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Exploring the Persistence of Fish Allergies: The Role of Parvalbumin

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Parvalbumins (PVs) form a major part of the fish skeletal muscle sarcoplasm, and were identified as the major cross-reactive allergens in a variety of fish species. Therefore, the intake of fish or fish-linked products may cause serious clinical problems. It is known that the biochemical and structural characteristics of a protein play a central role in determining its potential functions. This study is sought to evaluate the stability of PV's structure which would be the foremost reasons in keeping them responsive yet after cooking. Here, PVs obtained from a commonly consumed fish *Channa marulius* were used as the test protein. The protein, PV turned out to be fairly resistant to high temperatures (90°C) and set on in a typical single band of 10 kD in SDS-PAGE. This suggests that following heating only partial and /or reversible loss of PV structure occurred, which rendered it allergenic to predisposed individuals.

Key words: Parvalbumin, allergen, stability, fish.

INTRODUCTION

PVs are soluble low molecular weight proteins (10 - 12.5 kD) that bind free Ca^{2+}, thereby reducing the intracellular $[Ca^{2+}]$ in muscle (Arif et al., 2007; Arif, 2009). They facilitate relaxation in muscle and increase the rate of neuron firing. These proteins are expressed across many phyla, and have highly conserved Ca^{2+}-binding sites. PVs are of wide occurrence and their distribution varies in different fish species. A wide range (from zero to >1.5 mM) in total PV content has been reported by Gillis (1985). The number of PV-isoforms may range from two to eight throughout their development in various fish species. But most fish are found to express three to five isoforms of PV throughout their development (Gillis, 1985; Chikou et al., 1997; Huriaux et al., 2002; Focant et al., 2003; Arif et al., 2007; Arif, 2009).

Recently, Brownridge et al. (2009) reported the expression of eight PV-isoforms in carp muscle (at the same time), although only two to three were expressed in relatively large amounts.

PVs are becoming of great interest in nutritional and clinical studies as they have been reported to be the major food allergens in fish and other vertebrates like frog. Moreover, they are also one of the most common causes of food allergy in populations where fish consumption is high. In the last few decades, the incidents of hypersensitivity reaction to PVs have been noticed with increasing frequency (Aas and Elsayed, 1969; De Martino et al., 1990; Bernhisel- Broadbent et al., 1992; Pascual et al., 1992; O'Neil et al., 1993; Lindstroem et al., 1996; Bugajska-Schretter et al., 1998; Hamada et al., 2003; Hilger et al., 2004; Sten et al., 2004; Harumi 2006; Anibarro et al., 2007; Pascual et al., 2008; Ma et al., 2008; Griesmeier et al., 2009). The direct contact or intake of fish can cause mild to severe life threatening health problems in sensitized individuals, which may include dermatitis, asthma, diarrhea, acute urticaria, angioedema, and sometimes anaphylactic shock and/or death.

The cross-reactivity between PVs from different fish species and, between PVs from different sources like fish and frog has been recognized in several cases (Hilger et al., 2004; Sten et al., 2004; Hamada et al., 2004; Griesmeier et al., 2009). Here, the high sequence homology in their linear epitopes seems to be responsible

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for the allergen identity. This has been proved immunologically as well, where the IgE antibodies of fish-allergic patients were found to cross -react with frog PV because they share the IgE-binding epitopes (Hamada et al., 2004; Hilger et al., 2004; Sten et al., 2004; Swoboda et al., 2007). Therefore, the patients allergic to one fish could show sensitivity to PVs from other sources also.

Consequently, the patients afflicted by allergic reactions to one fish are recommended to refuse all fish unless they are diagnosed IgE-deficient for every PV homology.

The biochemical, structural and functional properties of PVs are investigative to their formative role in eliciting an allergic reaction. They are known to sensitize people either through direct contact or via the gastrointestinal tract (GIT) even after cooking (Bugajska-Schretter et al., 2000).

This suggests that PVs can resist and survive such harsh conditions as heating, acidic pH, denaturing effects of surfactants and proteolytic activities of the digestive enzymes of the GIT (Elsayed and Aas, 1971). Besides, the interaction of these proteins with other components of the food matrix might influence the rate of absorption and immune response. Once these proteins are absorbed through the gut they not only elicit an allergic response in a previously sensitized individual but can also direct an immune reaction in a new person.

