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Characterization of parvalbumin isotypes in white muscle from the barbel and expression during development

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ABSTRACT

Parvalbumin isotypes PA II, PA III, PA IVa, and PA IVb were isolated by chromatography from trunk white muscle of barbel and physicochemically characterized. Electrospray ionization mass spectroscopy revealed that PA II has a lower molecular weight than the other isotypes and that PA IVa and PA IVb each consist of two subforms. Isotype distribution was studied by polyacrylamide gel electrophoresis. In adult fish, the total parvalbumin titre decreased and the isotype distribution varied from the anterior to the posterior myotomes. In the course of barbel development, the total parvalbumin titre increased rapidly as fish standard length increased from 1-3 to 5 cm; then sloped down gently as the length increased to 60 cm. At least six parvalbumin isotypes were identified, three of which are different forms (a, b, and c) of PA II. These three forms were present together at the larval stage, but PA lie and chiefly PA lib appeared as early isotypes, contrary to PA IIa which was present until the adult period. Later PA IVb accounted for up to 90% of the total parvalbumin content; PA III and PA IVa are minor adult isotypes. Temporal and spatial variations in the total parvalbumin titre and in the differential expression of barbel parvalbumin isotypes very likely reflected the functional requirements of the fish axial musculature according to fish size and myotome location. Physiologically, the larval isotypes could promote faster relaxation of fast fibres than the adult isotypes, and hence favour shorter contraction times.

INTRODUCTION

The functional diversity of skeletal muscle cells is reflected by the vast array of protein isoforms found in differentiating, developing, and mature muscle fibres. In higher vertebrates, it is well known that muscle development is associated with sequential expression of a range of myofibrillar protein isoforms (see Swynghedauw, 1986; Pette & Staron, 1990; Bandman, 1992).

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Over the past few years, similar studies have focused on the myofibrillar proteins of several fish species (van Raamsdonk *et al.,* 1982; Scapolo *et al.,* 1988; Martinez *et al.,* 1991, 1993; Focant *et al.,* 1992, 1994*b*, 1995; Brooks & Johnston, 1993; Crockford & Johnston, 1993; Veggetti *et al.,* 1993). Few have dealt with the expression, during fish development, of the calcium-binding proteins called parvalbumins (Focant *et al.,* 1992; Rodnick & Sidell, 1995; Huriaux *et al.,* 1996).

Parvalbumins are polymorphic, highly acidic proteins with molecular weight values in the range of 10—12 kDa. These sarcoplasmic proteins can bind with a high affinity two calcium ions per molecule and are especially abundant in white muscle of cold-blooded vertebrates, where they may act as soluble relaxing factors (for a review, see Gerday, 1982). Fish usually display two to five parvalbumin isotypes, numbered according to their electrophoretic mobility on non-denaturing gels at alkaline pH. These isotypes constitute extremely valuable tools for distinguishing fibre types (Hamoir *et al.,* 1972) or fish species (Focant & Joyeux, 1988; Focant *et al.,* 1988, 1990, 1994a; Huriaux *et al.,* 1992).

The distribution of parvalbumins in the European barbel *Barbus barbus* L., has been investigated previously by glycerol-polyacrylamide gel electrophoresis (glycerol-PAGE) at pH 8-6. In adult fish, high amounts of PA II, PA III, and chiefly PA IV were found in trunk white fibres and low amounts of only PA I and PA IV in trunk red fibres; all four isotypes are present in head muscles but cardiac ventricle fibres are devoid of any parvalbumin (Huriaux *et al.,* 1990). The parvalbumin distribution is generally identical in wild and hatchery-reared barbels of the same size but differs between small and large barbels within a population. A preliminary study of parvalbumin synthesis in the axial white musculature of barbels during development and up to the adult stage (standard length: 20 cm) demonstrated considerable and unforeseen transitions in isotype distribution (Focant *et al.,* 1992). The PA II isotype occurs first after hatching, steadily augments during the larval stage, then decreases to the adult stage. PA IV appears a few days after PA II; PA III is detected during the juvenile stage; levels of both increase until the fish reach 10 cm in length, at which point they stabilize. The typical adult pattern is characterized by a strong predominance of PA IV. Differential expression of several isotypes suggests a specialization of these isotypes and may reflect specific functional demands that change as the fish grows.

Similar differential expression of parvalbumin isotypes has been described recently in two salmonids, the rainbow trout *Oncorhynchus mykiss* (Walbaum) and the brown trout *Salmo trutta* L. (Huriaux *et al.,* 1996). As in the barbel, PA II is the principal eleutheroembryonic and larval form and PA III, PA IV, or PA V the major adult form. In the serranid *Dicentrarchus labrax* L. or sea bass, the synthesis of both isotypes (PA II and PA V) is delayed and the larval form PA II remains the principal isotype in adult fish (Huriaux *et al.,* 1996). The various parvalbumin isotypes have been isolated from these three teleosts and their molecular properties characterized; the parvalbumins isolated from the two trout species are biochemically quite similar but they differ more markedly from those of the sea bass.

To extend this comparison, the parvalbumin isotypes present in barbel dorso-lateral white

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muscle were purified and characterized in this study. In the course of this work, additional isotypes were revealed. This study investigated how the level of each isotype varies as a function of fish standard length up to 60 cm, using non-denaturing and isoelectrofocusing—PAGE. The anteroposterior distribution of isotypes in adult fish was examined also. A preliminary note concerning this last study has been published already (Huriaux *et al.,* 1991).

MATERIALS AND METHODS

FISH SAMPLES

The numerous specimens of the cyprinid *B. barbus* were obtained from the experimental hatchery of the University of Liège (CERER-LDPA, Tihange, Belgium). In this work, all body lengths are expressed in terms of standard length (SL). The fish were anaesthetized with tricaine methanesulphonate (MS-222, Sandoz) and killed by decapitation.

To purify the parvalbumin isotypes, the trunk dorso-lateral white muscle was dissected free of any pigmented superficial fibres from fish 18-20 cm long.

