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Hollow-Fiber Affinity Partitioning Chromatography Using Affinity-Based Reversed Micelles as Stationary Phase

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ABSTRACT

An organic phase containing reversed micelles of Cibacron Blue F-3GA modified lecithin (CB-lecithin) was attached to microporous polypropylene hollow-fiber membranes. This led to the formation of a hollow-fiber column with immobilized dye-affinity reversed micelles within the membrane pores as the stationary phase. This column for chromatographic separation of proteins was called hollow-fiber affinity partitioning chromatography (HFAPC). With a column of 3 mL in net hollow fiber volume, lysozyme and bovine serum albumin (BSA) could be completely separated, and HFAPC experiments with overloaded lysozyme/BSA mixtures yielded a lysozyme fraction with a purity of 93.5% and a high yield. Furthermore, lysozyme was purified by the HFAPC from a crude chicken egg-white solution containing 9.56 mg proteins with a recovery yield of 98.2%. The purity of the lysozyme fraction was increased by 47.4-fold, reaching 85.3% of the total proteins.

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Key Words. Reversed micelle; Affinity partitioning; Chromatography; Hollow fiber; Lysozyme; Purification

INTRODUCTION

Column affinity chromatography with a variety of ligands immobilized to hydrophilic resins has been widely employed in the purification of proteins (1, 2). Highly selective separation can be achieved by affinity chromatography because the technology is based upon the specific association of molecules. Moreover, to make full use of the specificity of affinity interactions, alternative affinity-based separation processes are now under development. These include affinity precipitation (3), affinity crossflow filtration (4), and affinity partitioning based on aqueous two-phase systems (5). These techniques combine the advantages of the high selectivity of affinity interactions and the scalability and ease of continuous operation of precipitation, membrane separation, and liquid–liquid extraction. Furthermore, efforts have been made to incorporate bioaffinity ligands into reversed micelles formed by surfactants in apolar solvents (6–9). By introducing affinity cosurfactants to reversed micelles of ionic surfactants, the selectivity and/or protein transfer can be significantly increased in the range where electrostatic interactions between protein and the surfactants are alleviated (6, 8, 9). Most notably, the incorporation of affinity ligands into reversed micelles of nonionic surfactants has been proposed (10, 11). Because reversed micelles composed of nonionic surfactants have no ability to extract proteins, the introduction of an affinity ligand to a system could result in the extraction of the desired protein by biospecific interaction only (11). The authors recently developed a novel reversed micellar system composed of biocompatible crude soybean lecithin (12). The reversed micelles showed a low solubilizing capacity because the polar headgroups of the crude lecithin are zwitterions and the charge densities of the headgroups are pH-dependent, being small over a neutral pH range. By immobilizing an affinity ligand, Cibacron Blue F-3GA (CB), to the amino groups of the phosphatidylethanolamine contained in crude lecithin, the extraction capacity and selectivity was greatly improved. The partitioning of lysozyme, which has an affinity for CB, was five- to tenfold increased, while that of cytochrome *c*, which has no affinity for CB, was not affected by the CB introduction. Although bovine serum albumin (BSA) has an affinity for CB, it was little extracted to the reversed micelles containing CB because its high molecular mass results in a significant size-exclusion effect. Moreover, the recovered lysozyme exhibits an activity equivalent to the native lysozyme by backextraction, and its secondary structure is also unchanged (12).

In contrast to affinity chromatography, however, the batch processes of affinity extraction as well as of other alternative affinity purification tech-

niques are often less efficient due to their one-plate nature (13, 14). Therefore, separation processes or equipment with high efficiency should be developed to enhance the processing efficiencies of the purification methods. These approaches include the use of a multistage process in which the individual batches are connected in series (14, 15) and the organization of the column equipment (16), depending on differences in the process nature.

The objective of this study was to develop separation equipment for the efficient affinity purification of proteins with reversed micelles. We attached the reversed micellar solution composed of CB-lecithin described above to microporous polypropylene hollow-fiber membranes. This engendered a hollow-fiber affinity partitioning chromatography (HFAPC). The affinity-based reversed micellar solution within the micropores functions as the stationary phase in chromatography. This approach is similar to the hollow-fiber liquid chromatography reported by Ding et al. (16). In the present work, we studied the characteristics of HFAPC by separating lysozyme and bovine serum albumin. Lysozyme was purified by affinity chromatography from a chicken egg-white solution.

