Brazil Nut (*Bertholletia excelsa* H. B. K.) Proteins: Fractionation, Composition, and Identification of a Sulfur-Rich Protein

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An improved procedure was developed to fractionate the total protein from Brazil nut into three size classes of proteins, 11S, 7S, and 2S. The protein components and amino acid compositions of these protein fractions were analyzed. By sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the 11S protein fraction was resolved into six major polypeptides (M_r 24 000–32 000); the 7S protein fraction contained five major polypeptides (14 000–45 000); and the 2S protein contained two low molecular weight polypeptides (9000, 3000). All three protein fractions contain high concentrations of glutamine/glutamic acid and arginine. But the 2S protein contains exceptionally large amounts of the sulfur amino acids, 17.9% Met and 8.7% Cys. This sulfur-rich protein constitutes some 30% of the total extractable Brazil nut proteins. A gel filtration procedure was developed for preparation of large quantities (300 mg/run) of pure sulfur-rich protein for further studies.

Brazil nuts, the seeds of *Bertholletia excelsa* H. B. K., are produced and exported from the Amazon Basin region and are used most extensively in confections in countries of Europe and North America. Although known for their rich oil content (65–70%), the nuts are also a good source of protein (15–17% by fresh weight and about 50% by weight of its defatted flour) (Schreiber, 1950, Antunes, 1975).

Brazil nuts have unusually high levels of the sulfur-containing amino acids, 8.3% by weight (Antunes, 1975), and are probably the richest food source of these essential amino acids. This high sulfur content was noted as early as 1892 by Osborne (1924), when he compared the composition of the crystallized protein excelsin of the Brazil nut with that of other oilseed proteins. Antunes and Markakis (1977), through rat feeding experiments, studied the supplementary effect of defatted Brazil nut flour on navy beans, a poor source of sulfur amino acids, and found that the protein quality of navy bean could be largely improved by the addition of the Brazil nut flour. More recently, Youle and Huang (1981), in a survey of the low molecular weight proteins in a number of oilseeds, reported that a 2S albumin fraction in Brazil nut contains high levels of the sulfur amino acids, 17.37% methionine and 13.11% cysteine. Many vegetable proteins, especially those from legumes, are deficient in these essential sulfur amino acids (Yamaguchi, 1983). Thus, we were interested to learn whether the high levels of the sulfur amino acids were concentrated in a single protein species in the 2S protein fraction. This information would provide us with a basis for a molecular approach to improve the nutritive value of vegetable protein sources deficient in the sulfur amino acids.

Although Youle and Huang (1981) has reported the amino acid composition of the 2S albumin fraction in Brazil nut along with those in other oilseeds, there is no further information on the purity and properties of this 2S albumin or other protein fractions of the Brazil nuts. In this paper, we describe and compare various procedures for effective extraction, fractionation, and purification of the Brazil nut proteins from both defatted flour and fresh kernels. We present an analysis of the polypeptide components and amino acid compositions of the 11S, 7S, and 2S Brazil nut proteins. And, since we found that most of the sulfur amino acids are concentrated in the 2S protein, which contains only two low molecular weight polypeptides of 9000 and 3000, we have developed a method for the large-scale preparation of this sulfur-rich protein from Brazil nut for further studies, including the sequencing of this protein to obtain information for future identification and isolation of the gene(s) encoding this sulfur-rich protein.

MATERIALS AND METHODS

Preparation of Brazil Nut Defatted Flour. Two different lots of Brazil nuts were used in this study. One lot was obtained from Brazil through the courtesy of Bruce Nelson of the New York Botanical Garden, and the other lot was a gift of Dr. A. H. Huang of the University of South Carolina.

Brazil nut kernels were ground into a fine paste and defatted by extraction with hexane. After three extractions with fresh hexane, the residue was recovered by filtration under reduced pressure and dried under vacuum overnight. The resulting fine white powder was designated Brazil nut defatted flour (BNDF).

Extraction of Total Protein. BNDF (1 g) was ground in either of the following extraction buffers (10 mL): (a) sodium dodecyl sulfate (SDS) buffer, 0.5 M NaCl, 1% SDS, 2% 2-mercaptoethanol in 0.0625 M Tris-HCl buffer, pH 6.8; (b) salt buffer, 1 M NaCl in 0.035 M sodium phosphate buffer, pH 7.5. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 26000*g* for 30 min at 4 °C. The recovered supernatant fraction was again centrifuged at 26000*g* for 30 min, and the final supernatant fraction was designated as total extractable protein.

