Magnetic Cation Exchange Isolation of Lysozyme from Native Hen Egg White

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Summary

Two magnetic macroporous cellulose cation exchangers (Iontosorb MG CM 100 and Iontosorb MG SHP 100) were used for one-step isolation of lysozyme from native, undiluted hen egg white. Highly purified lysozyme (purity >96 %) with specific activity similar to that of commercial lysozyme preparations was obtained in both cases. Carboxymethyl--based cation exchanger exhibited substantially higher capacity for lysozyme; maximum adsorption capacity was 138 mg/mL. The lysozyme-depleted egg white can be used in the same way as the routinely used egg white because no dilution of this material was necessary during the purification process.

Key words: hen egg white, lysozyme, magnetic separation, cellulose cation exchangers

Introduction

Lysozyme (EC 3.2.1.17; peptidoglycan *N*-acetylmuramoylhydrolase; muramidase) hydrolyzes 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycans and between *N*-acetyl--D-glucosamine residues in chitodextrins. Lysozyme is widely distributed in animals and plants. The most extensively studied enzyme is obtained from hen egg white (lysozyme c).

Lysozyme has a number of possible applications; for example it can be used as an additive to baby milk or ophthalmic preparations, for treatment of ulcers, wounds and infections, as a potentiator of some antibiotics, *etc.* (*1*). The expanding potential for application of lysozyme in many fields of science and technology dictates the development of efficient and simple methods for lysozyme purification.

Different procedures have been used to isolate hen egg white lysozyme. They include ion exchange chromatography (*2–4*), affinity chromatography (*5–7*), dye--binding chromatography (*8*), affinity membrane separation (*9–11*), ultrafiltration (*12,13*), PEG/salt aqueous two-phase system (*14*), reverse micelles (*15*), metal-affinity precipitation (*16*) or adsorption to plant residues (*17*). However, severe limitations of many of these methods, including lengthy steps, high cost and dilution of the egg white during processing, have hampered their application.

Recently, magnetic separation techniques have been used to purify various proteins and peptides, including egg white lysozyme (*18,19*). These techniques in many cases enable simple, one-step separation of target proteins. In most cases magnetic affinity adsorbents are used. In this paper we have shown efficient one-step purification of lysozyme from native, undiluted hen egg

white using newly developed, commercially available magnetic ion exchangers.

Materials and Methods

Materials

The magnetic cation exchange adsorbents (Iontosorb MG CM 100 and Iontosorb MG SHP 100, particle size 50–80 µm) were obtained from Iontosorb, Usti nad Labem, Czech Republic. Highly purified hen egg white lysozyme was from Fluka, Germany (declared specific activity of 82 800 U/mg), while iron(II,III) oxide (magnetite) was from Aldrich, USA. Lyophilized *Micrococcus luteus* cells were from Serva, Germany. Common chemicals were from Lachema, Czech Republic. Hen eggs were obtained from a local market. The separated egg whites from several eggs were mixed carefully and the homogeneous material was filtered through a cheesecloth (Uhelon, produced by Silk and Progress, Czech Republic).

Preparation of magnetic microporous cellulose beads

The mixture of modified viscose cellulose and magnetite microparticles was added under intensive stirring into water non-miscible organic solvent and after heating to 60 °C the mixture was stirred for another 45 min. The formed magnetic macroporous cellulose particles, still in the xanthogenate form, were separated and then the hydrolysis process was performed. The beads were washed with distilled water until the pH was 7.0±0.2. Strongly and weakly acidic cation exchangers were prepared using the standard procedures used for the preparation of cation exchangers from microcrystallic cellulose [20]. During syntheses, sulphohydroxypropyl and carboxymethyl groups were attached to the basic cellulose matrix. In both cases the reaction was preformed at 50 °C for 3–5 hours. The beads were stored in 20 % ethanol solution at 4 °C.

