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Thermostability and in vitro digestibility of a purified major allergen 2S albumin (Ses i 1) from white sesame seeds (*Sesamum indicum* L.)

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Abstract

A major 2S albumin allergen, Ses i 1, from white sesame seeds was purified to homogeneity, characterized and identified using proteomic techniques. Ses i 1 exhibited a molecular weight of 12062 Da, although an extensive C-terminal clipping of the small subunit was observed. In addition, the N-terminal glutamine of the small subunit had been converted to pyroglutamate and a variant of the large subunit which had lost the N-terminal glutamine was also detected. The protein was thermo-stable up to 90 °C at neutral and acid pH, retaining its monomeric state and showing minimal alterations, which were reversible on cooling, in a predominantly α-helical secondary structure, as shown by circular dichroism and Fourier transform-infrared spectroscopy. Ses i 1 was also highly resistant to digestion using a physiologically relevant in vitro gastrointestinal model system. After 2 h of gastric digestion, the allergen remained completely intact and only the small subunit was cleaved during 2 h of subsequent duodenal digestion, leaving a major IgE epitope region of this protein intact. Neither prior heating of the Ses i 1 nor the presence of the physiological surfactant phosphatidylcholine affected the pattern of proteolysis. These findings are consistent with those found for the 2S albumin allergen from Brazil nut, Ber e 1, and suggest that Ses i 1 may preserve its structure from the degradation in the gastrointestinal tract, a property thought to be crucial for both a protein to sensitise the mucosal immune system and provoke an allergic reaction in a sensitised individual.
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Keywords: Food allergy; 2S albumin; Sesame; Digestion; Proteomic

1. Introduction

It is emerging that plant food allergens belong to only a limited number of protein families [1] with the prolamin superfamily, to which the non specific lipid transfer proteins, 2S albumins, α-amylase inhibitors and prolamin seed storage proteins of cereals belong, being particularly prominent. Underlying this association is the fact that the three-dimensional structures dictated by the primary sequence, confers properties which may potentate the allergenic properties of these proteins. Factors such as abundance in edible plant tissues (which determines the amount ingested), stability to processing and degradation in the gastro-intestinal tract may all play a role.

In order to investigate this premise further, we have been systematically studying the stability properties of members of the prolamin superfamily, including the 2S albumins [2]. These proteins are considered to be structural homologous, typically heterodimeric (small and large subunits of M_r about 4,000 and 9,000 respectively) globular proteins [3]. They have been described as major allergens in many plant food species, including sesame seeds [4]. Sesame is associated with immunoglobulin E (IgE) mediated food allergy [5–9] and is a cause for concern because of the severity of reactions it elicits.

The cDNAs of two 2S albumin precursors from sesame seeds have been sequenced [10], and the corresponding mature proteins, known as Ses i 1 and Ses i 2, have been reported to be major allergens [9,11]. Although the skeleton

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of cysteine residues is conserved, the proteins show a low sequence homology (46%) [9], Ses i 1 being a sulphur-poor protein whilst Ses i 2 is rich in methionine. Despite its potential importance, Ses i 1 has not been completely characterized and, to our knowledge, only two peptides of 5 and 17 residues derived from the small and large subunit of the intact M_r 9,000 protein have been described [11].

This study reports the purification and characterization of Ses i 1 using a combination of chromatographic and electrophoretic techniques, as well as proteomic approaches. The stability of the protein to thermal denaturation and proteolysis is described and compared with other allergenic 2S albumins.

2. Material and methods

2.1. Purification of the 2S albumin (Ses i 1)

2.1.1. Extraction of Ses i 1 from sesame seeds

White sesame seeds were purchased from a local retail outlet and milled in dry ice to yield a fine paste and defatted by extraction (four times) with petroleum ether (1 g:10 mL) at room temperature. Ses i 1 was extracted from the defatted flour by stirring for 2 h at 2 °C with 0.1 M sodium phosphate buffer pH 7.4 (1 g:15 mL), centrifuged at 7780×g for 30 min at 5 °C. The supernatant was collected, filtered through 8 μm pore

