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ESTRADIOL AFFINITY CHROMATOGRAPHY

APPLICATION TO PURIFICATION OF MURINE ALPHA-FETOPROTEIN

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SUMMARY

A one-step batch procedure is described for purification of murine alpha-fetoprotein (AFP) by estradiol affinity chromatography. Various ratios of carbodiimide (C), diaminononame (D) and estradiol hemisuccinate (E) were tested to determine optimal conditions for AFP purification. Although yields of AFP ranged from 15 to 44% depending on the reagent ratio employed, AFP isolates free of other protein contaminants were achieved at C:D:E ratios of 10:10:1 with a 29% yield. Both estrone and estradiol proved efficient as elution agents to free AFP bound to the estradiol–Sepharose beads, but higher yields were produced with estrone. After isolation the estrogen-eluted AFP preparations were analyzed by (1) estradiol-binding assays, (2) third-party radiocoprecipitation, (3) inhibition of radioimmunoassay for estrone and estradiol and (4) exchange of unlabeled for radiolabeled estradiol. These results indicated that the steroid remained attached to the eluted AFP molecule.

INTRODUCTION

A major difficulty in the purification of alpha-fetoprotein (AFP) has been its separation from albumin due to similarities in size, charge and isoelectric point. Only partial success has been achieved by combining such techniques as gel filtration, ion-exchange, isofocusing, and lectin and antibody affinity chromatography[1,2]. Until recently only cibacron blue–agarose chromatography had shown promise as a possible method for separating albumin from AFP in mammalian biological fluids[3]. However, since mouse and rat AFP have been shown to possess high binding affinity for estrogens[4,5], Uriel *et al.*[6] and Hassoux *et al.*[7] have used these properties to develop an estradiol affinity chromatographic technique for the purification of rodent AFP. While attempting to apply their methods, we have devised a one-step batch procedure for isolating murine AFP devoid of albumin. Our results also show a previously unreported property of the AFP isolated by estradiol-affinity chromatography.

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MATERIALS AND METHODS

Amniotic fluid

Amniotic fluid was drawn from Nya:NYLAR mice (15 to 18 days pregnant) with a tuberculin syringe fitted with a 26-gauge needle. The mouse amniotic fluid (MAF) was pooled, centrifuged and stored at —20°C.

Immunologic procedures

An AFP standard prepared as previously described for hepatoma fluids[8] was used to quantitate AFP and to provide antigen for immunization. AFP content was quantitated by the radial immunodiffusion method of Mancini *et al.*[9]. The production of rabbit antiserum to AFP, either from amniotic fluid or hepatoma derived, has been described[10,11]. Double diffusion in agar was performed by the method of Ouchterlony[12] and immunoelectrophoresis by the method of Scheidigger[13]. The minimum detection level of these agar procedures is 10 μg/ml. Protein was determined by the folin–phenol method of Lowry *et al.*[14]. Rabbit anti-mouse transferrin, γ-globulin, and albumin were purchased from Cappel Laboratories (Downington, PA, U.S.A.).

Gel electrophoresis

Analytical polyacrylamide disc gel electrophoresis (PAGE) was performed at 4°C with constant current (2 mA/tube) for 4 h or until the dye front was 1 cm from the end of the gel. The samples had been layered onto gel columns of 4, 6 and 8% acrylamide by the procedure of Joshi and Ebert[15]. The buffer was 0.15 *M* glycine, 0.02 *M* trizma base, at pH 8.6, and the gel stain was amido black. For maximal analytical sensitivity the gel tubes were loaded with 200 μl of the concentrated test samples to detect impurities.

Biochemicals

Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden); diaminononane (D) from Aldrich (Milwaukee, WI, U.S.A.); [2,4,6,7N-^{3}H]17β-estradiol (100 μCi/mmol) and [2,4,6,7N-^{3}H]17β-estrone (85 μCi/mmol) from Amersham-Searle (Chicago, IL, U.S.A.); 17β-estradiol-monohemiscuccinate (E) and unlabeled estrone (E_1) and estradiol (E_2) from Sigma (St. Louis, MO, U.S.A.); and 1-ethyl-3-(3-diethylaminepropyl)carbodiimide (C) from Ott Chemicals (Muskegon, MI, U.S.A.).

