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Gel Electrophoretic Analysis of Peanut Proteins and Enzymes. I. Characterization of DEAE-Cellulose Separated Fractions

John P. Cherry,[1] Joseph M. Dechary, and Robert L. Ory*

The total proteins of Virginia 56R peanuts were solubilized by extracting the seeds with dilute phosphate buffer. The individual components were separated by DEAE-cellulose chromatography into eight major fractions which were then characterized by polyacrylamide disk gel electrophoresis. Using immunoelectrophoretic analysis, the peanut trypsin inhibitor was localized in the albumins fraction. Seven enzyme activities were examined by polyacrylamide disk and starch gel electrophoretic techniques. Zymograms of these enzymes, similar to those in the total cotyledonary extracts, seemed to be confined mostly to the fractions containing albumins and smaller molecular weight globulins. These studies provided information that may be potentially useful in the preparation of high-quality protein concentrates and in identification of specific changes in proteins caused by conditions used during processing of peanut meals into concentrates or isolates.

There have been many investigations on the reserve proteins of peanuts because of their potential usefulness as food supplements. In general, these studies have shown that most of the proteins are easily extractable (Altschul *et al.*, 1961, 1964a,b; Dawson, 1971; Dechary and Altschul, 1966; Dechary *et al.*, 1961) and can be separated into two major fractions, arachin and conarachin (Daussant *et al.*, 1969a,b; Dechary *et al.*, 1961; Evans *et al.*, 1962; Neucere, 1969; Neucere and Ory, 1970). More extensive purification of these two fractions (Dawson, 1971; Evans *et al.*, 1962; Tombs, 1965; Tombs and Lowe, 1967; Neucere, 1969) has revealed that they are composed of complex large molecular weight globulins (α-arachin, α-conarachin), plus some other closely related components, not completely separated by the usual techniques.

Gel electrophoresis has been used extensively to characterize and identify proteins and enzymes in biological systems (Cantagalli *et al.*, 1971; Cherry *et al.*, 1970, 1971a,b, 1972; Dawson, 1971; Haikerwal and Mathieson, 1971; Minetti *et al.*, 1971; Neucere and Ory, 1970; Sastry and Virupaksha, 1967; Tombs, 1963) and to detect experimentally induced modifications in these molecules (Jensen, 1959; Neucere, 1972; Neucere *et al.*, 1972).

Cherry *et al.* (1971b) and Cherry and Ory (1973a,b,c) have shown that the electrophoretic protein and enzyme profiles of individual peanuts are very complex, with much variation within the cultivars examined. The complexity of these electrophoretic patterns made it difficult to identify specific proteins that varied qualitatively and/or quantitatively. Identification of groups of proteins showing polymorphism could provide information that might be used during the fractionation of peanut proteins to produce concentrates or isolates of good amino acid balance. Also, standardization of these electrophoretic protein patterns could help in identifying changes in proteins caused by processing conditions.

For this investigation, peanut protein extracts were separated into eight fractions by DEAE-cellulose chromatography. The protein and enzyme components of each fraction were further characterized by polyacrylamide disk and starch gel electrophoretic techniques.

MATERIALS AND METHODS

Seeds. Virginia 56R certified peanut seeds were obtained "in shell" from a commercial supplier in Holland, Va. The seeds were shelled and hand-selected for uniform size and quality through the courtesy of W. K. Bailey.

Fractionation of Peanut Proteins. Most of the fractions were prepared by the method of Dechary *et al.* (1961). Fraction IV was separated into V and VI by chromatography over DEAE-cellulose in pH 8.0, I = 0.06, phosphate buffer, with a linear gradient of 0–0.5 *M* sodium chloride. Protein content of each fraction was determined by the method of Lowry *et al.* (1951). Arachin was purified by cryoprecipitation as described by Neucere (1969).

Gel Electrophoretic Techniques. Protein samples were dissolved in phosphate buffer, pH 7.9, I = 0.01, and examined by polyacrylamide (Cherry *et al.*, 1970) and starch (Brewbaker *et al.*, 1968) gel electrophoresis. Each fraction was examined at three protein concentrations (1.0, 0.6–0.8, and 0.4 mg/gel) to determine the major as well as all minor electrophoretic bands in each preparation. Methods for determining enzyme activities within each fraction were described by Cherry and Ory (1973a,b).

Immunochemical Techniques. Peanut trypsin inhibitor activity was analyzed according to Daussant *et al.* (1969a).