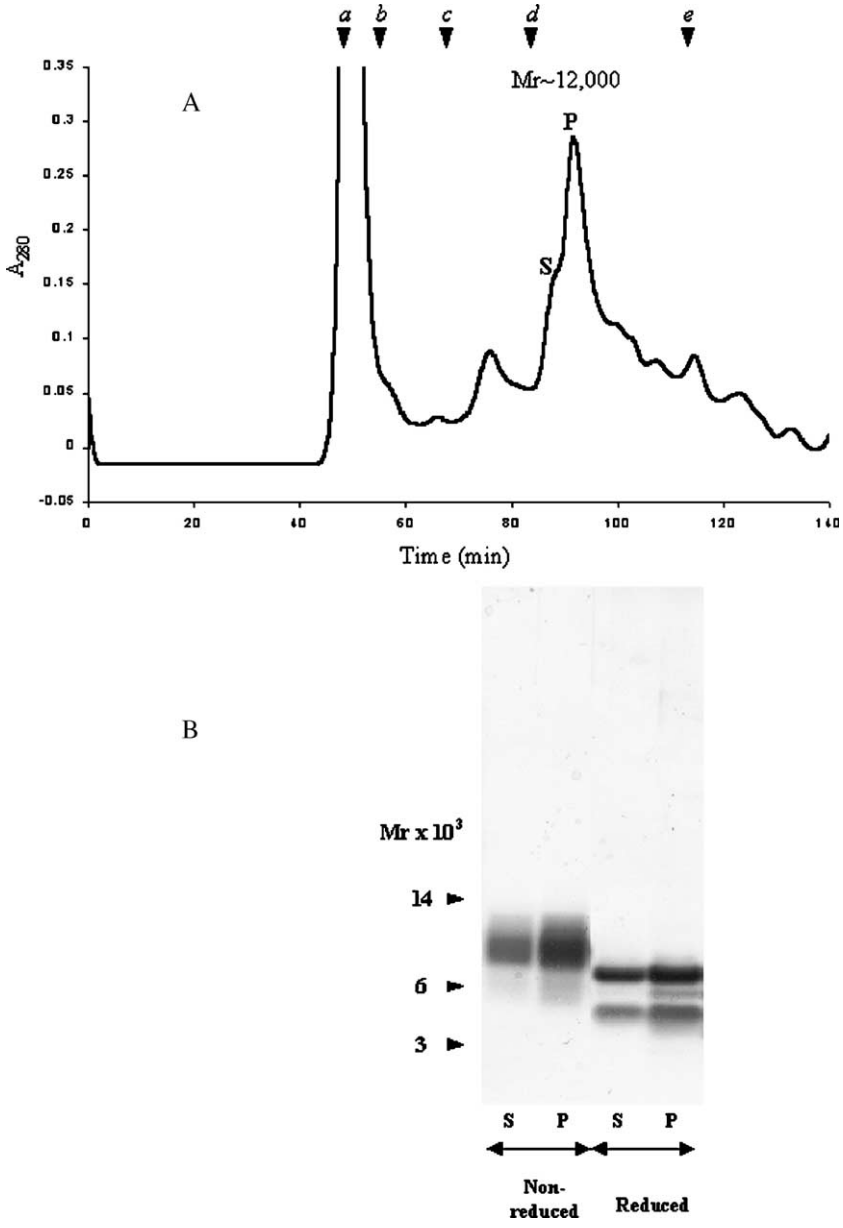

Fig. 1. (A) Gel-permeation chromatography profile of the sodium phosphate buffer extract of defatted sesame seeds. Elution positions of standard proteins are indicated by arrows: a, thyroglobulin (M_r 670000); b, bovine gamma globulin (M_r 158000); c, chicken ovalbumin (M_r 44000); d, equine myoglobin (M_r 17000); e, vitamin B-12 (M_r 1350). (B) SDS-PAGE analyses under non-reducing and reducing conditions of the gel-permeation fractions S and P.

size membrane filters (Mixed Cellulose Esters hydrophilic, Millipore, MA, USA), dialysed against water (5 L) at 2 °C for 48 h using a cellulose membrane of MW cut off: ca. 3500 and lyophilised.

2.1.2. Gel permeation chromatography

The extract was subjected to gel-permeation chromatography on a HiLoad 16/60 Superdex 75 preparative column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 0.05 M sodium phosphate + 0.15 M NaCl (pH 7) and attached to a Biocad Sprint (PerSeptive Biosystems, Framingham, USA) perfusion chromatography system. Sample (100 mg) was dissolved in 2 mL 0.05 M sodium phosphate + 0.15 M NaCl and Ses i 1 eluted in the same using a flow rate of 1 mL/min at room temperature. The column was calibrated using a set of molecular weight markers ranging from 1.35 kDa to 670 kDa (Bio-Rad, Hercules, USA).

2.2. Identification of the 2S albumin (Ses i 1)

2.2.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel permeation fractions (33 μL) were added to 42 μL of ultrapure water and 25 μL of 4× NuPAGE® LDS Sample buffer (Invitrogen, CA, USA) and heated at 70 °C for 10 min. When required, samples were reduced with 0.5 M DTT. Samples were loaded (20 μL) on a 12% polyacrylamide NuPAGE® Novex Bis-Tris pre-cast gel and a continuous MES SDS running buffer was used. Gels were run for 35 min at 120 mA/gel and 200 V and stained using Colloidal Blue Staining Kit (Invitrogen).

2.2.2. Reverse phase-HPLC-ESI-MS

Gel permeation fractions were diluted with solvent A and applied to either a protein (Phenomenex Jupiter 300 Å pore size, 5 μm particle size, 250 × 4.6 mm i.d.) or peptide (Phenomenex Jupiter Proteo 90 Å pore size, 4 μm particle size, 250 × 4.6 mm i.d.) column attached to a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) coupled to a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, CO-1560 column oven and UV-1575 UV detector (Jasco Ltd., Great Dunmow, UK). 2S albumin was eluted using 0.1% (w/v) trifluoroacetic acid in double-distilled water as solvent A and 0.085% (w/v) trifluoroacetic acid in double-distilled water/acetonitrile (10:90, v/v) as solvent B. The chromatographic and mass spectrometric conditions were those described by Moreno et al. [12].

2.2.3. Proteomic analysis

The protein bands (~1 mm gel plugs) from one-dimensional SDS PAGE gels were excised, destained, reduced, carbamidothylated and hydrolysed with modified porcine trypsin (specific activity: 5,000 u/mg, Promega, Madison, USA) following the method described by Moreno et al. [12].

Tryptic peptides were analysed by the joint IFR-JIC proteomics facility using matrix-assisted laser desorption/ionisation – time of flight mass spectrometry (MALDI-TOF-MS) using a Reflex III (Bruker) equipped with a SCOUT 384 ion source. All spectra were acquired in a positive-ion reflector. The acceleration voltage was set to 25 kV, the reflection voltage was set to 28.7 kV, ion source acceleration voltage to 20.9 kV, and the reflector-detector voltage to 1.65 kV. Peptide profiles were searched (on the basis of mass) against the NCBI nonredundant protein

Table 1
Summary of peptides identified by MALDI-ToF-MS following in gel trypsin digestion corresponding to the sequence of the allergenic 2S albumin of sesame, Ses i 1 (accession number ExPASy Q9AUD1)

Subunit	Mass	Sequence and position	Gel filtration fraction	
			S	P
Small	917.4	^{1}[a]QQSQQCR^{7}		X
	1570.7	^{1}[a]QQSQQCRQQLQGR^{13}	X	X
	1627.8	^{1}[a]QQSQQ[b]CRQQLQGR^{13}		X
	924.5	^{14}QFRSCQR^{20}		X
	1070.5	^{21}YLSQGRSPY^{29}		X
	1557.7	^{21}YLSQGRSPYGGEED^{34}	X	
Large	979.4	^{5}D[b]C[b]CQQLR^{11}	X	X
	907.4	^{17}CR[b]CEAIR^{23}	X	
	2723.4	^{24}QAVRQQQQEGGYQEGQSQQVYQR^{46}		X
	2269.0	^{28}QQQQEGGYQEGQSQQVYQR^{46}	X	X
	1566.5	^{53}R[b]CNMRPQQCAFR^{64}	X	X
	1369.5	^{54}CN[c]MRPQQCAFR^{64}	X	X
	1410.7	^{54}CNMRPQQ[b]CAFR^{64}	X	X
	1483.6	^{54b}CN[c]MRPQQ[b]CAFR^{64}		X

[a] Cyclization of glutamine to pyroglutamic acid.
[b] Carbamidomethylation of Cys.
[c] Oxidation of Met.

database using the Mascot program from Matrix Science (http://www.matrixscience.com/) and Viridiplantae as taxonomy. The search parameters were as follows: (1) tryptic digest was assumed to have a maximum number of one missed cleavage; (2) peptide masses were stated to be monoisotopic; (3) methionine residues were assumed to be partially oxidized; (4) the carbamidomethylation of cysteine residues was considered; (5) the mass tolerance was kept at 50 ppm.

