BIOLOGIA PLANTARUM (PRAHA)

33 (4) : 303–310, 1991

Acetylcholinesterase from Oat Seedlings. I. Preliminary Biochemical Characterization of the Enzyme*

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Abstract. The activity of acetylcholinesterase (AChE) isolated from coleoptiles of etiolated oat seedlings is strongly inhibited by neostigmine and less so by eserine. The optimum of the enzyme activity occurs at pH 7.2 and a temperature of + 36 °C. The enzyme Michaelis constant is 280 μM. Choline within the range of concentration from 0.001 to 10 mM does not affect the enzyme activity. Calcium ions at 5 mM concentration cause inhibition, while magnesium and manganese ions do not affect the enzyme activity.

AChE isolated from oat seedlings differs in a number of properties from AChE occurring in the tissues of other plants.

Additional index words: Acetylcholinesterase (AChE), *Avena sativa*, biochemical characterization, coleoptile, oat

Acetylcholine (ACh) is one of several neurotransmitters occuring in the nerve endings of animals (Lester 1978). It is also present in animals lacking a nervous system such as protozoa, and in wide variety of non-neural tissues in higher animals (Sastry and Sadavongvivad 1979). Furthermore, this compound has been detected in representatives of lower and higher plants as well was bacteria and fungi (Fluck and Jaffe 1974b, Hartmann and Gupta 1989, Tretyn and Kendrick 1991). The site of ACh synthesis is probably situated in young leaves (Jaffe 1970, Tretyn and Kendrick 1991).

In some plant species choline acetyltransferase (ChAT) activity has been found (Barlow and Dixon 1973, Smallman and Maneckjee 1981, Hadačová *et al.* 1981). This enzyme showing properties similar to animal ChAT, probably participates in ACh synthesis from its precursors, choline and acetyl-Coenzyme A (Barlow and Dixon 1973, Smallman and Maneckjee 1981).

Received March 7, 1990; accepted August 15, 1990

* This research was supported in part by grant CPBP 05.02.4.07.

Cholinesterase (ChE) activity has also been found in plant tissues. This enzyme decomposes ACh and exhibit properties similar to animal acetylcholinesterase (AChE; Fluck and Jaffe 1974b, Roshchina and Mukhin 1985, Roshchina 1988). The presence of both ChAT and AChE in plant tissues suggests that ACh undergoes similar metabolism in plants as it does in animals (Roshchina 1987; for review see Tretyn and Kendrick 1991).

To date (acetyl)cholinesterase activity has been found in the tissues of more than 100 plant species (Fluck and Jaffe 1974a, Miura *et al.* 1982). However, the absence of ChE hydrolysis has been recorded in 65 plant species (Hartmann and Gupta 1989).

The exact role of cholinesterases in plants is unclear. To overcome this lack of knowledge it is necessary to investigate their properties, especially in these plant species in which endogenous ACh exists. Therefore, this paper reports studies on the purification and properties of ChE isolated from coleoptiles of etiolated oat seedlings which also contained endogenous ACh (Tretyn *et al.* 1987).

MATERIAL AND METHODS

AChE was isolated from coleoptiles of 4d-old etiolated oat seedlings (*Avena sativa* L., cv. Diadem).

Oat caryopses were soaked in water at a temperature of 27 °C, then planted in sterile sawdust in plastic containers and grown in the dark at 27 °C for four days.

The enzyme was isolated at a temperature of about 4 °C by the Riov and Jaffe (1973) method. The coleoptiles were cut at a height of 3 cm from the apex and homogenized in 10 mM K-phosphate buffer (KPB), pH 7.0, using 2 ml of the buffer per 1 g of tissue. After stirring for 30 min the homogenate was centrifuged for 15 min at 6 000 *g*. After centrifugation the supernatant was discarded, and the residue was resuspended in 1.5 volumes (m/v) of 10 mM KPB containing 4 % (w/v) ammonium sulphate. The suspension was stirred for 30 min, and then after filtering through double nylon net again centrifuged for 15 min at 6 000 *g*. The clear yellow-orange supernatant contained soluble proteins. These proteins were precipitated by adding solid ammonium sulphate up to 80 % saturation and subsequently centrifugated at 20 000 *g* and redissolved in a small volume of 20 mM KPB (1 ml of buffer per 10 g of initial material). Solution of concentrated proteins was passed through a 20 mM KPB equilibrated column packed with Sephadex G-25 (3.5 × 25 cm). To the ChE activity

Abbreviations: ACh – acetylcholine; AChE – acetylcholinesterase; ATCh – acetylthiocholine; BTCh – butyrylthiocholine; ChAT – choline acetyltransferase; ChE – cholinesterase(s); DTNB – 5,5-dithiobis-(2-nitrobenzoic acid); iso-OMPA – tetraisopropyl pyrophosphoramide; KPB – K-phosphate buffer; PTCh – propionylthiocholine.

