Characterization of Methanol-Soluble and Methanol-Insoluble Proteins from Developing Peanut Seed

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The 85% methanol-soluble proteins are known to specifically contribute to the production of flavor of roasted peanut. To determine the nature of the 85% methanol-soluble proteins, they were isolated from the peanut seed, and the 85% methanol-soluble (MS) and 85% methanol-insoluble (MIS) fractions were characterized using polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis. The results showed that the 85% MS fraction contained lower amounts (9−10%) of protein than the MIS fraction (15−33%). Protein content of the MIS fraction increased more significantly during seed maturation than it did in the MS fraction. Unlike the protein, free amino acids and soluble sugars levels of the MS fraction decreased significantly during seed maturation. The 85% MS fraction contained predominantly low molecular weight (<20 kDa) proteins/polypeptides, whereas the MIS fraction contained a mixture of polypeptides with molecular weight between 14 kDa and 90 kDa. SDS−PAGE showed no major changes in the polypeptide composition of the MS fraction during seed maturation. Capillary electrophoretic analysis revealed major qualitative and quantitative changes in the protein and polypeptide composition of the MS and MIS fractions during seed maturation. Fatty acid analysis of these fractions indicated that the MS fraction is lipoprotein in nature and rich in oleic and linoleic acids.

Keywords: *Amino acids; electrophoresis; fatty acids; peanut; polypeptides; protein; sugars*

Roasted peanut seeds have a unique desirable flavor, and consumption of peanuts is greatly influenced by the flavor level of roasted peanuts. Previous studies have shown that free amino acids and carbohydrates are the precursors of roasted peanut flavor components (Pickett and Holly, 1952; Newell et al., 1967), being synthesized to pyrazine and carbonyl compounds (Mason et al., 1966, 1967; Johnson et al., 1971a, b) via Mailard sugar-amino type reactions. It is believed that amino acids involved in the browning reaction are released because of thermal breakdown of a protein/polypeptide fraction during roasting (Mason et al., 1969). Although a large amount of this fraction is known to be desirable for quality flavor characteristics, no major attempts have been made to identify and characterize the contributing components. Recently, Basha and Young (1996) have examined 10 protein fractions obtained following gel filtration of total peanut seed proteins, and found that only the Peak I protein fraction produced the headspace volatiles, especially several of the off-flavor components, following heating. They (Basha et al. 1998) also found that the Peak I protein fraction contains lipoproteins and is rich in oleic acid (63%) and palmitic acid (8%). Fractionation of Peak I proteins with 85% methanol and characterization of the resulting methanol-soluble and methanol-insoluble fractions showed that only the 85% methanol-soluble components contribute to the flavor. The 85% methanol-soluble fraction contained a mixture of low molecular weight proteins rich in glycine (11%), alanine (11%), proline (15%), phenylalanine (8%), and lysine (8%). It should be noted that the Peak I protein fraction characterized by Basha and Young (1996) was obtained following fractionation of an aqueous buffer (0.5 M NaCl, 0.01 M Tris-HCl, pH 8.2) extract of peanut seed and hence, it may not include all the components, especially those that contribute to roasted flavor and are insoluble in aqueous solvents (Basha et al., 1998). Peanut seed proteins soluble in aqueous solvents have been well characterized (Basha, 1979; Basha and Pancholy, 1981a and b; Cherry et al., 1973; Dawson, 1971; Neucere et al., 1978; Tombs, 1965). However, the nature and characteristics of the proteins soluble in nonaqueous solvents is unknown. Hence, the objective of this study was to isolate and characterize the peanut seed proteins soluble in nonaqueous solvents. For this purpose, peanut seed was extracted with a nonaqueous solvent, methanol, and the resulting fractions (soluble and insoluble) were characterized to determine differences in their protein content and to monitor changes in these components during seed maturation.

