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SOME ESTERASES OF THE PEA (*PISUM SATIVUM* L.)*

MARIDA J. NORGAARD AND M. W. MONTGOMERY

Department of Food Science and Technology, Oregon State University, Corvallis, Oreg. (U.S.A.)

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SUMMARY

1. Water-extractable esterases of the pea (*Pisum sativum* L.) have a pH optimum of 7. Acetyl, propionyl and *n*-butyryl esters of phenol, sodium 2-naphthol-6-sulfonate and glycerol were hydrolyzed by an aqueous extract of the pea. Hexyl, octyl, decyl and hexadecyl esters of sodium 2-naphthol-6-sulfonate, triolein, and acetyl-, propionyl- and *n*-butyrylcholine were not hydrolyzed or hydrolyzed slowly, which suggests the absence of lipases and cholinesterases.

2. Pea esterases showed the greatest activity toward the phenyl and propionyl esters.

3. Selective inhibition of the pea esterases by diethyl *p*-nitrophenyl thiophosphate (parathion), tetraethyl pyrophosphate and DFP, at concentrations ranging from 10^{-10} to 10^{-1} M, revealed the presence of at least 6 esterases in an aqueous extract of peas. 5 of these esterases were classified as carboxylesterases (carboxylic-ester hydrolase, EC 3.1.1.1).

INTRODUCTION

Selective inhibition by organophosphorus compounds has been used as a criterion for the classification of esterases from various animal tissues[1-4]. ALDRIDGE[1] employed aromatic esters and triglycerides as substrates and various concentrations of several organophosphorus compounds to classify the esterases of various tissues of the rat. The esterases of bovine milk[2] and mycobacteria[3] were also classified by this method. Studies by JOOSTE AND MORELAND[5] and SCHWARTZ and co-workers[6] indicate that plant tissues may contain esterase systems as complex as those of animals.

In the present work, the activities of the water-soluble esterases of the pea (*Pisum sativum* L.) were determined toward 9 different substrates in the presence of various concentrations of 3 different organophosphorus compounds. At least 6 esterases appear to be present in the pea that differ in their substrate and inhibitor specificities. The effect of bivalent cations on certain pea esterases was also studied.

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MATERIALS AND METHODS

Enzyme preparation

Field grown, freshly harvested peas (*Pisum sativum* L., variety Dark Skin Perfection) were lyophilized, flushed with nitrogen, sealed in cans in an atmosphere of nitrogen, and stored at −18°. Enzyme extracts were prepared by grinding the dried peas in a Waring Blendor for 2 min, diluting 1 part powder with 10 parts distilled water (w/v), and mixing. These slurries were centrifuged in the cold (3°) at 31 000 × *g* for 25 min. Appropriate dilutions of the supernatants were used as the enzyme preparations. Enzyme for the controls was prepared by heating portions of the preparations in a boiling water bath for 10 min and removing the precipitate by filtration (Whatman No. 12).

Substrates

Substrates used were phenyl acetate, phenyl propionate, phenyl *n*-butyrate, triacetin, tripropionin, tri-*n*-butyrin (Eastman), triolein, acetylcholine iodide, propionylcholine iodide, *n*-butyrylcholine iodide (K and K Laboratories), and acetyl, propionyl, *n*-butyryl, hexyl, octyl, decyl, and hexadecyl esters of sodium 2-naphthol-6-sulfonate (gift from T. L. FORSTER, Washington State University, Pullman, Washington). The concentrations of phenyl esters and triglycerides were 1 M with the exception of 0.23 M triacetin and 0.5 M triolein. Choline esters and naphthyl esters were 0.1 M and 0.06 M, respectively. All substrates were prepared in 3% (w/v) Triton X-155 (Rohm and Haas) and 0.1% (w/v) gum arabic (Matheson, Coleman and Bell) and homogenized for 2 min in a water-cooled microblender. Homogenization was omitted in the preparation of water soluble naphthyl esters.

Inhibitors

Organophosphorus inhibitors used were diethyl-*p*-nitrophenyl thiophosphate (parathion), DFP, and tetraethyl pyrophosphate (K and K Laboratories). They were prepared in 1% (w/v) Triton X-100 (Rohm and Haas). The 0.1 M parathion solution required 30 sec homogenization in a microblender to form a semi-stable emulsion. DFP and tetraethyl pyrophosphate were soluble at the concentrations used. Prescribed concentrations were prepared by serially diluting the more concentrated solutions or emulsions. Physostigmine sulfate (K and K Laboratories) was prepared in water.

