may be due to the polar, hydrophilic characteristics of DMA-2,4-D.

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# Immunochemical-Cytological Study of Proteins from Partially Defatted Peanuts

Navin J. Neucere* and Thomas Hensarling

To help open new markets for peanuts and peanut products, Southern Regional Research Laboratory is developing various processing conditions to obtain low-fat peanuts (Pominski *et al.*, 1970; Vix *et al.*, 1967). In this study, we report integrated analyses correlating cytological ultrastructure with immunochemistry and electrophoresis of proteins of seeds that were partially defatted by hydraulic pressing and reconstituted with hot water. Pressing alone does not appreciably affect the qualitative disk and immunoelectrophoretic patterns of the proteins; however, the hot water treatment does. Protein solubility is negligibly reduced by pressing out the oil but reconstitution with hot water reduced it twofold. Electron micrographs of the pressed and reconstituted seeds showed progressive disruption of subcellular organelles and membranes. Trypsin inhibitors were inactivated after the hot water treatment.

The conventional approach to preparing peanuts for snack use is either dry or oil roasting. For peanuts and some of the edible tree nuts, the process achieves desirable texture and flavor, and in some cases mild heat improves nutrition. A simple step to remove excess oil from peanuts, while increasing protein content, is one way of providing nutritious products for the general public. Though it is recognized that vitamins and minerals are also essential to good nutrition, high-quality protein foods are a special case where people are demanding more options at moderate prices.

Laboratory-prepared peanut flours subjected to heat, pressure, and steaming under several conditions of time and temperature have received much attention in recent years (Anantharaman and Carpenter, 1969; McOsker, 1962; Neucere *et al.*, 1972; Woodham and Dawson, 1968). Changes in physicochemical properties of peanut proteins under various processing conditions and general characterization of the proteins have also been reported (Daussant *et al.*, 1969; Dawson, 1971; Dieckert *et al.*, 1962; Neucere, 1972; Neucere *et al.*, 1969; Tombs, 1965). All of these studies revealed the complexity of peanut proteins as evidenced by results from several different types of analyses.

In this article we report an integrated study of cellular ultrastructure and electrophoretic-immunochemical properties of proteins from seeds that were deoiled by hydraulic pressing followed by reconstitution with hot water. Solubility differences and trypsin inhibition affected by processing are also discussed.

## MATERIALS AND METHODS

Virginia peanut cotyledons were hydraulically pressed and reconstituted with hot water as described by Vix *et al.* (1967). Seeds were pressed out at 2000 psi for 60 min and then placed in hot water from 3 to 8 min. The deoiling process removed about 75% of the oil, leaving seeds with 40–45% protein. The expansion step (hot water) resulted in seed containing about 40% moisture; the finished product is then achieved by roasting to remove most of the moisture.

For chemical analyses: (1) full-fat seeds were used as the control; (2) the seeds were pressed; and (3) the reconstituted deoiled seeds were defatted with equal volumes of cold acetone (5 g of seed per 20 ml of acetone). The peanut flours were then extracted in phosphate buffer, pH 7.9, ionic strength 0.2, with a hand homogenizer at room temperature (50 mg of flour per 1 ml of buffer). The homogenate was centrifuged at 35,000 × *g* for 20 min and the final supernatants were used in the chemical analyses.

Protein contents were determined by the method of Lowry *et al.* (1951). Immunoelectrophoresis on microscope slides was carried out according to Grabar and Williams (1953) in 1.5% Ionagar (Oxoid Ltd., London) in 0.025 *M* veronal buffer, pH 8.2, at room temperature with a voltage gradient of 4 V/cm for 2 hr. Disk electrophoresis was performed according to Steward *et al.* (1965) using 7.5% acrylamide in the running gel, and a modification of the standard Canalco procedure (Canalco Industrial Corporation, Bethesda, Md.) using 7.5% Cyanogum 41 (Fisher Scientific Company) gelling agent in the running gel and 3.0% of same in the stacking gel; tris-glycine buffer, pH 8.4, was employed. Fixation for electron microscopy was performed according to Luft (1956) and tissue embedding in epoxy resin was performed according to Spurr (1969).

## RESULTS

The morphological changes induced on the fine structure of the peanut by processing are shown in Figures 1, 2, and 3. A typical cell in the native dormant seed (Figure 1) shows the major subcellular organelles—aleurone grains, spherosomes, and starch grains. Spherosomes, which con-

Southern Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, New Orleans, Louisiana 70179.

