Protein Fraction Producing Off-Flavor Headspace Volatiles in Peanut Seed

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The free amino acids involved in the sugar−amine nonenzymatic reaction are believed to originate following thermal breakdown of unknown protein(s) during roasting. This study was conducted to identify the seed protein(s) involved in the production of off-flavor volatiles, especially the *n*-methylpyrrole, in roasted peanuts. Peanut (*Arachis hypogaea* L. cv. Florunner) seed proteins were separated into 10 fractions by gel filtration on a Sephacryl S-300 column. These fractions were heated, and the resulting headspace gasses were tested for flavor volatiles. The data showed that of the 10 protein fractions evaluated, only the peak I fraction produced the headspace volatiles. The volatile compounds included the ones responsible for off-flavors, as well as some unknown compounds. Peak I was found to be protein in nature and to contain sulfur. Further fractionation of peak I showed that the cryoprecipitate and dialysis-precipitate produced mainly hexanal, while the cryosupernatant and dialysis-supernatant produced a complex mixture of headspace volatiles. The data suggested that some of the peak I proteins appear to be responsible for the production of off-flavor roasted volatiles in peanut.

Keywords: *Flavor; gel filtration; headspace; peanut; protein; roasting*

Roasting is a cooking process applied to peanuts for development of a unique, desirable flavor. Free amino acids and free sugars were found to be the major flavor precursors in roasted peanuts (Newell et al., 1967) and gave rise to 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. Koehler et al. (1969) investigated the pathway for the formation of alkylated pyrazine compounds in amino acid−carbohydrate model systems of low water content. Their data supported the hypothesis of Dawes and Edwards (1966) that the carbon atoms of pyrazines arise from sugar degradation products. The carbonyl compound which contributes to roasted peanut flavor (Mason et al., 1967) was thought to result from a Strecker degradation or oxidative deamination of free amino acids (Schonberg and Moubacher, 1952).

The free amino acid content of raw peanuts varies among varieties, planting locations, and maturation (Young et al., 1974a,b; Oupadissakoon et al., 1980a,b). Buckholtz et al. (1980) found that roasting time had a significant influence on the strength of odor and flavor of roasted peanuts. Aspartic acid, glutamic acid, glutamine, asparagine, histidine, and phenylalanine were associated with the production of typical roasted flavor, while threonine, tyrosine, lysine, and an unknown amino acid were considered the precursors of atypical flavor (Newell et al., 1967). The unknown amino acid was later referred to as a peptide-2 and considered to contribute to the typical flavor. During roasting, this peptide is hydrolyzed to give the amino acid reactants and appears to be a major contributor to a good roasted flavor (Mason et al., 1969). A large amount of this peptide/protein would be desirable. The concentration of this peptide was shown to increase from 2 to 4 μmol/g of fat free meal with increasing maturity (Newell et al., 1967). An attempt was made for the purification and characterization of the peptide.

Studies of Basha and Young (1985) and Rodriguez et al. (1989) revealed that although total nitrogen and protein contents of the seed were not greatly affected, protein composition changed significantly during the 12−14 min roasting period. Their data also showed that the peanut pastes contained relatively lower amounts of the 90 000, 70 000, 50 000, and 32 000 Da polypeptides compared to the raw peanuts. Loss of these polypeptides during roasting suggests that some of the polypeptides may be providing the amino acid reactants involved in roasted flavor production.

The objective of this study was to isolate and identify the seed protein(s) associated with the production of off-flavor compounds during the roasting of peanuts.

MATERIALS AND METHODS

Protein Extraction and Fractionation. Peanut (*Arachis hypogaea* L. cv. Florunner) seeds were ground into a powder and extensively defatted with hexane (Basha et al., 1976). The defatted meal (3 g) was extracted with 10 mL of 0.5 M NaCl, 0.01 M Tris-HCl, pH 8.2, by homogenizing the meal in a Polytron homogenizer. The homogenate was centrifuged at 20 000*g*, and the supernatant was loaded on a Sephacryl S-300 column (2.5 cm × 135 cm) which was equilibrated with 0.5 M NaCl, 0.01 M Tris-HCl (pH 8.2) and 0.002% sodium azide (Basha and Pancholy, 1981a). Column eluates were collected in 5 mL fractions, and their protein content was determined by measuring absorption at 280 nm. The protein peaks were pooled, dialyzed against deionized water, and concentrated using a Speed Vac dryer. The dried protein samples were used for headspace analysis.