Binding of estradiol to Sepharose

E_2 was bound to Sepharose beads by a procedure modified from Arnon *et al.*[16]. Cyanogen–bromide-activated Sepharose 4B (10 g), swollen in 1.0 m*M* HCl (pH 3.0), was prehydrolyzed in 0.1 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.3) for 2 h. After centrifugation beads were added to the 9-carbon spacer (100 mg D) at a concentration of 2 mg of spacer/ml of basic buffer (pH 8.3; see above) and incubated at 4°C for 16 h. The beads were washed with alternating cycles of basic buffer and 0.1 *M* sodium acetate–1.0 *M* NaCl (pH 4.0). Using the trinitrobenesulfonic acid determination for amines, it was calculated that 72% of the spacer was attached to the beads.

The D–Sepharose complex was then linked to an E_2 ligand via the carboxylic group. C (500 mg), dissolved in 30 ml of distilled water (pH 4.5) and 30 ml of dioxane, was mixed with 30 mg of E in 30 ml of dioxane–water (pH 4.5) for 30 min at ambient

temperatures. The D–Sepharose beads were washed once with 50% dioxane–water (pH 4.5). After centrifugation the supernatant was removed from the packed beads, and 30 ml of E_2–C was added to 10 ml of packed beads. The reagents were mixed overnight at 4°C with gentle agitation. After centrifugation the supernatant was removed, and the packed beads were washed exhaustively with 50% dioxane–water until monitoring at 280 nm showed zero absorbance. The amount of estradiol bound to D–Sepharose, determined by monitoring 280 nm absorbance of the solution before and after the binding procedure, was calculated to be 30% (1.0 mg E/1.0 g dry sepharose). The E_2–Sepharose beads were washed and stored in phosphate-buffered saline (PBS; pH 7.0).

AFP isolation on E_2-affinity beads

Equal volumes of E_2–Sepharose beads in PBS and Millipore-filtered MAF were mixed and gently agitated overnight at 4°C. After centrifugation the supernatant was removed, and the packed beads were washed with PBS until a zero absorbance was attained. To remove the bound protein (AFP) from the beads, 6 ml of saturated E_2 (or E_1) in 15% dioxane–PBS was added, and the mixture was gently agitated for 2 h at ambient temperatures. The entire mixture was then transferred to a syringe, Millipore-filtered into dialysis tubing, dialyzed exhaustively against PBS to remove all traces of dioxane, and then analyzed for AFP. The maximal binding of AFP to the beads prepared in the study ranged from 500 to 1000 μg per ml of swollen E_2-linked Sepharose beads. We used the E_2–Sepharose beads only once and could not regenerate them.

Estrogen-binding assay

Estrogen binding of MAF and purified AFP was examined by a dextran-coated charcoal procedure similar to that used in previous studies of steroid hormone binding[17,18]. For each assay 0.05 ml of MAF or purified AFP was added to an appropriate number of ice-chilled 12 × 75 mm disposable glass culture tubes, followed by 0.40 ml of TED buffer (10 m*M* Tris, 0.5 m*M* EDTA, 0.5 m*M* DTT, pH 7.4) and 0.05 ml of $[^3H]E_2$ (80 nmol) in the same buffer. In inhibitor studies 0.05 ml of unlabeled E_2 (10 μmol) was substituted for buffer, and the mixture was incubated for 30 min before the $[^3H]E_2$ incubation. After incubation for 1.0 h in an ice bath 1.0 ml of dextran-coated charcoal particles (0.5%) suspended in TED buffer was added to the incubation mixture to adsorb free (unbound) $[^3H]E_{23}$ After 15 min the charcoal was sedimented at 1800 *g* for 10 min in a refrigerated centrifuge. The supernatant was decanted into a Scintiverse cocktail (Fisher Scientific, Pittsburgh, PA, U.S.A.), and radioactivity was determined in three 2-min counting cycles in a liquid scintillation counter (40% efficiency). In previous studies Scatchard analysis of data on binding of AFP to $[^3H]E_2$ showed an association constant of $0.16 \cdot 10^9$ M^{-1} and a binding-site concentration of $2.2 \cdot 10^5$ fmol/ml MAF[18]. E_2 bound to AFP was immunoprecipitated using a double-antibody radioimmunoassay called third-party radiocoprecipitation as previously described[18].

