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Studies of Lipid–Protein Interaction in Stored Raw Peanuts and Peanut Flours

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Peanuts readily undergo lipid oxidation because of their high polyunsaturated fatty acid content in both their triglycerides and polar lipid components. Defatted peanut flour contains residual lipids, which because of their various reactive groups can affect the ultimate quality of the flour destined for human consumption. The role of these lipids was investigated as to their involvement in protein–lipid interaction. Polar and nonpolar bound lipids were extracted from proteins with an acidic solvent system comprised of chloroform, methanol, and hydrochloric acid. The neutral fraction contained sterols, triglycerides, and esterified and free fatty acids. The polar fraction contained phospho- and glycolipids. The electrophoretic mobilities of the proteins that contained the bound lipids were changed after removal of the lipids by solvent extractions. Results of this study could be useful to understanding an important factor that affects the quality and stability of peanuts, products, and flours stored for long periods before their utilization.

INTRODUCTION

Lipid oxidation is known to be a major problem in the storage of fresh and processed foods. The oxidation process can adversely affect not only flavor, odor, and color qualities but also nutritive value. Lipid peroxides can cause damage to proteins, enzymes, and amino acids. The autoxidized products or precursors can possibly serve as toxic, mutagenic, or carcinogenic agents. These deleterious effects of autoxidation, as well as some that are beneficial, were previously described (Simic and Karel, 1980).

Peanuts readily undergo lipid oxidation because of their high polyunsaturated fatty acid content in both their triglycerides and polar lipid components. Lipoxygenase can oxidize lipids in raw peanuts, but its activity is lost during roasting (St. Angelo et al., 1979). Lipoxygenase, prepared from raw peanuts, was shown to catalyze the oxidation of linoleic acid and its methyl ester to form C-9 and C-13 hydroperoxides (St. Angleo et al., 1980a), which ultimately can degrade into secondary products that cause off-flavors (St. Angelo et al., 1980b). In roasted peanuts, lipids are oxidized by nonenzymic sources (St. Angelo et al., 1979). Hexane-defatted peanut flour was found to contain up to 2% polar lipids, which because of their reactive groups can also affect the ultimate quality of the flour destined for human consumption (St. Angelo and Ory, 1975).

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Lipid–protein interaction was examined in hexane-extracted meals and in the protein extracted from raw and roasted whole peanuts and peanut butter (St. Angelo and Ory, 1975a,b). Results on polyacrylamide gel electrophoresis (PAGE), with the use of a dual staining system, Amido Black for protein and Oil Red O or Rhodamine 6B for lipid, showed that at least three major protein components were associated with lipid. Upon storage, at least one of the lipid bands became noticeably denser and broader than the corresponding band in the fresh control samples. Extraction of the fresh and stored protein samples with hexane or chloroform/methanol (2/1) failed to remove any of the lipids that were associated with the three protein bands. Also, in each of these experiments, the electrophoretic protein banding patterns of extracts from the two solvent systems were identical. As part of our investigation on the effects of peroxidized lipids on food quality, and to better understand the nature of components responsible for adversely affecting food quality, we report in this paper on the interaction between lipids and proteins and a solvent system to remove bound lipids.

MATERIALS AND METHODS

Lipid standards were purchased from Supelco, Inc. (Bellefonte, PA). Catalase (C-10), insulin (I-550), ribonuclease-S-peptide (R-6125), and soybean lipoxygenase (L-7127) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA), albumin fraction V, and egg albumin were purchased from Nutritional Biochemicals (Cleveland, OH). Oil Red O (ORO) was purchased from Matheson, Coleman, and Bell (Nor-

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wood, OH). Infrared spectra of samples were obtained with a Perkin-Elmer Model 621 spectrophotometer.

