DISCUSSION
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Fish muscle consists of several types of fibers which determine its biochemical and functional characteristics primarily depending on the type of contractile apparatus they possess. Both of the characteristics are determined mainly by the type of myofibrillar proteins and to some extent with the participation of a few sarcoplasmic proteins. It is calcium that triggers muscle contraction by release into sarcomeres through T-channels and the relaxation depends on calcium removal from the contractile apparatus. This is carried out by Ca$^{++}$-regulators or internal and external exchangers of myofibrils. Parvalbumin that is located in the muscle sarcoplasm is the most likely external exchanger or the regulator of calcium ion levels (Gerday, 1982).

Parvalbumin (PA), a high affinity calcium binding protein of a multigene family (Heizmann et al., 1987) is quite abundant in muscle of cold blooded vertebrates specifically the fishes (Hamoir et al., 1972). It some cases it is the major fraction of soluble protein repertoire of the sarcoplasam. As supported by the published evidence and shown for species of genus *Channa* in this study (Fig.1), PAGE of sarcoplasmic proteins have largely served as species markers. A similar inference can be made by SDS-PAGE pattern (Fig.2), if the number of bands showing consistent presence are taken into account. SDS-PAGE has so far rarely been used to study biomarkers.

Contrary to the above generalization, the PAGE pattern of PA isotypes or isoforms have the application beyond species diagnostics (Focant and Joyeaux, 1988), that is, the identification of fiber types as
initially shown by Hamoir (1972). Huriaux et al. (1990) showed that in the trunk white fibers of European barbell PA II to PA IV occur while in red fibers low amounts of only PA I and PA IV. Fiber discriminatory pattern have been reported in other instances also (Focant et al., 1990; Huriaux et al., 1992).

Occurrence of two to five PA isoform (or isotype) is common to fish muscle (Boback and Slechta, 1988; Huriaux et al., 1992; 1997), but in snook as many as seven isoforms have been discovered (Ross, et al., 1997). The maximum number of PA isoforms in the species of genus *Channa* is three. Each of the four species has one PA as the major and 1 to 2 or the minor ones (Fig.3). The ratio of minor to major isoforms is species specific. In *C. punctatus*, *C. marulius* and *C. striatus* which have three isoform each the ratio is of PA I : II : III is 0.07 : 0.043:1, 0.05:0.54:1 and 0.9:0.2:1, respectively. The value for *C. gachua* which has two isoforms is 0.35:1. Since the ratios given above represent an average of 40-50 samples of each species, they may be taken as a reliable estimate for the muscle of the selected defined region. Published evidence otherwise suggest differences in, the ratio of the musculature in different regions of the trunk (Huriaux et al., 1991; Huriaux et al., 1993). In addition, ontogenetic (Sherwani et al., 2001) and developmental changes (Focant et al., 1992; Huriaux et al., 1996 and Huriaux et al., 1997) also influence the intraspecies isoforms ratio.

Cumulatively the amount of PAs as a fraction of soluble muscle proteins, is characteristically high in all four fish species of *Channa*. On an average the value for *C. punctatus*, *C. marulius*, *C. gachua* and *C. striatus* are: 33%, 46%, 36% and 38%, respectively (Table 8 A and B).
A degree of species specially is exhibited by the number as well as the type of the major isoforms that are typical to each species. Major isoform in *C. gachua* and *C. punctatus* is PA II but the total number is different because the former species lacks PA III (Fig.3). *C. marulius* and *C. striatus* have PA I as the major isoform but there is substantial difference in the relative amount of PA II. PA II in *C. marulius* is 55% of the total amount of all isoform as against 9% of *C. striatus* (Fig. 3).

During literature search, the report dealing with immunoblotting of fish PAs could not be listed. In the present attempt Western blotting was carried out with twin objective of : 1) identifying PA isoforms and, 2) exploring the possibility of a quantitative assessment that would facilitate an interspecies comparison of cross reactivity. By the results shown in Fig. 4 based on screening with anti-CpPAII-rabbit antisera only the first objective (the positive identification of entire set of isoforms in each species) could be achieved. Since the cross-reactivity of anti-CpPAII-rabbit antiserum with chick PA is almost of the same order and magnitude as that of fish PAs, for tracing phylogeny further work of quantitative nature is required to perfect the use of western blots developed with chemiluminiscent techniques.

