Asian Fisheries Science **14** (2001): 285-292
ISSN: 0116-6514
https://doi.org/10.33997/j.afs.2001.14.3.005

Asian Fisheries Society, Manila, Philippines

Changes in the Patterns of Parvalbumin Expression During the Development of the Catfish *Heteropneustes fossilis* (Bloch)

F. A. SHERWANI, A. HASNAIN and I. PARWEZ

Department of Zoology
Aligarh Muslim University
Aligarh 202 002
India

Abstract

Exceptional thermal stability of parvalbumins has been employed to follow the ontogeny of parvalbumin isoforms in air-breathing catfish *H. fossilis*. Out of three isoforms separable by polyacrylamide gel electrophoresis, isoform III occurs ubiquitously from unfertilized ova to adulthood. Expression of isoform II coincides with the appearance of somites, whereas isoform I that occurs in trace amounts is detectable only after the formation of a sufficient number of somites and their persistent contraction after hatching. The results thus suggest that isoforms I and II are exclusively skeletal muscle parvalbumins.

Introduction

Parvalbumin, one of the calcium-binding proteins, is abundant in the skeletal muscle of lower vertebrates including fishes (Gerday 1988) and considered homologous to light chains of myosin (Collins 1976). Published evidence indicates that multiplicity of parvalbumin isoforms as a biochemical genetic marker may prove valuable in fish resource management. For instance, polychromic species of genus *Symphodus* were sorted out with the help of parvalbumin isotypes (Focant et al. 1990), and cichlid species could be biochemically identified at the species level (Focant et al. 1994). Similarly, subspecies level genetic polymorphism has been recorded in sea bream (Taniguchi et al. 1982) and tench (Bobak and Slechta 1988). Employing parvalbumin polymorphism, Huriaux et al. (1992) demonstrated greater diversity in Asian barbel as compared to its European counterpart. Besides, in order to define its role, physicochemical characterization and phylogenetic interrelationships have also been studied in several fish species. Electrophoretic patterns in these cases exhibit species specific multiplicity as well as independence from nongenetic variables (Bobak and Slechta 1988, Focant et al. 1988, Focant and Vandewalle 1991). To establish a functional correspondence with different muscle types, the intraspecies structural variants of isoforms are receiving increasing attention (Huriaux et al. 1990, 1992).

However, for a better understanding of the morphofunctional aspect of parvalbumins, it is important that studies on intraspecific multiplicity should also be extended to isotypes in noncontractile tissues where these proteins occur in trace amounts (Gosselin-Rey 1974, Hamoir 1974, Hamoir et al. 1980).

Tissue specificities have long been explained on the basis of temporal expression during differentiation. As for the fishes, ontogeny of parvalbumin and its correlationship with tissue differentiation has not been extensively studied, possibly due to the lack of a suitable simple technique to permit localization in heterogeneous tissues extracts. Focant et al. (1992) and Huriaux et al. (1997) have employed immunohistochemical reactivity and electrospray ionization mass spectroscopy as the likely approaches to work out the ontogeny of parvalbumin isoforms in barbel (*Barbus barbus* L.) that revealed a definite correlation with muscle morphogenesis. As a simple alternative, we have explored the use of relative thermostability and electrophoretic comigration of these proteins to follow the ontogenic schedule of expression of isoforms in air-breathing catfish, *H. fossilis* (Bloch). This fish is ideally suitable for induced breeding under laboratory conditions and is being used in this study as a model to biochemically investigate the various aspects of its ontogeny.

Materials and Methods

Procurement and maintenance of the adult catfish

Mature specimens of catfish, *H. fossilis* (body weight: 60 to 100 g) were obtained from the local fish market at Aligarh during the breeding season (July to August). They were kept in glass aquaria (23 x 10 x 12") containing dechlorinated tap water and acclimated to laboratory conditions for about one week prior to initiation of experiments. They were fed *ad libitum* daily with Hindlever laboratory animal feed (Hindustan Lever Limited, Delhi, India). Water in the aquaria was replaced daily with equilibrated tap water.

