Journal of Chromatography, 417 (1987) 168–172
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3601

Note

Microheterogeneity of rat α-fetoprotein on a copper chelate column

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(First received October 31st, 1986; revised manuscript received January 5th, 1987)

α-Fetoprotein (AFP) is one of the major glycoproteins of the foetus, but it is normally absent in adults. The biological role of AFP has been intensively investigated over the past ten years, but its specific function remains unclear. It is known that AFP can bind various substances, such as steroids, fatty acids, bilirubin and copper ion. These binding capacities, as well as immunosuppressive activities, seem to be related to the biological role of AFP. However, recent discrepancies observed in both the oestrogen-binding capacity and the immunosuppressive activity raise the problem of the purity and heterogeneity of AFP preparations (for a review see ref. 1).

In this regard, the molecular microheterogeneity of AFP observed on polyacrylamide gel electrophoresis (PAGE) [2,3], ion-exchange chromatography [4] and affinity chromatography on lectins bound to an insoluble matrix (for a review see ref. 5) might be an important feature. These observations indicate that AFP might comprise molecular variants of differing size, charge and lectin-binding affinity. However, no molecular variants on other principles have ever been reported.

We applied purified rat AFP (RAFP) on the copper chelate column and observed microheterogeneity on this column. Furthermore, we compared the fractionation of RAFP by copper affinity chromatography with its electrophoretic microheterogeneity as shown by PAGE.

EXPERIMENTAL

Materials

RAFP was purified by the method of Wong et al. [6] from rat amniotic fluid of Wistar rats (Keari, Japan) of seventeen to nineteen days' gestational age.

0378-4347/87/$03.50

Instrumentation

Copper affinity chromatography was performed with a Pharmacia liquid chromatographic system (Pharmacia, Uppsala, Sweden) equipped with two pumps and a gradient profile. Samples were injected with a V-7 valve, and the absorbance of the eluent was monitored at 280 nm using a single-beam UV-1 monitor fitted with a 10-mm path cell. The diffusion sensitivity was between 0.02 and 0.5 a.u.f.s. Fractions were collected with a Pharmacia FRAC-100 fraction collector.

Gel permeation chromatography was performed in Tri-Rotar-VI high-performance liquid chromatographic (HPLC) system (Jasco, Japan) with TSK-gel G-3000SW (60×0.75 cm I.D.) (Toyo Soda, Japan). Chromatograms were recorded by monitoring the absorbance of the eluent at 280 nm using UVIDEC-100-VI (Jasco) at 0.005 to 0.1 a.u.f.s.

Preparation of the copper chelate column

Pharmacia Chelating Sepharose 6B was packed into a column (50×0.9 cm I.D.) with a total volume of 25 ml. The upper two thirds of the column were saturated with copper ions by frontal development with 30 ml of 20 m*M* copper sulphate. The bed thus prepared was washed with 0.01 *M* Tris–HCl (pH 7.5) in 0.1 *M* sodium chloride. The part of the column free of copper ions served to prevent the elution of copper ions from the column.

Fractionation of RAFP

Fractionation of RAFP was carried out on the Pharmacia liquid chromatographic system equipped with the copper chelate column. Buffers A and B were 0.01 *M* Tris–HCl (pH 7.5) containing 0.1 *M* sodium chloride and 0.1 *M* Tris–HCl (pH 8.0) containing 0.3 *M* ammonium chloride, respectively. A programmed linear gradient was used at a flow-rate of 0.3 ml/min. The appropriate peak fractions were collected, dialysed against distilled water and concentrated by Amicon Centricon-10 (Amicon, U.S.A.).

Polyacrylamide gel electrophoresis

Slab PAGE was performed with a Pharmacia GE-2/4 apparatus with 7.5% gels. Sample solutions for the electrophoresis were mixed with equal volumes of 50% sucrose solution, and 5 m*M* Tris–glycine (pH 8.6) was used as electrophoretic buffer. The gels were stained with 0.25% Coomassie Brilliant Blue G (Sigma, St. Louis, MO, U.S.A.) in a solvent (methanol–water–acetic acid, 150:150:50, v/v/v).

Gel permeation chromatography

A TSK-gel G-3000SW column and 0.05 *M* 1,3-bis[tris(hydroxymethyl) methylamino]propane (Bis-Tris propane)–acetic acid (pH 6.5) were used for gel permeation chromatography. The flow-rate was 0.8 ml/min, and the sample size was 20 μl.

Protein assay

Protein concentrations were determined by the Bio-Rad protein assay method [7] with bovine serum albumin as a standard.

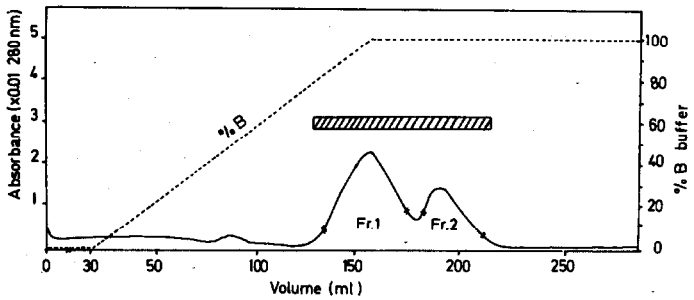


Fig. 1. Fractionation of 5 mg of RAFP on a copper chelate column. Buffer A, 0.01 *M* Tris-HCl (pH 7.5) containing 0.1 *M* sodium chloride; buffer B, 0.1 *M* Tris-HCl (pH 8.0) containing 0.3 *M* ammonium chloride; flow-rate, 0.3 ml/min; sensitivity, 0.1 a.u.f.s. The presence of RAFP in the fractions is shown by the hatched zone. The RAFP fractions collected are indicated by hash marks in the elution profile.