The molecular forces that determine the structure of PVs are appearing to play an important role in stabilizing these proteins. Circular dichroism (CD) measurements revealed that the refolding ability of Ca^{2+}-bound PV and the special structural features are responsible for their unique stability (Bugajska-Schretter et al., 2000). Hence, following heating only partial and reversible loss of helical structures was detected. However, from the present study, it can be hypothesized that not only the PV region that has the IgE-binding epitopes but also the PV structure (of at least a little protein) as a whole would remain almost undisturbed after heating as revealed from the electrophoretic (SDS-PAGE) profiles suggesting a key factor in determining their potential allergenicity. Overall, it can be assumed that PVs might be able to retain linear and potentially conformational epitopes after heating and therefore, their ability to trigger an allergic reaction would remain essentially unaltered.

MATERIALS AND METHODS

Preparation of fish muscle extracts

Live fish specimens of *Channa marulius* were obtained directly from the local fish market or nearby ponds of Aligarh, India. The fish were inactivated by stunning with a brief cerebral blow. Anterior most regions just behind the head were dissected out for taking out white dorso-lateral skeletal muscle. The obtained fresh muscle was then homogenized in prechilled 50 mM Tris -HCl, pH 7.5 containing 10% glycerol using electrical homogenizer (Model: 985-370; Type 2; 5000 - 30,000 rpm; Biospec Products, Inc.) by giving several bursts of about 30 s each (Arif et al., 2007). The resulting

homogenate mixture was spun at 12,000 rpm for 20 min in a refrigerated centrifuge maintained at 4°C. The supernatants were thereafter analyzed and processed immediately for PV purification and iced up at -20°C till analysis.

Purification of PVS

The clear soluble muscle extracts were subjected to acetone fractionation to obtain mixed-purified PVs by giving two cuts, 55 and 85% of acetone (Piront and Gerday, 1978). The precipitate obtained at the first cut was pellet out by centrifugation (4°C) at 10,000 rpm for 20 min, and the supernatant was given a subsequent cut of 85% acetone. The resulting mixture was centrifuged (4°C) again at 10,000 rpm for 20 min to pellet the precipitated protein. The so obtained pellet was partly dried at room temperature and dissolved in a reasonable volume of precooled 10 mM Tris- HCl buffer of pH 7.5. Now, this mixture of protein was heated in a water bath at 70°C for 20 min, and centrifuged (4°C) at 12,000 rpm for 30 min (Arif et al ., 2007). The clear supernatant containing the heat-stable proteins (mixed-purified PVs) was saved and analyzed.

Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE

The mixed- purified PV samples were screened following the system of Laemmli (1970) with the modification that the gels contained 10% glycerol and 0.01 M Tricine in the upper running buffer but no solution contained sodium dodecyl sulfate (SDS) (Huriaux et al., 1997; Arif et al., 2007). The samples were loaded on 10% polyacrylamide slab gels (100 × 150 × 1 mm) after a pre-run of about 30 min at 4 mA/gel, 50 V and 1 W. Initially, the gels were run at 4 mA/gel, 50 V and 1 W until the tracking dye (Bromo-phenol blue, BPB) entered into the separating gel. When the samples entered into the separating gel the settings were changed to a constant supply of 8 mA/gel, 70 V and 1 W for the entire duration of electrophoresis.

Denaturing PAGE

Denaturing PAGE or SDS-PAGE was performed exactly in the system of Laemmli (1970) to screen the heated mixed- purified PV samples. The screening was done on 15% (100 × 150 × 1 mm) slab gels. In this system, the running buffer as well as the gel contained 0.1% SDS; and the samples were prepared in the sample buffer containing SDS. The gels were pre- run at 4 mA/gel, 50 V and 1 W for about 15 min. However, after entry of the sample into the separating gel the settings were kept 8 mA/ gel, 120 V and 1 W throughout the run. The electrophoresis termination was followed by overnight washing of the gels in 5% acetic acid to remove SDS, and then staining on the next day with coomassie brilliant blue (CBB) R-250. The percentage of protein present in each lane was determined by GelPro software (Media Cybernetics, Silver Spring, MD) analysis.

