Partial Purification and Some Properties of 1-Sinapoylglucose: Choline Sinapoyltransferase ("Sinapine Synthase") from Seeds of *Raphanus sativus* L. and *Sinapis alba* L.

Walter Gräwe and Dieter Strack

Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41, Bundesrepublik Deutschland

Hydroxycinnamoyltransferases which catalyze the formation of 0-sinapoylcholine (sinapine) using 1-0-sinapoyl-β-D-glucose as acyl donor have been isolated from seeds of radish (*Raphanus sativus* L. var. *sativus*) and mustard (*Sinapis alba* L.) and purified 420- and 293-fold, respectively. The enzymes ("sinapine synthase") had apparent molecular weights of about 60,000 daltons and showed highest activities at pH 7.2 and 7.6, respectively, at 45 °C with apparent energies of activation at 53 kJ mol^{-1}. There were no requirements for divalent cations or sulfhydryl reagents. The apparent K_m's of the radish and mustard enzymes were 0.48 and 0.71 mM for 1-sinapoylglucose and 5.3 and 6.5 mM for choline, respectively. The ratios of the V_{max}/K_m values for 1-sinapoyl-, 1-feruloyl- and 1-*p*-coumaroylglucose were found to be 100:19:9 (radish) and 100:20:29 (mustard). 6-0-Sinapoylglucose, 3-0-sinapoylfructose, 1-0-benzoyl- and 1-0-galloylglucose were not accepted as donors.

Introduction

It has recently been shown with radish (*Raphanus sativus* L. var. *sativus*) seeds that the biosynthesis of sinapine (0-sinapoylcholine), which is stored in seeds of members of the Brassicaceae [1−3], is catalyzed by 1-sinapoylglucose : choline sinapoyltransferase (SCT) [4].

$$\text{1-Sinapoylglucose} + \text{Choline} \xrightarrow{\text{SCT}} \text{Sinapine} + \text{Glucose}$$
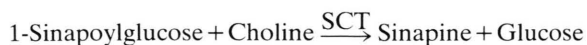
The enzyme exhibits high activities in immature radish seeds when sinapine is rapidly accumulated. During the last stages of seed ripening the activity decreases, but is still detectable in the mature seed. This activity has been surveyed in members of the Brassicaceae and it was found that SCT is widely distributed within this plant family and its activity is positively correlated with the quantity of sinapine stored in the seeds [5].

In this paper we report partial purification and some properties of SCT from two plant sources, seeds of radish and mustard.

Materials and Methods

Chemicals

Materials for column chromatography were obtained from Macherey, Nagel & Co., Düren, FRG (MN polyamide CC6), Pharmacia, Uppsala, Sweden (Carboxymethyl(CM)-Sepharose), and LKB, Gräfelfing, FRG (Ultrogel AcA 44).

1-Sinapoylglucose (1-0-sinapoyl-β-D-glucose) [7] and 1-feruloylglucose were isolated from 3-day-old radish seedlings; 1-*p*-coumaroylglucose was from petals of *Antirrhinum majus* [8] and 6,3'-di-0-sinapoylsucrose from radish seeds [7]. Extraction and procedures of chromatography were published elsewhere [9]. 6-Sinapoylglucose and 3-sinapoylfructose were produced by treatment of disinapoylsucrose with 1 N HCl for 15 min at 100 °C and the monoesters isolated by polyamide column chromatography and thin layer chromatography on microcrystalline cellulose [9]. 1,2-Di-0-sinapoyl-β-D-glucose was isolated from dark-grown radish seedlings [10]. 1-Benzoylglucose and 1-galloylglucose were gifts from Profs. W. Barz (Münster, FRG) and G. G. Gross (Ulm, FRG), respectively.

Plant material

Seeds of radish (*Raphanus sativus* L. var. *sativus* cv. Saxa) and mustard (*Sinapis alba* L.) were purchased from Zwaan & Co Samenzucht GmbH, Kleve, and Schmitz & Laux, Hilden, FRG, respectively. Growing conditions for the radish seedlings were the same as described previously [6].