MATERIALS AND METHODS

Plant Materials. Peanut (*Arachis hypogaea* L. cv. Florunner) seed of various maturities were collected from plants grown at the University of Florida Experiment Station, Marianna, FL, by Dr. D. W. Gorbet. The pods were classified into white (W), yellow (Y), orange (O), brown (B), and black (BL) maturity categories based on the Hull Scrape method (Henning, 1983), with white being the most immature pod and black being the most mature pod. The pods were hand shelled and the seeds were freeze-dried. Seeds were ground into a meal

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and extracted repeatedly (6×) with hexane (Basha et al., 1976) until they were essentially free from oil, and the resulting fatfree meal was stored at −20 °C for further analysis.

Extraction of Peanut Meal. Defatted peanut meal (1 g) was homogenized for 30 s (in two 15 s pulses) with 85% methanol (10 mL) using a Polytron (Brinkman Instruments) homogenizer . The homogenate was centrifuged at 20 000*g* for 20 min at 4 °C. The resulting supernatant and pellet were termed as methanol-soluble (MS) and methanol-insoluble (MIS) fractions, respectively. The MS and MIS fractions were dried and used for protein analyses as described below. Preliminary studies using various organic solvents showed that methanol is most effective in dissolving the flavor components. All the analyses were carried out in three replications of two independent experiments. Data represented in the results are means ± SE of at least six samples.

Total Protein. Protein was extracted from the MIS fraction (50 mg) with 1 M NaOH (5 mL) by homogenizing in a Polytron (high speed, 30 s) homogenizer. The homogenate was centrifuged at 20 000*g* for 20 min, and the supernatant was collected and used for protein analysis. To determine the protein content of the MS fraction, it (5 mg) was dissolved in 1 M NaOH and used for protein analysis. Protein analysis was conducted by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Amino Acids and Soluble Sugars. Free amino acids and soluble sugars content of the 85% methanol-soluble fraction was determined using an aliquot (50 μL) of the methanolic extract prepared as described above following the methods of Yemm and Cocking (1955) and Yemm and Willis (1954), respectively.

Polyacrylamide Gel Electrophoresis (PAGE). The MIS fraction (25 mg) was homogenized (Polytron) in the dissociation buffer (200 μL) containing 3% (w/v) sodium dodecyl sulfate (SDS), 3% (v/v) 2-mercaptoethanol, and 1.2% (w/v) Tris and boiled for 3 min. The soluble protein was collected following centrifugation at 20 000*g* for 20 min. An aliquot (500 μL) of the MS fraction was dried using the Speed Vac dryer (Savant, Farmingdale, NY), and the resulting residue was dissolved in the dissociation buffer as described above. Aliquots (100 μg of protein) of the dissociated samples of the MS and MIS fractions were subjected to electrophoresis in a 12.5% (w/v) polyacrylamide slab gel containing 0.1% SDS according to the procedure of Laemmli (1970). The protein was stained with Coomassie Blue R-250 and destained with 7% acetic acid and 10% ethanol.

Capillary Electrophoresis (CE). Differences in the protein and polypeptide composition of the MS and MIS fractions were also determined by capillary electrophoresis. For non-SDS CE, proteins were extracted from the MIS fraction (6 mg) by homogenizing for 2 min (30 pulses) with 500 μL of a buffer containing 0.3% sodium borate and 0.4% boric acid (pH 8.3) using a Polytron homogenizer. The homogenate was centrifuged at 20 000*g* for 20 min at 4 °C, and the supernatant was used for CE analysis. The MS fraction was dried in the Speed Vac dryer, dissolved in the borate buffer (0.3% sodium borate, 0.4% boric acid, pH 8.3), filtered through a 0.22 μm, filter and used for CE. Protein separations were performed (Basha, 1997) on a Beckman P/ACE 2100 CE system (Beckman Instruments, Palo Alto, CA) controlled by a computer equipped with System Gold software. The protein separation was performed in an uncoated fused silica capillary (75 μm i.d. × 57 cm) at 25 °C and a voltage of 10 kV, and the detector was set at 214 nm. Separation of protein was carried out in sodium borate buffer (0.3% sodium borate and 0.4% boric acid), at pH. 8.3. The capillary was rinsed between electrophoresis runs with 1% (w/v) NaOH (5 min), deionized water (5 min), and 0.3% sodium borate buffer, at pH. 8.3 (5 min).

