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PURIFICATION AND SUBSTRATE AND INHIBITOR SPECIFICITIES OF CARBOXYLESTERASES OF THE PEA (*PISUM SATIVUM* L.)*

M. W. MONTGOMERY, MARIDA J. NORGAARD AND P. S. VEERABHADRAPPA,
Department of Food Science and Technology, Oregon State University, Corvallis, Oreg. 97331 (U.S.A.)

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SUMMARY

1. Carboxylesterases (carboxylic ester hydrolase, EC 3.1.1.1) active toward phenyl propionate were purified from an aqueous extract of the pea (*Pisum sativum* L.) by precipitation of inactive material with protamine sulfate and chromatography on Sephadex G-100 and DEAE-cellulose. Three active fractions were obtained.

2. Inhibitor specificities determined with DFP, diethyl *p*-nitrophenyl thiophosphate and *p*-chloromercuribenzoate, and substrate specificity toward nine substrates indicated that two of these fractions were isozymes. The third fraction, which had differences in inhibitor and substrate specificities, showed one major esterase band on polyacrylamide-gel electrophoresis.

INTRODUCTION

Previous results from this laboratory have shown that the pea (*Pisum sativum* L.) contains six esterases[1]. Five of these esterases were classified as carboxylesterases (carboxylic ester hydrolases, EC 3.1.1.1) and one as an arylesterase (aryl ester hydrolase, EC 3.1.1.2). Esterase activity of 12 varieties of germinating peas have been separated by starch-gel electrophoresis into six bands[2]. Since the pea carboxylesterases have similar substrate and inhibitor specificities, purified preparations of these enzymes are necessary to study their properties in detail.

In the present study, the pea carboxylesterases hydrolyzing phenylpropionate were separated into three fractions. Data from inhibitor and substrate specificity studies and polyacrylamide-gel electrophoresis suggest that two of these fractions were isozymes. The third fraction revealed marked differences in these properties.

MATERIALS AND METHODS

Sources and preparation of the peas, substrates, and organophosphorous inhibitors were the same as those previously reported[1]. A 4 mM solution of *p*-chloro-

Abbreviations: PCMB, *p*-chloromercuribenzoate; parathion, diethyl *p*-nitrophenyl thiophosphate.

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mercuribenzoate (PCMB, K and K Laboratories) was prepared in water and serially diluted. All purification operations were carried out at 3°.

Enzyme extraction

Dried peas were powdered in a Waring Blendor for 2 min. Slurries of 1 part powder and 4 parts distilled water (w/v) were centrifuged at 31 000 × *g* for 25 min. The supernatant was decanted and 1 ml of 2% protamine sulfate (Sigma) was added to each 10 ml of supernatant. The precipitate was removed by centrifugation (18 000 × *g* for 10 min) and the supernatant was subjected to Sephadex chromatography.

Sephadex chromatography

Sephadex (Pharmacia) columns (2.5 cm × 95 cm) were prepared in accordance to instructions from the manufacturer (Technical Data Sheet No. 6) in 1 mM potassium phosphate (pH 6.9). 20 ml of enzyme extract treated with protamine sulfate were applied to the column and eluted with the above buffer. Eluent was continuously monitored at 280 mμ and collected in 10 ml fractions.

DEAE-cellulose chromatography

A column (2.0 cm × 35 cm) of DEAE-cellulose (Schleicher & Schuell, type 20, 0.8 mequiv/g) was prepared by the procedure of TING, MONTGOMERY AND ANGLEMIER[3] and equilibrated with starting buffer (1 mM potassium phosphate (pH 6.9)). The five most active fractions from 2 Sephadex columns were combined, lyophilized, dissolved in 5 ml of distilled water and applied to the DEAE-cellulose. The column was developed with starting buffer until the first protein peak appeared and then by a gradient from a Varigrad[4]. Each Varigrad chamber contained a total of 100 ml and the volume percentages of limit buffer (1 mM potassium phosphate–0.5 M NaCl (pH 6.9)) in each chamber were 0, 40, 35, 40, 45, 50, 55, 60, 70, respectively. The column eluent was continuously monitored at 280 mμ.

