ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2008) 26: 191-198

Identification of the Major Allergens of Indian Scad (*Decapterus russelli*)

Rosmilah Misnan[1], Shahnaz Murad[1], Meinir Jones[3], Graham Taylor[4], Dinah Rahman[4], Masita Arip[1], Noormalin Abdullah[1] and Jamaluddin Mohamed[2]

SUMMARY The purpose of this study was to characterize major allergens of Indian scad (*Decapterus russelli)* which is among the most commonly consumed fish in Malaysia*.* Raw and cooked extracts of the fish were prepared. Protein profiles and IgE binding patterns were produced by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using sera from subjects with fish allergy. The major allergens of the fish were then identified by two-dimensional electrophoresis (2-DE), followed by mass spectrometry of the peptide digests. The SDS-PAGE of the raw extract revealed 27 protein fractions over a wide molecular weight range, while the cooked extract demonstrated only six protein fractions. The 1-DE immunoblotting detected 14 IgE-binding proteins, with a molecular weight range from 90 to < 6.5 kDa. Three protein fractions with molecular weights of ~51, 46 and 12 kDa were identified as the major allergens of this fish. The ~12 kDa band was a heat-resistant protein while the ~51 and 46 kDa proteins were sensitive to heat. The 2-DE gel profile of the raw extract demonstrated >100 distinct protein spots and immunoblotting detected at least 10 different major IgE reactive spots with molecular masses as expected and isoelectric point (pI) values ranging from 4.0 to 7.0. A comparison of the major allergenic spot sequences of the 12 kDa proteins with known protein sequences in databases revealed extensive similarity with fish parvalbumin. In conclusion, this study demonstrated that a parvalbumin which is similar to Gad c 1 is the major allergen of Indian scad. Interestingly, we also detected heat-sensitive proteins as major allergenic components in our fish allergy patients.

Fish represents a valuable source of micronutrients, minerals, essential fatty acids and proteins in many countries.[1,2] Overall, fish provides more than 2.6 billion people worldwide with at least 20 percent of their average per capita intake of animal proteins.[3] In Malaysia, fisheries produced 1.42 million tons of fish in 2005 (1.21 and 0.21 million tons from capture and aquaculture, respectively).[3]

Fish, milk, egg, peanuts and shellfish are the most important allergens causing IgE-mediated food hypersensitivity worldwide.[4] Allergy to fish can cause symptoms such as urticaria, asthma and in severe cases, fatal anaphylaxis.[5] In addition, occupational seafood allergy has been reported among workers through direct contact with seafood products as well as inhalation of seafood aerosols during seafood processing.[3]

During the last 20 years, a number of major allergens have been identified in various seafoods.[3] The best-studied fish is cod and its major allergen is Gad c 1.[2,4] Subsequent molecular studies have char-

From the [1]Allergy and Immunology Research Centre, Institute for Medical Research, 50588 Jalan Pahang, Kuala Lumpur, [2]Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, 50300 Jalan Raja Muda Abdul Aziz, Kuala Lumpur, Malaysia, [3]Occupational and Environmental Medicine, Imperial College, Emmanuel Kaye Building, 1b Manresa Road, London SW3 6LR, United Kingdom, [4]Proteomics Facility, MRC Clinical Sciences Centre, Du Cane Road, London W12 0NN, United Kingdom.
Correspondence: Rosmilah Misnan
E-mail: rosmilah@imr.gov.my

acterized major allergens from various fish species, including salmon (Sal s 1),[6] Atlantic cod (Gad m 1),[7] carp (Cyp c 1)[8], mackerel (Sco j 1, Sco a 1 and Sco c 1)[9] and Alaska pollack (The c 1)[10]. All these allergens belong to the parvalbumins,[6,7,8,9,10,11] a group of muscle tissue proteins which control the flow of calcium in and out of cells and are only found in the muscles of amphibians and fish.[12] These proteins were identified as the major cross-reactive allergens among various fish species[12,13] and also between fish and frogs.[14,15]

