

Full Length Research Papers

Affinity purification of hen egg lysozyme using sephadex G75

Rizwana Islam^{1,2}, Jake Kite¹, Aaron S. Baker^{1,2}, Alejandro Ching Jr.³ and M. Rafiq Islam^{1*}

¹Department of Chemistry/Physics, ²Missouri Academy of Mathematics, Science and Computing, and ³Department of Agriculture, Northwest Missouri State University, Maryville, Missouri 64468, USA.

Accepted 25 September, 2006

We found lysozyme binds Sephadex G75, a dextran-based matrix routinely used for Gel-filtration chromatography, in a pH dependent manner. The binding is rapid and specific in a buffer containing 25 mM NaCl at pH 8.0, and requires only 0.1 ml of swollen Sephadex G75 suspension per mg of lysozyme. The bound lysozyme can be eluted with NaCl concentration over 0.15 M in the same buffer in a relatively pure form. Exploiting these binding properties with Sephadex G75, chromatographic and scaled-up methods were optimized to purify lysozyme from hen eggs with over 80% yield and over 70-fold purification. This also allows faster isolation of lysozyme compared to current methods in use.

Key words: Lysozyme purification, sephadex G-75, hen eggs.

INTRODUCTION

Hen egg white, known as albumen, contains more than 20 different proteins and is a rich source for a number of proteins with useful applications in health and pharmaceutical sectors (Desert et al., 2001). These proteins vary significantly in size, concentration and other properties. Thus, ovalbumin comprises over 50% of proteins present in albumen (Stevens, 1996; Desert et al., 2001), whereas ovotransferrin and lysozyme occur only in 10-12% and 1-2%, respectively (Stevens, 1996; Davis and Reeves, 2002).

Lysozyme (EC 3.2.1.17; muramidase), a single polypeptide chain with molecular weight of 14,300 Da and cross-linked by four disulfide bonds, is also present in tears, saliva, sweat, breast milk of humans and other animals, in plants, micro-organisms and viruses, and is assumed to be nontoxic (Masschalck et al., 2002). However, they cause hydrolysis of cell wall peptidoglycan

between N-acetyl muramic acid and N-acetyl-D-glucosamine. The cell walls in Gram-positive bacteria, contains roughly 40-90% of peptidoglycan, but only 10% in Gram-negative bacteria. Sensitivity to lysozyme action thus largely depends on bacterial type.

Although its exact function in egg whites is still unclear, its role in external secretion in humans is well established as the first non-immune defense that provides an early barrier against bacterial infections (Janeway, 2001; Ibrahim et al., 2001, 2002). It can inactivate certain viruses by forming an insoluble complex (Hasselberger, 1978) and modulate host immunity against bacterial and viral agents (Sava, 1996). The lysozyme absorbed systemically in the gut has also been shown to potentiate monocytes and macrophages (Roy et al., 2003a). Thus, it is not surprising that lysozyme has found wide applications, and often used in conjunction with other therapeutic drugs, applied topically or administered orally (Calvo et al., 1997). The antibacterial property has been exploited in a number of other applications such as eye drops and wound healing creams (Cowell et al., 1997). It is used as a food preservative to inhibit growth of *Clostridia* in cheese (Maullu et al., 1999), spoilage organisms in selected processed foods (Peck and Fernandez, 1995), and in wine as a substitute for sulfites (Leitch and Wilcox, 1998). It has also been used in

*Corresponding author. E-mail: islamr@nwmissouri.edu. Tel: 660-562-1210.

Abbreviations: EWL, commercially available egg white lysozyme; EWP, Egg White Prep; Buffer A, 20 mM Tris-HCl containing 25 mM NaCl, pH 8.0; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

gastrointestinal infections, post-radiation therapy, periodontosis and in the treatment of dry-mouth (Xerostomia) (Tenovuo, 2002; Roy et al., 2003a). According to Ventria Bioscience, metric tons quantity of lysozyme is required to meet its current demand (Ventria, 2005).