Activity and protein determinations

Esterase activity was determined by the method described by NORGAARD AND MONTGOMERY[1]. Phenyl propionate was used as a substrate to follow the esterase activity during purification. Protein was determined by absorption at 280 mμ (ref. 5).

Polyacrylamide-gel electrophoresis

Procedures for polyacrylamide-gel electrophoresis and detection of esterase activity were similar to that of NARISE AND HUBBY[6]. Briefly, the method was as follows. 150 ml of 7% cyanogum-41 (E-C Apparatus) were prepared in 17 mM Tris–HCl (pH 7.5) and filtered through Whatman No. 1 paper. 1 drop of Tween 80, 0.15 ml *N,N,N′,N′*-tetramethylethylenediamine (E-C Apparatus) and 150 mg of ammonium persulfate (E-C Apparatus) were mixed with this buffer. This solution was poured into a vertical gel electrophoresis cell (Model 470, E-C Apparatus, as described by RAYMOND[7]) and allowed to polymerize for 30 min at room temperature. Electrode buffers (0.3 M boric acid–0.1 M NaOH (pH 8.7)) were added to the upper and lower compartments, and 0.2 ml sample in 10% sucrose, containing a very small quantity of bromophenol blue (as marker), was placed in the sample slots with a syringe. A potential of 300 V was applied to this system for 2.5 h at 3°. After the initial 20 min of electrophoresis, the electrode buffers were circulated. Esterase activity was detected by placing the gel for 0.5 h at 37° in 100 ml of 0.1 M sodium acetate (pH 5.6) containing 70 mg of Fast Blue RR salt (K & K Laboratories) and 40 mg of α-naphthyl acetate (Sigma) dissolved in 2 ml of acetone.

RESULTS AND DISCUSSION

TABLE I

pH STABILITY OF PEA ESTERASES

pH	*Activity after 24 h (% of maximum)*
8.7	81
8.2	81
7.0	94
6.7	100
6.4	94
4.9	60
3.6	36

pH stability

To determine the pH of buffers to use in column chromatography, the pH stability of the phenyl propionate hydrolyzing esterases was studied. Portions of the pea extract were adjusted and held 24 h at 3°. Activities of the extracts, after re-adjustment to the original pH level (pH 6.5), are presented in Table I. These esterases had maximum stability at neutral pH levels; basic pH conditions were not as harmful to activity as acidic conditions.

Subsequent experiments revealed that 1 mM potassium phosphate (pH 6.7) did not interfere with the assay procedure or inactivate the esterases. Only 20% of the esterase activity was lost after storage for 6 days at 3° in the above buffer. Therefore, the esterases appeared to be sufficiently stable to withstand purification.

Purification

A summary of the data from the various purification steps is presented in Table II. Spectral analysis of the enzyme extract revealed an absorption maximum at 260 mμ. Treatment of the extract with protamine sulfate reduced the absorbance

TABLE II

PURIFICATION OF PEA CARBOXYLESTERASES HYDROLYSING PHENYL PROPIONATE

Step	*Vol. (ml)*	*Total units**	*Total protein (mg)*	*Spec. activity (units/mg protein)*	*Yield (%)*	*Purification (fold)*
1. Crude extract	38	809	3002	0.27		
2. Protamine sulfate precipitation	40	840	1620	0.52	104	1.9
3. Sephadex G-100	106	581	85	6.83	72	25.3
4. DEAE-cellulose						
Fraction I**	20	18	1.8	10.0	3	37.0
Fraction II**	20	20	2.1	9.6	3	35.5
Fraction III**	20	44	1.2	36.7	8	136

