Composition and Characteristics of Basic Proteins from Peanut (*Arachis hypogaea* L.) Seed

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Basic proteins from peanut (*Arachis hypogaea* L.) seed were isolated from total protein extract by using carboxymethylcellulose (CMC) at pH 8.2. The basic proteins constituted about 1% of the total seed protein. Gel filtration of basic proteins on Sephadex G-200 showed the presence of two protein peaks. Peak I had an apparent molecular weight of 70 000 ± 5000 and peak II eluted in the salt volume of the column. Ion-exchange chromatography of the basic proteins on a CMC column gave one major and five minor protein peaks. The basic protein fraction was rich in lysine (8.5%), glycine (27.9%), and methionine (1%) but was low in aspartic acid (5.3%) and glutamic acid (5.6%) compared to the total seed protein. The basic proteins were found to be glycoproteins and contained both the neutral (3.5%, glucose and mannose) and amino sugars (0.2%, glucosamine). One-dimensional gel electrophoresis toward the cathode at pH 4.5 showed four protein bands. Sodium dodecyl sulfate gel electrophoresis revealed six major (a–f) and seven minor polypeptide bands. Two-dimensional gel electrophoresis improved resolution between two sets of polypeptides (b and c; d and e) with close mobility and enabled calculation of their molecular weights. The apparent molecular weights of the six major polypeptides were 55 000, 30 000, 27 000, 22 500, 22 500, and 20 000, respectively.

The major storage proteins of peanut are arachin and conarachin (Johns and Jones, 1916; Jones and Horn, 1930). Several classical methods including ammonium sulfate precipitation (Johns and Jones, 1916; Jones and Horn, 1930; Dawson, 1968), $CaCl_2$ precipitation (Tombs, 1965), cryoprecipitation (Neucere, 1969; Basha and Cherry, 1976; Basha and Pancholy, 1982), NaBr precipitation (Shetty and Rao, 1974), ion-exchange chromatography (Dechary et al., 1961; Cherry et al., 1973; Neucere and Conkerton, 1978), and gel filtration (Basha and Pancholy, 1981) have been employed to isolate peanut storage proteins. Recently, Basha and Pancholy (1982) have constructed composite polypeptide maps for arachin and nonarachin proteins including their molecular weights and isoelectric points. DEAE chromatography and electrophoresis methods employed for purification and characterization of arachin and conarachin proteins indicated that most of the peanut seed proteins are acidic in nature. Since, arachin and conarachin together constitute approximately 96% of the seed protein (Irving et al., 1946), most of the earlier studies have been restricted to only these proteins. Thus, in previous studies no attempts have been made to identify and characterize the basic proteins of peanut seed. Hence, this study was initiated to isolate and identify the basic proteins of peanut seed. In this report we have described isolation of basic proteins from the peanut seed, their composition, and their characteristics.

MATERIALS AND METHODS

Seed Material. After removal of the seed coats and embryonic axes, cotyledons from peanut (*Arachis hypogaea* L.) cultivar Early Bunch were ground into a meal. The full-fat meal was defatted with cold diethyl ether and the defatted meal was stored at −20 °C until used (Basha and Pancholy, 1981).

Protein Extraction. Protein was extracted from the peanut meal with 50 mM Tris-HCl, pH 8.2 (1:20), containing 0.002% (w/v) NaN_3 and 2 mM phenylmethanesulfonyl fluoride (PMSF) by using a mortar and pestle. The homogenate was squeezed through a layer of cheesecloth and miracloth and centrifuged at 20000*g* for 15 min at 18 °C. The resulting supernatant was used for protein fractionation.

Protein Fractionation. Total protein extract was diluted, to reduce the ionic strength to 10 mM, and to this extract solid, preswollen carboxymethylcellulose (Whatman, New Jersey) equilibrated in 10 mM Tris-HCl (pH 8.2), 0.002% NaN_3, and 2 mM PMSF was added (1:10 v/v) and gently stirred for 5 min. The mixture was filtered through a Whatman No. 1 filter paper under suction. The carboxymethylcellulose (CMC) retained on the filter paper was washed thoroughly with 10 mM Tris-HCl, pH 8.2 (about 4 L), until the A_{280} absorbance of the filtrate was zero. Washed CMC was transferred quantitatively into a column (2.5 × 10 cm), and the CMC-bound protein was eluted with 10 mM Tris-HCl (pH 8.2) containing 1 M NaCl. So that any trace amounts of nonbasic proteins could be removed, the CMC-bound material was dialyzed (against 10 mM Tris-HCl, pH 8.2) and reloaded on a short CMC column. After the column was washed with 10 mM Tris-HCl (pH 8.2), bound protein was eluted with 1 M NaCl in 10 mM Tris-HCl (pH 8.2), and this fraction was designated as the basic protein (BP) fraction.

