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Note

Pitfalls in the isolation of α-fetoprotein by solid-phase immunoadsorption

Loss of fatty acids

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α-Fetoprotein (AFP) is a major component of serum in developing vertebrates[1]. Although the physiological function of this protein is still unclear, it is known that human AFP can bind long-chaing fatty acids[2] and particularly the polyunsaturated fatty acids arachidonic (20:4, n-6) and docosahexaenoic (22:6, n-3). This is, so far, the only property common to AFP from all species studied[3,4], and seems to be related to its physiological rôle. Indeed, it has been proposed that AFP may be the physiological carrier of polyunsaturated fatty acids such as arachidonic and docosahexaenoic, which are essential for the normal development of several animal tissues and especially the nervous system[4].

A method widely used for the isolation of AFP is the direct immunoadsorption of this protein with insolubilized antibodies and its elution at low pH[5,6]. However, as shown in this note, such a general procedure alters the amounts and the relative proportion of the different fatty acids bound to the protein. Consequently, it should be avoided in studies of the interactions between fatty acids and AFP under physiological conditions.

EXPERIMENTAL

AFP purification

Rat fetuses (gestation for 17–19 days) were homogenized with 0.01 *M* potassium phosphate–0.15 *M* sodium chloride, pH 7.4 (2:1, v/w). The homogenate was centrifuged (20000 *g*, 30 min, 4°C) and the supernatant was dialyzed against 0.025 *M* potassium phosphate–0.05 *M* sodium chloride, pH 6.2. The sample was applied to a column (30 × 3 cm) of DEAE-Sephadex A-50 equilibrated in the same buffer. Proteins were eluted with a linear gradient of sodium chloride, between 0.05 and 0.4 *M*, in the same phosphate buffer. Fractions were analyzed for their AFP content by immunodiffusion, pooled and submitted to forced dialysis against 0.01 *M* potassium

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phosphate, pH 7.0, with a Diaflo PM-10 membrane. The resulting fraction was chromatographed using the same pH 7.0 buffer, in a column of Cibacron Blue–Sepharose (25 × 2 cm), to achieve separation of AFP from albumin and other minor contaminants. Pig AFP was purified from fetal pig serum by a similar procedure which includes, as described in detail elsewhere[4], an additional chromatographic step on a trypsin-Sepharose adsorbent, to eliminate the α_1-antitrypsin.

The purity (95–98%) of AFP obtained was determined by polyacrylamide–agarose electrophoresis[7], followed by scanning of the gel in a densitometer (Vernon, France).

Affinity immunoadsorption of AFP

Antibodies to rat AFP were isolated from a specific rabbit antiserum and then insolubilized on cyanogen bromide-activated Sepharose as previously described[8]. Similar procedures were used to prepare specific immunoadsorbents for pig AFP. Purified rat or pig AFP (4 mg) was applied to columns (7 × 1 cm) of specific immunoadsorbent equilibrated in 0.05 *M* potassium phosphate, pH 7.4 containing 0.5 *M* sodium chloride. This amount of adsorbent was suitable for binding all the applied protein. The bound protein was quickly eluted at 4°C with 0.05 *M* glycine hydrochloride–0.5 *M* sodium chloride buffer pH 2.8. Fractions were collected in tubes containing a volume of 1 *M* Tris–HCl, pH 8.0 buffer, sufficient to neutralize the acidic effluent. The fractions were tested by immunodiffusion and those containing AFP were pooled and concentrated by ultrafiltration in Amicon cones (Centriflo CF-25).

Fatty acids analysis

Fatty acids bound to AFP were extracted from 3–4 mg of the purified protein with isopropanol–*n*-heptane–0.5 *M* sulphuric acid (4:1:1, v/v), according to the method of Parmelee *et al.*[2]. *n*-Heptadecanoic acid (10 μg) and 5 μg of BHT (2,6-di-*tert.*-butyl-4-methylphenol) were added as internal standard and antioxidant, respectively. The fatty acids were esterified under nitrogen with 0.5% sulphuric acid in anhydrous methanol for 2 h at 80°C. Fatty acid methyl esters were analyzed by gas–liquid chromatography as described previously[4].

RESULTS AND DISCUSSION

Table I shows the pattern of the fatty acids bound to rat or pig AFP before and after interaction of the purified proteins with immunoadsorbents. The initial samples of rat and pig AFP contained 1.63 and 2.60 mol of fatty acid per mol of protein, respectively. Among these fatty acids, arachidonic (20:4, n-6) and docosahexaenoic (22:6, n-3) acids together accounted for 0.5 mol per mol of rat AFP and about 1 mol per mol of pig AFP. After immunoadsorption and acidic elution, both AFP species lost about 40% of the initial fatty acids bound. However, this loss varied with the type of fatty acid considered. Whereas the relative loss was between 11 and 20% for saturated and monounsaturated fatty acids, it was 50–60% for arachidonic and 75–80% for docosahexaenoic acids, respectively. It seems that the acidic elution has a greater effect the longer and more unsaturated are the fatty acids.

In recent years it has been recognized that AFP binds long-chain fatty acids

TABLE I

CHANGES IN FATTY ACID COMPOSITION AFTER IMMUNOADSORPTION OF AFP

Fatty acids bound to AFP: (a) native protein; (b) after immunoadsorption and acidic elution. Data are expressed in mol of fatty acid per mol of protein. Column (c) indicates the difference between (a) and (b) expressed as percentage of (a). PUFA = polyunsaturated fatty acids.

Fatty acid	*Rat AFP*			*Pig AFP*		
	Control (a)	*Immunoad-sorbed (b)*	*% Loss (c)*	*Control (a)*	*Immunoad-sorbed (b)*	*% Loss (c)*
16:0	0.31	0.25	19	0.35	0.31	11
18:0	0.10	0.08	20	0.18	0.16	11
18:1, n-9	0.21	0.17	19	0.66	0.58	12
18:2, n-6	0.15	0.11	27	0.18	0.16	11
20:4, n-6	0.17	0.08	53	0.55	0.22	60
22:6, n-3	0.33	0.08	76	0.44	0.08	82
Other PUFA	0.21	0.10	52	0.14	0.05	64
Other fatty acids	0.15	0.10	33	0.10	0.06	40
Total	1.63	0.97	40	2.60	1.62	38

as does albumin, but unlike the latter, AFP contains important amounts of arachidonic and docosahexaenoic acids bound physiologically. This suggests that AFP posseses a higher affinity for polyunsaturated fatty acids than for saturated an monounsaturated ones. However, the experimental measurements are not conclusive in this respect[9,10]. The present results indicate that the interaction of polyunsaturated fatty acids with AFP is more susceptible to environmental changes than the corresponding interaction with saturated and monounsaturated acids. Thus, care should be taken in experiments in which it is important to preserve the pattern of the fatty acids bound to AFP, particularly during isolation of this protein. As we have illustrated here, immunoadsorption and acidic elution of AFP can seriously affect the pattern of the fatty acids bound to this protein. This could explain recently published results in which AFP from man and rat contained relatively low levels of polyunsaturated fatty acids[11,12] as compared with earlier results[2,13]. On the other hand, the relative proportion of saturated/unsaturated fatty acids bound to AFP may be essential in the physiological rôle of this protein. Recent results have shown that both saturated and unsaturated free fatty acids are needed for the blastic transformation of lymphocytes[14] and the uptake of AFP by these cells has also been reported[15]. Therefore, the preservation of the original fatty acid composition of AFP is probably a essential requirement in studies on the immunoregulatory rôle of this protein. This could be in part the reason for the contradictory results found in earlier studies[11,16].

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