Analysis of casein degradation in Tilsit cheeses

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1. Introduction

Tilsit cheese is a typical semi hard smear cheese which is produced in several European countries. The surface of these cheeses is coated with a so-called "Red Smear", which is a complex microflora of yeasts and bacteria protecting the surface from growth of undesirable microorganisms like moulds and pathogenic bacteria (7). Surface ripening begins with the growth of the yeasts which utilise lactate and increase the surface pH of the cheese (14,19,30). Above pH 6, *Brevibacterium linens*, other coryneform bacteria (e.g. *Arthrobacter*), staphylococci and micrococci begin to grow and eventually cover the whole cheese surface (3,4,24).

In contrast to other semi-hard cheeses not ripened with an additional surface flora, Tilsit cheeses develop a very intense aroma already after 4-6 weeks. The taste is still mild, but the smell is already very intense and contains a high degree of sulfur compounds produced by *Brevibacterium linens* and other coryneform bacteria (11,31). After 10-12 weeks of ripening, the taste is considerably stronger, probably the best time for consumption. After prolonged ripening (6 months), smell and taste have become so intense that these products are only acceptable by a minority of consumers.

Proteolysis is a key factor for aroma development in cheese. Therefore, the proteolytic system of lactic acid bacteria has been studied for a long time in order to understand and to improve proteolytic properties of lactic acid bacteria (2,23,27,32). A large number of proteinases and peptidases has been purified and their corresponding genes cloned and sequenced (for a review see (23)).

Part of the red smear bacteria are known to be strongly proteolytic. *B. linens* possesses extracellular proteinases (15,17,18,28) . Extracellular proteinases were also detected in other surface bacteria like *Arthrobacter* and staphylococci (4,5). The aim of the present study is to determine whether the observed enhanced aroma development of smear cheeses is related to a higher degree of casein degradation.

2. Materials and Methods

2.1 Sample preparation

Tilsit cheeses of 30 cm x 10 cm x 10 cm (2.5 kg) were used for the analyses. Samples were taken from the surface (0-0.5 cm) and from different depths (1.5-2 cm; 3-3.5 cm; 4.5-5 cm= core region). 0.5 g of Tilsit cheese were grinded in a 2 ml Eppendorf reaction vial. After addition of 1 ml protein extraction buffer (see 2.2), milk proteins were completely dissolved by shaking for 90-120 min at 37°C on a „Thermomixer" at maximum speed (Eppendorf, Hamburg, FRG). Insoluble components and fat were separated from the soluble milk proteins by centrifugation (5 min, 14000 x g, 4°C). The fat layer was removed from the surface, and additional 500 µl protein extraction buffer were added. After centrifugation (5 min, 14000 x g, 4°C) the supernatant was decanted, shock frozen in liquid nitrogen and stored at -20°C.

Small peptides were extracted by the same method using a different buffer (20 mM citrate buffer, pH 2.5, see 2.2). This prevented the complete extraction of milk proteins. For further purification of the peptide fraction, samples were centrifuged in Microcon-10 microconcentrators (molecular weight cut off at 10 kDa, Amicon, Witten, FRG) for 50 min at 9000 rpm and 4°C (centrifuge 5402, Eppendorf).

2.2 Electrophoresis buffers and solutions

All buffers were prepared in CE-grade water (Fluka) and were filtered before use (0.2 µm membranefilter, Millipore). For cleaning of capillaries, 0.1 M NaOH and for flushing before runs, CE-grade water was used.

Electrophoresis buffer: a stock solution of 8 M urea was purified from ionic contaminants by ion exchange (AG 501-X8; Biorad, Munich, FRG) according to the instruction given by the manufacturer. To avoid degradation of urea, the stock solutions were stored frozen in aliquots at -20°C. 10 mM tri-sodium citrate x $2H_2O$ and 0.05 % (w/v) methylhydroxy-propylcellulose (MHPC) were prepared in the urea stock solution; pH was adjusted to pH 2.5 with 2.5 M citric acid; the final concentration of citric acid was about 0.5 M and 6 M for urea. Electrophoresis buffers were stored at 4°C for 4-6 weeks.

Protein extraction buffer: 5 mM tri-sodium citrate x $2H_2O$ and 5 mM dithiothreitol were dissolved in 6 M urea, pH 8.0; the reducing buffer was stored in 2 ml aliquots at -20°C and thawed immediately before use.

