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# A Simple Purification Procedure for α-Fetoprotein by Immunoadsorbent Column Chromatography[1)]

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Human α-fetoprotein was purified from human cord serum by employing an immunoadsorbent column, consisting of Sepharose 4B coupled with the antibody to human α-fetoprotein. By using 0.2 M $Na_2CO_3$ solution (pH 11.6) as an eluent, a large amount of α-fetoprotein was obtained from the immunoadsorbent column in high purity. After gel filtration on Sephadex G-150, the purified α-fetoprotein was demonstrated to be homogeneous by polyacrylamide gel electrophoresis, SDS-gel electrophoresis and two-dimensional immunoelectrophoresis.

**Keywords**——α-fetoprotein; human cord serum; immunoadsorbent technique; oncodevelopmental protein; immunoelectrophoresis

## Introduction

α-Fetoprotein (AFP) was first discovered by Pederson[2)] in 1944 in fetal calf serum. Since it was detected in sera of patients with hepatocellular carcinoma or embryonal carcinoma,[3)] AFP was designated as a carcinofetal or oncodevelopmental protein. In recent years some properties of human AFP have been reported, and the proposed functions of AFP include the binding of some substances[4—6)] and immunoregulatory effects.[7,8)] However, the biological role of AFP has not yet been clarified, largely because homogeneous AFP cannot be easily obtained from human tissues in large quantities. For this reason, a simple and rapid purification procedure able to provide homogeneous AFP is desirable. It is albumin among various components in human tissue which mainly complicates the conventional purification methods because of its physicochemical similarity to AFP.[9)]

Recently, immunoadsorbent techniques for the purification of human AFP have been developed because of their simplicity and specificity.[10,11)] In our laboratory, α-antitrypsin, superoxide dismutase[12)] and kallikrein were purified from human tissues by employing such techniques; using $Na_2CO_3$ solution as an eluent, we obtained a high recovery. We have established a purification procedure for homogeneous AFP from human cord serum in two steps, *i.e.*, immunoadsorbent column chromatography and Sephadex G-150 gel filtration. Severe conditions would not be suitable for the elution of the adsorbed AFP, because degradation may occur, so we employed $Na_2CO_3$ solution as a mild eluent and obtained a high recovery of homogeneous AFP compared with others previously reported.[11,13,14)]

## Materials and Methods

### Materials

Activated CNBr-Sepharose 4B, Sephadex G-150 and Ampholyte (pH range 4.0—6.5) were purchased from Pharmacia Fine Chemicals, PM-10 membranes were obtained from Amicon Corp. and complete Freund's adjuvant was purchased from Difco Lab. DEAE-cellulose was purchased from Brown Co. Phosphorylase a and pyruvate kinase were obtained from Boehringer Mannheim GmbH and bovine serum albumin, ovalbumin and alcohol dehydrogenase were obtained from Sigma Co. Other chemicals were of analytical grade reagent.

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**Methods**

**Preparation of Anti-Human α-Fetoprotein Antibody**——A rabbit was injected 4 times with purified human AFP (100 μg) emulsified with an equal volume of complete Freund's adjuvant at 2-week intervals, and 2 weeks after the last injection the antiserum was collected. The antiserum was incubated at 56°C for 30 min, then saturated $(NH_4)_2SO_4$ solution (pH 7.0) was added to make 33% (v/v) and the mixture was stirred for 30 min at room temperature, then left overnight at 4°C. The antiserum solution was centrifuged at $12000 \times g$ for 20 min at 4°C. The precipitate was dissolved in and dialyzed against 20 mM potassium phosphate buffer (pH 8.0). The dialyzate was applied to a DEAE-cellulose column (3 × 20 cm) equilibrated with the above buffer, and the γ-globulin fraction was obtained.

**Preparation of Immunoadsorbent Column**——The activated CNBr-Sepharose 4B (15 g dry weight) was washed first with 1 mM HCl then immediately with 0.1 M carbonate buffer (pH 8.3) containing 0.5 M NaCl (coupling buffer) on a glass filter under suction. The γ-globulin fraction (*ca.* 450 mg) which had been dialyzed against the coupling buffer was then added to the activated Sepharose 4B slurry and the whole was gently agitated for 2 h at room temperature. Next, the slurry was washed with the coupling buffer on the glass filter until the effluent showed no measurable absorbance at 280 nm. An equal volume of 1 M ethanolamine previously adjusted to pH 7.0 with HCl was added as a blocking reagent and the mixture was agitated for 2 h at room temperature. This slurry was washed 3 times with 0.1 M acetate buffer (pH 4.0) containing 0.15 M NaCl, and 3 times with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, alternately.

