



## Article

# Distinct Animal Food Allergens Form IgE-Binding Amyloids

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**Abstract:** Several animal food allergens assemble into amyloids under gastric-like environments. These aggregated structures provide Gad m 1 with an enhanced immunoglobulin E (IgE) interaction due to the fibrillation of the epitope regions. However, whether these properties are unique to Gad m 1 or shared by other food allergens has not yet been addressed. Using Bos d 5, Bos d 12 and Gal d 2 as allergen models and Gad m 1 as the control, aggregation reactions and the sera of milk, egg and fish allergic patients have been analyzed, assessing the IgE interactions of their 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 native structure. Bos d 5 was not recognized under any fold by the IgE of the sera studied. These results suggest that the formation of IgE-binding amyloids could be a common feature to animal food allergens.

**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 a 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, with 95% of food allergies being caused by only eight foods including eggs, milk, fish, crustacean shellfish, peanuts, tree nuts, wheat and soybeans [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, a condition that is usually used in vitro to trigger the refolding of proteins and of their fragments 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]. An 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 the  $\beta$ -parvalbumin Gad m 1 from fish (*Gadus morhua*);  $\alpha$ -lactalbumin (Bos d 4),  $\beta$ -lactoglobulin (Bos d 5),  $\alpha$ S2-casein (Bos d 10),  $\beta$ -casein (Bos d 11) and  $\kappa$ -casein (Bos d 12) from bovine milk, and the ovalbumin (Gal d 2) and lysozyme (Gal d 4) from chicken egg-whites rapidly form amyloid aggregates under a variety of conditions, some of which are those of the gastric compartment [14–22]. Bos d 5 and Bos d 12 also form heterogeneous or mixed amyloid fibrils under ultraheat, mimicking treatments used in the dairy industry [23].

Gad m1 amyloids displayed an enhanced proteinase resistance and a 1000-fold increased IgE-specific binding compared to that of the monomer precursor [14–16]. Importantly, unrelated amyloid fibrils such as those formed by A $\beta$ 42 and PrP are not recognized by the IgE present in the sera of fish allergic patients, underlining 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 cores overlapped with the IgE epitopes [15,16]. Notwithstanding, whether this property is specific to Gad m 1 or is shared by other food allergens has not yet been addressed. Given their well-characterized aggregation processes, commercial availability and the knowledge of the major IgE epitopes, we have chosen Bos d 5, Bos d 12, and Gal d 2 as animal food allergen models to analyze the IgE-binding properties of their amyloid state using the sera of allergic patients.

## 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 Hospital La Paz in Madrid (Spain). Fish, milk, and egg allergic patients were selected based on case history, a 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 immunoglobulin E (IgE) profiles of the sera of food allergic patients recruited for the study.

Patient	Offending Food	Sex (M/F)	Age (year)	Symptoms <sup>a</sup>	IgE (kU/L) <sup>b</sup>							
					Total	Milk	Bos d 5	Bos d 12	Egg-White	Gal d 2	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

<sup>a</sup> A, anaphylaxis; AE, angioedema; E, eczema; OAS, oral allergy syndrome; RD, respiratory symptoms. <sup>b</sup> ImmunoCap determinations. nd, not determined.

### 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 database [24]. IgE binding epitopes were taken from previous SPOT-membrane and array assays [15,16,25–28]. Amyloid cores were predicted using the ZipperDB

algorithm [29,30]. 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. The relative organization (overlap/flank relation) between regions was visualized using a 3D stacking plot.

#### 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 (A51874) was prepared as described [31]. Before their use all proteins were extensively dialyzed at 4 °C against either 25 mM Tris, 0.1 M NaCl pH 7.5 or 0.1 M Gly pH 1.5 using dialysis membranes with an 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. Proteins at pH 7.5 were referred as the native states (N), whereas the solutions at pH 1.5 were used for amyloid formation.

#### 2.5. Amyloid Fibril Preparation

Protein solutions at 5–8 mg/mL solution in 0.1 M 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 an ultracentrifugation at 100,000× g for 1 h at 4 °C 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 taking 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 0.05 mg/mL in 2.5 mM Gly pH 1.5 were absorbed onto freshly cleaved mica via a 5–10 min incubation at room temperature. The surfaces were then rinsed with double-distilled water and dried. Images were obtained in the tapping mode using a JPK Nanowizard 2 microscope and HQXSC11 B (Mikromash) cantilevers (2.7 N/m force constant and 70 kHz resonance frequency). An AFM analysis was performed using the free program WSxM 4.0 (Nanotec)

#### 2.8. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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, Hercules, CA, USA), and the images were recorded with the Molecular Imager ChemiDoc XRS-Plus (BioRad, Hercules, CA, USA).

