



Prevalence and stability of lysozyme in cheese

N. Schneider^a, K. Werkmeister^b, C.-M. Becker^c, M. Pischetsrieder^{a,*}

^a Department of Chemistry and Pharmacy, Food Chemistry, Emil-Fischer-Center, University of Erlangen-Nuremberg, Schuhstr. 19, 91052 Erlangen, Germany

^b Bavarian Health and Food Safety Authority, Eggenreuther Weg 43, 91058 Erlangen, Germany

^c Institute of Biochemistry, Emil-Fischer-Center, University of Erlangen-Nuremberg, Fahrstr. 17, 91054 Erlangen, Germany

ARTICLE INFO

Article history:

Received 27 September 2010

Received in revised form 11 January 2011

Accepted 1 March 2011

Available online 5 March 2011

Keywords:

Cheese

Fluorescence detection

HPLC

Hen egg white lysozyme

Lysozyme degradation

ABSTRACT

The use of the preservative and potential allergen hen egg white lysozyme in cheese production has to be declared. In the present study, an HPLC method with fluorescence detection (HPLC-FLD) was optimised and validated for the analysis of lysozyme in cheese. Lysozyme was detected in concentrations between 30.8 and 386.2 mg/kg cheese in 30 out of 46 analysed commercial cheese samples. During cheese production and storage for 0–54 weeks a lysozyme satellite peak (LSP) was detected, which totals up to 18% of the lysozyme content. Mass spectrometry and peptide mass fingerprint revealed that LSP possesses the same primary structure as lysozyme. Since disulphide scrambling could not be detected, LSP was assigned to a conformational isomer of lysozyme. As a consequence, LSP was included in the HPLC-FLD analysis of lysozymes in cheese.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Lysozyme was discovered in 1922 by Alexander Fleming. The enzyme hydrolyses the cell wall of Gram-positive bacteria. Therefore, lysozyme can be used as preservative in cheese manufacturing to prevent the late gas defect, caused by *Clostridium tyrobutyricum* (Wasserfall & Teuber, 1979). Thus, lysozyme can be applied as an alternative to nitrate. France was the first country that allowed the industrial application of lysozyme in cheese production in 1981 (Busse et al., 1986). Today, the application of the preservative (E 1105) for ripened cheese is legal in the entire European Community according to the European Parliament Directive No. 95/2/EC and the Codex Alimentarius Standard 283-1978. The first methods developed for the detection of lysozyme were based on the lytic action of lysozyme. Lysis of the cell walls of *Micrococcus*

lysodeicticus was measured by turbidimetric analysis (Gorin, Wang, & Papapavlou, 1971; McKenzie & White, 1986). Furthermore, several enzyme-linked immunosorbent assay (ELISA) methods are described for the detection of lysozyme in food using polyclonal, mostly lab-made antibodies (Rauch, Hochel, & Kás, 1990; Rauch et al., 1995; Yoshida, Takagaki, & Nishimune, 1991). Recently, an ELISA procedure was developed based on a commercial monoclonal antibody, allowing the standardisation of the procedure (Schneider, Weigel, Werkmeister, & Pischetsrieder, 2010b). Lysozyme can also be detected and quantified by high performance liquid chromatography and fluorescence detection (HPLC-FLD), liquid chromatography–mass spectrometry (LC–MS), immunocapture mass spectrometry, or surface-enhanced mass spectrometry (Bärtschi, Muralt, Rieder, Kämpfer, & Schaller, 2006; Dragoni et al., 2010; Pellegrino & Tirelli, 2000; Schneider, Becker, & Pischetsrieder, 2010a). The HPLC-FLD method to detect lysozyme in milk and dairy products was recently published as an ISO standard (ISO, 2009). For commercial applications, lysozyme is extracted from hen egg white (HEW, 3.5% of the egg white proteins) (Guerin-Dubiard et al., 2005). Since eggs and egg products belong to the major food allergens, the use of lysozyme has to be declared as an allergen. In Europe, allergen labelling is regulated by the European Parliament and Council Directive 2003/89/EC, Annex IIIa amending Directive 2000/13/EC and in the United States by the “Food Allergen Labeling and Consumer Protection Act of 2004”. The classification of lysozyme as an allergen is, however, inconsistent. While some studies come to the conclusion that lysozyme is only a weak allergen (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994; Hoffman, 1983; Iaconelli et al., 2008),

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC-FLD, high performance liquid chromatography with fluorescence detection; LC–MS, liquid chromatography–mass spectrometry; HEW, hen egg white; MES, 2-(N-morpholino)ethanesulphonic acid; DHAP, 2,5-dihydroxyacetophenone; TFA, trifluoroacetic acid; DTT, dithiotreitol; HCCA, α -cyano-4-hydroxycinnamic acid; Tris, Tris(hydroxymethyl)aminomethane; DHAP, dihydroxyacetophenone; LSP, lysozyme satellite peak; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; MALDI-TOF-MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; ATCH, adrenocorticotrophic hormone.

* Corresponding author. Tel.: +49 9131 8524102; fax: +49 9131 8522587.

E-mail address: monika.pischetsrieder@lmchemie.uni-erlangen.de (M. Pischetsrieder).

others reason that lysozyme is one of the major allergens of HEW (Fremont, Kanny, Nicolas, & Moneret-Vautrin, 1997; Holen & Elsayed, 1990). Because of the allergenic potential of lysozyme, it is necessary to reliably detect and quantify this preservative in the cheese end product. Up to now, no data has been available on the stability of lysozyme during storage and ripening of cheese. Furthermore, it is necessary to know the structure of potential lysozyme decomposition products to evaluate their allergenicity as well as antimicrobial activity.

