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# Separation of Protein Mixtures by High-Performance Liquid Chromatography

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The mechanisms of protein separation by high-performance liquid chromatography (HPLC) using a Shodex OH pak B-804 column were examined, and an attempt was made to relate the separation behavior of some proteins to their molecular weight, isoelectric point and/or molecular size.

HPLC elution was carried out with several kinds of buffer solutions at different pH values to cover the range of protein isoelectric points, and the effect of the addition of salts on the elution time was tested. Elution was not necessarily in order of molecular weight or molecular size, and also varied according to whether or not protein-protein, protein-solute and/or protein-column packing interactions occurred in the buffer solutions used.

**Keywords**——protein; α-fetoprotein; high-performance liquid chromatography; separation mechanism; Shodex OH pak

High-performance liquid chromatography (HPLC) offers higher separation speeds for many organic small molecules, and is now also being widely applied for the separation of high molecular weight substances.

Exclusion chromatography (*i.e.* gel filtration or gel permeation, GPC) concerns the separation of sample molecules on the basis of size and shape differences. GPC is the simplest and most predictable chromatographic method. Solutes are separated by size with the large excluded molecules eluting first and the small totally included molecules eluting last. In practice, however, many extraneous mechanisms such as adsorptive, hydrophobic and ionic effects may affect the retention of a solute.[1]

Recently, HPLC has been successfully used for the separation of peptides and proteins, *e.g.* vasopressin, oxytocin, angiotensin II and substance P,[2-5] human leukocyte interferon,[6] albumin, lysozyme, myoglobin, cytochrome c and human globin[7-11] as well as hemoglobin chain variants.[12]

Although the results of these investigations indicate that protein mixtures can be separated by HPLC at least in some cases, the mechanisms of the protein separation have not been fully elucidated.

The authors examined the use of HPLC for the separation of several protein mixtures which are difficult to separate by using the usual eluting solvents. For several protein mixtures having a range of isoelectric points, association, dissociation and coagulation phenomena were observed by HPLC as a function of pH, and of the addition of salts or alcohols. The authors also found that elution is not necessarily in order of molecular weight or isoelectric point of proteins.

We have been attempting to determine and separate α-fetoprotein from other clinically important protein mixtures for the investigations of advanced tumors of the liver or testis. The proteins involved in these studies usually differ greatly in their physicochemical properties.

Since the physical and chemical properties of the proteins vary so widely, it is very likely that some will interact with any given support. It is important, therefore, to identify the mode of interaction and either eliminate it or take advantage of it when useful.

The authors carried out HPLC separation of "native" proteins without denaturation treatment by the addition of denaturing agents, such as urea and guanidine hydrochloride, or surface active agents, such as sodium dodecyl sulfate.

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### Experimental

**Reagents and Materials or Chemicals**——HPLC-quality methanol was from Wako Osaka, Japan. Bovine albumin (fraction V) was obtained from Wako Pure Chemical Ind. (Osaka, Japan), human hemoglobin from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.), human α-fetoprotein from Kainos Co. (Tokyo, Japan), and bovine γ-globulin (Cohn fraction II) and bovine fibrinogen from Povite Production N.V. (Amsterdam, Holland). The other proteins, as follows, were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.): ovalbumin, crystallized and lyophilized (G III); lysozyme, from egg white (Grade I); cytochrome c, from horse heart (Type III, VI); catalase, from beef liver; pepsin, from hog stomach mucosa 1: 60000; myoglobin, from skeletal muscle.

**Apparatus**——The liquid chromatograph used was a Hitachi HPLC model 635A (Hitachi Seisakusho, Japan) equipped with a multi UV detector (250 nm). The column was Shodex OH pak B-804 (B×500 mm) (Showa Denko K.K., Japan).

