

Your search term is: Identification allergens Indian fishes: Hilsa Pomfret exemplified ELISA immunoblotting

Indian Journal of Experimental Biology
Vol. 43, December 2005, pp. 1170-1175

Identification of **allergens** in **Indianfishes: Hilsa** and **Pomfret exemplified** by **ELISA** and **immunoblotting**

Arpita Das, Phuljhuri Chakraborti, UrmimalaChatterjee, Gautam Mondal & Bishnu P Chatterjee*

Department of Biological Chemistry

Indian Association for the Cultivation ofScience, Jadavpur, Kolkata 700 032, India

Received 27 December 2004; revised 3 August 2005

Enzymed-linkedimmunosorbant assay of **hilsa** and **pomfret** muscle extracts showed specific IgEbinding to ten allergic patients' sera, the results corroborated to that ofskin prick test. Comparison of allergen profiles of the two fish extracts by**immunoblotting** revealed a common antigenic protein of 50 kDa and some highmolecular weight fish **allergens** instead of low molecular weight parvalbuminfound in several fishes. Purified and well characterized fish **allergens** arealways considered better than crude fish extracts for diagnostic use.

Keywords: Allergen, **ELISA**, **Hilsa**, IgE reactivity, **Pomfret**,

Type I allergy is caused by therecognition of **allergens** by specific IgE antibodies leading to a cascade ofcellular inflammatory responses. Fish allergy is one of the most common foodallergies mediated by IgE antibody. Consumption of fish products could lead tosymptoms like skin rash, dermatitis, urticaria, angioedema, gastrointestinalproblems, diarrhoea, respiratory distress and even fatal systemic anaphylacticreactions¹⁻³. Though fish protein is the major cause ofhypersensitivity, it is a valuable source of polyunsaturated fatty acids, whichare highly recommended for health. Some common animal proteins from meat, egg,shrimp, crab, cow's milk^{4,5} have been identified and characterizedas major **allergens**. The only major allergen (*Pen a 1*) identified in shrimp is the muscle protein, tropomyosin⁶.The specific role of egg ovalbumin for binding IgE antibody has already beenelucidated⁷; has been found in patients allergic to cow milk,casein, along with other two milk proteins immunoreacted with IgE antibody⁸.Another vertebrate muscle protein, parvalbumin, which is a calcium-bindingprotein has been established as a major cross-reactive allergen in fishes likecod, mackerel, tuna, salmon and carp⁹⁻¹².

Consumption of fish is quite high in eastern and north easternIndia and the importance of fish in elicitation of hypersensitivity reactionsis well known, however there is no detailed study on **Indian**

fish **allergens**. Chaki¹³ has purified and characterized an allergen (molecular weight ~14 kDa) from rohu (*Labeo rohita*, Hamilton-Buchanan).

The present study deals with the **identification** of **allergens** in two highly consumed **Indian** fishes, viz., **hilsa** (*Tenualosa ilisha*, Hamilton-Buchanan) and **pomfret** (*Pampus argenteus*, Euphrasen) by determination of specific IgE in fish hypersensitive human sera, using enzyme-linked immunosorbent assay (**ELISA**). Also, profiles of different IgE binding proteins in these fish extracts by **immunoblotting** have been compared. This biochemical and immunological study of two fish **allergens** will help in understanding the allergenic/antigenic relationship between **hilsa** and **pomfret**.

Materials and Methods

Chemicals and biochemicals—Acrylamide, N,N',methylenebisacrylamide, sodium dodecyl sulfate, o-phenylenediamine, diaminobenzidine, anti-human IgE conjugated with horse radish peroxidase (HRP) and nitrocellulose membrane were purchased from Sigma-Aldrich, USA. All other reagents were of highest analytical grade and obtained locally.

Preparation of **hilsa and **pomfret** fish extracts**—**Hilsa** and **pomfret** fishes were purchased from New Market, Kolkata. The raw muscles were freed from bones and homogenized separately in 0.1 M PBS (pH 7.2). The individual extract after stirring overnight at 4° C was centrifuged at 10,000 *g* for 30 min. The supernatants were used as fish extracts for subsequent experiments. Protein concentration of the extracts was determined by the method of Lowry *et al*¹⁴.