In general, there are scarce reports on immunological characterization of fish parvalbumins. Gosselin-Rey et al. (1973) characterized PA III of the pike while muscle and compared the results with arginine residue modified conformational alternatives. In another study following electrophoresis in agar gels, Gadidae PAs were cross-reacted with anti-whitting-PAIII antibodies which recognized two groups of PA isoforms within this family (Piront and Gosselin-Rey, 1974). Identification of PA bands in gradient SDS-PAGE of snook muscle by
immuno cross-reactivity has been carried out in a later instance (Ross et al., 1997). In this study possibility of using ELISA assay to type PAs employing monoclonal antibodies was also explored.

Instead of the widely adopted method of Laemmli (1970) that stacks all of the PAs as the band of 10 kD, the system of Schagger & Jagow (1987) was preferred here, because in our laboratory it satisfactorily resolves the polypeptides as low as 6 kD in 10% gels (Fig.5). Gel-Pro analysis of the lanes show that the molecular weight of PA isoforms of *Channa* species has a range of 10 to 12 kD (Fig. 5). The purified PA isoforms of *C. punctatus* resolve as one broad band and at this loading it could not be assured, if it is in fact is one band. However, highly purified major isoform (eluted from the band in native PAGE) loaded in lane-4 stacks as the band of 10.5 kD (Fig. 5a). This is also true for the highly purified major isoform of *C. marulius*, though the entire set of its pure isoforms gives a broad band around 10 kD but with two minor bands of up to 12.5 kD are also visible. No further analysis was performed to determine the molecular weight of minor bands by eluting them individually. In the same figure the highly purified PA of chick loaded in lane 8 gives a higher value of 13.5 kD which is in agreement with its reported value (Heizmann and Strehler, 1979). Similar to *C. marulius* purified PA isoform of *C. gachua* stacks as two band of 10 and 12 kD respectively (Fig. 5b, lane 3) while the highly purified major isoform PA II stack as one band of 10 kD (Fig.5 b, lane 4). The results obtained from purified and eluted major isoform PA I of *C. marulius* ((lanes 6 &7, respectively) are the same as those of *C. gachua*. It is thus, likely that in this high resolution system 12 kD band represents the minor isoforms of each species. These results are to a certain extent in
conformity with those reported in literature. For instance, anti-PA antibody precipitable band of snook PAs in SDS-PAGE gave a value of around 11 kD (Ross et al., 1997). A relative molecular mass of about 11.2 to 11.5 kD has been reported for barbells PA isoform also (Huriaux et al., 1997).

Following the initial observations by the group of Hamoir, thermostability of parvalbumin has been principally used to help purification of its isoforms (Hamoir et al., 1972). Fish PAs have not otherwise been investigated for their behavior during heat treatment at high temperatures. One recent report by Rehbein et al. (2000) compared thermostabilities of several fish PAs by IEF but at the same temperature range (70°C) that is commonly used to remove most of other sarcoplasmic proteins during purification. Since it is the temperature around which fish is baked in developed countries, they suggested the use of this technique to identify cooked fish meat using PAs as the markers.

The extent of thermostability of PA isoform at a temperature and duration of incubation such as used in the present study has not so far been reported. Results shown in Fig. 7 to 12 and as already explained under “Results” highlight several new aspects of thermal stability of PAs in general and, in particular those of Channa species. This is important that the fish species of this genus inhabit rather high temperature tropical waters and a correlation between the ambient temperature and thermostability is a generally accepted view. The characteristic of heat treated PAs under the conditions applied here demonstrate that:

1. During heat treatment depending upon thermostability of each isoform, it is converted into soluble aggregates (since they do not precipitate out), migrating faster than the native ones resulting in the appearance of novel
bands, an observation supported by the fact that the appearance of novel bands correspond to the gradual disappearance of the PA III and PA II;

(2) The appearance of novel band during heat treatment depends on two factors: (a) the lack or extent of multiplicity in the native PA (e.g. chick where CBB stains only one isoform and no novel band appears vs fish where multiple PAs exist and novel bands appear) and, (b) the relative interspecies and intraspecies stability of PAs with the same number of isoform as apparent by the order: C. punctatus > C. striatus > C. marulius (Figs 7a, 8c and 7c, respectively);

(3) The novel bands are apparently soluble aggregates of different conformational states of PA isoforms and/or their heteromers, because their number and intensity is significantly affected by β-mercaptoethanol; (Fig. 7b and 8d & 7d respectively);

(4) The heat treatment leading to the appearance of novel aggregates and retardation or smearing effect apparently caused by heat denaturation is considerably reversed by β-mercaptoethanol.

