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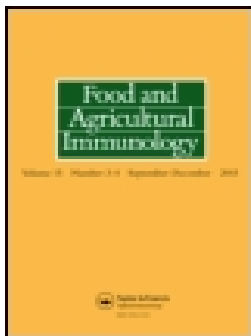
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Expression of recombinant parvalbumin from wolf-herring fish and determination of its IgE-binding capability

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ABSTRACT

In this study, we produced the recombinant form of parvalbumin from wolf-herring fish and determined its IgE reactivity. Parvalbumin cDNA was sub-cloned into pET28 and expressed in *Escherichia coli* BL-21. The immunoreactivities of the recombinant and native parvalbumins were compared, and the effect of calcium binding was determined by sera from 25 fish-allergic patients. ELISA and Western blotting confirmed similar IgE-reactivities of the recombinant and native proteins and confirmed that this phenomenon is highly dependent on calcium binding. The recombinant protein was 94.5% similar to carp parvalbumin (Cyp c1). Approximately 72% of patients reacted strongly with recombinant parvalbumin, 80% of them reacted with the native form and only 56% showed IgE reactivity with crude extract. Because the IgE-binding capacity of recombinant wolf-herring parvalbumin is retained and is highly similar to Cyp c1, the wild and hypoallergenic forms of this allergen could be used for diagnosis and immunotherapy of fish allergy, respectively.

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Parvalbumin; wolf-herring fish; food hypersensitivity; recombinant protein; disk ELISA; Western blotting

1. Introduction

Fish is a valuable source of essential fatty and amino acids. However, this crucial protein source contains allergenic molecules that occasionally result in food hypersensitivity and even anaphylactic shock, when eaten either raw or cooked (Hamzeh et al., 2015; Lehrer, Ayuso, & Reese, 2003; Sicherer & Sampson, 2010). Several studies demonstrated that parvalbumin is the major allergen in various fish species including cod fish (Gad c1), salmon (Sal s1), Atlantic cod (Gad m1), carp (Cyp c1) and mackerel (Sko j1) (Bugajska-Schretter et al., 2000; Das Dore, Chopin, Villaume, Fleurence, & Gueant, 2002; Hamada et al., 2003; Lindstrøm, Van Do, Hordvik, Endresen, & Elsayed, 1996; Swoboda et al., 2002). The firm

structure of this 11–12 kDa protein in different conditions increases the possibility of its allergenic property. It is thermostable. It is also stable in low pH and is resistant to degradation by proteolytic enzymes of the intestinal tract. These properties make fish parvalbumin one of the major allergens of most sea foods (Aiello et al., 2015; Kuehn, Swoboda, Arumugam, Hilger, & Hentges, 2014; Lindstrøm et al., 1996). It is also the main cross-reactive protein among fish species (Bugajska-Schretter et al., 1998; Swoboda et al., 2002). Based on this cross-reactivity, purified or recombinant parvalbumin could be applied for diagnosis and therapy of fish-allergic patients (Zuidmeer-Jongejan et al., 2015).

Parvalbumin is a calcium-binding protein with two EF-hand motifs and a free domain that covers the hydrophobic surface of the calcium-binding area (Breiteneder & Mills, 2005; Lewit-Bentley & Réty, 2000). Vertebrate parvalbumins are categorized into alpha and beta types. The beta-type parvalbumin shows more allergenic characteristics than the alpha-type (Lopata & Lehrer, 2009; Moncrief, Kretsinger, & Goodman, 1990) and was identified as a major cross-reactive structure in various fish species (Kuehn et al., 2014; Lopata & Potter, 2000).

Immunodiagnosis of fish allergy using diverse batches of crude extracts is often problematic because most of them have heterogeneous ingredients (Focke, Marth, Flicker, & Valenta, 2008; Vieths et al., 2001). This problem usually arises due to variations in the allergenic contents of the initial sources and also technical differences in preparation procedures. Moreover, resolving and purifying allergenic components from biological sources is laborious, time-consuming and sometimes impossible. In addition, some allergens may be denatured during the purification steps or present in very low concentrations in crude extracts. Availability of a sufficient amount of pure allergen is necessary for the development of *in vitro* and *in vivo* methods for diagnosis of allergic diseases (Peng et al., 2014; Tuano & Davis, 2015). Application of molecular techniques may provide us with the needed amounts of pure recombinant allergens to develop these methods (Deng et al., 2012; Hamada et al., 2004; Peng, Song, Liu, Kuang, & Xu, 2015; Vieths et al., 2001). Moreover, these techniques could help to determine the complete amino acid sequences and epitope map of the allergens (Hamada et al., 2003). Using molecular methods, parvalbumin from various fish species, including salmon (Sal s1), Atlantic cod (Gad M1) and mackerel, has been cloned and expressed in prokaryotic hosts and their IgE-binding characteristics studied (Hamada et al., 2003; Van Do, Hordvik, Endresen, & Elsayed, 1999, 2003).