Thus an efficient, fast, economical and scalable method for its purification is highly desirable. The methods in current use or reported recently include the classical crystallization (Green, 1970; Sigma 2002, 2003), gel-filtration chromatography alone (Fernandez-Souza and Rodriguez, 1977) or coupled with metal-affinity precipitation (Roy et al., 2003a), tandem chromatography on cation-exchanger followed by dye-linked cellulose beads (Roy et al., 2003b), ethanol treatment followed by chromatography on Alcohol-insoluble cross-linked pea pod solid ion-exchanger (Jiang et al., 2001) or using anionic polysaccharides (Yang et al., 1998). Generally, chromatographic methods are preferred for its inherent high resolution, but additional chromatographic and/or other steps are required to handle large initial volume or contaminating proteins (Hjorth, 1997). Here, exploiting our observation that Sephadex G75, a dextran based matrix commonly used for gel-filtration chromatography, binds lysozyme in a pH dependent manner, we optimized a purification scheme, used in both chromatographic and scaled-up batch, for rapid isolation of lysozyme from hen eggs.

EXPERIMENTAL

Materials

Lyophilized micrococcus lysodekticus cells, Sephadex G-75, egg white lysozyme, and recombinant human lysozyme were obtained from Sigma. Electrophoresis and protein assay reagents were purchased from Bio-Rad. Hen eggs were purchased from the local market. All other reagents used were of analytical grade.

Lysozyme and protein assays

The lysozyme activity in egg white prep and various fractions was determined using *M. lysodekticus* cell suspension (0.03%) in 10 mM phosphate buffered containing 50 mM NaCl, pH 6.3 at $25 \pm 1^\circ\text{C}$ (Shugar, 1952). A decrease in absorbance at 405 ($\square A_{405}$) of 0.001 is considered as 1 unit. The protein concentration in various samples was measured by the standard Bio-Rad dye-binding assay (Bradford, 1976) using BSA as standard protein.

Preparation of egg white Prep

The white albumen (35 ml) separated from an egg was combined with 165 ml of buffer A (20 mM Tris-HCl containing 25 mM NaCl, pH 8.0) and mixed. Insoluble materials, if produced, were removed by centrifugation (8000 g x 10 min). These precipitate materials could be easily removed by filtration through cheese clothe or glass wool, or by centrifugation. The resulting egg white prep (EWP) was stored

at 6°C .

Interaction of lysozyme with sephadex G75

Equal mass (1.2 mg) of EWL was dissolved in 0.6 ml of buffers of different pH ranging from 4.0 to 10.0. The buffers used were: 0.1 M Na-acetate, pH 4.0, 0.1 M Na-acetate, pH 6.0, buffer A and 0.1 M carbonate, pH 10.0. The pH equilibrated Sephadex G75 matrices were added with 0.5 ml lysozyme solution in respective pH, and were allowed to bind for 10 min with shaking at 25°C . The supernatants from each were collected by centrifugation at 16,300 g for 1 min and were assayed for lysozyme activity.

EWL (1.0 mg) in 0.5 ml of buffer A or EWP (0.5 ml) was incubated with 0.5 ml of Sephadex G75 pre-equilibrated in buffer A for the period 5 min to 60 min in a shaker at 25°C . At the end of each period, the mixture was centrifuged and the supernatant was assayed for lysozyme activity as above.

EWL (2 mg) in 0.5 ml of buffer A or EWP (0.5 ml) was added to 0.05 ml of pre-equilibrated Sephadex G75 suspension and shaken for 5 min. It was then centrifuged and the supernatant was assayed for lysozyme activity as above. The process was repeated each time with additional 0.05 ml of Sephadex G75 suspension until no activity was found in the supernatant.

Following binding of EWP to Sephadex G75, and removal of the supernatant (FT), the matrix was washed twice with 1 ml of buffer A (W). The bound materials were eluted using NaCl concentrations of 0.05 M to 1.0 M in buffer A. Each time the supernatant was separated by centrifugation and assayed for lysozyme activity.

Corboethoxylation with diethylpyrocarbonate (DEPC)

EWL (0.1 mg/ml 0.1 M phosphate buffer, pH 6.5) was treated with 1 mM DEPC over 20 min at 25°C and progress of the reaction was monitored by UV-difference spectra (Islam et al., 1997; Minami et al., 1998). The number of histidine residues modified was calculated using molar extinction coefficient of $3,200 \text{ cm}^{-1} \text{ M}^{-1}$ at 240 nm.

Microscale purification

EWP (1.0 ml) was allowed to bind with 0.5 ml pre-equilibrated Sephadex G75 matrix for 10 min. Following that, the supernatant was removed and the matrix was washed twice with the buffer A (1.0 ml). The bound proteins were eluted in two steps: first with 0.5 ml of 0.16 M NaCl in buffer A, followed by 0.5 ml of 1.0 M NaCl in the buffer A.

