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Analysis of lysozyme in cheese by immunocapture mass spectrometry☆

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ABSTRACT

The enzyme lysozyme is used as a preservative to prevent late blowing of ripened cheese, caused by *Clostridium tyrobutyricum*. Since the enzyme is extracted from hen egg white, lysozyme has to be declared on food product labels as a potential allergen. Here, a method is reported that combines immunocapture purification and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis for the detection of lysozyme in cheese samples. Cheese extracts were treated with magnetic particles coated with a monoclonal antibody directed against lysozyme. After immunocapture purification, lysozyme was detected by MALDI-TOF-MS. The limit of detection of the assay was about 5 mg/kg lysozyme in cheese. The method reliably distinguished between cheese samples which had been produced with and without lysozyme. Thus, the novel assay allows the reliable, sensitive, and specific detection of lysozyme in a food matrix. The assay could be easily adapted to other target peptides and proteins in complex food matrices and, therefore, has a broad application potential, e.g. for the analysis of allergens.

1. Introduction

The enzyme lysozyme (muramidase, EC. 3.2.1.17) shows lytic activity on the cell wall of some, mainly gram-positive bacteria [1]. Therefore, lysozyme is used in cheese manufacturing to prevent late blowing during the ripening of cheese caused by *Clostridium tyrobutyricum* [2]. Lysozyme is permitted as a preservative (E1105) in cheese according to the current EU legislation (European Parliament and Council Directive No. 95/2/EC) and the Codex Alimentarius (Codex Standard 283-1978). The estimated content of lysozyme ranges between 50 and 350 mg lysozyme per kilogram cheese [3]. The source of lysozyme used for cheese production is hen egg white. Since egg and egg products belong to the eight major food groups that account for 90% of food allergies, the use of lysozyme has to be declared according to the allergen labelling instructions of EU and FDA.

The role of the lysozyme itself as an allergen is still controversially discussed. Clinical reactions to hen egg white lysozyme have been described and IgE anti-lysozyme antibodies as markers for sensitization have been found in 35% of egg allergic consumers [4]. The evaluation of the allergenic potential of lysozyme ranges from weak allergen [5] to one of the major allergens of egg white [6].

For the analysis of lysozyme in food, the JECFA (Joint FAO/WHO Expert Committee on Food Additives) advises the turbidimetric determination of lysozyme. The method is based upon the lytic activity of lysozyme towards bacterial cells of *Micrococcus luteus* (*Micrococcus lysodeicticus*) quantified by turbidimetric analysis [7,8]. Other methods to detect the use of lysozyme include sodium dodecyl sulphate (SDS) gel electrophoresis [9], chromatography by either HPLC [10] or LC–MS [3], capillary zone electrophoresis [11] or immunoassays [12–14].

In the last years, mass spectrometry has become a powerful method for protein and peptide profiling in food analysis [15]. Both matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization mass spectrometry (ESI-MS) play an important role in the detection and characterization of proteins in milk and milk products. In particular, MALDI-TOF-MS has been successfully applied to detect adulteration [16,17] and thermal treatment [18] of milk and milk products. MALDI-TOF-MS offers several advantages compared to other ionization techniques. For example, it is highly sensitive, easy to handle and especially fast. However, the presence of high-abundant proteins in most of the complex samples impedes the

Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HEW lysozyme, hen egg white lysozyme; 2,5-DHAP, 2,5-dihydroxyacetophenone; HCCA, α-cyano-4-hydroxycinnamic acid; 2,5-DHB, 2,5-dihydroxybenzoic acid; TFA, trifluoroacetic acid; MB-IAC Prot G, magnetic bead based immunoaffinity chromatography on immobilized protein G.

