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Note

Fractionation of rat α-fetoprotein by high-performance liquid chromatography

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α-Fetoprotein (AFP) is a major plasma glycoprotein of the foetus [1]. It disappears after birth and is normally absent in adults [2, 3], although it reappears in association with certain pathological conditions [4—7]. Its function in the foetus is unknown, but it is thought to act as a binding protein for such ligands as steroid hormones, fatty acids and bilirubin, as well as having a role in immunoregulation [8].

It has been well documented that AFP is heterogeneous, comprising sub-populations of differing size, charge and lectin-binding behaviour (for a review see ref. 9). An extensively studied case of this heterogeneity is the existence of two charge variants of rat AFP (RAFP), distinguished by their migration as "fast" and "slow" bands in polyacrylamide gel electrophoresis (PAGE) [10—12].

The molecular basis of this difference, whether due to differences in primary structure, conformation or carbohydrate contents, remains unclear [9]. It is also unclear whether there are functional differences in ligand binding or immunoregulation by these variants. Elucidation of this heterogeneity, especially in the light of the possibility of changes in the function of AFP

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during development, should contribute greatly to our understanding of the biological role of this glycoprotein.

We have recently developed a high-performance liquid chromatographic (HPLC) methodology for the purification of AFP from rat amniotic fluid and foetal extract [13]. In this purification procedure, we have consistently observed heterogeneity of the rat AFP on the anion-exchange column. We have further studied this observation and we report here our results and compare the HPLC fractionation of RAFP with its electrophoretic heterogeneity as shown by PAGE.

MATERIALS AND METHODS

Materials

Rat amniotic fluid was obtained from Wistar rats (Charles River, St. Constant, Canada) of 17—19 days' gestational age. All reagents used were of analytical or reagent grade and purchased from local suppliers.

Instrumentation

HPLC was performed on a Pharmacia liquid chromatography system equipped with two pumps and capable of generating a gradient or step elution profile. Sample injections were carried out using a V-7 valve, and chromatograms were recorded by monitoring the absorbance of the eluent 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 0.02 and 0.1, as appropriate. Fractions were collected with a Pharmacia FRAC-100 fraction collector. The HPLC system was operated at room temperature.

Fractionation of RAFP

RAFP was purified from rat amniotic fluid by the HPLC method described previously [13]. Fractionation of RAFP was carried out on the HPLC system equipped with a Pharmacia Mono Q SI anion-exchange column (50 × 5 mm I.D., 10 μm particle size). Buffers A and B were 6.5 m*M* 1,3-bis[tris-(hydroxymethyl)methylamino]propane (Bis-Tris propane) (pH 9.5) containing 0.25 *M* and 0.50 *M* sodium chloride, respectively. A preprogrammed linear gradient was used for the chromatography, and the appropriate peak fractions were collected, dialyzed against distilled water and lyophilized.

Polyacrylamide gel electrophoresis

Slab polyacrylamide gel electrophoresis was performed on a PROTEAN double-slab electrophoresis cell (Bio-Rad Labs., Mississauga, Canada) using 10% and 5% gels as the stacking and separating gels, respectively. The gels were fixed with trichloroacetic acid and then stained with Coomassie blue. Bands in the gels were quantified by scanning with a DCD-16 digital densitometer (Gelman, Ann Arbor, MI, U.S.A.).

Protein assay

Total protein concentrations were determined by the Bio-Rad protein assay method [14] with bovine serum albumin as a standard. RAFP concentrations

were assayed by the radial immunodiffusion method of Mancini et al. [15].

RESULTS AND DISCUSSION

Following our HPLC purification of RAFP, we applied the resolving power of HPLC to the resolution of RAFP subpopulations. We found that strong anion-exchange HPLC of pure RAFP resolves two major fractions, corresponding to the well known charge variants of RAFP.

Fig. 1 shows the HPLC fractionation of pure RAFP on a Mono Q HR5/5 strong anion-exchange column. In the region associated with RAFP, two major peaks, 1 and 2, were resolved; integration showed that these peaks comprised ca. 59% and 30%, respectively, of the total RAFP. These peaks were isolated and rechromatography showed that they are genuine peaks with different elution volumes (Fig. 2).

Fig. 3 shows the characterization of the fractions by PAGE. Standard RAFP (track S) shows the typical fast and slow variants; peak 1 (track 1), comprising ca. 59% of the total RAFP, is essentially pure and co-migrates with the slow variant. The slow migration of this fraction toward the cathode in PAGE is consistent with its rapid elution from the cationic HPLC column. Peak 2 (track 2), on the other hand, is highly enriched with a molecular species (comprising ca. 80% of the fraction) which co-migrates with the fast RAFP variant. The minor (20%) band co-migrates with the slow variant. This appparent