Procurement of developmental stages

Catfish *H. fossilis* in various developmental stages (unfertilized egg, fertilized egg, morula, prehatching, and post-hatching) were obtained for a large number of specimens for two consecutive breeding seasons by artificial induction of spawning following the hypophysation method (Ramaswami and Sundararaj 1956). Various developmental stages are depicted in figure 1. The samples of various developmental stages were stored at –20°C until analyzed.

Preparation of muscle and larval extracts

White skeletal muscle from the anterio-dorsal region of the body was dissected out to free it of lateral line red muscles. Muscles as well as egg and whole embryonic stages were homogenized in 4 to 5 volumes of 50 mM Tris-HCl buffer of pH 7.5 and centrifuged at 10,000 rpm for 20 min. All proce-

dures were carried out at 2 to 5°C unless otherwise mentioned. Clear supernatants were subjected to electrophoresis (either directly or after heat denaturation) following the procedure of either Laemmli (1970) or Bobak and Slechta (1988) using 12.5 or 15% polyacrylamide gels, since larval extracts did not give better resolution on the gels of lower concentrations. As stated in the caption of figure 3, heat stable soluble fractions of egg and early larval stages had to be loaded several times more than the advanced stages due to trace amount of parvalbumin like protein in them. Protein bands were visualized by staining with coomassie brilliant blue. To confirm the parvalbumin nature of the proteins, heat denatured fractions concentrated by acetone fractionation (55 to 80%) were also run under identical electrophoretic conditions. Glycerol at a final concentration of 20% was added to store the sample at -2O°C.

Thermal denaturation and acetone fractionation

Clear supernatants were incubated at 7O°C for 30 min and after cooling down to room temperature, the precipitate was centrifuged at 4°C and 10,000 rpm. Since incubation of the extract, as such results in the appearance of a couple of additional bands that are noncomigratory with adult parvalbumin isotypes, the extract, in subsequent studies, was first subjected to acetone fractionation in a range of 55 to 80% as recommended for parvalbumins according to the procedure of Bhushana Rao et al. (1969). The same procedure was used to concentrate supernatants of larval extracts following the heat treatment. Traces of acetone from the precipitate were removed using vacuum evaporation while the dry pellet was dissolved in 50 mM Tris-HCl buffer (pH 7.5). The samples were extensively dialysed in benzoylated dialysis tubes. This procedure eliminated most of the background. That these properties as well as exceptional thermostability are typical of parvalbumins are elaborated under the section on discussion.

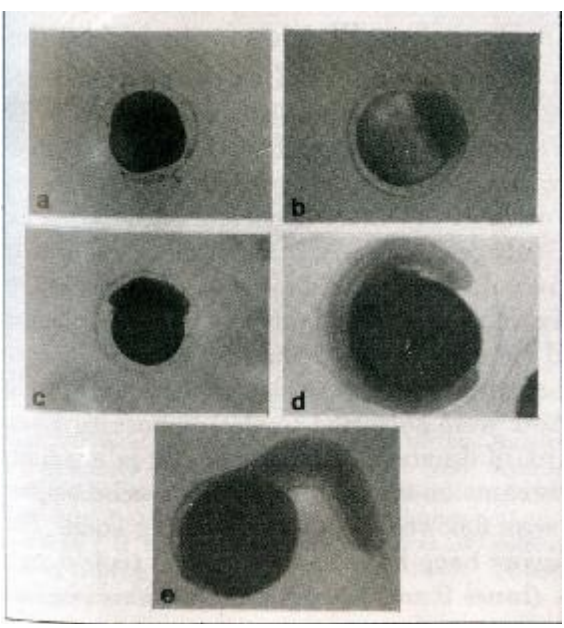

Fig. 1. Various developmental stages of *H. fossilis*. (a) unfertilized egg, x 30 (b) fertilized egg, x 35 (c) morula stage with compact mass of blastomeres, x 25 (d) prehatched larva occupying the entire perivitelline space, x 40 (e) newly hatched larva with laterally compressed body, x 30.