**Procedure**——Proteins were dissolved in the same solvent as the mobile phase to give concentrations of 0.1%. A 10 μl sample of each protein solution was injected into the HPLC machine. The solutions were kept at −5°C when not in use. Chromatography of protein mixtures was carried out at 24°C with a flow rate of 0.5 ml/min. All proteins were identified by their retention times, which were obtained by chromatography of individual preparations. The mobile phase was prepared by mixing the following solutions in an appropriate ratio to obtain a desired pH value: solution A, pH 4.70 buffer solution of 0.2 M acetic acid and 0.2 M sodium acetate; solution B, pH 6.72 buffer solution of 0.1 M $Na_2HPO_4$ and 0.01 M $KH_2PO_4$ containing 0.2 M potassium chloride; solution C, pH 7.20 buffer solution of M/30 $KH_2PO_4$ and M/30 $Na_2HPO_4$; solution D, solution C containing 0.2 M KCl; solution E, pH 7.80 buffer solution of 0.1 M $Na_2HPO_4$ and 0.01 M $KH_2PO_4$ containing 1% methanol; solution F, pH 8.50 buffer solution of 0.1 M $KH_2PO_4$ and 0.05 M sodium borate.

The mobile phase solvents were filtered through a 0.4 μm membrane filter and degassed prior to use.

## Results and Discussion

Proteins are not always eluted in order of molecular weight. The retention times of proteins in gel permeation chromatography (GPC) also depend on other interactions, such as hydrogen bonding, protein–protein association or dissociation, or protein–solute interaction in the buffer solutions, and the changes in these factors with pH and other conditions, as described in the introduction.

Thus, in investigating the separation mechanisms of protein mixture on adsorption and GPC at several pH values, it is necessary to take into consideration the isoelectric points of individual proteins, the ionic strength, and the effect of organic solvents as the mobile phase, among other factors.

The authors investigated the separation mechanisms of some proteins using a column of Shodex OH pak B-804, which is a spherical porous rigid polyester gel containing OH groups and is thus hydrophilic.

The isoelectric points[13] and molecular weights of proteins used in this investigation are summarized in Table I.

TABLE I. Isoelectric Point and Molecular Weight of Proteins

| Protein | Origin | Isoelectric point | | | Molecular weight |
|---|---|---|---|---|---|
| Albumin (Alb) | Human serum | 5.2 | 4.6—5.3 | | 65000 |
| Ovalbumin (Ova) | Egg white | 4.58 | 4.6—4.8 | | 45000 |
| γ-Globulin (γ-G) | | 7.0 | 6.3—8.4 | | 160000 |
| Lysozyme (Lyso) | Egg white | | 10.5—11.0 | | 14300 |
| | Human serum | | | | |
| Fibrinogen (Fib) | Human | 5.4 | | | 500000 |
| Cytochrome c (Cy-C) | | | 10.5—11.0 | | 12400 |
| Catalase (Cat) | Beef liver | 5.7 | 5.9 | | 244000 |
| Pepsin (Pep) | Pig | 2.2 | 2.8 | $<1$ | 35000 |
| α-Fetoprotein (α-FP) | Human serum | 4.8 | 4.7 | | 65000 |
| Hemoglobin (Hemo) | Human erythrocyte | | 7.0 | | 67000 |
| Myoglobin (Myo) | Horse muscle | 6.8 | 7.3 | | 17000 |

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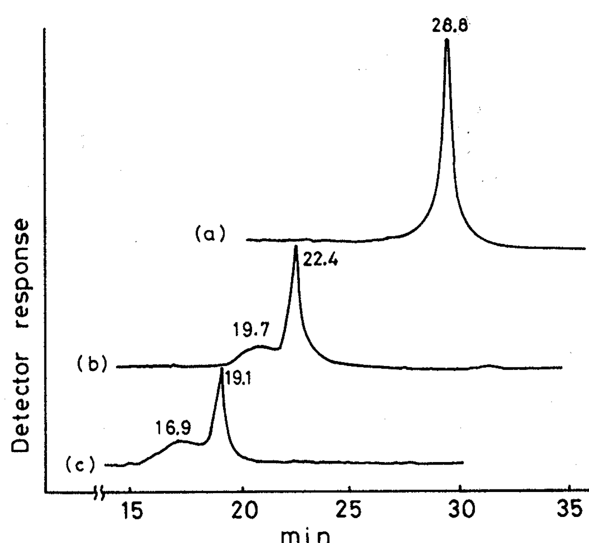


Fig. 1. HPLC of $\gamma$-Globulin at Different Concentration using Water as the Mobile Phase

(a) 10% $\gamma$-globulin.
(b) 1% $\gamma$-globulin.
(c) 0.1% $\gamma$-globulin.

