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Thin-layer ion-exchange chromatography of proteins

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Abstract

Thin-layer chromatography (TLC) is one of the simplest and most convenient techniques to separate small molecules. Of a variety of TLC separation modes, only size-exclusion was successfully used to separate proteins. In this paper, adsorption-TLC was used to separate proteins. The net charges were calculated for four model proteins, albumin, transferrin, lactoferrin and lysozyme, under different pH values. The suitable pH values for separation were determined according to the results from such calculations. Then, the adsorption isotherms of the four proteins were measured to deduce the ionic strength for appropriate elution conditions. Optimal conditions, 0.01 *M* bicine and pH 8.50, and a three-step elution process (1st step 0.01 *M* NaCl, 2nd 0.025 *M* NaCl, and 3rd 0.10 *M* NaCl), were obtained. Finally, the four model proteins were successfully separated under these elution conditions.

Keywords: Adsorption isotherms; Proteins; Albumin; Transferrin; Lactoferrin; Lysozyme

1. Introduction

Protein separation techniques, including sedimentation, gel electrophoresis (GE), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE), are highly developed [1,2]. However, a separation method which is simple, relatively inexpensive, utilizes little or no electrical energy, creates minimal waste, is applicable to diagnostics, and is gentle enough to isolate complex protein systems is still lacking [3].

Thin-layer chromatography (TLC), also called planar chromatography, is one of the simplest, the most popular, and widely used methods of separating small molecules [4]. TLC generally utilizes natural capillary forces to propel the mobile phase along a plate coated with a thin-layer sorbent. Pumps and related devices are not needed. TLC requires minimal amounts of sorbents and solvents and thus produces minimal waste. TLC is easier and more convenient to run than most other separation techniques [5]. Many chromatographic modes, including adsorption, size-exclusion, hydrophobic interaction, reversed-phase and affinity, and isoelectrofocusing and chromatofocusing have been used for separating both small and large molecules using column liquid chromatography (CLC). In TLC, most modes have been used only for separating small molecules. There have been few reports on the various modes in TLC used for separating proteins, except for size-exclusion [4,6,21].

Proteins vary in a number of their physical and chemical properties as a result of their amino acid sequences [7]. The amino acid residues attached to the polypeptide backbone may be positively or negatively charged, neutral and polar, or neutral and hydrophobic. In addition, the polypeptide is folded in definite secondary and tertiary structures to create a unique size, shape, and distribution of residues on the surface of the protein. By exploiting the differ-

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ences in properties between proteins in the mixture, a rational technique to separate them can be designed. Differences in surface charge properties are particularly effective due to the fact that the surface charges are strongly affected by pH and ionic strength (I) in solution [8–10]. There is a relation between isoelectric point (pI), pH, and net charge of protein [24].

Ion-exchange chromatography (IEC), one of the adsorption modes, has been the most widely used technique for the fractionation and purification of proteins, since the introduction of cellulosic ion exchangers in the 1950s [9]. IEC uses the charged groups on the surface of a protein to bind to an insoluble matrix of opposite charge. More precisely, the protein dipolar ion displaces the counterions of the matrix functional groups and will itself be displaced with an increasing proportion of counterion. This is usually done by increasing the concentration of ions in the elution buffer. The most important parameters in IEC are the choice of ion-exchange matrix and the initial conditions, including buffer type, pH, and ionic strength [11].

At equilibrium, the protein concentration in the stationary phase (C_s) is related to that in the mobile phase (C_m) and both are a function of the initial protein concentration and of the ionic strength in the mobile phase when the pH is fixed:

$$C_s = K(C,I)C_m \quad (1)$$

where K is a distribution coefficient between C_s and C_m [12]. Eq. (1) shows that if the protein is strongly adsorbed onto sorbent, in other words C_s is large, the K value will be large; the reverse is also true. In equilibrium theory, if zone spreading effects are ignored, the migration velocity of the protein zone along the sorbent layer can be expressed as:

