Characterization of Iraqi sheep milk lysozyme with respect to molecular weight and hydrolytic activity

Khalida Shakir, Marie Walsh, Salih Mohammed

To cite this version:

HAL Id: hal-01201446
https://hal.archives-ouvertes.fr/hal-01201446
Submitted on 17 Sep 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Characterization of Iraqi sheep milk lysozyme with respect to molecular weight and hydrolytic activity

Khalida Shakir · Marie K. Walsh · Salih A. Mohammed

Received: 26 March 2013 / Revised: 1 May 2013 / Accepted: 21 May 2013 / Published online: 20 June 2013 © INRA and Springer-Verlag France 2013

Abstract Lysozyme is an enzyme that can be found in egg white, milk of various species, as well as in animal tissues and secretions and it is antimicrobial to Gram-positive bacteria. The objective of this work was to purify and characterize lysozyme from Iraqi sheep milk with respect to molecular weight and the influence of pH and temperature on the hydrolytic activity. The purification procedure comprised of enzymatic separation of the casein from the whey proteins and filtration of the whey through a 100-kDa membrane. The permeate was applied to a size exclusion column, Sephadex G-75, and fractions were collected and assayed for lysozyme activity. Isoelectric precipitation of alpha-lactalbumin from the Sephadex fractions containing lysozyme activity resulted in a 28.6-fold purification from crude whey with a 21% yield. The molecular weight as determined by size exclusion was between 30 and 34 kDa and was 14.3 kDa as determined by SDS-PAGE analysis. Sheep milk lysozyme was active from 25 to 80 °C with the highest activity at 55–65 °C. The influence of pH on the activity revealed the highest activity at pH 7.5 and the lysozyme was active at a pH range between pH 4.5 and 9.5. This work demonstrated that Iraqi sheep lysozyme was similar to bovine milk lysozyme with respect to hydrolytic activity and molecular weight.

Keywords Lysozyme · Iraqi sheep milk · Lysozyme activity · Molecular weight

K. Shakir
Department of Food Science, College of Agriculture, University of Baghdad, Baghdad, Iraq e-mail: dr_khalida55@yahoo.com

M. K. Walsh (*)
Department of Nutrition, Dietetics and Food Sciences, Utah State University, 8700 Old Main Hill, Logan, USA e-mail: Marie.walsh@usu.edu

S. A. Mohammed
Biology Department, College of Sciences, The University of Mustansiriyah, Baghdad, Iraq
1 Introduction

Lysozyme (E.C. 3.2.1.17) cleaves the beta-(1,4)-glycoside linkages between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls and in chitin and other carbohydrate oligomers. This activity is antimicrobial to Gram-positive bacteria. In addition to glycosidase activity, lysozyme has transglycosylation activity and esterase activity (Kato 2003). Lysozyme exists in chicken egg white, milk from various species, and has been found in animal tissues and secretions. It has also been purified from various plant and microbial materials (Benkerroum 2008).

There are multiple types of lysozymes: the c-type is found in chicken egg white, the g-type is found in Embden goose, the i-type is found in plant, the i-type is found in invertebrates, the b-type is found in bacteria, and the v-type is found in viruses (Benkerroum 2008). Lysozymes differ in amino acid sequence, molecular mass, and extinction coefficients, yet the amino acids in the catalytic center (glutamic and aspartic acid residues) of the active site are conserved. Lysozymes in human, camel, bovine, and equine milks are considered to be the c-type because of their similarity to chicken egg white lysozyme (Benkerroum 2008). The g-type lysozymes have a molecular mass of approximately 20 kDa while the c-type are smaller, generally between 14 and 15 kDa. The isoelectric point for lysozymes ranges from pH 8.6 to pH 11.5 (Benkerroum 2008). Alpha-lactalbumin (α-Lb) also exists in all milks since it is required for lactose synthesis and has an average molecular weight of 14.2 kDa and an isoelectric point between pH 4.2 and 4.5. These properties are useful for the separation of lysozyme from α-Lb.

Chandan et al. (1968) reported that sheep milk contains lysozyme activity, yet no reported literature could be found on the molecular weight or hydrolytic activity of Iraqi sheep milk lysozyme. Therefore, this paper purified and partially characterized Iraqi sheep milk with respect to the influence of pH and temperature on the hydrolytic activity as well as the molecular weight of the enzyme via size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2 Materials and methods

2.1 Materials

Buffer salts, Coomassie blue dye, and Whatman filters were obtained from Thermo Fisher Scientific (Pittsburg, PA, USA). SDS-PAGE gradient gels (5 to 20% acrylamide) were from Jule Inc. (Milford, CT, USA). The bicinchoninic acid (BCA) protein determination kit, dialysis membranes, and SDS-PAGE molecular weight markers and SDS-PAGE sample loading buffer were obtained from Pierce Chemical Co. (Rockford, IL, USA) and molecular weight membranes were obtained from Millipore (Belrica, MA, USA). Bovine serum albumin, thyroglobulin, porcine pepsin, human insulin, chicken egg white lysozyme, lyophilized cells of Micrococcus lysodeikticus, and Sephadex G-75 were from Sigma-Aldrich (St. Louis, MO, USA).
2.2 Lysozyme purification