In this study, an HPLC-FLD method for the detection and quantification of lysozyme in cheese was optimised, fully validated, and applied to the analysis of commercial cheese samples. The stability of lysozyme was monitored during the storage of cheese for up to 54 weeks. A degradation product of lysozyme was detected by HPLC and characterised by mass spectrometry.

2. Materials and methods

2.1. Chemicals and buffers

HEW lysozyme, α -lactalbumin, β -lactoglobulin, 2-(*N*-morpholino)ethanesulphonic acid (MES), and trifluoroacetic acid (TFA) were supplied by Sigma–Aldrich (Taufkirchen, Germany). Modified trypsin (sequencing grade) and endoproteinase Asp-N (sequencing grade) were obtained from Roche (Mannheim, Germany). Acetonitrile was from Fisher Scientific (Schwerte, Germany) and dithiothreitol (DTT) from Roth (Karlsruhe, Germany). Tris(hydroxymethyl)aminomethane (Tris) was supplied by Acros (Geel, Belgium) and α -cyano-4-hydroxycinnamic acid (HCCA) as well as diammonium hydrogen citrate by Fluka (Taufkirchen, Germany). The peptide standard and 2,5-dihydroxyacetophenone (DHAP) were purchased from Bruker Daltonik (Bremen, Germany). Powdered lysozyme formulas, commercially available for cheese making, were supplied by Chr. Hansen (Nienburg, Germany), Optiferm (Kempten, Germany), and DSM Food Specialties (Duesseldorf, Germany).

2.2. Cheese samples

Commercial cheese samples were provided by the Bavarian Health and Food Safety Authority as part of the sampling plan or purchased in a local supermarket. For the storage experiments, the cheese was produced from 12,500 l milk and stored in a local cheese dairy. An aliquot of 1200 ml lysozyme-solution (optilact L80, $\geq 80,000,000$ U/ml, Optiferm, Kempten, Germany), diluted with 10 l water, was added to the milk. Three lots of a hard cheese, type parmesan, were produced from the kettle milk. As a control, the same type of cheese was produced without lysozyme. From each lot, two cheese wheels were stored at 12–16 °C and an air humidity of 80–85%. Samples were taken in intervals of 18 weeks with a cheese-drill. The holes were re-closed with a part of the cheese sample. One sample was taken from each stored cheese and the lysozyme content was analysed in two independent experiments.

2.3. Extraction of HEW lysozyme from the cheese samples

The extraction of lysozyme from the cheese samples was performed as described before (Schneider et al., 2010a). Briefly, 2 g of grated cheese and 20 ml of sodium chloride solution (1 M in water) were homogenised in a T18 basic Ultra Turrax (IKA, Staufen, Germany). The Ultra Turrax was rinsed with 20 ml sodium chloride solution. After combining the suspensions, the pH was adjusted to 6.0. The suspension was stirred for 1 h. Afterwards the pH was adjusted to 4.3. The suspension was first filtered through a fluted paper filter and then filtered through a membrane filter (0.22 μ m, Roth, Karlsruhe, Germany).

2.4. Determination of the dry matter of cheese

A nickel dish was filled with approximately 20 g of sea sand and a glass bar and dried in a drying oven (Ehret, Emmendingen, Germany) at 102 ± 2 °C overnight. The nickel dish was allowed to cool down to room temperature in a desiccator. Three grams of grated cheese were weighed into the nickel dish and carefully mixed with the sand using the glass bar. The mixture was dried for 3 h in the oven at 102 ± 2 °C, cooled down in the desiccator and weighed. Then the cheese was dried again in intervals of 1 h until it showed a constant weight. The dry matter of cheese was calculated. Lysozyme contents of cheese were expressed as mg lysozyme/kg fresh cheese unless otherwise stated.

2.5. HPLC-FLD analysis

HPLC separation was performed with an autosampler AS-2057, HPLC pump PU-1580, ternary gradient unit LG-1580-02, 3-line degasser DG-1580-53, fluorescence detector FP-920 (Jasco, Gross-Umstadt, Germany) on a reversed-phase polymeric column (PLRP-S, 250×4.6 mm, 300 Å pore size, 5 μ m) from Polymer Laboratories (Darmstadt, Germany). Elution solvents were water/0.1% TFA (v/v) (A) and acetonitrile/0.1% TFA (v/v) (B) with the following elution profile: 0–12 min, 30–37% B; 12–13 min, 37–70% B; 13–14 min, 70% B; 14–15 min, 70–30% B; 15–25 min, 30% B, a flow rate of 0.8 ml/min, a temperature of 40 °C, and an injection volume of 50 μ l. The fluorescence detector recorded at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Lysozyme and the lysozyme satellite peak (LSP) were quantified by an external calibration curve with standard solutions of 1, 2, 4, 8, and 12 μ g lysozyme/ml in 1 M aqueous sodium chloride. The method has previously been used to validate a lysozyme ELISA (Schneider et al., 2010b).

