KOREAN JOURNAL OF ZOOLOGY
Vol. 29, No. 4, pp.283-293 (1986)

Affinity Purification of Human Alpha-Fetoprotein

Shin Sung Kang, Ok Sun Bang and Tae Kyu Park
(Department of Biology, Kyungpook National University)

吸着 크로마토그라피法에 의한 사람 Alpha-Fetoprotein의 分離

姜 信 誠·方 玉 仙·朴 大 圭
(慶北大 自然大 生物學科)

(Received May 27, 1986)

요 약

사람의 alpha-fetoprotein(AFP)에 대한 모노클론 항체의 생산 및 분석을 위하여, 태아조직을 재료로 추출법, DEAE-cellulose 및 concanavalin A—Sepharose, Cibacron blue F3GA-agarose, immunoadsorbent column등의 흡착크로마토그라피법에 의해 AFP를 분리하였다. 총 534g의 태아조직에서 AFP의 量은 8.76 mg으로서 순수분리되었음을 확인하였다.

INTRODUCTION

Alpha-fetoprotein (AFP) is a major plasma α_1-glycoprotein present in the early stage of development in human and other animals, which virtually disappears in adult life. However, elevated serum AFP concentrations have been found in adults with hepatocellular carcinoma and teratoblastoma (Abelev, 1971; Ruoslahti & Seppala, 1979). The determination of AFP levels therefore has been used in diagnosis of adult cancers stated above as well as in prenatal diagnosis of certain pregnancy disorders such as neural tube defects, anencephalia, esophageal atresia, intrauterine fetal death and congenital nephrosis (Adniolfi *et al.*, 1975; Leighton *et al.*, 1975; Okuda *et al.*, 1975; Ommen, 1978; Ishiguro *et al.*, 1981; Chen *et al.*, 1984).

The AFP has a molecular weight of about 70,000 and is synthesized by the fetal liver and yolk sac (Gitlin *et al.*, 1972; Sell & Skelly, 1974). Although the same osmotic and carrier functions as those of serum albumin in adult, immunoregulation effect, blocking action of maternal estrogen and growth-control factor in fetus have been suggested, the

본 연구는 1985년도 문교부 학술연구조성비(유전공학연구소 지원분)의 일부로 이루어진 것임.

Abbreviations: AFP, alpha-fetoprotein; NHS, normal human serum; Con A, concanavalin A; SDS, sodium dodecyl sulfate

precise biological functions of this protein have not been clearly identified (review by Sell & Becker, 1978).

In order to elucidate the physiological functions of this protein the structural studies should be preceded, therefore, large quantities of pure AFP are required. Our another aim is to make monoclonal antibodies specific for AFP by hybridoma (Kang *et al.*, 1986), which can be valuable tools for characterizing the antigenic structures and for establishing the sensitive assay methods and the rapid purification of AFP by immunoaffinity chromatography.

In this report we focused on the purification of AFP from human fetal tissues through the DEAE-cellulose chromatography, concanavalin A-Sepharose, Cibacron Blue-agarose chromatography and immunoadsorbent affinity chromatography. The isolated AFP preparation showed a single band on polyacrylamide gel electrophoresis and a single precipitin arc against anti-human AFP and anti-human cord serum on immunoelectrophoresis.

MATERIALS AND METHODS

Materials

Three- to four-month-old fetuses immediately after therapeautic abortion were collected and kept frozen at -70°C until use. All chemicals used in this study unless otherwise indicated were of analytical grade. CNBr-activated Sepharose 4B and other Sepharose products were purchased from Pharmacia Fine Chemicals. Cibacron Blue F3GA-agarose from BRL; DEAE- and CM-cellulose from Whatman; goat anti-human α_1-fetoprotein, anti-human α_1-antitrypsin and anti-normal human serum from Cappel; human albumin, bovine albumin, human α_1-acid glycoprotein, Coomassie brilliant blue R and G, concanavalin A from Sigma.

Isolation of Alpha-fetoprotein

Purification of human AFP was performed at 4°C by the methods of Parmelee *et al.* (1978) and Wu *et al.* (1980a, b) with slight modifications.

