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Cholinesterase Activity in Some Species of the *Allium* Genus

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Abstract. In partly purified protein complexes obtained from 22 species of the *Allium* genus and 6 cultivars of *Allium cepa* the activity of cholinesterases was detected and measured using the method of Ellman et al. The degree of its inhibition with 10^{-4} M neostigmine was also tested. It was found that the activity of cholinesterase differed in individual species up to two hundred times, while the differences in the inhibitory activity of 10^{-4} M neostigmine occurred only in a few cases. Individual sections and cultivars could not be characterized on the basis of the differences in the activities of the cholinesterases. Of all the sections that of *Phyllodolon* shows the highest average activity. In the case of the tested cultivars distinctly the lowest activity was observed in cv. Kaštická. The values of the enzymatic activity measured by Ellman's method in this plant material include the activity of specific and unspecific cholinesterases and the part uninhibitable by neostigmine.

Similarly as in animals, enzymes were also found in plants, involved in the metabolism of acetylcholine (FLUCK and JAFFÉ 1974a). The isolation and the characterization of the enzyme cleaving acetylcholine and its structural analogues was carried out from the following species of *Fabaceae: Phaseolus vulgaris* (MANSFIELD *et al.* 1978, ERNST and HARTMANN 1980), *Phaseolus aureus* (RIOV and JAFFÉ 1973a, b), *Pisum sativum* (KASTURI and VASANTHARAJAN 1976) and *Cicer arietinum* (GUPTA and MAHESHWARI 1980). FLUCK and JAFFÉ (1974b) show that cholinesterase is distributed in many other families of dicotyledons. In repeated experiments aiming at cholinesterase activity determination in monocotyledons its occurrence could not be confirmed in the species investigated (FLUCK and JAFFÉ 1974b), with the exception of etiolated corn (maize) leaves.

In the preceding paper (HADAČOVÁ *et al.* 1981a) high cholinesterase activity was demonstrated in the seeds of *Allium altaicum*. The isolated and partly purified enzyme complex from these seeds cleaves acetylcholine iodide actively, but butyryl thiocholine chloride only to 20%, in comparison with acetylthiocholine iodide. It was further inhibited by the inhibitor physo-

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stigmine to 62% of its original activity at a 10^{-4} M concentration, and — at the same concentration — by the inhibitor neostigmine to 85% of the original enzyme activity. An inhibition with the specific cholinesterase inhibitor BW 284 C51 [1,5-bis-4-allyl-dimethylammoniumphenyl-pentan-3-one dibromide] could not be demonstrated. The enzyme complex consists of several molecular forms.

The aim of this study was to find whether cholinesterase can also be determined in the seeds of other accessible species of the genus *Allium* and whether a relationship between the activity and the taxons can be demonstrated. For example, in the set of the isoenzymes of unspecific esterases a relationship was observed at the level of cultivars, species and sections (HADAČOVÁ *et al.* 1981b).

MATERIAL AND METHODS

The following species were investigated

Section *Cepa* (MILLER) PROKHANOV:

Allium cepa L., cv. Alice, cv. Karmen, cv. Kaštická, cv. Moravanka, cv. Obrovská žlutá, cv. Všetatská, from Sempra, Praha
A. pskemense FEDTSCH. — BIN, Leningrad
A. praemixtum VVED. — BIN, Leningrad

Section *Phyllodolon* SALISB.:

A. altaicum PALLAS (I.) — BIN, Leningrad
A. altaicum PALLAS (II.) — Botanical Garden Novosibirsk
A. fistulosum L. — BIN, Leningrad

Section *Rhizirideum* G. DON ex KOCH:

A. schoenoprasum L. (I.) — BIN, Leningrad
A. schoenoprasum L. (II.) — Sempra, Praha
A. nutans L. (I.) — BIN, Leningrad
A. nutans L. (II.) — Botanical Garden Tbilisi
A. saxatile BIEB. — Botanical Garden Tbilisi
A. obliquum L. — BIN, Leningrad
A. angulosum L. — BIN, Leningrad
A. montanum SCHMIDT — Praha-Zlíchov — (limestone)
A. montanum SCHMIDT — Praha-Šárka — (lydite)
A. montanum SCHMIDT — Botanical Garden Lublin
A. senescens L. — BIN, Leningrad

Section *Melanocrommyum* WEBB et BERTH.:

A. aflatunense FEDTSCH. — BIN, Leningrad
A. stipitatum REGEL — Botanical Garden Dushanbe

Section *Acanthoprason* WENDELBO:

A. cristophii TRAUTV. — Botanical Garden Dushanbe

Section *Allium:*

A. porrum L. cv. Elefant — Sempra, Praha
A. porrum L. cv. Carrentan — Res. Breeding Inst. Vegetables, Olomouc
A. jajlae VVED. — BIN, Yalta, Crimea

