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Isoenzyme Polymorphism in Flowering Plants V. The Isoesterases of Maize: Tissue and Substrate Specificities, and Responses to Chemical Inhibitors

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INTRODUCTION

Esterases are among the most polymorphic enzymes of higher organisms. The discovery by Smithies (1955) that proteins could be separated electrophoretically and stained on gels provided a versatile new method for esterase studies. Esterase variants or isoenzymes under discrete genetic control can often be distinguished readily by this technique, and thus they provide biochemical fingerprints that are increasingly applicable to agriculture and medicine.

The esterases of maize were first studied on electrophoresced gels by Schwartz (1960) who described the E_1 allelic set of pH 7.5 isoesterases and called attention to other polymorphisms of this enzyme. The E_1 alleles interacted to produce hybrid enzymes, and this was interpreted to result from a dimeric structure of the E_1 esterases (Schwartz, 1962*a*, 1962*b*, 1967). Beckman, Scandalios, and Brewbaker (1964) and Macdonald (1969) subsequently showed that certain catalases and transaminases of maize also form hybrid isoenzymes. Schwartz and his colleagues later described esterases at the E_2, E_3, and E_4 loci (Schwartz, 1964, 1965; Harris, 1966, 1968). Seven loci (E_5 to E_{10}) were designated in studies related to those to be reported here (Macdonald, 1969; Macdonald and Brewbaker, 1974). Two additional esterase loci were proposed by Brown and Allard (1969).

Although early reports about maize esterases were confined to cathodal isoenzymes, the use of discontinuous buffers (Ashton and Braden, 1961) facilitated resolution of a large spectrum of discrete esterase isoenzymes that migrated to the anode on neutral or alkaline gels (Scandalios, 1964). These techniques were modified after tests on a

wide variety of species and tissues (Brewbaker, Upadhya, Makinen, and Macdonald, 1968) and are extended here to include diverse substrates and enzyme inhibitors.

Esterases have been divided into three major classes on the basis of selective inhibition and substrate specificity: the true lipases, the lipase-type hydrolases, and the ester hydrolases, the latter acting on a diverse group of predominantly soluble esters. Maize isoesterases appear to be entirely of this third group, which is further divided into cholinesterases (EC 3.1.1.7), pseudocholinesterases (EC 3.1.1.8), arylesterases (EC 3.1.1.2), carboxylesterases (EC 3.1.1.1), and acetylesterases (EC 3.1.1.6). Cholinesterases and pseudocholinesterases are distinguishable from other ester hydrolases by their sensitivity to eserine. Aldridge (1953*a*, 1953*b*) and Bergmann, Segal, and Rimon (1957) further proposed that arylesterases and acetylesterases could be differentiated from the carboxylesterases by their resistance to organophosphates and from each other by reactions to pCMB (parachloromercuribenzoate) and certain other sulfhydryl compounds.

All the principal maize isoesterases representing a very diverse germplasm have been identified in this study. Their tissue and substrate specificities and their reactions to selected substrate inhibitors and activators are described in relation to the Aldridge-Bergmann enzymatic classification (Bergmann et al., 1957).

EXPERIMENTAL METHODS

Methods used for the electrophoresis of maize esterases were quite similar to those described previously (Brewbaker et al., 1968). Most maize tissues were extracted by simple maceration in saline or tris-HCl buffer and applied to filter paper wicks. Immature endosperm was applied directly to the filter paper. Little loss of stainability occurred when tissues were frozen prior to sampling, and little difference was noted between extracts made in saline or in tris-HCl buffers. The gels contained 13.5 percent starch in a mixture of tris-citrate and boric acid-lithium hydroxide buffers with pH 8.2. The borate-LiOH buffer was used in electrode tanks, and the gels were electrophoresced about 4 hours in a refrigerator at 150 v and 50 ma. Resolution of individual bands was generally quite clear, with little difference noted on acrylamide gels.

Gels were stained at 37° C in 100 ml of a 0.1 M phosphate buffer solution at pH 6.5. Fast Blue RR salt was mixed with the buffer (100

mg in 100 ml) and filtered, and 3 ml of substrate solution (1 percent alpha-naphthyl acetate in 70 percent acetone) were added just prior to immersion of the gel. Inhibitor and activator studies were conducted by incubating the electrophoresced gel in the inhibitor or activator solutions (pH 6.5) prior to staining, by the addition of compounds directly to the stain, or by a combination of the two methods.

Seedling tissues were obtained from seeds germinated in the dark on moist filter paper and transferred to the light and maintained there for 8 to 10 days prior to extraction. Mature plant tissues were commonly collected from plants grown at the Waimanalo Research Station of the Hawaii Agricultural Experiment Station.

ESTERASE POLYMORPHISM AND TISSUE SPECIFICITY IN MAIZE

Relative Mobility and Genetic Polymorphism

The principal esterases in tissues from approximately 300 races, inbreds, and varieties of maize are presented in composite diagram in Figure 1, as they appeared following starch gel separation at pH 8.2 (*see* Figure 2, Brewbaker et al., 1968). A single group of esterases, the E_1 esterases (Schwartz, 1960), migrated toward the cathode under these conditions; all remaining isoenzymes migrated toward the anode. Studies of the cathodal polymorphisms confirmed the presence of the E_1^F (fast), E_1^N (normal), and E_1^S (slow) alleles and their hybrid bands. We did not attempt to verify activity of the alleles E_1^L, E_1^T, and E_1^W (of Colombian maize lines) or E_1^R (in teosinte) alluded to by Schwartz (1967). Since the E_1 locus had already been quite thoroughly researched, this study focused attention on the anodally migrating esterases.

