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Amino acid sequence, binding properties and evolutionary relationships of the basic liver fatty-acid-binding protein from the catfish *Rhamdia sapo*

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The complete amino acid sequence of a basic liver fatty acid-binding protein (L-FABP) from catfish (*Rhamdia sapo*) was determined. Alignment of sequences shows that it has more similarity to chicken basic L-FABP than to mammalian L-FABP. The phylogenetic analysis suggests that basic L-FABP from catfish, chicken and iguana diverged from the mammalian protein before the fish-tetrapod divergence, thus implying that the two types are encoded by different genes. Supporting this conclusion, a 14-kDa protein, structurally closely related to mammalian L-FABP, was isolated from catfish intestine, indicating the presence of the two genes in the same species. The catfish basic L-FABP binds only one fatty acid/molecule, while mammalian L-FABP bind two. The former has more affinity for *trans*-parinaric acid than for *cis*-parinaric acid, in contrast to the latter proteins.

Keywords: catfish; fatty-acid-binding protein; liver; *Rhamdia sapo*.

Different types of fatty acid-binding proteins (FABP) have been isolated from tissues with active fatty acid metabolism. The existence of various FABP in mammalian species and their distribution in different tissues with high lipid metabolism suggests that each of them fulfils specific functions according to the physiological characteristics of the tissue (Veerkamp et al., 1993; Banaszak et al., 1994). FABP isolated from the same tissue of different mammals show more primary structure similarity than proteins isolated from different tissues of the same species. Furthermore, the expression pattern of each FABP type seems to be conserved through evolution, on the basis of reports that FABP from frog (*Xenopus laevis*) intestine and from toad (*Bufo arenarum*) heart belong to the same subfamilies as mammalian intestinal FABP and heart (H)-FABP, respectively (Shi and Hayes, 1994; Schleicher and Santomé, 1994). However, Medzihradsky et al. (1992) isolated from the liver of nurse shark (*Ginglymostoma cirratum*) an FABP more closely related to mammalian H-FABP and adipocyte lipid-binding protein (A-LBP) than to liver (L)-FABP, and we recently found in catfish (*Rhamdia sapo*) liver two H-FABP (Di Pietro and Santomé, 1996). The biological significance of the presence of the H-FABP in nurse shark liver was attributed to the fact that shark liver behaves, in some aspects, like mammalian adipose tissue (Medzihradsky et al., 1992), whose FABP is more similar to the heart type. Catfish fat reserves are however not concentrated in the liver (Machado et al., 1988), as are those of the shark. A basic L-FABP was found in chicken (*Gallus domesticus*) liver, instead of the characteristic L-FABP present in mammals (Ce-

ciliani et al., 1994). Its amino acid sequence and results of phylogenetic analysis (Schleicher et al., 1995) suggest that avian and mammalian L-FABP and ileal lipid-binding proteins (I-LBP) belong to a subfamily that diverged from the other subgroups before the vertebrate-invertebrate split (Schleicher et al., 1995). Partial amino acid sequencing of an FABP from catfish liver suggested that this protein is of the L-FABP type previously reported for chicken (Di Pietro et al., 1996).

Several observations support the idea that mammalian L-FABP are structurally and functionally different from the other FABP types: mammalian L-FABP binds two fatty acids/molecule (Haunerland et al., 1984; Cistola et al., 1989; Nemezc et al., 1991a; Rolf et al., 1995; Richieri et al., 1996; Thompson et al., 1997), whereas the other FABP types have a single fatty acid-binding site (Nemezc et al., 1991a; Richieri et al., 1994). Additionally, L-FABP is able to bind a wide range of hydrophobic ligands (Burrier and Brecher, 1986; Khan and Sorof, 1990; Veerkamp et al., 1993; Epstein et al., 1994; Rolf et al., 1995), and, unlike other FABP, undergoes conformational changes when binding fatty acids (Nemezc et al., 1991b; Li and Ishibashi, 1992).

In a previous study, we isolated and partially sequenced a basic FABP from catfish liver (Di Pietro et al., 1996) and found that it is more closely related to the chicken L-FABP than to mammalian L-FABP. We now describe its complete amino acid sequence, expression pattern, and some ligand-binding properties. We present theoretical and experimental evidence confirming that basic L-FABP from catfish, chicken and iguana and mammalian L-FABP are encoded by two separate genes. Furthermore, we report the presence of a mammalian-type L-FABP in the catfish intestine.

MATERIALS AND METHODS

Materials. [1-¹⁴C]Palmitic acid (57 Ci/mol) was from Du Pont NEN. *cis*- and *trans*-parinaric acids were from Molecular

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Abbreviations. FABP, fatty acid-binding protein; L-FABP, mammalian type liver fatty acid-binding protein; H-FABP, heart fatty acid-binding protein; A-LBP, adipocyte lipid-binding protein; I-LBP, ileal lipid-binding protein; DEAE, diethylaminoethyl.

Note. The novel amino acid sequence data reported here have been submitted to the SwissProt data bank and are available under accession number P80856.

Probes, Inc. Horseradish-peroxidase-coupled goat antibodies against rabbit immunoglobulins were from Dako A/S. Centriprep concentrators were from Amicon. All other reagents were of analytical grade from Sigma, Applied Biosystems, Baker, Bio-Rad or Pharmacia Biotech.

Animals. Catfish (*R. sapo*) were collected from the Salado river (Chivilcoy, Argentina). Wistar albino rats (200–250 g) were used for this study.

Preparation of the cytosolic fraction including 12–16-kDa proteins from catfish and rat tissue. Tissues were extracted, cut into small pieces, suspended in 40 mM sodium phosphate, pH 7.4, 150 mM KCl, 2 mM EDTA, 4 mM dithiothreitol, and homogenized with the aid of a teflon potter. Homogenates were centrifuged at 20000×g for 15 min, and the resulting supernatants were centrifuged at 105000×g for 90 min in a Beckman XL-90 ultracentrifuge with a Ti 90 rotor. The 105000×g supernatants were loaded to a Sephadex G-75 column (2.5 cm×40 cm) equilibrated with 30 mM Tris/HCl, 1 mM dithiothreitol, 1 mM EDTA. The pH was either 8.5 (for western analyses or isolation of catfish L-FABP) or 9.0 (for purification of catfish basic L-FABP). Elution was performed at 4°C with the same buffer at 16 ml/h. Fractions including 12–16-kDa proteins were collected for further analysis.

Purification of basic L-FABP. The protein used for structural analyses was purified as described previously (Di Pietro et al., 1996). For functional studies, the purification procedure was modified as follows. The cytosolic fraction including 12–16-kDa proteins from catfish liver was applied directly to a DEAE-cellulose column (1.1 cm×11 cm) equilibrated with 30 mM Tris/HCl, pH 9.0. The unbound material was concentrated and the buffer changed to a 10 mM sodium acetate, pH 6.0, by using a Centriprep concentrator. The concentrated sample was loaded to a carboxymethyl-cellulose column (1 cm×5 cm) equilibrated with the same buffer. Pure basic L-FABP was recovered from the pass-through fraction. Before fatty-acid-binding analyses, the protein was delipidated by incubation at 37°C for 45 min with Lipidex beads (Glatz and Veerkamp, 1983), followed by a buffer change to 50 mM Tris/HCl, pH 7.4.

Fractionation of 12–16-kDa proteins from catfish intestine. The 12–16-kDa proteins from the cytosol of intestinal mucosa (see above) were incubated at 20°C with 0.2 µCi of [¹⁴C]palmitic acid for 20 min, then loaded to a DEAE-cellulose column (1 cm×6 cm) previously equilibrated with 30 mM Tris/HCl, pH 8.5. The material bound to the column was eluted with 10, 20, 30, 40, 50, 150 and 500 mM NaCl in the above buffer. Eluted fractions were analyzed by liquid scintillation counting and by SDS/PAGE and immunoblotting with an antiserum to rat L-FABP.