Standard enzyme assay and activity determination

The standard reaction mixture contained 30 µl of 100 mM potassium phosphate buffer (pH 7.2 for SCT from radish, pH 7.6 for SCT from mustard), 10 µl protein solution, 50 µl 1-0-sinapoyl-β-D-glucose (1.9 mM in H_2O), and 10 µl choline chloride (300 mM in H_2O). This mixture was incubated at 30 °C for 2 h and the reaction stopped by transferring to a freezer (−20 °C). Enzyme activities were determined by HPLC as described previously [5]. 20 µl of reaction mixture was injected to a prepacked RP-8-column (5 µm, 250 × 4 mm; Merck, Darmstadt, FRG) developed isocratically using 30% acetonitrile, 15% *glacial* acetic acid, 1% *ortho*-phosphoric acid, and 0.05% sodium dodecyl sulfate in water at a flow rate of 1 ml min^{-1}. Sinapine isolated from radish seeds [5] was used as the standard for quantifications. Identification of sinapine as reaction product was described elsewhere [4]. Other hydroxycinnamoylcholines formed were tentatively identified by reaction with Dragendorff's reagent on thin-layer plates and chromatographic behaviour in HPLC [5].

Protein estimation

Protein contents were determined by the method of Bradford [11] using bovine serum albumin as standard.

Gel electrophoresis

Sodium dodecyl sulfate-gel electrophoresis (slab gel SDS-PAGE) was performed according to Laemmli [12] and Laemmli and Favre [13].

Protein preparation

Step i: Preparation of crude extract. Seeds from radish or mustard (20 g) were frozen with liquid nitrogen and ground (15 min) in a precooled (4 °C) mortar in the presence of 10 g insoluble Polyclar AT, 20 g quartz sand and 380 ml potassium phosphate buffer (100 mM, pH 6.5). The homogenate was poured into a precooled beaker, allowed to stand for 45 min under continuous stirring, then passed through two layers of cheese cloth, and the filtrate centrifuged at 20,000 × *g* for 45 min.

Step ii: Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant solution (see step i) to obtain 35% saturation. The precipitate was removed by centrifugation (30 min at 20,000 × *g*) and the supernatant was raised to 65% saturation. After stirring for 30 min the precipitated protein was collected by centrifugation. The protein was dissolved in 32 ml of potassium phosphate buffer and the solution dialyzed for 12 h against 2 changes of 4 l of the same buffer.

Step iii: Heat treatment. The protein fraction obtained from ammonium sulfate precipitation was kept at 65 °C for 10 min, cooled down to 4 °C, and centrifuged at 48,000 × *g* for 20 min. The supernatant was used for the subsequent enzyme purification.

Step iv: Ion exchange on CM-Sepharose. The protein solution (see step iii) was chromatographed on a CM-Sepharose column (26 × 3 cm) at a flow rate of 60 ml h^{-1}. The column was washed with 180 ml citric acid buffer (0.02 M, pH 5.0) before the following linear gradient was applied: 200 ml 0.02 to 0.3 M citric acid buffer followed by 100 ml 2 M of the same buffer. Ten-ml fractions were collected. Fractions containing high SCT activities were pooled. Protein was concentrated by ammonium sulfate precipitation (70% saturation).

Step v: Molecular exclusion on Ultrogel AcA 44. The pooled, ammonium-sulfate precipitated protein obtained from chromatography on CM-Sepharose was dissolved in 3.8 ml 0.1 M potassium phosphate buffer (pH 7.0) and was applied to an Ultrogel AcA 44 column (85 × 2 cm). Protein was eluted with 0.05 M potassium phosphate buffer (pH 7) at a flow rate of 10 ml h^{-1}. Three-ml fractions were collected. Fractions containing high SCT activities were treated with ammonium sulfate (70% saturation) and the precipitated protein collected by centrifugation (48,000 × *g* for 20 min). This protein, redissolved in 0.1 M potassium phosphate buffer (pH 7.0) and dialyzed against this buffer, was used as the partially purified SCT for the investigation of its physical and kinetic properties.

Enzyme characterization

Molecular weight. The apparent molecular weights (daltons) of the SCT's were determined on an Ultrogel AcA 44 column (see above) using the following reference proteins (Serva, Heidelberg, FRG): cytochrome C (12,300), myoglobin (17,800), chymotrypsinogen (25,000), ovalbumin (45,000), bovine serum albumin (67,000), and aldolase (160,000).

pH Optimum. The following buffer systems (0.1 M each) were used to determine the pH optimum of SCT activities: potassium phosphate (pH 5.5 to 8.0), 2-(N-morpholino)ethanesulfonic acid (pH 5.0 to 7.0), 3-(N-morpholino)propanesulfonic acid (pH 6.5 to 8.0), N-tris(hydroxymethyl)methylglycine (pH 7.5 to 9.5), and glycine (pH 9.0 to 10.0).