The 28 major anodal esterases distinguished in maize are listed and discussed below in the order of their decreasing mobility on gels. Relative activities are given for these isozymes in the principal tissues studied in Table 1. These tissues include the seedling root, leaf, and coleoptile; the scutellum and endosperm of seeds; the mature leaves, nodes, and adventitious roots of plants; the ear husks and cobs; and the tassel, anther wall, and pollen. One major anodal isoenzyme, designated band 18, stained intensely in essentially all tissues and strains at a position of Rf 82, Rf being relative to the borate front. Band 18 thus proved to be a highly reliable reference band in maize for the calculation of relative mobility (Rm) values and was assigned Rm 1.00.

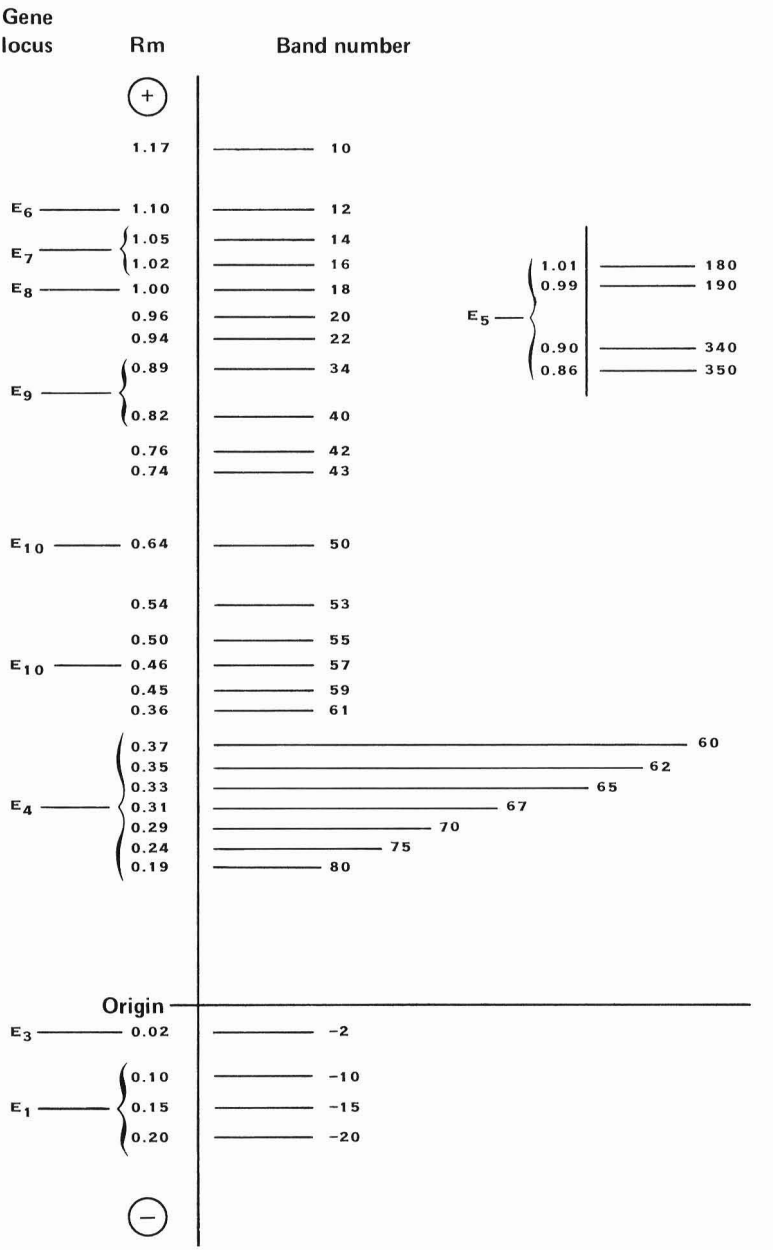

Figure 1. Diagram of the principal esterases of maize tissues, electrophoresced at pH 8.2, with mobility values relative to anodal band 18. Enzyme governed by E_2 locus is presumably near Rm 0.80.

Table 1. Relative activity of maize esterase isoenzyme bands in several tissues

Band	Gene locus	Tissue[a]													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
10	—	3[b]	0	0	0	0	0	0	0	0	0	0	0	0	0
12	E_6	1	2	1	0	0	2	1	0	0	0	1	0	0	0
14,16	E_7	1	1	0	2	1	0	0	0	0	0	1	0	1	1
18	E_8	3	3	3	3	3	3	3	3	3	3	3	3	3	3
180,190,340,350	E_5	2	2	0	2	0	0	0	0	0	0	0	0	0	1
20,22	—	2	0	0	0	0	0	0	0	0	0	0	0	0	0
34,40	E_9	3	3	3	2	3	3	3	3	3	3	3	3	3	3
42,43	—	2	1	1	3	1	1	1	1	1	1	1	1	1	1
50,57	E_{10}	0	0	0	0	0	2	0	0	0	0	0	0	0	0
53	—	0	0	0	0	0	1	0	0	0	0	0	0	0	0
55	—	0	0	0	0	0	0	0	0	0	0	0	0	0	3
59	—	0	1	0	1	0	0	0	0	0	0	0	0	0	3
61	—	0	0	0	0	0	0	0	0	0	0	0	0	0	3
60,62,65,67,70,75,80	E_4	3	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Numbers indicate tissues: 1—seedling root; 2—first leaf; 3—coleoptile; 4—scutellum; 5—mature endosperm; 6—immature endosperm; 7—mature leaf; 8—ear husk; 9—node and internode; 10—adventitious root; 11—immature ear; 12—tassel branch; 13—anther wall; 14—pollen.

