Phytochemistry, 1973, Vol. 12, pp. 283 to 289. Pergamon Press. Printed in England.

ELECTROPHORETIC CHARACTERIZATION OF SIX SELECTED ENZYMES OF PEANUT CULTIVARS

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(*Revised Received* 14 *July* 1972. *Accepted* 26 *July* 1972)

Key Word Index—*Arachis hypogaea*; Leguminosae; peanut; enzyme patterns; gel electrophoresis; variation within cultivars.

Abstract—The isozyme patterns (both anodic and cathodic) of esterase, catalase, leucine aminopeptidase, acid phosphatase, alcohol dehydrogenase and INT oxidase in individual seeds from several peanut cultivars (*Arachis hypogaea*) were characterized by polyacrylamide and starch gel electrophoresis in relation to the stages of seed development, maturity, and germination, geographic areas where grown and phylogenetic relationship. Of the six enzymes examined, only esterase contained cathodic isozymes, of which the patterns served to distinguish between the Spanish and the Virginia-type peanuts. Anodic esterase and acid phosphatase zymograms of early developing and germinating peanuts could be distinguished from those of predormant and mature seeds and the latter showed much intravarietal variation which was consistent among cultivars and the geographic areas where grown. Anodic isozymes of catalase, leucine aminopeptidase, alcohol dehydrogenase and INT oxidase were synthesized very early in peanut development and remained constant through maturity and to at least 24 hr germination; they were consistant within and between peanut cultivars and they were not influenced by the environmental conditions of the areas where the peanuts were grown. The consistency of the isozyme patterns within and between cultivars supports the suggestion that plant breeding programs used to develop superior cultivars have produced genetic uniformity in peanuts.

INTRODUCTION

GEL ELECTROPHORESIS of enzymes in higher plants have shown that many of these molecules exist in multiple molecular forms. These isozymes are widely distributed in higher plants[1,2] and exhibit Mendelian genetics,[3–5] making them useful as genetic markers at the molecular level. Qualitative and/or quantitative isozyme variations have been utilized in biochemical,[6–9]

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physiological,[10–16] pathological,[17,18] morphogenetic,[19–24] and chemotaxonomic,[1,25–30] investigations in a large variety of plants, but not in peanuts.

Cherry *et al.*[31] recently employed polyacrylamide gel electrophoresis to examine and characterize the total proteins of individual peanut seeds from a number of cultivars of *Arachis hypogaea* grown in five geographic areas of the United States. They showed that the protein banding patterns varied consistently among the different cultivated varieties grown in similar environments. This consistency in protein pattern made it difficult to distinguish the cultivars. However, major qualitative and quantitative protein differences were observed for the same peanut types grown in colder climates.

In this investigation, zymograms of six enzymes: esterase (E.C. 3.1.1.2), catalase (E.C. 1.11.1.6), leucine aminopeptidase (E.C. 3.4.1.1.), acid phosphatase (E.C. 3.1.3.2), alcohol dehydrogenase (E.C. 1.1.1.1) and INT oxidase (an enzyme that prevents the reduction of iodonitrotetrazolium-violet), from individual seeds of several peanut cultivars of *Arachis hypogaea* grown in different areas were compared. Included were cultivars from Spanish botanical, Virginia botanical and Virginia and Runner marketing types, these being representative of most of the commercial acreage in the United States.

RESULTS

Representative anodic zymograms for the following enzymes: alcohol dehydrogenase, INT oxidase, catalase, leucine aminopeptidase and acid phosphatase, were obtained by starch gel electrophoresis and the esterase isozyme patterns by polyacrylamide disc gel electrophoresis. The latter three enzymes are diagrammatically shown in Figs. 1–3. The cultivars examined and the areas in which they were grown are shown in Table 1.

A comparison of 84 seeds (four seeds from each of 21 plants) of Virginia 56R grown in Louisiana and 12 seeds (random samples of field grown plots) from each of the cultivars grown in the other areas (Oklahoma, Texas, Georgia, and Virginia) showed no qualitative and little quantitative intra- and intervarietal isozyme variation for alcohol dehydrogenase, INT oxidase and catalase. Only two major (dark staining) anodic and no cathodic isozyme bands were detected for each of these enzymes. In addition, these same isozyme patterns did not vary in either the developing or the germinating seeds.

