# Isolation and Purification of the Methionine-Rich Protein from Peanut

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A methionine-rich protein has been isolated and purified from the peanut (*Arachis hypogaea* L.) seed by gel filtration on a Sephacryl S-200 column followed by ion-exchange chromatography on DEAE-cellulose. The purified protein was found to be an acidic protein and has an apparent molecular mass of 118 000 Da. The protein is composed of two polypeptides with molecular mass of 20 500 and 18 000 Da. The MRP was found to contain 3.4% methionine and 3.3% cystine.

**Keywords:** *Amino acids; electrophoresis; methionine-rich protein; peanut; purification*

Protein quality and availability are functions of the amino acids present, and the amino acid composition determines the nutritive value of plant protein products to a large extent (Johnson and Lay, 1974; Kakade, 1974). Peanut seed is low in sulfur-containing amino acids such as cystine and methionine (Young et al., 1973; Young, 1979; Pancholy et al., 1978; Basha and Pancholy, 1981). This means that supplementing with free methionine and complementing with cereals is required to provide a diet with complete protein. To improve the utilization of peanut as a food protein source, it is important to increase the level of methionine-rich protein (MRP) in peanut. Because of limited genetic variability in methionine content (Heinis, 1971; Pancholy et al., 1978), the level of methionine in peanut seed could not be improved by traditional breeding. However, this goal may be achieved by genetic engineering an endogenous methionine-rich protein (MRP) gene into peanut. This may be attained either by incorporating an MRP gene from other crops or by overexpressing the MRP gene(s) in the peanut itself. We are therefore interested in isolating gene(s) specifying methionine-rich protein in peanut.

In this connection, we want to determine the amino acid sequence of MRP for synthesizing oligonucleotide probes to screen peanut cDNA libraries. Previously, we have identified a protein fraction rich in methionine and cystine (Basha and Pancholy, 1981) from Florunner peanut seed. However, no attempts were made to purify the protein. It was also found (Basha, 1991) that maximum accumulation of this protein occurred between the first (white) and third maturity (orange) stages, while it was minimal from the third (orange) to fifth (black) maturity stages. This paper describes the isolation and purification of MRP from peanut seed using gel filtration and ion-exchange chromatography and its polypeptide and amino acid composition.

## MATERIALS AND METHODS

**Seed Material.** Cotyledons from peanut (*Arachis hypogaea* L.) cv. Florunner were ground into a powder and defatted with hexane as described earlier (Basha et al., 1976). The defatted powder was stored at −20 °C until use.

**Protein Extraction.** Defatted peanut meal (3 g) was extracted with 10 mL of 2 M NaCl, 0.01 M Tris-HCl, pH 8.2, and 0.002% (w/v) sodium azide using a Polytron homogenizer. The homogenate was centrifuged at 20 000*g* for 20 min, and the supernatant was collected and used for protein fractionation.

**Protein Fractionation.** Protein fractionation was carried out according to the method of Basha and Pancholy (1981), except for the use of a Sephacryl S-200 column in place of a Sephacryl S-300 column. This column gave a better resolution between the MRP peak and adjacent peaks. The Sephacryl S-200 column (2.5 × 135 cm) was equilibrated with 0.5 M NaCl, 0.01 M Tris-HCl, pH 8.2, and 0.002% (w/v) sodium azide. About 8 mL of the protein extract was applied on the column, and the eluate was collected in 5-mL fractions. The protein content of the fractions was monitored by measuring their absorption at 280 nm. The fractions under each peak were pooled and designated peaks I–VIII. The pooled peaks were dialyzed and their amino acid compositions determined following hydrolysis with 6 N HCl as described below. On the basis of the amino acid analysis data, peak IV was found to be the methionine-rich protein. This is consistent with our previous paper (Basha and Pancholy, 1981) in which we had identified peak VI from Sephacryl S-300 (peak IV in Sephacryl S-200) to be the methionine-rich protein on the basis of amino acid analysis as well as [$^{35}$S]methionine labeling data.