* 1 Activity unit represents 1 μequiv of substrate hydrolyzed/min per ml of enzyme extract.
** Two most active tubes of each fraction.

at this wavelength by approx. 50%. Various levels of protamine sulfate were investigated and 1 ml of 2% protamine sulfate for 10 ml supernatant was found to be optimum. Extraction of the dried peas with suspensions of polyvinylpyrrolidone[8] did not appreciably lower the 260-mμ absorbance. Therefore, absorbance at 260 mμ was probably due to nucleic substances rather than phenolic compounds. The presence of the 260-mμ-absorbing material in the pea extract probably interfered with the determination of protein by absorbance at 280 mμ, which would cause the purification data (Table II) to be in error.

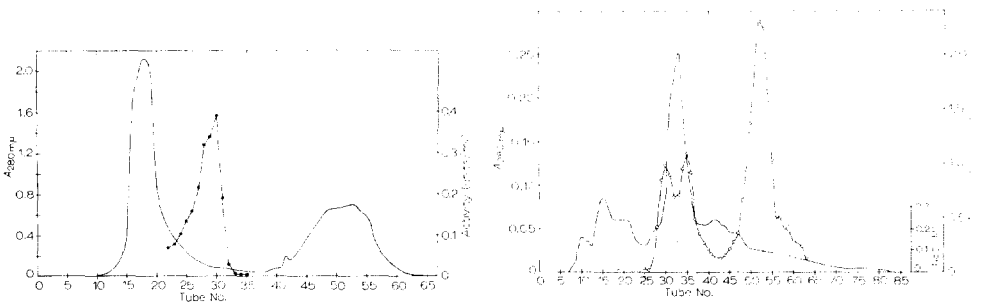

Fig. 1. Chromatography of protamine sulfate treated pea extract on Sephadex G-100. ——, absorbance at 280 mμ; ●—●, activity with phenyl propionate.

Fig. 2. Chromatography of pea carboxylesterases on DEAE-cellulose. ——, absorbance at 280 mμ; ○—○, activity with phenyl propionate; - - - -, NaCl concn.

A typical elution pattern from Sephadex G-100 is presented in Fig. 1. Esterase activity was eluted between the two major peaks. Preliminary trials showed that other areas of the chromatogram did not possess esterase activity. Since the ultraviolet spectrum of the esterase fraction showed maximal absorbance at 280 mμ, most of the 260 mμ absorbing material was removed by chromatography on Sephadex. Preliminary experiments with Sephadex G-50 and G-75 showed the activity was not well separated from the first major peak. With Sephadex G-150, however, the activity was found in the first portion of the second major peak.

Chromatography of the esterase activity from Sephadex on DEAE-cellulose (Fig. 2) revealed three esterase fractions which hydrolyzed phenyl propionate. Fractions I and II were eluted relatively close together and were not well separated, while Fraction III was separated from the other two fractions. Similar results were obtained in several experiments, however, ratios of the activities in the fractions varied from experiment to experiment.

Solutions in the peak tubes of each fraction and the pea extract were subjected to electrophoresis on polyacrylamide gel. The electrophoretic pattern of the pea extract (Fig. 3) revealed seven esterase bands, six evenly-spaced bands of relatively low mobility and a seventh band well-separated from the others. The even spacing of the first six bands suggests the possibility that these esterases were isozymes. The slower moving esterases were not completely separated on DEAE-cellulose. Esterase bands 1 to 3 were first to be eluted from the column and were found in Fraction I, while Bands 1 to 6 were in Fraction II. A more gradual NaCl gradient might have