Energy of activation. Initial velocities of enzyme-catalyzed reactions were determined at different temperatures (10 to 80 °C) and the apparent energies of activation were estimated from Arrhenius plots.

Kinetic properties. Apparent K_m and V_{max} values were graphically estimated according to Lineweaver and Burk [14]. Properties for the acceptor (choline) and the donor (1-sinapoylglucose) were determined at four fixed concentrations of the second substrate.

Results and Discussion

Enzyme purification

Table I summarizes preparations of SCT from seeds of radish and mustard. Typical purification steps (iv and v) by means of ion exchange and molecular exclusion column chromatography are shown in Figs. 1 and 2. The protein preparation was optimized with radish protein and was applied to mustard, yielding 420- and 293-fold SCT purifications at yields of 18 and 3%, respectively. The apparent molecular weights of the SCT's determined by molecular exclusion on Ultrogel AcA 44 were near 60,000 daltons. Slab gel SDS-PAGE still showed several protein bands after staining with Coomassie Brilliant Blue R250. We did not make an effort to purify SCT's further to achieve homogeneity.

When the partially purified enzymes from radish and mustard were stored at −20 °C for 14 days and repeatedly thawed and refrozen, no loss of the initial activities was observed. In crude extracts, however, both SCT's lost ca. 20% of activity when stored at −20 °C for 1 week. A marked difference in enzyme stabilities was observed when the effect of SDS was tested on the partially purified enzymes. While the SCT from radish exhibited at 0.05% SDS only 8%, the SCT from mustard still showed ca. 90% of the activity determined without the addition of SDS.

Properties of enzyme activity

Linearity. The formation of sinapine was linear with time up to at least 8 h and with protein concentration up to 400 μg of protein.

pH and temperature optimum. The effect of pH on the catalytic properties of SCT was tested from pH 5.5 to 10. Highest activities were found at pH 7.2 (radish) and pH 7.6 (mustard) in potassium phosphate buffer with constant activities at a range between 10 to 300 mM.

Maximal initial reaction velocities were obtained

Table I. Purification of SCT from radish and mustard seeds.

| Purification step	Total protein [mg]	Total activity [nkat]	Specific activity [nkat/mg]	Yield [%]	Purification (-fold)
Radish:					
1. Crude extract	1091	21.1	0.0193	100	–
2. Ammonium sulfate	416	15.9	0.0382	75	2
3. Heat	224	15.3	0.0683	73	3.5
4. CM-Sepharose	13.7	6.8	0.496	32	25.7
5. Ultrogel AcA 44	0.47	3.8	8.1	18	420
Mustard:					
1. Crude extract	1968	28.9	0.0147	100	–
2. Ammonium sulfate	426	13	0.0305	45	2.1
3. Heat	289	10.3	0.0356	36	2.4
4. CM-Sepharose	9.1	7.5	0.824	26	56.1
5. Ultrogel AcA 44	0.2	0.9	4.3	3	293
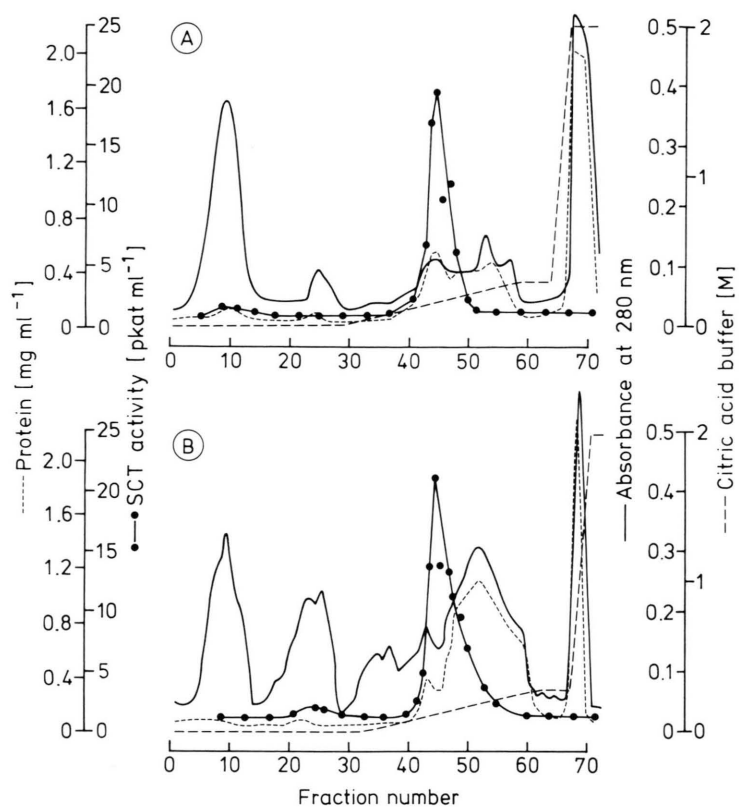
Fig. 1A, B. Chromatography of SCT from radish (A) and mustard (B) seeds, respectively, on CM-Sepharose using a citric acid buffer gradient; 10 ml fraction volume.
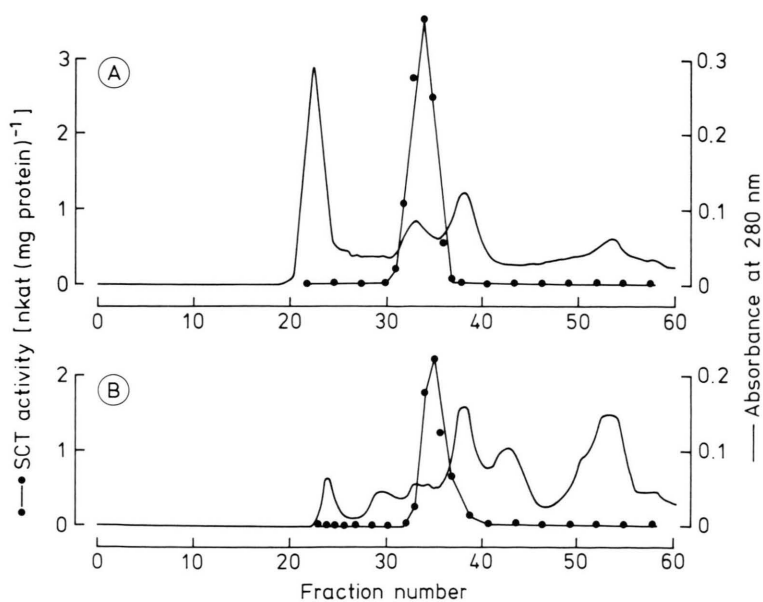