Peptide extraction buffer: 20 mM citrate buffer, pH 2.5 (Applied Biosystems, Weiterstadt, FRG)

2.3 Casein and whey protein standards

For identification, commercially available standards were used (Merck, Sigma) and dissolved in CE-grade water: Hammarsten-Casein (1 % w/v, pH 7.8); purified preparations of α_s-, β- and κ-casein, α-lactalbumin and β-lactglobulin (2 % w/v); peptides of the α-$_{s1}$ fraction (f 1-23, f 4-23 and f 11-23, 1 mM) were provided by the University College Cork, Dept. of Food Chemistry. Other peptides were identified by hydrolysing single casein-components with chymosin and subsequent CE analysis. Genetic variants of milk proteins were provided by the Institute of Chemistry and Physics of the Federal Dairy Research Center, Kiel, FRG. They were used to identify the genotype of the milk used for cheese making. This was necessary because different caseins can build more than one peak in electropherograms if taken from heterocygote cows.

2.4 Capillary electrophoresis

Capillary electrophoresis was performed with the model 270A of Applied Biosystems (Weiterstadt, Germany). Based on a method of DeJong et al. (13) for the analysis of milk proteins, a CE method for the separation of caseins and corresponding proteolysis products was developed.

For the separations, hydrophilic capillaries (CElect-P150, Supelco, Bad Homburg, FRG) were used. The length of the capillary was 50 cm (30 cm to the detector window) with an inner diameter of 50 µm. The cathode buffer (10 ml) was replaced weekly, the electrophoresis buffer, cleaning- and flushing solutions at the anode position (1.5 ml each) were replaced daily.

Washing, flushing, and equilibration cycles were performed by vacuum injection with 20 inch Hg. At the begin of a daily series the capillary was cleaned and primed for the separations (2 min 0.1 M NaOH, 4 min CE-grade water, 8 min electrophoresis buffer). The first run was always a blank run which was not used for analyses because of deviating peak areas and retention times.

For sample preparation, cheese extracts were diluted with extraction buffer (1:5 v/v) and incubated for 30 min at room temperature. Injection was performed by vacuum (2 s, 5 in Hg). Electrophoresis was performed at 30°C using +25 kV for 23-28 min. The wavelength for detection was set to 212 nm. Between runs, the capillary was flushed with CE-grade water for 0.2 min, and reequilibrated for 4 min with CE buffer (20 in Hg). When not in use, the capillary was stored in CE-grade water containing 0.1 % sodiumazide.

3. Results

3.1 Capillary electrophoresis

The protein extraction buffer containing 6 M urea, citrate and dithiothreitol was able to solubilise all milk proteins and large peptides. Electrophoresis buffer also contained urea, citrate, and dithiothreitol to prevent reaggregation of caseins and the formation of disulfide bonds. At pH 2.5 all proteins were separated as cations. Reproducibility of peak areas showed a standard deviation of s=2.8 % for α_{s1}-CN, s=2.7 % for β-A1-CN, and s=1.4 % for β-A2-CN (n=24 runs). Depending on the age of the electrophoresis buffer and the capillary a shift to shorter retention times was observed. Therefore, recalibration with standards was performed at the beginning and at the end of a series of analyses. Within one series of 4-8 runs the standard deviation of retention times was 2.9 % for all 3 caseins mentioned above.

For the separation of smaller peptides an acidic citrate buffer was used and peptide preparations were further purified from proteins over 10 kDa cut-off microconcentrators. Using this method it was possible to increase the time for vacuum injection 10fold (20 sec) and thus sensitivity was improved. Electrophoresis conditions were comparable to protein separations, however, runs were usually stopped at 15 min.

3.2 Proteolysis in Tilsit cheeses

First, proteolysis of commercial Tilsit cheeses was studied. Capillary electrophoresis of the cheese milk showed the separation of all major milk proteins. Apart from milk proteins, the plasmin degradation product of α_{s1}-casein (f24-199) and other not identified degradation products were detected (Fig. 1a). As expected, whey proteins were not detected in Tilsit cheese before brining (Fig. 1b). Since rennet was used for coagulation of the cheese milk the chymosin specific degradation product of κ-casein (paraκ-casein) was detected. One peak with a retention time similar of κ-casein could be attributed to a β-casein peptide through chymosin cleavage. This was verified by chymosin digests of purified β-casein (data not shown). After 5 weeks of ripening, samples from the surface and the core region were analysed (Fig. 1 c+d). In the core region, a significantly higher degree of α_{s1}-CN degradation was observed. At the surface, a slightly higher level of β-casein cleavage was observed (Fig. 1 c+d). The regions 1.5-2 cm and 3-3.5 cm from the surface were comparable to the core region and were therefore not included in Figure 1.

