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Egg White Ovalbumin Digestion Mimicking Physiological Conditions

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Gastrointestinal digestion of ovalbumin (OVA) was simulated using an in vitro system in two steps, which mimicked digestion in the stomach and duodenum, to assess the effect of different gastric pHs, different concentrations of proteases, and the presence of surfactants, such as phosphatidylcholine (PC) and bile salts (BS). OVA was very resistant to pepsin action at an enzyme/substrate ratio that would resemble a physiological situation (1:20 w/w, 172 units/mg) at pH values equal or above 2. The presence of PC did not change the susceptibility of OVA to proteolysis with pepsin. Fluorescence experiments showed that OVA interacted with PC vesicles, particularly at acidic pH, but it is likely that the protein maintained a high degree of conformational stability, resisting pepsin action. The presence of BS at physiological concentrations considerably increased the proteolysis of OVA by a mixture of pancreatic enzymes. The addition of PC made OVA even more sensitive to proteolytic degradation, suggesting that OVA could associate with the surfactants under duodenal conditions, increasing its exposure to pancreatic proteinases. Immunoreactivity against IgE from sera of allergic patients was retained after in vitro gastric digestion, depending on the reactivity of the sera, but it decreased considerably after in vitro duodenal digestion.

KEYWORDS: Ovalbumin; in vitro digestibility; pepsin; pancreatic enzymes; physiological surfactants; IgE-binding

INTRODUCTION

Egg represents, together with cow's milk, the most common cause of allergic reactions to food that affect approximately 6% of children and 3−4% of adults in the U.S., and ovalbumin (OVA, Gal d 2), the major protein in egg white (58% w/w), is considered a dominant allergen (*1*). OVA is a glycoprotein with a molecular mass of 45 kDa. Its sequence comprises 385 amino acids and includes six cysteines with a single disulfide bond between Cys_{73} and Cys_{120}. OVA partially resists hydrolysis with pepsin, a characteristic that is also shared by many allergenic proteins, which withstand processing and digestion in the gastrointestinal tract, mediated by proteolytic enzymes, low pH, and surfactants such as phospholipids and BS, and thus keep a certain degree of three-dimensional structural integrity to trigger the immune reaction (*2*). In fact, stability to digestion is used as a criterion to evaluate the allergenic potential of novel proteins, such as transgenic proteins.

While a general agreement on the proteolytic stability of many food allergens exists, a lack of correlation between in vitro digestibility and allergenicity has been reported by many authors (*3*−*5*). This is probably because the digestibility of a protein, as measured by an in vitro assay, is greatly influenced by the conditions used, which commonly imply protease to substrate ratios that are orders of magnitude greater than the ratios found in vivo or ignore the interactions of food proteins with other digestive components. In addition, even in the absence of intact protein, proteolytic fragments generated during digestion may have the potential to bind IgE and elicit an allergic response.

In this work we have simulated the gastrointestinal proteolysis OVA using an in vitro digestion system in two steps, which mimics the successive passage through the stomach and duodenum (*6*, *7*), assessing the effect of different gastric pHs, different concentrations of proteases, and the presence of surfactants, such as phosphatidylcholine (PC) and bile salts (BS). The influence of these processes on the digestibility of OVA and the IgE-binding properties of the resulting digestion products was examined.

MATERIALS AND METHODS

In Vitro Gastric Digestion. OVA (A-2512, grade VI, 99% purity, Sigma, MO, USA) was dissolved in simulated gastric fluid (SGF, 35 mM NaCl) at pH 1.2, 2, and 3.2, preheated for 15 min at 37 °C, and subjected to an in vitro gastric digestion at 37 °C with porcine pepsin (EC 3.4.23.1, 3440 units/mg, Sigma) at an enzyme/substrate ratio of 1:20 w/w (172 units/mg) (*6*, *7*). A highest ratio of 3:1 w/w (10320 units/mg) was also used (*8*). Aliquots were taken at 0, 60, 90, and 120 min, and the reaction was stopped by adding 1 M $NaHCO_3$, giving a final protein concentration of 5 mg/mL and pH 7.

Gastric digestions were also performed at pH 2 and a pepsin/OVA ratio of 1:20 w/w in either the absence or presence of PC (L-α-phosphatidylcholine, P3841; Sigma) as described by Moreno et al. (*6*, *7*). Phospholipid vesicles were prepared by dissolving PC in SGF pH 2 (9.58 mg/mL). To facilitate the homogeneous dispersion, the mix was incubated under agitation at 37 °C and vortexed periodically until complete dissolution. Then, it was sonicated in ice (5 min, raising the power from 10% to 50%,

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and 5 min at 60% power) not exceeding a sample temperature of 40 °C. OVA, dissolved in SGF pH 2, was mixed with the PC vesicle solution (1:1.2 v/v) and preheated, and pepsin was added as already described. In the digestions without PC, the PC solution was replaced by SGF pH 2. After 60 min of incubation, the reaction was stopped by raising the pH to 7 with 1 M $NaHCO_3$, the final protein concentration being 5 mg/mL.

At least triplicate digestions were conducted for each condition.

In Vitro Duodenal Digestion. Duodenal digestions were performed by using, as the starting material, pH 2, 60 min gastric digests (with a pepsin/OVA ratio of 1:20), adjusted to pH 7, as described above, with the addition of 1 M $CaCl_2$, 0.25 M bis-Tris pH 6.5, and a 0.125 M BS mixture containing equimolar quantities of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma) (*6*, *7*). After preheating at 37 °C for 15 min, trypsin (EC 232-650-8, 10100 BAEE units/mg protein, Sigma), α-chymotrypsin (EC 232-671-2; 55 units/mg protein, Sigma), porcine pancreatic lipase (EC 232-619-9, Sigma), and colipase (EC 259-490-1, Sigma) prepared in 35 mM NaCl adjusted to pH 7 were added to the duodenal mix. The final composition of the mixture was 4.15 mg/mL OVA, 6.15 mM of each BS, 20.3 mM bis-Tris, 7.6 mM $CaCl_2$; the enzymes referred to the quantity of protein were 40 units/mg trypsin, 0.5 units/mg α-chymotrypsin, 28.9 units/mg lipase and colipase (enzyme/substrate ratio 1:895 w/w). Duodenal digestions were also performed by replacing trypsin and α-chymotrypsin with a commercial pancreatic enzyme mix, Corolase PP (AB Enzymes GmbH, Darmstadt, Germany) at an enzyme/substrate ratio of 1:25 w/w. To test the influence of BS and lipases, these were replaced, when necessary, with 35 mM NaCl adjusted to pH 7. At least triplicate digestions were conducted for each condition.

