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One-step purification of rat heart-type fatty acid-binding protein expressed in *Escherichia coli*

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Abstract

Heart-type fatty acid-binding protein (H-FABP) is a member of a family of 14–15 kDa lipid binding proteins which are believed to enhance intracellular transport of lipids by facilitating their cytoplasmic diffusion. To obtain sufficient amounts of protein for in vitro studies, we expressed rat H-FABP in *Escherichia coli* and compared its biochemical properties with the protein isolated from rat heart. An effective method was developed to purify recombinant rat H-FABP from cell lysates in a single step using anion-exchange chromatography. This method also proved to be applicable for purifying heterologously expressed human H-FABP. Recombinant rat H-FABP, which made up approximately 25% of the soluble proteins in *E. coli*, was obtained in a yield of 30–40 mg/l culture. Characterization showed that recombinant rat H-FABP was indistinguishable from the protein isolated from rat heart regarding molecular mass and oleic acid binding. Some heterogeneity upon isoelectric focusing was observed, presumably due to differences in N-terminal processing of the proteins. In conclusion, a method is presented for efficient high-yield production of recombinant rat H-FABP.

Keywords: Proteins; Fatty acids; Heart-type fatty acid-binding protein

1. Introduction

Since 1971, cytoplasmic proteins exhibiting high-affinity binding of fatty acids have been isolated from several tissues [1]. These low-molecular-mass proteins (14–15 kDa), currently known as fatty acid-binding proteins (FABPs), are abundantly present in the cytoplasm of tissues with high fatty acid handling capacity, such as intestine, liver and heart (see, for a review, Refs. [2,3]). FABPs belong to a family of lipid binding proteins, which also encompasses the cellular retinoid binding proteins.

Heart-type FABP (H-FABP), initially identified in heart tissue and subsequently shown to be present in several other tissues such as skeletal muscle, kidney and brain [3], is implicated in the intracellular delivery of long-chain fatty acids to sites of metabolic conversion [4,5]. Moreover, roles in modulating fatty acid-mediated signal transduction pathways [6] and in protecting cells against detrimental effects of long-chain fatty acids have been proposed [7]. In

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addition to these intracellular actions, H-FABP was shown to function as an external growth regulator participating in reversible inhibition of bovine mammary carcinoma cell proliferation [8], differentiation of mouse mammary epithelial cells [9], and, possibly, in the induction of cardiac myocyte hypertrophy in the rat [10].

In vitro studies of FABP function require large amounts of protein, therefore FABPs from several species have been cloned and expressed in *Escherichia coli*. Among these are bovine heart FABP [11], rat intestinal FABP [12] and murine adipocyte FABP [13]. While this study was in progress, several other FABPs, including rat H-FABP, were expressed by Richieri et al. [14]. In all of these studies, procedures to purify recombinant FABPs from cell lysates were elaborate and time-consuming. In most cases, purification strategies consisted of ammonium sulphate precipitation followed by dialysis, ion-exchange chromatography and gel permeation chromatography (see e.g. Ref. [12]).

In this study we obtained high-level expression of rat H-FABP in *E. coli* and developed a one-step purification method, employing anion-exchange chromatography, to purify the recombinant protein. Purification of heterologously expressed human H-FABP was tested using the same procedure. The biochemical properties of recombinant rat H-FABP and H-FABP isolated from rat heart were compared to validate the potential use of recombinant rat H-FABP in in vitro studies.

2. Experimental

The expression vector pET3a and *E. coli* strain BL21(DE3)pLysS were obtained from Novagen (Madison, WI, USA). Restriction enzymes, T4 DNA ligase and Superscript II reverse-transcriptase (RTase) were obtained from Gibco BRL (Life Technologies, Gaithersburg, MD, USA). *Pfu* polymerase was purchased from Stratagene (La Jolla, CA, USA). For DNA sequence analysis the T7 deaza G/A sequencing kit from Pharmacia LKB (Uppsala, Sweden) was used. Chemicals were of analytical grade and were purchased from various vendors.

2.1. Construction of the expression vector

Total RNA was isolated from adult rat heart (male Wistar) by the guanidinium isothiocyanate method [15] and 2.5 μg was reverse transcribed with Superscript II RTase. Reverse transcription (RT) products were amplified using 25 cycles of polymerase chain reaction (PCR) consisting of 1 min denaturation (94°C), 1 min annealing (55°C) and 1 min elongation (72°C). PCR-products were separated on a 1.2% agarose gel. The resulting 422 bp fragment was purified from gel and ligated into *Sma*I-digested pUC19. The 412 bp *Nde*I–*Bam*HI fragment, encompassing the entire coding region, was isolated from this construct and ligated into the pET3a expression vector. The resulting construct will be referred to as pET3a–rHFABP.