EXPERIMENTAL

Materials

Crude soybean lecithin was purchased from Wako Pure Chemical Industries (Osaka, Japan). *n*-Hexane of analytical grade was produced by Tianjin Chemical Reagent Company (Tianjin, China). Chicken egg-white lysozyme (L 6876, 95%), bovine serum albumin (A 6793, 98–99%), and ethylene glycol chitin (E 1502) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Cibacron Blue F-3GA was a product of Fluka Chemie AG (Buchs, Switzerland). Coomassie brilliant blue G-250 was provided by Sino-American Biotechnology Company (Beijing, China). Other chemicals were commercially available reagents of the highest grade. All chemicals and reagents were used as received.

CB-Lecithin Reversed Micelles

The reversed micellar phase was prepared by dissolving crude soybean lecithin in *n*-hexane to make up a solution of 100 g/L. Cibacron Blue F-3GA (CB) was immobilized on the lecithin reversed micelles by the two-phase reaction method reported earlier (17). The CB-attached reversed micellar phase was diluted with *n*-hexane and mixed with the original lecithin/*n*-hexane solution to make the micellar phase contain 50 g/L of lecithin and 0.1 mM of CB. This CB-lecithin micellar solution was employed as the stationary phase by attaching it to hollow-fiber membranes as described below.

Preparation of Chicken Egg-White Solution

One gram of chicken egg-white from a fresh chicken egg was added to 150 mL of 5 mM sodium phosphate buffer (pH 8.0). The suspension was mixed by gentle agitation at 4°C for 30 minutes. Then the mixture was centrifuged at 3000 rpm to remove the insoluble components. The supernatant was used for the purification of lysozyme by HFAPC.

Hollow-Fiber Column and Reversed Micellar Phase Attachment

A hollow-fiber column for HFAPC was made at the Center of Membrane Separation Engineering, Tianjin Institute of Textile Science and Technology, Tianjin, People's Republic of China. It contained 122 microporous polypropylene fibers of 270 μm ID and was 62 μm thick. The fibers had a nominal pore diameter of 0.1 μm and a pore fraction of 75%. The net volume of the fibers in the column was calculated to be 3 mL. The fibers were plotted in an acrylonitrile–butadiene–styrene copolymer tube of 1.0 cm ID and 20 cm length with epoxy resin.

The CB-lecithin micellar solution described above was fed to the hollow fiber column from the column top until the organic phase flowed out of the column bottom. This procedure was repeated three times at 20-minute intervals. Then a stainless steel filter was installed to each end, and the column was fitted to column caps. The column was rinsed with 5 mM phosphate buffer (pH 8.0) to remove any excess organic phase before use. The mechanism for the organic phase attachment was similar to that for the preparation of a supported liquid membrane (18). That is, the micropores in the hydrophobic fibers were filled with the organic phase by capillary force.

Apparatus

Figure 1 describes the apparatus for our HFAPC experiments. An injection valve of 0.2 mL was used when a protein sample of 0.2 mL was to be loaded. Otherwise the protein samples were loaded from a sample reservoir through a double-piston pump. Protein samples were dissolved in 5 mM sodium phosphate buffer (pH 8.0). The same buffer was used as for contaminant protein washing of the mobile phase. Protein (lysozyme) elution was carried out by either stepwise or linearly increasing the mobile phase ionic strength (i.e., NaCl concentration). This was based upon the fact that lysozyme partitioning of the CB-lecithin micellar phase decreased drastically with increasing aqueous phase ionic strength (12). The solution coming out of the column was monitored by an UV detector at 280 nm. The absorbance data were acquired by computer via an analog to digital (A-D) converter.

