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Muscle parvalbumin isoforms of *Clarias gariepinus*, *Heterobranchus longifilis* and *Chrysichthys auratus*: isolation, characterization and expression during development

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ABSTRACT

The white-muscle parvalbumin isoforms of *Clarias gariepinus*, *Heterobranchus longifilis* and *Chrysichthys auratus* were purified and their physicochemical parameters determined. The three catfish isoforms are distinct but those of *C. gariepinus* and *H. longifilis* are more similar.

In the course of development, the successive appearance of larval and adult parvalbumins was observed. Larval isoforms (PA I, PA IIa, PA IIb) displayed a lower isoelectric point (pI) and molecular mass than adult ones (PA IIc, PA IIIa, PA IIIb, PA III, PA IV). The PA IIa isoform appeared as an omnipresent typical larval isoform. PA IIb appeared mostly larval, being insignificant in adult specimens; its physicochemical features were the same in the three catfish species. In *Chrysichthys auratus*, there were three PA II isoforms, one appearing as an adult isoform (PA IIc). The fact that the two types of parvalbumin isoforms appear at different times should reflect specific physiological needs (mobility, feeding) of different developmental stages.

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Introduction

Parvalbumins (PA) are soluble, low molecular weight (10 000–12 000 Da) calcium-binding proteins present in the sarcoplasm of vertebrate skeletal muscles (Focant & Pechère, 1965). They are also present in low amount in non-muscle cells, especially nerve cells (Baron *et al*., 1975). They are particularly abundant in the white fast-contracting muscles of amphibians and fishes (≤ 0·001 mol kg^{-1} tissue), present in lesser amount in slow-twitch and cardiac muscles, and absent from smooth muscles (Gerday, 1982). They display homology with other calcium-binding proteins such as troponin-C, calmodulin, and myosin light chains with which they share a common ancestry.

Parvalbumins from various sources share common properties: like many other calcium-binding proteins they all possess the EF-hand structural motif. They bind two molecules of Ca^{2+} per mole with high affinity (K_{ass} = 10^8 M^{-1}) or two molecules of Mg^{2+} per mole with a lower affinity (K_{ass} = 10^4 m^{-1}). They show a distinctive ultraviolet (UV) spectrum due to a high ratio of phenylalanine to tyrosine and tryptophan. They appear to act as calcium buffers. The positive correlation between the contraction–relaxation speed of a muscle and the concentration of parvalbumins in the muscle suggests a role of parvalbumins in muscle relaxation: these proteins can shuttle calcium from the contractile machinery to the storage site in the sarcoplasmic reticulum. In parallel with the primary mechanism of Ca^{2+} sequestration, the Ca^{2+} pump of the sarcoplasmic reticulum, they accelerate muscle relaxation by sequestering Ca^{2+} in exchange for Mg^{2+} (Gillis & Gerday, 1977; Heizmann *et al*., 1982; Ushio & Watabe, 1994; Jiang *et al*., 1996). This relies on competition of Mg^{2+} for Ca-binding sites on parvalbumins, its binding affinity being 10^4 times lower than that of Ca^{2+}. In cold-blooded animals where the Ca^{2+} pump of the sarcoplasmic reticulum is less efficient, parvalbumins act to promote relaxation at a rate limited by dissociation of Mg^{2+} from parvalbumins.

Parvalbumins, moreover, have been recognized as powerful allergens causing hypersensitivity in fish (Aas & Jebsen, 1967), as recently confirmed for several fish species (Bugajska-Schretter *et al*., 1998). This type of allergy can be a serious health problem in countries where fish farming accounts for a large percentage of the food supply.

Whereas mammals express a single muscle isoform, adult fish and amphibians express multiple, species-specific skeletal-muscle isoforms (up to five in some fish). These isoforms can belong to two sub-lineages: alpha-PA (isoelectric point, pI > 5·0) and beta-PA (pI < 5·0) (Goodman *et al*., 1979). They constitute a convenient and effective tool for identifying fish species (Focant *et al*., 1988).

Little is known about the role of particular isoforms. Ogawa & Tanokura (1986) suggest that the various parvalbumin isoforms found in amphibians may have kinetically different temperature-dependent Ca^{2+}- and Mg^{2+}-binding sites, reflecting molecular adaptation to changes in the temperature of their environment as the amphibians evolved (Gerday *et al*., 1991). Rodnick & Sidell (1995) have confirmed in muscles of striped bass *Morone saxatilis* (Walbaum) a change in total

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parvalbumin concentration linked to body size and thermal acclimation and affecting mainly one of the two isoforms present.

Parvalbumin regulation during ontogeny has been studied in frogs (Kay *et al*., 1987; Schwartz & Kay, 1988), chickens (Le Peuch *et al*., 1979), and mammals (Celio & Heizmann, 1982; Leberer & Pette, 1986). In the frog it was shown that parvalbumins are not involved in the early embryonic stages; they are detected first at a stage that coincides with the differentiation of myotomal muscles and with activity upon external stimulation. In chicken development, parvalbumins are not detectable until just before hatching, whereas the majority of contractile proteins are synthesized upon myoblast fusion. In the rat and rabbit, parvalbumin synthesis is switched on almost immediately after birth, synchronously with the onset of high-frequency neural activity and with the differentiation of immature fibres into fast-twitch glycolytic fibres.

For a few years we have been studying parvalbumin isoform distribution in the muscles of developing teleosts. Fish constitute a good material for studying the development and function of skeletal muscle, thanks to their continuous growth and to the homogeneity of their axial musculature. Given the polymorphism of parvalbumins, one of the most intriguing aspects is sequential expression of different isoforms according to the developmental stage. In four fish species examined [*Barbus barbus* (L.), *Oncorhynchus mykiss* (Walbaum), *Salmo trutta* L., and *Dicentrarchus labrax* (L.)], a similar pattern of differential parvalbumin isoform expression has been observed: the PA II isoform appears first and predominates during the larval stage; other isoforms are expressed later and are typical of juvenile and adult muscle (Focant *et al*., 1992; Huriaux *et al*., 1996). More precise examination has revealed three forms of PA II (PA IIa, PAIIb, and PA IIc) in the muscles of developing barbels; all three turned out to be present during the larval stages, but only PA IIc and chiefly PA IIb appeared as early forms, while PA IIa remained present into the adult period (Huriaux *et al*., 1997). We thus proposed that the two groups of isoforms (larval and adult parvalbumins) could have different Ca^{2+}-binding properties and selective effects on white-muscle contraction parameters, presumably linked to the physiological requirements of the developing muscle. These parvalbumin isoforms were isolated and analysed but no significant physicochemical difference was found.

To gain further insight into parvalbumin isoform differentiation and function, we isolated, characterized, and compared white-muscle parvalbumins from adult specimens of three African catfish species (Siluriforms). The species examined belong to two different families: the Clariidae, represented by *Clarias gariepinus* (Burchell, 1822) and *Heterobranchus longifilis* Valenciennes, 1840, and the Claroteidae, represented by *Chrysichthys auratus* (Geoffroy St. Hilaire, 1808). This work was a part of a multidisciplinary and multilaboratory study of the biology of African catfish. The three chosen species are bred commonly in Africa and have a great economic value for these countries. Using high-resolution polyacrylamide gel electrophoresis (PAGE) the kinetics of parvalbumin isoform appearance has been compared in the course of the larval, juvenile, and adult stages of the fish. Also the utility of parvalbumin isoform profiles was examined in species identification in particular from fish pieces or processed muscles. A short note has been published already on developing *C. gariepinus* muscle proteins (Focant *et al*., 1996).