Samples were taken from barbels ranging in size from 1-3 cm (larval stage) to 60 cm. Samples of fish measuring up to 5-0 cm were constituted by pooling several specimens: 1-3 cm (20); 1-6-3 0 cm (10); 3-5 cm (8) and 4 0-5-0 cm (3). Over this size, one fish was dissected. To study fish measuring up to 2-0 cm (beginning of the juvenile period, Krupka, 1988), the head, internal organs, and caudal fin were discarded; with larger fish, the trunk dorso-lateral white muscle was dissected. Only a piece of muscle located in front of the dorsal fin was selected from a fish of 26 cm; we demonstrated on the 21-cm specimen that the total parvalbumin content per mg sarcoplasmic protein and the isotype distribution at this place were the same as in the whole muscle. The material was weighed, minced, and suspended in 10 vol (or less for the two smallest samples) of a preservative solution containing 10 mM Tris-HCl, 50 HIM KC1, 10 mM dithiothreitol, 0-005% (w/v) NaN_3, 50% (v/v) glycerol pH 7-5. Samples were kept at 4° C for 24 h, mixed, and stored at — 20° C until used. For the anterior to posterior distribution study, the whole dorso-lateral muscle of an adult fish (21 cm) was cut into 16 1-cm samples, from head to tail, and treated as described above.

ISOLATION OF PARVALBUMIN ISOTYPES

The parvalbumin mixture was prepared by extraction of the sarcoplasmic proteins with 10 mM Tris-HCl, 2% (v/v) glycerol, 1 mM 2-mercaptoethanol (pH 8-7), precipitation with $(NH_4)_2SO_4$ between 50 and 90% saturation, heating at 60° C, and chromatography on a Sephacryl S-100 column equilibrated in 50 mM NH_4HCO_3, as previously detailed in Huriaux *et al.* (1996). The parvalbumin isotypes were separated on a diethylaminoethylcellulose (Whatman DE 52) column (2-5 x 30 cm) equilibrated in a buffer (pH 5-7) containing 15 mM piperazine-HCl and 1 mM 2-mercaptoethanol. The column was eluted with a linear NaCl gradient (400 ml buffer-400 ml buffer with 150 mM NaCl) at a flow rate of 35 ml h^{-1}. The fractions containing each isotype were pooled, concentrated on an Amicon YM3 membrane,

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desalted on a Biogel P2 column (1-5 x 45 cm) equilibrated in 50 mM NH_4HCO_3, and lyophilized.

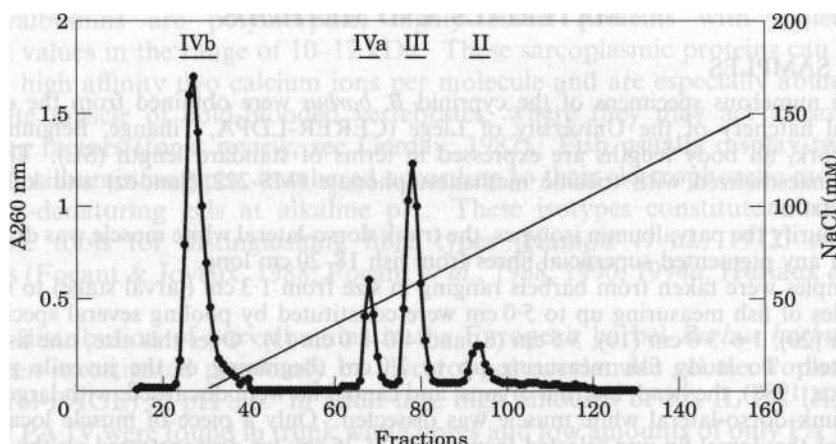
CRUDE PARVALBUMIN EXTRACTS

Samples in preservative glycerol solution were centrifuged at 17 530 g (Sigma 3K10 centrifuge) for 20 min at 4° C. The parvalbumin-containing supernatant was heated at 80° C for 5 min and again centrifuged. This last supernatant was used for electrophoretic analysis. Sarcoplasmic protein concentrations were determined before heating by the Bradford (1976) method, using a solution of bovine serum albumin as the standard.

ANALYTICAL METHODS

Polyacrylamide gel electrophoresis (PAGE) was performed under three sets of conditions: in a non-denaturing system (10% acrylamide, 10% glycerol pH 8-6); in the presence of sodium dodecyl sulfate (SDS) (20% acrylamide, pH 8-4 or 8-8); and in an isoelectrofocusing (IEF) system (7-5% acrylamide, 1-6% Pharmacia ampholine pH 4-6, 0-4% Pharmacia ampholine pH 3-5-10, 8 M urea). Conditions for electrophoresis, staining, densitometry, and estimation of isoelectric points (pI) have been described previously (Huriaux *et al.,* 1996). By computing the densitometer traces of parvalbumin electrophoretograms, it is possible to evaluate the relative content in each isotype (expressed in arbitrary units with respect to a same total sarcoplasmic protein content) and the stoichiometry of the several isotypes (percent contribution of each isotype to the total parvalbumin content). Measurements were taken from three gels. The marker proteins for estimating the apparent relative molecular mass (M_r) by SDS-PAGE were ribonuclease from bovine pancreas (Boehringer) (13 700 Da), cytochrome-C from equine heart (Calbiochem) (12 270 Da), and parvalbumin IV from carp muscle (11 430 Da, as calculated from the amino acid sequence data; Coffee *et al.,* 1974).

FIGURE 1. Chromatography of the barbel (18-20-cm fish) parvalbumin fraction (collected on Sephacryl S-100) on a DEAE-cellulose column (2-5 x 30 cm) equilibrated in a buffer (pH 5-7) containing 15 mM piperazine-HCl and 1 mM 2-mercaptoethanol. Gradient: 400 ml buffer-400 ml buffer plus 150mMNaCl; 6-ml fractions. Flow rate of 35 ml h^{-1}



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Electrospray ionization mass spectra (ESI-MS) were obtained on a VG Platform instrument (Fisons) equipped with an ESI ion source. The samples were dissolved in 50% acetonitrile, 0-1% formic acid. The molecular mass measurements were made in triplicate.