Thermal incubation of PVs

The mixed- purified PVs were subjected to thermal incubation in a water bath maintained at a constant temperature of 90°C for varying time intervals (Arif et al., 2007). During this heat treatment, aliquots of the protein were saved after a time period of 1, 2 and 3 h, and were placed immediately on ice. The saved aliquots of the protein were then centrifuged at 8000 rpm for 20 min at 4°C to
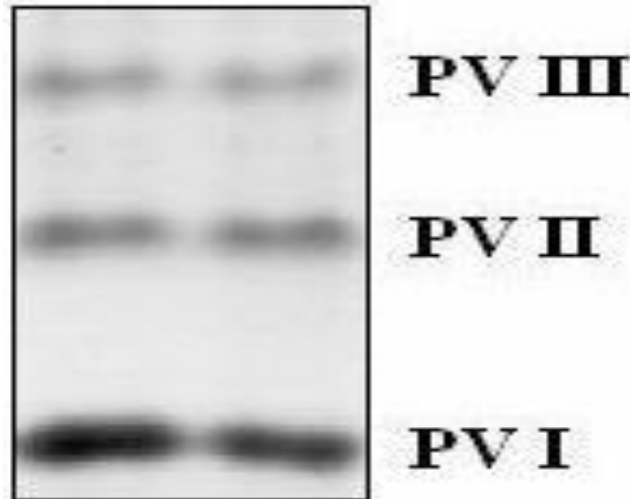
Figure 1. Screening pattern of the mixed-purified muscle PVs of *Channa marulius* on 10% nondenaturing polyacrylamide gel run in a modified buffer system by Arif et al. (2007). Anode at the bottom.

remove any denatured protein. Clear supernatants were electrophoresed on 15% denaturing polyacrylamide gels following the protocol as described below.

Addition of 2-mercaptaethanol to the mixed-purified PVs

To a final concentration of 5 mM, 2-mercaptoethanol was added to the mixed-purified PVs before thermal incubation (Arif et al., 2007).

RESULTS

The mixed-purified PVs from the fish *C. marulius* were first analyzed by using non-SDS polyacrylamide gels (Figure 1). Three PV-isoforms were noticed in this non-denaturing system. They were designated as PV I, PV II and PV III on the basis of their electrophoretic mobility in the non-SDS PAGE, where PV I being the fastest and PV III the slowest. Tricine was used as a trailing ion for better stacking of the protein (Arif et al., 2007). The clear supernatant containing the PVs (mixed-purified) was subjected to thermal incubation in a water bath kept at 90°C. The aliquots (of the protein) saved after 1, 2 and 3 h of incubation were screened on the SDS-polyacrylamide gel with standard markers (Figure 2a: lane, M) following the protocol by Laemmli, 1970. It was observed that all the three PV-isoforms (PV I, PV II and PV III) were stacked as a single protein band of 10 kD, in each case of the thermal incubation that is 1, 2 and 3 h (Figure 2a: lanes, 1, 2 and 3), where the lane 0 had the control (unheated) protein. Moreover, it was also noted that due to the heating effect, a part of the protein denatured resulting in the diminution of the protein band intensities with the increase in the time of incubation

(Figure 2a: lanes, 1 - 3). The corresponding decrease in the percent protein content with respect to the time of thermal incubation can be explicitly depicted by the bar diagram (Figure 2b).

Figure 3a shows the protein band pattern using SDS-PAGE for the PVs, if the samples contained 5 mM 2-mercaptoethanol before heating. It is interesting to note that the addition of 2-mercaptoethanol effectively prevented protein denaturation due to heating. As a result much prominent and intact protein (PVs) bands were obtained (Figure 3a: Lanes, 1, 2 and 3) at the position of 10 kD. In addition, the consequent change in the amount of protein at each time interval of thermal incubation can be elucidated from the bar diagram (Figure 3b).

DISCUSSION

Fish is an important component of the diet of the people especially in the coastal areas. IgE-mediated cross-reactions from several fish species indicated that more than 95% individuals were found allergic to fish (Bugajska-Schretter et al., 2000) while others can tolerate certain fish species (van Do et al., 2005a and b; Zapatero et al., 2005). In a number of fish species, like codfish, ocean pout, eelpout and eel it has been reported that the IgE-mediated allergy to fish is the most common reason of severe anaphylactic reactions (Sten et al., 2004). Here, PVs have been reported to be the major fish allergens (Bugajska-Schretter et al., 2000; Lim et al., 2008).