Results

Developmental stages

The eggs of the catfish were spherical, either green or brown and demersal. Differentiation between unfertilized (Fig. 1a) and fertilized (Fig. 1b) eggs was not possible until the process of cell division had initiated. Morula stage (Fig.1c) that was reached about two and a half hours post-fertilization was characterized by the presence of a compact mass of blastomeres without any increase in diameter. Shortly prior to hatching (Fig. 1d), almost the entire perivitelline space was occupied by the embryo. Hatching that was initiated 28 to 30 h post-fertilization was marked by frequent twitching movements of the embryo followed by violent jerky movements resulting in the rupture of the membrane and emergence of larva. The newly hatched larva (Fig. 1e) was transparent with laterally compressed body and a large almost round dark green yolk-sac. About 26 to 28 myomeres could be counted at this stage. The newly-hatched larva did not show much activity in the beginning but showed greater darting movements in the later stages of development.

Patterns of parvalbumin in adult and different developmental stages

Typical electrophoretic patterns of sarcoplasmic extract of dorsal muscle from four parents (two males and two females) used in the breeding experiments are shown in figure 2. This pattern reveals no significant individual variation; three major and five minor bands are detectable (lanes 5 to 8). However, following heat denaturation, only three bands persist - two major and one minor, detected after considerable overloading (lanes 1 to 4). These bands are recognized as parvalbumins as their electrophoretic comigrants appear with a consistency similar to acetone fractions (55 to 88%). Among the bands identified as parvalbumins, isoform III exists in the highest concentration followed by isoform II, while isoform I exists only in trace amounts. In the patterns of muscle extracts of adult fish, the band with the slowest mobility among the thick bands is not parvalbumin since it resolves into as many as three minor bands in low concentration gels exhibiting polymorphism (Pandey and Hasnain 1994). Electrophoretic patterns of extracts of selected developmental stages depicting major events related to parvalbumin expression are shown in figure 3 (lanes 3 to 8). Sometimes, considerable background smearing existed if whole extracts were heat denatured. Background smearing could be substantially reduced if extracts of various stages were first fractionated with acetone, incubated at 70°C for 30 min and soluble fractions thus obtained were subjected to electrophoresis. Even after this treatment, a little amount of denatured protein was still in a soluble state, that stacks in electropherograms as the band of slowest mobility just below the origin. The transition was followed up to post-hatching stage. For comparison, the representative lanes have been flanked by the typical patterns of the dorsal muscle extract (lanes 9 and 10) and parvalbumins purified

by thermal treatment were run under identical conditions (lanes 1 and 2). The correspondence of various isoforms with the comigrating bands reveals that parvalbumin III occurs ubiquitously from ova to adult stage. Band sharing comigration with isoform II exhibits temporal expression as its appearance coincides with the differentiation into somites (lane 6) that intensifies during the subsequent process of development. Trace amount occurrence of parvalbumin I makes any prediction in the extracts difficult. The obscurity may be due to lower sensitivity of the stain or the absence of crisp resolution in this region of the gel.

Discussion

Multiple forms of parvalbumin show considerable variation both in number as well as in the relative electrophoretic mobilities in the muscles of different species of lower vertebrates. For example, in carp (*C. carpio*) white muscle, four main components (II, III, IVa and IVb) are present (Gosselin-Rey et al. 1978) whereas in eel *(Anguilla anguilla*) skeletal muscle only three components (II, III and V) (Dubois and Gerday 1990) have been identified. In some other species, only one component is present, such as component IV in muskellunge (*Esox masquinongy*), component III in large mouth bass (*Micropterus salmoides*), component II in bar (*Morone labrax*) and in chub (*Luciscus cephalus*) (Bhushana Rao and Gerday 1973). In contrast, in muscles of mammals, including man, only a single isotype of parvalbumin has been so far detected (Heizmann 1984). The present study on the catfish *H. fossilis* skeletal muscle shows the presence of three components, I, II and III, with component III being present in largest amount, followed by component II while only traces of component I could be observed following polyacrylamide gel electrophoresis and visualization by staining with coomassie brilliant blue.