(1) Preparation of fetal extract

Approximately 530 g of chopped fetal tissues was homogenized in one-third volume of 0.15 M NaCl in waring blender. The homogenate was centrifuged at 5,000×g for 10 min and saved the supernatant. The precipitate was washed twice with the same volume of 0.15 M NaCl and centrifuged. The combined fetal extract was then passed through glass wool and dialyzed against 40 mM potassium phosphate buffer of pH 6.2, extensively. The aggregated material in the dialyzed extract was removed by centrifugation at 20,000×g for 30 min.

(2) DEAE-cellulose chromatography

The dialyzed fetal extract was mixed with 650 ml of packed DEAE-cellulose equilibrated with 40 mM potassium phosphate buffer, pH 6.2, and stirred slowly overnight at 4°C. The DEAE-cellulose slurry was packed into a column (4×60 cm) and washed with the above

buffer of pH 6.2 until the absorbance (A_{280}) of the effluent became less than 0.01. The adsorbed proteins were eluted with above buffer containing 0.5 M NaCl.

The protein fractions were pooled and concentrated by ultrafiltration followed by dialysis against 40 mM potassium phosphate buffer, pH 6.2. The sample was then loaded on a DEAE-cellulose column (3×60 cm) equilibrated with the same buffer, and nonadsorbed proteins were washed out extensively. The adsorbed proteins were eluted by a linear gradient of 0.05 M to 0.5 M NaCl in 40 mM potassium phosphate buffer, pH 6.2. Fractions of 15 ml were collected at a flow rate of 70 ml per hr. Eluates were monitored at 280 nm and the presence of the AFP was checked by double gel immunodiffusion (Ouchterlony, 1958) using anti-human AFP.

(3) Concanavalin A-Sepharose chromatography

The AFP-rich fractions from DEAE-cellulose chromatography were pooled, concentrated and dialyzed against 0.1 M sodium acetate buffer of pH 6.0 containing 1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.01% thimerosal. Concanavalin A was coupled to CNBr-activated Sepharose 4B (10 mg Con A/ml gel) through the procedures by manufacturer (Pharmacia), and the above preparation was applied to Con A-Sepharose column (2×35 cm) equilibrated with the above buffer. After the elution of the column with the same buffer, the adsorbed proteins were eluted by a linear gradient of 0 to 1% α-methylmannoside in the above buffer of pH 6.0 at a flow rate of 15 ml/hr. The adsorbed peak only reacted with anti-human AFP but still has a large quantity of serum albumin, therefore, this preparation was dialyzed against 0.05 M Tris-HCl buffer of pH 8.0 for further purification by Cibacron Blue F3GA-agarose (Affi-Gel Blue) affinity chromatography.

(4) Cibacron Blue-agarose affinity chromatography

The dialyzed preparation was loaded on a Blue-agarose column (2×28 cm) equilibrated with 0.05 M Tris-HCl buffer (0.5 M NaCl), pH 8.0, and eluted with the same buffer at a flow rate of 10 ml/hr. The eluate contained AFP, and the serum albumin retained in the column was subsequently eluted with 3 M NaSCN in the above buffer. The AFP-containing fraction was then dialyzed against 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl.

(5) Immunoadsorbent affinity chromatography

In order to remove trace amount of contaminant proteins further purification was carried out by passing the above preparation through an immunoadsorbent column containing IgG of rabbit anti-NHS-fraction antiboldies covalently coupled to Sepharose 4B followed the method of March *et al.* (1974). The NHS-fraction was prepared from 50 ml of normal human serum by batch DEAE-cellulose, column DEAE-cellulose and Blue-agarose chromatography as done by Wu *et al.* (1980b). This preparation contained most of the minor serum proteins but little γ-globulin and serum albumin. Rabbit antiserum against NHS-fraction was produced as explained below.

Production of Antisera

Antisera against human cord serum, human AFP and NHS-fraction were raised in rabbits

as reported elsewhere (Kang & Kang, 1983). Either protein antigen (1~2 mg) in complete Freund's Adjuvant was injected to rabbit subcutaneously every four weeks. Booster injections were given several times at intervals of 2 weeks. Rabbits were bled by cardiac puncture one week after the 5th inoculation, and the γ-globulin fraction of rabbit antiserum, if necessary, was prepared by $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography (Tung *et al.*, 1983).

