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PROPERTIES OF ACETYLCHOLINESTERASE FROM *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*: Leguminosae; pea; acetylcholinesterase; acetylcholine; fensulfothion.

Abstract—Acetylcholinesterase (AChE) from *Pisum sativum* purified 28 fold showed two closely moving protein bands on polyacrylamide gel electrophoresis, both of which have AChE activity. AChE activity occurs in roots, stem and leaves, that in roots varying with age. Activity is optimal at pH 9 and at 30°. The energy of activation is 9.82×10^3 J per mol and MW is greater than 200 000. Although the enzyme can hydrolyze both choline and non-choline esters, it has greater affinity for acetylthiocholine (ATCh) and acetylcholine (ACh). ATCh inhibits the enzyme at higher concentrations and the K_m is 0.2 mM with this as substrate. The enzyme is not as sensitive to Eserine as it is to Neostigmine. It is also inhibited by organophosphorus pesticides such as Fensulfothion, Parathion and Dimethoate. Treatment of the seeds with Fensulfothion [*O*, *O*-diethyl (*p*-methylsulfinylphenyl) phosphorothioate] affects growth and secondary root development. This might be explained by its inhibition of AChE and the consequent increase of endogenous levels of ACh.

INTRODUCTION

Organophosphorus pesticides are less persistent than the organochlorine pesticides and for this reason, they are finding increased use for crop protection. However organophosphorus pesticides affect the germination of seeds and enzyme activities [1–3].

Since ACh prevents the formation of secondary roots in much the same way as exposure to red light (660 nm), Jaffe [4] suggests that ACh may be involved in phytochrome mediated events. This inhibitory effect is reversed by far red (730 nm) irradiation. The involvement of ACh in phytochrome action suggests the possible occurrence of AChE in plants for the purpose of regulating the levels of endogenous ACh. Cholinesterase (ChE) from mung bean roots has been purified and characterized [5]. Jaffe and his associates [6–9] have reported the widespread occurrence of ChE in several genera of plants and also found that mung bean ChE is distinct from pectin esterase [10]. So, if there is an "acetylcholine–acetylcholinesterase" system operating in plants, playing a regulatory role in their development, then it is important to know the response of this system to organophosphorus pesticides, as it is well known that organophosphorus pesticides act by inhibiting AChE. Few reports are available on the effect of pesticides on plant AChE. This paper reports the studies on the purification and properties of AChE from pea roots, as well as the effect of Fensulfothion on lateral root development and AChE activity in the plant.

RESULTS AND DISCUSSION

AChE activity was detected both in the root and shoot systems of the pea plant. The AChE activity in roots varies with the age of the plant. There is no activity on the 3rd day of germination, but from the 4th day onwards the activity increases, up to day 15.

The steps employed for the purification of AChE from pea roots are given in Table 1. The preliminary extraction of the roots with low molarity buffer removes nearly 75% of the contaminating proteins without any loss of AChE activity. The enzyme is solubilized from the above residue using 5% $(NH_4)_2SO_4$ in 10 mM KPi buffer pH 7. Recovery increases during the $(NH_4)_2SO_4$ precipitation indicating the presence of endogenous inhibitor(s) in the crude preparation. AChE is eluted in the void volume of the Sephadex G-200 column. On polyacrylamide gel electrophoresis of this eluate, two protein bands are obtained. Both bands are positive when stained for

Table 1. Purification of acetylcholinesterase from pea roots

Fraction	Total protein	Total activity	Specific activity	Recovery	Purification
	mg	nkat	nkat/mg protein	%	fold
Buffer extract	295	0	0	0	0
5% $(NH_4)_2SO_4$ extract	62	1.40	0.023	100	1
30–80% $(NH_4)_2SO_4$ precipitate	12	3.25	0.28	232	12.5
Sephadex G-200	4	2.57	0.63	183	28

