Sinapine Esterase
II. Specificity and Change of Sinapine Esterase Activity During Germination of *Raphanus sativus*

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Following a 20 to 24 h lag-phase after sowing, the onset of both rapid degradation of sinapine (sinapolycholine) and rapid increase in sinapine esterase activity in cotyledons of *Raphanus sativus* was observed. After 2 days of germination maximal enzyme activity was reached and declined in subsequent germination stages as rapidly as it had appeared. Esterases, active against indophenyl acetate, showed highest activity in dry seeds, declining to more than 50% between the 1st and 3rd day of germination.

Starch gel electrophoresis showed that all protein extracts contained a multiplicity of esterases, active against α-naphthyl acetate. When gels were incubated with sinapine, one new band appeared, stainable with diazotized *p*-nitroaniline. This band represents sinapine esterase activity.

Tests for substrate specificity towards cinnamic acid choline esters showed highest enzyme activity with sinapine.

Studies on the occurrence of sinapine esterase in other Brassicaceae revealed that the enzyme activity coincides with the occurrence and degradation of sinapine.

Introduction

Most seeds of the Brassicaceae contain sinapine (sinapoylcholine) [1]. It was shown for *Sinapis alba* that this compound is accumulated during the main growth phase of developing embryos [2, 3], deriving from L-phenylalanine ammonia-lyase (PAL) mediated synthesis [3].

During early stages of germination of Brassicaceae seedlings, studied so far, sinapine is interconverted to sinapoylglucose [3–5]. The enzymes, involved in this pathway, were found to be sinapine esterase [6, 7] and UDP-glucose : sinapic acid glucosyltransferase [8].

It has been established that the sinapine esterase activity is not analogous with any esterase described in literature. The enzyme does not belong to the general class of plant cholinesterases [6, 7], as defined by Riov and Jaffe [9].

In this paper we report on specificity and change of sinapine esterase activity during germination of *Raphanus sativus* in comparison with general esterase activities. Some other Brassicaceae were tested for the occurrence of this enzyme activity.

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Materials and Methods

Plant material and culture conditions

Seeds of *Raphanus sativus* L. var. *sativus,* cv. Saxa were purchased from Zwaan u. Co's u. Komp., Delfter Marktgärtner-Samenzucht GmbH, Netherlands. Other seeds were harvested in the botanical garden of our institute. Culture conditions are described in ref. [5].

Chromatography

Column chromatography (CC) on polyamide is described in ref. [10]. Preparative thin layer chromatography (TLC) was carried out on microcrystalline cellulose (Avicel) (20×40 cm plates) in CAW, $CHCl_3$ – gl. acetic acid – H_2O (3:2, water saturated). Last purification was achieved on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) (90×2 cm) eluted with CH_3OH. The high performance liquid chromatograph used was Spectra-Physics (Santa Clara, Cal., USA) and is described in ref. [11]. The chromatographic column (5 µm, 250×4 mm) was prepacked with LiChrosorb Si 60 (Merck, Darmstadt). Sinapine was chromatographed with dichloromethane – methanol – H_2SO_4 (85:15:1) at 2 ml/min (t_R = 450 sec). Sinapine was extracted in 80% aq. methanol by treatment with an Ultra Turrax homogenizer. Before being applied to HPLC, extracts were taken to dryness and redissolved in methanol.

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Substrates

Sinapine was isolated from seeds of *Raphanus sativus* as described in ref. [10]. Other cinnamic acid choline esters were chemically synthesized as described below. Indophenyl acetate was purchased from Eastman Organic Chemicals and α-naphthyl acetate from Sigma Chemical Co.

Chemical synthesis of cinnamic acid choline esters

Choline esters were prepared from cinnamic acid chlorides. *p*-Coumaric, caffeic, ferulic, and sinapic acid (100 mg each), respectively, were dissolved in 4 ml pyridine and 2 ml $(CH_3CO)_2O$ were added to produce O-acetyl derivatives in order to protect phenolic hydroxyl groups from esterification. These derivatives were taken to dryness in a rotary evaporator at 60 °C. The acid chlorides were produced by dissolving cinnamic acid and the O-acetyl derivatives of hydroxycinnamic acids for 20 min in 3 ml oxalylchloride. The acid chlorides were taken to complete dryness and subsequently were reacted under stirring in darkness with choline chloride (200 mg) in 10 ml pyridine. Deacetylation of the obtained products was achieved by boiling under reflux with 0.12 N HCl in 75% aqueous dioxane. Purification of the cinnamic acid choline esters was carried out on polyamide CC, preparative TLC, and Sephadex LH-20. The yield of each product was approx 15–20 mg.

