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ANALYTICAL- AND PREPARATIVE-SCALE SEPARATION OF MOLECULAR VARIANTS OF α-FETOPROTEIN BY ANION-EXCHANGE CHROMATOGRAPHY ON MONOBEAD™ RESINS

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SUMMARY

A rapid and reliable purification procedure is described that is useful for both analytical detection and quantitative recovery of milligram amounts of individual molecular variants of mouse α-fetoprotein (AFP). The appropriate separation conditions were developed with an analytical-size Mono Q anion-exchange column linked to an automated Fast Protein Liquid Chromatography™ system. Effective separations of fetal-derived AFP variants was accomplished within 20 min under mild conditions with an L-histidine buffer. Employing the optimal separation conditions established on the Mono Q HR 5/5 column we upscaled the procedure by using a preparative Mono Q HR 16/10 column in order to obtain milligram quantities of each molecular variant of AFP. Seven distinct isomeric forms of AFP could be recovered on the preparative anion exchanger in a highly reproducible manner. Each of the seven protein peaks eluted from the Mono Q column were confirmed to be distinct isoforms of AFP by isoelectric focusing and Western blotting developed with monospecific anti-AFP antisera. This method in its scaled up version offers the benefit of providing milligram quantities of immunochemically pure AFP isomers for structure and function studies.

INTRODUCTION

α-Fetoprotein (AFP) is a well characterized onco-fetal molecule [1] that has attracted considerable attention for its potential as a diagnostic marker in a multiplicity of diseases [2–4] and because of the accumulating evidence supporting the concept that AFP plays an essential immunoregulatory role during normal embryonic development (reviewed in ref. 5). The classical approaches

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to AFP detection and purification have included a combination of both chromatographic and electrophoretic techniques (reviewed in ref. 6). Several such separation procedures have indicated that AFP exists as a heterogeneous population of molecules [7,8]. Thus, molecular variants of AFP have been observed with such procedures as polyacrylamide gel electrophoresis (PAGE) [9], isoelectric focusing (IEF) [7], ion-exchange chromatography [10] and lectin and hormone affinity chromatography [11,12]. Furthermore, reproducible changes in the ratios and concentrations of isomers was found to occur during normal development and in certain disease states [6]. In addition, investigations by Lester and co-workers [13,14] have demonstrated a positive correlation between the presence of certain molecular variants in fetal- or tumor-derived AFP samples and in vitro immunosuppressive strength. A major obstacle to further detailed structure and function studies of AFP molecular variants has been the lack of a suitable procedure for the purification of individual isoforms of AFP. In this paper, we describe both an analytical and preparative Fast Protein Liquid Chromatography™ (FPLC) anion-exchange procedure that is capable of meeting these requirements. Employing these new separation techniques, we have recently been able to establish a firm functional association between the presence of a unique isomeric form of mouse AFP and immunosuppressive activity [15].

The characterization of human AFP microheterogeneity is already of some practical clinical value as changes in the ratios of human AFP isomers have been correlated with several pathological processes [16,17]. The separation conditions described herein may therefore have clinical applicability in analytical-scale resolution of AFP isomers in biological fluids.

EXPERIMENTAL

Animals

Swiss Webster mice, purchased from Charles River, were bred and maintained in our own animal facilities.

AFP preparation

Murine AFP was purified from mouse amniotic fluid (MAF) extracted from pregnant Swiss mice in late stage of gestation (days 15–18). The isolation of AFP from MAF was performed by antibody-agarose affinity chromatography as previously described by Murgita and Tomasi, Jr. [18]. AFP preparations tested at 2.0 mg/ml failed to show detectable contamination with other serum or amniotic fluid proteins as determined by immunodiffusion and immunoelectrophoresis studies. The purity of AFP was further confirmed by physicochemical analyses including alkaline PAGE (APAGE) and sodium dodecyl sulfate PAGE (SDS-PAGE).

