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Gal d 6 Is the Second Allergen Characterized from Egg Yolk

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Only one allergen from the egg yolk, α-livetin (Gal d 5) has been described thus far. A new egg yolk allergen was detected studying 27 egg allergic patients. The study was performed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and IgE-immunoblotting and IgE-immunoblotting-inhibition assays. An egg yolk extract was fractioned by reverse-phase high-performance liquid chromatography (RP-HPLC), and the new allergen detected was characterized by N-terminal amino acid analysis. A total of 5 of the 27 patients (18%) detected a yolk allergen of an apparent molecular weight of 35 kDa by SDS−PAGE. Heating and reduction treatments did not affect its allergenicity, although digestion with simulated gastric fluid diminished the IgE-binding capacity of the allergen. The N-terminal amino acid sequence corresponded with the YGP42 protein, a fragment of the vitellogenin-1 precursor. Thus, a second egg yolk allergen has been described and designated Gal d 6 by the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee.

KEYWORDS: Egg allergy; food allergy; allergen detection; IgE; vitellogenin

INTRODUCTION

Egg allergy accounts for one of the most prevalent food hypersensitivities in industrialized countries. The estimated prevalence of egg allergy varies between 1.6 and 3.2% and, thus, makes it the second most common cause of food allergy in children (*1*). The omnipresence of egg and its derived components in manufactured food products renders the avoidance of egg difficult, and inadvertent exposure may lead to life-threatening anaphylactic responses in sensitized patients (*2*).

The majority of the relevant egg allergens have been identified in the egg white: ovomucoid (Gal d 1), a Kazal-type serine protease inhibitor; ovoalbumin (Gal d 2), a serine protease inhibitor; ovotransferrin (Gal d 3); and the egg lysozyme (Gal d 4) (*1*). A serum albumin, α-livetin (Gal d 5), is the only allergen found in the egg yolk. This egg yolk allergen has been involved in the bird-egg syndrome (*3*, *4*).

In the present study, 27 patients with egg allergy were investigated to identify and further characterize a new allergen detected in the egg yolk.

MATERIALS AND METHODS

Patient Sera. Sera from 27 patients (ages ranging from 2−74 years) with egg allergy [clinical history and both positive-specific IgE and skin prick tests (SPTs) to egg] and sera from 2 non-allergic subjects as negative controls were studied. Informed consent was obtained from the study participants. Experiments were performed in compliance with the appropriate laws and institutional guidelines.

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SPTs were performed with commercial extracts (Bial-Aristegui, Bilbao, Spain). Histamine dihydrochloride (1 mg/mL) and saline solution were used as positive and negative controls, respectively. A SPT response was considered positive if the largest wheal diameter was at least 3 mm greater than the produced by the negative control.

Prick-to-prick tests (PPTs) were performed with separate raw and cooked egg, white and yolk. Specific IgE determinations to egg white and yolk and their isolated allergens were performed by the CAP system and immuno solid-phase allergen chip microarray-based IgE detection (ImmunoCAP-ISAC) (Phadia, Uppsala, Sweden).

Preparation of the Extracts Used. *Egg Yolk Extract*. Raw egg yolk was defatted as described by Rodriguez-Perez et al., with some modifications (*5*). Briefly, 2 egg yolks were washed with distilled water to eliminate contamination by egg white and then were freeze-dried. The material obtained was defatted twice with acetone (1:10 wt/vol for 1 h at 4 °C), followed by ethanol/ether (1:3 vol/vol for 1 h at 4 °C). The dried material was then extracted with phosphate-buffered saline (PBS) buffer for 1 h at 4 °C. After centrifugation (4500*g* for 30 min), the supernatant was dialyzed against distilled H_2O (cutoff point of 3.5 kDa) and freeze-dried. The protein concentration was determined by the Protein Quantification Kit-Rapid (Fluka Chemie AG, Buchs, Switzerland).

Chicken Meat Extract. Raw chicken meat (10 g) was grounded and then extracted with PBS buffer for 1 h at 4 °C. After centrifugation (4500*g* for 30 min), the supernatant was dialyzed against distilled H_2O and freeze-dried.

