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Note

Simple purification procedure of rat α-fetoprotein by a combination of Cibacron Blue gel affinity chromatography and anion-exchange high-performance liquid chromatography

LAWRENCE T. WONG* and ZUO JIE XU

Department of Biochemistry, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 1A8 (Canada)

and

CARLETON J.C. HSIA

Department of Biochemistry, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 1A8 and Biosciences Division, Defence and Civil Institute of Environmental Medicine, Downsview, Ontario M3M 3B9 (Canada)

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α-Fetoprotein (AFP), a glycoprotein produced by the yolk sac, liver and gastrointestinal tract, is the principle plasma protein of the foetus [1, 2]. It is found only in very low concentration in normal adult serum [3]. Elevated levels of this protein in adult serum can be detected during pregnancy and in certain diseases, particularly liver cell carcinoma and germ cell tumours [4—7].

Previously, we have used a combination of Cibacron Blue gel affinity chromatography and anion-exchange high-performance liquid chromatography (HPLC) for the isolation of bovine AFP from foetal calf serum [8]. In the present paper, we report the adaptation of this methodology for the purification of AFP from rat amniotic fluid and rat foetal homogenate.

MATERIALS AND METHODS

Materials

Wistar rats of 15—19 days' gestational age were obtained from Charles River (St. Constant, Canada). Rat serum albumin (RSA) and rat gamma-globulin

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(RGG) were purchased from Sigma (St. Louis, MO, U.S.A.). Rat AFP (RAFP) and anti-RAFP and anti-RSA antibodies were gifts from Dr. H.F. Deutsch (University of Wisconsin, Madison, WI, U.S.A.). Cibacron Blue—Sepharose was obtained from Pharmacia (Montreal, Canada). All other reagents used were of analytical or reagent grade and purchased from local suppliers.

Instrumentation

HPLC was performed on a Pharmacia fast protein liquid chromatography (FPLC) system equipped with two pumps and capable of generating a gradient or step gradient elution profile. Sample injections were carried out using a V-7 valve and chromatograms were recorded by monitoring the absorbance at 280 nm using a single-path UV-1 monitor fitted with a 10-mm path cell. The absorbance unit full scale (a.u.f.s.) was set between 0.05 and 2.0 units as appropriate. Fractions were collected with a FRAC-100 fraction collector. The FPLC system was operated at room temperature.

Chromatography

The Cibacron Blue—Sepharose gel was washed first with 6 *M* urea and then with 6.5 m*M* 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris propane) buffer, pH 7.0. The washed gel was packed into a Pharmacia HR10/10 column (100 × 10 mm I.D.) and chromatographed using the FPLC system. A sample was injected, the column eluted with 6.5 m*M* Bis-Tris propane buffer, pH 7.0, and the unretained fraction collected. The fraction retained by the blue gel was then eluted using the same buffer containing 1.4 *M* sodium chloride. The column was regenerated with 6 *M* urea and equilibrated with the starting buffer before the next run.

The unretained protein fraction from above was used directly for HPLC on a Pharmacia Mono-Q SI anion-exchange column (50 × 5 mm I.D., 10 μm particle size). Buffer A was 6.5 m*M* Bis-Tris propane, pH 7.0. Buffer B was buffer A containing 0.35 *M* sodium chloride. A preprogrammed linear gradient was used for the chromatography and the appropriate peak fractions were collected, dialyzed against distilled water, and lyophilized.

Rat foetal material

Rat amniotic fluid was obtained from the amniotic sacs of rats. Each pregnant rat was anesthetized with diethyl ether and the uterus opened with a clean pair of scissors. The intact amniotic sac was punctured and the fluid collected. Approximately 4 ml of undiluted amniotic fluid was obtained from each rat. Contaminating blood cells were removed by centrifugation. The amniotic fluid was dialyzed against distilled water and lyophilized. Weighed samples were dissolved in 6.5 m*M* Bis-Tris propane buffer, pH 7.0, and used for Cibacron Blue gel chromatography.

Rat foetal homogenate was prepared from the foetuses by homogenizing them in 1 volume of 6.5 m*M* Bis-Tris propane buffer, pH 7.0. After centrifugation at 12,000 *g* for 10 min and Millipore filtration (0.22 μm), the supernatant was used for Cibacron Blue gel chromatography.

Protein assay

Total protein concentrations were determined by the Bio-Rad protein assay

method [9] using bovine serum albumin as a standard, or by UV absorption at 280 nm. RSA and RAFP concentrations were assayed by the radial immunodiffusion method of Mancini et al. [10]. The purity of the protein was determined by disc polyacrylamide gel electrophoresis (PAGE) [11]. Bands in the gels were quantified by scanning with a DCD-16 digital densitometer (Gelman, Ann Arbor, MI, U.S.A.).

