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ELECTROPHORETIC STUDY OF HEATING *IN SITU* ON PROTEINS AND ENZYMES IN GERMINATED *ARACHIS HYPOGAEA* COTYLEDONS: COMPARISON WITH DORMANT SEED

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Abstract—Cotyledons from 5-day germinated seed of *Arachis hypogaea* were heated in a moisturized chamber at temperatures from 25 to 121° Proteins were extracted in phosphate buffer and analyzed with horizontal starch gel electrophoresis to determine the effect of heat on migration patterns of soluble proteins, malate dehydrogenase, glutamate dehydrogenase, leucine aminopeptidase, peroxidases and nonspecific esterases The intensity of staining of soluble proteins from 5-day cotyledons began decreasing at 80–90°, very little staining occurred at 100° with the exception of a distinct band at R_f 1 0 Glutamate dehydrogenase and benzidine peroxidase retained some activity at 80° but other enzymes were inactivated at temperatures near 65° Differential heat sensitivities of isoenzymes were obvious Heat did not alter the R_f values of the bands of soluble proteins or enzymes but influenced the intensity of staining Two-year storage at 4° of viable seed and 33-month storage at −10° of frozen extracts from dormant seed had no influence upon migration patterns of soluble proteins and enzymes assayed

INTRODUCTION

LITTLE ATTENTION has been given to the effects of heat upon individual isoenzymes or migration patterns of enzymes analyzed with zone electrophoresis Differential heat sensitivity of multiple molecular forms of enzymes of lactate dehydrogenase (LDH) were studied [1–3] Thermal effects on isoenzymes of malate dehydrogenase (MDH) in crude, cell-free extracts from *Bacillus subtilis* were also analyzed [4] It was shown that certain isoenzymes of alkaline phosphatase[5] and acid phosphatase[6] reacted differently to heat than others Heating a partially purified extract from skim-milk at 60° for 5 min eliminated a band of nonspecific esterase in polyacrylamide gels [7]

The effects of heat upon proteins from dormant peanuts roasted at 145° were investigated using several techniques [8] The results of heating *in situ* at several different temperatures upon soluble proteins of imbibed and dry peanut seed were studied with disc electrophoresis and immunochemistry [9] Using samples prepared in the same manner, soluble

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[2] VESSEL, E S, FRITZ, P J and WHITE, E L (1968) *Biochim Biophys Acta* **159**, 236
[3] KNUDSEN, F U, KNUDSEN, H E and GORMSEN, J (1970) *Anal Biochem* **36**, 192
[4] ANTOHI, S, MORARU, I and COTAE, D (1970) *Biochem Biophys Res Commun* **39**, 226
[5] TAN, K K and AW, S E (1971) *Biochim Biophys Acta* **235**, 119
[6] EFRON, Y (1970) *Genetics* **65**, 575
[7] KITCHEN, B J (1971) *J Dairy Res* **38**, 171
[8] NEUCERE N J ORY R L and CARNEY W B (1969) *J Agric Food Chem* **17**, 25
[9] NEUCERL N J (1972) *J Agric Food Chem* **20**, 252

proteins and several enzymes were examined with starch gel electrophoresis.[10] It was shown that some enzymes produced lightly stained bands in gels containing electrophoresed extracts of seed soaked 16 hr and roasted at 110° for 1 hr. In general, gels containing samples from dry seed heated at 110° displayed darker bands, enzymes from dry seed heated at 130° produced bands that were barely visible. However migration patterns of soluble proteins in starch gels indicated a greater thermostability in soaked than in dry seed.

Since other studies[9,10] demonstrated that soluble proteins and some enzymes are not readily affected by *in situ* heating of whole dormant peanut seed and that *in vitro* studies indicated greater thermosensitivity of these same soluble proteins and enzymes,[11] it seemed feasible to investigate the effects of heating *in situ* upon cotyledons of germinated peanut seed. The primary objective was to determine the relationship between (a) electrophoretic profiles and thermal stability of soluble proteins and several enzymes from 5-day cotyledons heated at different temperatures under moisturized conditions and (b) profiles of soluble proteins and the same enzymes in heated and from nonheated dormant seed. The results were compared with those of other studies. For a more extensive analysis, freshly prepared extracts of dormant viable seed stored 2 yr at 4° and frozen extracts (stored up to 33 months at −10°) of dormant and germinated tissues were analyzed to determine the effects of prolonged storage upon migration patterns of soluble proteins and enzymes.