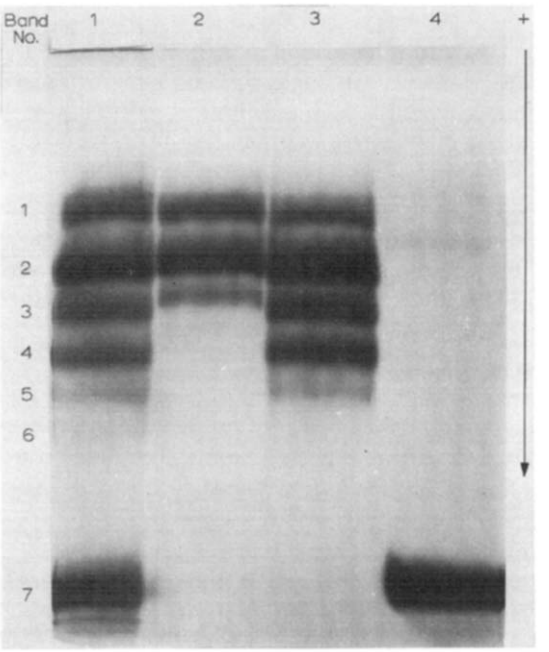

Fig. 3. Polyacrylamide-gel electrophoretic patterns of unfractionated pea carboxylesterases (1), Fraction I (2), Fraction II (3) and Fraction III (4). α-Naphthyl acetate was the substrate.

yielded better separation of these esterases. Fraction III was almost pure Band 7 with some slower moving components. Close examination of the gel (not clearly shown in the photograph) revealed that the mobility of these components was between that of the slower components of the pea extract. Slower bands of Fraction III were probably not visible in the pea extract due to possible overlapping with more active, slower components of Fraction II.

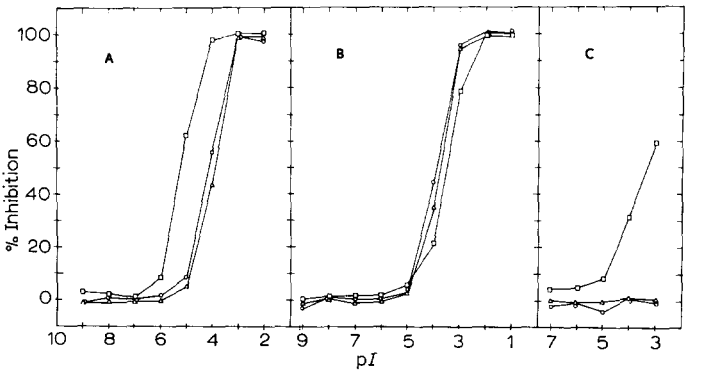

Fig. 4. Inhibition curves of the hydrolysis of phenyl propionate by three purified fractions of pea carboxylesterases with DFP (A), parathion (B) and PCMB (C). Percent inhibition is plotted against the negative $\log_{10}$ of the M inhibitor concentration (pI). ○—○, Fraction I; △—△, Fraction II; □—□, Fraction III.

Inhibitor specificity

Inhibition curves (Fig. 4) with DFP and parathion (diethyl *p*-nitrophenyl thiophosphate) for the three esterase fractions reveal single sigmoid curves. This is in contrast to previous work[1] which showed double sigmoid inhibition curves with parathion when pea extract was used as enzyme source. Although electrophoresis on polyacrylamide gel showed different esterases in Fraction I and II (Fig. 3), these fractions demonstrated approximately the same inhibitor specificities with the inhibitors used in this study. Therefore, esterases in Bands 4 to 6 had inhibitor specificities similar to esterases in Bands 1 to 3. Esterases of Fraction III, however, were more sensitive to DFP, slightly more resistant to parathion, and more sensitive to the higher concentrations of PCMB than Fractions I and II.

Comparison of the inhibition curves of DFP and parathion in Fig. 4 to those obtained with the whole pea extract[1] reveals that higher concentrations of DFP and parathion were required to cause complete inhibition of phenyl propionate hydrolysis in the purified fractions. This was contrary to the expected result. In the purified fractions, less chance existed for DFP and parathion to react with substances other than esterases, hence, inhibition at lower concentrations was expected. An explanation of this phenomenon is not readily apparent at this time.