Basic L-FABP cleavage and peptide purification. About 20 µg purified basic L-FABP was cleaved at methionine residues with CNBr as described by Montemartini et al. (1993). The resulting peptides were separated by HPLC on a Brownlee Aquapore RP 300 C₈ column (2.1 mm×220 mm) using a linear gradient from 0 to 80% (by vol.) acetonitrile in 0.1% (by vol.) aqueous trifluoroacetic acid. For Glu-C protease digestion, 70 µg of the pyridylethylated protein was incubated in 0.1 M Tris/HCl, 2 M urea, pH 7.9, with 3.5 µg enzyme at 20°C for 20 h. For chymotryptic digestion, 100 µg FABP was incubated with 3.5 µg chymotrypsin in the same buffer at 20°C for 14 h. Peptides resulting from both digestions were separated by HPLC on a Vydac C₁₈ column (4.6 mm×250 mm), also using an acetonitrile gradient in 0.1% (by vol.) aqueous trifluoroacetic acid.

Catfish L-FABP digestion and peptide purification. Isolated L-FABP from catfish intestine was digested with sequencing-grade trypsin following the in-gel procedure of Rosenfeld et al. (1992), as modified by Hellman et al. (1995). The resulting

peptides were recovered by passive elution and separated by HPLC on a Brownlee Aquapore RP 300 C₁₈ column (2.1 mm×220 mm) by using a combination of linear gradients of acetonitrile in 0.1% aqueous trifluoroacetic acid.

Purification of N-acyl-aminoacyl-peptidase from rabbit muscle. We followed the procedure described by Radhakrishna and Wold (1989).

Amino acid sequencing of blocked N-terminal peptides. Two procedures were followed. a) Chymotryptic peptide 4 (Fig. 1) was subjected to N-acyl-aminoacyl-peptidase digestion as described by Radhakrishna and Wold (1989) and sequenced by Edman degradation. b) Basic L-FABP was digested with Glu-C protease as described above. At the end of the incubation, Na₂CO₃ was added until pH 10 was reached. The procedure reported by Krishna et al. (1991) was used, with the following modifications, for succinylation of peptide amino groups and specifically unblocking of the N-terminal peptide and sequencing. Instead of ether extractions to remove succinic acid and salts, the succinylation product was loaded to a Vydac C₄ column (4.6 mm×25 mm) equilibrated with 0.1% (by vol.) trifluoroacetic acid in water. The column was developed at a flow rate of 0.8 ml/min with a linear gradient of 80% acetonitrile, 0.08% trifluoroacetic acid in water. Peptides with absorbance at 280 nm were collected and digested with N-acyl-aminoacyl-peptidase. The resulting mixture was directly applied to the sequencer.

Amino acid analysis and sequencing. Quantitative amino acid analysis and automated peptide sequencing were carried out in an Applied Biosystems 420A Amino Acid Analyzer and an Applied Biosystems 477A Protein Sequencer, respectively, according to the manufacturer's instructions.

Electrophoresis and immunoblotting. SDS/PAGE and immunoblotting were carried out as described (Di Pietro and Santomé, 1996). The preparation of antisera with specificity for catfish basic L-FABP (Di Pietro and Santomé, 1996) or rat L-FABP (Paulussen et al., 1990) has been reported previously.

Fluorescent fatty-acid-binding assay. We followed the procedure described by Nemezc et al. (1991a, b). The fluorescence intensity (excitation 324 nm, emission 415 nm) of either *cis*- or *trans*-parinaric acid was measured in 50 mM Tris/HCl, pH 7.4, 0.35–0.40 µM delipidated basic L-FABP, and 0.2–8 µM of each fluorescent fatty acid. Measurements were performed at 25°C in a Kontron SFM 25 spectrofluorometer (Kontron Instruments). Fatty acids were added in amounts of 0.5–1 µl from a solution in ethanol. The fluorescence intensity of a control solution without protein was subtracted from the total intensity at each fatty acid concentration. Corrected fluorescence-intensity values were fitted to a hyperbolic equation by non-linear regression as reported by Thumser et al. (1996). The data were transformed as described by Nemezc et al. (1991a) in order to obtain Hill plots of the binding isotherms.

Tryptophan fluorescence. The intrinsic emission spectrum of basic L-FABP was registered in the above spectrofluorometer. The excitation wavelength was set to 295 ± 5 nm. Measurements were performed at 25°C in a medium containing 50 mM Tris/HCl, pH 7.4, 1 µM delipidated basic L-FABP and 0–5 µM palmitic acid. The fatty acid was added in amounts of 0.5–1 µl from a solution in ethanol. Ethanol concentrations in the flow cell never exceeded 1%. Each spectrum was corrected by comparison with that of a protein-free control solution.

Fatty-acid-competition-binding assay. In 10 ml, 1 µM delipidated FABP was incubated at 25°C in 50 mM Tris/HCl, pH 7.4, 1% ethanol, and 1 µM of each of the following fatty acids: palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidic, arachidonic and eicosapentaenoic. After 20 min, the free-ligand fraction was removed by means of a PD-10 column. Fatty acids bound by FABP were extracted by the method of

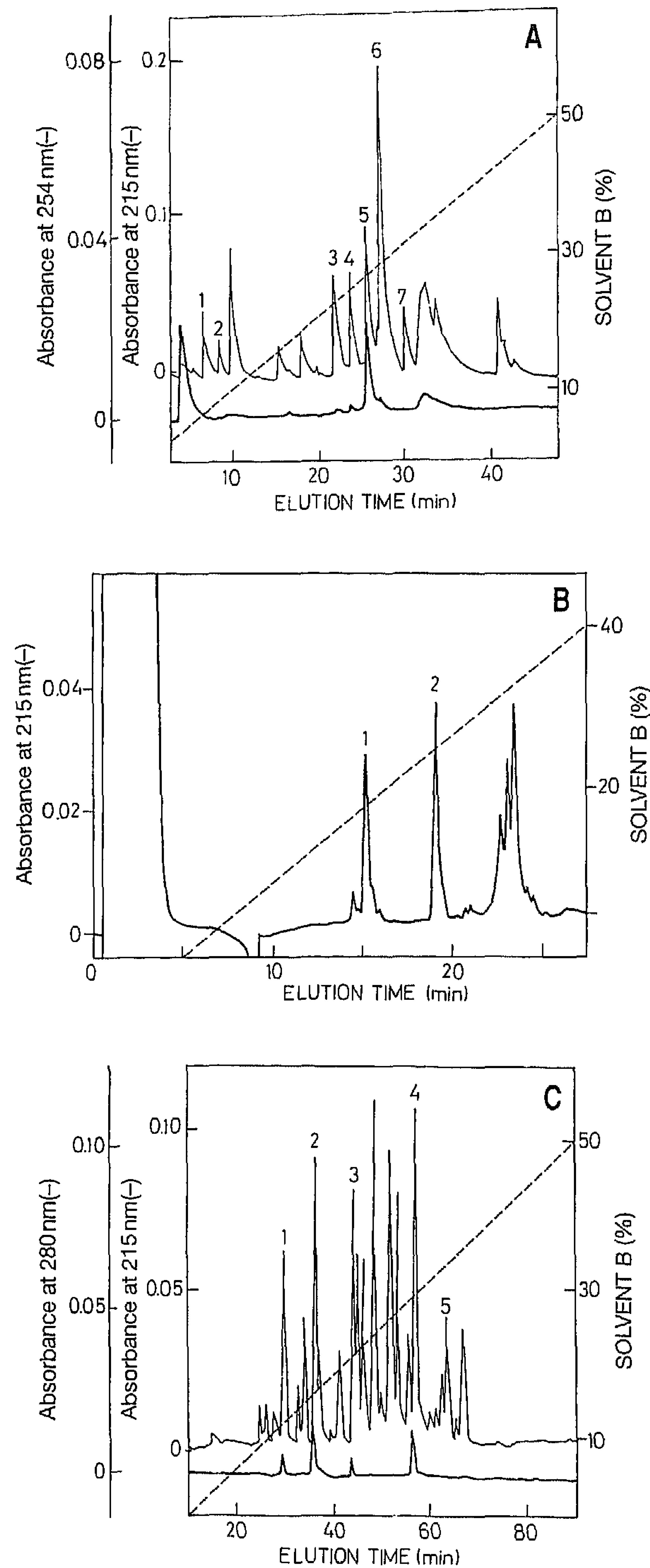


Fig. 1. HPLC separation of catfish basic L-FABP peptides generated by digestion with Glu-C endoprotease, CNBr, and chymotrypsin. (A) The peptide mixture obtained by Glu-C digestion was fractionated on a Vydac C₁₈ column (4.6 mm×250 mm) equilibrated with solvent A (0.1% trifluoroacetic acid in water) and eluted with a linear gradient (dashed line) of solvent B [80% (by vol.) acetonitrile, 0.08% (by vol.) trifluoroacetic acid in water]. Absorbance at 254 nm is indicated for detection of peptides containing pyridylethyl-Cys. (B) Peptides generated by CNBr chemical cleavage were isolated on a Brownlee Aquapore RP 300 C₈ column (2.1 mm×220 mm) equilibrated with solvent A and eluted with solvent B as indicated. (C) The products of chymotrypsin digestion were separated as described for the Glu-C peptide mixture. Absorbance at 280 nm is depicted for identification of peptides with Tyr and/or Trp residues. The amino acid composition of the peaks of the three digestions was determined (not shown). Only those peptides required to complete the primary structure were sequenced by Edman degradation and are numbered.