2.3. Secondary structure analysis

2.3.1. Circular dichroism spectroscopy

Far-ultraviolet CD spectra (260–190 nm) of Ses i 1 were recorded with a J-710 spectropolarimeter (Jasco Corporation, Tokyo, Japan) using a 0.2-cm pathlength quartz cell. The instrument was calibrated with ammonium d-10-camphorsulphonate. Spectra represent the average of 3 accumulations collected at 100 nm/min, with a 2 s time constant, 0.5 nm resolution and sensitivity of ±100 mdeg. Spectra are represented as molar CD (with respect to moles of amide bond), and analysis was carried out using the Contin method [13] with a basis set containing 29 proteins. An external water bath provided the means of temperature control for the cell, whilst a thermocouple measured the temperature in the cell's water jacket. Acquisition of spectra at each temperature was initiated 5 min after the temperature of the cell had equilibrated. Spectra were acquired at 20, 30, 40, 50, 60, 70, 80 and 90 °C during the heating phase then, following a 10-min holding time, at 90, 80, 70, 60, 50, 40, 30 and 20 °C in the cooling phase. Ses i 1 was dissolved at 0.2 mg/mL either in 10 mM sodium phosphate buffer, pH 7.0 or 0.15 M NaCl, pH 2.5.

2.3.2. Fourier transform infrared spectroscopy (FT-IR)

Ses i 1 solution (20 mg/mL) was prepared in 10.0 mM sodium phosphate buffer, pH 7.0. FT-IR spectra were collected using a Nicolet Magna-IR 860 spectrometer equipped with an MCT detector. The samples were placed in a thermostated horizontal ATR cell with a Ge crystal (Specac, Orpington, Kent). An external water circulation bath provided the means for the temperature control of the cell with an accuracy of ±1 °C. Spectra were recorded at 10, 20, 30, 40, 50, 60, 70, 80 and 90 °C during the heating phase, and, after holding at 90 °C for 10 min, at the same temperatures in reverse order in the cooling phase. Acquisition of spectra was initiated 5 min after the cell temperature had reached equilibrium. For each spectrum, 1000 scans at 4 cm^{-1} resolution were averaged. At each temperature, the spectrum of the protein solution in the region of 4000 to 800 cm^{-1} was referenced to the empty cell background at that temperature, then the buffer contribution was removed by digital subtraction of the buffer spectrum measured at the relevant temperature.

Since the broad amide I band is difficult to interpret, band narrowing by Fourier self-deconvolution (FSD) was carried out using the spectrometer software (WINIR Pro) with a bandwidth of 16 cm^{-1} and an enhancement factor of 1.3 or 1.8. IR band assignments were made in accordance with previously published work [14,15]. Estimates of the amount of secondary structure were made by fitting multiple Gaussian peaks to the FSD spectra using Peak fit v. 4 (SPSS Inc.) software and assuming identical extinction coefficients for all secondary structures.

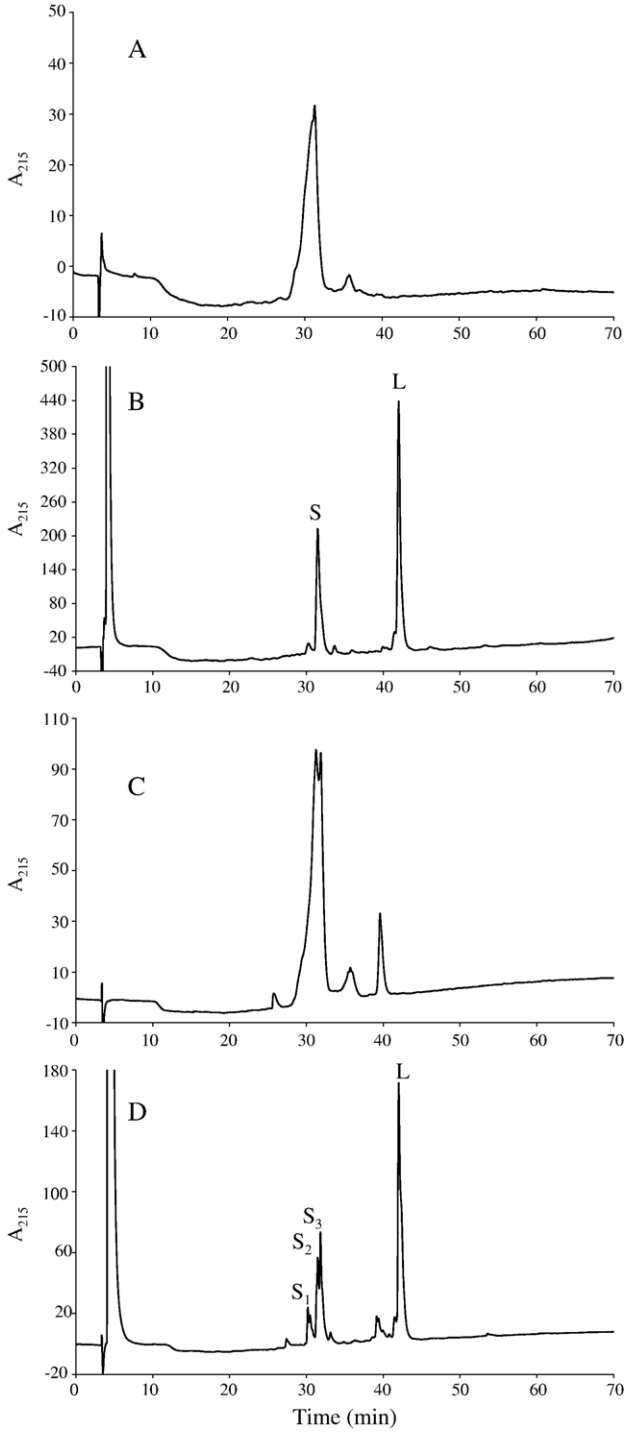

Fig. 2. Reverse phase-HPLC patterns using a protein column of (A) non-reduced fraction S (Ses i 1), (B) reduced fraction S, (C) non-reduced fraction P and (D) reduced fraction P. RP-HPLC peaks S, S_1, S_2 and S_3 correspond to the small and peak L to the large subunit of Ses i 1.