2.6. Validation of the HPLC-FLD method

For validating the method, repeatability, recovery, linearity, limit of detection (LOD), and limit of quantification (LOQ) were determined. LOD was calculated as $\text{LOD} = \text{blank} + 3.3 \times \text{SD}_{\text{blank}}$ and LOQ as $\text{LOQ} = \text{blank} + 10 \times \text{SD}_{\text{blank}}$ (blank = axis intercept of the calibration curve, SD_{blank} = standard deviation of six independent replicates of a lysozyme-free cheese). LOD and LOQ were experimentally confirmed by spiking a lysozyme-free cheese with different amounts of lysozyme. A signal-to-noise ratio of 3:1 for the LOD and of 9:1 for the LOQ was accepted. The determination coefficient R^2 of the calibration curve was 0.9998 over the whole concentration range; the Mandel test confirmed linearity between 1 and 8 μ g/ml ($p = 0.01$). The accuracy of the assay was specified by spiking lysozyme-free cheese samples with 50, 100, 200, and 400 mg/kg lysozyme, respectively. A quantity of 2 g grated cheese was weighed into a 100 ml flask and 100, 200, 400, and 800 μ l of a lysozyme standard solution (1 mg/ml in 1 M sodium chloride solution in water) were added to the cheese portions. Then, the extraction was performed as described above. Lysozyme-free cheese was spiked with the four standard solutions in six independent experiments. The repeatability of the HPLC method was determined by calculating the relative standard deviation (RSD) of six independent experiments.

2.7. Thermal degradation of a lysozyme standard

Lysozyme solutions (1 mg/ml in water) were heated in a thermomixer (Eppendorf, Hamburg, Germany) at 99 °C and 500 rpm for 0, 30, 60, and 120 min and then immediately cooled down on ice in two independent experiments. The solutions were diluted with 1 M sodium chloride in water to a concentration of 10 μ g/

ml and filtered through a 0.22 µm membrane filter. Each sample was analysed in duplicate by HPLC.

2.8. Isolation of lysozyme and LSP from a cheese sample

For this purpose, the HPLC method described above was applied with a slightly modified elution gradient. Elution solvents were solution A, water/0.1% TFA (v/v) and solution B, acetonitrile/0.1% TFA (v/v). Elution conditions are expressed as percentage of solvent B: 0–5 min 30%; 5–9 min 30–33.5%; 9–13 min 33.5%; 13–17 min 33.5–34%; 17–18 min 34–70%; 18–19 min 70%; 19–20 min 70–30%; 20–30 min 30%.

2.9. Peptide mass fingerprint of lysozyme and LSP

The isolated fractions of three HPLC runs of a heated lysozyme standard or cheese extract were pooled and freeze-dried. The proteins in the fractions were digested with trypsin or Asp-N. For digestion with trypsin, the lyophilisate was re-suspended in 10 µl Tris-HCl buffer (100 mM, pH 8.5). In order to measure the intact protein, 1 µl of the re-suspended lyophilisate was mixed with 1 µl DHAP matrix and 1 µl of 2% TFA. An aliquot of 1 µl of the mixture was spotted onto a stainless steel target and air-dried. The remaining 9 µl of the dissolved lyophilisate were mixed with 1 µl of trypsin (0.1 µg) and incubated for 5 h at 37 °C. After the digestion, 1 µl of the solution was mixed with 4 µl HCCA matrix, and 1 µl thereof was spotted onto a stainless steel target and air-dried in order to analyse the presence of disulphide bonds. The remaining 9 µl of the peptide solution were reduced with 1 µl DTT-solution in water (100 mM). After 30 min of incubation, 2 µl of the reduced peptides were mixed with 8 µl HCCA matrix, and 1 µl of the mixture was spotted onto a stainless steel target and air-dried. The same procedure was followed to digest the different fractions with the endoproteinase Asp-N. In contrast to the digestion with trypsin, the lyophilisate was dissolved in 10 µl MES buffer (50 mM, pH 6.0) and for digestion, 2.5 µl of Asp-N (0.1 µg) was added. The resulting peptides were reduced and spotted as described for trypsin digestion.

2.10. MALDI-TOF-MS analysis

To obtain a DHAP matrix, 7.6 mg (50 µM) DHAP was suspended in 375 µl ethanol. An aliquot of 125 µl aqueous diammonium hydrogen citrate solution (10 µM) was added to the DHAP suspension. The mixture was vortexed vigorously and sonicated for at least 15 min. For the HCCA matrix, saturated HCCA in 50%

acetonitrile/0.1% TFA was mixed with an equal volume of ammonium dihydrogen phosphate in 50% acetonitrile/0.1% TFA (10 mM).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed on a Bruker Autoflex (Bruker Daltonik, Bremen, Germany) equipped with a nitrogen laser ($\lambda = 337$ nm). Proteins and the tryptic digest were measured with delayed extraction (330 ns and 80 ns, respectively). External mass calibration was performed. For the proteins, bovine α -lactalbumin, HEW lysozyme, and bovine β -lactoglobulin were used. For peptides, angiotensin I and II, substance P, bombesin, adrenocorticotrophic hormone (ATCH) clip 1–17 and ATCH clip 18–39 were used as standards. Proteins and peptides were measured as positive ions. Proteins were accelerated by 20 kV in the linear mode and peptides by 19 kV in the reflector mode. At least 150 individual spectra for the proteins and 300 spectra for the peptides, obtained from several positions on a spot, were averaged.

2.11. Statistical data evaluation

The evaluation of the statistical data was carried out with Microsoft Excel 2003. Significance levels were calculated with a two-tailed, paired student *t*-test.

