Fractionation and Characterization of a Protein Fraction Producing Off-Flavor Volatiles in Peanut Seed

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A high molecular weight protein fraction (peak I) obtained from peanut (*Arachis hypogaea* L. cv. Florunner) seed was fractionated using ammonium sulfate and methanol to isolate the protein(s) involved in off-flavor volatile production during roasting of peanut. The results showed that maximum off-flavor volatile-producing activity is associated with the 20% ammonium sulfate precipitate and 85% methanol-soluble fraction of this protein. Peak I protein was found to be lipoprotein in nature and is rich in oleic acid (63%) and palmetic acid (8%). Furthermore, the 85% methanol-soluble fraction of peak I protein was found to contain a mixture of low molecular weight proteins and major amounts of glycine (11%), alanine (11%), proline (15%), phenylalanine (8%), and lysine (8%).

Keywords: *Amino acids; electrophoresis; fatty acids; fractionation; lipoprotein; off-flavor; peanut; protein*

During roasting, free sugars and free amino acids will react and produce pyrazine and carbonyl compounds (Mason et al., 1966, 1967; Johnson et al., 1971a,b; Shu and Waller, 1971; Walradt et al., 1971) via Maillard sugar−amine type reactions. Newell et al. (1967) identified the amino acids associated with the production of typical and atypical roasted flavor. It is believed (Mason et al., 1969) that during roasting, an unknown polypeptide undergoes thermal breakdown to give the amino acid reactants involved in flavor development. The concentration of this polypeptide was shown to increase from 2 to 4 μmol/g of fat-free meal with increasing maturity (Newell et al., 1967). Early studies of Basha and Young (1985) and Rodriguez et al. (1989) showed loss of several polypeptides during roasting of peanut, indicating that some of these proteins may be involved in roasted flavor production.

Recently, Basha and Young (1996) have evaluated 10 protein fractions from peanut seed and found that peak I protein fraction alone was capable of producing the headspace volatiles, especially several of the off-flavor components. Peak I protein was found to be a high molecular weight ($>1 \times 10^6$) sulfur-containing protein. Because peak I protein eluted in the void volume of Sephacryl S-300 column and showed a complex electrophoretic profile (Basha and Pancholy, 1981), it is believed to contain a mixture of high molecular weight proteins. Therefore, it was necessary to identify and isolate specific protein(s) in peak I protein fraction that is (are) responsible for the production of flavor volatiles.

The objective of this study was to fractionate, identify, and characterize the components of peak I protein fraction that are responsible for the production of roasted flavor volatiles.

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MATERIALS AND METHODS

Isolation of Peak I Protein. After the seed coat was removed, peanut (*Arachis hypogaea* L. cv. Florunner) seeds were ground into a meal using a mortar and pestle and defatted by repeated extraction with hexane (Basha et al., 1976). Peak I protein was isolated from defatted peanut meal essentially as described earlier (Basha and Pancholy, 1981). Briefly, 3 g of defatted meal was extracted with 10 mL of 0.5 M NaCl/0.01 M Tris-HCl, pH 8.2, by homogenizing the peanut meal in a Polytron (Brinkman) homogenizer. The homogenate was centrifuged at 20000*g*, and the supernatant was loaded on a Sephacryl S-400 (Pharmacia, Uppsala, Sweden) column (1.5 cm × 135 cm), which was equilibrated with 0.5 M NaCl/0.01 M Tris-HCl, pH 8.2, and 0.002% sodium azide. Column eluates were collected in 5 mL fractions, and protein content was determined by measuring their absorption at 280 nm. Peak I protein eluting between column fractions 50 and 57 (void volume) were pooled and used for ammonium sulfate fractionation. Alternatively, peak I was dialyzed against water, freeze-dried, and used in fractionation with methanol and headspace and amino acid analyses.

Fractionation of Peak I Protein. Since peak I protein eluted in the void volume of the Sephacryl S-400 column (exclusion range $> 1 \times 10^6$), it is believed to contain components with molecular weight greater than 1 000 000. The components of peak I were further separated using the techniques of ammonium sulfate precipitation and methanol fractionation as described below.