2.4. In vitro gastric (phase I) and duodenal (phase II) digestion models

The digestion of Ses i 1 was performed as described by Moreno et al. [2]. Briefly, in vitro gastric followed by duodenal digestion of Ses i 1 were performed with the presence or absence of the physiological surfactant egg L-phosphatidylcholine (PC, Lipid Products, Surrey, UK). Gastric digestion was performed at 37 °C for 120 min at pH 2.5 in the presence of porcine pepsin (Sigma, Dorset, UK; product No. P 6887) at a ratio of enzyme : substrate 1:20 (w/w); 182 U of pepsin per mg of Ses i 1. Aliquots were taken at 0, 2, 5, 15, 30, 60 and 120 min for SDS-PAGE and RP-HPLC analysis as described above. The digestion was stopped by raising the pH to 7.5 using 40 mM ammonium bicarbonate (BDH, Poole, UK).

In vitro duodenal digestion was performed using 120 min gastric digesta as starting material. The pH of the digesta was adjusted to 6.5 and duodenal digestion was performed following addition of a multi-component medium including a model bile salt mix, trypsin (Sigma, product No. T 7418) and α-chymotrypsin (Sigma, product No. C 7762) at ratios of Ses i 1 (as denoted by the initial concentration in phase (1): trypsin : chymotrypsin 1: 400: 100 (w/w); 1 mg: 34.5 U: 0.44 U. The digestion was performed at 37 °C and aliquots were taken at 0, 2, 5, 15, 30, 60 and 120 min for SDS-PAGE and RP-HPLC analysis. The digestion was stopped by adding a solution of Bowman–Birk trypsin-chymotrypsin inhibitor from soybean (Sigma product No. T9777) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

3. Results and discussion

3.1. Purification and identification of Ses i 1

A sodium phosphate buffer extract of defatted sesame flour was subjected to a Superdex 75 gel filtration chromatography and a broad peak M_r ~12,000 (P) with a pronounced shoulder (S) was observed, likely to correspond to the 2S albumin (Fig. 1A). When these fractions were analysed by SDS-PAGE, both comprised a prominent diffuse polypeptide M_r ~12,000, which on reduction gave rise to two bands of M_r above 3,000 and 6,000 likely to correspond to the small and large subunit, respectively, of the 2S albumin (Fig. 1B). The main fraction P also contained an additional minor polypeptide running between the large and small subunit after reduction. Coupled with the diffuse nature of the polypeptide analysed by SDS-PAGE under non-reducing conditions, this suggested that the main fraction P, is a heterogeneous mixture of at least two polypeptides of similar molecular weight, as has been observed for other 2S albumins [12,16–18].

A combination of in-gel trypsin digestion of polypeptides separated by one-dimensional SDS-PAGE followed by MALDI-ToF-MS analysis of resulting peptides was used to confirm the identity of the purified proteins as being the 2S albumin allergen Ses i 1. Regarding the small subunit, two long peptides covering the N- and C-termini were detected in the gel filtration fraction S, whereas five peptides were found in the gel filtration fraction P (Table 1). The different C-terminal peptides detected in the gel filtration fractions S (^{21}Y–D^{34}) and P (^{21}Y–Y^{29}) indicated that the C-

Table 2
Experimental molecular masses of the M_r ~12,000 gel filtration fractions S and P under non-reducing and reducing conditions determined by reverse phase-HPLC-mass spectrometry

Gel filtration fraction[a]	RP-HPLC Peaks	Observed molecular mass	Calculated molecular mass[b]	Putative N- and C-terminal amino acid sequences of small subunit by using ExPASy Q9AUD1	Putative N- and C-terminal amino acid sequences of large subunit by using ExPASy Q9AUD1
S	Non-Red[c]	12061.8	12061.8	^{1}Pyr[d] Q S Q Q C…^{27}S P Y G G E E D	^{1}Q S L R D C…^{61}C A F R V I F
	S[e] (Red)	4015.2	4013.9	^{1}Pyr Q S Q Q C…^{27}S P Y G G E E D	–
	L[e] (Red)	8056.0	8055.9	–	^{1}Q S L R D C…^{61}C A F R V I F
P	Non-Red[f]	11574.6	11574.6	^{1}Pyr Q S Q Q C…^{27}S P Y	^{1}Q S L R D C…^{61}C A F R V I F
		11818.1	11817.7	^{1}Pyr Q S Q Q C…^{27}S P Y G G E	^{1}Q S L R D C…^{61}C A F R V I F
		12062.3	12061.8	^{1}Pyr Q S Q Q C…^{27}S P Y G G E E D	^{1}Q S L R D C…^{61}C A F R V I F
		11315.3	11314.5	^{1}Pyr Q S Q Q C…^{27}S	^{1}Q S L R D C…^{61}C A F R V I F
	S_1[g] (Red)	3267.7	3266.6	^{1}Pyr Q S Q Q C…^{27}S	–
	S_2[g] (Red)	3771.1	3769.8	^{1}Pyr Q S Q Q C…^{27}S P Y G G E	–
		4015.4	4013.9	^{1}Pyr Q S Q Q C…^{27}S P Y G G E E D	–
	S_3[g] (Red)	3527.9	3526.7	^{1}Pyr Q S Q Q C…^{27}S P Y	–
	L[g] (Red)	8056.0	8055.9	–	^{1}Q S L R D C…^{61}C A F R V I F
		7927.9	7927.8	–	^{2}S L R D C…^{61}C A F R V I F

[a] Fractions described in Fig. 1A.
[b] Monoisotopic molecular mass values calculated from the primary amino acid sequence of unprocessed Ses i 1 [10] and considering the replacement of the ^{62}Glu by ^{62}Ala [11].
[c] Peaks described in Fig. 2A.
[d] Pyroglutamic acid.
[e] Peaks described in Fig. 2B.
[f] Peaks described in Fig. 2C and masses showed in decreasing order of abundance.
[g] Peaks described in Fig. 2D.

