

***In vitro* stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8**

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Summary

Background The ability of an intact protein to reach the circulatory system may be a prerequisite to allergenicity and many allergens, particularly those from plant foods, have been found to be consistently more resistant to digestion by pepsin than other proteins.

Objective This study assessed the pepsinolytic stability of native 2S albumins from Brazil nut and sunflower seed and their recombinant versions produced in *Pichia pastoris*. The physicochemical stability of native and recombinant Brazil nut 2S albumins and recombinant sunflower seed 2S albumin was also assessed. The immunoreactivity of native Brazil nut 2S albumin and recombinant 2S albumins was compared using serum from patients allergic to Brazil nuts and animals immunized with native 2S albumins.

Methods Digestibility was measured in simulated gastric fluid followed by SDS-PAGE. Circular dichroism spectra were used to analyse unfolding, as proteins were denatured by temperature, pH and guanidinium chloride. Immunoreactivity was assessed by immunoblot, RAST and ELISA.

Results Brazil nut 2S albumin was significantly more resistant to proteolytic digestion than other Brazil nut proteins. It was also resistant to thermally and chemically induced denaturation. Equally high resistance to proteolytic digestion was observed with sunflower seed 2S albumin. The recombinant albumins mirrored their native counterparts in stability and immunoreactivity.

Conclusion The important food allergen Brazil nut 2S albumin is as stable to digestion as is sunflower seed 2S albumin, whose allergenicity has yet to be determined. The 2S albumins and their recombinant counterparts could not be easily denatured by physicochemical treatments. The results suggest that 2S albumin is the only Brazil nut protein to reach the gut immune system intact. The production of properly folded recombinant proteins will facilitate mechanistic studies as well as diagnostic testing and antigen-based therapies.

Keywords allergy, Brazil nut, recombinant allergens, 2S albumins, stability, structure, sunflower

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Introduction

Amid rising concern among the world population about the safety and nutritional aspects of foods derived from biotechnology, a decision tree has been formulated and recommended for the evaluation of the allergenic potential of genetically modified foods [1, 2]. The stability of proteins or digestibility, as measured by resistance to proteolysis by simulated gastric fluid (SGF), is an important factor in the assessment of allergenicity of proteins that are either novel, from a source regarded as non-allergenic or where only low numbers of sera from allergic individuals are available [3].

The ability of an intact protein to reach the circulatory system may be a prerequisite to allergenicity [2] and many allergens, particularly those from plant foods, have been found to be consistently more resistant to digestion by pepsin

than other proteins [4, 5]. Among the most stable have been the representatives from the 2S seed storage albumins (e.g. Sin a 1 and Bra j IE from mustard [5]). Historically, 2S seed albumins have been associated with allergy in the oil milling (castor and rapeseed) and baking industries [6] and several proteins from this group have been characterized as clinically important allergens [7–11].

The methionine-rich Brazil nut 2S albumin (Ber e 1) is a particularly well-classified allergen [12] that gained notoriety when it was engineered into soybean in order to increase the nutritional value of the legume and hence became the first transgenic allergen [13]. In contrast, sunflower 2S albumin (SFA-8), although well characterized biochemically [14], is less well recognized as an allergen. The binding of IgE to sunflower albumins has been observed, but it is still unclear whether or not they are the main cause of allergic reactions in the low number of individuals sensitized to sunflower seeds [15, 16].

The 2S seed albumins are products of multigene families and therefore extracts from a single plant may yield several

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isoforms of 2S protein [17] impeding purification. Difficulties of this nature have been encountered with various allergens and result in poor standardization of extracts for mechanistic studies, diagnostic testing and possible antigen-based therapy. Recently, in order to overcome such complications, efforts have been made to produce homogeneous recombinant allergens with the same properties as the native protein [18–20].