The sense primer (5′-C*CATATG*GCGGACGCCTTTGTCGGTA) used in the PCR was identical to nucleotides 37–58 of the rat H-FABP cDNA sequence [16] except for some modifications which created a *Nde*I restriction site (italicised). The antisense primer (5′-TGACGGA*GGATCC*AGGTCACGCCT) used for first-strand cDNA synthesis and in the PCR was basically complementary to nucleotides 431–454 of the rat H-FABP cDNA sequence but contained a *Bam*HI restriction site (italicised). Primers were purchased from MWG Biotech (Ebersberg, Germany).

2.2. Expression of rat H-FABP in E. coli

E. coli strain BL21(DE3)pLysS [17] was transformed with pET3a–rHFABP. Cells were grown at 37°C in 250 ml 2×YT medium with ampicillin and chloramphenicol until an optical density of 0.6 at 600 nm was reached. Then isopropyl-β-thiogalactoside (IPTG) (Sigma, St. Louis, MO, USA) was added in a final concentration of 0.5 m*M*. Cells were grown for three more hours and harvested by centrifugation (15 min, 1500 *g*). Cells were resuspended in 15 ml ice-cold 10 m*M* imidazole (pH 7.0) and lysed by sonication. Chromosomal DNA was further sheared by repeated passage of the cell lysate through a small gauge needle.

2.3. Purification of recombinant rat H-FABP

The obtained cell lysate was cleared by centrifugation (15 min, 1000 *g*) and the supernatant was loaded onto a column (20×2 cm I.D.) containing the anion-exchanger Q Sepharose FF (Pharmacia LKB) equilibrated in 10 m*M* imidazole (pH 7.0). Following an initial washing step with 100 ml of this buffer, proteins were eluted with a linear gradient of NaCl (0–100 m*M* in 10 h) in 10 m*M* imidazole pH 7.0 (1 ml/min). All operations were performed at 4°C. Fractions of 7.5 ml were collected.

2.4. Identification of rat H-FABP containing fractions

Two methods were used to identify fractions containing rat H-FABP. The first employed the radiochemical Lipidex-1000 assay developed by Glatz and Veerkamp [18] which allows for a functional (fatty acid-binding capacity) screening of fractions. Briefly, fractions were incubated with [1-^{14}C]oleic acid (Amersham International, Little Chalford, UK) at 37°C. Next, Lipidex-1000 (Packard Instrument, Downers Grove, IL, USA) was added and incubation proceeded at 0°C. At this temperature non-protein- bound fatty acid is captured by Lipidex-1000, whereas protein-bound fatty acid remains unaffected. After centrifugation, radioactivity in the supernatant, representing protein-bound fatty acid, was determined.

Rat H-FABP containing fractions were also identified immunochemically, using the enzyme-linked immunosorbent assay (ELISA) described by Vork et al. [19]. Briefly, proteins from each fraction were directly coated onto PVC microtiter plates. After incubation with a biotinylated anti-rat H-FABP antibody, avidin-conjugated horseradish peroxidase was added. For staining of rat H-FABP containing fractions *o*-phenylenediamine was used.

Rat H-FABP containing fractions were pooled, concentrated by ultrafiltration through Amicon YM-3 filters (Amicon, Beverly, MA, USA), and stored at −20°C. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. Rat H-FABP concentrations were quantified with a sandwich ELISA method developed by Vork et al. [20]. H-FABP isolated from rat heart essentially according to the method of Van Nieuwenhoven et al. [21], was used as the standard.

2.5. Characterization of the recombinant protein

For N-terminal sequence analysis, recombinant rat H-FABP was subjected to Edman degradation and the first ten amino acids were determined. Sequencing was performed at the central sequencing facility of the Sonderforschungsbereich 310, Department of Physiological Chemistry, Münster, Germany.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% T, 2.7% C gels) was performed according to Laemmli [22] and gels were stained with Coomassie brilliant blue. Molecular mass markers were obtained from Pharmacia LKB. Western blots were immunostained, using (cross-reactive) peroxidase-conjugated monoclonal anti-human H-FABP antibodies [23], with tetramethylbenzidine as substrate.

Native isoelectric focusing (IEF) was performed as described earlier [24] with ampholytes (pH 4.0–6.5) from Pharmacia LKB. Both FABP isolated from rat heart (essentially according to Van Nieuwenhoven et al. [21]) and recombinant rat H-FABP were investigated. H-FABPs from other species were included in IEF analysis to study isoelectric heterogeneity. H-FABP was isolated from bovine heart [24] and human heart [25]. Recombinant bovine, human and mouse H-FABP were isolated according to Rump et al. [26]. All tissue-derived and recombinant H-FABPs employed in this study were homogenous in SDS-PAGE (at least 95% pure).