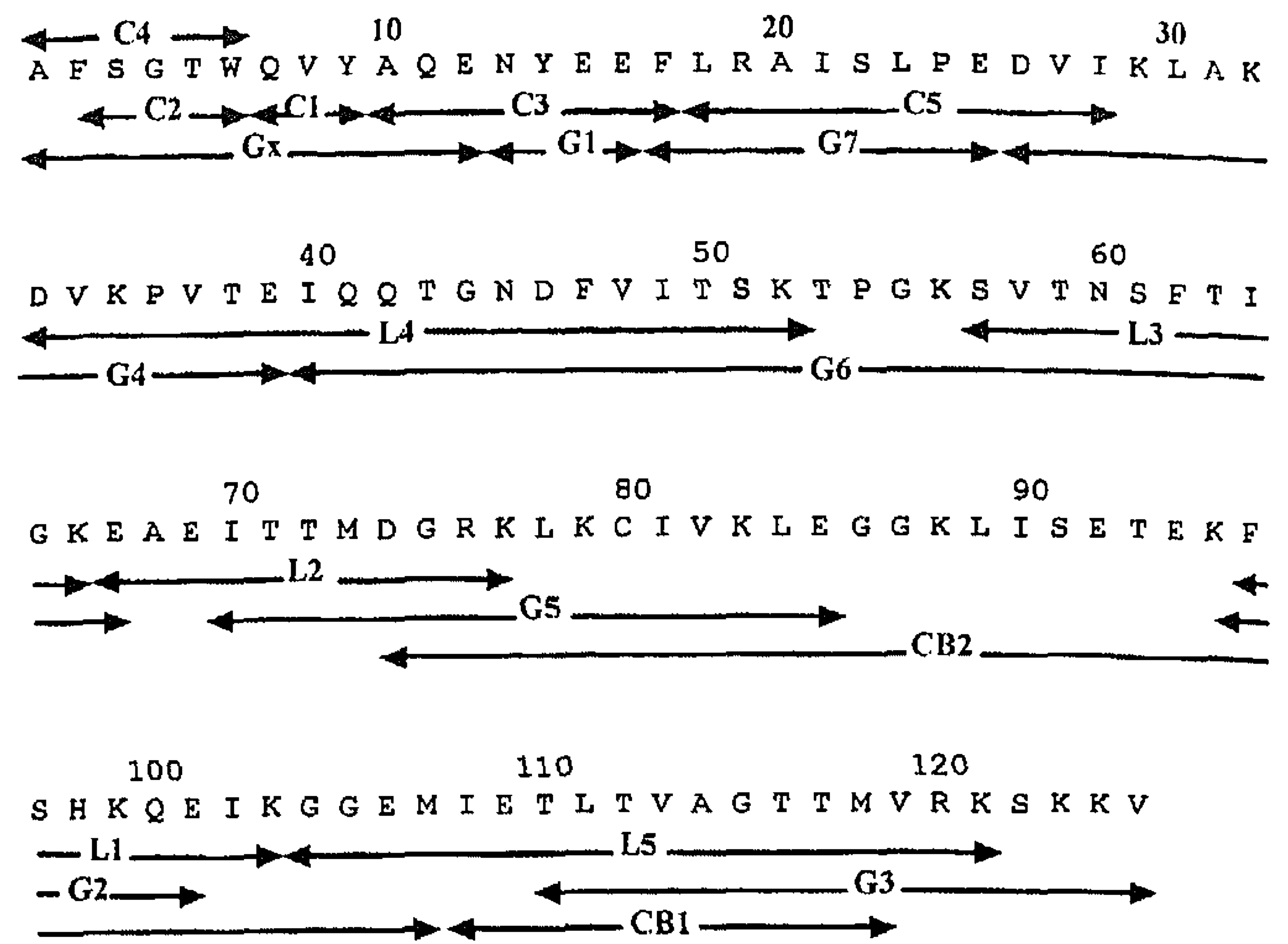


Fig. 2. Primary structure of catfish basic L-FABP. The numbering of peptides is consistent with that of Fig. 1. Peptide fragments (determined by Edman degradation) are indicated by arrows and labelled as follows: C, chymotryptic peptides; G, Glu-C protease peptides; Gx, peptide obtained by the procedure of Krishna et al. (1991); CB, CNBr peptides; L, Lys-C peptides whose amino acid sequences have been published elsewhere (Di Pietro et al., 1996).

Bligh and Dyer (1959). Fatty acid methyl esters were prepared by methylation with 2% (by vol.) sulfuric acid in methanol in a dry shaker at 60°C for 2 h. The fatty acid methyl esters were analyzed using a gas chromatograph (Series GC-8A) equipped with a 30 m×25 mm DR23 column.

RESULTS

Primary structure of catfish basic L-FABP. The amino acid sequence of several peptides obtained by digestion of the basic L-FABP with endoprotease Lys-C has been published elsewhere (Di Pietro et al., 1996). To complete the primary-structure determination, fragments were generated by enzymatic digestion with proteases Glu-C and chymotrypsin, and cleavage with CNBr (Fig. 1). On the basis of the amino acid composition of peptides shown in Fig. 2, it was possible to select those corresponding to the catfish-basic-L-FABP fragments whose amino acid sequences had not been determined previously (Di Pietro et al., 1996), and also the overlapping peptides required. A map obtained after reverse-phase HPLC of Glu-C peptides is shown in Fig. 1A. Numbered peaks correspond to sequenced peptides. Cleavage with CNBr was performed by employing a similar strategy. Fig. 1B shows the HPLC separation of the fragments obtained. Peptides represented by numbered peaks in the figure were sequenced by Edman degradation. The information obtained allowed completion of the total sequence except for the 12 N-terminal residues (Fig. 2). Because, according to amino acid determinations and already established sequences, tryptophan and tyrosine residues are found only in the N-terminal portion of catfish basic L-FABP, the protein was digested with chymotrypsin, the resulting peptides were fractionated by reverse-phase HPLC and those with absorbance at 280 nm selected (Fig. 1C). Four peptides (C1–C4) presented this characteristic and were subjected to sequencing. One of them yielded negative results by Edman degradation (C4), thus indicating a blocked α -amino group. Its amino acid sequence from the second residue was obtained following digestion with acyl-aminoacyl-peptidase, the first residue being established on the basis of the amino acid analysis. The overlapping of chymotryptic peptides C1, C2,