$$dZ_p/dt = u/[1 + K(V_s/V_m)] \quad (2)$$

where Z_p, u, V_s and V_m are the peak position of the protein, velocity of mobile phase, the stationary volume, and the mobile phase volume, respectively [12]. We should point out that K ($=C_s/C_m$) in Eq. (2) is valid if linear elution conditions exist. For analytical purposes such conditions can be easily satisfied because the capacity of the ion exchanger used is very high [700 mg bovine serum albumin (BSA)/1 dry g anion exchanger (Whatman, Clifton, NJ, USA)] and the deposited sample is very low (8 μg/deposited spot). Generally, the slope $(C_s-0)/(C_m-0)$ of the isotherms are roughly equal to the tangent, dC_s/dC_m, in the low protein sample range (see Fig. 2B). Eq. (2) only roughly describes the migration velocity decrease with an increase in the distribution coefficient K when K equals $(C_s-0)/(C_m-0)$. Our purpose in using Eq. (2) is only to roughly predict the migration differences between different proteins, not to exactly describe protein band migration in TLC. K values differ from protein to protein, reflecting the interaction forces between each protein and the stationary phase. This is the underlying principle of the separation and one of the most important factors affecting separation in liquid chromatography [12].

In this paper, we calculated the net charges for four model proteins, albumin, transferrin, lactoferrin and lysozyme, under different pH values to estimate optimum pH for separation. By measuring adsorption isotherms, the appropriate ionic strength ranges for optimal mobile phase conditions are obtained from the distribution coefficients. We developed a new, convenient, and efficient method to measure adsorption isotherms. The distribution coefficient is here defined as the initial distribution coefficient ($K_{ini.}$), which is different from the more common distribution coefficient, which is defined as $K_{equ.}$ in this paper (see Fig. 2). This new method works well. The separation of the four model proteins were carried out using such optimal elution conditions.

2. Experimental

2.1. Materials

The sorbent, diethyl aminoethyl (DEAE)-derivatized microgranular and preswollen type cellulose (anion exchanger), was manufactured by Whatman. Proteins, including BSA, human holo-transferrin (HHT), bovine milk lactoferrin (BML), and hen egg-white lysozyme (LYZ) were purchased from Sigma (St. Louis, MO, USA). The degree of saturation with iron ions in lactoferrin is about 20%. Generally, the stability of the holo-iron state is higher than that for the apo-iron state. Important

Table 1
Important properties of the proteins used in this study

Abbreviation	Protein	p*I* [18,19]	M_r [18,19]
BSA	Bovine serum albumin	4.6	66 400
HHT	Human holo-transferrin	5.0	79 600
BML	Bovine milk lactoferrin	7.8	80 800
LYZ	Hen-egg white lysosyme	10.7	14 300

properties of proteins are listed in Table 1. All other chemicals used were of analytical grade.

2.2. Solutions

Solutions used for equilibration of ion exchanger, for adsorption isotherms, and for chromatographic development (elution) contained sodium phosphate, bicine and sodium chloride (Table 2).

2.3. Preparation of resin

Before use, DEAE anion exchanger was equilibrated with the desired solutions. Five grams of ion exchanger is dispersed in 40 ml solutions. After manual stirring for about 1 min, pH is adjusted to the desired values (6.50 for phosphate buffer and 8.50 for bicine buffer) with 6 *M* HCl, the suspension is allowed to settle, and the supernatant liquid is decanted off. This process is then repeated three times. For isotherm adsorption use, the final suspension is filtered through Whatman filter paper No. 1, and the equilibrated ion exchanger dried in the air on the filter paper. The dried ion exchanger was stored in a covered container. For chromatographic use, supernatant fluid is then removed until the ratio of settled ion exchanger to supernatant volume is about 4:1. Such anion exchanger is stored for coating the plates.

2.4. Adsorption isotherm

The adsorption isotherm experiments were carried out in 1.5-ml polypropylene micro centrifuge tubes (Bio Plas, San Francisco, CA, USA). The ratio of solid (prepared anion exchanger) to liquid (suspension solution) is 1 (mg):1 (ml). The initial protein concentration was fixed at 800 μg/ml for all samples. NaCl concentration (ionic strength) range is 0–0.20 *M*. The different NaCl concentrations were obtained by different ratios of two different suspension solutions (PSI and PSII or BSI and BSII). After hydration of prepared ion exchanger in aqueous suspension for 5 min, the protein solution was added to the ion exchanger dispersion to make a total volume of 1 ml. Then the tubes were rotated end-over-end for 4 h. The samples were then centrifuged for 5 min at 13 000 rpm. The clear supernatant was measured by intrinsic UV fluorescence. The samples of diluted supernatant were analyzed using a PCI photon counting spectrofluorometer (ISS, Champaign, IL, USA) at $\lambda_{ex.} = 280$ nm and $\lambda_{em.} = 340$ nm. The maximum protein concentration was limited to less than 20 μg/ml to maintain a pseudolinear state between protein concentration and the fluorescence