Iraqi bulk sheep milk was obtained from the Research Center of Sheep & Goat Milk in Iraq. The casein was separated from the whey via coagulation using local chymosin; the whey fraction was obtained via filtration through a Whatman number 1 filter and was lyophilized. The whey was reconstituted in 50 mM phosphate buffer pH 7, and the protein concentration as determined by the BCA protein assay was 10 g protein.100 mL−1. The whey was then passed through a 100-kDa filter and the permeate was applied to a Sephadex G-75 size exclusion column (1.5×60 cm) with 0.1 mM phosphate buffer pH 7 as the eluant. Eluent fractions were collected each 3.5 mL and the absorbance at 280 nm and lysozyme activity (as described below) were determined for each fraction. The protein standards for size exclusion were thyroglobulin (660–690 kDa), bovine serum albumin (66 kDa), pepsin (34.6 kDa), chicken egg white lysozyme (14.3 kDa), and human insulin (5.81 kDa). A graph of the log molecular weight vs. the elution volumes of the standard proteins divided by the void volume was generated to calculate the molecular weight of lysozyme based on its elution. Ten purification runs were conducted and the fractions containing lysozyme activity were pooled, dialyzed against water with a 10,000 molecular weight cutoff membrane, and lyophilized.

Lyophilized protein (100 mg) was resuspended in 50 mM sodium acetate buffer, pH 4.2, and vortexed. The sample was centrifuged at 10,000×g for 15 min and the supernatant was removed. The pellet was resuspended in water and both the resuspended pellet and supernatant were dialyzed against 50 mM sodium phosphate buffer pH 7.5 and the lysozyme activity was determined as described below.

2.3 Lysozyme activity

Lysozyme activity was determined as described by Chandan et al. (1965) with *M. lysodeikticus* cells with the following modifications. The cells (9 g) were dissolved in 50 mL of 50 mM sodium phosphate buffer, pH 7.5. The cell solution (2 mL) was mixed with 0.2 mL of the G-75 fractions and initial absorbance at 450 nm (Biospec1601, Shimadzu Scientific Instruments, Japan) was taken. Samples were incubated at 37 °C for 60 min and the absorbance at 450 nm was recorded each 10 min. The hydrolytic activity (unit) was determined as the rate of decrease in absorbance at 450 nm.min−1 as described by Lesierowski et al. (2004). All experiments were carried out in triplicate and a blank without substrate was used as a control.

Purified lysozyme was assayed at incubation temperatures ranging from 25 to 90 °C as described above to determine the influence of temperature on activity with the activity expressed as units per milligram protein. Sodium phosphate buffer, pH 7.5, and 10 μg of lysozyme were used. The optimum pH for purified lysozyme activity was determined from the reaction rates over a pH range of 3.0–11 with 10 μg of lysozyme. The buffers used were sodium citrate buffer (pH 2–4), sodium acetate (pH 4–5), sodium phosphate buffer (pH 6–7), Tris–HCl buffer (pH 8–10.5), and glycine–HCl (pH 9–11). To determine pH stability, purified lysozyme (10 μg) was incubated in the buffers described above for 60 min then assayed in the same buffer as described above. All enzyme reactions were conducted in triplicate and the standard deviations were calculated.
2.4 SDS-PAGE analysis

For SDS-PAGE analysis, protein samples were mixed with sample loading buffer and heated for 1 min prior to analysis with polyacrylamide gel with a 5–20% gradient. Gels were run in accordance with manufacturer guidelines and approximately 20 μg of total protein was analyzed and stained with Coomassie blue. A graph of the log molecular weight of the protein standards vs. the relative migration distance was plotted. The molecular weight of lysozyme was estimated from interpolation of this graph.

3 Results and discussion

3.1 Lysozyme purification

The specific activity and purification of lysozyme at each purification step are given in Table 1. Size exclusion chromatography was successful in the separation of Iraqi sheep whey proteins although it had the most severe lost in lysozyme yield (28%). After isoelectric precipitation of the α-Lb, the specific activity of the sample increased from 287 to 700 U.mg\(^{-1}\) with a total purification factor of 28.6. We estimate the amount of lysozyme in Iraqi sheep milk to be about 0.1 μg.mL\(^{-1}\), which is similar to the amount found previously in sheep milk (Chandan et al. 1968) and bovine milk (Farkye 2003; Korhonen 2009) but lower than previously reported for Sardinian sheep milk (Moroni and Cuccuru 2001).