**Ion-Exchange Chromatography.** Peak IV (methionine-rich protein peak) from the Sephacryl S-200 column was pooled and dialyzed against 0.01 M Tris-HCl, pH 8.2. The dialysate was loaded onto a (diethylaminoethyl)cellulose (DEAE) column (1.5 cm × 30 cm) equilibrated with 0.01 M Tris-HCl, pH 8.2. Unbound protein was removed by washing the column with the equilibration buffer, and then the bound protein was eluted from the column using a linear NaCl gradient (0–0.3 M) in 5-mL fractions. Protein content of the fractions was monitored by measuring their absorption at 280 nm.

**Polyacrylamide Gel Electrophoresis.** The purity of the MRP preparations resulting from different purification steps was determined by subjecting them to one-dimensional gel electrophoresis. Electrophoresis under nondenaturing conditions was carried out in 10% polyacrylamide slab gel (8 × 7 cm) following the method of Davis (1964), while sodium dodecyl sulfate gel electrophoresis was conducted in 10% polyacrylamide slab gel (8 × 7 cm) according to the method of Laemmli (1970). After electrophoresis, the proteins were stained with Coomassie Blue R-250 and destained with 7% acetic acid and 10% methanol.

**Amino Acid Analysis.** Five hundred micrograms of lyophilized protein was hydrolyzed at 105 °C for 24 h with 6 N HCl in a Pico-Tag workstation (Waters, Milford, MA). Following hydrolysis, the samples were dried and phenyl isothiocyanate amino acid (PITC amino acid) derivatives prepared. An aliquot of the derivatized sample was analyzed using an HPLC system equipped with a Pico-Tag stainless steel column, a UV–vis detector, two Model 510 pumps, and an 820 data station (Basha, 1989). The amino acids were quantified using

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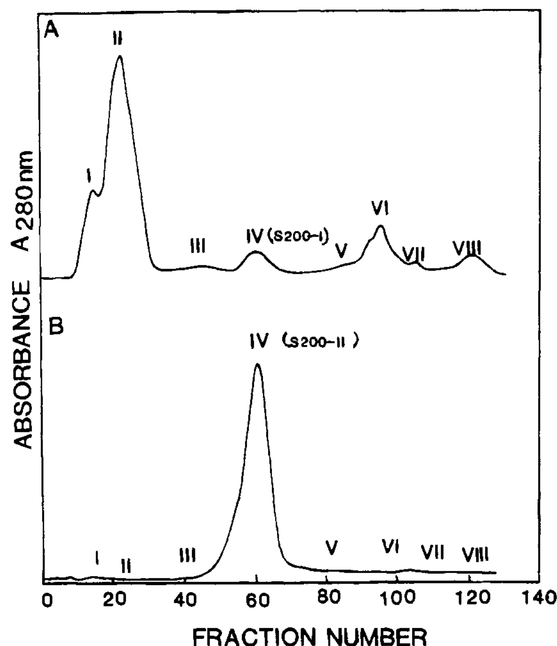


**Figure 1.** Gel filtration profile of peanut protein on Sephacryl S-200 column (2.5 × 135 cm). Seed protein was extracted with 2 M NaCl and 0.01 M Tris-HCl, pH 8.2, buffer and fractionated into eight peaks (I–VIII) on the column (A). Fractions 55–65 (peak IV) were pooled, concentrated, and rechromatographed on the same column (B) as described above. Fractions 55–65 (S200-II) were pooled and used for further purification.

an external amino acid standard and expressed as relative percentage of total amino acids.

**Molecular Mass Estimation.** Molecular mass of purified MRP was determined by calibrating the Sephacryl S-200 column with proteins of known molecular mass. Protein standards used were ferritin (440 000), catalase (232 000), aldolase (160 000), alkaline phosphatase (140 000), and bovine serum albumin (67 000).

The molecular mass of MRP polypeptides was determined by calibrating the SDS gel with protein standards after they had been dissociated with SDS and 2-mercaptoethanol. The protein standards used were bovine serum albumin (67 000), ovalbumin (43 500), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and lysozyme (14 300).