#### 2.9. Dot Blot Analysis

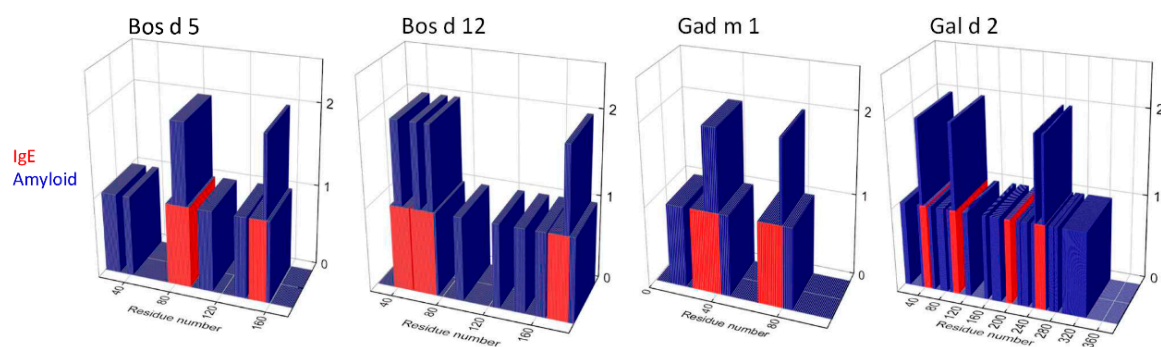
Samples of 0.1–0.2 µg protein/dot were loaded in duplicate onto nitrocellulose membranes. After blocking for 1 h in TBS-T (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Tween 20) containing 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at room temperature, the membranes were incubated for 2 h with either the distinct serum (diluted 1:10 in blocking buffer) or with the anti-amyloid fibril antibody (OC antibody) (AB2286 Merck Millipore, 1:2000 dilution, Darmstadt, Germany). After extensive washing with TBS-T, membranes were incubated for 1 h with horseradish

peroxidase (HRP)-labeled goat antimouse IgG (Sigma-Aldrich, 1:5000 dilution, St. Louis, MO, USA) or mouse monoclonal B3102E8 antihuman IgE (Abcam, 1:2000 dilution, Cambridge, UK) and developed using Clarity Western-ECL (BioRad, Hercules, CA, USA). 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 IgE-Binding and Amyloid-Forming 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 forming amyloid fibrils [15]. To test whether other food allergens share this feature the sequences of Bos d 5, Bos d 12, and Gal d 2 taken as allergen models were transformed into the binary (0/1) functions of IgE-binding epitopes and amyloid cores using previous epitope reports [15,16,25–28] and the predictions of the ZipperDB algorithm [29,30], respectively. To visualize their relative organization (flanking and/or overlapping), the obtained functions of the residue number were plotted using a 3D stacked bar representation (Figure 1).



**Figure 1.** Relative organization of the IgE-binding epitopes and the predicted amyloid cores 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. The regions predicted as amyloid cores are depicted as blue 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. It must be noted that ZipperDB yields hexapeptides as minimum cores [29,30], but the aggregation property can extend at their both N- and C-terminal side as experimentally shown [32]. The N-terminal IgE-binding epitope is overlapped by an amyloid core, whereas the C-terminal IgE-binding epitope is mainly flanked by aggregating segments with a partial C-terminal overlapped. Importantly, both regions contain polymorphic sites (Q75H, G80D and A134V) characteristic of distinct isoforms [24] and Cys residues which are engaged in disulfide bonds and may modify both IgE binding and amyloid formation features (Table 2). Bos d 12 is produced as a precursor chain (190 amino acids) with an N-terminal signal sequence (1–21 residues), the same as Bos d 5. The 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 minimal adhesive regions [33]. The 385 amino acid mature chain of Gal d 2 contains four IgE binding epitopes, three of which are overlapped by the predicted amyloid cores as depicted in Table 2.