Feathers Extract. A commercial mixture of extracts from chicken and duck feathers (ALK-Abelló, Madrid, Spain) was concentrated before use with Microcon filters YM10 (Millipore Corporation, Billerica, MA).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) Analysis and IgE Immunoblotting. Aliquots of 15 μg of protein from the egg yolk extract, the chicken meat extract, and the feathers extract were separated by SDS−PAGE on 12% acrylamide minigel, under standard conditions. After electrophoresis, proteins were stained with

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Coomassie Blue or electro-transferred onto nitrocellulose membranes (NitroPure supported, 0.45 μm, GE Osmonics Labstore, Minnetonka, MN) and incubated overnight with the sera (diluted 1:20). Specific IgE determination was performed with a monoclonal anti-IgE antiserum (Ingenasa, Madrid, Spain) and an alkaline phosphatase-labeled goat anti-mouse antiserum (Biosource International, Camarillo, CA). Finally, the signal was visualized with the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) system (Amresco, Solon, OH) for 30 min (*6*).

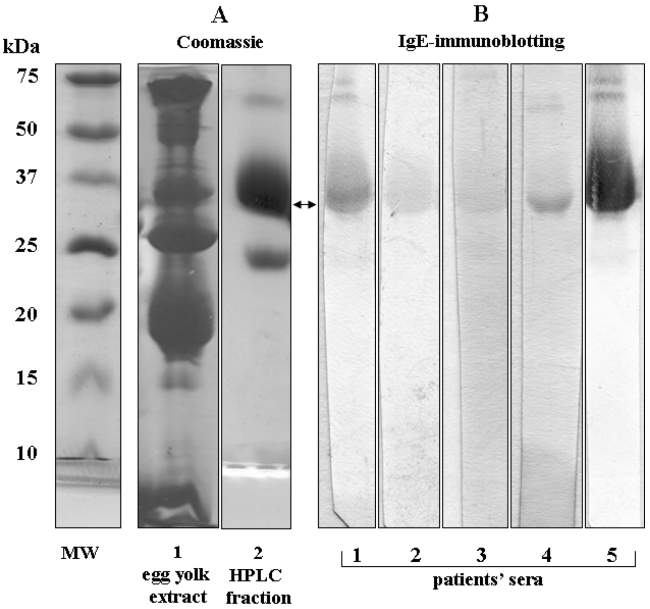

Figure 1. (A) Protein separation by SDS−PAGE and Coomassie staining. Lanes: (1) egg yolk extract and (2) HPLC fraction containing Gal d 6. (B) IgE immunoblotting performed with the HPLC fraction and the sera of the five patients detecting the allergen. MW = molecular-weight markers.

Reverse-Phase High-Performance Liquid Chromatography (*RP-HPLC*). The egg yolk extract (14 mg) was loaded on an ACE 5 C4-300, 250 × 4.6 column (Advanced Chromatography Technologies, Aberdeen, Scotland) and subjected to RP-HPLC. Elution was performed using a 60 min increasing linear gradient 0−100% of 70% acetonitrile containing 0.09% trifluoroacetic acid (TFA) and Milli-Q water containing 5% acetonitrile and 0.1% TFA, at a flow rate of 1 mL/min. Peaks containing allergens were identified by SDS−PAGE and IgE immunoblotting, performed with the sera of the patients.

Characterization of the New Egg Yolk Allergen. *N-Terminal Amino Acid Sequence*. The HPLC fraction containing the new allergen, Gal d 6, was electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Sequiblot, Bio-Rad) and stained with Coomassie Blue. The protein band was excised and submitted for N-terminal amino acid analysis in a Perkin-Elmer/Applied Biosystems Procise 494 microsequencer in pulse liquid mode at the Proteomic Service in Centro de Investigaciones Biológicas (CSIC, Madrid, Spain).

The SIB ExPASy BLAST2 interface (http://www.expasy.ch/cgi-bin/blast.pl) was used to analyze the N-terminal amino acid sequence in determining similarities to previously reported sequences.

Oxidation of Sugar Residues. The HPLC fraction enriched in Gal d 6 was electro-transferred onto 2 nitrocellulose membranes. One of the membranes was treated with sodium periodate, as described (*7*), the untreated membrane was used as a control. Then, both membranes were developed by IgE immunoblotting with a pool of sera from the patients who had detected the new allergen (pool of positive sera).

Heat Treatment, Reduction, and Digestion. Aliquots of 15 μg of protein from the HPLC fraction enriched in Gal d 6 were subjected to different treatments: heated at 100 °C for 15 min, reduced with 5% 2-mercaptoethanol at 100 °C for 5 min, or digested with simulated gastric fluid [12.8 μg/μL of pepsin A (Sigma, St. Louis, MO) in 50 mM HCl] at 37 °C for 30 min. Samples were then analyzed by SDS−PAGE and IgE immunoblotting with the pool of positive sera.

