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Uptake of 2S Albumin Allergens, Ber e 1 and Ses i 1, across Human Intestinal Epithelial Caco-2 Cell Monolayers

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We have investigated the absorption rates of two purified major allergen 2S albumins, Ber e 1 from Brazil nuts (*Bertholletia excelsa* Humb. & Bonpl.) and Ses i 1 from white sesame seeds (*Sesamum indicum* L.), across human intestinal epithelial Caco-2 cell monolayers following gastrointestinal digestion in vitro. The transport from apical to basolateral side in cell monolayers was evaluated by RP-HPLC-UV and indirect competitive ELISA methods, being confirmed by western-blotting analysis. Significant amounts (∼15−25 nmol μmol^{-1} initial amount/h) of intact Ber e 1 and Ses i 1 were found in the basolateral side. The absorption rates of both plant allergens through the cell monolayer were shown to be constant during the whole incubation period (4 h at 37 °C), verifying that the permeability of the membrane was not altered by the allergen digests. Our findings revealed that both purified 2S albumin allergens may be able to survive in immunologically reactive forms to the simulated harsh conditions of the gastrointestinal tract to be transported across the Caco-2 cell monolayers, so that they would be able to sensitize the mucosal immune system and/or elicit an allergic response.

KEYWORDS: Food allergy; 2S albumin; Caco-2 cell monolayers; gastrointestinal absorption; Brazil nuts; sesame seeds.

INTRODUCTION

Dietary proteins are mainly hydrolyzed by luminal enzymes in the gastrointestinal tract (GIT) to amino acids and small peptides, which are absorbable by enterocytes, but small amounts of proteins are subsequently absorbed with only partial digestion (*1*). Although the intestinal epithelium theoretically acts as a barrier restricting the permeation of macromolecules by tight cell junctions, certain proteins are enable to cross the intestinal barrier in an intact form (*2*, *3*). A clear example of that is given by food allergens. The relatively high stability of some food allergens to the harsh conditions present in GIT (acidic pH, digestive enzymes, and presence of surfactant agents) allows them to survive in a sufficiently intact form to be taken up by the gut and provoke immune-mediated hypersensitive reactions in allergic patients after sensitization (*4*−*6*).

The prevalence of food allergy is estimated to be as high as 6% in young children and approximately 3−4% in adults (*7*). Although the mechanisms by which food allergens sensitize an individual remain currently unclear, most of them are thought to sensitize via GIT (*8*−*10*). In vivo studies have demonstrated the gastrointestinal absorption of several food allergens, including bovine serum albumin (*11*), ovalbumin (*12*), Gly m Bd 30K from soybean (*13*), and proteins from digested peanuts (*14*). However, the complexity of GIT and the difficulty to obtain sufficient amounts of food allergens of high purity from natural sources has limited the capability to evaluate their transport and absorption rates in vivo. In recent years, Caco-2 cell monolayers have been routinely used in transport studies as an in vitro model which mimics the human intestinal epithelium (*15*). Caco-2 cells undergo in standard culture conditions a process of spontaneous differentiation that leads to the formation of a monolayer of cells, which separates two extremely different extracellular compartments. On confluence, these cells express several morphological and functional characteristics of the mature enterocyte, being functionally polarized, with apical brush border, microvilli, tight junctions between adjacent cells, and the excretion of brush border associated enzymes (*16*). When cultured on filter inserts, they form a tight differential monolayer, thus constituting a valuable in vitro permeability model which allows the rapid screening of drugs or food ingredients absorption under controlled environmental conditions. The predictive permeability information obtained from Caco-2 cell model seems to be well correlated with human oral absorption (*15*). The absorption of food allergens, from animal and plant sources, across Caco-2 cell monolayers has been recently reported. Milk (*17*, *18*), egg (*19*), and recombinant wheat (*20*) allergens were efficiently transported across the Caco-2 cell monolayers, showing remarkable differences in resistance against the attack of cellular peptidases. Milk allergens were reported to be

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susceptible of proteolysis with only small amounts remaining in intact form (*17*, *18*), whereas ovomucoid was completely resistant to cellular peptidases (*19*).

The 2S albumins form a major group of storage seed proteins in many dicotyledonous species. Although 2S albumins show certain level of polymorphism, a conserved heterodimeric structure comprising subunits of about 30−40 and 60−90 residues, respectively, and linked by two disulfide bridges is typically found in this protein family (*21*). The 2S albumins are becoming of increasing interest in clinical and nutritional studies as they have been described as major allergens in numerous plant foods (*22*, *23*). Ber e 1 and Ses i 1 have been reported to be major allergens in Brazil nuts (*Bertholletia excelsa* Humb. & Bonpl.) and sesame seeds (*Sesamum indicum* L.), respectively (*24*, *25*). They are associated with immunoglobulin E (IgE)-mediated food allergy (*26*−*28*) and are a major cause of concern due to the severity of reactions they elicit. In vitro gastrointestinal digestion studies have shown both allergens to retain sufficient three-dimensional structure after extensive digestion (*29*, *30*), suggesting that both proteins could reach the gut immune system in an immunologically active form. In this study, we have evaluated the absorption rates of both purified major plant food allergens, Ber e 1 and Ses i 1, across human intestinal epithelial Caco-2 cell monolayers following gastrointestinal digestion in vitro and discussed their transport mechanism.

MATERIALS AND METHODS

Materials. The human colon adenocarcinoma cell line Caco-2 at passage 40 was purchased from the European Collection of Cell Cultures (ECACC) and used in experiments at passages 45−50. The digestive enzymes pepsin (P-7000) and pancreatin (P-3292), bile salts (B-8756), high-glucose Dulbecco's modified minimal essential medium (DMEM), Hanks' buffered salt solution (HBSS), nonessential amino acids, and other cell culture-grade chemicals were obtained from Sigma (St. Louis, MO). A shaker (Certomat S) with a temperature controller (Certomat H), used for in vitro protein digestion, were both from B. Braun Biotech. Int. (Melsungen, Germany). Culture flasks and Transwell plates were purchased from Corning Costar (Cambridge, MA).