More recently, two new fish allergens have been identified by several studies.[16,17] Aldehyde phosphate dehydrogenase (APDH) was demonstrated as new allergen in codfish[16], while collagen has been identified as new fish allergen in the muscle of bigeye tuna and in the skin of several fish species.[5,17] More importantly, those studies demonstrated that fish muscle collagen is very termostable regarding its allergenicity.[5,17]

It should be noted that fish also contains minor allergens as demonstrated by immunoblots of various fish species. Additionally, species specific allergens with numerous molecular weights have been demonstrated, such as proteins of 25 kDa in swordfish,[18] 6-7 and 40 kDa in tropical sole[19] and 46 kDa in yellowfin tuna.[20] All these allergens are not well-characterized.

There are more than 20,000 different species of fish worldwide, and consumption depends on regional availability.[12] The Order *Perciformes* is the largest vertebrate order and contains the most diversified groups of bony fish. The Family *Carangidae* (scad and trevally) belongs to this order, and consists of fish which play a significant role in the commercial fisheries of Malaysia. *Decapterus* spp., locally known as *selayang* or *sardin* belong to this family.[1] This fish is commonly found in large quantities in the local market. It is also processed into fish crackers or *keropok lekor* (fish sausage) in the east coast of the Malay peninsula.[1] *Decapterus russelli* is very popular in Malaysia and other Asian countries.[1] This fish is found in the western Indian Ocean and the western Pacific Ocean including the South China Sea.[1] In 2004, local catch of *Decapterus* spp. amounted to 67.3 million tons making it one of the most common local catches.[21] The aim of this study was to characterize IgE-binding proteins and then identify the major allergens of this fish.

MATERIALS AND METHODS

Preparation of fish extracts

Uncooked extract of Indian scad was prepared following the method described in our previous studies.[22,23] Briefly, the fish meat was homogenized with 0.1M phosphate buffered saline pH 7.2 (PBS) in a 20% w/v solution. The homogenate was extracted overnight at 4°C with constant mixing, centrifuged and sterile-filtered. The supernatant was then lyophilized and stored at -20°C. Cooked extract was prepared to detect the heat-resistant and heat-sensitive proteins by boiling the homogenates for 15 minutes before extracting them in the same manner as the raw fish. The protein concentrations of both extracts were determined using the Total Protein Kit (Sigma, USA).

Allergen testing and collection of sera

The sera of 36 patients with a history of fish allergy and positive skin prick tests (SPT) to uncooked fish extract were used in this study. The SPTs were performed by a medical officer at the Ear, Nose and Throat (ENT) Clinic of the Hospital Kuala Lumpur. This study was approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia.

SDS-PAGE of fish extracts

The protein profiles of the uncooked and cooked fish extracts were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), following the method described by us previously.[22,23] In brief, the fish extract samples were denatured and heated at 95°C for 4 minutes in SDS reducing buffer (containing 0.06 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 25% glycerol and 0.01% bromophenol blue). Each sample and the prestained molecular weight markers (Biolab, UK) were resolved in a 12.0% separating gel with a 4% stacking gel using a Mini Protean 3 apparatus (Bio-Rad, USA). The gels were stained by Coomassie Brilliant Blue R-250 for protein determination. The molecular weights of the fish proteins were deter-

mined by comparing them to the molecular weight markers using an Imaging Densitometer GS800 and Quantity One Software (BioRad, USA).

One-dimensional IgE-immunoblots

Immunoblotting was performed in accordance with the protocol described in our previous studies,[22,23] using the above mentioned 36 sera. The serum from a non-allergic subject was used as negative control. Briefly, the unstained fish proteins separated by SDS-PAGE were electrophoretically transferred to a 0.45 μm nitrocellulose membrane using a mini trans-blot apparatus (BioRad, USA). After blotting, the membrane was cut into 3 mm wide strips, washed with tris-buffered saline (TBS) containing 0.05% Tween-20 (TTBS) and then blocked with blocking buffer containing 10% non-fat milk in TBS. The strips were then probed with patients' sera diluted 1:5 with a blocking buffer. The bound IgE on the strips were detected by incubation with biotinylated goat-anti-human IgE antibody (Kirkegaard and Perry Laboratories, UK) for 30 minutes, followed by incubation in streptavidin-conjugated alkaline phosphatase (BioRad, USA) for 30 minutes. After a final wash, the blots were developed using the alkaline phosphatase conjugate substrate kit (Bio-Rad, USA).