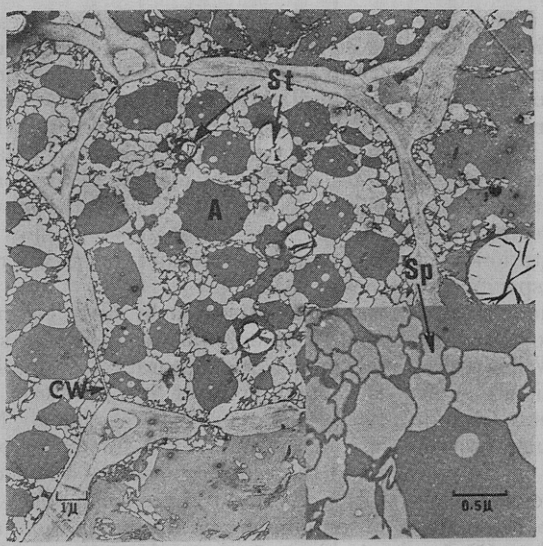


**Figure 1.** Electron micrograph of native peanut cotyledon cell showing major organelles. Nomenclature: A, aleurone grains; St, starch grains; Sp, spherosomes or fat bodies; and CW, cell wall.

tain oil, have been isolated and characterized by Jacks *et al.* (1967). They appear morphologically similar to the corresponding particles of intact plant and animal cells. In peanuts these lipid bodies contain less than 1% protein and approximately 1% phospholipid. The composition of aleurone grains has also been reported (Daussant *et al.*, 1969). After removal of oil by pressing (Figure 2), no major changes were observed for the starch grains, aleurone grains, and normal subcellular membranes, but the absence of fat bodies is obvious. Reconstitution with hot water induced more drastic changes in cellular integrity (Figure 3). Here all normal subcellular structures were disrupted; however, the cell walls remained intact. Starch grains were fused into irregular masses which stained blue with iodine.

Disk electrophoregrams of proteins in the control and the processed seed are shown in Figure 4. Identical samples in A and B were analyzed by two slightly different techniques mentioned earlier. Hydraulic pressing did not change the banding pattern appreciably, though a slight increase in electrophoretic mobilities (anodal shift) was apparent for all the protein zones (compare 3 and 4 to 7 and 8). After reconstitution with hot water, the zones appeared slightly more diffused and heterogeneous. Some of

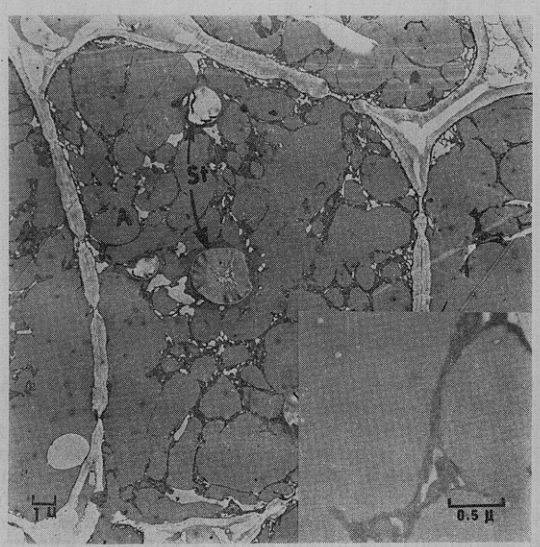


**Figure 2.** Electron micrograph of a typical peanut cotyledon cell after hydraulic pressing. Designations same as in Figure 1.

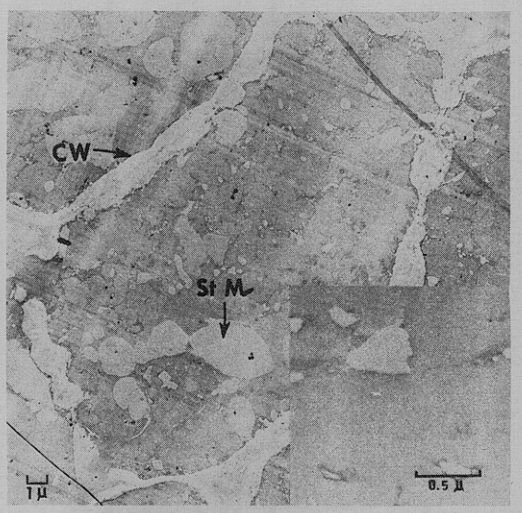


**Figure 3.** Electron micrograph of peanut cell after reconstitution with hot water. Note the crushed appearance of starch grains leaving a diffused mass (St M).

the faster migrating components (brackets in 8 and 12) decreased in concentration after the hot water treatment; note also the heavier staining at and near the origin of electrophoresis in samples 9 through 12.