RESULTS

Effect of reagent ratios on chromatographic yield

To determine the most efficient yields from E_2 affinity chromatography, various ratios of C, D and E ligand were tested in four sepharose experiments (Table I). Maximal yields resulted when 500 mg of C were combined with 100 mg of D and 30 mg of E_2 ligand (Table I, Seph 3) in a ratio of 16.6:3.3:1. The quantity of 9-carbon side-chain reagent (D) was kept constant in three of the four experiments. Moreover, yields of 20–30% could be consistently obtained when reagent ratios were employed at minimal concentrations (Table I, Seph 1). Excessively high amounts of reagents (Seph 4) proved inefficient due to increased binding of the beads for all protein moieties.

TABLE I

ESTRADIOL AFFINITY CHROMATOGRAPHIC YIELDS OF AFP FOR VARIOUS AMOUNTS AND RATIOS OF CARBODIIMIDE (C), DIAMINONONANE (D) AND ESTRADIOL HEMISUCCINATE (E)

Experiment	*C:D:E reagents*		*MAF–AFP total protein (mg/ml)*	*AFP Isolated (mg/ml)*	*Yield (%)*
	Amounts (mg)	*Ratio*			
Seph 1	100:100:10	10:10:1	0.974	0.300	29.00
Seph 2	200:100:20	10:5:1	0.974	0.150	15.40
Seph 3	500:100:30	16.6:3.3:1	1.120	0.500	44.60
Seph 4	2000:200:150	13:1.3:1	1.462	0.030	2.05

The impurities in the AFP isolates from the four Sepharose experiments are listed in Table II. The AFP isolated with Seph 1 (29% yield) was devoid of all impurities, including albumin. Seph 3 gave a higher AFP yield (44%), but the isolate contained traces of other protein moieties, mainly albumin. Subsequent immunization of rabbits with AFP isolated by Seph 1 resulted in the production of monospecific antisera.

TABLE II

ISOLATION PRODUCTS FROM ELUTION WITH ESTRONE (E_1) OR ESTRADIOL (E_2)

Agar techniques = immunodiffusion, immunoelectrophoresis; PAGE = polyacrylamide gel electrophoresis; ND = Not done.

Experiment	*C:D:E ratio*	*Elution steroid*	*Albumin*		*AFP*		*Other**	
			Agar	*PAGE*	*Agar*	*PAGE*	*Agar*	*PAGE*
Seph 1	10:10:1	E_1	—	—	+	+	—	—
		E_2	ND	ND	ND	ND	ND	ND
Seph 2	10:5:1	E_1	—	—	+	+	+	+
		E_2	—	—	+	+	—	—
Seph 3	16.6:3.3:1	E_1	+	+	+	+	—	—
		E_2	+	+	+	+	—	—
Seph 4	13:1.3:1	E_1	—	+	+	+	—	—
		E_2	—	+	+	+	—	—

* Serum or amniotic fluid component other than albumin or AFP.

Effect of elution steroid on chromatographic yield

Six pools of MAF with various C:D:E ratios were eluted with either E_1 or E_2. Saturated solutions of E_1 proved mostly superior or equal to E_2 (Table III). Yields of AFP with E_2 were predominantly 15–30% of the starting material depending on the C:D:E ratio. Yields with E_1 ranged generally from 15 to 45%.