(a) Ammonium Sulfate (AS) Precipitation. Peak I protein fraction (1 L) was brought to 0, 10, 20, 40, 60, and 80% AS saturation stepwise by adding appropriate amounts of solid AS. First, the peak I protein fraction was brought to 10% AS saturation by adding solid AS and then protein was allowed to precipitate for 2 h at 4 °C. The resulting protein precipitate was collected by centrifugation at 20000*g* for 20 min. The supernatant was then made to 20% AS saturation by adding solid AS and the precipitate collected as above. The above procedure was repeated to obtain the 40, 60, and 80% AS precipitates. The resulting pellets were taken up in 0.5 M NaCl/0.02 M Tris-HCl, pH 8.2, dialyzed against water, freeze-dried, and subjected to headspace analysis and polyacrylamide gel electrophoresis.

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(b) Solvent Extraction. To identify the suitable extraction media for solubilizing the flavor proteins, initially peak I protein (500 mg) was extracted (Polytron, low speed) with different combinations of organic solvents such as methanol, ethanol, and chloroform. The mixture was centrifuged at 20000*g* for 15 min, and the resulting supernatant was dried and tested for its ability to produce flavor volatiles by heating at 150 °C and analyzing the resulting headspace volatiles as described below by GC. The data showed that all of the soluble fractions obtained from the above treatments were capable of producing the flavor volatiles, whereas the insoluble fraction failed to produce the flavor volatiles. In this study the methanol extraction procedure was chosen over the other solvents because of the better solubility of methanolic extracts in subsequent experiments. To determine the optimum methanol concentration necessary for extracting the maximum amount of flavor proteins, peak I protein (500 mg) was extracted by homogenizing with different concentrations (0, 15, 25, 35, 45, 55, 65, 75, 85, and 95%) of methanol (1:5, w/v). The homogenates were allowed to settle on ice for 2 h and centrifuged at 20000*g* for 20 min. Resulting pellets and supernatants were dried in a Speed Vac concentrator (Savant, Farmingdale, NY) and used for headspace analysis as described below. Headspace analysis of these fractions showed that 85% methanol extract produced maximum amount of flavor volatiles. Hence, peak I protein was routinely extracted with 85% methanol and used in protein characterization studies. From here onward, the 85% methanol-soluble fraction of peak I protein will be referred to as MS, and the methanol-insoluble fraction will be referred to as MIS.

Gel Electrophoresis. Protein fractions were dissociated by boiling for 3 min in a buffer containing 1.2% (w/v) Tris, 3% (w/v) sodium dodecyl sulfate (SDS), and 3% (v/v) 2-mercaptoethanol. An aliquot (100 μg) of the sample was loaded on a 15% polyacrylamide slab gel and electrophoresed (Laemmli, 1970). After electrophoresis, proteins were visualized by staining with Coomassie Blue R-250.

Amino Acid Analysis. One milligram of protein was hydrolyzed with 6 N HCl containing 1% phenol at 110 °C for 24 h in a Pico-Tag workstation (Waters, Milford, MA). The hydrolysates were dried and derivatized with phenylisothiocyanate (PITC). An aliquot of the derivatized sample was analyzed using a waters 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 identified and quantified using an external amino acid standard and expressed as relative percentage of total amino acids.

Fatty Acid Analysis. Crude lipids were extracted from peak I protein using a modified Folch procedure (Folch et al., 1957) in which methylene chloride/methanol (2:1, v/v) was used as the extracting solvent system. Fatty acid methyl esters (FAME) were prepared as described by Morrison and Smith (1964). This involved transesterification using boron trifluoride in methanol, followed by extraction into hexane. Crude FAME were purified by placing extracted lipids into Pasteur pipets packed with Florisil (2 cm) followed by elution with hexane/ethyl ether (95:5, v/v). Purified FAME were analyzed on an HP model 5890 gas chromatograph (GC) (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector, an HP 3393A integrator, and a 30 m × 0.25 i.d. DB-225 fused silica capillary column (J&W Scientific, Folsom, CA) as described by Boyd et al. (1993). Column temperature was programmed from 180° to 230 °C at 2 °C/min with a final hold of 2 min. Injector and detector temperatures were set at 250° and 275 °C, respectively. Fatty acids were identified by comparison to authentic standards (Nu-Chek Prep, Elysian, MN). Fatty acid composition was expressed as weight percent of total FAME. Absolute response factors were calculated for each identified fatty acid peak using the normalization technique described by Sampugna et al. (1982).