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Materials and methods

MATERIALS

To make it easier to compare fish of different breeds, growth stages were expressed in relation to standard fish length rather than age. The morphological stages of post-hatching development were established by ourselves and by Surlemont & Vandewalle (1991) for *Clarias*, by Legendre & Teugels (1991) for *Heterobranchus*, and by Laleye & Philippart (1993) for *Chrysichthys*.

Adult *Clarias* specimens and fertilized eggs were from the University of Leuven. Two batches of eggs were reared in our laboratory under controlled conditions (t = 27° C) up to 11 cm (day 104). The larval-to-juvenile stage transition occurred around 1·5–2·3 cm (days 27–34) under our conditions. Two adult specimens were studied (15·5 and 32 cm).

Heterobranchus specimens were obtained from experimental hatcheries at CERER-LDPA, Tihange, Belgium and ORSTOM-GAMET, Montpellier, France. The latter batch of developing fish was sampled from 0·3 to 12·5 cm (day 1–day 70) and the former batch was sampled to 13 cm (day 100). The temperature was approximately the same in both cases (27° C). The larvae became juvenile at 1·5–1·8 cm (day 17). Adult specimens (length 28–52 cm) were obtained from CERER-LDPA.

Chrysichthys larvae up to 1·35 cm (day 22), juveniles from 3 cm, and adults from around 12 cm were fished and bred by P. Laleye (Cotonou, Benin). They were hatched under running water at 27° C. The larva-to-juvenile transition occurred between 1·3 and 3 cm.

All specimens were stored frozen at −18° C for weeks without significant changes in protein composition. Dissection of muscle samples from developing fish was adapted to specimen size. For fish ≤ 1 cm, 30 specimens were pooled and the head, yolk sac, and tail were removed with jeweller's forceps. For fish ≤ 5 cm, four to 20 specimens were pooled; from these, the entire trunk muscles were collected after removal of the skin along with the single layer of superficial muscle fibres. For each chosen length exceeding 5 cm, one fish was dissected. When the fish became much larger, only a piece (1 g) of muscle located in front of the dorsal fin was sampled; at this spot, the parvalbumin distribution and concentration are the same as in a total trunk-muscle extract (Huriaux *et al*., 1997). Samples were handled on ice for minimum proteolytic breakdown. Dissected muscles were treated and stored at −20° C in a preservative glycerol solution until used as described previously (Focant *et al*., 1992).

METHODS

CRUDE PARVALBUMIN EXTRACTS

Sarcoplasmic extracts were obtained by centrifuging (17 500 ***g*** for 20 min at 4° C) thawed muscle homogenates in preservative glycerol solution. They were heated at 100° C for 5 min to eliminate labile sarcoplasmic proteins, parvalbumins being unaffected, centrifuged (17 500 ***g*** for 10 min),

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diluted with 2 vol incubation solution, and electrophoresed on polyacrylamide gels. Total sarcoplasmic protein concentrations were measured before heating by the Bradford method (Bradford, 1976), using a solution of BSA as the standard.

ISOLATION OF PARVALBUMIN ISOFORMS

Parvalbumin isoforms of the three fish species were isolated according to Huriaux *et al*. (1996) from 200 g of fresh *Clarias* white trunk muscle (50 cm fish), 200 g of frozen *Heterobranchus* muscle (30 cm fish), and 88 g of frozen *Chrysichthys* muscle (15 cm fish). The length of these adult specimens used for the parvalbumin isolation was chosen in order to obtain the highest amounts of larval and adult isoforms at the same time. Parvalbumins were isolated from sarcoplasmic extracts by precipitation with $(NH_4)_2SO_4$ between 50% and 90% saturation, heating to 60° C, and chromatography on a Sephacryl S-100 column. The parvalbumin isoforms were separated on a DEAE-cellulose (Whatman DE 52) column (2·5 × 30 cm) equilibrated in a buffer containing 0·015 M piperazine-HCl and 0·001 M beta-mercaptoethanol (pH 5·7). They were eluted with a linear NaCl gradient (400 ml buffer–400 ml buffer plus 0·15 M NaCl). Fractions containing each isoform were pooled, concentrated on an AMICON YM3 membrane, desalted, and lyophilized.

In the case of *Chrysichthys*, an additional isoform separation was performed by preparative PAGE with the Model 491 Prep Cell from BIO-RAD. Continuous PAGE was carried out through a cylindrical gel (3·7 × 9 cm) under non-denaturing conditions [10% acrylamide, 0·26% bis-acrylamide, 10% (v/v) glycerol, 0·019 M Tris, 0·119 M glycine, pH 8·6 buffer] at room temperature with recirculating buffer. Approximately 60 mg of sample equilibrated in gel buffer was loaded on top of the gel after a pre-run, and continuous elution was monitored by UV absorbance. Peak fractions were pooled, desalted, and lyophilized.

ANALYTICAL METHODS

Analytical PAGE separations of parvalbumin isoforms were performed in a BIO-RAD Mini-PROTEAN II cell (vertical plate 0·075 × 8·3 × 6 cm) under various conditions. The non-denaturing system contained 10% acrylamide, 0·26% bis-acrylamide, 10% (v/v) glycerol, 0·019 M Tris, 0·119 M glycine, pH 8·6 (Focant *et al*., 1992). Isoelectric focusing (IEF-PAGE) was carried out on a 7·1% acrylamide, 0·4% bis-acrylamide gel containing 1·6% PHARMACIA ampholine pH 4–6, 0·4% PHARMACIA ampholine pH 3·5–10, and 8 M urea (Huriaux *et al*., 1996). Discontinuous SDS-PAGE was done in gels containing 20% acrylamide and 0·1% bis-acrylamide at pH 8·4 according to Laemmli (1970). Non-denaturing and SDS gels were stained with coomassie brilliant blue R-250 and IEF gels with a mixture containing 0·04% coomassie brilliant blue R-250, 0·05% crocein scarlet 7B, 0·5% $CuSO_4$, 27% isopropanol, 10% acetic acid. The relative contents in each isoform (expressed in arbitrary units for a same total sarcoplasmic protein content) and the per cent contribution of each isoform to the total parvalbumin content were evaluated by computing the densitometer traces of the electrophoretograms (Imaging Densitometer BIORAD model GS-670). Measurements were made on at least three gels.

Apparent relative molecular masses (M_r) were determined by two independent methods: SDS-PAGE in the presence of molecular weight standards (bovine pancreas ribonuclease from

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Boehringer, 13 700 Da; equine heart cytochrome-C from Calbiochem, 12 270 Da; carp-muscle parvalbumin PA IV, 11 430 Da) and electrospray ionization mass spectra (ESI-MS) obtained with a VG Platform instrument (FISONS) equipped with an ESI ion source after dissolving the samples in 50% acetonitrile, 0·1% formic acid.

Isoelectric points (pI), UV spectra, and sulphydryl groups were determined as described by Huriaux *et al.* (1996).