RESULTS AND DISCUSSION

Fractionation of Conarachin and Arachin. The complexity of the total proteins of peanut cotyledonary ex-

Oilseed and Food Laboratory, Southern Regional Research Laboratories, New Orleans, Louisiana 70179.
[1] Present address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843.

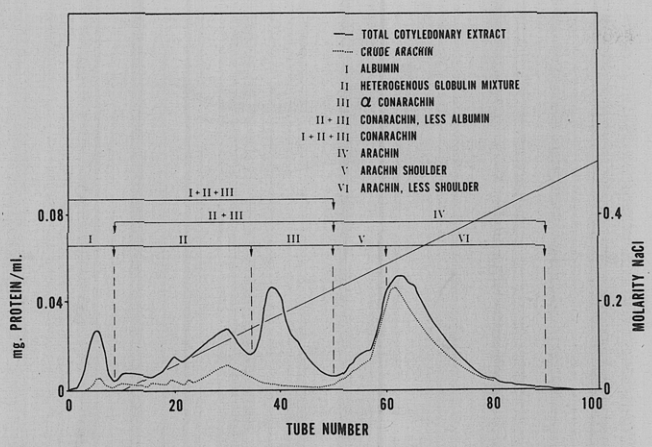

Figure 1. Chromatographic elution patterns of peanut proteins fractionated on DEAE-cellulose. The straight line indicates the NaCl gradient from 0 to 0.5 *M*.

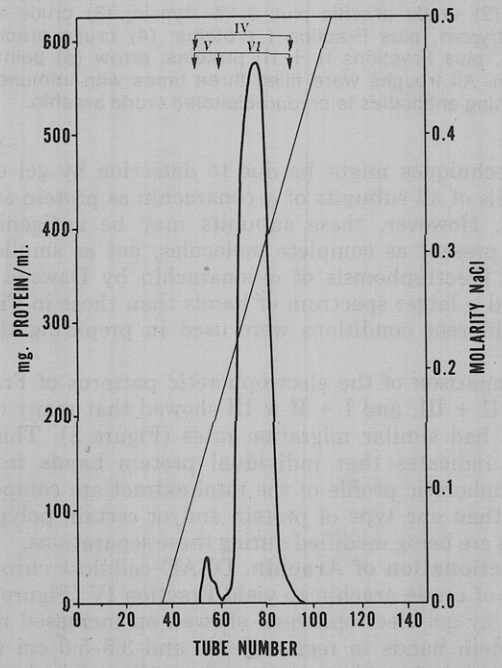

Figure 2. Chromatographic elution pattern for separation of Fraction IV peanut proteins into two components on DEAE-cellulose. The straight line indicates the NaCl gradient. Fractions IV, V, and VI proteins are identified in Figure 1.

tracts (*i.e.*, 10% sodium chloride or pH 7.9 phosphate buffer, I = 0.01, soluble fractions from acetone powders) is reflected in the chromatographic (Figures 1 and 2) and electrophoretic (Figures 3 and 4) patterns. This extract, containing albumins (Figure 1, I) and mixed globulins (Figure 1, II, III, and IV), was further separated into the two classic groups of proteins—arachin (Fraction IV: insoluble after dialysis against 0.9% sodium chloride or precipitated by 40% ammonium sulfate) and conarachin (Fraction I + II + III: soluble after dialysis against 0.9% sodium chloride or precipitated by 85% ammonium sulfate after removal of arachin).

DEAE-cellulose chromatography revealed that the conarachin fraction could be separated into three definable groups: Fraction I (albumins); Fraction II + III (heterogeneous globulin and enzyme mixture); and Fraction III (α-conarachin). Crude arachin had a chromatographic elution pattern similar to that of Fraction IV, but the latter was more purified (Figures 1 and 3). Further chromatography of Fraction IV (arachin) yielded Fractions V and VI (Figures 2 and 3).

