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# Identification of an IgE Reactive Peptide in Hen Egg Riboflavin Binding Protein Subjected to Simulated Gastrointestinal Digestion

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**ABSTRACT:** Riboflavin binding protein (RfBP) is a minor protein in hen egg; its potential involvement in egg allergy has seldom been studied. The aim of this work was to investigate the IgE binding capacity of RfBP before and after simulated gastrointestinal digestion. It was shown that digestion of RfBP mainly occurred during the gastric phase. The protein fragments resulting from the subsequent duodenal phase remained linked through disulfide bonds. Both the intact protein and its digests were subjected to inhibition ELISA with sera obtained from patients allergic to egg. The results revealed significant IgE binding to intact RfBP, whereas the digests showed reduced but substantial IgE binding levels, with serum-to-serum variability. The RfBP digests were then subjected to immunoblot with allergic patients' sera, and the IgE-reactive peptides were further analyzed by MALDI-TOF/TOF mass spectrometry for sequence determination. The RfBP sequence 41−84 was identified as a novel IgE binding peptide in patients allergic to egg.

**KEYWORDS:** allergen, egg white, riboflavin binding protein, digestion, IgE binding

## INTRODUCTION

Allergy to egg is very common among food allergies, particularly in children, affecting 1.5−3.2% of this population.[1] There are well-known egg allergens, such as ovalbumin (*Gal d 2*), ovomucoid (*Gal d 1*), ovotransferrin (*Gal d 3*), and lysozyme (*Gal d 4*) in the egg white or α-livetin (*Gal d 5*) in the egg yolk and, recently, a fragment of the vitellogenin-1 precursor has been identified as another yolk allergen (*Gal d 6*).[2] The allergic response to individual egg proteins varies from patient to patient in terms of specificity and degree of sensitivity. In addition, many proteins in egg remain poorly characterized,[3] and as such, the allergenicity of minor egg proteins, either as sensitizing proteins or through cross-reactivity, has not been investigated yet.

Riboflavin binding protein (RfBP), also called riboflavin carrier protein, ovoflavoprotein, or flavoprotein, is known for its involvement in reproductive biology.[4] In hen egg, RfBP is present in both white and yolk in amounts of 0.8 and 0.3% (w/w protein), respectively.[5] It is a small acidic and heat-stable protein, and its molecular properties have been reviewed elsewhere.[6−8] It is composed of 219 amino acid residues, its sequence exhibits an unusual pyroglutamic residue at the N-terminal end, and it contains linked carbohydrates that account for ∼14% of its molecular mass (30−35 kDa), phosphate moieties that contribute to its p$I$ of ∼4.0, and nine disulfide bonds, which make it very stable to thermal denaturation. It has been previously reported as a minor allergen;[9] however, current literature on its allergenic potential remains scarce. Some studies have reported cross-reactivity between bovine caseins and RfBP,[10] and previous work in our laboratory, using sera obtained from human patients allergic to egg (unpublished), revealed that RfBP exhibited high IgE binding, as shown by indirect ELISA analysis. In the latter work, sera of allergic patients with high IgE levels, but no clinical signs of allergy to egg, showed no binding to RfBP, like normal individuals, suggesting that the IgE of the egg-allergic patients specifically bound RfBP. The allergenicity of RfBP should be taken into consideration, not only because egg products are widely distributed in foods but also because recent studies have proposed the use of RfBP as an inhibitor of bitterness,[11] resulting in new food applications where it could be found as a hidden allergen.

It is generally accepted that digestion of a protein along the gastrointestinal (GI) tract significantly alters its allergenicity and, therefore, it is relevant to assess the IgE binding properties of a protein in this context. In vitro conditions mimicking the GI environment have been optimized in the past decade to introduce variables aiming to approach the physiological process of gastrointestinal digestion.[12] Such in vitro models include gastric and subsequent duodenal steps, a better fit of the pH and the enzyme/substrate ratio (E:S), and incorporation of other important components, such as phospholipids or bile salts, and have been used to study egg allergens.[13,14]

To investigate the contribution of RfBP to the allergenicity of egg proteins, the aim of this study was to evaluate the IgE binding capacity of intact RfBP and RfBP digestion products using an in vitro model that closely mimics physiological conditions.