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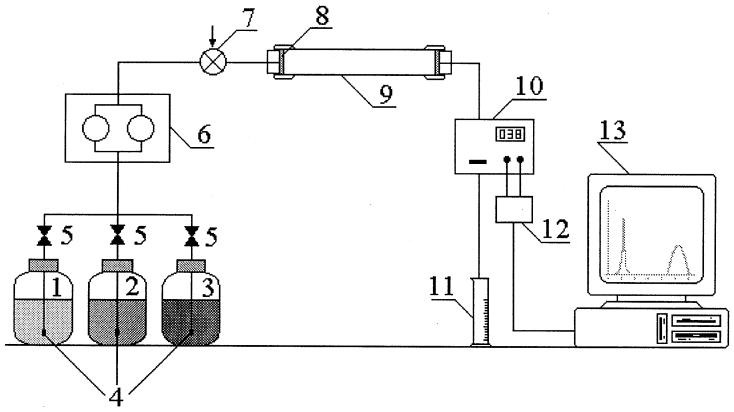

FIG. 1 Experimental apparatus: 1, protein sample; 2, washing buffer; 3, elution buffer; 4, stainless steel filters; 5, valves; 6, double-piston pump; 7, sample injector; 8, stainless steel filter (mobile phase distributor); 9, hollow-fiber column; 10, UV detector; 11, fraction collector; 12, A-D converter; 13, computer.

Teflon tubes of 1.0 mm ID were used for the connections of the apparatus. The volume of the tubes outside the column (i.e., from the sample or buffer reservoirs to the UV detector) was 4.8 mL. The interstitial flow rates of the mobile phase inside the column for sample loading, washing, and elution processes were all the same, 1.46 cm/min in most cases.

Analytical Methods

The concentration of the immobilized CB in the organic phase was measured by a spectrophotometer at 605 nm and determined with a molar extinction coefficient of 8.19×10^3 L/mol·cm. The concentrations of lysozyme, BSA and proteins in egg-white solution were determined by the Bradford method based on a protein dye (Coomassie brilliant blue G-250) binding (19). Lysozyme activity was assayed by the method of Imoto and Yagishita (20) using ethylene glycol chitin as the substrate (12). Lysozyme concentration in protein mixtures was determined by activity measurement.

RESULTS AND DISCUSSION

Chromatography of BSA and Lysozyme

We first performed the chromatography of BSA, lysozyme, and a BSA and lysozyme mixture by injection of 0.2 mL protein aliquots, respectively. Figure 2 shows the chromatograms of BSA (a), lysozyme (b), and the BSA and lysozyme mixture (c). Because of the very low partition coefficient of BSA between the reversed micellar phase and the aqueous phase (21), it was nearly