As shown in Fig. 1, decrease of $\gamma$-globulin concentration reduces the retention time in the Shodex OH pak column using distilled water as the eluting solvent. Similar phenomena also appeared in the chromatography of $\alpha$-fetoprotein. It seems that water has no buffer action.

The above result may be at least partly explained in terms of the gradual association of protein molecules with increasing dilution; the resultant larger molecules have smaller retention times. The separation of mixtures of lysozyme (M.W. 143000) and ovalbumin (M.W. 45000), and of lysozyme and $\alpha$-fetoprotein (M.W. 65000) was examined using phosphate buffer solution at pH 7.20 as the mobile phase. Neither of them could be separated, despite the great differences in molecular weights and isoelectric points, as shown in Fig. 2(a). On the other hand, the addition of 0.2 M KCl to the buffer solution permitted the complete separation of these mixtures (Fig. 2(b)). These phenomena indicate that the adsorption of lysozyme on the packing material is differently affected than that of ovalbumin and $\alpha$-fetoprotein by the addition of inorganic salts.

Crone *et al.* reported that the use of eluting buffer solution at ionic strengths above 0.1 eliminates most of the adsorption of proteins on controlled-pore glasses.[14]

As shown in Fig. 3, in the case of myoglobin and $\alpha$-fetoprotein mixtures, the addition of potassium chloride decreased the effective separation, and complete separation could not

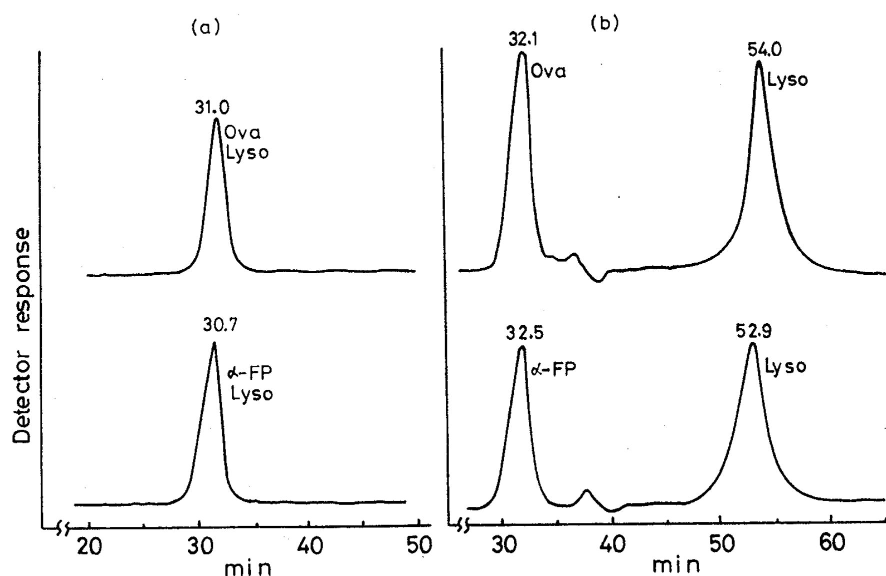


Fig. 2. Separation Behavior of Mixtures of Ovalbumin and Lysozyme, and of Lysozyme and $\alpha$-Fetoprotein

(a) mobile phase: pH 7.20 phosphate buffer.
(b) mobile phase: pH 7.20 phosphate buffer containing 0.2 M KCl.