## MATERIALS AND METHODS

Riboflavin binding protein, digestive enzymes, and all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

**In Vitro Gastroduodenal Digestion.** In vitro digestions were carried out in duplicate (i.e., two test tubes in the same experiment). Riboflavin binding protein from chicken egg white (Apo form) was digested according to the method reported by Moreno et al.[12] and modified by Martos et al.,[13] using the enzyme pepsin in the gastric phase and trypsin and $\alpha$-chymotrypsin for the subsequent duodenal phase. Aliquots were taken at 0 and 60 min of gastric digestion and at 30 and 60 min of duodenal digestion. Aliquots of digests were also taken after 24 h of gastric digestion. Eventually, all samples were diluted in Milli-Q water to a final protein concentration of 1.24 mg/mL and kept at −20 °C until further use.

**Reverse-Phase HPLC Analysis.** Aliquots taken during gastroduodenal digestion were filtered through 0.2 $\mu$m nitrocellulose filters and analyzed by HPLC,[13] using a Hi-Pore RP-318 (250 × 4.6 mm) column (Bio-Rad) in a Waters 600 HPLC (Waters Corp., Milford, MA, USA) equipped with a 717 Plus autosampler and a 2487 dual wavelength absorbance detector. The mobile phases used were 0.37% (v/v) trifluoroacetic acid (TFA) in Milli-Q water as solvent A and 0.27% (v/v) TFA in acetonitrile as solvent B. A linear gradient of 0−60% B was applied over 60 min. After 35 min at 60% B, 100% B was reached in 1 min, maintained during 10 min, and finally decreased to 0% in 1 min. The flow rate of the mobile phase was maintained at 1 mL/min, and peptides were detected by monitoring the absorbance at 220 nm. Data were processed by using Empower 2 software (Waters Corp.).

**SDS-PAGE Analysis.** Proteins and peptides were separated according to their molecular weight on Precast Criterion XT 4−12% Bis-Tris gels using the Criterion cell (Bio-Rad, Richmond, CA, USA). Samples were diluted 1:3 (v/v) in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) $\beta$-mercaptoethanol, and 0.025% (w/v) bromophenol blue. The same buffer without $\beta$-mercaptoethanol was used for separation under nonreducing conditions. Samples were then heated at 95 °C for 4 min, and 40 $\mu$L was loaded on a 4−12% Bis-Tris polyacrylamide gel (CriteriumTMXT, Bio-Rad). Electrophoretic separations were carried out at 100 V in a Criterion cell using XT-MES as running buffer (Bio-Rad). Gels were stained with Coomassie blue G-250.

**Collection of Allergic Patients' Sera.** Serum samples from 16 children with proven allergy to egg white were collected with written consent at the Hospital Gregorio Marañón (Madrid, Spain). All patients showed specific IgE levels against egg white proteins, as determined by the Immuno-CAP method (Phadia, Uppsala, Sweden) and were documented to exhibit clinical allergic symptoms upon oral challenge with egg protein-based products. All procedures regarding serum collection and patient's data confidentiality were carried out in accordance with current laws and approved by the ethics committee of the Gregorio Marañón Hospital.

For inhibition ELISA, a total of nine sera from allergic patients were used, distributed into three different pools, each one composed of three different sera. For Western Blot, one serum, pooled from the sera of seven allergic patients (Table 1), different from the above, was used.