SDS-PAGE. Samples were dissolved (1:1 v/v) in 10 mM Tris-HCl buffer, pH 8, containing 2.5% SDS, 5% 2-β-mercaptoethanol, and 10 mM EDTA and heated at 100 °C for 10 min. SDS-PAGE was performed on a PhastSystem Electrophoresis apparatus, using precast Homogeneous Gels 20% and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the electrophoretic and Coomassie Blue and silver nitrate staining conditions of the manufacturer. A LMW Calibration Kit for SDS (Amersham Biosciences) was used.

RP-HPLC. OVA hydrolysates, at a concentration of 2.5 mg/mL, were separated in a Hi-Pore RP-318 (250 mm × 4.6 mm internal diameter) column (Bio-Rad, Richmond, CA, USA), in a Waters 600 HPLC (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and UV detector. The digests were eluted by using 0.37% (v/v) trifluoroacetic acid in double-distilled water as solvent A and 0.27% (v/v) trifluoroacetic acid in acetonitrile as solvent B, at 1 mL/min, and 220 nm (*9*). Data were processed by using Empower 2 Software (Waters Corporation).

Mass Spectrometry Analyses. The protein band of interest was manually excised, and in-gel trypsin digestion was performed with Promega trypsin (Madison, WI, USA) (*10*). MALDI-MS analysis of tryptic peptides was performed on an Ultraflex TOF-TOF Instrument (Bruker Daltonics, GmbH, Bremen, Germany). The matrix material was CHCA on anchor-chip targets (Bruker Daltonics). Identification of the proteins was also carried out by TOF-TOF PSD fragmentation spectra. For ESI-MS/MS, an Esquire HCT (Bruker Daltonics) was used. Database searches were performed using the MASCOT program (Matrix Science).

Fluorescence Spectroscopy. The interaction between OVA, PC, and BS was studied by fluorescence spectroscopy (*11*). Fluorescence spectra between 300 and 380 nm (excitation, 280 nm) were recorded at room temperature on a Shimadzu RF-1501 spectrofluorophotometer. The binding of PC and BS was measured as the change in fluorescence. The following procedure was used for titration of OVA with the phospholipid. The PC vesicle solution was prepared both in SGF pH 2 and in 10 mM phosphate buffer pH 7, at a concentration of 4.1 mg/mL. Two milliliters of 0.15 mg/mL OVA in SGF or in phosphate buffer were placed in a cuvette, and every 5 min, under continuous shaking, 10 μL of the phospholipid solution was added. In order to eliminate the dilution of the OVA solution by the added phospholipid, a blank containing the protein solution, titrated with SGF or phosphate buffer, was monitored as described above. The fluorescence intensity changes of the blank were subtracted from the fluorescence intensity measurements of the OVA-PC mixture for every titration point. In all cases, before correction for the blank, fluorescence intensity of free OVA was normalized to 1. For the titration with BS, 10 μL of 15 mM sodium taurocholate and 15 mM glycodeoxycholic acid was added, either separately or both together.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were measured using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). The far (195−260 nm) and near (250−350 nm) UV CD spectra of OVA, in SGF pH 2 and phosphate buffer 10 mM pH 7, were recorded at 20 °C using cells with respective pathlengths of 0.1 and 0.2 cm. The instrument was calibrated with ammonium D-10-camphorsulfonate. Spectra represent the average of four accumulations collected at 100 nm/min, with a 2 s time constant, a 0.5 nm resolution, and a sensitivity of ±100 mdeg. The samples were dissolved at 0.2 mg/mL for the analysis in the far-UV region and at 2 mg/mL for the near-UV region. The buffer blanks were subtracted from each CD spectrum. PC was dissolved at 0.184 mg/mL or 1.84 mg/mL in either SGF or phosphate buffer, and sodium taurocholate and glycodeoxycholic acid were at 0.29 or 2.9 mM to maintain the ratios used during digestion. The data were expressed as mean residue ellipticity (10^3 deg cm^2/decimol). Empirical determinations of protein secondary structure were obtained employing the CDSSTR algorithm (*12*, *13*) and the reference database SP175 (*14*).

IgG Binding by Direct ELISA. Gastric and duodenal digests, as well as blanks containing the protein in SGF pH 2 or in a buffer containing 20.3 mM bis-Tris methane and 7.6 mM $CaCl_2$, pH 6.5, were properly diluted to a final protein concentration of 2.5 μg/mL and applied in triplicate to microplates (polystyrene microtiter plates, Corning, Cambridge, MA, USA). Each well was coated with 50 μL of the sample and incubated overnight under refrigeration. Afterward, the plate was washed using a Microplate Washer (Nunc, Roskilde, Denmark), and PBS-Tween 20 (PBST, 2.5%) was used as saturating agent to avoid nonspecific binding. After 60 min of blocking, 50 μL of polyclonal rabbit anti-OVA conjugated with horseradish peroxidase (HRP) (DakoCytomation, Glostrup, Denmark), diluted 1:55000 in PBST, was added per well and incubated for 60 min. *o*-Phenylene-diamine (OPD, Palex Medical SA, Sant Cugat del Vallés Barcelona, Spain) was used as substrate, and after 30 min of incubation, the reaction was stopped by the addition of 0.5 M sulphuric acid. Absorbance was measured at 492 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). A negative control without antigen (PBS) and positive controls (with different concentrations of OVA) were included each plate.

Triplicate determinations in two different ELISA plates were carried out, and measurements were averaged. Values are expressed as means ± SD. Significant differences ($P < 0.05$) were evaluated by one-way analysis of variance (ANOVA). PASW (formerly SPSS) was used for data processing (version 14.0, SPSS Inc., Chicago, IL, USA).