Dorab wolf-herring fish (*Chirocentrus dorab*) belongs to the Chirocentridae family and is extensively consumed in coastal regions of Iran. Despite its beneficial properties, this fish is responsible for the frequently observed fish allergy incidents in these regions. These emergency conditions most likely occur due to hypersensitivity to the cross-reactive parvalbumin. We previously demonstrated the allergenic properties of parvalbumin purified from this fish species (Mohammadi et al., 2016). In the present study, we cloned and expressed a recombinant form of wolf-herring parvalbumin in *Escherichia coli* and evaluated its allergenic characteristics with fish-allergic patients' pooled serum.

2. Materials and methods

2.1. Human and mice sera

Sera from 25 allergic patients with clinical histories of fish hypersensitivities and positive specific IgE results for a fish mix of cod, salmon and trout in disk ELISAs (Specific IgE

Disks, Dr Fooke-Achterrath laboratorien GmbH) were collected in Day General Hospital. We also included sera from seven non-allergic healthy individuals who declared no clinical symptoms following fish consumption and whose sera showed no significant immuno-reactivities with fish mix ELISA disks as negative controls (detailed demographic data published in Mohammadi et al., 2016). We also immunized two female BALB/c mice with native protein as described elsewhere (Soukhtanloo, Falak, Sankian, & Varasteh, 2011), and their sera were collected and used to evaluate IgG antibodies. The study was approved by the Medical Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran.

2.2. Fish extract preparation

Fresh dorab wolf-herring (*C. dorab*) filet was purchased from Bushehr, Iran. Raw fish muscle (500 mg) was homogenized with three volumes of 20 mM Tris-HCl buffer pH 7.5 containing 150 mM NaCl, 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 10% glycerol and 0.5% protease inhibitor cocktail (Sigma-Aldrich). The homogenate was vigorously stirred at 4°C overnight, centrifuged at $14,000 \times g$ for 30 min at 4°C and the clear supernatant was collected (Falak, Sankian, Tehrani, & Jabbari Azad, 2012). The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

2.3. Parvalbumin purification

As described elsewhere (Mohammadi et al., 2016), the heat-treated extract (100°C for 1 h in dry block) was lyophilized, reconstituted and dialyzed in 20 mM Tris-HCl pH 8.6, overnight. Proteins were resolved on an AKTA Prime Plus FPLC system (GE healthcare) using a diethylaminoethyl (DEAE) sepharose CL-6B column as the anion exchange matrix and eluted at a flow rate of 1 ml/min by a linear gradient mixture of starting buffer (20 mM Tris-HCl pH 8.6) supplemented with 1 M NaCl. Following SDS-PAGE, the desired fractions were selected for further studies (Falak et al., 2013; Falak, Varasteh, Ketabdar, & Sankian, 2014).

2.4. Cloning and expression of parvalbumin in prokaryotic system

Total RNA was isolated from 100 mg of fish muscle using a commercial RNA extraction kit (Geneon, GmbH, Deutschland, Germany), and the first strand of cDNA was synthesized according to the manufacturer's instructions (Thermo, Life Sciences).

Since there was no report about wolf-herring parvalbumin characteristics, we first purified the native parvalbumin and predicted its sequence by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as previously reported (Falak et al., 2013). Based on deduction from computed results, the fragments derived from enzymatic digestion of wolf-herring parvalbumin were highly similar to gold fish (*Carassius auratus*) parvalbumin (Accession No.: JN190495, GI: 379998732). Therefore, we designed the cloning primers based on the nucleotide sequence of this species. The parvalbumin gene sequence was amplified using the following primers

containing *Bam* H1 and *Xho*1 restriction sites, respectively: 5'-ATA GGA TCC GCA TTC GCT GGA ATT CTG AAT G-3' and 5'-ATA CTC GAG TGC CTT GAC CAG GGC AGC AAA CTC-3'.

