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PURIFICATION AND SOME PROPERTIES OF SORGHUM GRAIN ESTERASE AND PEROXIDASE*

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Abstract—A carboxylesterase from sorghum grain was partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 gel filtration. A 72 fold purification was obtained with 28% yield. The enzyme was identified as carboxylic ester hydrolase, (E.C. 3.1.1.1) by studying the inhibition response to DFP, eserine, and PCMB. A mol. wt. of 60,000 was determined by gel filtration. The isoelectric point at pH 6·6 was revealed from a single peak in a focusing electrophoresis gradient; however, two isoenzymes were observed on agarose gel electrophoresis at pH 5·5. These isoenzymes reacted differently with DFP and paraoxon. A peroxidase was also detected in the sorghum grain extract. Isoelectric focusing showed two basic peroxidase isoenzymes immobile at pH 9·0 and 10·0. A single peroxidase peak on gel filtration gave mol. wt. 43,000.

INTRODUCTION

ESTERASES are widespread in nature, but considerably less work has been done on plant esterases than on cholinesterases and carboxylesterases from animals. The nomenclature and distribution of animal esterases is also better understood, as a result of recent comparative studies using disc electrophoresis.[1,2] Similar studies on plant extracts[3-7] also show complex esterase isoenzyme patterns.

Esterase sensitivity to organophosphorus pesticides has prompted numerous studies on cholinesterase inhibition (see Refs. 8–10 for reviews); however, little work on plant esterase inhibition has been done. Because stored grain is often treated with pesticides to prevent insect infestation, plant esterases should be examined more closely for possible effects of their interactions with pesticides.

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Pectinesterase[11-14] citrus acetylesterase[15,16] chlorophyllase[17-19] and esterase from wheat germ[20-22] and pea[23,24] represent most of the known esterase purifications from plant sources. This report deals with the detection of a new esterase in sorghum grain extracts and describes a partial purification and some properties of the enzyme. A peroxidase was also detected and some of its properties are reported.

RESULTS AND DISCUSSION

Enzyme Activity Determination

Esterase activity in the sorghum grain homogenate was detected by the use of indophenyl acetate. Its enzymic nature was shown by inactivation after heating the homogenate at 95° for 5 min and also by specific inactivation using the organophosphates DFP (diisopropylphosphofluoridate). Electrophoresis of the crude grain homogenate followed by staining with 2-naphthol acetate plus Diazo Blue-B showed at least five colored bands. All but the two major esterase bands are removed by the fractionation procedure. Staining with guaiacol-hydrogen peroxide (10^{-3} M H_2O_2, 2×10^{-3} M guaiacol, pH 5·5) showed one broad basic peroxidase band.

An assay method for the esterase was developed by modifying that of Kramer and Gamson[25] so that the colored hydrolysis product could be measured spectrophotometrically in the reaction tube. A linear relationship between the absorbance (625 mμ) of the indophenol produced and the amount of enzyme used was observed; the rate of indophenol formation was also linear for 10 min. The non-enzymic substrate hydrolysis in pH 8·0 buffer was less than 0·02 absorbance after 10 min incubation, but at pH 9·0 it was appreciable.

Purification

Esterase activity was followed by hydrolysis of indophenyl acetate substrate and protein concentration was followed by measuring absorbance at 280 mμ. Buffers with pH values between 3·0 and 8·0 were tested for extraction efficiency. Sodium acetate buffer pH 5·5, 0·1 M, gave the highest specific activity and was chosen as the extraction medium. An overall 72 fold purification was obtained with 28% yield (Table 1). The increase in yield after dialysis of the 0·5–0·7 $(NH_4)_2SO_4$ precipitate against the 0·1 M acetate buffer might have been due to the removal of some inhibitor(s). The elution pattern of esterase through the Sephadex G-100 column is shown in Fig. 1. Fractions included by the solid bar were pooled and the specific activity determined. The enzyme was stable at room temp. for at least 12 hr and at 4° for at least 72 hr with little or no loss of activity. The pooled, G-100 treated,