Table 2
The compositions and properties of suspension solutions

Name	$NaH_2PO_4 \cdot H_2O$ (*M*)	$Na_2HPO_4 \cdot 7H_2O$ (*M*)	Bicine (*M*)	NaCl (*M*)	pH
PSI	0.00685	0.00315			6.50
PSII	0.00685	0.00315		0.20	6.50
BSI			0.01		8.50
BSII			0.01	0.20	8.50

intensity. The additives and pH in the sample solutions and standard solutions were identical. Temperature was controlled at 21±1°C.

2.5. Coating of plates

Glass plate sections 6.9 cm×8.8 cm in dimension were cleaned by soaking in chromic sulfuric acid solution for 6 h. They were then scrubbed by sponge, then rinsed by double deionized and filtered water. Such plates were placed in a vacuum oven and dried before coating with ion exchanger slurry. In order to control the thickness of sorbent, two narrow plastic strips (total 0.6 cm wide) were bound on the two-sides of the plate with two-sided tape. The thoroughly suspended ion exchanger slurry was poured into the plate at a thickness setting of 1 mm. After making the slurry even and smooth, the plate was dried overnight at room temperature. The thickness of dried sorbent on the plate is about 0.4 mm. The plate was stored in the open air.

2.6. Sampling

Five samples, including BSA, HHT, BML, LYZ, and a mixture composed of BSA, HHT, BML, LYZ were used on one plate. The concentration for all five samples was 4 mg/ml. The amount per sample is 2 μl. Every deposited spot on the plate contained 8 μg of protein. Before all samples are ready for depositing, the plate to be eluted is placed in the development container and the mobile phase is allowed to ascend about 1 cm in order for the samples to be deposited on a wet surface. After sampling, the plate was quickly returned to the container for elution.

2.7. Development (elution)

In these experiments ascending development was applied in a beaker covered with transparent plastic film. The mobile phase level in the beaker was about 0.4 cm. The rate of development, which varied to a small extent from plate to plate, averaged about 0.8 cm/min.

2.8. Detection

Fluorescamine was used as a label. Fluorescamine reacts with primary amine groups on proteins, unbound dye is nonfluorescent, and its sensitivity depends on the number of amines present [13]. The developed plate was dried in the air at room temperature. The dried plate was dipped into the solution of 0.05% fluorescamine in acetone. After about 1 h, the plate was placed under UV light of multiband UV-254/366 nm of Model UVGL-58 (^{U}UVP, Upland, CA, USA). The fluorescent spots were recorded as chromatograms with a Spectra AF camera (Polaroid, Cambridge, MA, USA), then the chromatograms were copied onto the transparent papers in order to make the chromatograms more clear.

3. Results and discussion

3.1. Adsorption isotherm

The batchwise method was used to measure adsorption isotherms in order to quickly screen the conditions for development (elution). The advantages of this method are convenience, rapidity and simplicity. In addition, this method is thrifty because only a very small amount of protein sample is required, which is very important for expensive proteins. The disadvantages are that the exact elution condition is not identical with that obtained from adsorption isotherms. So, such measurement can only predict the rough range of ionic strength required for elution.

Since ion exchanger was used as sorbent, it is necessary to analyze the properties of proteins and sorbent and to characterize the surface charge which plays an important role in ion exchange chromatography. The program ''p*I* protein 1.0v1'' (Internet: iho@biobase.aau.dk) was used to calculate the net charge of proteins under different pH values (Fig. 1). From Fig. 1, pH values 6.50 and 8.50 were selected because at these two pH values the different proteins have different net charge values. At the same time this is an optimal choice for the DEAE anion exchanger because the working pH range for anion exchanger is less than 9.00 [20].