Assay procedure

Esterase activity was determined manometrically using the Gilson differential respirometer at a constant pressure of 770 mm Hg and water bath temperature of 37°. The procedure used was similar to that of FORSTER, BENDIXEN AND MONTGOMERY[2]. One and a half ml $NaHCO_3$ buffer, 1.0 ml enzyme preparation, and 1.0 ml of either distilled water, 1% (w/v) Triton X-100, or inhibitor solution, depending on the experiment, were pipetted into the main compartments of the respirometer flasks. 0.1 ml $NaHCO_3$ buffer and 0.4 ml substrate were pipetted into the side arms. Bicarbonate concentrations required to give the desired pH were determined according to UMBREIT, BURRIS AND STAUFFER[7]. Flasks were attached to the respirometer and gassed for 10 min with a mixture of 5% CO_2 and 95% N_2 with the side-

arm vents open. After gassing, side-arm vents were closed, the pressure was adjusted to 770 mm Hg, and the flasks were equilibrated for 10 min. Side-arm contents were tipped into the main compartment at zero-time. Readings were taken at 10 min intervals for 30 min. Net μl CO_2 produced in 30 min were corrected as described by equation D of GREGORY AND WINTER[8].

RESULTS

Effect of pH

The change in activity with variation in pH was investigated to determine a suitable pH to be used throughout this work. Since a mixture of esterases were presumed to be present, a detailed pH study was not deemed necessary. Esterase activity was determined at various pH values between 6.0 and 8.0, the limits allowed by the assay procedure, using phenyl acetate, phenyl propionate, tripropionin, tri-*n*-butyrin as substrates. The pH optima were found to lie between 6.9 and 7.2

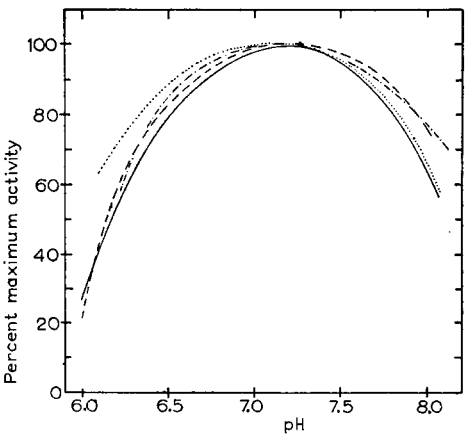

Fig. 1. Effect of pH on the esterase activity of aqueous pea extracts. - - -, phenyl acetate; ———, phenyl propionate; . . . ., tripropionin; ·—·—·, tri-*n*-butyrin.

(Fig. 1) and a pH of 7 was chosen to be used, since it was desirable for comparative purposes to maintain constant conditions. This contrasts with a pH optimum of 5.5 to 6.5 for citrus acetylesterase[9]. Most animal esterases have broad pH optima in the region of 7 and 8 (refs. 10, 11).

Substrate specificity

Activities of the enzyme extract toward the various substrates used in this study are presented in Table I. Phenyl esters were hydrolyzed at a greater rate than the triglycerides, naphthyl esters, and the choline esters. There was a general decrease in activity as the polarity of the alcohol moiety was increased. The least polar substrates, the phenyls, yielded the greatest activity. The most polar substrates, the cholines, showed the lowest activity, whereas, the intermediate polar triglycerides and naphthyls resulted in intermediate rates of hydrolysis. Hence, non-polar inter-

TABLE I

HYDROLYSIS OF VARIOUS ESTERS BY THE PEA EXTRACT

Substrate	*Number of replications*	*Activity units**	*Standard deviation*
Phenyl acetate	11	3.07	0.71
Phenyl propionate	7	6.92	1.13
Phenyl *n*-butyrate	3	6.81	1.01
Triacetin	5	0.73	0.17
Tripropionin	6	1.17	0.07
Tri-*n*-butyrin	8	0.66	0.14
Triolein	3	0.03	—
Acetyl ester of sodium 2-naphthol-6-sulfonate	8	0.82	0.09
Propionyl ester of sodium 2-naphthol-6-sulfonate	8	1.83	0.16
n-Butyryl ester of sodium 2-naphthol-6-sulfonate	6	1.06	0.08
Hexyl, octyl, decyl, hexadecyl esters of sodium 2-naphthol-6-sulfonate	1	0.00	—
Acetyl choline iodide	1	0.03	—
Propionyl choline iodide	1	0.02	—
n-Butyryl choline iodide	1	0.02	—

