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# Direct Purification of Lysozyme from Hen Egg White Using High Density Mixed Mode Adsorbent

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**Abstract** The high density mixed mode adsorbent known by the trade name of Mimo-AD was used to purify lysozyme directly from the hen egg white (HEW). The homogenized hen egg white was treated with the adsorbent in a stirred vessel for lysozyme adsorption, and then the adsorbent, easily separated from the HEW by sedimentation, was packed into a column. The remaining HEW and contaminant proteins were removed by washing with pH 11 distilled water in an expanded-bed state, and subsequently the elution was performed with pH 12 distilled water in a packed-bed state. By this simple and rapid adsorption, washing, and elution procedure, lysozyme was purified to >95% with an overall recovery yield of 66%. This process offers a great potential for industrial application by allowing the extraction of lysozyme while retaining the commercial value of HEW.

**Key words:** Lysozyme, hen egg white (HEW), mixed mode adsorbent, expanded bed

Lysozyme (1, 4,-β-*N*-acetylmuramidase; EC 3.2.1.17) is an enzyme with an antibacterial activity that causes cell lysis by breaking bonds in carbohydrate polymers found in cell walls [3]. Due to this antibacterial activity, lysozyme can be used as a food preservative, and can also be added to food packing films to render it antiseptic [1, 6]. In addition, lysozyme is used clinically as a therapeutic agent in the treatment of infectious diseases and wounds, and as a potentiator of antibiotics by increasing their efficacy and allowing lower concentrations of antibiotics to act for longer periods of time.

Lysozyme has been extracted from various species [4, 5, 9, 11, 17, 21], but is being commercially extracted from the hen egg white (HEW) due to better profitability. The important point to be considered in the commercial purification of HEW lysozyme is to retain the physical property of HEW after the extraction of lysozyme, since HEW itself is commercially valuable. Since lysozyme is a strongly basic protein of pI 10.5, it can easily be purified from HEW by being selectively bound on cation exchangers under alkaline conditions. However, direct capture of lysozyme from the crude HEW onto cation exchangers is hampered by its high ionic strength. Therefore, desalting or dilution steps are required to reduce its ionic strength prior to cation exchange chromatography [13, 14, 15], which eventually destroys the commercial value of the remaining HEW. In order to resolve this problem, affinity adsorbents have been used to purify lysozyme directly from crude HEW [7, 16, 19]. However, the commercial use of these affinity adsorbents in the purification of lysozyme has also been limited due to the operational difficulty in removing insoluble components contained in crude HEW.

To tackle these problems, we developed a process for direct and rapid purification of lysozyme from HEW using a high density mixed-mode adsorbent known by the trade name of Mimo-AD. This adsorbent is a matrix comprised of low molecular weight ligands which have both hydrophobic and ionic functionalities, and containing quartz to give a high density (1.4- 1.6 g/ml). Due to these properties, this adsorbent can capture the desired product without desalting or dilution steps which are generally employed prior to ion exchange chromatography. In addition, the direct capture of proteins from particle-containing feedstocks can be achieved by carrying out the column operation in an expanded state. In this study, lysozyme was purified to >95% by a very simple adsorption, washing, and elution procedure using the Mimo-AD adsorbent, offering the potential for commercial application of the process to the large-scale production of lysozyme.

## MATERIALS AND METHODS

### Materials

HEW was obtained from Poongrim Co. in Korea. Mimo-AD™ resin was purchased from UpFront Chromatography

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AS (Denmark). Lysozyme and other chemicals used in this study were obtained from Sigma Co. (St. Louis, U.S.A.).

