

Asian Fisheries Society, Manila, Philippines

Differences in Thermal Aggregability of Polymorphic Soluble Muscle Proteins of *Channa punctata* (Channidae: Channiformes)

RIAZ AHMAD¹, KHURSHEED A. KHAN², R.B. PANDEY¹ and A. HASNAIN^{1*}

¹Section of Genetics, Department of Zoology
Aligarh Muslim University, Aligarh-202 002 India

²Department of Basic Sciences, A.K. Tibbiya College
Aligarh Muslim University, Aligarh-202 002 India

Abstract

Electrophoresis in native 7.5% polyacrylamide gel made in Tris-HCl and run in Tris-borate revealed three parvalbumin phenotypes in soluble proteins of white skeletal muscle of spotted snakehead *Channa punctata*. Parvalbumins (PV) were initially recognized because of fast electrophoretic migration at alkaline pH (that indicates acidic nature), exceptional thermostability, relatively high concentration and acetone fractionation. Though constituted by isoforms PVIII, PVI or PVII, acetone purified each of the three phenotypes stacked as single bands of identical M_r of ~11 kD following SDS-PAGE. Phenotype-specific but soluble aggregates were formed by incubating muscle extracts at 70°C. These results demonstrate that modified PAGE protocols can reveal polymorphism, which may be obscure under alternative systems. Further, heat-stable soluble muscle proteins, specifically the isoparvalbumins may be good candidates for such screening.

Introduction

Muscle sarcoplasm is a rich source of soluble proteins. It embraces an entire range of enzymes and proteins of glycolysis and electron trans-

* Corresponding author. Tel.: +91 93 5821 8737; Fax: +91 57 1270 1239
E-mail address: absar.hasnain@gmail.com

port. As a result, extensive electrophoretic investigations have been made on soluble sarcoplasmic proteins of teleosts. In a number of instances, even genetic variations have been revealed by routine staining of sarcoplasmic protein electrophoregrams. The presence of two variants in soluble muscle proteins electrophoregrams of *Anoplopoma fimbria* and three in *Catostomus* sp. was reported by Tsuyuki et al. (1965; 1967). Population analysis of polymorphic isoforms of low molecular weight proteins of muscle was made in cases of *Menidia menidia* and rainbow trout (Morgan and Ulanowicz 1976; Jones and Glay 1997). *Tinca tinca* and several cyprinids were also shown to display genetic polymorphism within the above group of proteins (Boback and Slechta 1988; Huriaux et al. 1992). High resolution isoelectric focusing (IEF) was used by Taniguchi et al. (1982) to demonstrate polymorphism in soluble muscle proteins of black seabream. Most of the published work employing IEF has otherwise documented species specificity of investigated proteins with diagnostic importance of parvalbumins to identify even cooked fish (Basaglia 1992; Basaglia and Marchetti 1990; Esteve-Romero et al. 1996; Rehbein et al. 2000; Etienne et al. 2001; Berini et al. 2005). However, cost effective and convenient polyacrylamide gel electrophoresis (PAGE) remains a widely preferred technique of resolving protein complexes; specifically, when number of samples is large as in the case of population studies.

We had previously reported that a minor modification in PAGE protocols can help in the detection of isoloci in muscle extracts of air-breathing catfish *Heteropneustes fossilis* (Pandey and Hasnain 1994). This report describes that a change in the sieving property of polyacrylamide gel can detect polymorphism of acidic (fast migrating) muscle proteins of important food fish *Channa punctata* (Bloch). Further identification of this group of proteins was made by incubating muscle extracts at 70°C. Information on genetics of widely distributed spotted snakehead *C. punctata* has implications in aquaculture. It is one of the food fishes with a wide distribution from China, throughout Southeast Asia up to Iran. It carries further commercial importance as a component of integrated fish culture.

Materials and Methods

Source of fish samples and preparation of muscle extracts

Live fish (*Channa punctata*) samples of 15-16.5 cm were obtained from local (Aligarh) market and represented catches from two different

locations. The fish were stunned by cerebral blows and the white dorso-lateral muscle of the most anterior parts of the body were homogenized in 50 mM Tris-HCl of pH 7.5 at top speed of a mechanical homogenizer (Biospec, U.S.A., model 985-380). Following centrifugation at 10,000 rpm (4°C), clear supernatants were saved and either analyzed fresh or stored at -20°C until electrophoresis.