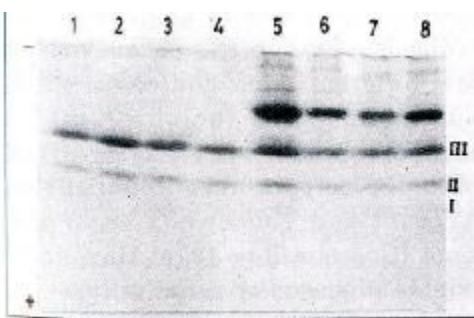

Fig. 2. Typical electrophoretic patterns of the whole muscle extracts (lanes 5 - 8) and heat resistant fractions (lanes 1 - 4) of four *H. fossilis* brooders. Bands designated as parvalbumin isoforms I, II and III consistently appear in all adults (see Fig. 3, lanes 1- 2; 9 - 10 for clearer appearance of isoform I band).

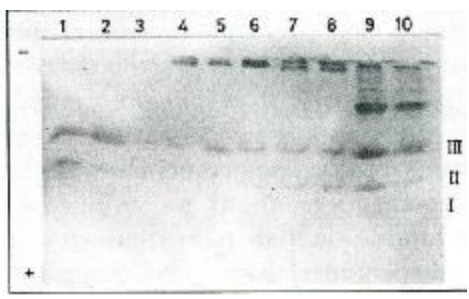

Fig. 3. Electrophoretic patterns of thermally treated and acetone fractionated parvalbumin isoforms of *H. fossilis.* Lanes 1 - 2: skeletal muscle of the adult; 3-unfertilized egg; 4-fertilized egg; 5-morula stage; 6-prehatching stage; 7 - 8-post-hatching stage. Lanes 9 - 10 are whole muscle extractsof the adult. In lanes 3, 4 - 5 respectively, 20, 10 & 5 times the volume of the sample volume used for lane 6 were loaded. I-III, assigned numericals for parvalbumin isoforms in the order of decreasing electrophoretic mobilities.

A recent study on *H. fossilis* (Hasnain et al. 1999) reveals that all three thermostable bands that have been identified as parvalbumins in the adult fish stack as a single band of ~12 KD in SDS-PAGE, the value assigned to parvalbumins in the documented literature (Huriaux et al. 1997). The above study also shows that the three bands marked as parvalbumin I-III prominently appear in PAGE patterns of soluble fraction of heat treated muscle extracts with other trace bands as shown in figures 2 and 3. Significantly, even these trace bands would not appear if acetone fraction (taken here as parvalbumins) was subjected to heat treatment before electrophoresis. This report thus, established beyond doubt that in so far as *H. fossilis* is concerned, acetone fractionation-cum-heat treatment yields parvalbumins of remarkable purity. Parvalbumin-like nature of these bands is further corroborated by the criteria such as acetone fractionation in the range typical of parvalbumins, solubility even after treatment with this organic reagent, compatible thermostability, electrophoretic comigration with adult parvalbumin isotypes, presence of a gradient concordant with the course of muscle development and reproducibility of results for two consecutive breeding seasons.