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TABLE 1. GEOGRAPHIC AREAS, CULTIVARS, GEL TYPES, AND FREQUENCIES OF LEUCINE AMINOPEPTIDASE (LAP), ACID PHOSPHATASE (AP), AND ESTERASE (EST) ZYMOGRAMS REPRESENTED IN FIGS. 1 AND 2

Cultivars* and areas where grown	Types and Frequency of Zymograms in: LAP Fig. 1*A*, gels: a	b	c	AP Fig. 1*B*, gels: a	b	c	d	e	f	EST Fig. 2*B*, gels a	b	c	d
Virginia-grown													
Tifspan		12				12					12		
Starr		12				12					12		
Early Runner	12				3	9					12		
Florunner	12			12							12		
Virginia Bunch 67	12				9	3					12		
Virginia 56R	12					12					12		
Virginia 61R		12				12					12		
Florigiant		12				12					12		
NC 17		12				12				12			
NC 5		12				12				2	10		
NC 2		12				12				12			
Georgia-grown													
Tifspan	12					12					12		
Starr		12				12					12		
Argentine	12					12					12		
Spancross	12					12					12		
Early Runner	12					12					12		
Florunner	12			12							12		
Florigiant	10	2				12					12		
Louisiana-grown													
Virginia 56R	47	37			21	36	20	6	1	24	34	21	5
Texas-grown													
Comet		12				12					12		
Starr		12				12				2	10		
Argentine		12				12					12		
Oklahoma-grown													
Comet	12					12				12			
Starr		12				12				12			
Argentine		11	1			12					12		
Spanhoma		12				12				6	6		
Totals	177	206	1	24	33	300	20	6	1	82	276	21	5

* Peanut cultivars examined: Spanish botanical: Tifspan, Starr, Argentine, Spancross Comet, Spanhoma; Virginia botanical: (a) Runner Market Type: Early Runner, Florunner, Virginia Bunch 67; (b) Virginia Market Type: Virginia 56R, Virginia 61R, Florigiant, NC 17, NC 5, NC 2.

Except for one gel of the Oklahoma-grown peanut, Argentine, which had a major band missing in region 3·8 cm (Fig. 1*A*, gel c), five major anodic and no cathodic leucine aminopeptidase bands were observed in all zymograms of the cultivars (Fig. 1*A*, gels a and b; Table 1). Variation within and between the cultivars was observed in the minor (light staining) band located in region 1·0 cm (Table 1). However, the minor bands in region 1·0–2·5 cm

were variable and difficult to discern in a number of cases. Quantitative differences distinguished the leucine aminopeptidase zymograms of the least developed (region 3·8–4·5 cm; decreased activity) and the germinated (region 3·0–4·5 cm; increased activity) seeds from those of mature peanuts.

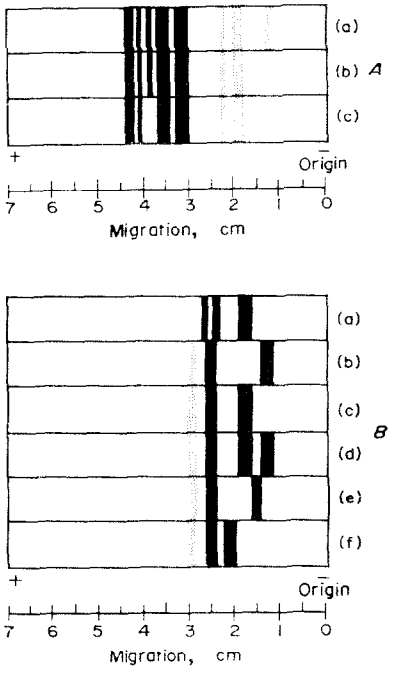

FIG. 1. DIAGRAMS OF GEL PATTERNS FOR LEUCINE AMINOPEPTIDASE (*A*) AND ACID PHOSPHATASE (*B*) ACTIVITIES IN MATURE PEANUT SEEDS. FREQUENCIES OF THE LAP PATTERNS (*A*, GELS a–c) AND THE AP PATTERNS (*B*, GELS a–f) BETWEEN CULTIVARS ARE LISTED IN TABLE 1.