After polymerase chain reaction (PCR) amplification, the parvalbumin coding sequence was ligated into the pET28a vector. Following transformation and amplification of the ligation product in *E. coli* TOP 10 cells (Invitrogen, Life Technologies, Carlsbad, CA, USA), the plasmid was purified using a DNA extraction kit (YTA, Iran). The size of the insert was checked by conventional PCR using T7 polymerase-specific universal primers. The inserted DNA was sequenced (Source BioScience, UK), and the recombinant plasmid was transformed into *E. coli* BL-21 (DE3) cells.

Recombinant parvalbumin was expressed in *E. coli* BL21 cells (Novagen). Briefly, the construct was transformed into *E. coli* BL21 cells, and a typical colony was selected and inoculated into Luria Bertani medium containing 0.005% ampicillin. The inoculated medium was incubated at room temperature (RT) until its absorbance reached 0.5 at 600 nm. Recombinant protein expression was induced by adding 1 mM isopropyl-thiogalactopyranoside to the suspension and incubating the cells at 18°C on a cold shaker-incubator at 200 rpm overnight. Finally, the bacterial suspension was centrifuged at 4000 RCF for 10 min, and the pellet was re-suspended in 5 ml of 50 mM Tris-HCl pH 7.0 containing 150 mM NaCl and 1 mM EDTA. The bacterial cells were disrupted by three freeze-thaw cycles at -80°C and 37°C, respectively. Insoluble material was removed by centrifugation at 14,000 × g for 30 min. Recombinant parvalbumin was purified from the soluble phase of the lysate (supernatant) by metal affinity chromatography (Ni-IDA agarose, ParsToos, Iran) according to the manufacturer's instructions (Falak et al., 2014).

2.5. SDS-PAGE and Western blotting

Proteins were separated by reducing SDS-PAGE as previously described (Falak et al., 2013). In brief, the samples were subjected to 15% SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue G-250 or alternatively, proteins were electrotransferred and immunoblotted.

For Western blotting, the proteins were transferred on PVDF membranes (Amersham Biosciences, Uppsala, Sweden) using a semidry transfer system as previously described (Falak et al., 2013). The membranes were cut into strips and blocked with 2% BSA for 4 h at 37°C. After washing 3 times for 5 min each with PBS containing 0.05% Tween 20 (PBS-T), the strips were incubated with patients' 1:3 diluted sera overnight at 4°C on a rocker. After washing (as above mentioned), the strips were incubated with 1:2000 diluted biotin-labeled anti-human IgE (Abcam, USA) for 2 h at RT. After another wash, the membranes were incubated with 1:30000 diluted horseradish peroxidase (HRP)-conjugated streptavidin (Sigma, USA) for 1 h at RT. The reactive bands were visualized with a chemiluminescent substrate and ultra-photosensitive film (Falak et al., 2013).

2.6. IgG- and IgE-binding assays of recombinant and native parvalbumin

We compared the IgE reactivity of the recombinant parvalbumin-coated disks with that of native parvalbumin-coated and crude extract-coated disks using fish-allergic patients' sera and healthy negative controls by disk ELISA (Abedini et al., 2011; Falak et al., 2013).

Nitrocellulose membranes were punched into 5 mm diameter disks and incubated with 10 µg/ml of the purified native or recombinant parvalbumin or 100 µg/ml of the crude extract in micro-titer plates for 3 h at 37°C. After washing 3 times 5 min each, as described above, disks were blocked with 2% BSA for 3 h at RT, then were washed and incubated with 1:5 diluted patients' sera for 2 h at RT. After one extensive wash, a 1:2000 dilution of biotinylated anti-human IgE was added and incubated for 2 h at RT. After another wash, a 1:30,000 dilution of HRP-conjugated streptavidin was added to the disks and incubated for 1 h at RT. The disks were then washed, and 100 µl of TMB/H₂O₂ substrate was added. Following a 20-min incubation at 37°C, the reaction was stopped and the optical densities (ODs) of the samples were measured at 450/630 nm. Means and standard deviations (SDs) were determined. All samples with ODs greater than the threshold (mean + 2 SDs) were considered positive. All disk ELISA procedures were performed with constant shaking at 300 rpm on an orbital shaker-incubator.

