

Identification of IgE-Binding Peptides in Hen Egg Ovalbumin Digested *In Vitro* with Human and Simulated Gastrointestinal Fluids

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S Supporting Information

ABSTRACT: The digestibility of the major egg allergen ovalbumin (OVA, Gal d 2) with human and simulated digestive fluids was assessed. Degradation of OVA was faster when treated with human fluids, particularly following duodenal digestion, leading to gastrointestinal digests with lower IgE binding. Gastric digestion with both systems yielded 52 identical cleavage sites and a similar peptide pattern with 47 peptides in common. Subsequent duodenal digestion showed that the human fluid released fewer and shorter peptides. Several high-frequency IgE-binding epitopes were detected among the fragments of molecular mass lower than 3 kDa identified in the digests: OVA (141–154) and OVA (164–176) in the gastrointestinal digests produced with human fluids; and OVA (125–134), OVA (159–172), OVA (141–154), OVA (188–198), OVA (326–336), and OVA (370–385) in the gastrointestinal digests produced with simulated fluids. The high binding frequency of the fragment OVA (370–385), which reacted with 80% of the sera from allergic patients used, was noteworthy.

KEYWORDS: egg allergy, ovalbumin, *in vitro* digestion, human digestive fluids, IgE, epitope

INTRODUCTION

Egg allergy is one of the most frequent food allergies in children below the age of three, with common symptoms frequently involving the skin as well as the gut and, in more severe cases, anaphylaxis.¹ A meta-analysis of double-blind placebo-controlled food challenges confirmed a prevalence of >1.7%,² with most children not outgrowing their egg allergy by school age but, instead, showing a long progression to egg allergy resolution.³

Ovalbumin (OVA, Gal d 2), the most abundant protein in egg white (up to 54% w/w of the protein content), is considered one of the dominant allergens.⁴ It is a phosphoglycoprotein with a molecular mass of 45 kDa, the sequence of which (with 385 amino acids) includes six cysteines, with a single disulfide bond between Cys₇₃ and Cys₁₂₀, a glycosylation site at Asn₂₉₂, and two phosphorylation sites at Ser₆₈ and Ser₃₄₄. As many other allergenic proteins, OVA partially resists hydrolysis with pepsin and pancreatic enzymes, an aspect that could contribute to its ability to sensitize or elicit allergic reactions.⁵

So far, studies investigating the gastrointestinal stability of OVA have been performed *in vitro* by the use of enzymes of bovine or porcine origin as part of different digestion models, ranging from simple one-step hydrolyses^{6,7} to advanced physiologically relevant systems in which subsequent gastric and duodenal digestions are conducted under conditions that mimic the *in vivo* processes in infants and adults.^{8,9} These latter studies have highlighted the effects that the enzyme to substrate ratio, pH, and concentration of physiological surfactants, such as phosphatidylcholine and bile salts, exert on the gastrointestinal stability of OVA and their influence in the resulting immunoreactivity of the digests. However, these relevant parameters regarding digestion, such as enzyme activity, volume of digestive juices secreted, pH, or surfactant level, vary widely among individuals and also with the type and amount of food

ingested and the time of the day, making *in vivo* conditions very difficult to simulate.¹⁰ In addition, human gastrointestinal juices contain complex mixtures of enzymes, which could present either broader or different specificities or vary in their functional parameters with respect to purified commercial enzymes from animal sources.¹¹ In the present work, the digestibility of OVA using human gastric and duodenal juices was assessed, the peptides released were characterized, and the IgE-binding properties of the most relevant digestion products were evaluated. Digestions were also conducted with simulated fluids for comparative purposes. This allowed the identification of relevant IgE-binding epitopes that resisted digestion in both systems and could contribute to the allergenic potential of the protein.

MATERIALS AND METHODS

Human Digestive Fluids. Human gastric and duodenal fluids were collected by gastroscopy of four different patients without any gastrointestinal pathology at the Hospital Universitario La Paz (Madrid, Spain) after 8 h of fasting. Gastric and duodenal fluids were separately pooled and frozen at –20 °C before use. Pepsin activity of the pooled gastric fluid (pH 2.13) was 728.9 U/mL. Trypsin and chymotrypsin activities of the duodenal fluid (pH 7.8) were 167.2 and 2.8 U/mL, respectively, with a bile salt concentration of 0.7 mM.¹²

Human Sera. Individual serum samples from 22 children with proven allergy to egg white proteins were kindly donated by the Hospital Infanta Sofía (San Sebastián de los Reyes, Madrid, Spain). The diagnosis of IgE-mediated egg allergy was made on the basis of objective symptoms of an acute reaction after egg ingestion, together with evidence of specific IgE antibodies against egg (Table S1 in the