[b] Numbers indicate intensity of stain on gels: 0—no stain; 1—weak stain; 2—medium stain; 3—intense stain.

Band 10 (Rm 1.17) appeared only in root tissues infected by common airborne fungi, including penicillium and aspergillus, and was inhibited by fungicidal treatments. The mercuric fungicide, Panogen, inhibited the appearance of band 10 at 5000 ppm, although it had no effect on the other bands. Band 10 was observed sporadically in partially senescent root tissues in the petri dish seedlings; fungal extracts did not show the esterase.

Band 12 (Rm 1.10) was a lightly stained band found in most tissues examined and in nearly all strains. Monogenic variants that lacked this band were found, and the controlling locus was designated E_6 (Macdonald, 1969; Macdonald and Brewbaker, 1974).

Bands 14 and 16 (Rm 1.05 and 1.02) were relatively weak bands observed in extracts from several tissues (for example, seedling root and leaf, scutellum, and pollen). They occurred together, never separately, in inbred lines and were designated the E_7 esterases (Macdonald, 1969; Macdonald and Brewbaker, 1974).

Band 18 (Rm 1.00) was an intensely stained band present in extracts of all maize tissues and in nearly all of the 300 strains tested. Several variants of this band were observed to have small displacements (for example, 0.03 Rm values) from band 18, but they were not studied genetically. A null type was observed in two tropical stocks. Genetic studies with the null type indicated a monogenic F_2 segregation of presence and absence, with presence dominant. This was designated the E_8 locus (Macdonald, 1969; Macdonald and Brewbaker, 1974).

Bands 180 and 190 (Rm 1.01 and 0.99) were weakly stained bands that occurred in seedling and pollen extracts. They occurred together in all lines and were never found separately. These bands could be observed only upon the chemical inhibition of band 18. Genetic studies revealed fast and slow variants of each band and the relationship of two sets of four bands to the 340 and 350 series. This complex was found to be under the control of a pair of genes (loci E_5-I and E_5-II), governing the presence of four bands (either 180^F, 190^F, 340^F, and 350^F or 180^S, 190^S, 340^S, and 350^S).

Band 20 (Rm 0.96) was observed only in seedling root extracts. It differed from neighboring band 18 in its reaction to several inhibitors.

Band 22 (Rm 0.94) was found only in roots of a single plant introduction (PI 240320) from the United States Department of Agriculture, and it appeared to be related to band 20.

Bands 340 and 350 (Rm 0.90 and 0.86) were lightly stained bands discernible in roots and pollen after the chemical inhibition of band 34. Fast and slow variants occurred in each band, and both variants were always observed together. Genetic studies revealed the relationship of bands 340 and 350 to bands 180 and 190 and the E_5 loci control.

Bands 34 and 40 (Rm 0.89 and 0.82) were relatively intensely stained bands found in all tissues examined, and they were never found together in inbred lines. They were strongly activated by atropine. Genetic studies of these bands in pollen indicated monogenic control, with the bands representing fast and slow variants of a single esterase, designated the E_9 esterase. The poorly defined E_2 locus of Schwartz (1965) could not be verified, but it is possibly the same as the E_9 locus.

Bands 42 and 43 (Rm 0.76 and 0.74) were weakly stained bands observed in all tissues examined. No inbreds were observed that lacked both bands.

Bands 50 and 57 (Rm 0.64 and 0.46) were strong bands observed only in endosperm extracts. They were found to be under allelic control and were designated the E_{10} esterases (Macdonald and Brewbaker, 1974).

Band 53 (Rm 0.54) was a weakly stained band observed only in endosperm extracts. No strains were observed to lack this faint band.

Bands 55, 59, and 61 (Rm 0.50, 0.45, and 0.36) stained intensely only in pollen. A weakly stained band of corresponding mobility to band 59 was also noted in scutellum and plumule tissues. Genetic studies were not made of these enzymes.

Bands 60, 62, 65, 67, 70, 75, and 80 (Rm 0.37, 0.35, 0.33, 0.31, 0.29, 0.24, and 0.19, respectively) have been reported under control of the multiple allelic E_4 locus (Harris, 1968). Each homozygous genotype was represented by a group of four or five major bands of decreasing stainability, with as many as eight observed in certain gels. Genotypes were designated by band number of the slowest migrating band, always the most intensely stained. Null genotypes were also observed, and their genetic control provided evidence for both null alleles and repressor genes (Macdonald, 1969). As diagrammed in Figure 1, the second band in group 80 coincided with the first band in group 75, the second band in group 75 coincided with the first band in group 70, and so forth. Two exceptions to this pattern were provided by the 67 and 62 groups of bands, which were displaced toward the origin from the 65 and 60 multiple bands. The E_4 esterases stained strongly only in seedling root tissues. Harris (1966) varied pH and starch concentrations in his studies of the E_4 complex, concluding that the multiple forms were not grossly different in molecular weight.

Differentiation and Tissue Specificity

The distribution of maize isoesterases in diverse tissues is summarized in Table 1. With few exceptions, the identity of bands with similar mobilities in different tissues was confirmed by electrophorescing adjacent samples. There was no evidence of tissue modification of isozyme Rf values.

Comparatively few maize isoesterases stained intensely in all tissues studied, most striking among these the ubiquitous and strong band 18 and the allelic bands 34 and 40. Bands 42 and 43 were also observed in all tissues; their concentration in seeds increased rapidly during germination.