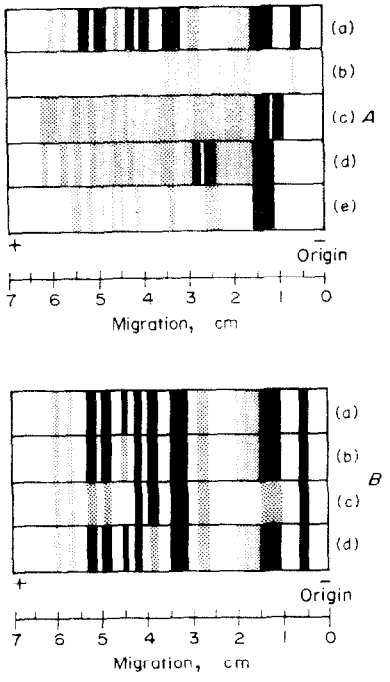

FIG. 2. DIAGRAMS OF GEL PATTERNS FOR ANODIC ESTERASE ISOZYMES IN MATURE SEEDS.
(*A*) Gel a, purified extract after $(NH_4)_2SO_4$ removal of arachin; gel b, arachin added back to purified fraction; gels c–e crude extracts without prior to ammonium sulfate precipitation of arachin. (*B*) Gels a–d, frequencies of the esterase zymograms between cultivars as listed in Table 1.

Much intravarietal anodic isozyme variation (no activity was detected in the cathodic zymograms) was evident for acid phosphatase activity from Virginia 56R peanuts grown in Louisiana (Fig. 1*B*; Table 1). The variation within and between most of the cultivars from the other areas was consistent and limited to three types of zymograms (Fig. 1*B*; gels a–c; Table 1). The two major bands in region 2·5 cm for Florunner seeds (Fig. 1*B*; gel a) appear as single, broader bands for the other cultivars (gels b–f), and may be a useful characteristic of this cultivar. One seed of Virginia 56R grown in Louisiana had a major band in region 2·0 cm which was not present in any other seed (Fig. 1*B*, gel f; Table 1).

The acid phosphatase isozyme patterns from immature and germinating peanuts were easily distinguished from the zymograms of mature seeds. Both qualitative and visibly quantitative increases in activity occur during seed development. Also, the one mature Virginia 56R seed with the major acid phosphatase band in region 2·0 cm (Fig. 1*B*, gel f) is

present as a faint band during the intermediate stages of development. In the germinated seeds, the isozyme band in region 2·5 cm decreases in intensity.

Our initial studies of peanut esterases produced unclear and variable anodic isozyme patterns (Fig. 2*A*, gels c–e) and efforts were made to improve the resolution of these bands. Ammonium sulfate (40% saturated) precipitation of the large molecular weight storage protein, arachin, from the protein extracts produced clear and repeatable isozyme patterns in the polyacrylamide gels (Fig. 2*A*, gel a). Addition of crude arachin back to the ammonium sulfate-treated fraction caused a striking decrease in this intensity (Fig. 2*A*, gel b), showing the masking effect of enzyme activities by arachin.

The intravarietal analysis of Virginia 56R yielded both qualitative and quantitative esterase isozyme variations which were limited primarily to regions 3·8 and 4·4 cm of the anodic zymograms (Fig. 2*B*, gels a–d; Table 1). Variations within and between the other cultivars were limited to quantitative differences in region 4·4 cm (Fig. 2*B*, gels a and b; Table 1).