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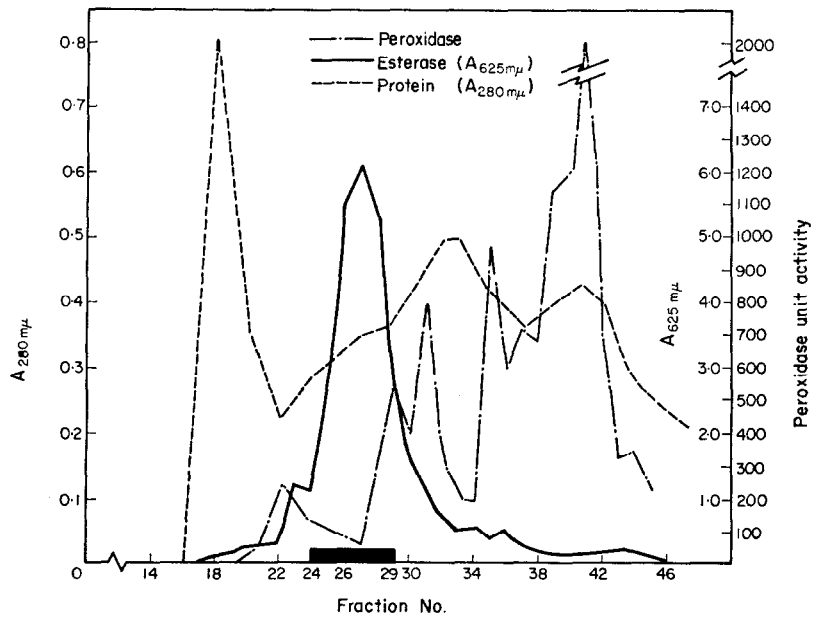

FIG. 1. ELUTION PATTERN OF ESTERASE THROUGH A SEPHADEX G-100 COLUMN.
The column (2·6 cm dia. × 42 cm long) was equilibrated and eluted with a 0·1 M NaOAc buffer pH 5·5. The effluent was collected in 2 ml fractions at the rate of 1·6 ml/hr/cm². The solid bar indicates pooled fractions.

esterase was concentrated in a dialysis bag against dry Bio-Gel P-6 and then electrophoresed on agarose gel strips at pH 5·5. Two positively charged bands with esterase activity were obtained (Fig. 4).

TABLE 1. PURIFICATION OF SORGHUM GRAIN ESTERASE

Steps	Total act. units	Spec. act. units/mg	Yield (%)
Grain extract 10,000 *g* supernatant solution	1425	0·32	100
0·5–0·7 $(NH_4)_2SO_4$ Precipitate before dialysis	535	1·02	38
0·5–0·7 $(NH_4)_2SO_4$ Precipitate dialyzed against 0·1 M acetate pH 5·5 and reprecicipated with saturated $(NH_4)_2SO_4$ [0·5–0·7 $(NH_4)_2SO_4$ fraction]	612	7·13	63
Sephadex G-100 effluent—pooled fractions	400	23·1	28

Molecular Weight

The esterase Ve/Vo was 1·56 and the peroxidase Ve/Vo was 1·70, which corresponds mol. wts. of 60,000 and 43,000, respectively (Fig. 2). Only one peak of esterase activity was detected from the Sephadex column, indicating that the esterase isoenzymes might differ only in charge and not size.

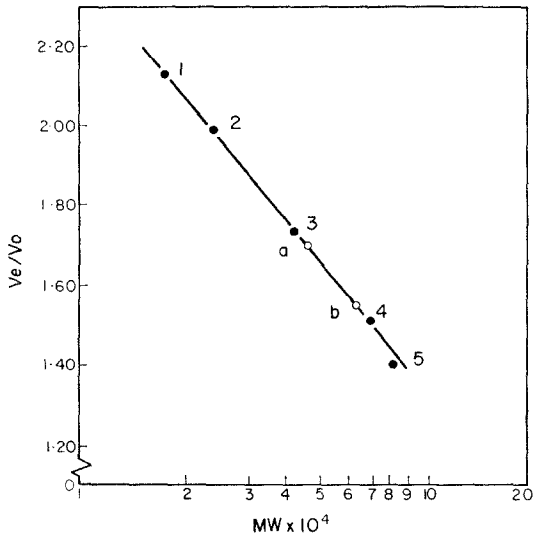

FIG. 2. CHROMATOGRAPHY OF SORGHUM GRAIN ESTERASE ON SEPHADEX G-100.
Mol. wt. standards are: (1) horse heart myoglobin (16,900), (2) trypsin (23,800), (3) β-lactoglobulin (40,000), (4) bovine serum albumin (68,000) and (5) lactoperoxidase (80,000). Ve/Vo = 1·00 for Blue Dextran-2000. (a) sorghum grain peroxidase; (b) sorghum grain esterase.