Protein Purification. The main 2S albumins from Brazil nut (Ber e 1; ExPASY entry P04403) and white sesame seeds (Ses i 1; ExPaSY entry Q9AUD1) were purified from natural sources to homogeneity by using gel filtration chromatography and gradient chromatofocusing in tandem. Further characterization by proteomic techniques was carried out as previously described (*29*, *31*).

In Vitro Digestion of 2S Albumins. The 2S albumins, Ber e 1 and Ses i 1, were digested in vitro by following the procedure described by Glahn et al. (*32*) with some modifications. A 10 mg amount of purified allergen (see above) was suspended in 8 mL of 120 mM NaCl, mixed, and allowed to stand at room temperature for 15 min. The pH was then adjusted to pH 2.0 with 5 N HCl, the volume brought to 10 mL with NaCl solution, and 0.1 mL of pepsin solution (5 mg in 2.5 mL of 0.1 N HCl) added to each sample. The flasks were placed in a shaker (100 oscillations min^{-1}) at 37 °C for 1 h. For the intestinal digestion step, the pH was raised to 6.0 with 1 M $NaHCO_3$ dropwise, and 2.5 mL of pancreatin-bile salts mixture (5 mg of pancreatin and 30 mg of bile extract in 50 mL of 100 mM $NaHC0_3$) was added. The pH was adjusted to pH 7.5 with 1 N NaOH and the volume brought to 15 mL with 120 mM NaCl. Intestinal digestion of allergens was carried out at 37 °C for 2 h. Control samples having only the digestive enzymes in buffered solution were obtained. Plant proteins highly susceptible to proteolysis by endopeptidases, such as the 11S fraction from chickpea seeds (*33*), were used to demonstrate the effectiveness of the in vitro digestion. After protein hydrolysis, digestive enzymes were inactivated by heat treatment at 85 °C for 5 min in a water bath. Digested samples were assayed to confirm the inactivation of proteolytic enzymes by using a highly sensitive universal substrate, casein resorufin-labeled (Roche, Pezberg, Germany), especially suited for the detection of traces of protease activity (<0.1 μg of enzyme). These assays were carried out by following the manufacturer's instructions. Proteases were considered not detectable when absorbance values at 574 nm were lower than 0.02. After digestion, samples were passed through a 0.22 μm filter and aliquots (1.5 mL) stored at −20 °C until use in cell culture experiments.

Cell Culture. Human intestinal epithelial Caco-2 cells were maintained by serial passage in 75 cm^2 plastic culture flasks. Cells were cultured in DMEM, supplemented with heat inactivated fetal bovine serum (15%), sodium bicarbonate (3.7 g L^{-1}), nonessential amino acids (1%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (15 mmol L^{-1}), bovine insulin (0.1 UI mL^{-1}), and 1% antibiotic−antimicotic solution (Sigma, A5955). The cells were grown under 5% CO_2 in humidified air atmosphere at 37 °C and given fresh medium every 2 or 3 days. For transport studies, Caco-2 cells were cultured on permeable polycarbonate filter supports (Transwell inserts of 3 μm pore size and diameter of 24 mm) in 6-well plates, following the procedure previously described by Rubio and Seiquer (*34*). Cell seeding was carried out at a density of 9×10^4 cells cm^{-2}, and cells were grown as above. The volume of culture medium was 1.5 mL on the apical and 2.5 mL on the basolateral side, being both replaced every 48 h. Cell monolayer integrity was monitored by transepithelial electrical resistance (TEER), using a Minicell electrical resistance system (Millipore, Bedford, MA), from day 5 after cell seeding until immediately before the digests were added to the apical side of the bicameral system (day 20). Three TEER measurements/well in days 5, 10, 14, 18, and 20 after cell seeding were taken, and values were determined by the following calculation: monolayer resistance minus resistance of a blank insert (without Caco-2 cells) × area of the Transwell (4.7 cm^2) = monolayer resistance (Ω cm^2). Blank electrical resistance values were usually in the range of 90−100 Ω cm^2. In addition to TEER measurements, the poorly absorbed marker phenol red, at concentration of 42 μM in culture medium, was used to confirm the integrity of Caco-2 cell monolayers. To evaluate the rates of phenol red absorption, 1.5 mL of culture medium to the apical side and 2.5 mL of transport solution (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7) in the basolateral side were added. After 1h incubation at 37 °C, spectrophotometric measurements (λ = 559 nm) of phenol red in both apical and basolateral samples following its adjustment at pH 10 with 5 N NaOH, where only the basic form of phenol red exists, were carried out. Cell monolayers were used in transport studies when values of TEER reached a plateau exceeding 600 Ω cm^2 and the leakage rates of phenol red were lower than 0.5%. Discarded monolayers were assumed to exhibit extensive leakage through imperfect occluding junctions or holes in the monolayer. After full period of incubation (4 h) of allergen digests, no significant changes in TEER and phenol red absorption rates were observed.