**Western Blot Analysis.** Samples of interest were subjected to electrophoresis as described above. Afterward, the gel was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min,and blotted on a nitrocellulose membrane using a Trans-Blot semidry system (Bio-Rad) for 30 min at 18 V. Then, blocking was carried out using 1% (w/v) bovine serum albumin in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T). After a 5 min washing with TBS-T, the membrane was dipped into 5 mL of patient's serum (a pool of sera with an averaged IgE titer of 13.2 kU/L for egg white, further diluted 1:30 in blocking buffer) and kept for 40 h at 4 °C. After four washes, it was incubated for 6 h at 4 °C with biotin-conjugated anti-human-IgE antibody (Clone HP6029, Southern Biotech, Birmingham, AL, USA) diluted 1:500 in blocking buffer. Again, the membrane was washed four times. Then, HRP−streptavidin (DakoCytomation, Glostrup, Denmark) diluted 1:3000 in blocking buffer was added and incubated for 1 h at room temperature. Finally, the membrane was washed and developed with the chemiluminescent substrate ECL Prime WB reagent (Amersham Biosciences, Inc.).

**Table 1. Data from the Egg-Allergic Patients, Whose Sera Were Pooled and Used for the Detection of Allergenic RfBP and Peptides by Western Blot**

| patient | age (years) | total and allergen specific IgE in kU/L (in parentheses) |
|---|---|---|
| 1 | 4 | total (38) |
| | | egg: white (8), ovalbumin (9), ovomucoid (9) |
| 2 | 6 | total (672) |
| | | egg: white (16), yolk (4) |
| | | nonegg |
| | | seafood: shrimp (>100), crab (>100), mussels (32), clams (14), squid (6) |
| | | air: olive tree (9), ryegrass (28), mite (15), lawn (8), mugwort (<1) |
| 3 | 2 | total (809) |
| | | egg: white (17), yolk (4) |
| | | nonegg |
| | | cow's milk (3): casein (2), $\beta$-lactoglobulin (<1), $\alpha$-lactalbumin (<1) |
| 4 | 7 | total (2146) |
| | | egg: white (20), yolk (2) |
| | | nonegg |
| | | seeds/beans: lentils (20) |
| | | animals: cat dandruff (71), dog dandruff (73) |
| | | air: olive tree (42), ryegrass (51), mite (<1), lawn (17), mugwort (1) |
| | | fungi: *Alternaria tenius* (59), *Aspergillus fumigatus* (19), *Cladosporium herbarum* (7) |
| 5 | 1 | total (358) |
| | | egg: white (8) yolk (1) ovalbumin (9) ovomucoid (2) |
| | | nonegg |
| | | cow's milk (35): casein (38), $\beta$-lactoglobulin (4), $\alpha$-lactalbumin (22), bovine serum albumin (24) |
| | | clinical symptoms with casein hydrolysates |
| 6 | 3 | total (80) |
| | | egg: white (15) |
| 7 | 1 | total (318) |
| | | egg: white (7), yolk (<1), ovalbumin (3), ovomucoid (7) |
| | | nonegg |
| | | cow's milk (8): casein (<1), $\beta$-lactoglobulin (4), $\alpha$-lactalbumin (4), bovine serum albumin (3) |
| | | seeds/beans: lentils (6), garbanzo beans (5), phasol beans (1), wheat (2) |
| | | vegetables/fruits: carrot (<1), potato (1), pear (22), apple (10), orange (3), banana (2) |

Image acquisition was carried out using a VersaDoc Imaging System (Bio-Rad) with an exposure time of 10 min.

**Mass Spectrometry Analysis of Peptide Bands.** The assignment of peptide bands to a specific protein was done by tryptic digestion and mass fingerprinting. The identification of the exact sequence of particular peptides was done by mass fingerprinting and MS/MS analysis of tryptic digests, in combination with intact molecular weight determination.

Bands of interest were manually excised from micropreparative gels using biopsy punches. Peptides selected for analysis were in-gel reduced, alkylated, and digested with trypsin.[16] After digestion, the supernatant was collected and spotted onto a MALDI target plate with $\alpha$-CHCA matrix for protein identification by peptide mass fingerprint. SwissProt DB v. 57.15 with taxonomy restriction to Metazoa (Animals) was searched using MASCOT 2.2 (matrixscience.com) through the Global Protein Server v3.6 from ABSciex. Search parameters were set on carbamidomethyl cysteine as fixed modification and oxidized methionine as variable modification, a peptide mass tolerance of 50 ppm, and an allowance for one missed trypsin cleavage site. The probability scores used for the identification

of sequences were in all cases over the significance threshold fixed by MASCOT, which has a default setting of 5%.

