Extraction of Esterases from Germinating Pea and Bean Seeds

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A procedure for the extraction of esterases of three varieties of bean (*Phaseolus vulgaris* L.) and one variety of pea (*Pisum sativum* L.) is outlined. The rates of enzymic hydrolysis of *p*-nitrophenyl propionate and *p*-nitrophenyl butyrate were determined. The esterases were markedly inhibited by malathion, methyl parathion, carbaryl, metepa, cobalt ion, manganese ion, and fluoride ion. Magnesium ion increased the activity of the esterases; sodium and calcium ions did not affect the enzyme activity.

The extracted esterases were detected also on starch gels with the substrates α-naphthyl and 5-bromoindoxyl acetates and were inhibited by Guthion oxygen analogue.

Plant esterases were studied in the crude extracts of seeds of pea, *Pisum sativum* L. (Frankel and Garber, 1965; Norgaard and Montgomery, 1968), of green beans, *Phaseolus vulgaris* L. (Schwartz *et al.*, 1964), and of wheat, *Triticum aestivum* L. (Jooste and Moreland, 1963). The enzymic hydrolysis of different esters and the effect of some inhibitors on enzyme activity were determined (Jooste and Moreland, 1963; Norgaard and Montgomery, 1968; Schwartz *et al.*, 1964). Frankel and Garber (1965), Jooste and Moreland (1963), and Schwartz *et al.* (1964) studied the electrophoretic patterns of these esterases. Norgaard and Montgomery (1968) found that pH 7 was optimum for pea esterase activity.

The activities of water-soluble esterases in the crude and purified extracts of germinating leguminous seeds were determined by using two substrates, various enzyme concentrations, four inhibitors, and six different ions. The esterases were extracted from three varieties of bean and one variety of pea to study the relationship between the esterases and substrates or inhibitors for future application in the determination and confirmation of pesticide residues.

EXPERIMENTAL

Esterase Extraction. The esterases were extracted from the germinating seeds of bean (*Phaseolus vulgaris* L.) var. Pacer, var. Round Pod Kidney and var. Strider and of pea (*Pisum sativum* L.) var. Director by following the procedure outlined in Figure 1. The stage of germination ranged from the emergence of the hypocotyl to the emergence of the epicotyl.

Enzyme Assay. The crude extracts were diluted 10 times with 0.01*M* Tris-maleate buffer solution at pH 6.3 before assay. The activity of the varying amounts of enzyme was determined by using a constant amount of substrate *p*-nitrophenyl propionate (PNPP) or *p*-nitrophenyl butyrate (PNPB). Two milliliters of substrate was added to the enzyme solution to make a 5-ml. final reaction medium with 94 μmoles of PNPP or 153 μmoles of PNPB; the solution was incubated at

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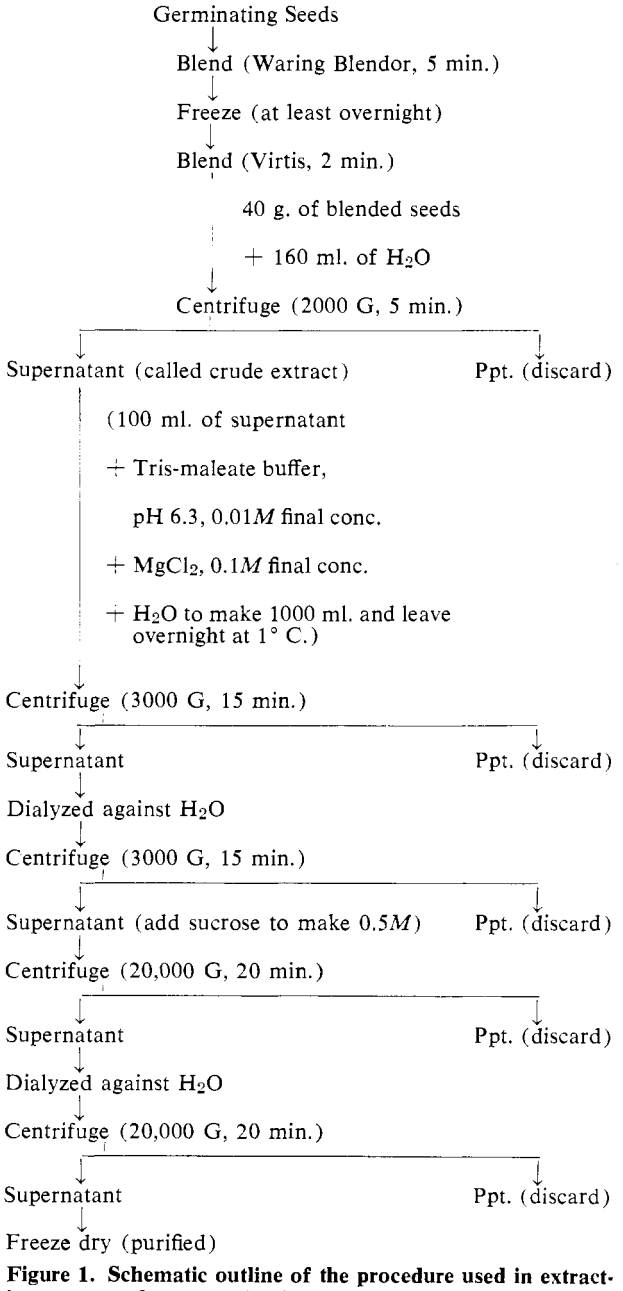

