Peptide Mapping of Peanut Proteins: Identification of Peptides as Potential Indicators of Peanut Maturity

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Proteins are the major source of peanut flavor precursors such as peptides and amino acids. Changes in the protein structure possibly would lead to a change in peanut flavor quality. As peanut maturity can affect peanut flavor quality, it was postulated that proteins between mature and immature peanuts could be structurally different. To support this postulation, peptide mapping of mature and immature peanut proteins was carried out. Peptide maps were produced by digesting peanut proteins with an arginyl endopeptidase. This was followed by treatment of the resultant protein digests with trichloroacetic acid (TCA) or an affinity column of immobilized anhydrotrypsin (IMAT). The TCA-soluble peptide fractions were then subjected to analyses by C_{18} reversed-phase high-performance liquid chromatography (HPLC), whereas fractions, which contained carboxyl- or C-terminal and non-C-terminal peptides, from the IMAT column were analyzed by capillary zone electrophoresis (CZE). For resolution purposes, fractions collected from the C_{18} column of HPLC were further analyzed by CZE. Results showed that peptide maps from immature peanut proteins contained peptides different from those from mature peanut proteins. Peptides such as peptide **I** (a C-terminal peptide) from immature peanut proteins and peptide **M** (a TCA-soluble peptide) from mature peanut proteins were identified. This difference in peptide patterns indicates that proteins between mature and immature peanuts were structurally different.

INTRODUCTION

Much research has been done to determine the change in levels between mature and immature peanut proteins (Basha et al., 1976; Basha, 1988, 1989, 1991; Rodriguez et al., 1989). However, little is known about the structural difference between these proteins. Here, protein structure refers to the arrangement, replacement, or modification of amino acids in an amino acid sequence. Proteins that are similar in composition can be structurally different if an alteration in the sequence occurs. This structural difference can be detected by an enzyme. For instance, cleavage of a substrate peptide with a sequence of A-X-X-X by an enzyme which specifically cleaves at A (where A and X represent various amino acids) will give a peptide fragment X-X-X and an amino acid A, whereas cleavage of another substrate peptide with a similar composition but differing sequence of X-A-X-X will give two different peptide fragments, X-A and X-X. As proteins are the major source of peanut flavor precursors such as peptides and amino acids (Newell et al., 1967), changes in the protein structure (i.e., amino acid sequence) possibly would lead to a change in peanut flavor quality. These structural changes have been shown to have an effect on the binding of flavor compounds to proteins (Damodaran and Kinsella, 1981; O'Keefe et al., 1991). Here, we postulated that proteins between mature and immature peanuts could be structurally different due to the fact that peanut flavor quality is affected by the maturity of peanuts (Sanders et al., 1989, 1990; Blankenship et al., 1989). The objective of this study is to examine these proteins and the difference in their structures.

To determine if proteins undergo structural changes, a "fingerprint" technique, namely, peptide mapping, was employed. Peptide maps were obtained by subjecting mature (black) or immature (yellow) peanut proteins to digestion by an arginyl endopeptidase which selectively cleaves proteins at the C-terminal side of arginine. This was followed by treating the digested proteins with trichloroacetic acid (TCA) and separating the resultant TCA-soluble peptide fragments with C_{18} reversed-phase high-performance liquid chromatography (HPLC). Despite its speed and resolution, HPLC has a drawback in that peptides often coelute, making it difficult to obtain a true peptide profile. As a complimentary technique to HPLC, capillary zone electrophoresis (CZE), which separates peptides on the basis of their charge and mass (Strickland, 1991; Landers et al., 1993), was used to separate the co-eluting peptides obtained from HPLC.