Results

PARVALBUMIN IDENTIFICATION

Parvalbumins from larval and adult catfish were separated by non-denaturing PAGE. The trunk muscle in *Clarias* larvae contains parvalbumins PA I and PA II [**Fig. 1(a)**]; in the adult, parvalbumins PA I, PA II, PA IIIa, and PA IIIb are found [representing respectively 7·9% (S.D. = 1·25), 29·9% (S.D. = 0·83), 52·4% (S.D. = 3·65), 9·8% (S.D. = 1·88), n = 4]. *Heterobranchus* also displays four parvalbumins, but PA IV replaces PA IIIb; PA III predominates in the adult (PA I: 6·1%, S.D. = 0·68; PA II: 14·3%, S.D. = 0·62; PA III: 71·1%, S.D. = 2·13; PA IV: 8·5%, S.D. = 2·46; n = 4). In *Chrysichthys*, the larvae possess only PA II; this form remains predominant in the adult but PA III is also present (PA II: 86·46%, S.D. = 0·75; PA III: 13·54%, S.D. = 0·75; n = 7). The presence of the reducing agent beta-mercaptoethanol (0·003 M) did not modify migration of the parvalbumins. All the bands detected after SDS-PAGE correspond to molecular masses in the range typical of parvalbumins (not shown). IEF-PAGE allowed an extra and quite different separation, highlighting the polymorphism of larval PA II [**Fig. 1(b)**]. The various isoforms were identified by cutting out the PA bands obtained on non-denaturing polyacrylamide gels at several development stages and subjecting them to IEF-PAGE.

Clarias and *Heterobranchus* larvae contain two PA II isoforms, PA IIa and PA IIb. Only PA IIa remains in the adult. In *Chrysichthys*, both PA IIa and PA IIb are present in the larvae, PA IIa and PA IIc in the adult. The evolution of parvalbumin isoform levels in the course of development is examined in more detail below.

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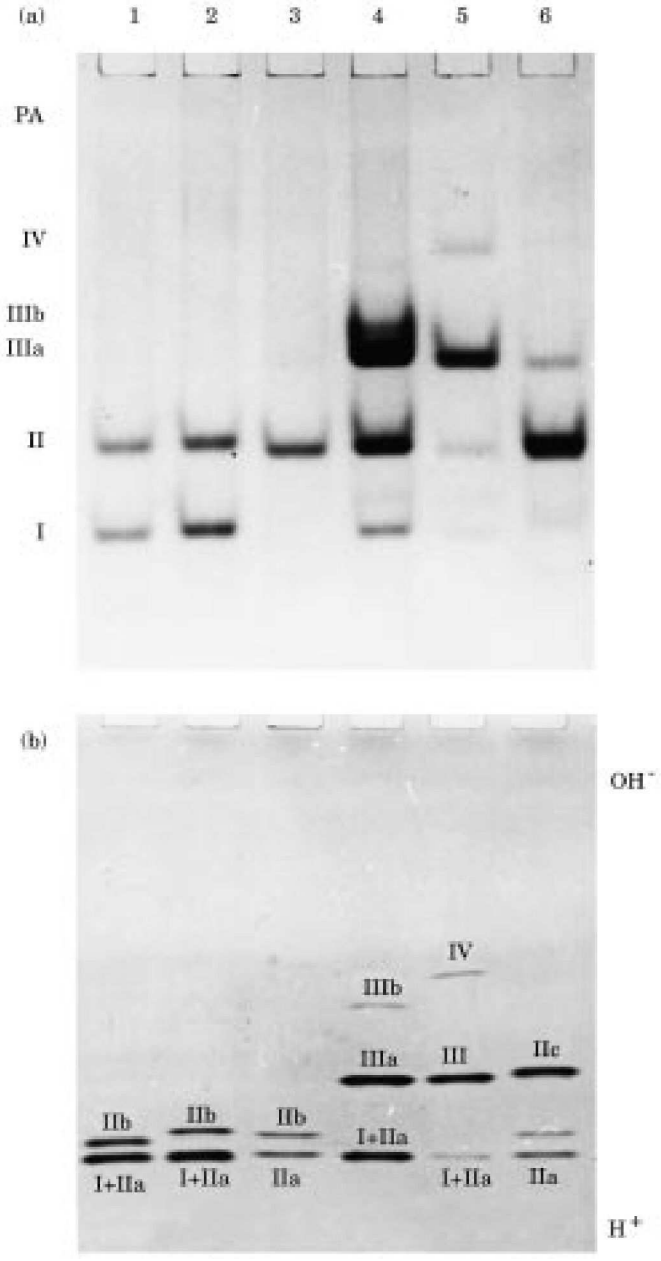
Fig. 1. Parvalbumin isoform distribution profiles for three catfish species (larval and adult). (a) non-denaturing PAGE, pH 8·6; (b) IEF-PAGE. (1) *Clarias*, 1·5 cm; (2) *Heterobranchus*, 1·2 cm; (3) *Chrysichthys*, 1·25 cm; (4) *Clarias*, 32 cm; (5) *Heterobranchus*, 28 cm; (6) *Chrysichthys*, 25 cm.



PARVALBUMIN ISOLATION

In *Clarias* [**Figs 2(a)** and **4(a)**], four isoforms eluted in the order: PA IIIb, PA IIIa, PA II, and PA I, corresponding respectively to 44, 163, 35, and 5 mg. In *Heterobranchus* [**Figs 2(b)** and **4(b)**], the forms PA IV (73 mg), PA III (246 mg), PA II (33 mg), and PA I (9 mg) were collected successively. In both of these fish, PA I was isolated in very little amount and on the non-denaturing gel it appeared

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contaminated by an additional band, slightly faster than PA II and representing 16% of the whole peak in *Clarias*-derived samples and 33% in *Heterobranchus*-derived samples. The pI of this band (4·62) and comigration experiments in IEF showed unambiguously that it is not PA IIb (pI 4·58) (**Table I**). The nature of this contaminant remains unknown; it could explain the abnormally high absorption of PA I near the end of the UV spectrum (290–300 nm). In these two fish, the PA II peak corresponds to the PA IIa isoform. In the case of *Chrysichthys* [**Figs 2(c)** and **4(c)**], two well-separated peaks eluted from the DEAE-cellulose column. When examined by non-denaturing PAGE, the second peak (45 mg) appeared to consist of PA II while the first peak (123 mg) appeared heterogeneous: it contained PA III but also another form of PA II, differing from the peak-2 PA II by a slightly lower electric charge [**Fig. 4(c)**, lanes 2–3]. IEF-PAGE revealed that the first peak contained the isoform PA IIc and the second peak PA IIa. In order to isolate the two parvalbumins of peak 1, we subjected this mixture to preparative PAGE, using analytical running conditions. This made it possible to distinguish PA III from PA II. The technique allowed separation of PA IIc and PA III with a good yield (38 and 44 mg, respectively) [**Figs 3** and **4(c)**, lanes 4–5].

IEF analysis of all parvalbumins isolated from the three catfish confirmed the purity of these proteins observed on non-denaturing PAGE, except for PA I with its unknown contaminant (not shown).

PHYSICOCHEMICAL CHARACTERIZATION

Parvalbumin isoforms PA IIb, present only during the larval stage (see below), was obtained by cutting the PA II band separated by non-denaturing PAGE or IEF-PAGE from larval specimens, then examined by IEF or SDS-PAGE (**Table I**).

Each isoform migrated as a single band on SDS-polyacrylamide gels, to a position corresponding with a molecular mass in the range of 11 300–12 000 Da. PA IIIb from *Clarias* and PA IV from *Heterobranchus* displayed the highest molecular masses. ESI-MS was used to measure very accurately the molecular mass of all isolated isoforms. The M_r values obtained by the two independent techniques were quite similar. The main discrepancies concerned PA I from *Clarias* and *Heterobranchus* and PA IIa from *Chrysichthys*, but PA I was not absolutely pure, being contaminated by foreign material as confirmed by the UV spectra.

After IEF-PAGE, each parvalbumin appeared homogeneous. Isoforms PA I and PA IIa focused at nearby positions, identical for the three catfish. The position at which PA IIb focused was also about the same for the three fish. *Clarias* PA IIIa cofocused with *Heterobranchus* PA III and with the adult isoform PA IIc of *Chrysichthys*. The only difference between the two Clariidae concerned *Heterobranchus* PA IV which exhibited a higher pI (5·21) than *Clarias* PA IIIb (5·05), as expected from the results of non-denaturing PAGE. *Clarias* PA IIIb and *Heterobranchus* PA IV probably belong to the alpha-parvalbumin family, characterized by pI > 5·0.