Two-dimensional electrophoresis (2-DE) and 2-DE IgE-immunoblots

The lyophilized samples containing fish proteins were solubilized in rehydration buffer (containing 8 M urea, 50 mM DTT, 4% CHAPS, 0.2% carrier ampholyte pH 3-10, 0.0002% bromophenol blue) in preparation for isoelectric focusing. Immobilized drystrips (BioRad, USA) of 7 cm length and a pH range of 3-10 were rehydrated overnight (12-14 hours) under mineral oil in 125 μl of the solubilized sample containing 50 μg of protein. Isoelectric focusing was performed using the Protean IEF Cell apparatus at 20°C (BioRad, USA) with the following voltage/time gradient: 100 V for 1 minute, 250 V for 30 minutes, 4,000 V for 2 hours and 4,000 V for 10,000 V-hours (V-hr). On completion, the strips containing focused proteins were treated sequentially with equilibration buffers (containing 65 mM dithiothreitol and then 135 mM iodoacetamide in 125 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 30% glycerol and 0.01% bromophenol blue). The strips were then placed on gradient 8-12% polyacrylamide gels and sealed in place using 1% agarose (containing 125 mM Tris-HCl, pH 6.8 and 0.1% SDS). The proteins were then separated using the Mini Protean 3 system (BioRad, USA) for 45 minutes or until the bromophenol blue dye reached the bottom of the gel. The protein spots profile was stained with Coomassie brilliant blue R-250 and then scanned using an Imaging Densitometer GS800 with PDQuest software (BioRad, USA).

For 2-DE immunoblotting, the unstained 2-DE gels containing protein spots were electrotransferred to the nitrocellulose membrane following the same protocol as the 1-DE immunoblotting. the 2-DE immunoblotting was performed using sera from 10 patients identified to have specific IgE to Indian scad.

Identification of major allergenic spots

The Coomassie-stained protein spots corresponding to those recognized by the above sera were manually excised and subjected to reduction, carbamidomethylation and in-gel tryptic digestion on a MassPrep workstation (Micromass, UK) using modified porcine trypsin (Promega, USA).

Peptide mass fingerprints were obtained using a 5% aliquot of the samples, analyzed on a MALDI-TOF instrument (M@LDI from Micromass, UK). The remaining extracts were concentrated in a Speed Vac and reconstituted in 1% formic acid (aqueous). This was subjected to LC-MS/MS analysis, performed on a QTOF II instrument (Micromass, UK) as previously reported.[24] The spectra were searched against the MSDB database using the Mascot program from Matrix Science.

RESULTS

SDS-PAGE profiles

Fig. 1A shows the Coomassie stained protein bands of the uncooked and cooked extracts of the fish. The uncooked extract produced the most protein bands. At least 27 protein bands of molecular weights from >175 to <6.5 kDa were observed in the uncooked extract while the cooked extract demon-

strated only 17 protein bands. Several protein fractions in the range of 25 to 90 kDa were sensitive to heat and were not present in the cooked extract. A ~12 kDa protein band which is equivalent in molecular weight to parvalbumin was detected in both extracts.