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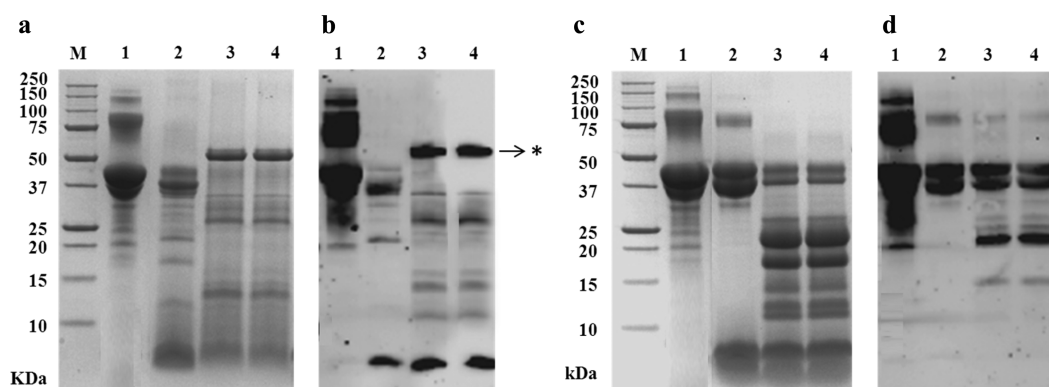


Figure 1. SDS-PAGE patterns (a, c) and Western blotting (b, d) of OVA after in vitro gastric and gastroduodenal digestions with human (a, b) and simulated (c, d) fluids. Electrophoresis was performed using Bis-Tris 12% acrylamide gel with reducing agents. Lanes: M, molecular mass marker (Precision Plus Protein standards (Bio Rad), ranging in molecular mass from 10 to 250 kDa; 1, OVA; 2, 60 min gastric digest; 3, gastroduodenal digest (60 min of gastric followed by 30 min of duodenal digestion); 4, gastroduodenal digest (60 min of gastric followed by 60 min of duodenal digestion). *, IgE-binding fragment originally present in the human duodenal fluid.

Supporting Information). Serum samples from two nonallergic donors were also used.

In Vitro Digestions. In vitro gastric and duodenal digestions with simulated fluids, based upon the procedure described by Moreno et al.,¹³ were performed as described in Benedé et al.,¹² using albumin (EC232-692-7) from chicken egg white grade V (Sigma-Aldrich, St. Louis, MO, USA). Gastric digestions were conducted at pH 2.0 for 60 min at 37 °C, with 182 U/mg OVA of porcine pepsin. Duodenal digestions were conducted on the 60 min gastric digests, readjusted to pH 6.5, and stopped after either 30 or 60 min at 37 °C with Bowman–Birk trypsin–chymotrypsin inhibitor. The final composition of the simulated duodenal mixture was 3.9 mg/mL OVA, 3.3 mg/mL phosphatidylcholine, 7.4 mM bile salts (BS; sodium glycodeoxycholate and sodium taurocholate), 7.6 mM CaCl₂, and 20.3 mM Bis-Tris, with pancreatic porcine lipase (24.7 U/mg OVA), pancreatic porcine colipase (1:895 mg/mg OVA), pancreatic bovine trypsin (34.5 U/mg OVA), and pancreatic bovine α -chymotrypsin (0.44 U/mg OVA), all from Sigma-Aldrich.

In vitro protein digestion with human fluids, mimicking gastric and duodenal phases, followed the method of Benedé et al.¹² OVA was mixed with the pool of human gastric fluids at pH 2.1 at a pepsin to OVA ratio of 182 U/mg. After 1 h of incubation at 37 °C, the pH was adjusted to pH 6.5 with 1 M NaHCO₃, and the pool of human duodenal fluids was added, so that the enzyme to substrate ratio was 34.5 U/mg trypsin and 0.49 U/mg chymotrypsin. The reaction was stopped after 30 and 60 min with Bowman–Birk trypsin–chymotrypsin inhibitor.

SDS-PAGE Analyses. OVA and its hydrolysates were diluted to 1 mg/mL in sample buffer containing 2% (w/v) SDS and 5% (v/v) β -mercaptoethanol and heated at 95 °C for 4 min. Samples were analyzed on Precast Criterion XT 12% Bis-Tris gels, and electrophoretic separations were carried out at 150 V using XT-MES as running buffer (Bio-Rad, Richmond, CA, USA) in the Criterion cell (Bio-Rad). Gels were stained with Bio-Safe Coomassie G-250 (Bio-Rad).

Western Blotting. After SDS-PAGE separation, the gels (loaded with 50 μ L of a 1 mg/mL sample solution) were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min and subjected to semidry transfer in a Trans-Blot SD (Bio-Rad) for 30 min at 18 V. Transfer effectiveness to the membrane was assessed by staining with Ponceau reagent. Western blotting was conducted as explained in Benedé et al.,¹² using pooled sera from six children with egg allergy (patients 7–12, Table S1 in the Supporting Information), diluted 1/50 in Tris buffer saline (50 mM Tris, 150 mM NaCl, pH 7.6, TBS), containing Tween 20 (0.05% v/v) (TBTS) and BSA 0.1% (w/v).

Human IgE Binding by Inhibition ELISA. Human IgE binding of OVA and its digests was assessed by inhibition ELISA as previously

reported Jiménez-Saiz et al.,¹⁴ using individual sera from patients 1–6 (Table S1 in the Supporting Information).