Analytical Techniques

Detection and purity of AFP in each purification step was examined by 7.5% polyacrylamide gel electrophoresis (Davis, 1964), immunoelectrophoresis (Scheidegger, 1955), double gel immunodifffusion (Ouchterlony, 1958) and SDS-polyacrylamide gel electrophoresis (Laemmeli, 1970). SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide slab gel in Tris-HCl buffer of pH 8.8 containing 0.1% SDS, and the gels were stained with 0.2% Coomassie blue R-5% methanol-7% acetic acid, and destained with 50% methanol-10% acetic acid.

The protein concentration was determined by the method of Pradford (1977) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

In human fetus the serum concentration of AFP reaches maximum of 3 mg/ml at 15 weeks of gestation (Sell & Becker, 1978). Hence, three-to four-month-old fetuses obtained immediately after therapeutic abortion were used as starting material. From 534 g of fetal tissues 470ml of fetal extract was obtained, which was identified to contain AFP (Fig. 1). This fetal extract contain a large quantity of proteins (24.44 g), which is not applicable to most column processes, a batch DEAE-cellulose chromatography therefore was performed to remove the majority of proteins. The non-adsorbed materials to DEAE-cellulose were washed off with 40 mM potassium phosphate buffer, pH 6.2, and the adsorbed pro-

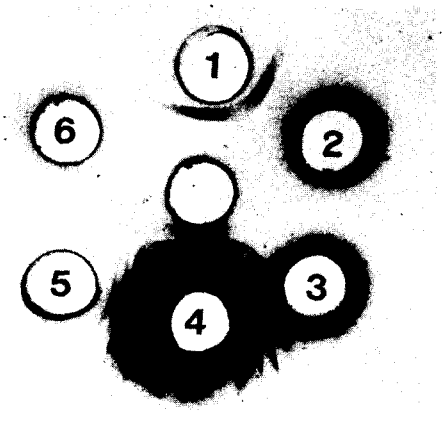

Fig. 1. Double gel immunodffusion test of fetal extract. Well 1: goat anti-human AFP; well 2 & 3: fetal extract; well 4: goat anti-normal human serum; well 5 & 6: NHS; center well: human cord serum. This test indicates that starting material contained AFP.

teins were eluted with the same buffer containing 0.5 M NaCl.

The AFP preparation from batch method was then loaded to a DEAE-cellulose column (3×60 cm) equilibrated with 40 mM potassium phosphate buffer of pH 6.2. After complete washing the column with the equilibrium buffer, a linear gradient elution of 0.05M to 0.5 M NaCl in the same buffer was carried out (Fig. 2). The first main peak fractions of the adsorbed contained AFP, but still had large quantity of other fetal proteins especially serum albumin as shown in Fig. 2.

Since AFP has very similar physicochemical properties to those of serum albumin (Ruoslahti & Engvall, 1976; Ruoslahti & Terry, 1976), it is difficult to separate AFP from albumin. However, albumin does not contain carbohydrates (Ruoslahti & Seppala, 1971), the above AFP-rich preparation was applied on a Con A-Sepharose column (Fig. 3). The first non-adsorbed fractions were the eluate with Con A-buffer (0.1 M sodium acetate buffer, pH 6.0, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$), and the second adsorbed

