Reversible Denaturation of Brazil Nut 2S Albumin (Ber e1) and Implication of Structural Destabilization on Digestion by Pepsin

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The high resistance of Brazil nut 2S albumin, previously identified as an allergen, against proteolysis by pepsin was examined in this work. Although the denaturation temperature of this protein exceeds the 110 °C at neutral pH, at low pH a fully reversible thermal denaturation was observed at ∼82 °C. The poor digestibility of the protein by pepsin illustrates the tight globular packing. Chemical processing (i.e., subsequent reduction and alkylation of the protein) was used to destabilize the globular fold. Far-UV circular dichroism and infrared spectroscopy showed that the reduced and alkylated form had lost its β-structures, whereas the α-helix content was conserved. The free energy of stabilization of the globular fold of the processed protein as assessed by a guanidine titration study was only 30−40% of that of the native form. Size exclusion chromatography indicated that the heavy chain lost its globular character once separated from the native 2S albumin. The consequences of these changes in structural stability for degradation by pepsin were analyzed using gel electrophoresis and mass spectrometry. Whereas native 2S albumin was digested slowly in 1 h, the reduced and alkylated protein was digested completely within 30 s. These results are discussed in view of the potential allergenicity of Brazil nut 2S albumin.

KEYWORDS: Protein stability; protein folding; denaturation; pepsin; allergenicity

INTRODUCTION

2S albumins are storage proteins in seeds and nuts that share common features regarding structural organization. They are globular proteins from which the structure is supported by disulfide bridges (*1*). Some 2S albumins, such as that from Brazil nut, are composed of a small and a large subunit (*2*). Both subunits originate from one gene, expressed as a single-chain peptide that is proteolytically processed. For the 2S albumin from Brazil nut (Ber e 1), the large subunit is 9 kDa and the small subunit is 3 kDa (*2*). The amino acid sequence of 2S albumins in different species is homologous, and the cysteine residues are conserved. Within the species Brazil nut, several isoforms of 2S albumin have been described with the largest heterogeneity in the light chain (*3*). Recently, a three-dimensional model was constructed for this protein on the basis of the conserved 2S albumin disulfide connectivity, together with both sequence and structural alignment with napin seed

storage protein (*4*). The tight globular packing of the protein is dominated by the presence of four disulfide bridges. On the basis of this model the protein is a member of the "four helices; folded leaf; right-handed superhelix; disulfide rich" fold family. In numerous studies it was shown that the presence of disulfide bonds increases thermostability (*5*), is often encountered in proteins of thermophilic bacteria (*6*), increases the conformational stability at ambient temperatures (*7*), and reduces the susceptibility for enzymatic digestion (*8*). In addition, the protein structure of Brazil nut 2S albumin supports the stability to heat and guanidinium-induced unfolding (*9*).

Another common feature of 2S albumins is their amino acid composition. They are rich in arginine, glutamine, asparagine, and cysteine. In particular, Brazil nut 2S albumin has high contents of cysteine (8%) and methionine (19%) as well (*3*). Sulfur-containing amino acids are essential for human and animal diets, and 2S albumin from Brazil nut is considered to be a valuable nutrient. The introduction of the Brazil nut 2S albumin gene using recombinant DNA techniques led to transgenic soy for animal feed purposes (*10*) with a balanced amino acid composition. However, Brazil nut is a known food allergen (*11*), and cases of severe allergic reactions including anaphylaxis have been described (*12−14*). 2S albumin from Brazil nut was identified as an allergen and classified as Ber

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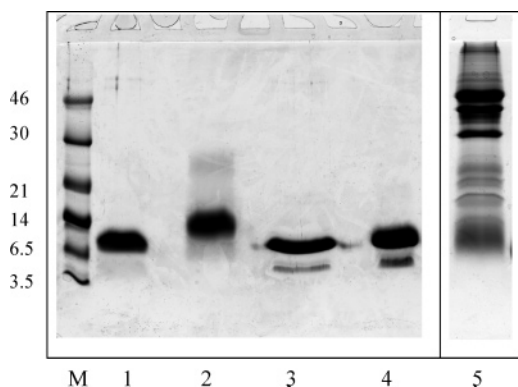**124** *J. Agric. Food Chem.,* Vol. 53, No. 1, 2005

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Figure 1. SDS-PAGE of Brazil nut 2S albumin: (lane 1) native 2S albumin under nonreducing conditions; (lane 2) native 2S albumin under reducing conditions; (lane 3) RA-2S albumin under nonreducing conditions; (lane 4) RA-2S albumin under reducing conditions; (lane 5) crude protein extract of Brazil nut; (lane M) molecular weight markers (indicted at left, kDa).

e1 (*15*). In fact, 2S albumin from Brazil nut was found to be an important IgE-binding protein in symptomatic Brazil nut-allergic patients, whereas sensitization to other IgE-binding proteins only was not associated with food allergic symptoms (*16*). The transgenic soy containing Brazil nut 2S albumin was indeed allergenic to patients (*17*), and therefore product development stopped.

Because the possible allergenicity of transgenic crops is a rising concern, a decision tree for the assessment of allergenic potential of foods derived from genetically engineered food crops was proposed (*18*) and further accentuated by the Food and Agriculture Organization and the World Health Organization (*19*). Common features of food allergens are their stability to acidic conditions and proteolysis (*20*, *21*), although there are exceptions such as the strong allergen casein that is easily denatured and digested and nonallergenic proteins that present very high stability to digestion and acidic treatment [reviewed by Bannon et al. (*22*)]. Although the relationship between stability and allergenicity is not undisputed, it is thought that digestion-stable proteins may pass the stomach in an intact form and induce allergic sensitization via the gut immune system.

The purpose of this study is to identify the structural constraints of Brazil nut 2S albumin and to test the digestibility of this protein by pepsin. Knowledge of the structure–stability relationship of 2S albumin may be used to further explain the allergic sensitization, as elaborated in a recent work (*23*).