It is, therefore, suggested that the heat induced changes are a SH-SS mediated aggregation. More so, because none of the novel bands is a degradation product due to total absence of any new band in SDS-PAGE and only one band of 10 kD appears in all the heat treated PA samples. The intensity of single band that stacks as 10 kD band in SDS-PAGE of samples incubated for prolonged duration greatly depends on the absence or presence of β-mercaptoethanol (Fig. 10a and b). The above observations are also supported by plots of these changes based on densitometric quantitation (Fig. 11 and 12). According to this data both inter and intraspecies variations in thermostabilities of PA isoforms between four fish species become evident.
This study is also the first of its kind where the effect of chelators on fish PAs has directly being demonstrated by the changes in 10% PAGE (Fig. 14 to 20). Some limited observations on of this nature on PA of bullfrog on 4% polyacrylamide gels have been reported by Tanokura et al., (1987). The results presented here demonstrate remarkable changes in the electrophoretic mobilities of PA isoforms of all four fish species over a wide range of EDTA (1 mM to 200 mM) or/and EGTA. With the description available under “Results” the main inferences can be summarized as:

(1) PA isoforms of all four species elicit a response to chelating affect which differs from species to species;

(2) Whereas in fish species mobility shift is towards the anode, chick PAs elicit an opposite response;

(3) Excepting EDTA treated PA isoforms of C. marulius where a mobility shift is initiated at 1 mM of EDTA, in all other fish species and chick it happens at 10mM EDTA;

(4) The observed changes are not due to salt effect (i.e. high chelator concentrations up to 200mM) since no major differences exist between the pattern throughout this range; and,

(5) The effect of EGTA is stronger than that of EDTA and if both of the chelators are simultaneously added, the effect of EGTA remains predominant.

The above changes to a limited extent are supported by difference spectra (Figs 13 to 20). Fish as well as chick PA isoforms show a prominent trough at 290 nm of almost of the same value of O.D. at only 10 mM EDTA.
Konosu *et al.*, (1965) demonstrated that UV difference spectra of carp white muscle PA is a classical example with a high Phe to other aromatic amino acids while Tyr and Trp were absent. The absorbance at 259-269 nm indicates Phe. The difference spectra showing peaks at 259 and 270 nm have generally been reported for cyprinids and (Piront and Gerday, 1973); cyprinids and the eel (Dubois and Gerday, 1988). Laforet *et al.*, (1991) showed that specific peaks of Tyr and Trp are not present in cardiac muscle PA isoform of Antarctic fish.

Similar to typical difference spectra of reported cases, PA isoforms of *C. punctatus*, *C. marulius* and *C. striatus* also showed low intensity peaks at 259 nm indicating the presence of Phe residues. In *C. gachua* these residue are in negligible amount because only after stretching the spectrum a low shoulder appears at this wavelength (Fig. 19). There are no signs of Tyr or Trp in this species whereas in very low amounts Tyr may be present in *C. punctatus* and *C. marulius* and still less in *C. striatus*. The later species may have Trp in very low amount as per height of the shoulder at 293 nm. It is thus evident that the species differences exist between amino acid compositions of PA isoforms of these four fish species, in particular in the residues discussed with respect to difference spectra.

Keeping in view that evolutionarily *C. gachua* has been the most generalized form according to classical data (Chandy, 1955), an abnormal profile of difference spectrum makes it an attractive model for further investigations to trace the phylogeny of fish parvalbumin isoforms. Its specialized evolutionary placement is revealed by another unique feature of UV spectrum of PA isoforms of this species and, where it differs from
others, is the appearance of a sharp peak at 259 with 10 mM EDTA indicating the presence of buried Phe.