Protein contents of muscle extracts were estimated following biuret protocol.

Purification of parvalbumin phenotypes

This was carried out by cold acetone fractionation of muscle extracts. Precipitate obtained between 65-85% was saved and acetone was removed by keeping in a dessicator overnight. The protein was dissolved in Tris-HCl buffer used to homogenize the muscle. Parvalbumin solution thus obtained was passed through 5x1.5 cm columns of Sepharose 6B. Main peak was collected and protein concentration was determined using the biuret method.

Native polyacrylamide gel electrophoresis (PAGE)

The modified buffer system as described by [Hasnain et al. \(1999\)](#) was used where separating gel is made in 0.375 M Tris-HCl (pH 8.6), 3 % stacking gel in 0.0125 M Tris-HCl (pH 6.8) and run in 0.0661 M Tris-0.0324 M boric acid (pH 8.3). Protein bands were stained with coomassie brilliant blue (CBB).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight (M_r) was estimated with the help of SDS-PAGE carried out essentially according to the protocol of [Schagger and Jagow \(1987\)](#). Separating gels were 10% or 12.5 % in acrylamide:crosslinker (49.5%:3%) and Tris-HCl. Running buffer in both reservoirs was 0.034 M Tris-glycine of pH 8.6. Upper reservoir also contained 0.001 M tricine. Low molecular weight (43-6.5 kD) markers were purchased from BangaloreGenei (India).

Thermal incubation

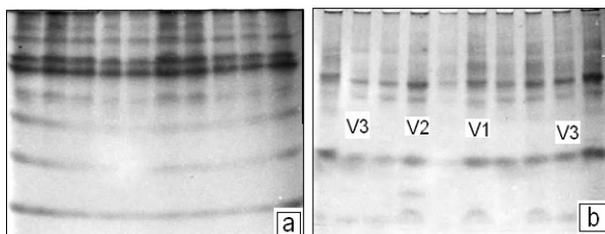
Thermal incubation was carried out at 70°C for 30 minutes as described previously ([Hasnain et al. 1999](#)). Precipitated heat-denatured protein was removed by centrifugation as described above. Clear supernatants containing soluble heat-stable proteins were subjected to electrophoresis.

Densitometric analysis

ScionImage software was used for densitometric tracing and Gel-Pro program (Cybernetics, USA) for quantitative estimation of peak areas of protein bands.

Results

In all, soluble proteins from 50 individuals of *Channa punctata* were analyzed. Typical 10 selected lanes that include 3 phenotypes are shown in figure 1. No qualitative difference was observed in the fastest migrating bands in 10% polyacrylamide (PA) gels (Fig. 1a). However in 7.5% gels, fast migrating bands marked I-III displayed variations in relative electrophoretic mobilities. On the basis of these differences three phenotypes phenotype-1, phenotype-2 and phenotype-3 were identified (Fig. 1b). The 2-banded phenotype-1 differed from phenotype-3 in slow migration of band PVI, while 3-banded phenotype-2 had an additional band (PVII) of intermediate mobility. Highest intensity band marked PVIII was common to all phenotypes. Out of 50 samples analyzed, relative phenotypic abundance of phenotypes 1-3 were: 0.32, 0.14 and 0.4, respectively. Densitometric tracings (Fig. 3) substantiated the qualitative observations on native PAGE profiles (Fig. 3, left hand panel).



Concentration of polymer in gels was 10% in (a) and 7.5% in (b), respectively. Separating gels were made in 0.375 M Tris-HCl (pH 8.6). In either case, 3 % stacking gel were in 0.0125 M Tris-HCl (pH 6.8) and compartments buffer 0.0661 M Tris-0.0324 M boric acid (pH 8.3). Gels were stained with CBB.

Fig. 1. Native polyacrylamide gel electrophoretic (PAGE) profiles of selected lanes of soluble muscle proteins of

Channa punctata showing polymorphism in gel-b.