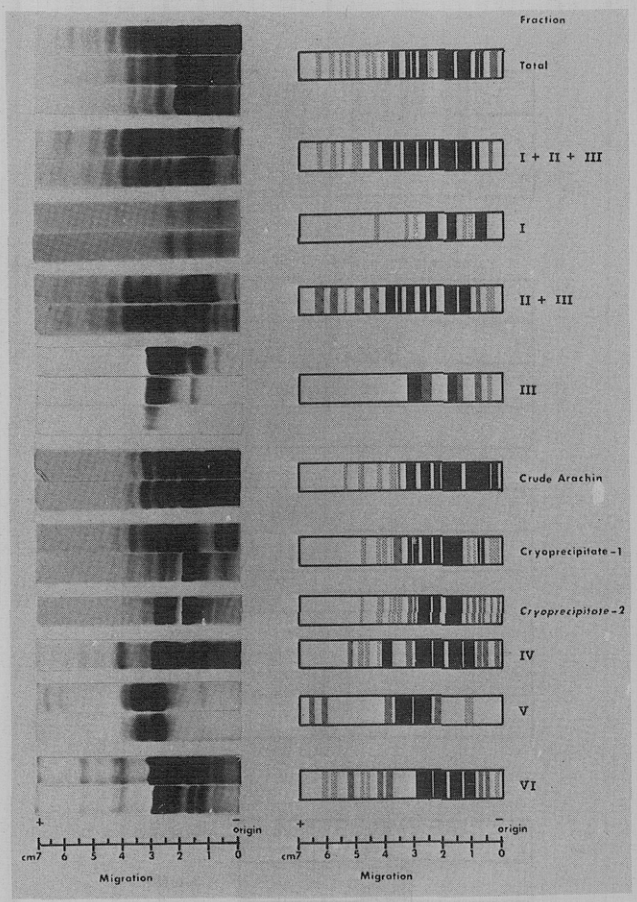

Figure 3. Polyacrylamide gel electrophoretic and diagrammatic patterns of peanut proteins fractionated on DEAE-cellulose and by cryoprecipitation. Identification of Fractions is shown in Figure 1.

The combination of gel electrophoresis with DEAE-cellulose chromatography confirmed that the separation of the two classical fractions (conarachin and arachin) was not complete (Figures 1 and 3). Many of the same protein bands (region 1.0–4.0 cm in Figure 3) were observed in Fractions I + II + III and IV and DEAE-cellulose chromatography showed arachin to be contaminated with conarachin proteins (Figure 1). Cryoprecipitation specifically separated arachin from conarachin (Figure 3; Neucere, 1969), but insoluble residues from the redissolved cryoprecipitate yielded a conarachin fraction that still contained some arachin. Also, electrophoretic patterns varied for different preparations of cryoprecipitates (Figure 3; cryoprecipitates 1 and 2), suggesting that purification of seed globulins by this technique may be some sort of equilibrium reaction dependent upon the conditions employed.

As reported by Dawson (1971), Tombs (1963, 1965), and Tombs and Lowe (1967), these data confirm that the peanut reserve proteins are primarily arachin and conarachin, which are themselves made up of smaller subunits difficult to separate by the usual fractionation procedures. An attempt to further isolate and characterize the proteins within the conarachin and arachin fractions follows.

Fractionation of Fraction I + II + III. A number of qualitative and apparently quantitative variations in major (dark staining; region 2.0–4.0 cm) and minor (light staining; *e.g.*, region 4.0–5.0 cm) protein bands distinguished the gel pattern of the total extract from that of conarachin, Fraction I + II + III (Figure 3). In addition, fewer bands were observed in region 4.0–6.0 cm of the conarachin fraction than in the total extract. Each of the eight bands observed in the pattern of the albumins