The commercial Tilsit cheeses analysed, were traditional „old-young" smeared cheeses. A defined surface starter culture which was developed by Bockelmann et al. (4) was used for experimental cheese making. The cheeses smeared and ripened with starter culture consisting of 4 bacterial and one yeast strain developed a typical aroma after 6 weeks of ripening. The biochemical analysis of proteolysis is shown in Figure 2. Except a slighty lower degree of β-casein degradation no significant differences were found in comparison to the analysed commercial Tilsit cheeses (Fig. 2a+b, 1 c+d).

To determine the specific influence of the surface flora on proteolysis some experimental Tilsit cheeses were plastic coated and ripened under the same conditions as the smeared cheeses. A surface flora could not grow on these cheeses. Proteolysis in the core region

of both types of cheeses was identical; compared to the rind, α_{S1}-casein was degraded to a higher degree (Fig. 2). Higher concentrations of the primary α_{S1}-CN cleavage product (f24-199) of chymosin were detected in the core region. The slightly enhanced β-casein hydrolysis as observed for the old-young smeared commercial cheeses was not observed here.

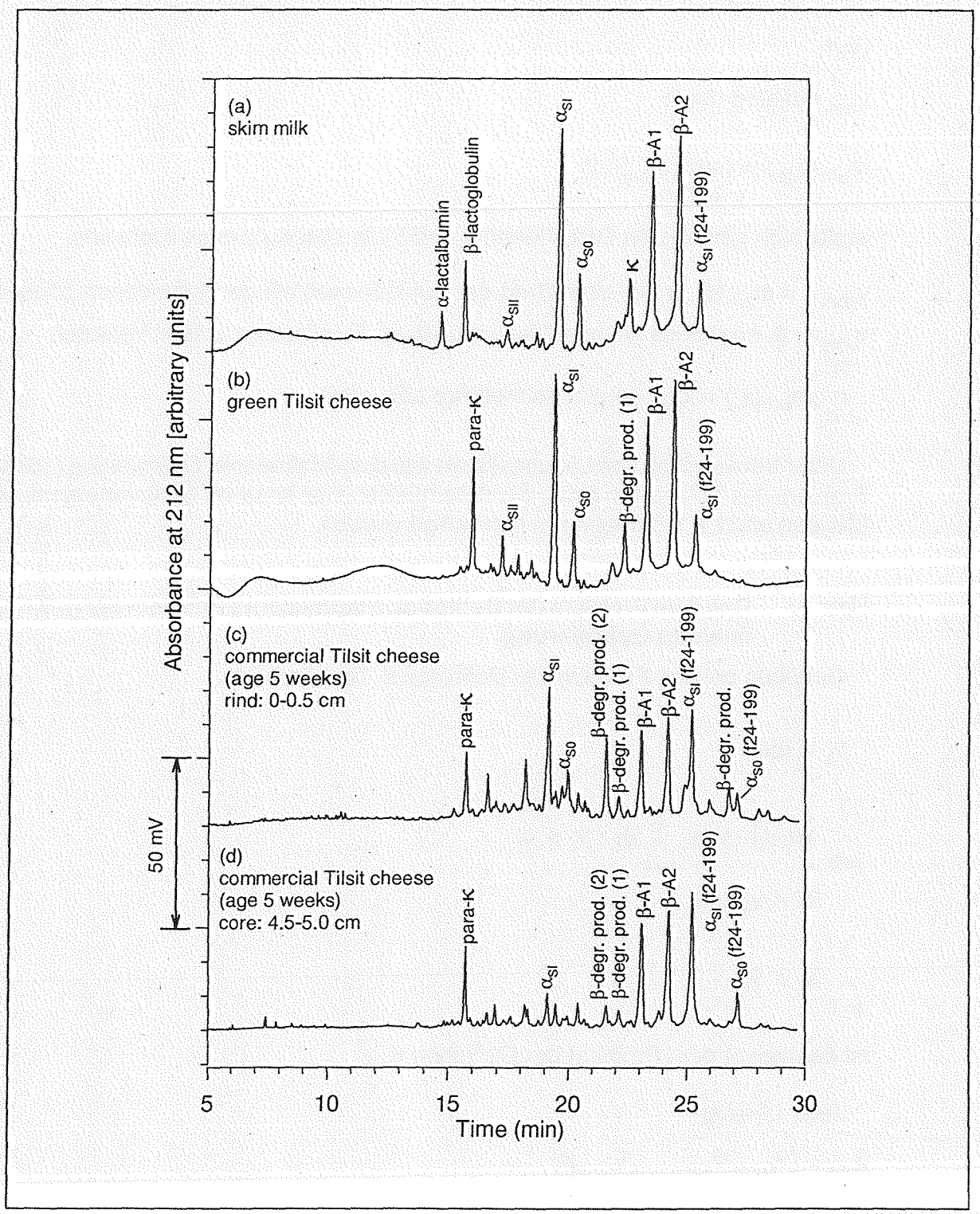

Fig. 1: Capillary electrophoresis of proteins and large peptides from milk and commercial, „old-young"-ripened (see Introduction) Tilsit cheeses. Cheese samples were taken from the surface and the core region to show the effect of the surface flora on casein degradation in relation to plasmin, rennet, and the proteolytic enzymes from lactic acid bacteria (core region).