Allergen Absorption Experiments. Preliminary cell viability assays were carried out to investigate the potential effect of allergen digests on integrity of the cell monolayer in the bicameral Caco-2 cell culture system. The procedure followed was as described by Clemente et al. (*35*), with some minor modifications. Following a period of growth in vitro, the viability of human colorectal adenocarcinoma Caco-2 cells was assessed by a neutral red (NR) cytotoxicity assay procedure, based on the ability of viable uninjured cells to incorporate actively NR, a supravital dye, into lysosomes. The 96-well microtitre plates were inoculated at a density of 10^4 cells/well in 100 μL of media which gave optimal cell growth. Caco-2 cells were maintained overnight to allow them to adhere to the wells. Growth media was removed and 100 μL of digested allergen solution (see above) added to the cells under sterile conditions. Control wells received growth media only. Caco-2 cells were harvested after 4 h exposure, during which time the growth media were not changed. Cell viability was assessed by staining with NR (2 h at 37 °C), followed by cell fixation (0.5% formaldehyde, 0.1% $CaCl_2$ for 30 s at room temperature). Microtitre plates were washed by three brief immersions in sodium phosphate-buffered saline (PBS, 0.01 M sodium phosphate buffer + 0.15 M NaCl) and cells lysed (50% ethanol containing 1% acetic acid overnight at 4 °C). The optical densities of the resulting solutions were measured at 550 nm using a BioRad model 550 microplate reader (BioRad, Hercules, CA). Cell viability data, expressed as a percentage compared to control data, were

obtained from at least two independent experiments ($n \geq 5$/experiment). The data were analyzed statistically by the Student's *t*-test, using the Minitab statistical software (State College, PA).

Absorption experiments were carried out 20 days after initial cell seeding. Spent culture media was aspirated from the apical and basolateral chambers, and both cell surfaces were washed three times with HBSS at 37 °C. A 2.5 mL volume of transport solution (see above) was added to the basolateral chamber, and aliquots of allergen digests (1.5 mL, three replicates/allergen tested) were added to the apical chamber. Buffered solution containing an enzyme mixture inactivated by heat treatment (see above) was used as negative control. Basolateral samples were collected every 1 h for a total period of incubation of 4 h at 37 °C under 5% CO_2 in humidified air. An equal volume of transport buffer to the basolateral side was replaced immediately after each sampling. Collected samples were stored at −20 °C until use. The functionality of the cell monolayer was monitored by the evaluation of TEER and phenol red leakage before and after the experiments as above. The apparent permeability coefficient (P_{app}) of digested allergens was expressed in cm s^{-1} and calculated as $P_{app} = \Delta Q/\Delta t \times 1/A \times 1/C_o$, where $\Delta Q/\Delta t$ is the transport rate (μg s^{-1}), A is the surface area of the membrane (4.7 cm^2), and C_o is the initial concentration of allergens in the apical chamber (μg mL^{-1}).

Determination of Allergen Absorption Rates by RP-HPLC-UV. Digested 2S albumins and control samples from apical and basolateral chambers were applied to a Phenomenex Jupiter Proteo (90 Å pore size, 4 μm particle size, 250 × 4.6 mm i.d.) column coupled to a Beckman binary gradient 125 pump HPLC (Beckman, Fullerton, CA) equipped with a Metrohm-Spark Triathlon autosampler and a Beckman 166-UV detector. Samples were eluted using 0.1% (w:v) trifluoroacetic acid (TFA) in double-distilled water as solvent A and 0.085% (w:v) TFA in double-distilled water−acetonitrile (10:90, v:v) as solvent B, as described by Moreno et al. (*30*). When required, 2S albumins (100 μL, 0.25 mg) were reduced with 10 μL of 100 mM dithiothreitol (DTT) dissolved in 25 mM sodium phosphate buffer, pH 7.4, and incubated at 65 °C for 30 min. Allergen quantification was performed by the external standard method using individual solutions of known concentrations of both purified allergens in the range 0.01−0.5 mg mL^{-1}. To build the corresponding calibration curves, integrated areas were plotted against the known amount injected. The corresponding calibration curves, $y = 8725.22x - 2.41$ for Ber e 1 and $y = 4827.71x - 0.36$ for Ses i 1, were linear over the range studied, and the determination coefficients obtained (r^2) were >0.99 in both cases.

Determination of Allergen Absorption Rates by Enzyme-Linked Immunosorbent Assay (ELISA). Two New Zealand white rabbits were immunized subcutaneously with 200 μg of purified allergens emulsified in Freund's complete adjuvant solution. The rabbits were administered a booster consisting of five injections of 100 μg of native protein in Freund's incomplete adjuvant given at intervals of 14 days. Blood samples (20 mL) were taken at 35, 49, 63, and 77 days after booster immunizations and centrifuged, and the plasma was removed and stored at −20 °C. Using different bleedings, the reactivity of the polyclonal antisera to bind Ber e 1 and Ses i 1 was evaluated by a noncompetitive ELISA. The antisera gave absorbance values over 1.5 at 450 nm when used at a dilution of 1:350 000 and 1:50 000 for Ber e 1 and Ses i 1, respectively. To determine the amount of allergen transported across the cell monolayers, an indirect competitive ELISA (*36*) for detection of Ber e 1 and Ses i 1 in samples collected from the apical and basolateral chambers was carried out. Polystyrene microtitration high-binding plates (Corning Inc., Corning, NY) were coated with 300 μL/well of purified Ber e 1 or Ses i 1 (1 μg mL^{-1} in 0.2 M sodium carbonate−bicarbonate buffer, pH 9.6). After overnight incubation at 4 °C, plates were washed four times with PBS, pH 7.4, containing 0.05% Tween 20 (PBST). A 150 μL/well amount of a 1% bovine serum albumin-PBS solution was added to the plates and incubated for 1 h at 37 °C before washing four times with PBST. A 100 μL/well amount of the diluted samples collected from the apical and basolateral chambers at different times of incubation (0−4 h) together with 100 μL/well of polyclonal rabbit antibody serum raised against Ber e 1 or Ses i 1 diluted 1:175 000 and 1:25 000 in PBST, respectively, was added to the plates and incubated for 1.5 h at 37 °C before washing four times with PBST. After incubation with 200 μL/well of HRP labeled goat antirabbit IgG (Sigma) diluted 1:20 000 and 1:10 000 for Ber e 1 and Ses i 1, respectively for 1.5 h, plates were again washed four times with PBST. Color development was with 100 μL of a solution of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma). After 15 min, 100 μL of 1 N HCl was added to each well to stop the reaction. The intensity of the resulting color was read in a microplate reader at 450 nm.