MATERIALS AND METHODS

Brazil Nut Protein Extraction and Liquid Chromatography. Brazil nuts (*Bertholletia excelsa*) were purchased as unshelled nuts from Imko Nut Products (Doetinchem, The Netherlands) and were stored vacuum-sealed at 10 °C until use. One kilogram of Brazil nuts was ground and extracted three times with petroleum ether. A portion of 368 g of defatted meal was extracted with 3680 mL of 20 mM sodium acetate, pH 5.5, for 2 h at room temperature. The insoluble fraction was discarded after centrifugation (30 min at 8000*g*). The extract was stored in small aliquots at −20 °C.

A portion of 2 L was applied to a Source S column (300 mL column volume; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM sodium acetate, pH 5.5 (loading buffer). After the column had been washed with loading buffer to remove unbound protein, the column was eluted with a 3 L gradient from 0 to 1 M sodium chloride in loading buffer. 2S albumin-containing fractions were pooled and stored in small portions for single use at −20 °C. All buffers used were filtered through 0.45 μm membranes. Under nonreducing conditions, purified 2S albumin migrates as a single band with an estimated purity of >95% at ∼12 kDa on SDS-PAGE (**Figure 1**). Purified 2S albumin was

dialyzed against demineralized water, lyophilized, and stored at −20 °C.

Protein Determination. Protein concentrations in crude extracts were measured using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Concentrations of purified 2S albumin were determined by measuring the A_{280}. A value for A_{280} (1 mg/mL) of 0.125 was used, based on the Web-based program ProtParam from SwissProt (www. expasy.org/tools/protparam.html, sequence code AB044391).

Reduction and Alkylation of 2S Albumin. Lyophilized protein (540 mg) was dissolved in 270 mL of 6 M guanidinium and 100 mM NH₄-HCO₃ (pH 7.8). After the solution had been warmed to 56 °C, dithiotreitol (DTT) was added (20 mM final concentration). After 60 min, the sample was allowed to cool to room temperature and a freshly prepared iodoacetamide solution (5.02 g in 27 mL of 6 M guanidine and 100 mM NH₄HCO₃, pH 7.8) was added. The alkylation was allowed to proceed for 90 min at room temperature in the dark. The sample was then dialyzed against demineralized water (3 × 10 L) in Spectro/Por 6 (1 kDa cutoff) dialysis tubing at 4 °C. As a control, 2S albumin was processed in the same way without the addition of reducing and alkylating agents.

Both the alkylated 2S albumin and the control protein were lyophilized. The akylated 2S albumin was obtained as a crystalline slightly yellow dense powder, and the control 2S albumin was obtained as a white fluffy powder. Both the alkylated and control 2S albumin were dissolved (2 mg/mL) in 100 mM NH₄HCO₃, pH 7.8. Four hundred microliters of protein solution and 100 μL of DTNB solution [50 mM 5,5′-dithiobis(2-nitrobenzoic acid) in NH₄HCO₃ pH 7.8] were mixed, and the absorption at 405 nm was measured after 10 min of incubation at room temperature. As a blank, 400 μL of buffer and 100 μL of DTNB solution were mixed. The number of free −SH groups was calculated using the molar extinction coefficient ($A_{405} = 13260$ M⁻¹ cm⁻¹) of the free 5-thio-2-nitrobenzoic acid (TNBS) anion. In both the reduced and alkylated 2S albumin and the control protein no free −SH groups could be detected. On SDS-PAGE the reduced and alkylated 2S albumin shows a large and a small subunit, at approximately 9 and 3 kDa, respectively (**Figure 1**). This material is further denoted RA-2S albumin.

The heavy and light chains of RA-2S albumin were isolated using size exclusion chromatography. The reduced, alkylated and lyophilized 2S albumin was dissolved in 20 mM sodium phosphate buffer containing 100 mM NaCl. The heavy and light chains were separated using an Äkta Explorer FPLC (Amersham Pharmacia Biotech) equipped with an SD-30 peptide gel filtration column (475 mL, Amersham-Pharmacia Biotech), calibrated with a mixture of globular proteins and peptides of known mass according to the instruction of the column manufacturer. Separation was performed batchwise (4 mL, 10 mg/mL portions) with a flow rate of 1 mL/min, and eluted proteins were monitored at 214 nm. Both heavy and light chains were pure (>95%) as judged on the chromatograms (not shown).

SDS-PAGE. SDS-PAGE was performed essentially according to the method of Laemlli (*24*) using a Bio-Rad Mini Protean II system (Bio-Rad, Hercules, CA) with 15% acrylamide gels (15 × 10 cm), under standardized conditions (*25*).

Secondary Structure. Far-UV CD spectra were recorded on a Jasco-715 spectropolarimeter maintained at 37 or 95 °C (±0.1 °C) in the spectral range from 190 to 260 nm with a step resolution of 0.5 nm, a scan speed of 100 nm/min, a bandwidth of 1 nm, and a response time of 0.125 s. Eight scans were accumulated and averaged. Typically, 0.25 mg/mL protein samples (20 μM) were prepared in 5 mM phosphate buffer (at either pH 2.0 or 7.0), containing 25 mM NaCl. Samples were equilibrated for at least 15 min at 37 °C prior to the first measurement. From each protein spectrum the corresponding spectrum of the protein-free buffer was subtracted. No noise reduction was applied to the spectra. Estimation of the secondary structure content was performed using a spectral nonlinear least-squares fitting procedure using reference spectra as described by de Jongh et al. (*26*). Typical root-mean-square values were below 4. All samples were prepared and analyzed at least in duplicate.

Attenuated total reflection infrared (ATR-IR) spectra were recorded on a Bio-Rad FTS 6000 equipped with a deuterated triglycine sulfate detector. Typically, 50 μL of a 1 mg/mL protein sample in 5 mM

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phosphate buffer (pH 2.0 or 7.0) containing 25 mM NaCl was transferred onto a germanium crystal (1 × 8 cm) and dried under air to remove excess water. Next, the crystal was placed in the light beam so that six total reflections were obtained. Spectra were accumulated at ambient temperature in the spectral region of 4000−400 cm⁻¹ with a spectral resolution of 2 cm⁻¹ prior to zero-filling and Fourier transformation, using a speed of 5 kHz and a filter of 1.2 kHz. Typically, 100 spectra were accumulated and subsequently averaged. A spectrum representing atmospheric water was subtracted from the sample spectra. All samples were prepared and analyzed at least in duplicate.