RESULTS

Soluble proteins

The major globulin of peanuts, α-arachin, was obvious in extracts from dormant cotyledons (Fig. 1, arrows 1, 2), proof of the position of α-arachin bands in starch gels has been described.[10] Diffuse indistinct bands between R_f 0.7 and 0.95 are not shown in the diagram. Arrow 3 probably indicates a primary band of arachin after 5 days of germination. From 25° to 90° no change occurred in the position of the bands but some decrease in intensity of staining was detected in bands at R_f 0.54 and 0.62. At 100° these two sites of activity had overlapped and were barely visible. After autoclaving the cotyledons at 121° only one band at R_f 1.0 remained (Fig. 1, sample 121°). This band was apparent in all samples of 5-day cotyledons but was weak in the extracts of dormant seed. It is noteworthy that after heating no new bands appeared anodic to arachin. Electrophoresed extracts of dormant seed heated *in vitro* showed a significant decrease of arachin from 95° to 100° and the appearance of at least three new bands with greater R_f values.[11] To further compare the heat stability of soluble proteins in 5-day cotyledons with soluble proteins in dormant seed, ungerminated seed were autoclaved (121°) for 15 min and the soluble proteins assayed. The electrophoretic pattern indicated that the reserve protein α-arachin from dormant cotyledons was little affected by autoclaving in contrast to the soluble proteins from autoclaved 5-day cotyledons.

Malate dehydrogenase (MDH E.C. 1.1.1.37) and glutamate dehydrogenase (GDH E.C. 1.4.1.2)

Extracts from 5-day cotyledons (and dormant cotyledons) exhibited five isoenzymes of MDH, including a weak band at R_f 0.16, a faint diffuse band at R_f 0.27, and three

[10] THOMAS D. L. and NEUCERE N. J. (1973) *J. Agric. Food Chem.* **21**, 479.
[11] THOMAS D. L. and BRIGHT J. F. (1973) *Can. J. Botany* **51**, 1191.

distinct sites of activity at R_f 0 42, 0 55 and 0 70 At 50°, the two bands nearest the origin,had disappeared Other than a slight decrease in intensity of staining, no significant change occurred in the distribution pattern between 50 and 60°, however, at 65° no bands were present Three intense anodic bands of GDH were obvious in extracts from both 5-day and dormant cotyledons Bands at R_f 0 22 and 0 32 were of equal intensity and were more obvious than the one at R_f 0 39 Occasionally a fourth region of very weak staining was visible at R_f 0 43 Band characteristics of the three major isoenzymes were unaffected by heat through 75° At 80°, the activity began to decrease and at 85° no formazan deposition was observed Malate dehydrogenase and GDH were assumed to be substrate specific, consequently, the bands in the patterns are referred to as isoenzymes

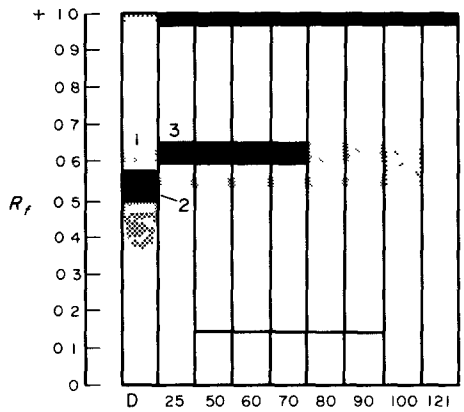

FIG 1 STARCH GEL ELECTROPHORESIS OF SOLUBLE PROTEINS FROM NON-HEATED, DORMANT SEED (D) AND HEATED (25–121) 5-DAY COTYLEDONS OF PEANUTS
Basal numbers represent temperatures of heat treatment Intensity of shading reflects density of staining in starch gels