TABLE III

ESTRADIOL AFFINITY CHROMATOGRAPHIC YIELDS WITH EITHER E_1 OR E_2 AS THE ELUTION STEROID

Different pools of MAF were used as starting material.

MAF pool	*C:D:E ratios*	*Elution steroid*	*MAF–AFP total protein (mg/ml)*	*AFP isolated (mg/ml)*	*Yield (%)*
MAF-A	10:5:1	E_1	0.974	0.150	15.4
		E_2	0.974	0.150	15.4
MAF-B	13:1.3:1	E_1	1.462	0.030	2.1
		E_2	1.462	0.025	1.7
MAF-C	10:10:1	E_1	0.701	0.160	22.8
		E_2	0.701	0.130	18.5
MAF-D	10:10:1	E_1	0.842	0.145	17.2
		E_2	0.842	0.159	18.8
MAF-E	16.6:3.3:1	E_1	1.34	0.475	35.4
		E_2	0.736	0.225	30.5
MAF-F	16.6:3.3:1	E_1	1.117	0.500	44.7
		E_2	1.130	0.350	30.9

Chromatographic yields from individual MAF samples

When various individual samples of MAF were chromatographed with C:D:E ratios of 10:10:1 (from Table I, Seph 1) followed by E_1 elution, the yields were strinkingly similar, ranging from 19.2 to 24.8% (Table IV). AFP concentrations of approximately 0.2 mg/ml were consistently obtained with an average yield of 21%.

TABLE IV

ESTRADIOL AFFINITY CHROMATOGRAPHIC YIELDS FOR INDIVIDUAL MAF SAMPLES (C:D:E RATIO, 10:10:1)

MAF sample	*Total protein (mg/ml)*		*AFP isolated (mg/ml)*	*Yield (%)*
	MAF	*AFP*		
MAF 1	3.245	1.070	0.220	20.4
MAF 2	1.510	0.498	0.125	24.8
MAF 3	3.346	1.115	0.235	21.0
MAF 4	3.050	1.006	0.195	19.2
MAF 5	2.210	0.736	0.150	20.4

E_2 binding assay of E_1-eluted AFP

The MAF and the E_1-eluted AFP were subjected to estrogen-binding assays, using the dextran-coated charcoal procedure. After adjustments for total AFP protein, the specific bound counts (cpm) in native MAF were 20 to 45% higher than

those in the E_1-eluted AFP isolates (Table V, experiments 1–5). Since the E_1-eluted AFP was partially inhibited from binding to the [^{3}H]E_2, some steroid apparently remained bound to the eluted AFP molecule.

In an attempt to mimic the experimental conditions which produced E_1-eluted AFP, native MAF was dialyzed against PBS, E_1 and E_2 (Table V). Dialysis against either E_1 or E_2 resulted in 30–45% less [^{3}H]E_2 binding than dialysis against PBS.

TABLE V

SPECIFIC E_2 BINDING OF NATIVE MAF, AFP PURIFIED BY E_2 AFFINITY CHROMATOGRAPHY, AND MAF DIALYZED AGAINST PBS OR STEROIDS

Native MAF was adjusted to AFP total protein content for comparison to radioactive counts obtained with E_1-eluted AFP. Specific [^{3}H]E_2 bound to native or PBS-dialyzed MAF was considered to be 100%.

Experiment number	*Dialyzed against*	*Specific [^{3}H]E_2 bound*			
		Native MAF		*E_1-isolated AFP*	
		*cpm**	%	*cpm**	%
1	None	6301	100	3850	62
2	None	5593	100	3078	55
3	None	7258	100	5760	80
4	None	6610	100	4750	70
5	None	6541	100	4600	70
6	PBS	36,663	100	—	—
7	E_1	19,516	53	—	—
8	E_2	24,859	67	—	—

* Specific count (cpm) = total bound cpm minus cpm inhibitable by estrone.

