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SEPARATION OF FATTY ACID BINDING PROTEIN BY HIGH-PERFORMANCE MIXED-MODE CHROMATOGRAPHY

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SUMMARY

Fatty acid binding protein (FABP) (14 kDa), can regulate the levels of tissue free fatty acids by binding them with high affinity. Since free fatty acids are known to accumulate in the ischemic myocardium, it is likely that FABP has a significant role in regulating their concentration in ischemic heart. FABP has recently been purified from other proteins, but the method requires several hours and special techniques. In this report, we describe a rapid high-performance liquid chromatographic method for separating and isolating the FABP from myocardial tissue biopsies.

About 25–50 μg of rat heart cytosol was incubated with 2 nmol of the potassium salt of [9,10-^{3}H]oleate (25 000 cpm) for 10 min at 37°C. This was then injected onto a Bio-Rad (Richmond, CA, U.S.A.) TSK-125 column. The sample was run using a low-salt isocratic mobile phase containing 10 m*M* potassium phosphate buffer (pH 7) and 1 m*M* dithiothreitol, and at a flow-rate of 0.8 ml/min. The heart cytosol, when incubated with isotopic oleate, was resolved into two radioactive peaks, one eluting in the area of serum albumin (retention time 9.6 min) and the other corresponding to a retention time of 12.9 min. The sodium dodecyl sulfate polyacrylamide gel electrophoretic profile of the later peak revealed a major protein band of *ca.* 14 kDa. Rat heart FABP purified by gel filtration and ion-exchange chromatography coeluted with the second radioactive peak. When increasing concentrations of cytosol were incubated with radioactive oleate and analyzed by high-performance liquid chromatography, a linear increase in the radioactive oleate-bound FABP peak (retention time 12.9 min) was obtained. These results suggest that FABP was separated from other proteins using the TSK-125 column, and hence this method can be used for a rapid recovery of FABP from biological tissues.

INTRODUCTION

Fatty acid binding protein (FABP), an intracellular non-enzymatic protein, has recently been found to possess several unique properties in controlling the levels of tissue free fatty acids (FFAs) and their thioesters by binding them with high affinity[1,2]. FABP has recently been isolated and characterized from several tissues, including heart, liver, kidney, adipose tissue, and intestinal mucosa, and found to possess a molecular weight of *ca.* 14 kDa[1,2].

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The purification of FABP requires skill and is time-consuming. The most common method of its purification includes G-75 gel-filtration followed by DEAE anion-exchange chromatography[3–5]. When eluted by potassium chloride gradient on DEAE chromatography, the FABP occasionally needs another gel-filtration step. The final purity is judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In this report, we describe a rapid method for separating the FABP from myocardial tissue using high-performance liquid chromatography (HPLC). The Bio-Rad TSK-125 column used in this study behaved as both gel filtration column and ion exchanger under our experimental conditions. This method can thus be used as a rapid single-step recovery of FABP from biological tissues such as heart.

EXPERIMENTAL

Materials

Fatty acid-free bovine serum albumin (BSA) and Sephadex G-75 were purchased from Pharmacia (Piscataway, NJ, U.S.A.). DE-52 was purchased from Whatman (Clifton, NJ, U.S.A.). Myoglobin standard was obtained from Bio-Rad (Richmond, CA, U.S.A.), and [9,10-^{3}H]oleate and Aquasol were from New England Nuclear (Boston, MA, U.S.A.). Dithiothreitol was obtained from Sigma (St. Louis, MO, U.S.A.), and Lipidex-1000 from Packard Instrument (Downers Grove, IL, U.S.A.). HPLC-grade water was obtained from a Millipore water system (Millipore, Bedford, MA, U.S.A.). Nylon-66 (0.45 μm) filters were purchased from Rainin Instrument (Woburn, MA, U.S.A.).