Peptide Sequencing by RP-HPLC-MS/MS. RP-HPLC-MS/MS analyses of the digested samples were performed as described in Benedé et al. on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) with an RP318 C18 column (250 $\times$ 4.6 mm, Bio-Rad). The HPLC system was connected online to an Esquire 3000 quadrupole ion trap (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization source. Mass spectra were recorded over the range of m/z 100–3000 for MS spectra. Tandem MS was applied by using a signal threshold of 10000, a window width of 4.0 m/z , and a voltage ramp from 0.3 to 2 V. Using Data Analysis (version 4.0, Bruker), the m/z spectral data were processed and transformed to spectra representing mass values. BioTools (version 3.1, Bruker) was used to process the MS(n) spectra and perform peptide sequencing.

Dot-Blot. Twenty-seven peptides representative of those identified in the in vitro gastroduodenal digests were commercially synthesized (JPT peptide Technologies, Berlin, Germany). Among the peptides selected, those with 8 and 9 amino acids were extended to 10 amino acids with the preceding and following amino acids of the protein chain. Peptides, at a concentration of 1 mM, were diluted 1/3 in TBS. Then, 1 μ L of each peptide was spotted onto the membrane and allowed to dry.

The nitrocellulose membranes were conditioned in transfer buffer for 20 min, and 1 μ L from each peptide was spotted onto the membrane and allowed to dry. Then, the nitrocellulose membranes were blocked with TBST containing 1% w/v BSA for 60 min and washed with TBST. Immunolabeling with 10 individual serum samples (patients 13–22, Table S1 in the Supporting Information) was conducted as described for the Western blotting experiments.

Ethical Concerns. All human samples in this study have been obtained with written consent of the subjects. The researchers involved in the process handled and used such material sensitively and responsibly. All procedures received ethical approval by CSIC.

RESULTS

In Vitro Digestion of Ovalbumin with Human and Simulated Fluids. Figure 1 shows the SDS-PAGE patterns (Figure 1a,c) and Western blots (Figure 1b,d) of OVA, after being subjected to two-step digestion protocols, mimicking the stomach and duodenal stages, by using human and simulated fluids. In general terms, after 60 min of incubation with human gastric fluid a remarkable amount of OVA remained undigested, whereas the intact protein virtually disappeared following the subsequent duodenal phase (Figure 1a). Gastric and, particularly, duodenal hydrolysates were much faster with

human fluids as compared with simulated fluids, with the appearance of more degradation products after the gastric phase, of ~40, 22, 18, 13, and 5 kDa, and fewer degradation products, within the range from ~25 to 5 kDa, after the duodenal phase (Figure 1a,c). As a result, there was less IgE binding from intact OVA and its 40 kDa fragment following gastroduodenal digestion with human fluids. Remarkably, a strong IgE-binding band of ~21 kDa, which formed during duodenal digestion with simulated fluid, was absent from the corresponding digests produced with human fluid, which, in turn, yielded lower molecular mass immunoreactive degradation products (Figure 1b,d). It should be mentioned that no differences in the degradation patterns were detected when 30 and 60 min of duodenal digestion were compared with either system.

In consonance with these results, inhibition ELISA (Figure 2) showed that the gastric and gastroduodenal digests produced

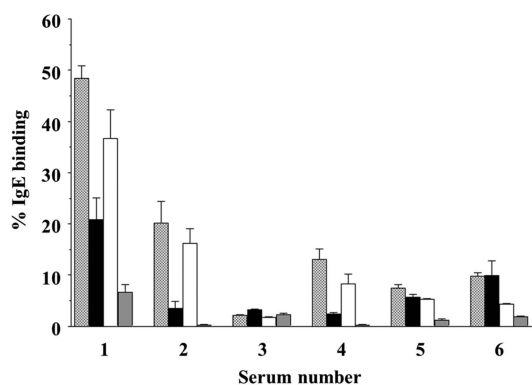


Figure 2. IgE binding of OVA, expressed as percentage of the EC_{50} of the intact protein, as estimated by inhibition ELISA with sera from six allergic patients (patients 1–6, Table S1 in the Supporting Information), after in vitro gastric digestion with simulated (gray dotted bars) and human (black bars) fluids and gastroduodenal digestion with simulated (white bars) and human (gray bars) fluids.

with human fluids exhibited the lowest IgE binding to three individual sera from egg-allergic patients (patients 1, 2, 4, and 5, Table S1 in the Supporting Information). With another two sera (patients 3 and 6, Table S1 in the Supporting Information), IgE reactivities toward the gastric digests produced by both systems were very similar, although the lowest IgE binding always corresponded to the human gastroduodenal digests. No IgE binding to sera from non-allergic donors was detected.

Identification of IgE-Binding Epitopes. Figure 3 shows the sequences of the peptides identified by RP-HPLC/MS-MS in the gastric (60 min) and gastroduodenal (30 min) digests of OVA. No peptides covering the first 50 amino acids of the protein sequence were found in either model. Following gastric digestion with human and simulated fluids, 84 and 118 peptides were identified, respectively, with 47 peptides in common. There were 52 identical pepsin cleavage sites in both systems, including Leu (83, 105, 114, 124, 183, 216, 232, 242, 252, 255, 282, 312, 321, and 365), Glu (70, 108, 143, 202, 248, 266, 274, 336, 340, and 357), Phe (59, 99, 134, 198, 217, and 261), Ala (141, 171, 177, 187, 195, 332, and 345), Met (210, 211, 222, 239, and 241), and Gln (89, 140, 203, and 325). Eighty-one of the 118 sequences identified after gastric hydrolysis with simulated fluid resisted subsequent duodenal hydrolysis, appearing as end products of digestion. Most of the remaining

