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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS: PURIFICATION OF α-FETOPROTEIN FROM FETAL CALF SERUM*

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SUMMARY

High-performance liquid chromatography was utilized for the purification of bovine α-fetoprotein (BAFP) from fetal calf serum (FCS). An initial step in the purification involved absorption of charcoal delipidated FCS on Cibacron Blue F3GA gel. The Cibacron Blue pre-purified FCS was then chromatographed on a Polyanion SI weak anion-exchange column. The BAFP isolated had a purity of >93% with an overall yield of 48% from FCS. The procedure was applicable for semi-preparative scale purification of BAFP.

INTRODUCTION

α-Fetoprotein (AFP), a serum glycoprotein produced by the yolk sac, liver and gastrointestinal tract, is the principle plasma protein of fetus during the early gestation period [1, 2]. AFP is maintained at high level throughout the gestation, but drops markly to trace amounts after birth [3, 4]. However, in certain pathological conditions particularly liver cell carcinoma and germ cell tumors [5—8], its concentrations may reappear in the serum to a significant level and is therefore of a great diagnostic significance [5—8].

In recent years, some of the properties of AFP have been unfolded. Its

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proposed functions [4] include bindings to ligands and immunoregulatory effects. However, the biological role of AFP has not yet been fully clarified, largely because homogeneous AFP is still difficult to be purified. For this reason, a simple and rapid purification procedure able to provide homogeneous AFP is desirable.

Various physicochemical methods have been suggested for the purification of AFP [4]. However, because of the structural similarity between AFP and albumin [9, 10] and that the concentration of the latter in serum is usually many folds higher, separation by these methods is rendered particularly difficult involving laborious multi-step procedures which often resulted in low recoveries of the products. In this respect, purification by an immunoadsorbent technique [11—13] is simple and efficient, but unfortunately it is still a long and tedious process and involves the prior availability of purified AFP for the generation of anti-AFP antibodies.

Because of the recently availability of high-performance liquid chromatographic supports specifically designed for the resolution, separation and recovery of biopolymers [14], high-performance liquid chromatography (HPLC) is now being used routinely in protein chemistry laboratories for isolation and purification of native polypeptides and proteins, and for protein structural and sequence analysis. The aim of this paper is to demonstrate the feasibility of using a combination of Cibacron Blue F3GA gel affinity chromatography and anion-exchange HPLC as a fast, simple and efficient means of isolating AFP from fetal calf serum (FCS).

EXPERIMENTAL

Materials

FCS was obtained as a gift from Dr. H.F. Deutsch (University of Wisconsin, Madison, WI, U.S.A.). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, U.S.A.). Bovine α-fetoprotein (BAFP) standard was kindly donated by Dr. E. Ruoslahti (City of Hope National Medical Center, Duarte, CA, U.S.A.). Cibacron Blue F3GA agarose (Affi-Gel Blue) was obtained from Bio-Rad Labs. (Mississauga, Canada). All other reagents used were of analytical or reagent grade and purchased from local suppliers.

Instrumentation

HPLC was performed on a Pharmacia FPLC (fast protein liquid chromatography) system equipped with dual pump capable of generating a gradient or step gradient elution profile. Chromatograms were recorded by monitoring the absorbance at 280 nm using a Pharmacia UV-1 monitor fitted with a 10-nm path length HR-cell. The absorbance unit full scale (a.u.f.s.) was set between 0.1 to 2.0 units, where appropriate. Fractions were collected with a FRAC-100 fraction collector and the operating temperature was ambient.

Delipidation of FCS

FCS was delipidated using a modified method according to Chen [15]. Lyophilized FCS (1 g) was reconstituted with 18 ml of 0.03% citric acid solution and dialyzed against distilled water. Activated charcoal was added

(600 mg), the pH carefully adjusted to 3.0 with 1 *M* hydrochloric acid, and the mixture incubated with shaking at 0°C for 2 h. The solution was then centrifuged at 15,000 *g* for 30 min at 0°C. The supernatant was decanted from the charcoal, adjusted to pH 7 with 0.5 *M* sodium hydroxide, and dialyzed against 0.02 *M* phosphate buffer, pH 7.2.