The sorghum grain esterase (60,000 mol. wt.) is considerably smaller than pig or ox liver carboxylesterases (160,000 mol. wt.) which contain two 80,000 mol. wt. subunits[26,27] and is somewhat smaller than chicken liver carboxylesterase, a monomer of mol. wt. 80,000.[28] Studies of chicken carboxylesterase amino acid sequences near the active serine appear to favor the view that it is a monomer[29] and similar studies should be useful for determining whether plant esterases are mono- or dimeric.

Only one peak of peroxidase activity was detected by gel filtration and its mol. wt. fell in the range of several other peroxidases: horseradish peroxidase 40,000;[29] chloroperoxidase 42,000;[30] Japanese radish peroxidase- C41,500.[31]

Isoelectric Point

Electrofocusing of the 0·5–0·7 $(NH_4)_2SO_4$ fraction in a pH 3–10 gradient showed only one major esterase component, isoelectric at pH 6·6 (Fig. 3), whereas two closely running

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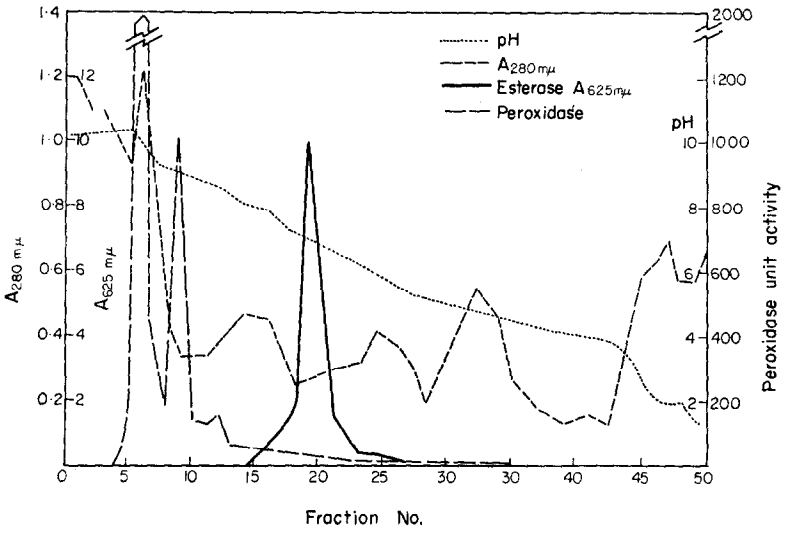

FIG. 3. THE ELECTROFOCUSING PROFILE OF THE SORGHUM GRAIN EXTRACT IN A pH GRADIENT OF 3–10, WITH CATHODE CONNECTED TO THE BOTTOM. FRACTIONS OF 2 ml EACH WERE COLLECTED.

bands of approximately equal stain intensitity were observed in the agarose gel electrophoresis (Fig. 4). It may be that one of the isoenzymes is inactivated on the ampholyte gradient or that separation would be observed in a focusing experiment using a shallower pH gradient.

An esterase-like activity was detected at the cathode (bottom) of the electrofocusing column but it was non-enzymic (active after treatment at 95° for 5 min) and was due to the basicity of the cathode electrolyte (> pH 10). As mentioned before, appreciable spontaneous hydrolysis of indophenyl acetate occurred in solutions above pH 9·0.

Peroxidases of isoelectric pH 9·0 and 10·0 were detected. Complete separation of esterase from peroxidase was achieved using the electrofocusing technique.

pH Optimum

The esterase pH optimum was 7 (Fig. 5) as determined by indophenyl-acetate assay (Method 2). The broad optimum region from pH 6·5–8 shows that assay methods within that range would give similar results. The progress curves were essentially linear in each assay from pH 3–10 indicating that the reaction kinetics were zero order over the entire pH range.

Characterization of Esterase

According to Holmes and Masters,[1] carboxylesterases (E.C. 3.1.1.1) are inhibited by organophosphates but not by eserine; arylesterases (E.C. 3.1.1.2) are not inhibited by organophosphates, but are inhibited by sulfhydryl reagents such as PCMB; cholinester hydrolases (E.C. 3.1.1.7–3.1.1.8) are inhibited by organophosphates and eserine, but not by sulfhydryl reagents, and acetylesterases (E.C. 3.1.1.6) are not inhibited by organophosphates,