Capillary Electrophoresis (CE). The protein compositions of different fractions were determined by CE using a Beckman P/ACE 2100 system (Beckman Instruments, Inc., Palo Alto, CA) controlled by a computer equipped with System Gold software. Protein separations were performed (Basha, 1997) using a Beckman gel-filled capillary (75 μm i.d. × 57 cm) and SDS 14-200 buffer kit (Beckman). Electrophoresis was performed at 25 °C and a voltage of 14 kV. The detector was set at 214 nm, and 6 nL (20 μg) of the sample (dissociated with SDS and 2-mercaptoethanol as described above) was injected (20 s). Separations were conducted in SDS 14-200 buffer. The capillary was rinsed for 5 min between electrophoretic runs with SDS 14-200 buffer.

Headspace Analysis. Peak I protein, AS precipitates, and MS and MIS fractions were subjected to headspace analysis as follows. Protein samples (0.5 g) were placed in a 12 mL screw-cap vial sealed with a Teflon-lined silicone disk in a Tekmar autosampler model 7000/7050. The sealed vial was heated for 12 min at 150 °C; 2 mL of headspace gas was collected from the vial and injected into a GC. The GC was fitted with a flame ionization detector and a 1 mm × 2 mm i.d. glass column packed with 80−100 mesh Porapak P. The carrier gas flow was adjusted to 40 mL/min. Initial column temperature was 120 °C, and it was programmed to increase to 200 °C at 20 °C/min. The injector/detector temperature was set at 200 °C (Young and Hovis, 1990). Peaks were integrated with a Hewlett-Packard HP 3309A integrator. The identities of the individual headspace peaks were confirmed by both coanalyzing with authentic compounds and comparing with the fragmentation patterns of standard references.

RESULTS AND DISCUSSION

Previously, Basha and Young (1996) have shown that peak I protein isolated from peanut seed is responsible for the production of off-flavor volatiles. Some of the off-flavor compounds produced by peak I protein are known to be responsible for musty aftertaste (peak II, pentane, acetone, dimethyl sulfide), fruity (peak IV, 2-methylpropanol), degree of roast (peak V, 2-butanone), tongue or throat burn (peak VI, pentanal), musty flavor (peak VII, *N*-methylpyrrole), and beany flavor (peak VIII, hexanal) of roasted peanuts (Young and Hovis, 1990). It should be noted that the peaks (II and IV−VIII) mentioned in parentheses above refer to the headspace volatile peaks. The ability of peak I protein to produce flavor components during roasting was determined by heating (without added oil or sucrose) peak I protein for different periods followed by headspace analysis of the resulting gases. The results showed (Figure 1) that flavor volatiles produced by peak I protein increased rapidly between 0 and 4 min (Figure 1a,b) of heating and then slowly up to 12 min (Figure 1d) of heating. As seen in the figure, levels of all 10 flavor components increased with increasing periods of heating, indicating that the amount of flavor components produced by peak I protein increased with heating period. Since the reaction vial contained only the protein without added oil or sugar, peak I proteins are believed to be the source of flavor volatiles. These data confirm our previous observation (Basha and Young, 1996) that peak I proteins are responsible for flavor volatile production during roasting of peanut.

