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Choline Esterases and Choline Acetyltransferase in the Seeds of *Allium altaicum* (PALL.) REYSE

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Abstract. In the seeds of *Allium altaicum* (PALL.) REYSE a set of enzymes was found, metabolizing choline esters, composed of active choline esterases and choline acetyltransferase. Choline esterase cleaving acetylcholine occurs in five isoenzymes. The enzyme preparation hydrolyses strongly acetylthiocholine and sinapine, but weakly butyrylthiocholine (20%) in comparison with acetylthiocholine. The hydrolysis of the substrates mentioned is inhibited by physostigmine and neostigmine, but it is not inhibited by the specific inhibitor of acetylcholine esterase (BW 284 C51). In addition to hydrolytic activity a strong catalytic activity of choline acetyltransferase was also observed during the synthesis of sinapine from sinapic acid and choline. The detection of the mentioned enzymes in some representatives of the *Allium* genus indicates that choline esterases are more widely distributed in monocotyledons than previously assumed.

As is evident from the last decade of research the acetylcholine system has an important function not only in animals, but in plants as well. It is assumed that acetylcholine in plants takes part in some reactions elicited by phytochrome, or that it displays a similar effect as phytochrome. This is for example an inhibition of the formation of secondary roots, an increase in the consumption of oxygen, a decrease of the ATP level, accompanied by an increase in the levels of ADP and Pi in secondary roots, the stimulation of the delivery of hydrogen ions, the increase in the germinating power of seeds (KOŠTÍŘ *et al.* 1965, FLUCK and JAFFE 1974a, b, PENEL *et al.* 1976). Acetylcholine also functions in the protonemas of ferns as an antagonist of ethylene (BÄHRE 1975).

In addition to acetylcholine the plants probably also contain a whole group of choline esters of hydrocinnamic acids (KUTÁČEK *et al.*, in press). Among these esters only sinapine is known as a natural component so far *i.e.* the choline ester of sinapic acid occurring in the plants of the *Brassicaceae* family. Sinapine enhances the effect of auxin on growth and it also functions as a protector of IAA (KUTÁČEK *et al.* 1976, KEFELI *et al.* 1977).

The natural level of choline esters is regulated by the ratio of their synthesis and hydrolysis. In plants the synthesis of acetylcholine was demonstrated merely in nettle, while in other plants the activity of choline

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acetyltransferase (E.C. 2.3. 1.6) known in animals, could not be demonstrated (BARLOW and DIXON 1973, FLUCK and JAFFE 1974a). Recently we found that pea possesses enzymatic activity of choline acetyltransferase, synthetizing both acetylcholine and choline esters of hydrocinnamic acids (KUTÁČEK *et al.*, in press).

Several studies were devoted to the isolation and characterization of plant choline esterases with the hydrolytic activity of acetylcholine and sinapine (for example RIOV and JAFFE 1973, TZAGOLOFF 1973, KASTURI and VASANTHARAJAN 1976, KASTURI 1978, KUTÁČEK *et al.*, in press). Pea was the most often studied plant. The content of acetylcholine esterases in individual families is surveyed in a table by FLUCK and JAFFE (1974b). The presence of choline esterase in the plants of the *Allium* genus is not mentioned in this survey or elsewhere in the literature either.

In a comparative study of enzymatic systems, including isoenzyme patterns in some representatives of the *Allium* genus, we found indications that choline esterases and choline acetyltransferase were present in the plants of this genus as well. These results are presented in this paper.