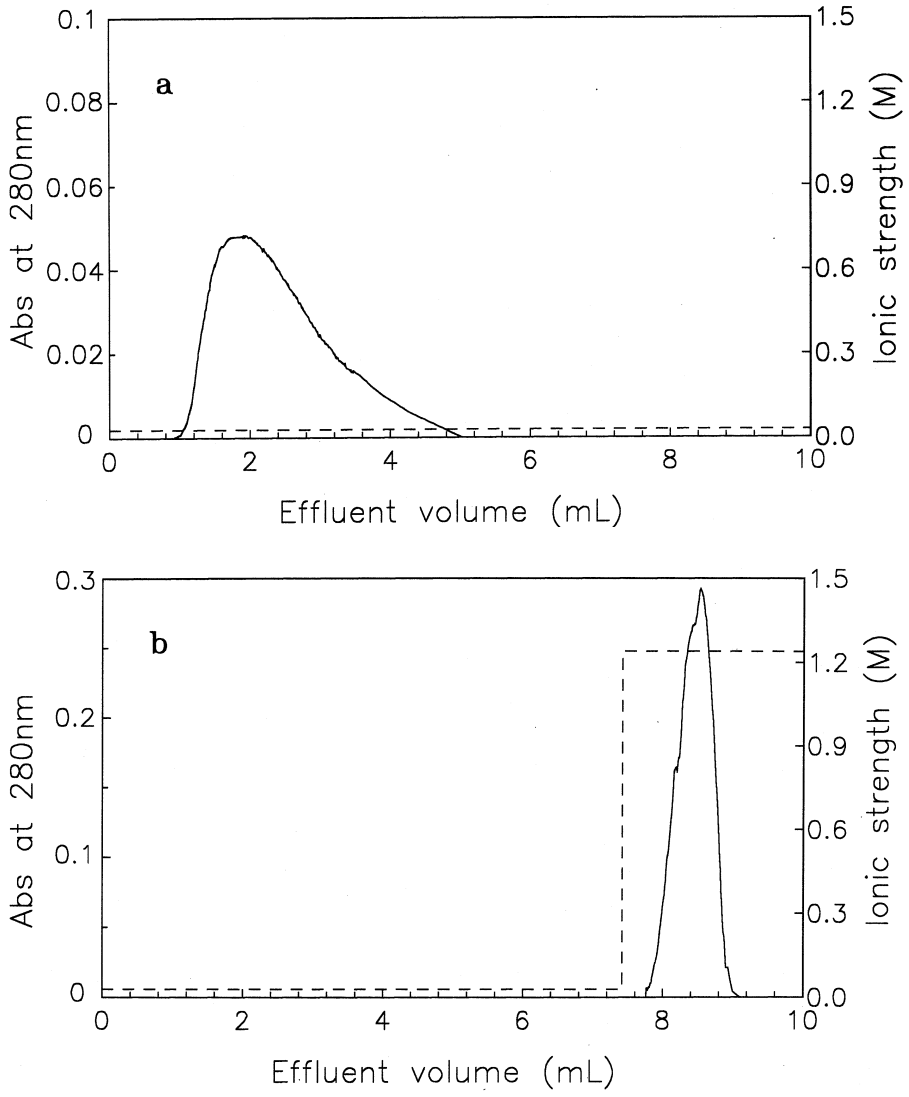

FIG. 2 Elution chromatograms of (a) BSA, (b) lysozyme, and (c) BSA and lysozyme mixture. Dashed lines represent the mobile phase ionic strength. The injected samples (0.2 mL each) contained (a) 0.8 mg/mL BSA, (b) 1.0 mg/L lysozyme, and (c) 0.4 mg/mL BSA and 0.5 mg/mL lysozyme. The mobile phase flow rate was 1.46 cm/min.

eluted at the void volume of the column (i.e., the volume from the sample injector to the UV detector, 1.9 mL) (Fig. 2a). Contrasted with BSA, lysozyme was difficult to elute with the washing buffer due to its high partition coefficient (21). However, when a stepwise increase of the mobile phase ionic strength was applied, we obtained a sharp chromatogram of lysozyme (Fig. 2b). Because of the small partitioning of BSA to the stationary phase, lysozyme and BSA did not affect each other during their chromatographic processing. Therefore, as indicated in Fig. 2(c), the elution behavior of the

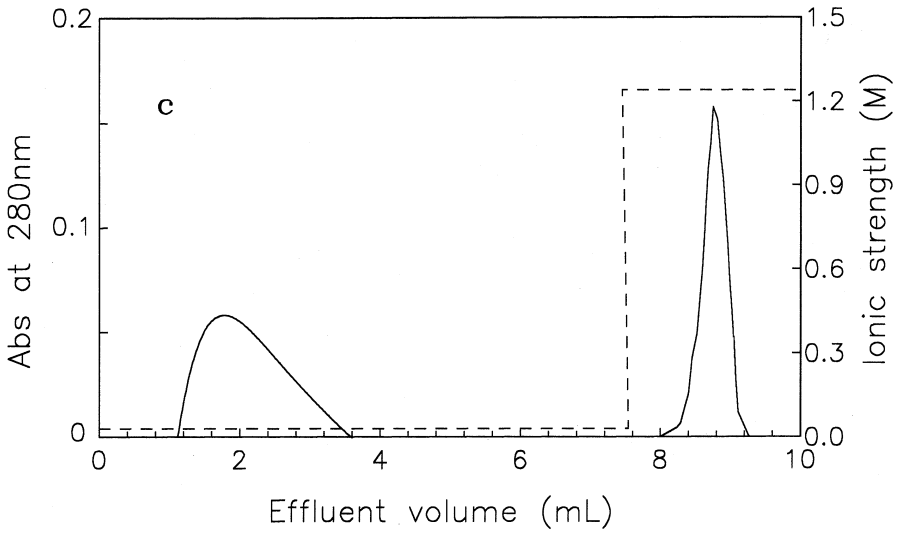

FIG. 2 Continued

lysozyme and BSA mixture was nearly the same as when they were separately loaded to the column. The results indicate that lysozyme and BSA can be completely separated by an HFAPC. Protein content and activity measurement of the collected lysozyme peak confirmed this.