Denaturation of specific proteins is more obvious as evidenced by immunoelectrophoresis depicted in Figure 5. Pressing alone does not change the typical precipitin arcs (compare 1 and 2 to 3) but reconstitution (sample 4) inactivates all the proteins antigenically except for $\alpha$-arachin (arrow A) and one other densely stained component (arrow C). Note also the trace of precipitin around the origin, which moved slightly toward the anode. Using immune serum against an isolated peanut fraction (classic fractions II and III from DEAE-cellulose chromatography of Dechary *et al.*, 1961), only two of the four antigenic proteins in that fraction showed marked precipitin lines (compare 5, 6, and 7) after deoiling. Note that the arc designated by arrow B in sample 7 is more intense than in the control (samples 5 and 6), suggesting a relative increase in solubility of this protein after pressing. After reconstitution, only a trace of proteins in the isolated fraction was antigenically active, as evidenced by analysis of sample 8. (Note a trace of precipitin around the well.)

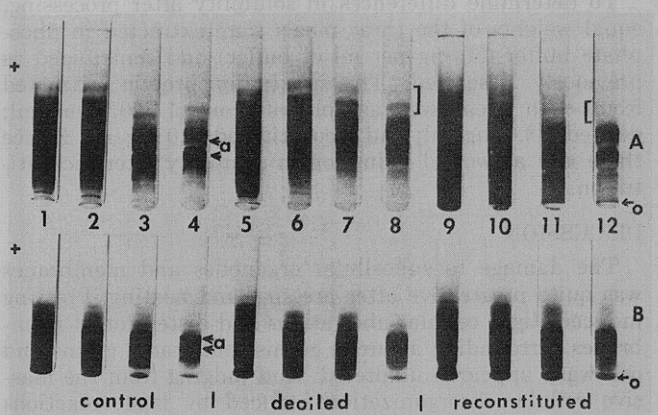


**Figure 4.** Disk electrophoresis of proteins in native (control) and processed seed. Nomenclature: A, Steward *et al.* (1965) technique; B, modified Canalco technique; samples 1–4, control seed; samples 5–8, deoiled seed; samples 9–12, reconstituted seed (serial dilution starting with 2.0 mg of protein for each sample); a, polymeric forms of $\alpha$-arachin; o, origin of electrophoresis. Electrophoresis in part A at 3.0 mA/tube for 1 hr and in part B at 2.0 mA/tube for 1 hr. Gels were stained with 0.1% Amido Black in 7.0% acetic acid and destained with 7.0% acetic acid.

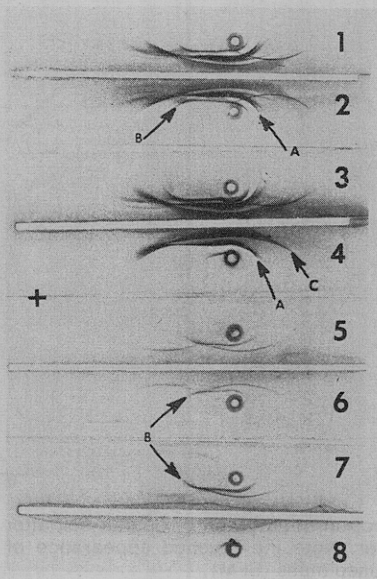


**Figure 5.** Immunoelectrophoreograms of proteins from native (control) and processed seed. Nomenclature: wells 1, 2, 5, and 6 contained protein extract from control seed; wells 3 and 7 contained protein extract from deoiled seed; wells 4 and 8 contained protein extract from reconstituted seed (0.2 mg of protein in each well); troughs below 1 and 3 were filled 4 times with immune serum against total peanut proteins; troughs below 5 and 7 were filled 4 times with immune serum against fractionated proteins (Gps II and III from Dechary *et al.*, 1961). Precipitin lines were stained with 0.1% Amido Black in 7.0% acetic acid and destained with 7.0% acetic acid after slides were dried. Arrows A point to $\alpha$-arachin, arrows B refer to a contaminant of $\alpha$-arachin, and arrow C refers to an unidentified component.