To determine the allergen content of the collected samples, standard curves of both purified allergens were constructed for the serially diluted Ber e 1 and Ses i 1 in the range of concentrations 0.0005−1000 μg mL^{-1}. The amounts of Ber e 1 and Ses i 1 contained in the apical and basolateral chambers were calculated from the standard curves and expressed as total μg or μg mL^{-1} (*36*) using first-order exponential decay equations ($y = 0.35 + 0.98 \exp(-x/1.14)$ for Ber e 1 and $y = 0.24 + 0.89 \exp(-x/0.07)$ for Ses i 1) provided by Origin 7.5 Version (OriginLab Corp., Northampton, MA). The limit of detection for both purified allergens was found to be below 5 ng mL^{-1}.

SDS-PAGE and Western-Blotting Analysis. For electrophoresis analysis, samples collected from the apical and basolateral chambers were concentrated (×20), diluted 1:1 in NuPAGE LDS sample buffer, and analyzed on Novex 12% Bis-Tris precast gels using NuPAGE MES as running buffer (Invitrogen, Paisley, U.K.). When required, samples were reduced with DTT and NuPAGE antioxidant was added to the upper buffer chamber to prevent reduced proteins from reoxidizing during electrophoresis. Proteins were visualized by Colloidal Blue staining kit (Invitrogen). After electrophoresis, proteins were transferred onto a 0.45 μm Invitrolon PVDF membrane using the XCell II Blot module (Invitrogen). Blots were blocked with TBST [0.05% v:v Tween-20 in Tris-buffered saline (TBS)] containing 10% (w:v) milk powder for 1 h at room temperature. After washing with TBST, blots were incubated with anti-Ber e 1 or anti-Ses i 1 sera diluted 1:1000 (v:v) in TBST, overnight at 4 °C. After washing, blots were incubated with HRP labeled goat antirabbit IgG (Sigma) diluted 1:1000 (v:v) in TBST containing 10% (w:v) milk powder for 1 h at room temperature. Blots were again washed five times with TBST and stained with a liquid substrate system for membranes of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma).

RESULTS

2S albumin allergens from different species, including those from Brazil nuts and sesame seeds, have been reported to be highly resistant to in vitro digestion (*29*, *30*, *37*−*39*). In good agreement with these findings, our results showed large fractions of Ber e 1 and Ses i 1 that remained intact after in vitro gastrointestinal digestion (**Figures 1A,B** and **2A,B,** respectively). In the case of Ber e 1, partial trimming of intact allergen gave rise to two chromatographic peaks with close but shorter retention times when compared with intact protein (**Figure 1B**). These peaks were previously identified as a large fragment of M_r 5000−6400 Da comprising a complex mixture of smaller peptides, derived mainly from the large subunit and linked by intrachain disulfide bridges, containing potentially active B- and T-cell epitopes (*30*). In addition, a mixture of minor and small peptides with retention times between 15 and 30 min was also observed as result of limited proteolysis of both allergens.

In microplates assays, native allergens in buffered solution did not have apparent effect on the viability of Caco-2 cells; however, the presence of active exogenous proteases in allergen digests decreased more than 90% cell viability. As a result, protease inactivation (85 °C, 5 min) was strictly necessary to obtain optimal cell viability (higher than 95%) to carry out the absorption experiments in Caco-2 cell monolayers. Under such thermal treatment, allergens were predicted to be structurally stable (*29*, *36*, *40*, *41*). Concentrations of digested allergens similar to those used in transport experiments appeared to have no significant effect on viability of Caco-2 cells ($P < 0.01$).

The apical to basolateral transport of Ber e 1 and Ses i 1 across Caco-2 cell monolayers was estimated using both RP-