The aim of the present study was to assess and compare the stability of both native and recombinant 2S albumins from Brazil nut (Ber e 1 and rBer e 1, respectively) and sunflower seed (SFA and rSFA-8) in SGF. The stability of Ber e 1, rBer e 1 and rSFA-8 was also assessed under various denaturing conditions, including temperature and guanidinium chloride. The immunoreactivity of recombinant proteins was assessed using serum from animals immunized with native Ber e 1 or native SFA. Further, the binding specificity of serum from Brazil nut-sensitive patients was assessed in an ELISA format.

Materials and methods

Extraction and purification of native and recombinant proteins

Total water-soluble protein and 2S albumin (Ber e 1) fractions were extracted and purified from Brazil nuts (*Bertholletia excelsa*) as described previously [21]. Native sunflower (*Helianthus annuus*) 2S albumin was kindly provided by Professor Peter Shewry (Institute of Arable Crop Research, Bristol, UK) as a mixture of the isoforms SFA-7 and SFA-8 (SFA). Recombinant Ber e 1 and SFA-8 (rBer e 1 and rSFA-8 respectively) were produced as secreted protein by the yeast *Pichia pastoris* and purified by FPLC using a heparin–sepharose column as previously described [22]. Bovine serum albumin (BSA), used as a non-plant albumin representative, was obtained from Sigma, Poole, UK.

SGF digests

SGF contained pepsin 0.32% (w/v) (activity: 3200–4500 U per mg of protein, product No. P 6887, Sigma) in 0.03 M NaCl, HCl 0.7% (v/v) and pH 1.2 [5, 23]. Digestions contained 120 µL of SGF and 23 µg of purified protein resulting in a pepsin to substrate ratio of 17:1, similar in range to ratios of 16:1 and 18.8:1 used in other studies [5, 23]. Incubations were performed at 37 °C. At each time-point, samples of the digestion reaction were removed and the reaction was quenched by the addition of Na₂CO₃ to a final concentration of 70 mM before freezing until electrophoresis.

Electrophoresis

Proteins were separated by discontinuous SDS-PAGE under reducing conditions using pre-cast NuPAGE[®] Novex 12% Bis-Tris gels (Invitrogen, Paisley, UK) with MES running buffer (Invitrogen) as per the manufacturer's instructions. Proteins were visualized by staining with Coomassie brilliant blue R250.

Circular dichroism measurements

Far UV CD spectra for the analysis of heat-induced and chemically induced unfolding were generated as previously described [22, 24, 25]. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and 0.03–0.32 mg/mL in 10 mM phosphate buffer pH 6.8 or 100-mM glycine buffer pH 2.2 was used in CD experiments.

Sera

Rabbit antisera from animals immunized with Ber e 1 and SFA were kindly provided by Dr Gary Wyatt (Institute of Food Research, Norwich, UK) and Professor Peter Shewry (Institute of Arable Crop Research), respectively.

Sera from 10 patients sensitized to Brazil nut were obtained at The David Hide Asthma and Allergy Research Centre, St Mary's Hospital, Isle of Wight, UK.

Immunoblotting

Proteins from SDS-PAGE gels were electroblotted to Immobilon-P PVDF membranes (Millipore, Watford, UK) using an Invitrogen X-cell blot module according to the manufacturer's instructions. PVDF membranes for immunoblotting were placed in a blocking solution of Tris-buffered saline (TBS, 100 mM Tris/HCl pH 7.4, 155 mM NaCl) and BSA 5% (w/v) for 1 h. Incubations were performed at 37 °C and membranes were washed three times for 5 min with TBS containing BSA 0.1% (w/v) between incubations. Membranes were incubated with rabbit anti-Brazil nut or rabbit anti-sunflower primary antiserum diluted at 1:7 500 (v/v) in TBS containing BSA 1% (w/v) and Tween-20 0.05% (w/v). Bound rabbit IgG was detected with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted at 1:20 000 (v/v). A final wash in Tween-20 0.1% (w/v) TBS was performed prior to incubation at room temperature with pre-mixed BCIP/NBT visualization solution (Sigma).