peptides found in the duodenal digests produced with simulated fluid could be products of hydrolysis of the low molecular mass peptides previously released by pepsin action, except for the C-terminal sequence HIATNAVLFGRGCVS, resulting from the trypsin cleavage at Lys369. In addition, in this digestion system, breakdown at the trypsin cleavage sites Lys (55, 92, 226, 228, 277, 279, 286, and 322) and Arg (50, 84, 104, 142, 158, and 276) was detected. Only 47 peptides were identified after gastrointestinal digestion with human fluids. In this case, there were several fragments that could not arise from the peptides previously identified after gastric digestion and, thus, they could derive from the hydrolysis of either intact OVA or the big proteolysis fragments that resisted pepsin hydrolysis. Only six peptides were coincident with those produced with simulated duodenal fluid and, in general terms, the human fluid gave rise to less numerous and shorter peptides. Hydrolysis by trypsin at Lys290 and Arg (50, 158, 284, and 359) was detected (Figure 3 and Table S2 in the Supporting Information).

Twenty-seven peptides, ranging from 10 to 16 amino acids, representative of those found in the gastroduodenal digests with simulated and human fluids were chemically synthesized, and their IgE binding to 10 individual sera from egg-allergic patients was assessed by dot-blot. As shown in Figure 3, all peptides were recognized by, at least, one serum, except for two fragments, OVA (299–308) and (351–360), which did not react with any of the patient's sera employed in the study. A high IgE binding corresponded to the region comprised between positions 125 and 176, because 40–60% of the patients reacted to the synthetic peptides OVA (125–134), OVA (135–144), OVA (141–154), OVA (159–172), and OVA (164–176). The fragments OVA (188–198) and OVA (326–336) were recognized by sera from 60 and 50% of the patients, respectively. The highest IgE binding corresponded to the C-terminal fragment OVA (370–385), which reacted with 80% of the sera.

DISCUSSION

Proteolyses of OVA with human and simulated digestive fluids were compared. The results obtained with simulated gastric fluid agree with previously published information showing that OVA is quite stable to porcine pepsin at an E:S ratio of 182 U/mg and pH 2.0.⁹ A digestion pattern with two main degradation products, of 40.1 and 4.1 kDa, has been described,^{7,15,16} the first one probably corresponding to the fragment Ala23–Pro385.⁹ Moreover, Dupont et al.,⁸ using a gastric model under similar conditions (E:S 182 U/mg, pH 2.5) reported, in addition to smaller peptides of ~5400 and 4600 Da, the appearance of digestion products of ~21700 and 17800 Da, which are analogous to those found in the gastric digests produced with human fluid in the present study (Figure 1a). In any case, and although there was intact protein remaining after the gastric phase in both systems, degradation of OVA was faster when digested with human than with simulated fluid (Figure 1a,c), an aspect that was already observed with β -CN.¹² This suggests a more efficient performance of pepsin of human origin despite specificity being similar, as judged by the existence of 52 identical cleavage sites and a similar peptide pattern with 47 peptides in common (Figure 3 and Table S2 in the Supporting Information). It is noteworthy that the in vitro digestion with human gastric juice released peptides with antihypertensive activity that either act as inhibitors of angiotensin-I-converting enzyme or exert a vasodilator action, such as FRADHPFL, YAEERYPIL, YRGGLEPINF, ESSINF, or YQIGL, which were



Figure 3. Peptide sequences, identified by RP-HPLC-MS/MS, in the gastric (60 min) and subsequent duodenal digests of OVA (60 min of gastric followed by 30 min of duodenal digestion), and IgE binding, estimated by dot-blot with 10 sera of egg-allergic patients (patients 13–22, Table S1 in the Supporting Information), of 27 synthetic peptides selected within the peptides identified in the simulated digestions.

previously produced from OVA by hydrolysis with porcine pepsin for 3 h and also appeared in the hydrolysates produced with simulated fluid.^{17,18}

Following in vitro duodenal hydrolysis with trypsin (E:S 40 U/mg) and chymotrypsin (E:S 0.5 U/mg), the appearance of digestion products of ~20700, 17000, 12500, 10400, and 6500 Da has been described,⁸ which could coincide with those formed during duodenal digestion with the simulated fluid in our study (Figure 1c). These degradation products were absent from the gastroduodenal digests produced with human duodenal fluid (Figure 1a), as this caused a much more extensive proteolysis of the gastric digests than the simulated fluid. A difference between both duodenal digestion systems laid in the concentration of BS, which was much lower in the pool of human fluids, probably due to the prolonged fasting that preceded their extraction. It has been found that BS, at the concentration present in the simulated duodenal fluid, which is typical of a fed state, considerably increase the proteolysis of OVA by pancreatic enzymes.⁹ However, despite the lower concentration of bile acids in the human duodenal fluid, it promoted very effectively the proteolysis of intact OVA and of the peptides generated from it by pepsin hydrolysis. This could be attributed to differences in the specificity between human trypsin and α -chymotrypsin and their bovine counterparts, as well as to the presence of peptidases in the human pancreatic extracts.¹⁹ In fact, it has been reported that intact OVA and its 40 kDa fragment partially resist hydrolysis by porcine trypsin and chymotrypsin, but they are more readily degraded by an enzyme preparation from the pig pancreas gland that, in addition to both proteases, contains amino and carboxypeptidases.⁹ Furthermore, comparison of the peptide patterns produced by both systems showed that in vitro gastroduodenal digestion with human fluids released fewer and shorter peptides, revealing a most extensive liberation of free amino acids from the short fragments previously formed during the gastric phase (Figure 3 and Table S2 in the Supporting Information).