"Antibands", or well-defined areas on the gel in which no background staining occurred, were apparent in gels stained for MDH and GDH from dormant seed These antibands were apparent between R_f 0 64 and 0 83 and were more obvious in gels remaining in the stain for several hours Furthermore, antibands were present in extracts (heated at 75° for 30 min) of dormant peanut seed[11] and in nonheated extracts of dormant and 5-day cotyledons stored at −10° for 3 yr and assayed for MDH and GDH (Thomas, unpublished) Antibands have also been observed in starch and acrylamide gels containing electrophoresed green algal extracts stained for MDH and GDH activity (Thomas, unpublished) and in starch gels stained for GDH from chlorosarcinacean algae [12]

Hall *et al* [13] observed antibands in acrylamide gels containing extracts from different plants A similar nonspecific reaction was noted when staining for malate dehydrogenase activity in crude cotton leaf enzyme preparations electrophoresed in polyacrylamide gels [14] Tetrazolium oxidase produces similar acromatic regions in dehydrogenase stains containing phenazine This phenomenom has been further discussed [11]

[12] THOMAS, D L and GROOVER, R D (1973) *J Phycol* **9**, 289
[13] HALL T C MCCOWN B H DISBOROUGH S MCLEESTER R C and BECK G E (1969) *Phytochemistry* **8**, 385
[14] O'SULLIVAN S A and WEDDING R T (1972) *Plant Physiol* **49**, 117

Leucine aminopeptidase (LAP, E C 3 4 1 1)

Four isoenzymes at R_f 0 73 0 78, 0 84 and 0 88 were present in extracts from dormant cotyledons, The major site of activity was at R_f 0 78 The band at R_f 0 73 was less intense but was slightly darker than the remaining two The patterns produced by extracts of 5-day cotyledons were similar to those of the dormant seed except the two fastest migrating bands were partially overlapped LAP activity was little affected by heating through 50° The intensity of staining of the slowest migrating band began to decrease between 50 and 55° and had disappeared at 60 The remaining bands were not present at 65 It was assumed that the only enzyme reacting with the substrate L-leucyl-β-napthylamide was LAP therefore, the bands of activity represent isoenzymes

Peroxidases (E C 1 11 1 7)

The term isoenzymes is not employed here because of the apparent lack of specificity of peroxidases for any one substrate This may be the case with the most cathodic band (R_f −0 52) in gels stained with solutions containing either pyrogallol or benzidine as hydrogen donors The major difference between this band which developed in each stain was the apparent greater reactivity of the peroxidase with benzidine as evidenced by the increased density of the band Little change in the anodic pyrogallol peroxidase (a long band at R_f 0 47) occurred with increasing temperature through 60 , but there was a considerable reduction in staining at 65 , and none at 70 The most cathodic band at R_f −0 52 was present but exceptionally weak at 60 , however, this band was also weak at lower temperatures Cathodic banding in the benzidine stain consisted of three strong bands (R_f −0 06, −0 44 and −0 52) and some very indistinct bands The effects of heat on these bands were not significant between 25 and 70 However there was an abrupt decrease in intensity of staining between 70 and 80 with only one band (R_f −0 06) remaining at the latter temperature and none at 90° The anodic pattern consisted of several weak bands exhibiting heat sensitivity similar to the cathodic bands, no staining occurred at 90° In the dormant seed, 24-hr-germinated seed, and cotyledons of dormant seed, there was little difference in pyrogallol peroxidase activity Each sample exhibited two overlapping bands (R_f 0 37 and 0 47) in contrast to the single band at R_f 0 47 in samples from the 5-day cotyledons Results of the assay for benzidine peroxidase in dormant cotyledons indicated a very weak anodic band near the origin (R_f 0 07) and a slightly darker cathodic band at R_f −0 06 Additionally seed germinated 24 hr exhibited two weak cathodic bands of greater mobility which corresponded to the two bands at R_f −0 44 and −0 52 in 5-day cotyledons These bands were more intense on the second day of germination and remained obvious from day 5–11 [15]

Nonspecific γ-esterases (γ-EST, E C 3 1 1 2)