### Purification of Lysozyme

The HEW was homogenized by a homogenizer (Heidolph, Diax600, Germany) at 9500 rpm for 5 min. Fifty grams (wet wt.) of Mimo-AD resin was washed with distilled water, suction-dried, and then added to 250 ml of the homogenized HEW. The mixture was stirred on a magnetic stirrer for 15 min, and then the resin particles were gravitationally sedimented. After the HEW supernatant was poured out carefully, the remaining resins were transferred, as a slurry, to a glass column (3×250 mm, Amicon Co., Deverly, U.S.A.). The end-plate screen attached to the upper adaptor of the column was replaced with a large pore screen for the expanded-bed operation. The residual HEW and contaminant proteins contained in the slurry were removed by washing the resin with pH 11 distilled water. During this operation, an expanded-bed state was maintained by applying an upward flow at a linear flow rate of 500 cm/h. After washing the column, the adaptor was moved down to pack the column. Subsequently, the flow was reversed to apply a downward flow of pH 12 distilled water for elution. The linear flow rate was maintained at 104 cm/min. The pooled fractions exhibiting a lysozyme activity were collected, dialyzed against 5 mM sodium phosphate buffer (pH 6.24), and lyophilized to dryness.

### Assay of Protein and Lysozyme

Assay of lysozyme activity was performed at 30°C in 66 mM potassium phosphate buffer (pH 6.24). Lysis of *Micrococcus lysodeikticus* (Sigma Co., U.S.A.) was monitored by measuring the decrease in absorbance at 450 nm. One unit is defined as the decrease of 0.001 optical density per 1 min. Total protein concentration was determined by the method of Bradford [2].

## RESULTS AND DISCUSSION

### Batch Adsorption of Lysozyme

The lysozyme adsorption rate was determined from batch experiments in an Eppendorf tube. A tube rotator (Scientific Equipment Products, Baltimore, U.S.A.) was used for mixing. Transient lysozyme uptake experiments were performed by quickly introducing 0.2 g (wet wt.) of Mimo-AD resin in 1 ml of the homogenized HEW solution which was preloaded in the Eppendorf tube. Twenty micro liters of the mixture was withdrawn from the Eppendorf tube, and the residual amount of lysozyme in HEW was determined by measuring the lysozyme activity as described in Materials and Methods. As shown in Fig. 1, about 75% of the lysozyme was adsorbed onto the Mimo-AD resin in 5 min, indicating a highly efficient adsorption capability of

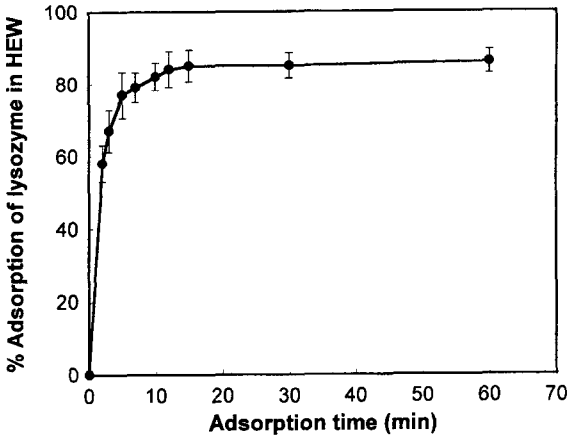


**Fig. 1.** Time course of adsorption of lysozyme in HEW on Mimo-AD adsorbent.

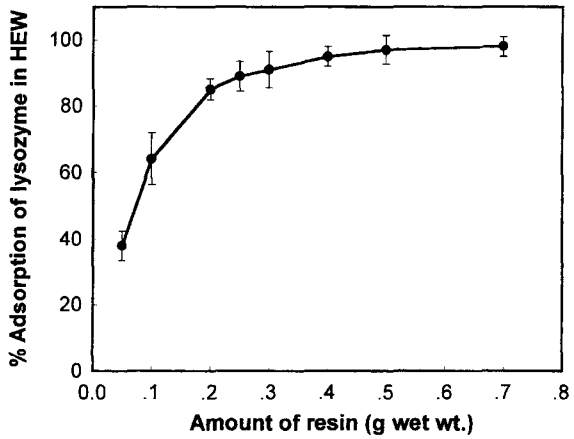


**Fig. 2.** Maximum adsorption of lysozyme in HEW on Mimo-AD adsorbent at various concentrations.

this resin with respect to lysozyme, even in the presence of HEW. After 5 min, the lysozyme uptake rate was greatly retarded, and an equilibrium state was attained at ca. 85% adsorption of the lysozyme contained in HEW.