In contrast, several isoenzymes characterized only a single tissue. Root-specific isozymes were those of bands 10, 20, and 22, and of the E_4 esterases. Bands restricted to young endosperm included band 53 and the E_{10} esterases (bands 50 and 57), while bands 55 and 61 were pollen-specific. This, in fact, left only four stained regions that were present in more than one tissue but not in all. These comprised band

12, band 59, and the bands under control of the E_5 (180, 190, 340, and 350) and E_7 (14 and 16) loci.

Differentiation of tissues was accompanied in some cases by both qualitative and quantitative changes in esterase activity, although this was more the exception than the rule (Figure 2). Germinating seeds of maize inbreds representing the full complement of esterase bands were tested for their esterase activity at different stages of germination. Roots taken between 3 and 8 days showed a rapid increase of band multiplicity of the unique E_4 bands, with the slowest or parental band staining darkly throughout. No other bands of roots or of seedling leaves showed major changes during germination. The scutellum and embryo, however, underwent several changes during the first week of germination, accompanying a general increase in esterase activity. Increasing stainability was noted especially for scutellum bands 59, 42, and 43 and the bands of the 340 series, while an apparently unique

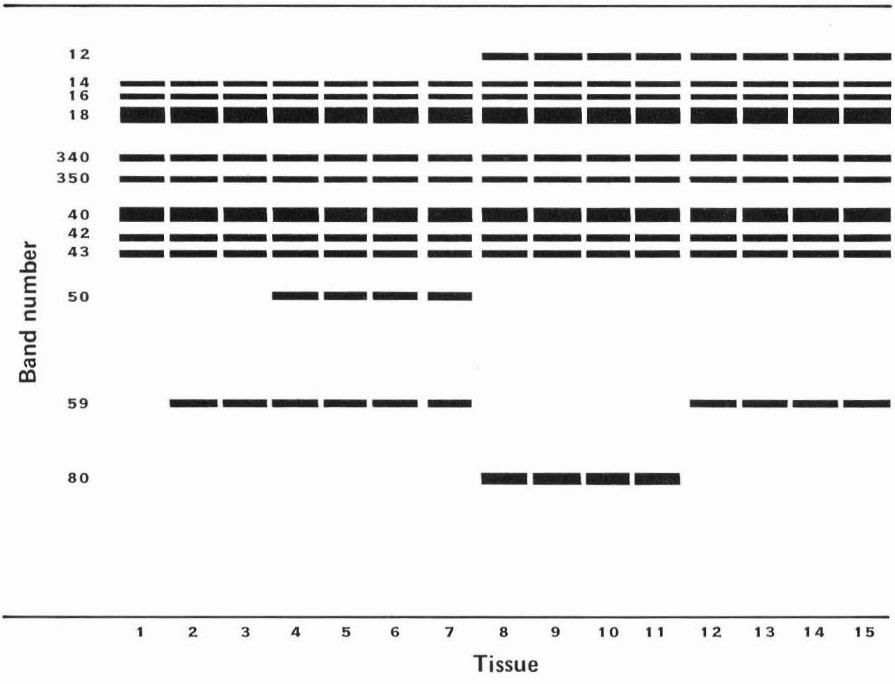

Figure 2. Esterase isoenzyme bands from tissues of germinating seeds of maize. Tissue legend: 1 to 6, scutellum (1, 3, 4, 5, 6, and 7 days); 7, embryo axis (3 days); 8 to 11, root (4, 5, 6, and 7 days); and 12 to 15, plumule (4, 5, 6, and 7 days).

band appeared at a position near band 43 (Rm 0.70). In the endosperm of seeds, bands 42 and 43 similarly increased in activity during germination, as did those of the E_7 locus (bands 14 and 16). Judging by the gel results, scutellum had the highest esterase activity of any maize tissue, a fact evidently associated with the high lipid content (40 percent) of this tissue.

SUBSTRATE SPECIFICITY IN MAIZE

Esterase staining on gels is dependent on the splitting of naphthyl esters and coupling of the naphthyl moiety to Fast Blue RR salt or other dye. A series of soluble naphthyl esters were utilized here as substrates in a study of the comparative effectiveness of maize esterases. The principal substrates used were the alpha and beta naphthyl esters of acetic, butyric, lauric, myristic, palmitic, and stearic acids (Table 2). All were used at the standard concentration for naphthyl acetate, 0.03 percent. The longer chain esters, palmitate and stearate, were of limited solubility in the stain solution.

Maize isoesterases appeared to utilize the alpha and beta naphthyl esters of acetic acid with equal effectiveness, and they were only slightly less effective on butyrate. However, staining time required for readout on laurate, myristate, palmitate, and stearate salts was very prolonged, up to as much as 15 hours. Judging by both staining time and intensity of stained bands, the maize esterases are not particularly effective as lipases, that is, in acting on the long chain esters in this study. Significantly, the scutellum isoesterases (especially the E_5, E_9 loci, and bands 42 and 43) were more efficient lipases than were those of roots and other tissues.

CHEMICAL INHIBITION AND ACTIVATION OF MAIZE ESTERASE ISOENZYMES

The effects of a large number of organic and inorganic compounds have been assessed on the activity of esterases from plant and animal tissues. Esterase classification is based to some extent on selective inhibition by certain of these compounds (Aldridge, 1953*a*, 1953*b*; Bergmann et al., 1957). Highly selective inhibitors were included in the present study of electrophoresced maize esterases, and representative data are summarized in Tables 3, 4, and 5.