Detection of IgE-binding proteins

The results of the IgE-immunoblots are given in Fig. 2 and Table 1. A total of 14 different IgE-binding proteins in the range of 90 to < 6.5 kDa were detected by various sera. A heat-resistant protein at ~12 kDa corresponding to fish parvalbumin was recognized as a major allergen by 50% of the sera tested. In addition, heat-labile proteins at 46 and 51 kDa were also identified as major allergens in 55.6 and 50.0% of the sera, respectively. Multiple heat-resistant and heat-labile proteins of various molecular weights were identified as minor allergens of

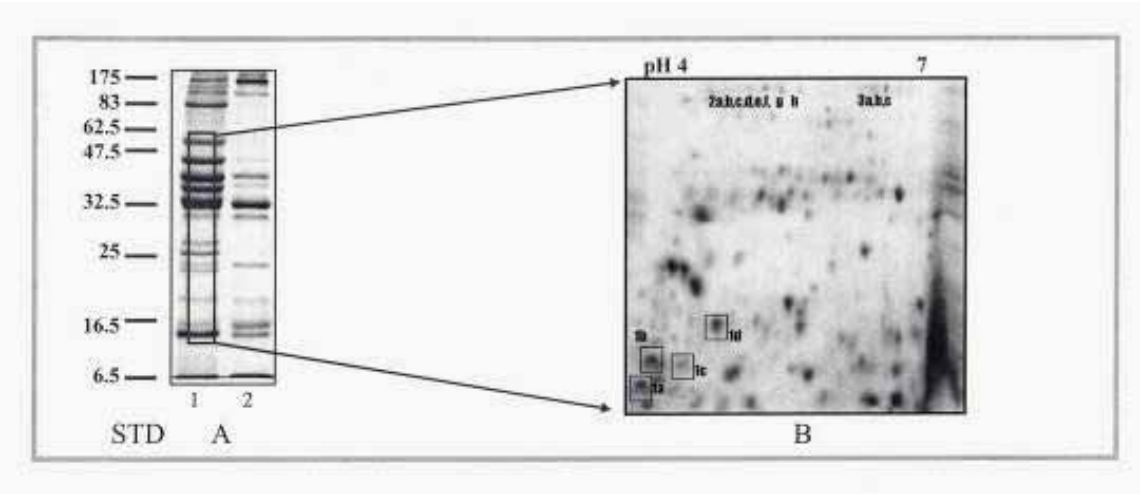

Fig. 1 Coomassie blue stain of SDS-PAGE (A) and 2 DE (B) gels showing the protein pattern of Indian scad extracts. 1 and 2 in gel A are the uncooked and cooked extracts, respectively, while the numbers in gel B show the position of allergenic spots of 12 (1a-1d), 46 (2a-2h) and 51 kDa (3a-3c). STD is the molecular weight marker in kiloDalton (kDa).

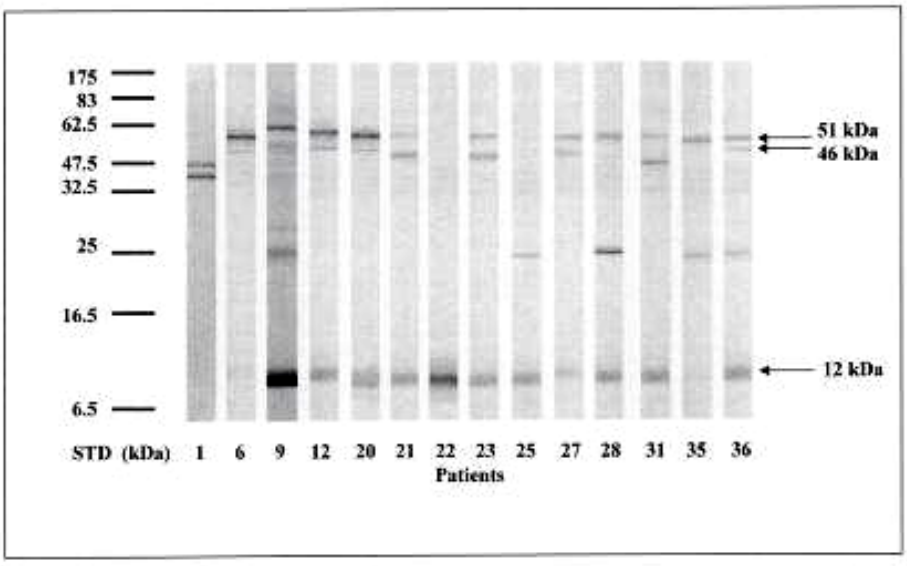

Fig. 2 Immunoblotting profile of the uncooked extract of Indian scad using sera from 14 fish-allergic patients. STD is the molecular weight marker in kiloDaltons (kDa).