Gel Filtration. The BP fraction was separated by gel filtration on a Sephadex G-200 column (1.5 × 240 cm). The column was equilibrated with a buffer containing 10 mM Tris-HCl (pH 8.2), 0.5 M NaCl, and 0.002% NaN_3. The BP fraction was concentrated and loaded (20 mg in 2 mL) on the column and eluted in 3-mL fractions. The flow rate (1 mL/5 min) of the column was regulated by using a peristaltic pump. Protein content of the fractions was monitored by measuring their absorption at 280 nm.

Carboxymethylcellulose Chromatography. The BP fraction was dialyzed against 10 mM Tris-HCl buffer (pH 8.2) and loaded (15 mg) on a CMC column (1.5 × 30 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.2). The protein was eluted from the column by using a linear gradient of 0–0.4 M NaCl in 10 mM Tris-HCl buffer, pH 8.2. The protein content of the fractions (4 mL) was determined by measuring their absorption at 280 nm.

Amino Acid Analysis. The BP fraction was dialyzed extensively against distilled, deionized water and lyophilized to dryness. The lyophilized protein (50 mg) was hydrolyzed with 6 N HCl for 18 h at 110 °C. The hydrolysate was neutralized and analyzed in a JEOL amino acid analyzer as described earlier (Pancholy et al., 1978).

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One-Dimensional Polyacrylamide Gel Electrophoresis. Electrophoresis of basic proteins was performed in 7.5% (w/v) polyacrylamide gels at pH 4.5 by using β-alanine buffer as described by Reisfield et al. (1962). About 100 μg of protein was loaded on each gel and electrophoresed at a constant current of 4 mA/gel for 2 h toward the cathode (−). Electrophoresis was also performed at pH 8.3 toward the anode (+) and cathode (−) by using the Tris–glycine system of Davis (1964). After electrophoresis the gels were stained with Coomassie blue R-250 and destained with 7% acetic acid containing 10% ethanol. Sodium dodecyl sulfate gel electrophoresis was carried out in 10% (w/v) acrylamide gels according to the method of Laemmli (1970).

Two-Dimensional Gel Electrophoresis. The BP fraction was subjected to two-dimensional gel electrophoresis following the methods of O'Farrell et al. (1977) and Basha et al. (1980). The first dimension was nonequilibrium pH gradient gel electrophoresis (NEPHGE) in 4% (w/v) acrylamide gel containing 9.3 M urea, 2% (v/v) nonidet P-40 and 2% (v/v) ampholines (1.4% pH 9–11, 0.4% pH 8–9.5, and 0.2% pH 6–8 ampholines). The sample (300 μg) was loaded on the acid (anode) end of the gel, and electrofocusing was carried out at 75 V for 15 min and 400 V for 3.5 h by using 0.4 M NaOH in the lower (cathode) chamber and 0.06 M H_2SO_4 in the upper (anode) chamber. The protein migration was toward the cathode. After NEPHGE, the gels were removed from the tubes, equilibrated for 10 min with a buffer containing 65 mM Tris-HCl, pH 6.9, 1% (v/v) 2-mercaptoethanol, and 1% (w/v) sodium dodecyl sulfate ($NaDodSO_4$). The equilibrated gels were subjected to $NaDodSO_4$ gel electrophoresis in the second dimension in 10% (w/v) acrylamide slab gels as described earlier (Basha, 1979).

Glycoprotein Determination. Fifty milligrams of lyophilized BP fraction was hydrolyzed in 5 mL of 0.05 N HCl along with 100 mg of Dowex 50W-X4 (H^+ form) resin at 100 °C for 24 h in sealed tubes under N_2. The neutral sugars released into the acid were collected after passing the hydrolysates through a short Dowex 50W-X4 resin column (4 × 20 mm). The column was washed with 5 mL of water, and the initial eluate and washings were pooled and analyzed for neutral sugar content by using the anthrone–sulfuric acid method of Yemm and Willis (1954). The amino sugars bound to the Dowex resin were eluted with 2 N HCl and measured by the method of Rondle and Morgan (1955). The types of neutral and amino sugars in the protein hydrolysates were identified by paper chromatography following the methods of Koshiyama (1966) and Basha and Beevers (1976).