**Analytical Gel Electrophoresis**——Electrophoresis on 7.5% polyacrylamide gel was carried out according to the method of Davis[15] and sodium dodecyl sulfate gel electrophoresis according to the method of Weber and Osborn.[16] Gels were stained with 0.5% Coomassie Brilliant Blue R-250 to visualize protein and with perchloric acid-Schiff reagent to visualize glycoprotein.

**Molecular Weight Determination**——The molecular weight was determined by SDS-gel electrophoresis according to the method of Weber and Osborn.[16] Phosphorylase a (M.W. 94000), bovine serum albumin (M.W. 67000), pyruvate kinase (M.W. 57000), ovalbumin (M.W. 43000) and alcohol dehydrogenase (M.W. 37000) were used as standard proteins.

**Isoelectric Focusing**——The isoelectric focusing was performed according to the method of Vesterberg and Svensson.[17] Ampholyte pH gradient of 4.0—6.5 was formed and electrofocusing was done at 800 V at 6°C for 48 h.

**Immunoelectrophoresis**——The rocket immunoelectrophoresis method and the two-dimensional immunoelectrophoresis method were employed with the antibody to human AFP, according to the Laurell procedure with a minor modification.[18]

**Purification of α-Fetoprotein**——Ten ml of human cord serum was applied to an immunoadsorbent column (5 × 12 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, and the column was thoroughly washed with the same buffer until the effluent showed no measurable absorbance at 280 nm. The adsorbed AFP was eluted with 0.2 M $Na_2CO_3$ solution (pH 11.6). The column was immediately washed with the above buffer in preparation for subsequent use. The fraction containing AFP was immediately neutralized with HCl and concentrated with an Amicon PM-10 membrane, then subjected to Sephadex G-150 gel filtration on a column (3.5 × 120 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl.

## Results

### Comparison of Recovery of α-Fetoprotein from an Immunoadsorbent Column with Various Eluents

Fresh human cord serum was applied to the prepared anti-human AFP antibody coupled Sepharose 4B. When the effluent showed no measurable absorbance at 280 nm, elution was started with various eluents: 0.2 M $Na_2CO_3$ solution (pH 11.6), 0.2 M glycine-HCl buffer (pH 2.5) containing 0.15 M NaCl, 3 M NaSCN solution (pH 5.1) and 6 M urea solution (pH 10.6). When

TABLE I. Comparison of Recovery of α-Fetoprotein from an Immunoadsorbent Column

| Eluent | Recovery of AFP (%) |
|---|---|
| 0.2 M $Na_2CO_3$ (pH 11.6) | 100 |
| 0.2 M Glycine–HCl, 0.15 M NaCl (pH 2.5) | 53 |
| 3 M NaSCN (pH 5.1) | 78 |
| 6 M Urea (pH 10.6) | 93 |

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the elution was finished, the eluate was immediately neutralized, dialyzed against distilled water and concentrated. The recovery ratio was calculated from the results of rocket immunoelectrophoresis. The use of 0.2 M $Na_2CO_3$ solution (pH 11.6) as an eluent gave a higher recovery as compared with other eluents, as shown in Table I, and the recovery of AFP was calculated as almost 100%. Its adsorption capacity was unchanged even after it had been used more than 100 times.

### Purification of α-Fetoprotein from Human Cord Serum

Human AFP shows a single major band on polyacrylamide gel electrophoresis, using the immunoadsorbent technique. A minor band was excluded by further purification by Sephadex G-150 gel filtration. Fig. 1 shows the results of polyacrylamide gel and SDS-gel electrophoresis of the purified AFP. The protein band and the sugar band appeared to coincide. Fig. 2 shows the results of analysis of the purified AFP by the Ouchterlony double diffusion method and by two-dimensional immunoelectrophoresis. A single precipitin line was seen against anti-human AFP antibody. Therefore the preparation was confirmed as homogeneous by electrophoretical and immunological investigation.

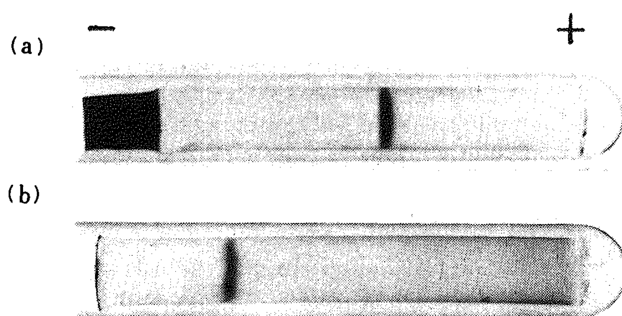


Fig. 1. Polyacrylamide Gel Electrophoretical Patternsof Purified α-Fetoprotein from Human Cord Serum

(a) 7.5% gel, (b) 7.5% gel in the presence of SDS and 2-mercaptoethanol.