Magnetic cation exchange separation of lysozyme

Magnetic cation exchanger (0.5 mL, settled volume) was added to 5 mL of the filtered egg white; usually no pH adjustment was necessary because of its appropriate value (pH around or slightly above 9). In exceptional cases pH was adjusted to 9.0 with ammonium hydroxide. The suspension was slowly mixed on a rotary mixer (Dynal, Norway) for 2 h. Then magnetic particles were separated on a test-tube magnetic separator MPC-L (Dynal, Norway) and the egg white was removed carefully using a Pasteur pipette. The magnetic ionex particles were repeatedly washed with water until almost no protein was detected in the washings. The adsorbed lysozyme was eluted with 5 mL of sodium chloride solution (0.5 mol/L) for 30 min.

Analytical cation exchange chromatography

Lysozyme purity was checked using an FPLC system (Pharmacia, Sweden), which comprised LCC-500 controller and two P500 pumps. The chromatography was carried out on a Mono S HR 5/5 column using 0.05 M acetate buffer, pH=4.4, as a mobile phase A, and the same buffer containing 1 M sodium sulphate as a mobile phase B. The flow rate was 1 mL/min. The elution profile was monitored at 280 nm with diode array detector (Agilent 1100 Series, Agilent, USA). Spectra between 190 and 400 nm were collected throughout the chromatography run.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [21] using a 12 % resolving gel and a 5 % stacking gel. Protein bands were stained with Coomassie Brilliant Blue R-250.

Equilibrium adsorption isotherms

Aliquots of 0.1 mL volume of settled ion-exchanger equilibrated with 50 mM sodium bicarbonate buffer, pH=9.2, were added to a series of test tubes. To each tube 3 mL of lysozyme solution (concentration of 0.2–6.0 mg/mL; dissolved in the same buffer) were added. The tubes were sealed and rotated overnight at 4 °C and then the supernatant was assayed at 280 nm for protein concentration. Using mass balance, the amount of lysozyme adsorbed to magnetic ion-exchanger was determined.

Protein determination and enzyme assays

Concentration of lysozyme in eluted fractions was determined by the spectrophotometric measurements (absorbance at 280 nm) in a Cintra 20 UV-VIS spectrophotometer (GBC, Australia) using the absorption coefficient of 2.64 mL/(mg·cm) [3,22]. Protein determination in crude solutions was carried out using the Bradford reagent (Sigma, USA) with either bovine serum albumin or ovalbumin as standards.

Lysozyme was assayed by adding enzyme solution (25 µL) to a 1.7-mL glass cuvette containing a suspension of lyophilized *Micrococcus luteus* cells (20 mg/mL) in phosphate buffer, pH=6.3 (1.5 mL). Enzyme activity was measured spectrophotometrically (30 °C) at 450 nm by monitoring the reduction in turbidity caused by the lysis of the cells by lysozyme. Results are expressed in units of enzyme activity where 1 unit is defined as the amount of enzyme causing a decrease in absorbance of 0.001 per min [23].

Results

New types of magnetic ion exchangers based on macroporous bead cellulose (distributed under the trademark Perloza®) have been developed and used throughout this work. Perloza is a hydrophilic, highly porous regenerated cellulose with heterogenous matrix (partially microcrystalline) and with polymer structure stabilized by hydrogen bonds only (there is no covalent cross-linking). This bead cellulose is produced in several porosity types and various particle sizes. Magnetic macroporous bead celulose (Perloza MG) is produced with the same technology as the classic macroporous bead cellulose (Perloza MT) with the only difference that Perloza MG has magnetite microparticles (30 %, related to dry matter) built in its matrix. The magnetite particles are homogeneously distributed in the cellulose matrix (see in Fig. 1).
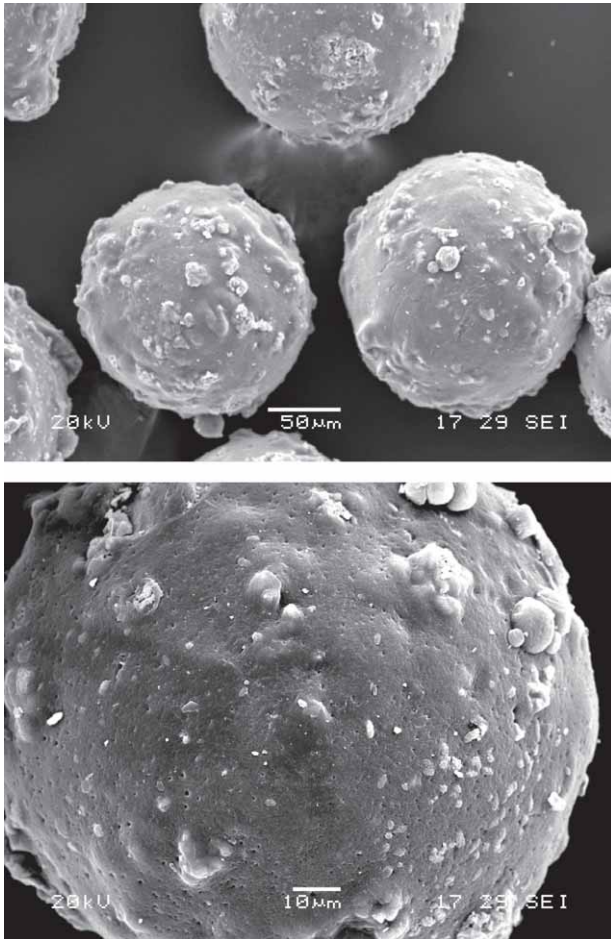
Fig. 1. Scanning electron microscopy of magnetic macroporous bead cellulose. Magnification: A – 300×, B – 1000×