Additionally, peptide maps were developed by subjecting carboxyl- or C-terminal peptides from mature or immature peanut proteins to CZE analysis. C-Terminal peptides are of particular interest due to the fact that they are susceptible to modifications (Henriksen et al., 1992; Harris et al., 1993), different between variants of the β amyloid protein in brains of aged patients with Alzheimer's disease (Jarrett et al., 1993), and reactive in many biochemical reactions such as binding of iron essential for the activity of soybean lipoxygenase (Boyington et al., 1993; Minor et al., 1993), protection of proteins against denaturation (Takemoto et al., 1993), regulation of enzyme activity (Kaliman et al., 1993; Morgan et al., 1993; Orellano et al., 1993), and interaction with other proteins (Mossakowska et al., 1993; Ortiz et al., 1993). C-Terminal peptides were obtained by applying the arginyl endopeptidase-digested protein mixture to an affinity column of immobilized anhydrotrypsin (IMAT) and then eluting from the void volume of the column. C-Terminal peptides did not bind

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to the column due to the fact that anhydrotrypsin is an inert (active site-altered) derivative of trypsin (Kumazaki et al., 1987; Ohta et al., 1991) which binds only peptides containing arginine or lysine residues at their C termini (these peptides were produced as a result of arginyl endopeptidase cleavage at the arginine). As C-terminal peptides had no arginine residue at their terminus, they were eluted from the void volume of the column.

In this study, we report on the finding of a difference in peptide patterns between mature and immature peanut proteins as detected by the technique of peptide mapping using arginyl endopeptidase, HPLC/CZE, and IMAT/CZE.

MATERIALS AND METHODS

Materials. Immobilized anhydrotrypsin was purchased from Pierce Chemical Co. (Rockford, IL). Arginyl endopeptidase was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Bio-Rad protein assay kit (catalog no. 500–0002) was purchased from Bio-Rad Laboratory (Melville, NY). YM3 membrane was purchased from Amicon, Inc. (Beverly, MA). Phenylmethanesulfonyl fluoride (PMSF) was purchased from Sigma Chemical Co. (St. Louis, MO). Peanuts (*Arachis hypogaea* L. var. Florunner) were planted at the USDA–ARS National Peanut Research Laboratory (Dawson, GA), dug 160 days after planting, sand blasted, shelled, sorted for maturity, and stored at −80 °C. Peanut maturity (e.g., black for mature and yellow for immature) was determined by using the hull-scrape method (Williams and Drexler, 1981).

Preparation of Peanut Proteins. Prior to protein extraction, mature (black) or immature (yellow) peanuts were deskinned and defatted by grinding in sequence with cold acetone and hexane. The resulting defatted peanut meals were air-dried. Proteins were extracted from the defatted meals with some modifications of the method of Chiou (1990). Briefly, defatted meals (1 g) were added to 0.02 M sodium phosphate (Na_2HPO_4) buffer (20 mL), pH 7.0, containing 0.5 M sodium chloride (NaCl), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% sodium azide (NaN_3). The mixture was allowed to stir for 18 h at 4 °C and centrifuged at 2000*g* for 15 min at 4 °C. After centrifugation, the supernatant was pooled and subjected to ammonium sulfate fractionation in the following order: 40, 40–60, and 60–85%. Proteins precipitated by ammonium sulfate at 60–85% saturation were dissolved in 0.1 M ammonium bicarbonate (NH_4HCO_3) buffer containing 2 M urea, pH 8, dialyzed against the same buffer at 4 °C, and used for enzyme digestion (see below). Protein concentration was determined using the Bio-Rad protein assay kit (catalog no. 500-0002).

Digestion by Arginyl Endopeptidase. Prior to digestion, the extracted proteins (obtained by 60–85% ammonium sulfate saturation) were diluted to a concentration of 10 mg/mL with 0.1 M NH_4HCO_3, pH 8. The diluted proteins were digested for 18 h at 37 °C with an arginyl endopeptidase [1 mg/mL; 100:1 (w/w)] in the same buffer containing 5 mM calcium chloride ($CaCl_2$). After digestion, the protein digests were treated with trichloroacetic acid (TCA) for C_{18} reversed-phase HPLC analysis or applied directly (after dilution) to an affinity column of immobilized anhydrotrypsin (see below).