Table 2. Relative activity of maize esterase isoenzymes on various substrates

Substrate	Isoenzyme complex												
	E_4 (60,62, 65,67, 70,75, 80)	E_5 (180,190, 340,350)		E_6 (12)	E_7 (14,16)		E_8 (18)		E_9 (34,40)		(42,43)		(59)
	Rt[a]	Sc	Rt	Rt	Sc	Rt	Sc	Rt	Sc	Rt	Sc	Rt	Sc
α - naphthyl acetate	3[c]	2	2	1	2	2	3	3	3	3	2	2	1
β - naphthyl acetate	3	2	2	1	2	2	3	3	3	3	2	1	1
α - naphthyl butyrate	3	1	1	1	1	1	3	3	2	3	1	1	1
α - naphthyl laurate[b]	1	0	0	0	0	0	2	2	2	2	1	1	0
β - naphthyl laurate[b]	1	0	0	0	0	0	2	2	2	2	1	1	0
α - naphthyl myristate[b]	1	0	0	0	0	0	2	2	2	2	1	1	0
β - naphthyl myristate[b]	0	0	0	0	0	0	2	2	2	2	1	1	0
α - naphthyl palmitate[b]	0	0	0	0	0	1	0	1	0	0	1	0	0
β - naphthyl palmitate[b]	0	0	0	0	0	1	0	1	0	0	1	0	0
α - naphthyl stearate[b]	0	0	0	0	0	1	0	1	0	0	1	0	0
β - naphthyl stearate[b]	0	0	0	0	0	1	0	1	0	0	1	0	0
β - carbonaphthoxy choline iodine	1	0	1	0	0	0	2	2	2	2	2	1	0

[a] Rt = root; Sc = scutellum.

[b] Data recorded after a prolonged staining time exceeding 12 hours.

[c] Numbers indicate intensity of stain on gels: 0—no stain; 1—weak stain; 2—medium stain; 3—intense stain.

Inorganic Compounds

Three inorganic compounds inhibited certain maize isoesterases specifically; these were fluoride, permanganate, and the phosphate compounds (Table 3). The fluoride ion partially suppressed band 18 at a concentration of 1 g/liter (as NaF) and inhibited it entirely at 5 g/liter. Following the treatments with 5 g/liter NaF, stained gels showing inhibition of band 18 were washed in running water for an hour and restained. Band 18 was reactivated by this treatment, indicating reversability of the inhibition. As noted earlier, inhibition of band 18 revealed that it masks two weakly staining bands, 180 and 190, at nearly the same position on the gels. Fluoride has been reported to inhibit cholinesterases (Pastor and Fennell, 1959; Heilbronn, 1965; Cimasoni, 1966), arylesterases (Komma, 1963) and possibly also carboxylesterases (Bolkova, Novak, and Skorepa, 1960). The reversibility of this inhibition was demonstrated by Heilbronn (1965) and Cimasoni (1966).

The phosphate ion similarly appeared to inhibit band 18 specifically. The staining solution used for these studies normally contained

Table 3. Relative activity of maize esterase isoenzymes in the presence of various inorganic inhibitors

Compound	Concentration (g/liter)	Isoenzyme complex						
		E_4	E_5	E_6	E_7	E_8	E_9	42,43
Na fluoride	1	2[a]	2	2	2	1	2	2
Na fluoride	5	2	2	2	2	0	2	2
K permanganate	5	2	2	2	2	2	1	2
Na phosphate	0.1 M	2	2	2	2	2	2	2
Na phosphate	0.5 M	2	2	2	2	1	2	2
K metaperiodate	10	0	0	0	0	0	0	0
Cu nitrate	10	0	0	0	0	0	0	0
Cu chloride	5	0	1	1	1	1	1	1
Hg chloride	1	2	1	1	1	1	1	1
Fe ammonium sulfate	5	1	1	1	1	1	1	1
Na ferricyanide	5	1	1	1	1	1	1	1
Na ferrocyanide	5	1	1	1	1	1	1	1
Fe chloride	5	2	2	2	2	2	2	2
Na cyanide	1	2	2	2	2	2	2	2
Na arsenate	1	2	2	2	2	2	2	2
Pb acetate	1	2	2	2	2	2	2	2
St chloride	1	2	2	2	2	2	2	2
Ca chloride	5	2	2	2	2	2	2	2

[a] Numbers indicate intensity of stain on gels: 0—no stain; 1—weak stain; 2—intense stain.

phosphate buffer. When the phosphate level was raised from the normal 0.1 M to 0.5 M, selective inhibition of band 18 occurred (Table 2). Phosphate has been reported as an inhibitor of cholinesterases at high concentrations (Cimasoni, 1966).

The permanganate ion appeared to be a specific inhibitor of related bands 34 and 40 (genetic locus E_9), which were partially but not completely suppressed by potassium permanganate at a concentration of 5 g/liter. Other inorganic compounds showed either a generalized inhibition of all bands or no effect at the concentrations tested. Limited tests were made with bands 20, 53, and the E_{10} locus bands 50 and 57; they were unaffected by fluoride, permanganate, and phosphate at the concentrations tested.