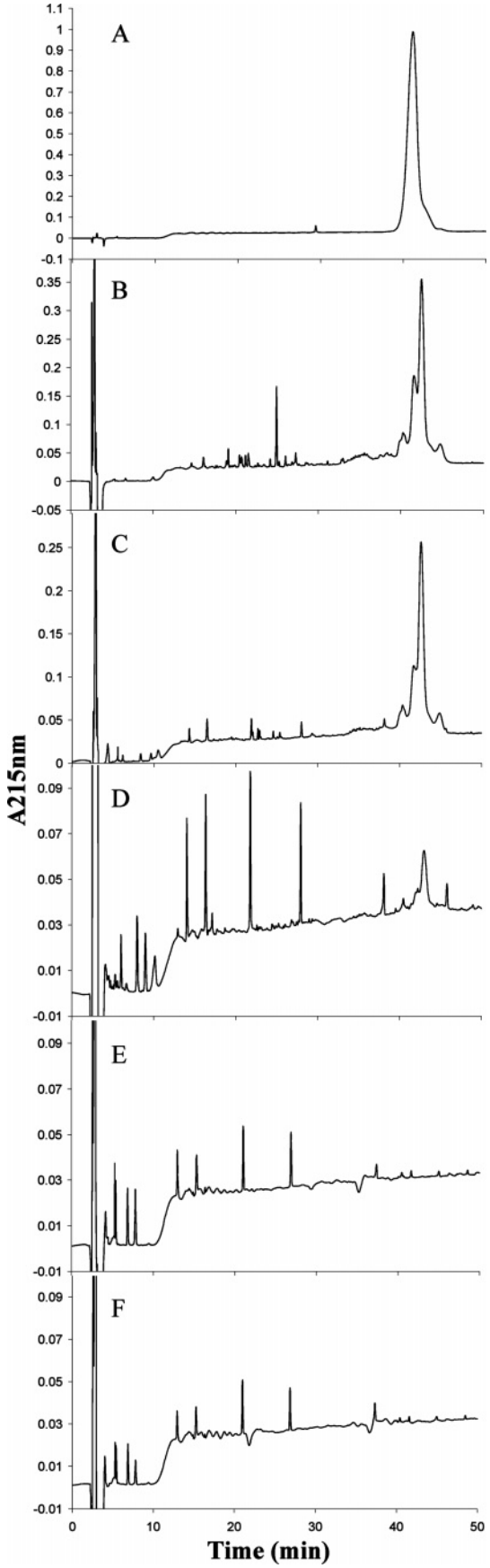

Figure 1. Reverse phase-HPLC-UV ($\lambda = 215$ nm) patterns of Ber e 1: (**A**) native; (**B**) digested before loading on Caco-2 cell monolayers; digested and collected from the (**C**) apical and (**D**) basolateral chambers after 4 h incubation at 37 °C. Control samples (with no allergens added) collected from the (**E**) apical and (**F**) basolateral chambers.

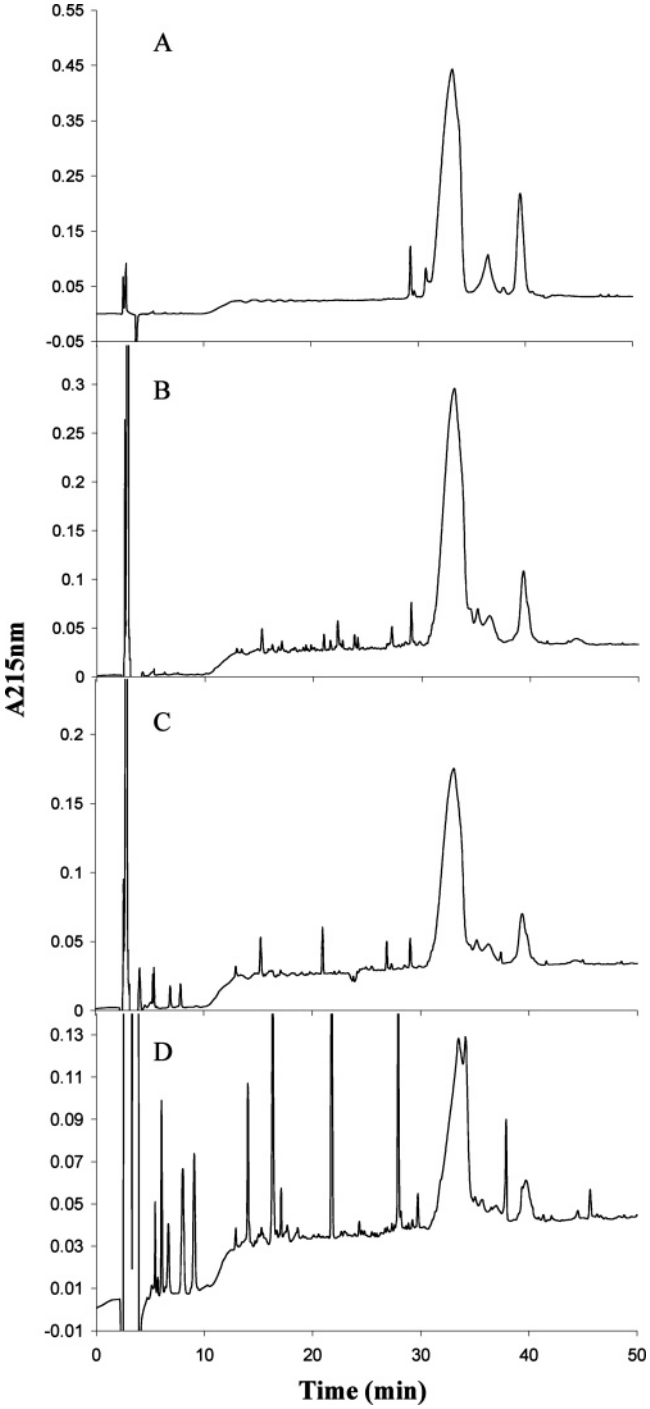

Figure 2. Reverse phase-HPLC-UV ($\lambda = 215$ nm) patterns of Ses i 1: (**A**) native; (**B**) digested before loading on Caco-2 cell monolayers; digested and collected from the (**C**) apical and (**D**) basolateral chambers after 4 h incubation at 37 °C.

HPLC-UV and ELISA methods. **Figures 1C,D** and **2C,D** show the RP-HPLC-UV profiles of the digested Ber e 1 and Ses i 1 taken from the apical and basolateral chambers following incubation of 4 h at 37 °C. No significant differences between RP-HPLC-UV profiles were observed for the whole period of incubation. The peptides resulting from the limited proteolysis of both allergens, and whose retention times were between 15 and 30 min, disappeared after the incubation of the digested allergens on the apical chamber (**Figures 1C** and **2C**). The absence of these small peptides is likely due to the hydrolytic action of peptidases present in the enterocytes brush border (*42*). As a result, only intact allergens, partially trimmed Ber e 1 and

Table 1. Detected Amounts of Intact and Trimmed Ber e 1 following in Vitro Gastrointestinal Digestion before Loading on Caco-2 Cell Monolayers (Apical 0 h) and Collected from the Basolateral Chambers at 1–4 h after Incubation at 37 °C

method	sample	total nmol	10^{-5} nmol s^{-1}	$10^{-6}P_{app}$[b] (cm s^{-1})	recovery/h (nmol μmol^{-1} initial amount)
ELISA	apical 0 h	7.598			
	basolateral 1 h	0.197 ± 0.014[a]	5.47	2.30	25.9
	basolateral 2 h	0.201 ± 0.009	5.58	2.30	26.5
	basolateral 3 h	0.183 ± 0.026	5.08	2.10	24.1
	basolateral 4 h	0.247 ± 0.026	6.86	2.90	32.5
RP-HPLC	apical 0 h	5.990			
	basolateral 1 h	0.128 ± 0.000	3.55	1.90	21.4
	basolateral 2 h	0.140 ± 0.004	3.88	2.10	23.4
	basolateral 3 h	0.145 ± 0.007	4.02	2.10	24.2
	basolateral 4 h	0.165 ± 0.002	4.58	2.40	27.5

[a] Mean ± SD ($n = 3$). [b] Apparent permeability coefficient.