Chromatographic purification

EWP (11 ml) was loaded on a column filled with Sephadex G75 (bed volume 5 ml) equilibrated with the buffer A. The matrix was then washed successively with buffer A (10 ml), 0.16 M NaCl in buffer A (5 ml) and buffer A (5 ml). The bound lysozyme was then eluted with 1.0 M NaCl in the buffer A. Maximum flow rate possible was maintained throughout the process.

Scaled-up batch purification

EWP (165 ml) was added to 30 ml of pre-equilibrated Sephadex G75 and shaken in an orbital shaker for 30 min at 25°C . After that, the mixture was filtered through a 60 ml sintered glass funnel appl-

ying gentle suction using a water vacuum system at a rate of 50 ml/min. The Sephadex G75 matrix on the funnel was washed successively with buffer A (30 ml), buffer A containing 0.16 M NaCl (30 ml) and buffer A (30 ml) by resuspending the matrix and removing the liquid by gentle suction each time. The bound lysozyme to Sephadex G75 matrix was then eluted by resuspending in 30 ml of buffer A containing 1.0 M NaCl as above.

Electrophoresis

Purity of various fractions obtained was monitored using 12% polyacrylamide gel electrophoresis (PAGE) containing sodium dodecyl sulphate (SDS) under reduced condition. The gel was stained with Coomassie Brilliant Blue R-250.

HPLC

HPLC of various fractions was carried out using 50 mM Na-Phosphate buffer containing 150 mM NaCl, pH 6.8 on a size-exclusion Bio-Sil SEC 400 column at a flow rate of 1.0 ml/min. The elution was monitored at 280 nm.

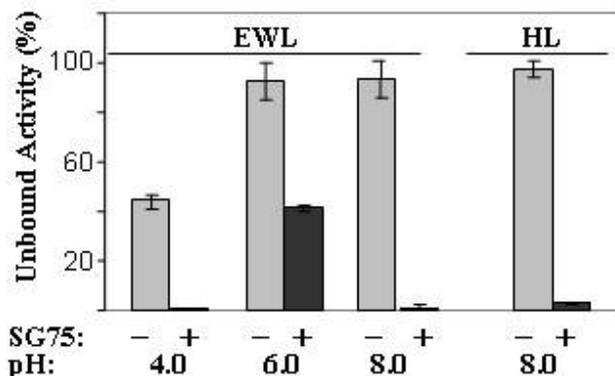


Figure 1. Binding of lysozyme to Sephadex G75 at different pHs. Equal mass (1.2 mg) of commercially available egg white lysozyme (EWL) or human lysozyme (HL) in 0.5 ml buffer of indicated pHs was incubated with or without previously equilibrated 0.5 ml Sephadex G75 (SG75) for 30 min. Following centrifugation for 1 min at 16,300g, lysozyme activity was assayed in the supernatants and presented as percentage. Each bar is the average \pm SD (Standard deviation) of three independent experiments.

RESULTS AND DISCUSSION

Binding of lysozyme to Sephadex G75

We observed complete binding of EWL to Sephadex G75 at pH 8 and partially (40%) at pH 6. It also binds completely at pH 4, even though it lost nearly half of its activity at this pH compared to pH 6 and 8. We also found that human lysozyme isolated from genetically modified rice binds strongly at pH 8. Previously, anomalous elution of EWL was reported in gel-filtration chromatography using dextran (Miranda et al., 1962; Whitaker, 1963) and

Sephadex G75 (Figure 1). (Fernandez-Souza and Rodriguez, 1977) (both used 50 mM phosphate buffer, pH 6.3), suggesting an interaction between lysozyme and these matrices. However, the abnormal elution in these studies was independent of ionic strength. To our knowledge, no study has been reported on its pH dependent interaction.

The complete binding of EWL at pH 4 and nearly half binding at pH 6.0, suggest a charge group with pK_a value near 6.0 may be involved in the binding. The possibility of histidine, whose pK_a is 6.0, was ruled out when modification of the single histidine present in EWL with DEPC could not abolish its binding to Sephadex G75 (Result not shown). Glutamate 35 (Horton et al., 2002) in the active site of lysozyme has been shown to be perturbed (abnormally ionized) and has a pK_a of 6.5, raising a possibility that Glu35 may be involved in Sephadex G75 binding at pH 4 and 6. However, further studies are necessary to establish this possibility. The regaining of complete binding at pH 8.0 is surprising but probably due to conformational change as shown by Kumata et al. (2003).