Implication of Gal d 6 in the Bird-Egg Syndrome. A SDS−PAGE was performed with the chicken meat extract, the feathers extract, and the HPLC fraction enriched in Gal d 6. The IgE-immunoblotting inhibition was performed as described above, except that the pool of positive sera was preincubated for 3 h at room temperature with the HPLC fraction (10 μg of protein).

Table 1. Clinical Data of the Five Patients Sensitized to the 35 kDa Egg Yolk Allergen[a]

patient	age	sex	symptoms after egg ingestion	SPT (mm)	PPT (mm)	CAP (kU/L)	ISAC (ISU)	total IgE (kU/L)	other allergies
1	13	M	urticaria	raw egg white 19 × 21 egg yolk 12 × 9	cooked egg white 22 × 21 egg yolk 9 × 11	egg white >100 egg yolk 41.2 Gal d 1 38 Gal d 2 86.6 Gal d 4 2	Gal d 1 40 Gal d 2 21 Gal d 3 25 Gal d 5 27	616	chicken, fish, grass pollen, *Alternaria alternata*, dog and cat danders
2	74	M	cutaneous pruritus	raw egg white 6 × 6 egg yolk 7 × 5	cooked egg white 5 × 4 egg yolk 4 × 4	egg white 4.03 egg yolk 1.46 Gal d 1 0.42 Gal d 2 0.62 Gal d 4 <0.35	Gal d 1 <0.35 Gal d 2 <0.35 Gal d 3 1.1 Gal d 5 <0.35	87	no
3	8	M	asthma	raw egg white 4 × 6 egg yolk 3 × 6	np	egg white 3.76 egg yolk 2.76 Gal d 10.74 Gal d 2 3.14 Gal d 4 <0.35	Gal d 1 0.9 Gal d 2 4.1 Gal d 3 <0.35 Gal d 5 <0.35	1873	fish, shellfish, hazelnuts, almond, pistachio, *A. alternata*, *Cupressus arizonica*, cat dander
4	3	F	pruritic exanthema, facial erythema, bronchospasm	raw egg white 5 × 5 egg yolk 6 × 14	np	egg white 34.9 egg yolk 22.4 Gal d 1 15.7 Gal d 2 17.8 Gal d 4 1.67	Gal d 1 11 Gal d 2 2.3 Gal d 3 9.6 Gal d 5 <0.35	204	no
5	16	M	oral allergy syndrome, digestive symptoms	raw egg white 10 × 16 egg yolk 15 × 10	cooked egg white 13 × 15 egg yolk 5 × 6	egg white >100 egg yolk >100 Gal d 1 >100 Gal d 2 >100 Gal d 4 62	Gal d 1 71 Gal d 2 23 Gal d 3 8.5 Gal d 5 69	1508	chicken, nuts, feathers of chicken and parrot, grass pollen, dog and cat danders

[a] SPT, skin prick testing (mean wheal in mm); PPT, prick−prick testing; CAP, serum-specific determination by the CAP method; ISAC, ImmunoCAP-ISAC, immuno solid-phase allergen chip microarray-based IgE detection; np, not performed.

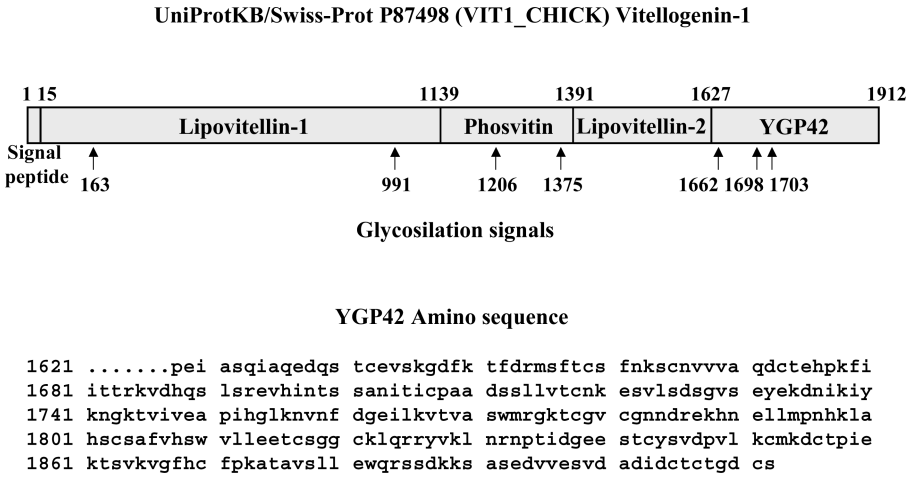

Figure 2. Schematic representation of VTG-1 and YGP42 protein.