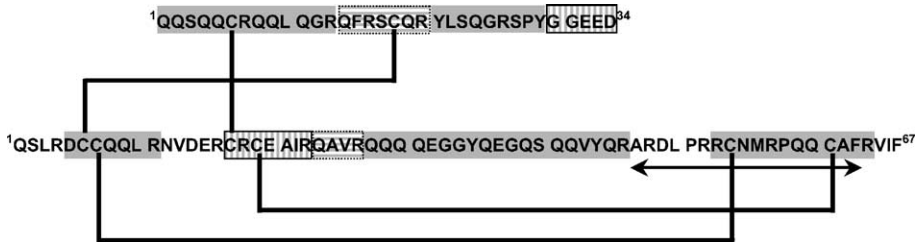

Fig. 3. Disulphide mapping and amino acid sequence of the small and large subunits of Ses i 1 identified by RP-HPLC-ESI-MS and MALDI-TOF-MS deduced from its precursor [10]. Amino acid residues matching with peptides found in both gel filtration fractions are in shaded areas. Amino acid residues corresponding to peptides found only in the gel filtration fractions S or P are in vertical lines (solid squares) or horizontal lines (dotted squares), respectively. The arrow indicates the peptide identified and sequenced by Pastorello et al. [11].

terminus of the small subunit had been clipped and, conversion of the N-terminal Gln of the small subunit into pyroglutamic acid could be confirmed in both fractions. Finally, six and seven peptides, respectively, in fractions S and P, were found which were consistent with their being derived from the large subunit (Table 1).

Further analysis of the P and S fractions was performed using RP-HPLC analysis. Under non-reducing conditions fraction S gave an asymmetric peak (Fig. 2A), which after reduction gave rise to two well-defined peaks eluting at 31.5 and 42 min (Fig. 2B). RP-HPLC-ESI-MS of this fraction indicated a mass of 12062 Da prior to

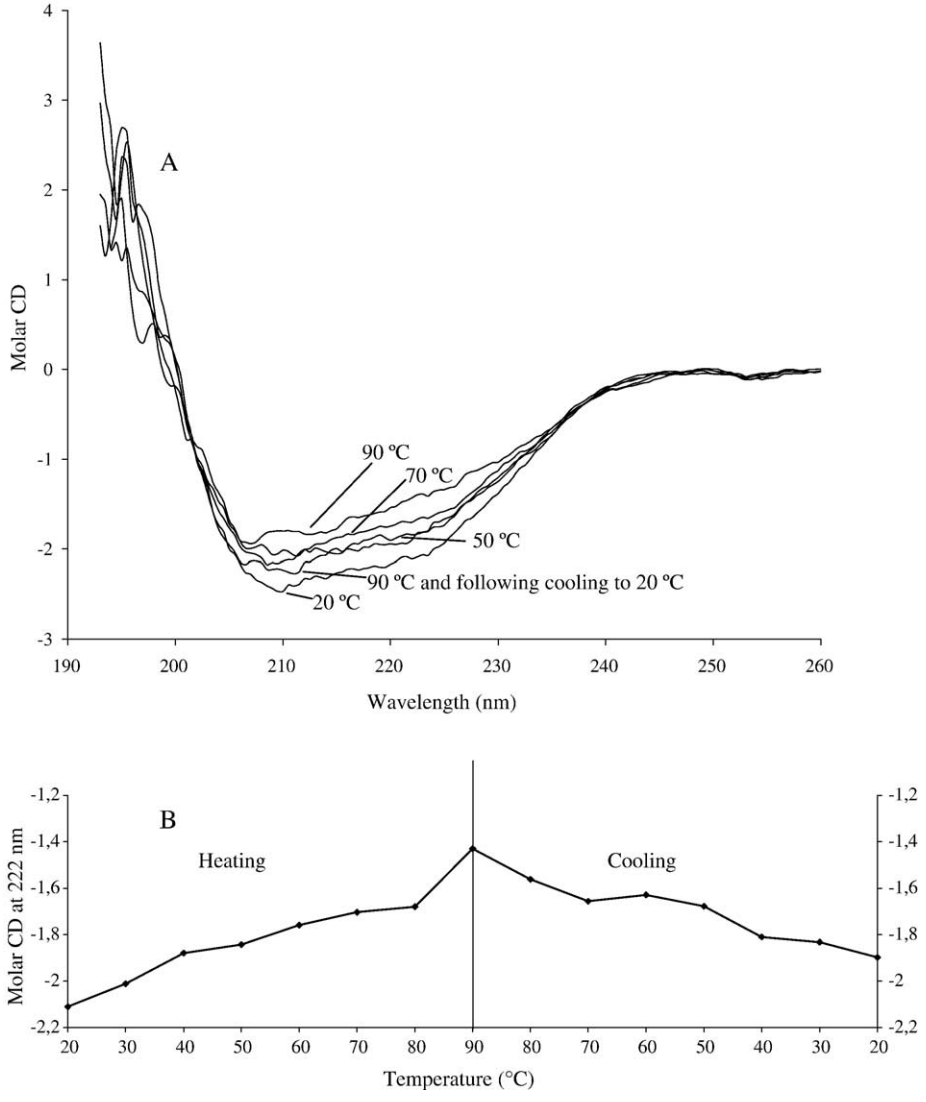

Fig. 4. (A) Far-UV circular dichroism (CD) spectra of Ses i 1 in situ during heating at neutral pH. (B) Change in molar ellipticity at 222 nm during heating and cooling.

Table 3
Protein secondary structure estimates for unheated, heated and heated-cooled down Ses i 1 from Contin analysis of CD spectra and Fitting Gaussian Curves to the FSD FT-IR spectra

Spectroscopy	Temperature (°C)	α-helix	Random coil	β-sheet	Intermolecular β-sheet	β-turn
CD	20	30%	28%	21%	21%	21%
	90	26%	25%	25%	25%	24%
	20 (heated+ cooled)	28%	27%	23%	23%	22%
FT-IR	20	53%	53%	21%	12%	15%
	90	51%	51%	17%	13%	19%
	20 (heated+cooled)	55%	55%	20%	11%	14%

reduction, with two polypeptides of masses 4015 Da and 8056 Da following reduction which corresponded to the calculated masses for the small and large subunits of the Ses i 1 sequence (Table 2).

