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ESTERASE ISOENZYMES FROM LEGUME ROOT NODULES

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Abstract—Multiple forms of esterases were separated by starch gel electrophoresis from root nodules of eight different legume species. Although nodules from each legume species had characteristic esterase-zymogram patterns, the reactivity of the various esterases with different substrates and inhibitors was quite similar. Most of the esterase activity was present in the supernatant fraction of the nodule extract. Zymogram patterns from young nodules were similar to those prepared from roots of the same plant but changes occurred in the nodule zymograms during the growth of the plant. Zymograms of esterases, proteases and dehydrogenases from the same nodule extract were also compared.

INTRODUCTION

GEL electrophoresis, combined with histochemical methods for localizing enzymic activity *in situ* on the gels, has been used extensively to separate multiple forms of esterases from mammalian tissues.[1–5] More recently, this technique has been used to study esterases from plant tissues[6–11] which have not been investigated as thoroughly as animal esterases.

Tissue specific esterase patterns have been applied to studies on the growth and development of human[12] and murine tissues.[2] It was decided to investigate legume root nodule esterases in some detail in relation to nodule development. This report is concerned with the heterogeneity of esterases from nodules of eight legume species. Brief reports about multiple forms of root nodule esterases have been published.[13, 14]

The following abbreviations are used:

NA, α-naphthyl acetate; NB, α-naphthyl butyrate; NP, α-naphthyl propionate; NC, α-naphthyl caprylate; BANA, benzoyl D-L-arginine-β-naphthylamide; DFP, diisopropyl-fluorophosphate.

RESULTS

Esterases from Different Legume Species

Zymograms prepared from nodules of eight different legume species all showed multiple forms of esterases (Fig. 1). The majority of the esterases migrated towards the anode but

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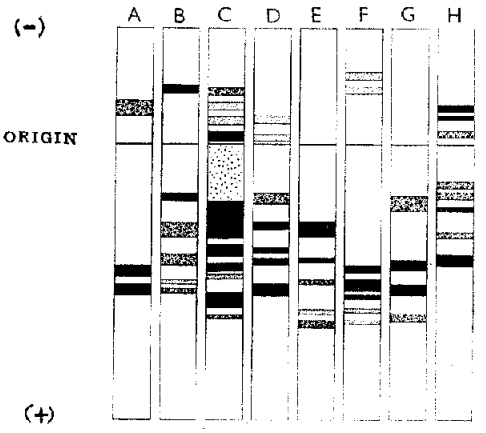

FIG. 1. DIAGRAMS OF ESTERASE ZYMOGRAMS PREPARED FROM NODULES OF EIGHT DIFFERENT LEGUME SPECIES. THE SUBSTRATE WAS α-NAPHTHYL ACETATE AND THE RELATIVE INTENSITIES OF THE ESTERASE BANDS ARE INDICATED BY SHADING.

A, *Pisum sativum;* B, *Lupinus luteus;* C, *Medicago sativa;* D, *Lotus corniculatus;* E, *Trifolium repens;* F, *Vicia faba;* G, *Galega officinalis;* H, *Glycine max.*

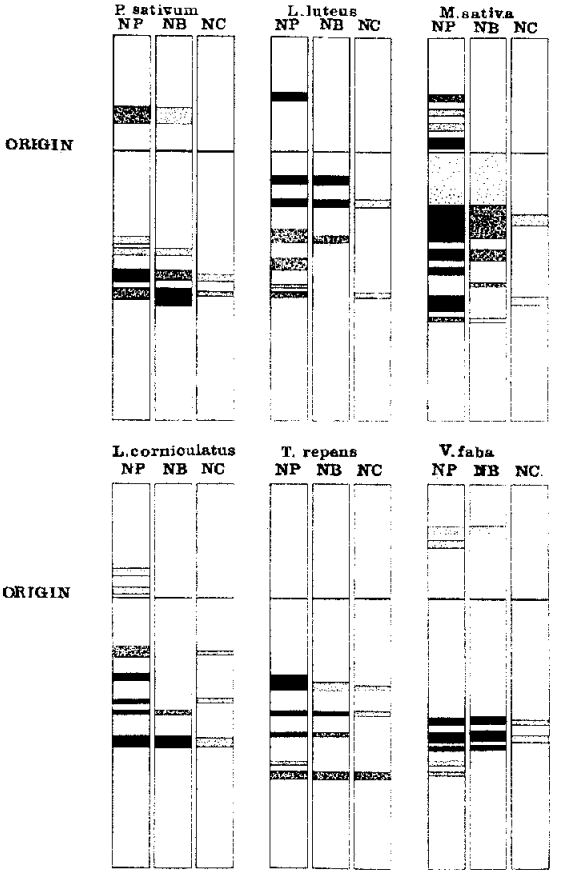

FIG. 2. DIAGRAMS OF ESTERASE ZYMOGRAMS PREPARED FROM SIX DIFFERENT LEGUME SPECIES. THREE SUBSTRATES WERE USED, NP, α-NAPHTHYL PROPIONATE; NB, α-NAPHTHYL BUTYRATE; NC, α-NAPHTHYL CAPRYLATE.