RESULTS

Identification of the New Allergen and Frequency of IgE Recognition. A new allergen with an apparent molecular weight around 35 kDa was identified from the yolk extract and enriched by RP-HPLC (**Figure 1A**). The HPLC fraction containing the allergen was analyzed by IgE immunoblotting with a total of 27 sera from patients allergic to egg. A total of 5 of the 27 patients studied (18% of the patients) showed specific IgE binding to the allergen (**Figure 1B**), and none of the negative controls recognized the allergen (data not shown). The clinical data of the five positive patients are reported in **Table 1**.

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence obtained for the allergen was PEIASQIAQEDQSTXEV. A homology search performed by BLAST revealed that the allergen was the yolk glycoprotein 42 (YGP42) protein, a fragment of the vitellogenin-1 (VTG-1) precursor (positions 1628−1912) (UniProtKB/Swiss-Prot P87498 (VIT1-CHICK) (**Figure 2**). The allergen has been designated Gal d 6.0101 by the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee. The molecular weight of Gal d 6 is 31 446.5 Da (ExPASy ProtParm tool).

Oxidation of Sugar Residues. The glycoprotein YGP42 has three glycosylation signals at positions: 1662, 1698, and 1703 (UniProtKB/Swiss-Prot P87498). Thus, we performed a periodate oxidation of sugar residues to determine whether carbohydrate epitopes were implicated in the IgE reactivity of Gal d 6. The results of the assay showed that periodate treatment did not cause a loss of IgE binding, indicating that the sugar residues are not involved in the allergenicity of Gal d 6 (**Figure 3**).

Heat Treatment, Reduction, and Digestion. We studied the allergenicity of Gal d 6 after different treatments to know its clinical relevance. Heating and reduction treatments did not affect the IgE reactivity of Gal d 6 (**Figure 4**); however, digestion with simulated gastric fluid abolished the reactivity of the allergen, as demonstrated by IgE immunoblotting (**Figure 5**).

Implication of the New Allergen in the Bird-Egg Syndrome. The egg yolk allergen Gal d 5 has been involved in the bird-egg syndrome, besides one of the patients who detected Gal d 6 (patient number 5 in **Table 1**) had urticaria and angioedema eating chicken meat and bronchial asthma with feathers of chicken and parrot. For these reasons, we studied the possibility that Gal d 6 could be implicated in the bird-egg syndrome. The IgE-immunoblotting-inhibition assay showed that Gal d 6 did not inhibit the allergens from the meat chicken extract and the feathers extract. Thus, Gal d 6 seems not to be involved in this syndrome (**Figure 6**).

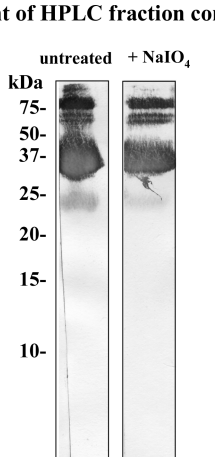

Figure 3. Oxidation of sugar residues. IgE immunoblotting performed with a pool of sera from the five positive patients after the periodate treatment of the HPLC fraction containing Gal d 6.

DISCUSSION

α-Livetin (Gal d 5) is the only allergen identified in the egg yolk thus far (*4*). In our study, 5 of 27 egg allergy patients detected a new egg yolk allergen Gal d 6, which we have characterized. It is important to emphasize that 3 of these 5 patients were negative to Gal d 5 by the ISAC determination, as shown in **Table 1** (patients 2, 3, and 4).

The new described Gal d 6 is heat-resistant but digestible by pepsin. It is possible to speculate that an allergen that could be degraded in the gastrointestinal tract after ingestion is responsible for milder symptoms than other pepsin-resistant allergens.

Gal d 6 is the yolk glycoprotein YGP42, a fragment of VTG-1. The VTG-derived proteins are the major yolk components; cleavage of VTG-1 and VTG-2 in the yolk produces apolipovitellins and phosvitins, which are components of the water-insoluble yolk granular lipoproteins. On the other hand, the C-terminal part of VTGs gives rise to yolk glycoproteins YGP40 and YGP42, which are major components of the yolk plasma (*8*).