Headspace Analysis. Headspace analysis was carried out by the modified method of Young (1994) and Young and Hovis (1990). Isolated protein samples 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 gas chromatograph. The GC was

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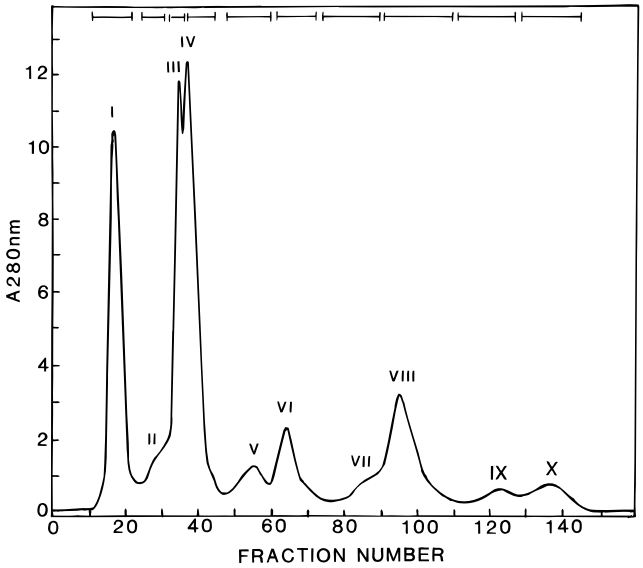

Figure 1. Gel filtration profile of peanut seed proteins fractionated by gel filtration on a Sephacryl S-300 column. Proteins falling under each peak were pooled and designated fractions I−X.

fitted with a flame-ionization detector or a sulfur detector and a 1 m × 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. Peaks were integrated with a Hewlett Packard HP3309A integrator. The identity of the individual headspace peaks was confirmed by both coanalyzing with authentic compounds and comparing with the fragmentation patterns of standard references.

Gel Electrophoresis. Peak I and its fractions, resulting following cryoprecipitation and dialysis, were subjected to polyacrylamide gel electrophoresis under nondenaturing conditions according to the method of Davis (1964). After electrophoresis, proteins were stained with Coomassie blue R-250. The polypeptide composition of the peak I fractions was determined by SDS−gel electrophoresis in 12.5% slab gels according to the method of Laemmli (1970). The polypeptides were visualized after staining with Coomassie blue R-250.

RESULTS AND DISCUSSION

Volatile compounds are responsible for the aroma and much of the flavor of roasted peanuts. Free amino acids and free sugars that are released during roasting are known to be major flavor precursors in roasted peanuts. The amino acids involved in this reaction are believed to originate following thermal degradation of an unknown protein/polypeptide during roasting. In this study an attempt was made to identify the protein(s) associated with the production of off-flavor compounds in roasted peanuts.

Fractionation of Seed Protein. Peanut seed proteins were resolved into 10 fractions based on their molecular weight, by gel filtration on a Sephacryl S-300 column. The gel filtration profile showed (Figure 1) that peaks I, III, IV, VI, and VIII were the major protein peaks, while peaks II, V, VII, IX, and X were present in relatively smaller amounts. Peaks III and IV represent the arachin (major peanut storage protein) protein, while peak VI is the methionine-rich protein (Basha and Pancholy, 1981a). Peak I eluted in the void volume of the Sephacryl S-300 column and thus mainly contains high molecular weight ($>1 \times 10^6$) proteins and protein aggregates.

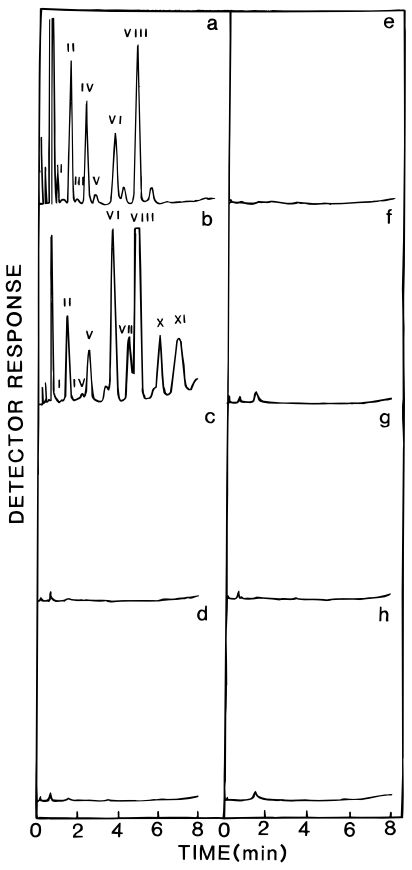

Figure 2. Headspace volatile profiles of peanut seed protein fractions detected using a flame-ionization detector: (a) whole peanut, (b) peak I, (c) peak III, (d) peak IV, (e) peak V, (f) peak VI, (g) peak VII and (h) peak VIII.