3. Results and discussion

3.1. Analysis of lysozyme in cheese by HPLC-FLD

Lysozyme was extracted from the cheese samples by a highly concentrated solution of sodium chloride as described before (Pellegrino & Tirelli, 2000; Schneider et al., 2010a, 2010b). After precipitation of the caseins by pH adjustment to 4.3 and filtration, the extract was directly injected into the HPLC unit. The content of lysozyme in the extracts was measured by HPLC with fluorescence detection. The method was adopted from Pellegrino and Tirelli (2000) with some modifications: instead of isocratic elution, gradient elution was performed. Gradient elution increased the robustness of the elution profile and resulted in narrower peaks. Furthermore, the runtime was reduced leading to a 43% lower solvent consumption. Fluorescence detection of lysozyme, which contains six tryptophan residues, highly improves the sensitivity compared to UV detection. In a very recent study, gradient elution has been applied to compare an HPLC and a commercial ELISA

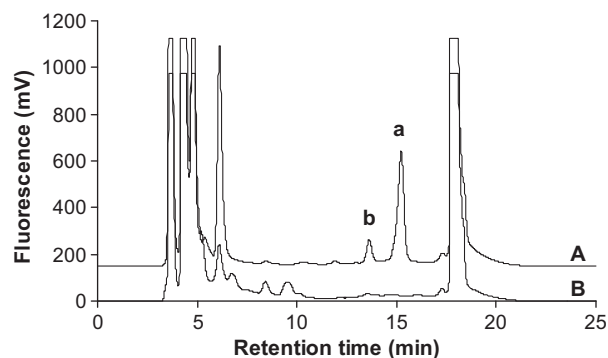


Fig. 1. HPLC-FLD chromatograms of cheese samples produced with (A) and without (B) lysozyme, after 54 weeks of storage ((a) lysozyme; (b) LSP). For the cheese sample produced with lysozyme, 1200 ml lysozyme solution ($\geq 80,000,000$ U/ml) was added to 12,500 l kettle milk.

Table 1
Lysozyme and LSP contents in commercial cheese samples.

Cheese type	Positive samples	Lysozyme content (mg/kg)	LSP content (mg/kg)	Total lysozyme (mg/kg) ^a	LSP (%) ^b
Grated hard cheese mixture (n = 18)	10	30.8–156.7	19.9–32.4	59.3–189.1	17.1–48.0
Grana Padano (n = 15)	15	170.9–247.2	46.1–69.3	220.5–316.5	19.1–23.8
Parmigiano-Reggiano (n = 5)	0	–	–	–	–
Cheese from sheep milk (n = 4)	2	95.0–165.7	15.6–22.3	110.6–187.9	11.9–14.1
Cheese from goat milk (n = 2)	2	252.0–386.2	54.6–123.3	375.3–440.8	12.4–32.9
Raclette cheese (n = 2)	1	241.2	56.3	297.5	18.9

^a Total lysozyme = lysozyme + LSP.

^b LSP (%) = LSP/(lysozyme + LSP) * 100.

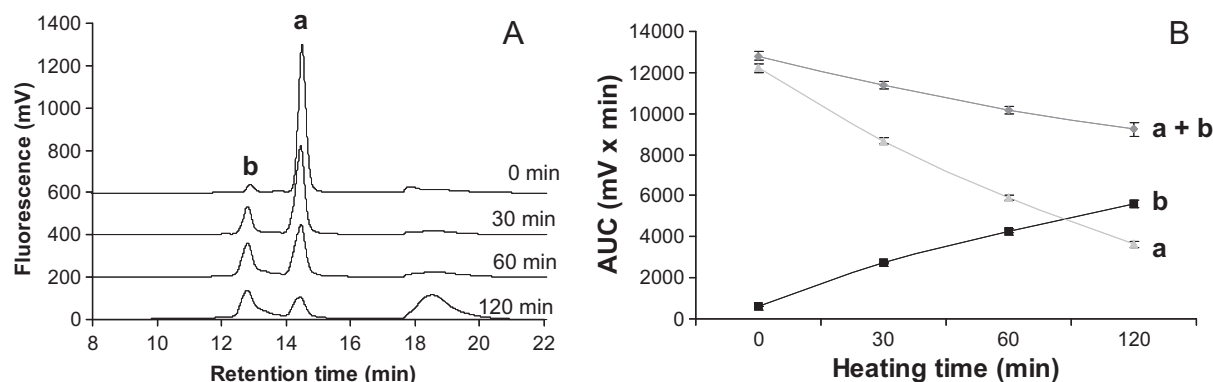


Fig. 2. Lysozyme solutions (1 mg/ml in water) were heated for 0, 30, 60, and 120 min. The presence of lysozyme (a) and LSP (b) was analysed by HPLC-FLD. (A) HPLC-FLD chromatograms of the heated lysozyme standards; (B) the diagram shows the areas of the lysozyme peak, LSP and the sum of both peaks. Data are presented as mean value $\pm$ standard deviation ($n = 4$) of the area under the curve (AUC).

method for lysozyme analysis in food (Kerkaert, Mestdagh, & De Meulenaer, 2010).