RP-HPLC profiles of the main gel filtration fraction P showed the presence of additional peaks under non-reducing (Fig. 2C) and reducing conditions (Fig. 2D), confirming its greater heterogeneity indicated by SDS-PAGE analysis (Fig. 1B). ESI-MS analysis of fraction P showed the presence of three additional masses before reduction of 11315, 11575 and 11818 Da species the latter two being more abundant than the intact Ses i 1 (12062 Da) (Table 2). After reduction, four masses were found that corresponded to the small subunit and two that corresponded to the large subunit. This pattern of masses can be explained by the presence of ragged C-termini in the small subunit, arising from post-translational processing of the protein, as has been observed for other 2S albumins [12,17–22]. Also, the observed masses indicated that the N-terminal Gln of the small subunit has been converted to pyroglutamic acid in all cases. This N-terminal blocking has also been described for 2S albumin from various plant species [12,17,19,21–23]. In addition, a very minor mass (7927.9) which corresponds to the large subunit with the loss of the N-terminal residue Gln was detected. Species lacking three N-terminal residues of the large subunit have been observed in the 2S albumin from Brazil nuts [19,24]. Lastly, no evidence of glycosylation was found.

Fig. 3 illustrates the primary structure of the sesame seed 2S albumin allergen, Ses i 1, deduced from the amino acid sequence of its precursor [10] and the RP-HPLC-ESI-MS and proteomic analyses. Overall, peptides covering 71.3% (peak S) and 70.3% (peak P) of the mature Ses i 1 could be identified by proteomic analysis. It should be noted that the peptide identified by Pastorello et al. [11] from the large subunit of Ses i 1 possessed an alanine instead of a glutamic acid residue at position 62, as described for the precursor [10]. RP-HPLC-ESI-MS data confirmed this substitution in the current study on the basis of the large subunit mass, and MALDI-ToF analysis identified a peptide corresponding to ^{53}RCNMRPQQCAFR^{64} (Table 1, Fig. 3).

3.2. Thermostability of Ses i 1

The effect of heating on the secondary structure of Ses i 1 was followed in situ using a combination of CD and FT-IR spectroscopy using dilute and more concentrated protein solutions, respectively. Fig. 4A shows the far-UV CD spectra recorded at different temperatures and neutral pH during both heating and subsequent cooling. The unheated Ses i 1 spectrum showed a strong positive maximum at 195 nm, and a double broad negative minima around 210 and

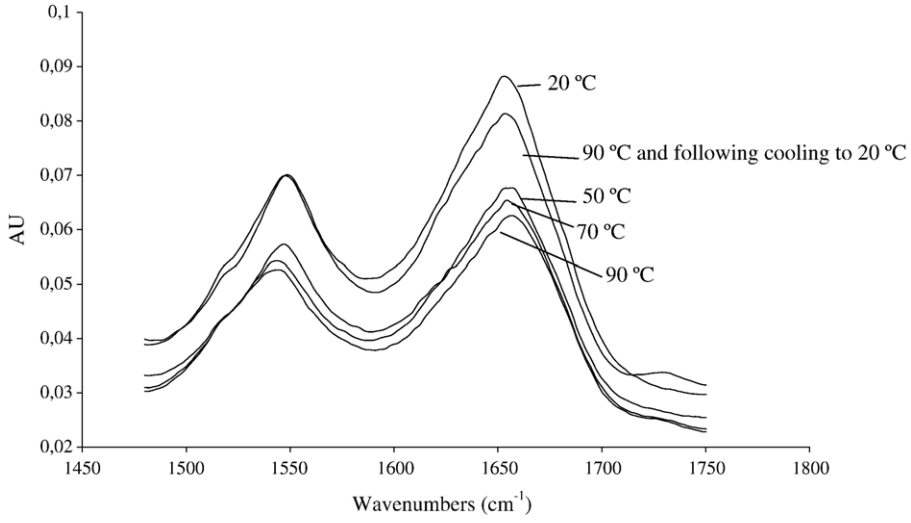

Fig. 5. Fourier transform-Infrared spectra of Ses i 1 in situ during heating at neutral pH.

220 nm, which is characteristic of α-helical proteins [12,25]. Secondary structure estimates indicated unheated Ses i 1 had a content of 30% of α-helix and 21% of β-sheet and β-turn, respectively. A decrease in molar CD was observed as temperature increased, consistent with a slight loss of helicity and a small increase in β-sheet and β-turn contents (Table 3). The ellipticity at 222 nm (Fig. 4B) became gradually more positive during the heating cycle and became more negative again on cooling. The loss of intensity of the 222 nm band is associated with a reduction in the α-helical content of the structure [26] and is in good agreement with the estimations of the secondary structure shown in Table 3. During heating, a slight decrease of 4% in α-helical structure accompanied by increases of 4% in β-sheet and 3% of β-turn was observed. However, after cooling, the proportions of these different secondary structures were essentially identical to those found for the unheated protein showing that Ses i 1 regained its original structure on cooling. No noticeable differences were found in secondary structure at acid (results not shown) and neutral pH, suggesting that the Ses i 1 conformation was not sensitive to pH.

Fig. 5 shows the alteration in secondary structure of Ses i 1 followed in situ during heating using Fourier-transform IR spectroscopy. The most useful FT-IR spectral band for analysis of secondary structure is the amide I region between 1620 and 1700 cm^{-1} [27,28]. Thus, the FT-IR spectrum of unheated Ses i 1 showed an amide I band maximum of 1655 cm^{-1}, which can be attributed to either α-helical or random structures [28,29]. No shift of this maximum was observed during heating and cooling, suggesting that secondary structure of Ses i 1 remained unaltered. This was confirmed by the negligible changes observed in the plots of intensity values at 1623 cm^{-1} (intermolecular β-sheet), 1635 cm^{-1} (intramolecular β-sheet) and 1669 cm^{-1} (β-turn) against 1655 cm^{-1} (data not shown). Consequently, minimal changes in the secondary structure estimations deduced from Fitting Gaussian curves of the FSD FT-IR spectra were observed (Table 3).

Both methods FT-IR and CD spectroscopy confirmed the mainly α-helical nature of Ses i 1 which is consistent with data from circular dichroism described for 2S albumins from yellow mustard [30], radish [31], rapeseed [32,33], sunflower seeds [34,35] and Brazil nuts [12]. The three-dimensional structure of 2S albumins from rapeseed [36], castor bean [37] and sunflower seeds [38] has been determined by NMR methods and they all adopt a compact structure comprising a bundle of five α-helices held together by the four disulphide bonds.

Finally, the ability of Ses i 1 to form aggregates following heating (100 °C/20 min) at neutral (7) and acid (2.5) pH was studied at low (0.2 mg/mL) and high (20 mg/mL) concentrations using gel permeation chromatography. A single peak corresponding to the monomer specie was detected in all cases, ruling out the formation of aggregates (data not shown).