Human IgE Binding by Inhibition ELISA. For inhibition ELISA, two individual serum samples from children with clinical allergic symptoms to egg white proteins were used. The sera were collected from the Maternal and Child Gregorio Marañon Hospital (Madrid, Spain). The patients had specific seric IgE levels toward OVA of 25.1 and 78.1 KU/L, as determined by CAP (GE HealthCare, Uppsala, Sweden).

Single wells of polystyrene microtiter plates were coated with 10 μg/mL of OVA solution in 0.01 M PBS, pH 7.4, and incubated overnight at 6 °C. Plates were washed with PBST using the Microplate Washer (Nunc). This washing system was used after each incubation step. Then, serial dilutions of each sample (not less than seven) were incubated during 120 min with patient's sera previously diluted in PBST (1:1 v/v), and 50 μL was added to each well. After 120 min of incubation, 50 μL of HRP-conjugated rabbit antihuman IgE, diluted 1:1000 in PBST, was added per well and incubated for 60 min. The tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Walthman, MA, USA). Finally, OPD was used as substrate, and after 30 min of incubation, the reaction was stopped with 0.5 M sulphuric acid. Absorbance was measured at 492 nm. A negative control without serum (native protein in PBST) and positive controls (sera diluted in PBS) were included in each plate.

A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal curve of inhibition dose-response with variable slope, from which the IC_{50} (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad PRISM 4 for Windows (www.graphpad.com). The IgE binding capacity was expressed as the percentage of the IC_{50} of the intact protein.

Western Blotting. Samples (prepared as described above) were run on a polyacrylamide Tris-HCl (10.5−14% gradient) gel in Tris-glycine-SDS

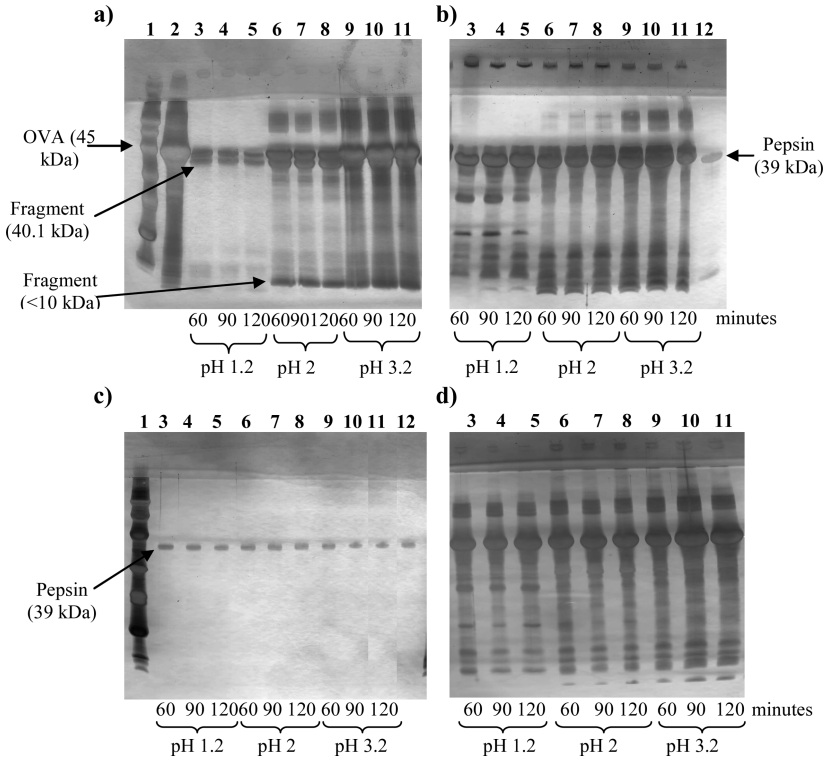

Figure 1. SDS-PAGE analysis of ovalbumin (OVA) digested with pepsin at enzyme/substrate ratios of 1:20 (**a**) and 3:1 (**b**) at different pH values and hydrolysis times and of pepsin at the same concentration as in plates **a** (**c**) and **b** (**d**), but without substrate. Lane 1: molecular mass markers; lane 2: OVA; lanes 3−5: digestions at pH 1.2 during 60, 90, and 120 min; lanes 6−8: digestions at pH 2 during 60, 90, and 120 min; lanes 9−11: digestions at pH 3.2 during 60, 90, and 120 min; lane 12: pepsin.

buffer (TGS), pH 8.3, at 150 V. The gel was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min. The semidry transfer took place in a Trans-Blot SD (Bio-Rad, Richmond, CA, USA) for 30 min at 15 V. Then, the nitrocellulose membrane was blocked with Tris Buffer Saline with Tween 20 (TBST, casein 1% w/v, Tween 20, 0.02% v/v), pH 7.6, for 60 min. The membrane was washed with TBST and dipped into a human serum (25.1 KU/L IgE), 1:20 diluted in TBST-casein (0.1%), overnight at 4 °C. After a new wash, it was incubated overnight at room temperature with HRP-conjugated antihuman IgE antibody (1:500 in TBST). Finally, the membrane was rinsed and detection with chemiluminescence was developed with luminol (Novagen, Darmstadt, Germany). Image acquisition (exposure time 10 min) was performed using the VersaDoc Imaging System (Bio-Rad).