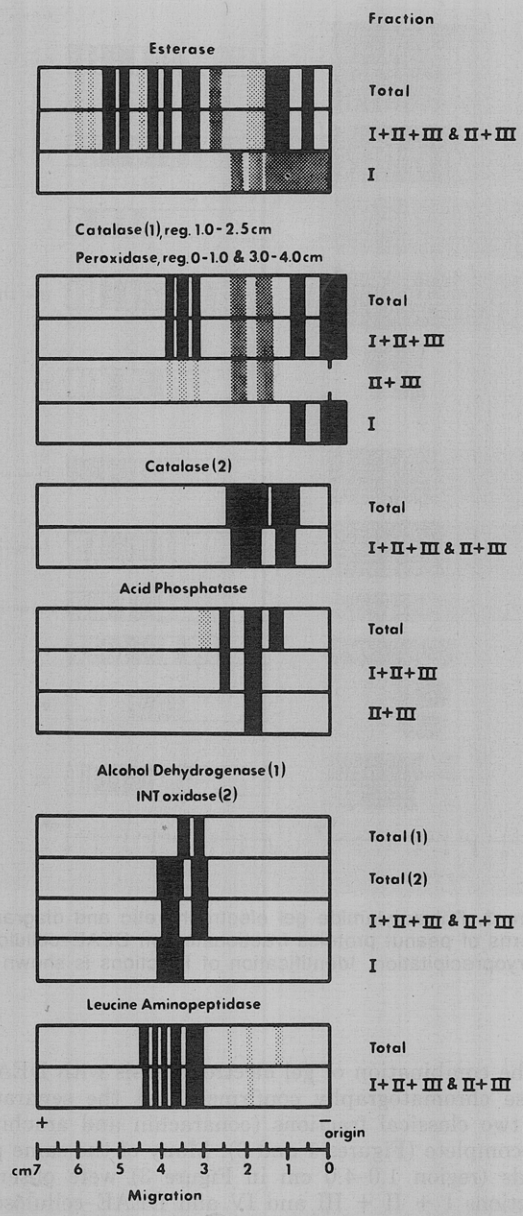

Figure 4. Diagrammatic sketches of polyacrylamide disk and starch gel zymograms of enzymes in peanut proteins fractionated by DEAE-cellulose. Identification of Fractions is shown in Figure 1.

(Fraction I) could be correlated with a specific region in Fraction I + II + III (Figure 3), though quantitative differences are apparent within the bands of these two profiles. Separation of Fraction I from the conarachin fraction produced some quantitative but no qualitative changes in the electrophoretic profile of the remaining proteins in Fraction II + III (Figure 3). Removal of the albumins rendered the protein bands in the lower half of the gel (region 4.0–7.0 cm) more distinct.

Further chromatographic separation of Fraction II + III globulins into its components (Fractions II and III) did not produce a different pattern for Fraction II, suggesting that these proteins are a heterogeneous mixture of low molecular weight globulins and α-conarachin that is difficult to fractionate. Chromatographic purification of α-conarachin, the Fraction III major peak in Figure 1, resulted in a much simpler pattern: two major (regions 1.5 and 3.0 cm) and two minor bands (regions 0.5 and 1.0 cm) in Figure 3. Immunoelectrophoresis of this fraction by Daussant *et al.* (1969a,b) produced only two major precitin arcs: an anodic protein (α_1-conarachin) and a cathodic protein (α_2-conarachin). The differences between these

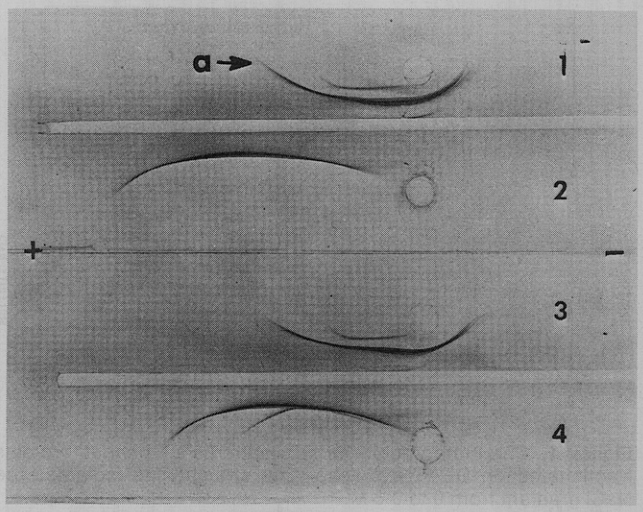

Figure 5. Localization of peanut trypsin inhibitor by immunoelectrophoresis. Samples in wells: (1) crude arachin (cryoprecipitate); (2) crude arachin plus 0.1% trypsin; (3) crude arachin, 0.1% trypsin, plus Fraction I proteins; (4) crude arachin, 0.1 trypsin, plus Fractions II + III proteins; arrow (a) points to α-arachin. All troughs were filled three times with immune serum containing antibodies to cryoprecipitated crude arachin.

two techniques might be due to detection by gel electrophoresis of all subunits of α-conarachin as protein-staining bands. However, these subunits may be antigenic only when present as complete molecules, not as smaller subunits. Electrophoresis of α-conarachin by Dawson (1971) showed a larger spectrum of bands than those in Figure 3, but different conditions were used in preparing the protein.

Comparison of the electrophoretic patterns of Fractions I, III, II + III, and I + II + III showed that many of their bands had similar migration rates (Figure 3). This similarity indicates that individual protein bands from the electrophoretic profile of the total extract are composed of more than one type of protein and/or certain polypeptide chains are being modified during these separations.