Muscle sarcoplasm of fish and other vertebrates contain abundant quantity of PVs, however, the amount and number of PV-isoforms varies in different fish
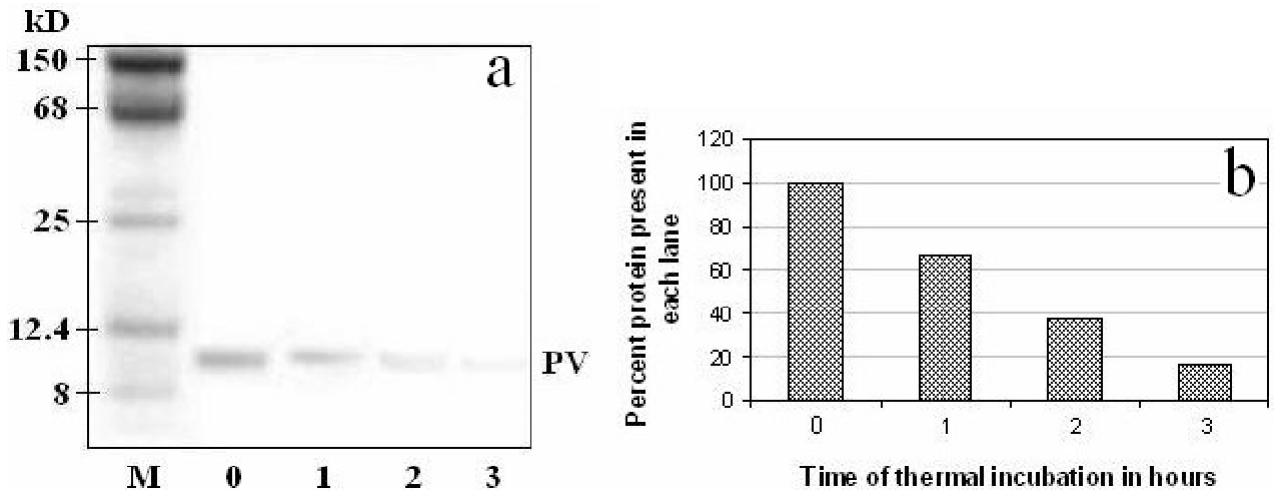
Figure 2(**a**) SDS-PAGE pattern of the heated mixed purified muscle PVs of *Channa marulius* on 15% gel (where, lane M: marker, lane 0: unheated PVs and lanes 1 to 3 contain PVs heated for the respective hours (1 to 3 h) of incubation at 90 °C run in the system of Laemmli (1970). Anode at the bottom. (**b**) The bar diagram showing percent protein present in each lane before (0 hour) and after (1 to 3 h) thermal incubation.
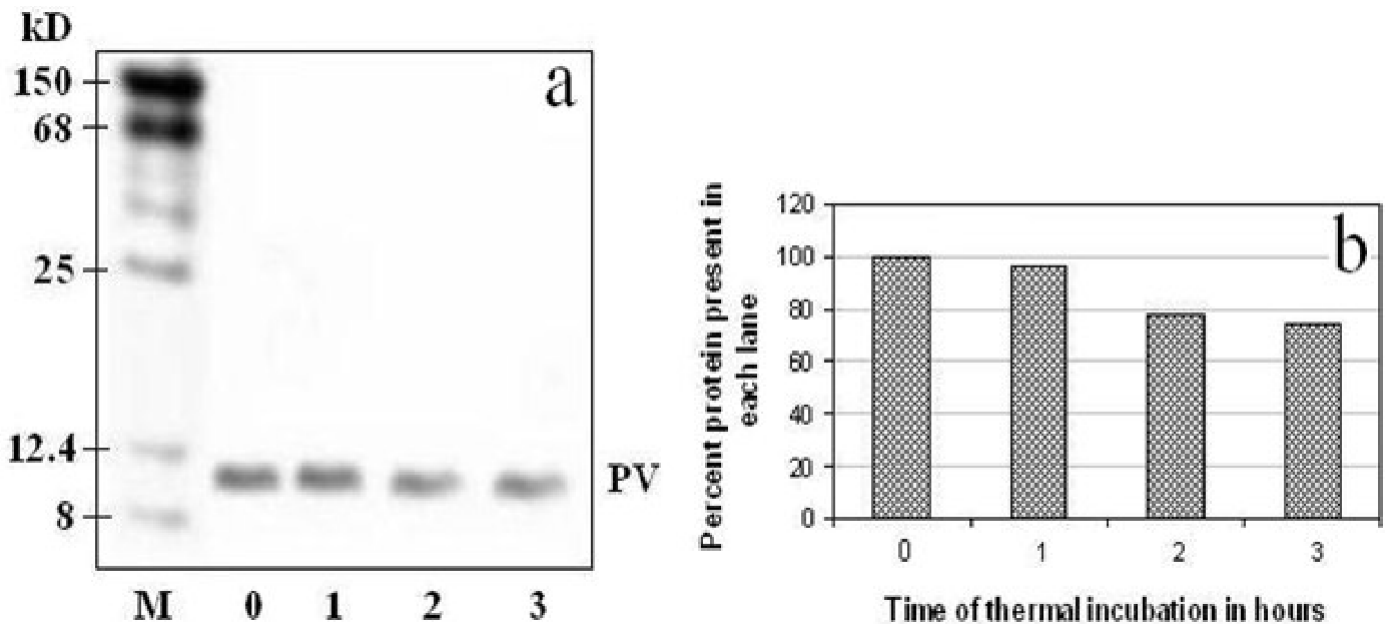