Peptide sequences were confirmed by MS/MS analysis. Search parameters were the same as described above. Mass tolerance for MS/MS fragments was set at 0.3 Da, and error tolerance for combined search (peptide mass fingerprint plus MS/MS spectra) was −80 ppm. Analyses were performed in a 4700 Proteomics MALDI-TOF/TOF Analyzer (Perspectives Biosystems, Framingham, MA, USA).

For the determination of intact molecular weights, proteins/peptides were electroblotted as described above onto a nitrocellulose membrane and detected by Ponceau S staining. The bands of interest were excised and directly analyzed after dissolution of the nitrocellulose in MALDI matrix solution prepared as a saturated solution of α-CHCA in acetonitrile/methanol (70:30) containing 1% TFA.[17] Samples were spotted on a Bruker Anchorchip target, and spectra were acquired in linear mode using a Bruker Autoflex Speed spectrometer (Bruker Daltonics GmbH, Bremen, Germany).

**Determination of IgE Binding by Inhibition ELISA.** Inhibition ELISA was conducted following a previously reported procedure.[15] Polystyrene microtiter plates (Corning, Cambridge, MA, USA) were coated with RfBP (10 μg/mL) in phosphate-buffered saline solution (PBS) and incubated overnight at 4 °C. Plates were blocked with PBS containing 2.5% Tween 20 and incubated overnight at 4 °C. On the other hand, 11 serial dilutions of the digested and undigested protein (starting at concentrations of 1.24 or 2 mg/mL, respectively) were prepared in duplicate. Each dilution was mixed with an equal volume of patient's serum (diluted 1:200 in PBS containing 0.05% Tween 20), incubated for 2 h at room temperature, and then added to the precoated wells. Plates were then incubated for 2 h at room temperature. Then, a 1:1000 dilution of rabbit anti-human IgE (polyclonal antibody against the ε chain of IgE; it does not bind human IgG, IgA, or IgM, as stated by the supplier, DakoCytomation) was added and incubated for 1 h at room temperature. This was followed by the addition of HRP-conjugated swine anti-rabbit IgG antibody (DakoCytomation, diluted 1:2000), incubated for 1 h at room temperature with the use of a tyramide−biotin−streptavidin−HRP amplification system (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Walthman, MA, USA) according to the manufacturer's instructions. A solution of 3,3′,5,5′-tetramethylbenzidine (TMB) was used as substrate for HRP, and the reaction was stopped with sulfuric acid. Absorbance was measured at 450 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

ELISA determinations were carried out with three different pools, each resulting from pooling the sera from three different allergic individuals with average IgE levels against egg white proteins of 39.76, 43.09, and 39.5 kU/L. A solution of antigen without antibody (patient's serum) was used as maximum inhibition, whereas a solution of antibody without antigen was used as minimum inhibition.

**Statistical Treatment.** A nonlinear adjustment of the ELISA data was performed using GraphPad Prism package 4 (GraphPad Software Inc., San Diego, CA, USA). The mathematical model was a sigmoidal curve of inhibition dose−response with variable slope. One curve was obtained for each pool and each sample (11 points in duplicate). The $IC_{50}$ and the 95% confidence interval were calculated by the software. The IgE binding capacity was expressed as $1/IC_{50}$.

## ■ RESULTS AND DISCUSSION

**Digestibility of RfBP.** RP-HPLC and SDS-PAGE analyses of the RfBP digests are shown in Figures 1 and 2, respectively. RfBP was mostly cleaved by pepsin throughout gastric digestion, giving rise to peptides that eluted at lower retention times in the chromatographic separation than the intact protein. These hydrophilic peptides increased in abundance as the digestion progressed into the duodenal phase (Figure 1). SDS-PAGE under nonreducing conditions showed the persistent presence of the intact protein after all steps of the simulated digestion (Figure 2, lanes 7−11). However, when the
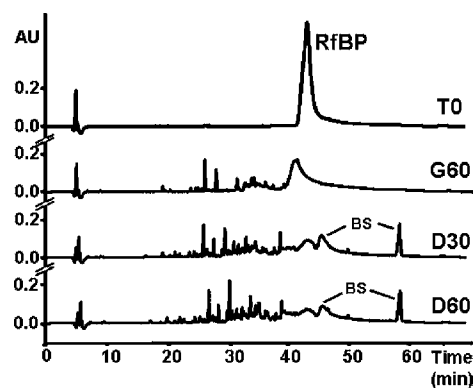