Cibacron Blue gel chromatography

Affi-Gel Blue was first washed with 8 *M* urea until the eluent became colorless and then washed extensively with 0.02 *M* phosphate buffer, pH 7.2. For quantitative runs, the washed gel was packed into a Pharmacia HR 10/10 column (100 mm × 10 mm I.D.) and chromatographed using the FPLC system. After eluting the unretained protein fraction with 0.02 *M* phosphate buffer, pH 7.2, the retained fraction was next eluted with phosphate buffer containing 1.4 *M* sodium chloride. The unretained and retained protein fractions were dialyzed against distilled water and lyophilized. The column was regenerated with 8 *M* urea and equilibrated with 0.02 *M* phosphate buffer, pH 7.2 before reused. For large scale runs, pre-washed gel was packed into a glass column (Bio-Rad Econo-column, 500 mm × 25 mm I.D.) and equilibrated with 0.02 *M* phosphate buffer, pH 7.2. The unretained and retained protein fractions were eluted with their respective buffers as above. The fractions were concentrated to a volume of 20 ml by means of a Diaflo PM-10 membrane, dialyzed against distilled water and lyophilized.

Polyanion SI chromatography

HPLC was carried out using either a prepacked analytical HR 5/5 Polyanion SI column (50 × 5 mm I.D., 6—7 μm particle size, Pharmacia) or a semi-preparative HR 10/10 column dry-packed with Polyanion SI, 17-μm particle size (Pharmacia). The A buffer for ion exchange was 12.1 g (0.1 *M*) of tris(hydroxymethyl)aminomethane (Tris) per liter, adjusted to pH 8 with 1 *M* hydrochloric acid while buffer B was 12.1 g Tris, pH 8, containing 40.8—68.0 g (0.3—0.5 *M*) of sodium acetate per liter. A preprogrammed linear gradient was used for the chromatography and the appropriate protein peaks were collected, dialyzed against distilled water and lyophilized.

Protein assay

Total protein concentrations were determined by the biuret method [16] using BSA as a standard or by UV absorption at 280 nm. BAFP and BSA concentrations were assayed by the radial immunodiffusion method of Mancini et al. [17]. The purity of the purified protein was determined by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) [18]. The gel was scanned with a DCD-16 digital computing densitometer (Gelman, Ann Arbor, MI, U.S.A.).

RESULTS

HPLC separation of BAFP and BSA

Attempts were made initially to establish an HPLC procedure for the separation of BAFP and BSA, the later being an anticipated major component of the

FCS which might interfere with BAFP in the purification. Fig. 1 depicts the separation of standard BAFP and BSA on a Pharmacia HR 5/5 Polyanion SI (weak anion-exchange) column. By employing a 0.1 *M* Tris buffer, pH 8, as the initial buffer and eluting with a 0.4 *M* sodium acetate gradient, BAFP and BSA were satisfactory resolved. Other eluting salts, such as sodium chloride, sodium sulphate, were tested but they gave a poorer resolution of the proteins. The pH of separation was also studied and found to be critical at around pH 8 with no separation of the two proteins at or below pH 6.

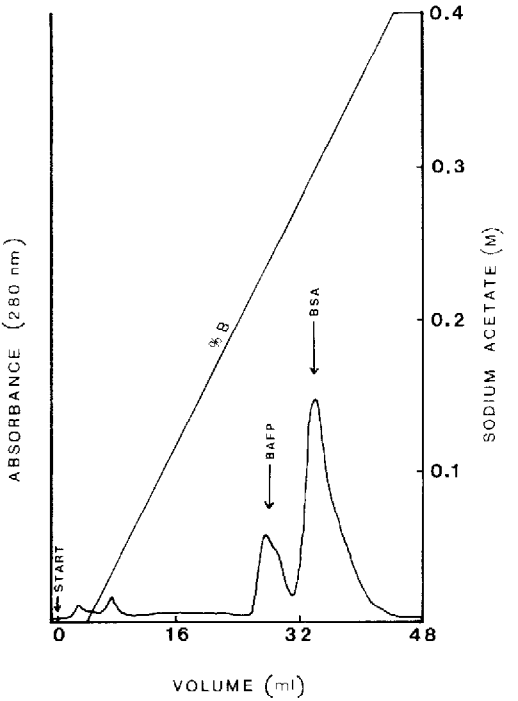

Fig. 1. HPLC separation of standard BAFP (200 μg) and BSA (500 μg) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.4 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 0.1.

