Sinapine Esterase
I. Characterization of Sinapine Esterase from Cotyledons of *Raphanus sativus*

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From cotyledons of *Raphanus sativus* (red radish) an esterase activity which catalyzes the hydrolysis of sinapine into sinapic acid and choline has been isolated. The enzyme, which has a near absolute specificity, is not analogous with any esterase described in the literature. The reaction has a pH optimum of 8.5 and the apparent K_m is 1.95×10^{-5} M. The enzyme is relatively insensitive to both physostigmine (eserine) ($K_i = 1.73 \times 10^{-4}$ M) and neostigmine ($K_i = 2.13 \times 10^{-4}$ M). Diisopropyl fluorophosphate (DFP) showed no inhibition and diethyl *p*-nitrophenylphosphate (E 600) only a slight inhibitory effect at 10^{-5} M, respectively. Choline (10^{-2} M) was inhibitory but acetylcholine (10^{-2} M) stimulated the enzyme activity.

Introduction

Sinapine occurs in seeds of most Brassicaceae [1]. Degradation of sinapine during germination has been demonstrated to occur in *Sinapis alba* [2, 3], *Brassica oleraceae*, *Lepidium sativum*, and among the plants investigated most rapidly in *Raphanus sativus* [4].

In respect to the occurrence of multiple forms of esterases [5–7] and the apparent difficulty of functional interpretations of these enzymatic activities [7–9] the interesting question arises, whether degradation of sinapine in Brassicaceae is catalyzed by general esterase activities, *e. g.* common cholinesterases, or by an esterase specific for the hydrolysis of sinapine.

In seedlings of *Sinapis alba* Tzagoloff [10] found a sinapine esterase activity which was not analogous with any esterase described in literature, however, his attempts to absolutely exclude unspecificity of the reaction were unsuccessful. The reaction exhibited a strong competitive feature with acetylcholine and the "physostigmine insensitivity" criterion used to exclude that the enzyme belongs to the general class of plant cholinesterases is equivocal [11].

In this paper we report some properties of a sinapine esterase from cotyledons of *Raphanus sativus*, which differs in many aspects from the sinapine esterase in *Sinapis alba*, and we conclude that the *Raphanus* enzyme possesses a near absolute specificity for the degradation of sinapine.

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Materials and Methods

Plant material and culture conditions

Seeds of *Raphanus sativus* L. *sativus*, cv. Saxa were purchased from Zwaan u. Co's u. Komp., Delfter Marktgärtner-Samenzucht GmbH, Netherlands. Seedlings were grown in a phytotron in petri dishes under fluorescent light (ca. 7,000 lux) at 22 °C.

Thin-layer chromatography

Hydroxycinnamic acids were chromatographed on microcrystalline cellulose (AVICEL) in TAW, toluene-gl. acetic acid-water (2:1, water saturated) and were detected under UV with and without NH_3-vapor and in the daylight with diazotized *p*-nitroaniline oversprayed with 2 N NaOH [12].

High-performance liquid chromatography

The chromatographic system used was obtained from Spectra-Physics (Santa Clara, Cal., USA) and is described elsewhere [13]. The chromatographic columns (4×250 and 3×250 mm) were prepacked with LiChrosorb RP-8 (Merck, Darmstadt, West Germany) and Spherisorb Silica (Spectra-Physics,

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Darmstadt, West Germany) respectively, both with a particle size of 5 μm.

Sinapine was chromatographed on Spherisorb Silica with dicloroethane, methanol, H_2SO_4 (85:15:1) and sinapic acid on LiChrosorb RP-8 with methanol, water, gl. acetic acid (35:60:5). Flow-rate was 1 ml/min, detection was at 312 nm, and the sample size was 10 μl.

Substrates and inhibitors

Sinapine and other sinapic acid esters were isolated from seeds and seedlings of *Raphanus sativus* [14, 15].

