

Article

# Animal food allergens share the formation of IgE-binding amyloids

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**Abstract:** Several animal food allergens assembly into amyloids under gastric-like environments. These aggregated structures provide Gad m 1 with an enhanced IgE interaction due to the amyloid assembly of the epitope regions. However, whether these properties are unique of Gad m 1 or common to other food allergens has not yet been addressed. Using Bos d 5, Bos d 12 and Gal d 2 as food allergen models and Gad m 1 as control, aggregation reactions and the sera of milk, egg and fish allergic patients we have analyzed the IgE interaction of the distinct amyloids. We found that amyloids formed by Bos d 12 and Gal d 2 full-length and truncated chains are recognized by the IgEs of milk and egg allergic patient sera. As with Gad m 1, in most cases amyloid recognition is higher than that of the precursor structure. Bos d 5 was not recognized under any fold by the IgE of the sera studied. These results support that formation of IgE-binding amyloids might be a common feature to animal food allergen.

**Keywords:** food allergens; egg allergy, milk allergy, fish allergy, amyloids, IgE-binding.

## 1. Introduction

More than 5 % of the population in industrialized countries suffer from Type I food allergy, an immunoglobulin E (IgE)-mediated hypersensitivity disease resulting from the loss of oral tolerance to food proteins [1-4]. A limited number of foods are responsible for the majority of reactions, being 95% of all food allergies caused by only eight foods including eggs, milk, fish, crustacean shellfish, peanut, tree nuts, wheat and soybean [3,5,6]. All these foods contain many proteins, but only a few of them are allergens [3,5,6]. Known food type I allergens have in general molecular weights below 70 kDa, are stable molecules that resist cooking and digestion, and stimulate the immune response inducing the production of allergen-specific IgE [3,5]. Regardless of the limited number of represented native structural scaffolds, sequence difference thresholds, abundance and stability properties, the molecular signature of protein allergenicity remains yet unresolved [3,5-9].

All food proteins are exposed to pH 1.3-2 at the gastric compartment during digestion, condition that is usually used *in vitro* to trigger the refolding of proteins into amyloid aggregates [10,11]. Amyloids are insoluble fibrillary protein structural states displaying protease resistance and, with one exception, a cross- $\beta$  sheet backbone [11-13]. Amyloid formation is a generic feature of many, if not all, natural polypeptide chains if placed under the appropriate environment and concentration

[10-12]. In fact, food allergens of animal sources such as *Gadus morhua*  $\beta$ -parvalbumin (Gad m 1), bovine milk  $\beta$ -lactoglobulin (Bos d 5) and  $\kappa$ -casein (Bos d 12), and the chicken egg-white ovalbumin (Gal d 2) rapidly form amyloids under gastric-like conditions [14-17]. For Bos d 5 and Bos d 12 the formation of heterogeneous amyloids has also been reported [18].

Gad m1 amyloids displayed a reduced proteinase sensitivity and a 1,000-fold increased IgE-specific binding compared to that of the monomer precursor [14-16]. Importantly, unrelated amyloids such as those formed by the neurodegeneration relevant A $\beta$ 42 and PrP are not recognized by the IgE present in the sera of fish allergic patients indicating the sequence specificity of the recognition process [14]. A battery of approaches including peptide arrays, proteomics and the use of mutant chains concluded that regions forming the amyloid core overlapped the IgE epitopes [15,16]. However, whether these properties are specific of Gad m 1 or common to other food allergens has not yet been addressed. Therefore, in this work, we have analyzed the IgE-binding properties of Bos d 5, Bos d 12, Gal d 2 and Gad m 1 amyloids using the sera of milk, egg and fish allergic patient sera.

## 2. Materials and Methods

**2.1. Ethics statement.** All experimental protocols and methods were performed following the guidelines of the University Hospital La Paz and the Spanish National Research Council and applied with approval from the Ethics Committee of the University Hospital La Paz (protocol number PI-3065). Anonymity was preserved as established in the ethical permission.

**2.2. Food allergic patient sera.** Sera were obtained from patients recruited from the Hospital La Paz in Madrid (Spain). Fish, milk, and egg allergic patients were selected based on case history, positive skin prick test with commercial extracts and determination of specific IgE (sIgE) by ImmunoCAP 100 (Thermo Fisher, Uppsala, Sweden) according to the manufacturer instructions (Table 1).