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Fig. 2. DEAE-cellulose chromatography of the parvalbumin fraction (collected on sephacryl S-100) obtained from (a) *Clarias*, (b) *Heterobranchus*, (c) *Chrysichthys*. The column (2·5 × 30 cm) was equilibrated in a buffer (pH 5·7) containing 0·015 M piperazine-HCl, and 0·001 M beta-mercaptoethanol. Gradient: 400 ml buffer–400 ml buffer plus 0·15 M NaCl (broken line). Fractions: 6 ml. Flow-rate: 35 ml h^{-1}. Fractions pooled are indicated.

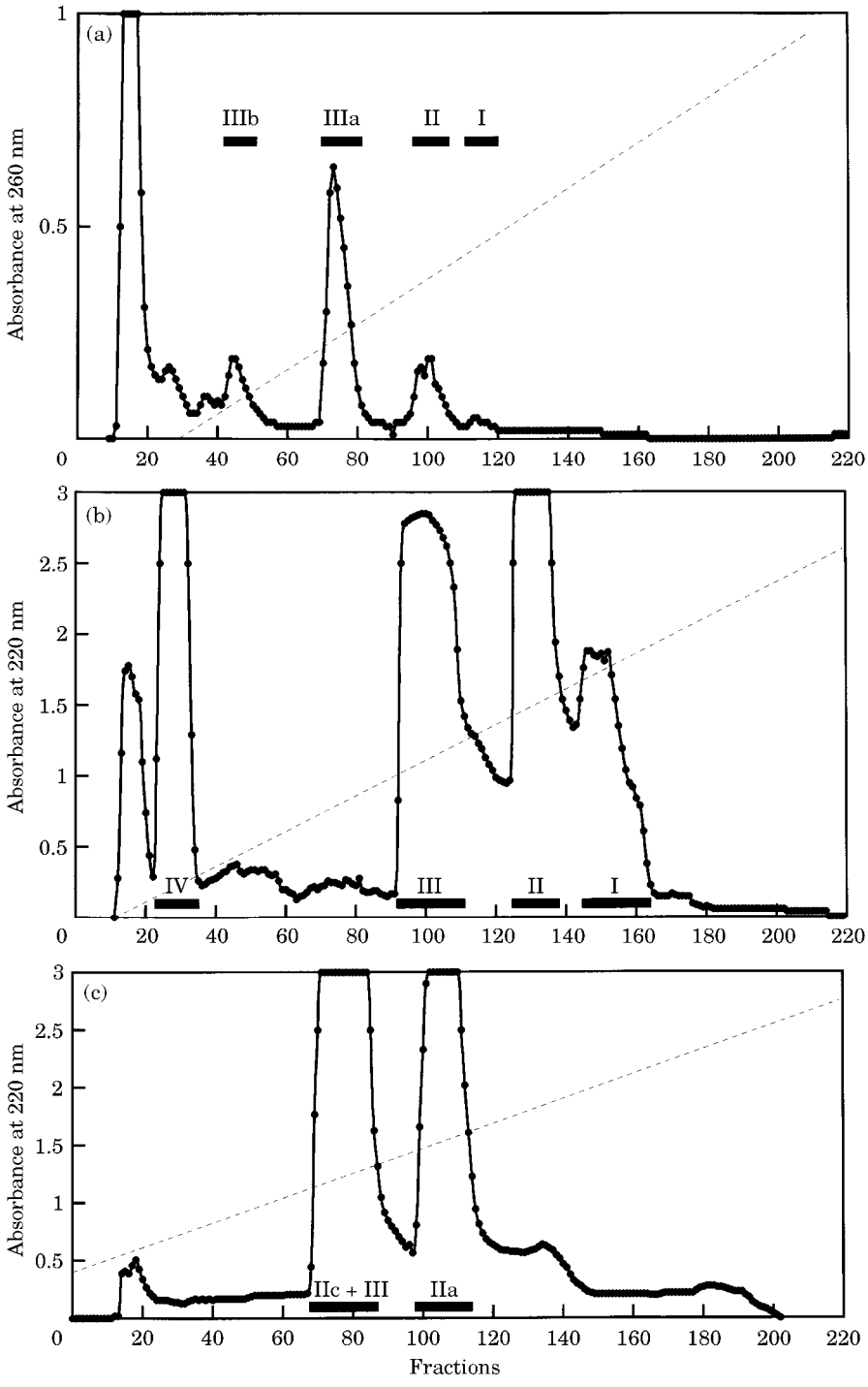

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Table I. Physicochemical properties of the isolated parvalbumin isoforms.

PA isoforms	Molecular mass		pI	Estimated no. of AA			PA type
	SDS	ESI-MS		Tyr	Trp	Cyst	
Clarias gariepinus							
PA IIIb	12 050 ± 30	12 063 ± 1·58	5·05 ± 0·02	0	0	0	A
PA IIIa	11 500 ± 40	11 525 ± 0·43	4·77 ± 0·04	0	0	1	A
PA IIb	11 410 ± 10		4·56 ± 0·04				L
PA IIa	11 340 ± 20	11 392 ± 1·08	4·51 ± 0·03	1	0	1	L
PA I	11 410 ± 20	11 284 ± 1·06	4·50 ± 0·03	1 (1·5)	0	1	L
Heterobranchus longifilis							
PA IV	12 000 ± 50	12 030 ± 1·83	5·21 ± 0·03	0	0	0	A
PA III	11 480 ± 30	11 481 ± 1·28	4·76 ± 0·05	0	0	1	A
PA IIb	11 420 ± 20		4·58 ± 0·05				L
PA IIa	11 380 ± 20	11 437 ± 0·75	4·51 ± 0·06	1	0	1	L
PA I	11 420 ± 20	11 316 ± 1·38	4·50 ± 0·06	1 (1·8)	0	1	L
Chrvsichthvs auratus							
PA III	11 470 ± 40	11 448 ± 0·86	4·58 ± 0·02	1	0	1	A
PA IIc	11 710 ± 40	11 648 ± 1·85	4·76 ± 0·02	0	0	1	A
PA IIb	11 370 ± 25		4·57 ± 0·02				L
PA IIa	11 510 ± 30	11 237 ± 4·78	4·51 ± 0·03	1	0	2	L

PA, Parvalbumin; SDS, sodium dodecylsulphate; ESI-MS, electrospray ionization mass spectrometry; AA, amino acid; Tyr, tyrosine; Trp, tryptophan; Cyst, cysteine; A, adult parvalbumin isoform; L, larval parvalbumin isoform.

Fig. 3. Preparative PAGE elution profile (non-denaturing conditions, pH 8·6) of *Chrysichthys* parvalbumin peak 1 (fractions 69–86 from a DEAE-cellulose column). Fractions: 2·5 ml. Flow-rate: 18 ml h^{-1}. Running conditions: 100 V constant voltage. Fractions pooled are indicated.