Acetylcholine chloride, neostigmine bromide and *p*-chloromercuribenzoic acid (PCMB) were purchased from Sigma Chemie, Taufkirchen, diisopropyl fluorophosphate (DFP) and choline chloride from Fluka, Neu-Ulm, phenyl acetic acid and physostigmine (eserine) from Merck, Darmstadt, and diethyl *p*-nitrophenylphosphate (E 600) was purchased from Serva, Heidelberg, West Germany.

Buffer solutions

Dependence of enzyme activity on pH was studied with a mixed buffer system of the following equimolar (20 mM) composition: 2(N-morpholino)ethanesulfonic acid (MES, pKa 6.1), 3-(N-morpholino)propanesulfonic acid (MOPS, pKa 7.2), N-tris(hydroxymethyl)methylglycine (tricine, pKa 8.2), glycine (pKa 9.9). All other enzyme assays contained tricine buffer (100 mM, pH 8.5).

None of the components of the mixed buffer system showed any inhibitory effect.

With the reaction temperature of 30 °C at pH 8.5 ca. 10% autocatalyzed hydrolysis of sinapine had to be taken into account.

Enzyme isolation

Seeds and seedlings (age between 48 and 60 h) were treated with an Ultra Turrax momogenizer for 2 min in water free, cold acetone (−20 °C) and the homogenate was centrifuged at 3,000 × *g* for 15 min. The pellet was washed 3 times with cold acetone, 2 times with cold ether, and then dried in a desiccator *in vacuo*.

The powder was suspended in a known volumn of tricine buffer (100 mM, pH 8.5). The suspension was centrifuged at 3,000 × *g* for 15 min and the supernatant was brought to 30% saturation by the addition of solid $(NH_4)_2SO_4$ and centrifuged at 50,000 × *g* for 20 min. The supernatant was brought to 80% saturation by further addition of solid $(NH_4)_2SO_4$ and centrifuged at 50,000 × *g* for 20 min. The pellet was dissolved in a known volumn of tricine buffer and was subjected to a Sephadex G-25 centrifugation. To determine if there was any loss in activity by treatment with acetone, the enzyme was extracted by homogenizing fresh plant material in tricine buffer in an ice bath. Subsequent purification was carried out as described above.

Enzyme assay and determination of activity

0.15 ml enzyme extract (0.37 to 0.49 mg protein, determined by the biuret method) was mixed with substrate and brought to 3 ml with buffer, giving a substrate concentration of 0.1 to 0.4 mM. Enzyme activity (at 30 °C) was determined spectrophotometrically by measuring the increase in absorbance at 290 nm (PMQ II, Zeiss) according to Tzagoloff [10]. Values were corrected for non-enzymatic hydrolysis. Alternatively the enzyme activity was determined by HPLC-analyses of substrate (sinapine) decrease and product (sinapic acid) increase.

The absorption of sinapic acid at 290 nm in the reaction mixture followed the Lambert-Beer law. During the first 50 min of two enzyme assays aliquots of the reaction mixture were taken in 10 min intervals and by HPLC calibration curves the molar sinapic acid production was determined and found to be linear to the absorption kinetics of the reaction mixture at 290 nm; $E_{290\ nm} = 1$ is equivalent to 83.26 nmol of sinapic acid. From this the absorbance coefficient of sinapine was determined to be 2.085×10^4 cm²/mmol.

Results

Product identification

In addition to the product identification by HPLC-authentic sinapic acid and the peak obtained from the reaction mixture had identical capacity factors (k′) of 2.17 – the product showed TL-chromatographic identity with sinapic acid in TAW ($hR_f = 63$) and exhibited identical detection behaviour – color changes under UV in the presence of NH_3-vapor (blue to blue-green) and in the daylight after spraying with diazotized *p*-nitroaniline (red-violet) oversprayed with 2 N NaOH (blue-violet).

Enzyme properties

In comparison with enzyme extraction from fresh plant material, there was only 10% less enzyme activity when prepared as an acetone powder.