Horizontal starch gel electrophoresis

Starch gel electrophoresis was carried out by a modified method, described in ref. [12]. 12.8% starch hydrolyzed (Connaught Labs., Canada) was prepared in a 4 mM citrate – 16 mM Tris buffer, pH 7.9. The electrode buffer was 0.3 M sodium borate, pH 8.6. Separation was achieved at constant voltage (150 V) under cooling with ice within 4 h. 20 µl (30–60 µg protein) was absorbed onto paper wicks and placed into gel slices [13]. Wicks were removed after 30 min. After 4 h development the gel was sliced horizontally into 2 slices. One slice was tested for α-naphthylacetylesterase activities using α-naphthyl acetate and tetrazotized o-dianisidine [14] and the second gel was incubated for 3–4 h with tricine buffer (0.1 M, pH 8.5) containing 0.1 mM sinapine. The gel was washed with water and free sinapic acid was stained with diazotized *p*-nitroaniline [15].

Protein preparation

Isolation of enzymatic activities was done as described in ref. [7].

Enzyme assay and determination of activity

Sinapine esterase activities towards sinapine were tested as described in ref. [7]. Determinations of the activity of this enzyme towards other choline esters of cinnamic acids were carried out by photometrical measurements of the isolated hydrolyzed cinnamic acids. Incubation medium was acidified with conc. HCl and the cinnamic acids extracted into ether. The ether was removed under an air jet and the acids redissolved in methanol. Quantitative values were obtained by photometrical comparison with absorbance of standard samples. K_m and V_{max} values were determined in dublicates by Lineweaver-Burk plots. Determination of general esterase activities was performed by a modified method of Kramer and Gamson [16] as described in ref. [9] using indophenyl acetate as substrate. Increase in absorbance at 625 nm was measured in order to determine free indophenol.

Results and Discussion

Kinetics of esterase activities

During germination of *Raphanus sativus* the kinetics of two esterase activities were examined (Fig. 1). Enzymatic hydrolysis of indophenyl acetate, which represents a wide range of esterase activities [17], exhibits its highest activity in dry seeds. This activity declines to more than 50% within 48 h between the 1st and 3rd day of germination. Zymograms (Fig. 2) of these protein preparations exhibit multiplicity and variation of esterases. This high hydrolytic capacity is inactive towards sinapine. With protein preparations from dry seeds, only trace amounts of sinapine were hydrolyzed. In subsequent germination stages hydrolytic activity towards sinapine rises rapidly, reaches a sharp maximum at 48 h, and decreases thereafter as rapidly as it had appeared.

Zymograms in Fig. 2, stained for sinapine esterase activities, exhibit one band with changing intensities, which cannot be ascribe to any α-naphthylacetylesterase activity.

The development of sinapine esterase activity in the cotyledons of *Raphanus sativus* seems to be in-

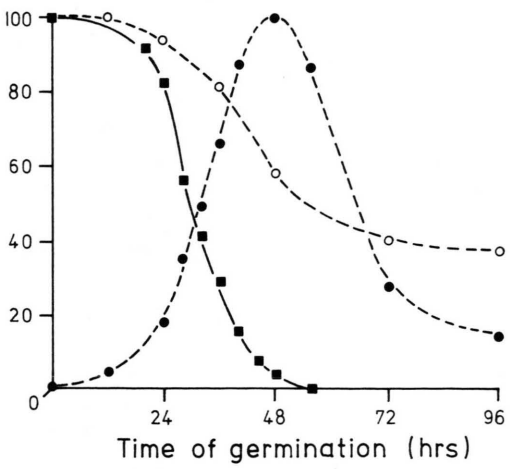

Fig. 1. Kinetics of sinapine degradation and esterase activities in cotyledons of *Raphanus sativus* during germination. Each datum represents the average from 3–4 replicates of 20–50 individuals. Ordinate: 100=160 nmol sinapine (■), 1.16 nmol sinapic acid min^{-1} from sinapine esterase (●), and 4.30 nmol indophenol min^{-1} from indophenylacetylesterases (○) per pair of cotyledons, respectively.

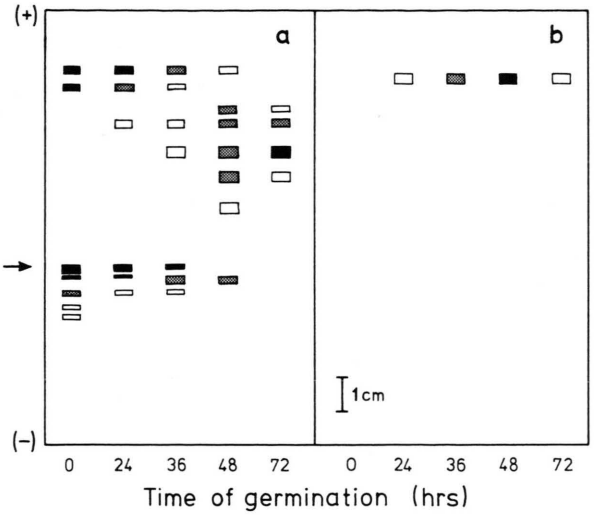

Fig. 2. Zymograms of protein extracts from cotyledons of *Raphanus sativus* at various stages of germination, stained for α-naphthylacetylesterases (a) and sinapine esterase (b). Intensity of bands is indicated by shading. Arrow shows point of sample application.