Our results demonstrate that parvalbumin isoforms in *H. fossilis* are expressed according to an ontogenic schedule and the exceptional thermostability of these proteins can be employed as a simple technique to qualitatively follow the developmental changes. The polyacrylamide gel electrophoresis of the extracts of various developmental stages (unfertilized egg, fertilized egg, morula, prehatching and post-hatching) of the catfish reveals that an electrophoretic equivalent of component III, being the most prominent and present in the largest concentration in the muscle of adult catfish, is present even in an unfertilized egg. The ontogenic expressions involve both enhanced expression of the preexisting isoform III and the temporal expression of isoform II. The presence of parvalbumin-like protein in ova of fish has not so far been reported. We have, however, detected the presence of this band (isoform III) applying the above mentioned criteria. Isoform III, therefore, apparently may not be entirely associated with the modulation of muscle contraction because it is present even in the unfertilized egg. However, parvalbumin II in the catfish seems to be involved in the above process as its appearance coincides with muscle differentiation and contraction. The findings about parvalbumin II is in line with the observations available on fish parvalbumin ontogeny (Focant et al. 1992) and frog (Schwartz and Kay 1988). Since parvalbumin isotypes have different distributions depending on muscle type (Focant and Vandewalle 1991) and may also exist in other nonmuscle tissue (Gosselin-Rey 1974; Hamoir et al. 1980), we have confirmed the invariable presence of three isotypes in the whole advanced larvae beyond hatching, pointing to a delayed expression of parvalbumin I or its raised level due to increase in the quantity of tissue where it exists, as the larvae grow. This supports the idea of asynchronous synthesis of various parvalbumin isotypes. The delayed expression of isoform I may presumably be correlated with the innervation schedule of differentiating muscle fibers and bundles

(Kulberg et al. 1977). These observations clearly suggest that parvalbumins in the catfish are expressed in the very early stage of development (unfertilized egg) and significant qualitative and quantitative changes follow as development proceeds.

The present study also demonstrates a positive correlation between the quantitative intensity of parvalbumin component in adult and the timing of their appearance during the developmental stages. For instance, component III which is most intense in adult first appears during the developmental stages and becomes progressively more prominent while component I which is the least intense even in adult appears last in preadult stages. This may possibly be associated with some functional significance of these components that becomes more important at specific times during development.

There is no parvalbumin IV in *H. fossilis*. The thick band of slowest mobility on the basis of applied criteria is not parvalbumin. Under modified electrophoretic conditions it resolves into 1 to 3 bands in some of the other stocks, exhibiting isoloci polymorphism (Pandey and Hasnain 1994) which is indicative of its enzyme nature.

Acknowledgment

The authors would like to thank Ms. Alka Kulshrestha and Ms. Meenu Nayyar for their assistance in this study. This work was partially supported by a research grant from the Council of Scientific and Industrial Research, New Delhi.

References

Bhushana Rao, K.S.P., B. Focant, Ch. Gerday and G. Hamoir. 1969. Low molecular weight albumins of cod white muscle (*Gadus callarias* L.). Comparative Biochemistry and Physiology 30: 33-48.

Bhushana Rao, K.S.P. and Ch. Gerday. 1973. Low molecular weight proteins of pike (*Esox lucius*) white muscle-I. Extraction and purification-Polymorphism. Comparative Biochemistry and Physiology 448: 931-937.

Bobak, P. and V. Slechta. 1988. Comparison of parvalbumin of some species of family cyprinidae. Comparative Biochemistry and Physiology 91B: 697-699.

Collins, J. H. 1976. Homology of myosin DTNB light chain with alkali light chains, troponin C and parvalbumin. Nature 259: 699-700.

Dubois, I. and Ch. Gerday. 1990. Soluble calcium-binding proteins: Parvalbumins and calmodulin from eel skeletal muscle. Comparative Biochemistry and Physiology 95B: 381-385.

Focant, B., C. Viladiu and P. Vandewalle. 1988. Biochemical analysis of parvalbumins and myosin light chains from Mediterranean Serranids: first applications to the systematics. Commission International pour l' Exploration Scientifique de la Mer Mediterranee. Rapports et Proces-Verbaux des Reunions 31: 258.

Focant, B., C. Michel and P. Vandewalle. 1990. Use of biochemical analysis of muscle proteins to help the classification of polychromic individuals of the genus *Symphodus*. Archives Internationales de Physiologie et de Biochimie 98: 87-93.

Focant, B. and P. Vandewalle. 1991. Comparison of myosin subunits and sarcoplasmic parvalbumins of white and red trunk muscles from various species of African cichlids. Application to an hybrid. Musee Royal de l' Afrique Centrale, Tervueren, Belgique. Annales, Sciences Zoologiques 262: 33-38.