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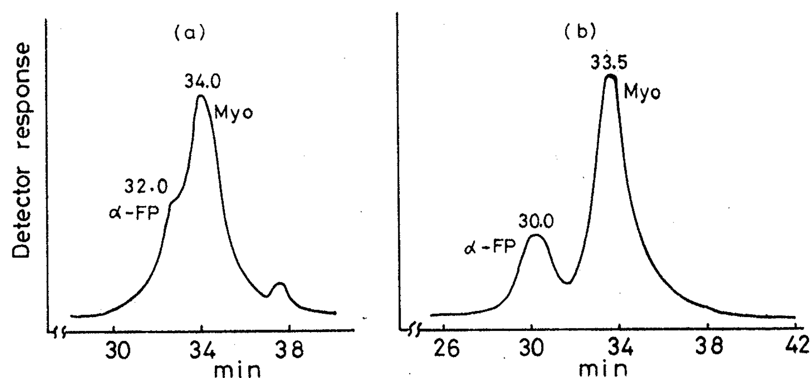


Fig. 3. Separation Behavior of α-Fetoprotein and Myoglobin

(a) mobile phase: pH 7.20 phosphate buffer containing 0.2 M KCl.
(b) mobile phase: pH 7.20 phosphate buffer.

be obtained. Thus, the addition of potassium chloride is not always effective for protein separations.

Pepsin (M.W. 35000) was separated from lysozyme in order of molecular weight using the same eluting solvent. Consequently, the GPC molecular weight calibration plot for ovalbumin, pepsin and lysozyme was linear on Shodex OH pak B-804 (Fig. 4).

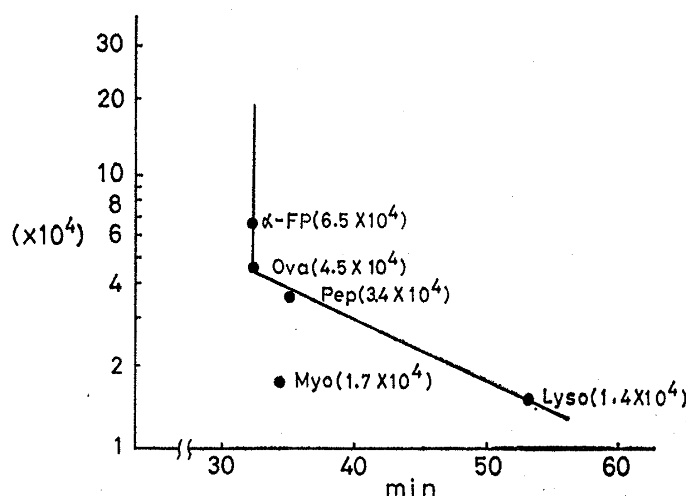


Fig. 4. The Relationship between Molecular Weight of Proteins and Elution Volume on Shodex OH pak B-804 using pH 7.20 Phosphate Buffer containing 0.2 M KCl

In some cases, mixtures of protein solutions form a precipitate; a mixture of 0.1% each of ovalbumin, pepsin and lysozyme solution deposited a white precipitate, as shown in Fig. 5. The supernatant of the solution showed a single peak on the chromatogram, which was identified as ovalbumin by running an authentic sample for comparison (Fig. 5(a)), while two chromatographic peaks were observed from the solution obtained by dissolving the precipitate in more basic buffer solution: one was pepsin, and the other lysozyme, as shown in Fig. 5(b).

Pepsin and lysozyme have fairly different pH values at their isoelectric points; the former has its isoelectric point at strongly acidic pH (2.2, 2.8) and the latter in the alkaline pH region (10.5—11.0). It is considered that the positively charged pepsin molecule interacts with the negatively charged molecule to yield a precipitate.

Kroeff *et al.* studied the retention and separation of short chain peptides on a $C_8$ bonded phase, and they found that the peptides are strongly retained in acidic and basic solutions and show minimum retention at the isoelectric pH.[15]

By the application of the present technique, ovalbumin can be isolated from the mixture of pepsin, lysozyme and ovalbumin. The combination of these three proteins also formed a precipitate when phosphate buffer solution containing 0.2 M KCl at pH 6.72 was used as an eluting solvent (Fig. 6), though the separation behavior was somewhat differed.