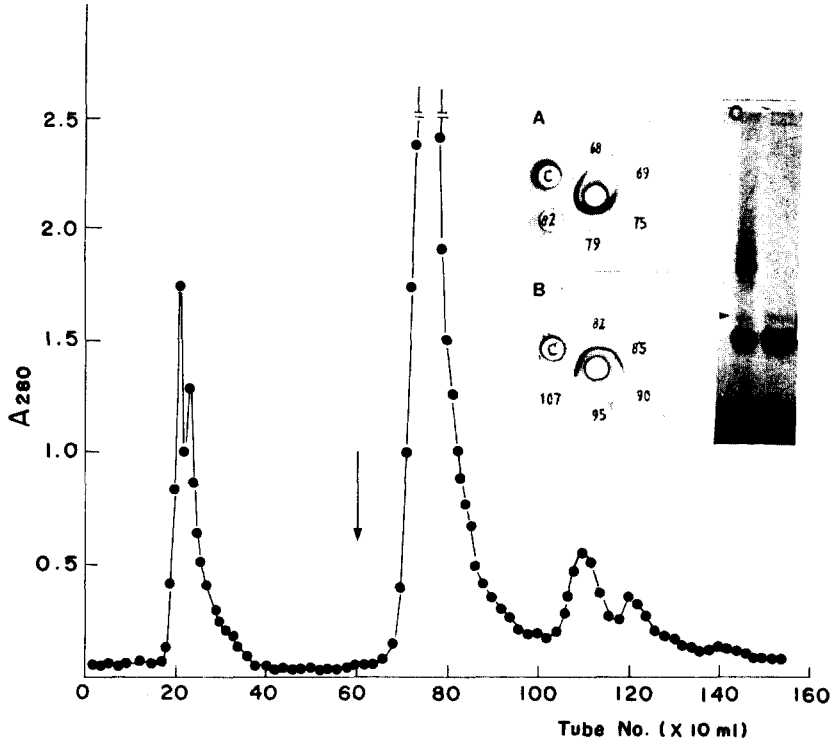

Fig. 2. DEAE-cellulose chromatography (3×60 cm) of AFP-rich preparation after batch DEAE-cellulose method. The nonadsorbed materials were washed off with 40 mM potassium phosphate buffer, pH 6.2, followed by a linear gradient elution of 0.05 M to 0.5 M NaCl in the same buffer (arrow). The adsorbed fractions were checked by immunodiffusion test (Inserts A & B, center well: anti-human AFP; C: human cord serum; other wells: 150 μl of each eluted fraction indicated). The AFP-containing fractions (70–92) were pooled and performed on polyacrylamide gel electrophoresis (Insert C, 1: fetal extract; 2: AFP preparation.)

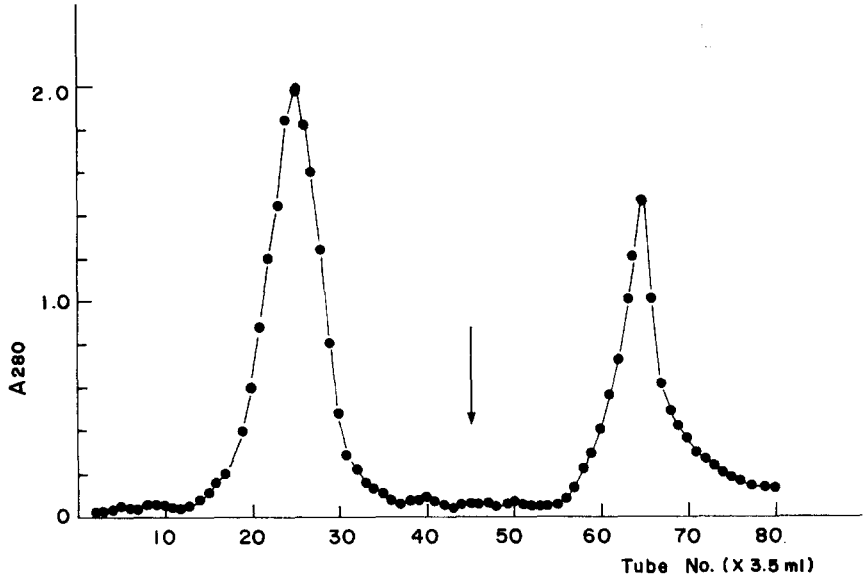

Fig. 3. Affinity chromatography of AFP-preparation after DEAE-cellulose on a Con A-Sepharose column (2×35 cm) equilibrated with Con A-buffer (see text). The first A_{280} peak was found to be pure ablumin, while the second peak eluted by the linear gradient (arrow) contained AFP as shown in Fig. 4.