RESULTS AND DISCUSSION

Simulated Gastric Digestion: Influence of the pH, Enzyme to Substrate Ratio, and Presence of Phosphatidylcholine. The SDS-PAGE analysis of OVA incubated with pepsin at an enzyme/substrate ratio of 1:20 and different pHs (**Figure 1a**) showed that pepsin hydrolysis increased with the incubation time, being much faster at the lowest pH. Using the sensitive silver staining, intact OVA was detectable at all pHs even after 120 min of incubation, although it could not be detected with Coomassie Blue after 60 min of hydrolysis at pH 1.2 (data not shown). In agreement with our results, OVA has been reported to be very stable to pepsin at enzyme to protein ratios close to 1:20 (*15*). This stability decreases at higher ratios (*5*), which points at the importance of the assay conditions when trying to correlate pepsin resistance with the allergenic potential of proteins (*16*). Although there is not a general agreement on the physiological enzyme to protein ratio, which might also be subjected to considerable intra- and interindividual variations, it seems clear that, in an in vivo situation, protein would always exceed pepsin (*16*). However, most studies on protein digestibility and, more specifically, OVA digestibility have used high enzyme to substrate ratios, such as 19:1 (*17*), 13:1 (*5*), 8:1 (*18*), or 3:1 (*8*, *19*). In order to check the effect of a higher pepsin concentration, we also tried an enzyme/substrate ratio of 3:1 (**Figure 1b**). In this case, OVA was detected only at pH 3.2 and only traces of intact protein were visible after 60 min of digestion at lower pHs. RP-HPLC analysis confirmed the absence of OVA after 90 min of hydrolysis at pH 1.2 and 2 at an enzyme to substrate ratio of 3:1 (results not shown). This is in argument with a collaborative study between nine laboratories (*8*) using a 3:1 pepsin/protein ratio that revealed that the disappearance of intact OVA and its fragments was not influenced by the pH value (either 1.2 or 2). However, according to our results, the pH seemed to be more relevant when lower relative amounts of enzyme were used. Although pepsin exhibits optimum activity over broad pH range (between 1.2 and 3.5), our results show that, at an enzyme to substrate ratio of 1:20, pH 2 did not particularly favor the enzyme activity. It is assumed that pH 1.5−2 prevails in the fasted stage of the stomach of healthy adults, but this value can increase to above 4 after food intake (*20*). Furthermore, the acidity of the infant stomach is much less, with a pH of around 4, what may lead to a poor and slow degradation of allergenic epitopes (*21*). Therefore, in children or adults with impaired stomach function, a slower and less efficient degradation of OVA could occur at pH higher than 1.2, at pepsin concentrations mimicking physiological conditions that could be of importance in terms of enhancing the sensitization or allergenic capacity of this protein.

It should be noted that the use of the sensitive silver staining made it difficult to interpret the electrophoretic pattern around 45 kDa, because both OVA and pepsin migrate closely in this area. To check this point, as well as to detect any interferences arising from the autodigestion of pepsin, the enzyme was incubated under the same conditions but without substrate. At the

lowest concentration, pepsin appeared as a faint band in the silver-stained gel and no shelf-degradation products were visible (**Figure 1c**). However, at the highest concentration, it became evident that most of the bands generated were fragments derived from pepsin autodigestion (**Figure 1d**). Thus, the main degradation products arising from pepsin action on OVA were more easily detected at the lowest enzyme/substrate ratio. These were a band of around 40 kDa of molecular mass that was quickly generated upon digestion and a band of less than 10 kDa that was observed even after 120 min of digestion at all pHs, which could correspond to an OVA fragment very resistant to pepsin (*9*) (**Figure 1a**). Thomas et al. (*8*); Dearman et al. (*18*), and Takagi et al. (*19*) described a similar digestion pattern, with the formation of two hydrolysis fragments of 40.1 and 4.1 kDa.

The 40.1 kDa band was sliced from the gel and subjected to trypsinolysis, ESI-MS/MS, and MALDI TOF-TOF analysis. **Figure 2** shows the peptide fragments identified in the primary sequence of OVA. According to Kitabatake et al. (*22*) pepsin cleaves OVA between His_{22} and Ala_{23}, this peptide bond being the only one cleft at pH 4. However, Tatsumi and Hirose (*23*) claimed that, even at pH 2, OVA undergoes a limited proteolysis at Ala_{351}, close to the canonical serpin cleavage site $Ala_{352}-Ser_{353}$. The fact that no peptides corresponding to the N-terminal region of OVA were found by mass spectrometry points out that the 40.1 kDa fragment might correspond to $Ala_{23}-Pro_{385}$. The formation of this fragment agrees with the results of a study on the effects of pepsinolysis of OVA on the surface hydrophobicity of the degradation products, carried out by Mine et al. (*24*).

1 GSIGAASMEF CFDVFKELKV HHANENIFYC PIAIMSALAM VYLGAKDSTR
51 **TQINKVVR**FD KLPGFGDSIE AQ**CGTSVNVH** **SSLR***DILNQI* ***TKPNDVYSFS***
101 ***LASR***LYAEER ***YPILPEYLQC*** ***VK***ELYR***GGLE*** ***PINFQTAADQ*** ***ARELINSWVE***
151 ***SQTNGIIRNV*** **LQPSSVDSQT** **AMVLVNAIVF** **K**GLWEK***AFKD*** ***EDTQAMPFRV***
201 ***TEQESKPVQM*** ***MYQIGLFRVA*** ***SMASEKMKIL*** **ELPFASGTMS** MLVLLPDEVS
251 GLEQLESIIN FEK***LTEWTSS*** ***NVMEERK***IKV YLPRMKMEEK YNLTSVLMAM
301 GITDVFSSSA NLSGISSAES LK***ISQAVHAA*** ***HAEINEAGR***E VVGSAEAGVD
351 **AASVSEEFR***A* ***DHPFLFCIKH*** ***IATNAVLFFG*** ***R*CVSP**

Figure 2. Primary ovalbumin sequence. Fragments in bold are the peptides recognized by mass spectrometry following in-gel trypsin hydrolysis. Underlined residues correspond to sequences identified by ESI-MS/MS and residues in italics to sequences identified by MALDI-TOF/TOF.

To assess the effect of other factors that may play a role in physiological digestion, such as the interactions between proteins and lipids (*25*), in vitro gastric digestion was also performed in the absence and presence of PC. As shown in **Figure 3a** and **b**, no effect of PC on OVA resistance to digestion was found. PC is secreted by the gastric mucosa and also occurs in the bile. The diet is also a supply of PC, and for instance, there is a high concentration of PC in egg yolk (aprox 1.7 mmol). Moreno et al. (*7*) reported for the first time that the interaction of certain proteins, such as α-La, with physiological surfactants can retard their proteolysis during gastric digestion, although other proteins are unaffected by the presence of PC during pepsin hydrolysis. The protective effect of PC on pepsinolysis was attributed to α-La adopting a partially unfolded state at acidic pH that would favor its partial penetration into PC vesicles. However, the digestion of a protein very resistant to pepsin, such as the 2S albumin from Brazil nut, with a compact 3D structure, is not affected by the presence of PC in SGF at pH 2.5 (*6*). Similarly, addition of PC does not affect the resistance of *β*-Lg to pepsin at pH 2.5 (*26*, *27*).