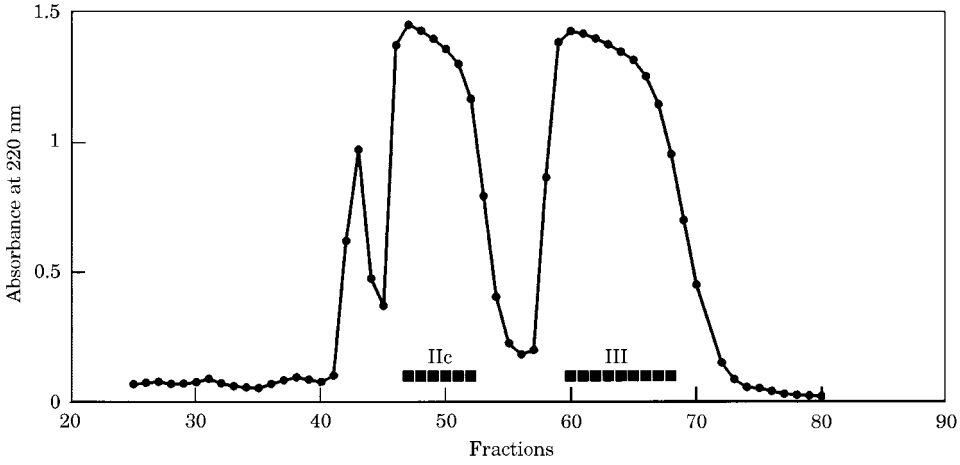

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Fig. 4. PAGE patterns (non-denaturing conditions, pH 8·6) obtained at the different stages of parvalbumin isoform purification. (a) *Clarias* (1) sephacryl S-100 parvalbumins, DEAE-cellulose fractions (2) PA IIIb; (3) PA IIIa; (4) PA II; (5) PA I + contaminant. (b) *Heterobranchus* (1) sephacryl S-100 parvalbumins, DEAE-cellulose fractions (2) PA IV; (3) PA III; (4) PA II; (5) PA I + contaminant. (c) *Chrysichthys* (1) sephacryl S-100 parvalbumins, DEAE-cellulose (2) PA IIc + PA III; (3) PA IIa; (4) and (5) preparative PAGE fractions, PA IIc and PA III.

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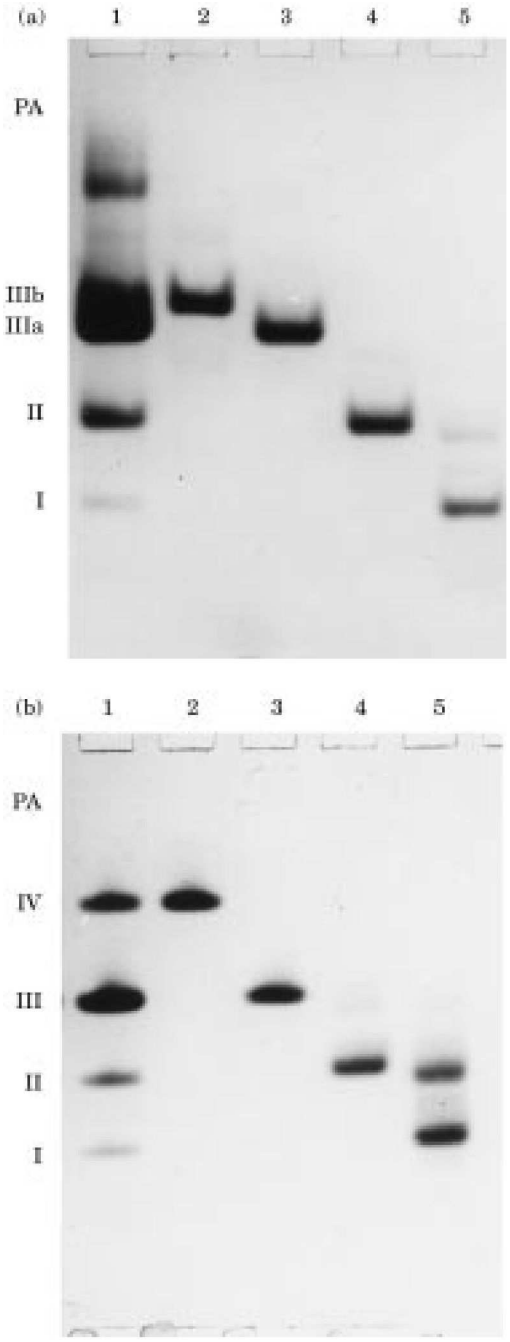

F . 4. (a) and (b).

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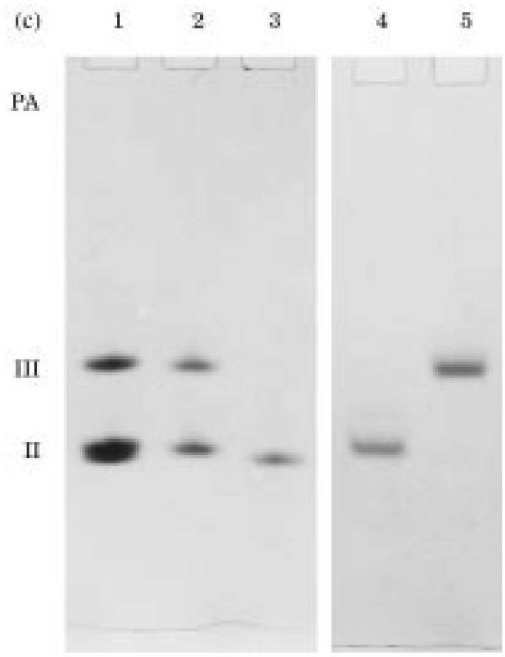

F . 4. (c).

The UV spectra of all isoforms displayed the usual bands typical of the phenylalanine residues that characterize parvalbumins [**Fig. 5(a)**–**(c)**]. *Clarias* PA I and PA IIa, *Heterobranchus* PA I and PA IIa, and *Chrysichthys* PA IIa and PA III each contain one tyrosine residue, calculated from the spectra. The PA I isoforms of *Clarias* and *Heterobranchus* appeared to have more than one tyrosine residue (1·5 and 1·8, respectively), but they probably contain only one, the excess being due, most likely, to the presence of a tyrosine-rich contaminant (more abundant in *Heterobranchus*, which would explain the higher value obtained). *Clarias* PA IIIb appeared devoid of tyrosine. The residual absorption near 280 nm was due to a contaminant which, as in barbel (Laforêt *et al*., 1993; Huriaux *et al*., 1997), could not be removed by an additional chromatography on a Sephadex CL 4B column. All isoforms appeared to lack tryptophan. Two cysteine residues were detected in *Chrysichthys* PA IIa and one in all other isoforms except *Clarias* PA IIIb and *Heterobranchus* PA IV, devoid of this amino acid. *Chrysichthys* PA IIa was the only catfish isoform studied to possess two cysteine residues; this might explain why the molecular masses determined by SDS-PAGE and ESI-MS differed by about 300 Da, which is not negligible.

CHANGE OF PARVALBUMIN ISOFORM COMPOSITION IN THE COURSE OF DEVELOPMENT

The change of parvalbumin isoform composition was followed from hatching to the adult stage. The level of each isoform was estimated by densitometric scanning of the gels from the total amount of parvalbumin, the result being adjusted for a same total sarcoplasmic protein content (relative parvalbumin concentrations).