Table 1 Immunoblot results of 36 fish-allergic patients

Patients	IgE-binding proteins (kDa)													
	90	60	~51	46	44	42	38	36	26	21	17	15	~12	6.5
1						■		■						
2			■											
3			■	■					■					
4			■						■					
5			■	■										
6			■	■										
7						■								
8		■				■		■						
9		■	■	■	■	■	■	■	■			■	■	
10	■									■	■	■		
11						■		■						
12			■	■									■	
13				■					■					
14			■						■				■	
15									■					
16			■			■								
17				■					■				■	
18			■	■			■	■					■	
19				■		■	■							
20			■	■									■	
21			■	■		■							■	
22													■	
23			■			■							■	
24			■										■	■
25									■				■	
26						■								
27			■	■									■	
28			■						■				■	
29				■					■					
30				■					■					
31			■	■		■							■	
32						■							■	
33			■	■									■	
34									■				■	
35			■	■					■					
36			■	■		■		■	■		■		■	
Frequency (%)	2.8	5.6	55.6*	50.0*	2.8	33.3	8.3	16.7	38.9	2.8	5.6	5.6	50.0*	2.8

* Major allergen

the fish.

2-DE profiles and immunoblots

Fig. 1B shows the Coomassie blue stained 2-DE gel of the uncooked extract of Indian scad. There were more than 100 protein spots of molecular weights between 10 to 51 kDa and pI of 4.0 to 7.0. The ~ 12 kDa single band in the SDS-PAGE was further fractionated to more than 20 protein spots, while the 46 and 51 kDa proteins were fractionated to 8 and 3 spots, respectively. The 2-DE IgE immunoblots using 10 different sera demonstrated that each subject had an individual IgE binding pattern with 6 to 40 different allergenic spots. Altogether, more than 50 different IgE-binding protein spots were detected (Fig. 3).

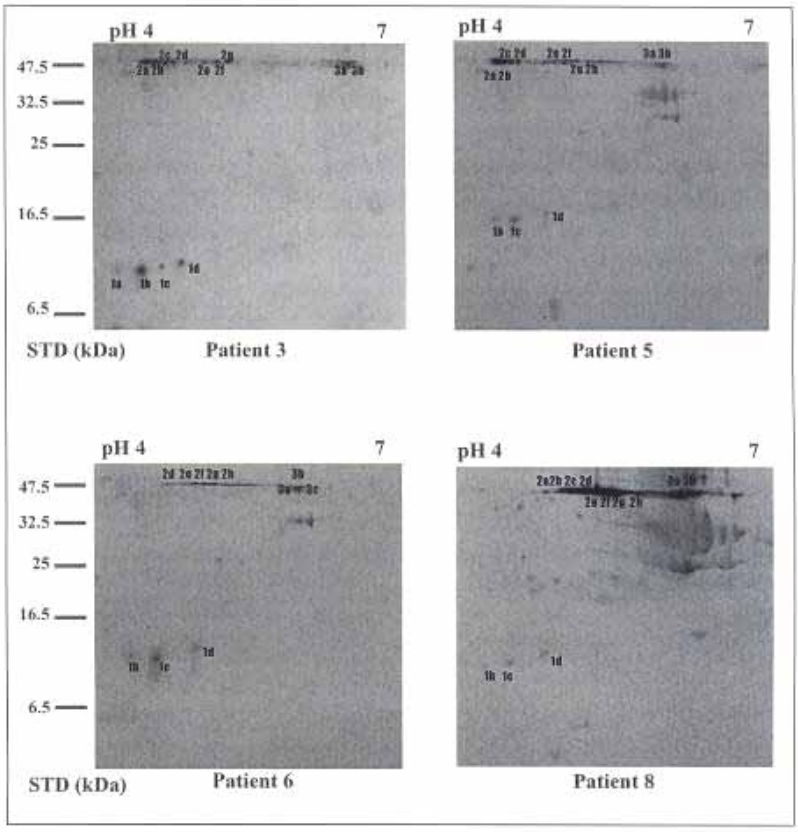

Fig. 3 Immunoblot profiles of 2 DE gels using 4 sera of patients known to have specific IgE to Indian scad allergens. STD is the molecular weight marker (kDa).

