RESEARCH ARTICLE

Epitope mapping of the major allergen from Atlantic cod in Spanish population reveals different IgE-binding patterns

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Scope: IgE-epitope mapping of allergens reveal important information about antigen components involved in allergic reactions. The peptide-based microarray immunoassay has been used to map epitopes of some food allergens. We developed a peptide microarray immunoassay to map allergenic epitopes in parvalbumin from Atlantic cod (Gad m 1), the most consumed cod species in Spain.

Methods and results: Sera from 13 fish-allergic patients with specific IgE to cod parvalbumin were used. A library of overlapping peptides was synthesized, representing the primary sequence of Gad m 1. Peptides were used to analyze allergen-specific IgE antibodies in patient sera. 100% of the patients recognized one antigenic region of 15 amino acids in length in Gad m 1. This region only partially correlated with one of the three antigenic determinants of Gad c 1 (Allergen M), parvalbumin from Baltic cod (*Gadus callarias*). In the 3D model of the protein, this region was located on the surface of the protein.

Conclusion: We have identified a relevant antigenic region in Gad m 1. This epitope could be considered as a severity marker and provides additional information to improve fish allergy diagnosis and the design of safe immunotherapeutic tools.

Keywords:
Cod parvalbumin / Epitope mapping / Fish allergy / Food allergy / Peptide microarray

1 Introduction

Food allergies have been increasing in prevalence over the last ten years. Fish and its derived products play an important role in human nutrition [1]. They are not only an important source of dietary proteins, but also a common cause of food allergies in fish-eating populations. Together with milk, egg, peanuts, tree nuts, and shellfish, fish are among the most important allergen sources causing IgE-mediated food hypersensitivity [2].

It is reported that 22% of food-hypersensitive patients in Spain react to fish [3]. In Spain the most consumed fish species are those whose lipid content in the flesh is less than 2%, known as flatfish, such as hake (*Merluccius merluccius*), whiff (*Lepidorhombus whiffiagonis*), and sole (*Solea solea*). However, there are some other species commonly consumed elsewhere that are increasingly consumed in Spain. This is the case for Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*), which are well known abroad and are increasingly used in Spanish cuisine, leading to new food-allergic sources among the Spanish population. Subjects with fish

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Abbreviation: RT, room temperature

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allergy are usually allergic to multiple fish species. The major fish allergen is parvalbumin, a 12 kDa soluble acidic protein. It belongs to the second largest animal food allergen family, the EF-hand family [4] and comprises three regions termed AB, CD, and EF; both CD and EF regions can bind two divalent cations (calcium and/or magnesium), whereas the AB region is thought to regulate the binding affinity of the two active sites [5–8]. Fish parvalbumins represent the second largest animal food allergen family, the largest being the tropomyosins of crustaceans and molluscs [9]. The binding of the calcium ligand was found to be necessary for maintaining parvalbumin's conformation suitable to bind IgE. Loss of calcium results in a change in conformation together with an associated loss of the protein's IgE-binding capacity [10, 11]. The first and best characterized fish parvalbumin is Gad c I, from Baltic cod (*Gadus callarias*) formerly named allergen M [12–15]. It is considered as a fish and amphibian pan-allergen and its main epitopes involved in the allergic reaction were identified by limited and selective tryptic hydrolyses [16]. Mimotopes generated by phage display technique have also been used for the identification of conformational epitopes in this same protein [17]. Atlantic cod is more frequently consumed in our environment and its parvalbumin, Gad m 1, was sequenced in 2002 [18]. Large homology was found with other fish parvalbumins, such as Sal s 1, parvalbumin from Atlantic salmon (75% sequence identity), or Cyp c 1, parvalbumin from carp (77.1% identity) [19]. Remarkably, 62.3% sequence identity was shown between Gad m 1 and Gad c 1, [18] even though they are both *Gadus* species.

The IgE epitope mapping of allergens might reveal relevant information about antigen structure, the patient's immune response, and fundamental data for designing safer hypoallergenic immunotherapeutics. Moreover, recent studies have described IgE-binding epitopes as biomarkers of food allergies [20, 21], such as fish allergy [22].