Materials

L-Histidine, Bis–Tris and piperazine were purchased from Sigma (St. Louis, MO, U.S.A.). The equilibration buffer for anion-exchange chromatography consisted of 20 m*M* L-histidine, pH 5.60, while the elution buffer contained 0.5 *M* sodium chloride in addition to L-histidine. All buffers were prepared with distilled water (Milli-Q^{TM} water purification system, Millipore, Montreal, Canada) and filtered through 0.22-μm filters prior to use. Ampholines (pH 4.0–6.5) required for isoelectric focusing were purchased from Pharmacia (Pharmalyte 4.0–6.5, Pharmacia, Dorval, Canada). Acrylamide, bisacrylamide, Tween-20, gelatin, p*I* markers and the alkaline phosphatase color development reagents were obtained from Bio-Rad (Bio-Rad, Mississauga, Canada).

Anion-exchange chromatography

All chromatographic separations were performed on an analytical Mono Q HR 5/5 or a preparative Mono Q HR 16/10 anion-exchange column linked to an FPLC system (Pharmacia Fine Chemicals, Dorval, Canada). For analytical separations, 200 μl of a 2.0 mg/ml AFP preparation in phosphate-buffered saline (PBS) were applied to the Mono Q HR 5/5 column previously equilibrated with L-histidine buffer (20 m*M*, pH 5.60). The pre-determined optimal conditions for eluting individual AFP isomers from the Mono Q column at a constant flow-rate of 1.0 ml/min was initially established with L-histidine buffer. After sample application, the column was washed with 0.5 ml of L-histidine buffer which allowed for the absorbance (UV at 280 nm) to return to the baseline. Bound proteins were eluted with a linear salt gradient extending from 0 to 0.15 *M* sodium chloride in 18.0 ml of L-histidine buffer. This was followed by a wash step of 0.5 *M* sodium chloride for a 2.0-ml volume. The entire procedure was developed with a volume as opposed to time programming base in order to facilitate the scale-up and optimization of runs on the preparative anion-exchange column. Fractions representing individual peaks were collected with a FRAC-100 fraction collector, dialyzed versus PBS overnight and kept frozen at $-20\,^{\circ}C$ until further analysis.

For all preparative-scale separations, 10.0-ml aliquots of purified AFP at 2.0 mg/ml in PBS were loaded onto the Mono Q HR 16/10 column employing a flow-rate of 8.0 ml/min. Samples were eluted from the Mono Q 16/10 columns with a linear salt gradient extending to 0.15 *M* sodium chloride over a total L-histidine buffer volume of 360 ml. Fractions corresponding to individual peaks were collected using a FRAC-100 fraction collector (Pharmacia). Both the threshold setting and tube advancement control were manually regulated to prevent the potential for cross-contamination between adjacent peaks. Tubes containing UV-absorbing peaks were pooled and concentrated on YM-10 membranes using an Amicon filtration unit (Amicon, Oakville, Canada). Samples were then dialyzed against PBS and the protein concentration was adjusted to 2 mg/ml by measuring the UV absorbance at 280 nm using an

extinction coefficient of 0.443 [18]. Fractions were analyzed by immunodiffusion, using rabbit antisera against mouse AFP, transferrin, albumin and normal mouse serum.

Isoelectric focusing

Analytical IEF was performed in 5% polyacrylamide gels using a Bio-Rad horizontal mini-IEF cell. Briefly, 2.0 ml of acrylamide monomer concentrate [24.25% (w/v) acrylamide, 0.75% (w/v) bis-acrylamide (25% acrylamide, 3% cross-linker)] were combined with 5.5 ml of Milli-Q-purified water, 2.0 ml of 25% (w/v) sucrose and 0.5 ml of ampholines (pH 4.0–6.5, Pharmacia). This solution was degassed for 5 min, and polymerization was subsequently initiated with 15 μl of ammonium persulfate (10%, w/v), 50 μl of riboflavin-5'-phosphate (0.1%, w/v) and 3 μl of N,N'-tetramethyleneethylenediamine (TEMED). Samples of 1 μl containing 1 μg of protein were loaded onto the gel and allowed to diffuse into the gel for 10 min. The samples were then electrophoresed at 4°C at a constant voltage of 100 V for 15 min, followed by an additional 15 min at 200 V and finally 450 V for 1 h. The gel was subsequently immersed in a fixative solution [5% (w/v) sulfosalicylic acid, 5% (w/v) trichloroacetic acid in Milli-Q-purified water] for 30 min and stained with Coomassie brilliant blue. The pH gradient was verified by measuring the pH of individual gel slices prior to fixing or by the inclusion of p*I* markers (Bio-Rad).