It should be noted that proteins can be adsorbed by synthesized polymer membranes (22). In other words, the retention of lysozyme observed above might be due to hydrophobic and/or electrostatic interactions with the membrane matrix. To confirm whether any nonspecific adsorption occurred in the present system, we carried out a control experiment on the column where the micropores in hollow fibers were filled with an organic phase containing no CB-lecithin micelles. We found little retention of lysozyme in this experiment. We conclude that lysozyme is retained by affinity partitioning to the CB-lecithin micelles rather than by hydrophobic or electrostatic interactions with the hollow-fiber matrix.

Overloading Behavior

This section describes the separation of lysozyme and BSA when a larger volume of protein sample is applied to the column for preparative purposes. Figure 3 displays the results of HFAPC experiments at different protein sample volumes. When 9.6 mL of the protein sample was loaded, the breakthrough of lysozyme occurred at an effluent volume of about 14 mL, after which the absorbance of the effluent increased drastically due to the appearance of lysozyme from the column, as seen in Fig. 3(c). After the loading and washing procedures, lysozyme was eluted by increasing the mobile phase

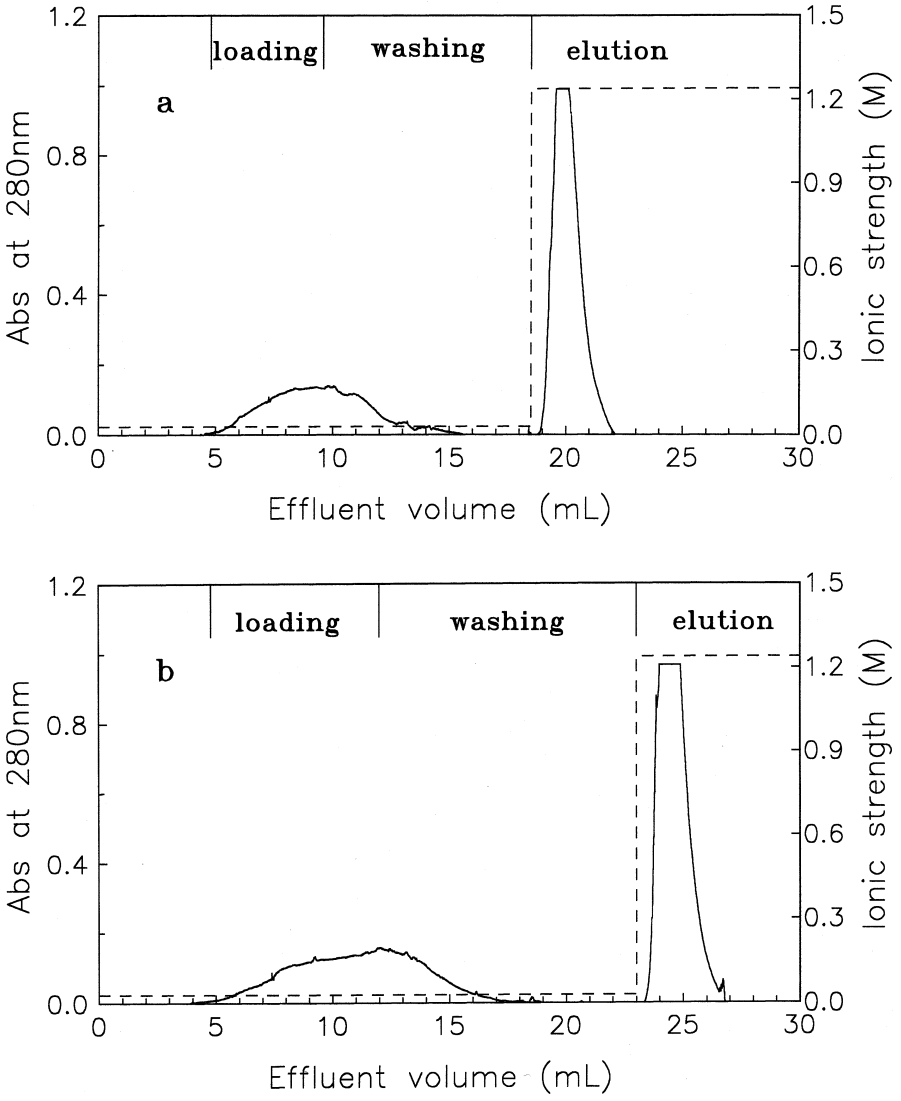

FIG. 3 Separation of BSA and lysozyme at different loading volumes: (a) 4.8 mL, (b) 7.2 mL, and (c) 9.6 mL. BSA and lysozyme concentrations in the samples were 0.4 and 0.25 mg/mL, respectively. The mobile phase flow rate was 1.46 cm/min. Dashed lines represent the mobile phase ionic strength.

ionic strength. Activity measurements of the collected lysozyme peaks, showed that the recovery yield of lysozyme decreased with an increase in the sample volume. The recovery yields of lysozyme in the three experiments were 92.1% (Fig. 3a), 86.4% (Fig. 3b), and 70.2% (Fig. 3c), respectively. The results indicate that when a larger volume of protein sample is applied, more lysozyme will be lost during the loading and/or washing processes.

We also performed a separation experiment at a mobile phase flow rate twice as high as that in Fig. 3. The result is shown in Fig. 4. Under the same

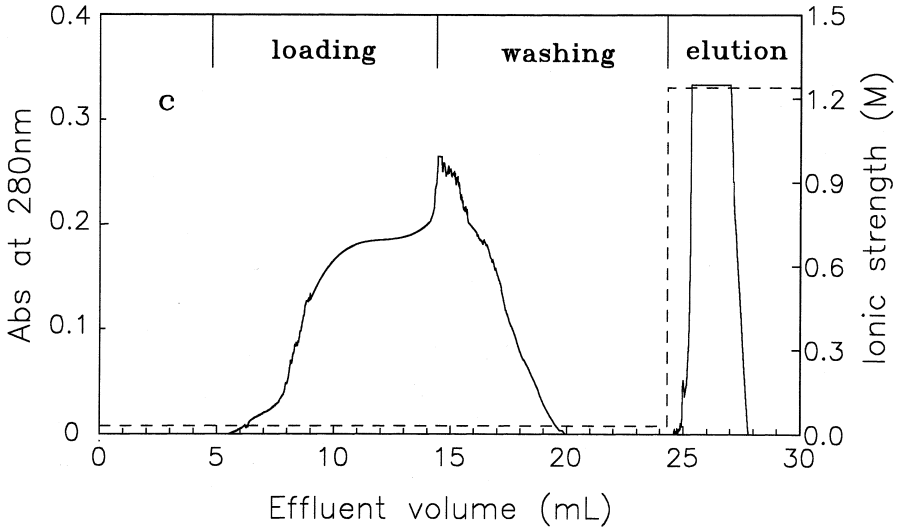

FIG. 3 Continued

conditions as those used in Fig. 3(a) except for the flow rate, we obtained similar BSA and lysozyme peaks in the two experiments. The recovery yield of lysozyme in Fig. 4 is 88.6%, only slightly smaller than that obtained in Fig. 3(a). Hence, the mobile phase flow rate has a negligible effect on the separation of lysozyme and BSA in the flow-rate range studied.