Sensitivity of the serum donors

In order to assess the sensitivity of patients to native Brazil nut, skin prick tests and labial/oral challenges were performed. Recombinant proteins were not employed in clinical testing.

Skin prick tests Skin prick tests to aeroallergens, nuts and egg using either Soluprick (ALK, Hørsholm, Denmark) or solutions from Bayer Corporation (Newbury, UK) were carried out. The mean of two diameters from the resultant weal was recorded and positive results were recorded according to the manufacturer's instructions.

Labial/oral challenge The labial challenge reduced the need to expose a strongly sensitive patient to higher doses of ingested allergen. The procedure employed native Brazil nut or placebo and was considered positive if definite symptoms were observed such as erythema, swelling or urticaria at the site of the nut challenge. If the labial challenge was negative or inconclusive, the patient proceeded to an oral challenge.

The double-blind placebo-controlled oral challenge consisted of feeding patients, in gradually increasing amounts, flapjack biscuits containing 0–0.53 g of native Brazil nut

protein (i.e. approximately one Brazil nut) per biscuit. The oral challenge was considered positive if signs or significant symptoms such as urticaria or rhinoconjunctivitis were reported during exposure to the nut, but not the placebo. After termination of the challenge, treatments necessary were administered and the patient observed prior to being accompanied home. Challenges were not performed in cases where there was a history of a serious, systemic or life-threatening reaction or where a good history of reaction had been recorded in the last 5 years.

RAST

RAST were performed using the Pharmacia Phadezyme RAST[®] RIA kit (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) and native Brazil nut extracts according to the manufacturer's instructions.

Indirect ELISA

Wells on 96-well plates (Dynatech Microfluor, VA, USA) were coated with either Ber e 1, rBer e 1, rSFA-8 or BSA (100 µL/well) at a concentration of 0.1 µg/mL diluted in PBS, covered with cling film and incubated overnight at 4 °C. The coated plates were subsequently blocked using 1% BSA in PBS and 0.1% Tween-20 (ELISA buffer) for 30 min at room temperature. Sera C, D and G diluted 1:4 in ELISA buffer were added to the plates in duplicate and serially diluted up to 1/64 for the assessment of the optimum serum dilution required. Other sera for analysis were added to the plates, in duplicate, diluted 1:10 in ELISA buffer. After incubation for 4 h at room temperature, plates were washed three times with 200 µL of PBS with 0.1% Tween-20. After washing, 200 µL of mouse monoclonal anti-IgE, alkaline phosphatase-linked antibody (GE-1, Sigma) was added to the plates at 1:1000 dilution and incubated for 1.5 h at room temperature. Plates were then washed (3 × 200 µL PBS+0.1% Tween-20) prior to the addition of 150 µL of SigmaFast pNpp (*p*-nitrophenyl phosphate) substrate and incubated for 16 h. The optical density was measured at 405 nm in an Anthos htII plate reader.

Results

Digestion in SGF

Total soluble proteins and specific 2S proteins were isolated from Brazil nuts. SDS-PAGE analysis of total protein revealed the presence of a number of polypeptides with sizes between 3 and 35 kDa. The higher molecular weight polypeptides included the 7S and 11S fractions [21], while the 2S fraction contained the remaining 9 kDa polypeptide (Fig. 1). An additional 3 kDa polypeptide from the 2S fraction [21] was also observed that was not visible under concentrations employed for SGF digests. Initial incubations in SGF demonstrated that the 7S and 11S fractions were highly susceptible to digestion as no fragments greater than 9 kDa were visible after approximately 5 s in SGF (Fig. 1). Products of degradation of the higher molecular weight polypeptides were observed between 3 and 6 kDa (Fig. 1). The 2S fraction (Ber e 1), constituting around 20% of the total