Western blotting of IEF gels

In order to perform immunoblotting of the IEF gel, the gel was poured vertically between two glass plates. Following polymerization, one plate was carefully removed and the gel was run under identical conditions as outlined for IEF gels except that 100 ng of protein were used per sample. After electrophoresis, the IEF gel was removed from the glass plate and equilibrated for 10 min in transfer buffer (12.5 m*M* Tris, 96 m*M* glycine and 20% (v/v) methanol, pH 8.2). The gel was applied to Immobilon (PVDF, Millipore) and proteins were transferred by electrophoresis using a Mini-transblot apparatus (Bio-Rad) in transfer buffer at 40 mA for 18 h. Following the completion of the transfer, the membrane was blocked for 1 h with gentle agitation in blocking solution [3% (w/v) gelatin in 20 m*M* Tris, 500 m*M* sodium chloride (Tris-buffered saline, TBS), pH 7.5]. Prior to incubation with the primary antibody, the membrane was washed twice for 5 min in TBS containing 5% (v/v) Tween-20 (TTBS). The membrane was then incubated for 1 h in a primary antibody solution containing a 1:1000 dilution of a monospecific rabbit anti-mouse AFP antibody preparation in antibody solution [1% (w/v) gelatin in TTBS, pH 7.5]. A second wash step of 2×5 min in TTBS was followed by a 1-h incubation with a 1:3000 dilution of a goat anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad). After three successive wash steps of 5 min each, twice with TTBS and once with TBS, the membrane was developed with the alkaline

phosphatase color development reagents BCIP (bromochloroindolylphosphate *p*-toluidine salt) and NBT (*p*-nitrotetrazolium chloride) (Bio-Rad).

RESULTS

Qualitative separations of mouse AFP molecular variants

Native AFP was purified from MAF by procedures that have been described in detail elsewhere [18]. Pure AFP preparations, as verified by conventional APAGE and immunodiffusion techniques, served as the starting material for all subsequent purification procedures. Fig. 1 illustrates the typical separation obtained for 400 μg of fetal mouse AFP with the 20 m*M* L-histidine buffer system. As shown in Fig. 1, AFP is resolved into seven distinct peaks, with one UV-absorbing fraction eluting in the void volume followed by six well defined peaks that appeared consecutively in the 18-ml linear salt gradient. Preliminary experiments indicated that both Bis–Tris and piperazine buffers could be substituted for L-histidine (data not shown). The individual fractions comprising each of the UV-absorbing peaks in the profile shown in Fig. 1 were isolated and examined for their AFP content by immunodiffusion studies using monospecific antisera against AFP. Six peaks from the Mono Q column which appeared in succession at retention volumes of 10, 11, 12, 13, 14 and 16 ml developed specific precipitin lines with anti-AFP antisera but not with antisera against albumin, transferrin or normal mouse serum. The six AFP frac-

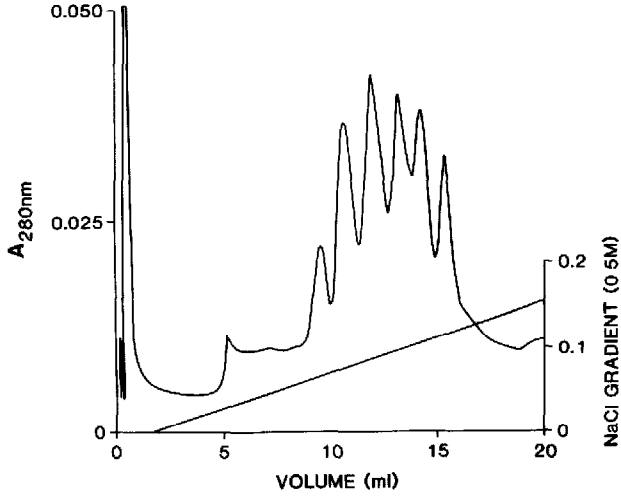

Fig. 1. Analytical-scale anion-exchange chromatography of 400 μg of purified mouse AFP with FPLC Mono Q columns. Mouse AFP was applied to the column that had been pre-equilibrated with 20 m*M* L-histidine, pH 5.60. Following sample loading, the column was rinsed and samples were eluted with a linear sodium chloride gradient extending to 0.15 *M*. The elution profile is based on the absorbance at 280 nm.