The presence of magnetite within the bead structure enables simple magnetic manipulation with these adsorbents.

Several derivatives of Perloza MG, known as Iontosorb MG, are currently available on the market. Two magnetic cation exchangers, namely Iontosorb MG SHP 100 (strongly acidic cation exchanger) and Iontosorb MG CM 100 (weakly acidic cation exchanger), were used in the course of subsequent experiments.

The separation experiments were performed with native egg white, without any dilution. The egg white was filtered through the cheesecloth in order to remove particulate matter (*e.g.* chalazae) and then it was mixed with appropriate cation exchanger. Usually no adjustment of the egg white pH value was necessary due to its natural slightly alkaline reaction (pH around or above 9). After finishing the adsorption process, magnetic beads were easily separated from the mixture using a magnetic separator or NdFeB permanent magnet. The repeated washing of the cation exchangers was done with distilled water; approximately 15 washing steps (using 5 mL of water each time) were necessary. Sodium chloride solution (0.5 mol/L; 5 mL) was used to elute the adsorbed lysozyme. The lysozyme purity was checked with FPLC (see Fig. 2). It can be clearly seen that both cation exchangers enabled very efficient isolation of a very pure lysozyme. The accompanying proteins present in the first major peak were almost completely eliminated during the isolation process. The purity of the separated lysozyme was more than 96 % in both cases, when calculation was based on the peak areas. The high purity of the separated lysozyme can also be observed on the diode-array detector spectral analysis of the eluted lysozyme peak (see Fig. 3). Five spectra scans of the lysozyme
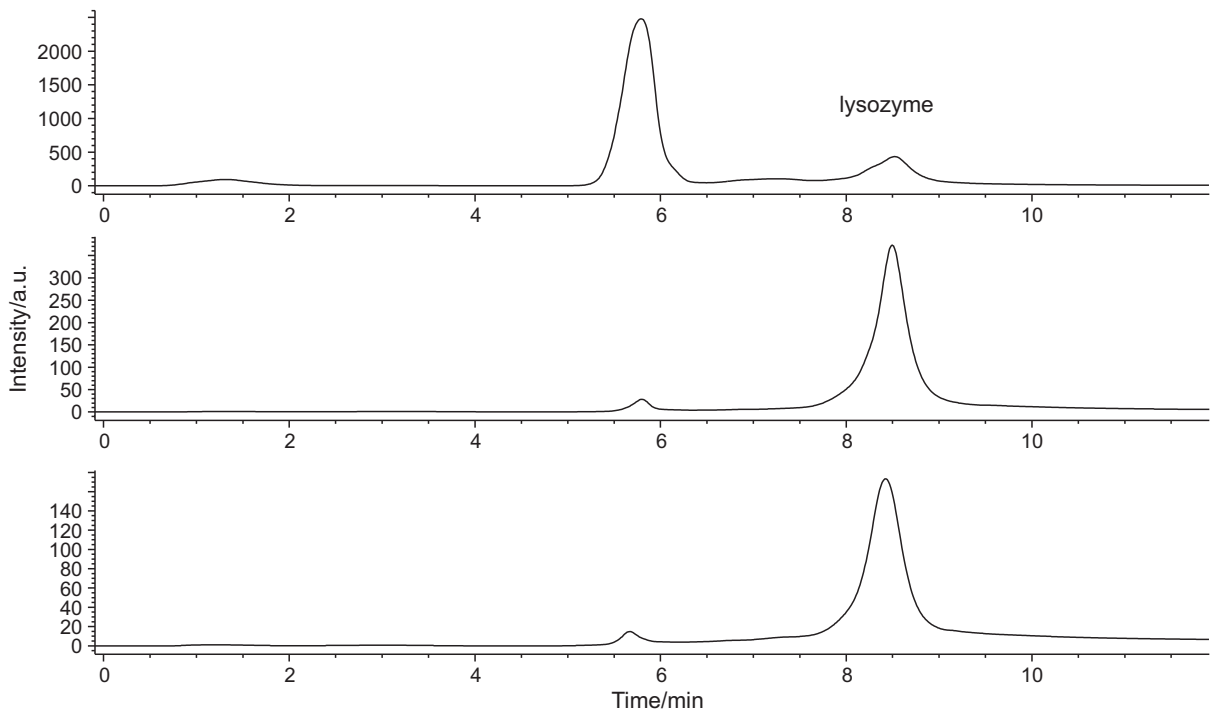