Table 1
Properties of cholinesterase from etiolated oat (*Avena sativa* L.) seedlings.

K_m	I_{50}		optimum		specific activity		
	neostigmine	eserine	pH	temp.	ATCh	PTCh	BTCh
[μM]	[μM]			[°C]	[μmol substr. min^{-1} mg^{-1}]		
280	2.5	280	7.2	36	0.126	0.123	0.023

containing fractions ammonium sulphate was added to 70 % saturation and these were then stored overnight at 4 °C. The precipitated proteins were then centrifuged for 30 min at 20 000 *g*. Obtained pellet was redissolved in several ml of 20 mM KPB and dialyzed against two changes of the same buffer for 24 h. Dialyzed enzyme was filtered through a Sepharose 6B column (3.3 × 41 cm) equilibrated with 100 mM KPB (flow rate; 30 ml h^{-1}). The ChE activity containing fractions (3 ml each) from the column were collected and used as the enzyme.

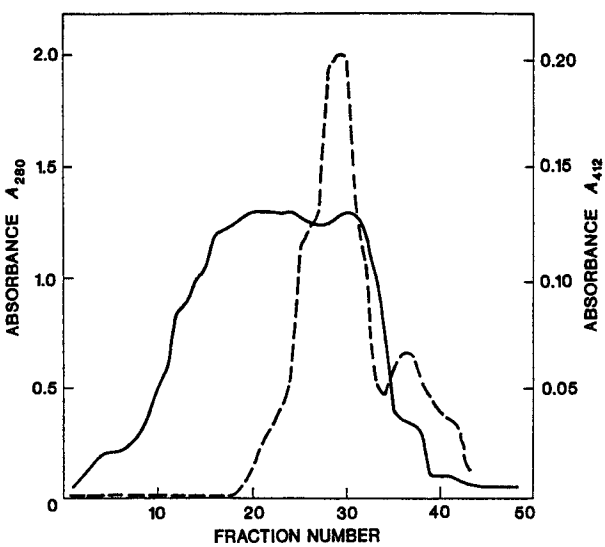

Fig. 1. Eluation profile of an enzyme isolated from etiolated seedlings on Sepharose 6B. Full line: UV absorbance of the eluate; dashed line: acetylthiocholine-hydrolyzing activity.

Cholinesterase activity was measured using the Ellman *et al.* (1961) method. The standard reaction mixture with the final volume of 0.6 ml contained: i) 0.2 ml of 0.5 M KPB, pH 7.0; ii) 0.1 ml of 0.6 mM DTNB (prepared in a buffer containing 9 mg of $NaHCO_3$/100 ml); iii) 0.1 ml of the enzyme; iv) 0.1 ml of water or water solution of the substance whose effect on the enzyme activity

was studied; and v) 0.1 ml of 3 mM either ATCh or PTCh or BTCh iodide dissolved in 10 mM KPB. The reaction was initiated by the addition 0.1 ml of the substrate. The mixture was incubated for 10 min at 32 °C and the increase in $A_{412\ nm}$ was measured. The activity was calculated as nmol of thiocholine ester hydrolyzed per min per mg protein, based on 1.36×10^{-4} for the molar extinction coefficient of the yellow anion (2-nitro, 5-thiobenzoate) formed during the reaction (Riov and Jaffe 1973).

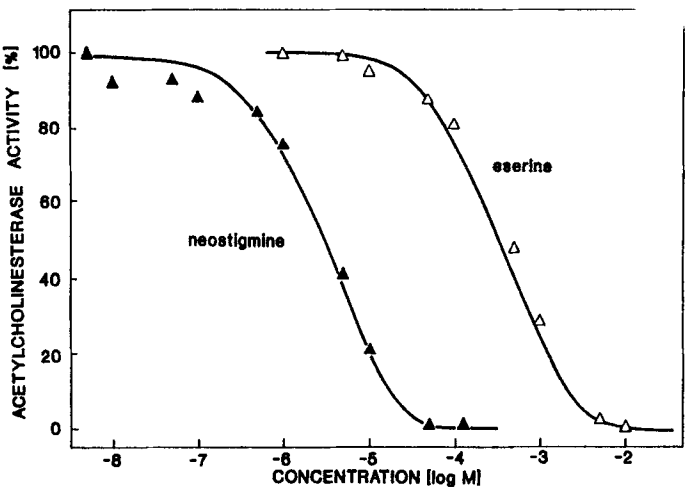

Fig. 2. Effect of eserine (open triangles) and neostigmine (full triangles) concentrations on oat acetylcholinesterase activity.