MATERIAL AND METHODS

The seeds of *Allium altaicum* (PALL.) REYSE from the Botanical Institute of the USSR Academy of Science in Leningrad were ground and defatted (see KLOZOVÁ *et al.* 1979). The isolation of the enzyme complex containing the activities of choline esterases and of choline acetyltransferase was carried out according to RIOV and JAFFE (1973). The powder (2 g) was extracted with 20 ml of a 10 mM K-phosphate buffer of pH 7.2 under stirring at +4 °C for 60 min. The suspension was centrifuged at 20 000 *g* and +4 °C to give a supernatant called "phosphate fraction" and a sediment which was extracted at +4 °C with a solution of 4 % ammonium sulfate in 10 mM K-phosphate buffer for 60 min. The suspension was centrifuged under the above conditions, the sediment was discarded and the supernatant saturated to 80% final concentration with solid ammonium sulfate. After 60 min standing the precipitate was centrifuged at 20 000 *g* and +4 °C, the sediment was dissolved in 20 ml of 10 mM K-phosphate buffer of pH 7.0, the solution was centrifuged as above and the supernatant dialyzed at +4 °C for 30 h using the same buffer as above, which was exchanged several times. After dialysis the content of the dialysis tube was centrifuged at 20 000 *g* and +4 °C. The supernatant indicated as "sulphate fraction" was used for further determination.

The activity of acetylcholine esterase was determined according to ELLMAN *et al.* (1961) using acetylthiocholine iodide or butyrythiocholine chloride as substrate. The incubation time was 20 min at 37 °C in 0.5 M phosphate buffer at pH 8.0. As a further substrate representing choline esters of hydrocinnamic acids, choline ester of sinapic acid, sinapine was used. The activity of the enzymatic hydrolysis of sinapine was tested spectrophotometrically (at 333 nm) by determining unreacted sinapine after its isolation by means of TLC (KUTÁČEK *et al.*, in press). The incubation time was 30 min at 31 °C in phosphate buffer of pH 7.5. The reaction mixture was 10 mM in K-phosphate buffer of pH 7.5, 1.5 mM in sinapine, 60 mM in choline chloride, 1 μM in acetylcoenzyme A, 0.33 mM in cytidine triphosphate. The incubation time was 60 min at 31 °C. For the following of choline acetyl-

TABLE 1

Comparison of the activities and the fraction of the soluble and cell-membrane bound enzymes of the metabolism of choline esters from the seeds of *Allium altaicum* (PALL.) REYSE

Extraction medium	Choline esterases				Choline acetyltransferase	
	Hydrolysis of acetylcholine [μmol $min^{-1}g^{-1}$]	[%]	Hydrolysis of sinapine [μmol $min^{-1}g^{-1}$]	[%]	Synthesis of sinapine [μmol $min^{-1}g^{-1}$]	[%]
10mM K-phosphate	150.0	69.8	93.2	41.7	65.1	51.2
10mM K-phosphate + 4% $(NH_4)_2SO_4$	65.0	30.2	130.0	58.3	62.2	48.8

The results of the enzymatic activity were calculated per 1 g of the weight of the defatted powder.

transferase activity the molar ratio of substrates is of prime importance (sinapic acid: choline should be 1 : 40).

The isoenzymes of choline esterases were separated by electrophoresis in polyacrylamide gel according to MAYNARD (1966). In some instances the isoenzymes were separated according to the method of DAVIS (1964). They permit the comparison of choline esterases with unspecific esterases and proteins. Choline esterases were detected according to KARNOVSKY and ROOTS (1964), using the modification by LOJDA *et al.* (1976), with the difference that the acetate buffer in the incubation medium was substituted by a 0.2 M tris-maleate buffer of pH 6.0. The gels were washed three times with this buffer before incubation. The isoenzymes of unspecific esterases were detected with Fast Blue RR according to SAHULKA and BENEŠ (1969) and with α-naphthylacetate as substrate. The protein sets on the gels were dyed with 0.2% Coomassie Brilliant Blue R-250.

For testing the specificity of enzymatic reactions the following inhibitors were used: physostigmine salicylate 10^{-4} M, neostigmine methylsulphate 10^{-4} M and anticholine esterase BW 284 C51 $4 . 10^{-4}$ M[1,5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide]. For the determination of the percentage of inhibition in *in vitro* experiments the enzyme preparation was preincubated with the inhibitor for 30 min and then incubated with the substrate. For the determination of the specificity of the isoenzymes of the esterases on gels a proper contact of the inhibitor with the enzyme had to be ensured. The enzyme preparation was first preincubated with the inhibitor for 60 min before the beginning of electrophoresis. After electrophoresis the gel was again preincubated for 30 min in a buffer with the inhibitor and the reaction proper took place over a further 30 min in the detection mixture containing both the substrate and the inhibitor. In view of the interaction of the inhibitors with the incubation medium according to KARNOVSKY and ROOTS (1964) the experiments with the inhibition of isoenzymes on gels were carried out only in the case of the detection of unspecific esterases.