A. rotundum L. (I.) — Nikitsk Botanical Garden, Yalta, Crimea
A. rotundum L. (II.) — BIN, Leningrad

Section *Butomissa* (SALISB.) KAMELIN:

A. ramosum L. (I.) — Central Inst. Genet. Cult. Plants Res. Acad. Sci. GDR, Gatersleben
A. ramosum L. (II.) — Botanical Garden Novosibirsk

The method of grinding and defatting the seeds is described in the paper by KLOZOVÁ *et al.* (1979). The extraction and the purification of the protein complex containing cholinesterase was carried out according to FLUCK and JAFFÉ (1974b), *i.e.* by repeated extraction with 4% ammonium sulphate in 0.01 M K-phosphate buffer. The cholinesterase activity was determined according to ELLMAN *et al.* (1961) using acetylthiocholine iodide as substrate and 25 min incubation time at 37 °C, in a 0.1 M K-phosphate buffer of pH = = 8.0. The reaction was terminated by cooling the sample to 0 °C. Change of absorbance was measured at 412 nm. The activity was expressed in nmol of the degraded substrate per mg of delipidated flour, or mg of proteins determined according to BRADFORD (1976). Parallel experiments were carried out in which the enzyme was preincubated with neostigmine (10^{-4}) at 26 °C for 30 min, before the enzyme activity determination. The results obtained were evaluated by applying variance analysis and the critical value of the difference of mean values in 4 measurements was calculated for a 5% level of significance.

RESULTS AND DISCUSSION

The determination of acetylcholinesterase according to Ellman is based on the reaction of the thiol groups with 5,5-dithio-bis(2-nitrobenzoate) (DTNB) under formation of the yelow 5-thio-2-nitrobenzoic acid (TNB). The species of *Allium* genus contain a lot of sulphur containing compounds (for example allicinase — GORYACHENKO (1952), alliinase — SELBY *et al.*, (1979)), which could react with DTNB. The possibility of interaction of these compounds under the conditions of Ellman's reaction is decreased by the procedure in which a protein complex was prepared from the seed flour, which was submitted to dialysis, and further the enzyme and the substrate control was subtracted. The activity in a sample after previous incubation with neostigmine — a cholinesterase inhibitor (see Fig. 1) was evaluated simultaneously. The evaluation of the methodical error and the reproducibility of the results is shown in Table 1 from which it is evident that the reproducibility is good.

In all the *Allium* species investigated a positive Ellman reaction was observed, and only exceptionally the 10^{-4} M neostigmine did not record the activity expressed by the Ellman reaction (for example see *A. ramosum*, *A. pskemense*). They were cases when the activity was very low. The average inhibition with 10^{-4} M neostigmine under the reaction conditions used is 80%. We consider that the values obtained by Ellman's method might include a certain part which is not the result of the activity of cholinesterases.

If only the value of the enzymatic activity determined on the basis of the Ellman reaction is taken into consideration, without regard to inhibition, the differences between the species are up to two-hundred-fold. In most

instances a high percentage of inhibition corresponds to the high values of the activity determined in this manner (*cf.* for example *A. altaicum*, *A. obliquum*, *A. fistulosum*, *A. rotundum*). It is also interesting that different cholinesterase activity is displayed by the seeds of the same species but of different origin (see for example *A. ramosum*, *A. rotundum* and *A. altaicum*). However, according to statistical evaluation the differences between localities are lower than those between species.