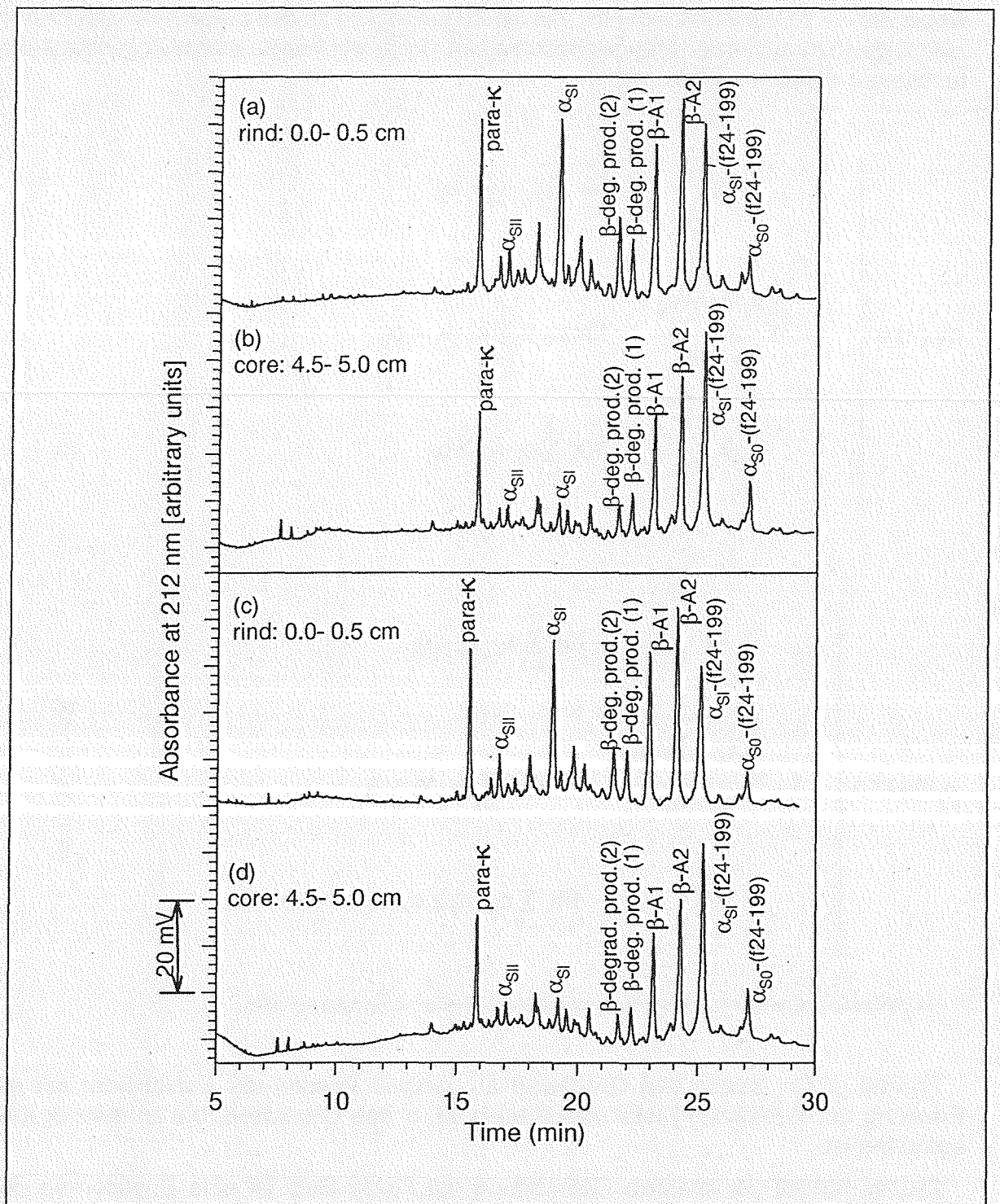

Fig. 2: Capillary electrophoresis of proteins and large peptides from Tilsit cheeses ripened with a defined surface starter culture (a,b) (4). For comparison, plastic-coated cheeses were produced from the same batch of milk and ripened without a surface flora (c,d).

Apart from the separation of caseins and large degradation products acidic extracts (pH 2.5) from cheeses were prepared to analyse smaller, fast migrating peptides. Here, the influence of the surface flora on proteolysis was more pronounced (Fig. 3). Plastic-coated cheeses showed similar peptide patterns in the core and surface region. In smeared cheeses, these peptides were not visible in the surface region. At the surface (0-0.5 cm) a bigger variety of peptides was present. Except for α_{s1}-CN (f1-23) an identification was not possible (Fig. 3).

