Identification of Parvalbumin and Two New Thermolabile Major Allergens of *Thunnus tonggol* Using a Proteomics Approach

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Key Words

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electrophoresis

Abstract

Background: The longtail tuna (*Thunnus tonggol*) is widely consumed in Asia. Parvalbumin, the main major allergen of fish, has been well identified in multiple fish species, yet little is known about the allergenic proteins in *T. tonggol*. Thus, the aim of this study was to characterize the major allergens of *T. tonggol* using a proteomics approach. **Methods:** Raw and boiled extracts of the fish were prepared. Fish proteins were separated by means of SDS-PAGE and two-dimensional (2-DE) electrophoresis. 1-DE immunoblotting of raw extract was performed with sera from fish-allergic patients. Ten sera were further analysed by 2-DE immunoblotting. Selected major allergenic protein spots were excised, trypsin digested and analysed by means of mass spectrometry. **Results:** SDS-PAGE of raw extract revealed 26 protein fractions, while boiled extract demonstrated fewer bands. The 2-DE gel profile of the raw extract further fractionated the protein bands to more than 100 distinct protein spots. 1-DE immunoblotting of raw extract exhibited two thermolabile protein

fractions of 42 and 51 kDa as the major allergens, while the boiled extract only revealed a single IgE-binding band at 151 kDa. 2-DE immunoblotting of raw extract further detected numerous major IgE-reactive spots of 11–13, 42 and 51 kDa. Mass spectrometry analysis of the peptides generated from the 12, 42 and 51 kDa digested spots indicated that these spots were parvalbumin, creatine kinase and enolase, respectively. **Conclusions:** In addition to parvalbumin, two new thermolabile allergens were identified as major allergenic proteins of *T. tonggol*. This study proved that both thermostable and thermolabile proteins are important in local tuna allergy and should be included in diagnostic strategies.

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Introduction

Tuna and tuna-like species are very important economically and a significant source of food [1]. True tuna belongs to the genus *Thunnus* that includes 8 species [1]. Longtail tuna, *Thunnus tonggol* (Bleeker) is the second smallest *Thunnus* spp. and grows to a maximum size of 142 cm total length and 35.9 kg [2]. The dorsal region of this fish is dark blue, and the lower region and abdomen

silver-white with colourless elongated oval spots along the body [3]. Locally known as 'tongkol', 'kayu' or 'aya', this fish is an economically important pelagic species inhabiting tropical and subtropical countries of the Indo-Pacific region, throughout the Indian Ocean and western Pacific Ocean [1]. This fish is the most frequently caught tuna in peninsular Malaysia; the local catch reached 29,714 tonnes in 2010, contributing to 48% of the total tuna landing in Peninsular Malaysia [4]. In Malaysia, this tuna is usually eaten fresh and a limited amount is canned for export [3].

However, proteins of the fish have been highlighted as potent allergens in sensitised children and adults, with clinical manifestations ranging from mild allergic responses to severe life-threatening anaphylactic reactions [5–8], especially in the coastal countries where fish consumption is high, such as Scandinavian countries and Japan [5]. Therefore, fish allergy has been listed as one of the 8 most frequent allergies, and the labelling of foods containing seafood including fish has already become mandatory in the USA, Europe and Japan, for example, which requires manufacturers to clearly list fish ingredients on product labels [6]. Various species of bony fish including tuna have also been updated as important seafood causing occupational allergy among seafood workers [9].

Our previous statistics showed that shellfish and fish were the most commonly implicated seafood in 44% of patients with asthma and allergic rhinitis [10]. Recent data from other Asian countries also emphasized seafood, particularly shellfish, and fish as an important allergens in up to 40% of children and 33% of adults [6]. Fish allergy was confirmed as a significant allergen in about 13% of children [11] and 4% of adults in Singapore [12], 4–12% among adults in India [13] and 1–3% among children in Taiwan [14]. Importantly, fish allergy tends to be lifelong, similar to peanut allergy [6, 15].