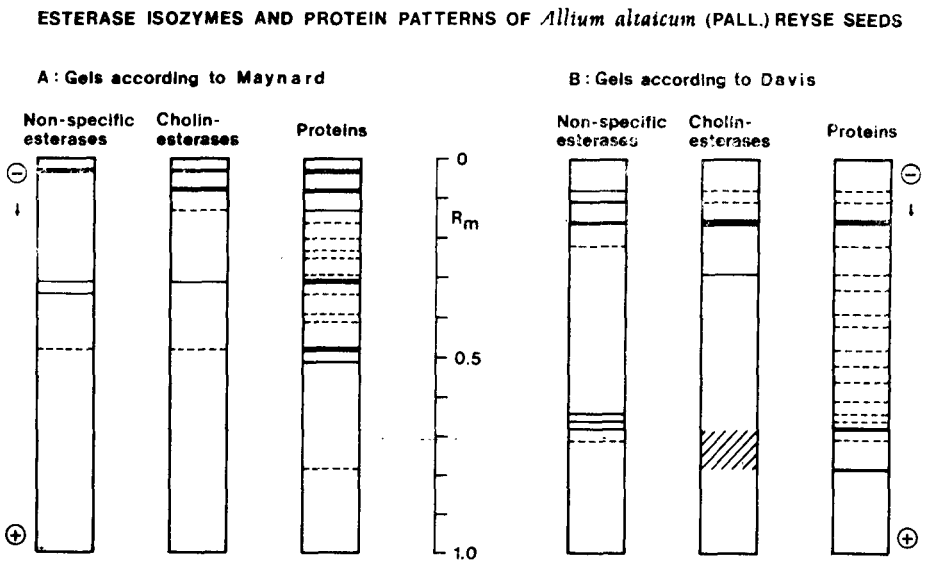

Fig. 1. Zymograms of non-specific esterases, choline esterases and protein patterns of *Allium altaicum* seeds.

RESULTS AND DISCUSSION

The presence of an active enzymatic system for the conversion of choline esters in the seeds of *Allium altaicum* is supported by the following findings:

1. High activity during the *in vitro* hydrolysis of both acetylthiocholine and sinapine with the enzyme preparation (see Table 1). Butyrylthiocholine was also cleaved with an activity of about 20% in comparison with acetylthiocholine.
2. Active synthesis of sinapine from sinapic acid and choline, catalysed with the enzyme preparation (see Table 1).
3. Inhibition of hydrolysis of acetylthiocholine and sinapine with the inhibitors of choline esterases, physostigmine and neostigmine (Table 2).
4. Positive detection of isoenzymes of choline esterases on gels and the inhibition of isoenzymes of esterases with corresponding Rm values by specific inhibitors of choline esterases (Figs. 1, 2).

The enzyme preparation prepared from the seeds of *Allium altaicum* catalyses both the synthesis and the hydrolysis of choline esters. Both these functions of the enzyme were found both in the soluble fraction (phosphate fraction) and to a considerable extent also in the fraction bound to the cell walls, which are set free only when buffers with a high ionic strength are applied (sulphate fraction) (Table 1). The difference in the proportions of individual activities in the soluble fraction and in the fraction bound to the cell walls also deserves attention (Table 1). These differences in extractibility and inhibition indicate that the individual processes under study are catalysed with specialized enzymes.

The synthesis of sinapine is catalysed by choline acetyltransferase which is clearly distinguished by the inactivity of physostigmine and neostigmine.