Esterases of Fraction III were inhibited by both DFP and PCMB (Fig. 4). Esterases from several plant sources[9], as well as the esterase activity of glyceraldehydephosphate dehydrogenase[10] (EC 1.2.1.12), were inhibited by relatively high concentrations of DFP and PCMB. SOHLER *et al.*[11] attributed the inhibition of catalase by high concentrations of PCMB to non-specific reactions of this inhibitor with protein and not with –SH groups. Other –SH reagents have been shown to react with histidine of ribonuclease, which contains no –SH groups[12]. Therefore, inhibition of the esterases of Fraction III may not have been due to the reaction of PCMB with an essential –SH group. AUGUSTINSSON[13] reported that blood plasma arylesterases were inhibited by PCMB, but not by organophosphorous inhibitors. Plasma carboxylesterases were inhibited by organophosphorous inhibitors, but not by PCMB. In light

TABLE III

RELATIVE ACTIVITY OF ESTERASE FRACTIONS TOWARD VARIOUS SUBSTRATES

Activity with phenyl propionate = 100.

Substrate	*Fraction I*	*Fraction II*	*Fraction III*	*Pea extract**
Phenyl acetate	24	26	60	44
Phenyl propionate	100	100	100	100
Phenyl *n*-butyrate	9	8	7	98
Triacetin	2	2	36	10
Tripropionin	15	15	19	17
Tri-*n*-butyrin	5	5	1	10
Acetyl ester of sodium 2-naphthol-6-sulfonate	6	6	44	12
Propionyl ester of sodium 2-naphthol-6-sulfonate	32	36	64	26
n-Butyryl ester of sodium 2-naphthol-6-sulfonate	6	6	2	15

* Calculated from ref. 1.

of the above discussion, the present authors suggest that the esterases of Fraction III be classified as a carboxylesterase.

Substrate specificity

The relative activity of the three esterase fractions from DEAE-cellulose and of the pea extract[1] toward nine substrates is presented in Table III. Substrate specificities of Fractions I and II were almost identical, demonstrating that the presence of esterase Bands 4 to 6 in Fraction II did not affect the substrate specificity of this fraction. Therefore, the substrate specificity of Bands 1 to 3 must have been similar to that of Bands 4 to 6. This evidence, as well as the similarity of the inhibitor specificities of these two fractions, suggest that the first six esterase bands in Fig. 3 were isozymes. Fraction III was considerably more specific for the acetyl esters than Fractions I and II, indicating specificity of Fraction III for the shorter-chain acyl groups.

Evidence from a previous study[1] suggested that the pea esterases, which hydrolyzed phenyl *n*-butyrate and phenyl propionate, were similar. However, comparison of the relative activities of the three fractions with that of the pea extract (Table III) reveals a large amount of phenyl *n*-butyrate hydrolyzing activity was lost during the fractionation. This activity may have been carried along in the fractionation but not detected since phenyl propionate was used as substrate to follow fractionation. Since all the esterase bands of the pea extract on polyacrylamide gel were found either in Fractions I, II or III and low activity toward phenyl *n*-butyrate was noted in these fractions, *α*-naphthyl acetate apparently was not hydrolyzed appreciably by the phenyl *n*-butyrate hydrolyzing esterases. Consequently, these esterases were not detected in the pea extract on polyacrylamide gel. AUGUSTINSSON[14] recently presented evidence that all plasma esterases would not be detected with *α*-naphthol esters. Data presented here demonstrate that all the carboxylesterases of the pea were not detected on polyacrylamide gel by *α*-naphthyl acetate.

Esterases described in this report were carboxylesterases according to established criteria[13]. However, differences found between the fractions noted above, demonstrates that a variety of carboxylesterases exists and a classification will have to be devised for these enzymes. The present authors suggest that nomenclature and a classification of these esterases be deferred until a more complete study of their properties is made.

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