Fig. 4. Polyacrylamide slab gel electrophoresis of AFP-preparation after Con A-Sepharose chromatography. Electrophoresis was carried out on 7.5% polyacrylamide gel of 0.05 M Tris-HCl buffer, pH 8.8, at 200 v for 3-4 hrs. Migration downward. Coomassie brilliant blue G in 2% perchloric acid stained. Slot 1: human α_1-acid glycoprotein (Sigma); 2: adsorbed fraction on Con A-Sepharose (AFP-preparation); 3: non-adsorbed fraction on Con A-Sepharose (fetal albumin); 4: human albumin (Sigma). All samples contained 20-25 μg of protein.

proteins were dissociated by a linear gradient of 0 to 1% α-methylmannoside in the Con A-buffer. On polyacrylamide gel and immunoelectrophoresis the first peak fraction was found to be almost pure albumin, while the second peak fraction contained most AFP (Fig. 4). Nevertheless the AFP-preparation after this purification step still had a small quantity of albumin as seen in Fig. 4. Other investigators had performed the CM-cellulose chromatography to remove the α_1-acid glycoprotein from AFP-preparation isolated from human cord serum (Wu *et al.*, 1980b; Kim *et al.*, 1984), but we didn't carry out this process because no α_1-acid glycoprotein could be found in our preparation (Fig. 4).

Affi-gel blue-agarose chromatography was done for the complete revmoval of the minor albumin contaminant (Ledden *et al.*, 1982). As the albumin only binds to blue-agarose, the AFP fraction was eluted out at the void volume (Fig. 5). Although the non-adsorbed AFP preparation appeared to have no albumin, trace amount of other proteins could be detectable (Fig. 4 & 7A).

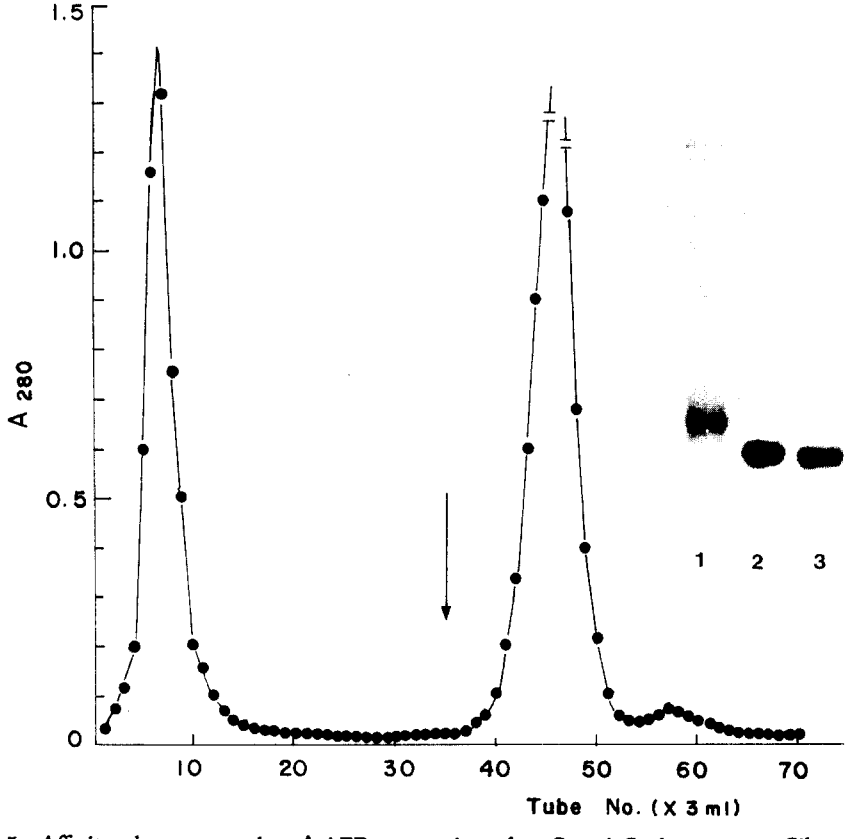

Fig. 5. Affinity chromatography of AFP-preparation after Con A-Sepharose on a Cibacron Blue-agarose column (2×28 cm) equilibrated with 0.05 M Tris-HCl buffer (0.5 M NaCl), pH 8.0. The first A_{280} peak appeared at the void volume contained AFP as shown in the figure inserted, and adsorbed albumin was eluted by 3 M NaSCN in the same buffer (arrow). Insert, 1: peak-1 fraction; 2: peak-2 fraction; 3: human albumin marker.