The enzyme preparation could be stored at −20 °C in tricine buffer for several weeks with no loss of activity. The production of sinapic acid in tricine buffer (100 mM, pH 8.5) was proportional to protein concentration up to 0.49 mg protein per assay (3 ml) and the reaction was linear for 50 min.

The effect of ionic strength of the tricine buffer on the reaction was examined between 10 and 200 mM. Highest reaction rate was reached at 70 mM and remained unchanged up to 200 mM. At 20 mM tricine buffer only 50% of the maximal reaction rate was observed.

The pH optimum of the enzyme activity was determined with a mixed buffer system and gave 8.5 (Fig. 1).

Effect of temperature on the initial reaction rate showed maximal hydrolysis rate at 35 °C; 50% rate was found to be at 25 and 40 °C.

The enzyme had no requirement for the metal ions Mg^{2+} or Ca^{2+}. There was no activation by sulfhydryl group reagents such as cystein or β-mercaptoethanol. PCMB was only effective at higher concentrations (18% inhibition at 10^{-3} M).

The effect of sinapine on the sinapine esterase activity was determined at 10 different concentrations ranging from 0.62 to 12.80×10^{-5} M in tricine buffer (100 mM, pH 8.5). The enzyme activity follows the typical Michaelis-Menten kinetics. With protein extracts obtained from seedlings at different germination stages K_m values were determined with Lineweaver-Burk plots and found to be around (± 15%) 1.95×10^{-5} M (Fig. 2).

Choline chloride inhibited enzyme activity: 10^{-2} M to 47% and 10^{-1} M to 60%.

Table I. Substrate specificity of the sinapine esterase activity.

Substrate (0.4 mM)	% activity
Sinapine (Sinapoylcholine)	100
Sinapoylglucose	25
Sinapoylmalate	0
Disinapoylsucrose	0
Sinapine + phenyl acetic acid (10^{-2} M)	100
Sinapine + acetylcholine (10^{-2} M)	120
Sinapine + acetylcholine (10^{-1} M)	140

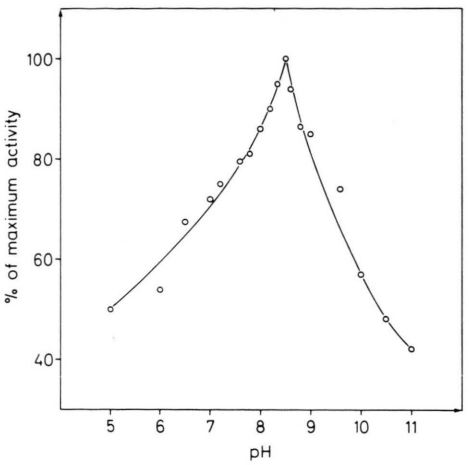

Fig. 1. Dependence of enzyme activity on pH. The buffer system includes MES, MOPS, tricine, and glycine.

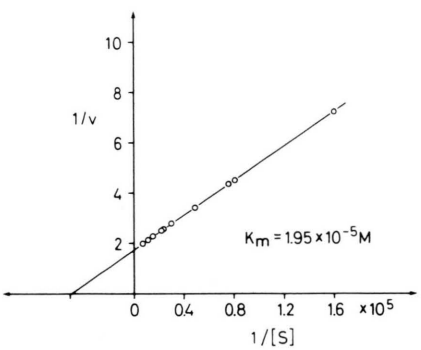

Fig. 2. Lineweaver-Burk plot of the dependence of enzyme activity (v = milliunits per seedling) on sinapine concentration (S = mol per l).

Highest enzyme activity could be extracted from 2 day-old seedlings (0.96 milliunits per pair of cotyledons). Dormant seeds exhibited only 0.03 and 3 day-old seedlings 0.28 milliunits.

Substrate specificity

The substrate specificity of the esterase activity was examined with a number of different sinapic acid esters and competitive assays with acetylcholine and phenyl acetic acid (Table I).

The enzyme exhibited high specificity towards sinapine. Phenyl acetic acid had no inhibitory effect, and acetylcholine stimulated enzyme activity.