Polypeptide composition of the MIS and MS fractions was determined by SDS−CE using a gel-filled capillary (75 μm i.d. × 57 cm) and SDS 14-200 buffer kit (Beckman Instruments, CA) at 25 °C and a voltage of 14 kV, and the detector was set at 214 nm. Protein from the MS fraction was dissociated by adding dissociation buffer (0.6 M Tris-HCl, pH. 6.6, 5% SDS, and 2.5% β-mercaptoethanol) and heating for 10 min at 37° C. A portion (6 mg) of the MIS fraction was homogenized in 200 μL of dissociation buffer, heated for 10 min at 37 °C, and centrifuged (20 000*g*, 20 min), and the supernatant was collected. The dissociated samples were filtered through a 0.22-μm filter, and an aliquot of the sample (6 nL) was injected into CE. The capillary was rinsed sequentially between electrophoretic runs with 1N HCl (1 min).

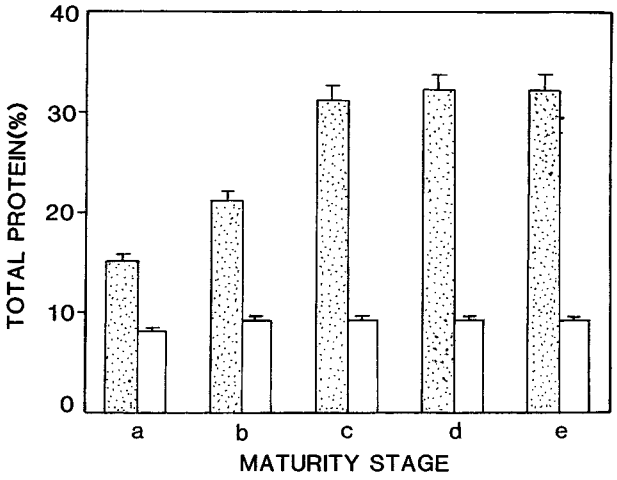

Figure 1. Changes in the total protein content (g/100 g of defatted meal) of the methanol-soluble (plain bar) and methanol-insoluble (dotted bar) fractions during peanut seed development. Maturity stages: (a) white, (b) yellow, (c) orange, (d) brown, and (e) black. Vertical lines on each bar represent + standard error.

Fatty Acid Analysis. To determine the lipoprotein nature, a portion (10 mL) of the MS fraction was dried using the Speed Vac dryer and analyzed for fatty acids. Boron trifluoride in methanol was used to transesterify crude fat extracts into fatty acid methyl esters (FAMEs) as described by Morrison and Smith (1964). Following partition into hexane and drying, crude methyl esters were dissolved in hexane/ethyl ether (95:5, v/v), and purified (on Florisil (2 cm) packed into Pasteur pipets) by elution with hexane/ethyl ether. Purified FAMEs were analyzed on an HP (Hewlett-Packard, Avondale, PA) Model 5890 gas chromatograph (GC) as described by Boyd et al. (1993). The GC contained a 30 m × 0.25 μm DB-225 fused-silica capillary column (J & W Scientific, Folsom, CA) and a flame ionization detector. Column temperature was programmed from 150° to 220 °C at a rate of 3°/min with an initial hold of 1 min and a final hold of 5 min. Data analysis was conducted on a Dell computer (Round Rock, TX) equipped with Chrom Perfect software (Justice Laboratory, Palo Alto, CA). Fatty acids were identified by comparison of unknown peaks to authentic standards (Nu-Chek Prep, Elysian, MN). The fatty acid composition was expressed as weight percent of total FAMEs. Absolute response factors were calculated for each identified fatty acids peak using the normalization technique.