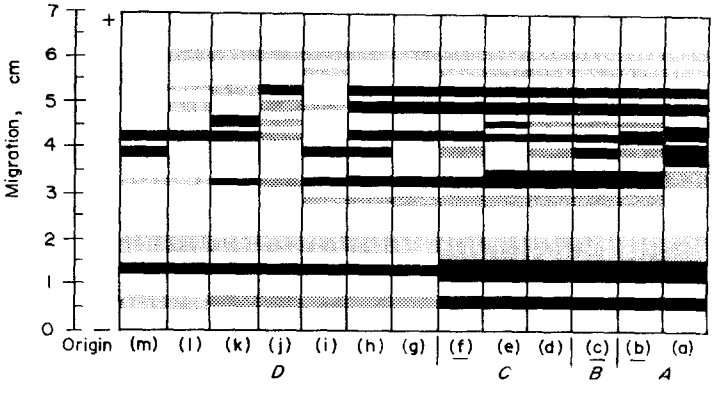

FIG. 3. DIAGRAMS OF GEL PATTERNS FOR ANODIC ESTERASE ISOZYMES IN DEVELOPING, MATURE AND GERMINATING SEEDS. ZYMOGRAMS SHOWN ARE:

(*A*) Gel a, 24-hr germinated seeds; gel b, seed after 4-hr imbibition of water; (*B*) gel c, a mature seed; (*C*) gels d–f, immature seeds passing screen sizes 09·6 × 1·92 to 0·79 × 1·92; (*D*) gels g–m, the least mature seeds passing screen sizes 0·48 × 1·92 to 0·77 × 1·92 and through size 0·48 × 1·92.

The anodic esterase zymograms from developing seeds showed much isozyme variation (Fig. 3, gels d–m). Starting from the least mature (smallest size) seeds (gel m), quantitative increases in activity occurred in bands at 0·5–2·0 cm as the seeds increased in age to predormancy (gels f–d), through dormancy (gel c), and to germination (gels a and b). Both qualitative and quantitative variations began to appear between regions 2·5–6·0 cm as the seeds matured (gels m–g). However, it should be stressed that seeds selected for each stage on the basis of size (with analyses done on seeds of the same size) may not be ontogenetically homogeneous. This could account for the gradual but inconsistent qualitative and quantitative band increases observed here. The later stages of development (gels f–d) showed less variation. The esterase intensity in region 3·5–4·5 cm of seeds imbibed for 4 hr (gel b) decreased quantitatively. At 24 hr germination, a large increase in activity appeared in this latter region, but was now present as two major bands (gel a). The isozyme intensity in regions 2·9 and 3·3 cm disappeared or decline noticeably.

Characterization of cathodic esterase activity showed that cultivars of Virginia and Spanish type peanuts could be distinguished by the presence of two major isozymes and one major and minor band in region 0·1–1·0 cm, respectively. The patterns did not change during seed development and germination and did not vary between the cultivars within each group grown at the different geographical locations.

DISCUSSION

One major problem encountered in interpretation of enzyme electrophoretic patterns from seeds is the lack of precise knowledge regarding the exact stage of maturity of the seeds. Thus, unless several individual seeds are separately examined at different stages of development, maturity and germination, as in the present investigation, it could be extremely difficult to determine and correctly interpret data for: (1) intra- and inter-specific molecular variation which would ascertain and verify the genetic relationships between typical species of plant populations; (2) the differential genetic expressions of alleles in the organism during development and differentiation; and (3) the collection of seeds at different stages of maturation or germination.

Alcohol dehydrogenase, INT oxidase and catalase showed no cathodic enzyme activity and no intra- and inter-varietal anodic isozyme polymorphism in developing, mature and germinating peanuts. Only quantitative variations in the early stages of seed development and germination distinguished most of the major anodic isozyme patterns of leucine aminopeptidase within and between cultivars. These observations indicated that these enzymes are synthesized very early in peanut development and remain constant through maturity and through 24 hr germination of the seeds. They do not vary between cultivars and are not influenced by environmental conditions.