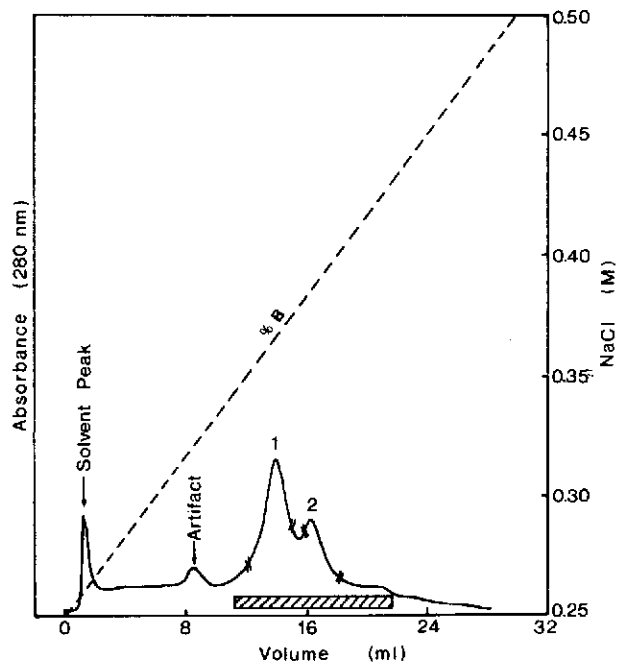

Fig. 1. HPLC fractionation of 1 mg of RAFP on a pre-packed HR5/5 Mono Q SI column. Buffer A, 6.5 m*M* Bis-Tris propane (pH 9.5) containing 0.25 *M* sodium chloride; buffer B, 6.5 m*M* Bis-Tris propane (pH 9.5), containing 0.5 *M* sodium chloride; flow-rate, 1 ml/min; a.u.f.s., 0.1. The presence of RAFP in the fractions is shown by the hatched zones. The RAFP peaks collected are indicated by hash marks in the elution profile.

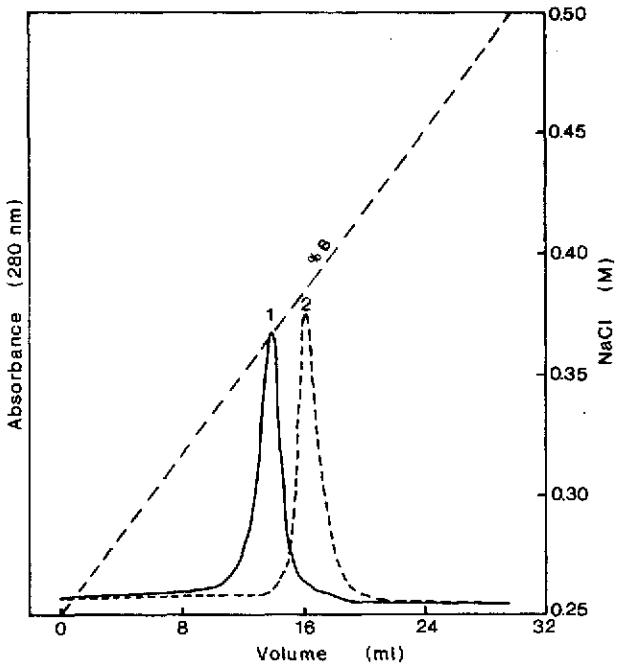

Fig. 2. HPLC re-injection of peaks 1 and 2 isolated in Fig. 1. Buffers A and B, as in Fig. 1. Flow-rate, 1 ml/min, a.u.f.s., 0.02.

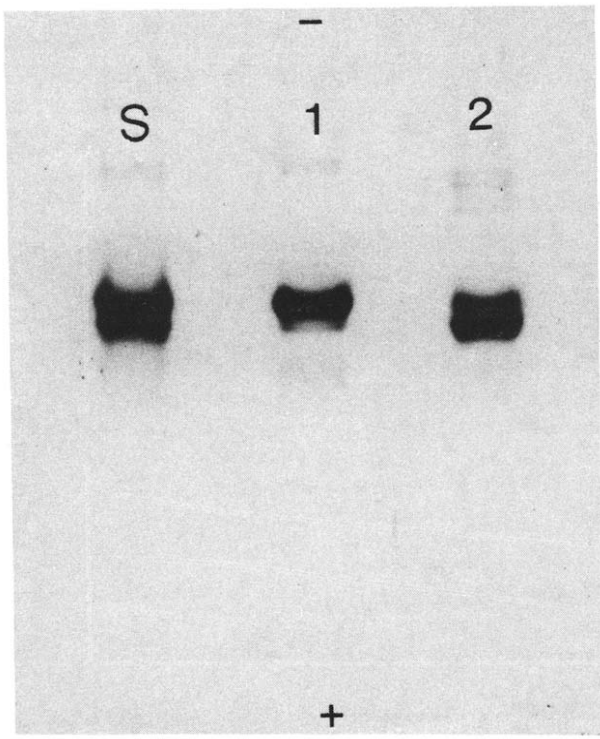

Fig. 3. Polyacrylamide gel electrophoresis of peak 1 (track 1) and peak 2 (track 2) isolated in Fig. 1, with purified RAFP (track S) as standard.

contamination of peak 2 by slow RAFP may be due to tailing from the larger peak 1, although this seems unlikely since fractions taken throughout peak 2 show the same PAGE pattern. The electrophoretic heterogeneity of this peak, therefore, seems genuine and requires further study.

To the best of our knowledge, this is the first method to allow the simple and rapid purification of large amounts of the major (slow) charge variant of RAFP. Previously this has been done only by preparative PAGE, which is rather tedious and which can accommodate only small amounts of protein. The slow variant seems the more interesting to study: it is more abundant than the fast AFP in the rat foetus [9] and it shows most of the oestrogen binding affinity of RAFP [12]. This simple, rapid and high-yield purification method should greatly aid the study of this RAFP subpopulation.

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