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used in this study. The system consisted of a WISP Model 710B injector, Model 720 system Controller, Model 730 data module, Model 490 programmable multi-wavelength UV detector, and Model 6000A pump. A Bio-Rad (Richmond, CA, U.S.A.) Bio-Sil TSK-125 HPLC column (30 cm × 7.5 mm I.D.) equipped with a Bio-Sil TSK guard column (7.5 cm × 7.5 mm I.D.) was used for the FABP separations.

Methods

Purification of rat heart cytosolic FABP. Isolation and purification of FABP from rat heart cytosol was performed according to the methods described by Fournier *et al.*[3] and Glatz *et al.*[6]. In short, hearts were obtained from pentobarbital (10 mg per rat intraperitoneal) anaesthetized male Sprague-Dawley rats weighing 300–325 g. Hearts were perfused with a buffer mixture containing 0.25 *M* sucrose, 10 m*M* potassium phosphate (pH 7.0), and 1 m*M* dithiothreitol (DTT). Hearts were then excised, blotted, minced, and homogenized in 3 ml of 10 m*M* potassium phosphate buffer containing 1 m*M* DTT, using a PTFE-glass Potter-Elvehjem tissue homogenizer (Kontes, Vineland, NJ, U.S.A.). The homogenates were centrifuged at 800 *g* for 15 min to remove nuclear fractions and cell debris, followed by centrifugation at 12 500 *g* for 20 min to remove mitochondria and 105 000 *g* for 60 min to settle the microsomes. The 105 000 *g* supernatant served as the source of FABP.

The FABP was then purified to homogeneity according to Ockner *et al.*[5] by

employing G-75 gel-filtration and DEAE anion-exchange chromatography. The FABP eluted with a potassium chloride gradient from the DEAE column was further purified by a second G-75 gel-filtration. The FABP fractions were collected, and SDS-PAGE was carried out under reduced and denaturing conditions using 4.0% stacking gel (pH 6.8) and 15% separating gel in SDS. The electrophoretic buffer contained 0.025 *M* Tris (pH 8.8), 0.192 *M* glycine, and 0.1% SDS. Gels of 15% were run at 10 mA/gel for 4–5 h, after which they were fixed and stained in a solution of 0.2% Coomassie brilliant blue in methanol–water–acetic acid (5:5:1). The relative mobility of purified FABP was compared with that of protein standards to determine molecular weight.

Preparation of heart cytosol for HPLC. The 105 000 *g* supernatant was used for HPLC. Cytosol (6–60 μg) was incubated with 2 nmol of the potassium salt of [9,10-^{3}H]oleate (*ca.* 25 000 cpm) for 10 min at 37°C, and a 25-μl volume was injected onto a TSK-125 column. A low-salt isocratic mobile phase was used, which contained 10 m*M* potassium phosphate buffer (pH 7) and 1 m*M* DTT. Using a flow-rate of 0.8 ml/min, the effluent was monitored at 280 nm, and 0.4-ml fractions were collected for scintillation counting.

Highly purified FABP standard, obtained as described above, as well as BSA and myoglobin (horse) standards, were also injected on the TSK-125 column and chromatographed separately under identical conditions. These peaks were compared with those obtained from chromatography of cytosol FABP.

Delipidation of purified FABP and assay of fatty acid binding. The purified FABP was subjected to delipidation using Lipidex 1000 as described by Glatz *et al.*[6]. In brief, 2 mg of FABP were loaded on a column of Lipidex (5 cm × 0.5 cm I.D.) and equilibrated with 100 m*M* Tris–HCl buffer (pH 7.4) at 37°C. The delipidated FABP emerged in the void volume. The oleate binding was determined essentially according to the procedure described by Offner *et al.*[7]. The reaction mixture contained increasing amounts of oleic acid (sodium salt), 24 μg of FABP, 1.5 m*M* DTT, and 100 m*M* Tris–HCl (pH 7.4) in a total volume of 0.35 ml. The binding was allowed to continue for 5 min at 37°C, after which the test-tubes were cooled in ice. The unbound fatty acid was removed by mixing with 200 μl of ice-cold Lipidex-1000 and buffer suspension (1:1, v/v) for 10 min at 0°C. The radioactivity remaining in the supernatant was determined to calculate the nmoles of oleate bound per nmole of FABP[7].