Total protein was also obtained by direct extraction of the fresh Brazil nut kernels using the procedure as described above for BNDF, except that, in order to obtain a clear protein extract with minimal lipid contamination, four centrifugations were carried out, and only a portion of the supernatant fraction between the pellet and the upper lipid layer was collected each time.

Fractionation of Total Protein. *I. Sucrose Gradient Centrifugation/Solubility Fractionation.* The sucrose gradient centrifugation procedure of Youle and Huang (1981) was used as a first fractionation step in this procedure. Each 1 mL of protein extract in salt buffer (prepared as detailed above) was applied on top of a 38-mL linear 5–30% sucrose gradient, prepared in 1 M NaCl and 0.035 M sodium phosphate, pH 7.5. The gradient was

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centrifuged at 25 000 rpm for 24 h at 4 °C in a Beckman L8-80 centrifuge using a SW28 rotor. After centrifugation, the gradient was fractionated into 1-mL fractions. The fractions containing the three size classes of proteins (11S, 7S, 2S) were pooled separately. To quantitate the relative amounts of the various proteins in each size class, the areas under the peaks of the sedimentation profiles (Figure 2A) were calculated. Since there was some cross-contamination of the protein fractons, the fractions were further purified by extensive dialysis against deionized water at 4 °C. For the 2S protein fraction, the contaminating globulin proteins precipitated by this step were removed by centrifugation at 26000*g* for 30 min. For the 11S and 7S protein fractions, the contaminating water-soluble albumin was eliminated from the precipitated 11S and 7S globulins by centrifugation.

II. Sephadex G-150 Gel Filtration. Total protein (3.8 mL) in 1 M NaCl and 0.035 M sodium phosphate buffer, pH 7.5, was separated at 4 °C on a 1.6 × 56cm column of Sephadex G-150. The column was equilibrated and eluted with 1 M NaCl in 0.035 M sodium phosphate buffer, pH 7.5. Fractions of 2 mL were collected, and proteins in the different fractions were analyzed by gel electrophoresis. The fractions (peak II) containing the 2S protein were pooled and dialyzed against distilled water at 4 °C overnight. The water-soluble 2S protein was concentrated by lyophilization.

III. Water Extraction/Acid Precipitation. BNDF (1 g) was extracted with distilled water (10 mL) by grinding in a mortar. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 26000*g* for 30 min. The supernatant fraction from this sedimentation was designated water-extractable protein. The water-extractable proteins were further fractionated by acidification to pH 5.0 with 0.1 M HCl, and the precipitated proteins were removed by centrifugation at 26000*g* for 30 min. The supernatant fraction was dialyzed against distilled water (adjusted to pH 5.0 with NaOH) overnight. After removal of the precipitated proteins by centrifugation, the dialysate was designated as the albumin protein fraction.

Polyacrylamide Gel Electrophoresis. SDS–20% polyacrylamide slab gel electrophoresis (SDS–PAGE) of Brazil nut proteins was essentially as described by Laemmli (1970).

Amino Acid Analysis. Amino acid analyses were carried out on a Dionex Durrum D-500 analyzer using a standard acid hydrolysis procedure. For determination of the sulfur-containing amino acids, the performic acid oxidation procedure was carried out (Hirs, 1967).

RESULTS AND DISCUSSION

Extraction of Brazil Nut Proteins. With the use of either the SDS buffer or the salt buffer for extraction, 0.5 g of total protein was obtained from 1 g of BNDF. This protein content (50%) in BNDF was close to the 54.5% determined earlier by Antunes and Markakis (1977). When fresh kernels (containing 65–70% oil) were used directly, both buffer extracts yielded substantially lower amounts of total protein, since some of the protein extract was sacrificed to avoid contamination with the lipid layer during separation by centrifugation (Materials and Methods).

Figure 1 shows the SDS–PAGE of the total protein extracts from BNDF and fresh kernels by the SDS buffer and the salt buffer extractions. The profiles of these extracts were basically identical (Figure 1, lanes A–D), and all the extractions were adequate procedures for obtaining the small polypeptides of the 2S albumin fraction (M_r 9000 and less). The addition of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), in the extraction buffer did not result in detectable changes in the polypeptide profile (data not shown).