dependent of the sinapine content. Maximal enzyme activity is reached when more than 90% of sinapine has disappeared. However, activity exceeds the *in vivo* degradation rate of sinapine at any stage of germination. Between 24 and 36 h *in vivo* degradation was found to be approx. 0.1 nmol/min/pair of cotyledons and enzyme activity increases at that time from 0.23 to 0.75 nmol hydrolyzed sinapine/min/pair of cotyledons. Mixing experiments excluded the presence of components in the protein preparations, which might affect apparent enzyme activity to be out of line with the *in vivo* degradation kinetics of sinapine.

Specificity of sinapine esterase activity

In the first study on *Raphanus* sinapine esterase [7] it was shown that the enzyme activity exhibits high specificity towards sinapine, compared to some other sinapoyl derivatives [18], tested. Sinapoylmalate and 6,3′-disinapoylsucrose were not hydrolyzed by protein preparations from 2-day old *Raphanus* seedlings; sinapoylglucose, however, to about 25%, compared to the rate of hydrolysis of sinapine. This hydrolytic activity, present in all protein preparations, is most likely due to contaminating hydrolases [19], unspecific towards sinapoyl derivatives. The activity does neither coincide with the development of sinapine esterase activity, nor with the interconversion kinetics of sinapoylglucose to sinapoylmalate, present in later stages of *Raphanus* germination [18, 19]. The same stands for the hydrolytic activity towards 6-sinapoylglucoraphenine (sinapic acid at C-6 of the thioglucose), which is metabolized between the 2nd and 3rd day of cotyledon growth. We have not yet been able to find an esterase activity, specific for the cleavage of the ester bond in this compound.

Tests for substrate specificity of the sinapine esterase at the level of cinnamic acid choline esters

Table I. Substrate specificity of sinapine esterase activity from 42 h old cotyledons of *Raphanus sativus*.

Substrate	$\frac{V_{max}}{K_m}$ [a]	K_m [μM]
Naturally occurring:		
Sinapine (sinapoylcholine)	426	22
Synthetic:		
Sinapine	428	20
Feruloylcholine	232	17
Cinnamoylcholine	212	32
p-Coumaroylcholine	194	17
Caffeoylcholine	75	38

[a] V_{max}, pmol/min/pair of cotyledons.

Table II. Occurrence of sinapine and sinapine esterase activity in some Brassicaceae seedlings. Each datum represents the mean of at least 2 determinations from 20–50 individuals.

Plant	Enzyme activity[a]	Sinapine[b]
Raphanus sativus var. *sativus*	1.16 (2)[c]	160 (1–2)[c]
Brassica oleracea var. *capitata*	0.73 (4)	120 (2–4)
Sinapis alba	0.42 (3)	156 (2–4)
Camelina sativa	0.19 (2)	7 (2–3)
Lepidium sativum	0.09 (3)	42 (2–6)
Lepidium densiflorum	0.06 (4)	trace
Brassica oleracea var. *gemmifera*	0.05 (3)	trace
Sisymbrium austriacum	0	trace
Lunaria annua	0	145 (no change)

[a] mU/pair of cotyledons;
[b] nmol/seed;
[c] days of maximal enzyme activity and maximal *in vivo* sinapine degradation, respectively.

(Table I) demonstrate, that the pattern of substitution in the aromatic ring system has a great influence on the rate of hydrolysis. The lack of one methoxy group (ferulic acid) decreases activity already to approx 50%, compared to the "best" substrate sinapine. The most polar caffeoylcholine was the least suitable substrate; K_m is almost 2 times higher and V_{max} approx 30%, compared to sinapine. These results support our conclusion that the enzyme, catalyzing the hydrolysis of sinapine, can be classified specifically as sinapine esterase.

Occurrence of sinapine esterase in Brassicaceae

Table II shows results on a study on the correlation between sinapine metabolism and the activity and kinetics of extractable sinapine esterase in the course of seedling development of some members of the Brassicaceae. Seedlings which show a rapid degradation of high amounts of sinapine exhibited high enzymatic activity with pronounced kinetics. Consequently, those plants which contain low amounts of sinapine like *Camelina sativa, Lepidium densiflorum, Brassica oleracea* var. *gemmifera,* or *Sisymbrium austriacum* had either none or only low extractable enzyme activities. The low level of activity in cotyledons of *Lepidium sativum* coincides with a very slow degradation of sinapine. *Lunaria annua* contains a high amount of sinapine, showing no significant change in concentration over 14 days of germination and no sinapine esterase activity was detected at any stage.

The results described in this paper suggest that degradation of sinapine in seedlings of Brassicaceae is controlled by a specific sinapine esterase. This enzyme is closely related to the developmental stage of the cotyledons.

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