RESULTS

Separation and identification of FABP from other proteins

About 37.5 μg of rat heart cytosol was incubated with 3 nmol of [9,10-^{3}H]oleate and injected onto a TSK-125 column as described in *Methods*. The effluent, monitored at 280 nm, revealed three major peaks, as shown in Fig. 1. Purified FABP, BSA, and myoglobin standards were injected separately on the same column and run under identical conditions. The tracings of these peaks were overlaid on the rat heart cytosol chromatogram shown in Fig. 1. As observed in the figure, the ^{3}H-labeled heart cytosols are resolved into two radioactive peaks, one eluting in the area of the BSA standard (t_R = 9.6 min) and the other corresponding to the area of the purified FABP peak (t_R = 12.9 min).

The later effluents corresponding to FABP peak were collected and SDS-PAGE

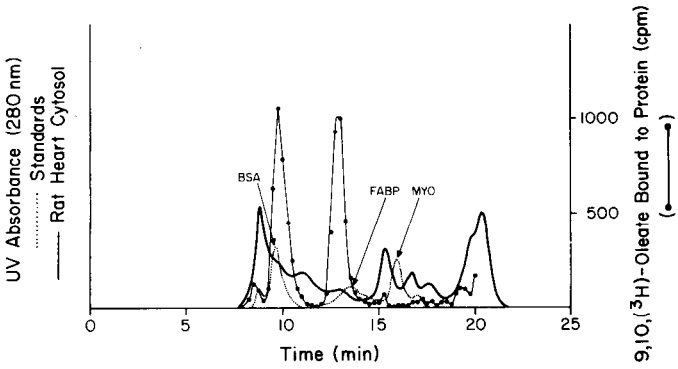


Fig. 1. HPLC chromatogram of rat heart cytosol and protein standards. Rat heart cytosol (37.5 μg) was prepared, incubated with 3 nmol of [9,10-^{3}H]oleate, and chromatographed as described in Experimental. A 25-μl volume of protein standards (BSA = bovine serum albumin, 10 μg; FABP = fatty acid binding protein, 5 μg; MYO = horse myoglobin, 6 μg) was run separately and the tracing overlaid on the rat heart cytosol chromatogram. Fractions were collected and counted for [9,10-^{3}H]oleate bound to rat heart cytosol as described in Experimental. UV absorbance of cytosol at 280 nm (———), UV absorbance of protein standards at 280 nm (– – –), and radioactivity bound to cytosol (●—●) were plotted against time (min).

was run on this sample. The SDS-PAGE profile revealed a major band of *ca.* 14 kDa molecular weight (Fig. 2), which corresponds to the molecular weight of rat heart FABP reported in the literature[1–7].

Binding of purified FABP with fatty acid

In order to confirm whether the peak identified as FABP in Fig. 1 is indeed FABP capable of binding FFAs, we assayed for oleate binding on the delipidated sample, as described in *Methods*. As shown in Fig. 3, when the sodium oleate concentration was increased, increasing amounts of oleate were bound to FABP and the saturation was reached at a value of 2 nmol of oleate bound per nmol of FABP. This binding is similar to the value recently reported in the literature[4,7]. The affinity of binding for [^{14}C]oleic acid with FABP was analyzed from a Scatchard plot. From the Scatchard analysis (Fig. 4), the maximum binding capacity for oleic acid was determined as 2.0 nmol/n mol FABP. The apparent dissociation constant (K_d) value was 0.48 μM for oleic acid. This K_d value compares favorably with those reported in the literature[6].