Separation by C_{18} Reversed-Phase HPLC. Prior to HPLC analysis, the arginyl endopeptidase-digested proteins were allowed to mix for 10 min at 4 °C with cold TCA at a final concentration of 5% and then centrifuged at 12000*g* for 25 min at 4 °C. After centrifugation, the supernatant (i.e., TCA-soluble peptides) was recovered and 10 μL of the supernatant was injected into a C_{18} reversed-phase column (0.46 × 25 cm; 218TP54, Vydac, Hesperia, CA) equilibrated with 0.1% trifluoroacetate (TFA). The HPLC system consists of an ISCO (Lincoln, NE) V^4 variable absorbance detector and Model 2350 pumps. Data acquisition and analysis as well as system control were achieved using ISCO's ChemResearch software and an IBM System 2 Model 30 personal computer. Peptides were separated at a flow rate of 1 mL/min using acetonitrile/TFA solvent mixtures and the following gradients: 0–25% acetonitrile in 0.1% TFA (25 min); 25–55% acetonitrile in 0.1% TFA (25–75 min). HPLC fractions were collected manually, treated by passage through C_{18} Sep-Pak cartridges (Waters, Milford, MA), and analyzed by capillary zone electrophoresis (CZE) (Beckman Instruments, Inc., Palo Alto, CA).

Fractionation by Immobilized Anhydrotrypsin. The arginyl endopeptidase-digested protein mixture was first diluted 1:20 with cold binding buffer (0.05 M sodium acetate buffer, pH 5.0, containing 0.02 M $CaCl_2$ and 0.02% NaN_3). Then the diluted sample (500 μL) was applied to an affinity column of immobilized anhydrotrypsin equilibrated with binding buffer at 4 °C. C-Terminal peptides (unbound to the column) were obtained by washing the column with 20 mL of the binding buffer. Peptides that were bound to the column were eluted with 15 mL of 0.1 M formic acid, pH 2.5. The resulting peptides were then separated on a YM3 membrane (3000 MW cutoff) into low and high molecular weight fractions. The fractions were concentrated by evaporating in a Savant Speed-Vac, treated by passage through C_{18} Sep-Pak cartridges, and analyzed by CZE.

Separation by Capillary Zone Electrophoresis (CZE). CZE was performed on an automated PACE 2050 (Beckman Instruments, Inc., Palo Alto, CA) controlled by a computer fitted with PACE System Gold and WINDOWS (Microsoft, Redmond, WA). All CZE separations were performed in uncoated fused-silica capillaries (75 μm i.d. × 57 cm). Electrophoretic separations were performed at 25 °C and a voltage of 15 kV. Peptides were diluted in 0.1% TFA and 1% acetonitrile, injected for 10 s, and detected at 210 nm. The capillary was rinsed sequentially between successive electrophoretic runs with 0.1 M sodium hydroxide (NaOH) (5 min), deionized water (5 min), 0.1 M phosphoric acid (H_3PO_4), pH 1.58 (10 min), and 0.1 M sodium phosphate (NaH_2PO_4), pH 2.5 (15 min).

RESULTS AND DISCUSSION

To reduce the complexity of the peptide maps, only a small fraction of the total proteins extracted from defatted peanut meals was used for digestion by arginyl endopeptidase. This fraction was obtained by precipitation of proteins at 60-85% ammonium sulfate saturation, which gave about 28% of the total proteins (Chiou, 1990). With a small fraction like this, peptide maps are expected to be clearer and less complex and, also, peptides are easier to separate and identify. The fraction obtained from 0 to 60% saturation was not investigated because it contained large amount of proteins, most of which were insoluble in buffers unless urea or salt was added in high concentration, which probably would lower the activity of arginyl endopeptidase. Moreover, with such large amounts of proteins, more peptides would possibly be generated by the enzyme, and as a result, peptide maps may become more complex and peptides more difficult to separate by HPLC or capillary electrophoresis. This was why 60–85% instead of 0–60% saturation was selected for peptide mapping.