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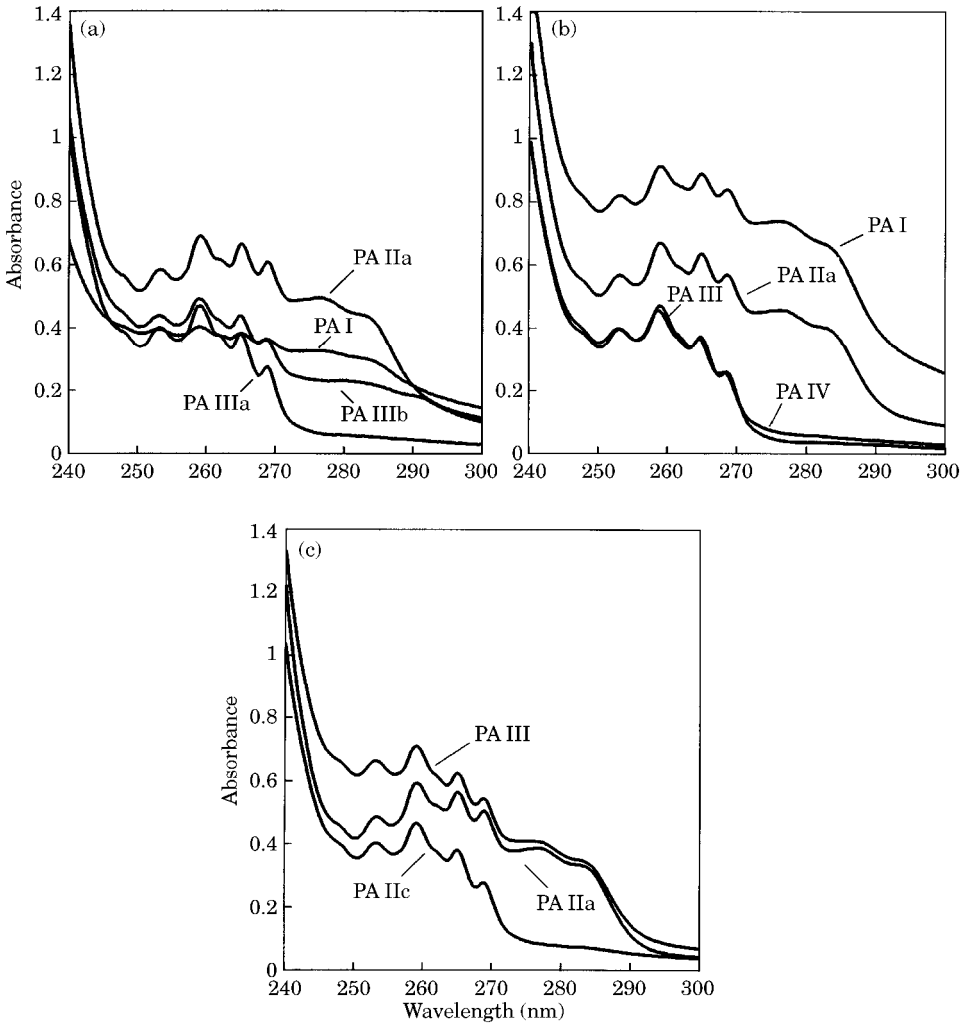
Fig. 5. Ultraviolet absorption spectra (1-cm light path) of the (a) *Clarias*, (b) *Heterobranchus*, (c) *Chrysichthys* parvalbumin isoforms (3 mg ml^{-1}) in 0·050 M NH_4HCO_3.



CLARIAS

Two batches of developing *Clarias* specimens from the same hatchery were analysed, with similar results. Parvalbumins were detected on non-denaturing polyacrylamide gels [**Fig. 6(a)**] as early as day 2, when the fish reached 0·6 cm. Concentrations of isoforms PA I and PA II increased rapidly from the end of larval stage (1·5 cm), peaking around 9 cm (day 90) with each representing 45% of the total parvalbumin content. Their concentrations then decreased gradually during later growth. PA IIIa appeared at the beginning of the juvenile stage (1·9 cm) and PA IIIb in a 15·5-cm adult specimen; levels of these parvalbumins rose slowly. PA IIIa appeared as the major adult isoform (55% at 32 cm) and PA IIIb as the minor one.

IEF-PAGE [**Fig. 6(b)**] confirmed this time course but it resolved the PA II band observed on non-denaturing gel into a major PA IIa band and a minor PA IIb band. At the outset, the level of each isoform paralleled the total PA II concentration, but while PA IIa remained present in considerable amount throughout the life of the fish, the PA IIb level tended progressively towards zero during the adult stage. These curves were computed taking into account the total PA content and the

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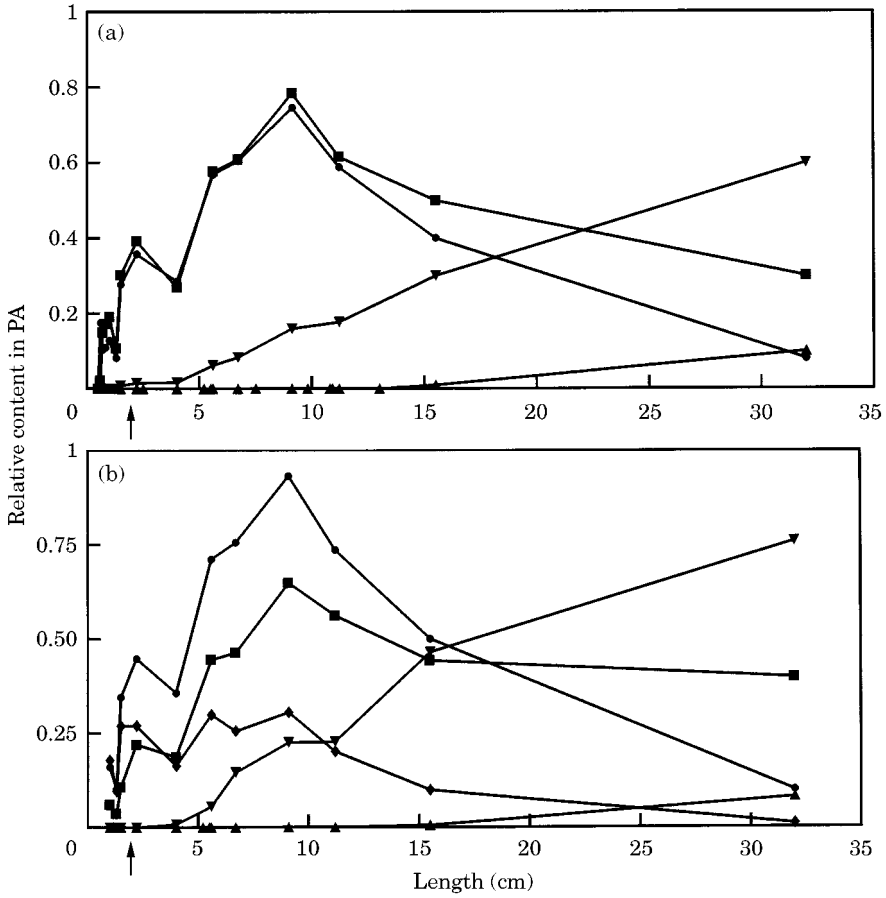
amount of PA I estimated from a non-denaturing gel, because PA I and PA IIa comigrated in IEF experiments, their pI values being very close (4·50/4·51).

Fig. 6. Evolution of the concentration of the parvalbumin isoforms (relative content in arbitrary units, measured by densitometry) in the course of development in *Clarias*, as a function of body length. (a) Non-denaturing PAGE; (b) IEF-PAGE. (a), ● PA I; ■ PA II; ▼ PA IIIa; ▲ PA IIIb; (b), ● PA I; ■ PA IIa; ◆ PA IIb; ▼ PA IIIa; ▲ PA IIIb. Arrow: larval-to-juvenile stage transition.