Identification of 12 kDa spots

In this study, we focused only on the identification of the major allergens at 12 kDa. The IgE-binding spots 1b, 1c and 1d of the 12 kDa protein showed the highest frequency of IgE-binding and therefore were selected to be identified by Q-TOF 2 MS/MS (Table 2). A comparison of the sequence information of the partial peptide sequence obtained from the Q-TOF analysis against various databases identified all 12 kDa allergenic spots as parvalbumin (Table 3).

DISCUSSION

Fish allergy is rather common especially in countries where fish represents a main dietary component.[2, 3] Local data reported that the prevalence of fish and shellfish allergy was 44% among patients with allergic rhinitis.[25] This is not surprising as fish is a major component of the local diet.[1] Interest in fish allergy has increased during recent years. Several major and minor allergens have been identified and characterized from various species of fish.[26,27,28] This study reports the IgE-binding proteins of *Decapterus russelli*, a commonly consumed fish in Asian countries.

Generally, fish contains many proteins, but only a few are allergenic.[29] Similarly, in this study we found that Indian Scad contains 27 protein bands, but only 14 bands were IgE-binders. It has been widely reported that major seafood allergens, like other food allergens, are heat-stable glycoproteins and therefore resist the effect of cooking, processing or digestive process.[27,29] Our SDS-PAGE gel also showed that most of the IgE-binding proteins, except for several proteins between a molecular weight of 25 to 90 kDa, were resistant to denaturation and were found in both uncooked and cooked extracts. The allergens between 40 to 90 kDa were reported to be heat-sensitive[27,29] and were repeatedly demonstrated

Table 2 Summary of IgE binding spots of the ~12, 46 and 51 kDa of Indian scad in 2-dimensional immunoblots

Spots	Molecular weight, pI (kDa), pI	Patients										Frequency (%)
		1	2	3	4	5	6	7	8	9	10	
1a	~11, 4.3	X		X							X	3 (30%)
1b*	~11, 4.5	X		X	X	X	X			X	X	7 (70%)*
1c *	~12, 4.8	X	X	X	X	X	X	X		X	X	9 (90%)*
1d*	~12, 5.3		X	X	X	X	X	X		X	X	8 (80%)*
2a	~46, 5.1		X	X	X	X		X	X	X		7 (70%)
2b	~46, 5.2		X	X	X	X		X	X	X		7 (70%)
2c	~46, 5.3			X		X		X	X			4 (40%)
2d	~46, 5.4			X		X	X	X	X			5 (50%)
2e	~46, 5.5			X		X	X	X	X			5 (50%)
2f	~46, 5.6			X		X	X	X	X			5 (50%)
2g	~46, 5.7			X		X	X	X	X			5 (50%)
2h	~46, 5.9					X	X	X	X			4 (40%)
3a	~51, 5.6	X	X	X		X	X	X	X		X	8 (80%)
3b	~51, 5.8	X		X		X	X	X	X			6 (60%)
3c	~51, 6.1						X					1 (10%)

*The major protein allergenic spots for identification by Q-TOF 2 MS/MS.