six of the eight legume species also had one or more bands which migrated towards the cathode. As shown in Fig. 1 the nodule zymograms were species specific and also the number of esterases on the zymograms varied from three bands in *Pisum sativum* to ten in *Medicago sativa*.

Substrate Specificity of Esterases

The reactivity of the nodule esterases with different substrates is shown in Fig. 2. Zymograms were prepared with substrates such as α-naphthyl propionate (NP), α-naphthyl butyrate (NB) and α-naphthyl caprylate (NC). When compared with the zymograms in Fig. 1 where α-naphthyl acetate (NA) was the substrate, it can be seen that several nodule esterases reacted equally well with NA, NP and NB. In general however, nodule esterases hydrolysed NA and NP in preference to NB but they had little reactivity towards NC (Fig. 2). Similar results were obtained with esterases from nodules of *Glycine max* and *Galega officinalis* which are not included in Fig. 2. Nodules from *G. officinalis*, *Trifolium repens* and *Vicia faba* were also tested for reactivity with α-naphthyl myristate but no bands were detected on the zymograms. Nodule esterases are therefore similar to mammalian esterases in that they preferentially hydrolyse esters of short-chain fatty acids.[12]

Fractionation of Nodule Esterases

Glycine max nodules were fractionated as described by Bergersen[15] to determine the distribution of esterase activity within the nodule. Each fraction was tested using NP as substrate. As shown in Table 1 the majority of the esterase activity was found in the supernatant fraction of the nodule extract. The bacteroid fraction contained considerable esterase activity whereas the membrane fraction had relatively little.

TABLE 1. ESTERASE ACTIVITY IN DIFFERENT FRACTIONS OF *G. max* NODULES

Fraction	Units* per ml	Percentage of total activity	Specific activity†
Supernatant	89·6	72	46·2
Bacteroid	27·8	22	38·7
Membrane	6·9	6	13·6

* One enzyme unit = amount of enzyme that liberates one μmole of α-naphthol per min from α-naphthyl propionate.

† μmoles of α-naphthol liberated per mg protein per min.

Comparison of Nodule, Shoot and Root Esterases

Esterase zymograms prepared from nodules, roots and shoots of the same legume plant are compared in Fig. 3. Root and shoot zymograms contained fewer esterases which were also of lower activity than those on nodule zymograms. Although the number of esterases on root and shoot zymograms was relatively small, it was nevertheless interesting that many of the latter esterases had similar electrophoretic mobilities to nodule esterases. For example, four esterases from the nodules and roots of *G. max* had similar electrophoretic mobilities (see Fig. 3). Likewise, a number of esterases from nodules of *V. faba* amd *M. sativa* had similar electrophoretic mobilities to esterases from the roots and shoots of these legumes.

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However, several esterases were present only in nodules of the aforementioned plants, and were not detected in roots or shoots.

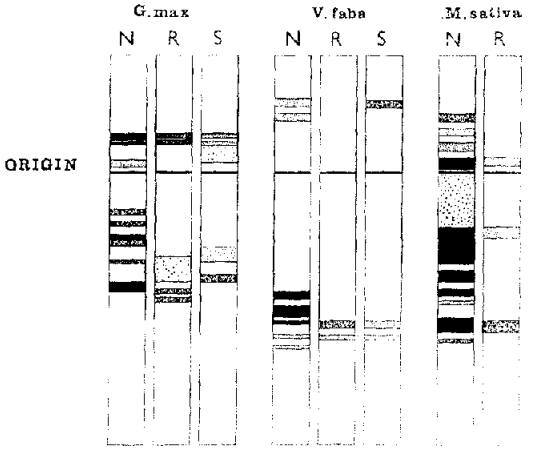

FIG. 3. COMPARISON OF ESTERASE ZYMOGRAMS PREPARED FROM NODULES (N), ROOTS (R) AND SHOOTS (S) OF DIFFERENT LEGUME SPECIES. THE SUBSTRATE WAS α-NAPHTHYL ACETATE. NO ZYMOGRAMS WERE PREPARED FROM THE SHOOTS OF *M. sativa*.