**Table 1. Clinical features and IgE profiles of the sera of food allergic patients recruited for the study**

<sup>a</sup> A, anaphylaxis; AE, angioedema; E, eczema; OAS, oral allergy syndrome; RD, respiratory symptoms. <sup>b</sup> ImmunoCap determinations. <sup>c</sup> Bos d 5. <sup>d</sup> fraction containing Bos d 12. <sup>e</sup> Gal d 2

Patient	Ofending food	Sex (M/F)	Age (y)	Symptoms <sup>a</sup>	IgE (kU/L) <sup>b</sup>							
					Total	Milk	BLG <sup>c</sup>	Casein <sup>d</sup>	Egg White	OVA <sup>e</sup>	Hake	Cod
1	milk	F	16	OAS,AE	391	7.1	0.2	0.7	<0.4	nd	nd	nd
2	milk	M	15	A	648	22.8	2.5	23.8	0.4	0.3	nd	nd
3	milk	F	19	A	1323	354	70.2	461	nd	nd	nd	nd
4	egg	M	20	RD	850	nd	nd	nd	325	62.1	nd	nd
5	egg	F	14	E	5774	nd	0.1	0.1	3.2	3.9	nd	nd
6	egg	M	15	E, A	3017	nd	nd	nd	6.1	5.6	nd	nd
7	hake	M	35	A	124	nd	nd	nd	nd	nd	2.7	1.0
8	hake	F	18	RD	222	nd	nd	0.1	nd	0.01	17.4	nd

**2.3. In silico studies.** Bos d 5 (P02754), Bos d 12 (P02668), Gal d 2 (P01012) and Gad m 1 (A5I874) sequences were retrieved from UniProtKB. IgE binding epitopes were taken from previous SPOT-membrane and arrays assays [15,19-22]. Amyloid forming regions were predicted using the ZipperDB algorithm [23]. IgE-binding regions and amyloid forming segments were used to generate binary functions (0,1) of the polypeptide chain (residue number including the signal peptide) using Origin 2019 software.

**2.4. Food allergens.** Gal d 2 (A-2512), Bos d 5 (L3908) and Bos d 12 (C0406) were purchased from Sigma-Aldrich. Gad m 1 (A5I874) was prepared as described [19]. Before their use all proteins were extensively dialyzed at 4 °C against either 25 mM Tris, 0.1M NaCl pH 7.5 or 0.1M Gly pH 1.5 using dialysis membranes with 8 kDa pore diameter (Spectra Por). Dialyzed solutions were centrifuged at 13,500 rpm at 4 °C for 20 min to clear possible existing aggregates and the protein concentrations of the supernatants were determined with the Bradford assay.

**2.5. Amyloid fibril formation.** Protein solutions at 5-8 mg/mL solution in 0.1M Gly pH 1.5 were incubated at 90 °C for 5 h. After the heating step, all protein samples were stored at room temperature for 36 h to allow fibril maturation. Fibrils were harvested in the pellet fraction of a 4 °C centrifugation at 100,000xg for 1h and resuspended in 50 mM Gly pH 1.5. When required, mature fibrils were placed in 1.5 mL eppendorf tubes and sonicated for 15 min in a sonicating water bath.

**2.6. Circular dichroism spectroscopy.** Circular dichroism (CD) measurements were performed using a Jasco J-820 spectropolarimeter equipped with a Peltier-controlled thermostatted cell holder. Far-UV CD spectra were recorded using a 0.3 mg/mL protein concentration solution in 25 mM Gly pH 1.5 at 25 °C. Spectra were corrected for the base line contribution and analyzed as described taken 110 Da as the residue average molecular weight [14].

**2.7. Atomic Force Microscopy (AFM).** For AFM visualization, 30 µl of the aggregate solutions prepared at 2 mg/ml in 0.1M Gly pH 1.5 and diluted 15 to 30 times were absorbed onto freshly cleaved mica via 5-10 min incubation at room temperature. The surfaces were then rinsed with double-distilled water and dried. Images were obtained in Tapping mode using a JPK Nanowizard 2 microscope and HQXSC11 B (Mikromash) cantilevers (2.7 N/m force constant and 70 kHz resonance frequency). AFM analysis was performed using the free program WSxM 4.0 (Nanotec)

**2.8. PAGE-SDS.** Typically, 3 µg of native and aggregated proteins were separated by SDS-PAGE under denaturing conditions using Mini-Protean® TGX Stain-Free™ (#456-8126) gels. Bands were visualized using Coomassie Brilliant Blue R-250 (CBB) staining solution (BioRad, USA), and the images were recorded with the Molecular Imager ChemiDoc XRS-Plus (BioRad, USA).