The real aim in measuring adsorption isotherms is to establish the relation between distribution coefficient (K), ionic strength and pH. However, there are two methods to express K. One has the same initial

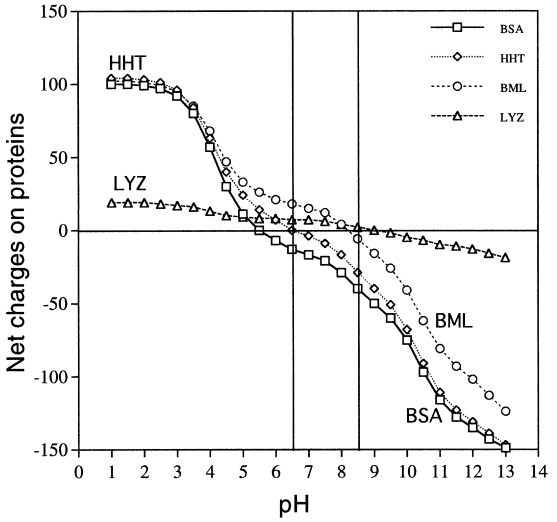

Fig. 1. The calculated relationship between charge number and pH of albumin, transferrin, lactoferrin and lysozyme.

protein concentration in the mobile phase ($C_{m,I.}$); such a K is called the initial distribution coefficient and termed as $K_{ini.}$ (Fig. 2A). The other is the more common K, which has the same equilibrated protein concentration in mobile phase ($C_{m,e.}$); such a K is called the equilibrium distribution coefficient and termed as $K_{equ.}$ (Fig. 2B). From our experiments, we found that it is more convenient and efficient to measure $K_{ini.}$. In such adsorption isotherms the sample is of low concentration and is near the linear adsorption range. So, the ratio, C_s/C_m, in the isotherms can roughly replace the tangent, dC_s/dC_m, which also avoids the minus values sometimes obtained with dC_s/dC_m. It seems more suitable to call such $K_{ini.}$ the partition coefficient [22,23]. In Fig. 2A, every point corresponds to a different ionic strength, which is very important for the step elution processes. However, in Fig. 2B, every curve corresponds to an ionic strength.

With pH fixed, the adsorption isotherm is measured at various sodium chloride concentrations, range 0.00–0.20 *M*, realized by changing the ratios of PSI/PSII or BSI/BSII. Phosphate and bicine were used as buffer salts for pH values 6.50 and 8.50, respectively (Table 2). Figs. 3 and 4 demonstrate the relations of initial distribution coefficients ($K_{ini.}$) of four proteins with ionic strength under different buffer salts and pH values. Figs. 3 and 4 show that

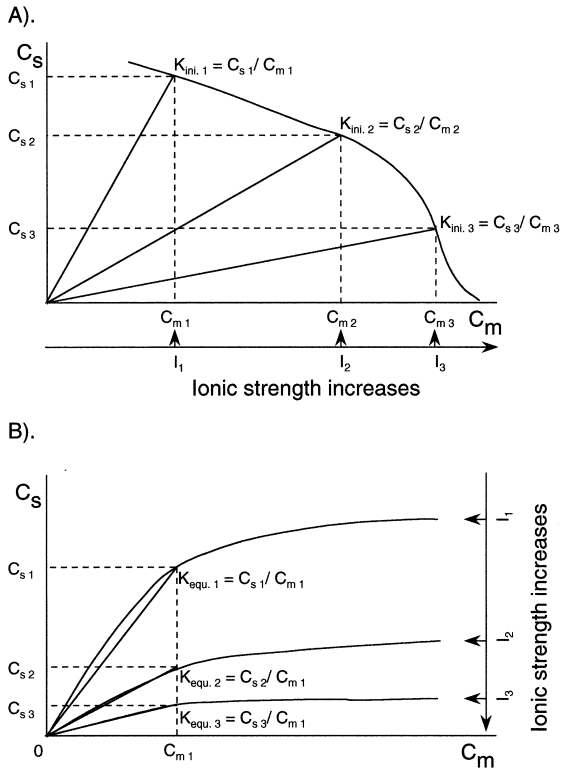

Fig. 2. Schematic diagrams of how to obtain the two different distribution coefficients. (A) $K_{ini.}$ is the initial distribution coefficient. Every point (C_s, C_m) in the isotherm corresponds to an ionic strength. When ionic strength increases, C_s decreases and thus $K_{ini.}$ reduces. C_s almost equals zero, or no protein is adsorbed, when ionic strength reaches a higher value. (B) $K_{equ.}$ is the equilibrating distribution coefficient.

