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Effects of Heat *in situ* on Electrophoretic Patterns of Reserve Proteins and Enzymes in Dormant Peanuts (*Arachis hypogaea* L.)

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The effects of heat on general (soluble) proteins and several enzymes in wet- and dry-roasted peanuts were determined by starch gel electrophoresis. Staining of general proteins was more obvious in extracts of seed wet-roasted at 155° than in extracts of seed dry-roasted at that temperature. No apparent migrational differences were observed for α-arachin with increasing temperatures. New fast moving bands did not appear at higher temperatures, but the migrations of proteins near the origin were slightly different from that in the control. Wet-roasting reduced enzyme activity more than dry-roasting for all enzymes assayed; most enzymes exhibited some activity in peanuts dry-roasted up to 130°, but differential thermostability of isoenzymes was observed. Aqueous extracts of seeds defatted with acetone–hexane showed general protein and enzyme electrophoretic patterns similar to extracts defatted by centrifugation of native seed.

The effects of heat on peanut proteins heated at 145° for 1 hr were investigated by chromatography on DEAE-cellulose, polyacrylamide gel electrophoresis, immunoelectrophoresis, and ultracentrifugation (Neucere *et al.*, 1969). Immunochemical and other analyses indicated that the major reserve globulin, α-arachin, remained antigenic during the heating process but showed a reduction in its solubility and diffusion coefficient in agar. The effects of heating *in situ* at several different temperatures on the general proteins of imbibed and dry peanut seed were studied by disk electrophoresis and immunochemistry (Neucere, 1972). The disk gels showed greater differences in protein migration after dry heat than after wet heat. Immunoelectrophoretic patterns indicated that α-arachin maintained antigenicity in both wet- and dry-heated seeds but other proteins were progressively inactivated antigenically.

Most work with heat sensitivity of isoenzymes has been performed *in vitro* with whole extracts of cells or tissues or with purified isoenzymes. Studies of animal tissues showed considerable variations in thermal stability of lactate dehydrogenase (LDH) isoenzymes (Fondy *et al.*, 1964; Knudsen *et al.*, 1970; Vesell *et al.*, 1968). Crude cell-free extracts from *Bacillus subtilis* heated at 60 and 70° for 30 min showed marked differences in stability of two bands of malate dehydrogenase (MDH) (Antohi *et al.*, 1970). Other studies of maize and HeLa cells showed differential heat sensitivities for acid phosphatase isoenzymes (Efron, 1970; Tan and Aw, 1971).

The aim of this report was to investigate, by starch gel electrophoresis, the general effects of heat *in situ* on the major proteins and several common enzymes in soaked and dry peanut seed.

EXPERIMENTAL SECTION

Preparation of Heated Samples and Controls. The heat treatment was described by Neucere (1972) and is briefly discussed here. One-year-old seeds of *Arachis hypogaea* L. (Virginia 56-R, 1968 crop) were roasted either dry or after full imbibition. One kilogram of dry seed was soaked 16 hr in distilled water at 25°, blotted, and placed for 1 hr in a shallow pan in a forced air temperature-regulated oven preset to designated temperatures (°C). Each batch of seeds was extracted with hexane–acetone (1:1) at a ratio of 1 g of tissue per 3 ml of solvent at 5°. Based on 0.1 g of meal per ml extracted at room temperature in phosphate buffer, pH 7.8, ionic strength 0.2, the following amounts of protein were solubilized from each sample (Table I).

The controls used in this study (samples 11 and 12) were prepared from acetone–hexane defatted seeds that were unheated and from full-fat unheated seeds; the same phosphate buffer was used for extraction. All extracts were frozen and stored at −20° before experimentation.

Preparation of α-Arachin. α-Arachin was partially purified from crude phosphate buffer extracts of peanut cotyledons by cold-precipitation (2°) according to Neucere (1969).