In accordance with the least extensive proteolytic degradation and although there were, as expected, differences in donor's specificity,²⁰ inhibition ELISA with individual sera from allergic patients revealed a comparatively higher IgE reactivity to the gastroduodenal digests produced with simulated fluids (Figure 2). This is probably because of the presence, in addition to intact OVA and its 40 kDa fragment, of a strong IgE-binding band of ~21 kDa and of other minor immunoreactive degradation products (Figure 1d). This pattern is similar to that previously observed after in vitro digestion of egg white with simulated gastroduodenal fluids that allowed the identification of several IgE-binding fragments with molecular masses in the range of 35–20 kDa, as well as others of 15 and 11 kDa, which derived from the hydrolysis of OVA.²¹ The corresponding digests produced with human fluids showed fainter and lower molecular mass IgE-binding degradation products and the virtual absence of intact protein and its high molecular mass fragment (Figure 1b).

In addition, several IgE-binding epitopes were detected among the peptide fragments of molecular mass <3 kDa identified in the digests by RP-HPLC-MS/MS (Figure 3). Considering the binding frequency to human sera of the 27 synthetic peptides that mimicked the products of the gastrointestinal digestion, 4 main areas of the protein were recognized by >40% of the sera from egg-allergic patients: 125–176, 188–198, 326–336, and 370–385.

Within the first one, 125–176, at least five distinct epitopes were found, including OVA (125–134) and OVA (159–172), as well as OVA (141–154) and OVA (164–176), which were present in the gastrointestinal digests produced with simulated and human fluids, respectively. The peptides OVA (188–198) and OVA (326–336), found in the gastrointestinal digests produced with simulated fluids that bound, respectively, 60 and 50% of the sera from allergic patients, are related to previously defined allergenic epitopes. OVA (188–198) partially coincides with one of the five dominant human IgE-binding epitopes described by Mine and Rupa,²⁰ (38–49, 95–102, 191–200, 243–248, and 251–260), whereas OVA (326–336) is comprised within the fragment 323–339, considered an immunodominant B and T cell epitope.²² The discovery of this latter epitope gave rise to a transgenic mouse carrying the MHC class II restricted rearranged T cell receptor transgene, Tg(DO11.10)10Dlo, specific for OVA (323–339), broadly used in in vitro studies of T cell differentiation and in vivo studies of tolerance and immune responses.²³ Finally, the C-terminal fragment, OVA (370–385), showed the highest binding frequency, reacting with 80% of the sera. Human IgE binding has been demonstrated to broader areas of the C-terminal region of OVA, such as 301–385²⁴ and 347–385.²⁵ On the other hand, the existence of, at least, two epitopes within the shorter fragment OVA (367–385) was implied from a positive basophil stimulation in one allergic patient.²⁵ Interestingly, the peptide OVA (375–384) was recognized by IgE from orally sensitized BALB/c mice but not from mice submitted to intraperitoneal or subcutaneous immunization,²⁶ which suggests that, even if it is protected within the native structure (Figure 4), it can be specifically exposed as a result of

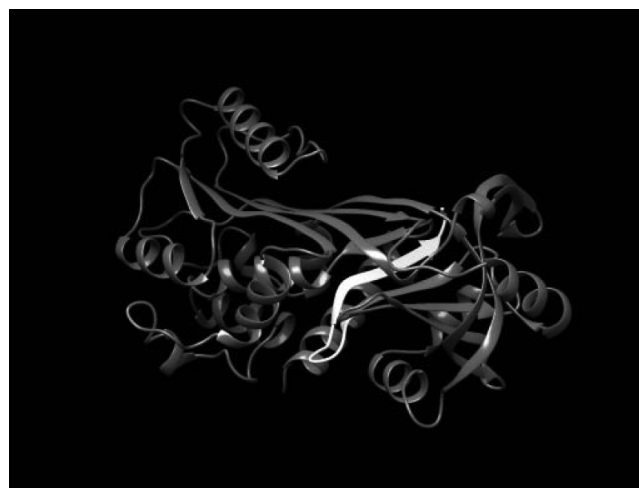


Figure 4. Representation of the IgE-binding epitope OVA (370–385) in the 3D structure of the protein obtained from the RCSB Protein Data Bank (PDB ID 1OVA) with the UCSF Chimera package.

digestion. This immunoreactive fragment was present in the gastrointestinal digests produced with simulated fluids, but it could not be found in the gastrointestinal digests produced with human fluids.