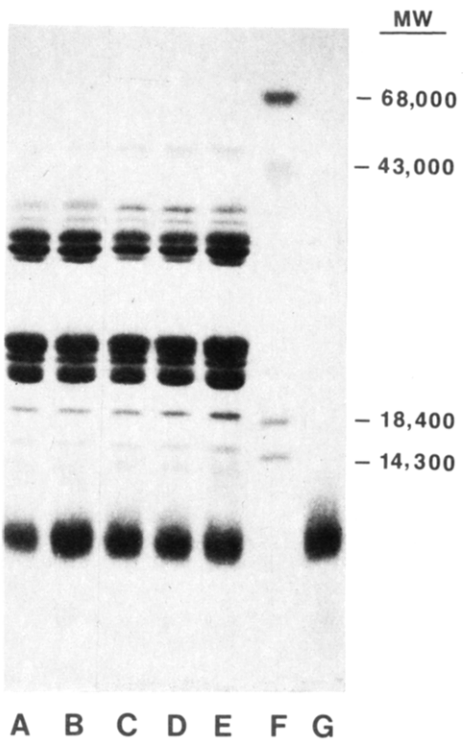

Figure 1. SDS–polyacrylamide gel electrophoretic profiles of proteins extracted from Brazil nut fresh kernels and defatted flour (BNDF) by different procedures (Materials and Methods). Key: A, SDS buffer extract, fresh kernels; B, SDS buffer extract, BNDF; C, salt buffer extract, fresh kernels; D, salt buffer extract, BNDF; E, water extract, BNDF; F, molecular weight marker proteins; G, 2S protein purified by the water extraction/acid precipitation procedure. Numbers on the right indicate molecular weights of the marker proteins. About 80 μg of protein was loaded on each lane. The gel was 20% acrylamide and 0.1% bisacrylamide.

On the basis of the above results, the salt buffer procedure was subsequently used for quantitative preparation of native proteins from BNDF. Both the salt buffer and the SDS buffer extraction procedures are adequate for the quick preparation of small quantities of total protein directly from fresh Brazil nuts for qualitative analysis.

Fractionation of Brazil Nut Proteins. By sucrose gradient centrifugation, Brazil nut proteins can be fractionated into three size classes of proteins, the 11S, 7S, and 2S, with a ratio of 60:10:30 (11S:7S:2S; Figure 2A). This result is similar to that reported earlier by Youle and Huang (1981). However, there was no evidence provided in their paper to demonstrate the purity of these protein fractions. We found that while the 11S and the 7S fractions can be separated by this centrifugation, the 7S and the 2S protein fractions are cross-contaminated (data not shown). We thus included an additional solubility fractionation step to further purify these protein fractions (Materials and Methods). As shown in Figure 2B, the three size classes of proteins isolated by this improved method show no overlapping bands by SDS–PAGE.

Components of Brazil Nut Proteins. The polypeptide components of the total extractable protein of Brazil nuts were analyzed by SDS–PAGE. As shown in Figure 1, the proteins in the total protein extract were resolved into polypeptide components of diverse molecular weights and abundance. The major polypeptide bands, 7–8 in number and amounting to some 80% of the total protein, were concentrated in regions of the gel with mo-

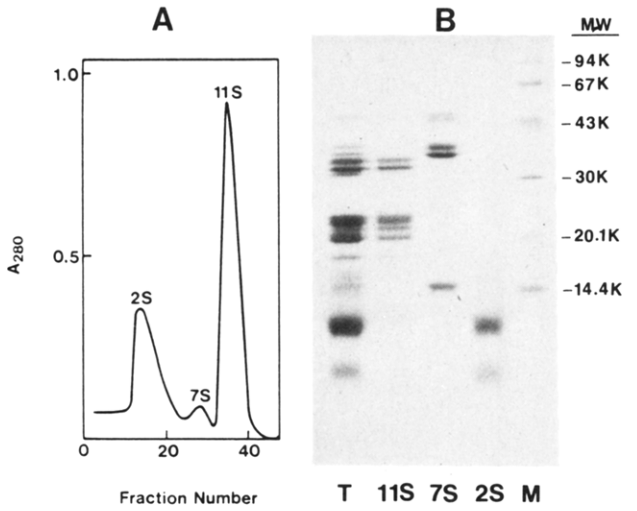

Figure 2. Fractionation of Brazil nut proteins. (A) Sucrose gradient centrifugation pattern of Brazil nut total proteins. The protein sample was separated on a linear gradient of 5–30% sucrose into three size classes of proteins, 11S, 7S, and 2S. (B) SDS–PAGE profiles of the 11S, 7S, and 2S Brazil nut proteins from (A) after further solubility purification (Materials and Methods); lane T, total protein. Numbers on the right indicate molecular weights of the marker proteins, lane M.

lecular weights around 35 000, 24 000, and 9000. Minor polypeptide bands of diverse molecular weights occurred throughout the gel.