Rs L-FABPb	..AFSGTWQVYAQENYEEFLRAIISLPEDVILKAKDVKPVTEIQQTGND	
ch L-FABPb	..AFSGTWQVYAQENYEEFLKALALPEDLTKMARDIKPIVLEIQKCGDD	
Ap L-FABPb	..AFNGTWQVYSQENYEDFLKATIALPDDILKAAKDVKPVTEIROTGMT	
hu L-FABP	..SFSGKYQLQSQENFEAFMKALGLPEELIQKGDIKGVSEIVQNGKH	
bo L-FABP	..NFSGKYQVQSQENFEAFMKAVGMPDDILQKGDIKGVSEIVQNGKH	
ra L-FABP	..NFSGKYQVQSQENFEAFMKAMGLPEDLQKGDIKGVSEIVHEGKK	
sk L-FABP	VEAFLGSWKLOKSHNFDEYMKNLDVSLAQRKVATTVKEKTIISLDGCV	
Rs L-FABPb	FVITSKTPGKSVTNSFTIGKEAEITTMGRRLLKCTVKLEG.GKLISET	
ch L-FABPb	FVITSKTPRQTVTNSFTLGKEADITTMGKRLKCTVHLAN.GKLVTKS	
Ap L-FABPb	FVITSKTPNKSVTNSFTLGKEADMTTMGKRVKCTVNLVD.GKLVAKS	
hu L-FABP	FKFIITAGSKVIQNEFTVGGEECELETMTGKVKVTVVQLEGDNKLVVTF	
bo L-FABP	FKFIITAGSKVIQNEFTVGGEECEMEFMTGKIKAVVQEGDNKLVVTF	
ra L-FABP	VKLIITYGSKVIHNEFTVGGEECELETMTGKVKAVVQEGDNKLVVTF	
sk L-FABP	ITIKTESTFKSTNIQFKLAEEFDETTADNRTTKTIVKLEN.GKLVQTO	
Rs L-FABPb	...EKFSHKQEIKGEMIEITLVAGITMVRKSKKV.	100
ch L-FABPb	...EKFSHEQEVKGNEMVETITFGGVTLIRRSKRV.	70
Ap L-FABPb	...DKFIHQEIVGNEMVETITSGSATFTRRSKRI.	68
hu L-FABP	...KNIKSVTELNCDIITNTMTLGDIVFKRISKRI.	40
bo L-FABP	...KGIKSVTEFNCDTIVTSTMTKGDVVFKRISKRI.	37
ra L-FABP	...KGIKSVTEFNCDTITNTMTLGDIVYKRISKRI.	38
sk L-FABP	RWDGKETTLVRELQDGKLLTCTMGDVVCTREYVREQ	28

Fig. 3. Sequence alignment of catfish basic L-FABP with other L-FABP. The amino acid sequences of catfish basic L-FABP (Rs L-FABPb), chicken basic L-FABP (ch L-FABPb), human L-FABP (hu L-FABP), cow L-FABP (bo L-FABP), rat L-FABP (ra L-FABP) and nurse shark L-FABP (sk L-FABP) were aligned according to Schleicher et al. (1995). The deduced amino acid sequence of the cDNA (GenBank accession code U28756) of iguana basic L-FABP (Ap L-FABPb) was included in the alignment. Amino acid residues identical with those in catfish basic L-FABP are shown with a black background. A dot indicates a deletion/insertion. Sequence identities to catfish basic L-FABP are indicated.

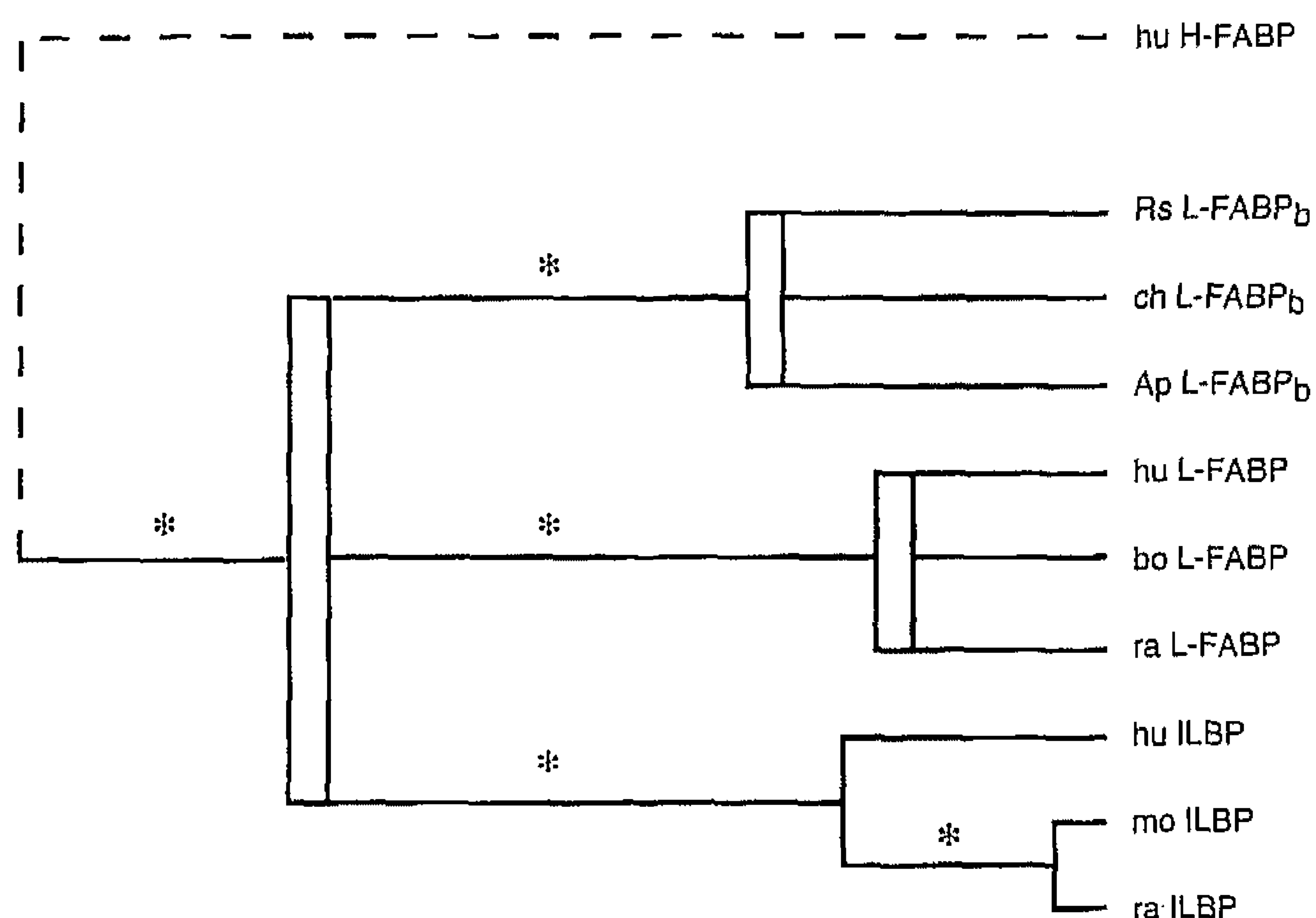


Fig. 4. Evolutionary relationship between members of the L-FABP/I-LBP subfamily. Three members of each branch are represented: catfish (Rs L-FABPb), chicken (ch L-FABPb) and iguana (Ap L-FABPb) basic L-FABP; human (hu L-FABP), cow (bo L-FABP) and rat (ra L-FABP) L-FABP; and human (hu I-LBP), mouse (mo I-LBP) and rat (ra I-LBP) I-LBP. Human H-FABP (hu H-FABP) is included as an outgroup sequence. Rectangles represent regions of the phylogeny where the branching order is unresolved (statistically non-significant). Asterisks denote the significance of each branch $P < 0.001$.

C3 and C4 was obtained by sequencing an additional peptide, Gx, obtained by the procedure of Krishna et al. (1991).

A summary of the sequence analyses and the resulting primary structure of catfish basic L-FABP are shown in Fig. 2. The protein consists of 125 residues and, on the basis of the sequence, the molecular mass was calculated as 13 899 Da. The calculated isoelectric point (9.1) agrees with that obtained experimentally (Di Pietro et al., 1996).

Evolutionary relationship. The alignment of the amino acid sequence of catfish basic L-FABP with those of FABP isolated from the livers of chicken, human, cow, rat and shark is shown

in Fig. 3. The deduced amino acid sequence of a cDNA (GenBank accession code U28756) from the iguana (*Anolis pulchellus*) was also included in the alignment. Identities between catfish basic L-FABP and the other FABP are indicated (Fig. 3). Catfish basic L-FABP appears to be more closely related to chicken and iguana basic L-FABP than to mammalian L-FABP. The low identity between catfish basic L-FABP and the FABP from nurse shark liver is not surprising since the latter does not belong to the L-FABP/I-LBP subfamily but to a subfamily including H-FABP and A-LBP (Medzihradszky et al., 1992; Schleicher et al., 1995).