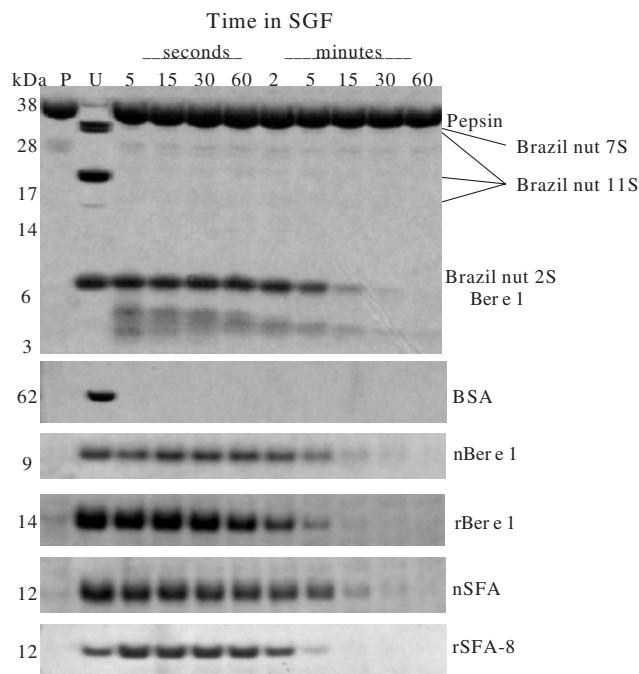


Fig. 1. *In vitro* digestibility of native and recombinant proteins in SGF. P – simulated gastric fluid alone, *U – protein sample untreated with SGF.

extract, had significantly greater stability in SGF persisting for over 15 min (Fig. 1). BSA, included as a non-plant protein albumin representative, was also found to be very unstable in the presence of SGF with the 66 kDa band disappearing after approximately 5 s (Fig. 1). On further investigation of purified fractions, Ber e 1 was observed to be stable to digestion in SGF for over 15 min, with no fragments visible after 30 min. This pattern of digestibility was duplicated in experiments utilizing the purified recombinant allergen rBer e 1. Although the difference in the digestibility characteristics of Ber e 1 and rBer e 1 was negligible, it was notable to observe that the recombinant protein was expressed as an unprocessed unit (14 kDa), whereas *in planta* the protein is processed and expressed as two subunits of 9 and 3 kDa [21, 22].

Native SFA was also significantly more stable than BSA and Brazil nut 7S and 11S proteins with bands and fragments persisting up to 30 min after the addition of SGF (Fig. 1). Experiments with the purified recombinant protein revealed that the characteristics of resistance to low pH and hydrolysis by pepsin found in SFA were closely mirrored by those of rSFA-8 (Fig. 1).

Stability to heat, low pH and guanidinium chloride

Synchronous investigations of Ber e 1, rBer e 1 and rSFA-8 using far UV CD spectroscopy revealed that each native and corresponding recombinant protein had a similar secondary structure [22, 26]. The CD spectra generated highlight the alpha-helical structure of the 2S albumins analysed (Fig. 2a). In the current study this technique was used to examine the stability of Ber e 1, rBer e 1 and rSFA-8 to denaturation by temperature and guanidinium chloride. The stability of rSFA-8 at low pH was also analysed. At pH 6.8, the secondary structures of both Ber e 1 and rBer e 1 were highly resistant to thermal unfolding. Neither protein showed any evidence of