## RESULTS AND DISCUSSION

**Purification of Methionine-Rich Protein.** Protein from Florunner peanut seed was extracted and fractionated on the basis of their molecular mass on a Sephacryl S-200 column. The column resolved seed proteins into eight (I–VIII) peaks (Figure 1A). Sephacryl S-200 was found to give improved resolution between the MRP peak (IV) and the adjacent peaks (III and V) compared to the Sephacryl S-300 employed in our previous study (Basha and Pancholy, 1981). Besides this, no major differences were observed in the protein resolution patterns between the two columns. Peaks I and II represent the arachin (major peanut storage globulin) protein, while peak IV has been identified as the methionine-rich protein on the basis of amino acid composition and radiolabeling with methionine (Basha and Pancholy, 1981). Since we used a column (Sephacryl S-200) in this study different from that in our previous study, the amino acid compositions of peaks I–VIII resulting from the S-200 column were determined to confirm the identity of the methionine-rich peak. The results showed (data not shown) that only peak IV was rich in methionine and cystine. Hence, protein fractions under peak IV (fractions 55–65) were

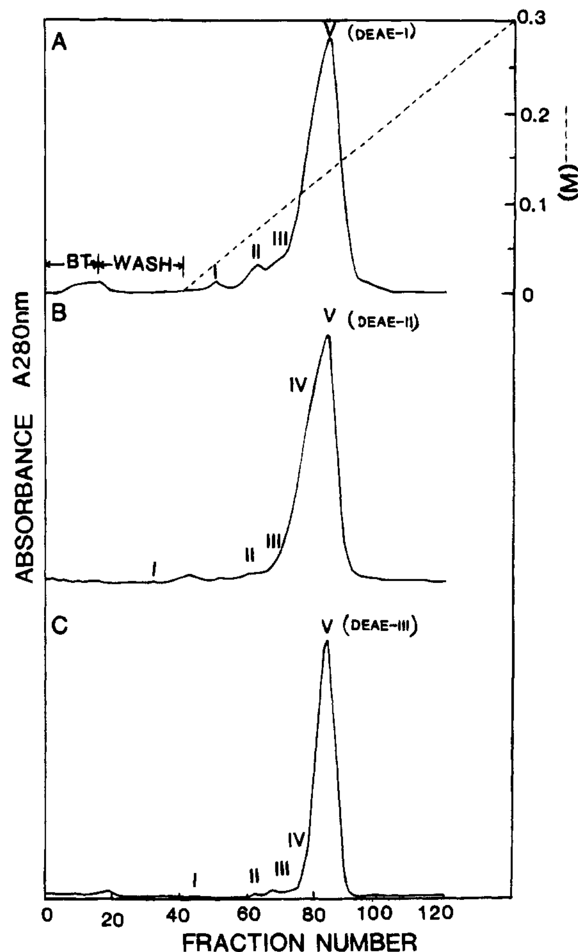


**Figure 2.** MRP sample (S200-II) resulting from the second Sephacryl S-200 chromatography was pooled, dialyzed, and loaded on a DEAE-cellulose column. The column was washed with the equilibration buffer (0.01 M Tris-HCl, pH 8.2) and the protein eluted with a linear NaCl gradient of 0–0.3 M. Protein eluting in fractions 80–90 was pooled, dialyzed, and rechromatographed on DEAE as described above. (A) First DEAE chromatography; (B) second DEAE chromatography; (C) third DEAE chromatography.

pooled, dialyzed, and used for further purification. The MRP sample resulting from this run was designated S200-I. The S200-I sample was concentrated and rechromatographed on the Sephacryl S-200 column. The Sephacryl S-200 chromatograph of S200-I is shown in Figure 1B. Rechromatography of S200-I showed the presence of a single peak corresponding to peak IV and the absence of other seed proteins seen in Figure 1A. From this run, fractions 55–65 were pooled and designated S200-II. This sample (S200-II) was dialyzed against 0.01 M Tris-HCl, pH 8.2, and loaded on a DEAE-cellulose column. The column was washed with 0.01 M Tris-HCl, pH 8.2, buffer and eluted with a linear NaCl gradient of 0–0.3 M. The elution profile of S200-II following DEAE-cellulose chromatography is shown in Figure 2A. Fractions 80–90 were pooled, dialyzed against 0.01 M Tris-HCl, pH 8.2, and designated DEAE-I. The DEAE-I sample was dialyzed and rechromatographed on a DEAE-cellulose column as described above, and the protein eluting in fractions 80–90 was pooled (Figure 2B). The pooled protein (DEAE-II) was rechromatographed for the third time on a DEAE-cellulose column, and the protein eluting in tubes 80–90 was pooled, dialyzed, and designated DEAE-III.