Protease inhibitors, in general, are of concern to nutritionists. The effect of pressing and reconstitution with hot water on trypsin inhibitors is shown in Figure 6. Interpretations of results are based on a previous report which showed that trypsin induced an anodal electrophoretic shift of the major protein, $\alpha$-arachin, after exposure for about 30 min (Daussant *et al.*, 1969). Comparison of samples 1 and 2 with samples 3 and 4 showed that the inhibitor(s) were not affected by pressing but were completely inactivated after reconstitution. Trypsin (0.1%) was added to extract 2 and 4 about 1 hr before electrophoresis. Note the shift of $\alpha$-arachin in sample 4.

To determine differences in solubility after processing, equal weights of the three meals were extracted in phosphate buffer (50 mg per ml of buffer) and centrifuged as previously described. The maximum protein extracted from each meal was as follows: control, 20.0 mg/ml; deoiled, 18.0 mg/ml; and reconstituted, 9.0 mg/ml. Hence there was a twofold reduction in solubility after reconstitution.

## DISCUSSION

The damage to subcellular organelles and membranes was quite progressive after pressing and heating. Pressing induced slight cellular aberrations and distention of membranes surrounding aleurone grains, but starch grains and cell walls appeared unaltered. And judging from the massive cellular disorganization induced by heat, reactions that followed were undoubtedly complicated and difficult to explain. Lipid–protein and carbohydrate–protein interactions in such systems are well known, but their exact chemical nature remains obscure. For amino acids and peptides reacting with sugars, the Maillard reactions have been studied extensively (Ellis, 1959). Though many of these reactions are unspecific, the progress of specific interactions can best be measured by spectroscopy, polarography, radioactive tracing, and other techniques.

In this study, a relationship of the protein changes determined with disk electrophoresis and immunoelectrophoresis was not readily apparent. Hence, minor conformational changes that inhibited antigen–antibody precipitin interactions were not necessarily reflected by migration in polyacrylamide gels and *vice versa*. The latter technique approximates changes in molecular size (or association–dissociation of proteins) and charge; the absence of precipitant arcs in immunoelectrophoresis implies disorientation of determinant groups that normally react with specific antibodies.

The process of protein denaturation by heat leading to insoluble aggregates has been an object of numerous studies. Whether the mechanism of denaturation is simple or multistate as heat is applied, however, is a matter of controversy (Brandts, 1969; Poland and Scheraga, 1965). Most globular proteins vary in the number of free SH groups, both on the surface and those buried inside the molecule. Therefore, each protein does not necessarily obey the same mechanism of unfolding and recombination after heating. Obviously, in the present study, more extensive investigations of individual proteins are required to theorize mechanisms. Although solubility was reduced twofold after reconstitution, qualitative analysis by immunochemistry of the soluble portion suggested that at least two of the proteins (including $\alpha$-arachin) retained native conformations. Consequently, since the bulk of protein in the seed consists of $\alpha$-arachin, only part of it underwent inversible denaturation. Apparently, the time of exposure to hot water was not long enough to affect all the molecules.

Because antigenic properties of proteins are destroyed, this does not necessarily mean that the nutritional quality of the product was impaired. In a previous study, for example, a meal from imbibed peanuts that were heated at 110° for 1 hr had a relatively high protein efficiency ratio (1.8 compared to 1.4 for the control and 2.5 for casein), yet all but two of the proteins retained their immunological properties (Neucere *et al.*, 1972). Though actual feeding experiments were not carried out in the present study, the final product probably corresponded to meals subjected to mild wet heat.

In vegetable and oilseed protein products, the presence of trypsin inhibitors and aflatoxins in large quantities can impair nutrition and have deleterious effects on humans and animals. Heating peanut meals at high moisture levels (comparable to reconstitution) has been shown to reduce the level of aflatoxins (Mann *et al.*, 1967). And as we have shown in this study, trypsin inhibitors are readily inactivated by reconstitution. Thus, this high protein peanut is excellent in these two respects.