Figure 2 shows the maximum adsorption of lysozyme on Mimo-AD resin at varying amounts of the resin. After 15 min of adsorption, the HEW was separated from the resins and the lysozyme activity was measured. The maximum adsorption capacity increased greatly with an increase in the amount of resin up to 0.2 g (wet wt.), but further significant increase was not observed above 0.2 g (wet wt). From these results, the optimal ratio of the amount of resin to the volume of HEW and the optimal adsorption time were determined to be 0.2 g wet wt. resin/ml HEW and 15 min, respectively.

### Optimization of Desorption Conditions

In most mixed mode adsorbents, the adsorption is governed by hydrophobic interactions and the desorption is caused by electrostatic repulsion between the support and the protein

[10, 12, 18, 20, 22, 23]. Such adsorbents are manufactured by attaching hydrophilic or ionic substituents onto the hydrophobic ligate immobilized on the solid support. By changing the pH of solvent, the density of positively-charged groups on the support increases. The same applies to proteins. Hence, highly efficient elution of the hydrophobically adsorbed protein can be achieved by increasing the electrostatic repulsion simply by changing the pH of the eluent.

To determine the optimal desorption condition, adsorption and desorption experiments were carried out in a batch mode. 0.2 g (wet wt.) of the resin was added to 1 ml of HEW. After mixing for 15 min, the resins were separated from HEW by centrifugation and then washed with distilled water to remove the residual HEW. The desorption of lysozyme was performed with distilled water adjusted to various pHs with 2 N NaOH or 2 N HCl. After desorption for 2 h, the supernatant was subjected to SDS-PAGE for analysis (Fig. 3). No lysozyme band was observed in the pH range of 3-11 (Fig. 3, lanes 3-8). In contrast, ca. 85% of lysozyme was desorbed at pH 12. To further increase the desorption yield, pH 12 distilled water containing NaCl at varying concentrations was used for desorption of lysozyme. As shown in Fig. 4, an increase in NaCl concentration did not improve the desorption yield. On the contrary, many contaminant proteins were coeluted with lysozyme. Therefore, pH 12 distilled water was adopted as the most preferred eluent.

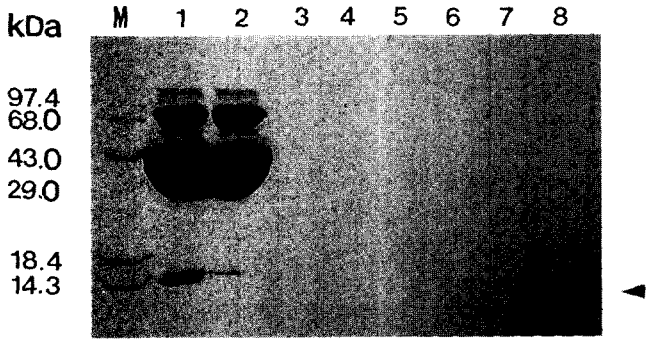


**Fig. 3.** SDS-PAGE analysis of the supernatants after desorption of lysozyme with distilled water adjusted to various pHs.
Lanes: M, molecular weight standards; 1, HEW; 2, HEW fraction after adsorption; 3, pH 3.0; 4, pH 4.7; 5, pH 8.7; 6, pH 10; 7, pH 11; 8, pH 12. Arrowhead indicates lysozyme.