Numerous fish allergens have been identified and characterized in a variety of fish species [6]. Most of these food allergens belong to parvalbumin, a calcium-binding sarcoplasmic protein with a molecular weight (MW) of about 12 kDa [5, 6, 8, 16]. The first and best-identified parvalbumin allergen is Gad c 1 from codfish *Gadus callarias* which has high cross-reactivity between different fish species [6, 17]. In subsequent molecular studies, parvalbumin has been identified as major allergen in multiple species of fish, such as salmon [18], Atlantic cod [19], carp [20, 21], mackerel [22, 23], Alaska pollack [24], blunt snout bream [25] and red stingray [26]. In addition to parvalbumin, within the last several

years some studies have identified higher-MW fish allergens, including aldehyde phosphate dehydrogenase [27], collagen [28–30], transferrin [31], enolase and creatine kinase [25].

Studies on tuna allergens have been conducted on several species of tuna such as *Thunnus albacares*/yellowtail tuna [28, 32, 33], *Thunnus alalunga*/albacore [16, 32], *Thunnus obesus*/bigeye tuna [29, 30] and *Thunnus thynnus*/bluefin tuna [31], while some other studies did not mentioned the tuna species [34]. To the best of our knowledge, there is only one short report on *T. tonggol* [35]. Therefore, the aim of this study is to characterize and identify the major allergens of local tuna, *T. tonggol*, using a proteomics approach.

Materials and Methods

Fish Protein Extraction

Protein extraction was conducted according to our previous study [23] with modifications. *T. tonggol* was purchased from a local market at Port Klang (Malaysia). To recognize thermostable and thermolabile bands, two types of *T. tonggol* extracts (raw and boiled) were prepared from a mixture of white and dark muscles. In brief, the fish muscles (40 g) were homogenized in 400 ml of 0.1 M phosphate-buffered saline (pH 7.2; diluted 1:10 in distilled water) using a Waring blender for 15 min, followed by overnight extraction at 4°C under shaking. The fish homogenates were then clarified by centrifugation at 4,500 and 14,000 r.p.m., both for 15 min at 4°C. The supernatant was collected, sterile filtered using a 0.22-µm syringe filter, freeze dried and stored at –20°C until use. In contrast, for boiled extract preparation, the homogenates were boiled on a hot plate for 15 min before being extracted as mentioned above. The protein content of the extracts was quantified using a total protein kit (Sigma, St. Louis, Mo., USA).

Experimental Sera

To identify IgE-binding proteins of *T. tonggol*, 67 sera from patients with a clinical history of fish allergy and positive skin prick test to raw extract of *T. tonggol* were used. The sera were obtained from patients referred to the Allergy Clinic, Kuala Lumpur Hospital, while several control sera were obtained from non-allergic subjects. The sera were stored at –80°C until use. This study was approved by the Medical Research and Ethics Committee of the Ministry of Health Malaysia.

SDS-PAGE Analysis

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was carried out following a method described previously [23]. The fish extracts were denatured by heating at 95°C for 4 min 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). The aliquots of fish protein samples (10 µg/well) were then resolved in a 12.0% separating gel with a 4% stacking gel at 120 mA for 45 min using a Mini-PROTEAN 3 system (BioRad, Hercules, Calif., USA). MW markers (Precision Plus Protein standards; BioRad) were run as a reference along with samples. After

running, the gels were stained by Coomassie brilliant blue R250 to visualise the protein bands. MW markers were used to determine MW of the bands using the Imaging Densitometer GS800 and Quantity One Software (BioRad).