The polypeptide components of the three size classes of Brazil nut proteins, 11S, 7S, and 2S, were separated by SDS–PAGE, as shown in Figure 2B. The 11S protein fraction consists of four major polypeptides with molecular weights around 24 000, two polypeptides about 32 000, and a few polypeptides around 17 000. The 7S protein fraction contains mainly five polypeptides: two about 45 000, two around 35 000, and one smaller polypeptide of 14 000. The 2S protein fraction consists of two low molecular weight polypeptides with sizes estimated at 9000 and 3000.

Amino Acid Composition. Total Brazil nut protein as well as the 11S, 7S, and 2S protein fractions were subjected to amino acid analysis (Table I). All of the protein fractions contain high concentrations of glutamine/glutamic acid and arginine, a characteristic of seed (storage) proteins. Also, the level of methionine in all protein fractions is generally higher than that of many other plant seed storage proteins (Payne, 1983). Except for a few amino acids (Ser, Phe, Lys, His), the 7S protein fraction is very similar to the 11S protein fraction in amino acid composition. However, the amino acid profile of the 2S protein is clearly different from those of the other fractions; particularly distinctive are the much higher content of the sulfur amino acids and the lower amounts of Phe, Ile, Val, and Thr. The levels of Met (17.9%) as well as Cys (8.7%) in the 2S protein are unusually high. Since the 2S protein constitutes some 30% of the total Brazil nut protein, the relatively high concentration of the sulfur amino acids in Brazil nut protein (9.1%) is largely due to this 2S sulfur-rich protein. Recently, Phillips and McClure (1986) reported that a 10-kDa zein-2 peptide in the maize mutant, sugary-1, contains 21 mol % Met and 3.1 mol % Cys. While the Met content of this maize protein is slightly higher than that of the Brazil nut 2S protein, this zein protein represents only some 3.0% of the total maize protein.

Comparisons of the amino acid profile of Brazil nut 2S protein with those of other 2S proteins from various oilseeds including cotton, sunflower, linseed, cucumber, lupine, hazelnut, rapeseed, and castor (Youle and Huang, 1981; Sharief and Li, 1982; Crouch et al., 1983) reveal that all these small and water-soluble seed proteins are high in cysteine (about 8%) and nitrogen content (glutamine/glutamate, asparagine/aspartate, arginine). In addition, both the rapeseed (Crouch et al., 1983) and castor bean (Sharief and Li, 1982) proteins, the two better characterized 2S proteins, show further analogies to the Brazil nut 2S protein in that they are also composed of two low molecular weight subunits. The nitrogen- and cysteine-rich 2S proteins have been suggested to function as seed storage proteins, with the additional role of providing sulfur reserves for seed germination (Youle and Huang, 1981). However, of these 2S proteins, only the Brazil nut 2S protein is unusually rich in methionine (18%), while the other oilseed 2S proteins contain only 2–4% methionine. At the present time, we do not know why Brazil nut might require such high levels of methionine, in addition to its rich cysteine content.

Table I. Amino Acid Composition of Brazil Nut Proteins[a]

amino acid	2S	7S	11S	total
Asx	4.31	9.53	9.58	7.67
Thr	0.47	2.00	2.81	2.36
Ser	4.36	1.72	4.28	4.62
Glx	25.32	22.93	21.04	22.17
Pro	5.60	4.83	5.00	5.10
Gly	4.17	5.78	5.84	5.50
Ala	1.21	3.53	3.96	3.24
Val	0.73	5.52	4.78	4.02
Cys[b]	8.73	1.02	1.66	2.65
Met[b]	17.92	3.71	3.52	6.53
Ile	0.71	3.53	3.61	2.80
Leu	5.55	7.07	7.25	6.75
Tyr	1.56	3.36	2.75	2.85
Phe	0.51	5.95	4.90	3.74
Lys	1.74	4.31	3.16	2.94
His	2.15	3.10	2.07	2.20
Arg	15.70	13.02	13.81	14.89

[a] Grams of amino acid/100 g of protein. [b] By the performic acid procedure.