Cibacron Blue F3GA gel pre-purification of FCS

Chromatography of neat FCS on the above described HPLC procedure (Fig. 2) showed that although BAFP and BSA were resolved from each other, the BAFP peak was massed by a number of other proteins having the same retention volume as BAFP. Thus a pre-purification of the FCS was necessary so as to concentrate BAFP and remove the majority of the interfering proteins.

Fig. 3 shows the chromatography of FCS on a Cibacron Blue F3GA gel affinity column. A single passage through the gel showed that 57% of BAFP and > 97% of BSA were bound (Table I). The retained fraction (fraction B) was subsequently eluted with sodium chloride and analyses revealed that it contained, in addition to BAFP and BSA, only a small amount of other proteins. The remaining BAFP (43%) was associated with the unretained fraction (fraction A) which contained also the majority of the other proteins

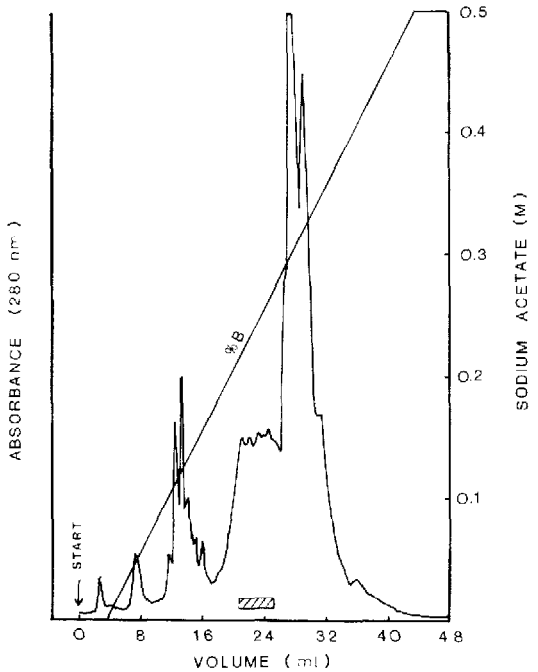

Fig. 2. HPLC separation of FCS (100 µl) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.5 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 0.2. The presence of BAFP in fractions is shown in hatched zones.

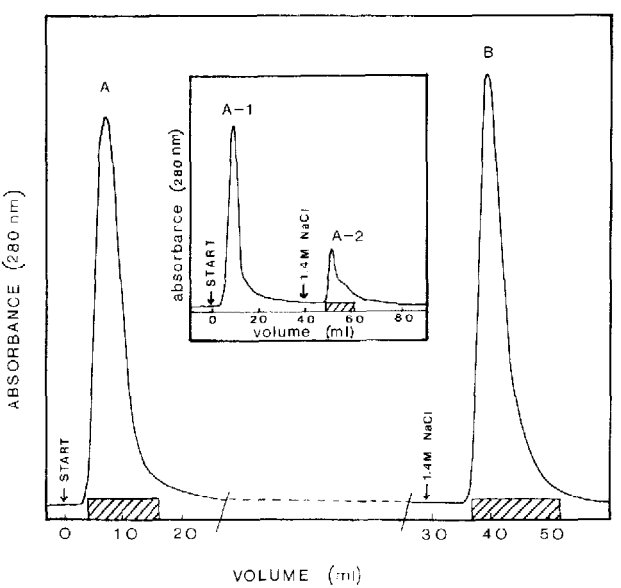

Fig. 3. Chromatography of FCS (1.5 ml) on a HR 10/10 Affi-Gel Blue column using 0.02 *M* phosphate, pH 7.2 as the eluent (1 ml/min). Insert: rechromatography of charcoal delipidated Fraction A on a HR 10/10 Affi-Gel Blue column using 0.02 *M* phosphate, pH 7.2 as the eluent (1 ml/min). The presence of BAFP in fractions is shown in hatched zones.