1-DE Immunoblot Analysis

Immunoblotting of raw extract was performed using sera from 67 fish-allergic patients as mentioned above. Briefly, the fish proteins resolved by SDS-PAGE were first electrophoretically transferred from the gel onto a 0.45- μ m nitrocellulose membrane using a mini trans-blot System (BioRad) for 70 min at a constant voltage of 100 V. The membrane was then rapidly stained with Ponceau S to visualise the protein bands, cut into 3-mm-wide strips, washed with TTBS buffer (TBS buffer containing 0.05% Tween-20) and then blocked for 1 h with 10% non-fat milk in TBS (blocking buffer). After blocking, the strips were probed with individual patient sera overnight at 4°C under shaking, followed by incubation with biotinylated goat-anti-human IgE as the secondary antibody (KPL, Guildford, UK) for 30 min. Finally, the strips were incubated in streptavidin-conjugated alkaline phosphatase (BioRad) for 30 min and alkaline phosphatase conjugate substrate kit (BioRad) for another 30 min to detect the IgE-binding proteins. To confirm the thermolabile properties of the IgE-binding proteins, immunoblotting of boiled extract was performed using sera from 10 patients identified to have specific IgE to raw *T. tonggol* in the immunoblotting above.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2-DE) gels were analysed using 7-cm immobilized dry strips (nonlinear, pH 3–10; BioRad). Briefly, 50 μ g of the raw fish proteins were solubilised in rehydration buffer (containing 8 M urea, 50 mM DTT, 4% CHAPS, 0.2% carrier ampholyte, pH 3–10, and 0.0002% bromophenol blue). The strips were then rehydrated overnight with the rehydration buffer, followed by isoelectric focusing at 100 V for 1 min, 250 V for 30 min, 4,000 V for 2 h and 4,000 V for 10,000 V-h using the PROTEAN IEF cell system (BioRad). After focusing, the strips were treated sequentially with equilibration buffers (containing 65 mM dithiothreitol and then 135 mM iodo-acetamide in 125 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 30% glycerol and 0.01% bromophenol blue). The strips were then subjected to gradient 8–12% polyacrylamide gels and sealed with 1% agarose (containing 125 mM Tris-HCl, pH 6.8, and 0.1% SDS). Proteins were then resolved by MW using the Mini PROTEAN 3 system (BioRad), stained with Coomassie brilliant blue R250, scanned using Imaging Densitometer GS800 and analysed using PDQuest software (BioRad).

2-DE Immunoblot Analysis

IgE-binding spots were detected by immunoblotting of the 2-DE gel using sera from 10 patients identified to have specific IgE to *T. tonggol* in the immunoblotting above. In brief, the unstained 2-DE gels were electrotransferred onto nitrocellulose membranes, followed by the same protocol of immunoblotting as mentioned above.

Mass Spectrometry Analysis

Coomassie-stained protein bands corresponding to those that reacted to patient sera were excised, stained and subjected to reduction, carbamidomethylation and in-gel tryptic digestion using modified porcine trypsin (Promega, Madison, Wisc., USA) carried

out on a MassPrep workstation (Micromass, Manchester, UK). Data of peptide mass fingerprints were obtained by MALDI-TOF instrument (Micromass) using a 5% aliquot of the samples. The remaining samples were concentrated in a Speed Vac, reconstituted in 1% formic acid (aqueous) and subjected to liquid chromatography tandem mass spectrometry, performed on a QTOF II instrument (Micromass) as previously reported [36]. The acquired tandem mass spectrometry spectra were searched against the MSDB database using the Mascot software (Matrix Science, London, UK).

Results

SDS-PAGE Analysis

SDS-PAGE of raw extract contains both heat-stable and thermolabile proteins (fig. 1a). At least 26 protein bands over a wide MW range (between 175 and 6.5 kDa) were exhibited in the raw extract. In the boiled extract, approximately only 9 of the bands, including a 12 kDa band, can still be visualised on SDS-PAGE gel, while the remaining bands between 16–25 and 40–90 kDa were missing (fig. 1b).

1-DE Immunoblot Analysis

Figure 1a and table 1 show 1-DE immunoblotting analysis of raw extract of the fish. Overall, all sera detected at least 1 IgE-binding protein. A total of 17 IgE-binding proteins, ranging in MW from 151 to 12 kDa, were detected by the various sera. Two protein bands with MW of 51 and 42 kDa have been identified as the major allergens of this fish. The 51 kDa band was recognized by IgE in 35 sera (52.2%), while the 42 kDa band was demonstrated by 37 sera (55.2%). Both proteins were found to be sensitive to heat in SDS-PAGE profiles. Moreover, a 46-kDa thermolabile band and a 36 kDa thermostable band were considered as potential minor allergens, as detected by 28.4 and 26.9% of the sera, respectively. In addition, other thermostable bands, including a low-MW (LMW) protein of 12 kDa and several high-MW (HMW) bands of 90, 115 and 151 kDa, were also recognized as minor allergens with a very low frequency of detection (1.5–6.0%).