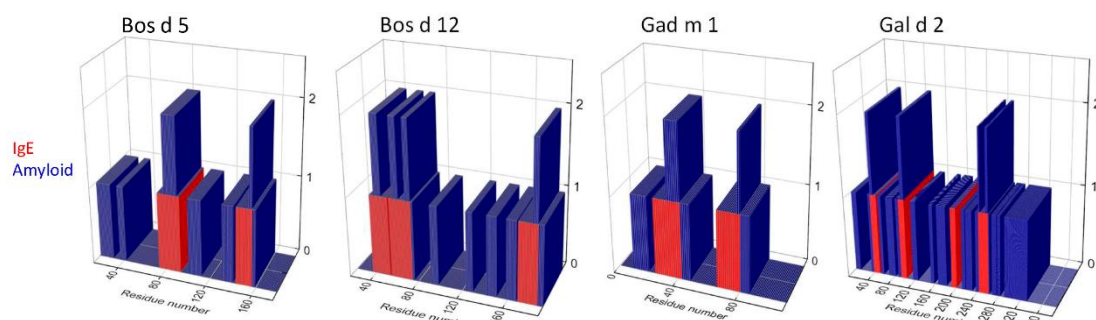
**2.9. Dot blot analysis.** Samples of 0.1-0.2 µg protein/dot were loaded in duplicate onto nitrocellulose membranes. After blocking for 1 h in TBS-T (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Tween 20) containing 0.5% bovine serum albumin (Sigma-Aldrich) at room temperature, the membranes were incubated for 2 h with either the distinct serum pools (diluted 1:10 in blocking buffer) or with the anti-amyloid OC (AB2286 Merck Millipore, 1:2000 dilution). After extensive washing with TBS-T, membranes were incubated for 1 h with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Sigma-Aldrich, 1:5000 dilution) or mouse monoclonal B3102E8 anti-human IgE (Abcam, 1:2000 dilution) and developed using Clarity Western-ECL (BioRad). The signals were recorded with the ChemiDoc XRS-Plus imager and analyzed using the volume tools provided by ImageLab software.

### 3. Results

#### 3.1. Relation between amyloid and IgE-binding and amyloid regions of milk and egg allergens.

Previous work with Gad m 1 showed a peculiar overlapping between the major IgE binding epitopes and the segments assembling into amyloid fibrils [15]. To test whether Bos d 5, Bos d 12, and Gal d 2 share this feature we used previous IgE-binding epitope determinations and the ZipperDB algorithm for predicting the amyloid forming regions [15,16,19-23]. To visualize the possible relations as flanking and/or overlapping, both IgE-binding epitopes and amyloid forming segments were used to generate binary (0/1) functions of the residue number and construct their stacked bar representation (Figure 1).

**Figure 1. Relation between IgE-binding epitopes and amyloid predicted regions in the sequences of Bos d 5, Bos d 12, Gad m 1 and Gal d 2 allergens.** IgE-binding epitopes are shown as red bars. Amyloid forming regions predicted by ZipperDB are depicted in blue stacked bars. Residue numbers correspond



to those of the precursor chains including the signal peptides.

As previously shown, the 109 amino acid chain of Gad m 1 contains two major IgE-binding epitopes which are overlapped and flanked at their C-terminus by regions predicted as amyloid cores [14-16]. These sequences are displayed in Table 2. Bos d 5, made by a 178 amino acid residues chain that undergoes the removal of the 1-16 N-terminal signal sequence, contains two major IgE-binding epitopes and six segments predicted to form amyloids. The N-terminal IgE-binding epitope is overlapped by an amyloid segment, whereas the C-terminal IgE-binding epitope is mainly flanked by aggregating segments with a partial C-terminal overlapped. However, both regions contain Cys residues which may be forming part of disulfide bonds and modify both IgE binding and amyloid formation features (Table 2). As Bos d 5, Bos d 12 is produced as a precursor chain (190 amino acids) with a N-terminal signal sequence (1-21 residues). Bos d 12 mature chain harbors three major IgE binding epitopes, being the two N-terminal epitopes overlapped by amyloid forming regions (Figure 1, Table 2). The C-terminal epitope which contains a large number of glycosylation sites, is mainly flanked by adhesive regions [24]. The 385 amino acid mature chain of Gal d 2 contains four IgE binding epitopes, three of which are overlapped by amyloid forming regions as depicted in Table 2.