RESULTS

We initially attempted to establish an HPLC condition for the separation of RAFP from RSA, the latter protein being an anticipated major contaminant of RAFP which has similar physicochemical properties. However, no satisfactory resolution of the two proteins could be achieved; by employing the optimal HPLC conditions described under Materials and methods, we could only partially resolve RAFP and RSA. It was therefore evident that before subjecting rat foetal material to HPLC separation, a pre-purification step to remove the RSA was necessary.

Fig. 1 shows the elution profile of rat amniotic fluid on a Cibacron Blue—Sepharose column. This affinity column was found to be effective in removing RSA from the injected sample. The unretained fraction I showed a solvent peak (a) and a major protein peak (b). Analyses of peak (b) by disc PAGE and radial immunodiffusion plate revealed that it contained RAFP

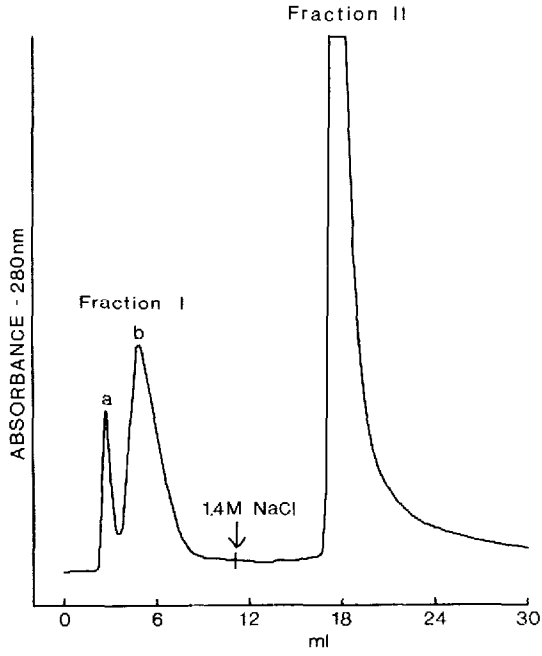

Fig. 1. Chromatography of rat amniotic fluid (10 mg) on an HR 10/10 Cibacron Blue-Sepharose column using 6.5 m*M* Bis-Tris propane buffer, pH 7.0, as the eluent (flow-rate, 0.2 ml/min, a.u.f.s., 2.0). Fraction I is the unretained fraction and fraction II is the retained fraction eluted with 1.4 *M* sodium chloride. Peaks: a = solvent; b = RAFP and other proteins.

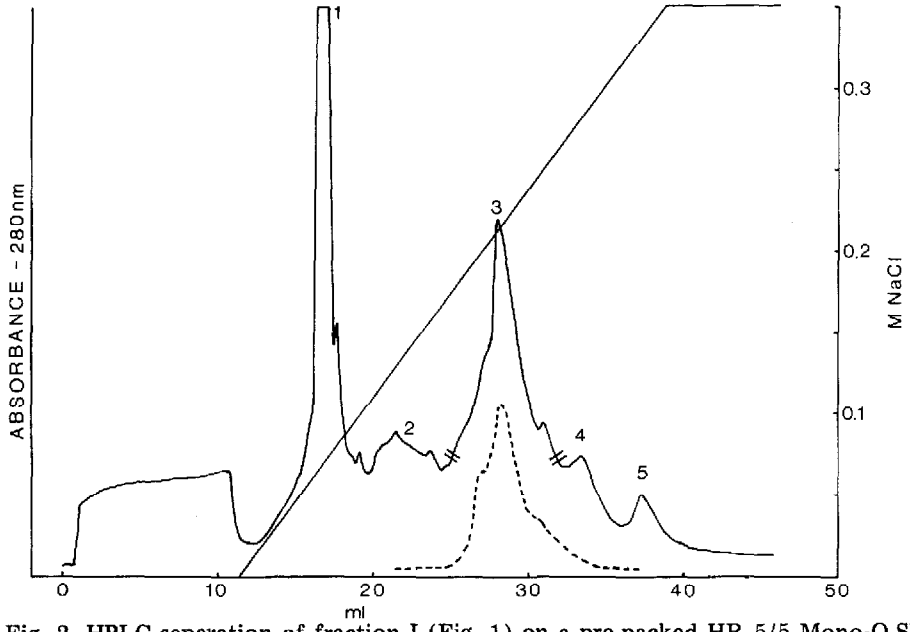

Fig. 2. HPLC separation of fraction I (Fig. 1) on a pre-packed HR 5/5 Mono-Q SI column. Buffer A: 6.5 m*M* Bis-Tris propane, pH 7.0; buffer B: buffer A containing 0.35 *M* sodium chloride. Flow-rate, 1 ml/min; a.u.f.s., 0.5. The RAFP fractions collected are indicated by hash marks in the elution profile. - - -, Elution profile of a standard solution of RAFP.