Further characterization of the binding with Sephadex G75 revealed over 99% of lysozyme activity present EWL and EWP were removed from the supernatant within 5 min (Figure 2a) in buffer A (pH 8.0) and required only 0.1 ml of Sephadex G75 per mg of EWL (Figure 2b). Elution of lysozyme activity and protein (Figure 3a) were observed in fractions containing 0.15 M NaCl or more in buffer A. The combined lysozyme activities in these fractions were 101% of EWP, suggesting a total recovery of lysozyme from the matrix. SDS-PAGE analysis of the fractions (Figure 3a) showed the presence of a protein band corresponding to lysozyme in 0.2 M, 0.25 M, 0.5 M and 1.0 M NaCl fractions. The 0.2 M NaCl fraction contained slight contamination of higher molecular weight proteins, but the other three fractions (0.25–1.0 M) were relatively pure, as no other protein band was visible in these lanes.

Microscale purification

Based on our observations for binding and elution from the Sephadex G75, we carried out microscale purification from EWP with only four steps: binding, washing and elutions with 0.16 M and 1.0 M NaCl - all in buffer A. As shown in Figure 3b, 89% activity was recovered in 1.0 M NaCl fraction that showed a strong lysozyme band free from other protein bands, suggesting this fraction contains most of the lysozyme present in EWP in relatively pure form.

Chromatographic and scaled-up batch purifications

To evaluate the microscale purification strategy on a larger sample volume, we performed a chromatographic

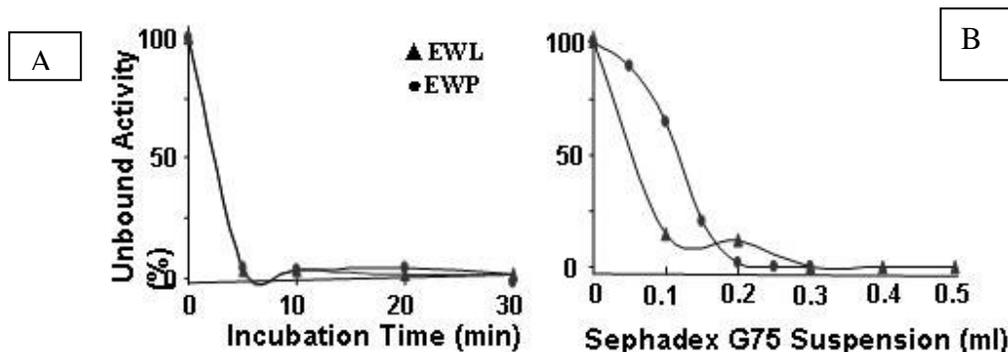


Figure 2. Incubation time and Sephadex G75 volume required for lysozyme binding. **(A)** EWL (0.5 mg) or EWP (3.0 mg) in buffer A was incubated with 0.5 ml of pre-equilibrated Sephadex G75 matrix for the time period indicated. Following that, the unbound activity in the supernatant was separated by centrifugation and assayed for lysozyme activity. **(B)** EWL (2.0 mg) or EWP (3.0 mg) in buffer A was incubated with increasing volume of pre-equilibrated Sephadex G75 for 5 min. At the end of each incubation period, the supernatant was collected and assayed for lysozyme as in Figure 1.

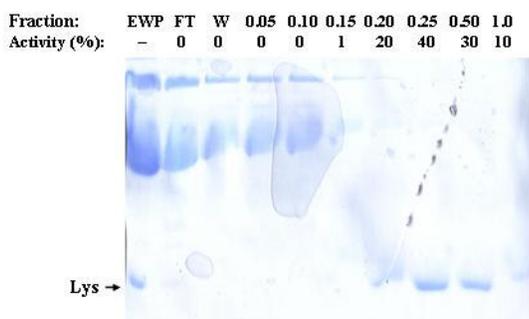


Figure 3A. Percent recovery in and SDS-PAGE analysis of fractions obtained in a microscale purification from EWP. As described in the methods, following incubation of 1 ml of EWP (6 mg proteins) with 0.5 ml Sephadex G75 in buffer A, the various fractions obtained in a microscale purification were: EWP supernatant, FT (Flow-through); wash with buffer A (W) and elution with increasing molarity (0.05, 0.1, 0.15, 0.20, 0.25, 0.50 and 1.0) of NaCl in buffer A. The fractions were analyzed in 12% gel and stained with Coomassie Brilliant Blue R-250.