Table 2. Detected Amounts of Intact Ses i 1 following in Vitro Gastrointestinal Digestion before Loading on Caco-2 Cell Monolayers (Apical 0 h) and Collected from the Basolateral Chambers at 1–4 h after Incubation at 37 °C

method	sample	total nmol	10^{-5} nmol s^{-1}	$10^{-6}P_{app}$[b] (cm s^{-1})	recovery/h (nmol μmol^{-1} init amount)
ELISA	apical 0 h	13.032			
	basolateral 1 h	0.358±0.050[a]	9.90	2.40	27.5
	basolateral 2 h	0.337±0.011	9.39	2.30	25.6
	basolateral 3 h	0.337±0.015	9.39	2.30	25.6
	basolateral 4 h	0.392±0.008	11.00	2.70	30.1
RP-HPLC	apical 0 h	13.053			
	basolateral 1 h	0.217±0.001	6.01	1.50	16.6
	basolateral 2 h	0.186±0.008	5.16	1.30	14.2
	basolateral 3 h	0.179±0.034	4.98	1.20	13.7
	basolateral 4 h	0.256±0.081	7.11	1.70	19.6

[a] Mean ± SD ($n = 3$). [b] Apparent permeability coefficient.

several chromatographic peaks corresponding to the control samples (inactivated enzymes in buffered solution) collected from the apical chambers (**Figure 1E**) were detected. Interestingly, the RP-HPLC-UV method was sensitive enough to detect and quantify both allergens in the basolateral chamber, demonstrating the transport of both proteins in intact form across Caco-2 cell monolayers (**Figures 1D** and **2D**). As mentioned above, other narrow peaks whose retention times coincided with those found in control samples collected from the basolateral chambers (**Figure 1F**) were also found. Under reducing conditions, both allergens gave rise two well-defined chromatographic peaks corresponding to the small and large subunits, confirming the presence of intact Ber e 1 and Ses i 1 in the basolateral side (data not shown).

Digested and native allergens were normalized for protein content and their immunoreactivities tested by indirect competitive ELISA. Digested allergen reactivity was found not to be significantly different when compared to native allergen (data not shown), which is in good agreement with the high resistance of antigenic determinants shown by the 2S albumins to thermal treatment and proteolysis by digestive enzymes. These data supported the suitability of the immunoassay to evaluate the total amounts of Ber e 1 and Ses i 1 in both apical and basolateral chambers. The permeability (P_{app} values) and recovery rates for Ber e 1 and Ses i 1, estimated by both RP-HPLC-UV and ELISA methods, are shown in **Tables 1** and **2**. Both allergens showed a constant absorption rate across Caco-2 cell monolayers during the whole period of incubation (4 h), confirming that the membrane permeability was not altered by the allergen digests. After 4 h of incubation, total recoveries of both allergens in the basolateral side of Caco-2 cell monolayers were measured. According to the ELISA method, total recovery values of both allergens were 109 nmol μmol^{-1} initial amount whereas values of 97 and 64 nmol μmol^{-1} initial amounts were obtained by RP-HPLC-UV for Ber e 1 and Ses i 1, respectively. Levels of allergens detected in the basolateral chambers by the ELISA method were always higher when compared to the chromatographic method. Such discrepancies could be due to the fact that the RP-HPLC-UV method measured only the intact (and the partially trimmed Ber e 1) allergen whereas the ELISA method detected the levels of transported allergens, intact or fragments, with the ability to bind the polyclonal antibodies.

The transport in intact form of both Ber e 1 and Ses i 1 to the basolateral side was confirmed by SDS-PAGE (**Figures 3A** and **4A**). Following SDS-PAGE analysis under nonreducing conditions, a faint and a prominent band of $M_r \sim 12\,000$ were observed in samples recovered from the basolateral chamber for Ber e 1 and Ses i 1, respectively (**Figures 3A** and **4A**, lane 5). Under reducing conditions, Ses i 1 gave rise to the M_r 9000 and 3000 polypeptides characteristic of the two subunit albumins (**Figure 4A**, lane 9), whereas the main polypeptides corresponding to Ber e 1 showed lower molecular weights (between $M_r \sim$ 6,000 and 3500) than those found prior to digestion (**Figure 3A**, lanes 6 and 9), which is indicative of limited proteolysis as previously reported (*30*). In addition, the detection of both allergens in basolateral samples by western-blotting demonstrated the presence of some antigenic determinants resistant to the harsh conditions of the digestive process but also with the ability to pass through the Caco-2 cell monolayer (**Figures 3B** and **4B**, lane 4).