**Table 2.** Sequence regions with IgE binding and amyloid forming properties. IgE-binding epitopes are depicted with grey background; amyloid forming regions are shown in blue.

Allergen	Sequence Regions
Bos d 5	74 LQKWENGECAQKKIAEKT <sup>93</sup> 131 QSLACQCLVRTP EVDDEALEKFDKAL <sup>159</sup>
Bos d 12	37 RFFSDKIAKYIPIQYVL <sup>53</sup> 55 RYPSYGLNYYQQKPVALINN <sup>76</sup> QF <sup>76</sup> 159 AVESTVATLE DSPEVIESPPEINTVQV <sup>190</sup> TSTAV <sup>190</sup>
Gad m 1	25 FDHKAFFTKVGLAAS <sup>48</sup> SADIKKVF <sup>48</sup> 67 FLQNFSAGARALSDA <sup>86</sup> ETKVF <sup>86</sup>
Gal d 2	39 AIMSALAMVYLGAKDST <sup>68</sup> RTQINKVV <sup>68</sup> 88 LNQITK <sup>104</sup> PNDVYSFSLAS <sup>104</sup> 238 GTMSMLVLLPDEVSGLEQLESIIN <sup>263</sup> FE <sup>263</sup>

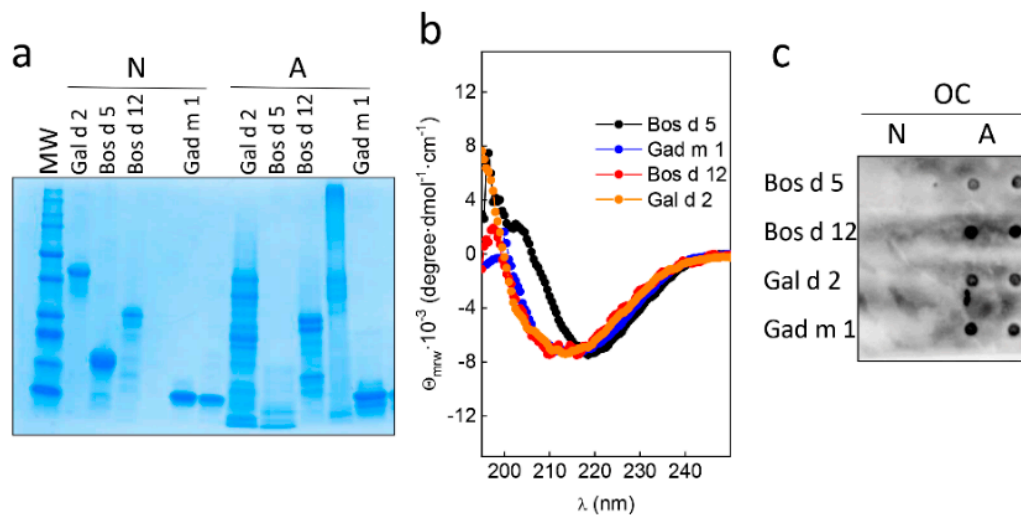
Then, this in silico analysis shows that the milk and egg allergen models share with the fish allergen Gad m 1 the sequence overlap of IgE binding epitopes and amyloid forming cores and suggests that their aggregates may harbor IgE-binding properties [15,16].