Eserine

Organic compounds tested for selective inhibition or activation of maize esterases (Table 4) included substances, such as eserine, that have been reported to be inhibitors of esterase activity. None of the maize

Table 4. Relative activity of maize esterase isoenzymes in the presence of various organic inhibitors

Compound	Concentration (M)	Isoenzyme complex						
		E_4	E_5	E_6	E_7	E_8	E_9	42,43
Eserine sulfate	1×10^{-2}	2[a]	2	2	2	2	2	2
Atropine alkaloid	1000 ppm	2	2	2	2	2	3	2
Atropine sulfate	2×10^{-3}	2	2	2	2	2	3	2
Tropine	4×10^{-2}	2	2	2	2	2	2	2
EDTA	1×10^{-3}	2	1	2	1	2	2	2
pCMB	2.5×10^{-2}	2	0	2	0	2	0	2
Cysteine	2×10^{-3}	1	0	0	0	1	1	1
Cysteine	8×10^{-3}	0	0	0	0	0	0	0
Thiourea	4×10^{-1}	1	1	1	1	1	1	1
Taurocholate	1×10^{-3}	2	2	2	2	2	2	2
Iodoacetamide	4×10^{-2}	2	2	2	2	2	2	2
Tannic acid	3×10^{-2}	0	0	0	0	0	0	0
Quinine sulfate	7×10^{-3}	2	2	2	2	2	2	2
Acetylcholine	5×10^{-3}	2	2	2	2	2	2	2
Cholic acid	1×10^{-3}	2	2	2	2	2	2	2
Lauryl sulfate	1×10^{-3}	2	2	2	2	2	2	2
Formamide	1	2	2	2	2	2	2	2

[a] Numbers indicate intensity of stain on gels: 0—no stain; 1—weak stain; 2—medium stain; 3—intense stain (activation).

esterases were inhibited by eserine sulfate at a concentration of 10^{-2} M, although band 18 appeared to have an altered staining reaction. In the presence of eserine, band 18 stained red rather than the normal blue-black, suggesting a change in activity induced by eserine. Eserine is often used to differentiate the cholinesterases, which are specifically inhibited at concentrations that do not affect other esterases (for example, 10^{-3} M).

Atropine and Tropine

Atropine was applied to gels both as a sulfate salt and as the alkaloid solution. The E_9 locus bands, 34 and 40, were strongly activated by atropine alkaloid at 0.1 percent and by atropine sulfate at 2×10^{-3} M (Table 4). These two bands stained within 3 minutes in the presence of 0.5 percent alkaloid, but took up to 30 minutes in the absence of atropine. No other bands were affected similarly by the alkaloid or sulfate solution. Atropine has been reported to inhibit the aromatic or aryl esterases (Marton and Kalow, 1960). Tropine had no effect on maize isoesterases at a concentration of 4×10^{-2} M.

Ethylenediaminetetraacetic Acid (EDTA)

EDTA repressed slightly the activity of bands under control of the E_5 and E_7 loci at a concentration of 10^{-3} M. At concentrations of 10^{-4} M and less, no inhibition was observed, but all bands were inhibited by 10^{-1} M treatments. This inhibition could be reversed by soaking gels for 1 hour in 5 percent aqueous solutions of calcium chloride.

EDTA is a strong chelating agent known to inhibit cholinesterases, arylesterases, and carboxylesterases (Marton and Kalow, 1960; Komma, 1963; Cimasoni, 1966). Low concentrations of EDTA were observed by Erdös, Debary, and Westerman (1960) and Komma (1963) to activate esterases. Keay and Crook (1965) reported no EDTA inhibition of liver esterases, but they noted activation by calcium chloride. Neither EDTA nor $CaCl_2$ activated or hastened staining of maize esterases in the present study.

Parachloromercuribenzoate (pCMB)

Treatments with pCMB selectively inhibited bands controlled by the E_5, E_7, and E_9 loci at a concentration of 2.5×10^{-2} M, while having no effect on other bands. The chemical pCMB has been reported to inhibit cholinesterases and arylesterases (Aldridge, 1953*a* and 1953*b;* Bergmann et al., 1957).

Cysteine

Cysteine proved to be a potent inhibitor of maize esterases, repressing all bands at 8×10^{-3} M. A small amount of staining activity was retained at 2×10^{-3} M by bands 18, 34, and 40, and by the E_4 locus (all of which were intensely staining regions). Cysteine has been reported as a generalized esterase inhibitor (Erdös and Laswick, 1961).

Other Organic Compounds

Taurocholate has been reported both to inhibit esterases (Pastor and Fennell, 1959) and to activate them (Komma, 1963); it had no effect on maize esterases at 10^{-3} M. Iodoacetamide, reported to activate esterases by Komma (1963), had no effect on maize esterases at concentrations as high as 4×10^{-2} M. Tannic acid, reported by Hall (1966) to inhibit tomato pectinesterases, completely repressed maize esterases at concentrations above 3×10^{-2} M, and a generalized inhibition of all bands occurred at 3×10^{-3} M. Quinine sulfate, reported to inhibit esterase activity by Augustinsson and Olsson (1959), failed to inhibit maize esterases at concentrations up to 7×10^{-3} M. Other organic compounds tested had no inhibitory effect at the concentrations applied.

Organophosphates

The implied importance of organophosphates in esterase classification, and the potency of many of these and the carbamates as insecticides, led us to initiate a study of the effects of various pesticides on maize esterases (Table 5). The anticholinesterase activity of many compounds is held responsible for their pesticidal properties (Meyers, Kemp, Tol, and DeJonge, 1957; O'Brien, 1966; Lewis, 1967; Bulmer and Fisher, 1967; van Asperen, 1960).

DDVP (dimethyl-2,2-dichlorovinyl phosphate) completely inhibited bands controlled by the E_6, E_9, and E_4 loci at a concentration of 25 ppm. Band 18 was highly inhibited by this treatment, and bands 42 and 43 were slightly inhibited. At concentrations as high as 100 ppm DDVP, activity was retained by bands 14 and 16 and by the bands of the E_5 locus.