Ultraviolet spectra were obtained and sulfhydryl groups titrated as in Huriaux *et al.* (1996).

RESULTS

ISOLATION AND CHARACTERIZATION OF PARVALBUMINS

Four parvalbumin isotypes eluted from a DEAE-cellulose column in the order: PA IVb, PA IVa, PA III, PA II (Fig. 1). The numbers attributed correspond to the electrophoretic mobilities observed in non-denaturing PAGE (Fig. 2), conditions under which all the bands seemed homogeneous, but PA IVa and PA IVb could not be distinguished. The presence of a reducing agent (2-mercaptoethanol) in the samples did not modify isotype migration. When subjected to IEF-PAGE (Fig. 3), the four isotypes focused at different positions: PA IVb, PA IVa, and PA III appeared homogeneous whereas PA II displayed an additional minor band (19%) migrating like PA III (see below).

When subjected to SDS-PAGE, the parvalbumin isotypes all exhibited apparent relative molecular masses of 11 400-11 500 Da (Table I). When the protein samples were diluted, PA IVb split into two bands with very similar mobilities, a phenomenon never encountered with other parvalbumins. A similar doublet pattern was observed with samples prepared from a single specimen (ranging from 10-6 to 60 cm) when PA IV was isolated by cutting the band obtained by glycerol-PAGE and then examined by SDS-PAGE. More precise molecular mass values were obtained by electrospray ionization mass spectroscopy (Table I).

FIGURE 2. Glycerol-PAGE: the different steps leading to the purification of the barbel parvalbumin isotypes. From left to right: (1) total extract of white muscle; (2) $(NH_4)_2SO_4$ precipitation, fraction precipitating at 50-90% saturation; (3) fraction stable at 60° C; (4) Sephacryl S-100 parvalbumins; (5) PA IVb; (6) PA IVa; (7) PA III; (8) PA II.

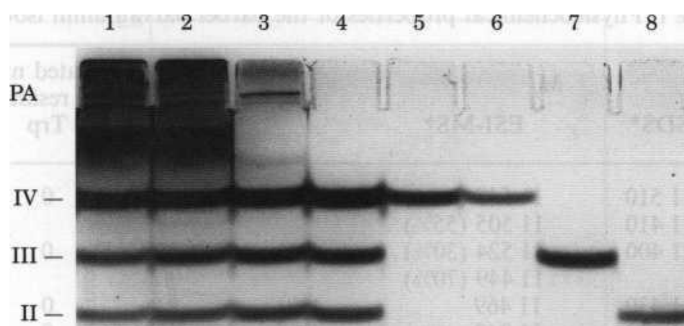

***FIGURE 3**. IEF-PAGE of the barbel parvalbumin isotypes isolated by chromatography on DEAE-cellulose. (1) PA IVb; (2) PA IVa; (3) PA III; (4) PA II.*

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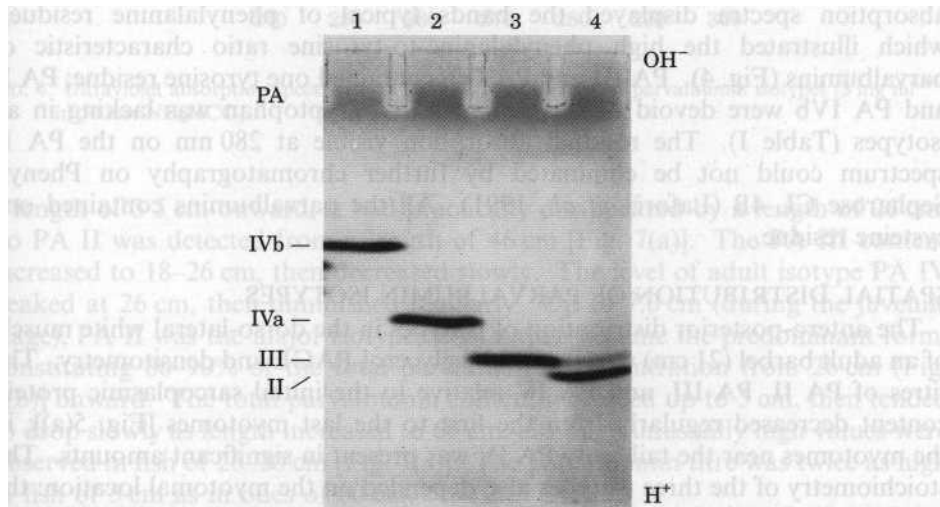

This method yielded a lower mass for PA II (11 248 Da) than for the three other isotypes. It also revealed that PA IVa and PA IVb each existed in two subforms, present in different proportions. No change was observed in the presence of 5 DIM ethylene glycol bis-N, N'-tetraacetic acid (EGTA), a calcium-chelating agent. In the presence of 5 HIM $CaCl_2$, the peak corresponding to each subform was replaced partially by a new peak corresponding to a 37-Da higher mass. PA IVa and PA IVb showed very different isoelectric points, in keeping with their chromatographic elution profiles at acidic pH. The ultraviolet absorption spectra displayed the bands typical of phenylalanine residues, which illustrated the high phenylalanine-to-tyrosine ratio characteristic of parvalbumins (Fig. 4). PA III and PA IVa contained one tyrosine residue; PA II and PA IVb were devoid of this amino acid. Tryptophan was lacking in all isotypes (Table I). The residual absorption visible at 280 nm on the PA II spectrum could not be eliminated by further chromatography on Phenyl- Sepharose CL 4B (Laforêt *et al.,* 1991). All the parvalbumins contained one cysteine residue.