Stored raw peanuts (Virginia, 56-R variety) were used in these studies. Fractionation of oil and proteins and solubilization of proteins from hexane-extracted meals were performed as described previously (St. Angelo and Ory, 1975a). The control samples were flours that had been extracted with hexane and then chloroform/methanol. "Bound" lipids (56 mg) were removed by extracting the hexane- and chloroform/methanol- (2/1) extracted flour (1 g) twice with 100 mL of chloroform/methanol/concentrated hydrochloric acid (2/1/0.03, CMH). After extraction, the precipitate was dried under nitrogen and then redissolved in either (a) 0.1 M Tris-HCl buffer, pH 7.2; (b) 0.05 M carbonate–bicarbonate buffer, pH 10.5; (c) the Tris-HCl buffer containing 1% sodium dodecyl sulfate (SDS); or (d) the carbonate–bicarbonate buffer containing 1% SDS. Protein content was determined by the method of Lowry et al. (1951). Buffer soluble and SDS buffer soluble protein solutions were analyzed for their protein and lipid banding patterns by electrophoretic methods described by St. Angelo and Ory (1977). In standard PAGE experiments, the running buffer was either Tris–glycine, pH 8.3 buffer, or the carbonate–bicarbonate, pH 10.5 buffer. Staining techniques for protein or lipid-associated protein fractions were as previously described (St. Angelo and Ory, 1975a). Electrophoretic migration was from the cathode (top) toward the anode (bottom). Buffer-soluble control and CMH-extracted protein fractions were also examined by IR spectroscopy.

The extracted lipid fraction was dried under nitrogen, transferred to stoppered vials, and placed in a freezer until used. Lipids were analyzed by thin-layer chromatography (TLC) on 20 × 20 cm standard silica gel G (0.250-mm) plates with a migration of 15 cm in a saturated chamber. For separation of neutral lipids, a solvent system containing petroleum ether/diethyl ether/acetic acid (90/30/1) was used. Polar lipids were chromatographed in solvent systems comparised of chloroform/methanol/water (87/32/4, 65/25/4). Spots were visualized and partially identified by spraying with cupric acetate/phosphoric acid solution, followed by heating for 20 min at 175 °C (Fewster et al., 1969). For polar lipid identification, other sprays were used on the TLC plates. These sprays included the following: Dragendorf reagent, Phospray, and Bromothymol Blue for phospholipids; diphenylamine and α-napthol for glycolipids.

RESULTS AND DISCUSSION

To determine whether the Oil Red O solution would stain rather pure proteins and to determine whether the dye is compatible with SDS, a series of experiments using PAGE-SDS and PAGE on several commercially available proteins (catalase, insulin, lipoxygenase, ribonuclease, bovine serum albumin) were conducted. In the SDS-PAGE system, ORO stained all of the protein bands, even after extraction with CMH, and thus showed that ORO could not be used on SDS gels.

Results from the PAGE system (without SDS) showed that catalase had four amido black (protein) bands, but no ORO-positive (lipid) bands; insulin had one protein band, but no lipid band; lipoxygenase had three protein bands, but no lipid bands; ribonuclease had one protein band, but no lipid band; bovine serum albumin had two protein bands, and, not surprising, one red-stained lipid band. Consequently, five different commercial bovine and egg albumin proteins were electrophoresed and stained for proteins and lipid. All proteins stained positive with both amido black and Oil Red O solutions. When the BSA was

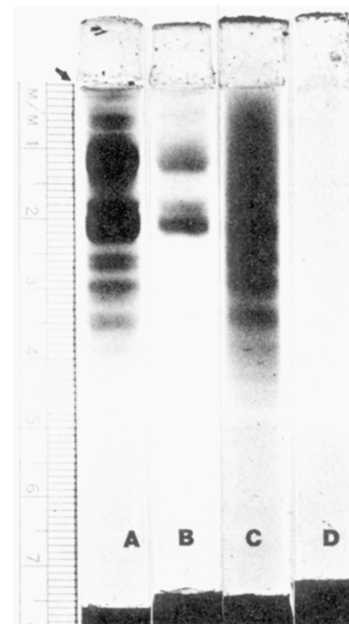

Figure 1. Protein and lipid banding patterns of raw stored peanuts electrophoresed in glycine–phosphate buffer, pH 8.3, without SDS. Samples: A and B, controls; C and D, CMH extract. A and C were stained with Amido Black; B and D were stained with Oil Red O. Protein concentration was 0.4 mg/gel.

extracted with the CMH solvent system, electrophoresed and then stained with the dual staining solutions, only the amido black protein bands were observed. There were no Oil Red O stained bands.