**Figure 1.** RP-HPLC chromatograms of RfBP before digestion (T0), after 60 min of gastric digestion (G60), and after 30 (D30) and 60 min (D60) of the subsequent duodenal digestion. BS, bile salts; AU, absorbance units.
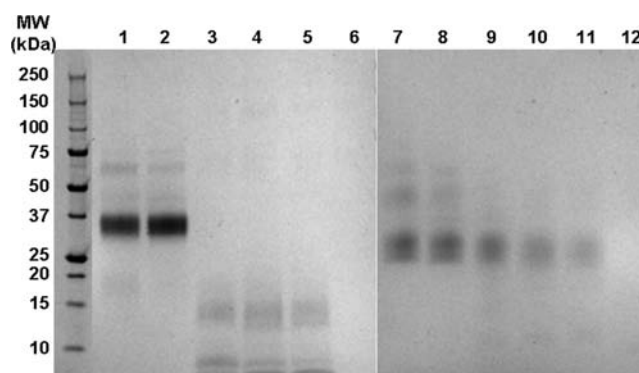


**Figure 2.** SDS-PAGE of RfBP gastroduodenal digests under reducing (lanes 1−6) and nonreducing (lanes 7−12) conditions. Lanes: 1 and 7, native RBP; 2 and 8, before digestion (T0); 3 and 9, aliquots collected at 60 min of gastric digestion (G60); 4 and 10, aliquots collected at 30 min of duodenal digestion (D30); 5 and 11, aliquots collected at 60 min of gastroduodenal digestion (D60); 6 and 12, aliquots collected at 24 h of gastric digestion.

digests were analyzed under reducing conditions (Figure 2, lanes 1−5), the main protein band completely disappeared after 60 min of the gastric digestion phase, giving rise to new bands, corresponding to digestion products with lower molecular weights (Figure 2, lane 3). As RfBP contains nine disulfide bridges within the molecule, these results suggest that the full-length protein was cleaved during the gastric digestion step, although its fragments remained linked together through disulfide bonds. In addition, within the time frame employed, the whole simulated physiological digestion led to fragments of considerable size (up to 15 kDa). After 24 h of gastric digestion, the protein and its fragments completely disappeared from the SDS-PAGE gels (Figure 2, lanes 6 and 12).

**Immunoreactivity of RfBP Digests.** To assess the human IgE recognition of RfBP before and after in vitro gastrointestinal digestion, inhibition ELISA assays were performed on the intact protein and on its digests, using three different serum pools from egg-allergic patients (Figure 3). Gastric digests showed reduced IgE binding capacity, when compared to the undigested protein. Duodenal digests also presented reduced immunoreactivity, albeit comparable to the gastric ones. However, in general, residual IgE binding was found in the digests, although its level greatly varied depending on the serum pool used. Serum pool 1, presenting the highest reactivity to
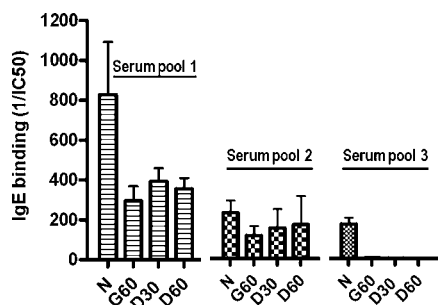
**Figure 3.** Inhibition ELISA of RfBP with three pooled sera from egg-allergic patients: IgE binding of native RfBP (N) and 60 min gastric (G60) and 30 and 60 min gastroduodenal (D30 and D60) digests. Error bars indicate the 95% confidence interval for the estimated value $(1/IC_{50})$.

the intact protein, exhibited also the highest reactivity to the digests. Serum pool 2 showed a high residual reactivity to the digests. Serum pool 3 did not react to any of the digests, despite presenting RfBP-specific IgE binding comparable to pool 2. Most interestingly, serum pools 1 and 2 from egg-allergic patients were able to recognize RfBP even after 60 min of simulated duodenal digestion.