for acidic proteins (albumin and transferrin), $K_{ini.}$ values under the conditions studied are very sensitive to NaCl concentrations or ionic strength of aqueous suspension, because Coulombic interactions dominate between acidic proteins and anion exchanger when pH>p*I* values (Table 1). For basic proteins (lactoferrin and lysozyme), the sensitivity to ionic strength is low. These are expected results because anion exchanger is more appropriate for separation of acidic proteins. For basic proteins, the adsorption is not strongly controlled by the Coulombic interaction force, therefore $K_{ini.}$ values of lactoferrin and lysozyme are small and do not change very much when ionic strength is changed. However, both lactoferrin and lysozyme are more hydrophobic than albumin and transferrin [14]. Also the fact that

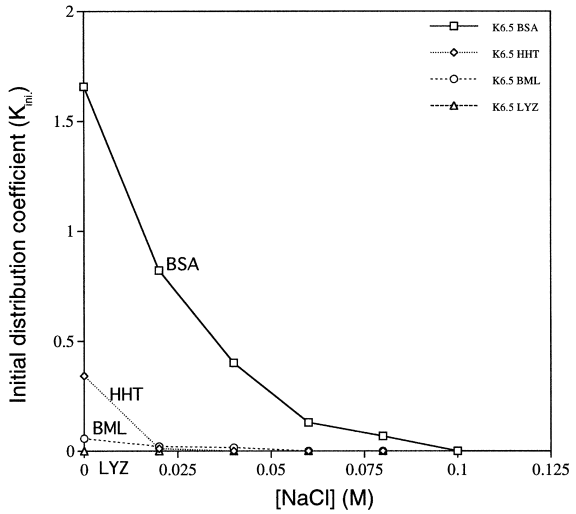

Fig. 3. The relation of initial distribution coefficient with ionic strength. Conditions for adsorption isotherms: 0.01 *M* sodium phosphate and pH 6.5.

lysozyme has a patch of negative charge [15] makes the behavior of lysozyme and lactoferrin on anion exchangers not easily predictable.

$K_{ini.}$ values of acidic proteins (albumin and transferrin) are strongly affected by buffer salt types. In sodium phosphate buffers $K_{ini.}$ values are small (less than 2). However, in bicine buffer the $K_{ini.}$ value sometimes is very large and the maximum reaches 137. Phosphate ions have the ability to complex with positively charged groups on the anion exchanger, plus multivalent charges on phosphate increase the ability to shield the surface charge on anionic exchanger, thus greatly reducing the ζ-potential of the charged groups on the anionic exchanger. This leads to a decrease of Coulombic interactions between acidic proteins and anion exchanger. Bicine is a zwitterionic buffer which is a dipolar ion molecule and does not complex with the anionic charges on the ion exchanger, as does phosphate. So in bicine buffer solution, the ζ-potential of the charged groups on the anionic exchanger was not strongly affected by bicine buffer ions.

Generally, if $K_{ini.}$ values are very large and $K_{ini.}$ is not sensitive to ionic strength, the elution of the adsorbed protein by increased ionic strength is difficult because the interaction between protein and sorbent is too strong. In addition, if $K_{ini.}$–I curves of two proteins overlap or the gap between curves is too narrow, these two proteins can not be separated or separation is difficult because they have the same or similar elution properties under this mobile phase condition. Based on these criteria, the conditions in Fig. 4 satisfy the elution requirements for separation. In thin-layer ion exchanger chromatographic development, the conditions in Fig. 4 were utilized.