Examination of Virginia 56R seeds showed that both qualitative and visible quantitative anodic isozyme variations exist between zymograms for acid phosphatase, ammonium sulfate purified esterase and the minor bands of leucine aminopeptidase. In most cases, comparisons of other cultivars from different geographic areas consistently produced isozyme patterns similar to those of Virginia 56R occurring in highest frequency. The few distinguishing isozyme variations observed for certain cultivars may be due to intravarietal polymorphism not yet detected in the other cultivars, as indicated in similar studies with cotton.[29,30]

Comparison of the anodic isozyme patterns for acid phosphatase and esterase from early developing and germinating peanuts to those at later stages of development and dormancy indicate that most of the variations present in the former stages do not occur in the latter. This suggests that isozyme patterns of acid phosphatase and esterase may be useful as indicators of seed maturity. It is interesting to note that similar anodic isozyme variations for acid phosphatase have been reported in genetic studies of maize.[32,33]

EXPERIMENTAL

Materials. A number of cultivars from the different peanut types, *Arachis hypogaea* L. subsp. *fastigiata* var. *vulgaris* (Spanish botanical type) and *A. hypogaea* L. subsp. *hypogaea* var. *hypogaea* (Virginia botanical and Virginia and Runner marketing types) grown in four areas (Virginia, Georgia, Texas, and Oklahoma) of the United States were analyzed as shown in Table 1. 12 seeds (from random samples of field-grown plots) of

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each cultivar were analyzed individually for enzyme content. 84 seeds (4 seeds from each of 21 plants) of Virginia 56R grown in Louisiana were similarly examined.

Experimental procedures. All procedures were carried out at 0–4°. The skins were removed from the seeds and each seed was ground individually in 1·4–2·8 ml of pH 7·9 phosphate buffer, I = 0·01, (depending upon the size of the seed), followed by centrifugation at 39 000 *g* for 30 min. After centrifugation the clear supernatants were examined for anodic and cathodic catalase, leucine aminopeptidase, acid phosphatase, INT oxidase, and alchol dehydrogenase activity by the starch gel electrophoretic technique of Brewbaker *et al.*[34] The enzymic stains for characterizing the catalase, leucine aminopeptidase, acid phosphatase and alcohol dehydrogenase isozyme patterns were those of Brewbaker *et al.*[34] and Scandalios.[3] INT oxidase activity was identified on the same gels as the alchol dehydrogenase as clear areas, while the latter enzyme appeared as dark blue bands on a light blue background.[35] To detect esterase activity, the supernatants were subsequently treated with 40% $(NH_4)_2SO_4$. After allowing the samples to stand for 30 min with occasional mixing, they were centrifuged for 10 min at 39 000 *g*. The clear supernatants were then dialyzed overnight in the cold against the extracting buffer to remove $(NH_4)_2SO_4$ prior to electrophoresis. The dialyzed samples were then examined by polyacrylamide gel electrophoresis using the combined methods of Steward *et al.*,[36] and Cherry *et al.*[37] Cathodic zymograms were developed on polyacrylamide gels simply by reversing the electrical poles. Esterase activity was detected by the procedure of Cherry and Katterman.[29]

Immature pods were shelled and the seeds screen-sorted into 10 categories. The screen sizes used were as shown under Fig. 3. In each of the 10 sized categories, the seed rode the indicated screen but passed through the next larger size with the last one passing through the screen 0·48 × 1·92 in. Schenk[38] reported on the week-by-week morphological characteristics of developing peanuts. An examination of his data indicated that the immature seeds used in our studies ranged in maturity from 3 to 12 weeks before dormancy. The cultivar represented in our investigation of immature seeds was Florigiant grown in Georgia. For studies of isozyme profiles in germinating seeds, Virginia 56R peanuts from Virginia were germinated for 4 and 24 hr in a moist atmosphere at 30°.

Acknowledgements—Seeds from different cultivars of peanuts grown in five geographical locations of the United States for use in this investigation were generously supplied by Mr. W. K. Bailey, Mr. J. I. Davidson, Dr. R. O. Hammons, Mr. J. A. Harris, Dr. A. L. Harrison, and Dr. J. S. Kirby. Immature seeds used in the developmental studies were a gift of Dr. R. O. Hammons.

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