We sought to develop a peptide microarray-based immunoassay to identify allergenic epitopes in Gad m 1 and compared them to those previously identified by other techniques in Gad c 1 to characterize a specific pattern of IgE-binding epitopes.

2 Materials and methods

2.1 Patient sera

Sera from 13 fish-allergic patients (3 adults, 10 children) from Fundación Jiménez Díaz Hospital and Niño Jesús University Hospital (Madrid, Spain) with specific IgE antibodies to cod parvalbumin were selected. Allergy diagnosis was based on the criteria described by Wood RA [23]. All patients had a history of symptoms elicited by eating fish, with positive skin prick tests to fish extracts [24, 25]. Specific IgE antibodies to fish were determined by CAP-System FEIA™ (Pharmacia Diagnostics AB, Uppsala, Sweden). Sera from 12 healthy volunteers with negative case histories for any type-I allergy were included as controls. The study was approved by the Hospital Ethics Committee and all patients gave written consent to participate in the study.

2.2 Purification of parvalbumins

Purification of parvalbumins was carried out as previously described [26] Briefly, raw fish muscle from cod (10 g) was homogenized under liquid nitrogen, dissolved in 80 mL of PBS containing PMSF 1 mM and extracted overnight at 4°C at constant stirring. Total muscle extract was boiled for 30 min and precipitated proteins were removed by centrifugation for 10 min at 4°C. Parvalbumin was enriched from supernatant by ammonium sulfate precipitation of contaminating proteins. Ammonium sulfate was removed by dialysis to water and the parvalbumin-enriched fraction was lyophilized, redissolved in Tris 10 mM, pH 7.5 and applied to a MonoQ column (MonoQ 5/50 GL, GE Healthcare, Uppsala, Sweden) in an ÄKTA System (GE Healthcare). Fractions containing purified parvalbumin isoforms were eluted with a linear salt gradient from 0 to 1 M NaCl.

2.3 IgE ELISA

For IgE ELISA experiment, 96 well flat bottom plates were used (Immunolon 4HBX, Thermo). Plates were coated in duplicate overnight at 4°C with 10 μg/mL of purified cod parvalbumin diluted in coating buffer (50 mM) carbonate bicarbonate buffer pH = 9.6). Plates were blocked with PBS containing 0.05% Tween-20 and 2% BSA for 1 h at room temperature (RT). After blocking, they were washed three times with PBS containing 0.05% Tween-20 before incubation for 2 h at RT with patients' sera diluted 1/5 in blocking buffer. The plates were washed again and incubated with the secondary antibody (Goat anti Human IgE/PO, Nordic Immunology Co. Ltd., Tilburg, The Netherlands) diluted 1/5000 with 2% BSA, 0.05% PBS-T for 1 h at RT. IgE reactivity was detected by addition of 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Fisher Scientific Inc., IL, USA) and measured at 620 nm. Sera from five nonatopic patients were used as negative controls and the median of their optical density (OD) values was used to establish the cutoff value. Parvalbumin sensitization was considered positive when patient's OD at 620 nm was two times higher than negative control's OD at the same absorbance.

For ELISA inhibition assays, a sera pool from allergic patients was preincubated for 4 h at RT with 100 μg of inhibitor per milliliter of serum. Inhibitors ("clipped" C-terminal peptides) were commercially synthesized by Biomedal S.L. (Sevilla, Spain).