3.2. Validation of the HPLC method

For validation of the method, detection and quantification limits, linearity, repeatability, and accuracy were determined. The LOD was calculated as 7.3 mg lysozyme/kg cheese and the LOQ as 22.3 mg lysozyme/kg cheese. LOD and LOQ were determined by using a lysozyme-free cheese as a blank matrix. The results were experimentally confirmed by spiking a lysozyme-free cheese with different amounts of lysozyme and determining the signal-to-noise ratio (data not shown). The five point calibration curve covered the concentration range of 1–12 μ g/ml lysozyme matching lysozyme concentrations in cheese between 25 and 300 mg/kg. The repeatability of the assay was evaluated by the extraction of cheese samples spiked with different amounts of lysozyme. The RSD value of the method ranged between 1.4% and 2.9%, determined at four different concentrations (50–400 mg lysozyme/kg cheese, $n = 6$). The accuracy was determined by calculating the recovery rates of the spiked cheese samples. The recovery ranged between 96.8% and 106.2% at four concentrations (50–400 mg lysozyme/kg cheese, $n = 6$) (Supplementary Table 1). The validation parameters are in good agreement with those previously reported. Pellegrino stated a LOD for lysozyme in cheese of 8.2 mg/kg, whereas the ISO protocol gives a LOQ of 5 mg/kg cheese (ISO, 2009; Pellegrino & Tirelli, 2000). Further, a recovery rate of 90% lysozyme from milk with a relative standard deviation of 1.47% was reported (Pellegrino & Tirelli, 2000). Thus, the validation parameters obtained in the present study indicate that the method is suitable for the quantification of lysozyme in commercial cheese samples.

3.3. Stability of lysozyme during cheese ripening

Four lots of independently produced cheese wheels were stored for 54 weeks to analyse the stability of lysozyme in the cheese products. Three lots were produced with lysozyme. The fourth lot was produced accordingly, but without adding lysozyme. Fig. 1 shows representative chromatograms of the cheese samples produced with and without lysozyme after 54 weeks of storage. The lysozyme content was determined every 18 weeks during storage. Since the water content of the cheese wheels decreased during storage, the lysozyme concentration of the samples was referred to the dry matter of the cheese. The dry matter increased from $60.4 \pm 1.1\%$ to $72.5 \pm 0.8\%$ during storage for 54 weeks. In the three lysozyme-containing cheese wheels, the content of lysozyme was relatively stable throughout the storage time. The level of lyso-

zyme in all samples was 282.5 ± 14.1 mg lysozyme per kg dry matter of cheese. This correlates with a content of lysozyme of 170.6 mg lysozyme/kg cheese and 204.8 mg lysozyme/kg cheese after 54 weeks of storage (both referring to the fresh products). In the lysozyme-free cheese, no peaks with the retention time of lysozyme could be detected during the entire storage time. In the samples produced with lysozyme, however, a satellite peak was detected that eluted with a retention time of about 13 min shortly before lysozyme (retention time ~ 15 min).

3.4. Analysis of lysozyme in commercial cheese samples

Forty-six commercially available cheese samples were investigated with the optimised HPLC method. The study focused on hard cheese produced in Italy. Lysozyme was clearly detected in 30 of the samples. The declaration of lysozyme was missing in seven cases. The concentration of lysozyme in these samples ranged from 30.8 to 386.2 mg lysozyme/kg cheese. The majority of cheese samples contained between 150 and 250 mg lysozyme/kg cheese. Lysozyme was typically detected in Grana Padano (15 out of 15) and grated hard cheese mixtures (10 out of 18). No addition of lysozyme was observed in any of the Parmigiano-Reggiano cheese samples. The highest amounts of lysozyme were found in semi-hard cheeses of goat. In contrast, the analysed Pecorino cheese samples made from sheep milk were free of lysozyme, whereas lysozyme was detected in two other products from sheep milk. Table 1 gives a summary of the results.

The chromatograms of the commercial cheese samples containing lysozyme always showed the satellite peak that had been observed before in the cheese samples stored for 54 weeks. The median area of the second peak was 28.0% of the lysozyme peak. This peak could also be detected in the lysozyme standard and in lysozyme preparations for cheese manufacturing. The nature of this peak, however, had not been determined (Pellegrino & Tirelli, 2000).

3.5. Origin of the LSP

In order to determine the origin of the satellite peak of lysozyme, the lysozyme standard was analysed. In the standard, the satellite peak was detected, but the ratio of the satellite peak to the lysozyme peak was much smaller (about 4%). The lysozyme standard was heated at 99 °C for different periods of time to analyse if the satellite peak represents a product of lysozyme. Heating of the standard led to a decrease of the lysozyme peak and an increase of the satellite peak (Fig. 2). Thus, it can be concluded that the satellite peak is formed from native lysozyme.

Furthermore, the lysozyme content and the ratio of the satellite peak to the lysozyme peak were investigated in commercially available lysozyme formula for cheese manufacturing (Supplementary Fig. 1). All preparations were in powder form and showed almost the same lysozyme content. The ratio of the two peaks was 6.5–11.1%, which was higher than in the lysozyme standard (about

4%), but lower than the ratios determined in the cheese samples (Table 1).

In the cheese samples stored for 0–54 weeks, a ratio of $12.6 \pm 0.1\%$ was determined immediately after cheese production. The ratio of the two peaks increased significantly to $17.6 \pm 1.0\%$ during the 54 weeks of storage (Fig. 3). It can be excluded that