Fractionation and Characterization of Peak I Protein. To isolate and identify the proteins of peak I that are associated with flavor volatile production, peak I protein was separated using ion-exchange chromatography (diethylaminoethylcellulose and carboxymethylcellulose). However, the resulting fractions failed (data not shown) to produce any flavor volatiles, indicating that the proteins responsible for flavor volatile production are lost during ion-exchange chromatography. This loss may be due to irreversible binding or precipitation of some of the peak I proteins (including flavor proteins)

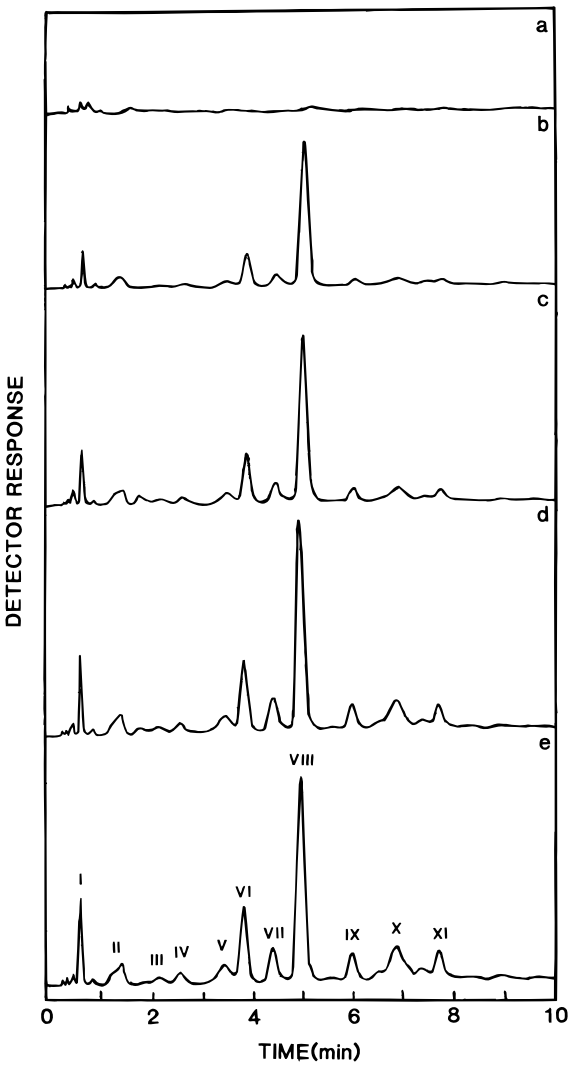

Figure 1. Headspace volatile profiles of peak I protein heated for different periods: (a) 0 min; (b) 4 min; (c) 8 min; (d) 12 min; (e) 16 min. Peak I (0.5 g) protein was heated at 150 °C, and headspace gases were collected and injected into a GC.

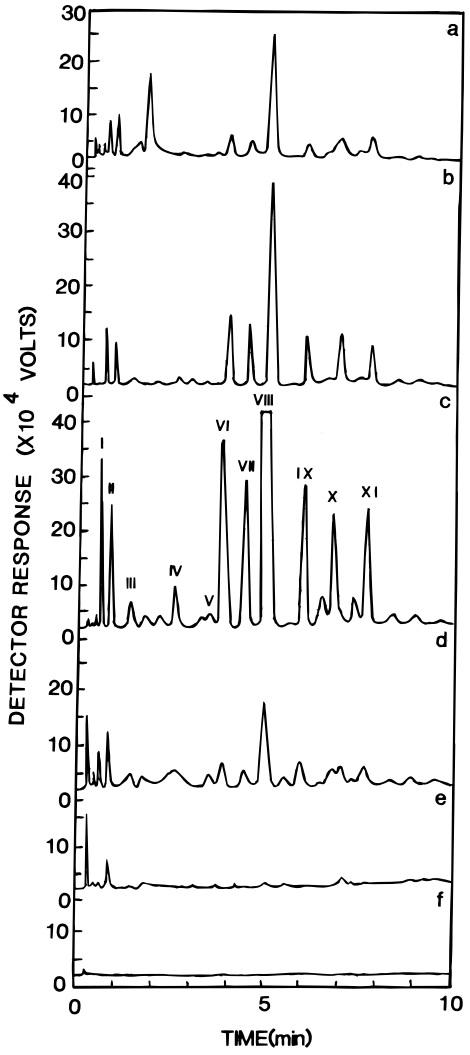

Figure 2. Headspace volatile profiles of proteins obtained at different AS saturations: (a) peak I; (b) 10%; (c) 20%; (d) 40%; (e) 60%; (f) 80%. Peak I protein solution was saturated stepwise with increasing concentrations of AS. Protein precipitating at each step was collected and subjected to headspace analysis.

on the ion-exchange column. Hence, classical protein fractionation techniques such as AS precipitation and organic solvent extraction were employed for peak I protein fractionation.