Application of Samples in Gels. To increase protein quantities for each sample, the application paper was dipped twice into the extract with drying between each dipping before inserting the sample into the gel. The dipping-drying procedure did not alter the banding patterns but effectively increased protein concentration; this procedure with α-arachin is discussed later.

Electrophoresis. Procedures for horizontal starch gel electrophoresis were similar to those of Smithies (1955) and Poulik (1957) and were described previously (Thomas and Brown, 1970).

Enzyme Assays. To demonstrate nonspecific α-esterases (α-EST), the starch slices were incubated 2 hr at 25° in 100 ml of 0.2 *M* phosphate buffer, pH 6.0, containing 75 mg of Fast Blue RR salt, 1.5 ml of 1% α-naphthyl acetate in acetone–water 1:1 (v/v), and 10 ml of 1-propanol (absolute). Leucine aminopeptidase (LAP) was detected by incubating the gels 2 hr in 100 ml of 0.2 *M* phosphate buffer, pH 4.4, containing 20 mg of L-leucyl-β-naphthylamide HCl, and 25 mg of Black Salt K. The solution for the malate dehydrogenase (MDH) assay consisted of 100 ml of 0.1 *M* Trisma Base buffer, pH 8.5, 3 ml of neutralized 2 *M* *dl*-malic acid, 50 mg of β-diphosphopyridine nu-

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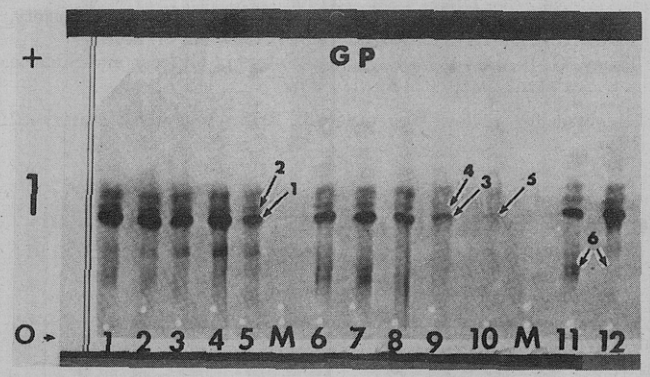

Figure 1. Zymograms showing general protein (GP) and enzyme patterns in wet-roasted, dry-roasted, and nonheated dormant peanuts. Numbers 1–12 are sample numbers given in experimental procedures and are used in all figures. Sample 13 refers to a concentrated solution of a total protein extract. M represents the bromophenol blue indicator measuring the front of electrophoresis. Stippling beside some zymograms corresponds to position of bands; degree of shading illustrates intensity of staining. These conditions also apply to Figures 2–5. General proteins (GP) stained with Amido Black.

cleotide, 50 mg of MTT tetrazolium, and 10 mg of phenazine methosulfate. The peroxidase stain consisted of 100 ml of 0.1 *M* phosphate buffer, pH 6.0, 1 ml of 3% hydrogen peroxide, and 0.5 g of pyrogallol as the hydrogen donor. All enzyme stains were prepared immediately prior to use and sonicated to maximize dissolution of components.

Consistent with isoenzyme studies of MDH, it was assumed that each band or bands represented a reaction with a specific substrate only. A basic assumption was that MDH was specific for *dl*-malic acid, hence enzymes producing two or more bands are referred to operationally as isoenzymes. For LAP it was assumed the enzyme system was specific for L-leucyl-β-naphthylamide. The peroxidases are referred to here as peroxidases or isoperoxidases and not isoenzymes since it is probable that the enzyme(s) oxidizing pyrogallol may also utilize one or more of several phenolic compounds as hydrogen donors. A large number of esterase enzymes may hydrolyze the common substrate α-naphthyl acetate, so each band may represent several different enzymes. These esterases are called nonspecific esterases or α-esterases.