Molecular Weight Estimation. Sephadex G-200 column (1.5 × 240 cm) was calibrated by using ferritin (500 000), catalase (232 000), alkaline phosphatase (140 000), transferrin (76 000), ovalbumin (46 000), trypsin (23 800), and lysozyme (14 900) as the molecular weight markers. The void and salt volumes of the column were determined by using Blue Dextran 2000 and sucrose, respectively. The molecular weight of unknown protein was estimated according to the method of Andrews (1964). The molecular weight of the unknown polypeptides was determined after $NaDodSO_4$ gel electrophoresis using the following protein standards: thyroglobulin (334 500), β-galactosidase (130 000), phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (46 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and cytochrome *c* (13 370).

RESULTS AND DISCUSSION

Protein Isolation. Carboxymethylcellulose (CMC), a cation exchanger, was employed to absorb the basic proteins from peanut seed protein extract. The ionic strength (10 mM) and pH (8.2) used for this purpose gave a satisfactory recovery of basic proteins. So that the microbial and proteolytic enzyme activities could be prevented, sodium azide (0.002%) and PMSF (2 mM) were included in the buffers. These measures enabled us to obtain basic protein (BP) fractions having consistent protein composition from different preparations. One milliliter (packed) of CMC per 10 mL of protein extract was found to be sufficient to bind all the basic proteins from the total protein extract. Thus, no more basic protein could be recovered after addition of fresh CMC to the CMC-negative material (filtrate of the CMC–total protein suspension), indicating that the CMC and protein extract ratio (1:10, respectively) used in this study was sufficient to absorb all the basic proteins. Reloading the dialyzed BP fraction on a new CMC column bound all the protein, indicating that the BP fraction did not contain any nonbasic proteins.

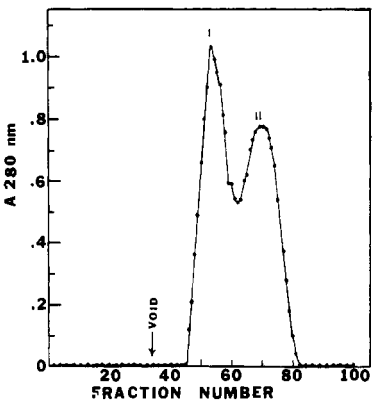

Figure 1. Gel filtration profile of peanut (cv. Early Bunch) basic proteins on a Sephadex G-200 column (1.5 × 240 cm) equilibrated with 10 mM Tris-HCl (pH 8.2) containing 0.5 M NaCl. The sample (20 mg in 2 mL) was loaded on the column and eluted in 3-mL fractions.

Gel Filtration. Gel filtration of BP fraction on Sephadex G-200 column showed (Figure 1) two protein peaks. Peak I eluted in the column fractionation range while peak II eluted in the salt volume of the column. Peak I contained about 30% more protein than the peak II. No protein was found in the void volume of the column. The apparent molecular weight of the peak I protein was 70 000 ± 5000.

Carboxymethylcellulose Chromatography. Ion-exchange chromatography of basic proteins showed (Figure 2) one major and five minor protein peaks. Peak I (major) eluted at an ionic strength of 0.12 M while peaks II, III, IV, V, and VI (minor) eluted at 0.17, 0.20, 0.22, 0.24, and 0.26 M, respectively.

Amino Acid Composition. The amino acid composition of basic, CMC-negative, and total protein are shown in Table I. As seen, the BP fraction is rich in basic amino acids such as lysine (8.5%) and histidine (3.7%) and low in acidic amino acids like aspartic acid (5.3%) and glutamic acid (5.6%). Interestingly, the basic proteins were also rich in glycine (27.9%) and methionine (1%) compared to the total protein. In contrast, the CMC-negative fraction was high in aspartic acid (12%) and glutamic acid (18.8%) and low in glycine (3.8%) and methionine (0.7%).