In the supernatant separated from the precipitate by centrifugation, some lysozyme is present with the main component, ovalbumin. The chromatogram of a solution of the precipitate in pH 7.70 phosphate buffer showed two peaks, one of which is lysozyme. The retention

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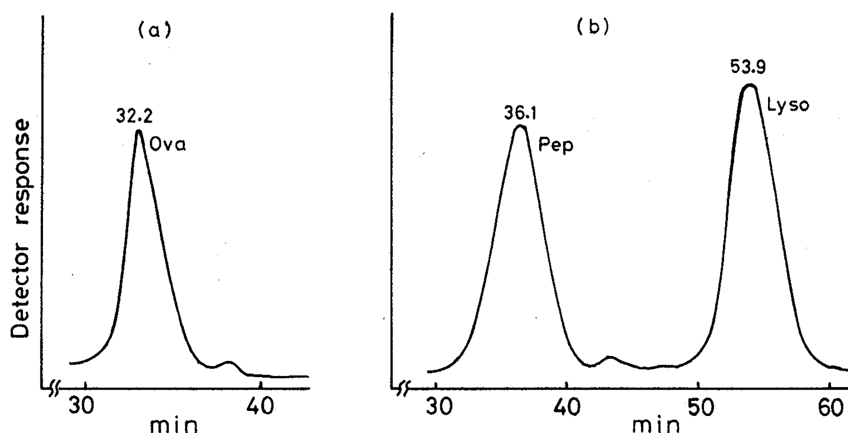


Fig. 5. HPLC of Ovalbumin, Pepsin and Lysozyme with pH 7.20 Phosphate Buffer containing 0.2 M KCl

(a) Supernatant of the mixture.
(b) Solution of the precipitate formed from the mixture.
The precipitate was dissolved in buffer solution with a higher pH value.

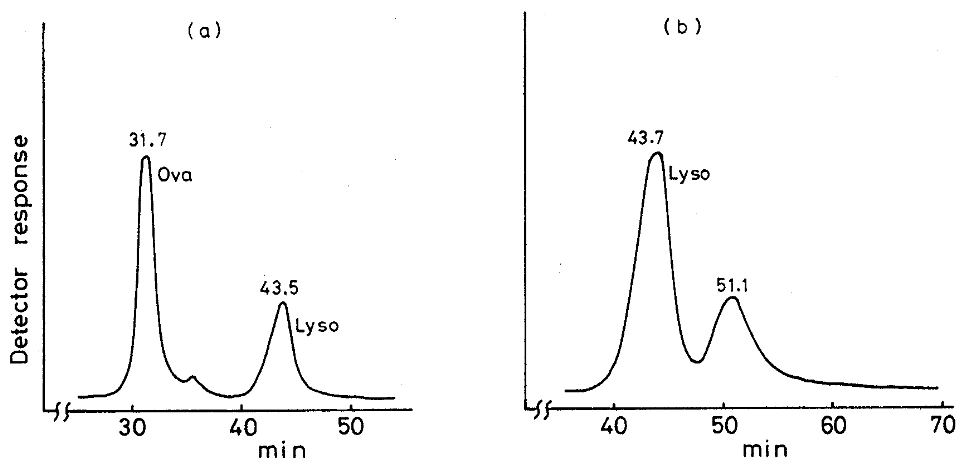


Fig. 6. HPLC of Ovalbumin, Lysozyme and Pepsin Mixture with pH 6.72 Phosphate Buffer containing 0.2 M KCl

(a) Supernatant of the mixture.
(b) Solution of the precipitate formed from the mixture.
The precipitate was dissolved in buffer solution (pH 7.80).

time of the other peak is 51.1 min and it is not identical with that of pepsin (Fig. 6), because pepsin has a retention time of 31—32 min in pH 6.72 phosphate buffer containing 0.2 M KCl. This indicated the possibility of interaction between the packing material and protein in protein solutions.

The use of a slightly more acidic buffer solution (pH 6.8) slows the retention time on Shodex OH pak B-804 in comparison with that of pH 7.20 buffer solution. The retention times of ovalbumin, pepsin and lysozyme were 32.2, 36.1 and 53.9 min in pH 7.20 phosphate buffer containing 0.2 M KCl, whereas they were 31.7, 31.8 and 43.5 min in pH 6.72 phosphate buffer containing 0.2 M KCl, respectively. Mönch *et al.*[16] reported that peptides are more strongly bound to ODS at acidic pH, and thus retention times are increased.