Simulated Duodenal Digestion: Influence of Lipases, Bile Salts, and Phosphatidylcholine. The samples obtained after 60 min of an in vitro gastric digestion with pepsin (enzyme/substrate 1:20) at pH 2 were further subjected to a process mimicking duodenal digestion with trypsin and α-chymotrypsin (*6*, *7*). **Figure 4a** shows that both intact OVA and its 40.1 kDa fragment resisted hydrolysis by these enzymes for at least 60 min. According to Fu et al. (*5*), OVA is stable to SIF (simulated intestinal fluid, consisting of pancreatin in alkaline medium). However, Takagi et al. (*19*) reported that the original band of OVA rapidly decreases, while a small amount of the fragment band (40.1) persists for 120 min. As

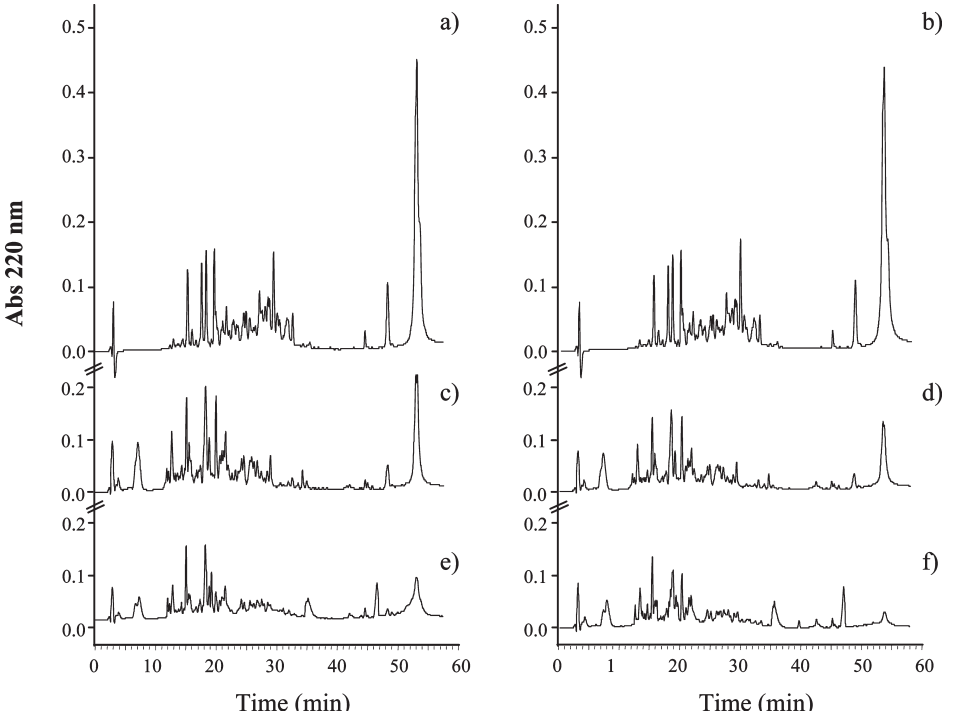

Figure 3. RP-HPLC chromatograms of ovalbumin (OVA) hydrolyzed under different conditions. In vitro gastric digestions with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min, in the absence (**a**) and presence (**b**) of phosphatidylcholine (PC), followed by in vitro duodenal digestions with Corolase PP for 60 min: without BS (BS) and PC (**c**); without BS but with PC (**d**); with BS but without PC (**e**); and with BS and PC (**f**).

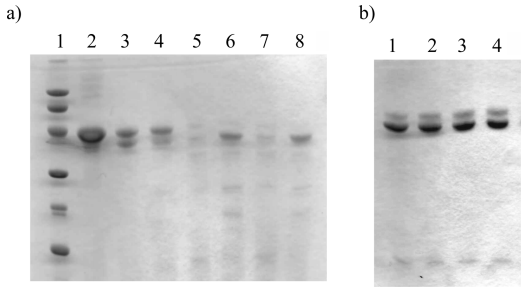

Figure 4. (**a**) SDS-PAGE analysis of ovalbumin (OVA) digests under different conditions. Lane 1: molecular mass markers; lane 2: OVA; lane 3: OVA digested with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min; lane 4: duodenal digestion with trypsin and chymotrypsin for 60 min; lanes 5−8: duodenal digestions with Corolase PP for 60 min with lipases and bile salts (BS) (lane 5), with lipases but without BS (lane 6), without lipases but with BS (lane 7); and without lipases or BS (lane 8). (**b**) SDS-PAGE analysis of OVA digested with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min incubated for 5 (lane 1), 10 (lane 2), and 20 (lane 3) min in duodenal media containing lipases and BS but without proteases.

is the case in SGF, in vitro hydrolysis in SIF can be greatly influenced by the amount and type of enzymes used. In fact, Corolase PP, an enzyme preparation from the pig pancreas gland that in addition to trypsin and chymotrypsin contains amino and carboxypeptidase activities, caused a more extensive proteolytic degradation of both OVA and its fragment (**Figure 4a**, lanes 5−8). In subsequent experiments, Corolase PP and an incubation time of 60 min were used to evaluate the influence of BS, PC, and lipases in the duodenal medium on OVA digestibility.

Parallel in vitro duodenal digestions, following gastric digestion without PC, were carried out, giving rise to four digestion samples: in the presence of all of the duodenal components, in the absence of BS, in the absence of lipases, and in the absence of BS and lipases. Lipases did not prove to have any effect on protein digestibility; however, SDS-PAGE (**Figure 4a**) and RP-HPLC analyses (**Figure 3e** and **f**) revealed that BS considerably enhanced the hydrolysis of the intact protein and the large fragment generated after pepsin digestion (which coelutes with OVA in this system). Gass et al. (*28*) recently reported that BS accelerate the cleavage by trypsin and chymotrypsin of some but not all of a spectrum of dietary proteins. According to these authors, 10 mM of a bile acid mixture (containing taurocholate and glycodeoxycholate) considerably promotes the digestion rates of β-Lg, myoglobulin, and BSA, probably through the destabilization of their tertiary structure. However, unlike the present results, Gass et al. (*28*) reported that chicken OVA remains unaffected by the BS.