The esterases in the 5-day cotyledons were characterized by four anodic bands (R_f 0 79, 0 82, 0 86 and 1 0) and one cathodic band near the origin Band patterns indicated that activity was lost at two sites (R_f 0 79 and 1 0) at approximately 50 Bands at R_f 0 82 and 0 86 were barely visible at 55 and were not apparent at 60 Esterase activity in the cathodic section was obvious through 60 but was not present at 65 Migration patterns of esterases extracted from the dormant seed were similar to those in the 5-day cotyledon, the dormant cotyledons exhibited only three anodic bands (R_f 0 86 0 91 and 1 0) and the

[15] THOMAS D L and NEUCERE N J (1973) *Am J Botany* in press

same cathodic band as the 5-day cotyledon The esterases are not referred to here as isoenzymes because a number of esterase enzymes may hydrolyze the common substrate α-napthyl acetate Consequently, each band may represent more than one esterase

Stability in storage

Storage of extracts from dormant seed and dormant cotyledons at $-10°$ up to 33 months and similar storage of extracts (for 2 yr) from 24-hr germinated seed had no influence upon the migration patterns of soluble proteins or enzymes Two-year storage of extracts ($-10°$) from 5-day cotyledons did not affect the intensity of staining or the number of bands produced by MDH, LAP, pyrogallol peroxidase, or α-EST but most bands had a slight increase in R_f values No effect upon benzidine peroxidase was observed No significant difference occurred in the patterns of soluble proteins or enzymes of fresh extracts assayed at one date and fresh extracts prepared 2 years later from the same batch of viable peanuts maintained in refrigerated storage (4°) during the interval This indicates a remarkable ability of soluble proteins and these enzymes to remain stable when stored under such conditions

It was noted that cold storage of tubers of *Solanum* species and freezing of protein extracts from the tubers had no influence upon the protein patterns [16] However, the authors did not report the maximum time of storage used In another study, storage of protein extracts at $-25°$ for 6 weeks considerably altered the electrophoretic pattern of *Solanum* tubers [17]

DISCUSSION

According to band patterns in zone electrophoresis, storage proteins in heated, dormant peanut seed are highly resistant to thermal denaturation [8–10] The antigenic properties of α-arachin, the major peanut globulin, were not significantly altered after roasting the seed at 145° for 1 hr [8] The globulin also remained antigenic in both soaked and dry seed heated at 155° for 1 hr [9] In this current electrophoretic analysis, the soluble proteins in 5-day-old cotyledons subjected to heat at 100° for 30 min had almost disappeared, based upon the lack of discreet banding in the gel (Fig 1) This probably indicates that proteins in the 5-day cotyledon are much more thermosensitive than those in the imbibed, dormant seed which exhibited distinct bands when heated to 155 for 1 hr [10]

The band pattern of the reserve proteins in the 5-day cotyledons (Fig 1 samples 25–121) was significantly different from that in the dormant cotyledons (Fig 1, sample D) Two important alterations occurred in the distribution of the soluble proteins in 5-day cotyledons as compared with dormant cotyledons First, the comparative amounts of protein in the major bands of arachin changed and the position of the most anodic band of arachin in the dormant cotyledon (Fig 1, arrow 1) greatly increased in staining intensity in the extracts of the 5-day cotyledons (Fig 1, arrow 3) In contrast, the most intensely stained band of arachin in the dormant cotyledons (arrow 2) decreased noticeably in the 5-day cotyledons Second, a weak band at R_f 1 0, in the dormant cotyledons (Fig 1, sample D) increased in density in extracts from the 5-day cotyledons These observations suggest that the reserve proteins in the germinated cotyledons were enzymatically hydrolyzed causing an increase in electrophoretic mobility and possibly producing small peptides which migrated with the front

[16] DISBOROUGH S and PELOQUIN S J (1966) *Phytochemistry* **5**, 727
[17] ERIKSEN L and SKUDE G (1970) *Hereditas* **64**, 294

In an immunoelectrophoretic analysis (IEA) of reserve proteins, α-arachin electrophoretically shifted toward the anode after 1 day of germination [18] The shift remained obvious at days 5 and 9 It was suggested that this was due to progressive deamidation of glutamine and asparagine The IEA study indicated that α-arachin in cotyledons of germinating seed is composed of molecules with varying electrophoretic mobilities but possessing the same antigenicity This shift in mobility may offer a partial explanation for the observed increase in migration velocity and the changes in migration patterns of reserve proteins in dormant cotyledons as compared with 5-day germinated cotyledons (Fig 1) It would be revealing to perform an IEA study on α-arachin from heated 5-day cotyledons to determine if the decrease in this reserve protein observed at 100 (Fig 1) was accompanied by a destruction of the antigenic sites