Focant, B., F. Huriaux, P. Vandewalle, M. Castelli and G. Gossens. 1992. Myosin, parvalbumin and myofibril expression in barbel (*Barbus barbus* L.) lateral white muscle during development. Fish Physiology and Biochemistry 10: 133-143.

Focant, B., P. Laleye and P. Vandewalle. 1994. Biochemical attempts to characterize thirteen fish species by their muscular parvalbumins. Archives Internationales de Physiologie et de Biochimie 102: 135-138.

Gerday, Ch. 1988. Soluble calcium binding proteins in vertebrate and invertebrate muscles. In: Calcium and Calcium Binding Proteins (eds. Ch. Gerday, R. Gilles, and L. Bolis), pp.23-39. Springer-Verlag, Berlin, Heidelberg.

Gosselin-Rey, C. (1974). Fish parvalbumins: Immunochemical reactivity and biological distribution. In Symposium on calcium-binding proteins (eds. W. Drabikwski, H.Strzelecka Golaszewska and E. Carafoli). Elsevier, Amsterdam and PWN-Polish Scientific Publications, Warszawa. 679-701.

Gosselin-Rey, C., A. Piront and Ch. Gerday. 1978. Polymorphism of parvalbumin and tissue distribution characterization of component I, isolated from red muscles of *Cyprinus carpio* L. Biochimica Et Biophysica Acta 532: 294-304.

Hamoir, G. 1974. Earlier and recent work on parvalbumins. Symposia Biologica Hungarica 17: 17-33.

Hamoir, G., N. Gerardin-Otthiers and B. Focant. 1980. Protein differentiation of the superfast swim bladder muscle of the toad fish *Opsanus tau.* Journal of Molecular Biology 143: 155-160.

Hasnain, A., R.B. Pandey and S.H. Arif. 1999. An electrophoretic evaluation of thermostable proteins of muscle myogen as biochemical markers of *Heteropneustes fossilis* Bloch. Journal of Applied Zoological Research 10: 155-159.

Heizmann, C.W. 1984. Parvalbumin an intracellular calcium-binding protein: distribution, properties and possible roles in mammalian cells. Experientia 40: 910-921.

Huriaux, F., P. Vandewalle, J.C. Philippart and B. Focant.1990. Electrophoretic comparison of myosin light chains and parvalbumins of trunk and head muscles from two barbel (*Barbus barbus*) populations. Comparative Biochemistry and Physiology 97B: 547-553.

Huriaux, F., P. Vandewalle and B. Focant 1992. Polymorphism of white muscle myosin and parvalbumins in the genus *Barbus* (Teleostei:Cyprinidae). Journal of Fish Biology 41: 873-882.

Huriaux, F., S. Collins, P. Vandewalle, J.C. Philippart and B. Focant. 1997. Characterization of parvalbumin isotypes in white muscle from barbel and expression during development. Journal of Fish Biology 50: 821-836.

Kulberg, R.W., T.L. Lentz and M.W. Cohen. 1977. Development of myotomal neuromuscular junction in *Xenopus laevis*: an electrophysiological and fine-structural study. Developmental Biology 60: 101-129.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T_4. Nature 227: 680-685.

Pandey, R.B. and A. Hasnain. 1994. Protein polymorphism in muscle myogens of *Heteropneustes fossilis*. Current Science 66: 870-871.

Ramaswami, L. S. and B. I. Sundararaj. 1956. Induced spawning in the catfish. Science 125: 1080.

Schwartz, L.M. and B.K. Kay. 1988. Differential expression of Ca^{2+}-binding protein parvalbumin during myogenesis in *Xenopus laevis*. Developmental Biology 128: 441-452.

Taniguchi, N., K. Sumantadinata, A. Suzuki and J. Yamada. (1982). Genetic variations in isoelectric focusing pattern of sarcoplasmic protein of black seabream. Bulletin of the Japanese Society of Scientific Fisheries. 48:139-141.

Manuscript received 21 July 2000; Accepted 06 February 2001