In agreement with a previous study comparing the in vitro digestibility of bovine β -casein with human and simulated digestive fluids¹² and despite human fluids leading to a much faster proteolytic degradation, a high degree of similarity within the peptide patterns arising from gastric digestion was detected, which was not so evident following duodenal digestion, likely

because of the presence of active exopeptidases in the human duodenal extract. The accumulation of large degradation fragments and low molecular mass peptides with IgE-binding properties could contribute to the stronger residual IgE-binding capacity of the gastroduodenal digests produced with simulated fluids as compared with those produced with human fluids, although the gastrointestinal digests produced with human fluids also contained IgE-binding epitopes that could play a role in the allergenic potential of OVA. Among the epitopes released, OVA (370–385) seemed to be of special importance, as it was able to bind IgE of 80% of the sera employed in the study. More research is needed to elucidate the biological activity of this fragment.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

OVA, ovalbumin; BS, bile salts; BSA, bovine serum albumin

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Table S1. Specific IgE levels (kU/L) towards egg white, yolk and ovalbumin of the sera used in the inhibition ELISA (patients 1 to 6), Western blotting (patients 7 to 12) and dot blot (patients 13 to 22).

Patient	Age (years)	IgE levels (kU/L)		
		White	Yolk	OVA
1	6	40.4	15.8	16.7
2	6	>100	>100	>100
3	7	20	1.67	25.1
4	3	75.9	14.1	65.6
5	4	50.9	16.2	57.7
6	7	41.7	25.7	7.29
7	12	14.7	6.07	2.16
8	3	1.62	0.82	2.89
9	9	11.5	7.25	9.35
10	7	41.7	25.7	7.29
11	11	13.1	6.78	8.33
12	3	>100	90.9	73.7
13	11	54.7	32.9	26
14	7	27.6	20.6	12.3
15	7	41.7	25.7	7.29
16	3	73.2	42.5	46.4
17	12	66	42.9	35.6
18	9	92.7	63.8	76
19	7	>100	>100	70.9
20	3	>100	90.9	73.7
21	6	>100	>100	60.9
22	8	>100	>100	>100

Table S2. Peptide sequences identified by RP-HPLC-MS/MS in the gastric (60 min, G60) and gastroduodenal digests of OVA (60 min of gastric followed by 30 min of duodenal digestion, D30), with simulated and human fluids.