Headspace Analysis. The protein fractions obtained following gel filtration were heated without adding any oil or sucrose, and the resulting headspace gases were analyzed to identify the protein fraction(s) producing flavor volatiles. Oil and sugar were excluded from the reaction because we were interested in determining whether protein alone can produce flavor volatiles. The results showed (Figure 2) that of the 10 protein fractions only peak I fraction (Figure 2b) produced flavor volatile compounds. The other nine protein fractions (II−X) failed to produce any flavor volatiles. This would suggest that only peak I fraction contained the protein component(s) capable of producing the flavor volatiles during roasting. Since the reaction vial contained only the protein, it is believed to be the source of flavor volatiles. Some of the flavor volatiles produced by peak I protein were known to be responsible for off-flavors such as 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) (Young and Hovis, 1990). The identities of the compounds eluting beyond peak VIII are unknown, but they are believed to contain mainly the on-flavor volatiles. Studies are in progress to develop methodology for their identification.

The musty flavor is usually the major off-flavor found in raw peanuts and peanut products. Although it is known (Young and Hovis, 1990) that lipid is responsible for several off-flavor compounds, the source of musty flavor (caused by *N*-methylpyrrole) is unknown. The musty flavor is thought to be associated with the protein

(Young and Hovis, 1990), while the other peaks are mostly associated with autoxidation products. Results of this study indicate that in addition to musty flavor, the proteins may be also associated with other flavor compounds. In whole peanut, volatiles such as pentane, acetone, methylpropanol, butanone, pentanal, and hexanal can come from peanut oil. It is realized that the presence of trace levels of oil in the samples can produce some of the volatile compounds. However, we believe that our protein fractions were not contaminated with oil since they were prepared first by extracting total protein from extensively defatted peanut meal followed by high-speed (20000*g*) centrifugation, filtration, fractionation on a 135 cm long gel filtration column, and dialysis. Moreover, if the protein samples were contaminated with oil, some of the protein fractions other than peak I should also contain oil and would be able to produce flavor volatiles. Repeatedly, only the peak I fraction produced the flavor volatiles, while the other fractions (II−X) consistently failed to produce any flavor volatiles, indicating that the observed flavor volatiles are coming from the protein and not from the contaminants. The reactions containing fractions II−X can be considered as controls since all the 10 fractions were heated under identical conditions and subsequent headspace analysis revealed production of flavor volatiles only by the peak I fraction.

Since sulfur is believed (Watkins, 1987) to enhance roasted flavor characteristics by forming hydrogen sulfide, the headspace volatile compounds resulting from heating of protein fractions were also monitored with a sulfur detector, to detect the sulfur-containing compounds. The data revealed that only fractions I (Figure 3Ba) and VI (Figure 3Bc) produced the sulfur-containing compounds, indicating that these two protein fractions contained sulfur amino acids. These results are consistent with the report of Basha and Pancholy (1981b) who have shown the presence of significant levels of sulfur amino acids in fractions I and VI using [^{35}S]methionine-labeling studies and amino acid analysis (Basha and Pancholy, 1981a).

To confirm that proteins in the peak I fraction are indeed responsible for the production of observed flavor volatiles, the peak I fraction was treated with TCA, ether, or ethanol and the resulting protein precipitates and supernatants were analyzed for their ability to produce headspace volatiles. The results showed (data not shown) that only the protein precipitates produced the flavor volatiles, while the supernatants (should contain oil contaminants, if any) failed to produce any volatile compounds. The above results indicate that the component(s) responsible for the production of flavor volatiles in the peak I fraction are nondialyzable and precipitatable. This would also suggest that the component(s) producing flavor volatiles is protein in nature and that it may possess the unique amino acid composition necessary for flavor volatile production during roasting of the peanut.