HETEROBRANCHUS

The numerous specimens were obtained from two different hatcheries. As the results expressed as a function of fish length were similar, we have chosen to show here only those obtained with the CERER specimens because this batch contained several adult fish. On non-denaturing polyacrylamide gels [**Fig. 7(a)**], PA I and PA II were detected together early (0·7 cm, day 7); they remained predominant to 11 cm. Their levels increased rapidly through the larval and juvenile stages (the latter staring around 1·6 cm). They peaked around 5 cm, then decreased gradually to become negligible in adult fish. The PA III isoform made its appearance at the beginning of the juvenile stage and became the major adult parvalbumin (80% of the total parvalbumin content). Finally, PA IV appeared in adult fish but its level remained low, even at 52 cm.

IEF-PAGE [**Fig. 7(b)**] confirmed these results and enabled us to distinguish two forms of PA II as in *Clarias*. The larval PA IIb disappeared earlier than in *Clarias*.

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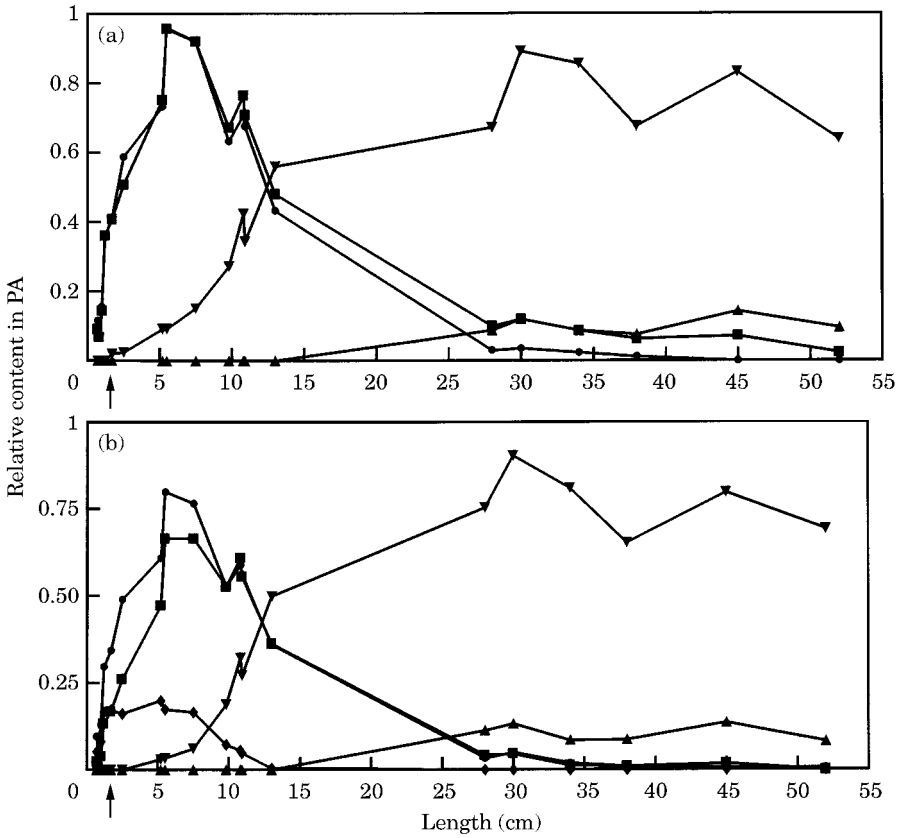
Fig. 7. Evolution of the concentration of the parvalbumin isoforms (relative content in arbitrary units, measured by densitometry) in the course of development in *Heterobranchus*, as a function of body length. (a) Non-denaturing PAGE; (b) IEF-PAGE. (a), ● PA I; ■ PA II; ▼ PA III; ▲ PA IV; (b), ● PA I; ■ PA IIa; ◆ PA IIb; ▼ PA III; ▲ PA IV. Arrow: larval-to-juvenile stage transition.



CHRYSICHTHYS

On non-denaturing polyacrylamide gels *Chrysichthys* displayed only two parvalbumin isoforms, PA II and PA III. In the course of development, parvalbumin levels changed according to a different pattern than in the clariid species [**Fig. 8(a)**]. PA II was detected at 0·75 cm (day 5); its relative concentration increased rapidly through the larval stage and the beginning of the juvenile stage (around 2 cm) and peaked at 11 cm. PA II remained the major parvalbumin throughout the life of the fish (80–90% of the total parvalbumin content). PA III was detected at 3 cm; the proportion of PA III increased slowly until the adult stage, reaching 20% of the total parvalbumin content, then decreased to 10% in large fish.

As described above, there were surprises in our attempts to separate *Chrysichthys* isoforms. On non-denaturing gels loaded with a small amount of material, it was possible to distinguish two forms of PA II of slightly different electric charge. They are summed in **Fig. 8(a)**. Further resolution by IEF revealed three different PA II isoforms. **Figure 8(b)** shows the appearance and concentration variations of these three PA II isoforms during growth and into the adult stage. The relative isoform concentrations were calculated from densitometric measurements of total PA II on non-denaturing gels. The entire PAII band was cut from the non-denaturing gel and subjected

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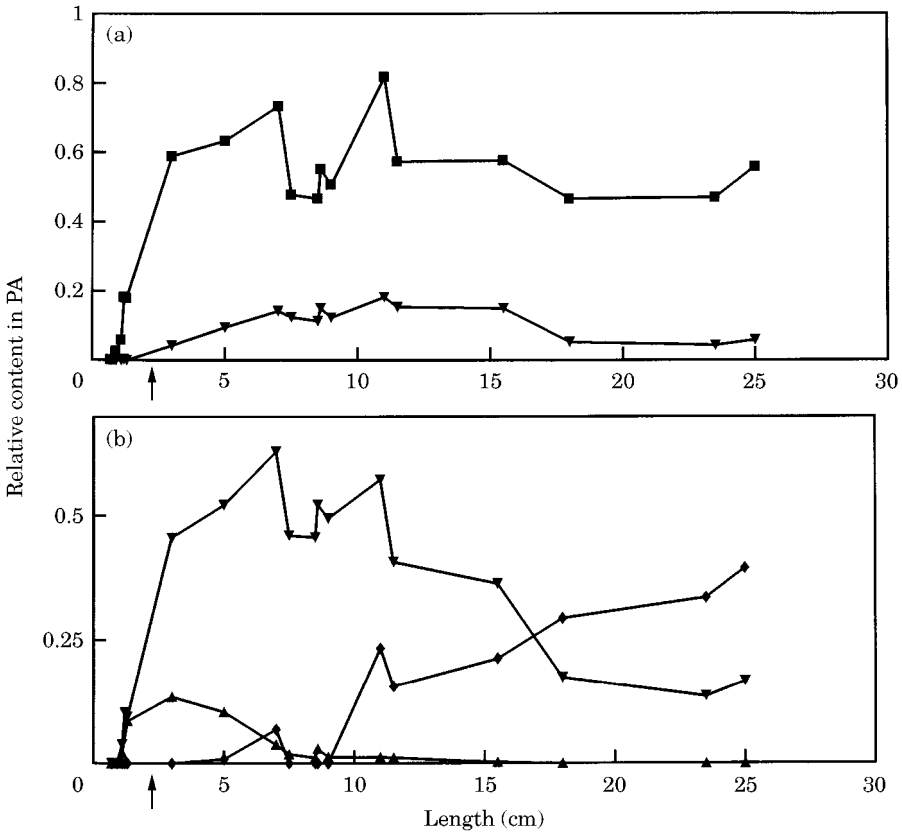
to IEF. It was impossible to evaluate the PA IIb content from an IEF pattern obtained with a total parvalbumin sample because its pI is too close to that of PA III. The major PA II isoform PA IIa remained the main component of PA II to at least 15 cm. PA IIc appeared as a typical adult isoform, appearing late (at 4–5 cm) but persisting during later growth. The minor transient larval isoform PA IIb appeared early with PA IIa but became negligible from 9 cm.