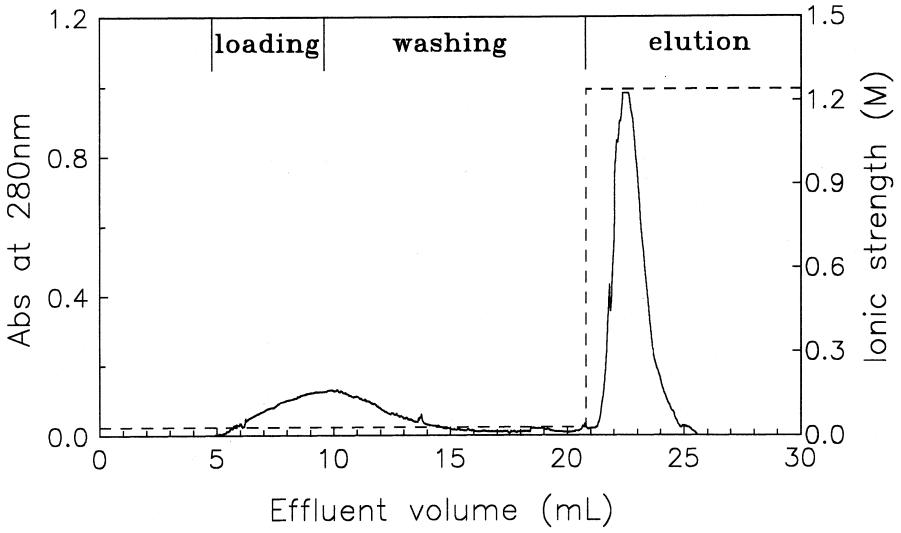

FIG. 4 Separation of BSA and lysozyme at a mobile phase flow rate of 2.92 cm/min. The other conditions were the same as those in Fig. 3(a).

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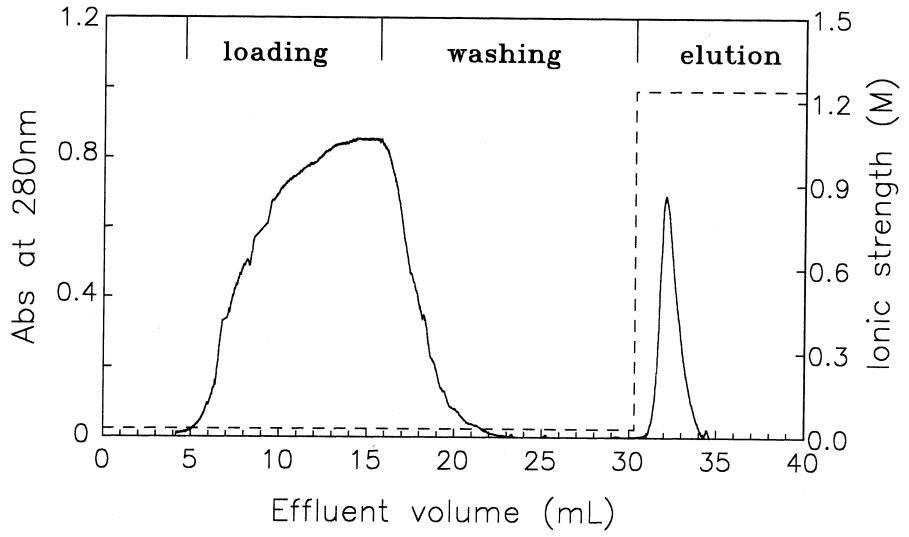

FIG. 5 Purification of lysozyme from crude egg-white solution by stepwise elution. The sample loading volume was 11.1 mL. The mobile phase flow rate was 1.46 cm/min.

Purification of Lysozyme from Egg-White Solution

Lysozyme purification from crude chicken egg-white solution was carried out by overloading HFAPC experiments. The crude egg-white solution had a protein concentration of 0.86 mg/mL and a lysozyme concentration of 0.015 mg/mL. Figures 5 and 6 show the results when different elution protocols were employed. The stepwise elution of lysozyme gave a sharper lysozyme peak due to the rapid release of lysozyme from the stationary phase (reversed micellar