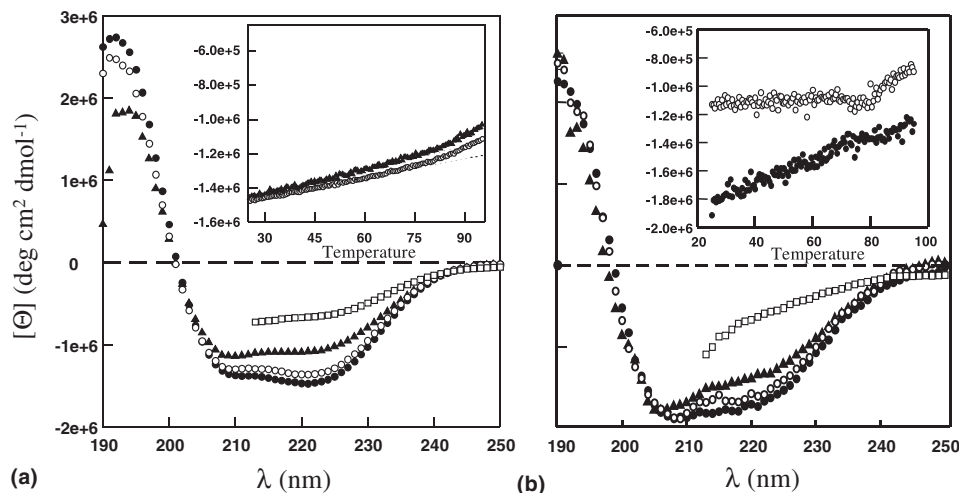


Fig. 2. CD spectra of (a) rBer e 1 (0.146 mg/mL) and (b) rSFA-8 (0.32 mg/mL) in 10 mM phosphate buffer pH 6.8 under various conditions. ● – 25 °C upon heating; ▲ – 95 °C; ○ – 25 °C upon cooling and □ – 6 M guanidinium chloride. *Insets.* Heat-induced unfolding transition followed by far UV CD measurements at 222 nm of (a) ○ – Ber e 1 and ▲ – rBer e 1, dotted lines represent the slope of the pre-transition and (b) rSFA-8 ● – pH 6.8 and ○ – pH 2.2 (10 mM glycine buffer).

unfolding at temperatures lower than 75 °C (Fig. 2a inset) and both retained most of their secondary structure at 95 °C (Fig. 2a). For each protein, spectra obtained at pH 6.8 and 25 °C were superimposable to those recorded at pH 2.2 by Alcocer et al. [22], confirming that these proteins are also highly resistant to acidic pH. The two proteins were also highly resistant to chemically induced unfolding. No changes in the secondary structure were observed up to a concentration of at least 4.5 M guanidinium chloride (data not shown) and the protein exhibited a significant amount of secondary structure in 6 M guanidinium chloride (Fig. 2a).

Recombinant SFA-8 also retained most of its secondary structure at 95 °C and pH 6.8 (Fig. 2b), corresponding to behaviour similar to that described for the native protein [26]. rSFA-8 was slightly less resistant than Ber e 1 and rBer e 1 to unfolding induced by acidic pH (data not shown) and increasing concentrations of guanidinium chloride (Fig. 2b).

Immunochemical characterization

IgG originating from rabbits immunized with SFA or Ber e 1 reacted positively both with the native and corresponding recombinant protein (Fig. 3). Low or no detectable cross-reactivity was observed between the two proteins, i.e. IgG recognizing SFA did not react with Ber e 1 or rBer e 1 and IgG directed against Ber e 1 did not react with SFA or rSFA-8 (Fig. 3).

All patients from whom sera were obtained had both a positive skin prick test and positive labial/oral challenge, unless a recent convincing reaction had been observed in which case challenge was deemed unnecessary, when presented with native Brazil nut extracts. All except one (a) had positive RAST assay results when collected sera were presented with native Brazil nut extracts *in vitro* (Fig. 4).