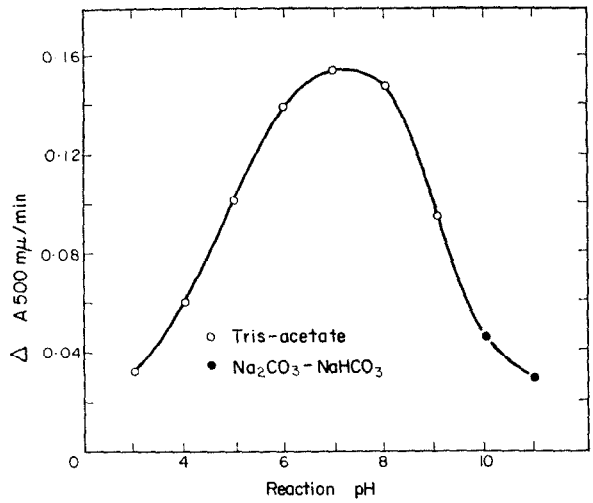

FIG. 5. pH OPTIMUM OF SORGHUM GRAIN ESTERASE. ASSAY WITH INDOPHENYL ACETATE (METHOD 2). Non enzymic hydrolysis has been subtracted. Enzyme was 0·1 ml of 0·5–0·7 $(NH_4)_2SO_4$ fraction.

eserine or sulfhydryl reagents. The cholinester hydrolases can be distinguished by inhibition of acetylcholine hydrolase (E.C. 3.1.1.7) in the presence of acetylcholine iodide. This scheme, plus information from substrate preference, the effect of heat and treatment with urea has added considerable order to the extremely complicated nomenclature of animal esterases. Plant esterases seem equally complicated[3–7] and as a start, we have used the classification based on inhibitors to characterize sorghum grain esterase. Much additional work and some changes in nomenclature will be required before such a classification proves acceptable for non-animal systems. Citrus acetylesterase for example is not inhibited by eserine[15] but is inhibited by organophosphates[16,32] and is therefore inconsistent with the Holmes and Masters nomenclature. Wheat esterase[20] is inhibited by sulfhydryl reagents and may perhaps be classed as an arylesterase.

Figure 4 shows the electrophoretic pattern of sorghum grain esterases on agarose gel at pH 5·5 and the results of treatment by eserine (B), paraoxon (C), PCMB (D), acetyl-choline iodide (E), and DFP (F). Strips (C) and (F) show inhibition resulting from treatment with paraoxon and DFP, respectively; eserine, PCMB, and acetylcholine iodide has no effect on these esterases, which therefore, can be classified as carboxylesterases. It should be noted, however, that the sorghum carboxylesterase isoenzymes differ in susceptibility toward paraoxon and DFP, both of which are organophosphates.

EXPERIMENTAL

Materials

Sorghum grain Frontier 400, a regionally-adapted hybrid variety grown in Kansas, was used. Indophenyl acetate (Eastman 7739) was recrystallized from heptane. Reagent guaiacol was distilled before use.

Esterase Assay

Routine analysis of esterase activity (Method 1) was performed by a modification of the Kramer–Gamson method.[25] To 4·6 ml of 0·05 M phosphate buffer pH 8·0 in a test tube (16 mm path) was added 0·2 ml of

