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Antigenic and Electrophoretic Changes of α-Arachin after Heating *in Vitro*

The two major protein zones detected in the cold-insoluble fraction of the peanut by electrophoresis consist of subunits of α-arachin. After heating dry for 1 hr, α-arachin showed indistinct electrophoretic and immunologic properties above 145° and was nonantigenic after heating above 175°. Between 110 and 195°, decreased solubility of α-arachin followed an exponential-like curve. After heating the cold-insoluble fraction in solution for 1 hr, immunoprecipitation of α-arachin was partially identical to that of the control at 100° but was completely inhibited when heated above that temperature. These results suggested modifications in the steric arrangements of determinant groups in α-arachin.

The immunological characterization of some of the peanut proteins was reported by Daussant *et al.* (1969). From this study the major protein in the classic arachin fraction of Johns and Jones (1916) was named α-arachin. Analysis by acrylamide electrophoresis showed that the two major protein zones contained in the cold-insoluble fraction of the peanut proteins were protomers of α-arachin (Neucere, 1969, 1972). Previous studies (Neucere *et al.*, 1969; Thomas and Neucere, 1973) showed differential stability of general proteins and enzymes after applying wet and dry heat to whole seeds, and α-arachin was the most stable. The purpose of the present research was to investigate further the effects of wet and dry heat on "isolated" α-arachin as measured by electrophoresis, immunochemistry, and solubility changes.

PROCEDURE

Heat Treatments and Protein Extractions. Preparation of the cold-insoluble fraction from Virginia 56-R peanuts was reported previously (Neucere, 1969). The material used in the present study was the precipitate before ammonium sulfate fractionation (P_2 in above reference). Five samples (freeze dried) of 100 mg each were dissolved in 1.0 ml of phosphate buffer, pH 7.9, ionic strength 0.2, sealed in vials and were heated for 1 hr at 80, 90, 100, 110, 120, 130, 145, and 155°, respectively. Similar samples were dry-heated in open Petri dishes for 1 hr at 110, 120, 130, 145, 155, 175, and 195°, respectively. Each of these was extracted in 1.0 ml of the above buffer for analysis.

Analytical Methods. Protein contents were determined by the method of Lowry *et al.* (1951). Immunoelectrophoresis (IEA) and simple electrophoresis were carried out according to Grabar and Williams (1953) in 1.5% Ionagar gel (Oxoid, Ltd., London) and Agarose (simple electrophoresis). (It is not the policy of the Department to recommend the products of one company over those of any other engaged in the same business.) Electrophoresis was conducted in 0.025 *M* veronal buffer, pH 8.2, with a voltage gradient of 4 V/cm for 2 hr at room temperature. Antibody-in-gel electrophoresis was done according to Laurell (1966) in 1.5% Ionagar employing 10 V/cm for 16 hr at room temperature. Double diffusion was performed according to Ouchterlony (1949). Disk electrophoresis was conducted by the method of Davis (1964) using 7.5% acrylamide in the running gel and 3.0% in the stacking gel; the electrophoresis was performed at a constant current of 3 mA per tube for approximately 1 hr at 5°. All protein zones on Agarose slides, disk gels, and immunoprecipitates were stained with 0.1% Amido Black in 7.0% acetic acid and destained with 7.0% acetic acid.

RESULTS AND DISCUSSION

The immunoelectrophoretic analysis of the dry-heated samples is shown in Figure 1. Up to 130° (sample 4), essentially no precipitin variation from that of the control was observed. However, the minor antigenic protein or the so-called α-arachin contaminant (arrow 1) was inactivated at higher temperatures. At 155°, the precipitin line of α-arachin appeared diffused with a slight anodic shift. Analysis after heating at 175° showed a wide indistinct line that formed a double arc (arrow 2 in sample 7), but all antigenic activity was destroyed at 195°.

From the solubility changes induced by dry heat presented in Figure 2, it is evident that denaturation was not an "all" or "none" process. At 130°, for example, solubility was decreased to one-half, yet some antigenic species of α-arachin remained in solution. The relationship of solubilized protein and temperature is approximately exponential within the range indicated.