The amount of protein in the sample was determined by the Bradford (1976) method for specific activity measurements, or by recording the absorbance at 280 nm for column chromatography effluents. The spectrophotometric measurements were taken with a Specord UV/VIS spectrophotometer.

RESULTS AND DISCUSSION

The enzyme isolated from etiolated oat seedlings and then purified was able to hydrolyze ATCh (Fig. 1). It hydrolyzed also PTCh at a similar rate. However, the decomposition of BTCh by this enzyme was by about 80 % slower than ATCh destruction rate (Table 1). It has been found that its activity is inhibited by eserine and neostigmine, two well known inhibitors of animal ChE (Riov and Jaffe 1973). The inhibitory effect of eserine on enzyme activity was weaker than that of neostigmine (Fig. 2, Tab. 1). Inhibition constants (*i.e.* concentrations of the inhibitor at which the enzymatic activity is 50 % inhibited) were 2.5 μM and 280 μM, respectively. On the other hand, oat ChE was insensitive to the specific inhibitor of (pseudo)cholinesterase, iso-OMPA, used at 10 mM concentration.

The optimum pH of the enzyme isolated from etiolated oat coleoptiles was about 7.2 (Fig. 3) and the temperature optimum for enzymatic ATCh hydrolysis was at about 36 °C (Fig. 4). Choline within the concentration range from 0.001 to 10 mM has no effect on the enzyme activity. Calcium ions at concentration 5 mM inhibit, and manganese and manganese and magnesium ions have no effect on ChE activity (data not shown).

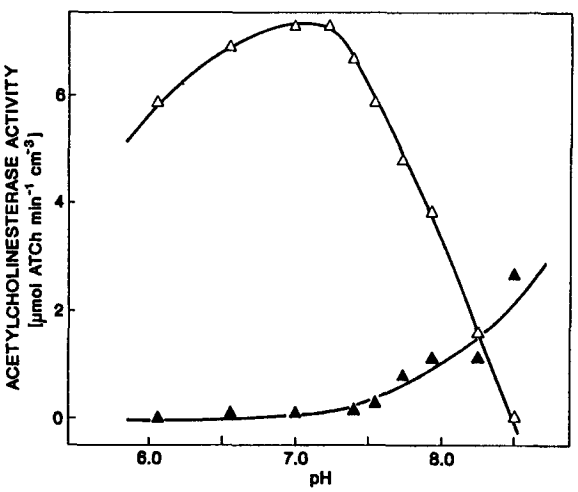

Fig. 3. Effect of pH (open triangles) oat acetylcholinesterase activity. Full triangles represents nonenzymatic hydrolysis of acetylthiocholine with increasing pH.

Fig. 5 illustrates the dependence of the cholinesterase activity of the isolated enzyme on ATCh concentration. The Michaelis constant based on it was 280 μM. In each assay a parallel control was used, in which the same amount of buffer was added instead of the enzyme. This allowed to obtain the values of nonenzymatic ATCh hydrolysis under these conditions. The values have been marked on the diagrams.

Judging from the behavior of the enzyme during filtration on Sepharose 6B gel (Fig. 1), where activity appears in effluent volume nearly equal to the effluent volume of the stationary phase, it can be assumed that the molecular mass of ChE from etiolated oat seedlings is lower than 100 kilodaltons.

The classification of plant cholinesterases presents many difficulties. On the basis of kinetic properties, substrate specificities, and sensitivity for different inhibitors, ChE in animals have been classified into two major groups: the specific, or "true" ChE or AChE (E.C. 3.1.1.7) and non-specific or "pseudo"

ChE (E.C. 3.1.1.8) (Oosterbaan and Janisz 1965). For animal esterases, it is accepted that an enzyme which is not inhibited by 10 μM concentration of eserine cannot be considered to be a cholinesterase (Augustinson 1963). Some authors adopted this criterion for the classification of cholinesterases from plants (Sae *et al.* 1971). However, only few enzymes studied so far correspond with this criterion. Most of them show an affinity to ACh comparable to that of animal enzymes and strong inhibition by another inhibitor of animal ChE – neostigmine (Fluck and Jaffe 1974b, Roshchina 1988).