tions were homogeneous and maintained identical retention volumes when rechromatographed on the same Mono Q column (data not shown). No amniotic fluid or serum proteins could be detected in the UV-absorbing material present in the void volume or at a retention volume of 5.0 ml. A seventh AFP peak was detected in the void volume when individual fractions from several analytical runs were combined. This seventh AFP peak was readily identified when larger quantities of native AFP were applied to preparative anion-exchange columns.

Isoelectric focusing and immunoblot analysis of Mono Q-defined AFP fractions

In order to determine whether the seven distinct peaks separated on the Mono Q column represented unique molecular variants of AFP, the individual fractions were characterized by IEF. Due to the limited quantities of peaks purified using analytical runs, most IEF studies were performed on AFP peaks obtained from preparative anion-exchange columns.

Unfractionated fetal AFP subjected to narrow-range IEF (pH 4.0–6.5) could be resolved into six distinct variants in a p*I* range of 4.7 to 5.1 (Fig. 2A). The analysis of the seven individual FPLC-purified AFP peaks revealed six major isoelectric variants corresponding in mobility to the six isoforms of control AFP. The terminology used in this study (i.e. AFP-1 through AFP-7) defines the seven AFP peaks separable on the anion-exchange column. AFP-1, appearing in the Mono Q fall-through, had the most basic p*I* of 5.1, whereas AFP-7 was the most acidic (p*I* 4.7) (Table I). It is notable that two isomers, AFP-4 and AFP-5, although distinguished by their FPLC retention volumes, exhibited an identical p*I* of 4.85. Immunoblot analysis of the protein in each of the seven FPLC peaks was performed using a rabbit antiserum that recognized the seven AFP isomers. The results showed that the isoelectric microheterogeneity

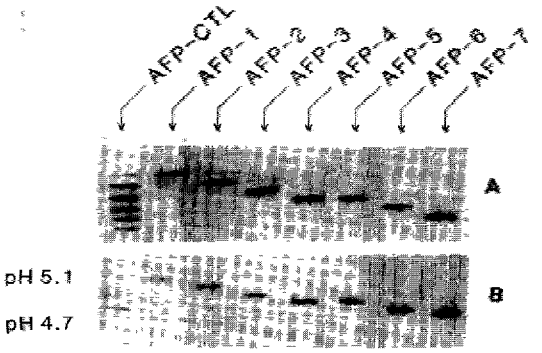

Fig. 2. Isoelectric focusing and immunoblot analysis of FPLC-purified AFP molecular variants. (A) Native AFP and the FPLC-generated AFP isomers designated AFP-1 through AFP-7 were analyzed on narrow ampholine range IEF gels (p*I* 4.0–6.5) that were Coomassie-stained. (B) For Western blots, 100 ng of control AFP or AFP-1 through AFP-7 were separated on narrow pH range IEF gels (p*I* 4.0–6.5) and immunoblotted with a monospecific anti-AFP antibody.

TABLE I

QUANTITATIVE RECOVERIES OF AFP ISOMERS

AFP fraction[a]	Retention volume[b]	Isoelectric point[c]	Yield of individual isomers[d] (mg)
AFP-1	25 ml	5.1	0.2
AFP-2	189 ml	5.0	0.52
AFP-3	211 ml	4.9	0.79
AFP-4	236 ml	4.85	1.3
AFP-5	268 ml	4.85	0.8
AFP-6	295 ml	4.8	1.3
AFP-7	318 ml	4.7	0.3
		Total	5.21

[a]AFP fractions were defined by the FPLC retention times, immunodiffusion analyses and IEF patterns.
[b]The retention volumes are based on the FPLC elution profile of 20.0 mg of AFP similar to that shown in Fig. 2.
[c]The isoelectric points of the individual isomers was estimated from IEF gels which had p*I* markers alongside native AFP.
[d]Fractions representing individual AFP peaks were dialyzed and concentrated, and the amount of protein was calculated as described in Experimental.

of the seven major immunoreactive bands was identical to control AFP (Fig. 2B) and confirmed that the proteins recovered from each Mono Q peak represented unique isoforms of AFP.