Table 1. Summary of clinical histories and laboratory data

Patient no	Age (years)	Sex	Symptoms	Specific IgE CAP FEIA, kU/L[a)]				Sensitization to parvalbumins[b)](OD $\geq$ 0.2)				Reactive history to other fish species
				codfish	Salmon	Whiff	Sole	Gad m 1	Sal s 1	Whiff	Sole	
1	19	M	U, AE	0,4	<0,35	<0,35	<0,35	0,23	<0,2	<0,2	<0,2	Anchovy and tuna
2	7	M	U	0,35	1,75	<0,35	<0,35	0,48	0,24	<0,2	<0,2	-
3	14	M	U, AE	0,94	0,47	1,05	0,5	0,40	<0,2	<0,2	<0,2	Hake, conger, sardine, tuna
4	7	M	U	0,48	<0,35	<0,35	<0,35	0,39	<0,2	<0,2	<0,2	Sardine and sole
5	38	F	OAS	60,8	61,5	54,9	43,7	1,10	1,07	1,93	0,77	Sole
6	7	F	U, OAS	6,14	3	1,92	2,46	0,34	0,23	0,23	0,41	Sole, tuna, and whiff
7	15	M	AE, OAS	11,3	6,42	8,69	6,17	0,21	<0,2	0,44	<0,2	Tuna
8	19	M	A, AE, OAS, GIS	6,35	6,83	7,28	6,7	0,36	0,63	0,68	0,36	Anchovy, hake, and sardine
9	9	M	OAS	6,85	5,35	5,95	3,83	0,52	0,23	0,74	0,36	Anchovy, hake, and whiff
10	14	M	U, OAS, GIS	13,8	7,85	10,6	7,85	0,38	0,32	0,78	0,42	Hake and sole
11	5	F	U	0,42	<0,35	0,87	0,46	0,32	<0.2	0,36	0,23	Whiff
12	15	M	A, AE, GIS	90,6	95,6	78,8	47,3	0,50	0,54	1,15	0,60	Anchovy, hake, salmon, sardine, sole, swordfish, trout, and whiff
13	13	M	U, A, AE, GIS	32,5	25,9	19,9	21,5	0,88	0,87	1,97	0,83	Sardine, sole, tuna, and whiff

a) Specific IgE CAP-FEIA expressed in kU/L. All of the patients have IgE-mediated allergy.
b) IgE reactivity was considered positive when OD 620nm $\geq$ 2$\times$OD of negative controls (0.2).
A, asthma; AE, angioedema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; U, urticaria.

2.4 Microarray

2.4.1 Peptides and immunolabelling

A library of overlapping peptides, consisting of 15 amino acids with an offset of three, corresponding to the primary sequence of Gad m 1 was commercially synthesized using PepStar® technique by JPT Peptide Technologies (Berlin, Germany) at a concentration of 2 mM. Peptides were diluted (1:2) with Protein Printing Buffer (TeleChem International, Inc., Sunnyvale, CA, USA), and printed in two sets of triplicates onto SuperEpoxy glass slides (TeleChem International, Inc.) using the NanoPrint™ Microarrayer 60 (TeleChem International, Inc.).

In addition, printed arrays included: Protein Printing Buffer alone as spot negative control for background normalization and fluorochrome-labelled (GC-BSA) as reference for the grid alignment (positional grid controls).

Immunolabelling was performed as previously described [27–30], with some modifications [22].

2.5 Data analysis

Data analysis was performed as previously described [22, 30]. Briefly, we considered a region as a major allergenic epitope when at least two overlapping peptides were involved, peptides were bound by antibodies greater than 50% of patients with fish allergy and peptides were significantly recognized in patients and not in control individuals ($p < 0.01$).

2.6 3D structural modeling

Structure of cod parvalbumin was generated by homology modeling using the crystal structure of carp parvalbumin with a pI of 4.25 (data base entry code 4cpvA) as template. The Swiss-Model server (http://swissmodel.expasy.org) was used to create the 3D model [31–33] and POV-Ray program to improve images.

2.7 Peptide microarray inhibition experiment

A peptide inhibition assay was carried out in which the array was immunolabelled as described earlier except that the serum pool (25 μL diluted 1:10 in PBS-T/HAS) was preincubated with 1 μL of peptide at a concentration of 1 mM (1 peptide per array) for 2 h at RT and gentle agitation. Serum pool without peptide was processed in parallel under the same conditions as a control.

3 Results

Commercially synthesized peptides of 15 amino acids in length with an offset of three, corresponding to the sequence