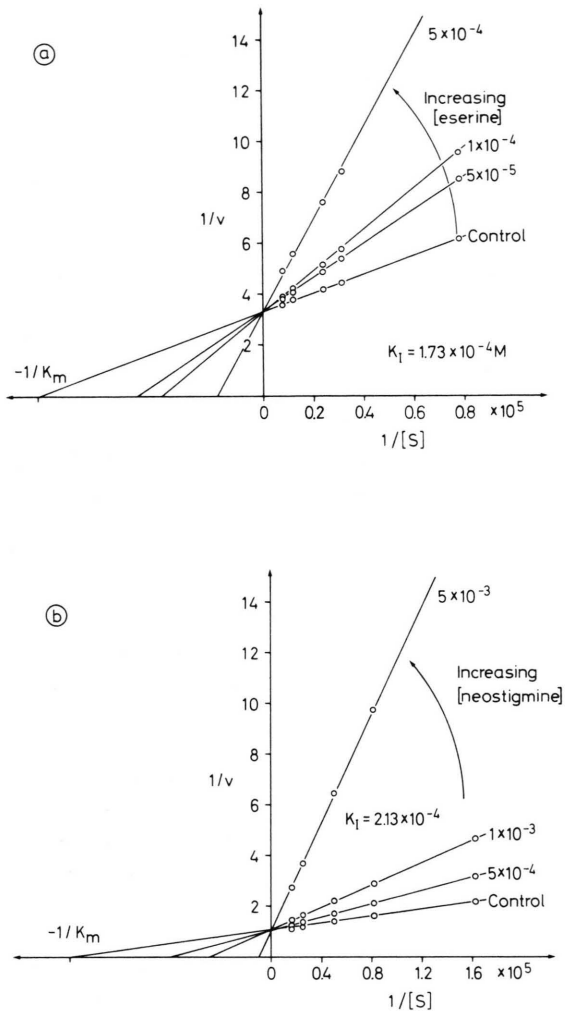

Fig. 3. Lineweaver-Burk plots of enzyme activity for the determination of K_i values (a) for eserine and (b) for neostigmine. At different inhibitor concentrations (M) the apparent K_m values were determined for the use in Dixon plots.

Enzyme localization

We examined the sinapine esterase activity in roots and cotyledons of *Raphanus* seedlings and found 91.3% of the activity in cotyledons, in the organs in which *in vivo* sinapine degradation occurs.

Effect of esterase inhibitors

To substantiate our suggestion, that sinapine hydrolysis in cotyledons of *Raphanus sativus* is catalyzed by a specific sinapine esterase, we examined the effects of the following esterase inhibitors: eserine, neostigmine, E 600, and DFP.

Eserine and the organophosphates E 600 and DFP are widely applied esterase inhibitors used to distinguish between different species of animal esterases [16, 17]. The adaption of these inhibitors to investigations on plant esterases [18] must be done with care [11]. Neostigmine was found to be a specific inhibitor for plant cholinesterases [9, 11].

All four inhibitors showed relatively weak inhibitory effects on *Raphanus sinapine* esterase activity (Table II). In order to compare our results with those found in literature [10, 11] we adjusted our assay-substrate concentration, in terms of multiples of K_m, to the range which had been used in these studies.

Mechanisms of inhibition were examined with Lineweaver-Burk plots. As to be expected, the organophosphates exhibited noncompetitive inhibitions; only inhibition with E 600 (10^{-4} M) was dependent on the time of preincubation with the enzyme: 20 min gave 30%, 50 min 50%, and 120 min 75% inhibition. The Lineweaver-Burk plots indicated that E 600 acted as a pure noncompetitive inhibitor, however, DFP seemed to act hyperbolic noncompetitively [19].

Eserine and neostigmine inhibited the sinapine esterase activity competitively (Fig. 3a and 3b) and

Table II. Effect of inhibitors on the sinapine esterase activity in comparison with the effects of these inhibitors on serum cholinesterase [16], *Phaseolus* cholinesterase [11], and on *Sinapis* sinapine esterase [10]. Values are expressed in % of control (sinapine alone).