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analysis of lower abundant proteins. Furthermore, salts also interfere considerably with mass spectrometry due to the formation of adducts, as well as impaired ionization, co-crystallization and mass resolution [19]. Therefore, MS-based methods for the analysis of complex protein mixtures require extraction steps before analysis, thus limiting their application for high throughput screening. To obtain higher specificity and sensitivity, an immunoaffinity isolation step can be used prior to MALDI-TOF-MS measurement of trace levels of defined proteins in complex samples. Whereas conventional immunoaffinity chromatography is rather time consuming [20], the use of antibodies coupled to magnetic beads (immunocapture) allows fast and specific purification of small sample volumes. Immunocapture purification using magnetic beads has been developed for the cleanup of biological fluids, e.g. ascites, serum or protein extracts from cultured cells or tissue [21,22].

In the present study, immunocapture mass spectrometry was applied for the rapid detection of the allergen and preservative lysozyme in food products.

2. Experimental

2.1. Chemicals

The immunocapturing kit MB-IAC Prot G (magnetic bead based immunoaffinity chromatography on immobilized protein G), 2,5-dihydroxyacetophenone (2,5-DHAP) and the peptide standard were purchased from Bruker Daltonik (Bremen, Germany). α-Cyano-4-hydroxycinnamic acid (HCCA), 2,5-dihydroxybenzoic acid (2,5-DHB), sinapic acid, diammonium hydrogen citrate, dimethyl pimelimidate dihydrochloride, and dithiotreitol were obtained from Fluka (Taufkirchen, Germany). Hen egg white (HEW) lysozyme, trifluoroacetic acid (TFA), α-lactalbumin, and β-lactoglobulin were supplied by Sigma–Aldrich (Taufkirchen, Germany). Ammonium dihydrogen phosphate and tris(hydroxymethyl)aminomethane were from Acros (Geel, Belgium), methanol and acetonitrile from Fisher Scientific (Schwerte, Germany). Monoclonal mouse anti-chicken lysozyme antibody was supplied by Biotrend (Cologne, Germany) and C18 Zip Tips by Millipore (Schwalbach, Germany). Modified trypsin of sequencing grade was obtained from Roche (Mannheim, Germany).

2.2. Cheese samples

Cheese samples (Manchego, Grana Padano, Parmigiano Reggiano and hard cheese mixtures) were purchased in a local supermarket.

2.3. Extraction of HEW lysozyme from cheese

An amount of 2 g of grated cheese was weighed into a 100 mL flask and 20 mL of sodium chloride solution (1 mol/L in water) was added. The sample was homogenized with a T18 basic Ultra Turrax (IKA, Staufen, Germany). The homogenizer was rinsed with 20 mL of sodium chloride solution (1 mol/L), which was then added to the homogenate. The pH of the combined suspensions was adjusted dropwise with sodium hydroxide solution (1 mol/L) to pH 6.0. The mixture was stirred at room temperature for 1 h in a closed flask and the pH was then adjusted dropwise with hydrochloric acid (1 mol/L) to pH 4.3. Sodium chloride solution (1 mol/L) was added to the suspension to a total volume of 50 mL. After standing for 15 min, the suspension was filtered through a fluted paper filter (Macherey-Nagel, Dueren, Germany) and then through a membrane filter (0.22 μm; Roth, Karlsruhe, Germany).

2.4. Spiking of cheese samples

A standard solution of lysozyme was prepared by dissolving lysozyme in 1 mol/L sodium chloride in water to a concentration of 1 mg/mL. To spike a cheese sample with a specific amount of lysozyme, 2 g of a lysozyme-free cheese was weighed into a 100 mL flask and 300 μL of the standard lysozyme solution were added, corresponding to a concentration of 150 mg lysozyme per kilogram cheese. Afterwards, the cheese sample was extracted as described before.

2.5. Purification of the cheese extract by C18 Zip Tips

The cheese extract was further purified by C18 Zip Tips before MALDI-TOF-MS analysis. Before purification, 9 μL of the extract was mixed with 1 μL of 1% TFA. The Zip Tips were wetted twice with 10 μL acetonitrile and equilibrated with 0.1% TFA. Proteins were bound to the material by aspirating the sample 10 times. Following a wash step by aspirating 10 μL of 0.1% TFA five times, peptides were eluted by aspirating 10 μL of 50% acetonitrile/0.1% TFA 10 times.