TABLE I

PURIFICATION OF BAFP FROM FCS BY CIBACRON BLUE F3GA GEL AFFINITY CHROMATOGRAPHY

Fraction	Total protein (mg)	BAFP (mg)	BSA (mg)	Other protein (mg)	Percent BAFP in protein
Whole	48.0	2.0	16.4	30.6	4.2
Unretained (A)	27.0	0.86	<0.05	26.1	3.2
Retained (B)	21.5	1.10	15.75	4.6	5.1
A - 1*	28.4	—	—	28.4	—
A - 2*	10.4	1.2	<0.05	9.2	11.5

*Data obtained from a combination of two unretained (A) fractions, delipidated and rechromatographed on Affi-Gel Blue. A-1 and A-2 were unretained and retained fractions, respectively (see Fig. 3).

in FCS. When fraction A was subjected to delipidation by the charcoal method of Chen [15] and rechromatographed on the Cibacron Blue gel column (Fig. 3, insert), nearly all of the BAFP now was retained by the column but the majority of the other proteins remained unretained (Table I). From these results, it was established that a purified FCS fraction containing predominantly BSA and BAFP could be obtained by subjecting the serum first to charcoal delipidation followed by chromatography on Cibacron Blue gel.

HPLC separation of Cibacron Blue gel purified FCS

When delipidated Cibacron Blue gel purified FCS was subjected to HPLC separation using the procedure described above, the chromatogram (Fig. 4) showed that a total of four peaks were resolved. The retention volumes of peaks 3 and 4 corresponded to that of standard BAFP and BSA, respectively, and their identities were confirmed by radial immunodiffusion plate analyses. A similar chromatogram (Fig. 5) showing the complete resolution of BAFP and BSA was also obtained on chromatography of a non-delipidated Cibacron Blue purified FCS fraction (fraction B, Fig. 3).

The purity of the BAFP thus separated was analyzed by subjecting various fractions of the peak (Fig. 4, fractions a—c) to SDS—PAGE. Fig. 6 shows the densitometer tracing of the gels of the fractions together with that of a standard of BAFP—BSA mixture. Fraction a, which comprised of about 5% of the total peak absorbance, showed the presence of a major BAFP peak (47%) and two other minor peaks. The major b fraction (80%) consisted predominantly of BAFP (> 93%) together with a trace amount of a protein(s) of slower electrophoretic mobility. Fraction c, which comprised of the remaining 15% of the total absorbance, had a BAFP peak with purity of about 70%. The purity of fraction b was further tested by comparing the size of the immunodiffusion ring of a 0.5 mg/ml solution of the protein to that of a standard BAFP sample isolated from an immunoabsorbent method [13]. The result confirmed a protein purity of > 93%. The extinction value $E^{1\%}_{1\,cm}$ (280 nm) of fraction b was 4.2 which was similar to that reported for BAFP isolated from an immunoabsorbent method [$E^{1\%}_{1\,cm}$ (280 nm) = 4.1] [19]. Table II summarizes the

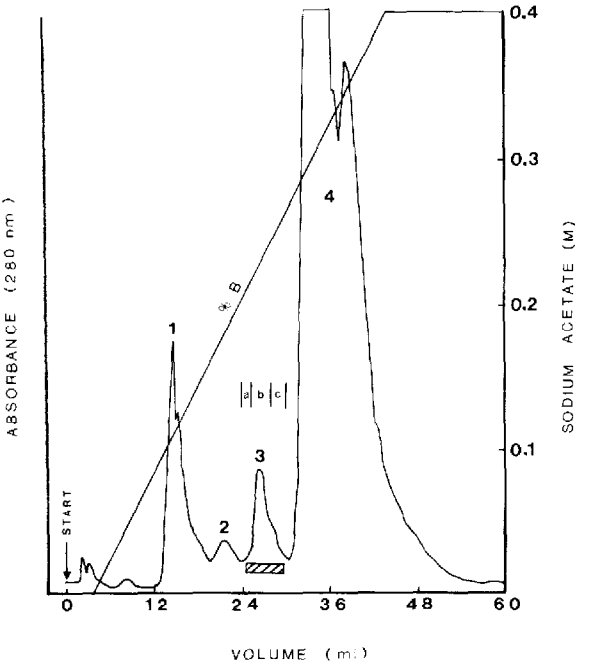

Fig. 4. HPLC separation of delipidated Cibacron Blue gel purified FCS (5 mg) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.4 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 0.2. The presence of BAFP in fractions is shown in hatched zones. Peaks: 3 = BAFP; 4 = BSA.