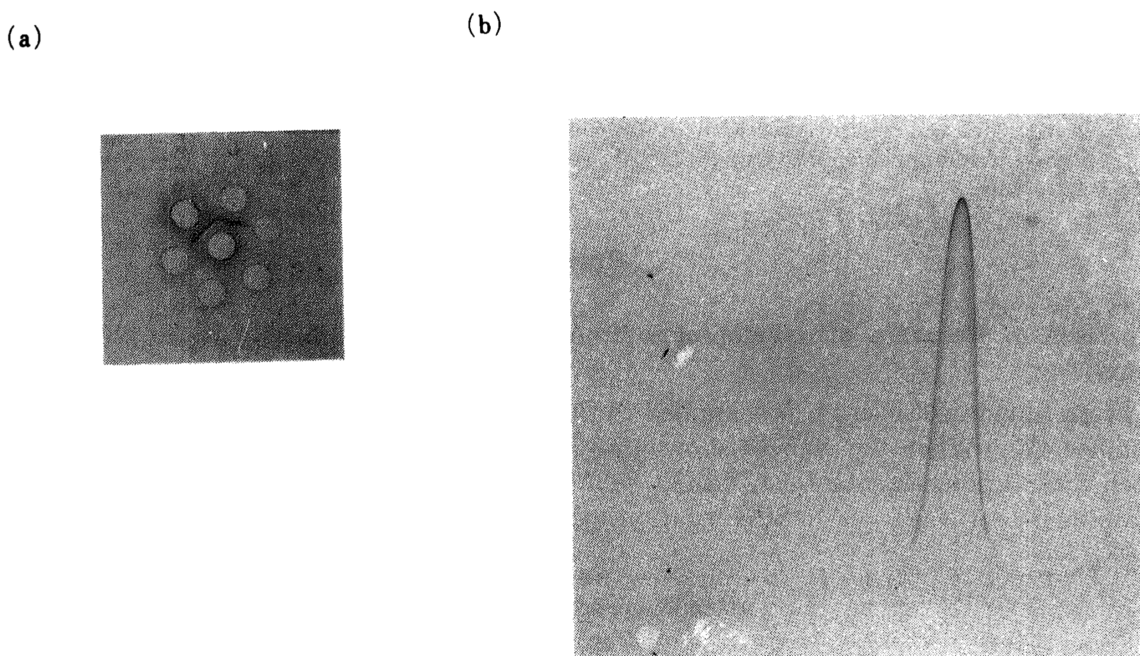


Fig. 2. (a) Ouchterlony Double Diffusion Pattern of α-Fetoprotein from Human Cord Serum

Inner well; anti-human α-fetoprotein antiserum, outer well; purified α-fetoprotein.

(b) Two-Dimensional Immunoelectrophoretical Pattern of α-Fetoprotem from Human Cord Serun

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**Physiological Properties of α-Fetoprotein**

The molecular weight was calculated as 70000 by SDS-gel electrophoresis and the p*I* was found to be 4.7 by isoelectric focusing. These values agree with those previously reported.[3] Thus the AFP purified by means of the immunoadsorbent column was demonstrated to be a native form.

## Discussion

Although several procedures are available for the purification of human AFP[19–21] most of them are complicated and the yield of AFP is usually poor, because the loss is cumulative over the whole course of the purification.[19] In recent years, the immunoadsorbent technique has been applied to the purification of various materials, even in minute quantities. In this technique, the antibody specificity is most important, and the antigen can be purified in one step.

However, some problems remain in the use of the immunoadsorbent technique, such as antibody specificity, elution reagents, support materials, *etc*. In general the antigen-antibody complex can be effectively dissolved under alkaline or acidic conditions and in the presence of chaotropic anions. The nature of the elution reagent required depends on the stability of the antigen and is very important. Up to now, 0.2 M glycine-HCl buffer (pH 2.5) containing 0.15 M NaCl,[11] 3 M NaSCN solution (pH 5.1) and 6 M urea solution (pH 10.6)[13,14] have been employed for eluting adsorbed AFP from the immunoadsorbent column. In our experiment, the use of 0.2 M glycine-HCl buffer (pH 2.5) containing 0.15 M NaCl gave a poor recovery of human AFP, and 3 M NaSCN solution (pH 5.1) and 6 M urea solution (pH 10.6) both necessitate immediate separation in order to avoid the denaturation of human AFP. However, when 0.2 M $Na_2CO_3$ solution (pH 11.6) was employed as an eluent, a separation procedure was not necessary if the pH of the eluate was neutralized with HCl. Thus this eluent offered a higher recovery than other eluents and it was considered to be a mild and effective eluent for the purification of human AFP by the immunoadsorbent technique.

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