Figure 1b and table 2 show the immunoblotting results of boiled extract. The test detected only 1 thermostable IgE-binding band at 115 kDa.

2-DE and 2-DE Immunoblot Analysis

Protein bands of 10–51 kDa of *T. tonggol* were further fractionated to more than 100 distinct protein spots between pIs of 4.0–7.0 (fig. 2a). The single band of 51 kDa

Fig. 1. SDS-PAGE gel and IgE immunoblotting results of raw (**a**) and boiled (**b**) *T. tonggol* proteins. R and C are SDS-PAGE profiles of raw and boiled extracts, respectively, and strips 1–67 are immunoblotting strips of sera from 67 fish-allergic patients. B = Blank; N = negative control; Std = MW markers. Arrows indicate the position of major allergens of *T. tonggol* (**a**) and the ~115-kDa protein (**b**).

Fig. 2. **a** 2-DE gel stained with Coomassie blue. **b** Immunoblotting of 2-DE profiles of *T. tonggol* using sera from patients known to have specific IgE to the fish allergens (No. 20–64 correspond to the subjects listed in table 1). Std = MW; pH = approximate pI (along the top axis). All identified allergens are encircled and numbered (spots 1–3).

Table 1. IgE immunoblotting analysis of raw *T. tonggol* using sera from 67 patients with fish allergy

Subject No.	MW (kDa)																
	151	115	90	70	60	51	48	46	42	38	36	28	26	25	17	15	12
1		√			√	√	√		√								
2										√							
3				√					√								
4									√								
5				√													
6				√	√	√											
7									√								
8								√									
9								√	√								
10		√	√			√		√	√								
11										√	√						
12					√						√	√	√	√			
13						√			√								
14				√	√	√		√	√	√	√						
15									√				√				
16				√					√		√	√					
17				√		√											
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34					√				√		√						
35									√								
36					√	√			√								
37			√		√												
38						√							√				
39											√						
40											√						
41								√	√	√	√						
42						√			√		√						
43						√	√	√	√		√		√			√	
44					√	√		√	√								
45						√	√		√			√	√				
46					√	√		√					√				

Table 1. (continued)

Subject No.	MW (kDa)																
	151	115	90	70	60	51	48	46	42	38	36	28	26	25	17	15	12
47						√						√	√				
48					√	√			√								
49						√		√									
50					√	√		√	√								
51						√											
52	√	√				√	√										
53													√	√			
54						√		√	√		√						
55	√	√				√			√				√				
56						√	√		√								
57						√	√	√	√								
58								√	√		√		√				
59						√		√	√								
60									√								
61								√									
62						√											
63						√			√								
64						√		√	√	√							
65					√	√		√	√		√		√				
66						√											
67											√						
Total n	2	4	2	7	15	35	8	19	37	5	18	5	12	2	1	1	1
%	3.0	6.0	3.0	10.4	22.4	52.2	11.9	28.4	55.2	7.5	26.9	7.5	17.9	3.0	1.5	1.5	1.5

√ = IgE-binding protein. The two major allergens are shown in italics.

Table 2. IgE immunoblotting analysis of boiled *T. tonggol* using sera from 10 patients with fish allergy

Subject No.	MW (kDa)																
	151	115	90	70	60	51	48	46	42	38	36	28	26	25	17	15	12
1		√															
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10		√															
Total n	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
%	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

√ = IgE-binding protein.