Fig. 4 depicts the evolutionary relationships between the various members of the L-FABP/I-LBP subfamily, with human H-FABP being included as an outgroup sequence. The phylogeny inferred implies the early divergence of three branches corresponding to the basic L-FABP from catfish, chicken and iguana, the L-FABP from mammals, and the I-LBP. The branch corresponding to mammalian L-FABP appears to have diverged from that comprising catfish, chicken and iguana basic L-FABP before the fish-tetrapod split. This branching order suggests that basic L-FABP from lower vertebrates are not orthologs of mammalian L-FABP but products of a separate gene. To rule out the possibility that the inferred branching order is a consequence of unequal mutation rates, we compared the evolutionary distances between an outgroup protein (human H-FABP) and the different members of the L-FABP/I-LBP subfamily. This was done by means of the statistical test described by Shearer and Johnson (1993). No significant differences were found ($P > 0.1$ in all cases), implying that the mutation rates of members of the L-FABP/I-LBP subfamily are statistically indistinguishable.

The expression of basic L-FABP is restricted to liver. To research the expression pattern of the basic L-FABP in catfish tissues, the cytosolic fractions including 12–16-kDa proteins from heart, brain, stomach mucosa, intestinal mucosa, skeletal muscle, skin and liver were submitted to electrophoresis and immunoblotting. The rabbit anti-(catfish basic L-FABP) Ig did not detect the protein in any tissue other than liver thus indicat-

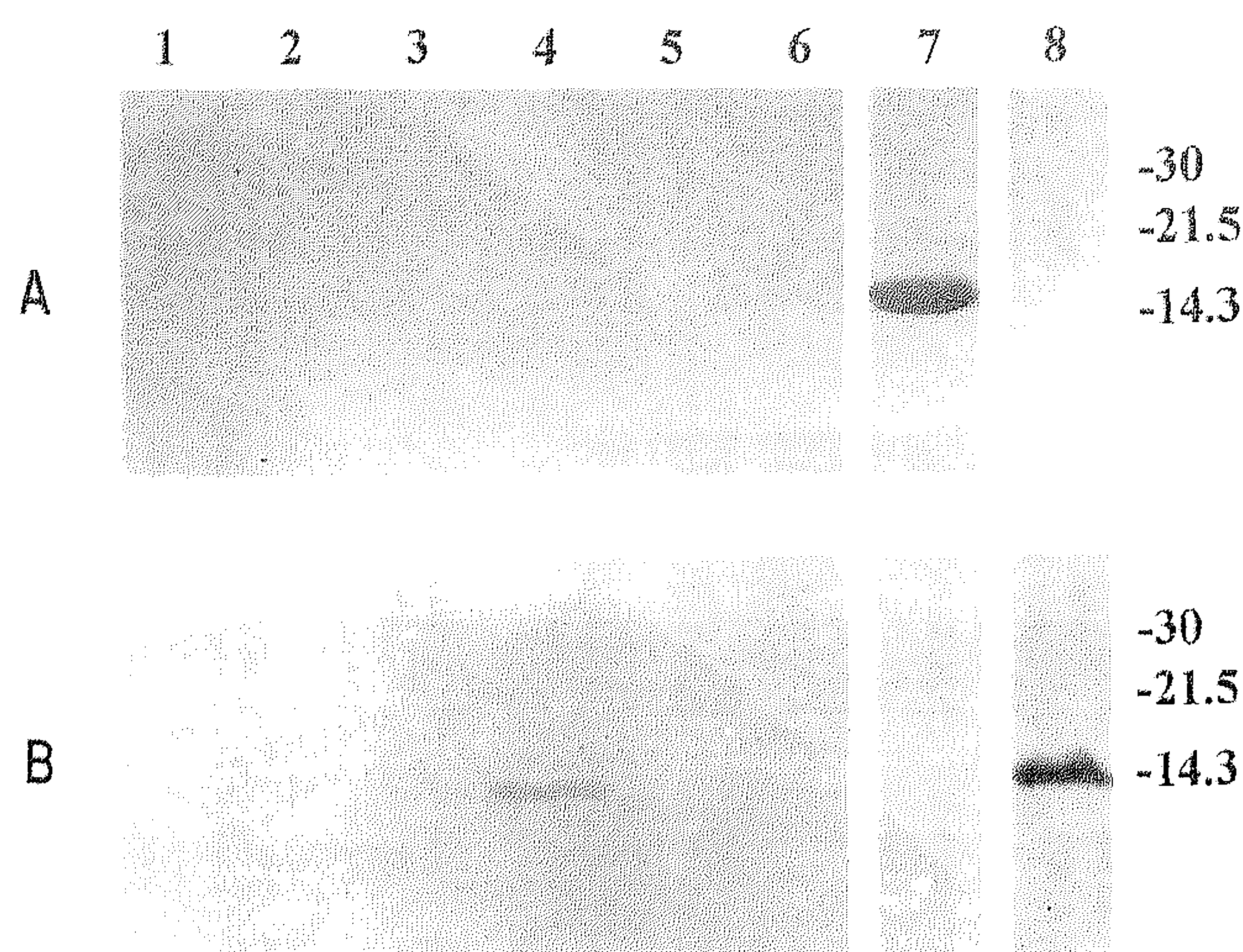


Fig. 5. Western blot analysis of the cytosolic fractions including 12–16-kDa proteins from different catfish tissues and from rat liver. About 5 μ g of the 12–16-kDa cytosolic proteins from catfish heart (lane 1), brain (lane 2), stomach mucosa (lane 3), intestinal mucosa (lane 4), skeletal muscle (lane 5), skin (lane 6) and liver (lane 7), and from rat liver (lane 8) were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Immunodetection was carried out by incubation with a polyclonal antibody to catfish basic L-FABP (A) or to rat L-FABP (B), then with goat anti-(rabbit Ig) Ig coupled to horseradish peroxidase. Immunocomplexes were visualized with 4-chloro-1-naphthol. The apparent molecular mass of marker proteins is indicated in kDa.

ing that basic L-FABP is expressed only in the liver (Fig. 5A). Additionally, the antibodies did not cross-react with rat L-FABP.

Identification of a 14-kDa protein from catfish intestine as a L-FABP. To check the possibility of an ortholog of mammalian L-FABP occurring in any catfish tissue, immunoblotting analyses were performed with antibodies to rat L-FABP (Fig. 5B). As reported previously (Di Pietro et al., 1996) these antibodies did not cross-react with catfish basic L-FABP. The antibodies recognized a 14-kDa protein from intestinal mucosa. To further characterize this protein, the 12–16-kDa fraction from intestinal cytosol was incubated with radiolabeled palmitic acid and subjected to anion-exchange chromatography (Fig. 6). As inferred from immunoblotting analysis with anti-(rat L-FABP) Ig, the 14-kDa protein was recovered in a fraction that displayed significant fatty-acid-binding activity (Fig. 6). For internal amino acid sequencing, the 14-kDa band was submitted to in-gel tryptic digestion following the procedure of Rosenfeld et al. (1992). The peptide mixture was fractionated by reverse-phase HPLC and four peptides were sequenced: YQLESQEGFVPP; SVSEIEQR; VTVTTGSK; and TVVTL.