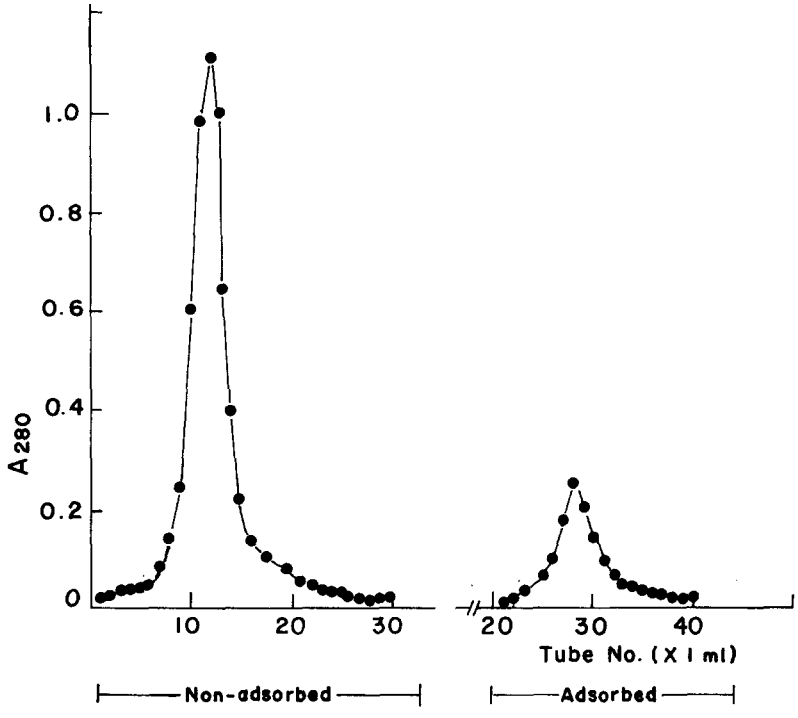

Fig. 6. Immunoaffinity chromatography of AFP preparation after Blue-agarose on a anti-NHS-fraction coupled Sepharose 4B column (1.2×20 cm). See text for details.

The immunoadsorbent affinity chromatography was performed for the final purification (Fig. 6). Since the portion of normal serum proteins was used to make antiserum for immunoadsorbent column, this immunoaffinity chromatography is very effective to remove the trace contaminants in AFP-preparation (Wu *et al.*, 1980b; Tung *et al.*, 1983).

The final product showed a single band on polyacrylamide gel electrophoresis and a single precipitin arc on immunoelectrophoresis against anti-human cord serum and anti-human AFP (Fig. 7A & B), which indicates that our AFP preparation is pure. As shown in Fig. 8, our AFP also displayed a single band on SDS-polyacrylamide gel electrophoresis. Since all the purification procedures were carried out in mild conditions, it can be said that the biological activity of AFP will be remained intact. The recovery of AFP was 8.76 mg total, and with this AFP the production of monoclonal antibodies and the persuits of its structure are in progress.

SUMMARY

For the preliminary step to make and characterize the monoclonal antibodies of human alpha-fetoprotein (AFP) was purified from 534 g of human fetal tissues through the pro-