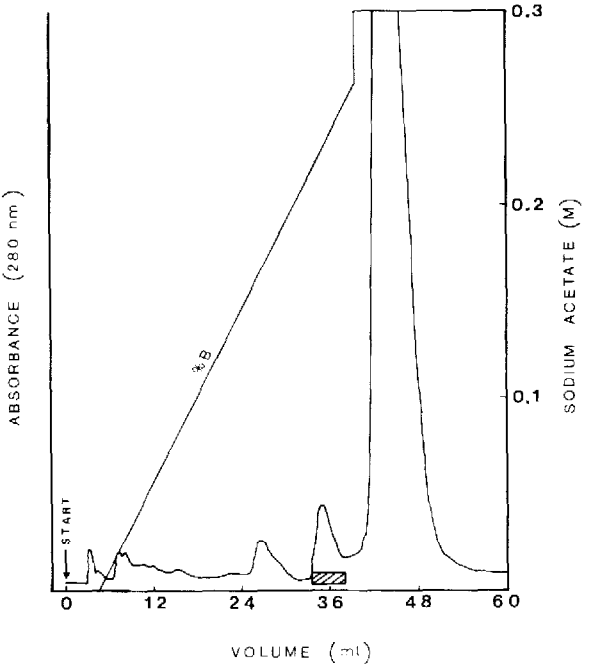

Fig. 5. HPLC separation of non-delipidated Cibacron Blue gel purified FCS (5 mg) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B; 0.1 *M* Tris, pH 8 containing 0.3 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s: 0.2. The presence of BAFP in fractions is shown in hatched zones.

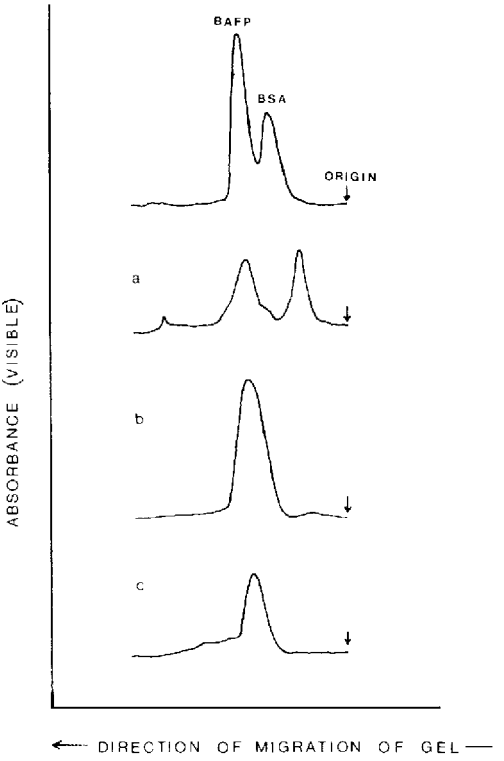

Fig. 6. Densitometer tracing of SDS—PAGE of BAFP peak fractions a, b and c shown in Fig. 4 together with that of a standard BAFP—BSA mixture.

TABLE II

SUMMARY OF PURIFICATION OF α-FETOPROTEIN FROM FETAL CALF SERUM

Method	Total protein (mg)	BAFP (μg)	Percent yield (overall)
Fetal calf serum (neat, 0.33 ml)	9.92	621	100
Delipidation and Cibacron Blue gel chromatography	5.10	460	74
HR 5/5 Polyanion SI chromatography	0.33	302	48.6

recoveries of the two-step BAFP purification procedure. A 48% overall yield of BAFP from FCS was obtained.

Semi-preparative scale purification of BAFP

Fig. 7 depicts the chromatographic separation of a delipidated Cibacron Blue purified FCS on a dry-packed HR 10/10 Polyanion SI (17 μm) column. The column gave a seven-fold increase in loading capacity compared to the analytical HR 5/5 Polyanion SI column with adequate resolution of the BAFP and BSA peaks still being maintained, thereby affording semi-preparative scale