RESULTS AND DISCUSSION

Previous studies (Basha et al., 1998) using protein isolates have suggested that in peanut the protein components involved in flavor production during heating are associated with the 85% methanol-soluble fraction. To evaluate the nature of 85% methanol-soluble components in whole peanut seed, they were isolated from the peanut seed and characterized to determine their composition and characteristics.

Characterization of Methanol-Soluble and Methanol-Insoluble Fractions. *Protein.* Protein contents of the MS and MIS fractions are shown in Figure 1. Protein level of the MIS fraction was significantly higher than that of the MS extract from peanut seed of all the maturities. In addition, in maturing seed, the protein

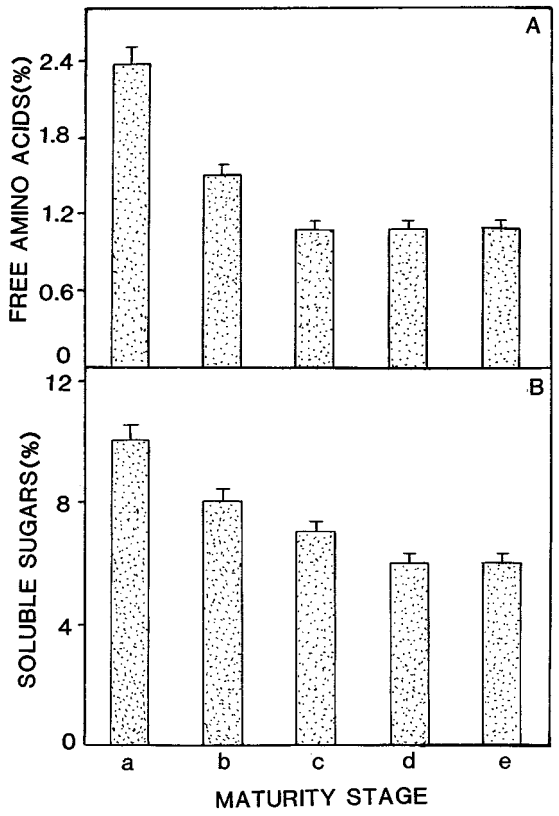

Figure 2. Changes in the free amino acids (A) and soluble sugars (B) content (g/100 g of defatted meal) of the methanol-soluble fraction during peanut seed development. Maturity stages: (a) white, (b) yellow, (c) orange, (d) brown, and (e) black. Vertical lines on each bar represent + standard error.

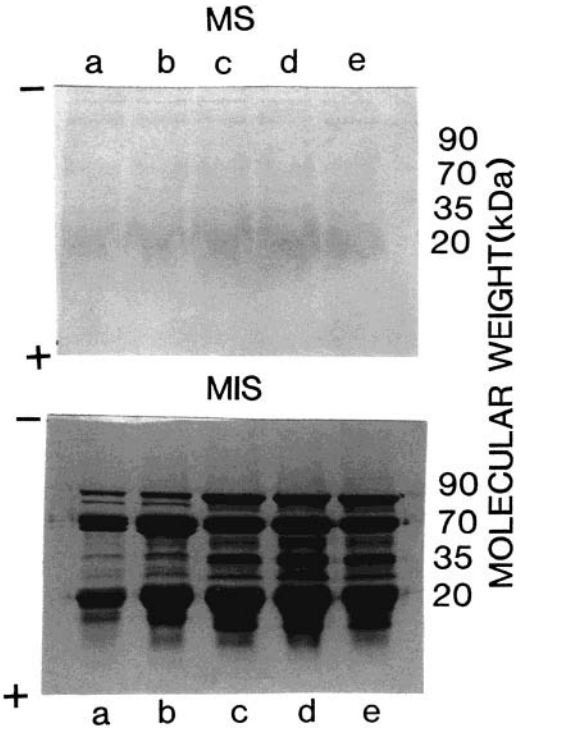

Figure 3. SDS−polyacrylamide gel electrophoretic (SDA−PAGE) rofiles of methanol-soluble (MS) and methanol-insoluble (MIS) fractions from developing peanut seed. Maturity stages: (a) white, (b) yellow, (c) orange, (d) brown, and (e) black.