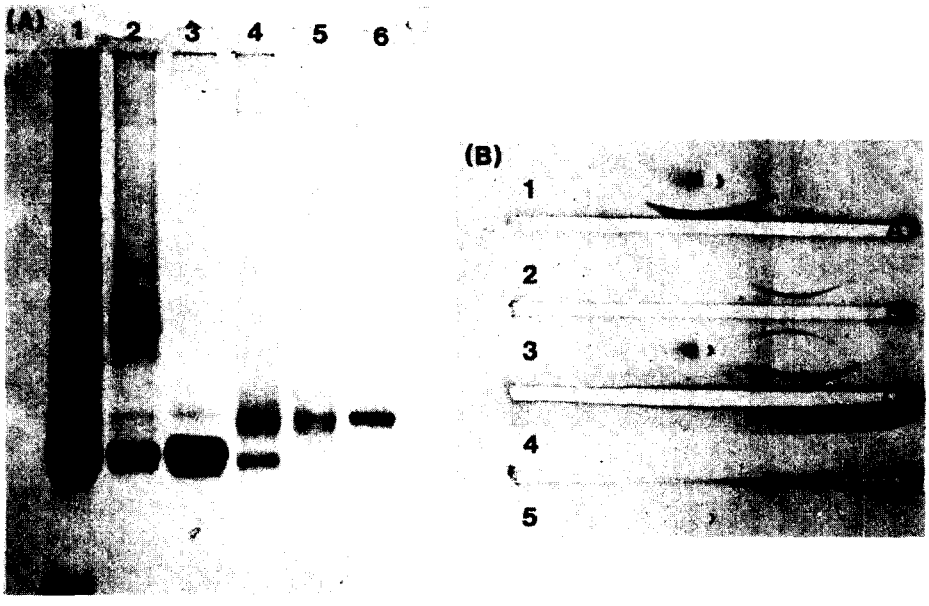

Fig. 7. (A) Polyacrylamide slab gel electrophoresis of AFP-preparation after each purification step. Electrophoresis was done as described in Fig. 4. Slot 1: human cord serum; 2: human fetal extract; 3: AFP-preparation after DEAE-cellulose chromatography; 4: AFP-preparation after Con A-Sepharose chromatography; 5: AFP-preparation after Blue-agarose chromatography; 6: finally purified AFP after immunoadsorbent chromatography.
(B) Immunoelectrophoretic pattern of purified AFP. Migration to the right (anode). Amido black 10B stained. Throughs, A: rabbit anti-human cord serum; B: goat anti-human AFP; C: goat anti-NHS; D: rabbit anti-human cord serum absorbed with NHS. Antigen holes, 1: human cord serum; 2: purified AFP; 3: human cord serum; 4: purifed AFP; 5: NHS. Our AFP showed a single precipitin arc against anti-human cord serum, anti-human AFP and anti-human cord serum absorbed with NHS, but no precipitin arc against anti-NHS.

cedures of tissue extraction, DEAE-cellulose, concanavalin A-Sepharose, Cibacron Blue F3GA-agarose and immunoadsorbent affinity chromatography. The isolated AFP preparation showed a single band on polyacrylamide gel electrophoresis and a single precipitin arc against rabbit anti-human cord serum and anti-human AFP on immunoelectrophoresis. Our AFP also displayed a single band on SDS-polyacrylamide gel electrophoresis. The recovery of AFP was 8.76mg total.

REFERENCES

Abelev, G.I., 1971. Alpha-fetoprotein in oncogenesis and its association with malignant tumors. *Adv. Cancer Res.* 19: 295-358.

Adniolfi, A., M. Adniolfi and M.H. Lessof, 1975. Alpha fetoprotein during development and in disease. *J. Med. Genet.* 12: 138-151.

Fig. 8. SDS-polyacrylamide gel electrophoreiss of purified AFP. The samples were dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing 0.1% SDS and 1% 2-mercaptoethanol, and heated on boiling water bath for 2 min prior to electrophoresis on 0.1% SDS-10% polyacrylgamide gel. Migration downward. Coomassie brilliant blue R stained. Slot 1: molecular weight marker protein mixture (m.w. 14,000-70,000); 2: purified fetal albumin; 3: purified AFP. AFP was appeared as a single band having a slightly slower mobility than that of fetal albumin.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **49**: 248-254.

Chen, D.S., J.L. Sung, J.C. Sheu, C.S. Lai, S.W. How, H.C. Hsu, C.S. Lee and T.C. Wei, 1984. Serum α-fetoprotein in the early stage of human hepatocellular carcinoma. *Gastroenterology* **86**: 1404-1409.

Davis B.J., 1964. Disc electrophoresis: Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**: 321-369.

Gitlin, D., A. Perricell and G.M. Gitlin, 1972. Synthesis of α-fetoprotein by liver, yolk sac and gastrointestinal tract of the human conceptus. *Cancer Res.* **32**: 979-982.

Ishiguro, T., Y. Yoshida, T. Tenzaki, M. Ohshima and H. Suzuki, 1981. AFP in yolk sac tumor and solid teratoma of the ovary. *Cancer* **48**: 2480-2484.