Calculated mass	Sequence	OVA Fragment	Simulated Fluids		Human Fluids	
			G60	D30	G60	D30
474,244	TQIN	51-54	-	+	-	+
1103,645	TQINKVVRF	51-59	+	-	+	-
874,539	INKVVRF	53-59	-	+	-	-
1327,798	INKVVRFDKLP	53-63	-	-	-	+
647,412	KVVRF	55-59	+	+	-	-
519,317	VVRF	56-59	+	+	-	-
677,386	RFDKL	58-62	+	-	-	-
1550,773	RFDKLPGFGDSIEA	58-71	-	-	-	+
1176,567	DKLPGFGDSIE	60-70	+	+	+	-
1247,604	DKLPGFGDSIEA	60-71	-	-	-	+
413,264	KLPG	61-64	+	-	-	+
1292,571	LPGFGDSIEAQCG	62-74	-	-	+	-
664,285	AQCGTSV	71-77	-	-	-	+
465,190	CGTSV	73-77	-	-	-	+
754,398	VNVHSSL	77-83	+	+	+	-
655,329	NVHSSL	78-83	+	+	-	-
1719,963	VHSSLRDILNQITKP	79-93	-	-	+	-
689,371	SSLRDI	81-86	-	-	-	+
629,350	RDILN	84-88	-	-	-	+
757,409	RDILNQ	84-89	+	+	+	-
943,534	DILNQITK	85-92	+	+	-	-
813,435	NQITKP	88-94	-	-	-	+
928,462	NQITKPND	88-95	-	-	-	+
1027,530	NQITKPNDV	88-96	-	-	+	+
1190,593	NQITKPNDVY	88-97	+	+	-	-
1277,625	NQITKPNDVYS	88-98	+	+	+	-
1424,694	NQITKPNDVYSF	88-99	-	-	+	-
686,360	ITKPND	90-95	-	-	-	+
785,429	ITKPNDV	90-96	+	+	+	+
948,492	ITKPNDVY	90-97	+	+	-	-
1035,524	ITKPNDVYS	90-98	+	+	+	-
672,345	TKPNDV	91-96	-	-	-	+
743,313	NDVYSF	94-99	-	-	+	-
829,386	DVYSFSL	95-101	-	-	-	+
792,450	FLASRL	99-105	+	-	-	-
645,381	SLASRL	100-105	+	+	+	-
445,265	LASRL	101-104	+	+	-	-
445,265	ASRL	102-105	-	-	+	-
381,154	YAE	106-108	+	+	-	-
829,361	YAEERY	106-111	+	+	+	-
1152,582	YAEERYPIL	106-114	+	+	+	-
1378,677	YAEERYPILPE	106-116	-	-	-	+
1215,614	AEERYPILPE	107-116	-	-	-	+
676,355	ERYPI	109-113	-	-	-	+
789,439	ERYPIL	109-114	+	+	+	-
1063,566	KELYRGGLE	122-130	+	-	-	-
1277,677	LYRGGLEPINF	124-134	+	-	-	-
693,345	YRGGLE	125-130	-	-	-	+
1164,593	YRGGLEPINF	125-134	+	+	+	-
740,418	RGGLEPI	126-132	-	-	-	+
854,461	RGGLEPIN	126-133	-	+	-	-
1001,530	RGGLEPINF	126-134	+	+	+	-
845,429	GGLEPINF	127-134	-	-	+	-
1416,689	LEPINFQTAAADQA	129-141	-	-	+	-
632,277	QTAADQ	135-140	+	+	+	+
859,415	QTAADQAR	135-142	-	+	-	-
988,458	QTAADQARE	135-143	+	+	+	-
688,314	ADQARE	138-143	-	-	-	+
617,277	DQARE	139-143	-	+	-	-
801,435	ARELINS	141-147	+	+	+	-
987,514	ARELINSW	141-148	-	-	+	-
1645,806	ARELINSWVESQTN	141-154	-	-	-	+
730,398	RELINS	142-147	+	+	-	-
445,254	LINS	144-147	+	+	-	-
519,233	WVES	148-151	-	-	-	+
862,382	WVESQTN	148-154	-	+	-	-
919,404	WVESQTNG	148-155	+	+	+	-
733,325	VESQTNG	149-155	+	+	-	-
400,280	IIR	156-158	-	+	-	-
514,323	IIRN	156-159	-	-	+	-
1224,719	IIRNVLPSSV	156-166	+	-	-	-
1554,837	IIRNVLPSSVDSQ	156-169	+	-	-	-
1726,922	IIRNVLPSSVDSQTA	156-171	+	-	+	-
1857,962	IIRNVLPSSVDSQTAM	156-172	+	-	-	-
812,451	RNVLPQS	158-164	-	-	-	+
1344,652	NVLPSSVDSQTA	159-171	-	-	+	-
1475,693	NVLPSSVDSQTAM	159-172	-	+	-	-
656,350	NVLQPS	159-164	-	-	-	+
542,307	VLQPS	160-164	-	-	-	+
728,407	VLQPSSV	160-166	+	+	-	-
843,434	VLQPSSVD	160-167	-	-	+	-
1058,525	VLQPSSVDSQ	160-169	+	+	-	-
1230,609	VLQPSSVDSQTA	160-171	-	-	+	-
1361,650	VLQPSSVDSQTAM	160-172	+	+	-	-
631,282	QPSSVD	162-167	-	-	+	-
1349,650	SSVDSQTAMVLVN	164-176	-	-	-	+
651,254	DSQTAM	167-172	+	+	-	-
675,432	IVFKGL	178-183	+	-	+	-
861,512	IVFKGLW	178-184	+	-	+	-
830,465	KGLWEKA	181-187	+	-	-	-
532,265	WEKA	184-187	+	+	-	-
1841,830	WEKAFKDEDQTAMPF	184-198	+	-	-	-
753,318	FKDEDIT	188-193	-	-	+	+
952,414	FKDEDITQA	188-195	+	+	+	-
1180,507	FKDEDITQAMP	188-197	+	+	-	-
1327,576	FKDEDITQAMPF	188-198	+	+	+	-
1812,836	FKDEDITQAMPFRVTE	188-202	-	-	+	-
1940,894	FKDEDITQAMPFRVTEQ	188-203	-	-	+	-
878,432	MPFRVTE	196-202	+	-	-	-
1006,491	MPFRVTEQ	196-203	+	-	+	-
1805,881	MPFRVTEQESKPVQM	196-210	+	-	-	-
875,450	PFRVTEQ	197-203	-	-	+	-
1577,787	FRVTEQESKPVQM	198-210	+	+	-	-
503,271	RVTE	199-202	+	+	-	+