Structural Stability. To evaluate the free energy of unfolding of the different protein samples at 37 °C, the ellipticity at 222 nm was monitored on a Jasco-715 spectropolarimeter as a function of the concentration denaturant in the sample. Samples of 190 μL of guanidinium hydrochloride in phosphate buffer at pH 2.0 or 7.0 were prepared, and subsequently 10 μL of a 5 mg/mL protein solution (in pH 2.0 or p7.0 phosphate buffer) was added. Final concentrations of guanidinium ranged from 0 to 7 M. Next, the samples were equilibrated for 2 h. This incubation time was shown to be sufficient to reach equilibrium conditions because longer incubation times did not affect the data. The ellipticity of the sample was measured in a 0.2 mm quartz cell maintained at 37 °C by monitoring the signal during 10 min using a bandwidth of 2 nm and a response time of 8 s and by linear regression of the data points to determine the average value of the ellipticity. The accuracy of the determination is within 0.05 millidegrees. The experiments were repeated at least three times.

The thermal stability of the protein secondary structure was monitored using the spectropolarimeter using ellipticity at 222 nm of 0.25 mg/mL samples in phosphate buffer either at pH 2.0 or at 7.0 that were heated at 1 K/min from 30 to 95 °C with a resolution of 0.1 °C. A 1 mm path length was used, and the signals were averaged for 4 s. In all cases a heating−cooling−heating cycle was recorded.

The thermodynamic changes of the samples during heating were monitored using differential scanning microcalorimetry using a VP-DSC (Microcal Inc., Northampton, MA). The heating or cooling rate was typically 1 K/min, and each run was a heating−cooling−heating cycle between 30 and 110 °C, with 15 min of equilibration time at the extreme temperatures. Typically, 2 mg/mL samples in phosphate buffer either at pH 2.0 or at 7.0 and the reference buffer samples were degassed extensively prior to the experiments. Baseline corrections were applied manually to the recorded heat flow profiles. The experiments were repeated twice.

Pepsin Digestion Experiments. The stability of 2S albumin toward pepsin hydrolysis was investigated in accordance with the protocol suggested by the FAO (*19*). Pepsin was from Sigma (St. Louis, MO) and had a specific activity of 3000 units/mg, with 1 unit defined as an increase in A_{280} of 0.001 per min at 37 °C at pH 2.0 measured as TCA-soluble products using hemoglobin as a substrate. 2S albumin (2.5 mg/mL) or RA-2S albumin (2.5 mg/mL) in 30 mM NaCl, pH 2.0, was incubated with pepsin (3.2 mg/mL, 9600 units/mL) at 37 °C for several periods of time. Samples for SDS-PAGE were taken and immediately diluted in SDS-PAGE sample buffer (1:1); samples for MALDI-TOF analyses were immediately frozen using liquid nitrogen and stored at −80 °C. Lower pepsin concentrations [E/S = 1/100 (w/w), 75 units/mL] were used for MALDI-TOF analysis as well because the prescribed high concentration resulted in digestion of, particularly, RA-2S albumin that was too rapid for proper sampling.

Proteolysis of 2S albumin combined with far-UV CD spectroscopy was performed in appropriate cuvettes, and the contribution of pepsin [E/S = 1/100 (w/w)] to the secondary structure was found to be negligible (not show).

MALDI-TOF-MS Analyses. 2S albumin digests were mixed in a 1:20 volume ratio with a matrix solution consisting of 20 mg/mL of 2,5-dihydroxybenzoic acid in 0.1% trifluoroacetic acid/acetonitrile 2:1. One microliter of the mixture was deposited on the MALDI target plate and allowed to dry. All MALDI-TOF measurements were performed on a Bruker BIFLEX III mass spectrometer (Bruker Instruments, Bremen, Germany) equipped with a nitrogen laser emitting pulsed UV light at 337 nm and operated in the delayed extraction reflectron mode, using an acceleration potential of 19 kV. Tuning and external calibration of the mass spectrometer were performed using a standard peptide
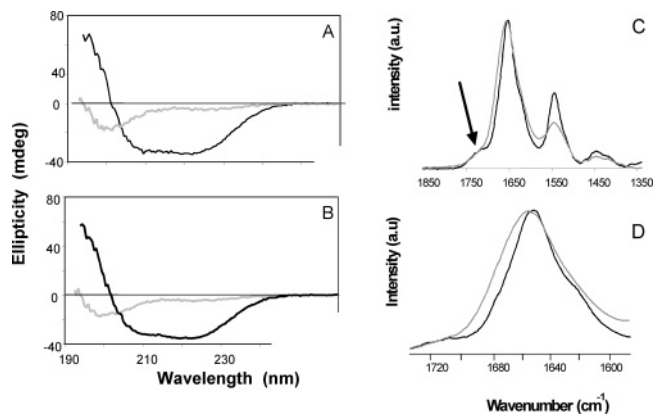

Figure 2. Far-UV CD and near-IR spectra of native and RA-2S albumin. Far-UV CD spectra of 0.25 mg/mL protein samples were recorded at pH 2.0 (**A**) and at pH 7.0 (**B**). Panel C shows the near-IR amide I region from 1350 to 1900 cm⁻¹ of protein films. The arrow indicates the protonated carboxylic acid vibration of the side chains. Panel D is an enlargement of panel C. The black line represents native 2S albumin and the gray line, RA-2S albumin.

mixture (five peptides, M_w range of 1045.5−3146.7). Spectra were acquired in positive ion mode, and the signal was accumulated and averaged over 100−200 shots.