Tests on estrogen-eluted AFP

The results to this point suggested that estrogen becomes attached to the AFP molecule during elution with saturated steroid solutions. To test this hypothesis, 50 μl of [^{3}H]E_2 (120 nmol) was added to the saturated E_2 solution just prior to sepharose E_2–MAF exposure. After elution and subsequent washing procedures, the eluted AFP was subjected to third-party radiocoprecipitation[18] using antisera monospecific to murine AFP, and 1160 cpm of radioactivity were detected. Since no other [^{3}H]E_2 was added, this radioactivity had to originate from solutions during the elution procedures. In non-specific binding studies using non-immune rabbit serum in lieu of anti-AFP serum, 197 cpm was precipitated.

Radioimmunoassays (RIAs) specific for either E_1 or E_2 were used to further investigate whether the steroid remained bound to the eluted AFP. Native MAF and steroid-eluted AFP were used as inhibitors in these assays. While native MAF did not inhibit the formation of complexes, E_2-eluted AFP inhibited 55% of the anti-E_2 counts (576 *versus* 1286 cpm), and E_1-eluted AFP inhibited 22% of the anti-E_1 counts (992 *versus* 1280 cpm). To further demonstrate the presence of steroid on AFP derived from E_2–Sepharose beads, an exchange of unlabeled for radiolabeled steroid was performed (Fig. 1). When E_2-eluted AFP was incubated at 37°C in the presence of [^{3}H]E_2 (80 nmol), after a slight initial decrease in binding, an increase in the counts occurred from 4 to 8 h incubation. Native MAF previously unexposed to estrogens

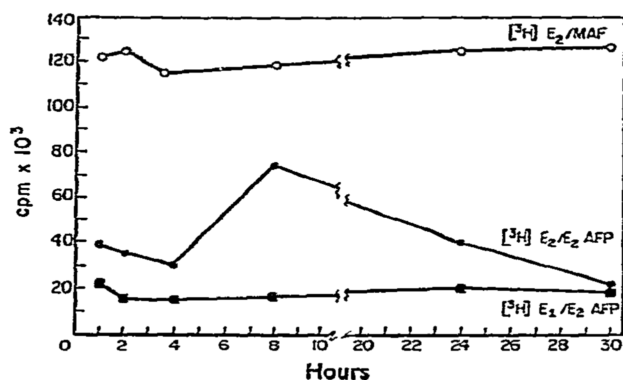

Fig. 1. Exchange of unlabeled for radiolabeled estrogen ([^{3}H]E_1 or [^{3}H]E_2) on native murine amniotic fluid (MAF), estradiol-eluted alpha-fetoprotein (E_1AFP) and estrone-eluted alpha-fetoprotein (E_2AFP). Samples were incubated at 37°C in a water bath with [^{3}H]E_2 or [^{3}H]E_1 for 1, 2, 4, 8, 24 or 30 h, then with dextran-coated charcoal, and assayed as described in the text.

showed a similar decrease at 2 to 3.5 h incubation, but remained essentially stable for 30 h thereafter.

Attempts to exchange unlabeled E_2 for radiolabeled E_1 with E_2-eluted AFP proved unsuccessful. After an initial decrease, the counts remained unchanged for the subsequent 28 h of incubation.

DISCUSSION

Our demonstration of a one-step batch procedure for purification of mouse AFP by E_2-affinity chromatography differs in several ways from the work by Arnon *et al.*[16]. (a) Various combinations of reagents were tested to determine the best C:D:E ratios for optimal AFP yields; (b) E_1 and E_2 were used to elute AFP bound to the sepharose beads, AFP yields proved E_1 to be the better eluting agent; (c) when the eluent (saturated E_2) was spiked with [^{3}H]E_2-bound AFP was precipitated; (d) for our one-step procedure no columns were necessary; (e) AFP was eluted at room temperature rather than 4°C; (f) the entire mixture, including the Sepharose beads was transferred to a syringe and Millipore-filtered directly into dialysis tubing. This step removed all protein attached to the beads and minimized protein loss during transfer.