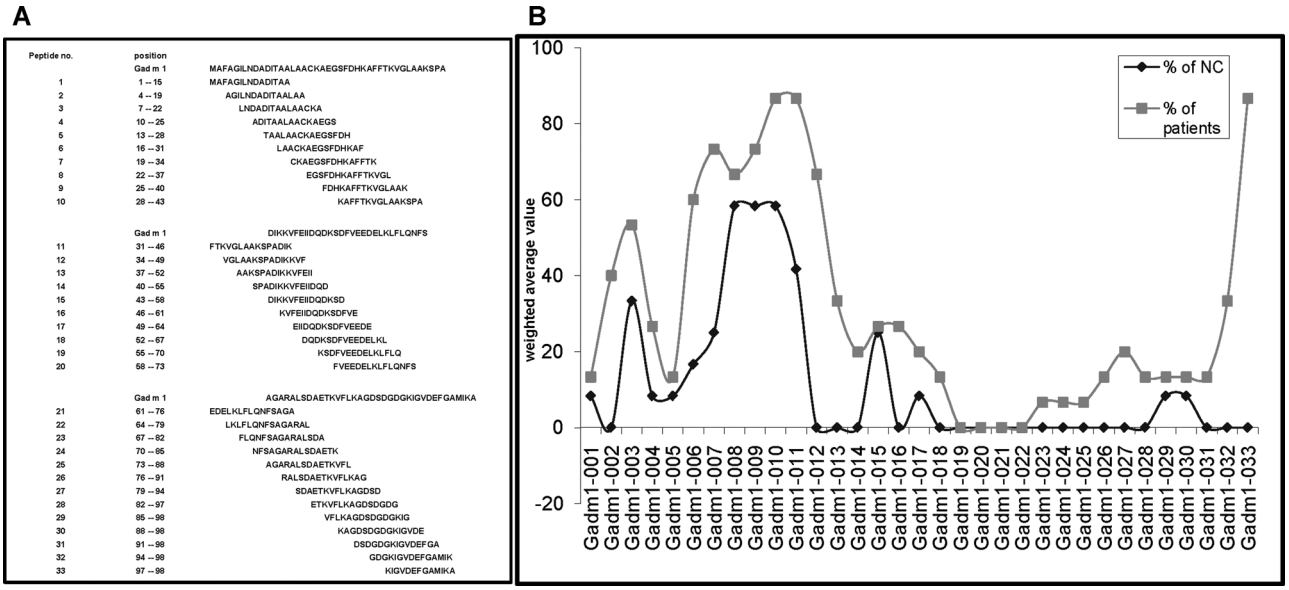

Figure 1. (A) Amino acid sequences of the 33 overlapping, synthetic peptides (length: 15 amino acid, offset: 3 amino acids) used for the identification of IgE-binding regions in Atlantic cod parvalbumin, Gad m 1. (B) Comparison between percentage of patients (grey line) and negative controls (black line) that recognized each overlapping peptide in Gad m 1.

of Gad m 1, were robotically arrayed in two sets of triplicate and site-specifically attached to epoxide derivatized glass slides. Thirty-three peptides were assayed in parallel with sera from 13 patients allergic to fish and 12 nonallergic controls. All of the patients included in the study were diagnosed with fish allergy, experienced at least one of the typical clinical symptoms (urticaria, angioedema, diarrhea, asthma, anaphylactic reaction) after contact with fish proteins and had specific IgE antibodies to purify cod parvalbumin. The median (Q_1 – Q_3) quantity of specific IgE antibodies to cod extract determined by CAP-FEIA was 6.49 kU_A/L (0.46 – 18.47) and for cod parvalbumin, determined by IgE ELISA was 0.46 kU_A/L (0.37 – 0.59). Data are summarized in Table 1.

For binding comparison between patients and control subjects to each overlapping peptide of the protein, the weighted average Z value was calculated as $Z = 0.25 \times Z_{-1} + 0.5 \times Z_{0+}0.25 \times Z_{+1}$, where Z_{-1}, Z_0, and Z_{+1} are the Z-values of the previous and following peptides. This formula could be used because peptides were overlapped by 12 amino acids, which means overlapping peptides might share the same epitopes. Subsequent analysis is carried out based on the weighted average index. For comparison of Z scores between patients and control groups, a Wilcoxon test was applied ($p < 0.01$).

Several peptides were recognized by most of the patients, the number of positive IgE peptides per individual ranged from 4 to 16 (median 10), but peptides representing a significant difference in pattern recognition between patients and controls were only two, peptides 32 and 33 (AAs 94–109), corresponding to the last 16 amino acids of the primary sequence of the protein: GDGKIGVDEFGAMIKA (Fig. 1). Peptide 32 was recognized by 5/13 patients and peptide 33 by 13/13 patients (see Table 2). To confirm the sequence specificity of the IgE signal, pooled sera were incubated for 2 h with individual peptide prior to immunolabelling, as described earlier. A peptide from the region of interest was chosen for inhibition studies (peptide #33). A 55% and 30% inhibition of signal was seen in peptides #32 and 33, respectively. Signal from overlapping peptides was attenuated using single peptides as inhibitors confirming this region as a specific IgE-binding site. Therefore, an epitope could be identified in the C-terminal region of Gad m 1 protein.