2.6. Selective purification of the cheese extract by immunocapture based on the use of magnetic beads

Alternatively, the cheese samples were purified by IAC Prot G magnetic beads. The purification was carried out according to the manufacturer's instructions using a magnetic separator. An aliquot of 15 μL of the protein G carrying magnetic particles was washed twice with 100 μL of antibody immobilization buffer. The magnetic beads were resuspended in 35 μL of antibody immobilization buffer and 15 μL of monoclonal mouse anti-chicken lysozyme antibody (1 mg/mL). After incubation for 1 h at room temperature under gentle agitation, the magnetic beads were washed twice with antibody immobilization buffer and twice with antigen binding buffer. Then, the magnetic beads were incubated in 10 μL of antigen binding buffer and 5 μL of the filtered cheese extracts for 2 h at room temperature under gentle agitation. The beads were washed twice with 100 μL antigen binding buffer and twice with 100 μL of antigen wash solution. For elution, 10 μL of elution solution was mixed thoroughly with the magnetic beads. After 20 min, the supernatant containing antibodies and antigen was transferred into a fresh tube.

Possibly co-eluted IgG did not interfere with mass analysis, when the intact lysozyme was eluted from the IMAC beads. However, when the proteins were enzymatically hydrolyzed after immunocapture purification, many interfering signals from peptide fragments were recorded impeding spectrum analysis. To elute only the antigen, an additional crosslinking was performed after the antibodies were bound to the protein G beads, when lysozyme was enzymatically digested after elution. For this purpose, the beads were rinsed twice with 100 μL of crosslinking buffer after the incubation of the magnetic beads with the antibodies. The beads were then resuspended in 50 μL of 10 mM dimethyl pimelimidate dihydrochloride and incubated under gentle agitation for 30 min. To stop the reaction, 50 μL of blocking reagent was added and incubated with the beads for 15 min. Then, the normal procedure was followed as described above.

2.7. Enzymatic protein hydrolysis

For the enzymatic protein hydrolysis, 2 g of grated cheese was extracted as described in Section 2.3, using, however, only half the volume of the sodium chloride solution, so that the final volume of the suspension amounted to 25 mL. After purification of the cheese extracts with the crosslinked immunocapture beads, the solution was freeze-dried and then dissolved in 6 μL of 100 mM Tris–HCl

buffer (pH 8.5). After addition of 3 μL of trypsin (0.3 μg), the solution was incubated for 15 h at 37 °C. The resulting peptides were reduced by adding 1 μL of 100 mM dithiotreitol and left for 30 min at room temperature. The tryptic digest was further purified by C18 Zip Tips before MALDI-TOF-MS analysis. The digest was mixed with 1 μL of 1% TFA. The Zip Tips were wetted twice with 10 μL acetonitrile and equilibrated with 0.1% TFA. Peptides were bound to the material by aspirating the sample 10 times. Following two washing steps by aspirating 10 μL 0.1% TFA five times and then 10 μL 5% methanol/0.1% TFA five times, the peptides were eluted by aspirating 5 μL of 50% acetonitrile/0.1% TFA 10 times.

2.8. MALDI-TOF-MS analysis

For MALDI-TOF-MS analysis of the intact proteins, 2 μL of the protein solutions was mixed with 2 μL of 2% TFA in water and 2 μL of a DHAP matrix. For the DHAP matrix, 7.6 mg (50 μmol) of 2,5-DHAP was suspended in 375 μL ethanol. After addition of 125 μL aqueous diammonium hydrogen citrate solution (10 μM), the suspension was vortexed vigorously and sonicated for at least 15 min. An aliquot of 1 μL was spotted twice onto a stainless steel target and subsequently air-dried.