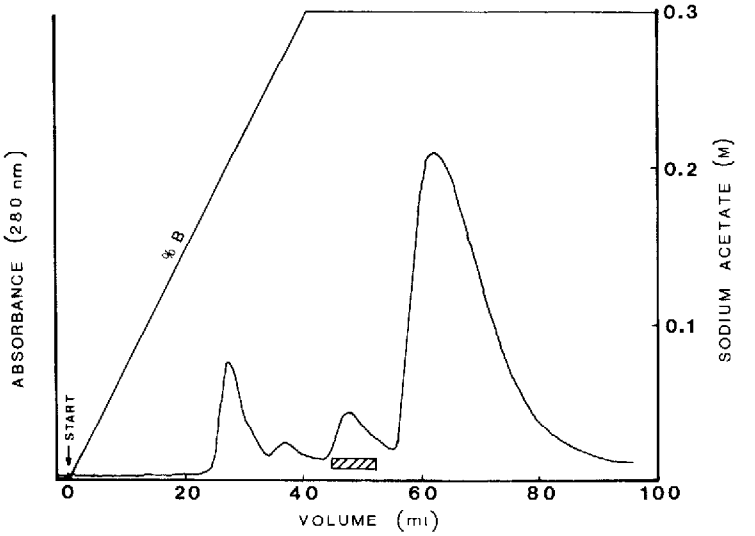

Fig. 7. HPLC separation of delipidated Cibacron Blue gel purified FCS (35 mg) on a dry-packed HR 10/10 Polyanion SI 17-μm column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.3 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 1.0. The presence of BAFP in fractions is shown in hatched zones.

purification of BAFP from FCS. Radial immunodiffusion plate analysis of the BAFP thus isolated showed a purity of 70—80%.

DISCUSSION

BAFP has been isolated from FCS by successive procedures of Con A-Sepharose, DEAE-Sephadex and SP-Sephadex chromatography followed by preparative disc PAGE [20]. More recently, Ruoslahti [13] reported the purification of BAFP from FCS using a combination of immunoadsorbent and gel chromatographic techniques. Although these methods are adequate for the purification of BAFP, they are however laborious and complicated multiple-step procedures.

The use of HPLC for the separation and purification of biopolymers has received considerable attention in recent years. Its advantage over conventional methods of separation is obvious. It is fast, convenient, flexible and also affords semi-preparative scale capability. Herein, we describe a simple two-step procedure involving Cibacron Blue gel pre-purification followed by anion-exchange HPLC separation for the isolation of BAFP from FCS.

Initially, attempts were made to develop an HPLC separation condition for the resolution of BAFP and BSA. Because of the similarity in molecular weight between the two proteins [9, 10], steric exclusion chromatography was not suitable for their separation. Reversed-phase (hydrophobic) chromatography, although being widely used for the separation of peptides and proteins, was also not employed because of the undesirable large amount of organic solvents needed in the separation procedure. The amphoteric character of BAFP and BSA makes ion-exchange chromatography a more suitable tool for their separation. Adequate resolution of the two proteins was attained with the use of an anion-exchange column.

Direct separation of neat FCS using the anion-exchange HPLC procedure showed that although BAFP and BSA could be resolved from each other, the BAFP peak was extensively massed by other protein peaks. Thus a pre-purification of the FCS was necessary so as to remove the majority of the masking proteins before subjecting to HPLC separation. By using a Cibacron Blue gel column which selectively absorbed BAFP and BSA but not the other proteins, a BAFP—BSA enriched fraction of FCS could be obtained. The use of Cibacron Blue gel as an affinity column for the separation of human AFP and serum albumin has recently been reported by Young and Webb [21]. In contrast to our findings in the bovine, these authors found that human AFP from cord serum was unbound while albumin was bound by the blue gel. The exact reason(s) for the discrepancy in binding between bovine and human AFP towards Cibacron Blue F3GA remains unclear and further investigations into these areas are thus warranted.

Although HPLC separation of the Cibacron Blue purified FCS on the HR 5/5 Polyanion SI analytical column could afford BAFP of > 93% in purity, the methodology was adequate only for the isolation of small (low mg) quantities of BAFP by repeated injections. The analytical column had a loading capacity of about 5 mg of the BAFP—BSA enriched protein mixture and therefore approximately 300 μg of BAFP could be purified in a single injection. In order to adapt this methodology for semi-preparative scale purifications of BAFP, a HR 10/10 Polyanion SI (17 μm) column was tested and found to have a seven-fold loading capacity compared to the analytical column. Thus in a single injection using this column, approximately 2 mg of BAFP could be purified. Although the BAFP isolated had a purity of 70—80%, its purity however could be easily upgraded by rechromatography on the analytical column.

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