Additionally, peanut proteins are known to contain cryoproteins. These cryoproteins may present a problem when incubated in the column of immobilized anhydrotrypsin at 4 °C; that is, precipitates may form and affect the performance of the column. To prevent this, cryoproteins were removed from the protein samples by preparing and dialyzing at 4 °C the protein extracts and fractions (60–85% ammonium sulfate saturation), followed by centrifugation. The resultant supernatants were then used for fractionation on the column of immobilized anhydrotrypsin or for TCA/HPLC and CZE analyses.

Reversed-Phase HPLC Separation and Analyses by CZE. Samples analyzed here contained low molecular weight peptides obtained from the TCA precipitation of arginyl endopeptidase-digested proteins. Figure 1 shows the C_{18} reversed-phase HPLC separation of these TCA-soluble peptides from immature and mature peanut proteins. Approximately 16 peaks were detected in each

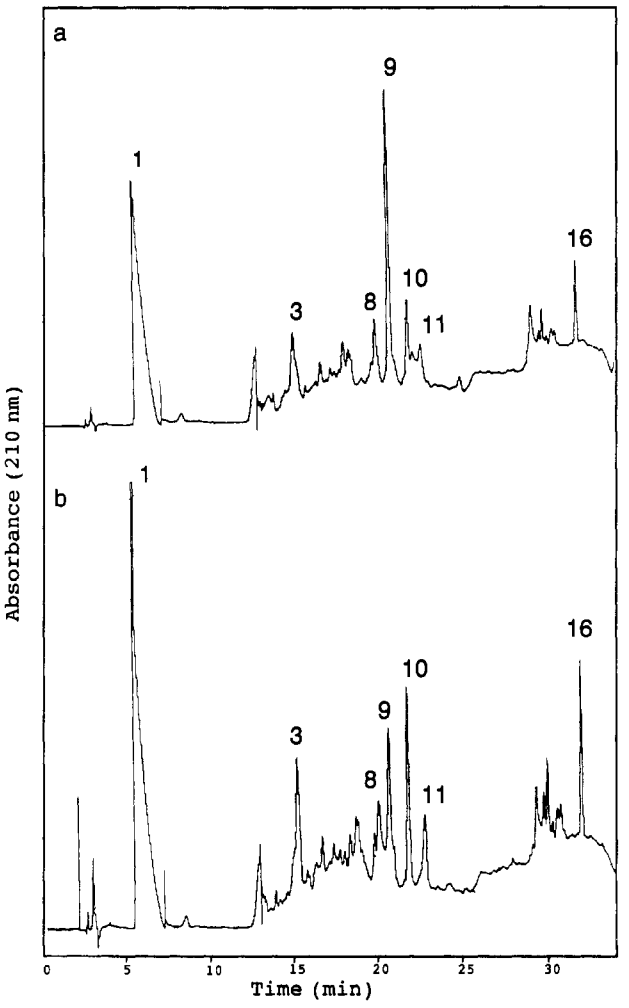

Figure 1. C_{18} reversed-phase HPLC separation of TCA-soluble peptides from mature and immature peanut proteins. TCA-soluble peptides were obtained following treatment of arginyl endopeptidase-digested proteins with TCA (final concentration of 5%) and centrifugation of the resultant mixture. (a) Immature peanuts; (b) mature peanuts.