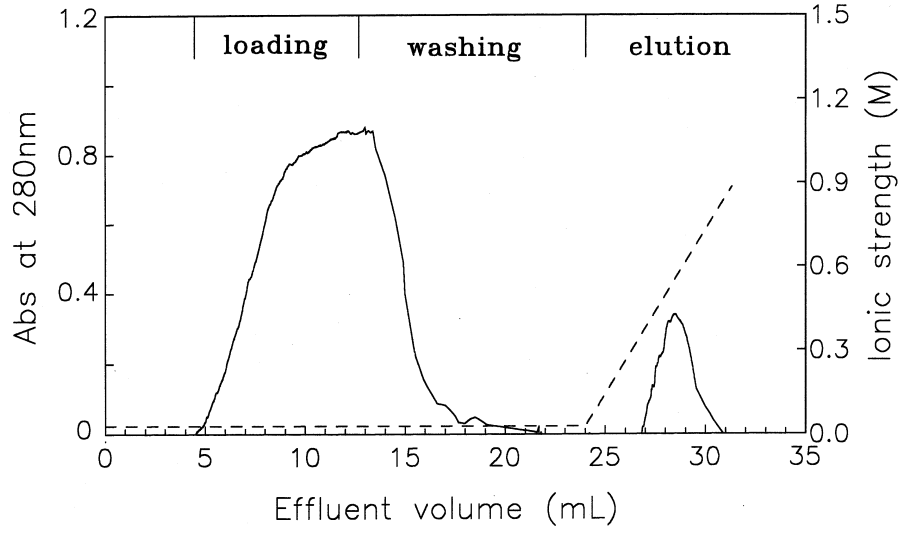

FIG. 6 Purification of lysozyme from crude egg-white solution by linear gradient elution. The sample loading volume was 8.2 mL. The mobile phase flow rate was 1.46 cm/min.

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solution), while gradient elution by linearly increasing the mobile phase ionic strength yielded a broader peak. The lysozyme peak in Fig. 5 was recovered and analyzed by protein measurements as described in the following section.

DISCUSSION

We analyzed the recovered lysozyme peaks in Figs. 3(c) and 5 by protein content and lysozyme activity measurements. Table 1 summarizes the results of the two experiments. The chromatography of the BSA and lysozyme mixture produced a lysozyme solution of 93.5% (i.e., 0.935 mg/mg protein). By loading 11.1 mL of the crude egg-white solution containing 9.6 mg proteins, lysozyme purity was increased 47.4-folds reaching 85.3% (i.e., 0.853 mg/mg protein). The concentrations of the purified lysozyme from the BSA/lysozyme mixture and the egg-white solution were increased by 2.7 and 5.5 times, respectively.

The above HFAPC results were obtained within 2 weeks, with a total chromatographic operation time of 52 hours. Changes in the retention of lysozyme were not observed during the experiments, which is obvious by inspecting the chromatograms shown in Figs. 2 to 6. This means that the hollow-fiber column containing immobilized reversed micelles was stable in this period. In longer-term operation, however, the loss of the organic solvent, lecithin, and CB-lecithin by dissolving in the aqueous mobile phase may result in a decrease of column efficiency. Column efficiency can be recovered by replenishing the reversed micellar solution to the hollow-fiber membranes.

TABLE 1
Purification of Lysozyme from Synthesized Protein Solution and Crude Egg-White Solution

	BSA/lysozyme solution	Egg-white solution
Sample loading:		
Volume (mL)	9.6	11.1
Total protein (mg)	6.2	9.56
Lysozyme concentration (mg/mL)	0.25	0.015
Lysozyme purity (%)	38.7	1.8
Recovered lysozyme:		
Recovery yield (%)	70.2	98.2
Purity (%)	93.5	85.3
Concentration (mg/mL)	0.68	0.082
Purification factor[a] (—)	2.4	47.4
Concentration factor[b] (—)	2.7	5.5

[a] Purification factor was defined as the ratio of the purity recovered lysozyme to that in the sample loaded.

[b] Concentration factor was defined as the ratio of the concentration of the recovered lysozyme to that in the sample loaded.

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The results presented above indicate that lysozyme and BSA can be completely separated by HFAPC, and lysozyme can be purified from crude egg-white solution with a high purification factor under overloading conditions. Because the straightforward flow of the mobile phase in hollow fibers enables the chromatograph to operate at a very low pressure (16), the main advantage of the technique will be the scalability of the hollow fiber column. Further work should be directed toward scaleup and such column improvements as increasing the packing capacity of fibers in the column. This may lead to an increase of the loading capacity of proteins for preparative separation purposes.

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