### 3.2. Native and Amyloid Folds of Milk, Egg and Fish Allergens

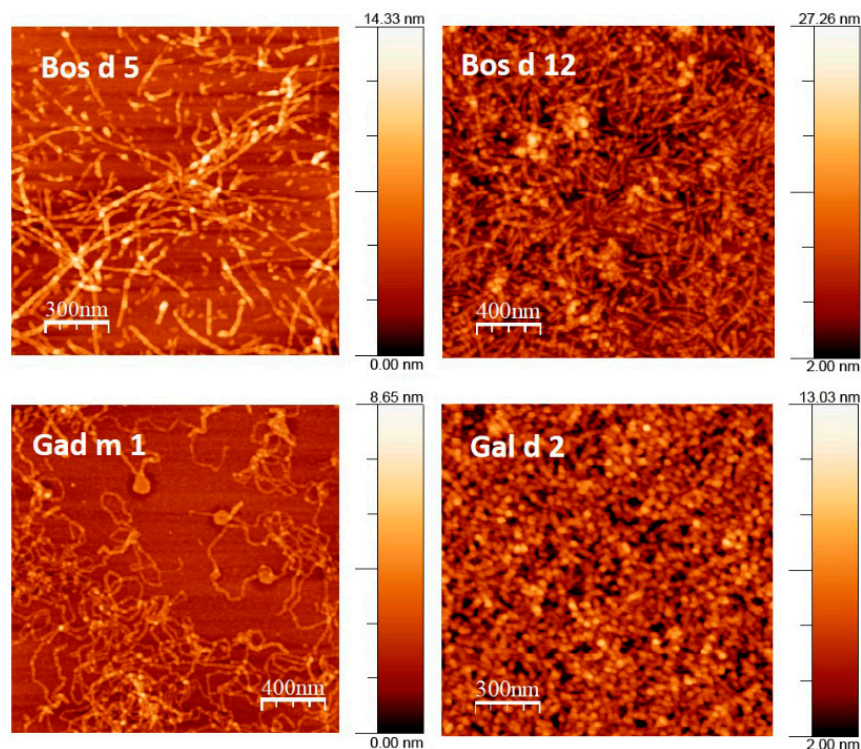
To analyze the interaction of the distinct food allergens with the IgE contained in the sera of food allergic patients, both their native and amyloid structures were prepared. The native fold (N) required as the control was prepared by an extensive dialysis in 25 mM Tris, 0.1 M NaCl pH 7.5, followed by centrifugation to clear the solutions from unspecific aggregates. Regarding the amyloid fold (A), a variety of conditions in acid media differing in the temperature and length of incubation, ionic strength, presence or absence of alcohols, protease treatments and protein concentration have been specifically used for each of the food allergens [14,17–22]. Aiming to find a balance between yield and kinetics, reduce the working protein concentration below 10 mg/mL and find a general application to all proteins, we tailored a procedure consisting of the use of dialyzed protein solutions prepared at 5–8 mg/mL in 0.1 M Gly pH 1.5 (removal of ligands), heating at 90 °C for 5 h (denaturation), followed by 36 h of growth at room temperature and isolation of the aggregates by ultracentrifugation.

The use of this procedure allowed the isolation of insoluble aggregates amounting to about 10% of the initial precursors and consisting in a mixture of full length and truncated chains (Figure 2a). The isolated aggregates displayed far-UV CD spectral features of secondary structures governed by a  $\beta$ -sheet (Figure 2b) and specifically reacted with an anti-amyloid fibril OC antibody in dot-blot assays (Figure 2c). In addition to the general amyloid features provided by the anti-OC reactivity, AFM imaging showed a variety of fibrillary shapes (Figure 3). Bos d 5 aggregates consisted of thick and linear fibrils filaments with variable lengths (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 lateral associations. On the contrary, Gad m 1 insoluble aggregates appeared as long flexible thin curved fibrils different from the fibrillary polymers generated under an acid pH in the absence of heating treatment [14].





**Figure 2.** Amyloid aggregation of the milk, egg and fish allergens. (a) Typical analysis by SDS-PAGE and Coomassie Brilliant Blue R-250 (CBB) staining of the distinct food allergens before (N) and after aggregation (A). Lanes of interest are labelled with the corresponding food allergen. MW correspond to the prestained protein ladder with the molecular weights (bottom to top): 10, 15, 20, 25, 37, 50, 100, 150 and 250 kDa. The unlabeled lanes were left to display the original gel. (b) Far-UV Circular dichroism (CD) spectra of Bos d 5 (black), Bos d 12 (red), Gal d 2 (orange) 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.