LC-MS/MS. 2S albumin samples were diluted in demineralized water to give a protein concentration of 100 μg/mL. Portions of 10 μL of the resulting solutions were then injected for LC-MS/MS analyses. Analyses were conducted on a 15 cm 800 μm i.d. column packed with Spherisorb C18 reverse phase material, 5 μm particle size, purchased from LC Packings (Amsterdam, The Netherlands). Gradient elution was performed by using the following mobile phases: (A) 10 mM ammonium acetate in water/formic acid 99.9:0.1 (v/v); (B) 10 mM ammonium acetate in water/acetonitrile/formic acid 19.9:80:0.1 (v/v/v). The eluent flow of 25 μL/min was provided by an Eldex micro LC pump (Separations). The gradient used was as follows: 3 min at 5% B, raised in 10 min from 5 to 30% B, in 15 min from 30 to 60% B, and in 10 min from 60 to 100% B.

MS measurements were conducted on an ion trap LCQ DECA (Thermoquest, San Jose, CA) using conventional ESI source in positive ion mode detection. The instrument was tuned using a 5 pmol/μL solution of angiotensin II (Sigma) dissolved in a 2:1 mixture of mobile phases A and B. The spray voltage was set to 4 kV and the heated capillary temperature to 300 °C. All MS/MS measurements were performed using a 3 *m/z* isolation width and 35% collision energy (manufacturer units).

RESULTS

In view of the potential allergenicity, the most relevant conditions for the study of the conformational properties and pepsin digestibility of Brazil nut 2S albumin are stomach conditions (i.e., pH 2 and 37 °C). For that reason this condition was chosen, next to pH 7.0 as a reference. First, the structural aspects of the protein are described and, next, the digestibility is studied.

Structural Aspects of Brazil Nut 2S Albumin. *Structure.* **Figure 2** shows the far-UV CD spectra of native 2S albumin. The spectra both at pH 2.0 (panel A) and at pH 7.0 (panel B) display a zero-crossing around 201.5 nm and two negative extremes around 222 and 208 nm, indicative of a high helical content in the protein. The larger extreme at 222 nm compared to that at 208 nm indicates that the β-strand contribution dominates. This is also evident from the secondary structure estimation based on a spectral fitting procedure as described under Materials and Methods. The native protein has a comparable secondary structure content of about 30% α-helix and about 40 and 46% β-strand at pH 2 and 7, respectively.
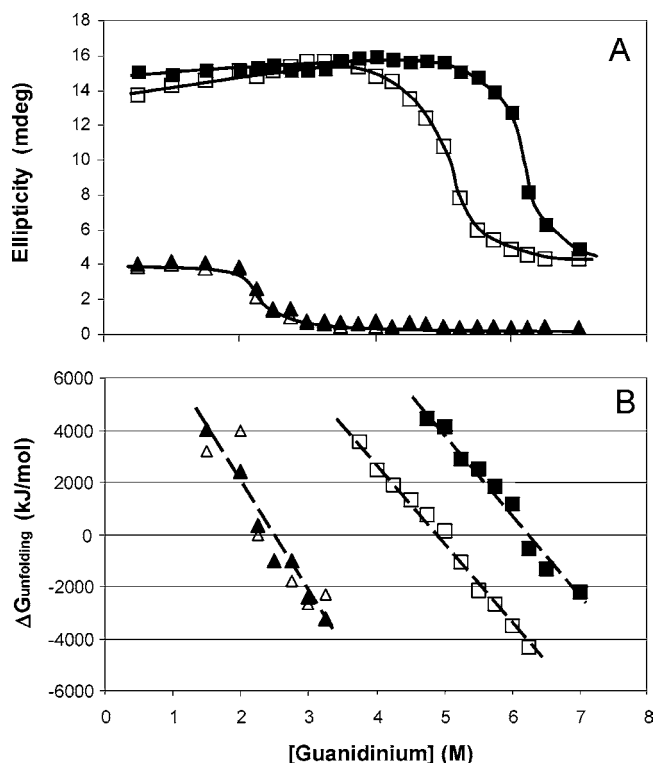
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Figure 3. Guanidinium titration studies of 2S albumin using CD: (**A**) ellipticity as a function of the concentration of guanidinium; (**B**) free energy of unfolding (ΔG) as a function of the concentration of guanidinium assuming a two-state unfolding model. Native 2S albumin (squares) and RA-2S albumin (triangles) are shown at pH 2.0 (open symbols) and at pH 7.0 (solid symbols).

Table 1. Thermodynamic Properties of Native 2S Albumin, RA-2S Albumin, and Control 2S Albumin at pH 2.0 and 7.0

	pH 2.0			pH 7.0		
	native	RA	control	native	RA	control
ΔG^{H_2O} ^{a} (kJ/mol)	15.2	9.8	14.8	19.9	10.3	18.7
T_d ^{b} (°C)	82.5	81.3	82.3	>110	>110	>110
ΔH^c (kJ/mol)	320	56	305	nd^d	nd	nd

^{a} Free energy change of unfolding at 37 °C, as determined from guanidinium-titration studies monitored by the CD ellipticity at 222 nm as representative for the secondary structure. Reproducibility for the native and control samples was typically ±0.3, whereas for the reduced/alkylated samples this is ±0.6 kJ/mol. ^{b} Denaturation temperature as determined by the maximal heat flow in a DSC thermogram. Accuracy and reproducibility of the T_d values in ±0.2 °C. ^{c} Enthalpy change is determined by integration of the area belonging to the changes in heat flow as function of temperature. For all samples the error was typically ±15 kJ/mol. ^{d} Not determined.

unfold the protein. Analysis of these data, assuming a two-state unfolding model (*31*), yields **Figure 3B**. According to $\Delta G = \Delta G^{H_2O} - m$[guanidinium], from the slopes of these lines the free energy change of unfolding (ΔG^{H_2O}) in the absence of denaturant can be extrapolated (to 0 M denaturant), and these values are summarized in **Table 1**. Whereas for native 2S albumin at pH 2.0 a free energy of stabilization of ~15 kJ/mol is found, at pH 7.0 this is ~25% higher.