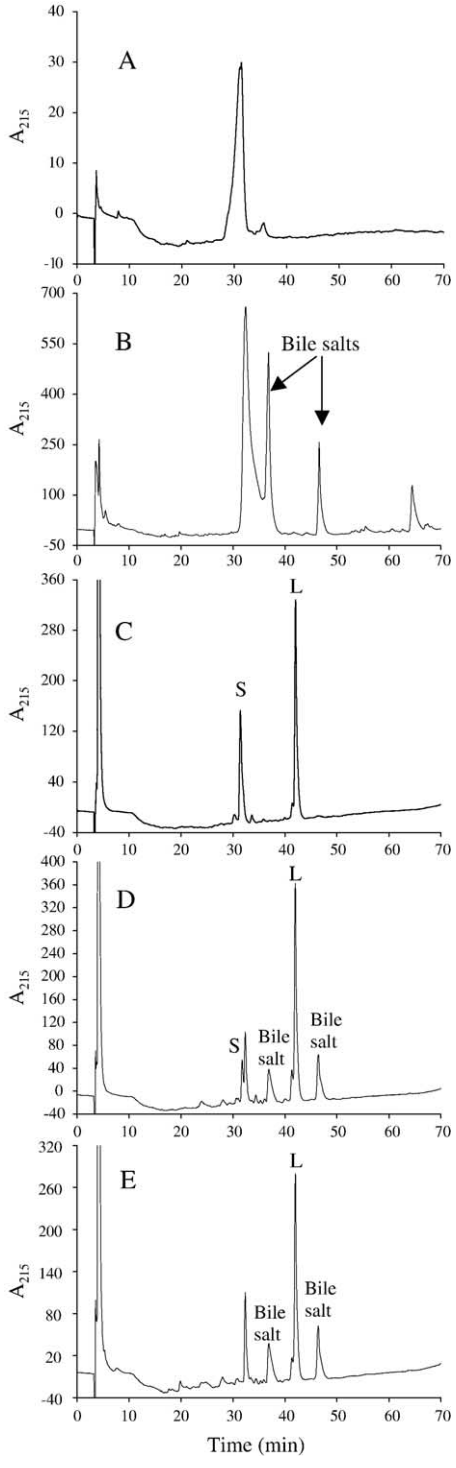

Fig. 6. Reverse phase-HPLC patterns using a protein column of (A) non-reduced Ses i 1 gastric digested (phase 1) for 120 min, (B) non-reduced Ses i 1 gastric (120 min) and duodenal digested (phase 1+2) for 15 min, (C) reduced Ses i 1 gastric digested (phase 1) for 120 min, (D) reduced Ses i 1 gastric (120 min) and duodenal digested (phase 1+2) for 15 min, (E) reduced Ses i 1 gastric (120 min) and duodenal digested (phase 1+2) for 120 min. Peaks S and L correspond to the intact small and large subunit of Ses i 1, respectively.

3.3. In vitro digestibility of Ses i 1

Ses i 1 was unaffected by pepsinolysis, remaining intact after 2 h of phase 1 (gastric) digestion, as determined by HPLC and SDS-PAGE respectively (Figs. 6A and 7A), the large and small subunits of the undigested protein remaining unaltered as shown by HPLC analysis under reducing conditions (Fig. 6C). Pre-heating (either at pH 7 or 2.5) of the 2S albumin did not reduce this resistance to digestion, nor was it affected by the presence of phosphatidylcholine (PC) (data not shown).

After 2 h of gastric digestion, the pH was increased and trypsin and chymotrypsin added with bile salts in order to simulate a duodenal environment (phase 1+2). No noteworthy differences in digestion patterns were found between native and pre-heated 2S albumin and the presence or absence of PC (data not shown). Even after phase 1+2 digestion, a prominent band corresponding to intact Ses i 1 could still be detected by SDS-PAGE (Fig. 7B). However, RP-HPLC profiles obtained on two different columns during duodenal digestion under non-reducing conditions showed a main peak (Figs. 6B, 8B and C) whose retention time and shape was slightly different to that obtained for undigested Ses i 1 (Figs. 2A and 8A) indicating some limited proteolysis. This was accompanied by the appearance of peaks corresponding to peptide fragments resolved using a peptide column (Fig. 8C), suggesting that Ses i 1 was eventually hydrolysed. Following reduction, RP-HPLC analyses (Figs. 6D and E) showed that whilst the large subunit remained intact during duodenal digestion (phase 1+2), the small subunit was gradually broken down with a new peak of very similar retention time to that of the small subunit appearing (Fig. 6D). This new peak completely replaced the small subunit peak after 2 h of duodenal digestion (Fig. 6E). These results clearly indicated that the large subunit was more resistant to proteolytic attack than the small one. However, despite hydrolysis, the remnants of the small subunit remained disulphide linked to the large one as judged by SDS-PAGE under reducing conditions

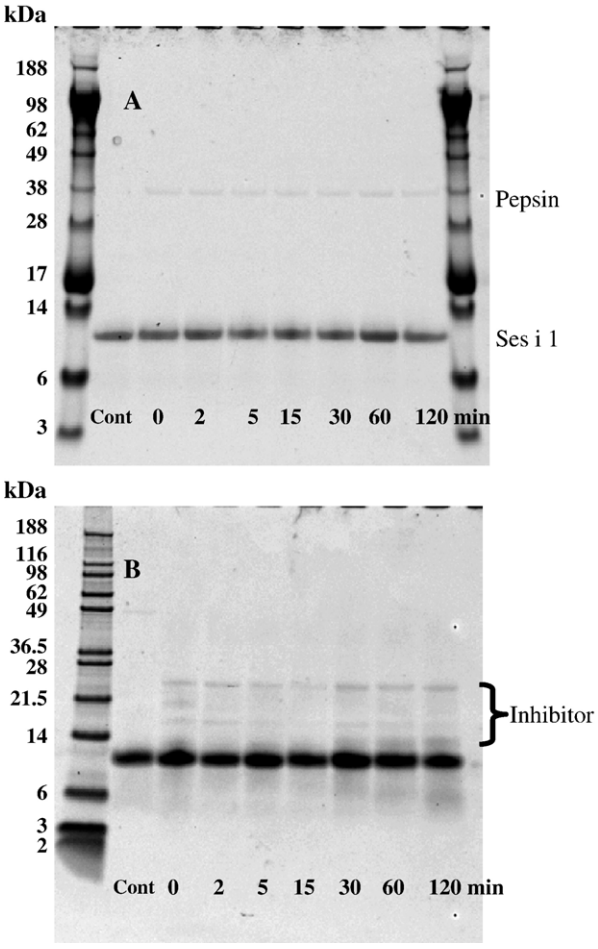

Fig. 7. SDS-PAGE analyses showing the (A) gastric digestion (phase 1) and (B) gastric and duodenal digestion (phase 1+2) of Ses i 1 under non-reducing conditions.