TABLE I. Physicochemical properties of the barbel parvalbumin isotypes

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PA	M_r SDS*	M_r ESI-MS†	pI‡	Estimated number of residues: Tyr	Trp	Cyst
IVb	11 510	11 547 (45%)	4·98	0	0	1
	11 410	11 505 (55%)				
IVa	11 400	11 524 (30%)	4·69	1	0	1
		11 449 (70%)				
III	11 430	11 469	4·59	1	0	1
II	11 400	11 248	4·54	0	0	1

*Average of four gels; S.D. ≤40.
†Average of three values; S.D. ≤3.
‡Average of four gels; S.D. ≤0·06.
PA, parvalbumin; M_r, relative molecular mass; SDS, sodium dodecyl sulfate; ESI-MS, electrospray ionization mass spectroscopy; pI, isoelectric point; Tyr, tyrosine; Trp, tryptophan; Cyst, cysteine; S.D., standard deviation.

SPATIAL DISTRIBUTION OF PARVALBUMIN ISOTYPES

The antero-posterior distribution of isotypes in the dorso-lateral white muscle of an adult barbel (21 cm) was studied by glycerol-PAGE and densitometry. The titres of PA II, PA III, and PA IV relative to the initial sarcoplasmic protein content decreased regularly from the first to the last myotomes [Fig. 5(a)]; in the myotomes near the tail, only PA IV was present in significant amounts. The stoichiometry of the three isotypes also depended on the myotomal location: the percentage of adult form (PA IV) increased from head to tail at the expense of the percentages of larval form PA II and of PA III [Fig. 5(b)], At all locations, PA IVa constituted a very minor proportion of the total PA IV (not shown).

DEVELOPMENTAL EVOLUTION OF PARVALBUMIN ISOTYPE DISTRIBUTION

Glycerol-PAGE

A previous study (Focant *et al.,* 1992), monitored how the titres of isotypes PA II, PA III, and PA IV evolved according to fish age or size, limiting the focus to fish not exceeding 20 cm. Here, parvalbumin distribution was monitored according to fish size, from 1-3 cm (larval stage) to 60 cm. The electrophoretic patterns of parvalbumins were shown at different growth stages [Fig. 6(a)], After an early fast increase, synthesis of the larval isotype PA II clearly decreased from a length of 3-5 cm onward; it had practically disappeared by a length of 26 cm; no PA II was detected from a length of 46 cm [Fig. 7(a)], The PA III content increased to 18-26 cm, then decreased slowly. The level of adult isotype PA IV peaked at 26 cm, then diminished regularly. Up to 7.0 cm (during the juvenile stage), PA II was the major isotype; PA IV then became the predominant form, constituting 80-90% of the total parvalbumin concentration from 26 cm (Fig. 7(b)] onward. The total parvalbumin content increased up to 5

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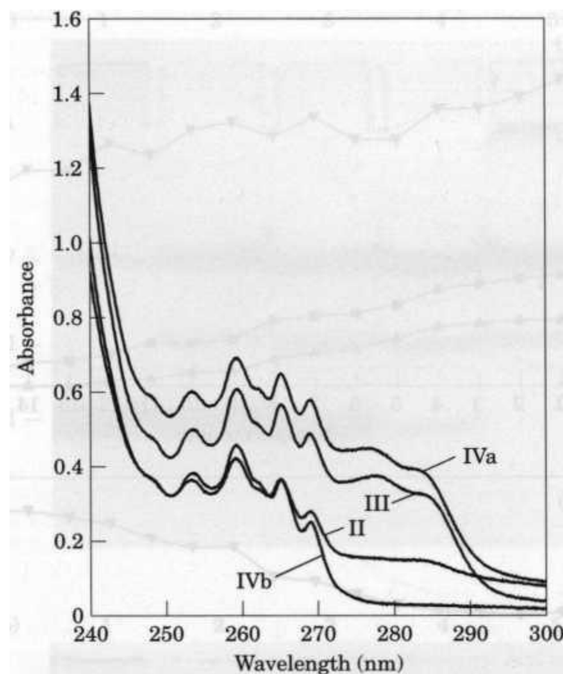
cm, then tended to drop slowly as length increased to 60 cm, although unusually high values were observed in fish of 26-30 cm [Fig. 7(a)], The parvalbumin titre was twice as high in fish of 5 cm as in ones of 60 cm.

FIGURE 4. Ultraviolet absorption spectra (1-cm light path) of the barbel parvalbumin isotypes (3 mg ml^{-1}) in 50 mM NH_4HCO_3.



IEF-PAGE

The isoelectrofocusing patterns of barbel parvalbumins corresponding to different body lengths showed a maximum of five protein bands [Fig. 6(b)]. The two bands with the highest isoelectric points were identified as PA IVa and PA IVb; the two bands with the lowest isoelectric points behaved like PA II and PA III from adult fish (cf. Fig. 3). When IEF was used to monitor parvalbumins in the course of barbel development PA IVb always appeared as the predominant PA IV form (Fig. 8). The level of PA IVa increased slowly up to 6-4 cm, then diminished; it approached the method's limit of detection at 30 cm. As the three more acidic bands of Fig. 6(b) appeared more intense in young specimens, it was endeavoured to identify them more precisely by cutting out the PA II band obtained by glycerol-PAGE and subjecting it to IEF-PAGE. A maximum of three bands was observed. These were numbered PA IIa, PA IIb, and PA lie (Fig. 9). PA IIa and PA lib respectively focalized at the same levels as the PA II (pI: 4-54) and PA III (pI: 4-59) isolated from 18-20 cm barbels, whereas PA lie had a higher pI of 4-66. When the three PA II bands obtained by IEF-PAGE were cut out and subjected to SDS-PAGE, PA IIa and PA lib displayed a same molecular mass of 11 400 Da; PA lie had a higher molecular mass of 11 600 Da.