Ontogeny of Nodule Esterases

Esterase zymograms prepared from *G. max* nodules at different times during plant growth are shown in Fig. 4. A striking resemblance was found between zymograms prepared from *G. max* nodules and roots (see Fig. 3) 17 days after the plants were sown. Additional esterase bands were detected on nodule zymograms prepared from plants which were 22 days old. Zymograms were also prepared from nodules harvested when the plants were 29 days old. A considerable increase was seen in the intensity of many of the esterases on the latter zymograms when compared with the other zymograms in Fig. 4. Increased esterase activity has also been reported during the growth and development of several mammalian tissues.[12]

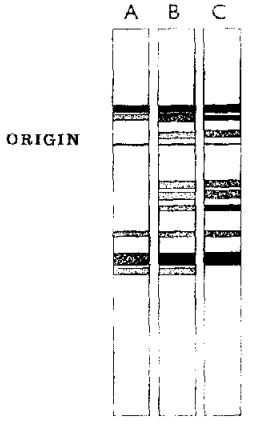

FIG. 4. DIAGRAM OF ESTERASE ZYMOGRAMS OF NODULES FROM *Glycine max* PREPARED AT DIFFERENT TIMES AFTER SOWING. A, 17 DAYS; B, 22 DAYS; C, 29 DAYS.

Esterases compared with Other Nodule Enzymes

Since many proteolytic enzymes possess esterase activity[16, 17] a comparison was made between nodule zymograms prepared with NA and benzoyl DL-arginine-β-naphthylamide (BANA). Although the latter substrate is hydrolysed by proteolytic enzymes such as trypsin,[18] very little activity was detected on nodule zymograms. As shown in Fig. 5, only one band was visible on nodule zymograms prepared from *M. sativa* and *T. repens* while zymograms prepared from *G. max* nodules contained two bands. However, the intensity of the bands was very weak when zymograms were prepared with BANA, and little change was noticed when the zymograms were developed at pH 5·0 instead of pH 6·2. These results suggest that the multiple forms of nodule esterases are not due to proteolytic enzymes.

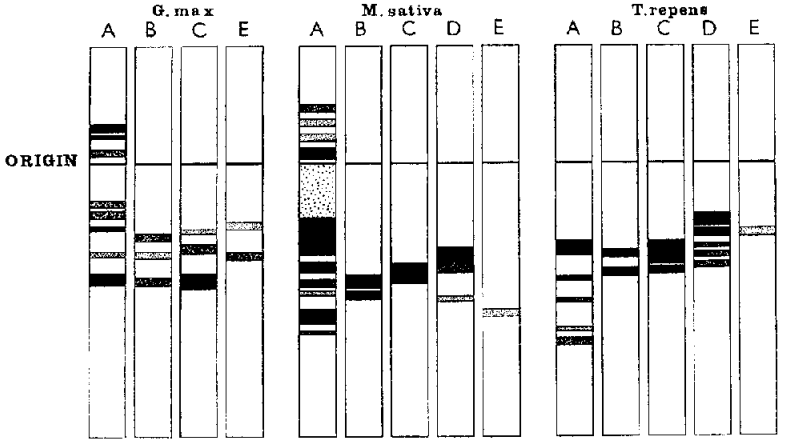

FIG. 5. DIAGRAMS OF ESTERASE ZYMOGRAMS PREPARED FROM NODULES OF THREE DIFFERENT LEGUME SPECIES COMPARED WITH DEHYDROGENASE AND PROTEINASE ZYMOGRAMS FROM THE SAME NODULES. A, ESTERASES (α-NAPHTHYL ACETATE); B, LACTATE DEHYDROGENASES; C, MALATE DEHYDROGENASES; D, GLUCOSE-6-PHOSPHATE DEHYDROGENASES; E, PROTEINASES.

Nodule zymograms which show isoenzymes of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase; 1.1.1.49), lactate dehydrogenase (L-lactate: NAD oxidoreductase; 1.1.1.27) and malate dehydrogenase (L-malate: NAD oxidoreductase; 1.1.1.37) are also included in Fig. 5. Many of these dehydrogenase isoenzymes were found to have similar electrophoretic mobilities to the esterases when zymograms were prepared from the same nodule extract.

Effect of Inhibitors

In order to obtain some information about the type of esterase present in nodules, the effect of inhibitors such as diisopropylfluorophosphate (DFP) and physostigmine (eserine) on the activity of the enzymes was studied. Esterase zymograms prepared from nodules of *M. sativa*, *T. repens*, *P. sativum*, *V. faba* and *L. luteus* were used in these studies. At 10^{-4} M DFP many of the esterase bands were inhibited while at 10^{-3} M DFP almost complete inhibitions of all esterase bands was obtained. One esterase band, however, in each of the three legumes *L. luteus*, *V. faba* and *M. sativa* was resistant to 10^{-3} M DFP. Physostigmine

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at a concentration of 10^{-5} M did not inhibit any of the nodule esterases but at 10^{-3} M and $4 \cdot 10^{-3}$ M many of the esterases were inhibited.