Concentration of polymer in gels was 10% in (a) and 7.5% in (b), respectively. Separating gels were made in 0.375 M Tris-HCl (pH 8.6). In either case, 3 % stacking gel were in 0.0125 M Tris-HCl (pH 6.8) and compartments buffer 0.0661 M Tris-0.0324 M boric acid (pH 8.3). Gels were stained with CBB.

As an acetone-fraction almost pure parvalbumin accounted for 15-25% of total soluble muscle proteins. Since in 10% SDS-PA gels purified parvalbumins stacked as single band just above the buffer line (Fig. 2a), we analyzed purified preparations using 12.5% SDS-PAGE (Fig. 2b). Al-

though in 12.5 % SDS-PA gels the variants stacked as single band of ~11 kD above 6.5 kD (aprotinin) band of low M_r marker standards, there is no other band beyond it above or below PV band.

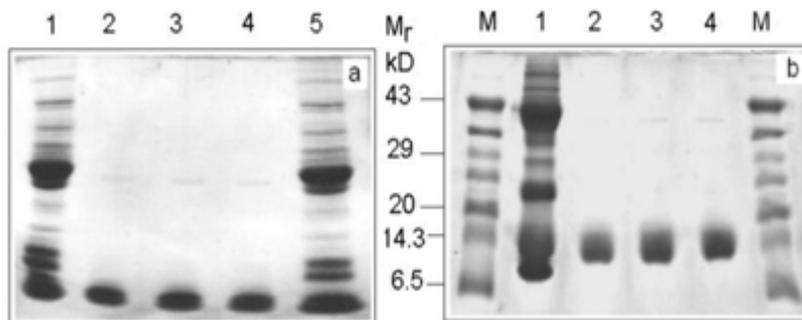
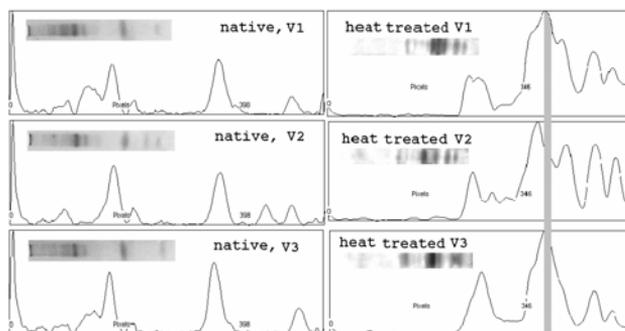


Fig. 2. SDS-PAGE profiles showing staking of purified parvalbumin phenotypes in 10% (a) and 12.5% gels (b). All isoforms are stacked as single band of ~11 kD in both systems. Loading sequence in 2a: purified parvalbumin phenotypes 1-3 are loaded in lanes 2,3 and 4, respectively. Flanking lanes 1 and 5 are soluble muscle proteins. Loading sequence in 2b: M (molecular weight markers), 1 whole soluble muscle proteins; 2, 3 and 4 are phenotypes 1-3 while the last lane is of Markers (M).

Fig. 3. Densitometric tracing of native PAGE profiles showing relative intensities and electrophoretic mobilities of soluble aggregates formed during incubation at 70°C for 30 minutes. Native lanes are shown in the left hand panel, while corresponding heat treated variants are shown on the right. ScionImaging software was used.



Electrophoretic conditions are the same as in figure 1.

Polymorphic protein bands of *C. punctata* muscle extracts were also tested for thermostability, which is an established characteristic of parvalbumins (Sherwani et al. 2001; Arif et al. 2007; Carrera et al. 2006). As shown in figure 3, following heat treatment each of the three phenotypes generated type-specific aggregates. The aggregated products remained soluble at low ionic strength of the extraction buffer. Soluble products also retained satisfactory stacking property in PAGE (Fig. 3). Densitographic tracing of PAGE profiles of each heat-treated phenotype (Fig. 3, right hand panel) supports the differences with its corresponding native

form (Fig. 3, left hand panel). A quantitative comparison of the relative intensities of most prominent bands has been made by GelPro software as shown in figure 4. Though for each phenotype the maximum stacking of soluble products was in the same range (bands, # 5-7), individual positional differences in corresponding peak areas of the phenotypes 1-3 are obvious.