Polyacrylamide gel electrophoresis was used to study the separation of proteins from flours extracted from stored raw peanuts. Figure 1 shows the protein banding patterns from peanuts that were stored for 2 years at room temperature (25 °C) and electrophoresed in Tris–glycine buffer, pH 8.3. The first two gels, 1A and 1B, represent the control samples, whereas the second two, 1C and 1D, represent the CMH-extracted protein. Two of the gels were stained for protein, 1A and 1C, and two were stained for lipid, 1B and 1D. The control sample contained at least three major protein bands (11, 18, and 20 mm) and several minor bands. Although the CMH-extracted sample contained several protein bands throughout the gel, at least two (11 and 18 mm) of the three major proteins found in the control, 1A, were absent. In the gels stained for lipid, the three major protein bands were stained red in the control sample, but no lipid bands were observed in the CMH-extracted samples; compare 1B with 1D. These data indicate that the CMH solvent was able to extract the lipid from the proteins and, in so doing, either dissociated the proteins or at least changed their electrophoretic mobility and solubility. When the two protein samples were extracted with chloroform/methanol (2/1), the banding patterns observed were identical with those found in the hexane-extracted controls, i.e. there were no apparent changes in the protein banding patterns; the major proteins were present and the lipid-stained bands appeared when the gels were stained with Oil Red O. These results confirmed those reported previously (St. Angelo and Ory, 1975a). The absence of the major protein bands in the extracted protein may be attributed to the fact that the extracted protein is less soluble in the Tris buffer, pH 7.2, than the control protein sample. After redissolving the dried CMH-extracted protein with Tris buffer and centrifuging, the clear supernatant was applied to the gels. The large precipitate that remained in the centrifuge tube

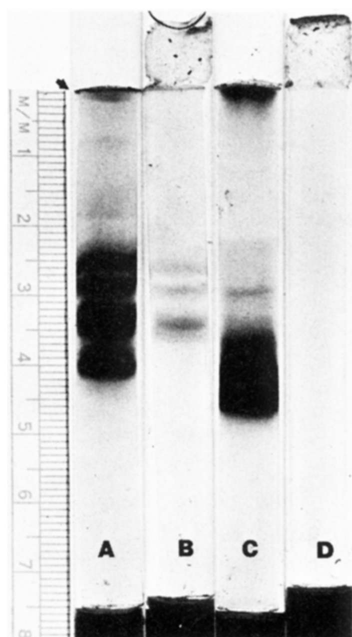

Figure 2. Protein and lipid banding patterns of raw stored peanuts electrophoresed in bicarbonate buffer, pH 10.4, without SDS. Samples: A and B, control; C and D, CMH extract. A and C were stained with Amido Black: B and D were stained with Oil Red O. Protein concentration 0.36 mg/gel.

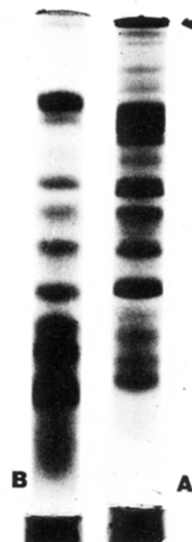

Figure 3. Protein banding patterns of raw stored peanuts electrophoresed in Tris-HCl buffer, pH 7.2, in SDS system. Samples: A, control; B, CMH extract. Protein concentration was 0.16 mg/gel in sample 3A and 0.33 mg/gel in sample 3B.

had similar banding patterns as the control after characterizing by PAGE-SDS gel electrophoresis.