Preparative-scale separation of seven molecular variants of AFP

Although the protein capacity for the analytical Mono Q column is estimated to be 25.0 mg, or 5.0 mg per single peak [19], we noted that the fine resolution of individual peaks of AFP began to decrease when quantities of AFP greater than 1–2 mg were loaded onto the column. Therefore a preparative-scale Mono Q HR 16/10 anion-exchange column with a twenty-fold greater protein capacity than the analytical column was employed in order to generate larger yields of each isomer for further biochemical studies.

The separation parameters optimized for the analytical column were modified with the FPLC LCC-500 programmable control unit to accommodate the preparative column. In the scale-up mode the gradient volume was expanded twenty-fold to 360.0 ml and the flow-rate increased from 1.0 to 8.0 ml/min. Fig. 3 shows the separation profile of 20.0 mg of AFP on the Mono Q HR 16/10 column equilibrated with 20 m*M* L-histidine, pH 5.60. The seven major UV-absorbing peaks shown in Fig. 3 were fractionated and analyzed by double immunodiffusion with a panel of monospecific antisera. One AFP peak was identified in the flow-through on the preparative column. The six major peaks

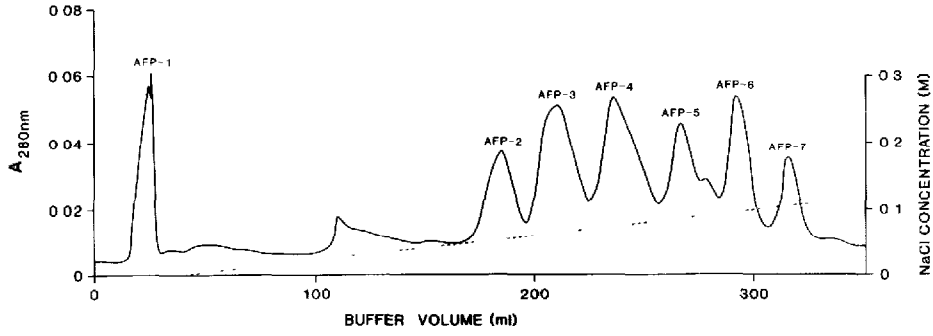

Fig. 3. Preparative-scale anion-exchange chromatography of 20.0 mg of AFP. A 10-ml volume of mouse AFP at 2.0 mg/ml was loaded onto the Mono Q HR 16/10 column After protein application, the column was rinsed with 20 m*M* L-histidine, pH 5.60, and bound proteins were eluted with a linear sodium chloride gradient over a 360-ml buffer volume. The absorbance was measured at 280 nm and the seven individual AFP fractions are indicated as AFP-1 through AFP-7.

that eluted sequentially in the salt gradient extending from 0.05 to 0.15 *M* sodium chloride were also identified as AFP-containing fractions.

Therefore, a total of seven AFP-containing peaks could be purified in separation times of 50 min with the preparative anion exchangers. These seven AFP isoforms, as defined by their specific isoelectric points and FPLC retention volumes (Table I), migrated as single 70 000-daltons bands on SDS-PAGE, showing a molecular mass identical to control unfractionated AFP (data not shown).

Quantitative recoveries of seven AFP molecular variants

In order to calculate the amount of each isoform of AFP purified with the preparative column, individual peaks were fractionated, concentrated, dialyzed against PBS and assayed for their AFP content spectrophotometrically. By over-riding the automatic mode on the FRAC-100 fraction collector and manually regulating the fractionation parameters, including the threshold setting and tube switching, it proved feasible to selectively isolate tubes containing homogeneous AFP peaks corresponding to individual isomers while avoiding those areas on the chromatogram containing cross-contaminated isomers. Beginning with 20.0 mg of starting material, the amount of each molecular variant purified ranged from a low of 0.2 mg for AFP-1 to a maximum of 1.3 mg for AFP-4 resulting in a total recovery of 25% (Table I). Due to the extremely high reproducibility of individual chromatographic separations, larger quantities of each isoform could be obtained by combining corresponding peaks from several 20.0-mg runs of AFP. By manually controlling the fraction collector, many tubes contained cross-contaminated AFP isoforms which had been intentionally excluded on initial chromatographic runs. These tubes could be

pooled and re-run over the Mono Q column in order to significantly increase the final yield of the seven individual AFP isomers.