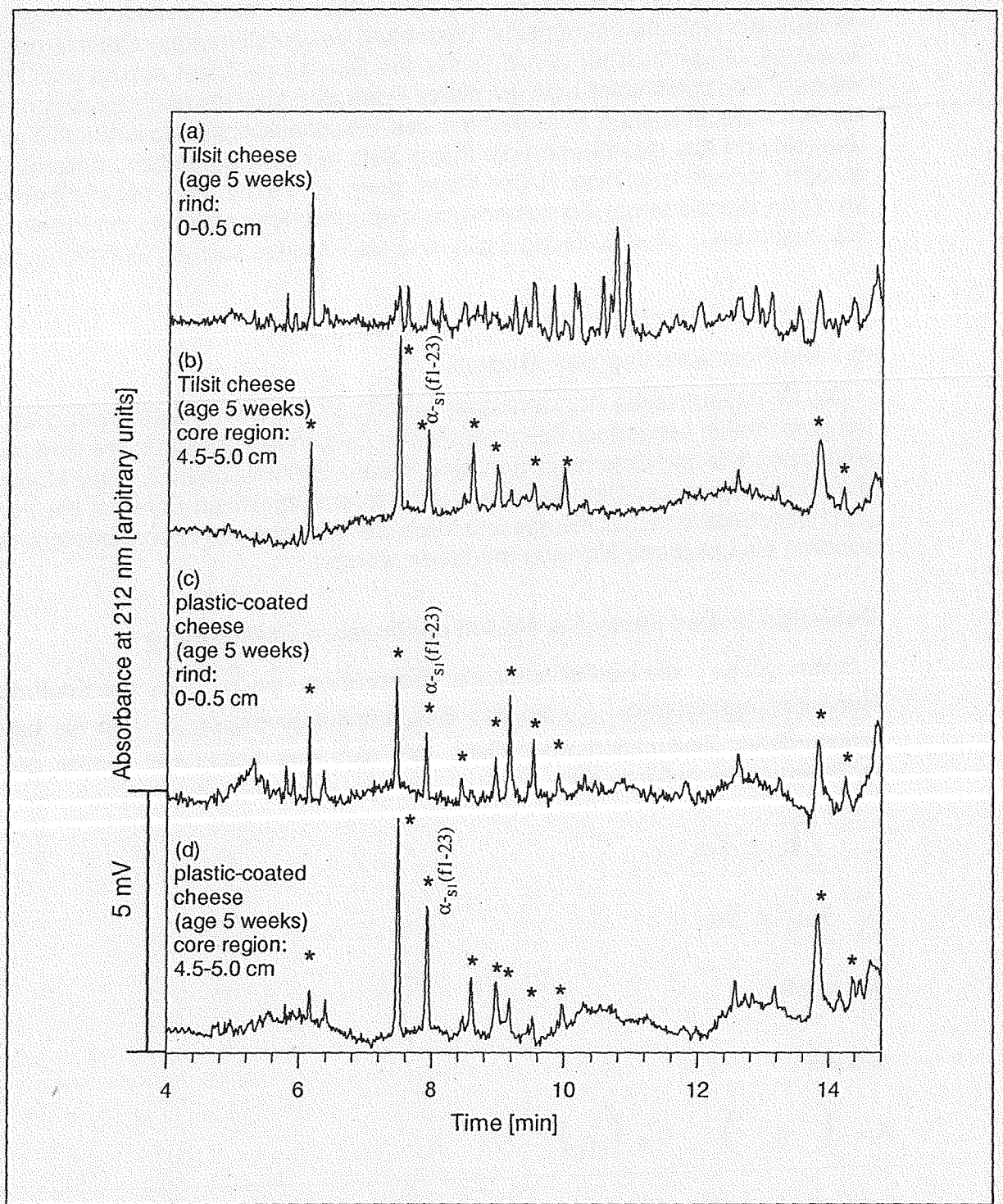

Fig. 3: Capillary electrophoresis of low molecular weight peptides from Tilsit cheeses (a,b) and from a plastic-coated control cheese (c,d). Peptides liberated from casein without the influence of the surface flora are marked with an asterisk

4. Discussion

Capillary electrophoresis is an ideal tool for the separation of milk proteins. For solubilisation of caseins, high concentrations of urea have to be used which have a corrosive effect on pump heads and seals in HPLC. This problem does not occur in CE because only glass capillaries are exposed to the urea containing separation buffers. In recent years several methods were published for the separation of milk proteins by capillary electrophoresis (1,9,10,12, 21,25,26,29).

Based on a method of De Jong et al. (12) capillary electrophoresis was used to analyse proteolysis in Tilsit cheeses. The method allowed the separation of whey proteins and caseins as well as large and small peptides in one electrophoretic run. Not reported by De Jong et al. (12) was a strong effect of electrophoresis buffers on the baseline. To get more or less steady baselines for analysis, blank subtraction was always necessary. The very good repeatability of retention times given by De Jong et al. (12) was not observed. Depending on the age of the capillary and electrophoresis buffers a shift of retention times was observed. For identification of proteins, a frequent recalibration was performed with purified marker proteins. Reproducibility of peak areas (3 %) was comparable to the values given by De Jong et al. (12).

The CE method used, was able to separate small peptides and milk proteins in one run. However, sensitivity was too low, when injection conditions were optimised for proteins. With higher injection volumes applied, proteins had a negative effect on the separations. Therefore, the extraction of peptides from cheese was performed in a different buffer (pH 2.5), extracting small amounts of caseins only. Extracts were further purified by size exclusion filtration (MW cut-off 10 kDa). Thus a 10 fold higher amount of sample could be injected and analysed by CE.