Subjects—Ten patients, age ranging from 18 to 65 years, with a clinical as well as family history of hypersensitivity to fish were selected in this study at the “Allergy and Asthma Research Clinic”, Kolkata. This study was approved by the Institutional Ethical Committee and informed consent was obtained from each subject.

Skin prick tests—The skin prick tests (SPT) were performed by placing a drop (1:20 w/v) of the fish extracts on the volar aspect of the forearm with a disposable (26 gauge) hypodermic needle. The site was observed after 20 min and a wheal of 3 mm diameter surrounding erythema was regarded as positive response; 50% glycerol in PBS was taken as negative control.

Enzyme-linked immunosorbent assay (ELISA**)**—To evaluate the IgE binding activity of patients' sera with fish allergen, **ELISA** was performed as per Voller *et. al*.¹⁵. Briefly, each well of a microtiter plate was coated with 100 µl (5 µg/ well) of either fish extract in 10 mM carbonate buffer (pH 9.6), and incubated overnight at 4°C. The plates were washed with PBS-T (10 mM PBS, pH 7.4 containing 0.05% Tween 20) and incubated with 1% BSA in PBS at 37°C for 1 hr followed by incubation with patients' sera (100 µl, 1:10) at 37°C. After washing, the wells were incubated with 100 µl of goat-anti-human IgE-HRP (1:1000) in PBS at 37°C for 1 hr. The colour was developed after incubation with 100 µl of o-phenylenediamine (1 mg/ml in 0.05 M citrate phosphate buffer with 0.01% H₂O₂, pH 5) for 30 min in the dark at 25 °C. Absorbance was measured at 492 nm in an **ELISA** reader (Flow Laboratory, UK) after adding 3 N sulfuric acid as stop solution.

For **ELISA** inhibition study, either **hilsa** or **pomfret** extract (5 µg/well) was coated onto the microtiter wells. Following the procedure as above, the wells were incubated with pooled patients' sera, pre-incubated overnight at 4 °C with different concentrations (7.5-150 µg) of either **hilsa** or **pomfret** extract. The IgE binding inhibition was detected using anti human IgE - HRP as described above. The above experiments were performed in triplicate and the data were expressed as the mean values.

Immunoblotting—The crude extracts (30 µg) were subjected to SDS-PAGE (10%) as per Laemmli¹⁶ under reducing condition with 2-mercaptoethanol. After electrophoresis, the separated proteins were stained with Coomassie Blue and destained with 50% acetic acid for visualization of peptide bands. **Immunoblotting** was performed by electrophoretic transfer of separated proteins of each fish extract to nitrocellulose membranes (0.45 µ pore size) in Tris-glycine buffer containing 25% methanol¹⁷. The membranes were cut into strips and free sites were blocked with 3% BSA. Each strip was incubated overnight at 4 °C with individual patient's sera (1:10). The membrane was washed thrice with PBS-T and incubated with anti-human IgE-HRP (1:1,000) for 3 hr at room temperature. Finally, the color was developed with diaminobenzidine and 0.01% H₂O₂ in sodium acetate buffer (pH 5).

Results

SPT reactivities and specific IgE of patients—Clinical history and skin test results of ten selected fish allergic individuals are summarized in Table 1. These patients had histories of allergic reactions following the ingestion of fish. **ELISA** studies showed elevated specific IgE levels to both **hilsa** and **pomfret** extracts in all ten patients (Fig. 1). The results are in concordance with the clinical symptoms as well as SPT results of the patients. No increased IgE level was detected in case of two non-allergic healthy individuals. **ELISA** inhibition studies were carried out to test cross-reactivity between **hilsa** and **pomfret**. Preincubation of the pooled patients' sera with either **hilsa** or **pomfret** protein extract (upto 150 µg) did not show any inhibition suggesting that the two fishes are allergenically quite distinct from each other (Fig. 2).

Table 1—Clinical summary of patients selected for the study

Subject No.	Age	Sex	Symptoms	SPT*	Family history
1	18	M	Skin rash	++	Nil
2	65	F	Breathlessness	+++	Nil
3	36	M	Skin rash	+++	Nil
4	19	F	Skin rash	+	Asthma
5	53	M	Breathlessness, diarrhoea	+++	Asthma

6	43	F	Breathlessness	++	Nil
7	50	M	Severe skin rash	+	Nil
8	46	F	Breathlessness, skin rash	+++	Asthma
9	36	F	Breathlessness	++	Asthma, Eczema
10	35	F	Breathlessness, diarrhoea	++	Eczema

*Wheal diameter: + = 3-5 mm, ++ = > 6 mm, +++ = >6 mm with pseudopodia.