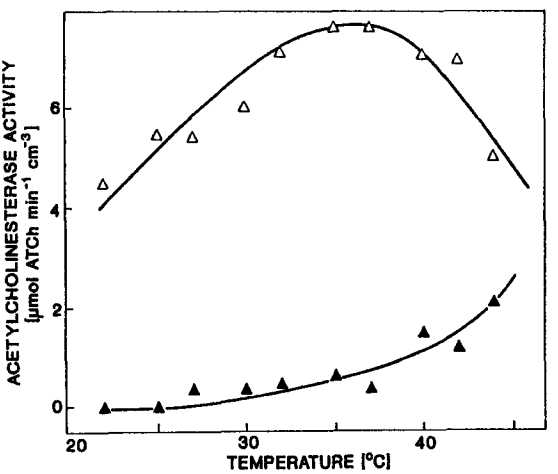

Fig. 4. Effect of temperature (open triangles) on oat acetylcholinesterase activity. Full triangles represents nonenzymatic hydrolysis of acetylthiocholine with increasing temperature.

The properties of the enzyme isolated from etiolated oat seedlings confirm the wide diversity of plant ChE. Generally speaking, this enzyme is not identical with any of those described earlier. Many authors (Riov and Jaffe 1973, Kasturi and Vasantharajan 1976, Mansfield *et al.* 1978, Vacková *et al.* 1984, Roshchina 1988) report that higher concentrations (about 1–2 mM) of the substrate inhibit enzymatic activity. The enzyme from oat seedlings was not inhibited even by 10 mM concentration of ATCh (Fig. 5). Also choline which stimulates (Riov and Jaffe 1973, Vacková *et al.* 1984) or inhibits (Ernst and Hartmann 1980) ChE from different plant species was without influence on oat enzyme. Furthermore, the oat enzyme differs from all the other ones (Riov and Jaffe 1973a,b, Kasturi and Vasantharajan 1976, Ernst and Hartmann 1980, Vacková *et al.* 1984) in having a comparatively low optimum pH, 7.2. This is important in so far as the optimum pH obtained in other experiments lies within the pH range in which both ACh and ATCh are

unstable (Tretyn and Kendrick 1991) and become rapidly nonenzymatically hydrolyzed.

Oat cholinesterase, however, in some biochemical properties, is similar to other plant ChE. Like in many other cases (Riov and Jaffe 1973, Kasturi and Vasantharajan 1976, Ernst and Hartmann 1980, Gupta and Maheshwari 1980), this enzyme was inhibited much more effectively by neostigmine than by eserine. Its activity was also inhibited by Ca^{2+} (Ernst and Hartmann 1980). Similarly to other plant ChE the affinity of oat enzyme for substrate analogs increased from BTCh to PTCh to ATCh (Riov and Jaffe 1973, Ernst and Hartmann 1980, Gupta and Maheshwari 1980, Roshchina 1988) and its highest activity was between 30 and 36 °C (Kasturi and Vasantharajan 1976, Ernst and Hartmann 1980).

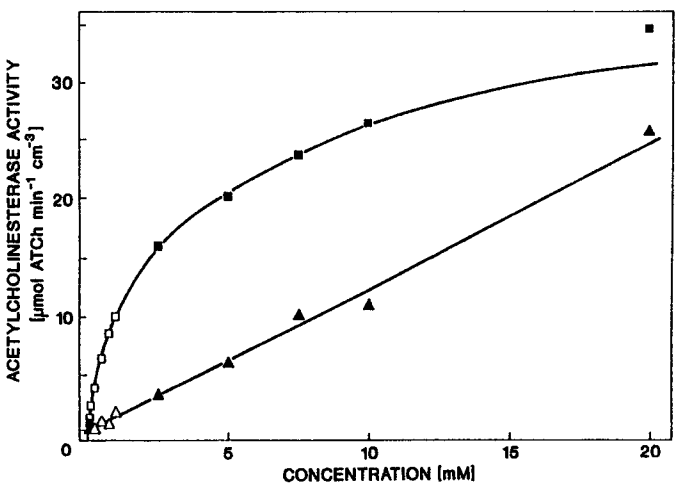

Fig. 5. Effect of substrate concentrations (full squares) on oat acetylcholinesterase activity. Full triangles represents nonenzymatic hydrolysis of substrate acetylthiocholine with increasing its concentration.

Considering that the enzyme isolated from the coleoptiles of etiolated oat seedlings hydrolyses ATCh five times as rapidly as BTCh, is also inhibited by both eserine and neostigmine, and is insensitive to iso-OMPA (the specific inhibitor of pseudocholinesterases (E.C. 3.1.1.8), it can be concluded that it is AChE (E.C. 3.1.1.7).

The physiological role of enzymes hydrolyzing specifically choline esters in plants is so far unclear. Using gas chromatography and nuclear magnetic resonance, endogenous ACh has been found in oat seedlings (Tretyn *et al.* 1987). It is therefore suggested that the function of AChE in oat consists in regulating the ACh level (Tretyn and Tretyn 1991).

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