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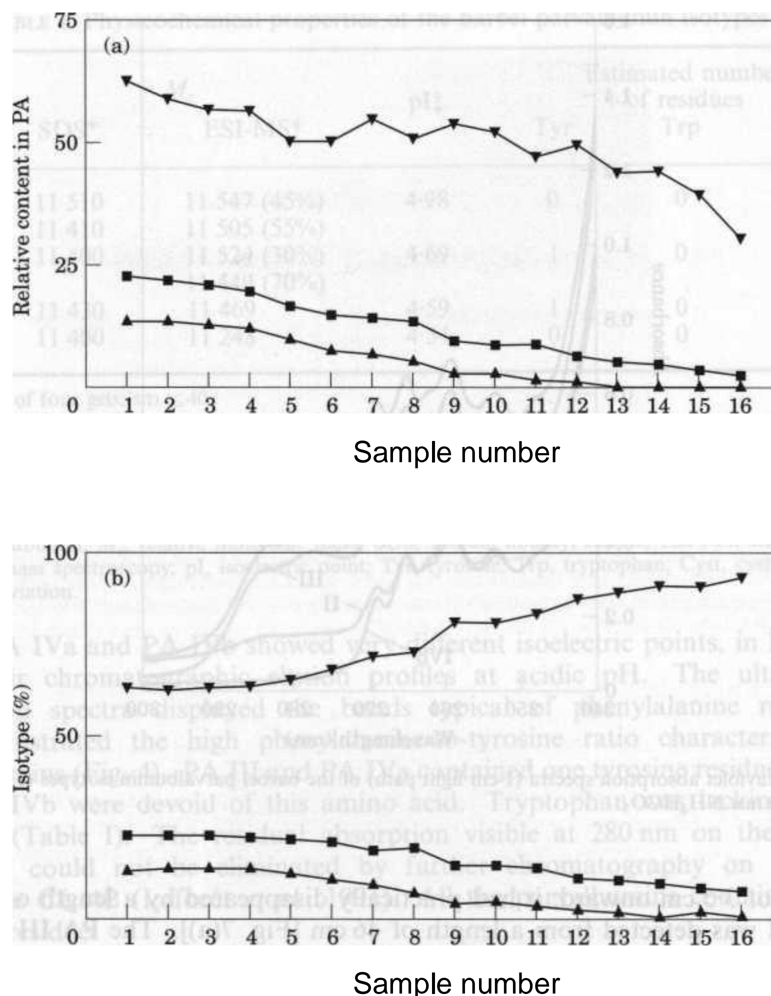
FIG. 5. Variation of the concentration of each parvalbumin isotype in the dorso-lateral muscle of barbel (21 cm) as measured by densitometry after glycerol-PAGE. The muscle was cut into 16 1-cm samples, from head (sample 1) to tail (sample 16). (a) Relative content (with respect to sarcoplasmic proteins), the total parvalbumin titre in sample 1 being set arbitrarily at 100. (b) Stoichiometry, i.e. percentages of each isotype with respect to the total parvalbumin titre. ▲, PA II; ■, PA III; ▼, PA IV.



All three PA II isotypes occurred during early development. The amount of PA lib quickly rose in fish up to 2-5 cm, then dropped, but this isotype remained the major one up to 5 cm; it was barely detectable in fish 21 cm long (Fig. 10). The PA lie content evolved like the PA lib content, but remained constantly lower. PA IIa appeared as a late-larval and juvenile isotype; it increased in fish up to 7-5 cm and remained the only PA II component present in 21-cm adult fish. These observations explain why PA II appeared heterogeneous in the experiment depicted in Fig. 3: the sample contained both PA IIa (81%) and PA lib (19%), the latter focalizing at the level of PA III (18-20 cm).

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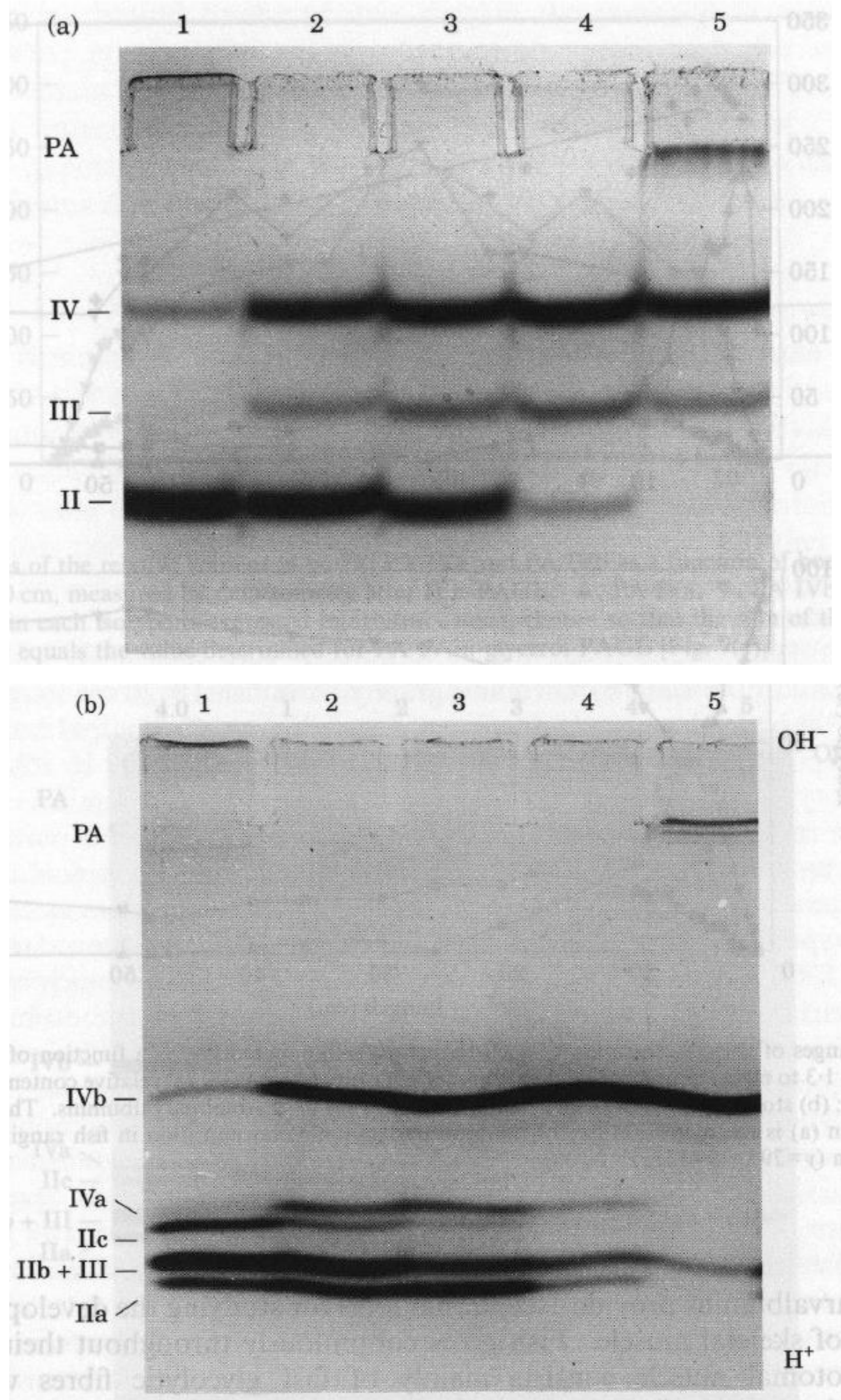
FIGURE 6. (a) Glycerol-PAGE and (b) IEF-PAGE of parvalbumin isotypes of barbels from 2-0 to 60 cm long. (1)2-0 cm; (2) 4-5 cm; (3) 7-5 cm; (4) 21 cm; (5) 60 cm. Samples corresponding to equal amounts of sarcoplasmic proteins were loaded into each well.