Figure 1. Schematic outline of the procedure used in extracting enzymes from germinating seeds (ppt. = precipitates)

32° C. for 1 hour before reading the absorbance at 325 mμ with a Beckman Model B spectrometer. The effect of calcium, cobalt, magnesium, manganese, and sodium chlorides (each at 0.02*M*) and sodium fluoride (0.016*M*) on the activity of varying amounts of crude extracts of var. Director was also observed. The frozen 20,000-G supernatant of var. Director (Figure 1) was assayed with PNPP and the freeze-dried 20,000-G supernatants (1 mg. of powder per 1 ml. of buffer) with PNPP or PNPB at 37° C. for 1 hour.

Inhibition of Esterases by Pesticides. The effect of four pesticides (malathion, methyl parathion, carbaryl, and metepa) on enzyme activity was determined by incubating the enzyme with each pesticide for 15 minutes before adding the substrate. The solution of purified enzyme preparation from either variety Pacer or Strider was used at the rate of 2 ml. per 5 ml. of reaction medium; 2 ml. of Pacer and of Strider enzyme solution contained approximately 195 and 245 μg. of protein, respectively. To stop the reaction, 0.05 ml. of approximately 6*N* HCl was added to the medium. For controls, the enzymes were incubated with HCl for at least 5 minutes before adding the substrate. The amount of the reaction product, *p*-nitrophenol (PNP), was determined at 325 mμ wavelength.

Protein Determination. Protein content was determined by the method of Lowry *et al.* (1951).

Electrophoretic Procedure. Horizontal starch gel electrophoresis was performed in a Plexiglas cell (inside dimensions 18.5- × 10.5- × 0.6-cm.) with a jacket for the circulation of cold water. The procedure of Kristjansson (1960, 1963), as modified by MacRae and Randall (1965), was followed in the preparation of the gels. The gel buffer was Tris-HCl, pH 7.5, and the bridge buffer was boric acid–NaOH, pH 8.6 (MacRae and Randall, 1965). Each sample was applied with a filter paper insert and 165 volts was applied for 15 minutes before removing the paper strip. The electrophoresis was continued under approximately 350 volts until the borate boundary migrated 8 cm. from the slit. The gel was sliced before incubation in a 0.15% solution of a substrate dissolved in ethanol (1 part) and diluted with buffer used in the preparation of the gel (4 parts). The substrates were α- or β-naphthyl acetate, α-naphthyl laurate, or 5-bromoindoxyl acetate. Azoene Fast Blue RR salt was used with naphthol esters. Amido Black 10B (1 gram) dissolved in 1 liter of methanol–acetic acid–water (5:1:5) solution was used to detect protein. After the reaction, the gel was rinsed with methanol–acetic acid–water (5:1:5) solution to remove the excess stain.

RESULTS AND DISCUSSION

Hydrolysis of Substrates PNPB and PNPP at Varying Enzyme Concentrations. Enzymes from different sources varied in the rate of hydrolyzing PNPP and PNPB. Enzymes from varieties Pacer, Strider, and Director had higher activity than those from var. Round Pod Kidney. Under excess substrate condition, enzymes from var. Pacer hydrolyzed more PNPB than PNPP. On the other hand, enzymes from var. Director and Round Pod Kidney hydrolyzed more PNPP than PNPB. The hydrolysis of both substrates by enzymes from var. Strider was comparable. Enzymes in the crude extracts

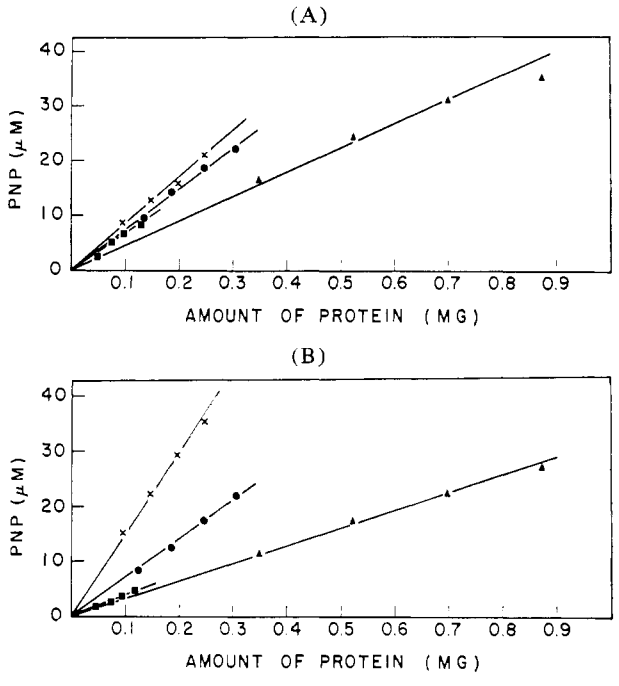

Figure 2. Rates of hydrolysis of (A) *p*-nitrophenyl butyrate and (B) *p*-nitrophenyl propionate by esterases from four leguminous seed sprouts

