useimmat tunnistetuista peptideistä olivat peräisin β -kaseiinin N- tai C-terminaalista päästä tai α_{s1} -kaseiinin N-terminaalista päästä.