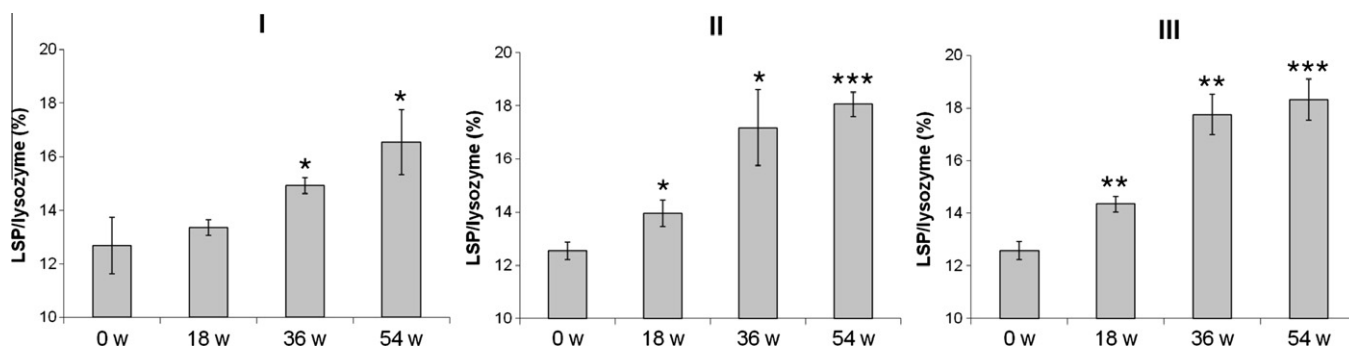


Fig. 3. Peak ratio of LSP and lysozyme in the three different lots of cheese (I–III) during storage for 0, 18, 36, and 54 weeks. Mean $\pm$ standard deviation ($n = 4$) is presented (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to 0 week).

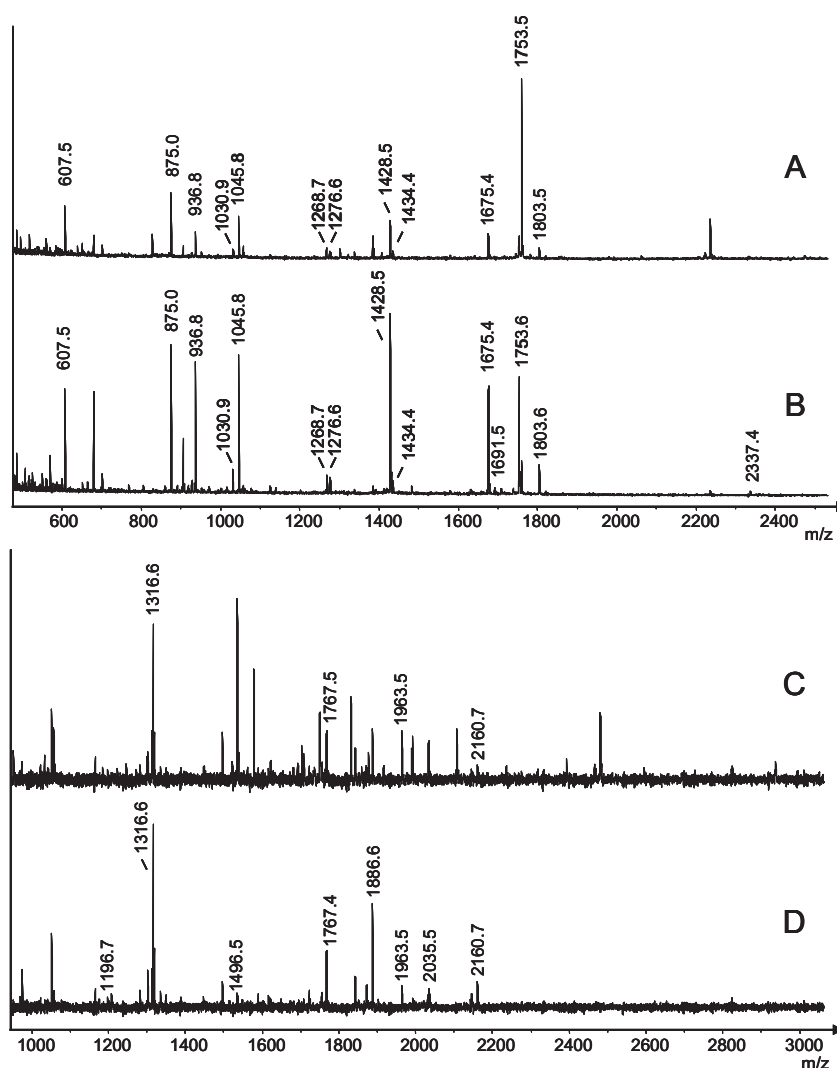


Fig. 4. Peptide mass fingerprint of LSP and lysozyme isolated by HPLC from a lysozyme containing cheese sample. The mass spectra were obtained by MALDI-TOF-MS. (A) LSP digested with trypsin; (B) lysozyme digested with trypsin; (C) LSP digested with Asp-N; (D) lysozyme digested with Asp-N.