DISCUSSION

In this paper, we have reported that Ber e 1 and Ses i 1 showed a significant thermal stability and resistance to proteolysis by digestive enzymes in vitro, remaining their immunoreactivity unaffected. Thermal treatment might change the intestinal transport properties of some allergens as recently shown for the native and heat-denatured β-lactoglobulin (*18*). However, in the case of 2S albumins the high stability to intense

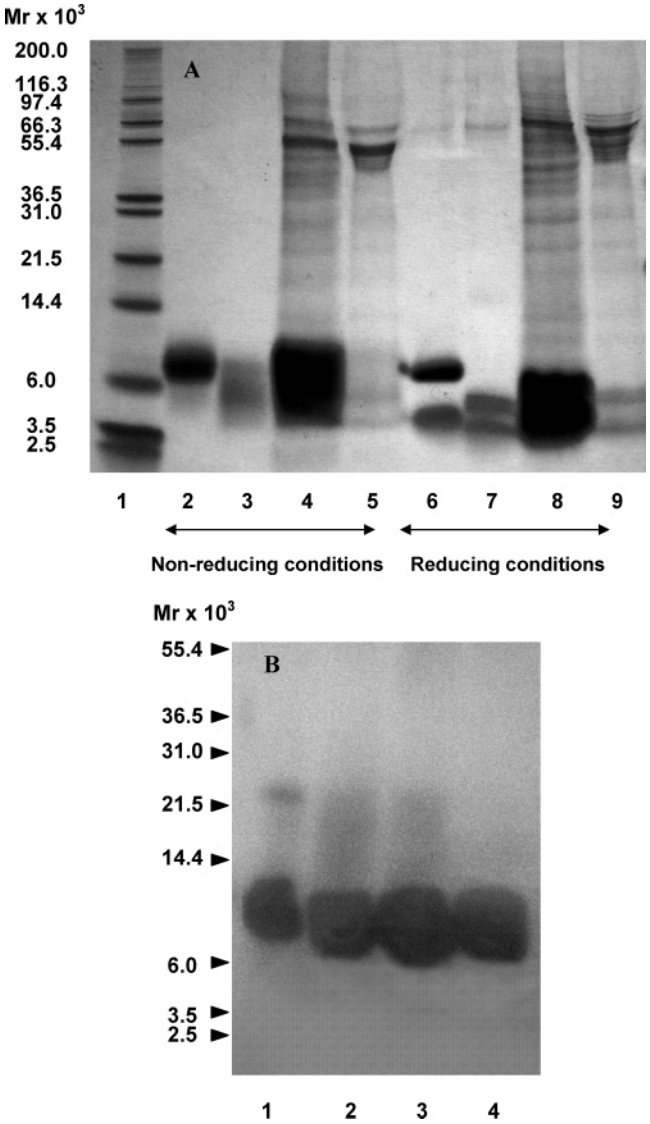

Figure 3. (**A**) SDS-PAGE of Ber e 1: (lanes 2, 6) native; (lanes 3, 7) digested before loading on Caco-2 cell monolayers; digested and collected from the (lanes 4, 8) apical and (lanes 5, 9) basolateral chambers under nonreducing and reducing conditions, respectively. (**B**) Immunoblot analysis with a rabbit polyclonal anti-Ber e 1 antibody of (lane 1) native Ber e 1, (lane 2) digested Ber e 1 before loading on Caco-2 cell monolayers, and digested and collected Ber e 1 from the (lane 3) apical and (lane 4) basolateral chambers under nonreducing conditions.

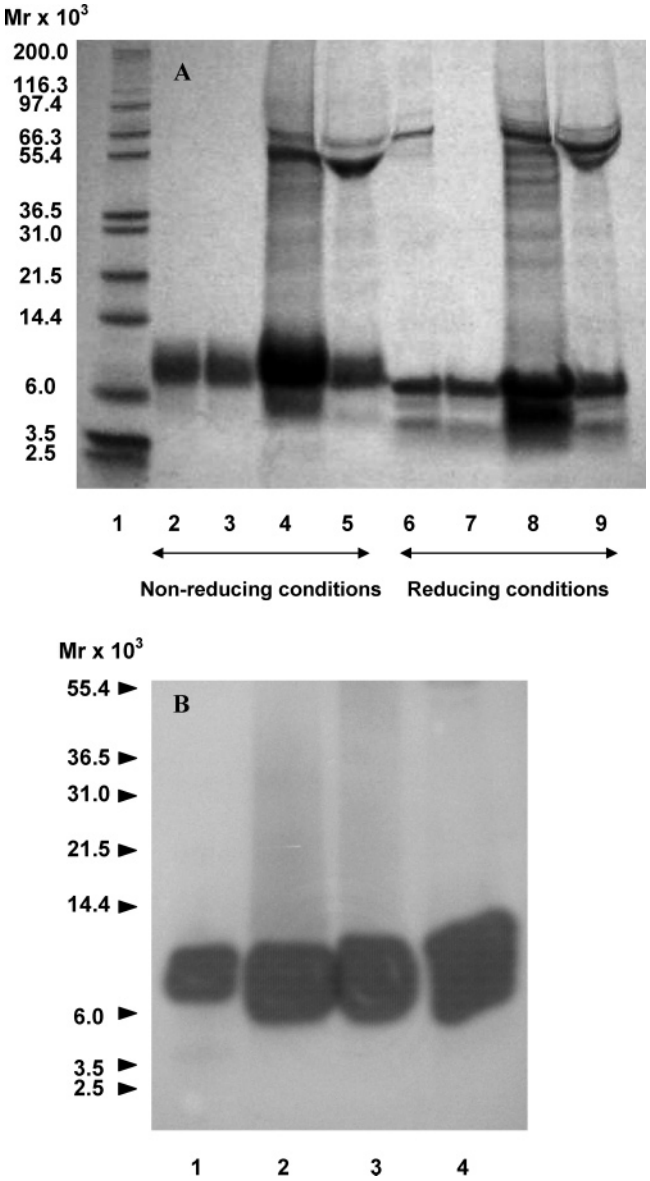

Figure 4. (**A**) SDS-PAGE of Ses i 1: (lanes 2, 6) native; (lanes 3, 7) digested before loading on Caco-2 cell monolayers; digested and collected from the (lanes 4, 8) apical and (lanes 5, 9) basolateral chambers under nonreducing and reducing conditions, respectively. (**B**) Immunoblot analysis with a rabbit polyclonal anti-Ses i 1 antibody of (lane 1) native Ses i 1, (lane 2) digested Ses i 1 before loading on Caco-2 cell monolayers and digested and collected Ses i 1 from the (lane 3) apical and (lane 4) basolateral chambers under nonreducing conditions.