Our yields were compatible with those in previous studies with E_2-linked Sepharose beads. Uriel *et al.*[6] reported yields of 42% for mouse AFP and 25% for rat AFP. However, these investigators achieved higher binding capacities (70 g/0.3 ml of beads) than we did (40 g/1.0 ml beads); differential E_2 binding in various rodent species may exist, and/or procedural differences in Sepharose ligand bead preparation may account for this disparity. Later studies by Hassoux *et al.*[7] found absorbent binding capacities of 0.5–0.6 mg/g of swollen beads, but their procedure follows that of Cuatrecases *et al.*[19] in using AH-Sepharose 4B and so is not directly comparable to ours.

We have demonstrated that some elution steroid remains bound to the AFP molecule after estrogen chromatography. The AFP isolated with E_2–Sepharose beads

consistently bound less [^{3}H]E_2 than equivalent amounts of AFP in MAF. AFP was eluted with saturated steroid solutions spiked with [^{3}H]E_2 and assayed for specific immunoprecipitation of the isolated AFP bound to the radiolabeled steroid. In these precipitates, free of exogenous [^{3}H]E_2, radiolabeled E_2 was present. Further studies indicated that AFP isolated by E_2 chromatography could inhibit complexing in E_2 RIAs; the AFP prepared by our procedure could neutralize up to 55% of the antibodies directed against either E_1 or E_2. If estrogen remained attached to the eluted AFP, an exchange of unlabeled for radiolabeled estrogen should be demonstrable. Such an exchange occurred after 4–8 h incubation at 37°C. Thus the AFP molecule did not readily exchange bound estrogen. Finally cross-exchange experiments showed that unlabeled E_1 was not readily exchangeable with labeled E_2.

Nevertheless, elution steroid remains demonstrably bound to at least some of the eluted AFP. Further support for this observation is provided by the findings of Keller *et al.*[20], who compared AFP preparations isolated by various E_2 affinity procedures. In their cell-culture studies involving immunoregulation, the various AFP isolates appeared to contain E_2 bound to the AFP molecule. In contrast, Hassoux *et al.*[7] found that AFP isolated by E_2 affinity chromatography has an affinity constant of 0.5 to $0.6 \cdot 10^8$ M^{-1} and a molecular combining ratio of less than unity (0.30–0.64). No mention was made of elution steroid attachment or suppression of [^{3}H]E_2 binding to the AFP molecule. However, their exchange procedure involved equilibrium dialysis at 4°C for 24 h, whereas our non-equilibrium dextran-coated charcoal procedure took place at 37°C for 30 h with the exchange at 4–8 h. It is conceivable that unlabeled E_2 was exchanged for [^{3}H]E_2 inadvertently during the equilibrium dialysis, as its incubation period would provide sufficient time for completion of steroidal exchange.

E_2-linked Sepharose chromatography is a valuable tool for rapid one-step purification of AFP with yields of 20–40% of the starting material. Our yields of rat AFP (15–20%) are lower than mouse AFP, in agreement with the studies of Uriel *et al.*[6]. We have also used this procedure to purify AFP from hepatoma-bearing mice, which have high concentrations of serum AFP[21]. The yields to date have been low (10–15%), possibly due to the heavy protein concentrations of the tumor sera, which may compete for binding sites on the Sepharose beads. In addition, we have performed E_2–Sepharose chromatography of human AFP to provide an injection vehicle for animal immunization, as described by Arnon *et al.*[16]. The immunizations produce antisera suitable for use in immunologic assays, provided the antisera are first absorbed with normal adult human serum.

Finally, the E_2 affinity beads can be used to remove specific anti-AFP immunoglobulin G from total immunoglobulin G. After AFP is attached to the beads and prior to elution with E_1, rabbit anti-AFP immunoglobulin G can be applied to the beads and incubated. The mixture is centrifuged to remove non-binding protein components. The remaining solution decanted from the beads contains immunoglobulin G devoid of anti-AFP antibodies.

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