The thermal stability of the protein samples was evaluated using differential scanning calorimetry (DSC) and by monitoring the ellipticity as a function of temperature. The DSC thermogram of the native protein at pH 2.0 displays a symmetric endothermic transition centered at ~82.5 °C (not shown), with a change in enthalpy of 320 kJ/mol. The thermodynamic characteristics are summarized in **Table 1**. Reheating the sample of native 2S albumin provided a similar heat-flow profile (not shown), indicating that the thermally induced unfolding of the protein is reversible. The midpoint of the transition is not significantly affected, but the transition is somewhat broader compared to the first heating run. Apparently, the cooperativity of unfolding has decreased by the first heating run. The total enthalpy of the transition was, however, not significantly affected. The reversible thermal unfolding process of native 2S albumin was confirmed by monitoring the CD ellipticity at 222 nm during heating and subsequent cooling as illustrated in **Figure 4A**. It can be clearly seen that during heating the native protein at pH 2.0 loses its secondary structure in a cooperative manner, as illustrated by the sigmoidal shape of the curve, with a midpoint of unfolding at 82.6 °C (as evaluated by taking the first derivative of the profile shown in **Figure 4A**; not shown). Also, the cooling curve displays such a sigmoidal shape with a midpoint at 80.4 °C. Furthermore, it can be seen that the ellipticity at ambient temperature after heating and cooling is similar to that prior to the heat treatment. The change in ellipticity of the native protein at pH 7.0 upon raising the temperature is given by the gray line. The absolute ellipticity steadily decreases with temperature, but no "transition" can be observed, in agreement with the DSC results.

The spectra of native 2S albumin at 37 °C prior to and after heating (**Figure 4B**) are very comparable, showing that there is a similar secondary structure content, whereas the spectrum taken of the protein at 95 °C shows significantly less defined secondary structure. In the latter case spectral fitting does not give unambiguous results. CD spectra of both a protein with a secondary structure content of 45% α-helix and 55% random and a protein consisting of 21% α-helix, 20% β-strand, and 59% random coil describe the experimental spectrum adequately. It

To further investigate the secondary structure, near-infrared absorption spectra were recorded. **Figure 2C** shows the amide I (1700−1600 cm^{-1}; mainly C=O vibrations) and amide II band (1600−1500 cm^{-1}; mainly N−H vibrations) of native and RA-2S at pH 2.0. Remarkable is the presence of the shoulder around 1730 cm^{-1}, which originates from the protonated carboxylic acid groups (*27*), indicative of the preserved original pH in the film, as reported previously (*28*). Evaluation of the band shape of the amide I (enlarged in **Figure 2D**) of these protein samples provides qualitatively comparable results on the secondary structure content compared to far-UV CD data. The spectrum of the native protein displays a band centered at 1655 cm^{-1} with a shoulder around 1630 cm^{-1}, the first representative for helical structures and the latter indicative for β-strands (*29, 30*).

Recording near-UV CD or tyrosine fluorescence spectra to obtain information on the tertiary packing of the protein under the conditions studied was not successful. The single tyrosine present in the protein and the absence of phenylalanines or tryptophans prohibit the recording of proper spectra. Size exclusion chromatography was applied to obtain information on the quaternary structure of the proteins (results not shown). On the basis of the separation of a calibration mixture of globular proteins and peptides, the native 2S albumin has an apparent molecular mass of 12 kDa and did not show a difference between pH 2 and 7.

Structural Stability. To investigate the structural stability of the proteins at 37 °C, guanidinium titration studies were performed to derive the free energy of unfolding for this protein (*31*). From **Figure 3A** it can be seen that native 2S albumin at pH 2.0 requires ~5 M guanidinium to unfold. The native protein at higher pH displays a stronger resistance toward unfolding as evidenced by the higher concentration denaturant required to

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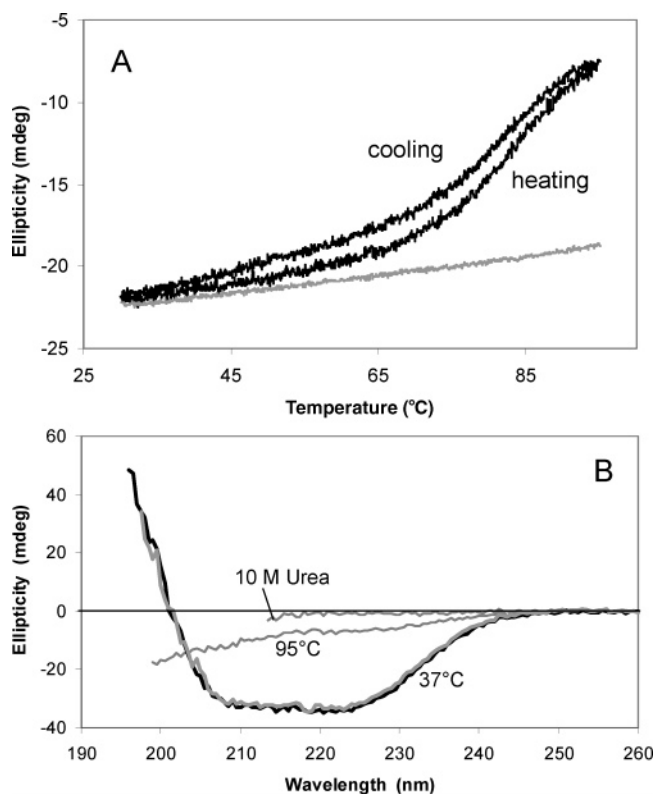*J. Agric. Food Chem.,* Vol. 53, No. 1, 2005 **127**

Figure 4. Far-UV CD analysis of native 2S albumin after temperature treatment: (**A**) temperature traces of the ellipticity at 222 nm of a 0.25 mg/mL protein sample at pH 2.0 upon heating and cooling at 1 K/min (the change in ellipticity for the native protein at pH 7.0 upon heating is indicated by the gray line); (**B**) far-UV CD spectra of native 2S albumin at 37 °C prior (black line) and after (gray line) heat treatment at 95 °C. For comparison, also the spectrum of the protein in the presence of 10 M urea is shown.

0   ¼   ½   1   2   4   8   16   30   60 min   0   ¼   ½   1 min

Figure 5. Pepsin digestion of 2S albumin; SDS-PAGE analysis of digested samples: (**A**) native 2S albumin; (**B**) RA-2S albumin. Incubation time is indicated below each lane (in minutes).