Fig. 1—Quantification of the level of specific IgE by **ELISA**. OD values at 492 nm of the IgE binding of patients' sera to **hilsa** and **pomfret** extracts. Each value is the mean of three determinants.

Fig. 2—Result of **ELISA**-inhibition. **ELISA**-inhibition of pooled patients' sera (—■—) with different concentration of **hilsa** allergen (7.5-150 μ g) in plate coated with **pomfret** allergen (5 μ g/well) and (—○—) with different concentration of **pomfret** allergen (7.5-150 mg) in plate coated with **hilsa** allergen (5 mg/well).

SDS-PAGE of fish extracts—On 10% SDS-PAGE, the **hilsa** extract showed at least 14-15 polypeptides (Fig. 3, lane 1). The majority of bands in the **hilsa** extract were in the molecular weight range of 26-62 kDa, though some high molecular weight bands were also present. The most prominent doublet seemed to be present in equal intensity in the molecular weight range of 41-42 kDa. The **pomfret** extract showed completely different polypeptide profile than that of **hilsa** extract (lane 2, Fig. 3). The majority of bands in the **pomfret** extract were in the range of 30-96 kDa though a low molecular weight (~14 kDa) polypeptide also appeared. The two most prominent Coomassie stained polypeptide bands were at 32 and 35 kDa, respectively.

Fig. 3—SDS-PAGE of **hilsa** and **pomfret** extracts. [Each of 30 μ g of **hilsa** and **pomfret** extract was analyzed by 10% SDS-PAGE. Lane M: molecular weight markers, Lane 1, **hilsa** extract and lane 2, **pomfret** extract.]

Immunoblot analyses of IgE reactivities of patients' sera—To confirm the SPT and **ELISA** results, the **hilsa** and **pomfret** extracts were immunoblotted with sera from ten allergic and two non-allergic individuals. All of the allergic patients' sera yielded almost identical blotting profile for either in **hilsa** or **pomfret** extract, though there was a subtle difference among them (Fig. 4, Table 2). In case of **hilsa** extract, the majority of the allergic patients' sera showed very strong binding to the most prominent 41-42 kDa doublets and also to other two polypeptides at 50 and 62 kDa respectively. Whereas, the 29 kDa polypeptide in the **hilsa** extract was recognized by six patients (sera numbers 1-3 and 7-9). On the other hand, in case of **pomfret** extract, most of the patients' sera recognized the two polypeptides of 32 and 35 kDa and another higher molecular weight polypeptide of 50 kDa. In

addition to that, all of the patients' sera except patient No. 3, showed IgE binding to a high molecular weight polypeptide (~97 kDa). Patient number 1, 2 and 9 showed some other additional minor bands, which the rest of the sera did not recognize.

Fig. 4—IgE immunoblot analysis using sera from allergic and non-allergic subjects. [The samples were analyzed by 10% SDS-PAGE and transferred to nitrocellulose membrane. The binding of serum was detected by HRP-conjugated anti-human IgE. Lane 1-10 fish sensitive patients' sera, lane 11-12, control sera, lane 13, BSA.]

Table 2—IgE-binding proteins (molecular weight in kDa) of **Hilsa** and **Pomfret**

Hilsa

Patients' sera	29	38	41	50	62	94
1	X	X	X	X	X	-
2	X	X	X	X	X	-
3	X	X	X	X	X	-
4	-	X	X	X	X	-
5	-	-	X	X	X	-
6	-	-	X	X	X	-
7	X	X	X	X	X	X
8	X	X	X	X	X	X
9	X	-	X	X	X	X
10	-	-	X	X	X	X

Pomfret

Patients' sera	32	35	43	50	68	97
1	X	-	X	-	X	X
2	X	-	X	-	X	X
3	X	X	X	X	-	-
4	X	X	-	X	-	X

5	X	X	X	X	-	X
6	X	X	X	X	-	X
7	X	X	-	X	-	X
8	X	X	X	X	-	X
9	X	X	X	X	X	X
10	X	X	-	X	-	X

'X' = presence of band.