(a) AS Fractionation. Protein precipitates resulting from different AS saturations were subjected to headspace analysis to identify the AS fraction(s) producing flavor volatiles. Figure 2 shows flavor profiles of different AS precipitates. Comparison of flavor profiles of AS fractions showed that 20% AS precipitate produced the maximum amount of flavor volatiles followed by 10% AS precipitate. The headspace volatile profile produced by 20% AS precipitate (Figure 2c) was found to be similar to the peak I protein and contained all of the off-flavor volatile components produced by total peak I protein. This would indicate that the 20% AS fraction contained the maximum amount of protein(s) responsible for flavor volatile production. The 10 and 40% AS precipitates produced relatively smaller amounts of flavor volatiles than the 20% AS precipitate. It is believed that the flavor volatiles produced by the 10 and 40% AS precipitates may be due to the presence of small amounts of 20% AS precipitatable proteins that may have coprecipitated along with the 10 and 40% AS protein precipitates. Furthermore, it is common knowledge that AS precipitation does not yield pure protein fraction but is generally employed to enrich certain proteins. Thus, it appears that the majority of the flavor proteins precipitated at 20% AS saturation, while small amounts of these proteins precipitated at 10 and 40% AS saturation. Interestingly, no flavor volatiles were produced by the proteins precipitated at 60 and 80% AS saturation. It should be noted that the graphs in the figure were plotted to a lower scale for enhancing the flavor profiles (if present) of all the AS fractions, which caused some peaks (peak VIII, Figure 2c) to go off-scale. These data suggested that the compounds responsible for flavor volatile production are protein in nature and that they precipitate between 10 and 20% AS saturation.

Variation in the protein composition of AS precipitates was monitored by SDS gel electrophoresis. The data showed (Figure 3) several differences in the polypeptide composition among the AS precipitates. In the case of 10 and 20% AS precipitates (parts c and d of Figure 3, respectively), except for small quantitative differences, no major variations were observed between them. Interestingly, both the 10 and 20% AS precipitates contained a diffused band (shown with an arrow)

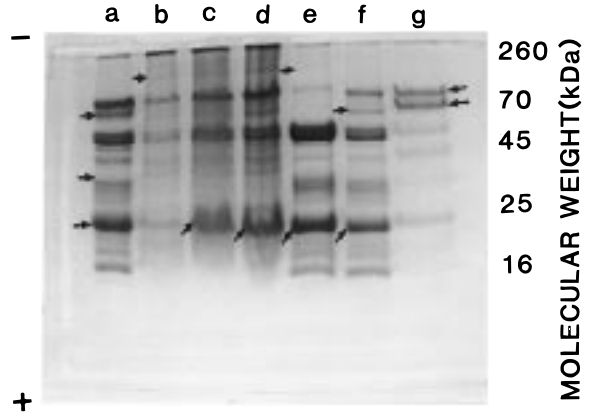

Figure 3. Differences in the polypeptide composition of AS precipitates: (a) peanut meal; (b) peak I; (c) 10%; (d) 20%; (e) 40%; (f) 60%; (g) 80%. Protein was dissociated using SDS and 2-mercaptoethanol, and polypeptides (75 μg) were resolved by SDS slab gel electrophoresis.