DISCUSSION

Esterases have been classified on the basis of their substrate specificity and sensitivity to various inhibitors.[19,20] Using these criteria the majority of esterases in legume root nodules are probably aliesterases, i.e. B-esterases according to the classifications proposed by Aldridge and Augustinsson.[19,20]

The similarity in substrate specificity and sensitivity towards inhibitors strongly suggests that many nodule esterases are isoenzymic forms[21] of the same enzyme. Esterase isoenzymes have been isolated also from maize,[8] cucumber[7] and *Solanum* tubers.[11] Desborough and Peloquin,[11] as a result of genetic studies on *Solanum* tubers have suggested that the esterase molecule in this plant might be a tetramer composed of one, two or three different types of monomers. They envisage that the three monomers, controlled by different alleles would combine to give 15 esterase isoenzymes. A similar model has been proposed for lactate dehydrogenase isoenzymes which have a tetrameric structure, the monomers being controlled by two or three structural genes.[22–24]

Esterases, unlike lactate dehydrogenases, represent a complex group of enzymes whose substrate specificity and biological function have not been fully defined. Since root nodules had little activity with BANA (see Fig. 5), it is unlikely that nodule esterase activity is due to proteolytic enzymes.

Although the physiological significance of esterase isoenzymes in legume root nodules is unknown, they might nevertheless prove useful for investigating the interaction between plant and *Rhizobium* during nodule development. In this regard the changes which took place in the esterase isoenzymes during the development of *G. max* are interesting (Fig. 4). Some of these isoenzymes may have been derived from the bacterial component of the nodule which contains 22 per cent of the total esterase activity (Table 1). Several attempts were made to prepare esterase zymograms from bacteroid fractions. But probably because of the small quantity of material available, the esterase bands were too faint to allow a valid comparison to be made with the supernatant fraction. However, esterase zymograms have been prepared from extracts of cultures of the strain of *Rhizobium japonicum* used to inoculate *G. max* and at least two of the esterase isoenzymes from the nodules and the *Rhizobium* had similar electrophoretic mobilities.[25] Thus, some of the esterase isoenzymes in the nodules are probably of rhizobial origin while others may have originated from the legume.

EXPERIMENTAL

Plant Material

The following legume species were grown in quartz sand in a greenhouse as described previously;[26] *Glycine max* L., var. Lincoln, *Medicago sativa* L., var. du Puits, *Trifolium repens* L., var. S100, *Lotus corniculatus* L., var. Viking, *Vicia faba* L., var. Giant Green Windsor, *Galega officinalis* (Goat's Rue), *Lupinus luteus* L., var. Weiko, *Pisum sativum* L., var. Onward.

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Preparation of Extracts

Nodule extracts were prepared as described before.[26] Root and shoot extracts were concentrated eightfold with carbowax[27] (polyethylene glycol, M.W. 4000) before being used for electrophoresis.

Electrophoresis

The experimental details for starch gel electrophoresis[28] have been described previously.[29]

Staining for Enzymes

The methods for locating esterases[29] and dehydrogenases[26] on the gels were described before. Using α-naphthyl propionate as substrate the optimum pH for nodule esterases was found to be pH 6·2.[25] Benzoyl DL-arginine β-naphthylamide (BANA) was used to detect proteolytic enzymes.[18]

Fractionation of Nodules

Bergersen's procedure[15] was used to fractionate *G. max* nodules. The bacteroid and membrane fractions were washed with cold 0·06 M phosphate buffer (pH 6·2) and esterases were extracted from these fractions by grinding with 1 ml of the latter buffer in a small mortar.

Inhibitors

The procedures for investigating the effects of inhibitors such as physostigmine salicylate and DFP have been described before.[29]

Quantitative Esterase Assay

The method of Nachlas and Seligman was used.[30] An enzyme unit was the amount of enzyme which liberated one micromole of α-naphthol per minute at 25°.

Determination of Protein

The method of Lowry *et al.*[31] was used.

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Note added in proof—Grimes and Masterson (personal communication) recently inoculated *Pisum sativum* with two different strains of *Rhizobium leguminosarum* (strains A and B). The experiment was conducted in a growth chamber under aseptic conditions. Esterase zymograms of nodules from plants inoculated with strain A were different from those inoculated with strain B.