Dibrom (dimethyl-1,2-dibromo-2,2-dichloroethyl phosphate) inhibited all bands except 18, 42, and 43 at a concentration of 50 ppm. Higher levels reduced stainability of bands 42 and 43 but did not affect band 18.

Table 5. Relative activity of maize esterase isoenzymes in the presence of several organophosphate and carbamate inhibitors

Compound[a]	Concentration (ppm)	Isoenzyme complex						
		E_4	E_5	E_6	E_7	E_8	E_9	42,43
DDVP	25	0[b]	2	0	2	1	0	1
DDVP	50	0	2	0	2	0	0	0
Dibrom	50	0	0	0	0	2	0	1
Dibrom	100	0	0	0	0	2	0	0
Fenitrothion	1000	2	2	2	2	2	2	2
Phosphamidon	1000	2	2	2	2	2	2	2
Carbaryl	200	0	2	0	2	1	0	2
Carbaryl	400	0	2	0	2	0	0	2
Pyramat	400	2	2	2	2	2	2	2
Pyrolan	400	2	2	2	2	2	2	2
Bayer 39007	400	2	2	2	2	2	2	2

[a] Insecticide trade names; for active compound names, see text.
[b] Numbers indicate intensity of stain on gels: 0—no stain; 1—weak stain; 2—intense stain.

Fenitrothion (0,0-dimethyl-0-(3 methyl-4 nitrophenyl) phosphorothionate) had no observable effects on maize esterases at concentrations up to 1000 ppm. Sulfonated organophosphates of this type evidently must be metabolized *in vivo* to oxygenated analogues before they can act as esterase inhibitors (O'Brien, 1966).

Phosphamidon (dimethyl-2-chloro-2-diethylcarbamoyl-1-methylvinyl phosphate) showed no inhibition of maize esterases at concentrations up to 1000 ppm, despite its relationship to organophosphatic inhibitors, DDVP and Dibrom.

Carbamates

Carbaryl (or Sevin) was the only one of four carbamates tested that showed selective inhibition of maize esterases. At a concentration of 200 ppm, carbaryl inhibited fully all bands except those under control of the E_7 and E_5 loci and bands 42 and 43. Band 18 was reduced in activity at 200 ppm and was inhibited entirely at 400 ppm. Carbaryl has been reported to be metabolized by specific esterases in cotton (Moustafa, Hassan, and Zayed, 1966). Three other carbamates—Pyramat, Pyrolan, and Bayer 39007—were not effective as maize esterase inhibitors at the two concentrations tested.

DISCUSSION

These studies have confirmed the high polymorphism of maize esterases, revealing 28 anodally migrating bands or zones of activity in maize. Several additional cathodally migrating bands have been reported in previous studies to result from the action of loci E_1 and E_3. Evidence for eight gene loci was obtained or confirmed in the present study, in addition to the E_2 and E_3 loci of Schwartz (1964, 1965). In all studies of tissue, substrate, and inhibitor specificities, bands under the control of alleles at a single locus acted identically and could be defined as isoenzymes in the strictest sense.

Maize esterase polymorphisms may thus be reduced, upon consideration of present genetic information, to ten regions controlled by loci E_1 to E_{10} plus at least five other major regions (Figure 1). These five regions include band 10, inducible in roots by disease; the faint bands 20 and 22 of roots; the faint bands 42 and 43 of all tissues; a faint endosperm band 53; and the strong pollen bands 55, 59, and 61. Only two of these 15 regions, specified by the E_1 and E_3 loci, migrated cathodally at pH 8.2.

The scutellum of maize displayed the highest esterase activity of tissues we studied, as indicated by the intensity of stains. The E_5, E_6, E_8, and E_9 loci and bands 42 and 43 were particularly active in this tissue. A great increase in esterase activity occurred during the germination process in scutellum and endosperm tissues; this may be related to the metabolism of stored scutellar lipids. The conversion of plant triglycerides to free fatty acids and glycerol is accomplished by lipases, and this is a first step in the production of acetyl CoA and carbohydrates from lipids (Beevers, 1961; Stumpf and Barber, 1956). As much as 40 percent of the dry weight of maize scutellum is lipoidal (Zeller, 1957). These lipids disappear during germination, while sugars and starch granules simultaneously accumulate (Toole, 1924; Ingle, Beevers, and Hagemann, 1964; James and James, 1940). Lipid catabolism in maize was greatest between 3 and 5 days after germination (Ingle et al., 1964), at a time when locus E_5 and bands 42 and 43 were activated. It is tempting to assign lipase activity to certain of the maize esterases, especially those active in scutellum. Scutellum esterases could hydrolyze the longer chained substrates, albeit slowly (Table 2), and this is traditionally considered indicative of lipase activity (Hunter and Strachan, 1961). However, the very low substrate specificity of maize esterases makes a clear distinction between lipase-like esterases and ester hydrolases very difficult.

Our evidence concerning the cholinesterase activity of maize esterases can be variously interpreted. The cholinester, beta carboxy naphthoxy choline iodide, was hydrolyzed by all major maize esterases. In addition to hydrolyzing the cholinester, band 18 also was inhibited reversibly by fluoride and phosphates, a reported characteristic of cholinesterases (Heilbronn, 1965; Cimasoni, 1966). However, no convincing evidence of inhibition by the cholinesterase inhibitor, eserine, was obtained for maize isoesterases (Table 4). Furthermore, when acetylcholine was added to staining solutions on the alpha-naphthyl acetate substrate, no diminution of staining was observed, as one might predict if there were substrate competition for active sites on a cholinesterase. Although the data do not warrant the classification of any maize esterase as a cholinesterase, some bands, such as band 18, might be considered pseudocholinesterases ("S-type cholinesterases") acting on acetylcholine and a wide range of aromatic and aliphatic esters (Hawkins and Mendel, 1949; Fulton and Mogey, 1954).