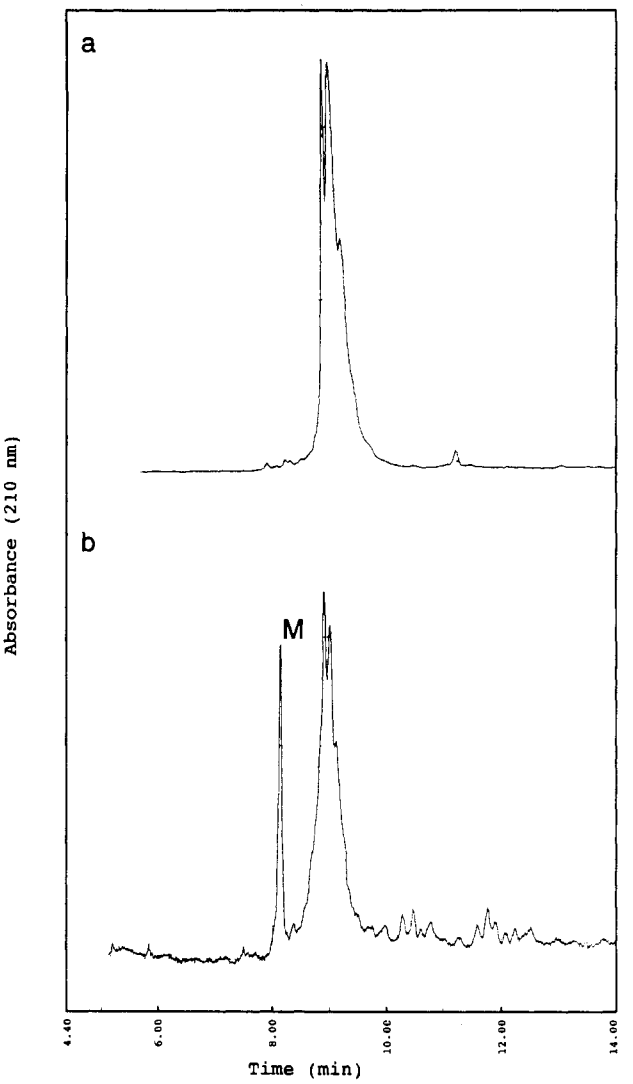

Figure 2. Capillary electrophoresis of peak 9 obtained from HPLC in Figure 1. Electrophoretic conditions are given under Materials and Methods. (a) Immature peanuts; (b) mature peanuts.

case. Except for a quantitative difference in peak areas, peptide patterns between mature and immature peanut proteins were very similar. Among the peaks, peaks 1, 3, 10, 11, and 16, except for peak 9 from mature peanut proteins, were shown to be more pronounced than those from immature peanut proteins. Analyses of the peaks by CZE showed (data not shown) that each consisted of several peptides rather than one single peptide and that peptide patterns between mature and immature peanuts were very similar in each case except for those of peak 9. The peptide pattern of peak 9 (analyzed by CZE) from mature peanut proteins was shown to contain a peptide **M** (Figure 2b) which was absent in immature peanut proteins (Figure 2a). Peptide **M** was considered not a contaminant from other peaks based on the following reasons: (a) Fraction (i.e., peak 9) collection started and ended approximately in the middle of the peak height (peak height was known on the basis of preliminary runs). By doing so, contaminants introduced from other peaks into the fraction were avoided. (b) If peak 9 contained contaminants, the source of contaminants could have been peak 8 or 10. Analysis of peak 8 by CZE showed that peptide patterns between mature and immature peanut proteins were almost identical (Figure 3). Similar identical profiles were also observed with peak 10 (data not shown). This suggests that if peptide **M** were a contaminant, it should have been present in the peak 9 profiles of both mature and immature peanut proteins. Instead, peptide **M** was shown to appear only in mature peanut proteins, suggesting that it was a product or a fragment released from a protein digest rather than a contaminant from peak 8 or 10. In addition to peptide **M**, other minor peaks that were absent in immature peanut proteins were also detected in mature peanut proteins (Figure 2). This difference in peptide patterns suggests that proteins between mature and immature peanuts were structurally different.