General Protein (GP) Stains. Soluble proteins were stained with 0.1% Amido Black in 3% acetic acid or by using a bromophenol blue stain, according to Bailey (1967). In the latter procedure, a treating solution contained 10 g of mercuric chloride, 0.5 g of bromophenol blue, and 20 ml of glacial acetic acid made to 1 l. with water. Gels were immersed in this solution for 1 hr and then stained for 1 hr with 0.1 g of bromophenol blue, 50 g of zinc sulfate, and 50 ml of glacial acetic acid made to 1 l. with water. Because background staining was much less for this stain than for Amido Black, it was used as a counter stain. All gels were destained in 3% acetic acid.

Table I.[a]

	Wet heat (1–5)					Dry heat (6–10)				
Sample	1	2	3	4	5	6	7	8	9	10
Temp heated, °C	110	120	130	145	155	110	120	130	145	155
Soluble protein per ml	30	22	24	22	13	39	30	23	15	8

[a] Lowry *et al.* (1951).

RESULTS

Compared to the controls, no significant differences in the density of the bands or the distribution pattern of general proteins were observed for soaked seed heated up to 145° (Figure 1). At 155°, however, the intensity of staining of the reserve protein α-arachin (Figure 1, arrows 1, 2) decreased slightly but the position of the bands remained the same. The general proteins of the dry-heated seed showed a similar profile up to 130°, but at 145° a noticeable decrease in band intensity of the major globulin was observed (Figure 1, arrows 3, 4) with no apparent mobility changes or the appearance of new bands. The trace of diffuse staining at 155° (Figure 1, arrow 5) indicated possible modification of native α-arachin at that temperature. Reduction of protein solubility at 155° was evident from the lack of other stained zones and by the test for soluble protein. No cathodal banding occurred.

In extracts from wet-roasted seed, MDH activity occurred at 100° and 120° only (Figure 2, arrows 1, 2). Extracts of dry-roasted seed heated at 110° showed activity comparable to the controls (Figure 2, arrows 3–7), with significantly decreased enzyme reaction in the slowest migrating band near the origin (Figure 2, arrows 7, 7A). The activity of the upper bands of MDH in the dry-roasted seeds became increasingly weaker up to 130° and was not evident at 145°. Cathodally migrating MDH was not observed.

Analysis for nonspecific esterases showed no activity in the wet-heated seeds (Figure 3). However, in the seed dry-heated at 110°, activity was similar to that in the two controls but declined progressively up to 145°. Activity was not apparent at 155°. Three bands with maximum mobility (Figure 3, arrows 1–3) exhibited weak staining. Note the cathodal migration typical of nonspecific esterases in crude extracts. Another sample from dormant seed containing higher protein contents (65 mg/ml) revealed more

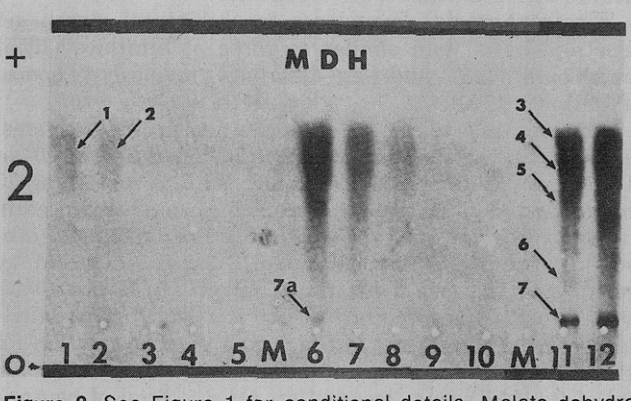

Figure 2. See Figure 1 for conditional details. Malate dehydrogenase (MDM) stained gel.