For evaluation of the IgG reactivity of the recombinant parvalbumin, we used sera from native parvalbumin-sensitized mice. For this purpose, a couple of female BALB/c mice were immunized with subcutaneous administration of 100 µg of native parvalbumin and twice subsequent 50 µg boosters with 2-week intervals as previously described (Soukhtanloo et al., 2011). Then sera were collected and used for evaluation of parvalbumin-specific IgG reactivity and inhibition studies. In brief, the IgG reactivity of non-absorbed or recombinant parvalbumin absorbed mice sera was studied by dot blot method. We added 100 µl of a 1:1000 dilution of pooled sera from native parvalbumin-immunized mice, or a pre-absorbed one (50 µl of whole sera was pre-incubated with 1 or 4 µg of recombinant parvalbumin for 1 h on a shaker and then diluted 1:1000 with BSA 1%), to native parvalbumin-coated disks (as described above) in ELISA plates and incubated them for 3 h at RT. Then after an extensive washing step, 100 µl of 1:3500 dilution of HRP-conjugated rabbit anti-mouse IgG H&L antibody (Abcam, Cambridge, MA, USA) was added to each well. After an hour, the disks were extensively washed, and their peroxidase reactivity was analyzed by electrochemiluminescent substrate (Abcam, Cambridge, MA, USA) and ultra-photosensitive film (Figure 4).

2.7. Evaluation of the effect of calcium on IgE- and IgG binding of parvalbumin

The lyophilized native parvalbumin was incubated in 5 mM CaCl₂ or 20 mM EDTA solutions at 37°C for 1 h. The effects of calcium on IgE and IgG binding to parvalbumin were determined with fish-allergic patient sera and sera from native parvalbumin-immunized mice.

3. Results

The protein concentration of the total extract was approximately 1 mg/ml, and the extract appeared as a pale smear with some distinct bands ranging from 11 to 200 kDa in 15% polyacrylamide gel (Figure 1).

Following cDNA synthesis, the parvalbumin coding sequence was amplified with *Bam*H1- and *Xho*1-specific restriction sites at the ends of the PCR product. The PCR product and the pET28b vector were purified from agarose gels, digested with *Bam*H1 and *Xho*1 and the double-digested molecules were ligated. Sequencing of the insert

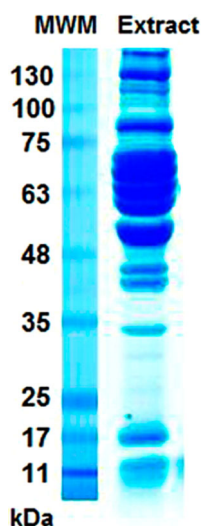


Figure 1. SDS–PAGE of wolf-herring fish muscle extract on 15% polyacrylamide gel. MWM, Protein molecular weight marker; Extract, Crude extract of wolf-herring fish.

revealed a 327 base-pair (bp) fragment with 94.55% identity to gold fish (*C. auratus*) parvalbumin.

Recombinant parvalbumin was expressed in *E. coli* BL21 cells and purified by metal affinity chromatography. SDS–PAGE of the bacterial lysate revealed expression of an apparent 14 kDa protein, with approximately 11.9 kDa belonging to parvalbumin and 2 kDa to the histidine tag. The lysate was applied to Ni-IDA agarose, and the column was washed and the protein eluted. SDS–PAGE of the eluted contents showed a single 14 kDa band (Figure 2).

The amino acid sequence of the recombinant protein showed 93.52% similarity to the *C. auratus* parvalbumin sequence. The deduced parvalbumin protein encoded by wolf-herring fish contained 109 amino acids with a predicted molecular weight of 11.9 kDa (Figure 3). The amino acid sequence of the cloned parvalbumin was 95.37% identical to *Cyprinus carpio* parvalbumin, too.