Two non-allergic individuals showed very weak binding to some antigens present in both the extracts (lanes 11-12). No IgE binding was observed in absence of serum (lane 13).

Discussion

The two fishes, **hilsa** and **pomfret** are widely consumed in India and considered to be the frequent causes of IgE mediated hypersensitivity. The molecules present in fish extracts play a major role in fish induced hypersensitive reactions. Therefore, the **identification** and characterization of fish **allergens** is an essential step towards the understanding of molecular basis of fish allergy.

Allergens isolated from two **Indian** fishes, **hilsa** and **pomfret** were identified. **ELISA** results revealed that all fish allergic patients, who showed positive reactivity in SPT, had increased level of specific IgE. **Immunoblotting** experiments also showed that the allergenic proteins present in both the fish extracts reacted with all ten fish allergic subjects. A calcium binding vertebrate muscle protein, parvalbumin has been identified and characterized as a major allergen in several fishes⁹. Surprisingly, in the immunoblot experiments, none of the patients' sera could recognize any low molecular weight protein like parvalbumin. An earlier observation indicated the presence of a low molecular weight IgE binding protein of 14 kDa from rohu fish¹³, though the identity of that protein with parvalbumin had not yet been confirmed. The absence of parvalbumin in **hilsa** and **pomfret** suggests the presence of species specific IgE epitopes.

Cross-reactive **allergens** among fish species were reported earlier. Gad c1 or parvalbumin was found to be a common cross-reactive allergen in different fish species¹⁸. Though the immunoblot results showed the presence of one shared protein of 50 kDa in both fish extracts (Table 2 and Fig. 4), there was no cross - reactivity even when 150 µg of either fish extract was used in **ELISA** inhibition studies. Therefore, the occurrence of this allergen in **hilsa** and **pomfret** doesn't clearly show whether the presence of this common protein in both the extracts represents one common allergen having same allergenic determinants or protein of same molecular size, but with different allergenic epitope. When

an immunoblot experiment was carried out with several **Indian** fish extracts viz. bhetki (*Lates calcarifer*, Bloch), parse (*Liza parsia*, Hamilton-Buchanan), rohu (*Labeo rohita*, Hamilton-Buchanan), magur (*Clarias batrachus*, Linnaeus), **pomfret** and **hilsa** using a rabbit anti-**hilsa** antibody, almost no cross reactivity was detected between **hilsa** and **pomfret**, whereas other fishes showed high cross-reactivity with **hilsa** antibody. Therefore, this experiment also points to the fact that no cross-reactivity exists between the two **fishes: hilsa** and **pomfret**.

The major cross-reactive fish allergen parvalbumin was purified and cloned from carp¹⁸, salmon¹⁹ and mackerel²⁰. Parvalbumin is not the only major fish allergen that was studied in detail, but also other proteins from several fishes were identified and characterized. Das Dore et al²¹ identified and purified an allergic protein of 41 kDa from codfish. Fish gelatin (type 1 collagen) was identified and purified as an allergen and specific IgE antibody to this protein was also reported²². In this context, the present work on **identification** of **allergens** from two **Indian** fishes is very relevant as identified major **allergens** can be purified and characterized for further studies. This kind of detailed study of **Indian** fishes has not been reported till date. Therefore, the present investigation involving the **identification** of allergenic molecules in two highly consumed **Indian** fishes is a first step towards the improvement of the quality of allergen extracts. The purified and recombinant **allergens** are always better substitutes for crude extracts, which contain a number of unwanted substances. In the management of fish allergy, the role of well-characterized relevant **allergens** is very important because these **allergens** may replace crude allergen extracts in allergy diagnosis.

Acknowledgement

This work was supported by a grant from ICMR, New Delhi. The authors are grateful to Mr. Soumya Samanta, for technical assistance.

References

- 1 Aas K, Fish allergy and the cod fish allergen model, in Food allergy and intolerance, edited by J Brostoff and S J Challacombe (Balliere Tindall, London), 1987, 356.
- 2 Pascual C, Martin E M & Crespo J F, Fish allergy: Evaluation of the importance of cross-reactivity, *J Pediatr*, 121 (1992) S29.
- 3 O'Neil C, Helbling A A & Lehrer S B, Allergic reactions to fish, *Clin Rev Allergy*, 11 (1993) 183.
- 4 Crespo J F, Blanco C, Contreras J, Pascual C & Martin E M, Food allergy: A clinical and epidemiological study (abstract), *J Allergy Clin Immunol*, 89 (1992) 192.