The mechanism for the appearance of peptide **M** and other minor peaks is not known. However, since this (i.e., transition from immature to mature) was a maturation process, it is possible that peptide **M** and other minor peptides could arise as a result of modification of existing proteins through posttranslational processes such as transpeptidation, glycosylation, or oxidation by free radicals generated from lipid peroxidation or transition metals (Cheng et al., 1991; Sellak et al., 1992; Lodge et al., 1993; Gebicki and Gebicki, 1993). All of these processes could occur during peanut maturation and could render the proteins more resistant or susceptible to cleavage by arginyl endopeptidase. As a result, the peptide patterns from the modified proteins could be different from those of the native proteins. This study showed that peptide **M** could be a potential component in determining the

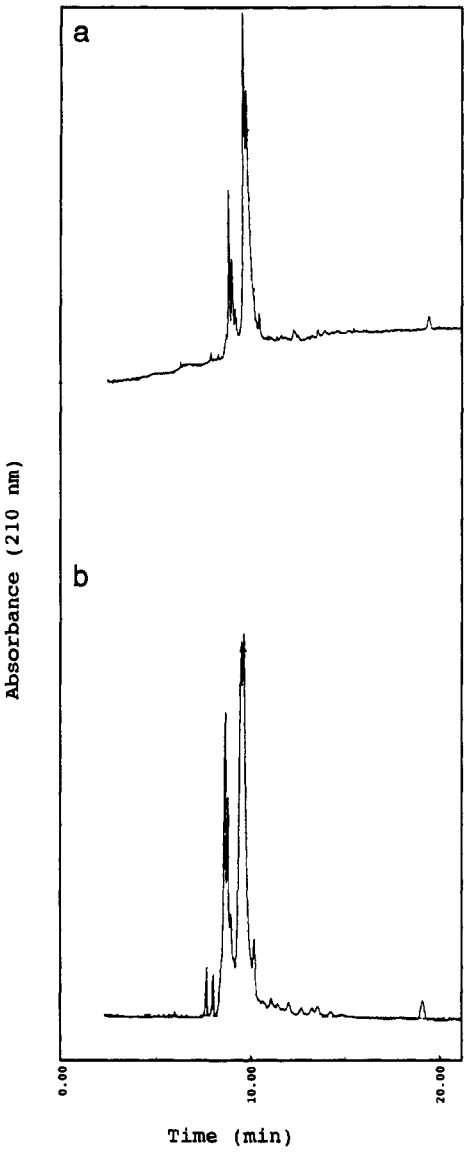

Figure 3. Capillary electrophoresis of peak 8 obtained from HPLC in Figure 1. (a) Immature peanuts; (b) mature peanuts.