Table 3. Identification of major allergenic proteins of *T. tonggol* (spot no. 1–3) by mass spectrometry analysis

Spot No.	Observed MW, kDa/pI	Peptides/number of matched peptides	Sequence coverage, %	Protein identity	Homologous proteins
1	12/4.4	SGFIEEDELK/1	9.2–9.4	parvalbumin	PRV2_SALSA, PARV_BRARE, Q7ZT36, AAF78471, PRVA_CYPCA, PVCAB, 1B8CA, IB8RA, Q8UUS2, Q8UUS3, 1B9AA, CAA66404, Q90YL0, PRVB_MERBI, PVPK2, Q8AYB4, Q8AYB3, PVTFB3, Q804Z0, Q8AVD4
2	42/5.1	TDLNFENLK, GGDDLDPNYVLSSR, GFTLPPHNSR, DLLDPVISDR, GTGGVDTASVGGVFDISNADR, LMVEMEK, GEAIMSMIPAQK, FCVGLQK/8	6–22	creatine kinase (EC 2.7.3.2)	Q8JH38, Q6XGY4, Q9YI15, Q7T1J3, Q804Z2, Q7T306
3	51/5.3	YDLDFK, KACNCLLLK, IGAEVYHNLK, ACNCLLLK, VNQIGSVTESIQACK, AAVPSGASTGVHEALELR, KFSVVEQEK, FMLELDGTENK, KACNCLLLK, DATNVGDEGGFAPNILENNEALELLK, YNQLMR, HIADLAGHK, EVILPVPFNVINGGSHAGNK, GGKYDLDFK/14	11–22	enolase (EC 1.1.1.17)	Q9DDH0, Q6TH14, Q9PTX5, Q9U5F7, Q7ZZM5, Q9DDH5, Q9DDG7, ENO_XENLA, ENO_ALLMI

was fractionated to at least 7 spots, while the 42 kDa band was fractionated to approximately 11 spots. Numerous protein spots were also visualised in the LMW protein region of 10–13 kDa. Immunoblotting of 2-DE gels using sera from 10 different sera demonstrated that each patient had individual IgE antibodies with different binding capacities (fig. 2b). At least 5 different IgE-reactive spots were detected by each serum. Overall, at least 20 different spots between 6.5 and 175 kDa were able to bind to IgE antibodies (data not shown). However, only several spots of 51 and 42 kDa were exhibited as IgE-binding spots. Surprisingly, all sera were found to have specific IgE antibodies to LMW protein spots of 10–13 kDa.

Mass Spectrometry Analysis

This study focuses on further identification of the allergenic spots of 51 and 42 kDa proteins, the major allergens of *T. tonggol*. However, the 12 kDa protein was also further characterized as the protein spots were also recognized as the major IgE-binding spots in immunoblotting of 2-DE profiles. Table 3 summarized the results of mass spectrometry analysis of the spots. Sequence comparisons of a 51 kDa spot (spot 3) and a 42 kDa spot (spot 2) with known protein sequences in databases have identified these spots as fish enolase and creatine kinase, respectively, while the major allergenic spot of 12 kDa (spot 1) is identical to parvalbumin of various species of fish including *Sal s 1*.

Discussion

Tuna plays an important role in nutrition worldwide, including Asian regions [6]. Characterization of new allergenic proteins could be effectively performed through recent advances in proteomics technology [17, 25, 37]. Thus, we used this approach to identify the allergenic proteins of local longtail tuna, *T. tonggol*.

Teleost fish, including tuna, has two types of muscles: white and dark muscles, and the allergenicity of the muscles mostly depends on the parvalbumin content [5, 38]. Parvalbumin, the major and cross-reactive allergen in multiple fish species, is found more abundant in the white than in the dark muscle, and therefore the white muscle is more allergenic [5, 35, 38]. The parvalbumin content also varies significantly among different fish species [5, 38, 39], and it was much lower in yellowtail tuna than in other species of fish [5, 38]. It was also reported that *Gad c 1*, *Sal s 1*, *The c 1*, herring and wolffish contained the most potent cross-reacting allergens, whereas tuna, together with halibut, flounder, and mackerel, were the least allergenic fish [16]. Although it is thought that the dark muscle of the fish is less allergenic than the white muscle, we prepared the *T. tonggol* extracts from a mixture of white and dark muscles as the dark muscle is often ingested with the white muscle.