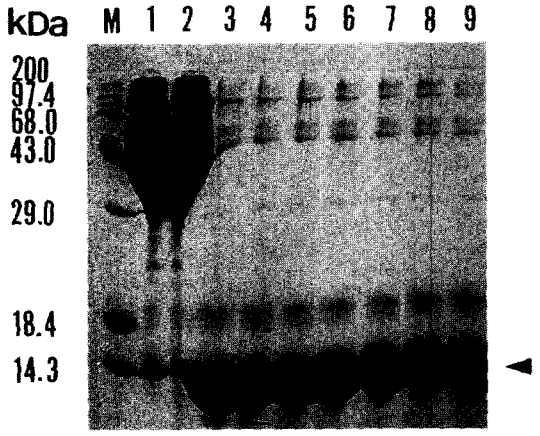


**Fig. 4.** SDS-PAGE analysis of the supernatants after desorption of lysozyme with pH 12 distilled water containing NaCl at various concentrations.
Lanes: M, molecular weight standards; 1, HEW; 2, HEW fraction after adsorption; 3, 0.05 M; 4, 0.1 M; 5, 0.15 M; 6, 0.3 M; 7, 0.5 M; 8, 0.7 M; 9, 1.0 M. Arrowhead indicates lysozyme.

## Semi-Preparative Scale Purification of Lysozyme

Schemes for purification of lysozyme were first developed on a small scale, and then scaled-up to a semi-preparative scale. For semi-preparative scale purification of lysozyme, 250 ml of the homogenized HEW was mixed with 50 g of Mimo-AD resin in a stirred vessel. After 15 min of adsorption, the mixing was terminated, and then the resins were sedimented very quickly. The fast sedimentation of the resins is attributed to their high density property. These resins were easily collected by removing the resin-free top phase. Resins were transferred to a glass column (32×250 mm, Amicon Co., U.S.A.). To remove the residual HEW and contaminant proteins, washing was performed with pH 11 distilled water prior to elution. During the washing step, an upward flow was applied with pH 11 distilled water at a linear flow rate of 500 cm/h which was high enough to maintain the column bed in an expanded-bed state. After the unbound material was washed out, the flow was stopped, and the bed was allowed to settle. Subsequently, the upper adaptor was moved down to pack the column, and elution was performed in a packed-bed mode using a downward flow at a decreased velocity (linear velocity=104 cm/h) with pH 12 distilled water. Elution may be performed in an expanded-bed mode, but this can result in an increase in the volume of the eluted fraction

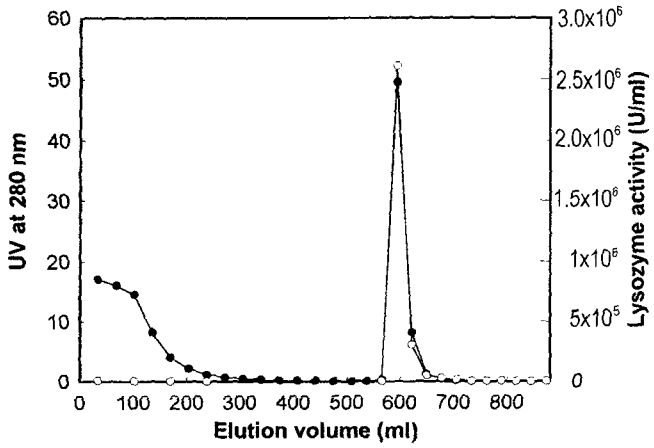


**Fig. 5.** Elution of lysozyme using pH 12 distilled water in a packed-bed state.
Symbols: ○, UV absorbance at 280 nm; ●, lysozyme activity.