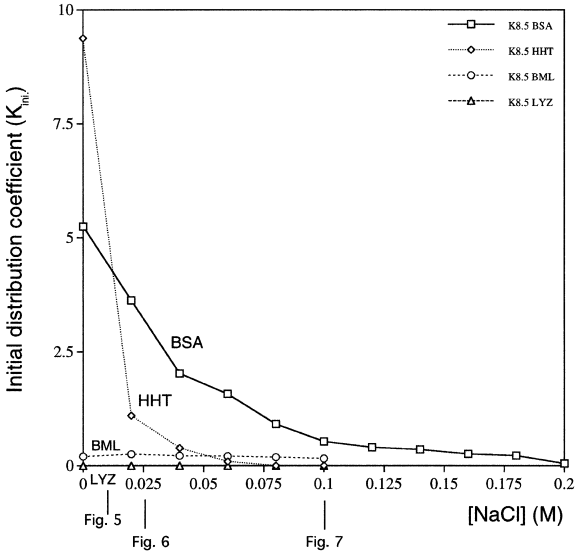

Fig. 4. The relation of initial distribution coefficient with ionic strength. Conditions: 0.01 *M* bicine and pH 8.5. Elution conditions are based on this figure.

3.2. Chromatograms

Fig. 1 shows that when pH = 8.50 lysozyme and lactoferrin have small numbers of net positive charge and net negative charge, respectively; albumin has maximum net negative charge number, and the net negative charge number of transferrin is in between lactoferrin and albumin. Generally, the order of Coulombic interaction force is the same as that of the net charge number when the anionic surface charge is fixed. So, for the four model proteins, the elution strengths (ionic strength) are different for different proteins. The larger the net charge number of protein, the higher the elution strength [16]. This is consistent with the results in Fig. 4. Eq. (2) demonstrates that the larger the $K_{ini.}$ value, the smaller the protein migration velocity (dZ_p/dt). It can be

expected that in chromatograms lysozyme should be in the front, followed by lactoferrin, followed by transferrin, with albumin last.

At the same time, the most important differences between small molecules and proteins arise from the polyelectrolyte character of the latter. The polyelectrolyte properties of proteins often result in multiple binding between the solute and the stationary phase. Multibinding tends to result in very high or very low distribution coefficients between phases, with an abrupt transition between extremes [17]. The retention mechanism behaves as ''all or none''. In addition, the gradient method may possess higher resolution at the cost of considerable tailing, while the discontinuous method will provide sharp zones at the expense of resolution, especially if the changes in effluent concentration are large. That is why discontinuous elution is required in TLC ion-exchange chromatography of proteins.

Figs. 5–7 are the chromatograms from a three-step elution. When ionic strength is 0.01 *M* NaCl (Fig. 5), only lysozyme migrates in the same step with effluent; when ionic strength is 0.025 *M* NaCl, not only does lysozyme continue to move together with effluent but lactoferrin begins to migrate (Fig. 6); when ionic strength in the third step was increased to 0.10 *M* NaCl, only albumin was kept in the sampling area, the other three proteins migrate at their own velocities (Fig. 7). The mixture sample in the middle (Fig. 7) was separated into four proteins. This discontinuous elution method clearly showed that the elution of one protein corresponds to one ionic strength and different proteins require different ionic strengths. It should be pointed out that the ionic strength required for elution is much higher than that shown in Fig. 4. These differences are caused by the different ratios (20 times) of solid volume divided by liquid volume of suspension solution or mobile phase between the chromatographic and batchwise methods, which was introduced simply in Section 3.1.

The concentration of NaCl in equilibrated solvent for the ion exchanger and in the mobile phase is of critical importance in controlling the migration of

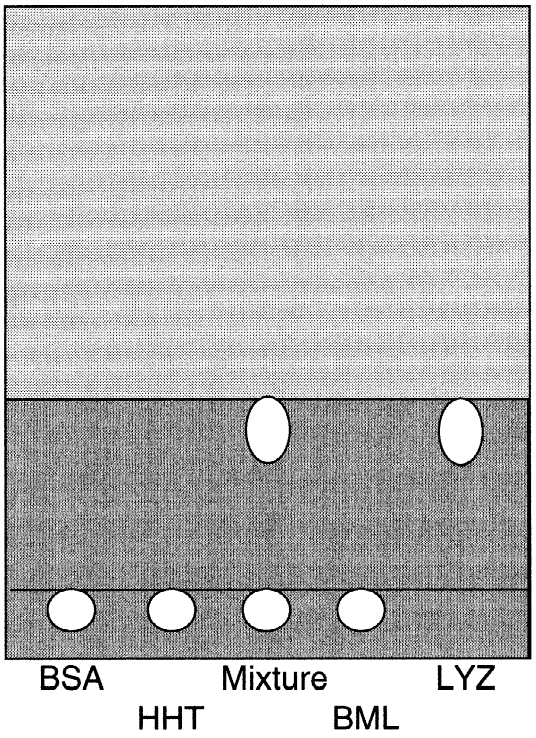

Fig. 5. The chromatogram after second elution step.