* One activity unit represents one μequiv of substrate hydrolyzed/min per ml of pea extract.

actions would appear to be important in the binding of these substrates to the enzymes. This would agree with the theory proposed by DIXON AND WEBB[12]. Propionyl esters were hydrolyzed at a greater rate than the acetyl and butyryl esters of the triglycerides and naphthyls. Specificity for the short-chain esters is indicated by the absence of activity toward the soluble longer-chain esters of sodium 2-naphthol-6-sulfonate. Thus, steric effects might have been involved in the binding of the acyl portion of the substrate to the enzymes. This is in contrast to horse liver carboxyesterase which has been reported to show steric effects for the alkyl portion of the ester molecule[12].

Lipase (glycerol-ester hydrolase, EC 3.1.1.3) attacks insoluble triglycerides of long-chain fatty acids[13]. Although LEE AND WAGENKNECHT[14] reported a lipase in peas, the low activity observed with triolein as substrate suggests the absence of lipase in the enzyme extract. It is possible that either the pea lipase was not extracted, was not active under experimental conditions used in this study, or that the pea lipase reported was actually an esterase.

Inhibitor specificity

Plots of pI *versus* percent inhibition or percent activity have been used to differentiate esterases. ALDRIDGE[15] was able to differentiate 2 types of esterases in several mammalian sera which hydrolyzed *p*-nitrophenyl acetate, propionate, and butyrate. One type, which he called A, was not inhibited by paraoxone, while the the other, which he called B, was inhibited by concentrations as low as 10^{-8} M. A sigmoid curve was obtained when activity of the latter was plotted against pI. Esterases which have different inhibitor sensitivities and are present in the same preparation, hydrolyzing the same substrate, can also be differentiated. In this case the curve obtained would be expected to approximate a double sigmoid curve.

MYERS, TOL AND DE JONGE[3] obtained such a curve in their work on the esterases of mycobacteria. A plateau was reached where increasing amounts of inhibitor had little effect until sufficient inhibitor was present to start inhibiting the least sensitive esterase.

Effects of the inhibitors, parathion, tetraethyl pyrophosphate, and DFP at pI 1 to 10 on esterase activity of the pea extract are presented in Figs. 2, 3, and 4. A complex mixture of esterases is revealed. In general, parathion and tetraethyl pyrophosphate showed more selective inhibition toward pea esterases than DFP. The esterases more sensitive to parathion hydrolyzed the less polar phenyl esters to

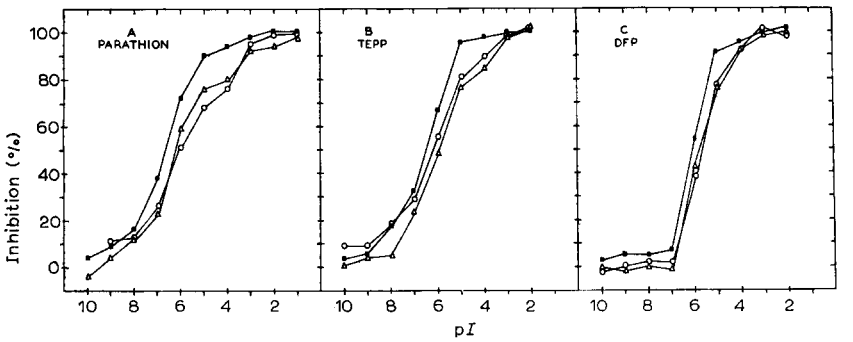

Fig. 2. Inhibition curves for the hydrolysis of phenyl esters by aqueous extracts of the pea. Percent inhibition is plotted against the negative $\log_{10}$ of the M inhibitor concentration (pI). Number of replications for A, B, and C, respectively, are given in parentheses. △, phenyl acetate (3, 7, 2); ○, phenyl propionate (2, 6, 3); ■, phenyl *n*-butyrate (4, 3, 2). TEPP, tetraethyl pyrophosphate.

a greater extent than the more polar triglycerides and naphthyls. Also, the hydrolysis of the less polar butyryl esters was more sensitive to all the organophosphorus compounds.