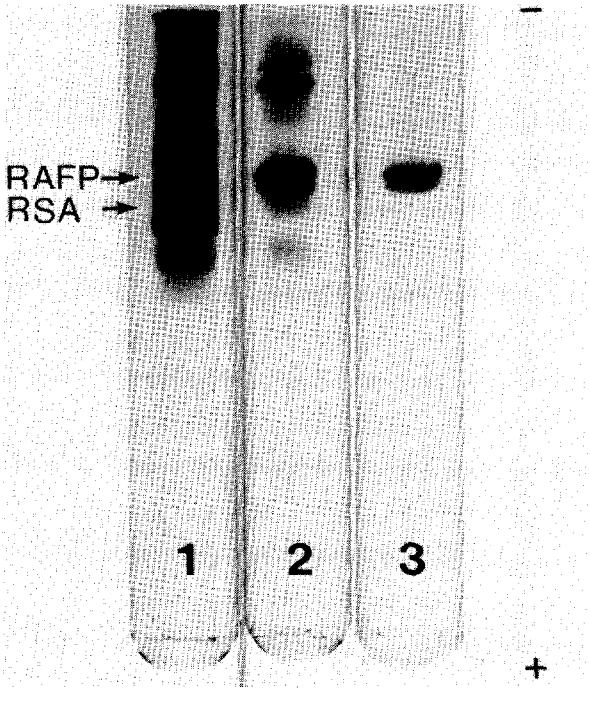

Fig. 3. Disc PAGE of (1) rat amniotic fluid; (2) rat amniotic fluid after Cibacron Blue—Sepharose chromatography (fraction I, Fig. 1); and (3) rat amniotic fluid after Cibacron Blue—Sepharose chromatography, anion-exchange HPLC and repeat Cibacron Blue—Sepharose chromatography. The arrows indicate the relative positions of standard RAFP and RSA.

(and other proteins) but no detectable amount of RSA. The retained fraction II (eluted with 1.4 *M* sodium chloride), on the other hand, contained RSA almost exclusively.

The RSA-depleted fraction (I) of rat amniotic fluid was next subjected to anion-exchange HPLC. A total of five peaks was resolved on the chromatogram (Fig. 2). Peak 1 was identified as RGG by comparison with a standard sample. RAFP was eluted as peak 3 at a sodium chloride concentration of between 0.17—0.25 *M*. The identities of the other minor peaks were not established.

The purity of the RAFP thus isolated was analyzed by disc PAGE, the results of which are shown in Fig. 3, with those of neat rat amniotic fluid and RSA-depleted fraction I also shown for purposes of comparison. The purified RAFP was quantified to have a purity of > 95% with no detectable amount of RSA. It shows the familiar pattern of fast and slow bands [12]. Its extinction coefficient ($E^{1\%}_{1\,cm}$, 280 nm) was 4.1, similar to that reported for RAFP isolated by an immunoabsorbent method ($E^{1\%}_{1\,cm}$, 278 nm = 4.27) [12]. The maximum loading capacity of rat amniotic fluid through this two-step chromatographic procedure was 20 mg, giving a yield of 3 mg (50% overall yield) of purified RAFP per injection.

The methodology was also tested for the purification of RAFP from rat foetal homogenate. Fig. 4A depicts the chromatogram of the Cibacron