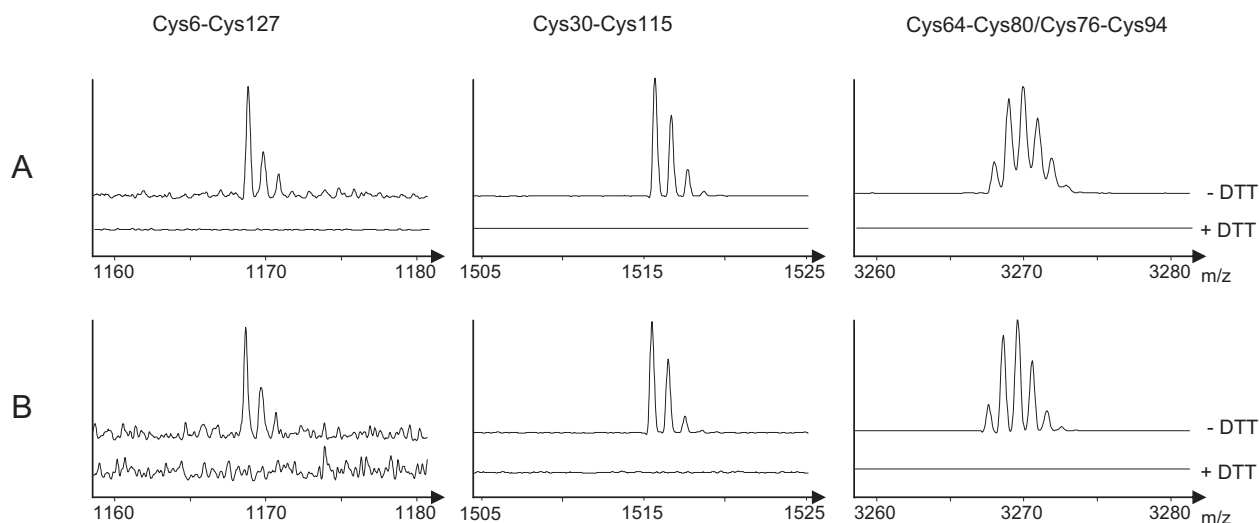


Fig. 5. Peptide mass fingerprint of LSP (A) and lysozyme (B) isolated from a heated lysozyme standard without (–DTT) and with (+DTT) reduction with DTT. The spectra show the signals of the disulphide containing peptides Cys6–Cys127 ($m/z = 1168.6$), Cys30–Cys115 ($m/z = 1515.7$), and Cys64–Cys80/Cys76–Cys94 ($m/z = 3268.5$).

the increase of LSP was induced by sample preparation or the solvent used for HPLC analysis, because all samples were prepared and analysed identically. Thus, it can be concluded that a minor part of LSP is already present in the lysozyme preparation added to the kettle milk, originating most likely from the technical preparation of the formula. Additional lysozyme degradation occurs during cheese production or ripening.

3.6. Characterisation of the LSP

To obtain information about the structure of the LSP, it was further analysed by mass spectrometry. For this purpose, lysozyme and LSP were isolated by HPLC from an industrially produced lysozyme-containing cheese and from a heated lysozyme standard. First, the protein mass was measured by MALDI-TOF-MS. All the fractions contained a main component with m/z 14,313, corresponding to the mass of lysozyme (data not shown). The primary structure of both proteins was further studied by their peptide mass fingerprints. The proteins were digested with the enzymes trypsin or Asp-N, respectively, and analysed by MALDI-TOF-MS (Fig. 4). The peptide mass fingerprints of both proteins showed very good agreement. The sequence coverage for lysozyme using trypsin was 71.3% for the degradation product and 89.1% for the native lysozyme. Using Asp-N, the sequence coverage of 58.1% for the degradation product and 86.1% for the native lysozyme was found. The combined sequence coverage was 84.5% for LSP and 100% for the native lysozyme. The lower sequence coverage of LSP was most likely caused by the lower signal to noise ratio preventing the detection of some low intensity signals. Both the mass spectra of the intact proteins as well as the peptide mass fingerprint prove that LSP possesses the same primary structure as lysozyme, excluding proteolytic processes or the formation of nonenzymatic posttranslational modifications (Humeny, Kislinger, Becker, & Pischetsrieder, 2002).

The differences in the chromatographic behaviour of both proteins can either be explained by disulphide scrambling or by the generation of a conformational isomer. Chang and Li (2002) investigated disulphide scrambling in lysozyme, which was treated with denaturants or heat. Among 104 possible scrambled isomers, the three most predominant ones have been identified. Whereas native lysozyme is characterised by the disulphide bonds (Cys6–Cys127, Cys30–Cys115, Cys64–Cys80, and Cys76–Cys94), the scrambled isomers differed from the native protein by the presence of (Cys6–Cys30, Cys64–Cys76, Cys80–Cys94, and Cys115–Cys127),

(Cys6–Cys30 and Cys115–127), or (Cys6–Cys30, Cys64–Cys76, Cys80–Cys115, and Cys94–Cys127).

In order to analyse disulphide scrambling, the peptide mass fingerprint of lysozyme and LSP was recorded before and after reduction with DTT. Peptides containing a disulphide bridge disappear in the spectrum after reduction with DTT and two new reduced peptides appear instead. Thus, disulphide containing peptides can easily be identified and characterised. For both proteins, the same disulphide bridged peptides were detected after tryptic digest (Fig. 5). Thus, the most predominant scrambling from Cys6–Cys127/Cys30–Cys115 to Cys6–Cys30, which was present in all the scrambled isomers described by Chang and Li, could be excluded (Chang & Li, 2002). Scrambling among Cys64/Cys76/Cys80/Cys94 is more difficult to study, because the amino acid sequence 76–80 is not prone to enzymatic cleavage using common endoproteases. Nevertheless, it was shown that LSP does not correlate to any of the scrambled lysozyme isomers described in literature and was, therefore, assigned to a conformational isomer of lysozyme. Circular dichroism spectroscopy could not be applied to compare the conformation of lysozyme and LSP because too little substance was available after HPLC fractionation. As a consequence, it is proposed that LSP should be taken into consideration, when lysozyme is analysed in cheese by HPLC.

To estimate the amount of LSP in commercially available cheese products, LSP was quantified using lysozyme as external standard (Table 1). Further, the content of total lysozyme (native lysozyme + LSP) is given. In average, the quantity of LSP amounted to 22% of the total lysozyme content.