Vitellogenins have previously implicated in fish roe allergy. The sturgeon vitellogenin was related to IgE-mediated allergic reactions and anaphylaxis with Beluga caviar (*9*, *10*). In this sense, IgE cross-reactivity between fish roe and chicken egg has been studied in a series of 27 patients with fish allergy. Two of these patients presented salmon roe anaphylaxis, and the authors of the study demonstrated cross-reactivity between salmon and herring roe

Heat treatment and reduction of the HPLC fraction containing Gal d 6

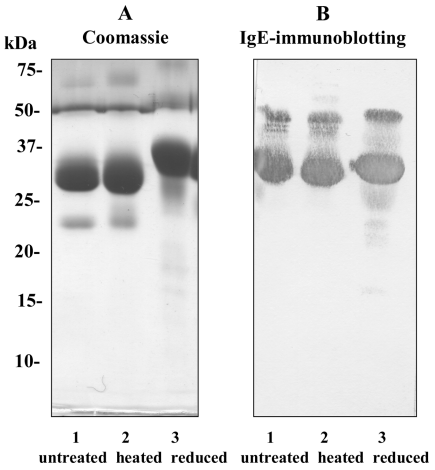

Figure 4. Heat treatment and reduction of the HPLC fraction containing Gal d 6. (A) SDS−PAGE and Coomassie staining. (B) IgE immunoblotting performed with the pool of sera. Lanes: (1) HPLC fraction untreated, (2) fraction heated at 100 °C for 15 min, and (3) fraction reduced with 5% 2-mercaptoethanol at 100 °C for 5 min.

Digestion of the HPLC fraction containing Gal d 6

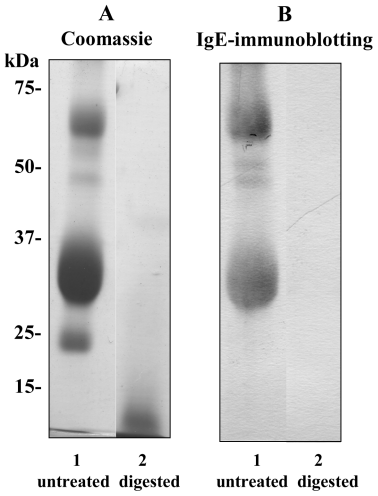

Figure 5. Digestion of the HPLC fraction containing Gal d 6. (A) SDS−PAGE and Coomassie staining. (B) IgE immunoblotting performed with the pool of sera. Lanes: (1) HPLC fraction untreated and (2) fraction digested with simulated gastric fluid.

but not between salmon roe and chicken egg (*11*). The patients who suffered anaphylaxis reacted against a protein identified as a fragment of the vitellogenin for its similarity with the rainbow trout vitellogenin. In fish roes, the allergenic fragment of the vitellogenin is the β component, which is a common major allergen in salmonid fish-roe-induced hypersensitivity (*12*, *13*). Teleost fish vitellogenin has the β component in the C-terminal region of the molecule, whereas bird vitellogenin has the yolk glycoproteins (YGP42) at this C-terminal region. There are important differences between the amino acid sequences of the β component and the yolk glycoproteins. In an analysis performed with the program LALING at http://www.ch.embnet.org, with the amino acid sequences of YGP42 and salmon β component, we found 31% sequence identity between them. This low identity explains the lack of cross-reactivity between fish roes and chicken egg.

IgE-immunoblotting-inhibition assay

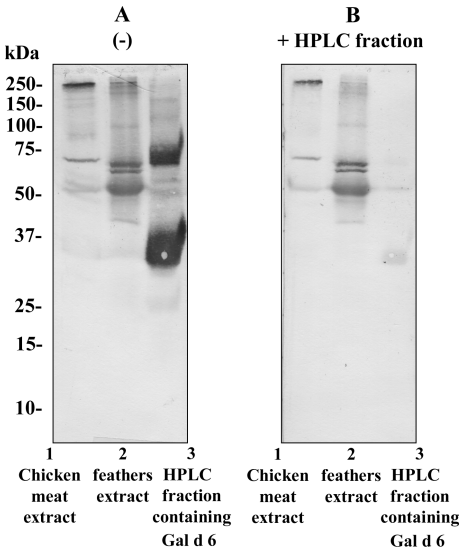

Figure 6. IgE-immunoblotting-inhibition assay performed with the pool of sera. (A) No inhibition. (B) Inhibition with the HPLC fraction containing Gal d 6. Lanes: (1) chicken meat extract, (2) feathers extract, and (3) HPLC fraction containing Gal d 6.