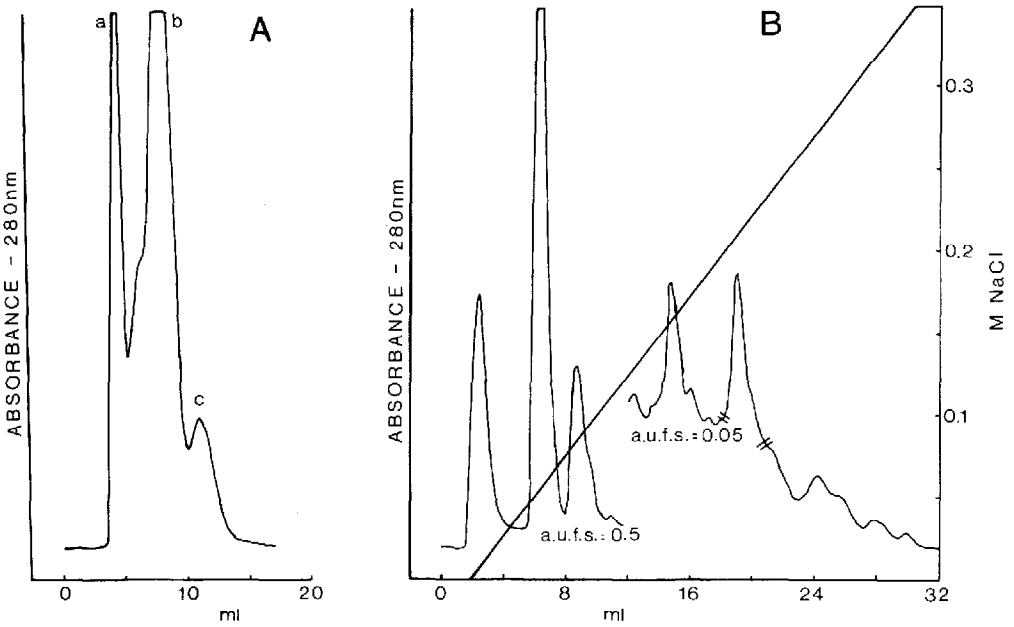

Fig. 4. (A) Unretained fraction of rat foetal homogenate (125 μl) chromatographed on an HR 10/10 Cibacron Blue—Sepharose column using 6.5 m*M* Bis-Tris propane, pH 7.0, as the eluent. Flow-rate, 0.2 ml/min; a.u.f.s., 0.5. Peaks: a = solvent; b = RAFP and other proteins; c = predominantly haemoglobin. (B) HPLC separation of peak b (Fig. 4A) on a pre-packed HR 5/5 Mono-Q SI column. Buffer A: 6.5 m*M* Bis Tris propane, pH 7.0; buffer B: buffer A containing 0.35 *M* sodium chloride. Flow-rate, 1 ml/min. The RAFP fractions collected are indicated by the hash marks in the elution profile.

Blue—Sepharose unretained fraction of rat foetal homogenate while Fig. 4B shows its chromatographic profile after anion-exchange HPLC. Because of the greater complexity of the protein constituents in rat foetal homogenate, the RAFP thus isolated was found to have a purity of only 80—90%. The purity, however, could be increased by re-chromatography on the anion-exchange column.

DISCUSSION

The binding properties of AFP have been extensively studied, but there exist in the literature discrepant reports of these binding functions, involving, for example, the number of types of oestrogen-binding sites and the oestrogen-binding specificity of human AFP [13]. A possible explanation for this confusion may lie in the different methods of AFP purification used, which may select sub-populations of AFP or cause modification due to harsh conditions. Thus it is reasonable to suggest that for studying the binding properties of AFP, a simple and mild method capable of purifying the protein in its most native form is highly desirable.

Rat amniotic fluid has been found to be a good source of RAFP, as RAFP constitutes about 30—40% of the total protein in this fluid [14]. RAFP has been purified from rat amniotic fluid by isoelectric focusing [14] and preparative PAGE [15], but these procedures are tedious and often give low recoveries of the product. In this respect, purification by a direct immunoadsorbent affinity chromatography [16, 17] and an indirect immunoadsorbent precipitation [18] method is fast and efficient and offers high recovery of RAFP. However, immunochemical methods are initially costly and time consuming, as they require the production of specific antibodies; they also have the inherent difficulties associated with immunoadsorbent chemistry, such as the low pH used in the recovery of ligand from the immunoadsorbent. In contrast with these methods of purification, the procedure described here is simple, mild, and efficient; using it, one can obtain milligram quantities of purified RAFP in less than 1 h.

RAFP and RSA are difficult to separate by physicochemical means because of their similarities in physical and chemical properties. Attempts to develop an HPLC condition for the complete resolution of these proteins proved unsuccessful. The Cibacron Blue affinity column, on the other hand, was found to be efficient in separating these proteins since RSA, but not RAFP, was retained by the column. These observations have previously been reported by other workers [19, 20].

In the present purification procedure, the efficiency of the Cibacron Blue gel column is critical if one is to obtain completely albumin-free RAFP. We have occasionally observed albumin contamination of the sample which we attribute to the deterioration of the affinity column after prolonged use. This contamination, however, is usually small ($< 2\%$) and can be completely removed by re-chromatography of the sample on the Cibacron Blue gel column.

A major difficulty in the purification of AFP lies in obtaining large quantities of the protein. Of the existing methods reported for the isolation of RAFP, the immunoabsorbent technique is probably the only one that can

readily give milligram quantities of the product. By employing the presently described methodology, one can obtain milligram quantities of purified RAFP through repeated injections. Furthermore, the present method has the potential to be automated for the production of RAFP by running the two chromatographic steps in series; we are now working toward this end.

ACKNOWLEDGEMENTS

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