When tested for IgE via ELISA, there was little discrimination among the sera between Ber e 1 and rBer e 1 (Fig. 4). All sera, except for H, reacted more strongly with Ber e 1/rBer e 1 than BSA with samples showing up to 13 times more activity with Ber e 1/rBer e 1. Sera showed high cross-reactivity with

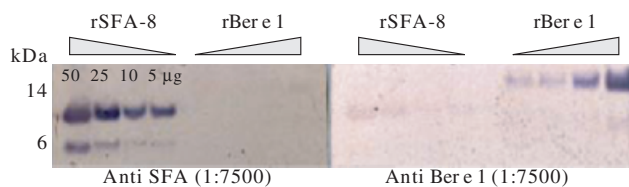


Fig. 3. 2S albumins transferred onto PVDF membranes, 50 µg of protein transferred and probed with rabbit anti-Ber e 1 1:7500 or rabbit anti SFA-8 1:7500 as indicated.

rBer e 1. While generally much less total serum IgE cross-reactivity was observed with SFA-8, serum I was found to be considerably more specific for SFA-8 (Fig. 4).

Discussion

We have demonstrated, for the first time, that the clinically most important allergen from Brazil nut (Ber e 1) [12] is also the water-soluble Brazil nut protein least susceptible to digestion by SGF. In the model employed, the 2S fraction from Brazil nut total protein extracts persisted for up to 30 min, whereas other components were completely hydrolysed after a few seconds. This suggests that the 2S albumin is likely to be the only intact Brazil nut protein that comes into contact with cells of the immune system found in the gut mucosa. The results demonstrating Ber e 1 to be more stable to pepsin hydrolysis than BSA are consistent with previous literature [5, 27]. For example, Astwood et al. [5] found 2S albumins from mustard seeds to be intact after incubation for 60 min in SGF. In conjunction with the mustard proteins previously described [5], the current results demonstrate that at least three important allergens from the 2S albumin group are highly stable to digestion by pepsin. This observation fits the model in which stability in the gastrointestinal tract is a prerequisite to allergenicity and could possibly be extended to other members of the 2S family. In order to test this

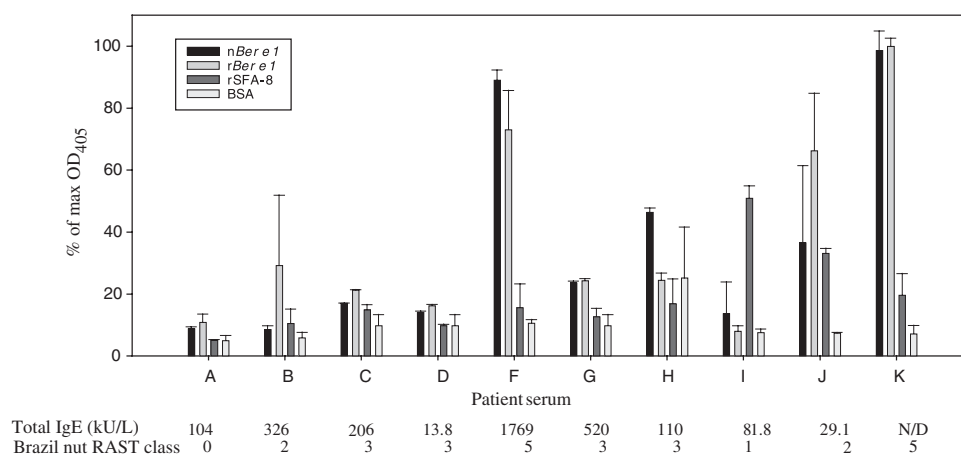


Fig. 4. IgE ELISA and RAST data obtained from 10 Brazil nut-sensitized patients. Sera were diluted 1 : 10 for ELISA analysis; error bars represent standard error of the mean.

hypothesis, the digestibility of a 2S albumin not yet defined as an allergen was studied.

Recent IgE-binding studies indicate that the methionine-rich 2S albumin, found in sunflower seed, may be allergenic [15]. However, reports of adverse reactions and anaphylaxis to sunflower seed are rare, particularly given its common use and consumption [22, 28, 29]. In the present study, 2S albumin from sunflower seed was found to be highly stable to hydrolysis by SGF, demonstrating similar digestibility characteristics to Ber e 1. Our results are in agreement with recent work demonstrating that food allergens could be more, equally or less susceptible to SGF than non-allergenic proteins of similar cellular function [30].