In conclusion, we report that a fragment of the VTG-1 precursor is the new egg yolk allergen named Gal d 6.

ABBREVIATIONS USED

ImmunoCAP-ISAC, immuno solid-phase allergen chip microarray-based IgE detection; PBS, phosphate-buffered saline (pH 7.5); PVDF, polyvinylidene difluoride; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VTG-1, vitellogenin-1; YGP42, yolk glycoprotein 42.

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LITERATURE CITED

(1) Mine, Y.; Yang, M. Recent advances in the understanding of egg allergens: Basic, industrial, and clinical perspectives. *J. Agric. Food Chem.* **2008**, *56*, 4874–4900.

(2) Añibarro, B.; Seoane, F. J.; Mugica, M. V. Involvement of hidden allergens in food allergic reactions. *J. Invest. Allergol. Clin. Immunol.* **2007**, *17*, 168–172.

(3) Szépfalusi, Z.; Ebner, C.; Pandjaitan, R.; Orlicek, F.; Scheiner, O.; Boltz-Nitulescu, G.; Kraft, D.; Ebner, H. Egg yolk α-livetin (chicken serum albumin) is a cross-reactive allergen in the bird-egg syndrome. *J. Allergy Clin. Immunol.* **1994**, *93*, 932–942.

(4) Quirce, S.; Marañón, F.; Umpierrez, A.; de las Heras, M.; Fernández-Caldas, E.; Sastre, J. Chicken serum albumin (Gal d 5) is a partially heat-labile inhalant and food allergen implicated in the bird-egg syndrome. *Allergy* **2001**, *56*, 754–762.

(5) Rodriguez-Perez, R.; Crespo, J. F.; Rodriguez, J.; Salcedo, G. Profilin is a relevant melon allergen susceptible to pepsin digestion in patients with oral allergy syndrome. *J. Allergy Clin. Immunol.* **2003**, *111*, 634–639.

(6) Moneo, I.; Caballero, M. L.; Gomez, F.; Ortega, E.; Alonso, M. J. Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. *J. Allergy Clin. Immunol.* **2000**, *106*, 177–182.

(7) Moneo, I.; Audicana, M. T.; Alday, E.; Curiel, G.; del Pozo, M. D.; Garcia, M. Periodate treatment of *Anisakis simplex* allergens. *Allergy* **1997**, *52*, 565–569.

(8) Mann, K.; Mann, M. The chicken egg yolk plasma and granule proteomes. *Proteomics* **2008**, *8*, 178–191.
(9) Untersmayr, E.; Focke, M.; Kinaciyan, T.; Poulsen, L. K.; Boltz-Nitulescu, G.; Scheiner, O.; Jensen-Jarolim, E. Anaphylaxis to Russian Beluga caviar. *J. Allergy Clin. Immunol.* **2002**, *109*, 1034–1035.
(10) Perez-Gordo, M.; Sanchez-Garcia, S.; Cases, B.; Pastor, C.; Vivanco, F.; Cuesta-Herranz, J. Identification of vitellogenin as an allergen in Beluga caviar allergy. *Allergy* **2008**, *63*, 479–480.
(11) Kondo, Y.; Kakami, M.; Koyama, H.; Yasuda, T.; Nakajima, Y.; Kawamura, M.; Tokuda, R.; Tsuge, I.; Urisu, A. IgE cross-reactivity between fish roe (salmon, herring and pollock) and chicken egg in patients anaphylactic to salmon roe. *Allergol. Int.* **2005**, *54*, 317–323.
(12) Hiramatsu, N.; Hiramatsu, K.; Hirano, K.; Hara, A. Vitellogenin-derived yolk proteins in a hybrid sturgeon, bester (*Huso huso* × *Acipencer ruthenus*): Identification, characterization and course of proteolysis during embryogenesis. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2002**, *131*, 429–441.
(13) Shimizu, Y.; Nakamura, A.; Kishimura, H.; Hara, A.; Watanabe, K.; Saeki, H. Major allergen and its IgE cross-reactivity among salmonid fish roe allergy. *J. Agric. Food Chem.* **2009**, *57*, 2314–2319.

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