Apparent dissociation constants (K_d) for oleic acid–rat H-FABP complexes were determined, using the Lipidex procedure, as described by Vork et al. [19]. Fixed amounts (125 pmol) of (non-delipidated) recombinant rat H-FABP were used, while oleic acid quantities varied from 0 to 500 pmol. Binding data (sixteen data points obtained in two individual experiments) was evaluated by methods described by Zivin and Waud [27].

To quantify the amount of fatty acids present in recombinant rat H-FABP preparations, fatty acids were extracted from 250 nmol protein, esterified and

analyzed by gas chromatography as described elsewhere [28].

3. Results and discussion

3.1. Cloning of rat H-FABP cDNA

Rat H-FABP cDNA was cloned by RT–PCR and subjected to sequence analysis. The obtained sequence was identical to the one published by Heuckeroth et al. [16]. Next, rat H-FABP cDNA was cloned into the pET3a vector and expressed in *E. coli*. The pET expression system utilizes an engineered *E. coli* strain [e.g. BL21(DE3)pLysS] which contains a chromosomal copy of the T7 RNA polymerase gene under control of an IPTG-inducible promoter, and a vector which allows transcription of genes from a strong T7 promoter [17].

3.2. Expression and purification of rat H-FABP

Mid-log phase cultures of *E. coli* strain BL21(DE3)pLysS, transformed with the expression vector pET3a–rHFABP, were induced by addition of IPTG. Fig. 1a shows the marked induction of a 15 kDa protein in cell lysates from induced cultures (lane 3) in comparison with cell lysates from induced cultures lacking pET3a–rHFABP (lane 2). This protein comigrated with H-FABP isolated from rat heart (lane 7) and was confirmed to be rat H-FABP by Western blotting (Fig. 1b, lanes 3 and 7).

Cleared cell lysate was applied to an anion-exchange column and proteins were eluted with a linear gradient of 0–100 m*M* NaCl in 10 m*M* imidazole (pH 7.0). The elution profile, monitored by measuring absorption at 280 nm (Fig. 2, thick line), showed four peaks. Peak A represents proteins which eluted from the column during washing (Fig. 1a, lane 4). Peak B represents unidentified substances which do not appear to be proteins as judged by SDS-PAGE (data not shown). Fractions which contained rat H-FABP were identified both immunochemically by ELISA and by the Lipidex binding assay (Fig. 2). Rat H-FABP (peak C) eluted at an ionic strength of 35–45 m*M* NaCl. Peak D contained proteins (Fig. 1a, lane 6) which eluted from the column by rinsing with 2 *M* NaCl.

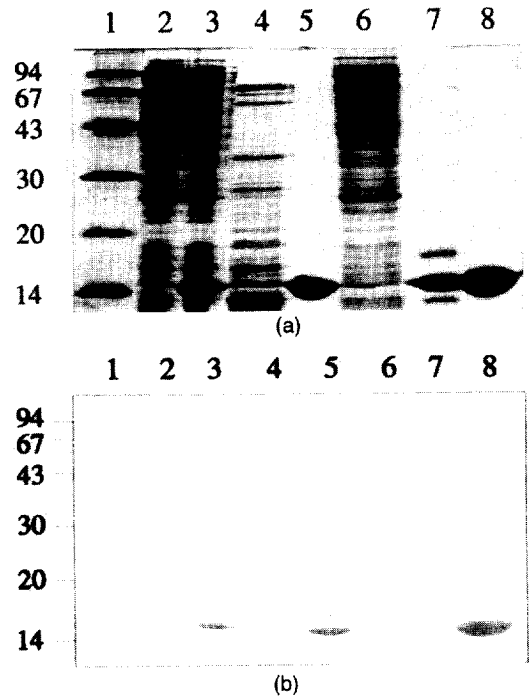

Fig. 1. Expression of rat H-FABP in *E. coli* and progress of the purification procedure. SDS-PAGE analysis of equal amounts of protein (10 μg) with (a) Coomassie blue staining and (b) immunostaining. For the latter, following electrophoresis, proteins were electroblotted onto nitrocellulose filter and probed with a horseradish peroxidase-conjugated monoclonal anti-human H-FABP antibody. Lane 1, molecular mass markers (masses shown in kDa); lane 2, untransformed *E. coli* lysate after induction; lane 3, pET3a–rHFABP transformed *E. coli* lysate after induction; lanes 4–6, fractions eluted at 0, 40 and 2000 m*M* NaCl, respectively; lane 7, H-FABP isolated from rat heart; lane 8, 25 μg recombinant rat H-FABP.