**Table 2. Sequence regions with IgE binding and amyloid forming properties.**

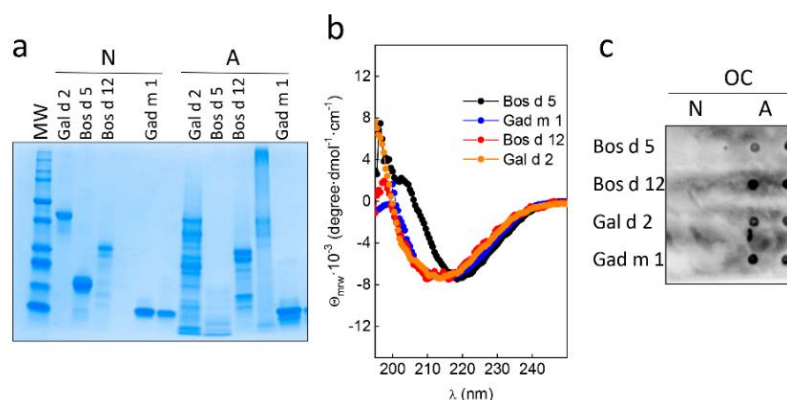
Allergen	Sequence regions <sup>a</sup>
Bos d 5	<sup>74</sup> LQKWENGECAQKKIIAEKTK <sup>93</sup> <sup>131</sup> QSLACQCLVRTPEVDDEALEKFDKALKAL <sup>159</sup>
Bos d 12	<sup>37</sup> RFFSDKIAYIPIQYVL <sup>53</sup> <sup>55</sup> RYPsyGLNYYQQKPVAlNNQF <sup>76</sup> <sup>159</sup> AVESTVATLEDSPEVIESPPEINTVQVTSTAV <sup>190</sup>
Gad m 1	<sup>25</sup> FDHKAFFTKVGLAAKSSADIKKVF <sup>48</sup> <sup>67</sup> FLQNFsAGARALSDAETKVF <sup>86</sup>
Gal d 2	<sup>39</sup> AIMSALAMVYLGAKDSTRTQINKVV <sup>68</sup> <sup>88</sup> LNQITKPNDVYsFSLAS <sup>104</sup> <sup>238</sup> GTMSMLVLLPDEVSGLEQLESINFE <sup>263</sup>

<sup>a</sup>IgE-binding epitopes are depicted with grey background; amyloid forming regions are shown in blue.

Then, this *in silico* analysis shows that the milk and egg allergen models share with the fish allergen Gad m 1 the sequence overlap of IgE binding epitopes and amyloid forming cores and suggests that their aggregates may harbor IgE-binding properties [15,16].

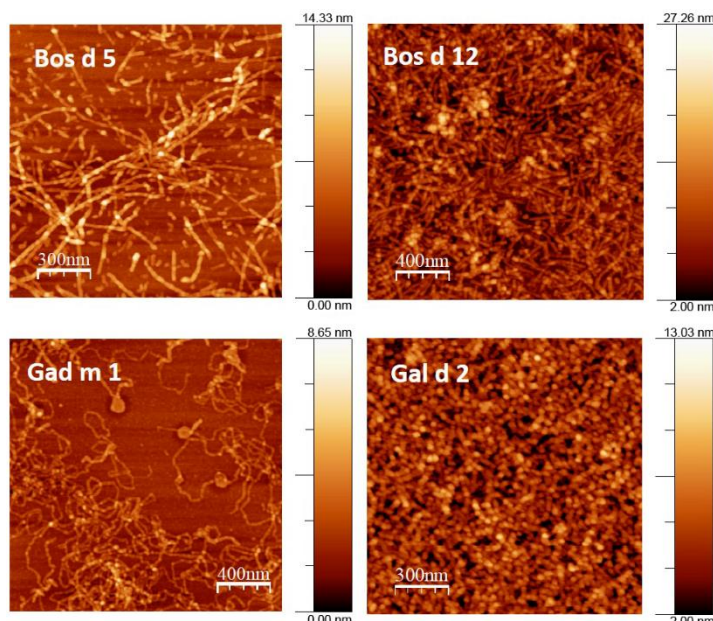
**3.2. Amyloid aggregates of milk, egg and fish allergens.** Amyloid aggregates of distinct food proteins have been formed at acid media with a variety of conditions differing in the temperature and length of incubation, buffer composition and protein concentration [17]. Aiming the balance among yield, kinetics and the general application to all proteins we choose a procedure consisting in heating at 90 °C for 5 h protein solutions prepared at 5-8 mg/mL in 0.1M Gly pH 1.5, followed by 36 h of maturation at room temperature and the isolation of the aggregates by ultracentrifugation. Since this protocol differs from that used for the preparation of Gad m 1 fibrils in previous studies, the fish allergen was also included as control.