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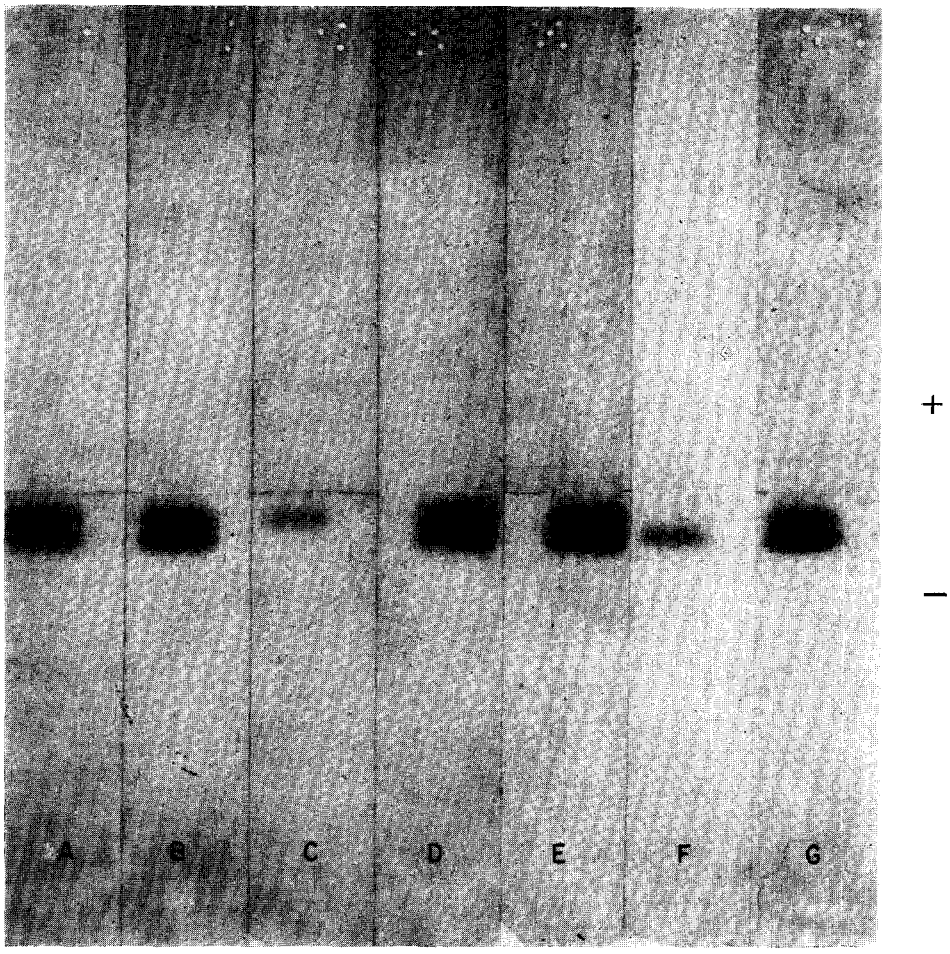

FIG. 4. AGAROSE GEL ELECTROPHORESIS OF PARTIALLY PURIFIED SORGHUM GRAIN ESTERASE AND ITS CLASSIFICATION.

Gel strips except G were treated with inhibitors before staining with 2-naphthyl acetate plus Diazo Blue-B. A. Tris buffer pH 7·4, 0·025 M; B. Eserine (10^{-5} M); C. Paraoxon (10^{-4} M); D. PCMB (10^{-3} M); E. Acetylcholine iodide (10^{-3} M); F. DFP (10^{-4} M); G. Not treated, i.e. no Tris buffer pre-soak.

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substrate (10^{-3} M indophenyl acetate in 95% ethanol). The substrate-buffer mixture was preincubated in a 30° water bath and the reaction was inititated by adding 0·2 ml of enzyme. Absorbance was measured at 625 mμ. Zero absorbance was set with a similar mixture except that 0·2 ml buffer replaced enzyme. Unit activity was defined as that amount of enzyme which produced a net absorbance of 1·0 after 10 min at 30°.

Another indophenyl acetate assay (Method 2) was devised to utilize the indophenol absorbance near 500 mμ and thus remove pH dependence for color development. The resulting method was used to determine the enzymic pH optimum but it is also useful as a general assay method. The substrate was prepared by adding 1 ml of a 1·5% ethanolic indophenyl acetate solution to 99 ml of distilled water; this solution was stable up to 3 days at 4°. To 1·0 ml of the appropriate buffer (in a 1-cm cuvet) was added 1·0 ml of aqueous substrate solution and 0·4 ml distilled water. A 25° constant temperature block held the cuvet in a Coleman Autoset spectrophotometer and it was stirred from below with a miniature magnetic stirring bar. The buffered substrate mixture was set to zero absorbance at 500 mμ. The reaction was then initiated by blowing in 0·1 ml of enzyme and the absorbance recorded on a Heath strip chart recorder at 30–60 sec/in. For assays above pH 8 all ingredients had to be rapidly combined (less than 30 sec) for accurate measurement of spontaneous hydrolysis. Buffers employed were 0·1 M Tris-acetate from pH 3 to 9 and 0·1 M carbonate-bicarbonate from pH 10 to 11. The initial velocity was obtained from the progress curves and expressed as change in absorbance at 500 mμ/min. Unit activity was defined as that amount of enzyme which caused an absorbance change of 1·0/min under the assay conditions.

Peroxidase Assay

A guaiacol method[33] was used with some modification. Buffered substrate was prepared by mixing equal volumes of 0·54 M NaOAc buffer pH 5·5 and $5{\cdot}4 \times 10^{-3}$ M guaiacol. Enzyme and/or buffer (0·5 ml total) was added to 2 ml of buffered substrate and the tubes (1 cm path) equilibrated to 30°. Zero absorbance was set at 470 mμ and the reaction was initiated by adding 0·2 ml of 0·0135 M H_2O_2. Unit activity was defined as the amount of enzyme which produced a fixed absorbance change of 0·2 in 1·0 sec. The time (sec) required to attain 0·2 A was measured and $1/t$ was proportional to enzyme concentration within the limit 15–150 sec reaction time.