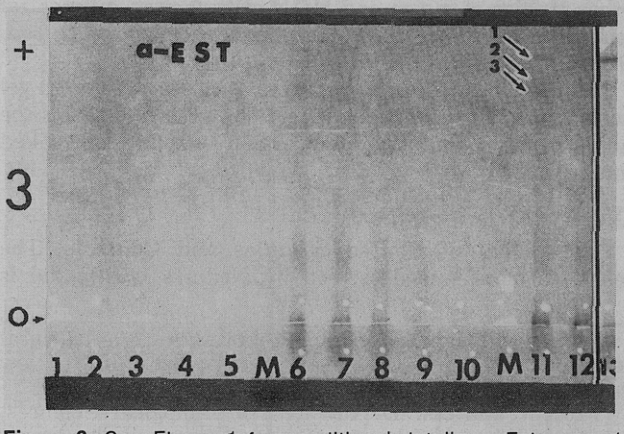

Figure 3. See Figure 1 for conditional details. α-Esterases (α-EST) stained gel.

intense staining for α-esterases, as shown in sample 13; horizontal arrows point to anodal bands.

Compared to the control, only the seed dry-roasted at 110° exhibited a trace of peroxidase activity (Figure 4, arrow 1); the controls displayed two diffused overlapping bands (Figure 4, arrows 2, 3) that were densely stained.

The LAP reaction was minimal in the wet-roasted seed (Figure 5, arrow 1). In contrast, the seed dry-heated at 110° exhibited more activity but this decreased with increased heat; only light staining occurred at 130° (arrows 2, 3) and no reaction was observed at 145°. The controls displayed four bands (arrows 4–7), varying in intensity. No LAP activity appeared in the cathodal section. The bands in the lower half of the gel (bracketed area near the origin) are probably due to nonspecific staining of some general proteins and are discussed later. The lightly stained band (light brown) in Figure 5, arrow 8, corresponds to α-arachin (arrow 1 in Figure 1).

To establish the relative position of α-arachin in a soluble protein extract, compared to its migration after partial purification, samples 11 and 12 were run on the same gels with the isolated fraction and stained with bromophenol blue general protein stain (Figure 6B, 6C). The number of dippings for each sample paper into a solution of α-arachin is designated by NX. A good correlation between the density of each band and the amount of α-arachin applied is apparent. Evidence indicates that the major bands of soluble protein in samples 11 and 12 (Figure 6B, arrows 2, 3) corresponds to α-arachin in the isolated fraction (Figure 6B, arrows 4, 5). Furthermore, this was verified by antigenic detection on acrylamide gels (Neucere, 1972). Figure 6D is a photograph using dark-field illumination (di) of part of the gel in 6A before staining with bromophenol blue general protein stain. It shows α-arachin (arrows 1, 2) as a white precipitate in the LAP stain solution. Counterstained with a general protein stain, the results are the same as in Figure 6B and 6C (sample 8×).

DISCUSSION AND SUMMARY

Starch gel electrophoresis of general proteins from soaked seed heated to 155° showed patterns corresponding to the control. The noticeable decrease in staining observed after dry heating over 145°, however, is a concentration effect. Semiquantitative analysis of α-arachin by electrophoresis in agar containing antibodies produced to α-arachin (Neucere, 1972) revealed greater solubility of the reserve globulin in the wet-roasted than in the dry-roasted samples. The data correspond reasonably well with the visual banding patterns observed here. In a separate *in vitro* study, banding patterns of proteins in phosphate buffer extracts of dormant seed heated at 95° for 15 min showed a significant decrease in the staining density of α-arachin and detected new bands near the anode (Thomas and Bright, 1972), suggesting protein hydrolysis or release of subunits. Another study on six commercial brands of roasted peanuts and peanut butter revealed the position and staining density of α-arachin and general protein in starch gels comparable to seed dry-roasted at 130° in this study (Thomas and Batulis, 1972).

One would normally expect most enzymes to be inactivated by heating seeds at temperatures over 100° for 1 hr. However, this study showed that even wet heat at 120° did not completely inactivate one of the enzymes analyzed, MDH. The thermal stability of proteins and enzymes varies with seeds of different plants. Chawan (1971), for example, reported that seeds of desert species of *Sida* pretreated at various high temperatures (70 to 100°) for different intervals of time ensured better germination. Undoubtedly, when seeds are dry-heated at 110° for 6 or 12 hr (the conditions which gave 100% germination of seeds of *Sida grewioides*), the internal and ambient temperatures become equivalent. One may therefore assume that the seed proteins and enzymes were not denatured by heating at 110°.