A computer search for those fragments in the National Center for Biotechnology Information data banks, using the BLAST E-mail Server (Altschul et al., 1990) with a BLOSUM 62 matrix, showed considerable similarity with the corresponding sequences of other FABP. The highest scores corresponded to mammalian L-FABP. Peptide sequences were aligned with the corresponding fragments of the L-FABP/I-LBP subfamily members according to Schleicher et al. (1995) (data not shown). The sequences obtained for the catfish intestinal protein averaged 60% identity with the corresponding fragments in mammalian (human, cow, pig and rat) L-FABP, 40% with those of catfish, chicken and iguana basic L-FABP, and 25% with mammalian (pig, rat and mouse) I-LBP. Additionally, the tyrosine residue of peptide 1 corresponds to Tyr6 of mammalian L-FABP, a position that in most FABP, including basic L-FABP, contains a highly

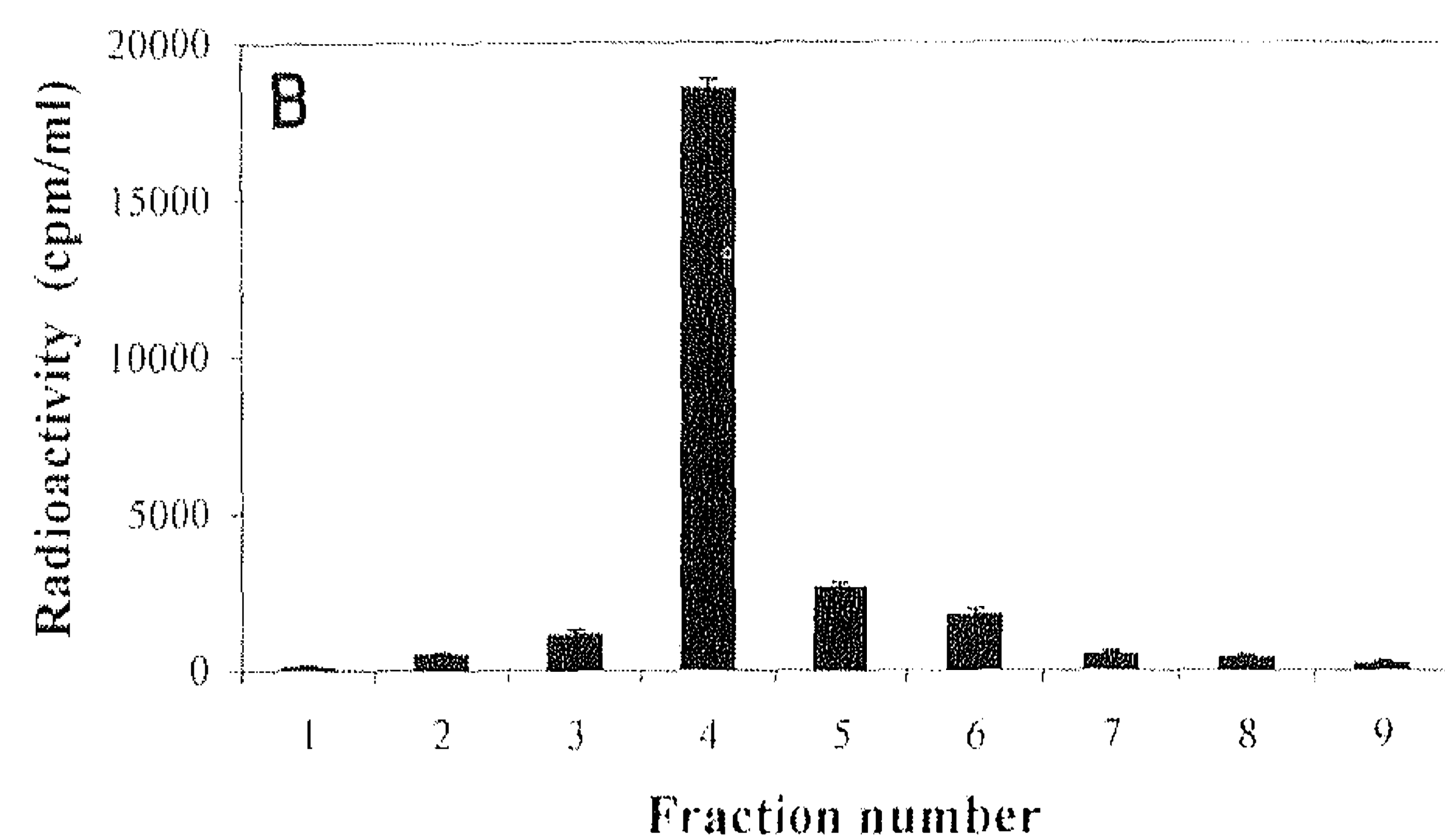
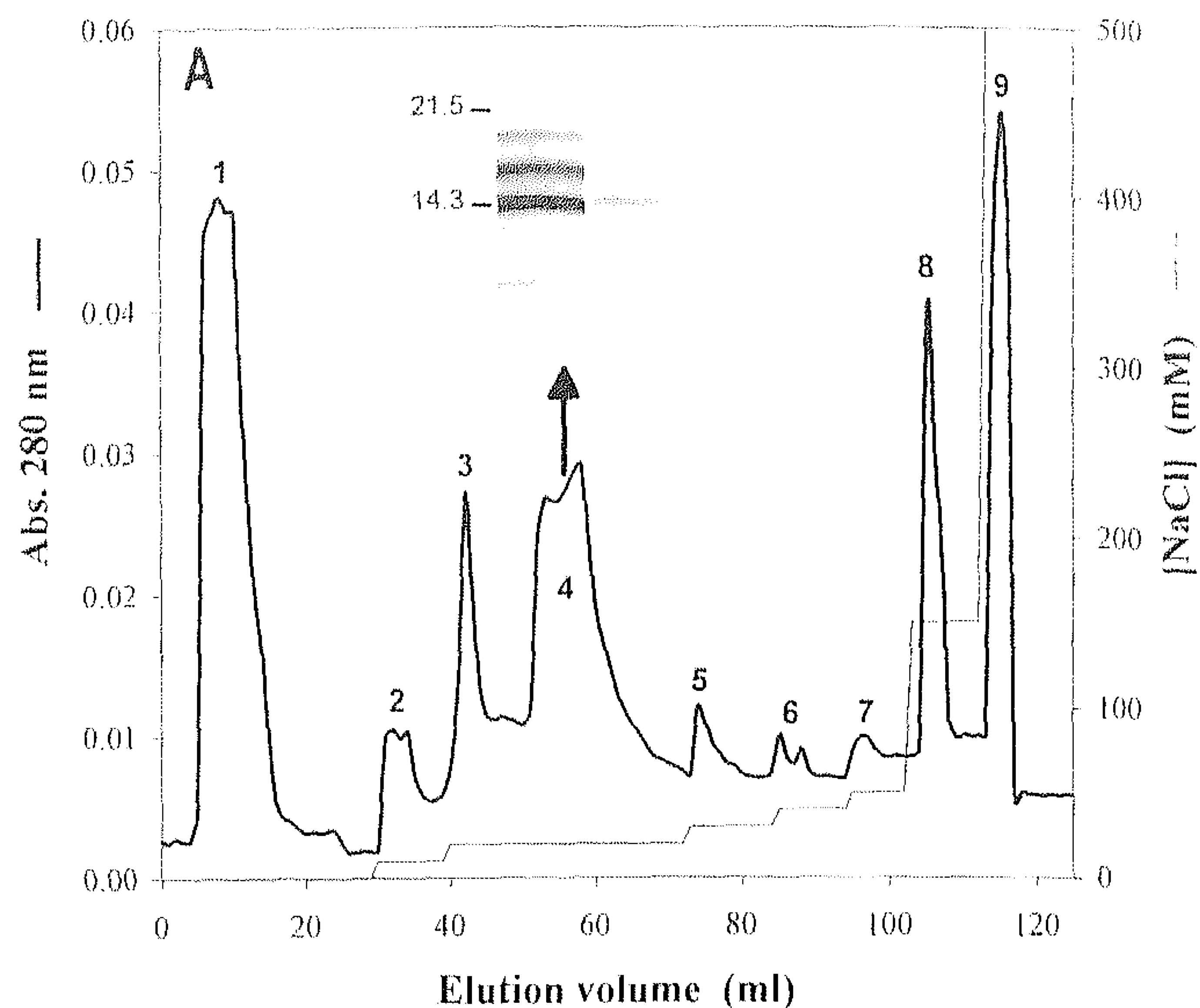


Fig. 6. L-FABP from catfish intestine. The cytosolic fraction containing 12–16-kDa proteins from catfish intestinal mucosa was incubated with [14 C]palmitic acid and loaded onto a DEAE-cellulose column previously equilibrated with 30 mM Tris/HCl, pH 8.5. The material bound to the column was eluted with 10, 20, 30, 40, 50, 150 and 500 mM NaCl in the same buffer. The eluted fractions were analyzed by absorbance (Abs.) at 280 nm. (A) and liquid scintillation counting (mean \pm SEM, $n = 2$) (B). Fraction 4 contained the bulk of the radioactivity and was studied by SDS/PAGE (A, inset, left lane) and immunoblotting (A, inset, right lane) with an antiserum to rat L-FABP. This fraction presents five bands, one of them recognized by the anti-(rat L-FABP) serum. The apparent molecular mass of marker proteins is indicated in kDa.

conserved tryptophan residue. Therefore, the 14-kDa protein isolated from catfish intestine probably represents the fish ortholog of mammalian L-FABP.