α-Arachin is stored in small membrane-bound particles termed aleurone grains [19 20] It is possible that these organelles confer some protection upon the proteins inside During protein utilization in the germinating seed, the protection these membrane-bound bodies provide may be considerably decreased thereby increasing the susceptibility of this reserve protein to heat

Enzymes in 5-day cotyledons were much more sensitive to heating *in situ* than those in the dormant seed [10] A similar relationship was observed by Sisler and Johnson in which studies of heating *in situ* indicated a marked influence of the moisture present on the stability of *O*-diphenol oxidase in tobacco leaves the lower the moisture content the slower was the inactivation [21] In general the enzymes in the dormant seed roasted at 110 for 1 hr showed good activity [10] whereas the same enzymes in the 5-day cotyledons were inactivated at a much lower temperature In the 5-day cotyledon benzidine peroxidase and GDH were the most heat stable exhibiting limited activity at 80 The two areas of benzidine peroxidase activity displayed at R_f −0 44 and −0 52 were present also in cotyledons, epicotyl hypocotyl and radicle of 11-day-old seedlings [15] Presumably, the enzymes responsible for these bands perform a similar role in each of these organs and may be associated with the oxidation of phenolic compounds These enzymes are probably functional in the early stages of germination the two bands were present at 24 hr of germination The peroxidase using pyrogallol as a hydrogen donor is present in the dormant and germinated seed

γ-EST MDH and LAP exhibited similar reactions to heat They were active to some extent at 60 but not at 65 These three enzymes displayed approximately half of the heat resistance in the heated 5-day cotyledon as in the heated dormant seed [10] and about the same thermostability as in extracts of dormant seed heated *in vitro* [11]

The range of temperatures for good growth of peanuts (a summer annual) is *ca* 30–35° One may assume that if the enzymes extracted from cotyledons heated at 55 60 are functional in stain solutions, they would exhibit activity at a similar or higher temperature in the living organism Thermophilic bacteria and blue-green algae are known to live in hot springs at optimum temperatures of 50–70 [22 23] Ljunger [24] briefly reviewed literature

[18] DAUSSANT J NEUCERE N J and CONKERTON L J (1969) *Plant Physiol* **44**, 480
[19] DAUSSANT J NEUCERE N J and YATSU L (1969) *Plant Physiol* **44**, 471
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[21] SISLER E C and JOHNSON W H (1965) *Plant Cell Physiol* **6** 645
[22] BROCK T D (1967) *Science* **158**, 1012
[23] BROCK T D (1969) *Phycologia* **8**, 201
[24] LJUNGER C (1970) *Physiol Plant* **23**, 351

pertaining to the nature of heat stability of thermophilic bacteria and noted that several enzymes extracted from thermophiles were more heat stable than those from mesophiles Many xerophytic plants survive at elevated temperatures Chawan[25] reported that seeds of desert species of *Sida* pretreated at various high temperatures (70–110°) for different intervals of time ensured better germination Apparently the seed proteins and enzymes were not denatured by heating at these temperatures

Studies of the biochemical and physiological characteristics of soluble proteins and enzymes from organisms adapted to living at different temperatures may lead to a better understanding of thermostability of proteins and of growth and survival in various environments

EXPERIMENTAL

Select shelled seed of *Arachis hypogaea* variety 56-R were obtained from the Agricultural Research Service Crops Research Division Beltsville Maryland All seed were of the 1968 crop and were maintained in refrigerated storage 3 yr prior to extraction