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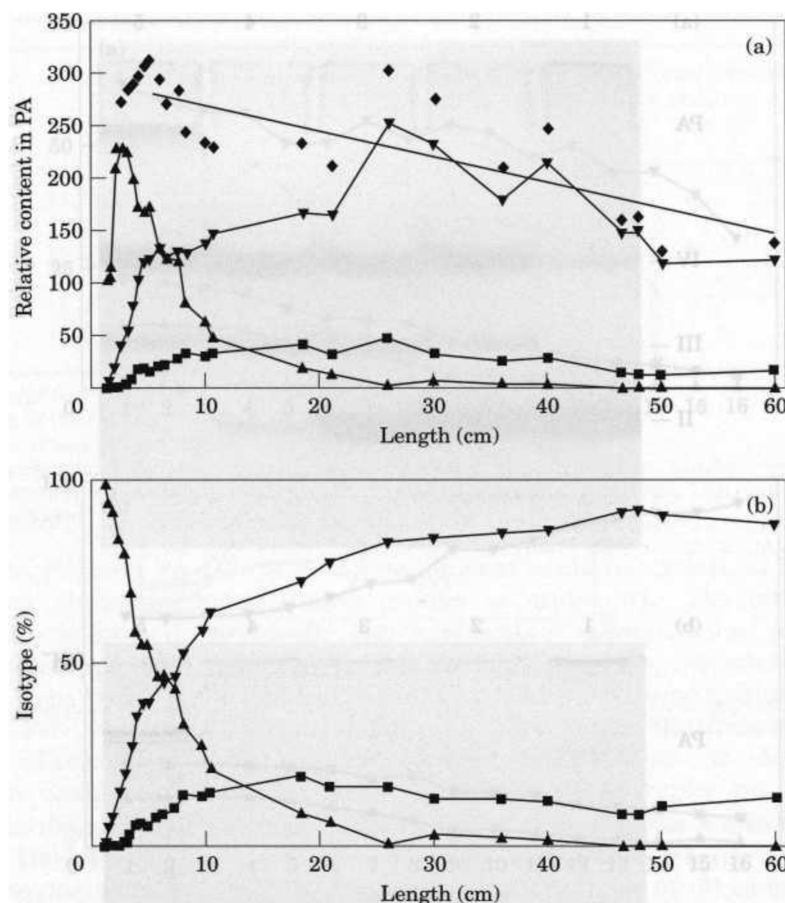

***Figure 7**. Changes of the concentration of each barbel parvalbumin isotype as a function of body length from 1 -3 to 60 cm, measured by densitometry after glycerol-PAGE: (a) relative content in arbitrary units; (b) stoichiometry. ▲, PAII; ■. PA III; ▼, PA IV; ♦, total parvalbumins. The upper solid line in (a) is the regression line of the total parvalbumin concentration in fish ranging from 5 to 60 cm (y=295 - 2-453x, r=0-790).*

DISCUSSION

Fish parvalbumins provide exceptional tools for studying the development and function of skeletal muscle. Fish grow continuously throughout their lifespan; their myotomal muscle consists mainly of fast glycolytic fibres which are segregated anatomically and very rich in parvalbumins; unlike those of mammals, these calcium-binding proteins display several isotypes. In previous papers, we described for the first time the differential expression of parvalbumin isotypes in axial white muscle from barbel, trout, and sea bass (Focant *et al.,* 1992; Huriaux *et al.,* 1996). In all three cases, PA II appeared as the predominant larval form and PA III, PA IV, or PA V generally as the major adult form. With a view to comparing the

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physicochemical properties of larval and adult isotypes from several fish, here we have purified the parvalbumin isotypes of barbel.