We also challenged the cytosol with radioactive oleate by incubating various concentrations of the cytosol (6 μg, 12.5 μg, 25 μg, 37.5 μg, 50 μg, and 60 μg) with a constant amount (2 nmol) of [^{3}H]oleate, followed by HPLC. The radioactivity found in the FABP peak was plotted against the concentration of cytosol. As shown in Fig. 5, we found a linear response in the radioactive oleate-bound FABP peak (t_R = 12.9 min) when increasing amounts of cytosolic protein (6–60 μg) were used.

DISCUSSION

The abundant presence of FABP in the cytosolic compartments of many biological tissues, including heart, kidney, liver, lung, brain, intestine, skeletal muscle,

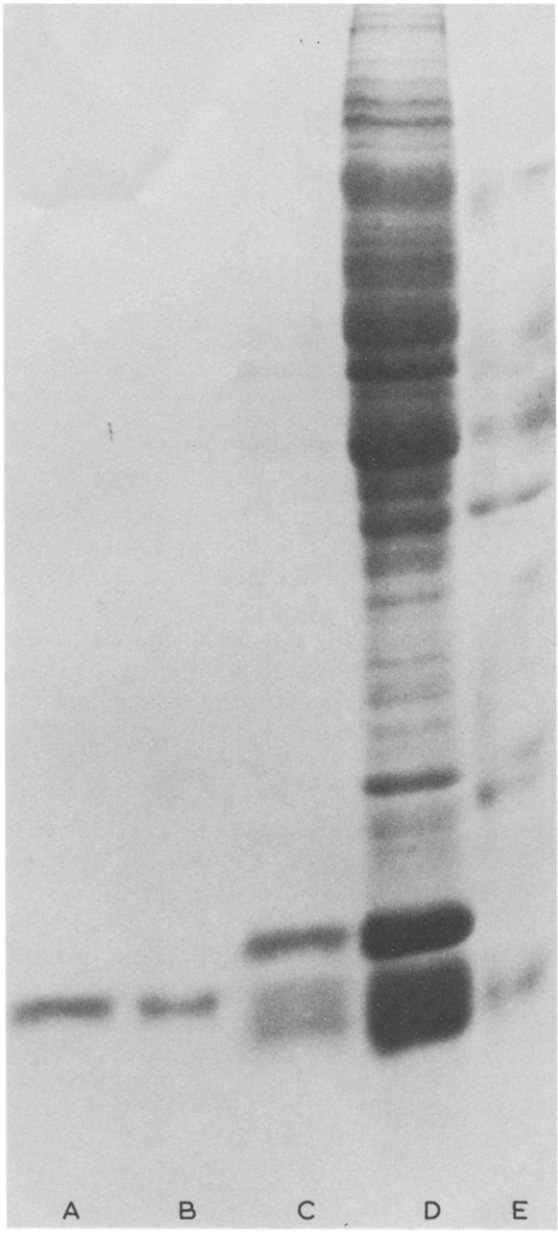


Fig. 2. SDS-PAGE profiles. (A) Effluent from TSK-125 column corresponding to FABP peak; (B) FABP standard purified by gel-filtration and anion-exchange chromatography; (C) G-75 pooled during purification of FABP (purified protein is given in B); (D) rat cytosolic proteins (this protein was then subjected to G-75 gel-filtration as shown in B, and subsequently purified to homogeneity as in (A); (E) marker proteins (67 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa, 14 kDa).