Glycoproteins. The BP fraction was also found to contain glycoproteins as suggested by the presence of neutral and amino sugars in the protein hydrolysates. The neutral sugar content was significantly higher (3.5%) than the amino sugar content (0.2%). Glucose and mannose

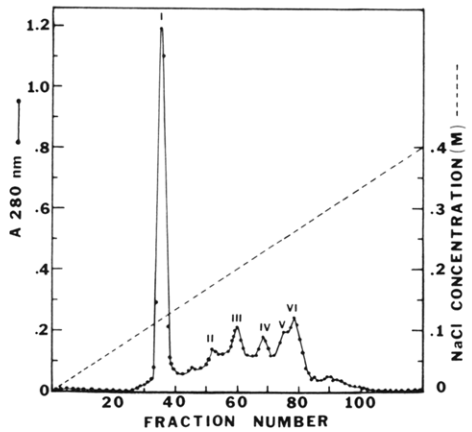

Figure 2. Carboxymethylcellulose chromatography pattern of peanut basic proteins. The protein (15 mg) was loaded on the column (1.5 × 30 cm), washed with 10 mM Tris-HCl, pH 8.2, and then eluted with 500 mL of a linear NaCl gradient (---) of 0–0.4 M, in 4-mL fractions.

Table I. Amino Acid Composition of Basic, CMC⁻, and Total Protein Fractions from Peanut Seed

	%[a] (g/100 g of protein)		
amino acid	basic	CMC⁻ [b]	total
lysine	8.51	4.14	3.49
histidine	3.70	3.21	2.46
NH_3	1.04	6.67	2.37
arginine	3.58	13.97	12.39
aspartic acid	5.32	11.59	11.98
threonine	6.87	3.88	4.33
serine	6.21	4.07	4.81
glutamic acid	5.60	18.85	18.79
proline	2.10	4.90	5.16
glycine	27.90	3.85	6.00
alanine	4.29	3.00	4.62
cystine	3.75	trace	trace
valine	3.12	4.89	4.21
methionine	1.00	0.30	0.74
isoleucine	4.23	2.88	3.18
leucine	4.26	5.84	6.38
tyrosine	4.70	4.37	3.90
phenylalanine	3.05	3.99	5.19

[a] Average of three analyses. [b] CMC⁻ = carboxymethylcellulose-negative fraction.

were found in the neutral sugar fraction, and glucosamine was found in the amino sugar fraction.

Gel Electrophoresis. One-dimensional gel electrophoresis of basic proteins toward cathode at pH 4.5 showed (Figure 3a) the presence of four protein bands (A–D). The major protein bands A and B have similar electrophoretic mobility and, hence, appear as a diffused band. When electrophoresis was performed at pH 8.3 toward the anode, no protein entered the gel (Figure 3b). On the other hand, electrophoresis of basic proteins at pH 8.3 toward the cathode did cause protein mobility into the gel (Figure 3c). This would indicate that the BP fraction contained only the cathodic proteins. Sodium dodecyl sulfate gel electrophoresis of the basic proteins showed (Figure 3d) six major (a–f) and seven minor protein bands. Of the major polypeptides, polypeptides b and c and d and e migrated very close to each other, suggesting that they may be of similar molecular weight. For comparison, the $NaDodSO_4$ gel of the total peanut protein is also included (Figure 3e). No major polypeptides with similar electrophoretic mobility as the polypeptides b–e were seen in the $NaDodSO_4$ gel of total protein.

Two-Dimensional Gel Electrophoresis. The two-dimensional gel electrophoresis system greatly improved

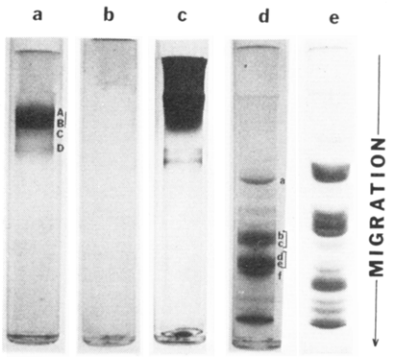

Figure 3. One-dimensional polyacrylamide gel electrophoretic profiles of the peanut protein fractions: (a) electrophoresis toward the cathode (–) in pH 4.5 gels; (b) electrophoresis toward the anode (+) in pH 8.3 gels; (c) electrophoresis toward the cathode (–) in pH 8.3 gels; (d) $NaDodSO_4$ gel electrophoresis of basic proteins toward the anode (+); (e) $NaDodSO_4$ gel electrophoretic profile of the total peanut protein after electrophoresis toward the anode (+). About 100 μg of protein was applied on each gel.

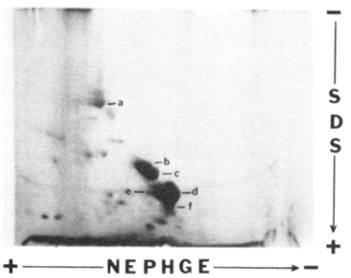

Figure 4. Two-dimensional gel electrophoretic profile of peanut basic proteins. The basic protein (300-μg) fraction was subjected to nonequilibrium pH gradient electrophoresis toward the cathode (–) in the first dimension and $NaDodSO_4$ (SDS in the figure) gel electrophoresis in the second. Polypeptides a–f represent the major polypeptides of the basic protein fraction.