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FIGURE 8*. Changes of the relative content of barbel PA IVa and PA IVb as a function of body length from L6 to 60 cm, measured by densitometry after IEF-PAGE. ▲, PA IVa; ▼, PA IVb. The relative content in each isotype is expressed in arbitrary units, chosen so that the sum of the two isotype contents equals the value determined for PA IV in glycerol-PAGE [Fig. 7(a)].*

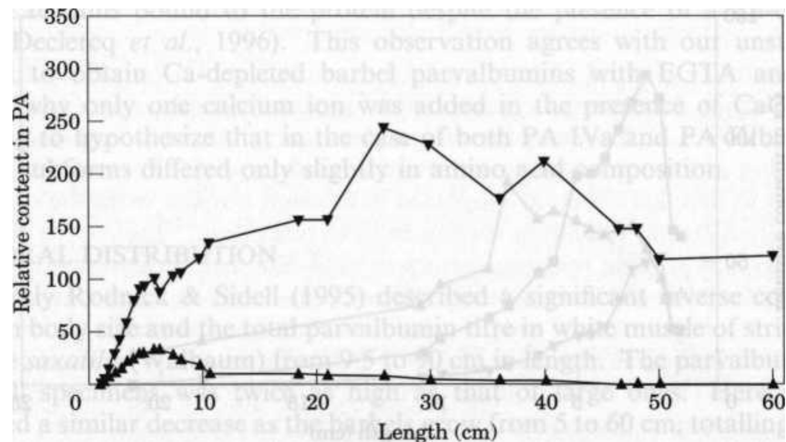
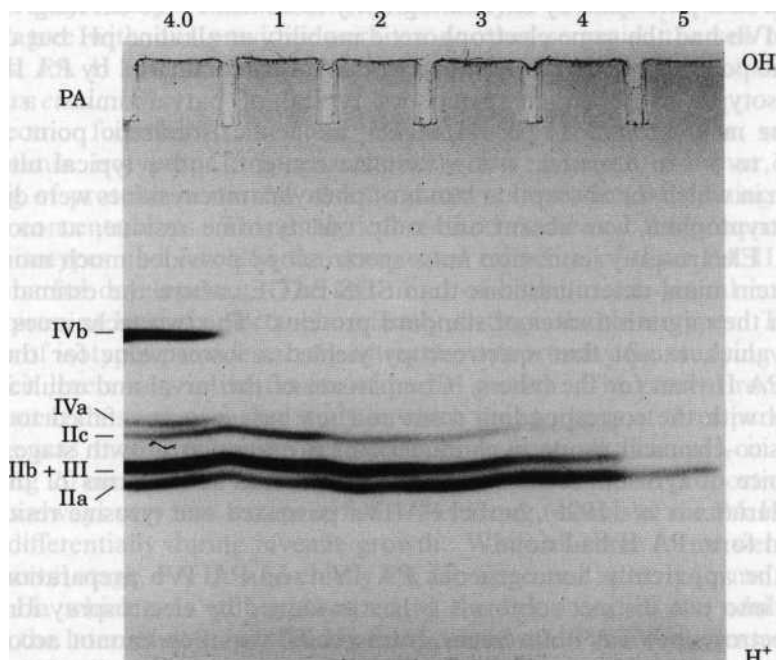


FIGURE 9*. IEF-PAGE of PA II isolated from gels after glycerol-PAGE of barbel parvalbumins obtained from specimens from 2 0 to 21 cm long. Lanes: (1) 2-0 cm; (2) 4 0 cm; (3) 5-9 cm; (4) 9-9 cm; (5) 21 cm. Lane 4.0 was loaded with total parvalbumins from a fish 4 0 cm long. The amounts of PA II loaded into each well are not equivalent.*



PHYSICOCHEMICAL PROPERTIES

While PAGE carried out under non-denaturing conditions at alkaline pH revealed only three isotypes (Huriaux *et al.,* 1990; Focant *et al.,* 1992), four were separated in a pure state by chromatography from fish 18-20 cm long. PA I Va and PA IVb had the same electrophoretic

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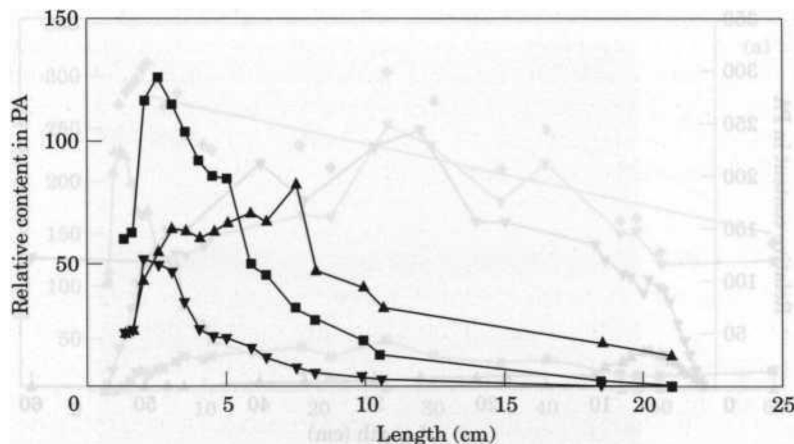
mobility at alkaline pH but different isoelectric points. PA II corresponded to PA IIa contaminated by PA lib. The barbel isotypes exhibited characteristics typical of parvalbumins: a relative molecular mass around 11 200-11500 Da; an acidic isoelectric point ranging from 4-5 to 5-0 in 8 M urea; a low cysteine content; and a typical ultraviolet spectrum in which the absorption bands of phenylalanine residues were distinctly visible (tryptophan was absent and only one tyrosine residue, at most, was present).

FIGURE 10. Changes of the relative content of barbel PA IIa, PA lib, and PA IIe as a function of body length from 1-3 to 21 cm, measured by densitometry after IEF-PAGE. ▲, PA IIa; ■, PA lib; ▼, PA He. The relative content in each isotype is expressed in arbitrary units, chosen so that the sum of the three isotype contents equals the value determined for PA II in glycerol-PAGE [Fig. 7(a)].



Electrospray ionization mass spectroscopy provided much more accurate protein mass determinations than SDS-PAGE, where the estimates were based on the migration rates of standard proteins. The two techniques yielded similar values, except that spectroscopy yielded a lower value for the larval isotype PA II than for the others. Comparison of the larval and adult isotypes of barbel with the corresponding trout and sea bass isotypes, failed to discern any physico-chemical property characterizing a particular growth stage. While the absence of tyrosine seemed characteristic of the adult forms of the latter fishes (Huriaux *et al.,* 1996), barbel PA I Va possessed one tyrosine residue and the larval form PA II had none.