Characterization of Peak I Fraction. In our attempts to fractionate the peak I proteins, initially we had separated peak I proteins (which produced headspace volatiles) into four protein peaks by ion-exchange chromatography on a DEAE cellulose column. However, the protein fractions resulting following DEAE chromatography failed to produce headspace volatiles after heating (data not shown), indicating that either the peak I proteins lost their headspace volatile-producing property during ion-exchange chromatography or

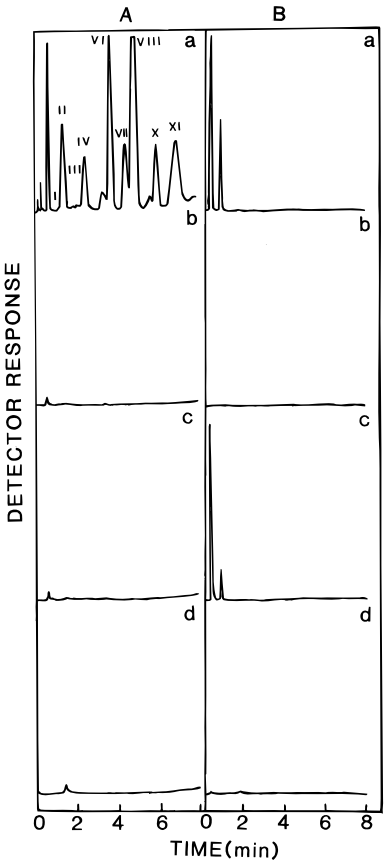

Figure 3. Headspace volatile profiles of selected peanut seed protein fractions detected using a flame-ionization detector (A) and a sulfur detector (B): (a) peak I, (b) peak III, (c) peak VI and (d) peak VIII.

the proteins possessing this property were bound to the DEAE column and failed to elute off the column. Hence, nonchromatographic methods such as cryoprecipitation and dialysis were employed for separation of proteins in peak I, to preserve their flavor volatile compound-producing property. Evaluation of these four peak I protein fractions by headspace analysis showed that hexanal (peak VIII) was the major headspace volatile compound produced by the cryoprecipitate (Figure 4b) and dialysis-precipitate (Figure 4d). In contrast, the headspace volatile profiles of the cryosupernatant (Figure 4c) and dialysis-supernatant (Figure 4e) were more complex and contained several off-flavor and unknown volatile compounds. Hexanal is usually a product of lipid oxidation. We found that hexanal was produced only by peak I (Figure 2) and its fractions (cryosupernatant and cryoprecipitate, dialysis-supernatant and dialysis-precipitate) (Figure 4). No hexanal was produced by peaks II−X (Figure 2). This would suggest that only peak I proteins are capable of producing hexanal and that it is not coming from oil contaminants (if any) in the fractions. Although the source of hexanal is unknown at this time, it is possible that the peak I and its fractions may contain lipoproteins and, hence, are able to produce some of the flavor volatiles of lipid origin. Studies are in progress to determine the lipoprotein nature of these proteins. In addition to hexanal, *N*-methylpyrrole was present in large amounts in both the cryosupernatant and dialysis-supernatant compared to the cryoprecipitate and dialysis-precipitate. Furthermore, the amount of unknown compounds eluting beyond hexanal was higher in cryosupernatant and dialysis-supernatant than in cryoprecipitate and dialysis-

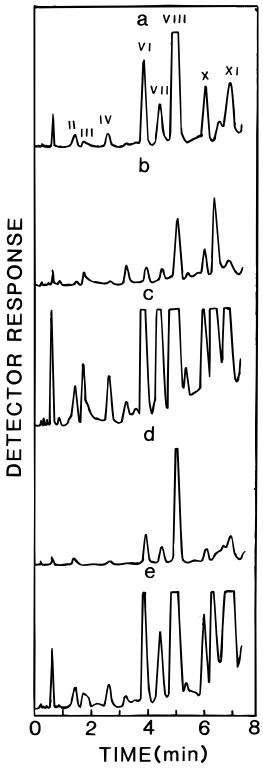

Figure 4. Headspace volatile profiles (flame-ionization detector) of different protein fractions obtained after fractionation of peak I: (a) total peak I, (b) cryoprecipitate, (c) cryosupernatant, (d) dialysis-precipitate, and (e) dialysis-supernatant; peak VII, *N*-methylpyrrole; peak VIII, hexanal.