The data similarly do not permit a simple classification of maize esterases as carboxylesterase, arylesterase, or acetylesterase, following traditional considerations of reactions to inhibitors. The Aldridge-Bergmann method of classifying esterases is summarized in Table 6 (Aldridge, 1953*a*, 1953*b*; Bergmann et al., 1957). Two different organophosphates, DDVP and Dibrom, selectively inhibited different maize esterases (Table 5), precluding their direct classification as carboxylesterases. However, bands resistant to DDVP were those of the E_5 and E_7 loci, which had also been unique in their susceptibility to the thiol reagent, pCMB (Table 4). They thus appeared to be classical arylesterases (*see* Bergmann et al., 1957), discounting their sensitivity to Dibrom. Band 18, under control of the E_8 locus, was unique in its resistance to Dibrom at concentrations inhibiting all other bands. As noted previously, band 18 was also unusual in its sensitivity to fluoride

Table 6. Aldridge-Bergmann classification of esterases by sensitivity to inhibitors[a]

	Inhibitor	
Class of esterase	Organophosphates	pCMB
Arylesterase ("A type")	0	+
Carboxylesterase ("B type")	+	0
Acetylesterase ("C type")	0	0

[a] 0 = no effect; + = strongly inhibited.

Table 7. Major groupings of maize esterases by sensitivity to inhibitors[a]

Loci or bands	Inhibitor					Possible classification
	Fluoride and phosphate	pCMB	DDVP	Dibrom	Carbaryl	
E_8	+	0	+	0	+	pseudocholinesterase
E_5,E_7	0	+	0	+	0	arylesterase
42,43	0	0	+	+	0	carboxylesterase
Others	0	0	+	+	+	carboxylesterase
E_9	0	+	+	+	+	(unknown)

[a] 0 = no effect; + = strongly inhibited.

and phosphate inhibition, and it showed strong pseudocholinesterase activity.

Although the data permit no direct classification, they leave little question of the enzymatic dissimilarity of many maize esterases, with the emergence of four major groupings according to their sensitivity to inhibitors (Table 7). The status of the E_9 esterases is enigmatic. Possible classifications in Table 7 are based principally on the fluoride, DDVP, and pCMB data. Similar applications of the Aldridge-Bergmann method of classification have indicated the relative complexity of plant esterase systems (Jooste and Moreland, 1963; Schwartz, Biedron, Holdt, and Rehm, 1964; Norgaard and Montgomery, 1968). Norgaard and Montgomery used manometric methods of separation on pea extracts, differentiating six esterases on the basis of selective inhibition by three organophosphates, and classifying five of the six as carboxylesterases. Since physical or electrophoretic methods of separation were not used, however, the six esterases may have been mixtures of more than one isoenzyme, and the confirmation of these results on zymograms would be desirable.

SUMMARY

The esterases of maize were separated by gel electrophoresis, and isoenzyme polymorphisms were identified in relation to genetic, tissue, substrate, and inhibitor variations. Among approximately 300 maize varieties and inbreds, 38 major zones of activity have been observed,

and our studies were confined to 28 zones that migrated anodally under the alkaline conditions imposed. Genetic studies confirmed or revealed that ten governing loci accounted for 24 of these zones of activity. The remaining bands aggregated in six apparent groups. The data are summarized in reference to esterase zones characterizing these ten loci (E_1 to E_{10}) and six groups (identified by bands 10, 20 and 22, 42 and 43, 53, 59, and 55 and 61).

Studies of 17 tissues revealed that many isoesterases of maizes are tissue-specific, confined to tissues, such as the root (E_4 locus, bands 10, 20, and 22), immature endosperm (E_{10} locus, band 53), and pollen (bands 55 and 61). Activity was shown in all 17 tissues by esterases of the E_8 and E_9 loci, and bands 42 and 43. Four remaining zones represented esterases that were active in some tissues and inactive in others (E_5 and E_7 loci, bands 12 and 59). Only one tissue variation was noted in relation to disease—the appearance of band 10 in infected roots. Other variations in tissues during maturation, during senescence, or in relation to light and growth conditions were small; however, a major increase in esterase activity occurred in the scutellum of the embryo during the germination process.

All maize esterases were highly active on substrates of acetate and butryate esters, but they utilized laurate, myristate, palmitate, and stearate esters much less efficiently. The embryo and scutellar isoesterases appeared to be more active on the longer chained esters, and they can be viewed as lipases of comparatively low activity.

Series of inorganic and organic compounds were tested as inhibitors or activators of maize isoesterases. Selective fluoride and phosphate inhibition occurred in band 18, which also showed a modified staining reaction in the presence of eserine, suggesting the probable classification of band 18 as a pseudocholinesterase (EC 3.1.1.8); however, it was highly resistant to pCMB.

Reactions were variable to other organic compounds, and to sulfhydryl and organophosphatic inhibitors in particular, with several examples of zone specificity of an inhibitor or activator. The data did not permit direct classification of maize isoesterases as arylesterases, carboxylesterases, or acetylesterases. Considering the results especially of the DDVP and pCMB studies, however, the E_5 and E_7 esterases were postulated to be arylesterases (EC 3.1.1.2) and the remaining enzymes to be carboxylesterases (EC 3.1.1.1).

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