As judged from SDS-PAGE analysis (Fig. 1a, lane 5), no contaminating proteins were observed in FABP-containing peak fractions even when large amounts (25 μg) of protein were loaded on the gel (lane 8). Taking into consideration the detection limit of our electrophoresis system (approximately 100 ng per band), it was estimated that the protein was at least 99% pure. Moreover, ultraviolet spectra recordings proved the absence of contaminating nucleic acids (data not shown). Hence, rat H-FABP expressed in *E. coli* could be purified from cell lysates in a single step. The recombinant protein

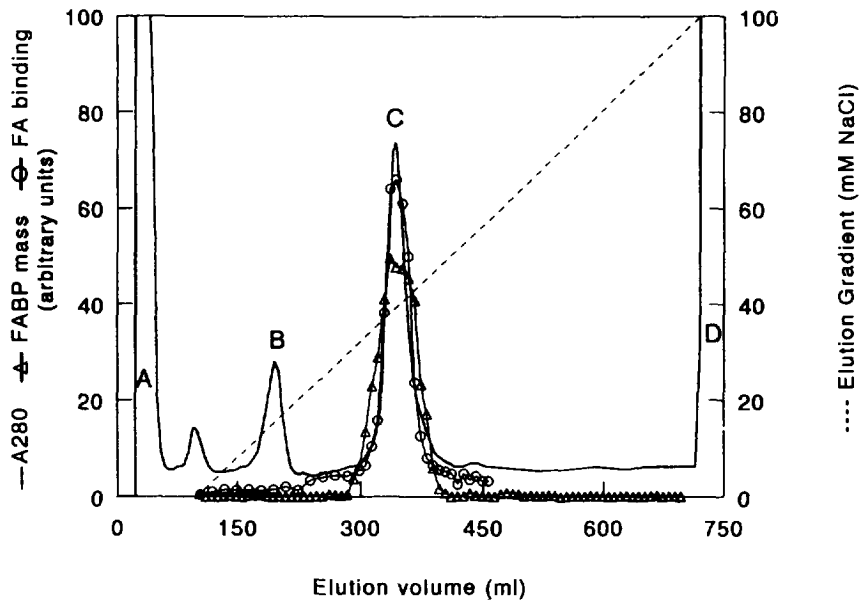

Fig. 2. Anion-exchange chromatography on Q Sepharose FF of bacterial cell lysate. Rat H-FABP was eluted with a linear gradient of 0–100 m*M* NaCl (dashed line) in 10 m*M* imidazole (pH 7.0). Elution was monitored by measuring absorption at 280 nm (thick line). Fractions were screened for rat H-FABP both immunochemically (▲) and by fatty acid-binding activity (○).

made up approximately 25% of the total soluble protein in *E. coli* and was obtained in yields ranging from 30–40 mg/l culture.

Recombinant rat H-FABP isolated from *E. coli* was found to contain fatty acids. Gas chromatography analysis revealed that approximately 0.2 mol fatty acid was bound per mol H-FABP (data not shown). Oleic acid constituted circa 40% of fatty acids complexed to recombinant rat H-FABP.

3.3. Expression and purification of human H-FABP

Contributing to the success of our purification method is the high-level expression of rat H-FABP driven by the pET system. To test whether our purification scheme was suitable for the purification of another recombinant FABP as well, we expressed human H-FABP in *E. coli* using the pET system and followed the same chromatographic protocol to purify the recombinant protein. Heterologously expressed human H-FABP eluted at an ionic strength of 25–35 m*M* NaCl and, as judged by SDS-PAGE, was over 99% pure (data not shown).

3.4. Characterization of recombinant rat H-FABP

As expected, recombinant rat H-FABP and H-FABP isolated from rat heart have similar molecular masses as concluded from SDS-PAGE analysis (Fig. 1). However, in IEF gels two isoforms (p*I* 5.0 and p*I* 5.2) were visible for recombinant rat H-FABP (Fig. 3, lane 6) while the tissue-derived protein migrated