Analyses of samples containing equal protein concentrations heated in solution are shown in Figures 3 and 4. Part A of Figure 3 showed the relative changes in electrophoretic migration of the precipitin complexes. At 80°, a slight increase in migration of the conical peaks were observed for both α-arachin (a) and the arachin contaminant (ac). However, at 90° the reverse was observed for α-arachin and the contaminant was completely inactivated; only a trace of activity was observed at 100° and no reaction occurred at 110°. The increase in migration could be due to either an increase in charge of the antigens or a reduction of active determinant groups on the antigens. On

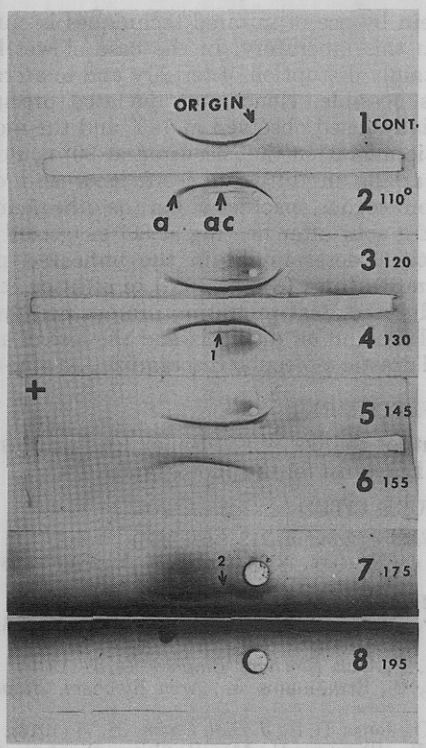

Figure 1. Immunoelectrophoresis (IEA) of proteins in the cold-insoluble fraction after heating dry for 1 hr. Nomenclature: sample 1, control; other samples heated at designated temperatures; a, α-arachin, ac, arachin contaminant. Each trough was filled three times with immune serum against the total peanut proteins and each well contained approximately 0.5 mg of protein for electrophoresis.

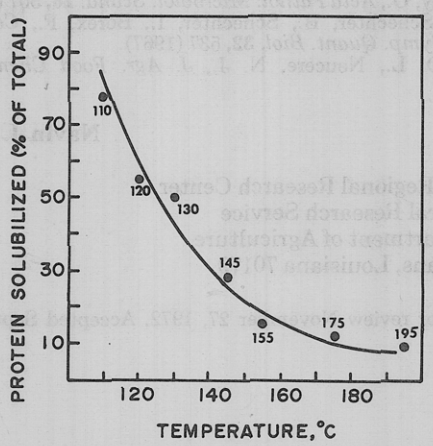

Figure 2. A plot of protein solubilized *vs.* temperature on dry samples of the cold-insoluble fraction heated for 1 hr. Protein assay on supernatants from samples containing 100 μg of dry powder (17.3% N) per ml of phosphate buffer pH 7.9, ionic strength 0.2.

the other hand, reduction in migration reflects decreased overall molecular charge or possibly the exposure of additional antigenic sites. Double diffusion of the same samples in parts B and C showed similar results. The reaction of partial fusion after heating at 100° (wells 4 in B and C) suggested major molecular disorganization without complete destruction of determinant groups. Note also the slight spur (dashed arrow) which formed between the control and the sample heated at 80° (wells 1 and 2 in B) indicating a reaction of partial identity.