with a molecular weight around ~18 000. Likewise, the polypeptide compositions of 40 and 60% AS precipitates were similar (parts e and f of Figure 3, respectively), except for the absence of a 60 000 polypeptide (shown with an arrow) in the 40% AS precipitate. The polypeptide profiles of 10 and 20% AS precipitates were quite different from those of the 40 and 60% AS precipitates. The 80% AS precipitate (Figure 3g) contained two high molecular weight (~70 000) polypeptides and trace levels of three other polypeptides. Comparison of polypeptide profiles of flavor volatile-producing (10 and 20% AS precipitates, Figure 3c,d) and nonproducing (60 and 80% AS precipitates, Figure 3f,g) fractions showed that the flavor volatile-producing fractions contained a low molecular weight (<20 000) polypeptide (Figure 3c,d) which has a distinct appearance (diffused band) and staining property (purple) (shown with an arrow). In contrast, the 20 000 polypeptide of the 40 and 60% AS precipitates was sharp and lacked the streaking property (Figure 3e,f, shown with an arrow) observed with the 10 and 20% AS precipitates.

(b) Fractionation Using Methanol. Peak I protein obtained following gel filtration of total peanut seed protein extract on the Sephacryl S-400 column is typically turbid and has a milky appearance. This characteristic of peak I protein fraction is unaffected even after repeated gel filtrations, AS precipitation, high-speed centrifugation, and heating. Peak I protein appearance and its ability to produce flavor volatile compounds suggested (Basha and Young, 1996) that peak I protein may be a lipoprotein. Figure 4 shows the flavor volatile profiles of the MS and MIS fractions. For comparison, headspace volatile profiles of whole peanut seed (Figure 4a) and peak I protein (Figure 4b) are also shown. As seen in the figure, only the MS fraction (Figure 4c) produced the flavor volatiles, whereas the MIS fraction (Figure 4d) failed to produce the flavor volatiles. These data showed that only the MS fraction of the peak I protein contained the components capable of producing flavor volatiles.

(c) Lipid Composition. To determine the lipoprotein nature of peak I protein, it was subjected to fatty acid analysis. The data showed (Table 1) that peak I protein fraction contained a large amount (63.4%) of oleic acid (18:1) followed by palmitic acid (16:0, 8.4%). Total lipid content of the peak I fraction was found to be ~4% (w/w). In addition, the peak I protein fraction was also