Table 2. Peptides Derived from the Pepsin Treatment of Native 2S Albumin[a]

peptide	monoisotopic molecular mass	peptide sequence[b]
1	567.5	30-MMRM-33
2	861.8	48-RRMMRL-54
3	1104.6	37-EMQPRGEQM-45
4	1775.8	31-MRMQQ**Q**EMQPRGEQ-44
5	1776.8	32-RMQQEEMQPRGEQM-45
6	1907.8	31-MRMQQ**Q**EMQPRGEQM-45

[a] Peptides were determined by MALDI-TOF MS and LC-MS analysis. Their sequences were then derived by interpretation of targeted LC-MS/MS experiments. [b] The numbers indicate the residue numbers in the mature 2S albumin (according to the sequence data in P04403) heavy chain. Note that the sequence of peptides with molecular masses of 1775.8 and 1907.8 contain a Gln (bold) instead of Glu.

is therefore fair to say that upon raising the temperature from 20 to 95 °C the defined secondary structure content lowers from 75 to ~40–45%. That the protein at elevated temperature is still secondary structured is illustrated by the spectral comparison with a sample in 10 M urea (also indicated in **Figure 4B**), reflecting the signal expected to belong to an entirely nonstructured protein.

Pepsin Digestion of Native 2S Albumin. Following the suggested protocol outlined by the FAO/WHO for pepsin digestion, degradation of native 2S albumin required 8–16 min as shown by SDS-PAGE analysis (**Figure 5A**). Because the enzyme/substrate ratio prescribed in the FAO/WHO protocol is high, dilutions of pepsin were tested as well. A pepsin-to-substrate ratio of 1:100 (w/w) was used to identify the site(s) where pepsin initiates the hydrolysis of native 2S albumin. Samples of this hydrolysis reaction in time were analyzed with LC-MS/MS and MALDI-TOF. At least eight products were formed during the first hour of the digestion, of which six could be identified by the fragmentation pattern in the MS/MS spectra. The identified peptides are listed in **Table 2**. All peptides originate from the heavy chain of the protein. The sequences of peptides with molecular weights 1775.8 and 1907.8 appear to contain a Gln (see **Table 2**, bold) instead of a Glu. This could be due to the fact that the protein was not obtained via heterologous expression, but by isolation from natural Brazil nut, and therefore may have some nonisogenic heterogeneity. Protein databases also contain conflicting sequence data (see comments in the Protein DataBank entry P04403). Heterogeneity especially in the heavy chain has been observed (*3*), and in
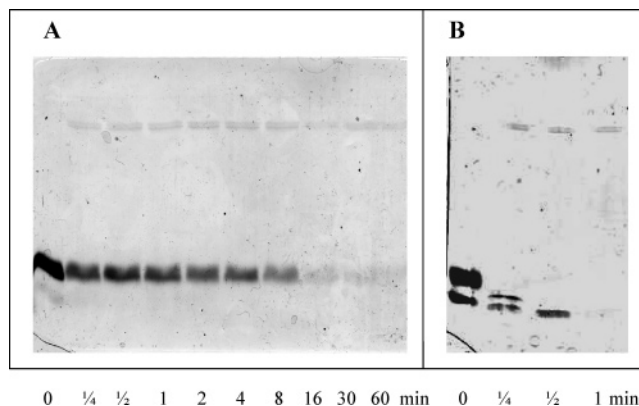
particular both Glu and Gln have been found at position 36 of the heavy chain (*3*).

RA-2S Albumin. The reduction and alkylation cycle has a major effect on the structure of the protein, as illustrated in **Figure 2A,B** by the altered shape of the CD spectrum (gray line), where a single negative extreme is apparent around 200 nm and a zero-crossing, around 194 nm. Spectral analysis reveals that at both pH values the α-helix content appears to be retained, whereas the β-structures are completely lost. The control protein, subjected to the same processing conditions in the absence of reducing and alkylating agent, showed results identical to those of native 2S albumin (not shown). Reduction and alkylation also results in a spectral change in the infrared spectra (**Figure 2C**, gray line), where the amide I band becomes broader and where a shoulder appears around 1645–1650 cm^{−1}. Both of these aspects are indicative for the appearance of random structures after processing (*30*). The control protein had a spectrum comparable to that of the native protein (not shown).

To investigate which subunit contributes to the secondary structure of RA-2S albumin, the material was separated using size exclusion chromatography, yielding two peptides with a purityof >95% as judged by the chromatograms. The far-UV CD spectra of these two polypeptides at pH 2.0 are shown in **Figure 6A**. The shapes of the spectra of the polypeptides differ slightly (**Figure 2A**). The spectrum of the light chain points to a slightly higher zero-crossing (around 4 nm) than that of the heavy chain (around 190 nm). Recombination of the spectra of the heavy and light chains in a 1:1 molar ratio yields a spectrum that is comparable to that of the RA-2S albumin (**Figure 6B**).
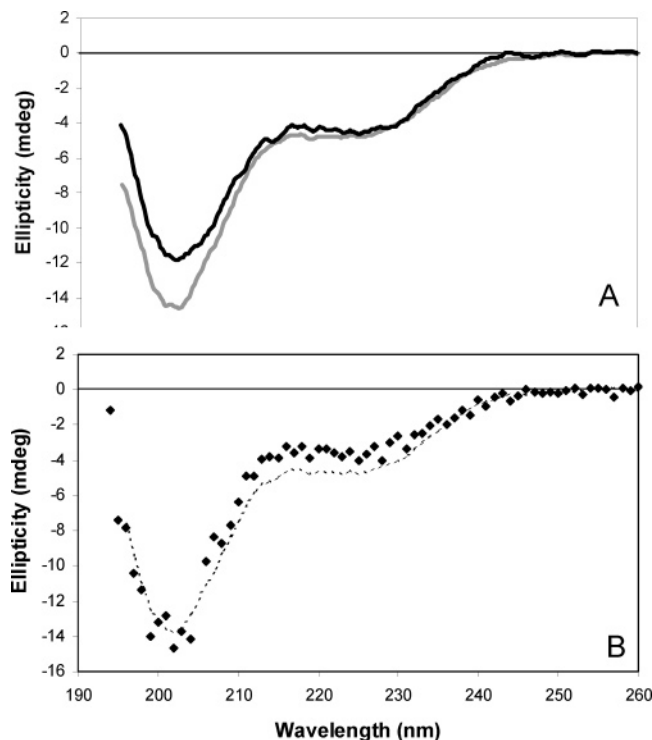
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Figure 6. Far-UV CD spectra of the heavy and light chains of 2S albumin at pH 2.0: (**A**) spectra of 0.25 mg/mL samples of the heavy (gray line) and light (black line) chain were recorded at 37 °C; (**B**) weighted sum of a 1:1 molar ratio of the spectra of the heavy and light chains of panel A (dotted line) compared to that of the nonfractionated RA-2S albumin (symbols).