Analysis of samples heated at higher temperature is shown in Figure 4. Both agarose and disk electrophoresis indicated that some of the protein did not migrate appreciably after being heated at 110° and 120° (note the arrows in samples 2 and 3). At 130° and at higher temperatures,

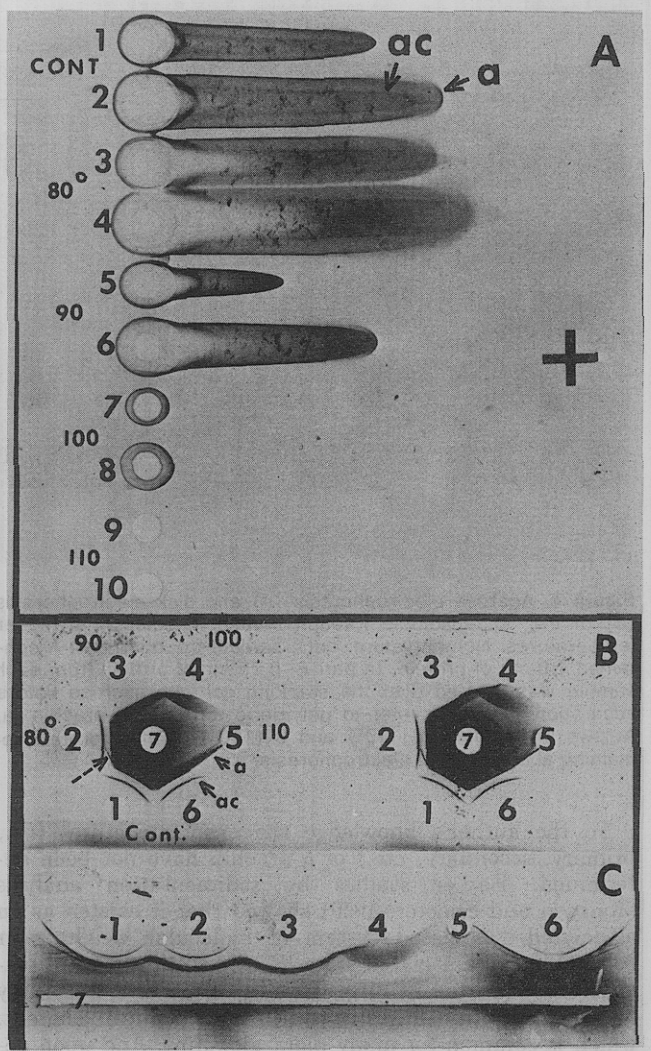

Figure 3. Antibody-in-gel (A) and double diffusion (B and C) analyses of samples heated in solution for 1 hr. Nomenclature: in part A, the odd- and even-numbered wells contained 30 and 60 μg of protein, respectively; 2% immune serum against the cold-insoluble fraction was mixed in the agar. In part B, all antigen wells on the left contained 300 μg of protein and 600 μg on the right. The antibody wells (7) were filled three and six times, respectively, with the same immune serum. C is identical to B (left) using a different diffusion pattern; the trough (7) was filled three times with immune serum. Reference sample numbers are the same in B and C. a refers to α-arachin and ac to the arachin contaminant.

a smear of protein positive material that progressively increased in mobility was observed.

For most globular proteins, partial or complete loss of antigenic specificity is generally due to modifications of sequential and/or conformational determinants. A sequential determinant is maintained by a distinct amino acid sequence in random coil form, whereas conformational specificity depends crucially on disulfide bonds and/or side chain polar groups that maintain steric structure of the antigen (Sela *et al.*, 1967). A study of heat denaturation of ovalbumin in solution by Arat (1966) showed that even if the secondary structure of the native antigen was modified, some determinant groups reacted weakly with the native antibody. However, the reactions of native antigen with its denatured antibody were stronger than that of the denatured antigen. Hence, the immunologic behavior of denatured protein depends on specific structure and cannot be generally described. As suggested by Sela *et al.* (1967), the lack of cross-reaction or any reaction deviating from the "normal" precipitin reaction must be due to modification(s) of the antigen molecule.