Comparison of SDS-PAGE results between raw and boiled extracts revealed that the boiling process has significantly modified the protein profiles of *T. tonggol*. The raw extract contains 26 protein fractions, generally sim-

ilar to the protein profile of raw yellowtail tuna [28, 33], whereas the boiled extract has only 9 protein bands, probably due to denaturation of thermolabile proteins of 16–25 and 40–90 kDa. This protein pattern is also in accordance with other studies which reported thermolabile proteins between 20–100 [40] and 40–90 kDa [41] in multiple fish species. Allergic reactions to fish most commonly occur upon ingestion of cooked fish [6, 8], but it can also be triggered by raw fish, particularly after direct contact with the raw fish, ingestion of raw or undercooked fish, or inhalation of fish odours or fumes, as proven by other researchers [41–43]. It was reported that almost 10% of patients with seafood allergy including fish have more severe reactions caused by inhalation or skin contact than by ingestion [44]. Therefore, in this study, the raw extract was selected to be used in skin prick and both 1-DE and 2-DE immunoblotting tests, predominantly to avoid missing IgE-binding proteins, as the raw extract contains both thermostable and thermolabile proteins, whereas the boiled extract comprises only a few thermostable bands. However, to confirm the thermostability properties of the bands, we repeated the 1-DE immunoblotting test with boiled extract using 10 sera.

Although *T. tonggol* contains numerous protein bands, only 17 bands are recognized as putative allergens based on the 1-DE immunoblotting results. Importantly, our study found that both groups of proteins (thermolabile and thermostable proteins) were able to bind with IgE antibodies of the tested sera, demonstrating that both proteins were significantly implicated in allergy to this fish. The thermolabile properties of the bands were also successfully confirmed by the immunoblotting results of boiled extract of the fish. Initially, seafood allergens are often reported as heat-stable proteins and therefore resist the effect of cooking, processing or digestive processes [17]. However, other findings have confirmed the presence of thermolabile allergenic proteins in multiple species of fish [28, 32, 41], and canned tuna is considered the least-allergenic form of fish [33]. Hence, allergenicity of the thermolabile proteins seems to be reduced by heat (boiling, frying or grilling) [40, 44], as well as by drying, salting, smoking and chemical processing [33].

In this study, more than half of the tested sera detected IgE-binding bands at 42 and 51 kDa, thus, these thermolabile proteins were identified as the major allergens of this local tuna. Mass spectrometry analysis of the peptide fragments isolated from the digested spots of 42 and 51 kDa were identical to the fish creatine kinase subunit and enolase fragment, respectively. Interestingly, a very re-

cent finding by Liu et al. [25] also reported enolase and creatine kinase as new allergens of freshwater fish blunt snout bream, conforming well to our study.

Creatine kinase, also known as creatine phosphokinase, is an important enzyme expressed by various tissues, particularly tissues with high energy demands such as skeletal and cardiac muscles, neurons, photoreceptor cells, spermatozoa and electrocytes. This enzyme catalyses the reversible conversion of creatine and ATP to phosphokinase and ADP, thereby helping maintain energy homeostasis in the cells [45, 46]. Creatine kinase, a dimeric enzyme with two identical subunits of approximately 40 kDa as estimated by SDS-PAGE [47], is a highly conserved enzyme with a sequence that is 60% identical across all species [45], while arginine kinase, the creatine kinase homologue found in invertebrates including shellfish, is ~45% identical and ~55% similar with various creatine kinase subunits [45]. Importantly, arginine kinase has been identified as the major allergen in multiple shellfish and invertebrates [48], whereas the creatine kinase subunit at 41 kDa was recently discovered as an allergenic protein in a freshwater fish [25]. On the contrary, earlier investigation by Das Dores et al. [27] has also identified a new fish allergen at 41 kDa in codfish, but as aldehyde phosphate dehydrogenase, a cytoplasmic enzyme involved in glycolysis and gluconeogenesis.