Fluorescent fatty-acid-binding measurements. Basic L-FABP displayed high affinity for *cis*- and *trans*-parinaric acids; fluorescence intensity increased in a fatty-acid-concentration-dependent manner and was saturable. A representative binding experiment for each fluorescent fatty acid is shown in Fig. 7. Non-linear-regression analyses yielded apparent dissociation constants (K_d) of $1.20 \pm 0.23 \mu$ M and $0.55 \pm 0.11 \mu$ M for *cis*- and *trans*-parinaric acid, respectively (mean \pm SEM, $n = 3$). Hill plots of these binding curves are also shown in Fig. 7. The slope of these Hill plots, the apparent order of the binding, was very close to 1 in both cases: 1.05 ± 0.10 for *cis*-parinaric acid and 0.94 ± 0.07 for *trans*-parinaric acid. Taking into account that FABP have a β -barrel structure with a central cavity in which two fatty acids cannot be independently accommodated, these results indicate a 1:1 binding stoichiometry (i.e. the protein contains only one fatty-acid-binding site for parinaric acids).

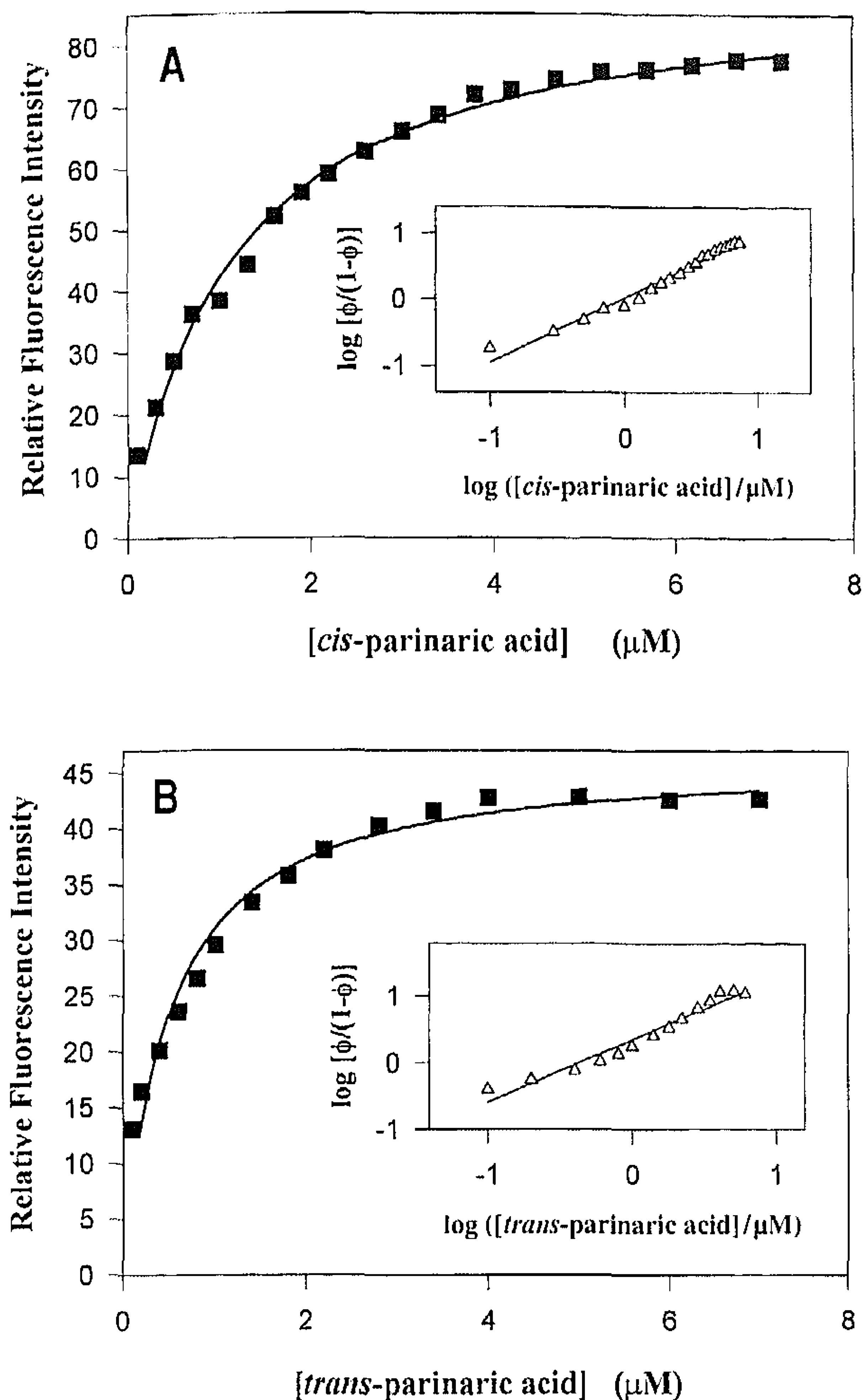


Fig. 7. Fluorescent-fatty-acid interaction with catfish basic L-FABP. Binding of *cis*- (A) and *trans*- (B) parinaric acid to basic L-FABP was determined by the increase in fluorescence intensity. Measured values of one representative experiment out of three (for each fatty acid) are shown, and the results of non-linear regression are shown as curves. To obtain the corresponding Hill plots (insets), the data were transformed as indicated in Materials and Methods.

Tryptophan-fluorescence studies. The effect of fatty acid binding on the conformation of catfish basic L-FABP was investigated by measuring the intrinsic fluorescence emission of its single tryptophan residue. The emission spectrum showed a maximum at 325 nm (data not shown). Neither the emission maximum nor the fluorescence intensity were affected by the addition of palmitic acid (up to 5 μM). This result suggests that basic L-FABP does not undergo any major conformational change upon fatty acid binding.

Fatty-acid-competition-binding assay. When catfish basic L-FABP was incubated with a solution containing 1 μM various fatty acids, basic L-FABP bound preferentially palmitic, stearic and oleic acids (Fig. 8). The affinity for long-chain polyenoic acids appears to be low.

DISCUSSION

According to their primary structure, known FABP from the livers of different species can be divided into three types: L-FABP from mammals; L-FABP from catfish, chicken and iguana; and the FABP from nurse shark. As mentioned above,