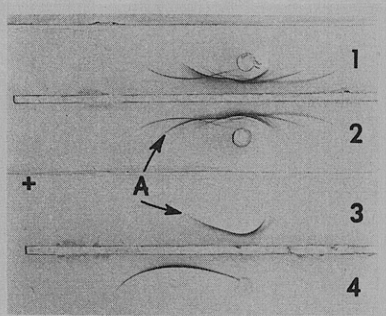


**Figure 6.** Immunoelectrophoreograms of proteins showing the effects of hydraulic pressing and reconstitution with hot water on trypsin inhibitor activity. Nomenclature: samples 1 and 2 correspond to peanut proteins from seeds hydraulically pressed, and samples 3 and 4 refer to the proteins from reconstituted seed. 0.1% trypsin was added to samples 2 and 4. Trough below 1 was filled with immune serum against the total proteins of the peanut and the trough below 3 was filled with immune serum against $\alpha$-arachin. Slides were finished as described in Figure 5. Arrows A point to $\alpha$-arachin.

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# Stachydrine: Content in Alfalfa and Biological Activity in Chicks

Margaret A. Connor,* J. Benjamin Stark, James C. Fritz,[1] and George O. Kohler

Stachydrine (proline betaine) has been identified as the major quaternary nitrogen base in alfalfa. Its content in dehydrated alfalfa meals has been positively correlated with protein contents. More stachydrine was present in the leaves than in the stems. A chick feeding study showed that stachydrine decreased the incidence of perosis.

Some confusion has existed over the identity of the quaternary nitrogen bases present in alfalfa. Steenbock (1918) first isolated and identified stachydrine (proline betaine) as a component of alfalfa hay, and later Vickery (1925) found that it was the principal quaternary base present in alfalfa. However, a recent report, "A Study of the Major Nutritional Constituents of Dehydrated Alfalfa" (American Dehydrators Association, 1965), lists the major base to be betaine (glycine betaine). Due to the possible physiological differences of the two bases, studies were conducted in this laboratory to identify the quaternary bases present, to determine which are the major constituents, to develop a satisfactory method for their determination, and to examine their biological activity.

## EXPERIMENTAL SECTION

**Identification of Quaternary Ammonium Compounds Present in Alfalfa.** A partially deproteinated pressed juice from fresh alfalfa, concentrated to 54.2% solids (Bickoff *et al.*, 1968), was fractionated according to the ion-exchange procedure of Stark (1962). The fraction containing the quaternary ammonium compounds was collected and concentrated (organic base concentrate).

A 95-mg sample of this organic base concentrate in 2.5 *N* HCl was fractionated on Dowex 50W (200–400 mesh), according to the procedure of Christianson *et al.* (1960). The order of elution and extent of separation of stachydrine, betaine, and choline were determined by chromatography of control materials.

Four 500-ml fractions and one 1-l. fraction of effluent were scanned for quaternary nitrogen compounds by measurement of the ultraviolet absorption of the periodide derivative (Wall *et al.*, 1960). Further identification was carried out by thin-layer chromatography on silica gel/Kieselguhr (25/75 by weight) using water (100%) or ethanol–ammonia (95:5) as the developing solvent systems. A modified Dragendorff reagent (Bregoff *et al.*, 1953) was used for detection. Identification of bands was carried out by comparison of $R_f$ values of known samples of choline, stachydrine, betaine, and trigonelline (betaine of *N*-methyl nicotinic acid).

Trigonelline in the organic base fraction was determined using the spectrophotometric procedure of Moores and Greninger (1951).

**Routine Procedure Developed for Determination of Stachydrine in Alfalfa.** An ion-exchange procedure was used to separate choline and stachydrine as follows. Dehydrated alfalfa (10 g) was blended with 500 ml of 80° water in a blender for 15 min. Approximately 10 g of Celite was added and the extract was filtered with suction. The filter cake was washed three times with 100-ml portions of water. Water was added to the combined filtrate to reach a final volume of 1 l. and 500 ml was passed through a 20-cm$^3$ column of Dowex 50 (H+) X-8, 50–100 mesh resin. The resin was washed with 60 ml of water and eluted with 100 ml of 1 *N* ammonium hydroxide, followed by 40 ml of water applied in small aliquots. The combined ammonia eluate and subsequent washings were evaporated on a rotary evaporator to remove free ammonia and the remaining solution was acidified with 2 ml of 6 *N* HCl and made to 100 ml with water. Analyses were carried out using the method of Focht *et al.* (1956). Concentration was estimated from a standard curve prepared from stachydrine–HCl reineckate.

Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Berkeley, California 94710.
[1]U. S. Food and Drug Administration, Bureau of Science, Washington, D. C. 20204.