TABLE 2

Comparison of the effect of the inhibitors on the activity of the enzymes of the metabolism of choline esters in the seeds of *Allium altaicum* (PALL.) REYSE

Inhibitor/conc.	Choline esterases		Choline acetyltransferase
	Hydrolysis of acetylcholine	Hydrolysis of sinapine	Synthesis of sinapine
	% of inhibition		
Physostigmine conc. 10^{-4} M	62.5	76.7	7.4
Neostigmine conc. 10^{-4} M	84.4	90.0	-
Anticholine esterase conc. $4 \cdot 10^{-4}$ M	0	13.4	-

Acetylcholine and acetylthiocholine are hydrolysed by acetylcholine esterase. Since the proof of acetylcholine esterase consists in addition to the cleavage of these substrates also of the inhibition by a specific inhibitor, such as anticholine esterases (PEARSE 1972) — which was not clearly demonstrated in our case — we are so far speaking of choline esterase. Choline esterase was studied with respect to its isoenzyme composition when separated on polyacrylamide gels according to MAYNARD (1966) and after detection according to KARNOVSKY and ROOTS (1964). The preparation extracted from the seeds contains 5 isoenzymes of choline esterases with $R_m = 0.03$, 0.08, 0.13, 0.31 and 0.48 (see Fig. 1a) of which the isoenzymes with $R_m = 0.08$ and 0.13 are absent in unspecific esterases. In the gels according to DAVIS (1964) of eight esterases detected in a purified preparation of *A. altaicum* four possess choline esterase activity, *i.e.* the isoenzymes with $R_m = 0.08$, 0.11, 0.16 and 0.68. The zone with $R_m = 0.29$, detectable according to KARNOVSKY and ROOTS was absent in unspecific esterases, but it corresponds to one of the protein fractions. The zone with $R_m = 0.68$, detected according to KARNOVSKY and ROOTS (1964) is not inhibited by neostigmine (Fig. 1b).

On detection according to KARNOVSKY and ROOTS (1964) the reaction with acetylthiocholine takes place about 10 times more slowly than on detection of unspecific esterases. The reaction with butyrylthiocholine is also positive but much weaker than with acetylthiocholine, *i.e.* about 20 %.

The differentiation of choline esterases or acetylcholine esterases from unspecific esterases on gels by means of inhibitors is represented in Fig. 2. Neostigmine, the inhibitor of choline esterases, inhibits isoenzymes with low mobility of $R_m = 0.08$, 0.11 and 0.16 at a 10^{-4} M concentration. It does not inhibit more mobile isoenzymes of unspecific esterases. So far it can only be determined with difficulty whether there exist some relative quantitative differences in the inhibition of the activity of individual isoenzymes. When anticholine esterase was used, which is a specific inhibitor of the human and animal acetylcholine esterase (PEARSE 1972), a certain weakening of the activity of the same isoenzymes was observed as in the case of neo-

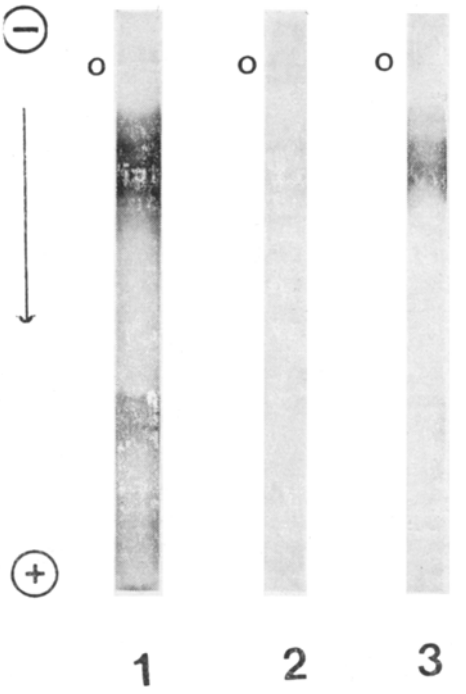

Fig. 2. Influence of the choline esterase inhibitors neostigmine and BW 284 C51 on non-specific esterase zymograms of *Allium altaicum* seeds.
(From left: 1 — control, 2 — neostigmine 10^{-4} M, 3 — BW 284 C51), o — origin.