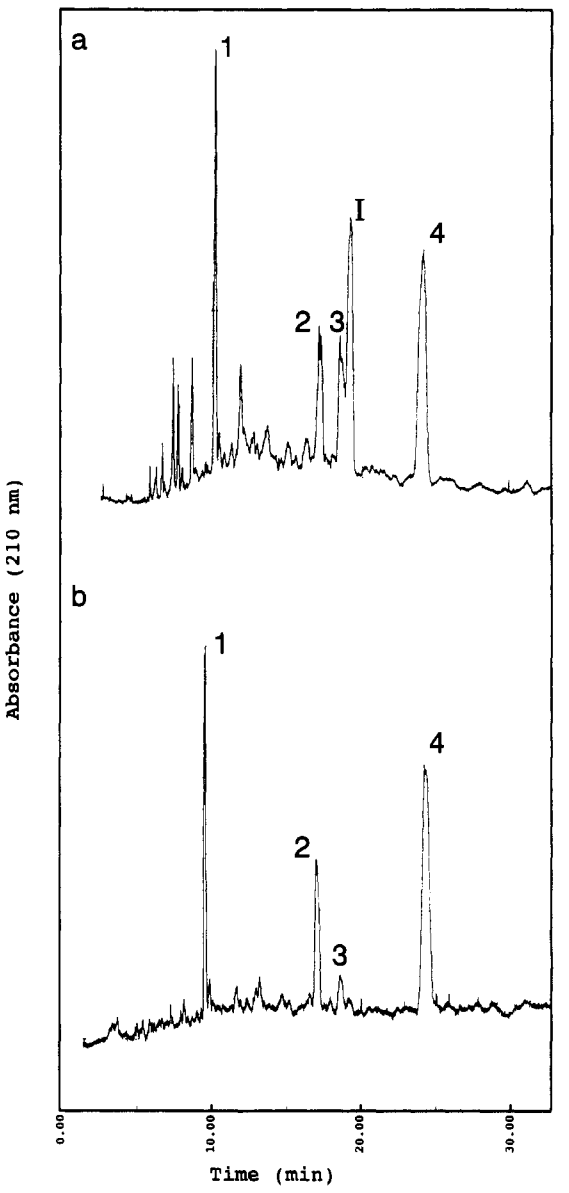

Figure 4. Capillary electrophoretic separation of C-terminal peptides from mature and immature peanut proteins. C-Terminal peptides were obtained by applying an arginyl endopeptidase-digested protein mixture to a column of immobilized anhydrotrypsin and eluting in the void volume with 0.1 M sodium acetate buffer, pH 5.0. (a) Immature peanuts; (b) mature peanuts.

maturity of peanuts. Because of its solubility in 5% TCA and being a low moleular weight fragment from a protein digest, peptide **M** is believed to be different in composition from the protein that Basha (1990) identified as an indicator of peanut maturity. In that study, proteins underwent no treatment (i.e., not digested by an enzyme) and only large molecular weight polypeptides were involved.

Fractionation by Immobilized Anhydrotrypsin and Analyses by CZE. This gave fractions containing C-terminal and non-C-terminal peptides. Analysis of the C-terminal peptides (<3000 Da) from immature peanut proteins by CZE showed that there were approximately nine peaks in the peptide map (Figure 4a). Of these peaks, peaks 1–4 were also shown in the map of mature peanut proteins (Figure 4b). However, the remainder of the peaks, such as peptide **I** and four other minor peaks, appeared to be missing or barely detectable in mature peanut proteins (Figure 4b). This difference in the C-terminal region suggests that proteins between mature and immature peanuts were structurally different. The role of the C-terminal peptides, particularly peptide **I**, in peanut maturity is not known. However, since a recent study (Chung and Bordelon, 1993) has shown that peanuts contain arginine-binding proteins, it is possible that the C-terminal peptides could be part of these proteins and involved in the binding of arginine through an ionic interaction between the carboxyl groups of the peptides and the amino/guanidine groups of arginine. In the case of soybean lipoxygenase, it has been shown (Boyington et al., 1993; Minor et al., 1993) that the C-terminal region of the enzyme is involved in the binding of iron, which is essential for its activity. Another example includes the binding of a farnesyl group to the C-terminal region of a Ras protein, the modification of which by the farnesyl group is known to lead to the transformation of normal cells to cancerous cells (James et al., 1993; Travis, 1993).

Additionally, fractions containing non-C-terminal peptides (peptides that bound to the anhydrotrypsin column) were examined. Analysis of these fractions (>3000 Da) by CZE showed that peptide patterns between immature and mature peanut proteins were similar (data not shown but similar to Figure 5b). Despite this, fractions from mature peanuts were believed to contain peptide fragments different from those from immature peanuts due to the