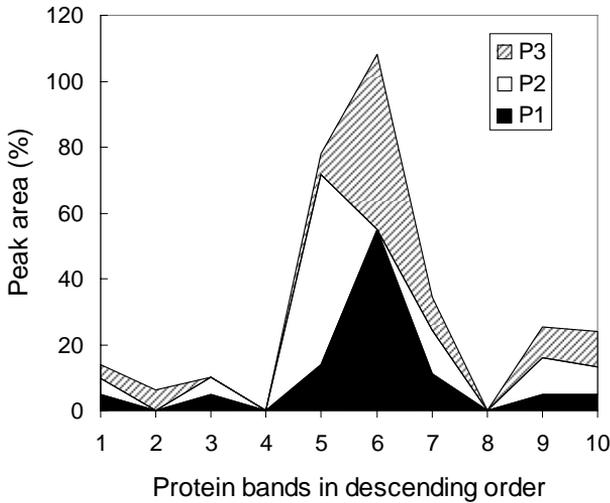


Fig. 4. Area diagram of the representative lanes of soluble aggregates in native PAGE profiles showing differences in relative intensities and mobilities of major bands of the aggregates.

Discussion

More extensive information is available on species specificity of electrophoretic profiles of fish parvalbumins (Basaglia 1992; Focant et al. 1990; 1994; Esteve-Romero et al. 1996; Rehbein et al. 2000; Etienne et al. 2001; Berini et al. 2005). In a modified system, Hurliaux et al. (1992; 1997) demonstrated polymorphism in parvalbumins of *Barbus* sp. We had also previously shown that PAGE with minor modifications in discontinuous buffer systems can display heterogeneity of muscle proteins with or without specificity (Pandey and Hasnain 1994; Hasnain et al. 1999; Hasnain et al. 2005). The present report is another example where polymorphism of soluble muscle proteins was detected by a simple change in the sieving property of the gel (Fig. 1b). On the basis of criteria outlined in the next paragraph, they have been identified as parvalbumin isoforms (Bhushan-

Rao and Gerday 1973; Hurliaux et al. 1992). Polymorphism was not detected in gels of higher polymer concentration (Fig. 1a). It is general practice to type parvalbumin (PV) isoforms using Roman numerals *I-III* or more, in a decreasing order of relative electrophoretic mobilities (Hurliaux et al. 1992).

Polymorphic protein bands displayed three distinct characteristics of parvalbumin, which is a calcium-binding protein of abundance in skeletal muscle of fish and other cold blooded vertebrates (Bhushan-Rao and Gerday 1973; Hamoir 1974; Hurliaux et al. 1992; Hasnain et al. 1999; Erickson et al. 2005). The established characteristics are: (i) high concentration in muscle extract; (ii) fast electrophoretic migration at alkaline pH that suggests their acidic nature; (iii) exceptional thermostability; (iv) precipitation by acetone; and, (v) a molecular weight value of ~11-12.5 kD (Fig. 2). We have recently reported the immunological identification and biochemical characterization of parvalbumins of four species of genus *Channa* that also included the most common PV phenotype-3 (Arif et al. 2007). Since two new phenotypes (1 and 2) being reported here share five typical biochemical characteristics of parvalbumins, the observed phenotypic variations are not artifacts. To the best of our knowledge, this is the first credible report on polymorphism in fast muscle isoparvalbumins of spotted snakehead *C. punctata*. Phenotype-1 and phenotype-2 are novel variants which have not so far been reported. The differences between stacking of PV isoforms by native PAGE suggest variations in the isoelectric pH (pI). This, in turn, indicates differences in the primary structure of individual parvalbumin isoforms.

This report is also the first that demonstrates variant-specific aggregation of parvalbumin isoforms. Since generally, thermal treatment of proteins leads to aggregation and insolubility, solubility of PV aggregates in low ionic strength buffer is a remarkable property. Figure 3 and its densitometric analysis also showed that thermal incubation generated extra bands (aggregates) which were phenotype-specific. Highest intensity bands of aggregates also displayed clear differences in relative electrophoretic mobilities (Fig. 4).