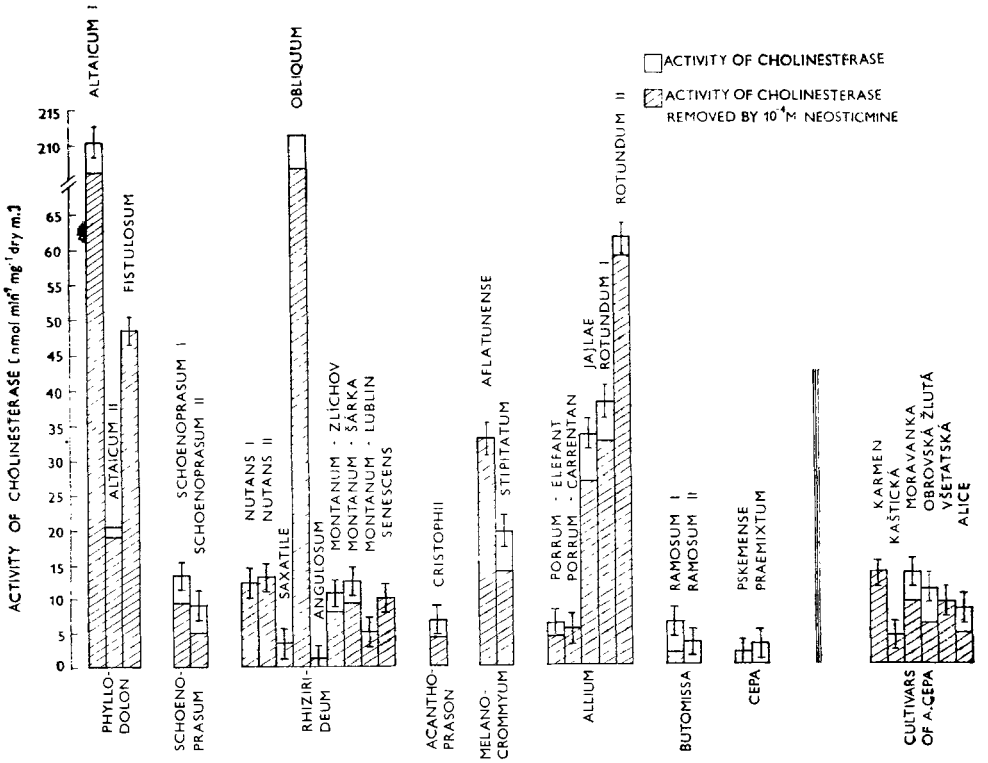

Fig. 1. Cholinesterase activity and the inhibitory effect of 10^{-4} M neostigmine in some representatives of the *Allium* genus and the cultivars of *A. cepa* L., expressed in nmol min^{-1} mg^{-1} of delipidated flour of the seeds, with the reliability interval at a 5% significance level.

$(t_{[3]}\, c = 0.05 \times s_{\bar{x}} = 3.18 \times 0.704 = 2.24)$

Critical value of the difference of the averages of the samples in 4 measurements for $\alpha = 0.05d = t_{[3]}$

$$\alpha = 0.05 \times s_{\bar{x}} \times \sqrt{2} = 3.18 \times 0.704 \times \sqrt{2} = 3.17\,.$$

As regards the relationship between the cholinesterase activity determined on the basis of the Ellman reaction and the relatedness on the level of individual sections, the statistical evaluation has shown that there are no significant differences between individual sections. High values were found in the section *Phyllodolon* and *Allium*, very different activity values for individual species were observed in the section *Rhizirideum* (1.26 to 211.15 nmol min^{-1} mg^{-1} delipidated flour). The high activity value in *A. obliquum* (211.15 nmol min^{-1} mg d.fl.) distorts the average activity value of the

TABLE 1
Overall evaluation of cholinesterase activity in the species of the *Allium* genus and cv. of *A. cepa* investigated, using the variance analysis

Source of changeability	SS	df	MS	F
Samples	244709.94	31	7893.87	3977.96
Within samples (residual)	182.56	92	1.98	s = 1.41
Total	244892.51	123		

$s_{\bar{x}} = 0.7043$; $n = 4$; F 0.05 30/80 = 1.60

section *Rhizirideum*. The statistical evaluation of the differences between individual sections is also distorted to a certain degree by the uneven number of species represented in individual sections, which is due to the accessibility of the material. In the subgenus of *Rhizirideum*, the sections *Phyllodolon* and *Cepa*, which were combined earlier, are very different in their average activity values of cholinesterases (the average value of the activity in the section *Phyllodolon* is 107.7 nmol min^{-1} mg^{-1} d.fl., in the section *Cepa* it is 8.58 nmol min^{-1} mg^{-1} d.fl.).

Among the cultivars of *Allium cepa* (Figs. 1, 2) a relatively low significance of the differences in cholinesterase activity was observed, with the exception of the cv. Kaštická, which is characterized by its low activity. It is interesting that a similar observation was also made in the case of the isoenzymes of unspecific esterases (HADAČOVÁ *et al.* 1981b) and the isoenzymes of malate dehydrogenase (not yet published).

The enzyme complex studied in the *Allium* genus, hydrolysing acetylcholine and — to a small extent — also butyrylcholine, is called by us cholinesterase and not acetylcholinesterase because we were unable to demonstrate an unambiguous inhibition by the specific inhibitor of the animal acetylcholinesterase BW 284 C51 (HADAČOVÁ *et al.*, 1981a), which represents according to PEARSE (1972) in addition to the hydrolysis rate of the mentioned substrates, the main criterion for the classification of the animal enzyme as acetylcholinesterase. To what extent this standpoint can be applied for the plant enzyme is not quite clear so far. ERNST and HARTMANN (1980) point out the increased inhibitory effect of neostigmine in comparison with physostigmine when tested on the enzyme from the beans, hydrolysing acetylcholine. This is just the opposite to what was observed in enzymes of animal origin. This was also confirmed in the case of the enzyme complex from *Allium altaicum* (HADAČOVÁ *et al.* 1981a). During the electrophoretic separation of cholinesterase from this species we also proved the presence of several isoenzymes with the character of acetylcholinesterase.