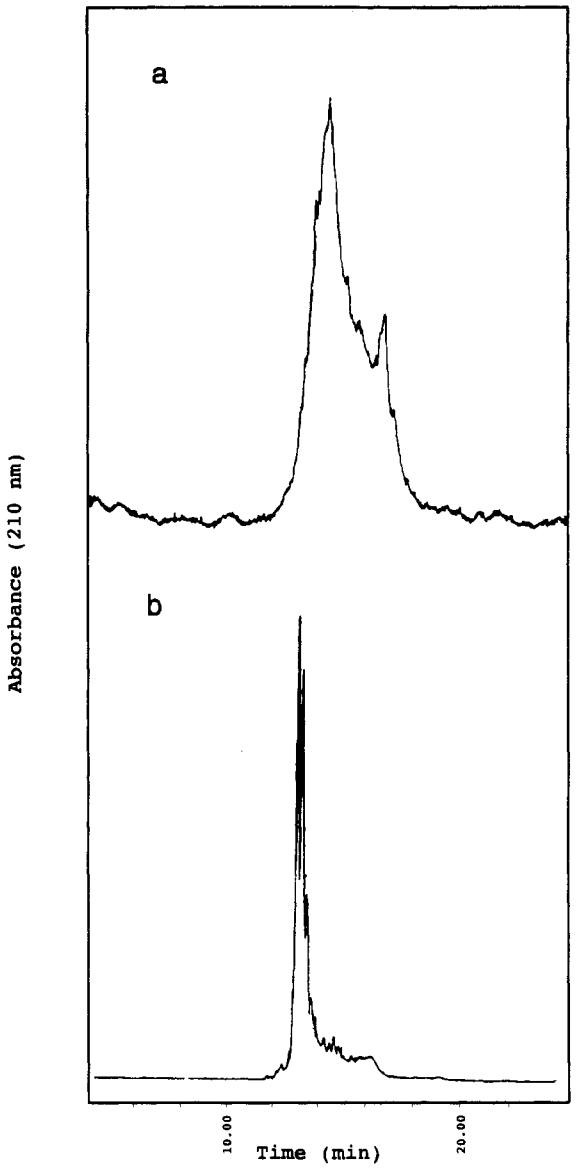

Figure 5. Capillary electrophoretic separation of non-C-terminal peptides from proteins of mature and immature peanuts treated by curing. Peanuts were cured by windrow drying for 24 h prior to protein extraction and digestion. Non-C-terminal peptides were obtained by applying an arginyl endopeptidase-digested protein mixture to a column of immobilized anhydrotrypsin, washing the column with 0.1 M sodium acetate buffer, pH 5.0, and eluting with 0.1 M formic acid, pH 2.5. (a) Immature peanuts; (b) mature peanuts.

fact that when treated by curing (i.e., windrow drying for 24 h), mature and immature peanuts were shown to differ in the peptide patterns (Figure 5; here, proteins from treated peanuts were prepared in the same way as those from the untreated). In the case of immature peanuts treated by curing, peaks were broad in shape (Figure 5a) as compared to the sharp peaks in mature treated peanuts (Figure 5b) or in untreated peanuts (data similar to Figure 5b). The similarity of peptide patterns (i.e., sharp peaks) between treated (mature) and untreated peanuts (immature or immature) indicated that curing has no effect on mature peanut proteins. In contrast, proteins from immature peanuts were affected by curing, possibly in a way that they changed in structure and became resistant to digestion by arginyl endopeptidase. As a result, large polymers instead of peptide fragments were produced, and when analyzed by CZE, they could appear as inseparable and broad peaks like those in Figure 5a. This change in peptide pattern caused by curing was an indication that immature peanut proteins are different in structure from those of mature peanuts.

Conclusion. This study applied the technique of peptide mapping to characterizing proteins from mature and immature peanuts. By digesting the proteins with an arginyl endopeptidase, treatment of the digested proteins with or without TCA, and subsequent analyses with such techniques as C_{18} reversed-phase HPLC, immobilized anhydrotrypsin (for preparing C-terminal peptides), and capillary zone electrophoresis, we demonstrated that proteins (prepared at 60–85% ammonium sulfate saturation) between mature and immature peanuts were structurally different on the basis of the difference in their peptide maps. Individual peptide fragments from protein digests such as those labeled as peptide **I**, a C-terminal peptide, from immature peanuts, and peptide **M**, a TCA-soluble peptide, from mature peanuts were identified. Further investigation is under way to determine the potential role of these peptides in peanut maturity.

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