Table 3 Identities of protein spots of Indian scad identified by amino acid sequencing using Q-TOF 2 spectrometry

Spots	Database searched peptides	Identified protein (protein homologue in database - www.matrixscience.com)
1b, 1c, 1d	AFAIIDQDK, SGFIEEEELK, IGVDEFAAMVK	Parvalbumin (PRVB_SCOJP, BAC66618, Q804V8, PRVB_THECH, AAK63089)

as IgE-binders in other studies but never well characterized.[19,27,29]

Most papers published in this field focus on the characterization of allergens homologous to parvalbumin.[2,4-6] Parvalbumins are calcium-binding sarcoplasmic proteins with molecular masses of approximately 12 kDa. These proteins are resistant to heat, denaturing chemicals, and proteolytic enzymes.[6,8-10] Even though they are not the predominant protein in fish, parvalbumins are major allergens as IgE from over 90% of all fish-allergic patients bind to them.[30-32] Thus, these proteins have been well-documented as the major fish allergens in various species of fish.[2,4,6,8-10,28,30-32] In this study we found a 12 kDa heat-resistant protein to be the major allergen. The peptide fragments isolated from the digested 12 kDa spot in this study were identified as parvalbumin.

In contrast, in our previous studies of IgE-binding proteins of patients allergic to three species of local fish, *Lutjanus argentimaculatus, Lutjanus johnii* and *Scomberomorus commerson,* no 12 kDa protein was identified as major allergen. Instead, we identified three heat-sensitive major allergens of higher molecular weights, ~51, 46 and 42 kDa.[22,23] In this present study, the ~51 and 46 kDa proteins were also identified as major allergens of Indian scad. It should be noted that other studies have also reported potential allergens at ~51 and 46 kDa, but they were not well-characterized.[7,20]

Concluding our study demonstrated that the major IgE-binding proteins for Indian scad were a ~12 kDa heat-resistant protein corresponding to fish parvalbumin, and two heat-labile allergens at ~51 and 46 kDa. These findings show that both heat-labile and heat-resistant proteins play an important

role in the allergy to Indian scad among our local patients. The identification of the heat-labile major allergens is on-going using proteomics.

ACKNOWLEDGEMENTS

We would like to thank the Director of the Institute for Medical Research for the permission to publish this paper. RM acknowledges the support from the Ministry of Science, Technology and Innovation of Malaysia (MOSTI) for the National Science Fellowship Award.