The obtained insoluble aggregates amounted to about 10% of the native precursors and consisted in a mixture of full length and truncated chains [Figure 2a]. Their far-UV CD spectral features agree with secondary structures governed by  $\beta$ -sheet [Figure 2b]. Dot-blot assays using the anti-amyloid fibril OC antibody showed the specific recognition of the aggregates confirming their amyloid features [Figure 2c].



**Figure 2. Amyloid aggregation of the milk, egg and fish allergens.** (a) Typical analysis by SDS-PAGE and CBB staining of the distinct food allergens before (N) and after aggregation (A). Lanes of interest have been labelled with the corresponding food allergen. MW correspond to the pre-stained protein ladder with the molecular weights (bottom to top): 10, 15, 20, 25, 37, 50, 100, 150 and 250 kDa. (b) Far-UV CD spectra of Bos d 5 (black), Bos d 12 (red), Gal d 2 (green) and Gad m 1 (blue) aggregates in 25 mM Gly pH 1.5. (c) Typical dot blot membranes probed the anti-amyloid fibril OC antibody. Dots were performed in duplicates using 0.1  $\mu$ g of protein of both their native (N) and aggregated (A) states.

Despite the general amyloid features, AFM imaging showed a variety of shapes for the distinct amyloid aggregates [Figure 3]. Bos d 5 aggregates consisted in thick and linear fibrils filaments with variable length (70 nm to 1  $\mu$ m length). Bos d 12 and Gal d 2 formed fibrils of 200 nm length and 5 nm diameters with a high number of side associations. On the contrary, Gad m 1 insoluble aggregates appeared as long flexible thin curved fibrils different from the fibrillary polymers generated under acid pH in the absence of heating treatment [14].



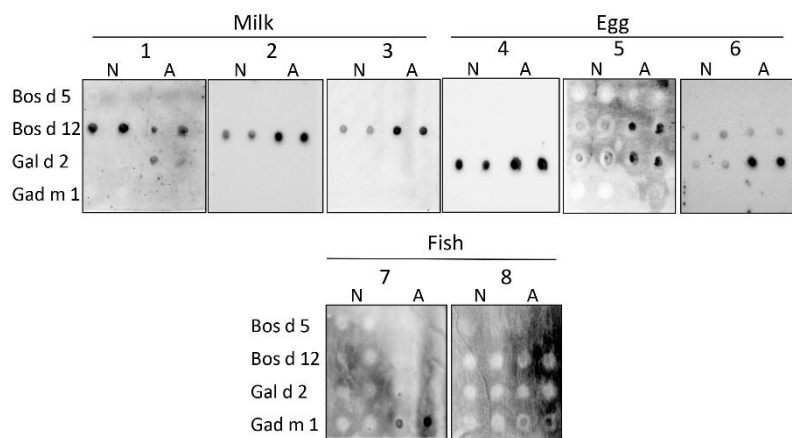
**Figure 3. AFM imaging of the insoluble aggregates formed by the milk, egg and fish allergens.** X and Y dimensions are indicated in the white bar, whereas the Z-axis color scale is shown at the right hand side of each panel.

**3.3. Interaction of the amyloids with the IgE of sera of milk, egg and fish allergic patients.** To analyze the recognition of the formed amyloids with the IgE of the sera of the food allergic patients described in Table 1 we used dot blot assays (Figure 3). In these assays 0.1  $\mu\text{g}$  of both native and amyloid folds of the distinct allergens were dotted in duplicates and the interactions with the sera were studied using blocking buffers containing BSA. It must be underlined that the serum of allergic patients contains a collection of IgE (total), some of which specifically but variably recognizes the offending food allergen (sIgE) and that the intensity of the recognition is modulated by the existence of neutralizing IgG<sub>4</sub> [3].

Since Gad m 1 is a major allergen in fish allergy, membranes were first probed with the sera of fish allergic patients (Figure 4). As previously shown, Gad m 1 amyloids are specifically recognized by the IgEs contained in the sera of fish allergic patients [14]. Interestingly, no signal was detected when the membranes were probed with the IgEs of the sera from milk and egg allergic patients.