Enzymer	Inhibitor			
	E 600*	DFP	eserine (10^{-4} M)	neostigmine (10^{-5} M)
Serum cholinesterase	50 at 2.1×10^{-3} M	50 at 10^{-7} M	100	–
Phaseolus cholinesterase	70.7 at 10^{-6} M	–	ca. 23	100
Sinapis sinapine esterase	–	–	7.7	–
Raphanus sinapine esterase	30 at 10^{-5} M	30 at 10^{-2} M	21	0

* Preincubation for 30 min.

K_i values were determined with Dixon plots to be 1.73×10^{-4} M and 2.13×10^{-4} M, respectively.

Aliquots of inhibited enzyme reaction mixtures were also analyzed by HPLC determinations of sinapine and sinapic acid and identical results were obtained.

Discussion

Adaption of animal esterase classification [16, 17] to plant esterase activities must be done with care. Sae *et al.* [18] found inconsistency with animal nomenclature (see also ref. 20). Riov and Jaffe [11] demonstrated that for plant cholinesterase identification, the criterion "eserine sensitivity" [17] cannot be applied. Even more difficult and often unsuccessful is the attempt to reveal the function of esterase activities in plant tissues [7, 8, 9, 21].

Tzagoloff [10] described an esterase activity, which might possess a specific function in the secondary metabolism of *Sinapis alba*, that of hydrolyzing sinapine, however, acetylcholine was also a good substrate. Tzagoloff concluded that the enzyme had a specificity towards choline esters, but did not belong to the general class of cholinesterases mainly because of the relatively small inhibition (20%) attained with eserine at 10^{-3} M. However, it has been shown, that cholinesterase activity in *Phaseolus* was also poorly inhibited by eserine [11] (ca. 23% at 10^{-4} M), whereas neostigmine inhibited 100% at 10^{-5} M.

Our results, that both eserine and neostigmine showed relatively weak inhibitory effects excludes that the *Raphanus* sinapine esterase belongs to the general class of cholinesterases and is not related to the enzyme described by Fluck and Jaffe [9] to occur in roots of *Raphanus sativus*. The poor inhibition with organophosphates also supports this statement (see Table II).

Furthermore we can exclude general cholinesterase activity because Mg^{2+} and Ca^{2+} showed no stimulatory effects and choline chloride inhibited enzyme activity, which is in agreement with the *Sinapis* esterase [10]. Activation of cholinesterases by divalent metal ions was reported by Massart and Dufait [22]. *Phaseolus* colinesterase exhibited a strong activation by choline at pH 8 [11].

In comparison with the *Sinapis* sinapine esterase we found a marked difference in the enzyme localization. Tzagoloff [2] found only 43% in cotyledons of *Sinapis* – where the substrate sinapine is located –, and we found 91% of the sinapine esterase activity in cotyledons of *Raphanus*. Furthermore the *Raphanus* enzyme exhibits a much higher substrate specificity (see Table I). Acetylcholine, which markedly slowed the rate of sinapine hydrolysis by the *Sinapis* enzyme, had no such effect on the *Raphanus* sinapine esterase activity; it even exhibited a stimulation. The pH optima of both enzymes are also markedly different; the *Sinapis* enzyme is near 10, whereas the *Raphanus* activity exhibits an optimum of 8.5. Other discrepancies are the *Sinapis* enzyme activation with cystein and the short time linearity of the reaction.

In conclusion we assume that the *Raphanus* sinapine esterase possesses the specific function of hydrolyzing sinapine into choline and sinapic acid during early stages of germination. This conclusion is supported by the organ localization and the fact that dormant seeds and seedlings older than 3 days exhibit low enzyme activites, whereas a high activity is found to be extractable in a germination stage where a high decline of sinapine concentration in the cotyledons occur [14].

In joining communications we will report on detailed studies on the correlation between sinapine esterase activity and the substrate sinapine and on a screening study of the enzyme activity in Brassicaceae.

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