Fig. 8. Evolution of the concentration of the parvalbumin isoforms (relative content in arbitrary units, measured by densitometry) in the course of the development in *Chrysichthys*, as a function of body length. (a) Non-denaturing PAGE; (b) IEF-PAGE (PA II only). (a), ■ PA II; ▼ PA III; (b), ▼ PA IIa; ▲ PA IIb; ◆ PA IIc. Arrow: larval-to-juvenile stage transition.



Discussion

Catfish are economically one of the most important groups of brackish- and freshwater fish in the world. Some species are much appreciated by fish breeders for their fast growth and many are prized by aquarium hobbyists. The clariid *C. gariepinus* and *H. longifilis* and the claroteid *C. auratus* represent priceless sources of proteins for native African populations. Several pilot projects have focused on optimizing their breeding, offering researchers in many disciplines a select and abundant material. Here we have analysed the polymorphism and distribution of white trunk muscle parvalbumins in adult specimens of these three species and during their development.

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The non-denaturing PAGE profiles obtained with adult-muscle parvalbumin preparations appear species-specific. Both qualitative and quantitative differences between species are noted. Clariid muscles show four isoforms (PA I, PA II, PA IIIa, and PA IIIb in *Clarias* and PA I, PA II, PA III, and PA IV in *Heterobranchus*) with similar physicochemical characteristics, except for the small difference in charge between PA IIIb and PA IV. The claroteid *Chrysichthys* is distinguishable by the presence of only two parvalbumins, PA II and PA III as stated previously by Chikou *et al*. (1997). Both the between-family and within-family differences are clear-cut. Because PAGE is such a fast and easy method, it should prove useful in identifying catfish species mainly in the case of processed fish muscles. Within-family and especially between-family differences have also been observed among Serranidae, Cichlidae, Cyprinidae, and Salmonidae (Focant *et al.*, 1988, 1994; Huriaux *et al.*, 1992, 1996).

We have isolated and purified white trunk muscle parvalbumins from adult specimens of all three species. The larval isoform IIb, detectable only in larvae and juveniles, could not be prepared because too many specimens would have been required. All pure isoforms displayed typical parvalbumin features: molecular mass around 11 300–12 000 Da, pI ranging from 4·50 to 5·21, high phenylalanine-to-tyrosine ratio, lack of tryptophan.

Isolating *Chrysichthys* parvalbumins was complicated by the fact that one of the two parvalbumin peaks eluting from the DEAE-cellulose column contained two isoforms, PA III and a PA II distinct from the PA II contained in the other DEAE-cellulose peak. For the first time we successfully resolved this heterogeneous peak into its components by preparative PAGE under non-denaturing conditions and thus obtained PA III and PA II. IEF-PAGE confirmed, as observed previously in barbel (Huriaux *et al.*, 1997), the existence of several PA II isoforms. Despite their different pI values (4·51/4·76), PA IIa and PA IIc do not separate well on a non-denaturing gel at pH 8·6. The unusually high pI of PA IIc may explain its elution with PA III on DEAE-cellulose at pH 5·7, because the electric charges of these two isoforms are nearly the same at this pH.

Some of the physicochemical properties (**Table I**) are worth stressing. The less acidic isoforms PA IIIb of *Clarias* and PA IV of *Heterobranchus*, with a molecular mass of 12 000 Da, have a pI above 5·0 and lack both tyrosine and cysteine residues. These adult parvalbumin isoforms should belong to the alpha-parvalbumin family, characterized by pI > 5·0 (Goodman *et al*., 1979). At the other end of the pI range, the larval PA I and PA IIa of *Clarias* and *Heterobranchus* and the larval PA IIa of *Chrysichthys* display the lowest molecular masses (11 300/11 500 Da on SDS gels and 11 240/11 440 Da measured by electrospray ionization mass spectroscopy).

We have examined further how parvalbumin isoform distribution evolves in the course of fish development by computing the densitometer traces of parvalbumin electrophoretograms obtained from fish at various stages of development. We measured both the relative content in each isoform (amount corrected for a same total sarcoplasmic protein content) and the stoichiometry of the different isoforms, i.e. the percentage of the total parvalbumin concentration represented by each. For a more synthetic view, we have chosen to show only the relative content data as calculated from non-denaturing and IEF gels, plotting it against the standard length of the fish specimens. The IEF technique was used extensively because it had previously enabled us to

distinguish multiple PA II isoforms in the developing barbel. While the very principle of this technique makes it less accurate quantitatively (concentration of the protein at its pI, non-linear relationship between staining intensity and protein concentration), it provided at each developmental stage additional information not obtainable by the other technique.

In all three fish, the larger specimens displayed only a slightly lower relative parvalbumin content than the smaller ones. This contrasts with findings concerning other fish species, where a considerable decrease was observed (Rodnick & Sidell, 1995; Huriaux *et al*., 1997). Parvalbumins appeared sequentially in the course of development, as already noted in barbel, trout, and sea-bass (Huriaux *et al.*, 1996, 1997). In catfish, parvalbumins were detectable during the early larval stages. Previously we showed that they appear in *Clarias* at a stage when well-developed myofibrils are discernible by electron microscopy (Focant *et al*., 1996). In *Clarias* and *Heterobranchus*, we saw a quick rise in PA I and PA II isoforms which we have called larval as opposed to the adult isoforms PA IIIa, PA IIIb, PA III, and PA IV which appear later and predominate in the adult. For the claroteid *Chrysichthys*, there appeared no clear-cut distinction between larval and adult isoforms after PAGE under non-denaturing conditions, confirming the observations of Chikou *et al*. (1997). With isoelectric focusing, however, isoform separation was carried a step further, revealing in *Clarias* and *Heterobranchus* a short-lived larval PA IIb and in *Chrysichthys*, no less than three PA II isoforms: PA IIa, a typically larval isoform, PA IIb, an early and short-lived larval form, and PA IIc an adult form. This is the first time a PA II isoform has been detected in an adult. The presence of this form explains the unexpected non-denaturing-PAGE profile obtained for adult muscle (the high proportion of PA II).

The fish length corresponding to the highest proportion of larval isoforms depended on the species (10 cm in *Clarias*, 5 cm in *Heterobranchus*, juveniles in *Chrysichthys*). We observed no correlation with developmental-stage transitions. The larval isoforms were distinguishable by a more acidic pI, a lower molecular mass, and always one tyrosine and at least one cysteine residue. These chemical features should affect the physiological function of these isoforms and may underlie the contractile characteristics of the propulsive musculature in the first life stages where locomotion and feeding are intense.

As regards phylogeny, it is worth noting that clariid *Clarias* and *Heterobranchus* share similar properties: their parvalbumin isoforms differ only by the charge and pI of PA IIIb and PA IV. In the course of development, the transition from larval to adult parvalbumins is faster and more evident in *Heterobranchus*. The claroteid *Chrysichthys* displays a quite different parvalbumin composition evolving differently in time. It lacks PA I but contains three forms of PA II, one being an adult isoform.

This work strengthens the view that parvalbumins can be valuable tools in fish systematics and in monitoring fish development. It confirms that fish parvalbumin polymorphism is probably a subtle means of adapting muscle performance in growing fish to the environment.

Since parvalbumins are known as allergens, it would be interesting to see whether all isoforms are allergenic or only some of them. PA IIb appears as the first larval isoform in all three catfish, as previously observed in the barbel. This isoform displays the same molecular mass and pI in all

three fish. Our next goal will be to purify it from juvenile fish, to undertake its physicochemical characterization, and to compare it with adult isoforms.

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