The serum IgEs of milk allergic patients recognized the amyloid fold of Bos d 12. For patients 2 and 3, the IgE binding to the Bos d 12 amyloid is 5-fold higher of that of the native state as judged from signal quantitation. On the contrary, the IgE of the sera of patient 1 interacts largely with the native fold of Bos d 12 and also recognizes the amyloid fold Gal d 2. Importantly, increasing the relative loads of both Bos d 5 native and amyloid folds using both the similar membrane format and membranes lacking the load of Bos d 12 forms did not alter the result. Absence of Bos d 5 recognition by the sera IgE excludes this allergen as relevant for the milk allergic patients under studied [19,20].

Similar to the recognition by IgE of Bos d 12 and Gad m 1 amyloids, Gal d 2 aggregates are the general binding targets of the IgEs contained in the sera of egg allergic patients. For sera 5 and 6 the IgE interaction with Gal d 2 amyloids 10-fold higher than with the native fold, whereas in serum 4 the preference is 3-fold higher. Interestingly, serum 5 which contains the higher level of total IgEs also recognized Bos d 12 amyloids.



**Figure 4.** Dot-blot analysis of the IgE interaction of native and amyloid folds of milk, egg and fish allergens using the sera of food allergic patients. Sample dotting was performed in duplicates using 0.1  $\mu\text{g}$  of the native (N) and amyloid (A) structures of the food allergens. Sera are indicated in numbers (from 1 to 8) and grouped according to the offending food (milk, egg and fish).

Taken together these results showed that, as previously described for Gad m 1, Bos d 12 and Gal d 2 amyloids are allergen structural states recognized by the IgEs present in the sera of milk and egg allergic patients, respectively. Notwithstanding, the specificity of the amyloid IgE recognition varies among the distinct food allergies.

#### 4. Discussion

In this study, we sought to investigate whether the formation of IgE-binding amyloids as found for Gad m 1 is a common feature of animal food allergens. Our results showed that Bos d 12 and Gal d 2 yield amyloids which are recognized by specific IgEs contained in the sera of milk and egg allergic patients, respectively. For most patient sera, the amyloid fold accounts for the major state recognized by their IgEs. Bos d 5 behaved distinctly since both native and amyloid folds did not react with the IgE of the milk allergic patient sera used, despite the low sIgE levels provided by the ImmunoCAP characterization.

The search of the molecular signatures that predispose food proteins to become allergens has yielded several characteristics including abundance in food, structure, resistance to processing and digestion and the presence of multiple linear IgE binding epitopes [5-9]. These features have been established omitting the effects that transit through the gastric compartment can cause. One of the effects is the pH-induced refolding of allergens into their amyloid states [10,11]. The efficiency of this refolding will mainly depend on the sequence and abundance of the protein [12]. Notwithstanding, stability factors such as those due to the presence of ligands bound to the native precursors and of anti-amyloid compounds might modulate the efficiency of the refolding process [12]. Animal food allergens such as Bos d 5, Bos d 12, Gal d 2 and Gad m 1 undergo such refolding processes under acid treatment. Despite their general amyloid features, the aggregated state of the allergens is not unique and differ in their shape and surface reactivity as shown by AFM imaging and IgE binding. Also, the aggregates may display different dissociation trend [12,14].

*In-silico* analysis using previously described IgE-binding epitopes and the amyloid forming segments predicted by the ZipperDB algorithm shows that each allergen chain contains multiple regions with a functional overlap. This behaviour was described for the segments <sup>25</sup>FDHKAFFTKVGLAAKSSA<sup>42</sup> and <sup>67</sup>FLQNFSAGARAL<sup>78</sup> of Gad m 1 using anti-fibril OC-antibody and sera IgE interactions [15,16]. Interestingly, the N-terminal region of each of the segments (DHKAFFTKV and FLQNFS) form part

of helical structures in the native state of Gad m 1 [26]. This conformational multiplicity of the regions forming the IgE-binding epitopes questions the validity of mapping the epitopes singly on the native 3D structures of food allergens. In fact, refolding and amyloid assembly of the IgE-binding epitopes will impede their hydrolysis by proteases and will amplify the avidity and affinity of any ligand binding process [12,14,15,25]. On the other hand, the identified segments significantly differ in the sequence which allows specificity in the IgE binding. It merits comment that each food allergen contains more than one segment with functional overlap permitting then multiplicity in the interacting IgEs.

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