Kang, S.S., O.S. Bang, T.K. Park, D.T. Cho and B.H. Song, 1986. Monoclonal antibodies of human alpha-fetoprotein. *Kor. J. Zool.* (In preparation).

Kang, Y.S. and S.S. Kang, 1983. Immunochemical, and biochemical evidence for the presence of shared idiotypic determinants between IgM and IgG in a single patient. *Genetica Breeda* **2**: 59-67.

Kim, S.W., B.S. Tchai, H.K. Chung, S.C. Park, J.B. Park, J.B. Park, P.C. Suh, I.G. Kim, Y.S. Juhn and H.L. Kim, 1984. Purification of α-fetoprotein from human cord serum. *Kor. J. Biochem.* **16**: 31-38.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T_4. *Nature* (London) **227**: 682-685.

Ledden, D.J., R.C. Feldhoff and S.K. Chan, 1982. Characterization of fragments of human albumin purified by Cibacron Blue F3GA affinity chromatography. *Biochem. J.* **205**: 331-337.

Leighton, P.C., Y.B. Gordon, J.J. Kitau, A.E. Leek and T. Chard, 1975. Levels of alpha-fetoprotein in maternal blood as a screening test for fetal neural-tube defect. *Lancet* **ii**: 1012-1015.

March, S.C., I. Parikh and P. Cuatrecases, 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* **60**: 149-152.

Okuda, K., K. Kotoba, H. Obata, N. Hayashi, T. Hisamitsu, M. Tamya, Y. Kubo, F. Yakushiji, E. Nagata, S. Jinnouchi and Y. Shimaokawa, 1975. Clinical observations during a relatively early stage of hepatocellular carcinoma, with special reference to serum α-fetoprotein levels. *Gastroenterology* **69**: 226-234.

Ommen, G.S., 1978. Prenatal diagnosis of genetic disorders. *Science* **200**: 952-958.

Ouchterlony, O., 1958. Diffusion-in-gel method of immunological analysis. *Progr. Allergy* **9**: 1-78.

Parmelee, D.C., M.A. Evenson and H.F. Deutch, 1978. The presence of fatty acids in human α-fetoprotein. *J. Biol. Chem.* **253**: 2114-2119.

Ruoslahti, E. and E. Engvall, 1976. Immunological cross reaction between alpha-fetoprotein and albumin. *Proc. Nat. Acad. Sci.* **73**: 4641-4644.

Ruoslahti, E. and M. Seppala, 1979. α-Fetoprotein in cancer and fetal development. *Adv. Cancer Res.* **29**: 275-364.

Ruoslahti, E. and M. Seppala, 1971. Studies of carcinofetal proteins. III. Development of a radioimmunoassay for α-fetoprotein: Demonstration in serum of healthy human adults. *Int. J. Cancer* **8**: 374-383.

Ruoslahti, E. and W.D. Terry, 1976. Alpha-fetoprotein and serum albumin show sequence homology. *Nature* **260**: 804-805.

Scheidegger, J.J., 1955. Une micro-methode de l'immunoelectrophorese. *Int. Arch. Allergy* **7**: 103-115.

Sell, S. and F.F. Becker, 1978. Alpha-fetoprotein, *J. Natl. Cancer Inst.* **60**: 19-26.

Sell, S. and H. Skelly, 1976. Tissue sites of synthesis of alpha-fetoprotein by the rat during pregnancy and hepatomy growth. *J. Natl. Cancer Inst.* **56**: 645-648.

Tung, E., J.M. Goust, W.Y. Chen, S.S. Kang, I.Y. Wang and A.C. Wang, 1983. Cytotoxic effect of anti-idiotype antibody-chlorambucil conjugates against human lymphoblastoid cells. *Immunology* **50**: 57-64.

Wu, J.T., L.H. Wu and A.C. Madsen, 1980a. Isolation of α-fetoprotein in milligram quantities from human cord serum. *Biochem. Med.* **23**: 336-349.

Wu, J.T., S.M. Monir-Vaghefi and W.J. Waterhouse, 1980b. An improved procedure for the isolation of milligram quantities of α-fetoprotein from human cord serum. *Biochem. Med.* **24**: 210-217.