The double sigmoid curves in Figs. 2A, 3A, and 4A demonstrate that at least 2 esterases were hydrolyzing the acetyl and propionyl esters of phenol, glycerol, and sodium 2-naphthol-6-sulfonate and were selectively inhibited by parathion. The

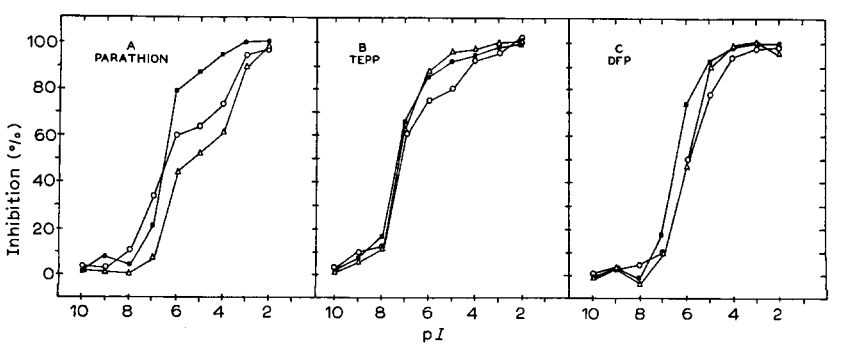

Fig. 3. Inhibition curves for the hydrolysis of triglycerides by aqueous extracts of the pea. Percent inhibition is plotted against the negative $\log_{10}$ of the M inhibitor concentration (pI). Number of replications for A, B, and C, respectively, are given in parentheses. △, triacetin (3, 2, 2); ○, tripropionin (3, 2, 2); ■, tri-*n*-butyrin (3, 4, 2). TEPP, tetraethyl pyrophosphate.

leveling-off of the butyryl ester curves in the region p*I* 2 to 5 or 6 indicates that the 2 esterases also were hydrolyzing these substrates. Further study of these figures shows that the more parathion sensitive esterase that hydrolyzed the phenyl esters (Fig. 2A) and *n*-butyryl ester of sodium 2-naphthol-6-sulfonate (Fig. 4A) was not the same as the more parathion sensitive esterase that hydrolyzed the triglycerides (Fig. 3A), acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate (Fig. 4A). The curves for the latter group reached the leveling-off point at p*I* 6, but the curves for the former group did not reach this point until p*I* 5, while a substantial amount of inhibition occurred between p*I* 5 and 6. This is most clearly seen in Fig. 4A, where the inhibition of the activity toward acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate did not increase, while the inhibition of the activity toward

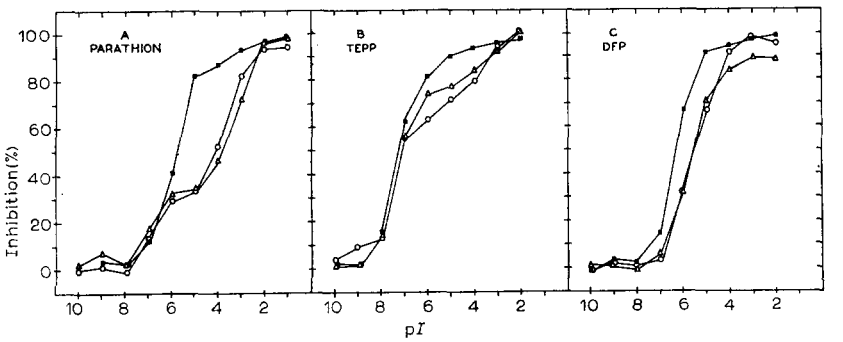

Fig. 4. Inhibition curves for the hydrolysis of esters of sodium 2-naphthol-6-sulfonate by aqueous extracts of the pea. Percent inhibition is plotted against the negative $\log_{10}$ of the M inhibitor concentration (p*I*). Number of replications for A, B, and C, respectively, are given in parentheses. △, acetyl ester (4, 2, 2); ○, propionyl ester (4, 2, 2); ■, butyryl ester (3, 2, 2). TEPP, tetraethyl pyrophosphate.

n-butyryl ester of sodium 2-naphthol-6-sulfonate was increased approx. 40%. The enzyme hydrolyzing the triglycerides also were more sensitive to tetraethyl pyrophosphate than those hydrolyzing the phenyl esters (Figs. 2B and 3B). Therefore, a third esterase, which hydrolyzed the phenyl esters and *n*-butyryl ester of sodium 2-naphthol-6-sulfonate, but not the triglycerides or the acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate, is indicated.