× = var. Pacer, ● = var. Strider, ■ = var. Director, ▲ = var. Round Pod Kidney

of var. Director had lower activity for PNPP than the purified enzymes.

The precipitates obtained from 20,000-G centrifugation were practically devoid of esterase activity; the precipitates from other centrifugations were too small for enzyme assays.

Figures 2A and B show the activity of enzyme (expressed in μmoles of PNP per mg. of protein) in relation to the seed varieties and substrates used. Enzymes from var. Pacer hydrolyzed PNPB (Figure 2A) more effectively than PNPP (Figure 2B). The rates of hydrolysis of PNPB and PNPP by esterases from var. Strider were comparable. Esterases from var. Round Pod Kidney and Director hydrolyzed PNPP faster than PNPB.

Effects of Ions on Activity of Esterases. The effects of different ions on the ability of the pea esterases, var. Director, to hydrolyze PNPP are shown in Figure 3. Magnesium ions increased and sodium ions did not affect the activity of the enzyme. Marked inhibition of enzyme activity was effected by cobalt, manganese, and fluoride ions. Calcium ions slightly depressed the enzyme activity.

Effects of Organic Pesticides on Esterases. Malathion, 1 μg. per 5 ml. of reaction medium, gave 50 and 31% inhibition of enzymes from Pacer and Strider, respectively. Inhibition of enzymes by 1 μg. of methyl parathion was 68% for Pacer and 21% for Strider; inhibition by 2 μg. of carbaryl was 69% for Pacer and 50% for Strider; and inhibition by 2 μg. of metepa was 64% for Pacer and 30% for Strider. The inhibition of the esterases by metepa concurs with the inhibition of phosphatases by apholate (Mendoza and Peters, 1968) and by tepa (Grushina, 1957).

Gel Electrophoresis. All the crude and purified extracts of the germinating leguminous seeds gave a strong

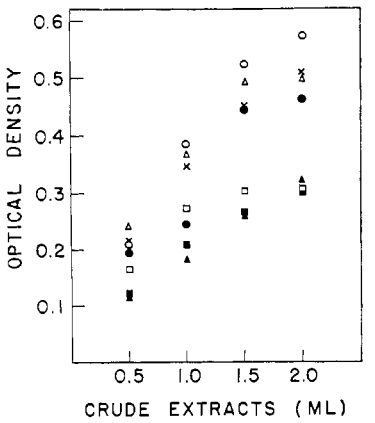

Figure 3. Effects of different ions on activity of esterases from crude extract of seed sprouts, var. Director

× = blank, ○ = magnesium chloride, ● = calcium chloride, □ = manganese chloride, ■ = cobalt chloride, △ = sodium chloride, ▲ = sodium fluoride

band of esterase activity when the substrates α-naphthyl acetate and 5-bromoindoxyl acetate were used. The band of esterase activity moved immediately behind the borate boundary. A single protein band which corresponded to the band with esterase activity was detected. The crude extracts usually exhibited streaking. The purified extract of var. Director also gave a single band with esterase activity when incubated with β-naphthyl acetate; no band was observed when the gel was incubated with α-naphthyl laurate. Complete inhibition of esterase activity was obtained when the gel was incubated in a solution containing 4.6×10^{-5} moles of Guthion oxygen analogue for 45 minutes and was incubated again in a solution containing α-naphthyl acetate, Fast Blue RR, and 4.5×10^{-5} moles of Guthion oxygen analogue.

General Comments. Some purification of esterases was indicated by the increase in enzyme activity as the protein content decreased after dialyses and 20,000-G centrifugations. The difference between esterases based on PNPP and PNPB hydrolysis has yet to be investigated, along with other possible substrates. The effects of ions on esterases of the germinating seeds showed that the activity can either be stimulated or depressed. The inhibitory effect of organic pesticides parallels the reports of Norgaard and Montgomery (1968). It is of interest to note the inhibition of the esterases by metepa, a compound belonging to the aziridinyl group with mutagenic and alkylating properties (Auerbach, 1958).

Further studies to simplify the method of enzyme extraction and to characterize some of these esterases are in progress. The results indicated that the esterases merit further studies in conjunction with the detection of particularly weak inhibitors such as metepa and with the thin-layer chromatographic-enzyme inhibition technique (Mendoza *et al.*, 1968).

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