**Protein and Polypeptide Composition.** The protein and polypeptide composition of MRP preparations

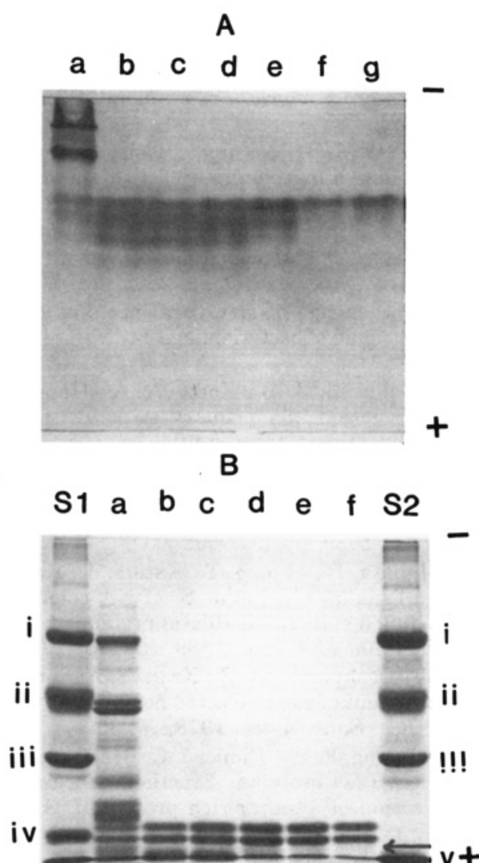


**Figure 3.** One-dimensional gel electrophoretic profile of methionine-rich protein fractions obtained after each purification step. (A) Nondenaturing gel: (a) total protein; (b) Sephacryl S200-I; (c) Sephacryl S200-II; (d) DEAE-I; (e) DEAE-II; (f, g) DEAE-III, 20 and 40 μg, respectively. (B) Denaturing gel: (a) total protein; (b) Sephacryl S200-I; (c) Sephacryl S200-II; (d) DEAE-I; (e) DEAE-II; (f) DEAE-III; (S1, S2) protein standards, (i) BSA (67 000), (ii) ovalbumin (43 500), (iii) carbonic anhydrase (30 000), (iv) soybean trypsin inhibitor (20 000), (v) lysozyme (14 300). About 40−50 μg of methionine-rich protein obtained at the end of each purification step was loaded on the polyacrylamide gels, electrophoresed, and stained with Coomassie Blue R-250.

from different purification steps is shown in Figure 3. Following electrophoresis under nondenaturing conditions, peanut seed proteins were resolved into two major (arachin) and several minor bands (non-arachin). MRP (S200-I) obtained following the first Sephacryl S-200 chromatography contained only the fast migrating non-arachin bands (Figure 3Ab). Subsequent chromatographic steps removed more acidic and faster moving non-MRP proteins (Figure 3Ad−f). Major enrichment of MRP protein occurred following the second DEAE-cellulose chromatography (Figure 3Ae). The third DEAE-cellulose chromatography yielded the purest MRP protein as evident from a single band on the gel (Figure 3Af,g). MRP moved about one-third into the gel, toward the anode, and hence appears to be acidic in nature.

Changes in the polypeptide composition of MRP samples were monitored by SDS gel electrophoresis (SDS−PAGE). The results showed (Figure 3B) that S200-I obtained after the first Sephacryl S-200 chromatography was devoid of most of the non-MRP proteins (Figure 3Bb). Additional chromatographic steps on DEAE enabled removal of very low molecular mass (<16 000) polypeptides moving slightly above the tracking dye (shown with an arrow ←).

**Molecular Mass.** The apparent molecular mass of the purified MRP was found to be 118 000 Da. Thus, the peanut MRP appears to be a relatively large protein compared to the MRPs isolated from Brazil nut (Altenbach et al., 1987; Sun et al., 1987), sunflower (Kortt and Caldwell, 1990; Lilley et al., 1989), corn (Esen et al., 1983; Kirihara et al., 1988), rice (Musum et al., 1989), and soybean (Aswathi and deLumen, 1991). SDS gel electrophoretic analysis of purified peanut MRP revealed the presence of two polypeptides with apparent molecular mass of 20 500 and 18 000 Da. The peanut MRP appears to be similar to the MRP isolated from an African cereal acha (*Digitaria exilis*), which has been reported (de Lumen et al., 1986) to contain 4.8% methionine and 2.5% cysteine. Acha was also found to contain two MRP polypeptides with molecular mass of 19 000 (M1) and 17 500 (M2) (de Lumen et al., 1993).