Apart from genetic identification, parvalbumin typing by native PAGE has been used to trace ontogeny of some fish species (Hurliaux et al. 1997; Sherwani et al. 2001). Recent studies confirm a relaxation role for parvalbumins in physiological functioning of muscle (Wilwert et al. 2006). Other applications include use of PAGE and IEF profiles as biomarkers in identifying cooked and processed fish food products (Esteve-Romero et al.

1996; Etienne et al. 2001) and as standard IEF markers (Rehbein et al. 2000; Berini et al. 2005). Clinical importance of parvalbumins as the major allergen of fish food has also prompted wide range investigations on these proteins of fish species (Sten et al. 2004).

Though, polymorphism involving as many as 7 PV isoforms existed in *Centropomus undecimalis* (Ross et al. 1997), an intraspecies Mendelian combination of isomorphs is yet to be reported. Molecular studies and sequence comparisons confirm that vertebrate PV has α and β , two distinct evolutionary lineages (accessible from CABP Data Library).

Conclusions

Polymorphism in parvalbumin isoforms can be detected by native PAGE using gels of suitable sieving properties or switching over buffers. Anodic stacking in electrophoregrams due to acidic nature, remarkable thermostability and high concentration help initial recognition of these proteins. These criteria helped to identify three phenotypes in soluble muscle extracts of spotted snakehead *Channa punctata*. Acetone fractionation and M_r verified parvalbumin nature of the polymorphic proteins. Significantly, aggregates of heat-treated parvalbumin phenotypes produced variant-specific native PAGE profiles.

Acknowledgements

Authors are grateful to the Aligarh Muslim University for providing the necessary laboratory facilities. The help of Dr. A. L. Bilgrami, Rutgers University (USA) is also acknowledged.

References

- Arif, S.H., M. Jabeen and A. Hasnain. 2007. Biochemical characterization and thermostable capacity of parvalbumins: The major food allergen. *J. Food. Biochem* 31:121-137.
- Basaglia, F. 1992. Comparative examination of soluble red muscle proteins of fifteen Sparidae species. *J. Fish. Biol.* 40: 557-566.