To elucidate whether BS could have any effect on protein solubility, we incubated a gastric digest with the duodenal digestion mixture without Corolase PP. As shown in **Figure 4b**, exposure to the simulated duodenal conditions without proteases did not alter the band pattern. On the other hand, it has been proven that bile acids do not have any effect on the activity profile of trypsin or chymotrypsin (*28*).

According to Gass et al. (*28*), physiologic bile acids can exert their effect as individual molecules (below the critical micelle concentration, 3.5 mM), but this is more pronounced when they act as micelles. The concentration of amphiphilic bile components, including BS and PC, increases after a meal. In that sense, the composition of our duodenal digestion mixture resembles that described for a fed state SIF, which can be simulated with 15 mM sodium taurocholate and 3.75 mM PC, while a simulated fasted state SIF contains 3 mM sodium taurocholate and 0.75 mM PC (*29*).

Gastric digests obtained in the presence of PC were also subjected to duodenal digestion. RP-HPLC analysis revealed a somehow higher proteolysis degree when PC was present, particularly in combination with the bile salt mixture (**Figure 3d** and **f**). The protective effect of PC on gastric digestion of α-La reported by Moreno et al. (*7*) is lost in a subsequent duodenal digestion, an observation that the authors attributed to the disruption of the vesicular structure of PC by the BS present in the duodenal digestion mix. On the other hand, PC was found to protect β-Lg from degradation under duodenal conditions (*26*). In this latter case, the protective effect could be ascribed not to the insertion of the protein into the PC vesicles, as the bile salt-PC components are exclusively present as mixed micelles in this system, but to the lipids binding to the secondary fatty acid binding site of β-Lg, which would block the proteinase action for steric reasons (*26*).

Interaction of Ovalbumin with Phosphatidylcholine and Bile Salts. The conformational state of OVA was analyzed by circular dichroism (CD) spectroscopy at pH 2 (in the absence and presence of PC) and pH 7 (in the absence and presence of PC and/or BS). As for the far-UV spectra (**Figure 5a**), the secondary structure analysis revealed a slight difference between both pH conditions, showing an approximately 5% α helix loss and a 5% β strand gain when changing from an acidic to a neutral environment. No effect on the secondary structure motif composition was observed by the addition of PC and/or BS at any pH tested. The featureless near-UV spectrum around zero for all wavelengths obtained at pH 2 (**Figure 5b**) clearly indicated the tertiary structure loss, typical of the molten globule state adopted by OVA at this pH. At pH 7, the characteristic spectrum of the protein was observed. These data reflected very different tertiary structures at both pH values, but no influence of PC, BS, or their combination.

The CD spectra of OVA show that its secondary structure content is essentially the same at pH 2 and pH 7, but the native tertiary interactions are almost completely disrupted at acidic pH (*23*, *30*). α-La, which is claimed to be protected from pepsin hydrolysis by insertion into PC vesicles, in the absence of PC undergoes a significant proteolysis by pepsin in the molten globule state induced by acidic pH (*7*). However, the molten globule-like state of OVA induced at pH 2 is resistant to pepsin digestion under conditions similar to those used for α-La, suggesting a much more limited disorder in the tertiary structure (*23*). The observation that OVA assumes a highly ordered molten globule conformation at pH 2.2, with the intrachain disulfide bond adding a high conformational stability to its structure (*30*), may explain why it does not adopt enough flexibility to penetrate into PC vesicles.

Fluorescence experiments were performed to evaluate any conformational change that could increase the exposure of Tyr and Trp residues to the solvent as a consequence of the interaction of OVA with PC or BS. The fluorescence intensity of OVA at pH 2 was lower than at pH 7, but the maximum wavelength of 334 nm was unchanged (**Figure 6a**). The lower intensity at acidic pH is due to the quenching effect exerted by the protonated Glu residues located in the vicinity of the three Trp residues in the OVA sequence (*31*). Addition of PC decreased the fluorescence intensity at pH 2 and increased the fluorescence intensity at pH 7, but did not change the emission maximum. The titration experiments (**Figure 6b**) showed that the fluorescence intensity at the emission maximum progressively changed with the concentration of added PC, particularly at pH 2. Fluorescence quenching upon PC addition at pH 2 could be due to binding of PC to the protein, which would cause the transfer of resonant energy between the excited aromatic rings and the ligand, while fluorescence increase at pH 7

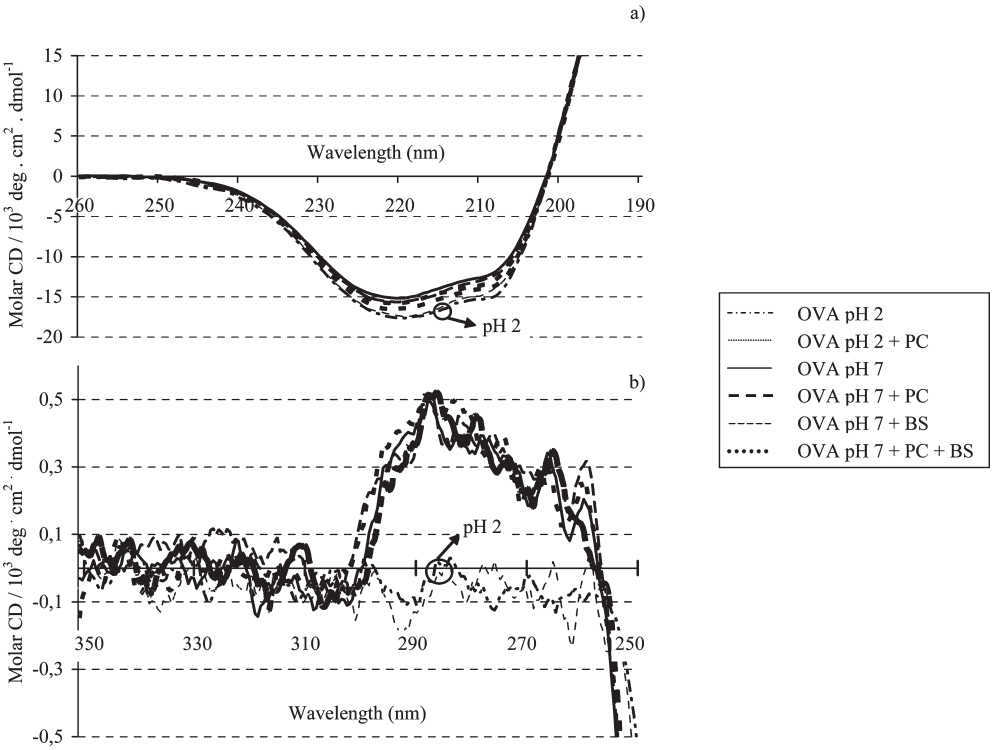

Figure 5. Circular dichroism spectra in the far (**a**) and the near (**b**) UV region of ovalbumin (OVA) at pH 2 in the absence and presence of phosphatidylcholine (PC) and at pH 7 in the absence and presence of PC, bile salts (BS), and their combination.