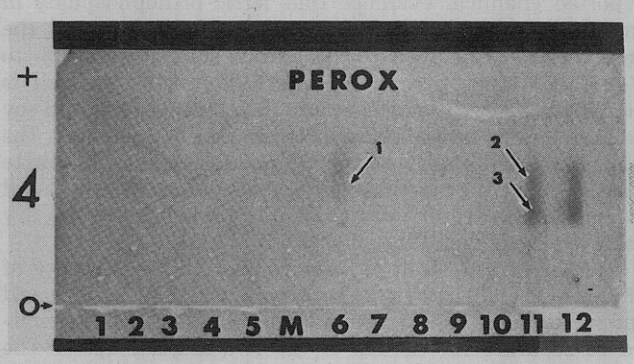

Figure 4. See Figure 1 for conditional details. Peroxidase (PEROX) stained gel using pyrogallol as substrate.

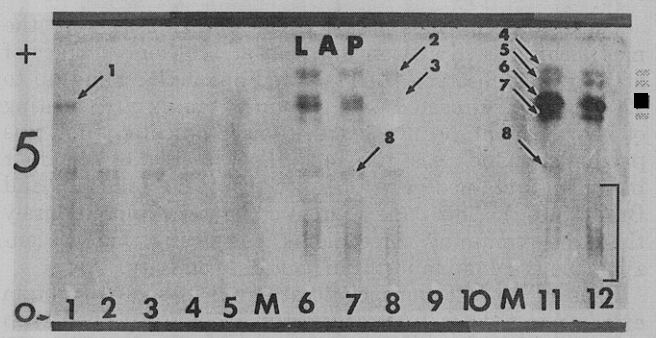

Figure 5. See Figure 1 for conditional details. Leucine aminopeptidase (LAP) stained gel.

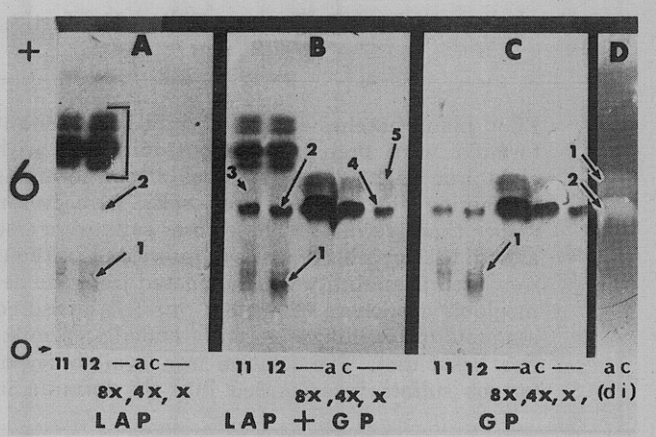

Figure 6. Four zymograms depicting relationship of leucine aminopeptidase (LAP), α-arachin, and nonspecific staining in extracts of dormant peanuts. Samples 11 and 12 are the same as in previous figures. "ac" represents partially purified α-arachin in different concentrations (8X, 4X, and X) applied in gel (refer to text). **6A:** Gel stained for LAP. Four LAP bands in samples 11 and 12 (controls) are bracketed. Nonspecific staining (arrow 1). Partially purified α-arachin present but no LAP activity observed in this fraction (arrow 2 points to normal position of α-arachin). **6B:** Gel similar to 6A but stained for LAP and counterstained with bromophenol blue general protein stain (LAP + GP). Presence of α-arachin in the controls (samples 11, 12) and the major and minor α-arachin bands in different concentrations of partially purified α-arachin denoted by arrows 2, 3, 4, and 5. Note staining at arrow 1. **6C:** Gel similar to 5A but stained for general proteins (GP) with bromophenol blue stain only. Note absence of LAP bands but presence of staining at arrow 1. **6D:** Darkfield illuminated (di) photograph of part of the same gel shown in Figure 6A (LAP stain) which was photographed in transmitted light. Note two bands of precipitated α-arachin (arrows 1, 2) at 8× concentration which did not stain in any of the samples in 6A.