**Detection and Identification of IgE-Reactive RfBP Fragments.** To identify the RfBP fragments that were responsible for the binding to human patients' IgE, an immunoblot of digested RfBP was performed using a pool of sera (Figure 4). Prior to digestion, RfBP showed a strong
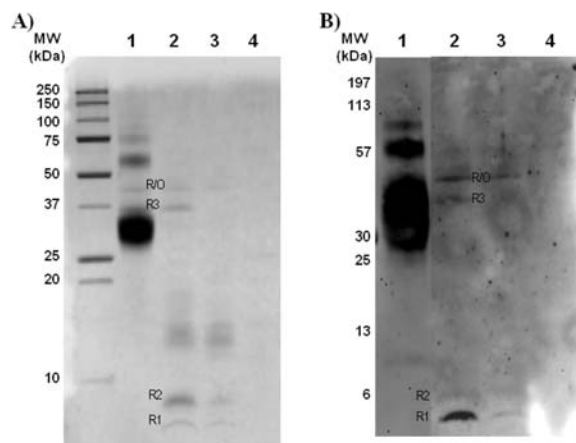


**Figure 4.** SDS-PAGE (A) and immunoblot with allergic patients' pooled sera (B) of RfBP digests. Lanes: 1, before digestion (T0); 2, 60 min gastric digest (G60); 3, 30 min subsequent duodenal digest (D30); 4, 24 h gastric digest. R1, R2, R3, and R/O labels correspond to bands that were assigned to a particular protein by peptide mass fingerprinting: R1, R2, R3, and R/O bands corresponded to RfBP; R/O also contained ovalbumin.

binding to IgE (Figure 4B, lane 1). After digestion, four bands with apparent molecular masses of 4, 5, 36, and 40 kDa (labeled in Figure 4 as R1, R2, R3, and R/O, respectively), still preserved IgE binding epitopes, as evidenced in the immunoblot (Figure 4B, lanes 2 and 3). It was only after 24 h of incubation with pepsin that all IgE binding sites were destroyed (Figure 4B, lane 4).

As shown in the immunoblot, two bands with molecular weight higher than that of RfBP showed IgE reactivity (labeled R3 and R/O in Figure 4), possibly due to the presence of

residual amounts of other allergens in the RfBP samples. By peptide mass fingerprinting, we found fragment R/O to contain both RfBP and ovalbumin, whereas the other three bands (R1, R2, and R3) were identified as RfBP. Therefore, we cannot preclude that the presence of a small amount of ovalbumin contributed to the IgE binding capacity. However, fragments R1 and R2, recognized by serum IgE of allergic patients, were unequivocally assigned to different fragments of digested RfBP.

We next sought to determine the sequence of fragments R1 and R2. These peptides were transferred from the gel onto a nitrocellulose membrane for intact mass determination, giving approximate masses of 3.9 and 5.1 kDa, respectively. Tryptic peptides produced after in-gel digestion of the two fragments are shown in Figure 5. R2 was unequivocally identified as fragment 41−84, whereas R1 contained residues 63−84, but it was not possible to find all peptides covering the whole sequence. However, according to the estimated molecular mass of 3.9 kDa, it is very likely that this band corresponds to residues 52/53−84. Therefore, this region of the protein could contain one or multiple IgE binding epitopes that would partially resist gastrointestinal digestion to be long enough to potentially cross-link IgE at the surface of mast cells.