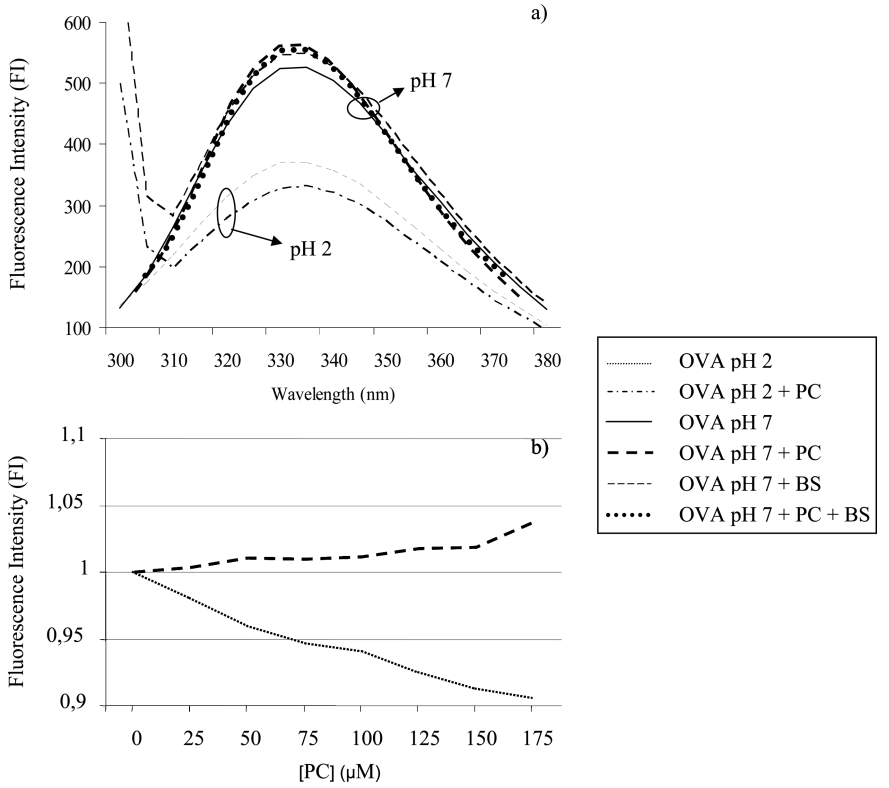

Figure 6. (**a**) Fluorescence spectra of ovalbumin (OVA) at pH 2 in the absence and presence of phosphatidylcholine (PC) and at pH 7 in the absence and presence of PC, bile salts (BS), and their combination. (**b**) Corrected OVA titration curves with PC at pH 2 and pH 7.

could reflect a decrease in polarity in the neighborhood of the excited residues caused by water displacement by the ligand (*32*). Mine et al. (*33*) reported that OVA can interact with PC vesicles at PC/OVA ratios from 10 to 40 M:M, showing a higher affinity for PC at acidic than at basic pH.

As has been described for other proteins, a simple electrostatic attraction of OVA with zwitterionic phospholipids, such as PC, would provide a loose association, so that binding would be reinforced at a low pH, when the protein would normally unfold (*34*−*36*). In fact, the interaction between OVA and PC

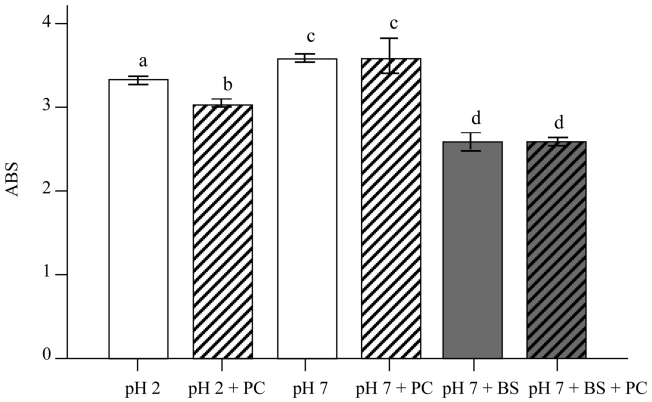

Figure 7. ELISA response against IgG of ovalbumin (OVA) at pH 2 in the absence and presence of phosphatidylcholine (PC) and at pH 7 in the absence and presence of PC, bile salts (BS) and their combination. Error bars correspond to 95% confidence intervals. Different letters above the bars indicate significant differences ($P < 0.05$).

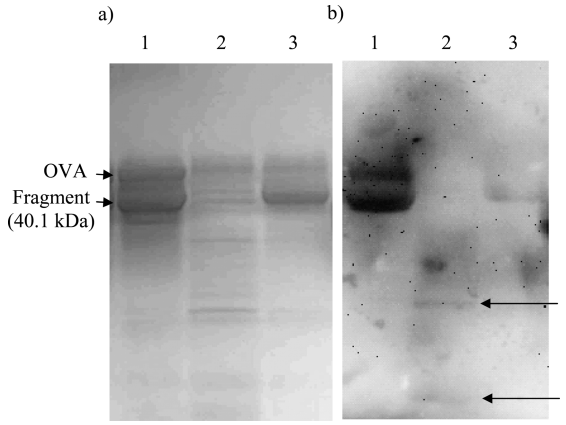

Figure 8. SDS-PAGE (**a**) and Western blot (**b**) of ovalbumin (OVA) digests. Lane 1: OVA digested with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min; lane 2: subsequent duodenal digestion with Corolase PP for 60 min in the presence of bile salts; lane 3: duodenal digestion with Corolase PP for 60 min in the absence of bile salts.