Subsequently, the HPLC separation of several protein mixtures was examined in pH 7.80 phosphate buffer containing 1% methanol. Good resolutions of the samples were achieved in several cases. $\gamma$-Globulin, fibrinogen, cytochrome c, and myoglobin were distinctly separated, as shown in Fig. 7. When $\alpha$-fetoprotein was added to these four proteins, overlapping of $\alpha$-fetoprotein with $\gamma$-globulin was observed and separation was impossible.

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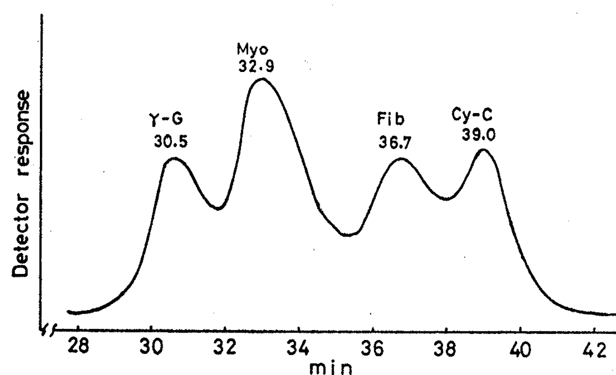


Fig. 7. HPLC of Protein Mixtures with pH 7.80 Phosphate Buffer containing 1% MeOH

Table II. HPLC Behavior of Proteins with pH 8.50 Buffer Solution

| Protein | $t_R$ (min) |
|---|---|
| Alb | 29.8 |
| α-G | 30.2 |
| Lyso | 29.7 |
| Cat | 28.5 |
| Pep | 28.4 |
| α-FP | 29.1 |
| Myo | 32.5 |

Table III. Separation of Proteins with pH 7.80 Phosphate Buffer containing 1% MeOH

| Alb | Ova | γ-G | Lyso | Fib | Cy-C | Pep | α-FP | Myo |
|---|---|---|---|---|---|---|---|---|
| | + | | + | | | | | |
| | + | | | | | + | | |
| | | | ± | | | | ± | ± |
| | | | +* | | | +* | | |
| | +* | | +* | | | +* | | |
| | | + | | + | + | | | + |
| | | − | | + | + | | − | + |
| +* | | | +* | | | +* | | |

+ : separated.
± : partially separated.
+*: separated after dissolution of precipitated proteins.
− : not separated.

Borate buffer at pH 8.50 prepared from 0.1 M $KH_2PO_4$ and 0.05 M sodium borate was used as the mobile phase and seven individual proteins were eluted from the Shodex column. As can be seen in Table II, the retention times of the tested proteins other than myoglobin are almost the same (29—30 min), regardless of the isoelectric point and molecular weight. Therefore, these proteins could not be separated.

It was recognized that the elution time of the proteins studied did not completely parallel their molecular weights. The albumin peak was observed at $t_R$ 58 min using 0.2 M acetate buffer solution at pH 4.70, while other proteins showed no distinct peaks on the corresponding chromatograms.

Dimenna *et al.*[17] examined the separation of bovine skim milk proteins by gel-permeation HPLC using Toyo-Soda TSK-GEL (Type SW) columns. They reported that the elution volumes of BSA (69000 daltons) and IgG (161000 daltons) were similar, though their molecular weights are markedly different. They also showed that each chromatographic peak of protein contained or was contaminated with the other proteins, and no protein beands were obtained with several peaks.

On the other hand, Hearn *et al.*[18] pointed out that the peptide chain proper probably makes only a very small contribution to the retention process for peptidases on reverse-phases under low pH elution conditions.

In the examination of molecular weight determinations and molecular weight calibration curves of polymer or polypeptides and proteins, it is necessary that the solute should not interact with the gel matrix. However, it is still difficult to relate the elution times of proteins on HPLC to molecular weight, isoelectric point, molecular size or molecular structure.

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