Table 2. IgE-binding epitopes identified and patient's symptoms

Patient no.	Symptoms	Peptide 32	Peptide 33	Peptides recognized by patient
1	U, AE	–	+	5
2	U	–	+	11
3	U, AE	–	+	8
4	U	+	+	10
5	OAS	–	+	6
6	U, OAS	–	+	4
7	AE, OAS	+	+	11
8	A, AE, OAS, GIS	–	+	9
9	OAS	–	+	12
10	U, OAS, GIS	+	+	16
11	U	–	+	10
12	A, AE, GIS	+	+	10
13	U, A, AE, GIS	+	+	12

A, asthma; AE, angioedema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; U, urticaria.

The C-terminal epitope (peptides 32–33) belongs to a region in Gad m 1 described as the calcium binding EF motif. To assess the role of Ca^{2+} on the IgE-binding capacity

Table 3. ELISA inhibition assay to evaluate essential residues involved in IgE recognition; Percentage of inhibition using C-terminal clipped peptides

Inhibitor	Sequence	% Inhibition
Peptide 98–109	^{98}IGVDEFGAMIKA^{109}	26.3
Peptide 100–109	^{100}VDEFGAMIKA^{109}	35.5
Peptide 102–109	^{102}EFGAMIKA^{109}	39.5
Peptide 104–109	^{104}GAMIKA^{109}	28.3
Cod parvalbumin		97.7
BSA		0

of peptides 25–33, IgE-binding assays were performed in the presence and absence of calcium (Ca^{2+}-containing buffer and EGTA-containing buffer, respectively) using sera pool from two allergic patients. Experiments were performed at different Ca^{2+} and EGTA concentrations (0.5, 1, 5, and 10 mM for Ca^{2+} and 5, 10, 20, and 50 mM for EGTA) following the method described by Bugajska-Schretter et al. [11]. No differences in IgE binding were found in assays performed with Ca^{2+} containing buffer compared with EGTA.

To evaluate essential residues for IgE recognition additional "clipped" C-terminal peptides were generated to address this question and an ELISA inhibition assay was performed (Table 3). Plates were coated with 0.5 μg of purified cod parvalbumin and sera pool from allergic patients was preincubated with each peptide. Peptide 98–109 inhibited around 26%. In peptides 100–109 and 102–109 inhibition increased to 35% and 39%, respectively and decreased to 28% in peptide 104–109. Probably, inhibition was favored in peptide 102–109 by a better exposure of the residues essential for IgE recognition. These data confirmed residues 102 to 109 crucial for IgE recognition. It is well known that an antibody-binding site must have size enough to accommodate a peptide of 6–8 aminoacids length, which is virtually identical with the core identified in the current work.

Although differences were found around peptides 12–14 in terms of meaningful recognition between patients and controls, it was not considered an epitope because the statistical significance did not meet the criterion previously established ($p < 0.01$). Some signal associated with peptides in the *N*-terminal side of the molecule (peptides 8–10) was seen in both allergic individuals and negative controls. Nonspecific binding artifact was suspected. The nonspecificity of these signals was confirmed by immunolabelling substituting patient's sera with blocking solution. Without serum incubation, fluorescence was absent in the C-terminal region of the molecule, but was still present in the *N*-terminus.

None of adult patients (aged over 14) recognized to peptide 32 (Table 2), although only three cases have been included, and recognized a smaller amount of peptides than children (aged under 14) (Table 2). Fish-allergic children recognized a greater number of peptides than controls and a correlation was seen between symptom severity and the number of peptides recognized by each patient: patients that recognized 10 IgE-binding peptides, including peptide 32, had a tendency for the most severe reactions. In summary, peptide 32 binding and the number of IgE-binding peptides should serve as severity marker.