It is also interesting that all fish PAs (Fig. 14 to 17) including that of chick (Fig. 20) responded to 10 mM EDTA in a similar manner showing a negative and prominent shift between 287 to 296 nm. The only difference is that the intensity of negative shift in the spectrum of chick PA is roughly half of that noted for PA isoforms of the fish species all of which show a change of almost similar magnitude (Fig. 14 to 17). A general similarity in the spectral behavior of the all the investigated proteins confirms their parvalbumin nature as well as a degree of structural homology; specifically, within the regions of the molecules that interact with EDTA (where tyr and trp may be buried).

In the spectral profiles of PAs of all fish species, positive low magnitudes shifts of a resembling pattern are characteristic of the change at 200 mM EDTA. Chick PA was an exception where at 200 mM of EDTA a sudden shift in O.D. of a magnitude of 2.5 units was noted after 233 nm that showed two maxima, one at 259-260 nm and the other at 270-280 nm showing the perturbation of the residues typical of these peaks.

Electrophoretic changes (shown at the top of Figs. 14-17) are in conformity with the spectral behavior of fish PAs, since the major shift in electrophoretic mobility occurs at the same concentration of EDTA (10 mM) as that in UV spectra. The only exception is C. marulius PA where the shift in PAGE patterns is apparent at 1 mM EDTA, but the difference spectrum at this concentration is not available at the moment. It is, however, noteworthy, that during heat treatment C. marulius PA shows
an early and more extensive response (Fig. 7c) and our unpublished data shows that during storage even with 2 mM β-mercaptoethanol additional bands (multimers) appear further supporting that it is most labile PA out of all four species.

The low intensity change or shift, though species specific, is observed when Ca$^{2+}$ specific chelater EGTA was used. EGTA induced changes were in accordance with the increasing concentration of the chelater. As shown in Fig. 18 and 19 the order of the change was, *C. marulius* > *C. striatus* > *C. punctatus* or *C. gachua*. Another important feature is the persistence of the peak of about that appeared in these vertically expanded spectra of all species at about 259 nm. The corresponding changes in the PAGE patterns were reflected by more crisp resolutions as shown in the inset of each diagram. If the two chelators were combined the changes in electrophoretic patterns become predominantly of the type when EGTA alone was present (Fig. 20, 21). This shows stronger affinity of EGTA with regions of PA isoforms it interacts as well as a similarity in the mechanism of the interaction of the two chelaters.

As for the evolutionary lineage, the PA isoforms of all *Channa* species appear to be of β-lineage because their pI values are below 4.8 (Pechère *et al.*, 1973; Goddman *et al.*, 1977). Due to the lack of comprehensive information about lineages some controversy exists regarding the numerical typing (nomenclature) of PA isoforms. Some authors have numbered the isoforms with Roman numericals on the basis of over all numerical strength of PA isoforms present within a family or
genus (Boback and Slechta, 1988; Huriaux et al. 1997). Five PA isoforms bands of differing mobilities can be counted within genus Channa. That would mean that at least 2 to 3 isoforms do not exist in one or the other species since the maximum number in any case is three (marked in this study as PA I, PA II and PA III). If the presence or absence of comigration is taken into account and the species-wise Roman numericals assigned in this study are altered taking overall number of bands within the genus as the final figure, an erroneous dendrogram is constructed.

It is contended here that the dendrogram constructed according to the numbering system of PA isoforms used in the present study appears to be reasonable (Fig. 23). According to it, C. punctatus is of the most remote origin, and C. striatus and C. marulius being more similar as per OTU clustering share a close phylogenetic relationship. PA isoforms of C. gachua that according to classical data was the most generalized form (Chandy, 1955), have a similarity with PA isoforms of C. marulius. C. striatus and PAs of all the three species might have evolved from those of C. punctatus.

These results show that the evolution of PA isoforms took a different course than that of transferrins (Tfs) which showed more identity between C. gachua and C. marulius proteins. Immunologically, Tfs of C. gachua and C. striatus have been shown to be more similar followed by C. marulius and all these Tfs exhibit least similarity with Tf of C. punctatus (Nabi, 1999).