The parathion inhibition curve for phenyl acetate (Fig. 2A) is suggestive of a triple sigmoid, which would indicate the presence of another esterase that hydrolyzed phenyl acetate. Approx. 8% of the activity toward phenyl acetate, which was inhibited between p*I* 1 and 3, would represent a fourth esterase present in the pea extract.

Leveling-off of the acetyl ester of sodium 2-naphthol-6-sulfonate curve (Fig. 4C) before 100% inhibition was reached, reveals the presence of a fifth esterase, which was not inhibited by DFP and was responsible for 11% of the activity toward the acetyl ester of sodium 2-naphthol-6-sulfonate. This esterase did not hydrolyze any of the other substrates used in this study, since 100% inhibition was observed with these substrates at the higher concentrations of DFP.

There is some evidence that the inhibition observed between p*I* 6 and 8

parathion and tetraethyl pyrophosphate was not due to inhibition of the same enzyme. A larger portion of the activity toward acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate and triacetin was inhibited at p*I* 6 by tetraethyl pyrophosphate than by parathion. If the same enzyme was inhibited, the percent inhibition would be expected to be the same at the plateau. However, the percentage is different with the 2 inhibitors. For example, 30% of the hydrolysis of acetyl ester of sodium 2-naphthol-6-sulfonate was inhibited at p*I* 6 parathion (Fig. 4A) while 70% of the hydrolysis was inhibited by tetraethyl pyrophosphate at the same concentration (Fig. 4B). This reveals the presence of a sixth esterase in the pea extract, which is more sensitive to tetraethyl pyrophosphate than to parathion and hydrolyzes acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate and triacetin. There is the possibility that the esterase more sensitive to parathion was the same as the one least sensitive to tetraethyl pyrophosphate. In this case, another esterase would not have been present.

The above indicate the presence of 6 esterases in the pea extract. If esterases had the same substrate and inhibitor specificity, they would not have been detected by this method. Hence, there is the possibility of more than 6 esterases present in the pea extract. Since these esterases were differentiated on the basis of substrate and inhibitor specificity, they were not isozymes. The effect of 10^{-5} M physostigmine sulfate on esterase activity was determined with each of the 9 substrates. No inhibition was observed. Since 10^{-5} M physostigmine completely inhibits cholinesterases[16], none of the activity toward these substrates was due to a cholinesterase. This is supported by the very low activity noted with the choline esters (Table I).

With bovine milk, a decrease in inhibition was noted at the higher concentrations of *N,N'*-diisopropyl phosphorodiamidofluoridate and tetraethyl pyrophosphate[2]. Although this was not explained in the paper, it was later shown to be due to the hydrolysis of these organophosphorus compounds by the esterases of the milk (T. L. FORSTER, personal communication). No decrease in inhibition was noted in Figs. 2, 3, and 4 at the higher inhibitor concentrations, therefore, it would appear that the esterases of the pea were not capable of hydrolyzing the inhibitors used in this study. However, a note of caution should be mentioned. Since the enzyme extract was quite dilute in most of these determinations, a higher concentration of enzyme might have shown hydrolysis of the organophorus compounds.

Effect of bivalent metal ions and metal complexing agents

During substrate specificity studies, various concentrations of the enzyme extract were used with the different substrate to obtain reaction rates which were constant with time. In experiments where too high a concentration was used, reaction rates decreased with time. When phenyl propionate and phenyl *n*-butyrate were used as substrates, concentrations low enough to give constant reaction rates resulted in reduced activities. The effect was quite pronounced using phenyl *n*-butyrate as substrate. The average of 3 replications gave 3.11 units of activity at a 1:20 dilution of the extract and 6.81 units at a 1:10 dilution when the rate for the first 10 min was used as an estimation of the activity. The presence of an inhibiting substance in the distilled water of the dilution of an activator might have caused this reduction in activity. A study or the effect of heavy metal ions and metal complexing agents supports the former interpretation.