The main difference between smeared cheeses and cheese without an additional surface flora is the fast aroma development of smear cheeses. Within weeks, cheese develop a strong smell and taste (8,30). The reason for fast aroma development was not an accelerated casein degradation. A slightly higher degree of β-casein hydrolysis observed in the analysed commercial cheeses could not explain the differences between the aroma of smeared and non-smeared cheeses. It is known that some surface bacteria like *Brevibacterium linens* and *Arthrobacter spec.* possess strong extracellular proteinase activity (4,6,15,16,28). Thus the barely detectable effect of the smear on casein degradation was unexpected.

The analysis of smaller peptides showed more differences. Peptides of plastic-coated control cheeses showed similar patterns in the core (depth 5 cm) and the surface region (0-0.5 cm, Fig. 3) caused by the action of plasmin, chymosin, and starter enzymes alone. In smear ripened cheeses, a further degradation of these peptides was observed in the surface region only. No effect was visible in areas from 1 cm depth to the core of cheese (5 cm) which shows that diffusion of peptides is quite low. It is known that low molecular weight compounds like lactic acid migrate well in cheese along a concentration gradient from the center to the surface of cheeses caused by the degradation of lactic acid by the smear flora (20). Migration of proteolytic enzymes from the surface into the cheese matrix is limited to about 2 mm because of their size (20). Results indicate that the influence of the surface flora on casein degradation is rather limited. The further degradation of peptides to amino acids and amino acid conversion to aromatic compounds seems to be the major reason for the observed differences of smear cheeses (11,22,31).