For the measurement of the peptides obtained by enzymatic hydrolysis, 2 μL of the reduced digest was diluted with 4 μL of a matrix consisting of a 1 + 1 mixture of a saturated solution of HCCA in 50% acetonitrile/0.1% TFA and a solution of 10 mM ammonium dihydrogen phosphate in 50% acetonitrile/0.1% TFA. An aliquot of 1 μL was spotted twice onto a stainless steel target and air-dried.

The MALDI-TOF-MS analysis was performed on a Bruker Autoflex (Bruker Daltonik, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm). Measurements of intact and digested proteins were carried out using delayed extraction (330 and 80 ns, respectively). Laser desorbed positive ions were analyzed after acceleration by 20 kV in the linear mode for the intact proteins and by 19 kV in the reflector mode for the peptide digest. For the intact proteins, external calibration was performed using a mix of bovine α-lactalbumin, HEW lysozyme and bovine β-lactoglobulin variants A and B. For peptides, external calibration was carried out with angiotensin I and II, substance P, bombesin, adrenocorticotropic hormone (ACTH) clip 1-17 and ACTH clip 18-39. For each displayed mass spectrum, at least 300 individual spectra obtained from several positions on a spot were averaged.

3. Results

3.1. Analysis of lysozyme in cheese by MALDI-TOF-MS after C18 purification

The purpose of the study was to develop a rapid method for the detection of the preservative and potential allergen lysozyme in cheese. During cheese manufacturing, lysozyme is transferred to the curd, probably by association of the enzyme with the caseins [23]. Lysozyme added to kettle milk binds to the caseins [24], but also to the whey proteins α-lactalbumin and β-lactoglobulin [25]. The binding is particularly caused by electrostatic interactions between the positively charged lysozyme and the milk proteins, which are negatively charged at the pH-value of about 7 in the kettle milk. Since temperature has only a small effect on the association, it is unlikely that hydrophobic interactions play a substantial role in this process. Therefore, the association of lysozyme to caseins must be disrupted prior to analysis. In the present study, a highly concentrated solution of sodium chloride was used to desorb lysozyme [10]. In the presence of the high salt concentrations, caseins could subsequently be re-precipitated without co-precipitation of lysozyme.

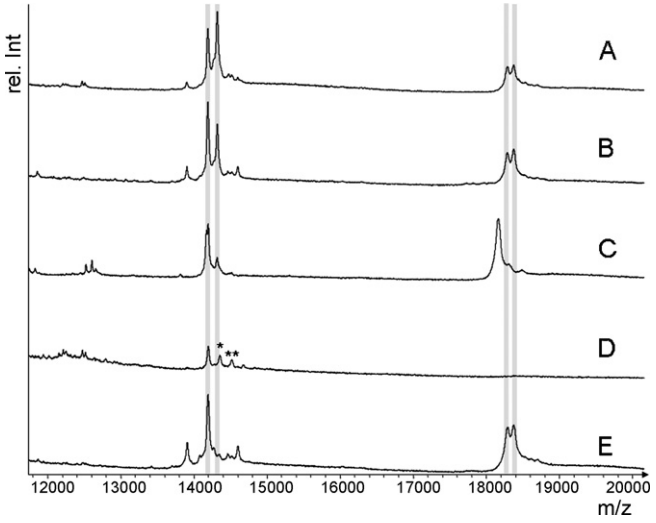

Fig. 1. MALDI-TOF mass spectra of different cheese samples after extraction with sodium chloride and purification with C18 Zip Tips. (A) Grana Padano; (B) Raclette cheese; (C and D) hard cheese mixture; (E) Parmigiano Reggiano. The use of lysozyme was declared on the labels of samples A–D, sample E did not contain lysozyme and served as control. The proteins α-lactalbumin (m/z = 14,186), β-lactoglobulin (variant A m/z = 18,366, variant B m/z = 18,281), and lysozyme (m/z = 14,313) are highlighted by grey boxes. The signals of the hexose adduct (*) and lactose adduct (**) of lactalbumin are marked.