631,329	RVTEQ	199-203	+	+	+	+
1171,620	RVTEQESKPV	199-208	-	-	-	+
1299,679	RVTEQESKPVQ	199-209	+	+	-	-
1430,719	RVTEQESKPVQM	199-210	+	+	-	-
1561,760	RVTEQESKPVQMM	199-211	+	+	-	-
686,360	QESKPV	203-208	-	-	-	+
945,459	QESKPVQM	203-210	+	+	+	-
7076,500	QESKPVQMM	203-211	+	+	-	-
558,302	ESKPV	204-208	-	-	-	+
686,360	ESKPVQ	204-209	+	+	-	-
817,401	ESKPVQM	204-210	+	+	+	-
948,441	ESKPVQMM	204-211	+	+	-	-
723,363	MYQIGL	211-216	-	-	+	-
592,322	YQIGL	212-216	+	+	+	-
748,423	GLFRVAS	215-221	-	-	-	+
709,358	FRVASM	217-222	+	+	-	-
562,290	RVASM	218-222	+	+	+	-
693,301	VASMASE	219-225	-	-	+	-
1193,615	VASMASEKMKI	219-229	-	-	+	-
692,353	ASEKMK	223-228	+	+	+	-
805,437	ASEKMKI	223-229	-	-	+	-
1160,648	ASEKMKILEL	223-232	+	-	+	-
631,409	KMKIL	226-230	-	-	+	-
745,441	MKILEL	227-232	-	+	+	-
486,306	ILEL	229-232	-	+	-	-
709,311	PFASGTM	233-239	+	+	+	-
927,383	PFASGTMSM	233-241	+	+	+	-
797,454	LVLLPDE	242-248	+	+	+	-
896,522	LVLLPDEV	242-249	-	-	-	+
684,370	VLLPDE	243-248	+	+	-	-
1040,576	VLLPDEVSGL	243-252	+	+	+	-
828,423	LPDEVSGL	245-252	+	+	-	-
631,318	VSGLEQ	249-254	-	-	-	+
574,297	ESIIN	256-260	-	-	-	+
721,365	ESIINF	256-261	+	+	+	-
618,323	EKLTE	262-266	+	+	+	+
804,402	EKLTEW	262-267	+	+	-	-
1193,557	EKLTEWTSN	262-271	-	-	+	-
823,335	TewTSSN	265-271	-	-	+	-
593,245	WTSSN	267-271	+	+	+	-
1307,727	MEERKIKVYL	273-282	+	-	-	-
1063,603	EERKIKVY	274-281	+	-	-	-
1176,687	EERKIKVYL	274-282	+	-	-	-
672,429	ERKIK	275-279	+	+	-	-
934,560	ERKIKVY	275-281	+	-	-	-
1047,644	ERKIKVYL	275-282	+	-	+	-
387,285	KIK	277-279	-	+	+	-
649,417	KIKVY	277-281	-	+	-	-
762,501	KIKVYL	277-282	-	-	+	-
521,322	IKVY	278-281	-	+	-	-
634,406	IKVYL	278-282	-	+	-	-
887,560	IKVYLP	278-284	-	-	+	-
774,425	LPRMKM	282-287	+	-	-	-
661,341	PRMKM	283-287	+	-	-	-
666,272	MKMEE	285-289	-	-	-	+
794,367	MKMEEK	285-290	-	-	-	+
826,390	KMEEKY	286-291	-	+	-	-
698,295	MEEKY	287-291	-	+	-	-
567,254	EEKY	288-291	-	+	-	-
836,377	MAMGITDV	298-305	+	+	-	-
983,446	MAMGITDVF	298-306	+	-	-	-
852,405	AMGITDVF	299-306	-	+	-	-
781,368	MGITDVF	300-306	+	+	-	-
650,328	GITDVF	301-306	+	+	-	-
823,408	VFSSANL	305-312	+	+	-	-
725,323	FSSADL	306-312	+	+	+	-
1079,510	SSSANL SGISSA	307-318	+	+	-	-
736,324	SGISSAES	313-320	-	-	-	+
849,408	SGISSAESL	313-321	+	+	-	-
803,439	ESLKISQ	319-325	+	+	-	-
1460,774	ESLKISQAVHAAHA	319-332	+	-	-	-
1945,986	ESLKISQAVHAAHAEINE	319-336	+	-	-	-
3243,612	ESLKISQAVHAAHAEINE	319-350	+	-	-	-
587,365	LKISQ	321-325	-	-	+	-
459,306	LKIS	321-324	-	-	-	+
1729,911	LKISQAVHAAHAEINE	321-336	-	-	+	-
781,445	KISQAVH	322-328	-	+	-	-
1131,615	KISQAVHAAHA	322-332	+	-	+	-
1616,827	KISQAVHAAHAEINE	322-336	+	-	+	-
2030,030	KISQAVHAAHAEINEAG	322-340	+	-	-	-
655,350	ISQAVH	323-328	-	+	-	-
675,346	AVHAAHA	326-332	+	+	+	-
1160,558	AVHAAHAEINE	326-336	+	+	+	-
1231,595	AVHAAHAEINEA	326-337	-	-	+	-
1573,760	AVHAAHAEINEAGRE	326-340	+	-	-	-
1986,987	AVHAAHAEINEAGREV	326-345	+	-	-	-
853,393	AAHAEINE	329-336	+	+	-	-
1266,596	AAHAEINEAGRE	329-340	+	+	-	-
1679,823	AAHAEINEAGREV	329-345	+	+	-	-
916,425	EINEAGRE	333-340	+	+	-	-
374,180	INE	334-336	-	-	-	+
844,441	AGREVVGSA	337-345	+	+	+	-
973,483	AGREVVGSAE	337-346	+	-	+	-
1315,637	AGREVVGSAEAGVD	337-350	+	+	-	-
902,435	VVGSAEAGVD	341-350	+	+	-	-
631,282	AEAGVDA	345-351	-	-	+	-
1628,743	VDAASVSEEFRADHP	349-363	-	-	+	-
691,303	AASVSEE	351-357	+	+	+	-
1750,832	ASVSEEFRADHPFLF	352-366	-	-	+	-
1130,551	EFRADHPFL	357-365	+	-	-	-
741,356	FRADHP	358-363	-	-	-	+
888,425	FRADHPF	358-364	-	+	-	-
1001,509	FRADHPFL	358-365	+	-	+	-
854,440	RADHPFL	359-365	+	+	-	-
438,187	ADHP	360-363	-	-	-	+
681,418	IKHIAT	368-373	+	+	-	-
795,461	IKHIATN	368-374	-	-	+	-
866,498	IKHIATNA	368-375	-	-	+	-
1730,893	HIATNAVLFFGRVCSP	370-385	-	+	-	+