**Figure 3.** Atomic Force Microscopy (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.

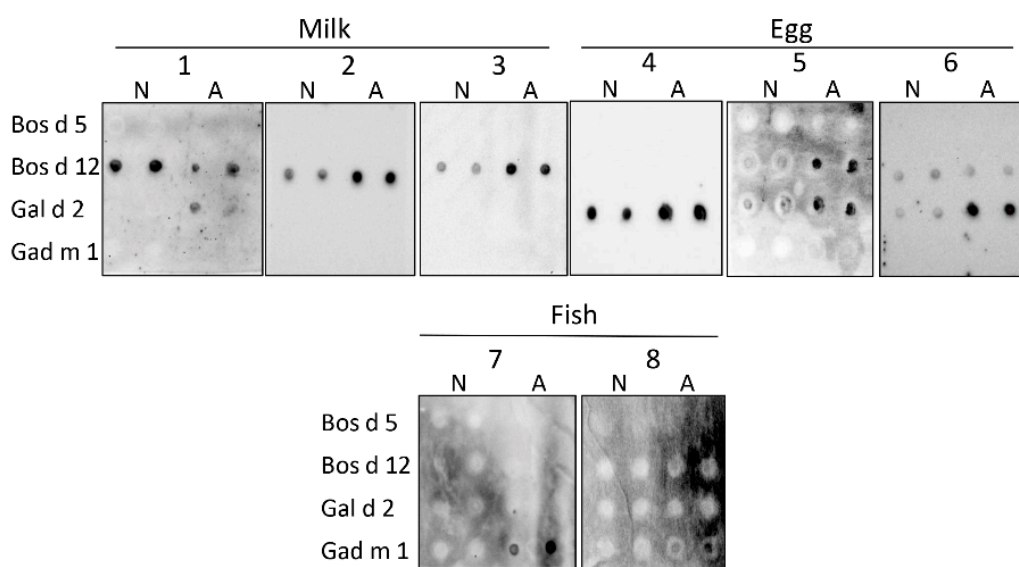
Then, the use of simple and systematic procedures permits the preparation of the native and amyloid folds of all the food protein allergens for their further analysis.

### 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 and compare it with the corresponding native folds, we used dot blot assays (Figure 3). In these assays, 0.1 µg of both the native and amyloid folds of the distinct allergens were dotted in duplicate and the interactions with the sera probed using blocking buffers containing bovine serum albumin (BSA).

It must be underlined that the serum of allergic patients contains a collection of IgEs (total), some of which specifically but variably recognize the offending food allergen (sIgE) [3]. In this sense, the intensity of the recognition might be modulated by the existence of neutralizing IgG<sub>4</sub> [3]. For these reasons and despite the fact that a higher number of sera were used in a preliminary analysis targeting individual allergens, the collective study was performed with the reagents displayed in Table 1 with a single use of the 1/10 dilutions.

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, for a similar load of Gad m 1 structures amyloids are specifically recognized by the IgEs contained in the sera of fish allergic patients [14,15]. As expected, no signal was detected when the membranes were probed with the IgEs of the sera from milk and egg allergic patients.



**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 µg 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).

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 in the native state as judged from signal quantitation. On the contrary, the IgEs of the sera of patient 1 interact largely with the native fold of Bos d 12 and also slightly recognize the amyloid fold Gal d 2. These recognition differences between the sera of patient 1 and the sera of patients 2 and 3 might result from the large differences in the anti-Bos d 12 IgE levels (Table 2). Importantly, Bos d 5 under native and amyloid folds was not recognized by the sera IgE of any of the patients despite the ImmunoCap revealed presence of anti-Bos d 5 IgEs (Table 2). Increasing the relative loads of both Bos d 5 folds using both the similar membrane format and membranes devoid of Bos d 12 forms did not alter the result. The absence of Bos d 5 recognition by the sera IgE suggests differences in the isoform composition of the product provided by Sigma-Aldrich and the reagent used in the ImmunoCap approach [24].

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

Taken together, these results show that, as described for Gad m 1 in fish allergy, Bos d 12 and Gal d 2 amyloids are the structural states highly recognized by the IgEs present in the sera of milk and egg allergic patients, respectively. Notwithstanding, the relative specific recognition of the amyloid state compared to the native fold by the sera IgEs varies among the distinct food allergic patients.

#### 4. Discussion

In this pilot study, we sought to investigate whether the formation of IgE-binding amyloids as found for Gad m 1 is a feature shared by other animal food allergens. Our results show 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 represents the major IgE-reactive state. 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 sIgE levels provided by the ImmunoCAP characterization, suggesting differences in the isoform composition of both reagents [24].