On the basis of these results we assume that the values of the enzyme activities measured by the Ellman method include in our material several types of cholinesterase activity (to a smaller extent the specific acetylcholinesterase, unspecific cholinesterase activity, which can differ by its sensitivity to various inhibitors). The sulphydryl groups of various substances present in the isolated protein complex may interfere in the determination especially in the case of some proteins (see for example RIDDLES

et al. 1979, VERHEUL *et al.* 1982). On the other hand, in some instances substances could be detected in the extract which decolorize the yellow TNB anion, as observed by FLUCK and JAFFÉ (1974b).

Our results cannot be confronted yet with the findings of other authors, because with the exception of the study by FLUCK and JAFFÉ (1974b) who carried out a screening of representatives of various families on their cholin-

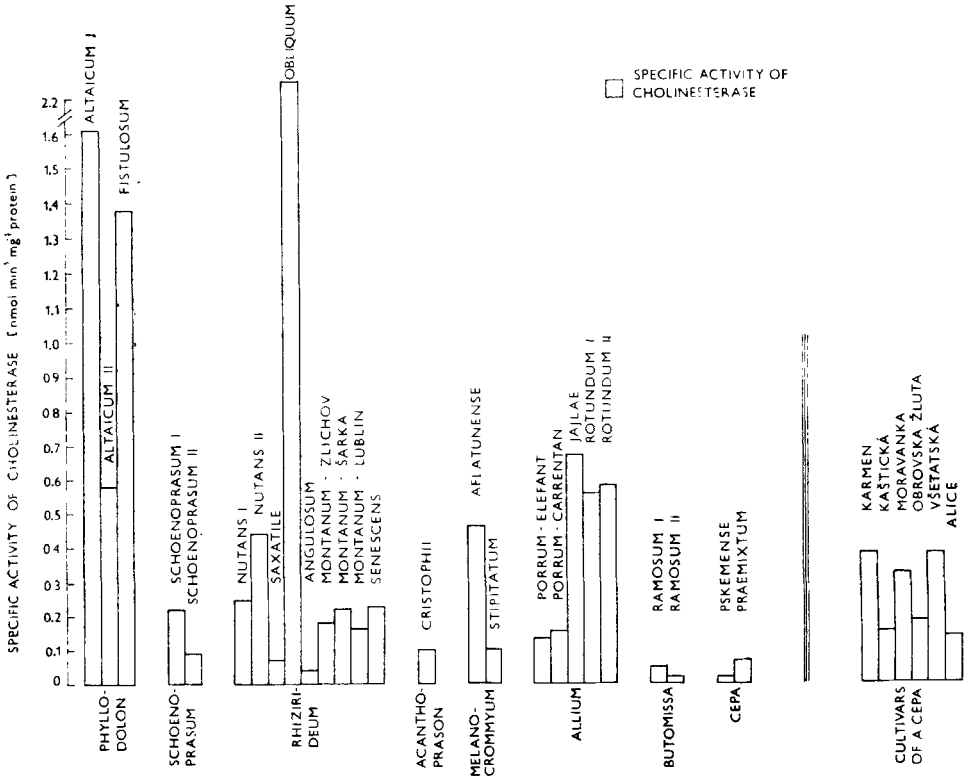

Fig. 2. Cholinesterase activity in some representatives of the *Allium* genus and cultivars of *A. cepa*, expressed in nmol min^{-1} of proteins.

esterase activity, this enzyme has not yet been studied from the chemotaxonomic point of view in plants. The authors mentioned found activity in the majority of the representatives of *Leguminosae*, exceptionally in *Solanaceae* and *Brassicaceae*, and among monocotyledons only in etiolated maize leaves. The screening of various species of a single genus has not yet been carried out. From the chemotaxonomical point of view this enzyme was investigated by Voss (1981) in insects. He found either identical or very similar values of the inhibition pattern in closely related species, while the species of various orders differed considerably. The author demonstrated the possibility of using this enzyme at the level of higher taxonomic units, such as the order, in the selection and the application of selective insecticides.

When summarizing all the facts observed from the chemotaxonomic point of view, it is evident that in all the species of the *Allium* genus investigated

a cholinesterase activity was found, and that the activity differences between the species are up to two-hundred-fold. The differences in the activities of the samples of the same species from various localities are lower than those between the species. A great variation of values is also evident among species of the same section, which is most pronounced in the section *Rhizirideum*. An unambiguous significance of the differences in the activities at the level of sections could not be demonstrated. Relatively low values occur in cultivated species. Among the cultivars the cv. Kaštická is exceptional in its low value. In the genus *Allium* cholinesterase activity in not a suitable marker at the level of sections, but it is such at the level of species.

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