Table 2. Hydrolysis of various esters by pea root acetylcholinesterase

Substrate	Specific activity nkat/mg protein
1. Acetylthiocholine	0.60
2. Acetylcholine	0.65
3. Propionylthiocholine	0.27
4. Butyrylthiocholine	0.00
5. Butyrylcholine	0.03
6. Indoxylacetate	0.40
7. α-Naphthylacetate	0.10

Table 3. Effect of inhibitors on pea root acetylcholinesterase

Inhibitor	Concentration (M)	Inhibition (%)
Eserine	9×10^{-4}	50
	6×10^{-3}	100
Neostigmine	6×10^{-7}	50
	4×10^{-6}	100
Ethionamide	8×10^{-5}	100
Parathion	4×10^{-5}	50
	1×10^{-4}	100
Dimethoate	1×10^{-3}	50
	5×10^{-3}	100
Fensulfothion	5×10^{-4}	50
	5×10^{-3}	100

activity. Pretreatment of the gels with Eserine or Parathion prevented the appearance of the bands.

The rate of hydrolysis of ATCh is proportional to the concentration of the enzyme. When a suitable concentration of ATCh is used, hydrolysis of ATCh is linear for the first 5 min. The pH and temperature optima for the AChE are 9.0 and 30° respectively. The energy of activation for the reaction is 9.82×10^3 J per mol. The purified AChE is stable for one week without any loss in activity at 4°.

Substrate specificity. A number of thiocholine, choline and non-choline esters were used to ascertain the substrate specificity of AChE (Table 2). Among the choline esters examined acetyl esters are hydrolyzed faster than propionyl ester, while the butyryl esters are not hydrolyzed. Increase in the chain length of an ester probably hinders the activity of the enzyme. Among the non-choline esters indoxyl acetate is broken down at a much faster rate than α-naphthyl acetate.

Effect of substrate concentration. High concentrations are inhibitory to the enzyme. The V vs S plot yields a bell shaped curve. Animal AChE [11,12], as well as ChE [5] from mung-bean plants behave similarly. The optimum substrate concentration is between 0.08–1 mM. From the Lineweaver–Burk and Dixon plot K_m and K_i for ATCh are 0.2 mM and 4 mM respectively.

Inhibitors. The AChE from pea is inhibited by the inhibitors which are known to inhibit AChE from animal sources (Table 3). The enzyme from pea is highly inhibited by Neostigmine and is inhibited only by relatively high concentration of Eserine. Ethionamide (an antitubercular drug) which inhibits serum cholinesterase of animals, also inhibits the pea enzyme. Organophosphorus pesticides like Fensulfothion, Parathion and Dimethoate also inhibit the enzyme as shown in the Table 3.

Effect of Fensulfothion on secondary root development and AChE activity in the roots of pea plant. The effect of Fensulfothion, an organophosphorus pesticide, on AChE activity, ACh levels of the roots and secondary root formation are shown in Table 4. On the 3rd day of germination there is no detectable AChE activity in the roots of both control and pesticide treated seedlings. On the 6th day AChE activity cannot be demonstrated in the treated seedling roots. Secondary roots start to appear in the control seedlings, but there is no sign of secondary root initiation in the treated plants. When compared to the untreated seedlings, ACh level in the treated seedling roots is 60% greater. On the 9th day AChE activity is still low in treated seedling roots. An inactive protein similar to the active enzyme is present in the Fensulfothion treated seedlings showing that the pesticide only inhibits the activity of the enzyme. Secondary roots are well formed in the untreated seedlings whereas they are very few and stunted in the case of Fensulfothion treated seedlings. ACh level is 45% more than in untreated seedling roots. Increase in the endogenous ACh level is probably responsible for the inhibition of formation of secondary roots.

However, this enduring inhibitory effect of Fensulfothion on AChE and the consequent increase of endogenous levels of ACh indicate that the phytotoxicity of the organophosphorus pesticides might be due to their effect on this system in plants.