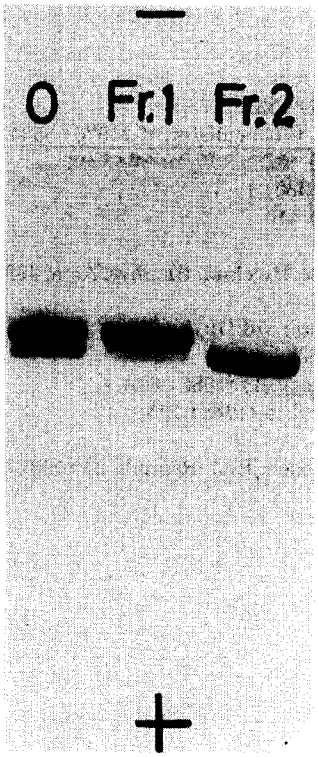
RESULTS AND DISCUSSION

We applied the pure RAFP on the copper chelate column and found that it was resolved into two major fractions, 1 and 2 (Fig. 1). A 10-ml sample (5 mg of pure RAFP dissolved in starting buffer A) was applied. Protein assay showed that these fractions contained ca. 74 and 21%, respectively, of the total RAFP. The use of samples sizes up to 15 ml did not produce any differences in the chromatographic pattern of RAFP.

Fig. 2 shows the characterization of the fractions by PAGE. Original RAFP (track 0) shows the typical fast and slow variants. The electrophoretic pattern of fraction 1 (track Fr. 1) shows one major and one minor band, which co-migrate with the slow and fast band, respectively. Fraction 1 was highly enriched a molecular species that co-migrated with the slow variant. The slow migration of this fraction toward the cathode in PAGE was consistent with its rapid elution from the chelate column. Conversely, fraction 2 (track Fr. 2) was essentially pure and co-migrated with the fast variants. A limited contamination of fraction 1 by the fast RAFP variant may be due to contamination from fraction 2, although this seemed unlikely since fractions taken throughout fraction 1 showed the same PAGE pattern. Further studies are needed of this electrophoretic microheterogeneity of fraction 1.

Fig. 3 shows the gel permeation patterns of RAFP and fractions 1 and 2. The original RAFP showed an asymmetric single peak. The gel permeation peaks of fraction 1 and 2 were symmetric. The peak of fraction 1 was eluted faster than that of fraction 2. When a mixture of 74 parts of fraction 1 and 21 parts of fraction 2 was applied on the gel, the elution profile showed the same pattern and same elution time as the original RAFP.

This is the first report of the separation of AFP variants by metal affinity chromatography. Our results show that RAFP might be a mixture of at least two variants. These two variants differ in their affinity for copper ion and in molecular mass, and are co-migratory in PAGE. Kerckaert and co-workers [8,9] have reported that RAFP contains two variants with different molecular mass.



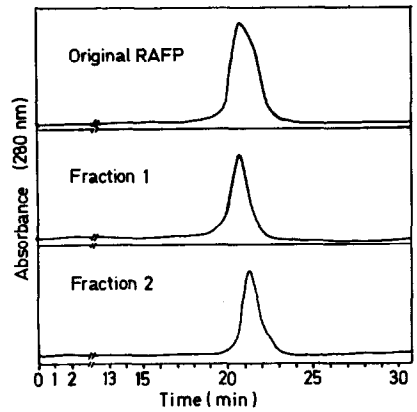


Fig. 2. Polyacrylamide gel electrophoresis of fraction 1 (Fr. 1) and fraction 2 (Fr. 2) in Fig. 1, with purified RAFP (track 0) as standard.

Fig. 3. Gel permeation pattern of fraction 1 and fraction 2 in Fig. 1, with purified RAFP (original RAFP). Column, TSK-gel G-3000SW; buffer, 0.05 *M* Bis-Tris propane-acetic acid (pH 6.5); flow-rate, 0.8 ml/min.

Aoyagi et al. [10] showed that AFP exhibited a characteristic binding of copper ion and suggested that the protein might be a carrier protein for copper in the foetus. Vormann et al. [11] reported that the concentration of zinc was drastically reduced in maternal serum and only slightly reduced in foetal serum during treatment of pregnant rats with a zinc-deficient diet, and suggested that it might be due to the high affinity of zinc ion for AFP. These reports indicate that AFP might play a role as a metal carrier in the foetus.

It has been well documented that the ratios of molecular variants on PAGE [3] and lectin affinity [12–14] change during foetal development. Our data suggest that the ratio of the metal affinity variants of RAFP might change during foetal development. This may be very important for understanding the role of AFP in metal metabolism in the foetus.

ACKNOWLEDGEMENT

The authors thank Dr. S. Kanoh for helpful discussions and encouragement.

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