The present amino acid data obtained for the Brazil nut 2S protein is generally in agreement with that reported by Youle and Huang 1981), except that they reported a higher value for Cys (13.11%). The 2S protein used in this study was purified more extensively than that described by Youle and Huang (1981) and thus was free from contaminating 7S protein (Materials and Methods), but the 7S proteins do not appear to contain large amounts of cysteine (Table I). At this time we do not know the reason for this discrepancy. We also found that the content of the sulfur amino acids in the total extractable Brazil nut protein (9.1%), as determined in this study, is slightly higher than the value (8.3%) obtained for the defatted Brazil nut flour by Antunes (1975).

Purification of Sulfur-Rich Protein by Gel Filtration. The identification of the sulfur-rich 2S protein is of particular interest since it is abundant in seeds (30% of total protein), exceptionally rich in the sulfur amino acids (26%) and consists of only two subunit polypeptides of 9000 and 3000. However, the sucrose gradient centrifugation/solubility fractionation procedure can yield only about 3 mg of the sulfur-rich protein per centrifugation. In order to obtain larger quantities of this protein for further studies, we developed a water extraction/acid precipitation method and a gel filtration technique (Materials and Methods).

Since the 2S protein is soluble in water, an attempt was made to extract the BNDF with water for enrichment of the 2S albumin fraction. However, as shown in Figure 1

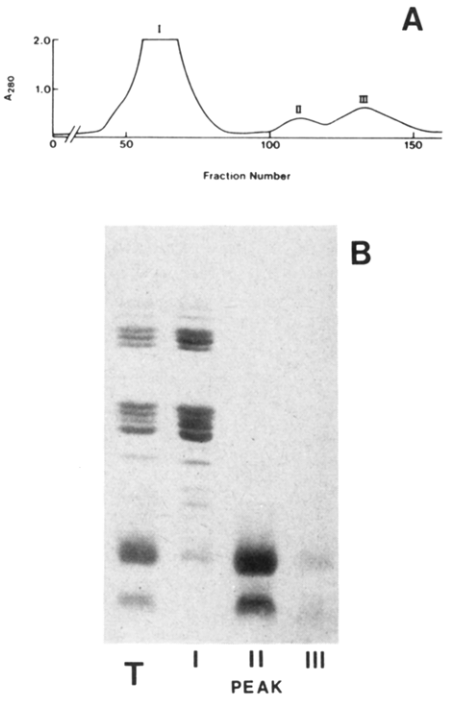

Figure 3. Purification of Brazil nut 2S protein by gel filtration. (A) Chromatography of Brazil nut total proteins on a Sephadex G-150 column. Experimental details are described under Materials and Methods. (B) SDS–PAGE profiles of the protein fractions (peaks I–III) obtained from gel filtration; lane T, total protein.

lane E, there is no preferential extraction of the 2S albumin fraction; the water extract contains the same protein components as those extracts made with other buffers. Brazil nuts have an unusually high content of several elements, particularly Ba, Br, Co, Cs, Ca, Mg, Sr, and Se (Furr et al. 1979); these elements, plus other ions in the seeds, may be sufficient to cause the solubilization of the globulins during the water extraction. But, we have been able to remove the globulins in this water extract by acid precipitation (Sun and Hall, 1975) and a subsequent step of extensive dialysis against deionized water. As shown in Figure 1, lane G, after such a solubility fractionation, the water extract now contains only the low molecular weight 9000 and 3000 polypeptides.

Parts A and B in Figure 3 show the elution and electrophoresis profiles of the protein fractions separated by the gel filtration technique (Materials and Methods). Peak I essentially contains all the 11S and 7S polypeptides plus a minor amount of the 2S protein, while peak II contains only the 2S polypeptides. A small quantity of the 2S protein, together with a large amount of UV-absorbing nonprotein materials, appears in peak III. Using a 5 × 24 cm column, we were able to obtain 0.3 g of pure 2S protein per chromatography run, a 100-fold increase over the amounts obtained by the centrifugation technique. As compared to the water extraction/acid precipitation method described above, the gel filtration technique is less time consuming and results in a higher yield of the sulfur-rich protein.

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