EXPERIMENTAL

Pea seeds (*Pisum sativum*) obtained from the local market were soaked in running H_2O for 4 hr and surface sterilized

Table 4. Effect of Fensulfothion on the acetylcholinesterase activity, ACh levels in the roots and on the secondary root development of pea seedlings

Age of plants (days)	Fr wt of roots/plant mg		Acetylcholine mg/g fr wt of roots		Lateral roots		Acetylcholinesterase activity nkat/mg protein	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
3	30.0	31.6	2.47	2.62	A	A	—	—
6	100.0	101.0	0.65	1.05	P	A	0.04	0
9	176.0	128.0	0.64	0.91	P	A†	0.07	0.01

* AChE activity in the 30–80% $(NH_4)_2SO_4$ pellet. † In a few plants poor growth of secondary roots was seen. A absent; P present.

using 0.01% $HgCl_2$ for 10 min. Then the seeds were washed well in sterile H_2O and germinated in trays. In the case of Fensulfothion treatment, the concentration of pesticide used was 50 ppm.

Extraction and assay of ACh. ACh was extracted from the roots of germinating seeds by grinding and extracting with 10 mM KPi buffer pH 7 followed by centrifugation at 20 000 *g* for 10 min. The ACh extracted in the supernatant does not contain any AChE activity. The supernatant was therefore used for the determination of ACh content and buffer residue was used for the AChE extraction. The amount of ACh present in the buffer extract was determined [13].

AChE extraction and purification. Buffer residue was reground and extracted with 5% $(NH_4)_2SO_4$ in 10 mM KPi buffer pH 7 which solubilizes AChE from the residue. Extract was centrifuged for 10 min at 20 000 *g*. AChE was precipitated out between 30–80% $(NH_4)_2SO_4$ saturation on centrifugation at 20 000 *g* for 10 min. The AChE pellet was dissolved in 10 mM KPi buffer pH 7 and dialyzed against two changes of the same buffer for 12 hr. Dialyzed enzyme was loaded onto a Sephadex G-200 column (1 × 63 cm) previously equilibrated with 20 mM KPi buffer pH 7. The enzyme was eluted with the same buffer (3 ml fractions). $A_{280\ nm}$ and AChE activity were determined. The fractions showing high AChE activity were pooled and used as the enzyme.

Assay of AChE using thiocholine esters. Enzyme activity was measured by the method of ref. [14] and the mixture contained in a final vol of 3 ml, 0.2–0.3 mg of protein, 0.33 mM 5,5′ dithiobis-(2-nitrobenzoic acid), 1–3 mM substrate, 0.1 M KPi buffer pH 8. After preincubation at 30° for 5 min or for 30 min in the presence of inhibitors, the reaction was initiated by the addition of substrate. The mixture was incubated for 10 min at 30° and the increase in $A_{412\ nm}$ was measured. Activity was calculated as nmol of thiocholine ester hydrolyzed per sec based on E: 1.36×10^4 for the yellow anion [14,15] (2-nitro, 5-thiobenzoate) formed during the reaction.

Assay of AChE using choline esters. AChE activity was measured by the modification of the method of ref. [16] using bromothymol blue as the indicator. Activity was expressed as nmol of acid produced sec^{-1}.

Assay of AChE using Kramer's esterase assay. Esterase activity was determined by modification of the method of ref. [17] using indoxyl acetate as a substrate. The amount of substrate hydrolyzed was calculated using E of indophenol as 1.38×10^4.

Disc electrophoresis was carried out according to the method of ref. [18] using Tris–glycine buffer pH 8.3 on 6% polyacrylamide gels. 30–50 μg of protein was used. Gels were stained for 30 min with Coomassie blue and destained with 7% HOAc for 12 hr.

Activity staining for pea AChE. The AChE activity regions in the polyacrylamide gels were stained by the method of refs. [19,20] as modified in ref. [21]. The effect of Eserine and Parathion were studied by pre-incubating the gels for 30 min in 0.01 mM soln of the inhibitors, before staining for activity.

The protein content was determined by the method of ref. [22].

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