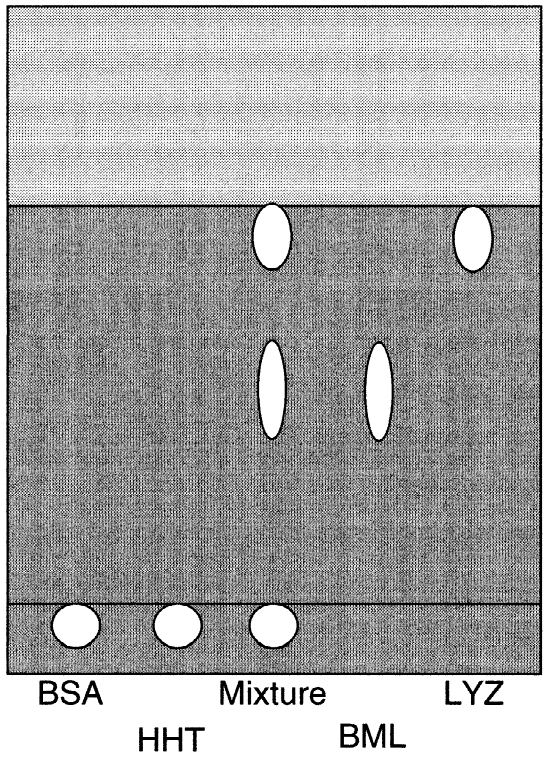

Fig. 6. The chromatogram after first elution step.

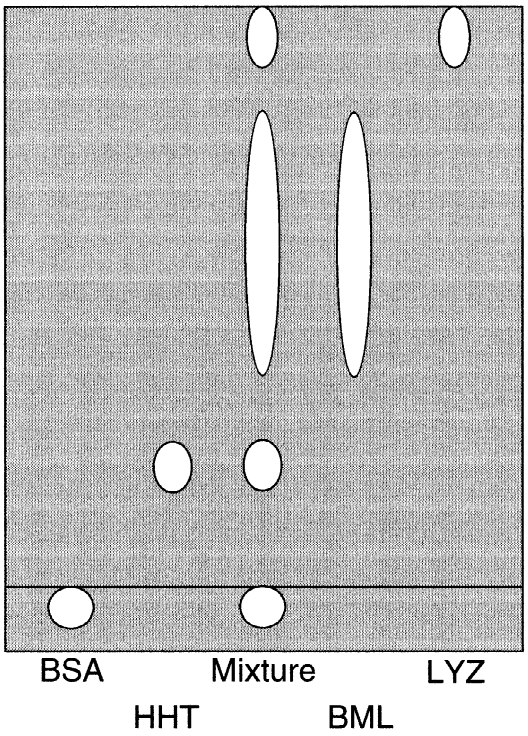

Fig. 7. The chromatogram after third elution step.

proteins. The significant value is the sum of the salt concentration in the ion exchanger and in the mobile phase. As this sum increases, the migration velocity of the samples also increases, but the increase in mobile phase is more effective according to our experiments. The optimal sum was semiempirically obtained. For the plates used in Figs. 5–7, the NaCl concentration in anion exchanger is 0.025 *M*.

4. Conclusion

Our studies were designed to obtain the basic information needed to develop a process for protein separation using adsorption TLC. Through establishing the relation between protein net charge and pH, the suitable pH values for elution were determined. Then by measuring adsorption isotherms for model proteins, the ionic strength ranges for elution were obtained from the initial distribution coefficients of the four proteins. From these data, 0.01 *M* bicine was selected as the mobile phase and a three-step (first step 0.01 *M* NaCl, second 0.025 *M* NaCl, and third 0.10 *M* NaCl) elution process was applied. Finally, the four proteins were thus successfully separated. This process represents a successful separation of proteins by adsorption-TLC. Future work will focus on optimizing the design of a separation device to make the development and visualization more convenient. Adsorption-TLC should be capable of separating proteins in complex mixture samples.

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