Testing the apparent size of RA-2S albumin using size exclusion chromatography yielded for the individual light and heavy chains masses of 3.8 and ∼17 kDa, respectively (not shown). The latter mass is ∼2-fold higher than expected. This could be due to either the loss of globular structure and more extended conformation of the heavy chain or a dimerization. These results were found both at pH 2.0 and at pH 7.0. SDS-PAGE performed under both nonreducing and reducing conditions shows that the heavy chain migrates somewhat above the 6.5 kDa marker, and well below the 14 kDa marker, negating the option of dimerization (data not shown). For RA-2S albumin a significantly lower free energy change is found from the guanidium titration studies (**Figure 3**). It appears to be ∼10 kJ/mol at both pH 2.0 and 7.0 (**Table 1**). The free energy of stabilization obtained for the separated polypeptides, having a comparable secondary structure content (**Figure 6**) compared to the mixed sample, displayed also values comparable to the mixed sample of RA-2S albumin (results not shown). Calorimetric analysis of the reduced and alkylated protein shows a lower denaturation temperature (∼81.3 °C) with a significantly lower enthalpy change of 56 kJ/mol (**Table 1**). Remarkably, at pH 7.0 none of the samples exhibited enthalpy changes in the temperature region studied here (results not shown). No significant effect was observed by the processing conditions alone (results not shown).

Figure 5B demonstrates that the RA-2S form of the protein is degraded by pepsin (shown on a second time scale) much more quickly than the native protein. It is unclear whether the low molecular weight band on the gel reflects a more resistant light chain or defined degradation products of the heavy chain.

DISCUSSION

Gastrointestinal protein digestibility is assumed to be a key factor for the safe uptake of nutrients in the human body. Poor digestibility is associated with allergenicity because intact proteins or large fragments thereof may be exposed to the gut immune system, thus inducing type I, IgE-mediated food allergy (*20*). However, exemptions to this rule are documented as well and have been reviewed recently (*22*). The reported allergenicity of Brazil nut 2S albumin, even after processing, could be related to poor digestibility by gastric fluid. This work aims to report on the stability aspects of this protein under the relevant conditions for digestion in the stomach (acidic pH). Another recent study (*23*) shows how the Brazil nut 2S structural stability relates to allergenic potential in vivo.

Structure and Stability of Brazil Nut 2S Albumin. Because the relevant conditions to test susceptibility for pepsin digestion for this study are at pH 2.0 and 37 °C, first the differences at a structural level between the protein's ambient condition (near-neutral pH) and acidic pH should be compared. The data demonstrated that the protein secondary structure at 37 °C is not affected when the pH is lowered from 7 to 2 (**Figure 2**). This is remarkable, because many globular proteins are known to denature under such conditions, mainly driven by protonation, and thereby neutralization of negatively charged carboxylate groups on the protein surface. That this protonation readily occurs is shown by the intensity at 1730 cm^{-1} shown in the infrared spectra of the protein (**Figure 2**). It is evident from evaluation of the secondary structure at pH 2.0 that a large proportion of β-stranded structures is present in Brazil nut 2S albumin (**Figure 2**), in contrast to the structure of the homologous protein napin seed storage protein, the structure of which is known (*32*). Prediction algorithms, like AGADIR (Serrano group at EMBL accessible via www.ebi.ac.uk), indicate that regions predicted to be helical also display a strong β-strand propensity. The sequence homology with napin 2S might therefore suggest a similarity on the basic global fold with the four disulfide bridges as structural anchors; the local folds and hydrogen bonding network might be quite different. The far-UV CD spectra presented by Alcocer and co-workers (*4*) for this protein do show, however, a great similarity to those presented in this work (despite the small differences in conditions used). The enhanced electrostatic repulsion at pH 2.0 does, however, result in a lower stability of the structure, as demonstrated by guanidinium titration studies (**Figure 3**; **Table 1**). At pH 7.0 and elevated temperatures (up to 95 °C) the protein does not undergo any cooperative unfolding event, in agreement with the observations of Murtagh et al., who found no unfolding up to 75 °C (*9*). At pH 2.0, however, a thermodynamic transition at 82.5 °C is apparent (**Figure 4A**; **Table 1**), resulting in a partial unfolded state. This unfolded state still contains significant secondary structure (**Figure 6B**). Remarkable is the full reversibility of the transition, most likely pointing to the dominating role of the four disulfide bridges that reduce the entropic gain upon unfolding significantly. Altogether it can be stated that the structural stability of the globular Brazil nut 2S albumin is governed by the presence of disulfide bridges.

In view of the above-described globularity of the protein, it is interesting to compare this with the separated heavy and light chains that are obtained upon reduction and subsequent alkylation of the protein. Disruption of the disulfide bridges results in a major loss of secondary structure in the protein; in fact, only the helical regions remain intact, whereas all β-strands become unstable (**Figure 2**). The helical propensity in these polypeptides must, however, be that strong so that even at low

Light chain:

QEE**C**REQMQRQQMLSH**C**RMYMRQQMEES

Heavy chain:

H**C**RRGMEPHMSE**CC**EQLEGMDES**C**R**C**EGLRMMMMRMQQEEMQPRGEQMRRMMRLAENIPSR**C**NLSPMR**C**PMGGS

Figure 7. Amino acid sequence of Brazil nut 2S albumin. The mature light chain and heavy chain are given according to the sequence data in protein DataBank entry P04403 in a single-letter code. In bold are presented the nine cysteine residues. The arrows indicate the pepsin cleavage positions based on the mass spectrometric analysis of fragments presented in **Table 2**.

pH they remain unaffected by the protonation. The structure of the polypeptides, however, differs significantly from that of the native protein at elevated temperature (95 °C) (**Figure 4**). Both the heavy and light chains have lost a great deal (but not all) of their original secondary structure and exhibit a significantly lower stability toward unfolding (**Figure 3**; **Table 1**).