Specific Activity

The esterase specific activity is defined as units/mg protein where protein is measured by 280 mμ absorbance assuming $E^{1\%}_{1\,cm} = 10{\cdot}0$. Sorghum grain esterase has max. at 280 mμ but its $E^{1\%}_{1\,cm}$ was not determined. Peroxidase specific activity was determined similarly.

Esterase Purification

40 G of sorghum grain and 100 ml of cold 0·1 M acetate buffer pH 5·5 were placed in a Sorvall Omnimixer lowered into an ice-water bath, and homogenized at maximum speed for 2 min. The homogenate was centrifuged for 30 min at 10,000 *g* and the precipitate discarded. The supernatant solution was brought to 0·5 saturation with $(NH_4)_2SO_4$, centrifuged as before and the precipitate again discarded. The 0·5 saturated supernatant solution was raised to 0·7 saturation with $(NH_4)_2SO_4$ and centrifuged as before. The precipitate was retained, dissolved in 0·1 M acetate buffer pH 5·5, and dialyzed against the same buffer. The retentate was concentrated (precipitated) by further dialysis against a saturated $(NH_4)_2SO_4$ solution, and the precipitate was then dissolved in a small volume of 0·1 M acetate buffer pH 5·5. This material is defined as the 0·5–0·7 $(NH_4)_2SO_4$ fraction. Sephadex G-100 was employed for further purification.

An aliquot of the 0·5–0·7 $(NH_4)_2SO_4$ fraction was applied to a Sephadex G-100 column (2·6 cm dia./42 cm height) which had been equilibrated with 0·1 M acetate buffer pH 5·5 at room temp. The enzyme was eluted using the same buffer at a flow rate of 1·6 ml/hr/cm^2. Fractions containing esterase activity were pooled.

Peroxidase Purification

Peroxidase activity was concentrated in the 0·5–0·7 $(NH_4)_2SO_4$ fraction, and no attempt was made to fractionate it further. Separation of peroxidase from esterase was achieved using Sephadex and isoelectric focusing electrophoresis.

Molecular Weight Determination

Gel filtration through Sephadex G-100 was employed.[34] The column was 1·0 cm dia. × 112 cm high and rinsed with 0·2 M acetate buffer pH 6·0 in reverse flow mode using a peristaltic pump at 1/min. The flow rate was 5·4 ml/hr/cm^2. After calibrating the column with known proteins and Blue Dextran-2000, an aliquot

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of the 0·5–0·7 $(NH_4)_2SO_4$ fraction was applied. Protein in the effluent was monitored at 254 mμ. Fractions (2 ml) were collected for esterase and peroxidase determinations. Void volume and sample volume were estimated from the peak midpoints.

Isoelectric Focusing

The 0·5–0·7 $(NH_4)_2SO_4$ fraction was dialyzed against 1% glycine solution. About 5 mg of protein from that sample was applied to a 110-ml LKB electrofocusing column. A sucrose density gradient with pH 3–10 ampholyte was prepared and layered into the column as fraction 13. The column had anode on top and cathode at the bottom. Starting voltage was 300 V; it was raised to 400 V after 30 min and maintained for an additional (25·5) hr at 15–17°. Fractions, (2 ml) collected from the bottom of the column, were analyzed for 280 mμ absorbance, esterase, peroxidase, and pH.

Characterization of Esterases

The method used to characterize and classify esterases was that described by Holmes and Masters.[1] The partially purified esterase was electrophoresed on 3·0% agarose in 0·025 M potassium acetate buffer, pH 5·5 at 80 V, approximately, 8 mA/strip, which measured 15 cm × 2 cm × 0·3 cm, for 1·5 hr. The gel strips were soaked for 10 min in either 0·025 M Tris buffer pH 7·4 (control) or the same buffer containing inhibitors; diisopropylphosphorofluoridate (DFP) (10^{-4} M), paraoxon (10^{-4} M), eserine (10^{-5} M), *p*-chloromercuribenzoate (PCMB) (10^{-3} M) or acetylcholine iodide (10^{-3} M). All gels were then stained with 2-naphthol acetate and Diazo Blue-B.[35]

Acknowledgements—The technical assistance of Mr. Charles Anderson III is greatly appreciated.

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