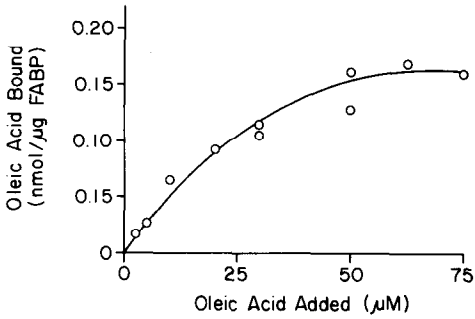
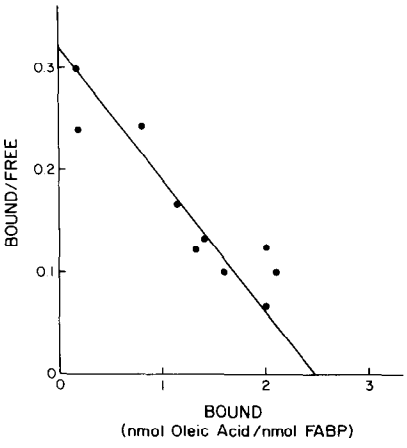


Fig. 3. Binding of radiolabeled oleic acid to rat heart FABP. Varying amounts of oleic acid were incubated with 24 μg of FABP for 5 min at 37°C. After equilibration, protein-bound and unbound oleic acids were separated by the use of Lipidex at 0°C as described in Experimental. Values have been corrected for non-specific binding at each point and represent an average of four experiments.

and adipose tissue, suggest some physiological significance of this protein in tissue[1]. However, most of the significance still remains under speculation, except that FABP has been found to bind with high affinity with FFAs and their esters and to shuttle them through the cytosolic compartment[7].

FFAs and their esters are known to accumulate in the biological tissue in a variety of disease processes. For example, prolonged ischemic insult results in the accumulation of FFAs[8]. Accumulation of FFAs is a common occurrence in myocardial infarction or during open-heart surgery[9]. Accumulation of FFAs and their es-



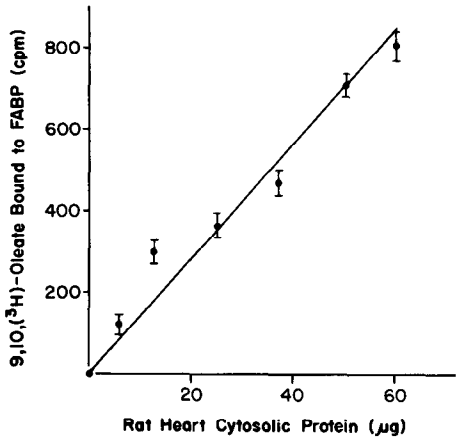


Fig. 4. Scatchard analysis of the saturation curve shown in Fig. 3. Values have been corrected for non-specific binding determined at each point and represent an average of four experiments.

Fig. 5. Incorporation of [9,10-^{3}H]oleate into FABP of rat heart cytosol. Increasing amounts of rat heart cytosol was incubated with [^{3}H]oleate and chromatographed as described in Experimental. Values have been corrected for non-specific binding determined at each point and represent an average of three experiments. The radioactivity found in the FABP peak was plotted against the concentration of cytosol used in the assay.

ters, particularly the long-chain thioesters, tends to cause myocardial dysfunction by their detergent-like action and enzyme-inhibitory properties[10]. Although serum albumin also binds to fatty acids, this protein, being of extracellular origin, is incapable of binding with the intracellularly accumulated FFAs and their esters. FABP, on the other hand, can not only bind them, presumably protecting the tissue from the detrimental effects of FFAs, but also shuttle them through the cytosolic compartment[5].

The separation and detection of FABP is a necessary step for performing any experiment concerning the physiological or biochemical roles of FABP. As mentioned earlier, the only method available for the separation of FABP involves several sophisticated chromatographic steps, including gel filtrations and anion-exchange chromatography. In addition, these techniques take over 24 h to separate FABP from the cytosol. The method described in this report is an extremely simple HPLC technique. The sample is run with an isocratic mobile phase by a single-step elution. The total separation time is 22 min.

In addition, the results of our study also suggest that Bio-Rad TSK-125, a gel filtration column, also behaves as an ion exchanger under our experimental conditions supporting the results from a previous study[11]. In the present study, at low ionic strength, myoglobin with a higher molecular weight than FABP eluted later from the column. This method can thus be used for a rapid single-step quantitation of FABP from biological tissues such as heart.

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