IgE-reactivities of the recombinant and native proteins were compared by immunoblotting. The fish-hypersensitive patients' pooled sera showed a considerable immunoreactivity

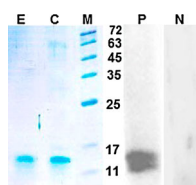


Figure 2. SDS–PAGE and Western blots of products with patient sera. Lane E: Purified recombinant parvalbumin; lane C: Supernatant of bacterial cell lysate of *E. coli* BL21 harboring the recombinant protein; Lane M: Protein molecular weight marker; Lane P: IgE Western blotting of recombinant parvalbumin with pooled sera from fish-allergic patients; Lane N: Western blotting of recombinant parvalbumin with pooled sera from healthy individuals.



Figure 3. Alignment of the deduced sequence of wolf-herring parvalbumin with parvalbumin from other fish species. Chi.D, *Chirocentrus dorab* or Wolf-herring; Cyp.C, *Cyprinus carpio* or carp (Accession No.: CAC83659, GI:17977827); Car.A, *Carassius auratus* or gold fish (Accession No. AET79255, GI:379998731); Dan.R, *Danio rerio* or zebra fish (Accession No.:CAK10745,GI:94733410); Sin.C, *Siniperca chuatsi* or Chinese perch (Accession No.: ACM07328, GI:221158262); Hyp.M, *Hypophthalmichthys molitrix* or silver carp (Accession No.: ACI95745, GI:209902357); Cam.C, *Campylomormyrus compressirostris* or elephant fish (Accession No.: AHI42560, GI:582040284). Red and yellow areas represent α -helices and β -strands, respectively, and the blue circles represent calcium-binding sites.

with recombinant parvalbumin on Western blots (Figure 2, lane P), but pooled sera from healthy individuals did not show significant reactivity (Figure 2, lane N).

As **Figure 4** (column 1) shows, sera from fish-sensitive patients contained specific IgE against purified native wolf-herring fish parvalbumin. Pre-absorption of the patients' sera with 1 or 4 μg (**Figure 4**, columns 2 and 3) of the recombinant parvalbumin significantly inhibited IgE binding to the native protein in dot blotting.

Immune sera from native parvalbumin-sensitized mice contained high titer of specific IgG which was significantly blocked after pre-absorption of the serum with recombinant parvalbumin in dot blotting.

No significant difference was observed in IgE reactivity of fish-allergic patient sera to recombinant and native parvalbumin (sera from 18 of 25 and 20 of 25 patients reacted with the 2 proteins, respectively) (Figure 5(B,C)). The mean ODs $\pm$ SDs of the wells incubated with recombinant and purified native parvalbumin were 0.520 ± 0.298 and 0.550 ± 0.309 , respectively. Fewer crude extract-coated disks reacted with fish-allergic patients' sera (14 out of 25) than with recombinant or native parvalbumin (Figure 5(A)). The mean OD in this experiment was 0.341 ± 0.280 . The mean ODs obtained with healthy individuals' sera for recombinant parvalbumin, native parvalbumin and crude extract-coated disks were 0.060 ± 0.036 , 0.082 ± 0.026 and 0.06 ± 0.013 , respectively. Overall, the mean ODs of the recombinant and native parvalbumin-coated disks were higher than those of the crude extract-coated ones (Figure 5).

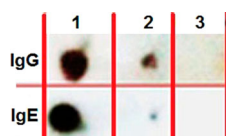


Figure 4. IgG- and IgE-binding and inhibition assays with native and recombinant parvalbumin. The upper row demonstrates serum IgG reactivity of parvalbumin-sensitized mice with native parvalbumin, while, the bottom row shows IgE reactivity of fish-allergic patients' sera with native protein. Column 1 represents the immunoreactivity of the mentioned sera with native protein, while, column 2 shows inhibition assay after pre-absorption of each serum sample with 1 μ g of recombinant parvalbumin. Column 3 depicts a similar inhibition assay after pre-absorption of the sera with 4 μ g of recombinant parvalbumin. Comparison of the columns point out to concentration dependency of the inhibition assay.

IgE reactivity was significantly greater with calcium-bound (CaCl_2 -treated) parvalbumin than with the calcium-depleted (EDTA-treated) protein (Figure 6). The mean ODs for the calcium- and EDTA-treated parvalbumins were 0.63 ± 0.29 and 0.30 ± 0.24 , respectively. These treatments had no significant effects on IgG reactivity (not shown).