**Table 1.** Comparative data of crude HEW and purified lysozyme.

| | Total lysozyme activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification fold | Recovery yield (%) |
|---|---|---|---|---|---|
| HEW | $1.16\times10^{8}$ | 9998 | $1.16\times10^{4}$ | - | - |
| Pooled fraction | $7.65\times10^{7}$ | 342 | $2.24\times10^{5}$ | 19.3 | 66 |

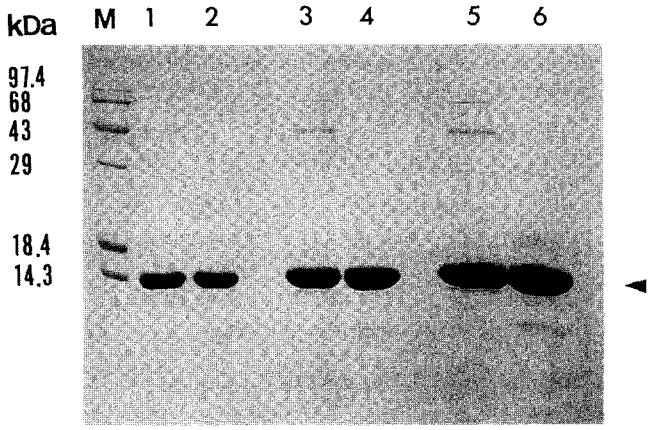


**Fig. 6.** SDS-PAGE analysis of the commercial lysozyme (odd-numbered lanes) and purified lysozyme (even-numbered lanes). Lanes: M, molecular weight standards; 1 and 2, 5 μg; 3 and 4, 10 μg; 5 and 6, 20 μg. Arrowhead indicates lysozyme.

[8]. The major peak exhibiting a lysozyme activity was collected (Fig. 5). The collected solution was dialyzed against 5 mM sodium phosphate buffer (pH 6.24) and lyophilized. Table 1 shows the comparative data of HEW and the purified lysozyme. The purification factor and the recovery yield obtained by this process were 19.3 and 66%, respectively. The recovery yield was relatively low, compared to previous results (≈90%) [14, 15]. However, this process has a distinct advantage over other previous methods in enabling the extraction of lysozyme while retaining the commercial value of HEW.

### Purity and Activity of the Purified Lysozyme

Figure 6 shows the SDS-PAGE of the commercial lysozyme and purified lysozyme. The purity of the purified lysozyme was >95% as judged by SDS-PAGE. The cell lysis activity of the purified lysozyme was measured and compared to that of the commercial lysozyme (Sigma Co., U.S.A.). As a result, the cell wall lysis activity of the purified lysozyme was identical to that of the commercial lysozyme (data not shown).

In summary, the flow chart of the process is shown in Fig. 7. The processing time from adsorption to elution was 59 min. One of the major advantages of this process is the reduction of the processing time by eliminating the steps to remove particulate matter and to reduce the ionic strength of the feedstock. The high productivity obtained from the reduced processing time results in a low production cost, rendering the desired product more competitive in the market [7]. In addition, the equipments involved in the unit operations to remove the insoluble matter are not required in this process. Therefore, it is expected that the process developed in this study can be efficiently employed for the mass production of lysozyme.

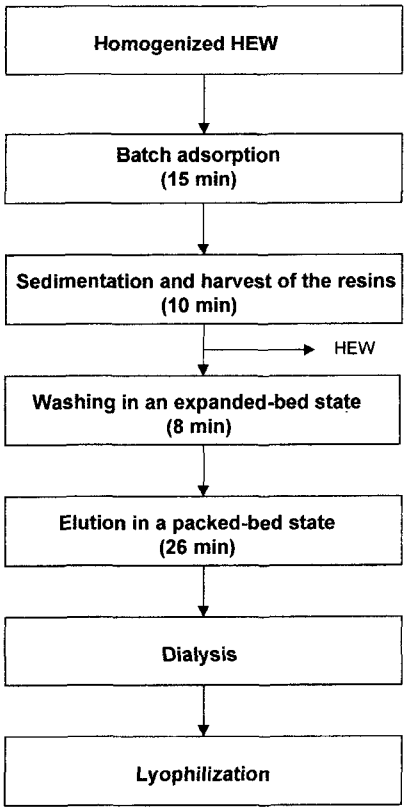


**Fig. 7.** Process flow diagram for direct purification of lysozyme from HEW.

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