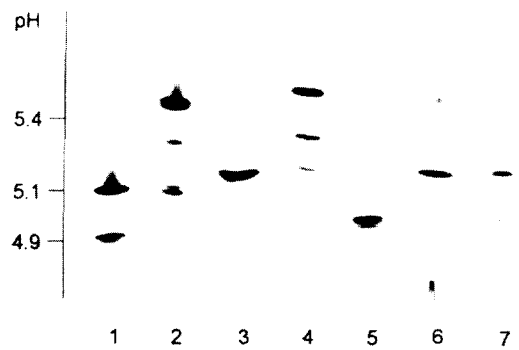

Fig. 3. Isoelectric heterogeneity of recombinant and tissue-derived H-FABPs. Coomassie stained IEF gel (pH 4–6.5). Lanes 1, 3 and 5, H-FABP isolated from bovine, human and rat heart, respectively; lanes 2, 4, 6 and 7, recombinant bovine, human, rat and mouse H-FABP, respectively.

as a single p*I* 5.0 isoform (Fig. 3, lane 5). Isoelectric heterogeneity, due to the existence of N-terminal variants in recombinant protein preparations, was also observed when comparing recombinant H-FABPs (Fig. 3, lanes 2, 4 and 7) and tissue-derived H-FABPs (Fig. 3, lanes 1 and 5) from other species.

The two bands observed for H-FABP from bovine heart (Fig. 3, lane 1) represent the two isoforms described by Unterberg et al. [29] which contain either Asp98 (p*I* 4.9 isoform) or Asn98 (p*I* 5.1 isoform). FABPs isolated from tissue seem resistant to Edman degradation, indicating that they are N-terminally blocked. For example, the N-terminus of human H-FABP consists of N-acetylvaline [30]. Apparently, the most acidic variants of the recombinant proteins comigrate with the FABPs isolated from tissue, suggesting that they have a blocked N-terminus as well. For heterologously expressed bovine H-FABP this variant corresponds to unprocessed N-formylated protein [31]. The unblocked isoforms have an additional free amino group at the N-terminus, making the protein more basic.

N-Terminal sequences of the recombinant H-FABPs are shown in Table 1. Recombinant rat H-FABP starts with alanine, thus the N-terminal methionine is removed by methionyl aminopeptidase (MAP). The faint, more acidic species visible in IEF, presumably originating from formylated rat H-FABP, did not yield a sequence in Edman degradation. The same result was found for recombinant mouse H-FABP. This is consistent with findings that MAP activity depends on the amino acid adjacent to the starter methionine, small amino acids increasing, and bulky and charged amino acids decreasing its activity [32]. In the case of recombinant bovine and human H-FABP the N-terminal methionine was incompletely removed by MAP. Accordingly, a secondary sequence can be read upon N-terminal Edman degradation, starting with valine (Table 1). Thus, the small amino acid valine seems to be an exception to the rule as was also observed by Flinta et al. [33]. Furthermore, the incomplete cleavage of the starter methionine increases the number of possible variants [31].

Table 1
N-Terminal sequences of recombinant H-FABPs

Species	Predominant sequence	Minor sequence
Bovine	MVDAFVGTWK	VDAFVGTWKL
Human	MVDAFLGTWK	VDAFLGTWKL
Mouse	ADAFVGTWKL	Absent
Rat	ADAFVGTWKL	Absent

Purified recombinant HFABPs were subjected to Edman degradation and the first ten amino acids were determined.

In order to functionally characterize the recombinant protein, the binding of oleic acid to recombinant rat H-FABP was determined. The apparent dissociation constant (K_d) was 0.29 ± 0.11 μM and the binding stoichiometry (B_{max}) was 0.85 ± 0.21 mol oleic acid per mol H-FABP. Taking into account the small amount of endogenously bound fatty acids, which results in an under-estimation of B_{max}, these results are in agreement with the results obtained for H-FABP isolated from rat heart ($K_d=0.33$ μM, $B_{max}=1.11$ mol/mol) as reported by Vork et al. [19].

4. Concluding remarks

High-level expression of rat H-FABP in *E. coli* was achieved. The recombinant protein constituted approximately 25% of the proteins in cell lysates and yields varied from 30–40 mg/l culture. Moreover, time-consuming procedures for purifying the recombinant protein were eliminated by the development of a one-step purification method. In addition, another recombinant FABP (human H-FABP) expressed with the same expression system, could be purified using this purification scheme.

In contrast to H-FABP isolated from rat heart, the recombinant protein exhibited isoelectric heterogeneity due to the existence of N-terminal variants. As deduced from the tertiary structure of human H-FABP and other FABPs [34], the N-terminus is located on the exterior of the protein and does not seem to take part in protein function. Binding data confirms this assumption, since the binding stoichiometry and the apparent dissociation constant for oleic acid-binding were similar for recombinant rat H-FABP and H-FABP isolated from rat heart. Therefore, the recombinant rat H-FABP seems suitable for use in in vitro studies.

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