stigmine, but the inhibition with anticholine esterase was less pronounced than in the case of neostigmine. When both the findings concerning anticholine esterase, obtained by studying the enzyme *in vitro*, and the relatively longer time of incubation of the enzyme with the inhibitor in gels are taken into account, the conclusion may be drawn that the observed weakening of the activity with anticholine esterase is not specific and that probably a partial denaturation of the enzyme is involved. The prolongation of the time of incubation of the enzyme separated in the gel with the inhibitor was necessary in order to enable the contact of both substances. The addition of the inhibitor into the gel, as described by PAUL and FOTTRELL (1961) was not successful. However, using the application method described by us it must be checked whether the added inhibitor does not change the separation, *i.e.* the R_p of isoenzymes, to a certain extent. In our experiments visible changes in mobility could not be observed. The isoenzymes of acetylcholine esterase of plant origin in pea are discussed by KASTURI and VASANTHARAJAN (1976). They found two close isoenzymes which could be inhibited by physostigmine.

As regards compartmentation of the enzyme in the cell, no difference was observed between the sets of isoenzymes from the total extract and the enzymatic preparation obtained by elution of the enzyme from the cell walls with a buffer of higher ionic strength. In parallel comparison of the isoenzymes of unspecific esterases two weak, most mobile, isoenzymes of $R_m = 0.78$ and 0.82 disappeared from the preparation.

When summarizing the results obtained by the study of the enzymes producing the conversions of the choline esters of the type of acetylcholine

and sinapine in the seeds of *Allium altaicum*, we may say that the seeds of these plants contain both choline esterases and choline acetyltransferase. Choline esterases from these plants, although actively splitting acetylthiocholine, do not react distinctly with the inhibitor anticholine esterase, which characterizes the "true", *i.e.* narrowly specific acetylcholine esterase of animal and human origin. By studying the isoenzyme composition of the enzyme using the KARNOVSKY and ROOTS detection (1964) it was found that it contained 5 isoenzymes of $R_m = 0.08$, 0.11, 0.16, 0.29 and 0.68. The isoenzyme of $R_m = 0.68$, which has a similar position as the isoenzyme of one of the unspecific esterases and which cleaves acetylthiocholine, is not inhibited by neostigmine. The enzymes of the conversion of choline esters are also present in further species of the *Allium* genus, as demonstrated by preliminary experiments with the seeds of *Allium cepa* and *Allium porrum*. These findings indicate that choline esterase is also more widely distributed in monocotyledonous plants than assumed so far.

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BOOK REVIEW

HANKS, R. J., ASHCROFT, G. L.: APPLIED SOIL PHYSICS. SOIL WATER AND TEMPERATURE APPLICATIONS. Advanced Series in Agricultural Sciences 8. — Springer-Verlag, Berlin—Heidelberg—New York 1980. Pp. 159. DM 39.50, US $ 22.20.

At the present time the Advanced Series of Agricultural Sciences is well known to every reader and this book devoted to soil moisture and temperature as the factors affecting crop production is the eighth volume of it.

It is divided into five parts. The first part deals with the soil water as a component of hydrological cycle. The main attention is paid to measurement and calculation of the soil water content. The second part is devoted to the soil water potential, the pressure potential, the solute potential and the matric potential, and to the relationships between these potentials and the soil water content. Also in this section particular attention is given to the methods. Practical utilization of them in scheduling irrigation is taken into account, too. The steady-state and transient-state water flow, the horizontal and vertical infiltrations are the main items considered in part three. In addition, evaporation and the movement of water vapour in soils are also discussed. The fourth part "Soil-Plant-Atmosphere Relations" deals mainly with the evapotranspiration, methods of its estimation, relation of evapotransporation to soil moisture and effect of it on the plant growth and production. The steady-state and nonsteady-state heat flow, the soil temperature and factors influencing it are discussed in the fifth part.

The authors, university professors of soil physics, bring a wealth of thirty years' knowledge gained in research and teaching and this book is an excellent example of a text-book. It is characterized by a modern approach to the problems to be solved, presented in an easy, understandable form. The other advantages are: numerous figures, tables and practical examples. It is only a pity that SI units are not used consistently.

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