**Table 1. Amino Acid Composition[a] of Protein Fractions Obtained during Different Purification Steps**

| amino acid | protein fractions | | | | | |
|---|---|---|---|---|---|---|
| | total protein | S200-I | S200-II | DEAE-I | DEAE-II | DEAE-III |
| Asx[b] | 12.3 | 12.7 | 12.8 | 12.7 | 11.9 | 11.9 |
| Glx[c] | 16.0 | 23.0 | 24.4 | 23.9 | 25.1 | 23.1 |
| serine | 6.8 | 7.1 | 7.0 | 7.7 | 7.3 | 8.1 |
| glycine | 11.6 | 6.3 | 5.6 | 6.0 | 6.1 | 6.7 |
| histidine | 2.2 | 1.2 | 1.2 | 1.2 | 1.3 | 1.2 |
| arginine | 8.7 | 12.7 | 12.4 | 13.1 | 13.5 | 12.6 |
| threonine | 2.7 | 1.1 | 0.9 | 0.9 | 0.9 | 0.9 |
| alanine | 6.4 | 3.2 | 3.0 | 3.1 | 3.2 | 3.2 |
| proline | 5.7 | 4.8 | 4.3 | 4.9 | 5.1 | 5.2 |
| tyrosine | 3.5 | 3.5 | 3.1 | 3.8 | 3.8 | 4.1 |
| valine | 3.5 | 2.2 | 2.1 | 1.7 | 1.5 | 1.7 |
| methionine | 1.2 | 4.0 | 3.8 | 3.5 | 3.4 | 3.4 |
| cystine | 1.1 | 3.4 | 3.9 | 3.1 | 3.1 | 3.3 |
| isoleucine | 2.4 | 2.1 | 2.0 | 2.0 | 1.7 | 1.7 |
| leucine | 7.0 | 8.4 | 7.8 | 8.8 | 8.3 | 8.7 |
| phenylalanine | 4.4 | 1.9 | 2.0 | 1.9 | 1.9 | 1.8 |
| lysine | 4.1 | 2.2 | 2.5 | 2.3 | 2.4 | 2.1 |

[a] Relative percent to total. [b] Asx, asparagine plus aspartic acid. [c] Glx, glutamine plus glutamic acid.

**Amino Acid Composition.** The amino acid composition of MRP preparations obtained during various purification steps is shown in Table 1. Total seed protein was rich in glutamic acid (16%), aspartic acid (12.3%), glycine (11.6%), and arginine (8.7%), while it was low in methionine (1.2%) and cystine (1.1%). It should be noted that, in many seed proteins, glutamine and asparagine represent most of the glutamic acid and aspartic acid values obtained by amino acid analysis of the acid hydrolysates. The MRP (S200-I) obtained after the first Sephacryl S-200 chromatography of total protein extract contained more than 3-fold methionine and cystine compared to the total protein. In addition, this protein (S200-I) was found to be high in glutamic acid and relatively low in glycine, arginine, alanine, and phenylalanine compared to the total seed protein. Further purification on Sephacryl S-200 and DEAE showed only minor changes in its amino acid composition, indicating that the MRP preparation is pure and contained no major protein contaminants. Interestingly, MRP samples obtained following DEAE chromatography (DEAE-I, DEAE-II, and DEAE-III) were slightly lower in methionine and cystine compared to the MRP samples obtained following Sephacryl S-200 chromatography (S200-I and S200-II). This decrease may be due to removal of some protein components rich in methionine and cystine during the DEAE purification steps or due to the breakdown of some of the fragments from the MRP during DEAE chromatography.

Currently, studies are in progress to isolate the individual polypeptides of MRP for determining the amino acid sequence of these polypeptides to synthesize oligonucleotide probes for screening the peanut cDNA library.

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