While some food allergens have been observed to be stable to cooking and processing [31], the extent of their thermal stability is unclear. The proteolytic stability of Ber e 1 was reflected not only in the thermal stability but also in the resistance to unfolding displayed when the protein was exposed to the chemical denaturant guanidinium chloride. Interestingly, at pH 6.8 or pH 2.2, none of the proteins (Ber e 1, rBer e 1 and rSFA-8) was fully denatured even at 95 °C and, importantly, the proteins largely regained their original structure upon cooling [22]. These results are not only relevant to the inherent stability of the 2S albumins but are also relevant to food processing techniques possibly employed to destroy allergens.

Recombinant 2S proteins were successfully expressed at high levels in the yeast *P. pastoris* as described previously [22]. Eukaryotic expression systems have unique benefits regarding folding and post-translational modifications not found in bacterial systems. However, while no differences were found between SFA expressed *in planta* and rSFA-8 expressed in *P. pastoris*, differences in processing and glycosylation were apparent between Ber e 1 and rBer e 1 [22]. Despite these differences, recombinant versions of the 2S proteins closely reflected their native counterparts when subjected to incubation in SGF. Further physicochemical analysis demonstrated that rBer e 1 had a very similar stability profile to Ber e 1 during thermally, acidically and chemically induced unfolding. These observations are not unique, as several allergens expressed in *P. pastoris* have been noted to display both non-

native glycosylation and native biological activity [18, 32, 33]. However, whether or not this difference will be important during sensitization studies remains to be discovered. The large-scale production of purified biologically active recombinant allergens offers considerable possibilities regarding allergen manipulation, specific diagnosis and immunotherapy.

Allergens, recombinant or otherwise, are defined by their immunoreactivity. Immunoblot analysis established that recombinant 2S allergens were equally well recognized by specific animal IgGs. In addition, human-specific IgE ELISAs showed little discrimination between recombinant and native Ber e 1. ELISA results largely reflected the clinical RAST measurements. Sera from individuals with an allergic response to Brazil nuts reacted strongly with Ber e 1 corroborating the finding by Pastorello et al. [12] that the 2S albumin is the major allergen in this food. IgE ELISA revealed sera from sensitized patients to cross-react, to varying degrees, with rSFA-8. Due to the low concentration of IgE in sera and taking into consideration the inability of indirect ELISA to elucidate individual IgE cross-reactivities such results are not surprising. It was of some interest that one patient's serum (I) had a reaction highly specific to rSFA-8, demonstrating the potential value of employing purified recombinant proteins for diagnostic purposes.

Although it has been demonstrated that 2S seed albumins form a major group of proteins in a diversity of plant species, their function remains largely unknown [6, 34]. The 2S albumin/amylase trypsin inhibitors/lipid transfer protein superfamily is characterized by a similar three-dimensional structure (SCOP – <http://scop.mrc-lmb.cam.ac.uk/scop/>) involving a bundle of four alpha helices cross-linked by four conserved disulphide bridges [17]. This non-glycosylated and compact structure may explain the thermal and chemical stability observed as well as resistance to digestion by SGF. Given their ubiquity, one would expect 2S seed albumins to be widely consumed from many different food sources. However, allergy to 2S albumins is restricted to relatively few individuals. This suggests that the overall stability of the allergen is only one of a number of factors contributing to the reaction between proteins and individuals that ultimately results in allergy.

In conclusion, we have demonstrated the considerable stability of 2S albumins Ber e 1 and SFA-8 to hydrolysis in SGF and to unfolding induced by temperature, acidity and chemical denaturant. In addition, the immunoreactivity and physicochemical properties of recombinant versions of these proteins produced in *P. pastoris* closely reflected those of the corresponding native protein.

Acknowledgements

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