Fractionation of Arachin. DEAE-cellulose chromatography of crude arachin to yield Fraction IV (Figure 1) followed by gel electrophoresis showed an increased number of protein bands in regions 0–2.0 and 3.5–5.0 cm (Figure 3) of the latter when compared to the original arachin. This dissociation became more evident upon further purification of Fraction IV into Fractions V and VI (Figures 2 and 3). An increased number of smaller proteins was observed in the lower half of the gel for Fraction VI (region 3.5–7.0 cm, Figure 3). Also, the major large molecular weight proteins in region 0–3.0 cm were further dissociated into distinct major and minor bands. The minor protein band in region 3.0–3.5 cm of Fraction IV was greater in Fraction V but was absent in Fraction VI (Figure 3). Proteins in region 2.5–3.0 cm were common to all three fractions.

Purification of the crude arachin fraction by both DEAE-cellulose chromatography and cryoprecipitation, then analyzed by electrophoresis (Figures 1–3), shows, as have others (Dawson, 1971; Tombs, 1963, 1965; Tombs and Lowe, 1967) the complexity of the different proteins in peanuts. Chromatography of arachin over DEAE-cellulose apparently alters the surface charge(s) of the intact molecule, resulting in a gradual release or fragmentation of subunits. This could cause the variations in electrophoretic patterns for total protein extracts compared to the corresponding proteins in separated fractions.

Characterization of Selected Enzymes. The isozyme patterns of esterase, catalase, peroxidase, acid phosphatase, leucine aminopeptidase, alcohol dehydrogenase, and INT-oxidase (iodophenyl-nitrophenyl-tetrazolium violet

oxidase) produce achromatic regions of enzyme activity on the same gels with alcohol dehydrogenase (Brewer and Sing, 1970; Larson and Benson, 1971) of total cotyledonary extracts were compared in all of these different fractions (Figure 4). Most enzyme activity was found in the conarachin fraction (Fraction I + II + III) and was similar to that of the total extract. Some diffuse activity was associated with arachin but this may be simple adsorption of enzyme molecules to this globulin (Evans *et al.*, 1962). Most of the peroxidase activity was located in Fraction I (region 0–1.0 cm). Three other peroxidase isozymes (region 3.0–4.0 cm) had little activity and seem to be present in Fraction II + III (small globulin fraction). Peroxidase has been employed in food quality control as an index of blanching temperatures (Gardner *et al.*, 1969) and, with certain other enzymes, has been implicated in the development of off-flavors during food storage (Acker and Beutler, 1965; Wagenknecht, 1959). Since most of the peroxidase activity is localized in Fraction I (albumins), analysis of this fraction may, therefore, be a useful tool in quality control during the preparation of protein isolates or concentrates. Alternatively, the early removal of Fraction I may prevent or retard the production of off-flavors.

Further purification of the conarachin fraction showed that the esterase, catalase, and leucine aminopeptidase activities were virtually unchanged from the patterns present in the total protein extracts. Except for some diffuse esterase activity in region 0–2.0 cm of Fraction I (albumins), these three enzymes were localized in the smaller globulins of the conarachin fraction. Fraction I also contained one of the two INT-oxidase bands at 3.5–4.0 cm, whereas both bands were present in conarachin (Fractions I + II + III plus II + III). Alcohol dehydrogenase activity was faint in the crude DEAE-cellulose separated Fraction I + II + III, but activity of this enzyme may be adversely affected by the isolation techniques. Other potential uses of the enzymes described here may provide additional biological indicators useful in food processing.

Localization of Peanut Trypsin Inhibitor. Immunoelectrophoretic analysis for the presence of peanut trypsin inhibitor is shown in Figure 5. Daussant *et al.* (1969b) showed that the major peanut globulin, α-arachin, shifted anodically after exposure to 0.1% trypsin. This effect was inhibited by addition of soybean trypsin inhibitor to the reaction mixture. In the present study, when Fraction I proteins were added to a 0.1% trypsin solution to which α-arachin was subsequently added, they inhibited trypsin hydrolysis of arachin. Such inhibition was not affected by other protein fractions (Figure 5), indicating that the peanut trypsin inhibitor is located in Fraction I. Separation of Fraction I proteins may be utilized to obtain large quantities of peanut trypsin inhibitor for further study or to remove it from protein isolates.

A thorough understanding of the nature, stability, and interaction of proteins obtained from seed meals is essential in developing new products. The effects of thiol-reducing reagents and/or frozen storage on certain properties of peanut proteins, presented in an adjoining paper (Cherry and Ory, 1973c), offer an additional means of obtaining useful information during the preparation of high-quality protein products from oilseeds.

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