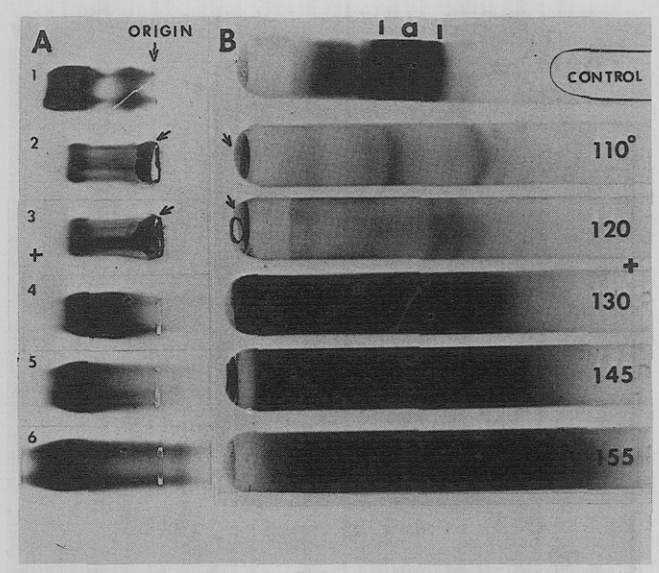

Figure 4. Agarose electrophoresis (A) and disk electrophoresis (B) of the cold-insoluble fraction heated in solution at higher temperatures. Nomenclature: each sample trough in part A contained 1.0 mg of protein. In part B, 0.75 mg of protein from each sample was layered over the stacking gel with sucrose before separation. Brackets refer to polymeric forms of α-arachin, a. Arrows in samples 2 (110°) and 3 (120°) point to protein remaining at the origin of electrophoresis.

To the author's knowledge the exact structures (*i.e.*, primary, secondary, etc.) of α-arachin have not been determined. Earlier studies by sedimentation analysis (Johnson and Shooter, 1950) showed that it existed as an associated–dissociated system of molecular weight near 380,000 in the associated state. Rotatory properties of arachin were reported by Jirgensons in 1958. From this study his calculations suggested very little helical content, which is common for many plant globulins. And upon denaturation of globular proteins in general, an increase in levorotation or in molecular disorder is often observed (Jirgensons and Straumanis, 1957).

Consequently, only qualitative interpretation of the data from the present study was possible. In the case of dry heat, the diffused precipitin reaction for α-arachin that revealed a coalesced double arc could reflect depolymerization of subunits that have weak and overlapping antigenicity. Further analysis on the effects of dry heat on this protein by use of infrared techniques is currently underway in this laboratory. In the case of wet heat below 110°, certainly disruption of tertiary and quaternary structures was possible. Hence, the deviated precipitin reaction partially fused observed at 100° and the reduced electrophoretic migration that occurred at 90° could be due to species varying in diffusion coefficients and charge but having homologous specificity. On the other hand, the formation of a spur after heating at 80° suggested a serologically distinct determinant in the unheated protein. At higher temperatures (110 to 155°) in solution, the appearance of diffused, fast migrating protein positive zones on agarose slides and on disk gels and the loss of antigenicity suggested drastic molecular disorganization of α-arachin.

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Direct Determination of Carbaryl by Gas–Liquid Chromatography Using Electron Capture Detection

A reproducible direct method for simultaneous determination of small quantities (0.2–15 ng) of carbaryl and its hydrolysis product, α-naphthol, is described. Gas–liquid chromatography using electron capture detection, which improves sensitivity over other direct methods of carbaryl determination, has not been previously described.

Carbaryl (1-naphthyl-*N*-methylcarbamate) is presently determined by a variety of gas–liquid chromatographic methods using Coulson conductivity and flame ionization (Coulson, 1966; Riva and Carisano, 1969; Zielinski and Fishbein, 1965). Another technique, involving conversion of the pesticide to various derivatives and subsequent gas–liquid chromatographic analysis, though more sensitive, is indirect and time consuming.

Electron capture detection, described below, combines high sensitivity and efficiency in the determination of carbaryl and its hydrolysis product, α-naphthol.

MATERIALS

A Tracor MT-220 gas–liquid chromatograph equipped with a nickel-63 high temperature electron capture detector was used. All solvents were from Burdick and Jackson