Digestion of Brazil Nut 2S Slbumin. According to the protocol provided by the FAO and WHO to demonstrate potential allergenicity, we investigated the pepsin sensitivity of native and reduced/alkylated 2S albumin from Brazil nut. The high enzyme/substrate ratio of around 1 (w/w) resulted in a degradation of native 2S on a time scale of 1 h, although some intact protein with a molecular weight of around 12 kDa was present for the rest of the time course of the experiment. In contrast, the reduced and alkylated 2S albumin was digested completely within 1 min. Typical degradation times for other allergens investigated with this protocol such as those from peanut, egg, and milk require between several minutes to >1 h (*20*), whereas common plant proteins that are not allergenic, such as rubisco from spinach and lipoxygenase from soy, are degraded completely within 30 s (*20*). Thus, native Brazil nut 2S albumin has a digestion stability that is comparable with that of common allergens, whereas the reduced and alkylated 2S albumin degrades on a time scale observed for nonallergenic proteins. This illustrates that the loss of conformational integrity in 2S albumin by reduction and subsequent alkylation of the disulfide bridges leads to an enhanced protease susceptibility.

Whereas the proteolytical breakdown of reduced and alkylated 2S albumin is fast, native 2S albumin degrades gradually (**Figure 5**). To explain the remarkable stability of Brazil nut 2S albumin toward pepsin, the peptides originating from pepsin digestion were investigated. Applying LC-MS/MS and MALDI-TOF MS on a digest prepared such that intermediate hydrolysis products could be detected as well, we identified six peptides, all from the heavy chain (**Table 2**), whereas the light chain was unaffected. **Figure 7** illustrates the observed cleavage sites in the native protein. The observation that the light chain is not hydrolyzed by pepsin may be explained by the absence or inaccessibility of the Tyr−Met bond, the preferred pepsin cleavage site (*33*), in our protein preparation. From the data in **Table 2** and **Figure 7** it is clear that pepsin cleaves only the heavy chain in native 2S albumin at multiple sites. Met30/31−Met31/32 and Gln44−Met45 appear to be the initial cleavage sites because they appear to be involved in the majority of formed fragments. In a homology model (1GYS.pdb) based on the experimentally determined structure of napin storage protein (1PNB.pdb) Met30/31−Met31/32 are part of the globular core of the protein. These cleavage sites have relative low solvent accessibilities (~15%) in the homology model, which may further explain why pepsin hydrolysis is slow.

Reduction and alkylation of Brazil nut 2S albumin results in a protein preparation with a strongly diminished structure and stability. As a consequence, the susceptibility for pepsin increases as demonstrated by a 30−60-fold faster proteolysis (**Figure 5**). Apparently in the reduced and alkylated form all potential cleavage site are readily available, leading to a large pool of small peptides that are difficult to identify (not shown).

Relationships between Structure Stability and Allergenicity of Brazil Nut 2S Albumin. IgE-binding epitopes on 2S albumin from Brazil nut have not been mapped yet. For walnut 2S albumin, highly homologous to Brazil nut 2S albumin (*34*), one IgE-binding epitope was recognized by a pool of serum from a U.S. population of walnut-allergic patients (*35*). This epitope, located on the heavy chain, however, is not present on 2S albumin from Brazil nut (*3, 34*). Cosensitization for different nuts and peanuts is a common clinical phenomenon, especially in older patients (*11*). IgE toward Brazil nut, hazelnut, and peanut was recently measured in 731 patients, and the majority (391 patients) had IgE that was specific for both tree nuts and peanut (*36*). Due to the limited knowledge of the specificity of IgE binding to Brazil nut 2S albumin, the relevance of the remaining peptides of 2S albumin after proteolysis cannot be established. The size of these peptides, up to 14 amino acids, is large enough for interaction with IgE. Interestingly, in the middle part of the heavy chain of the 2S albumin, three prominent T-cell epitopes were found when 92 healthy donors were tested (*37*). T-cell activation is a prerequisite for developing an allergic immune response, and the fact that the pepsin-resistant peptide 31−44 of the heavy chain resembles a prominent T-cell epitope (*37*) may contribute to the understanding of relationships between the stability of 2S albumin and its allergenicity.

In another study (*23*) the allergenic potential of native and RA-2S albumin from Brazil nut as described in this paper was reported. Because both preparations have an identical amino acid sequence, providing the same potential IgE epitopes, the structural stability and proteolysis are markedly different, as shown in this work. Using a Brown Norway rat model for testing allergenic potential via the oral route (*38*), native 2S albumin was found to be a potent allergen leading to high IgE titers, confirming the potent allergenic character of 2S albumin, whereas the reduced and alkylated form did not lead to IgE sensitization at all.

In summary, the data presented in this work show that the disulfide bonds in Brazil nut 2S albumin contribute to protein structure on secondary and tertiary folding levels and to the stability of this protein structure. Thermal denaturation may unfold the protein at elevated temperatures, but does not lead to an irreversible conformational change upon cooling. Consequently, Brazil nut 2S albumin is highly resistant toward proteolysis by pepsin not only because of its "disulfide-locked" structure but also due its reversible denaturation characteristics, making extensive technological processing ineffective. As a result, the poorly degraded protein and derived large fragments that exist after prolonged proteolysis are able to induce an

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allergic immune response, providing an explanation for the high allergenic potential of this class of plant storage proteins.

ABBREVIATIONS USED

RA-2S, reduced and alkylated 2S albumin; CD, circular dichroism; FTIR, Fourier transform infrared.

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