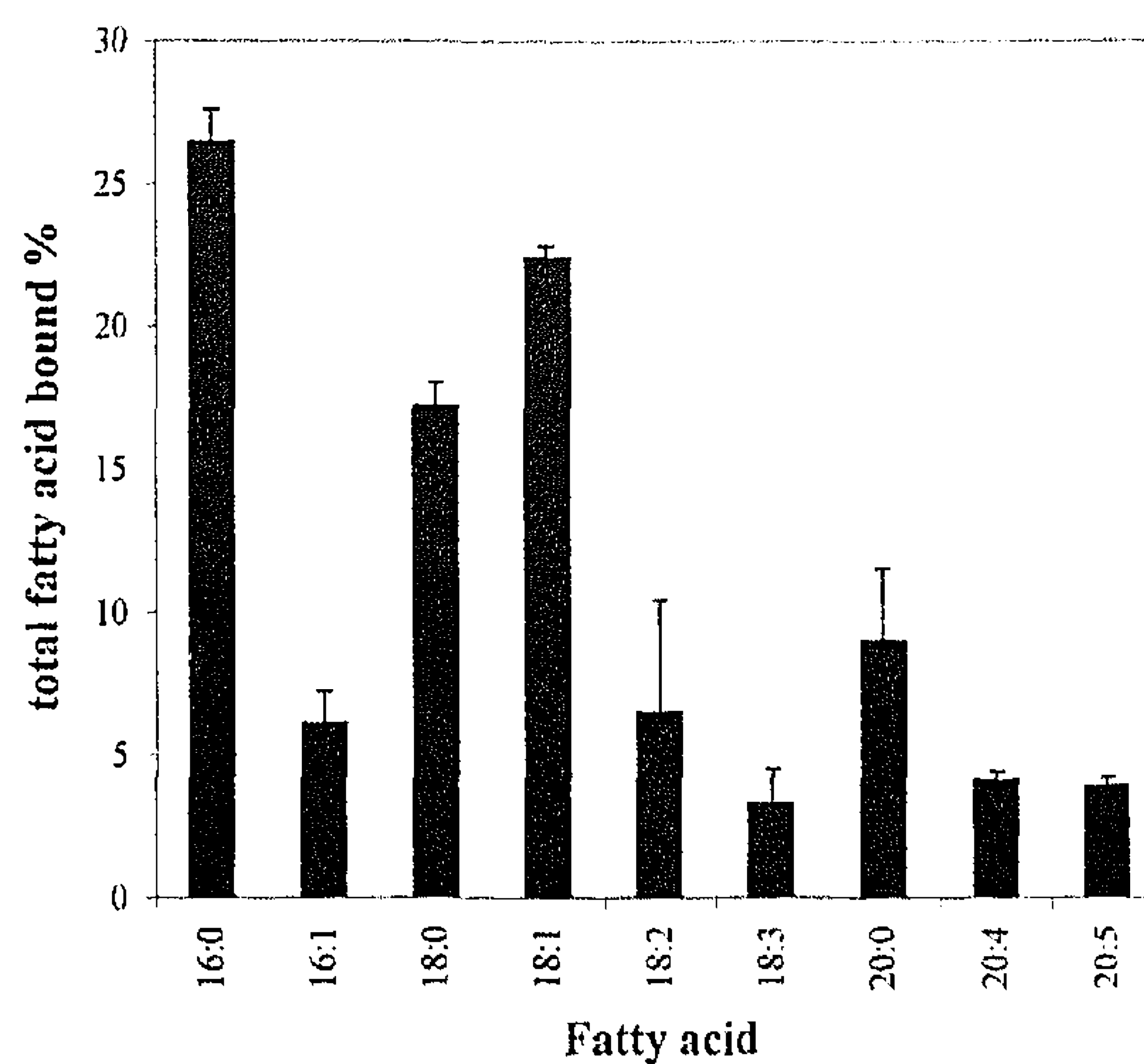


Fig. 8. Fatty-acid-competition-binding assay. 1 μM delipidated catfish basic L-FABP was incubated in 50 mM Tris/HCl, pH 7.4, containing 1% ethanol, and 1 μM palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidic, arachidonic and eicosapentaenoic acids. The free ligand fraction was removed and the fatty acids bound by basic L-FABP were methyl-esterified and analyzed by gas chromatography. The means ± SEM are given for three experiments.

the latter FABP does not belong to the L-FABP/I-LBP subfamily but is evolutionarily related to H-FABP and A-LBP (Medzihradszky et al., 1992). While this protein appears to be the only FABP expressed in the nurse shark liver (Medzihradszky et al., 1992), we have found recently that catfish liver contains, in addition to the basic L-FABP, two FABP that are closely related to H-FABP (Di Pietro and Santomé, 1996). Partial amino acid sequencing of an FABP from toad liver (Schleicher and Santomé, 1996) suggests that it belongs to the same group as catfish, chicken and iguana basic L-FABP (≈60% identity over 89 residues; data not shown).

Phylogenetic analyses of the family of intracellular lipid-binding proteins have shown previously that mammalian L-FABP and I-LBP belong to a subfamily that diverged from the other subgroups before the vertebrate-invertebrate split (Schleicher et al., 1995). Although the chicken basic L-FABP is part of this subfamily, it has been suggested that it is unlikely to be the avian counterpart of mammalian L-FABP but the product of a separate gene (Ceciliani et al., 1994; Schleicher et al., 1995). Results presented in this study provide further support for this proposal. Thus, the primary structure of the catfish basic L-FABP is more similar to that of the chicken basic L-FABP than to those of mammalian L-FABP (Fig. 3). Our phylogenetic analysis suggests that L-FABP from mammals diverged from basic L-FABP from catfish, chicken and iguana before the fish-tetrapod divergence, thus implying that L-FABP from mammals and those from catfish, chicken and iguana are paralogous proteins, i.e. they are encoded by two different genes. In support of this conclusion, we identified a 14-kDa protein from catfish intestine that is structurally more closely related to mammalian L-FABP and may correspond to the fish L-FABP ortholog. Further investigations will be required to establish whether a basic L-FABP homolog exists in mammals.

Catfish basic L-FABP differs from mammalian L-FABP not only in its primary structure but also in the following biochemical characteristics.

In the current study, *cis*- and *trans*-parinaric acids were used to characterize the ligand-binding properties of catfish basic L-FABP. Our results indicate that catfish basic L-FABP binds only one fluorescent fatty acid (Fig. 7), while rat L-FABP binds two

cis- or *trans*-parinaric acids/molecule (Nemecz et al., 1991a), and that catfish basic L-FABP has more affinity for *trans*-parinaric acid than for *cis*-parinaric acid in contrast to the rat L-FABP (Nemecz et al., 1991a). We were not able to detect, by tryptophan-fluorescence measurements, conformational changes in basic L-FABP when it bound palmitic acid though such changes had been reported for rat L-FABP (Nemecz et al., 1991b; Li and Ishibashi, 1992).

Catfish basic L-FABP competition-binding assays show more affinity for saturated and monounsaturated fatty acids (Fig. 8) while rat L-FABP presents more affinity for polyunsaturated fatty acids (Maatman et al., 1994). These results agree with those obtained using fluorescent fatty acids on account of *cis*-parinaric acid being used as a probe for unsaturated fatty acids and *trans*-parinaric acid for saturated ones (Nemecz et al., 1991a).

The immunological properties differ too. Catfish basic L-FABP is not recognized by the anti-(rat L-FABP) Ig, and rat L-FABP is not recognized by anti-(catfish basic L-FABP) Ig (Fig. 5).

Thompson et al. (1997) determined the crystal structure of rat L-FABP with two oleic acid molecules bound within the central cavity. The carboxylate of one oleic acid interacts with Arg122, while the carboxylate of the other oleate is exposed to the solvent but is involved in a network of hydrogen bonds. According to these authors, the required increase in FABP cavity volume to allow the binding of two fatty acid molecules, possibly by reductions in size or position changes at seven key residue locations, including Ser100 and Thr102. Larger residues are found in other FABP types. Thr102→Gln substitution in catfish basic L-FABP may account for the binding of only one fatty acid, possibly its steric effect may be increased, apart from the influence of other residues, by Ser100→His replacement. Scapin et al. (1990) reported the three-dimensional structure of chicken basic L-FABP and placed the fatty acid carboxylate close to Lys29 (Ceciliani et al., 1994). Catfish basic L-FABP has the same residue in this position. The fatty acid location proposed by Scapin et al. (1990) for chicken basic L-FABP differs from those of both oleic acids in rat L-FABP (Thompson et al., 1997). On the basis of the close amino acid sequence similarity between the basic L-FABP from chicken, catfish and iguana, it is likely that they have a similar binding site.

Taking into account that there are three FABP in catfish liver, it may be that each of them has a specific fatty-acid-binding affinity. Catfish basic L-FABP presents *in vitro* a marked specificity for C16:0, C18:0 and C18:1 (Fig. 8). It should be interesting to study the binding behavior of the other two FABP in catfish liver.

In conclusion, results reported here demonstrate that the basic L-FABP from catfish is structurally and functionally different from mammalian L-FABP, and that L-FABP and basic L-FABP are present in catfish, but in different tissues (intestine and liver). Although the physiological significance of two types of L-FABP in animals remains to be determined, their functional differences may be related to changes in lipid metabolism in the liver during the evolution of vertebrates.

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