DISCUSSION

The data presented in this paper indicate that an automated FPLC system employing Mono Q anion-exchange columns can be suitably adapted for the detection and recovery of molecular variants of mouse AFP.

The optimal chromatographic conditions for separating AFP variants were initially established on an analytical Mono Q column with three aqueous buffers, L-histidine, Bis–Tris and piperazine, all having pK_a values ranging from pH 5.0 to 6.0. At pH values in this range, about 1 pH unit above the range of isoelectric points for the native population of AFP molecules (pH 4.7–5.1), the net charge differences between the molecular variants of AFP appeared optimal to effect their resolution on anion-exchange columns. Chromatography of mouse AFP in buffers of varying pK_a values either more acidic or more basic than the pH 5.0–6.0 range confirmed that the pH chosen was essential for effective separations. Although similar separation patterns were obtained with all three buffers set at pH 5.6, the L-histidine buffer was selected for all chromatographic procedures described herein. This is partly because both Bis–Tris and piperazine are likely to have toxic and/or other adverse effects on lymphocyte tissue culture systems that we are presently employing to study the immunoregulatory properties of AFP isomers [15].

During the course of these investigations, we noted that the resolution of individual peaks began to decrease when protein loads greater than 1.0 mg of AFP were applied to the analytical column. This may be partly attributed to the finding that AFP isomers are known to have almost identical amino acid and carbohydrate compositions and thus the distinguishing characteristics of each are likely to be rather subtle [11]. In addition, the resolution of AFP peaks was severely diminished if separations were performed on MAF as opposed to purified AFP preparations. To generate larger quantities of individual AFP peaks, the purification conditions developed for the analytical column were adapted for the preparative Mono Q anion-exchange column. Deploying the preparative column, mouse AFP was efficiently separated into seven well resolved isoforms, as defined by the consistent retention volumes, the unique isoelectric points of individual isoforms compared to the typical isoelectric microheterogeneity of AFP and the immunoblotting studies with anti-AFP antisera. That the additional AFP isomer resolved from the void volume of the HR 16/10 column was not as readily detected in chromatographic runs on the analytical column is probably due to quantitative factors since this isomer represents less than 5% of the total number of native AFP molecules.

The identification of seven distinct mouse AFP isomers described in this study has not been reported previously. Earlier investigations on AFP micro-

heterogeneity revealed the presence of a maximum of six isoelectric variants during normal fetal development which focused over a p*I* range of 4.8–5.2 [20]. It is notable in this regard that the AFP subspecies defined in this study extended over a range of isoelectric points very similar to that described by Gustine and Zimmerman [20]. However, two of the FPLC-defined isomers identified here, AFP-4 and AFP-5, were found to have identical p*I* values, and this close isoelectric similarity may have hampered previous efforts to identify all of the native molecular variants of AFP.

The variants of mouse AFP have been shown to undergo reproducible changes in their concentrations with ontogeny [9,10,21]. These precisely regulated developmental shifts in the numbers and ratios of AFP isomers may be consistent with an immunoregulatory function for AFP operative at certain critical stages of gestation. Previous investigations have ascribed this potential physiological activity to the broad population of native AFP molecules (reviewed in refs. 5 and 22). We have recently determined that only one of the seven mouse AFP molecular variants described in this report is able to exert potent immunosuppressive activity in vitro [15]. Further studies to characterize this isomer are currently in progress using both biophysical and molecular genetic approaches [23].

The detection of human AFP in the adult is a common diagnostic measure for many diseases including fetal distress syndromes [4], acute and chronic liver diseases [24,25] and germ cell tumors and gastrointestinal neoplasm [2]. It is noteworthy that the detection of differences in the numbers and ratios of individual isomers in relation to certain diseases has been applied for presumptive diagnoses [26]. Based on these findings, it is interesting to speculate that the efficient analytical separation protocol of mouse AFP isomers described here may be extended to human AFP in the classification of malignant versus non-malignant diseases associated with aberrant re-synthesis of AFP.

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