The lysozyme extracts, which also contain residues of the whey proteins, were then subjected to MALDI-TOF-MS analysis. Since the presence of sodium inhibits the co-crystallization of the protein sample with the matrix and leads to sodium adducts, which impede the interpretation of the MALDI-TOF mass spectra [26], the extracts were desalted prior to mass spectrometry using C18 Zip Tips. For MALDI-TOF-MS analysis, the matrices sinapic acid, 2,5-DHB, and 2,5-DHAP were tested. Among those, 2,5-DHAP showed the best signal intensity and the best signal-to-noise ratio and was therefore used for further analysis.

With this method, several commercially available cheese samples were analyzed, for which the use of lysozyme was declared on the label (Fig. 1). In the mass spectrum, several peaks are clearly visible in the range of 12–20 kDa. The peaks were assigned to the whey proteins α-lactalbumin (m/z 14,186) and β-lactoglobulin variant B (m/z 18,281) and variant A (m/z 18,367). In samples A and B, additionally a clear signal was detected at m/z 14,313 for lysozyme. In sample C, a distinct, but minor signal for lysozyme was visible. In contrast, lysozyme was not detected in sample D, although it was declared on the label. Instead, signals for a hexose adduct and the lactose adduct of α-lactalbumin appeared in the mass spectrum [27], which probably disguise the signal for lysozyme. Glycated forms of α-lactalbumin are most likely caused by thermal treatment during cheese production. Sample E, a cheese without lysozyme, served as control.

3.2. Analysis of lysozyme in cheese by MALDI-TOF-MS after immunocapture purification

In order to improve the specificity and sensitivity of the method, the samples were prepurified by magnetic bead based immunoaffinity purification (MB-IAC Prot G). The general scheme of the method is shown in Fig. 2. In the first step, a commercial monoclonal mouse anti-chicken lysozyme antibody is immobilized on protein G carrying magnetic beads. These particles are then incubated with the cheese extracts. Only lysozyme binds highly specifically to the antibody on the magnetic beads. All other proteins and interferents are removed by washing. After elution, the