Acknowledgement

The financial support by the Bavarian State Ministry of the Environment and Public Health is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.03.010.

References

- Bärtschi, F., Mural, L., Rieder, K., Kämpfer, U., & Schaller, J. (2006). Bestimmung von Lysozym in Käse mittels LC-MS. *Mitteilung aus dem Gebiet der Lebensmitteluntersuchung und Hygiene*, 97, 478–488.

- Bernhisel-Broadbent, J., Dintzis, H. M., Dintzis, R. Z., & Sampson, H. A. (1994). Allergenicity and antigenicity of chicken egg ovomucoid (Gal d III) compared with ovalbumin (Gal d I) in children with egg allergy and in mice. *Journal of Allergy and Clinical Immunology*, 93(6), 1047–1059.
- Busse, M., Heilmeyer, J., Klostermeyer, H., Teuber, M., Krusch, U., Prokopenk, D., et al. (1986). *Fortbildungsseminar Lysozym in der Käseherstellung*. Weihenstephan: Sonderdruck der dmz deutsche molckerei-zeitung.
- Chang, J. Y., & Li, L. (2002). The unfolding mechanism and the disulfide structures of denatured lysozyme. *FEBS Letters*, 511(1–3), 73–78.
- Dragoni, I., Balzaretto, C., Rossini, S., Rossi, L., Dell'Orto, V., & Baldi, A. (2010). Detection of hen lysozyme on proteic profiles of Grana Padano cheese through SELDI-TOF MS high-throughput technology during the ripening process. *Food Analytical Methods* doi:10.1007/s12161-02010-19146-12164.
- Fremont, S., Kanny, G., Nicolas, J. P., & Moneret-Vautrin, D. A. (1997). Prevalence of lysozyme sensitization in an egg-allergic population. *Allergy*, 52(2), 224–228.
- Gorin, G., Wang, S. F., & Papapavlou, L. (1971). Assay of lysozyme by its lytic action on *M. lysodeikticus* cells. *Analytical Biochemistry*, 39(1), 113–127.
- Guerin-Dubiard, C., Pasco, M., Hietanen, A., Quiros del Bosque, A., Nau, F., & Croguennec, T. (2005). Hen egg white fractionation by ion-exchange chromatography. *Journal of Chromatography A*, 1090(1–2), 58–67.
- Hoffman, D. R. (1983). Immunochemical identification of the allergens in egg white. *Journal of Allergy and Clinical Immunology*, 71(5), 481–486.
- Holen, E., & Elsayed, S. (1990). Characterization of four major allergens of hen egg-white by IEF/SDS-PAGE combined with electrophoretic transfer and IgE-immunoblotting. *International Archives of Allergy and Immunology*, 91(2), 136–141.
- Humeny, A., Kislenger, T., Becker, C. M., & Pischetsrieder, M. (2002). Qualitative determination of specific protein glycation products by matrix-assisted laser desorption/ionization mass spectrometry peptide mapping. *Journal of Agricultural and Food Chemistry*, 50(7), 2153–2160.
- Iaconelli, A., Fiorentini, L., Bruschi, S., Rossi, F., Mingrone, G., & Piva, G. (2008). Absence of allergic reactions to egg white lysozyme additive in Grana Padano cheese. *Journal of the American College of Nutrition*, 27(2), 326–331.
- ISO (2009). *ISO/TS 27105:2009 (IDF 216: 2009) milk and milk products – Determination of hen's egg white lysozyme by HPLC*. Geneva, Switzerland: International Organization of Standardization.
- Kerkaert, B., Mestdag, F., & De Meulenaer, B. (2010). Detection of hen's egg white lysozyme in food: Comparison between a sensitive HPLC and a commercial ELISA method. *Food Chemistry*, 120, 580–584.
- McKenzie, H. A., & White, F. H. Jr., (1986). Determination of lysozyme activity at low levels with emphasis on the milk enzyme. *Analytical Biochemistry*, 157(2), 367–374.
- Pellegrino, L., & Tirelli, A. (2000). A sensitive HPLC method to detect hen's egg white lysozyme in milk and dairy products. *International of Dairy Journal*, 10(7), 435–442.
- Rauch, P., Hochel, I., & Kás, J. (1990). Sandwich enzyme immunoassay of hen egg lysozyme in foods. *Journal of Food Science*, 55(1), 103–105.
- Rauch, P., Poplstein, M., Hochel, I., Fukal, L., Ferri, E., Abagnato, C. A., et al. (1995). Enhanced chemiluminescent sandwich enzyme immunoassay for hen egg lysozyme. *Journal of Bioluminescence and Chemiluminescence*, 10(1), 35–40.
- Schneider, N., Becker, C. M., & Pischetsrieder, M. (2010a). Analysis of lysozyme in cheese by immunocapture mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, 878(2), 201–206.
- Schneider, N., Weigel, I., Werkmeister, K., & Pischetsrieder, M. (2010b). Development and validation of an enzyme-linked immunosorbent assay (ELISA) for quantification of lysozyme in cheese. *Journal of Agricultural and Food Chemistry*, 58(1), 76–81.
- Wasserfall, F., & Teuber, M. (1979). Action of egg white lysozyme on *Clostridium tyrobutyricum*. *Applied and Environmental Microbiology*, 38(2), 197–199.
- Yoshida, A., Takagaki, Y., & Nishimune, T. (1991). Enzyme immunoassay for hen egg white lysozyme used as a food additive. *Journal of the Association of Official Analytical Chemists*, 74(3), 502–505.