Based on the elution of the protein standards via size exclusion chromatography, the molecular weight of Iraqi sheep lysozyme was calculated to be between 30.4 and 34 kDa. The SDS-PAGE analysis of the crude whey and purified lysozyme is shown in Fig. 1. Lane 2 shows the original whey that contains proteins corresponding to β-Lg and α-Lb/lysozyme. The calculated molecular weights are 18.6 and 14.3 kDa, respectively. There are four other bands with calculated molecular weights of 174, 86.5, 78, and 58 kDa from the top to middle of the gel. Lane 3 shows the purified lysozyme with a calculated molecular weight of 14.3 kDa.

SDS-PAGE analysis showed the presence of high (>150 kDa) as well as well as low (<18 kDa) molecular weight whey proteins in the sheep whey. We used a 100-kDa filter to remove the high molecular weight proteins prior to size exclusion chromatography and isoelectric precipitation. Interestingly, with these techniques, we did have a protein of approximately 24 kDa which co-purified with the lysozyme.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U.mg(^{-1}))</th>
<th>Total protein (mg)</th>
<th>Specific activity (U.mg(^{-1}))</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL whey</td>
<td>4,950</td>
<td>61.11</td>
<td>81</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Permeate &lt;100 kDa</td>
<td>3,672</td>
<td>31.93</td>
<td>115</td>
<td>74.2</td>
<td>1.4</td>
</tr>
<tr>
<td>G-75 active fractions</td>
<td>1,388</td>
<td>4.84</td>
<td>287</td>
<td>28.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Isoelectric precipitation</td>
<td>1,015</td>
<td>1.45</td>
<td>700</td>
<td>21.0</td>
<td>28.6</td>
</tr>
</tbody>
</table>
The molecular weight of Iraqi sheep lysozyme was calculated to be 14.3 kDa via SDS-PAGE, which is similar to the c-type lysozymes, which range from 14 to 15 kDa. As a comparison, the molecular weights of camel, hen egg white, and bovine milk lysozymes are 14.4 kDa, while human and goat milk lysozymes are 15 kDa and buffalo milk lysozyme is 16 kDa (Elagamy et al. 1996; Priyadarshini and Kansal 2002; Farkye 2003). Since the size exclusion chromatography gave a molecular weight of approximately of 32.2 kDa, there is a possibility that the Iraqi sheep lysozyme exists as a dimer in milk. Lysozyme generally exists as a monomer, but this enzyme has been reported to exist as a reversible dimer between pH 5.0 and 9.0. The dimerization and the formation of higher molecular weight polymers depend on pH, enzyme concentration, and temperature (Sophianopoulos 1969; Cegielska-Radziejewska et al. 2008; Lesnierowski and Cegielska-Radziejewska 2012).

3.2 Lysozyme activity

Iraqi sheep lysozyme showed high enzymatic activities at temperatures from 40 to 70 °C, with the highest activity obtained at 60–65 °C (Fig. 2a). Iraqi sheep lysozyme was inactivated at temperatures greater than 80 °C. No literature could be found on the effect of temperature on bovine milk lysozyme activity, but egg white lysozyme
shows a sharp optimum at 50 °C with an activity range of 20 °C to greater than 60 °C (Hikima et al. 2003).

The activity of lysozyme showed a maximum at pH 7.5 and was active between pH values of 4.5 and 9.5 (Fig. 2b). The sheep lysozyme also maintained 50% of its activity after incubation for 1 h at pH values less than 6 and greater than 9 and greater than 70% of its activity at pH values between 6 and 8.5. Chandan et al. (1965) showed that bovine milk lysozyme had a pH optimum at 7.9 and was active over a wide pH range of 3.5 to 9.5. The bovine lysozyme maintained 50% of its activity between the pH values of 6 and 9.5. We showed similar results for Iraqi sheep milk lysozyme which maintained approximately 50% of its activity at pH values between 4.5 and 6 and greater than 50% of its activity at pH values between 6 and 9.5. In contrast, egg white lysozyme was shown to have an optimal pH at 6.2 and a range of pH 3.5 to 9 and maintained 50% of its activity at pH values between 4.5 and 8 (Chandan et al. 1965). The pH optimum for human milk lysozyme has been reported to be at pH 6.2 (Parry et al. 1969).
4 Conclusion

This is the first reported study on the purification and partial characterization of Iraqi sheep milk lysozyme. This enzyme is found in concentrations of approximately 0.1 μg.mL⁻¹ in Iraqi milk and has a molecular weight of 14.3 kDa similar to c-type lysozymes under denaturing conditions and between 30 and 34 kDa in whey. The pH optimum is basic, pH 7.5, and is active over a range of pH values (pH 4.5 to pH 9.5).

Acknowledgments  This project was partially supported by the Utah Agricultural Experiment Station, Utah State University and approved as journal paper number 8492.

References