The search for 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 and omit the effects that transit through the gastric compartment can cause. One of the effects is the pH-induced refolding of the allergens and of their fragments into their amyloid states [10,11]. Indeed, of almost all the proteins contained in their sequence, at least one segment capable of forming amyloid fibrils [34]. However, only 5.3% of these segments are found in the surface of the native 3D structures and less than 0.1% of them displayed the proper sticky geometry [34]. Consequently, the formation of amyloid fibrils under physiological conditions is common for intrinsically disordered proteins such as Bos d 12 [19,35]. However, globular proteins such as most food allergens require a partial unfolding or a nicking process to expose the amyloid forming segments otherwise buried in the structure interior or stabilized by bound ligands. The efficiency of this refolding mainly depends on the sequence, the abundance of the protein and the interference of off-pathways such as those driving the less favorable amorphous aggregates and nonproductive truncations [11,12,17,36]. For complex mixtures such as food, factors such as processing treatments and the presence of native state ligands and of anti-amyloid compounds might also modulate the efficiency [12,17,19]. Tailoring a protocol using acid pH, low protein concentrations, short incubations and random truncations, for general use, facilitates the amyloid fibril purification required for their systematic characterization.

The isolated Bos d 5, Bos d 12, Gal d 2 and Gad m 1 amyloid states are not unique and differ in their shape, polymorphism degree and surface reactivity as shown by AFM imaging and IgE binding. Indeed, the multiplicity of potential fibrillation-prone segments predicted by the ZipperDB algorithm and the fragmentations showed by SDS-PAGE entails complex aggregation events far beyond those uniquely containing IgE-binding sites. On the other hand, the formed aggregates may display different elongation and dissociation rates which will dictate the landscape of molecular species existing under gastrointestinal-like conditions [12,14]. In this sense, easy dissociating amyloid fibrils will function as IgE-binding epitope depots, whereas those displaying a slow dissociation will function as epitope backbones. These nonexclusive possibilities might coexist in any of the allergens if fibrillation is seeded through different sticky segments.

An *in silico* analysis using previously described IgE-binding epitopes and the amyloid cores predicted by the ZipperDB algorithm detected multiple regions with a functional overlap in each allergen chain. The overlap degree should be taken in qualitative terms, since each of the variables used employ different length windows to define function. In this sense, the considered IgE epitopes were



determined using 20 amino acid residues sequential peptides with a three-amino acid shift and sera from paediatric patient cohorts [25–28]. On the contrary, the prediction of amyloid cores uses a six-amino acid peptide window. Studies with Gad m 1 using 12-length peptides and a two-amino acid sequential shift between two consecutive peptides, an immobilization density of  $\approx 10$  nmol per spot ( $\approx 400$  nmol/cm<sup>2</sup>), sera from fish allergic patients for IgE-epitope mapping and the antifibril OC-antibody for amyloid detection identified the segments <sup>25</sup>FDHKAFFTKVGLAAKSSA<sup>42</sup> and <sup>67</sup>FLQNFSAGARAL<sup>78</sup> as the segments with dual functions [15,16]. Both sequences are N-terminal shifted to the predicted sticky segments, indicating the expansion of the aggregation properties beyond the detected cores. Using a similar approach for milk and egg allergens and the sera from a varied allergic patient cohort will allow a closer view of the functional overlap.

The potential conformational multiplicity of the regions forming the IgE-binding epitopes questions the validity of mapping the epitopes using the native 3D structures of food allergens as unique template. In fact, the IgE binding sequences DHKAFFTKV and FLQNFS form parts of helical structures in the native state of Gad m 1 whereas the amyloid fold adopts a cross  $\beta$ -sheet structure [16,37]. In fact, consideration of the IgE-epitope amyloid folds will simultaneously explain their protease resistance and higher avidity and affinity for any ligand binding process [12,14,15,32]. On the other hand, the sequence differences of the segments with functional overlaps identified in the distinct allergens sustain the binding specificity of the IgE repertoire present in the sera of the allergic patients sensitized to distinct foods. Additionally, for the given food allergen differences in the covalent structure (isoforms, covalent modifications, etc.), the concentration in the food source and local industrial processing may impact the functionality of the aggregating regions and therefore their stability. Dynamic changes in the quality of the offending foods may explain variations in the IgE-epitope repertoires of different patient cohorts.

In summary, this work provides the pilot proof of concept by which the amyloid fold of segments of animal food allergens can define the novel structural properties of IgE-binding epitopes. Notwithstanding, their solid assignment will require a deeper study with a higher number of sera samples, as will their simultaneous characterization using SPOT-membranes/arrays using a similar design (peptide length and offsets, and surface density, among other critical parameters) and the synthetic reconstruction of the segments of interest as performed with Gad m 1 [14–16]. Indeed, IgE-epitope repertoires depend on the regional origin of the patient cohort and change with the patient's age.

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