Fig. 2. Fast protein liquid chromatography of original egg white (top), eluate from Iontosorb MG CM 100 (middle) and eluate from Iontosorb MG SHP 100 (bottom)
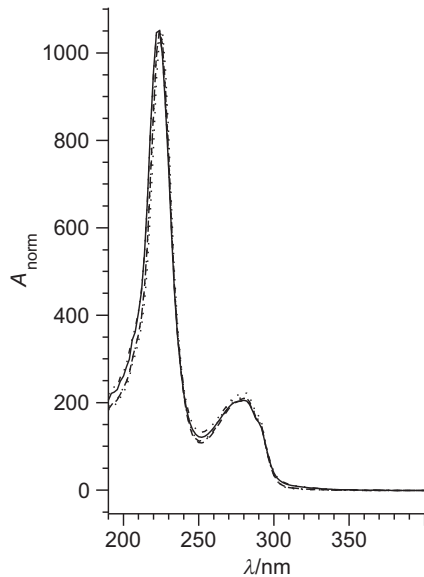
Fig. 3. Set of five overlapping spectra of a lysozyme peak (retention time 8.424 min; see Fig. 2) between 190 and 400 nm obtained after Iontosorb MG SHP 100 isolation. The spectra were taken at 8.068, 8.255, 8.415, 8.575 and 8.735 min, respectively

peak (Iontosorb MG SHP 100 used as a cation exchanger) taken between 8.068 and 8.735 min (*i.e.* covering the whole peak) clearly demonstrate the lysozyme purity. High purity of the isolated lysozyme was also confirmed by SDS electrophoresis in polyacrylamide gel. One main very intensive band corresponding to lysozyme was observed, which was accompanied by a very faint band of accompanying proteins (Fig. 4). Both the commercial pure lysozyme and the isolated lysozyme gave similar electrophoretic pattern. Lysozyme was isolated in the active form, as checked by the enzyme assay using lyophi-
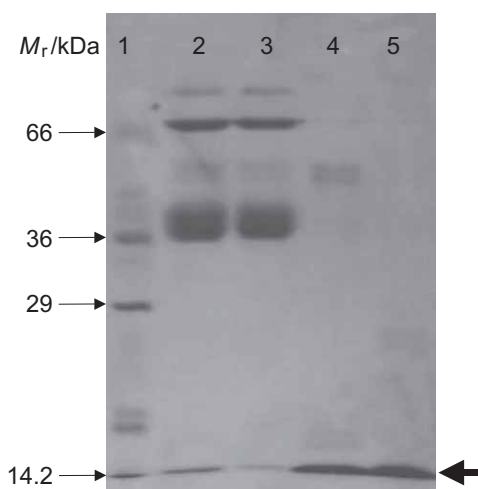

Fig. 4. Electrophoretic patterns of egg white proteins and lysozyme isolated by Iontosorb MG CM 100. Lane 1, molecular mass markers; lane 2, egg white (9.3 μg of proteins loaded); lane 3, egg white after finishing the adsorption process (8.1 μg of proteins loaded); lane 4, lysozyme after elution from Iontosorb MG CM 100 (8.75 μg of proteins loaded); lane 5, commercial lysozyme (Fluka, Germany, 12 μg of proteins loaded). Arrow indicates the lysozyme position

lized *Micrococcus luteus* cells as a substrate; its specific activity was comparable to the specific activity of commercial lysozyme.