4. Discussion

In this study, we cloned wolf-herring parvalbumin in pET28a and expressed the recombinant protein in *E. coli* BL21 cells. The recombinant allergen was purified by metal affinity chromatography and compared with the native protein. The bacterial expression system yielded approximately 2 mg of recombinant parvalbumin per 100 ml of the bacterial culture, which was sufficient for our experimental procedures. Western blots revealed that the expressed parvalbumin possesses measureable IgE-binding capacity and could be applied in diagnostic procedures. Inhibition disk ELISAs showed similar IgE- and IgG binding of the recombinant and native parvalbumin with human and immunized

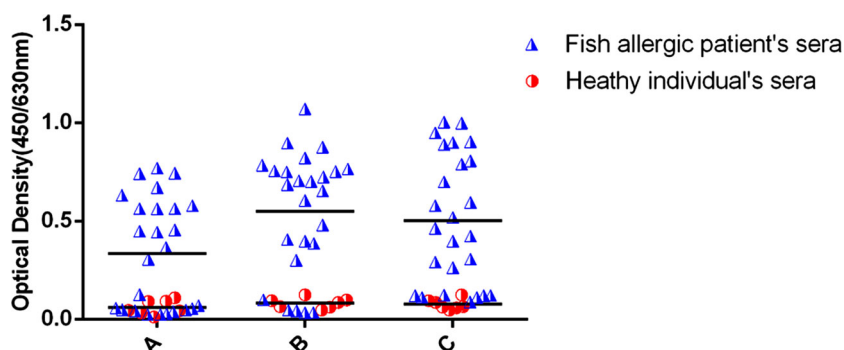


Figure 5. Comparison of IgE-reactivities of wolf-herring crude extract, native and recombinant parvalbumins. Nitrocellulose disks were coated with crude extract, purified native parvalbumin and recombinant parvalbumin and used for comparison of the IgE reactivity of fish-sensitive patients' and controls' sera. (A) Wolf-herring fish extract-coated disk, (B) native parvalbumin-coated disk and (C) recombinant parvalbumin-coated disk. The upper lines represent the mean optical density (OD) of patients' samples versus bottom lines which refer to healthy individuals.

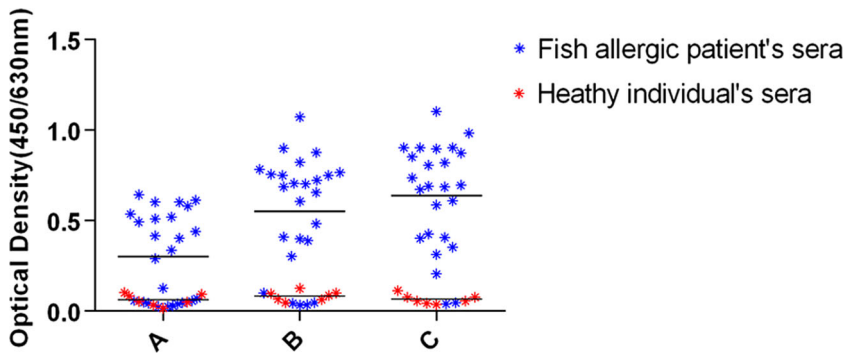


Figure 6. Effect of calcium on IgE reactivity of wolf-herring parvalbumin in disk ELISA. Nitrocellulose disks were coated with different preparation of wolf-herring parvalbumin and their IgE reactivity with fish-sensitive patients was compared with that of healthy controls. (A) Disks were coated with EDTA-treated parvalbumin; (B) disks were coated with native parvalbumin and (C) disks were coated with calcium-treated parvalbumin. The upper lines represent the mean optical density (OD) of patients' samples versus bottom lines which refer to healthy individuals.