Figure 3(**a**) SDS-PAGE pattern of the heated mixed purified muscle PVs (containing 5mM of 2-mercaptoethanol) of *Channa marulius* on 15% gel (where, lane M: marker, lane 0: unheated PVs and lanes 1 to 3 contain PVs heated for the respective hours (1 to 3 h) of incubation at 90 °C run in the system of Laemmli (1970). Anode at the bottom. (**b**) The bar diagram showing percent protein present in each lane before (0 hour) after (1 to 3 h) thermal incubation.

species (Heizmann, 1984). Furthermore, the high sequence identity of amino acids among different fish species PVs; and, between fish and frog PVs has resulted in allergic reactions common to either patients sensitive to fish or frog (Hilger et al., 2004; Hamada et al., 2004; Griesmeier et al., 2009). So, it is wise to consume fish or frog cautiously after receiving any related allergic reactions; and the patients allergic to fish should not consume frog earlier than they are diagnosed frog-IgE-deficient.

In the present study, the identified three PV-isoforms of *C. marulius* (Figure 1) were subjected to thermal distress in order to recognize their stability through SDS polyacrylamide gels. Since, PVs have shown exceptional resistance to heat; in this study, we reasoned a step ahead that why they remain reactive yet after boiling?

PVs of the fish, *C. marulius* were boiled at 90°C for 3 h as in Arif et al *.* (2007) . The saved aliquots (each after 1, 2 and 3 h of thermal incubation) were electrophoresed on SDS-polyacrylamide gels to assess the stability of their intrinsic protein structure. They were found to be pretty stable to heating since the saved heated protein resolved into clear intact bands on the SDS gels (Figures 2a and 3a). However, degradation of the protein has been found to be the function of time of heating which can be depicted in the form of low intensity PV bands (Figure 2a; lanes 1, 2 and 3). Nevertheless, the percent degradation of the protein can be fairly low if the protein contained 5 mM of 2-mercaptoethanol before heating (Figure 3a; lanes 1, 2 and 3). Interestingly, it was shown experimentally that the addition of micro amounts of 2-mercaptoethanol to the living systems (such as laboratory animals) has many useful effects; for example, it improves the life activities and other related characte-ristics of life in the trial organisms (Penzes et al., 1988). In Addition, Yu et al*.* (1998) demonstrated in another experiment that the introduction of 2-mercaptoethanol in the experimental animals could enhance the median and maximum life spans of the treated mice. These experiments revealed the fact that 2-mercaptoethanol would have beneficial effects on the tissues at protein level. However, we for the first time have reported the beneficial effects of 2-mercaptoethanol directly on protein protection (Arif et al., 2007). While, it can be further established from the present study that PVs are able to resist the denaturation changes also in the absence of 2-mercaptoethanol as they set on in a typical single band of 10 kD on SDS gels (Figure 2a). But this effect can be considerably reduced if the sample contained 2-mercaptoethanol before heating (Figure 3a). This could be easily observed by comparing the corresponding decrease in the protein amounts (with the time of incubation) in the two sets (Figures 2b and 3b).

Structurally, PVs have paired EF-hands whose domain partnering provides them a more stable structure and function (Kretsinger and Nockolds, 1973). Besides, in another study conducted by Bugajska-Schretter et al*.* (1998) PVs were also found stable to the proteolytic action of the enzymes present in the GIT. This fact therefore further strengthens the idea that the distinctive stability of their intrinsic protein structure help resists the harsh temperature (during cooking) and the enzymatic attack (of the digestive enzymes secreted in GIT).Thus, being resistant to denature these proteins are able to cross the gut mucosal barrier unchanged and can sensitize the immune system and/or elicit an allergic response. As a result, the ability of PVs to trigger an allergic reaction after heating and/or ingestion would remains unaltered keeping them potentially allergenic food (fish) components. In addition, from this study it is clearly evident that the heating of PVs at 90°C could not rocked up the protein (PV) structure as they stacked in a single intact band of their characteristic molecular weight (10 kD). Consequently, PVs are able to preserve their typical structural properties even after experiencing an extensive thermal stroke, and can work as active allergens and remain sensitive to susceptible people even after cooking.

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