In a different medium, bicarbonate buffer at pH 10.5, the protein banding patterns observed (Figure 2) were not only different from one another but also different from those obtained in the pH 8.3 buffer system. The banding patterns for the controls, 2A and 2B, showed four amido black stained protein bands of deep intensity and three lipid-stained bands, respectively. The CMH-extracted protein sample, 2C and 2D, indicated one very broad faster moving protein band (39–46 mm) of high-staining intensity, a smaller band (38 mm) of less intensity, and a small protein band (30 mm) of low intensity. This protein banding pattern of the CMH-extracted sample was different from the control and much faster moving, possible due in part to the neutralization of the positive-charged amino groups in the extracted protein and/or possibly to mild oxidation of the disulfide bonds. The proteins found in the CMH-extracted sample moved as one large protein band. When the gels were stained for lipid with Oil Red O, three red bands were observed in the control, but none were observed in the experimental sample; compare 2B with 2D. These data again suggested that the CMH was able to remove lipids from the proteins, while changing the electrophoretic mobility of the proteins.

Whereas the CMH-extracted protein appeared to become less soluble in pH 7.2 Tris buffer, it was very soluble in the pH 10.5 carbonate buffer system. The major proteins that were not observed in Figure 1C were possibly the proteins that comprised the broad band from 39 to 46 mm. High concentrations of proteins were intentionally applied to the gels (Figures 1 and 2) to show that the failure to detect any lipid-stained bands on the CMH-extracted proteins was probably not a concentration effect.

To further investigate the change in the electrophoretic mobility observed and to compare the monomeric forms of the two proteins, the control and the CMH-extracted protein sample were dried under nitrogen, extracted with 1% SDS/mercaptoethanol solutions, and electrophoresed on SDS-PAGE gels. The major banding patterns and the electrophoretic mobilities of the control and CMH-extracted protein samples are shown in Figure 3, parts A and B, respectively. These results showed that the control protein was dissociated into its monomeric form, which is comprised of several major and minor bands, including a large protein band that did not migrate into the gel. After the control protein was extracted with CMH, the electrophoretic pattern, 3B, indicated that almost all of the extracted proteins were solubilied by the SDS solution, owing to less protein remaining at the origin when compared to that of 3A. Moreover, the proteins that were soluble in SDS were dissociated into smaller monomeric forms that gave a different banding pattern than observed in 3A. These results are similar to those reported by Jacks et al. (1982), who showed that arachin, the major peanut storage protein, exposed to 30% hydrogen peroxide was dissociated into smaller monomeric subunits than when exposed to lesser harsh treatment such as peroxide-free oil, organoleptically rancid oil, or 3% hydrogen peroxide. They also showed conformational changes in arachin not only by the change in its electrophoretic mobility but also by changes in the antigenicity and in the far ultraviolet circular dichroic spectra of the protein. Later, Jacks et al. (1983) showed that arachin, exposed to acidic hexane (comprised of 5 or 20% acetic acid), increased electrophoretic mobility in PAGE gel systems, but not in SDS-PAGE gels. The exposed protein showed no change in infrared or circular dichroic spectra. They interpreted their results as an irreversible dissociation of arachin into subunits, each of which maintained the native secondary structure. From our results, as judged from the changes in electrophoretic mobility of the acidic solvent extracted protein, the increase in electrophoretic migration was not caused by the oxidation of any "bound" lipid but was probably due to the effects of the hydrochloric acid solvent system. According to Kates (1972), lipids in hydrophobically associated form may be extracted with relatively nonpolar solvents such as chloroform, whereas membrane-associated lipids require polar solvents such as methanol to disrupt hydrogen bonding or electrostatic bonds between lipids and proteins. Covalently bound lipids must be first cleaved by acid or alkaline hydrolysis. Consequently, the lipids in this study were possibly extracted owing to the ability of the acid to increase polarity, which causes a disruption

of hydrogen bonds or electrostatic forces between lipids and proteins. In addition, covalently bound lipids could have been cleaved from the complex by acid hydrolysis and then removed by solvent extraction.