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6. Summary

Bockelmann, W., Hoppe-Seyler, T., Lick, S., Heller, K.J.:**Analysis of casein degradation in Tilsit cheeses.** Kieler Milchwirtschaftliche Forschungsberichte **50** (2) 105-113 (1998)

56 Casein degradation (lactic bacteria, Tilsit cheese)

Extraction methods for proteins and peptides from cheese have been optimized for the subsequent analysis of the ripening of Tilsit cheeses. Proteins were extracted with a reducing alkaline buffer containing high concentrations of urea. Small peptides (< 10 kDa) were extracted with an acidic citrate buffer. Analysis of casein breakdown revealed only a small additional effect of the surface flora. This was only visible in the surface region (0-0.5 cm depth). Most of the casein degradation was shown to be caused by plasmin, rennet, and starter or non-starter proteinases. It was independent of the presence of a surface flora. However, analysis of low molecular weight peptides showed a large number of additional peaks in the surface region of smeared Tilsit cheese (0-0.5 cm depth).

Results indicate that the very intense aroma of smear cheeses after only weeks of ripening is due to the accelerated turnover of peptides and probably amino acids to aromatic compounds.

Zusammenfassung

Bockelmann, W., Hoppe-Seyler, T., Lick, S., Heller, K.J.: **Analyse des Caseinabbaus in Tilsiterkäse.** Kieler Milchwirtschaftliche Forschungsberichte **50** (2) 105-113 (1998)

56 Caseinabbau (Milchsäurebakterien, Tilsiterkäse)

Extraktionsmethoden für Proteine und Peptide wurden für die Analyse in der Kapillarelektrophorese optimiert und zur Beschreibung der Käsereifung von Rotschmierekäsen eingesetzt. Der reduzierende Extraktionspuffer für Proteine enthielt hohe Konzentrationen von Harnstoff zur Solubilisierung von Casein. Peptide (<10 kDa) wurden in einem Citratpuffer pH 2.5 extrahiert. Der Vorteil war, daß Casein nur geringfügig extrahiert wurde und so die Trennung in der Kapillarelektrophorese nicht störte. Kapillarelektrophorese von Tilsiter Käsen zeigte nur einen minimalen Einfluß der proteolytisch aktiven Oberflächenflora auf den Abbau von Casein und dies auch nur an der Käseoberfläche (0-0.5 cm Tiefe). Die Herkunft der hochmolekularen Caseinabbauprodukte im gesamten Käse konnte im Wesentlichen auf die Wirkung von Plasmin, Chymosin, und Proteasen der Milchsäurebakterien zurückgeführt werden. Wesentliche Unterschiede zwischen geschmierten und nicht geschmierten Käsen zeigten Elektropherogramme von niedermolekularen Peptiden. Hier war eine gesteigerte Proteolyse in Randbereichen (0-0.5 cm Tiefe) geschmierter Käse deutlich nachweisbar.

Résumé

Bockelmann, W., Hoppe-Seyler, T., Lick, S., Heller, K.J.: **Analyse de la dégradation de la caséine des fromages Tilsit.** Kieler Milchwirtschaftliche Forschungsberichte **50** (2) 105-113 (1998)

56 Dégradation de la caséine (bactéries lactiques, fromages Tilsit)

Les méthodes d'extraction pour protéines et peptides ont été perfectionnées pour l'analyse consécutive de l'affinage des fromages à croûte lavée. Des protéines furent extraites par extracteur réducteur, à forte concentration d'urine pour garantir une meilleure solubilité de la caséine. Les peptides (<10 kDa) furent extraits par extracteur à citrate à une valeur pH de 2.5. Comme uniquement un minimum de caséine fut extrait, cela n'avait pas d'effet néfaste sur la séparation dans l'électrophorèse capillaire. L'électrophorèse des fromages Tilsit n'a montré qu'une influence minimale de la flore superficielle à activité protéolytique sur la dégradation de la caséine. Cet effet fut d'ailleurs uniquement remarqué sur la surface du fromage (0-0.5 cm de profondeur). L'origine des produits à haute concentration moléculaire de dégradation de caséine dans tout le fromage a pu être attribuée dans l'essentiel à l'effet de plasmine, chymosine et protéases des bactéries lactiques. Des électrophérogrammes de peptides à faible concentration moléculaire ont relevé des différences considérables entre des fromages mous et des fromages non mous. Dans ces cas, une protéolyse élevée des fromages mous était nettement détectable sur les bords (0-0.5 cm de profondeur).