4 Discussion

Better knowledge about the IgE reactive sites is essential for the design of safe immunotherapy. The role of IgE epitope mapping has been widely described [34, 35] and the potential to become an important tool for the diagnosis and treatment of food allergy.

There are no studies describing the main regions involved in allergic reactions to Atlantic cod. In contrast, Gad c 1, parvalbumin from Baltic cod (*Gadus callarias*) has been extensively studied and three allergenic regions were described with tryptic hydrolysates of the protein using Nordic patients [16]. The immunologically reactive sites were located on the junction between the AB and CD domains (AAs 33–44), on the junction between CD and EF domains (AAs 65–74) and on the calcium-binding loop of the EF domain (AAs 88–96). These results were partially confirmed in 2006 by Untermayrs et al. [17] with computational mimotopes matching using the 3D model of carp parvalbumin. Furthermore, in this study they suggested that all three regions together may be involved in the antigen–antibody interaction. In our study, we have identified one unique reactive site in Gad m 1, recognized by 100% of patients, located at the end of the primary sequence of the protein: AAs 95 to 109, corresponding to the EF domain. This region, where the second Ca^{2+}-binding loop of the protein is located, was previously confirmed to be an immunogenic area in other parvalbumins [17, 36] and it has also been confirmed that Ca^{2+} depletion results in a reduced IgE-binding capacity of parvalbumin [11, 17], which may be ascribed to dramatic conformational changes induced upon Ca^{2+} binding [37]. Many proteins able to bind Ca^{2+} ion with high affinity, contain a calcium-binding site consisting of a helix-loop-helix motif called EF hand. This motif has a characteristic 3D structure in which several residues (particularly Asp and Glu) located in specific positions within the loop, play a key role coordinating Ca^{2+} ions [38]. Calcium lost results in a change in conformation and decrease the protein's IgE-binding capacity. But this conformation-dependent motif is lost in the linear sequence of peptide described in this work and thus, the presence or absence of Ca^{2+} is irrelevant in the IgE-binding of those peptides.

In order to support the identification of a major IgE-binding region in Gad m 1, an inhibition assay was performed. It was confirmed that preincubation of patients' sera with a peptide selected from the area of interest showed a clear inhibition of IgE binding to that peptide, and due to sequence overlap, inhibition of the adjacent peptide. Furthermore, the region identified as major epitope in Gad m 1 is adjacent to the third antibody-binding site described in Gad c 1 [11] (Fig. 2, panel A). This third matching site in the EF domain area has identical sequence in Gad m 1, Gad c 1, and