Enolase, a 51 kDa major allergen, is also a ubiquitous cytoplasmic enzyme involved in glycolysis and gluconeogenesis [49]. Importantly, enolase (molecular mass ranging between 46 and 51 kDa) has been identified as a highly conserved allergen in fungal species and *Hevea* latex [50, 51]. It should be pointed out that α -enolase at 50–52 kDa has been identified as an auto-antigen associated with severe asthma [52] and rheumatoid arthritis [53]. Therefore, the clinical relevance of enolase as well as creatine kinase in tuna allergy should be further investigated.

It should be noted that some earlier studies have reported HMW allergens at 34–38, 40–42, 45, 46, 50–51 or 63 kDa in multiple species of fish, including tuna, but they are not well characterized [16, 28, 32, 54–57]. Consistent with this, we have previously also reported major allergens at higher MW (42, 46 and 51 kDa) in local snappers [58] and mackerels [23, 59]. Similarly, in addition to the major allergens at 42 and 51 kDa, we also found that more than one fourth of the tested sera indicated potential allergenic proteins of 36 and 46 kDa of *T. tonggol*.

Immunoblotting of both 1-DE and 2-DE gels using sera from 67 and 10 different sera, respectively, demonstrated that each patient had individual IgE antibodies

with different binding capacities and patterns. This divergent pattern of reactivity suggests that patients responded to both common and different antigenic epitopes. A significant relationship between symptoms and the epitope profiles in salmon-allergic patients has been observed recently [60], as well as in other allergic patients [37, 61, 62]. It was highlighted that IgE epitope mapping of allergens might provide information about the antigenic structure and a patient's immune response, and thereby has as a potential to become an additional tool for the diagnosis or prognosis of food allergy and for the development of safe hypo-allergenic immunotherapeutic agents [60, 61].

Overall, the results of 1-DE immunoblotting were in agreement with 2-DE immunoblotting findings except for several spots of LMW protein regions of 11–13 kDa. In 1-DE immunoblotting, the 12 kDa band was only detected by 1 serum, but in 2-DE immunoblotting, all 10 sera demonstrated at least 3 allergenic spots of 11–13 kDa. This could be due to the higher sensitivity of 2-DE immunoblotting than 1-DE immunoblotting as the 2-DE separation method has further fractionated the proteins to higher-resolution protein spots [37, 63], thereby probably rendering the protein epitopes more detectable by IgE antibodies of tested sera. It was reported that IgE-binding epitopes that were hidden within the protein may be uncovered in 1-DE immunoblotting [64]. As predicted, analysis of the peptide fragments isolated from 1 of the 12 kDa spots revealed that this spot was homologous to the segments of multiple fish parvalbumin in the protein databases, indicating that parvalbumin is also a major allergenic protein of *T. tonggol*.

Besides parvalbumin, collagen and transferrin, HMW thermostable proteins were considered to be important

fish allergens in several species of fish, including tuna [29–31, 34, 39]. However, in this study, only 4 sera (6.0%) reacted to the HMW bands of 115 and 151 kDa, while only 2 sera (3.0%) reacted to the 90 kDa bands in 1-DE immunoblotting. Notably, in this study, the raw extract was used in skin prick tests, therefore collagen, transferrin as well as parvalbumin may not be successfully detected as those proteins were significantly more reactive in the heated extracts [29, 31, 39]. For that reason, the use of boiled extracts in future studies should also be considered to avoid missing thermostable allergens.

In conclusion, we have characterized new major allergens from *T. tonggol*, creatine kinase and enolase, which are two thermolabile enzymes with a high level of homology in different animal species. In addition, parvalbumin, the well-documented thermostable fish allergen, was also identified as a major allergenic spot of this fish. Therefore, we suggest that both thermostable and thermolabile proteins are important in local tuna allergy and should be included in both in vivo and in vitro tests to avoid the potential of misdiagnosis. Moreover, the identification of all these major allergenic proteins is the first step toward generating recombinant allergens for use in future immunotherapeutic approaches.

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