REFERENCES

1. Mohsin AKM, Ambak MA. Marine fishes & fisheries of Malaysia and neighboring countries. Serdang, Universiti Putra Malaysia, 1996.
2. Lehrer SB, Ayuso R, Reese G. Seafood allergy and allergens: a review. Marine Biotechnol 2003; 5: 339-48.
3. Malaysian Fisheries Department (DOF). Fisheries Statistics Year 2005.
4. Swobada I, Bugajska-Schretter A, verdino P, *et al.* Recombinant carp parvalbumin, the major cross-reactive fish allergen: a tool for diagnosis and therapy of fish allergy. J Immunol 2002; 168: 4576-84.
5. Hamada Y, Nagashima Y, Shiomi K, *et al.* Reactivity of IgE in fish-allergenic patients to fish muscle collagen. Allergol Int 2003a; 52: 139-47.
6. Van Do T, Hordvik I, Endresen C, Elsayed S. Expression and analysis of recombinant salmon parvalbumin, the major allergen in Atlantic Salmon (*Salmo salar*). Scand J Immunol 1999; 50: 619-25.
7. Das Dores S, Chopin C, Romana A, *et al.* A new oligomeric parvalbumin allergen of Atlantic cod (Gad m 1) encoded by a gene distinct from that of Gad c 1. Allergy 2002a; 57: 84-74.
8. Bugajska-Schretter A, Grote M, Vangelista L, *et al.* Purification, biochemical and immunological characterization of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin. Gut 2000; 46: 661-9.
9. Hamada Y, Tanaka H, Ishizaki S, *et al.* Purification, reactivity with IgE and cDNA cloning of parvalbumin as the major allergen of mackerels. Food Chem Toxicol 2003b; 41: 1149-56.
10. Van Do T, Hordvik I, Endresen C, Elsayed S. Characterization of parvalbumin, the major allergen in Alaska pollack, and comparison with codfish allergen M. Molecul Immunol 2005; 42:345-53.
11. Jeebhay MF, Robbins TG, Lehrer SB, Lopata AL. Occupational seafood allergy. Occup Environ Med 2001; 58: 553-62.
12. Lopata AL, Potter PC. Allergy and other adverse reactions to seafood. Allergy Clin Immunol Int 2000; 12: 271-81.
13. O'Neil CE, Lehrer SB. Seafood allergy and allergens: a review. Food Technol 1995; 49: 103-16.
14. Hilger C, Thill L, Grigioni F, Lehners C, *et al.* IgE antibodies of fish allergic patients cross-react with frog parvalbumin. Allergy 2004; 59: 653-60.
15. Hamada Y, Nagashima Y, Shiomi K. Reactivity of serum immunoglobulin E to bullfrog *Rana catesbeiana* parvalbumins in fish allergic patients. Fisheries Science 2004; 70: 1137-43.
16. Das Dores S, Chopin C, Villaume C, *et al.* IgE-binding and cross-reactivity of a new 41kDa allergen of codfish. Allergy 2002b; 57: 79-83.
17. Hamada Y, Nagashima Y, Shiomi K. Identification of collagen as a new fish allergen. Biosci Biotech Biochem 2001; 65: 285-91.
18. Kelso JM, Jones RT, Yunginger JW. Monospecific allergy to swordfish. Ann Allergy Asthma Immunol 1995; 77: 227-8.
19. Asero R, Mistrello G, Roncarolo D, *et al.* True monosensitivity to a tropical sole. Allergy 1999; 54(11): 1228-9.
20. Yamada S, Nolte H, Zychlinsky E. Identification and characterization of allergens in two species of tuna fish. Ann Allergy Asthma Immunol 1999; 82: 395-400.
21. Malaysian Fisheries Department. Malaysian Fisheries Statistics. 2004.
22. Rosmilah M, Shahnaz M, Masita A, *et al.* Characterization of immunoglobulin E-binding proteins (IgE) of *Scomberomorus commerson* Lacepede. Malaysian J Health Sciences 2005a; 3: 79-87.
23. Rosmilah M, Shahnaz, M, Masita A, *et al.* Identification of major allergens of two species of local snappers: *Lutjanus argentimaculatus* (merah/ red snapper) and *Lutjanus johnii* (jenahak/ golden snapper). Trop Biomed 2005b; 22: 171-7.
24. Wilm M, Shevchenko A, Houthaeve T, *et al.* Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 1996; 379: 466-9.
25. Shahnaz M, Gendeh BS, Nasuruddin A. Skin test reactivity to inhalant and food allergens in patients with allergic rhinitis. Int Med Res 2001; 5: 69-73.
26. Hansen TK, Bindslev-Jensen C, Skov PS, Poulsen LK. Codfish allergy in adults: IgE cross-reactivity among fish species. Ann Allergy Asthma Immunol 1997; 78: 187-94.
27. Bernhisel-Broadbent J, Scanlon SM, Sampson HA. Fish hypersensitivity: *In vitro* and oral challenge results in fish-allergic patients. J Allergy Clin Immunol 1992; 89: 730-7.
28. Ree-Kim L, Lehrer SB. Seafood allergy. Curr Opin Allergy Clin Immunol 2004; 4: 231-4.
29. Porcel S, Leon F, Cumplido J, *et al.* Contact urticaria caused by heat-sensitive raw fish allergens. Cont Dermatitis 2001; 45: 139-42.
30. De Martino M, Novembre E, Galli L, *et al.* Allergy to different fish species in cod-allergic children: *in vivo* and *in vitro* studies. J Allergy Clin Immunol 1990; 44: 909-14.
31. Lindstroem CDV, van Do T, Hordvik I, *et al.* Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (*Salmo salar*). Scand J Immunol 1996; 44: 335-44.
32. Bugajska-Schretter A, Elfman L, Fuchs T, *et al.* Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca2+ depletion. J Allergy Clin Immunol 1998; 101: 67-74.