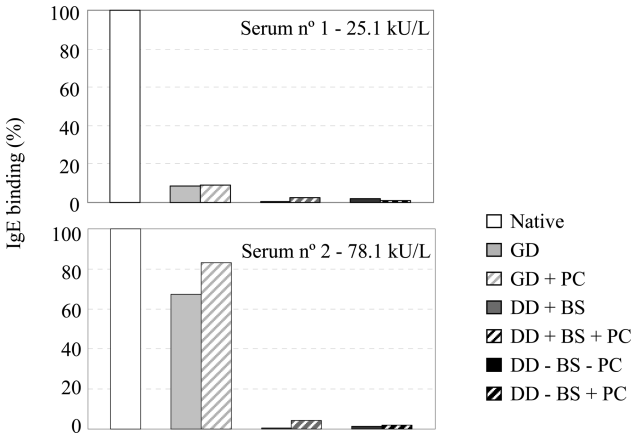

Figure 9. IgE binding estimated by inhibition ELISA, with two human sera of patients allergic to egg, of native ovalbumin (OVA) and its gastric digests at a pepsin/substrate ratio of 1:20, at pH 2, for 60 min (GD) in the presence (+ PC) and absence of phosphatidylcholine (−PC) and of duodenal digests with Corolase PP for 60 min (DD), in the presence (+BS) and absence of bile salts (−BS) and their combinations with PC.

was promoted at acidic pH, when the surface hydrophobicity of the protein was higher. However, the observation that PC did not change its susceptibility to proteolysis by pepsin suggests that, by virtue of its high conformational stability at acidic pH, either OVA did not became inserted into PC vesicles, or such insertion did not provide the protein with extra protection on incubation with the enzyme.

At neutral pH and in the absence of BS, electrostatic binding of OVA to PC may cause OVA to associate with the vesicles surface, increasing the exposure of the protein to pancreatic proteinases. Mogensen et al. (*36*) found that Bet v1, the major allergen from birch tree pollen, interacts with micelles forming lysophospholipids in a pH-depending manner. At pH 3.9, Bet v1 is inserted deeply into the membrane, and this prevents a general degradation of the protein on incubation with pepsin. At pH 7.2, Bet v1 associates to the membrane without loosing its integrity, and this makes it more sensitive to proteolytic degradation with trypsin.

BS (sodium taurocholate and sodium glycodeoxycholate were evaluated separately) did not exert any noticeable effect on the fluorescence emission of OVA (data not shown). In any case, our fluorescence experiments could not rule out the binding of protein to bile acids, as the maximum concentration used was below 0.5 mM. However, IgG binding to the protein, basically unchanged in the presence of PC at pH 7, was significantly ($P < 0.05$) decreased in the presence of BS (**Figure 7**), which points to structural changes due to mutual interaction. It is suggested that OVA could associate with the surfactants or with the mixed bile salt-PC micelles present, an effect that has been proposed to occur to proteins in the duodenum (*25*), increasing its exposure to pancreatic proteinases.

Immunoreactivity of OVA and Its Digests. Immunoblotting of gastric and duodenal digests of OVA in the absence and presence of BS is shown in **Figure 8**. Intact OVA and its 40.1 kDa fragment exhibited a considerable IgE-binding activity, which virtually disappeared after duodenal digestion and, particularly, in the presence of BS because of the higher digestibility promoted under those conditions. The immunoblotting suggests that the duodenal digestion products could still retain IgE-binding epitopes (indicated by arrows in **Figure 8b**), and this was further checked by competition ELISA with sera from patients allergic to eggs (**Figure 9**).

IgE binding decreased, depending on the patient serum, after in vitro gastric digestion and was further reduced after duodenal digestion. In vitro duodenal digestion under all conditions resulted in low reactivity against IgE from sera of allergic patients, although the detectable IgE binding response suggested the presence of peptides containing intact binding sites recognizable by serum IgE. It remains to be elucidated whether these fragments contain single IgE epitopes or more than one IgE binding site, which would make them capable of cross-linking IgE bond receptors and cause cell degranulation. Furthermore, the effect of surfactants on the proteolytic stability of the degradation products deserves further investigation. Thus, it has been suggested that during the digestion of whole egg, hydrolysis fragments of OVA could emulsify with the egg yolk PC, which would protect them from digestion and increase their intestinal absorption (*37*).

CONCLUSIONS

OVA was very resistant to pepsin action at an enzyme/substrate ratio that would resemble a physiological situation (1:20 w/w, 172 units/mg), at pH values equal to or above 2, typical of a fed state and, particularly, of the stomach of children. Under these conditions, the intact protein and its main degradation product, a 40 kDa fragment resulting from cleavage at the

N-terminal side, persisted for at least 2 h of digestion. The presence of the physiological surfactant PC did not change the susceptibility of OVA to proteolysis with pepsin. Fluorescence experiments showed that OVA interacted with PC vesicles, particularly at acidic pH, when the protein adopts a molten globule state devoid of tertiary structure interactions, but when it likely maintains a high degree of conformational stability, resisting pepsin action.

The presence of BS at physiological concentrations, typical of the duodenal fed state, considerably increased the proteolysis of OVA by a mixture of pancreatic enzymes. In the presence of BS, the CD spectra of OVA did not show any sign of tertiary structure destabilization that could make the protein more prone to proteinase action; however, antibody binding to the protein was considerably decreased in the presence of BS, what points to structural changes due to mutual interaction. The addition of PC made OVA even more sensitive to proteolytic degradation under duodenal conditions, suggesting that OVA could associate with the surfactants or with the mixed bile salt-PC micelles present in the duodenum, increasing the exposure of the protein to pancreatic proteinases.

Immunoreactivity against IgE from sera of allergic patients was retained after in vitro gastric digestion, depending on the reactivity of the sera, but it decreased considerably after in vitro duodenal digestion under all conditions. Nevertheless, the persistence of peptides containing intact binding sites recognizable by serum IgE could not be discarded.

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