mice sera, respectively. This finding indicates maintenance of IgE and IgG epitopes in the recombinant protein. Parvalbumin is a calcium-binding protein, and it was demonstrated that calcium binding may induce a conformational change in its structure (Bugajska-Schretter et al., 1998, 2000); therefore, we evaluated the effects of calcium on IgE- and IgG binding of the purified native and recombinant proteins. Both calcium-depleted proteins were less IgE-reactive than the Ca^{2+} -containing forms, but these treatments had no effect on IgG binding. This finding highlights the key role of conformational epitopes in the allergenicity of parvalbumin, as previously reported by Bugajska-Schretter et al. (1998, 2000). Of course, validation of the findings in the present study needs further biophysical evaluation of the folding and structure of the protein prior and after calcium binding steps. Meanwhile, comparable IgE reactivity of the recombinant and native forms suggest that some post-translational modifications, such as glycosylation, do not contribute to parvalbumin allergenicity. Similar findings were also reported for other allergens (Falak et al., 2014; Rosmilah, Shahnaz, Masita, Noormalin, & Jamaludin, 2005). Therefore, expression in prokaryotic systems, which usually do not provide glycosylation, did not abolish IgE binding of wolf-herring parvalbumin. This finding is consistent with previous studies conducted by Hamada et al. (2004) and Swoboda et al. (2002), which confirmed the IgE-binding capacity of *E. coli*-expressed parvalbumin of Pacific mackerel and carp, respectively. We found that the deduced amino acid sequence of wolf-herring parvalbumin shared 94.5% identity with *C. carpio* (Carp) parvalbumin. According to reports of Swoboda et al. (2002), recombinant carp parvalbumin (Cyp c1) is considered as the main cross-reactive fish allergen and could be used for diagnosis and immunotherapy of fish allergy. Based on our *in silico* studies, wolf-herring and carp parvalbumin are highly similar. Therefore, it seems that due to high similarity of the recombinant wolf-herring parvalbumin to Cyp c1, this protein, as a major cross-reactive fish allergen, could be another substitute in diagnosis and therapy of fish allergy. Therefore, similar to recent studies on development of the mutated forms of Cyp c1 that lead the allergists to successful production of the hypoallergenic form of this allergen (Zuidmeer-Jongejan

et al., 2015) and promising application of this protein in clinical trials (Zuidmeer-Jongejan et al., 2012), we believe that efforts in generation of the hypoallergenic form of this protein could be useful. However, because parvalbumins from other sources were not available during the experiments, we did not measure its cross-reactivity with parvalbumins from other species. Therefore, further characterization is still necessary to confirm that the wolf-herring parvalbumin could be a suitable substitute for the well-known Cyp c1 molecule.

Disk ELISA results demonstrated that crude extract was bound by IgE from fewer of fish-allergic patients than purified parvalbumin. This difference could be due to better management of the major allergen content and parvalbumin enrichment on recombinant and native parvalbumin-coated disks and the purity of the contents, as well as lack of non-allergic substances in recombinant and native parvalbumin-coated disks. Food allergens are usually categorized as thermoresistant or thermolabile. It was demonstrated that parvalbumin is the exclusive allergen in cooked fish, due to its thermoresistance (Arif & Hasnain, 2010; Arif, Jabeen, & Hasnain, 2007; Lewit-Bentley & Réty, 2000; Rosmilah et al., 2005, 2013). Therefore, due to the absence of thermolabile allergens and the presence of parvalbumin as the major allergen in cooked fish, purification of parvalbumin from heat-processed fish or production of its recombinant form via molecular genetic techniques can be a forward step for efficient diagnosis and safe immunotherapy of cooked fish-sensitive patients. The variability of allergenic extracts, which are routinely employed in IgE-specific *in vitro* and *in vivo* assays, is usually due to allergen extraction methods, manufacturing processes and storage conditions (Abedini et al., 2011). These differences may lead to inconsistent outcomes of the described analyses (Jeong, Hong, Lee, & Park, 2011). Expression and purification of recombinant proteins may help us to overcome these problems. However, in patients who are sensitive to minor allergens, this approach could be problematic (Jeong et al., 2011). In such patients, application of crude extracts may be acceptable.

5. Conclusion

The present study was designed to study IgE binding of *E. coli*-expressed parvalbumin. The following conclusions can be drawn from the present study: (1) the *E. coli*-expressed parvalbumin retained proper folding and allergenicity characteristics, (2) calcium binding has a key role in the formation of IgE-specific conformational epitope, (3) due to high amino acid sequence similarity of wolf-herring fish and *C. carpio* (Carp) parvalbumin, it seems that the former could be used as tool in diagnosis and therapy of fish allergy and (4) the mean ODs of recombinant and native wolf-herring parvalbumin were greater than that of crude extract. Therefore, application of purified proteins may facilitate and improve the diagnosis and immunotherapy of fish allergy.

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Disclosure statement

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