content of MIS fraction increased more than that of the MS fraction. For example, the protein content of the MIS fraction increased from 15 to 33% whereas that of MS fractions increased from 9 to 10% (Figure 1) between white and black maturity stages. Protein content of the MIS fraction increased rapidly between white (immature) and orange maturity stages and then remained stable. In contrast, protein content of the MS fraction increased by 1% between white and yellow maturity stages and then remained unchanged (Figure 1).

Free Amino Acids and Soluble Sugars. Changes in the free amino acids and soluble sugars contents of the MS fraction in developing peanut seed are shown in Figure 2. These components decreased in the MS fraction as the seed matured. Major decreases were especially noted in the level of free amino acids and sugars between white and orange maturity stages. Decreases in the level of free amino acids and soluble sugars during seed maturation are consistent with the previous findings (Basha et al., 1976; Basha and Pancholy, 1981b). The MIS fraction contained only trace levels of free amino acids and soluble sugars (data not shown).

SDS Gel Electrophoresis. Polypeptide composition of MS and MIS fractions was determined by SDS−PAGE. Figure 3 shows the electrophoretic profiles of MS (Figure 3a) and MIS (Figure 3b) fractions. The MS fraction shows one rapidly migrating low molecular weight (<18 000 daltons) broad diffuse band at all the five maturity stages that stained poorly (purple) with Coomassie Blue R-250. The diffused banding and purple staining characteristics may be due to the lipoprotein nature of the MS fraction resulting in slight variation in their size, mobility, and staining characteristic. The staining pattern and appearance of the MS fraction from peanut seed (Figure 3a) was found to be similar to the staining pattern of the MS fraction obtained from the Peak I protein isolate (Basha et al. 1998). Results of this study also showed that, like the MS fraction from Peak I protein isolate, the MS fraction from whole peanut seed also contained a mixture of low molecular weight proteins, and stained purple as evidenced by the diffuse banding pattern. Staining intensity of the protein bands of the MS extracts from peanuts of various stages of maturity was similar, indicating that the protein level in the MS fractions remained similar during seed development. This finding is consistent with the protein content (Figure 1) of the MS fractions. Unlike the MS fraction, the MIS components separated into several distinct polypeptides, and they stained normal (blue) with Coomassie Blue R-250. Molecular weights of these polypeptides ranged from 14 000 to 90 000 daltons. MIS fractions from developing seed showed five polypeptides with molecular weights around 70, 50, 40, and 20 kDa, which increased with increasing maturity. Maximum accumulation of these polypeptides occurred between the white and orange maturity stages and then remained unchanged.

Capillary Electrophoresis (CE). MS and MIS fractions were analyzed by CE (Basha, 1997) to determine differences in their protein/polypeptide compositions. The CE profiles of the MS and MIS fractions are shown in Figure 4. CE successfully resolved proteins of the MS fraction into more than fifteen components (Figure 4). These data show that the MS fraction is composed of a mixture of low molecular weight proteins. Comparison of CE profiles of MS fractions from developing seeds showed major changes in their protein composition between the orange and brown maturity stages. For example, peak I increased as the seed matured to orange