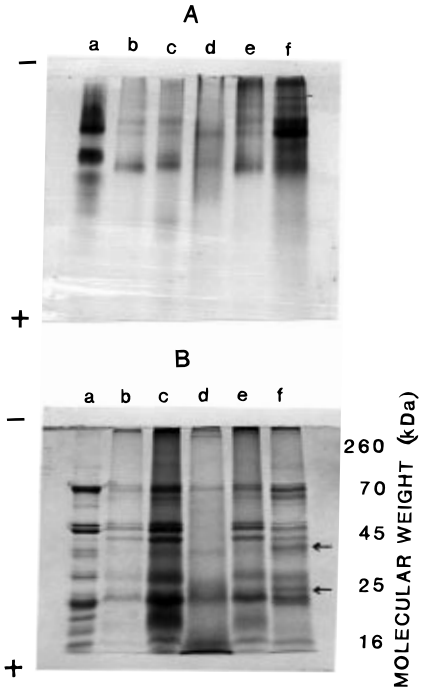

Figure 5. Gel electrophoretic profiles of peak I protein fractions under nondenaturing (A) and denaturing (B) conditions. About 75−100 μg of protein was loaded in each lane and electrophoresed toward the anode. Protein was detected by staining the gels with Coomassie blue R-250: (a) total protein, (b) peak I, (c) cryosupernatant, (d) cryoprecipitate, (e) dialysis-supernatant, and (f) dialysis-precipitate.

precipitate indicating that they differ in their ability to produce flavor volatiles.

Electrophoresis. Differences in the protein composition of peak I and its cryoprecipitation and dialysis fractions was determined by electrophoresis under nondenaturing and denaturing conditions. The nondenaturing gel electrophoresis data showed (Figure 5Ab) the presence of one major and two minor protein bands moving toward the anodic end of the gel indicating that peak I is composed of more than one protein. Since peak I is eluted in the void volume of the Sephacryl S-300 column, it is expected to contain a mixture of high molecular weight ($>1 \times 10^6$) proteins and protein aggregates (Basha and Pancholy, 1981a). Comparison of electrophoretic profiles of different protein fractions showed little difference in the protein composition between peak I (Figure 5Ab) and the cryosupernatant (Figure 5Ac). In contrast, the protein composition of the cryoprecipitate appeared to be quite different from that of the cryosupernatant (Figure 5Ad). The cryosupernatant was further separated into the dialysis-supernatant and dialysis-precipitate by dialyzing it against deionized water. Electrophoretic analysis of these fractions showed major differences in their composition. The dialysis-supernatant (Figure 5Ae) contained one major and two minor proteins, while dialysis-precipitate (Figure 5Af) contained several proteins. Variation in the polypeptide composition of these protein fractions was determined by SDS−gel electrophoresis. The data showed (Figure 5B) that peak I (Figure 5Bb) contained several polypeptides with molecular weights between 25 000 and 70 000 Da. The SDS gels revealed major differences in the polypeptide composition between the cryosupernatant (Figure 5Bc) and cryoprecipitate (Figure 5Bd). The cryoprecipitate contained one major and several minor polypeptides. Likewise, there were also small differences in the polypeptide composition of the dialysis-supernatant (Figure 5Be) and dialysis-precipitate (Figure 5Bf). The dialysis-precipitate contained two polypeptides (shown with arrows) that were absent in the dialysis-supernatant. Interestingly, the polypeptide profiles of the cryosupernatant (Figure5Bc) and dialysis-supernatant (Figure 5Be) appeared to be very similar suggesting that the cryosupernatant contained predominantly the water-soluble proteins. This is consistent with our observation that only a small amount (<5%) of protein precipitated during cryoprecipitation and dialysis. This may be the reason for the presence of only small differences in the protein composition among the peak I, cryosupernatant and dialysis-supernatant fractions. These differences in the protein composition may be responsible for the observed variations in their flavor volatile-producing ability (Figure 4). Although all four fractions of peak I showed (Figure 5) variation in their protein and polypeptide composition, since all of them produced various levels of flavor volatiles (Figure 4), it was not possible to clearly identify the specific protein or polypeptide associated with a certain flavor volatile. Currently we are isolating individual proteins and polypeptides for use in determining the specific flavor volatile produced by each protein component.

In summary, of the 10 protein fractions evaluated for their ability to produce flavor volatiles, only the peak I fraction was capable of producing these compounds. Protein appears to be the source of flavor volatiles in the peak I fraction, especially the *N*-methylpyrrole. Studies are in progress to isolate the individual proteins of the peak I fraction for determining the relationship between the proteins/polypeptides and specific off-flavor volatiles associated with them.

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