separation and a scaled-up purification starting from a whole egg. In the latter, all the centrifugation steps were replaced with filtrations through a sintered glass funnel applying gentle suction to separate liquid from the matrix in order to reduce the time. As presented in Figure 4A and B, essentially similar results were obtained in terms of percent yield and purity compared to the microscale purification.

Analysis of the 1.0 M NaCl fractions obtained in both by SDS-PAGE (Figure 4) showed the presence of only lysozyme band, suggesting again a relatively pure lysozyme. This is also supported by the presence of a single major peak in HPLC chromatogram (Figure 5). Specific

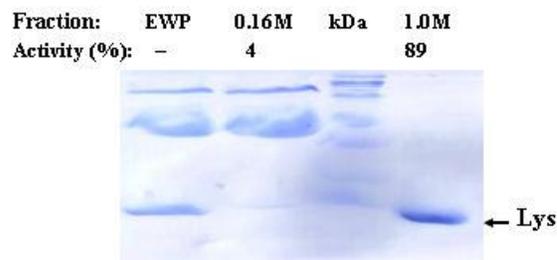


Figure 3B. The experiment was similar as in (A), but after a wash with buffer A, only three elution steps were carried out: 0.5 ml of the buffer A containing 0.16 M NaCl, then with 0.5 ml buffer A containing 1.0 M NaCl. The samples containing lysozyme activity were analyzed by SDS-PAGE in 12% gel stained with Coomassie Brilliant Blue R-250. Activity (%) is the percent lysozyme activity recovered in various fractions.

activities determined in these fractions were comparable to the EWL (Table 1) yielding approximately 72-fold purification in a single step. The overall yield obtained in this simple method was ~80% (Table 1), which is similar to 77% yield obtained using Streamline SP followed by PD-10 and Dye-Ligand Chromatography (Roy et al., 2003b) and 80% yield in a method where metal-affinity precipitation with Cu^{2+} followed by gel filtration chromatography on Sephadex G75 (Roy et al., 2003a). In the former, two chromatographic separation steps and one desalting step were used, and in the later, as lysozyme remains in the solution after protein precipitation with Cu^{2+} , there was no net reduction of sample volume to be gel-filtered on Sephadex G75 (50 mM potassium phosph-

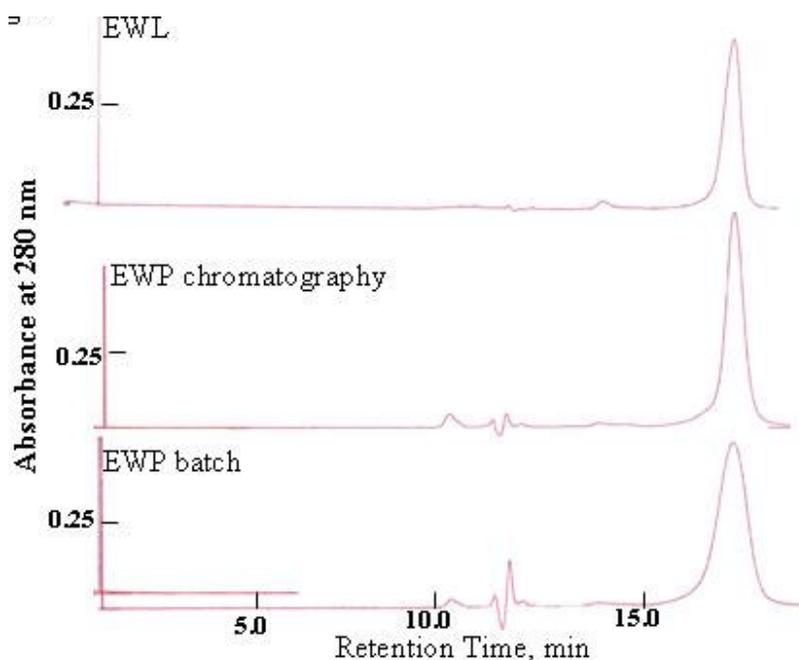


Figure 5. Purity of the lysozyme containing fractions obtained in chromatographic and scaled-scale batch purifications. Size-exclusion HPLC was carried out on a Bio-sil SEC 400 column (300 x mm) in 50 mM Na-phosphate, pH 6.8 containing 150 mM NaCl at a flow rate of 1.0 ml/min.