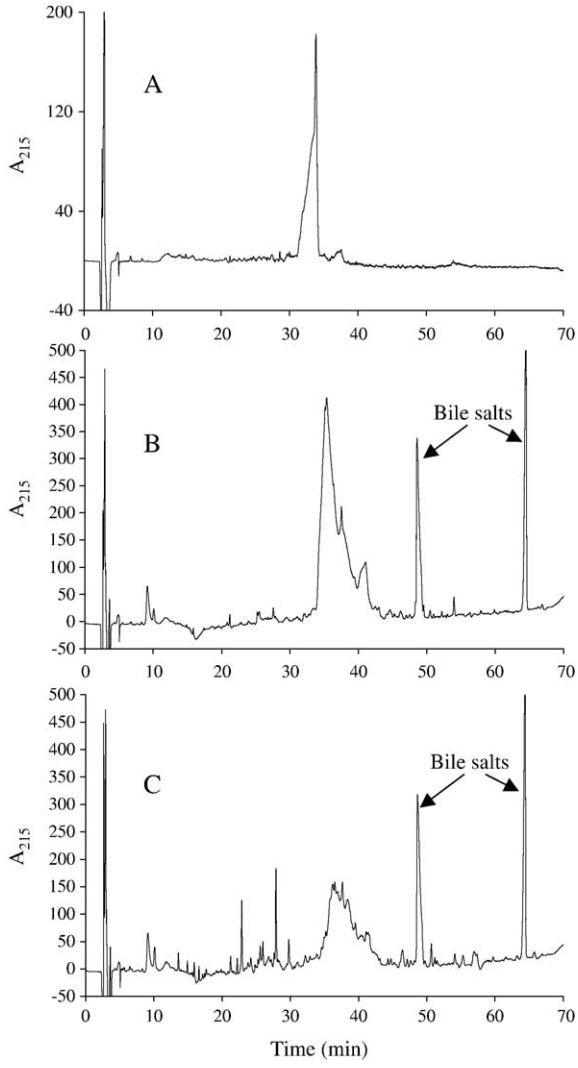

Fig. 8. Reverse phase-HPLC patterns using a peptide column of non-reduced (A) native gel filtration peak 1 (Ses i 1), (B) Ses i 1 gastric (120 min) and duodenal digested (phase 1+2) for 15 min, (C) Ses i 1 gastric (120 min) and duodenal digested (phase 1+2) for 120 min.

(data not shown). Further identification of peptides generate from the limited proteolysis of the Ses i 1 during the duodenal digestion by mass spectrometry was unsuccessful. This may be due to the multi-component medium used for phase 2, including bile salts, lipase, colipase, enzymatic inhibitor, etc., which interfered with the ionisation of these peptides [2].

4. General discussion

In contrast to other 2S albumins from other plant species, such as Brazil nut [12,19], radish [31], sunflower seed [39,40] and rapeseed [33,41] which are codified by a large number of genes leading to numerous isoforms, only a single isoform (Ses i 1) of white sesame 2S albumin was found. Ses i 1 exhibited common features of the 2S albumin family such as a blocked N-terminus, C-terminal truncations of the small subunit, the absence of glycosylation and an α-helical nature. Ses i 1 has also been shown to be extremely stable to acid conditions, thermal processing and in vitro gastrointestinal digestion. Such unusual high stability is mainly attributed to the rigid structure of 2S albumin family dominated by the well-conserved skeleton of cysteine residues [3,42]. Thus, although Ses i 1 unfolded to a limited extent on heating, it refolded on cooling to an almost native structure (Figs. 4 and 5). This can be explained by the fact that the disulphide bonds reduce the conformational entropy of the protein in the denaturated state, increasing the stability of the folded protein [43–45]. Hence, Ses i 1 may be able to retain both linear and conformational epitopes following severe heat-treatments and their ability to trigger an allergic reaction in a sensitised individual will be essentially unaltered.

This structural stability could explain the fact that Ses i 1 digestion was not affected by pre-heating at 100 °C, either at acid or neutral pH. Following in vitro gastric digestion Ses i 1 remained completely intact and then when followed by duodenal digestion remained largely intact with only the small subunit being broken down (Figs. 6D and E). Despite the limited proteolysis observed in the duodenal phase, intact small subunit, and therefore intact Ses i 1, could be still detected after 120 min gastric digestion followed by 15 min of duodenal digestion (Fig. 6D). This is of particular importance since it shows that largely intact allergen is likely to remain in the gut lumen as it moves down the duodenum and into the jejunum/ileum to the site of the first Peyer's patch. It has been claimed that the Peyer's patches are likely to be the major site of sampling for food antigens and allergens (through the activities of constituent M cells) and hence may play a key role in the development of food allergy [46,47].

The greater resistance to the gastrointestinal digestion shown by the large subunit could explain the fact that all the IgE-binding epitopes characterized so far in 2S albumin allergens have been located in the large chain [48–50]. Also, in a recent study, the main digestion-resistant fragments of 2S albumin allergen Ber e 1 from Brazil nuts corresponded to the large subunit [2]. A recent investigation of the IgE epitopes of the methionine-rich 2S albumin allergen from sesame, Ses i 2, indicated that whilst one of the IgE epitopes coincides with those observed in the large subunit of other 2S albumins, two others located in the small subunit were observed [51]. However, Ses i 2 does belong to a different type of 2S albumin and it maybe that it has different stability to digestion of the S-poor albumins. This evidence suggests that the intrachain disulphide bonds of the large subunit make a greater contribution than the inter-chain bonds to the stability of the protein against proteolysis.

In conclusion, although it is not possible yet to determine exactly what makes a protein an allergen, it is evident that the stability of food allergens to low pH, surfactants such as bile salts and proteolytic enzymes in the gastrointestinal tract is needed in order to reach (and stimulate) the immunological system. Findings from this study have shown that Ses i 1 could retain sufficient three-dimensional structure surviving the degradative environment of the gastrointestinal tract in order to sensitise a naïve individual, enhancing its general immunogenicity and/or provoke an allergic reaction in a sensitised individual.

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