thermal treatments seems not to modify their proteolysis pattern (*29*, *30*); consequently, thermal treatment is unlikely to affect their absorption rates. The rigid structure of 2S albumins consisting of a well-conserved skeleton of cysteine residues, which form two inter- and two intramolecular disulfide bonds, is thought to be responsible for the unusual high stability of these proteins to harsh conditions, as described by the allergenic 2S albumins from mustard seeds and rapeseeds (*39*, *43*). The presence of disulfide bonds might prevent structural changes induced by food processing, as occur with the protease inhibitors of the Bowman-Birk family (*35*), making possible such proteins to reach the intestinal mucosa in intact form.

The absorption rates of digested allergens were evaluated by using a Caco-2 cell monolayer model system. Strikingly, our results revealed substantial amounts of both allergens in the basolateral side of the Caco-2 cell monolayers, suggesting that these proteins are able to reach the mucosal immune system in intact form and initiate an immunogenic response. From our knowledge, neither in vitro nor in vivo studies have been previously published in relation to the gastrointestinal absorption of plant proteins from the allergenic 2S albumin family. Nevertheless, Oñaderra et al. (*44*) showed the interaction of the mustard seed 2S albumin allergen Sin a 1 with acid phospholipid vesicles which could result in a protective mechanism against proteolytic digestion of the allergen. The extracellular leaflet of the intestinal brush border membranes has larger amounts of such phospholipids (*45*) indicating that the interaction of Sin a 1 with the epithelial gut membranes could allow an increased cellular uptake of the protein.

Intestinal absorption and immune responses of food antigens are highly interdependent, and the nature of the antigen can dictate the route and the type of immune response generated

(*46*). In the case of food antigens, two different mechanisms of transport across the intestinal epithelium enterocytes have been described, named paracellular and transcelullar. The former route, which implies the diffusion of food antigens through the tight junctions joining the enterocytes, has been reported to be negligible under physiological conditions since the integrity of tight junctions is maintained even at cell extrusion sites (*47*, *48*). The use of purified allergens ruled out the possibility that other factors could favor the paracellular leakage of macromolecules through tight junctions (*19*), as demonstrated by the low absorption rates of the marker phenol red (<0.5%) observed in our experiments. The transcellular pathway has been previously reported in the uptake of bovine milk allergens, β-lactoglobulin and α-lactalbumin, through enterocytes (*17*, *18*). Such mechanism has two functional routes, a major lysosomal degradative pathway and a minor nondegradative pathway one. Our chromatographic, electrophoretic, and immunoblotting data indicated that Ber e 1 and Ses i 1 were transported in an intact form across the Caco-2 cell monolayers, although hydrolysis of the peptides resulting from the gastrointestinal digestion was also observed on the apical surface of the Caco-2 cell monolayers. The capacity of enterocytes to internalize and transport small amounts of intact proteins or large fragments with antibody binding capacity which could be involved in the immunological sensitization to food allergens has been previously reported (*3*, *49*, *50*). Such is the case of Gly m Bd 30K, a major soybean allergen; after oral administration in mice, intact allergen could be detected in plasma (*13*). Other intact food allergens such as ovomucoid and ovalbumin have been found in human breast milk, suggesting an uptake of the antigen without degradation across the human intestinal epithelial pathway (*51*, *52*).

Although digestion stability is relevant for a protein sensitizing via GIT, it might be that relatively large proteolytic fragments generated during the digestion also plays a role in sensitization (*53*). That would imply that some proteolytic fragments originated either from the exogenous digestive and/or the brush borders Caco-2 proteases could retain sufficient structure to bind the polyclonal antibodies and, therefore, to be detected by ELISA but not by RP-HPLC-UV analysis. In this sense, Terpend et al. (*54*) indicated that enterocytes are able to process horseradish peroxidase into peptides potentially capable of stimulating the immune system with a molecular mass ~1100 Da, which is compatible with a binding to major histocompatibility complexes restriction molecules. Van Beresteijn et al. (*55*) found the minimum molecular mass required for whey peptides necessary to elicit an immunological response was between 3000 and 5000 Da. Similarly, Huby et al. (*56*) stated that an allergen must contain at least two IgE binding sites or epitopes, each of which with a minimum of 15 amino acid residues long, in order to make possible the antibody binding.

In conclusion, our results suggest that significant amounts of 2S albumin allergens from Brazil nuts and sesame seeds are structurally stable to the gastrointestinal digestion and, subsequently, could be absorbed in an immunologically active form by the gut, facilitating the exposition of these allergens to the immune system to sensitize a naive individual and/or elicit an allergic response in a sensitized individual. Although the influence of the food matrix on the gastrointestinal absorption and the stimulation of the immune system remain to be determined, and other functional and physicochemical properties may also play an important role in promoting allergenicity of food proteins (*57*), the high resistance to gastrointestinal digestion and subsequent gut absorption of intact forms seem to underlie the inherent allergenicity of the 2S albumin family.

ABBREVIATIONS USED

DMEM, Dulbecco's modified minimal essential medium; DTT, dithiothreitol; GIT, gastrointestinal tract; HBSS, Hanks' buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NR, neutral red; P_{app}, apparent permeability coefficient; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; TEER, transepithelial electrical resistance; TFA, trifluoroacetic acid.

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