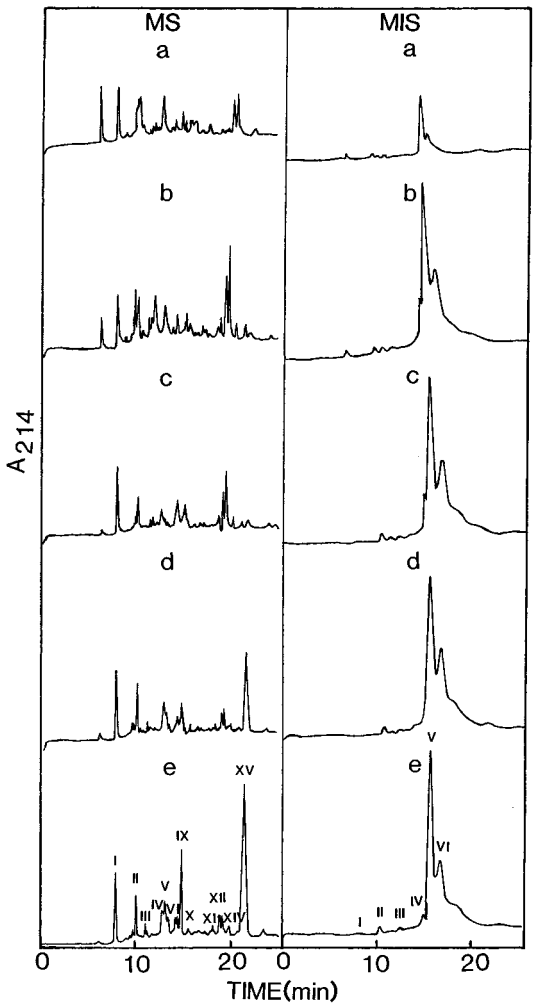

Figure 4. Capillary electrophoretic profiles of proteins from methanol-soluble (MS) and methanol-insoluble (MIS) fractions from developing peanut seed. Maturity stages: (a) white, (b) yellow, (c) orange, (d) brown, and (e) black.

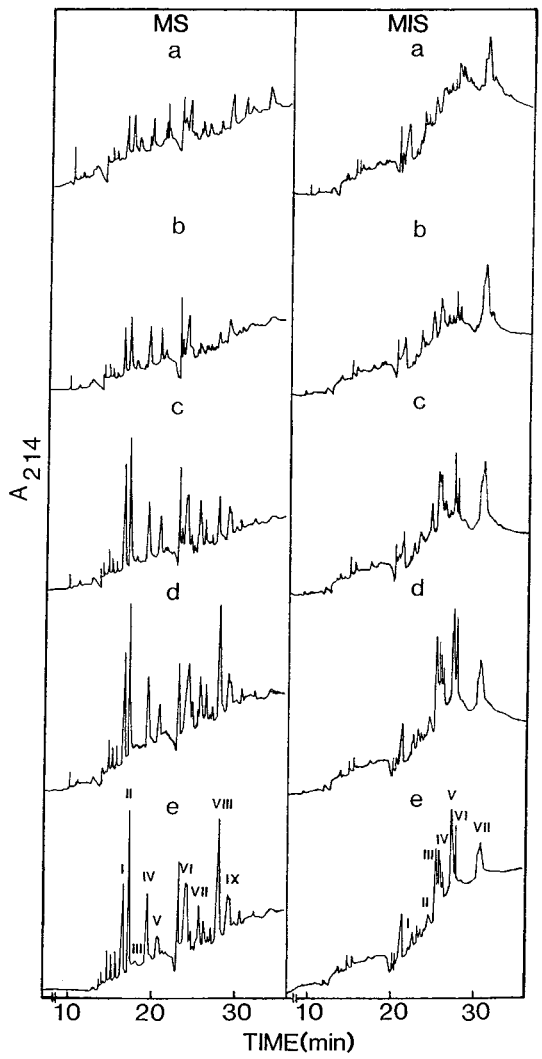

Figure 5. SDS−capillary electrophoretic profiles of methanol-soluble (MS) and methanol-insoluble (MIS) fractions from developing peanut seed. Maturity stages: (a) white, (b) yellow, (c) orange, (d) brown, and (e) black.

stage, and then remained high in brown and black maturity stages (Figure 4d and 4e). Peaks VIII and XIII increased rapidly between white and yellow maturity stages and then decreased as the seed matured from orange to the black maturity stage. Peak XV was present only in trace amounts until the orange stage, and then it increased rapidly. In addition, several other peaks showed minor quantitative and qualitative changes with increasing seed maturity. Unlike the MS fraction, MIS fraction resolved (Figure 4) into two major (V and VI) and four minor protein peaks. The two major peaks increased rapidly between the white and yellow maturity stages. Protein profiles of the MIS fraction remained similar between the orange and black maturity stages (Figure 4c through 4e).