From the chromatogram in Fig. 2, it can be seen that the recovery of lysozyme (under identical conditions) was higher in the case of carboxymethyl-based cation exchanger. The binding capacity of both adsorbents was further studied in equilibrium adsorption experiments (see Fig. 5). Using both linear and non-linear regression calculations it is obvious that the adsorption isotherms could be described by the Langmuir equation:

$$q_{eq} = \frac{Q_{max}bC_{eq}}{1 + bC_{eq}} \qquad /1/$$

where Q_{max} is maximum adsorption capacity of the adsorbent (mg/mL), b is a constant related to the affinity of the binding sites (L/mg), C_{eq} is the concentration of the free (unbound) lysozyme (mg/mL) and q_{eq} is the amount of lysozyme bound to volume unit of adsorbent (mg/mL). Maximum adsorption capacities for lysozyme were calculated for both cation exchangers; this value for Iontosorb MG CM 100 is 138 mg/mL and for Iontosorb MG SHP 100 it is 61 mg/mL. These data correspond to the chromatography measurements.
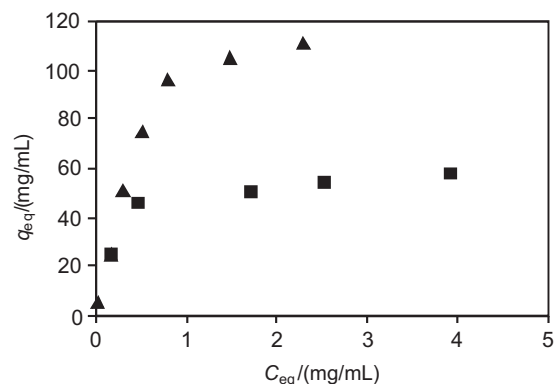

Fig. 5. Equilibrium adsorption isotherms of hen egg white lysozyme using Iontosorb MG CM 100 (▲) and Iontosorb MG SHP 100 (■) as adsorbents. C_{eq} – equilibrium liquid-phase concentration of the unadsorbed (free) lysozyme; q_{eq} – equilibrium solid-phase concentration of the adsorbed lysozyme

Discussion

Magnetic separations represent an efficient tool for the isolation of proteins and peptides (*18*). In most cases different types of magnetic affinity adsorbents have been used and the separations were performed in the laboratory scale.

Lysozyme has been selected several times as an interesting model enzyme for the development of magnetic separation procedures (*18*). Usually diluted egg white or partially purified lysozyme were used as the starting materials. The use of native, undiluted egg white for magnetic separation of lysozyme has not been described yet. In this paper, however, new, commercially available magnetic cellulose cation exchangers were successfully used for this purpose. Magnetic properties of the particles enabled simple magnetic separation of the adsor-

bents from this extremely viscous material. In one step highly purified lysozyme (purity >96 %) was obtained; the purity was checked both by liquid chromatography and electrophoresis. The specific activity of isolated lysozyme was comparable to the specific activity of commercial enzyme. The maximum adsorption capacity of 138 mg/mL for Iontosorb MG CM 100 is sufficiently high in order to consider possible lysozyme separation in larger scale. Also, relatively low price of commercially available magnetic Iontosorb materials can stimulate industrial-scale separation of lysozyme and other target proteins of different origin.

The egg white remaining after the magnetic separation process (lysozyme-depleted egg white) can be used in the same way as the routinely used egg white for the manufacture of the products because no dilution of this material was necessary during the purification process.

Conclusion

The study clearly shows that magnetic cation exchangers can be efficiently used for one-step isolation of very pure lysozyme from native (undiluted) egg white. The lysozyme-depleted egg white can be further processed in the standard way.

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