- 5 Kajosaari M, Food allergy in Finnish children aged 1 to 6years, *Acta Paediatr Scand*, 71 (1982) 815.
- 6 Daul C B, Slattery M, Reese G & Lehrer S B, **Identification** of the major brown shrimp (*Penaeus aztecus*) allergen (Pen a 1) as the muscle protein tropomyosin, *Int Arch Allergy Clin Immunol*, 105(1994) 49.
- 7 Anet J, Back J F & Baker R S, **Allergens** in the white and yolk of hen's egg, *Int Arch Allergy Clin Immunol*, 77 (1985) 367.
- 8 Szabo I & Eigenmann P A, Allergenicity of major cow's milk and peanut proteins determined by IgE and IgG **immunoblotting**, *Allergy*, 55 (2000) 42.
- 9 Bugajska-Schretter A, Elfman L, Fuchs T, Kapiotis S, Rumpold H, Valenta R & Spitzauer S, Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca^{2+} depletion, *J Allergy Clin Immunol*, 101 (1998) 67.
- 10 Bugajska-Schretter A, Grote M, Vangelista L, Valent P, Sperr WR, Rumpold H, Pastore A, Reichelt R, Valenta R & Spitzauer S, Purification, biochemical and immunological characterization of a major food allergen: Different immunoglobulin E recognition of the apo- and calcium-bound forms of parvalbumin, *Gut*, 46 (2000) 661.
- 11 Shiomi K, Hamada Y, Sekiguchi K, Shimakura K & Nagashima Y, Two classes of **allergens**, parvalbumins and higher molecular weight substances, in Japanese eel and bigeye tuna, *Fisheries Sci*, 65 (1999) 592.
- 12 Elsayed S & Bennich H, The primary structure of allergen M from cod, *Scand J Immunol*, 4 (1975) 203.
- 13 Chaki S, *Molecular and immunological characterization of Ig-E – binding component(s) in **Indian** major carp and study of its cross-reactivity with other such food components*, Ph.D. thesis, Jadavpur University, Kolkata, December 2003.
- 14 Lowry O H, Rosebrough, N J, Farr A L & Randall R J, Protein measurement with the folin-phenol reagent, *J Biol Chem*, 193 (1951) 265.
- 15 Voller A, Draper C, Bidwell D & Barlett A A, Microplate enzyme linked immunosorbent assay (**ELISA**) for Chagas' disease, *Lancet*, 1 (1975) 426.
- 16 Laemmli U K, Cleavage of structural protein during the assembly of the head of bacteriophage T4, *Nature*, 227 (1970) 680.
- 17 Towbin H, Staehelin T & Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, *Proc Nat Acad Sci USA*, 76 (1979)

4350.

- 18 Swoboda I, Bugajska-Schretter A, Verdino P, Keller W, Sperr WR, Valenta P, Valenta R & Spitzauer S, Recombinant carp parvalbumin, the major cross-reactive fish allergen: A tool for diagnosis and therapy of fish allergy, *J Immunol.*, 168 (2002) 4576.
- 19 Lindstrom C D-V, Van Do T, Hordvik I., Endresen C, & Elsayed S, Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of atlantic salmon (*Salmo salar*), *Scand J Immunol*, 44 (1996) 335.
- 20 Hamada Y, Tanaka H, Ishizaki S, Ishida M, Nagashima Y & Shiomi K, Purification, reactivity with IgE and cDNA cloning of parvalbumin as the major allergen of mackerels, *Food Chem Toxicol*, 41, 8 (2003) 1149.
- 21 Das Dore S, Chopin C, Romano A, Gallard - Irmoli A- V, Quarantino D, Pascual C, Fleurence J & Gueant J L, IgE - binding and cross-reactivity of a new 41 kDa allergen from codfish, *Allergy*, 57 suppl. 72 (2002) 84.
- 22 Hamada Y, Nagashima Y & Shiomi K, **Identification** of collagen as a new fish allergen, *Biosci Biotechnol Biochem*, 65 (2001) 285.

*Correspondent author

Phone: +91-33-2473-4971

Fax: +91-33-2473-2805

E-mail: bcbpc@mahendra.iacs.res.in