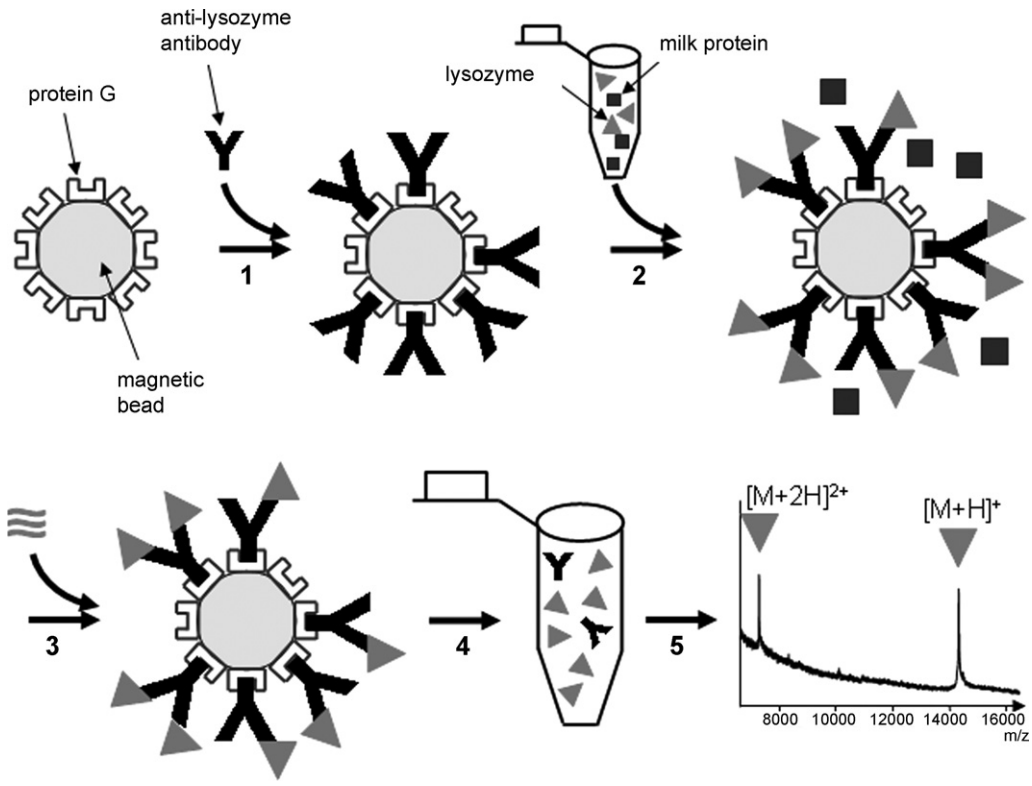

Fig. 2. Scheme of the detection of HEW lysozyme in cheese by immunoaffinity capture purification and MALDI-TOF-MS. (1) Immobilization of the antibodies on the magnetic beads; (2) antigen capturing procedure; (3) removal of non lysozyme components by a wash step; (4) elution of lysozyme and antibodies; (5) analysis by MALDI-TOF-MS.

extracts are analyzed by MALDI-TOF-MS. A cheese sample was spiked with a technical relevant lysozyme concentration. Fig. 3 shows the mass spectra obtained from the cheese sample before (A) and after (B) immunocapture purification. After immunocapture purification, only the signal of lysozyme at m/z = 14,313 was visible in the spectrum, indicating a highly specific enrichment of lysozyme. Furthermore, a signal for double charged lysozyme appeared at m/z = 7157 (data not shown). A signal for α-lactalbumin was not detected indicating that the antibody does not cross-react with α-lactalbumin, which has a sequence similarity of about 60% to lysozyme [28].

In the next step, the detection limit of the method was determined. For this purpose, lysozyme-free cheese was spiked with different concentrations of lysozyme. The resulting mass spectra are shown in Fig. 4. Concentrations as low as 5 mg/kg could still be readily detected with a signal-to-noise ratio of higher than 5:1, indicating the detection limit of the method. In contrast, the limit of detection was 50 mg/kg, when the samples were purified by C18 Zip Tips (data not shown). Since the technologically relevant concentrations of lysozyme as preservative in cheese range between 50 and 350 mg/kg, the limit of detection should be sufficiently low to distinguish between cheese samples produced with and without lysozyme.

Thus, the cheese samples, which had been analyzed before by MALDI-TOF-MS after C18 Zip Tip purification (see Fig. 1), were reinvestigated with this improved method. After immunocapture MALDI-TOF-MS analysis, lysozyme, which was identified by specific binding to the antibody as well as by its specific mass, was clearly detected in all samples (Fig. 5), for which lysozyme has been declared. The control sample E, which was produced without lysozyme, did not show a signal.

3.3. Confirmation of protein identity by its peptide mass fingerprint

The protein identity was further confirmed by its peptide mass fingerprint. For this purpose, lysozyme was subjected to partial

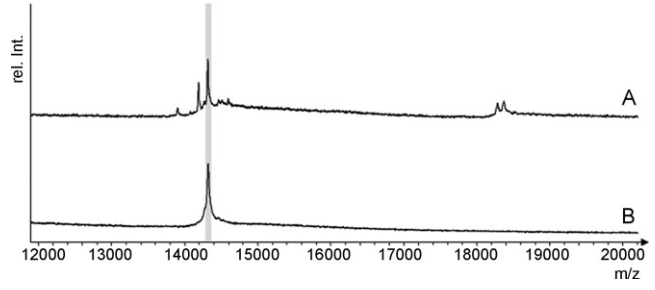

Fig. 3. MALDI-TOF mass spectrum of lysozyme-spiked cheese (150 mg/kg cheese) before (A) and after (B) immunocapture purification. Lysozyme is highlighted by a grey box.