Of all the enzymes assayed, the peroxidases were the most heat sensitive. After dry heat at 110°, only a trace of activity was observed. LAP showed one active band up to 120° in the dry-roasted seed. Results from *in vitro* heating of the dormant peanut extracts also indicated that this particular band was the most heat stable of the four bands and retained very weak activity at 65° (Thomas and Bright, 1972). This band reproducibly stains more densely than the remaining three bands, suggesting that the enzyme plays a dominant role in peptidase activity.

Reasons for the nonspecific staining in soluble protein extracts tested for LAP activity and counterstained with bromophenol blue stain are somewhat obscure. The diffused nonspecific banding appeared more densely stained with bromophenol blue after the gel was immersed in LAP stain (Figure 6B, arrow 1). The bands were also observed in acid phosphatase stain in acetate buffer (pH 4.6), and in α-EST stain (pH 6.0), but not consistently.

In summary, the electrophoretic separations of general proteins in starch gels reported here showed less difference in banding patterns after wet heat than after dry heat. The enzyme data suggested that dry seeds give better heat protection to these functional proteins than do imbibed seeds. Some of the isoenzymes were differentially sensitive to heat, suggesting possible differences in both their structure and function.

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Effect of Processing on Availability of Iron Salts in Liquid Infant Formula Products. Experimental Milk-Based Formulas

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Pilot plant batches of liquid milk-based infant formula were prepared without iron and with eight iron salts added. Portions of the formulas containing three of the iron salts were frozen rather than sterilized. These three salts were also added to lyophilized formula processed without iron. Iron availability was calculated from the hemoglobin responses of anemic rats fed measured amounts of lyophilized formula added to a milk-free basal diet. The relative iron availability of ferrous sulfate incorporated into the formula in these three ways was 114 to 129, expressed as a percentage of the hemoglobin response to standard ferrous sulfate added to the milk-free basal diet. Sterilization increased the relative iron availability of ferric pyrophosphate from 75 to 125, and of sodium iron pyrophosphate from 40 to 60. Formulas containing five other iron salts had relative iron availabilities of 118 to 148. Milk-based formulas containing ferrous sulfate and produced in production equipment had relative iron availabilities of 136 to 143.

The relative availability of the iron of various iron salts, when added to milk or to processed milk-based liquid products, is controversial. For instance, Niccum *et al.* (1953) added either ferrous sulfate or ferric ammonium citrate to high-protein milk-based formulas fed to infants and found higher hemoglobin values in those infants fed the formula supplemented with ferrous sulfate. In contrast, Pla *et al.* (1971) reported that the iron of ferric ammonium citrate added to milk before or after pasteurization was utilized as well as standard ferrous sulfate by iron-depleted rats and chicks.

These conflicting reports suggest that many factors affect the biological availability of iron in milk and in processed milk-based liquid products. Duration of exposure of the iron salt to the liquid milieu is a factor. Hodson (1970) reported chemical evidence that ferric orthophosphate in liquid dietaries dissolves over a period of time and the iron therein is reduced to the ferrous state during a 2- to 5-month storage period. An effect of processing itself is suggested by our previous report that processing liquid soy isolate infant formula products markedly increased the biological availability of the iron of ferric pyrophosphate and sodium iron pyrophosphate, two sources of iron found to have mediocre or poor availability when added as dry salts to a basal diet (Theuer *et al.*, 1971).

In the present study we determined the availability of the iron of eight iron salts incorporated into a milk-based infant formula. With three of these salts, we also attempted to separate the effects of the two major steps in the processing of a liquid infant formula (physical mixing of ingredients and heat sterilization) on the availability of

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