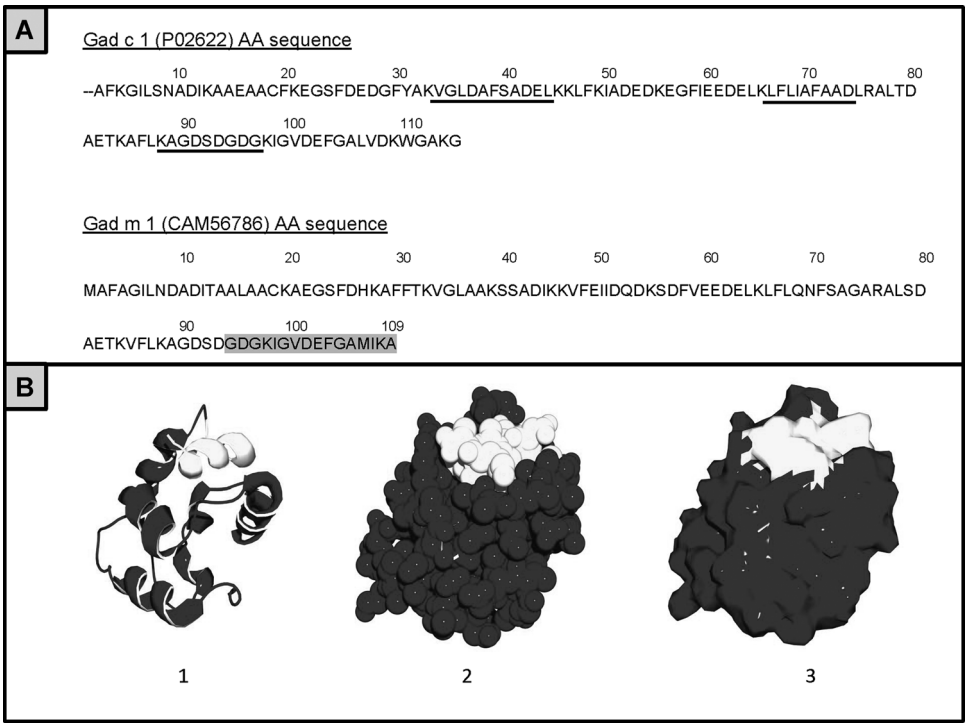

Figure 2. (A) AA sequence of Baltic cod (Gad c 1) and Atlantic cod (Gad m 1) parvalbumins. IgE-binding epitopes identified by peptide-based microarray immunoassay are highlighted in grey. Those reported previously are underlined. (B) Ribbon (1) and molecular surface (2–3) diagrams of carp parvalbumin used as model (4cpvA) to represent main epitopes found in Gad m 1 (highlighted in white: residues 95–109).

Cyp c 1 molecules, strengthening the relevance of this area as an allergenic epitope region.

We suggest that this allergenic region identified in Gad m 1 might be considered as a reactive epitope. A single lineal IgE-binding epitope was identified in Gad m 1. Complete allergens must have at least two IgE-binding epitopes in order to cross-link IgE antibodies on the surface of mast cell. In the manuscript, we have studied linear epitopes, but presumably there would be more conformational epitopes involved in IgE binding.

Differences observed between Gad c 1 and Gad m 1 epitopes may be due to several factors. Gad m 1 and Gad c 1 are very close phylogenetically, however they share only 62.3% sequence identity. Remarkably, identity was higher (75%) with *Salmo salar* parvalbumin (Sal s 1). Das Dores and colleagues [18] suggested that Gad m 1 and Sal s 1 might be encoded by the same gene in two different species and that Gad m 1 and Gad c 1 correspond to two distinct genes which may be present in both Gadus species.

Furthermore, different techniques used for epitope identification might also explain the differences in epitope recognition. In the case of Gad c 1, epitopes were identified by spot membrane technique from tryptic hydrolysates of the protein, where cleavage is made between lysine and arginine amino acids [39]. This is a limitation when trying to identify new epitopes because lysine and arginine could be located in the middle of the sequence of a putative epitope, and trypsine would cleave between them, rendering the region unable to bind IgE. In this sense, very important information can be lost and an allergenic region may not be considered as allergenic epitope with IgE-binding capacity. In the case of Gad m 1, synthetic peptides were designed taking into consideration 15 amino acid length with an overlap of 12 and therefore the concern about trypsin cleavage is avoided. Also, determination of allergens IgE-binding epitopes using the microarray technique offer several advantages over other techniques such as phage display. The results obtained from the microarray immunoassays were found to be very consistent and the high throughput platform of this system allows screening of thousands of target peptides in parallel using microliter quantities of serum, greatly reducing the biological sample cost of individual assays.

Based on structural data of parvalbumins, and their high sequence homology with carp parvalbumin (77.1% identity shared with Gad m 1), and taking into consideration that carp parvalbumin has been examined by X-ray diffraction and the tertiary structure was completely resolved [19], it was used as a template to see where the epitope identified in cod parvalbumin was located in the 3D model of the molecule (Fig. 2, panel B). When looking at the 3D model of the protein we could see that the antigenic region identified is located in the periphery of the protein, as is expected.

An important application of IgE epitope mapping is the search for peptides as biomarkers of persistence or severity. In the case of salmon parvalbumin, a peptide related to the severity in patients allergic to fish has been described [22]. In the case of Gad m1, cod-allergic children who recognized ten or more peptides, including peptide 32, showed a greater number of symptoms, some of them serious (Table 2). Therefore this peptide can be considered as a biomarker of

severity in parvalbumin-mediated allergic reactions to fish in the Spanish population.

In conclusion, this is the first time that IgE B-cell epitopes of Atlantic cod parvalbumin have been studied. We have identified an antigenic determinant of 16 amino acids in length in Gad m 1, confirmed by inhibition assay, which should be considered as major epitope of the protein.

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