The IR absorbance studies of the control and CMH-extracted peanut samples showed small but noticeable differences between them. There was a broad band indicating absorption at 1100 cm^{-1} and a sharp band at 985 cm^{-1} for the control sample, whereas there were no corresponding bands in the extracted sample. In the extract, there was a slight but definite increased absorption at 1585 cm^{-1} relative to 1650 cm^{-1}. In the control, there was a slight but definite increased absorption at 1240 and 1060 cm^{-1}. The IR spectra of the two samples showed that amides I, II, and V, bands occurring at 1650, 1540, and 700 cm^{-1}, respectively, were essentially the same. Therefore, any conformational changes sufficient to affect the spectral locations of those bands were not induced by the acidic solvent extraction system. However, examination of the IV and VI amide bands, areas from 555 to 630 cm^{-1}, showed some differences between the two samples. In the control, there were bands at 629, 580, and 555 cm^{-1}. The CMH-extracted sample showed bands at 613 and 521 cm^{-1}. The 555-cm^{-1} band found in the control completely disappeared in the CMH-extracted sample. In the control, the intensity of the amide IV band (near 620 cm^{-1}) was higher than the amide VI band (near 600 cm^{-1}) whereas this phenomenon was not found for those bands in the CMH-extracted sample. These results were similar to that reported by Jacks et al. (1975), who showed that predominance shifted from amide VI to amide IV as arachin was heated. They also showed by circular dichroism (CD) studies that the conformational mode of the protein had shifted from order to disorder as the temperature increased. Results from our IR analyses for the amides I, II, and V bands did not indicate that a conformational change occurred after extracting the protein with the acidic solvent. However, the change in the amides IV and VI bands suggested a change in secondary structure based on the combination of IR and CD studies of Jacks et al. (1975).

Upon removal of the lipids by the CMH solvent system, a preliminary study was initiated to characterize the residual extracted lipids. This study showed that the lipid fraction contains both nonpolar and polar lipids. By comparing the R_f's of known nonpolar standard compounds, the nonpolar fraction was found to contain several compounds, which included the following: a long-chain fatty acid, an esterified fatty acid, a cholesteryl ester, and mono-, di-, and triglycerides. Upon examination of the polar lipids, the presence of phospholipids and glycolipids in the extracts was confirmed by applying to the TLC plates particular stains that are specific for phosphorus-containing compounds or for sugars, as described in Materials and Methods. Attempts to characterize the polar lipid fractions on TLC by comparing R_f's to those of known polar lipid standards (phosphatidylethanolamine, -serine, -choline, -inositol, -glycerol, and lysolecithin, phosphatidic acid, monogaloactosyl diglyceride, diagalactosyl diglycride) were unsuccessful. In their preparation of oil and protein from glanded cottonseed by solvent extraction with mixtures of hexane and 2–25% acetic acid, Hensarling et al. (1974) and Hensarling and Jacks (1975) were able to extract ca. 9.7% more total lipids than with hexane. Of this total, 3–4% were neutral lipids. Later, Hensarling and Jacks (1983), again with the acetic acid/hexane solvent system reported on extracting 6% more neutral lipid from soybeans and from a 5- to 35-fold increase in phospholipids from cottonseed and soybeans than with hexane alone. These authors had earlier concluded that the extra amount of lipids that was extracted can be attributed to the increased permeability of the membrane caused by the hexane/acetic acid solution (Hensarling et al., 1974). Moreover, Jacks et al. (1974) showed an ultrastructural disparity between cottonseed tissue extracted with hexane and tissues extracted with acidified hexane. The membranes appeared fuzzy and diffuse rather than crisp and neat as in hexane-extracted tissue. They concluded that the disorganized membranes allowed passage of solvents and the solvent–lipid mixtures through membrane-bound, cytoplasmic structures. In our system, we used approximately 1% concentrated HCl. However, we have been able to decrease the acid content to as little as 0.33% and still remove the bound lipid.

In conclusion, we have shown that Oil Red O and SDS are not compatible and, therefore, should not be used simultaneously. We have also shown that extracting peanuts with either hexane or chloroform/methanol solutions does not remove all of the lipids. Due to the secondary structures of the proteins, some of the lipids are difficult to extract and are possibly inaccessible to those solvents. A possible source of rancidity or at least a potential cause for decreased shelf life exists since some of the lipids remain in the flour. However, when an acid is added to the solvent system, the lipids are removed concomitantly with an alteration in the protein secondary structure. Those residual lipids were characterized as both polar and nonpolar. Of future interest are their isolation and identification.

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