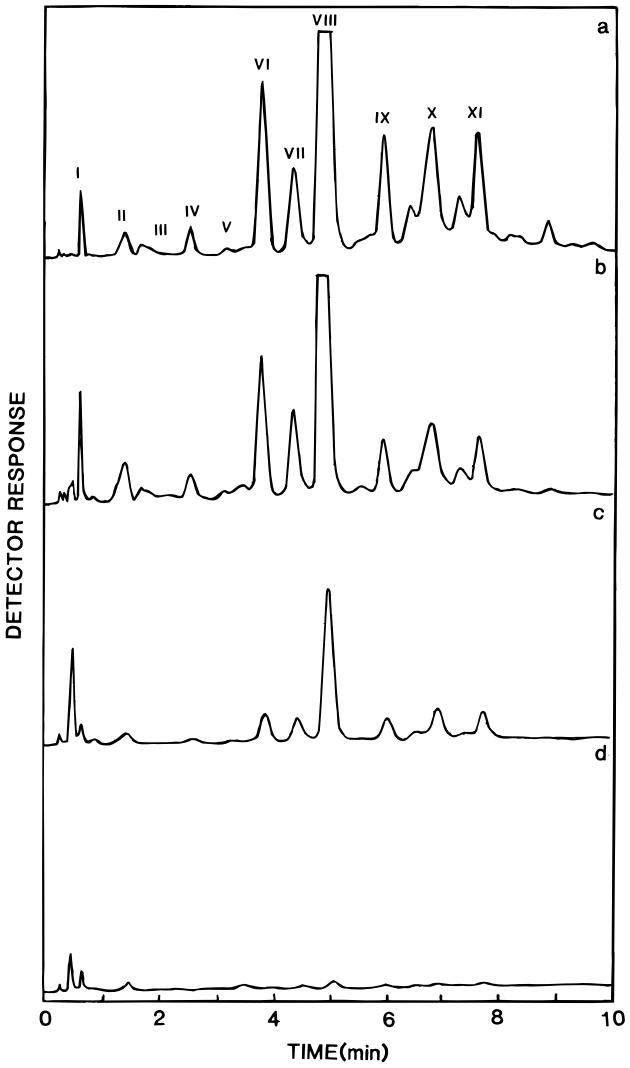

Figure 4. Headspace volatile profiles of protein fractions obtained following fractionation of peak I protein with 85% methanol: (a) peanut meal; (b) total peak I; (c) 85% MS fraction; (d) 85% MIS fraction. For comparison, flavor profiles of peanut meal and total peak I protein are also included.

Table 1. Fatty Acid Composition (Grams per 100 g of Protein) of Peak I Fraction Obtained following Separation of Peanut Seed Proteins

fatty acid	%	fatty acid	%
16:0	8.4	20:0	2.6
16:1	1.7	20:1	2.2
17:0	1.6	22:0	5.5
18:0	3.7	24:0	5.5
18:1	63.4		
18:2	5.3		

found to contain phospholipids and triglycerides (data not shown). These data confirm that peak I protein contains lipoprotein, which may be responsible for its milky appearance.

(d) Nature of the MS Fraction. The MS fraction obtained from peak I protein was subjected to gel and capillary electrophoresis to determine its nature and composition. The gel electrophoresis data showed (Figure 5) the presence of protein in the MS fraction (Figure 5d), which was different from that of peak I and MIS fractions. Peak I protein (Figure 5b) and MIS fraction (Figure 5c) contained ~15 polypeptides. Compared to the whole seed protein (Figure 5a), peak I protein and the MIS fraction contained lower amounts of 150 000,

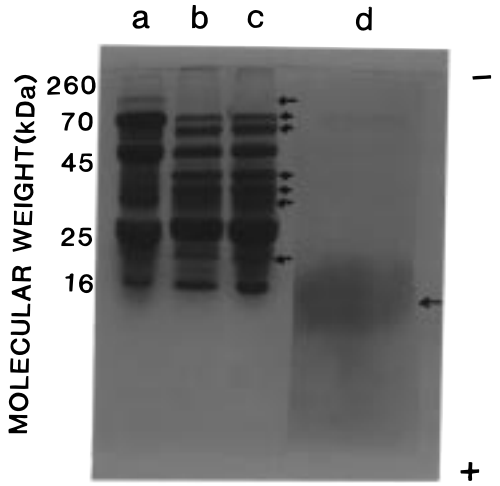

Figure 5. Polypeptide composition of protein fractions obtained following fractionation of peak I protein with 85% methanol. Protein fractions were dissociated with SDS and 2-mercaptoethanol, and an aliquot (75 μg) of the protein was loaded on the 15% (w/v) acrylamide slab gel. Proteins were visualized by staining with Coomassie Blue R-250. Polypeptides showing variation among the fractions are marked with arrows.

Table 2. Amino Acid Composition[a] of Protein Fractions from Peanut Seed

amino acid	defatted meal	peak I	85% methanol supernatant	85% methanol pellet
Asx[b]	11.05	10.19	5.33	8.45
Glx[c]	17.22	16.03	4.20	14.30
Ser	6.73	6.26	7.13	6.06
Gly	13.70	7.94	11.03	7.73
His	2.0	1.86	1.65	2.01
Arg	6.65	8.22	2.02	7.78
Thr	3.27	3.54	5.07	4.28
Ala	5.87	6.45	11.12	7.45
Pro	7.52	8.64	15.05	13.21
Tyr	2.04	3.64	2.80	3.74
Val	4.11	4.48	5.80	4.67
Met	0.50	0.45	1.12	0.13
Cys	0.65	0.26	0.71	tr[d]
Ile	3.00	3.55	4.96	3.50
Leu	6.86	7.60	9.60	7.20
Phe	3.94	5.41	8.57	5.40
Lys	4.92	5.25	8.35	3.52

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

70 000, and 50 000 polypeptides and higher amounts of 60 000, 45 000, 40 000, and 18 000 polypeptides. However, no major qualitative and quantitative differences were found between the polypeptide profiles of peak I protein and the MIS fraction. Unlike peak I protein and the MIS fraction, the MS fraction contained one rapidly migrating low molecular weight (<18 000) diffused band. This band consistently showed diffused appearance and stained purple with Coomassie Blue R-250. It should be recalled that 20% AS precipitate (Figure 2d) also contained a low molecular weight polypeptide with a similar appearance and staining property.