In addition to protein, changes in polypeptide composition of the MS and the MIS fractions were also monitored by the SDS−CE (Figure 5). SDS−CE resolved the MS fraction into more than nine polypeptides. As seen in the figure, polypeptide composition of the MS fractions changed greatly during peanut seed maturation. In the MS fraction, peaks I, II, IV, V, VI, VII, VIII and IX were the major peaks, whereas the other peaks were present in relatively smaller amounts. Among the major peaks, peaks I, II, IV, V and VIII increased with increasing seed maturity becoming maximum between orange and brown maturity stages. The SDS−CE profile seen in Figure 5 further supports the conclusion that the MS fraction of peanut seed contains a mixture of small molecular weight proteins and polypeptides whose compositions change during seed development. SDS−CE resolved the MIS fraction into more than seven major polypeptides (Figure 5). During seed maturation the polypeptide content and composition of the MIS fraction changed rapidly between the white and orange maturity stages and then remained similar. This is consistent with the protein profiles of MIS seen in Figure 4. Of the nine peaks, peaks III, IV, V, and VI increased more than other peaks during seed development. Unlike the other peaks, peak VII appeared to remain unchanged during seed development.

Lipid Composition. The MS fraction was subjected to fatty acid analysis to determine its lipoprotein nature. The data showed (Table 1) that the MS fraction contained a large amount (44.8−64.3%) of oleic acid, followed by smaller amounts of linoleic acid (13.9−30.6%) and palmitic acid (11.5−13.8%), indicating that it is lipoprotein in nature. These findings are consistent with the report of Basha et al. (1998), who also noted large amounts of oleic acid and palmitic acid in the MS fraction from Peak I protein isolate.

Table 1. Changes in the Fatty Acid Composition of Methanol-Soluble Fraction from Maturing Peanuts (g/100 g of Total Fatty Acids Identified)

	developmental stage				
fatty acid	white	yellow	orange	brown	black
16:0	11.5	13.8	11.8	10.6	11.5
16:1	0.1	0.1	0.1	0.1	0.1
17:0	0.1	0.1	0.1	0.1	0.1
18:0	2.2	2.9	2.8	2.5	0.6
18:1	44.8	53.4	52.9	50.0	64.3
18:2	30.6	17.0	22.4	28.6	13.9
18:3	0.1	0.1	0.1	0.1	0.1
20:0	1.3	1.8	1.5	1.3	1.7
20:1	2.0	2.0	1.6	1.4	1.6
20:2	0.1	0.2	0.6	0.4	0.1
22:0	4.7	5.4	3.4	2.9	3.6
22:1	0.2	0.1	0.1	0.1	0.2
24:0	2.3	3.2	2.2	1.9	2.3
saturates	22.1	27.2	21.8	19.3	19.8
monoenes	47.1	55.6	54.7	51.6	66.2
PUFA[a]	30.7	17.1	23.1	29.1	14.1

[a] PUFA, polyunsaturated fatty acids.

In summary, the SDS−PAGE, CE, and lipid data indicated that the MS fractions obtained from the whole peanut seed and Peak I protein isolate appeared to be similar in nature and composition. In addition, this study also revealed that the protein and polypeptide composition of MS fraction changed significantly during seed maturation. Studies are in progress to isolate the protein/polypeptide components of MS fraction for determining the role of individual protein and polypeptide components in flavor production.

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