- Basaglia, F. and M.G. Marchetti. 1990. A comparative study of some soluble proteins of the genus *Diplodus* (Sparidae: Teleostei). *Comp. Biochem. Physiol.* 95B: 653-656.
- Berini, A., V. Tepedino, V. Borromeo and C. Secchi. 2005. Isoelectric focusing identification of four freshwater fish commercially labeled "Perch". *J. AOAC International*. 88 : 670-672.
- Bhushan-Rao, K.S.P. and C. Gerday. 1973. Low molecular weight proteins in Pike (*Esox lucius*) white muscle-1. Extraction and purification-polymorphism. *Comp. Biochem. Physiol.* 44B : 931-937.
- Bobak, P.E and V. Slechta. 1988. Comparison of parvalbumins of some species of family Cyprinidae. *Ibid.* 91B: 697-699.
- Carrera, M., B. Cañas, C. Piñeiro, J. Vázquez and J.M. Gallardo. 2006. Identification of commercial hake and grenadier species by proteomic analysis of the parvalbumin fraction. *Proteomics*. 6 : 5278-5287.
- Erickson, J.R., B.D. Sidell and T.S. Moerland. 2005. Temperature sensitivity of calcium binding for parvalbumins from Antarctic and temperate zone teleost fishes. *Comp. Biochem. Physiol. Part A*, 140: 179-185.
- Esteve-Romero, J., U.M. Ynam and P.G. Righetti. 1996. Fish species identification by isoelectric focusing of parvalbumins in immobilized pH gradients. *Electrophoresis*. 17: 1380-1385.
- Etienne, M., J. Fleurence, H. Rehbein, R. Kundiger, E. Memdes, H. Costa and I. Martinez. 2001. Species identification of formed fisheries products and high pressure-treated fish by electrophoresis: a collaborative study. *Food Chem.* 72:105-112.
- 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*. *Arch. Inter. Physiol. Biochem.* 98: 87-93.
- Focant, B., P. Lalhe and P. Vandewalle. 1994. Biochemical attempts to characterize thirteen fish species by their muscular parvalbumins. *Arch. Inter. Physiol. Biochem.* (now *Arch. Physiol. Biochem.*) 102: 135-138.
- Hamoir, G. 1974. Earlier and recent work on parvalbumins. *Symp. Biol. Hung.* 17:17-33.
- 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. *J. Appl. Zool. Res.* 10:155-159.
- Hasnain, A., S.H. Arif, R. Ahmad and R.B. Pandey. 2005. Identification of species-marker bands in native and SDS-PAGE patterns of soluble muscle proteins of four species of genus *Channa* (Gronov) (Channidae:Channiformes) with evidence of some intraspecies heterogeneity. *Asian Fish. Sci.* 18:49-58.
- Huriaux, F., P. Vandewalle, J.C. Philippart and B. Focant. 1992. Polymorphism of white muscle myosin and parvalbumins in genus *Barbus* (Teleostei : Cyprinidae). *J. Fish. Biol.* 41: 873-882.
- Huriaux, F., S. Collin, P. Vandewalle, J.C. Philippart and B. Focant. 1997. Characterization of parvalbumin isotypes in white muscle from the barbel and expression during development. *J. Fish. Biol.* 50: 821-836.
- Jones, M.W. and D. Glay. 1997. Genetic relationships among populations of wild and hatchery resident of anadromous brook trout. *Comp. Biochem. Physiol.* 51B:29-40
- Morgan, R.P. and N.I. Ulanowicz. 1976. The frequency of muscle protein polymorphism in *Menidia menidia* (Atherinidae) along the Atlantic coast. *Copeia* 2:356-360.

- Pandey, R.B. and A. Hasnain. 1994. Protein polymorphism in muscle myogens of *Heteropneustes fossilis*. Curr. Sci. 66 : 870-871.
- Rehbein, H., R. Kundniger, C. Pineiro and R.I. Perez-Martin. 2000. Fish muscle parvalbumins as marker proteins for native and urea isoelectric focusing. Electrophoresis 21:1458-1463
- Ross C., R.W. Tilghman, J.X. Hartman and F. Mari. 1997. Distribution of parvalbumin isotypes in aduly snook and their potential applications as species biomarkers. J. Fish Biol. 51 :561-572.
- Schagger, H. and G.V. Jagow. 1987. Tricine-Sodium doedecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kD. Analyt. Biochem. 166:368-379.
- Sherwani, F. A., A. Hasnain and I. Parwez. 2001. Changes in the patterns of parvalbumin expression during the development of the catfish *Heteropneustes fossilis* (Bloch). Asian Fish. Sci. 14: 285-292.
- Sten, I., K. Hansen, S.P. Stahl, S.B. Andersen, A. M. Torp, U. Bindsev-Jensen and J.C. Poulsen. 2004. Cross-reactivity to eel, eelpout and ocean pout in codfish-allergic patients. Allergy. 59 : 1173-1180
- Taniguchi, N., K. Sumantadinata, A. Suzuki and J. Yamada. 1982. Genetic variations in isoelectric focussing pattern of sarcoplasmic protein of black seabream. Bull. Japan. Soc. Sci. Fish. 48 : 139-141.
- Tsuyuki, H., E. Roberts and W.E. Vanstone. 1965. Comparative zone electropherograms of muscle myogens and blood hemoglobins of marine and freshwater vertebrates and their application to biochemical systematics. J. Fish. Res. Bd. Canada 22:203-211.
- Tsuyuki, H., E. Roberts and R.H. Kerr. 1967. Comparative electropherograms of the family Catostomidae. Ibid. 24 : 299-304.
- Wilwert, J.L., N.M. Madhoun and D. Coughlin. 2006. Pravalbumin correlates with relaxation rate in the swimming muscle of sheepshead and kingfish. J. Exp. Biol. 209:227-237.