The diffused banding pattern of the MS fraction suggested that it consists of a mixture of low molecular weight (<18 000) polypeptides. Hence, the MS fraction was further characterized by CE. The CE profiles of various protein fractions are shown in Figure 6. CE resolved peanut meal, peak I protein, and the MIS fraction into 3−5 components, whereas the MS fraction (Figure 6d) was separated into >30 components. These data suggested that the MS fraction is composed of a

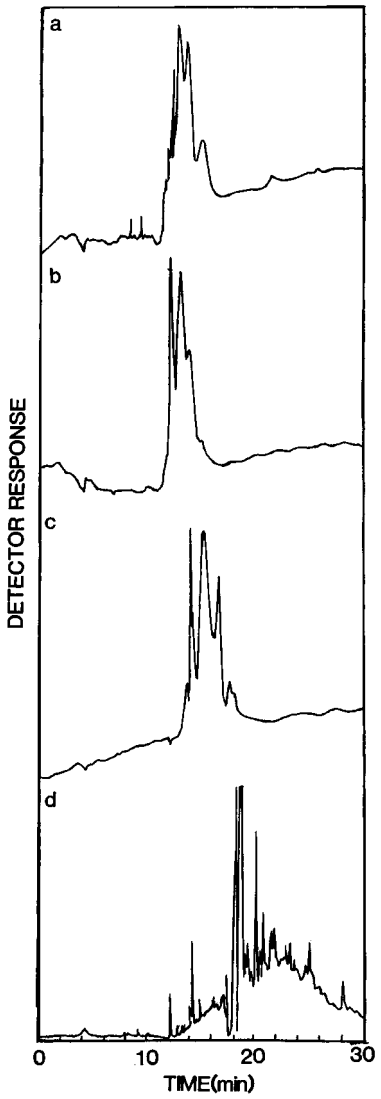

Figure 6. CE profiles of protein fractions obtained from 85% methanol fractionation of peak I protein. Protein fractions were dissociated with SDS and 2-mercaptoethanol and fractionated on a gel-filled capillary using Beckman SDS 14-200 gel buffer.

heterogeneous mixture of polypeptides, whereas the MIS fraction contains about five major components.

(e) Amino Acid Composition. The amino acid compositions of different protein fractions are shown in Table 2. Comparison of amino acid profiles of peanut meal and peak I protein showed quantitative differences in glycine, alanine, arginine, tyrosine, and phenylalanine levels, whereas the amounts of other amino acids were similar. The amino acid compositions of peak I protein and the MIS fraction were very similar except for a lower amount (8.6%) of proline in peak I protein. In contrast, the amino acid composition of the MS fraction was quite distinct. It contained low amounts of Asx (asparagine plus aspartic acid, 5.3%), Glx (glutamine plus glutamic acid, 4.2%), and arginine (2.0%) and large amounts of glycine (11.0%), threonine (5%), alanine (11%), proline (15%), methionine (1.1%), isoleucine (4.9%), leucine (9.6%), phenylalanine (8.6%), and lysine (8.3%). These data indicated that the MS fraction of peak I protein is proteinaceous in nature and that it has an amino acid profile distinct from that of total peak I protein and the MIS fraction.

In summary, the results of this study indicated that peak I protein is a lipoprotein and that the flavor

volatile-producing property appears to be associated with its MS fraction. Currently, studies are in progress to further separate the MS fraction of peak I protein and to determine the effect of heating on individual polypeptides for identifying the polypeptide(s) associated with specific flavor components.

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