Germination and heat treatment of cotyledons The 3-yr-old seed were surface sterilized 15 min in 10% Chlorox (commercial 5% NaOCl) rinsed soaked 2 hr in H_2O and germinated in steam sterilized vermiculite for 5 days at 25° The testae were removed and the cotyledons excised and frozen 3–6 days until further treatment The cotyledons were thawed to 25° and placed between absorbent paper satd with H_2O in a Petri dish, covered with Al foil and placed in a forced-air temp regulated oven preset to the designated temp from 50 to 100° The approximate temp at the surface of the cotyledons was determined by replacing the cotyledons with a thermometer in a similar apparatus After the desired temperature inside the Petri dish was reached the cotyledons remained in the oven for 30 min Additional cotyledons were autoclaved at 121° for 15 min and immediately brought to room temp For comparison dormant seed were autoclaved in the same manner

Extraction of proteins The cotyledons were homogenized in 15 phosphate buffer (1 g/2 ml), pH 7 8 ionic strength 0 2 (0 008 M NaH_2PO_4 H_2O, 0 064 M Na_2HPO_4), for 5 min The homogenate was filtered through cheese cloth and the filtrate centrifuged at 34 800 *g* for 20 min at 20° The lipid layer and pellet were discarded and the remaining liquid fraction recentrifuged under the same conditions The liquid fraction was frozen and assayed within 3 weeks The extract from the 5-day cotyledons contained 47 mg of protein per ml[26] using bovine serum albumin as a standard To compare soluble proteins and enzymes in fresh extracts with the stability of those in frozen extracts (−10°), 24-month-old samples of frozen extracts (prepared in the above manner) from cotyledons of 5-day germinated seed were analyzed In addition, 24-month-old frozen extracts of 1-yr-old dormant seed 1-yr-old dormant cotyledons and 24-hr germinated seed were assayed Some samples had been extracted at 1 g per 2 0 ml buffer yielding a protein concentration of 87 mg per ml others were extracted at 1 g per 2 9 ml and contained 67 mg protein per ml Extracts of 24-hr germinated seed contained 53 mg protein per ml Other extracts (67 mg protein per ml) from dormant cotyledons and dormant seed were stored frozen for 33 months and assayed All extracts used to determine the effects of frozen storage upon proteins were assayed prior to storage Autoclaved (121° for 15 min) dormant seed were extracted at 1 g (dry seed) per 2 8 ml of buffer The above variety of samples provided additional data for comparison

Electrophoresis Procedures for horizontal starch gel electrophoresis were similar to those of Smithies[27] and Poulik[28] The gel was covered with a thin plastic sheet to prevent evaporation and was kept cool with a pan of ice on top 50 mA were applied until the bromphenol blue marker dye had migrated 6–7 cm R_fs were relative to this marker band

Enzyme assays To demonstrate nonspecific α-EST, the starch slices were incubated 2 hr at 25° in 100 ml 0 2 M phosphate buffer containing 75 mg Fast Blue RR salt, 1 5 ml 1% α-naphthyl acetate in acetone–H_2O (1/1) and 10 ml *n*-propanol (absolute) at a final pH of 6 0 Leucine aminopeptidase was detected by incubating the gels 2 hr in 100 ml 0 2 M phosphate buffer containing 20 mg L-leucyl-β-napthylamide HCl and 25 mg Black Salt K The final pH was 4 4 The standard solution for the MDH assay consisted of 100 ml 0 1 M Trisma Base (pH 8 5), 3 ml neutralized 2 M DL-malic acid, 50 mg β-NAD 50 mg MTT tetrazolium and 10 mg phenazine methosulfate The assay for GDH was the same as that for MDH except 3 0 ml neutralized 2 M L-glutamic acid was substituted for the malic acid substrate Two different assays were employed to determine peroxidase activity In one assay benzidine DI-HCl was used as the hydrogen donor in a stain solution

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[26] LOWRY, O H, ROSEBROUGH, N J, FARR, A L and RANDALL R J (1951) *J Biol Chem* **193**, 265
[27] SMITHIES, O (1955) *Biochem J* **61**, 629
[28] POULIK M D (1957) *Nature* **180**, 1477

described[29] except that only 1 7 ml 3% H_2O_2 per 100 ml stain was employed The other assay used pyrogallol and consisted of 100 ml 0 1 M phosphate buffer (pH 6 0) 1 ml of 3% H_2O and 0 5 g pyrogallol The general proteins were stained in 0 1% amido black in 7% HOAc and destained in 3% HOAc

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