Table II. Apparent Molecular Weights of the Major Polypeptides of Peanut Basic Proteins As Determined by One- and Two-Dimensional Gel Electrophoresis

polypeptide	one-dimensional gel electrophoresis (±2000)	two-dimensional gel electrophoresis (±2000)
a	56 000	55 000
b	30 000	30 000
c		27 000
d	23 000	22 500
e		22 500
f	21 000	20 000

the polypeptide resolution of basic proteins and showed the presence of six major and about 30 minor polypeptides (Figure 4). Unlike the one-dimensional gel electrophoresis the two-dimensional gel electrophoresis gave a better resolution between polypeptides b and c and d and e. Polypeptides d and e appeared to have similar molecular weight but different isoelectric points.

Molecular Weight Estimation. The molecular weights (M_r) of the major polypeptides of peanut basic proteins were estimated by using both one- and two-dimensional gel electrophoresis. As seen in the Table II the M_rs calculated by using these two systems were very similar (21 000–56 000 vs. 20 000–55 000 from one- and two-dimensional gel electrophoresis, respectively). However, the two-dimensional gel electrophoresis improved the resolution between polypeptides b and c and d and e and enabled calculation of their M_r's. The minor polypeptides

in general had a wide range of M_r's (between 14 000 and 60 000) and pI's. Since, in the NEPHGE system the pH gradient is not allowed to reach equilibrium to prevent cathodic pH drift (O'Farrell et al., 1977; Horst et al., 1980), it will not be possible to obtain accurate pI's of the polypeptides. Hence, pI's of the polypeptides were not determined.

These data indicated that the basic proteins of peanut are heterogeneous and constitute only a small portion (about 1%) of the peanut protein. Of particular interest is the presence of two sets of polypeptides (b and c and d and e) differing slightly in their M_r's and pI's. In addition, the basic proteins appear to be unique in that they are rich in lysine and glycine, are low in acidic amino acids, and are glycosylated. Currently experiments are in progress to purify the major basic proteins of peanut seed and to identify the carbohydrate-containing polypeptide(s).

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Effect of Succinylation on the Protein Quality and Urinary Excretion of Bound and Free Amino Acids

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The net protein ratio (NPR) of whey protein concentrate succinylated at four different levels was determined in order to establish an appropriate level of modification that will not adversely affect the protein quality. The utilization of the absorbed succinyl amino acids was also determined in rats by examining the urinary excretion of bound and free amino acids. Succinylated whey concentrate (SWC) with 37% succinylation is still a good quality protein with NPR higher than that of casein. At higher levels of succinylation, the NPR was adversely lowered. Rats fed SWC had high urinary nitrogen excretion, but little of the succinyllysine and none of the succinylcysteine and succinylthreonine were recovered as bound amino acids in the urine. Apparently these amino acids were partially catabolized to forms that were unavailable to the body. Further metabolic and toxicity studies of the succinyl amino acids are recommended.

Succinylation of heat-denatured cheese whey protein concentrates (WC) has been described as a means of improving the protein's functional properties and utilization in food systems (Thompson and Reyes, 1980). The potential use of such modified protein in food makes information on the nutritional and toxicological properties important. As reported previously (Siu and Thompson, 1982), succinylation affects the digestibility of amino acids. However, the relatively high in vivo amino acid digestibilities did not correspond with the poor in vitro results. For example, with exhaustively succinylated whey protein, there was a 98% decrease in in vitro lysine digestibility but only a 14% decrease in the in vivo value. The differences between in vitro and in vivo results were attributed to the absorption of succinylated amino acids or succinylated dipeptides in vivo.

The utilization of the succinylated amino acids or succinylated dipeptides depends on the ability of the cytoplasmic enzymes to release the dipeptides to individual succinylated or free amino acids and the ability of the organ's enzymes to deacylate the succinyl groups. Mammalian tissues contain acylases that act on a number of *N*-acyl amino acids (Endo, 1978; Paik and Benoiton, 1963), but their specific activities toward succinylated amino acids have not been reported. In a study of acylated methionine derivatives (Boggs, 1978), the specificity of the acylase appeared to be a function of the chemical group attached to methionine. The enzyme does not hydrolyze N^{α}-

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