That the apparently homogeneous PA I Va and PA IVb preparations each resolved into two distinct subforms (when examined by electrospray ionization mass spectroscopy) was unforeseen. Intraspecific variation cannot account for this (although the parvalbumin isotypes were prepared by pooling muscles of several fish), since PA IVb likewise gave rise to two bands in SDS-PAGE even when it was isolated from a single specimen (this was done at several different lengths). Nor can the two subforms correspond to different amounts of calcium bound to the same protein: in the presence of additional calcium, the number of peaks doubled; the shift of each new peak with respect to its twin was compatible with the supplementary binding of one calcium ion. A recent investigation of a pike *Esox lucius* L. parvalbumin by X-ray crystallography has shown that calcium remains bound to the protein

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despite the presence of a Ca-chelating agent (Declercq *et al.,* 1996). This observation agrees with our unsuccessful attempt to obtain Ca-depleted barbel parvalbumins with EGTA and might explain why only one calcium ion was added in the presence of $CaCl_2$. It is tempting to hypothesize that in the case of both PA I Va and PA IVb, the two distinct subforms differed only slightly in amino acid composition.

TEMPORAL DISTRIBUTION

Recently Rodnick & Sidell (1995) described a significant inverse correlation between body size and the total parvalbumin titre in white muscle of striped bass *Morone saxatilis* (Walbaum) from 9-5 to 90 cm in length. The parvalbumin titre of small specimens was twice as high as that of large ones. Here we have observed a similar decrease as the barbels grow from 5 to 60 cm, totalling 50% by the end of this period, but the decrease was not regular; the rather high value recorded in fish 26-30 cm long may be due either to interindividual variations or to the choice of a muscle sample (at this body size, only a piece of muscle was dissected—see Materials and Methods). Two parvalbumin isotypes with pI values of 4-63 and 4-90 have been identified in striped bass. The one with a pI of 4-63, the major isotype in small fish, is thus likely to correspond to PA II. These results agree with our previous work on another serranid, the sea bass: two isotypes, PA II (pI: 4-56) and PA V (pI: 4-99) have also been characterized (Huriaux *et al.,* 1996).

Isoelectric focusing is a more discriminating technique for separating parvalbumin isotypes. It is quantitatively less accurate, however, because focusing of proteins in narrow zones induces a saturation of staining for high protein concentrations. This analytical method confirmed the presence of two PA IV isotypes and revealed the unexpected existence of three PA II isotypes during barbel development. The fast fibres of barbel dorso-lateral muscle can thus express at least six electrophoretically distinguishable isotypes. This large number of isotypes provides a very attractive opportunity for studying precisely how their levels vary in the course of barbel growth. The present results aptly complement those of an earlier study in which the synthesis of three parvalbumin isotypes was monitored only by electrophoresis at alkaline pH, in fish not exceeding 20 cm (Focant *et al.,* 1992). Here, we see that the proportion of PA IVa was always very low, except in fish 4-8 cm long. Levels of the PA II isotypes varied differentially during juvenile growth. While all three forms were present at the larval stage, PA lie and chiefly PA lib appeared as early forms, contrary to PA IIa which was still synthesized at the beginning of the adult period. During the later adult stage, PA IIa disappeared and PA IVb increased to 90% of the total parvalbumin content. PA III remained a minor adult isotype as did trout PA I (Huriaux *et al.,* 1996).

The pI differences observed between the PA II isotypes did not seem to happen during sample preparation because the level of each form varied regularly during development and independently of one another. A precise physicochemical comparison would require the isolation of PA lib and PA lie from very small specimens, which would be difficult in consideration of the low amount of muscle available. On the other hand, in our previous study on barbel myosin structure no sample was contaminated by red fibres, even in the first

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stages of development (Focant *et al.,* 1992).

The contraction speed (K) required for maximum power output by fish white muscle during swimming decreases with increasing body size (Anderson & Johnston, 1992). This should entail a decreasing tail-beat frequency and a diminishing maximum swimming length-specific speed. Parvalbumins, very abundant in fish fast fibres, are involved in calcium transfer from the myofibrils to the sarcoplasmic reticulum during muscle relaxation. They have therefore been viewed as soluble relaxing factors in cold-blooded vertebrates (Gerday & Gillis, 1976; Gillis & Gerday, 1977). Their higher titre in small barbels (5 cm) may lead to a higher rate of muscle relaxation and thus reduce the active state of muscle contraction, favouring a high tail-beat frequency, as Rodnick & Sidell (1995) assumed to be the case in the striped bass. The sequential appearance of different isotypes in the course of development probably reflects the changing functional requirements of the axial musculature as the fish grows; thus it suggests a specific physiological role for each isotype in muscle relaxation. The larval isotypes should favour faster relaxation of fast fibres and thus shorter contraction times than the adult ones.

SPATIAL DISTRIBUTION

The total parvalbumin titre also decreased from the first to the last myotomes in the adult barbel. In cod, the relaxation time is reportedly shorter for fast fibres from rostral than from caudal myotomes, although the volume and surface densities of T-tubules and sarcoplasmic reticulum do not differ significantly according to the myotomes (Davies *et al.,* 1995). Both the higher total parvalbumin titre in the anterior myotomes of the barbel and the higher percentage of isotype PA II, the most efficient relaxation factor, at this location could mean that the relaxation time is shorter there than in the caudal myotomes of this fish.

In conclusion, parvalbumin isotypes are differentially expressed, both spatially and temporally. The in-depth study of this differential expression should contribute considerably to understanding muscle contraction in fish. Further insight into the functions of particular isotypes could be gained by comparing their calcium-binding properties and any specific effects on calcium flux in the sarcoplasm and on fast-fibre contraction.

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