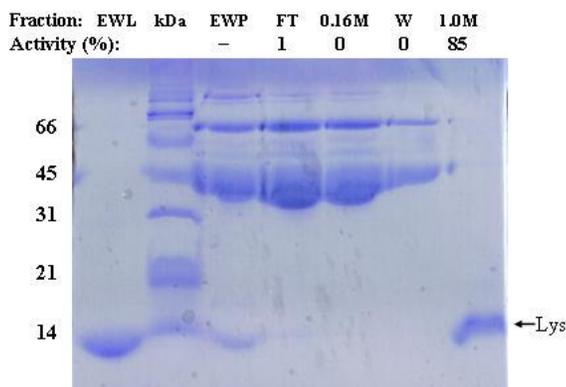


Figure 4A. Percent recovery in and SDS-PAGE analysis fractions obtained in purification from EWP using Sephadex G75. EWP (11 ml) was chromatographed on a column filled with Sephadex G75 (Bed volume 5 ml) and equilibrated with buffer A. Following a wash with buffer A, elution was carried out as in 3B and the fractions were analyzed SDS-PAGE. Activity (%) is the percent lysozyme activity recovered in various fractions.

phate buffer, pH 6.3). The method described here is much simpler as it exploits a pH where lysozyme selectively binds Sephadex G75 allowing scale-up to process larger sample volume at once. Additionally, les-

Fraction:	EWP	FT	W	0.16M	W	1.0M	EWL	kDa
Activity (%)	-	0	1	1	3	83		

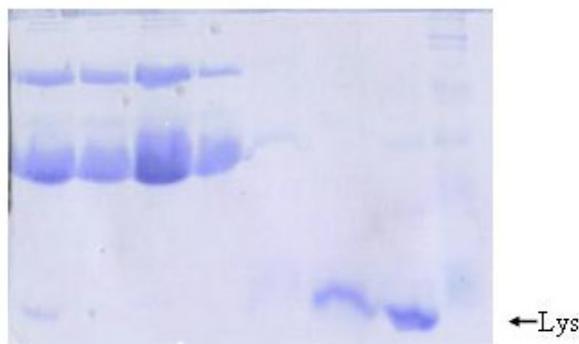


Figure 4B. EWP (165 ml) obtained from a whole hen egg were mixed with 30 ml of Sephadex G75 for 30 min. Then, the supernatant (FT) was separated on a sintered glass-funnel applying gentle (water) vacuum pressure. The matrix on the funnel was resuspended in 30 ml of buffer A, which was again removed by applying gentle suction. This resuspension and removal steps were repeated successively for buffer A containing 0.16 M NaCl, buffer A and buffer A containing 1.0 M NaCl as in Fig 3B. The fractions were analyzed by SDS-PAGE using 12% gel stained with coomassie R250. Activity (%) is the percent lysozyme activity recovered in various fractions.

Table 1. Yield, specific activity and fold-purification of the lysozyme isolated from hen eggs in two different ways using Sephadex G75.

	Yield (%)	Specific Activity (unit/mg protein)	Purification (fold)
EWP	-	954	1
Chromatography	85	72,457	76
Scaled-up batch	83	68,725	72
EWL	-	56,384	-

ser steps reduce the time for purification. This method achieved similar extent of purification (Table 1) compared to the 6 x crystallographic purification procedure, but in less steps and time.

CONCLUSION

Selective and strong binding of lysozyme to the Sephadex G75 matrix at pH 8.0 allowed us to enrich lysozyme from egg whites in a relatively pure form that is comparable to commercially available lysozyme. The procedure described here is simple, rapid and scalable. Strong binding was also observed at pH 4.0 (Figure 1) that may allow isolation of lysozyme, if worked out, from sources that require extraction in acidic pH. To our knowledge, this is the first report of isolation of a protein in a single step using Sephadex G75 as affinity matrix.

ACKNOWLEDGEMENTS

This work was supported by Faculty Research Fund (1-22178 to MRI) and Applied Research Fund (1-22124 to MRI).

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