The carbohydrate moieties of RfBP and the dominant egg allergen ovomucoid show high similarity,[18] and it has been suggested that they may be involved in the cross-reactivity of the two proteins with a monoclonal antibody.[19] However, other authors have ruled out the presence of epitopes on ovomucoid carbohydrates.[20,21] In RfBP, the N-linked carbohydrates are found on residues Asn 36 and 147.[22] These positions are not included in the immunoreactive peptide 41−84. Therefore, IgE binds the peptide chain, rather than the carbohydrate moieties, suggesting that a potential IgE cross-reactivity of RfBP and ovomucoid would not be likely related to their carbohydrate moieties.

Despite extensive research aiming at characterizing egg allergens, many questions remain to be addressed. Whereas most studies have focused on the main allergens, ovalbumin and ovomucoid, the role of other proteins present in egg at lower concentrations remains to be elucidated. In the present study, we assessed the IgE binding properties of intact RfBP using sera obtained from egg-allergic patients and report a relevant IgE binding capacity, which points at RfBP as an allergen. In addition, we showed that the intact protein was cleaved by pepsin in the simulated gastric digestion, but its fragments remained linked through disulfide bridges. This could maintain together epitopes far apart in the sequence that could bind various IgE molecules. It was also found that following the simulated duodenal digestion, an RfBP fragment that corresponded to residues 41−84, long enough to trigger IgE cross-linking, was reactive against patient's serum IgE. Subramanian and Adiga[23] have identified by PEPSCAN analysis some antigenic B-cell epitopes for chicken RfBP included in this sequence, particularly residue sequences 42−49 and 68−83. In addition, the fragment 64−83 has been reported to contain a bimodal T-cell epitope.[24] Altogether, these observations support the idea that this region of the protein may be relevant in the context of sensitization to RfBP and/or egg proteins.

In conclusion, it has been demonstrated that RfBP contains human IgE binding epitopes and that, despite the significant enzymatic fragmentation occurring during the gastrointestinal digestion, particularly during the gastric phase, at least one

**A)**

| Fragment | Tryptic peptide (Ion Mass) | Protein residues | Sequence |
|---|---|---|---|
| **R2 (5.1 kDa)** | | | |
| | 878.4 | 41-48 | LAHSPIIK |
| | 1025.5 | 49-56 | VSNSYWNR |
| | 1882.8 | 63-66 | SC*EDFTKKIEC*F |
| | 1015.5 | 70-76 | KIEC*FYR |
| | 781.4 (798-17) | 77-83 | C*SPHAAR |
| | 967.4 (984-17) | 77-84 | C*SPHAARW |
| **R1 (3.9 kDa)** | | | |
| | 1563.7 | 63-74 | SC*EDFTKKIEC*F |
| | 1882.8 | 63-76 | SC*EDFTKKIEC*FYR |
| | 781.4 (798-17) | 77-83 | C*SPHAAR |
| | 967.4 (984-17) | 77-84 | C*SPHAARW |

**B)**

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         10         20         30         40         50         60
    QQYGCLEGDT HKANPSPEPN MHECTLYSES SCCYANFTEQ LAHSPIIKVS NSYWNRCGQL

         70         80         90        100        110        120
    SKSCEDFTKK IECFYRCSPH AARWIDPRYT AAIQSVPLCQ SFCDDWYEAC KDDSICAHNW

        130        140        150        160        170        180
    LTDWERDESG ENHCKSKCVP YSEMYANGTD MCQSMWGESF KVSESSCLCL QMNKKDMVAI

        190        200        210        220
    KHLLSESSEE SSSMSSSEEH ACQKKLLKFE ALQQEEGEERR
```

**Figure 5.** Peptides identified by MALDI-TOF/TOF mass spectrometry after in-gel tryptic digestion of the immunoreactive RfBP fragments R1 and R2 (see Figure 4): (A) ion mass of detected peptides, their sequence and location in the protein (C* indicates carbamidomethylated cysteine); (B) primary sequence of mature RfBP (lines correspond to the sequences identified within R1 (dotted) and R2 (solid)).

fragment, that is, amino acid sequence 41−84, retains its allergenicity.

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### Notes
The authors declare no competing financial interest.

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