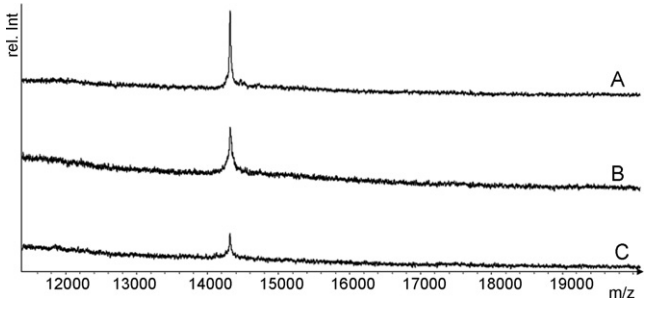

Fig. 4. MALDI-TOF mass spectra of cheese samples spiked with different amounts of lysozyme. A = 50 mg/kg, B = 25 mg/kg, C = 5 mg/kg lysozyme. The cheese extracts were purified by immunoaffinity capture before mass spectroscopy. A concentration of 5 mg/kg lysozyme was determined as the detection limit.

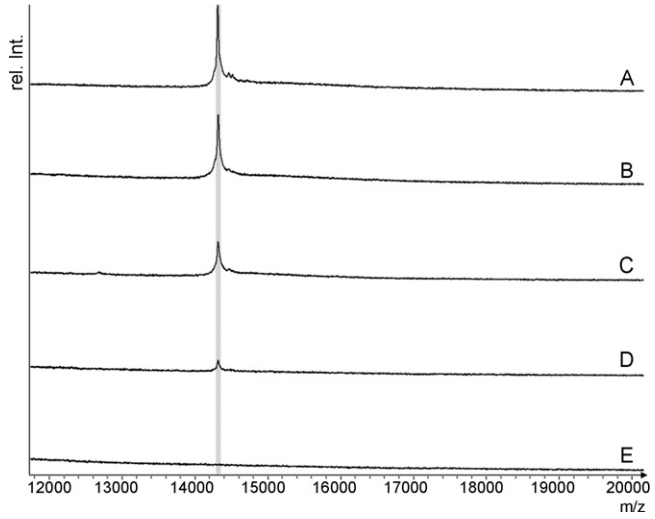

Fig. 5. MALDI-TOF mass spectra of different cheese samples after extraction with sodium chloride and purification with immunocapture beads. (A) Grana Padano; (B) Raclette cheese; (C and D) hard cheese mixture; (E) Parmigiano Reggiano. The use of lysozyme was declared on the labels of samples A–D, sample E did not contain lysozyme and served as control. Lysozyme (m/z = 14,313) is highlighted by a grey box.

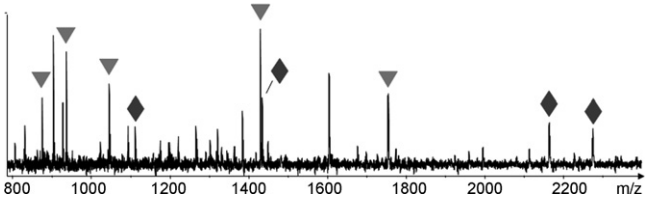

Fig. 6. Verification of the lysozyme identity by peptide mass fingerprint. MALDI-TOF mass spectrum of a cheese sample containing lysozyme was recorded after extraction by crosslinked antibody capture beads and digestion with trypsin. The signals of lysozyme (▼) and self-digest of trypsin (♦) are marked.