The effects of 7 heavy metal ions on pea esterase activity were determined using phenyl propionate as substrate. Esterases which hydrolyze phenyl propionate were sensitive to all of the ions studied (Table II). The degree of sensitivity varied with Zn^{2+} and Cd^{2+} being most inhibitory, followed by Ni^{2+} and Co^{2+}, while Mn^{2+}, Ca^{2+}, and Mg^{2+} were inhibitory only at the higher concentrations. These esterases were activated by EDTA, cysteine and glutathion. EDTA and cysteine showed maximal activation at 1 mM while glutathion showed maximal activation at 10 mM. Addition of both EDTA and glutathion to the reaction mixture at optimal concen-

TABLE II

EFFECT OF HEAVY METAL IONS ON ESTERASE ACTIVITY USING PHENYL PROPIONATE AS SUBSTRATE

Compound	*Concentration (mM) in final reaction mixture*	*Percent inhibition*	*Compound*	*Concentration (mM) in final reaction mixture*	*Percent inhibition*
$CdCl_2 \cdot 4H_2O$	100	84	$MnCl_2 \cdot 4H_2O$	100	28
	10	62		10	14
	1	44		1	9
$CoCl_2 \cdot 6H_2O$	100	56			
	10	37	$CaCl_2$	100	20
	1	32		10	7
$NiCl_2 \cdot 6H_2O$	100	63	$MgCl_2$	100	10
	10	40		10	0
	1	34		1	0
$ZnCl_2$	100	97			
	10	60			
	1	37			

trations did not produce greater activation than with either of the substances alone. Hence, it would appear that the glutathion was acting by metal ion removal rather than by the reduction of essential sulfhydryl groups.

8 replications with and without 0.1 mM EDTA were performed using phenyl propionate as substrate. Average activity without EDTA was 5.63 units with a S.D. of 1.11, while the same enzyme preparations showed 8.30 units with a S.D. of 0.52 with EDTA. Therefore, the addition of EDTA to the reaction mixture not only increases the activity of the pea esterases hydrolyzing phenyl propionate, but also decreases the variation between the enzyme preparations. The values presented in Table I for phenyl propionate and probably for phenyl *n*-butyrate are low. This may be true for some of the other substrates, also.

DISCUSSION

Esterases of animal systems have been characterized and classified[17,18]. Carboxylesterases (carboxylic-ester hydrolases, EC 3.1.1.1), formerly known as aliesterases or B-esterases, have wide specificity, hydrolyze both aliphatic and aromatic esters, but not choline esters, and are sensitive to most organophosphorus inhibitors,

but resistant to physostigmine (pI = 5). Arylesterases (aryl-ester hydrolase, EC 3.1.1.2), formerly known as A-esterase, hydrolyze aromatic esters, but normally not aliphatic esters and are resistant to most organophosphorus compounds and physostigmine. In addition, they are sensitive to *p*-hydroxymercuribenzoate.

An acetylesterase (acetic-ester hydrolase, EC 3.1.1.6), purified from citrus fruits, was shown to hydrolyze esters of acetic acid and was inhibited by DFP (ref. 8). More recently, JOOSTE AND MORELAND[5] obtained evidence from electrophoretic mobilities and substrate and inhibitor specificities that cucumber, soybean, wheat, and corn contain complex esterase systems. Both carboxylesterases and arylesterases were found to be present. These authors[19] also reported a phenoxyesterase in soybeans that was activated by DFP (pI = 3), not inhibited by *p*-chloromercuribenzoate and specific for 2-naphthol-phenoxyacetate. SCHWARTZ *et al.*[6] found esterases in green beans, cabbage, potato tuber, citrus albedo and flavedo and fruits of many cucurbits which varied in different species, in different strains of the same species, and in different parts of the same plant. FRANKEL AND GARBER[20] reported 6 bands of esterase activity which were separated by starch gel electrophoresis from extracts of 12 varieties of germinating peas. However, the effect of inhibitors was not reported and the esterases were not classified.

From the data presented in Figs. 2–4, 5 of the 6 esterases in the pea extract were carboxylesterases, while the esterase active toward acetyl ester of sodium 2-naphthol-6-sulfonate and not inhibited by DFP may be classified as an arylesterase. However, the fact that this esterase was inhibited by parathion and tetraethyl pyrophosphate makes such a classification questionable. Similar problems have occurred in the classification of animal esterases[1].

Since the literature already contains an over abundance of names for various esterases, the present authors are reluctant to name the carboxylesterases reported in this paper. It would appear wise to delay the naming of these esterases until the natural substrates are known or until the esterases are purified and characterized. Work leading to this end is currently under way in this laboratory.

ACKNOWLEDGEMENTS

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