enzymatic hydrolysis by trypsin after immunocapture purification and before MALDI-TOF-MS analysis. Since peptides derived from the antibodies coupled to the magnetic beads may interfere with the peptide mass fingerprint of lysozyme, a slightly modified immunocapture method was applied. For this purpose, the anti-lysozyme antibodies were crosslinked to the protein G via the bifunctional coupling reagent dimethyl pimelimidate (DMP). Thus, it was possible to elute pure lysozyme from the magnetic beads, whereas signals from antibody fragments were hardly detected. The spectrum of lysozyme that was extracted from a cheese sample by these antibody capture beads and digested with trypsin is shown in Fig. 6. Five peptides derived from lysozyme appeared in the mass spectrum, corresponding to a sequence coverage of 40%. The software "PeptideMass" (http://www.expasy.org/tools/peptide-mass.html) was used for calculating the sequence coverage. Further peak identification by the peptide mass fingerprint is not really essential for the analysis of lysozyme in cheese in routine analysis, because lysozyme could be correctly identified in all samples by its specific binding to the antibody as well as by its mass. Nevertheless, the option to verify signal identity by peptide mass fingerprint can be interesting if very similar proteins have to be distinguished, which have similar masses and epitopes [29].

4. Discussion

Thus, a simple and rapid method to detect the addition of lysozyme in cheese was developed. For this purpose, magnetic bead based immunoaffinity purification on immobilized protein G for a highly specific cleanup of the cheese extracts was used prior to MALDI-TOF-MS analysis. Thus, it was possible to distinguish reliably between lysozyme-free cheese and cheese to which lysozyme was added during manufacturing.

Immunoassays, mainly in the ELISA format, are broadly used in food analysis, because the tests are usually easy to handle and fast. However, the complexity of the food matrix bears the risk of unspecific binding of the antibody to matrix components or cross-reactivity of the antibody with related structures. Using ELISA techniques, unspecific binding and cross-reactivity cannot be easily distinguished from specific binding, which may lead to false results. On the other hand, mass spectrometry provides highly reliable results due to the detection of the specific mass of the analyte. As mass spectrometry is often interfered by matrix components, such as salts or high-abundant compounds, several purification steps, mostly including liquid chromatography, have to be applied prior to mass spectrometric analysis of food components. Thus, mass spectrometry based methods in food analysis are very reliable, but often rather time consuming and complicated. The coupling of MALDI-TOF-MS with immunoaffinity purification by magnetic beads combines the advantages of both methods. Lysozyme was identified by its specific binding to the anti-lysozyme antibody as well as by its specific mass, rendering the detection of lysozyme in the complex food matrix very reliable. Additionally, lysozyme could be fast and effectively enriched from an aqueous cheese extract using magnetic immunobeads. Combined with MALDI-TOF-MS, the method is, therefore, very rapid and allows a high throughput.

Copper-coated magnetic beads (immobilized metal affinity chromatography, IMAC) have been used before in food analysis. Cu-ions bind to acidic groups and electron donor groups in proteins, so that IMAC was used to enrich peptides and proteins effectively from milk and dairy products prior to MALDI-TOF-MS analysis [18,30]. Immunocapture beads, i.e. magnetic beads coated with specific antibodies, have been developed mainly for clinical chemistry [31]. However, there are few examples for their application in food analysis. Staphylococcal enterotoxin B has been analyzed in milk samples using magnetic beads coated with specific antibodies for purification and MALDI-TOF-MS for detection [32]. In a similar approach, ricin, the toxin of *Ricinus communis*, was analyzed in spiked milk and castor beans [29]. In that case, ricin identification was achieved after tryptic digest using three characteristic peptides to increase further specificity. Antibody coated magnetic nanoparticles were also applied as part of an ELISA for sulphonamide [33].

5. Conclusion

The present study shows that immunocapture MALDI-TOF-MS can be used to reliably detect the addition of lysozyme to cheese. The sensitivity of the method was sufficient for commercial samples and the assay proved to be very rapid and easy to handle. It can be concluded that the presented method can be more generally applied for the analysis of peptide analytes in food, such as other protein based additives or allergens.

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