Fig. 2A, B. Elution of SCT from radish (A) and mustard (B) seeds, respectively, from Ultrogel AcA 44; 3 ml fraction volume.

at 45 °C and the apparent activation energy (Arrhenius) was found to be at 53 kJ mol^{-1}. Arrhenius plots showed no discontinuity.

Divalent cations and sulfhydryl reagents. Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} had no influence on enzyme activities at concentrations up to 1 mM and exhibited, with the exception of Mg^{2+}, strong inhibitions above 5 mM. EDTA at 10 mM inhibited the activity of the SCT from radish by ca. 55%, however, the SCT from mustard only by ca. 15%. There was also an inhibition when sulfhydryl reagents, such as DTT (dithiothreitol) or DTE (dithioerythritol), were included in the reaction mixtures, showing ca. 50% inhibition at 2 mM with total inhibition at ca. 20 mM for both enzymes.

Substrate specificity. Both enzymes showed an absolute acceptor specificity towards choline (compare ref. [4]) and a pronounced donor specificity towards 1-sinapoylglucose (Table II). The apparent K_m values, as graphically determined according to Lineweaver and Burk [14] were 0.48 and 0.71 mM for 1-sinapoylglucose and 5.3 and 6.5 mM for choline for the radish and mustard enzyme, respectively. The ratios of the $V_{max\ app.}/K_{m\ app.}$ values for 1-sinapoyl-, 1-feruloyl- and 1-*p*-coumaroylglucose were found to be 100:19:9 (radish) and 100:20:29 (mustard). It is interesting to note that 6-0-sinapoylglucose was not accepted at all as donor. This result substantiates the hypothesis that from a thermodynamic point of view the formation of an ester via the energy-rich 1-0-acyl glucoside could be predicted (*e.g.* [15]). Surprisingly, both SCT's showed highest activities of sinapine formation when the diester 1,2-disinapoylglucose was tested as donor (not included in Table II), exhibiting $K_{m\ app.}$ of 0.11 and 0.2 mM and $V_{max\ app.}$ of 8.9 and

7.3 nkat (mg protein)$^{-1}$ for radish and mustard, respectively.

The SCT's obviously possess strict specificities towards $C_6 - C_3$ acid esters. No activity was observed with 1-0-acyl glucosides of $C_6 - C_1$ acids, such as 1-benzoyl- or 1-galloylglucose tested (Table II).

The kinetic properties of both enzymes were tested for the possibility of a sequential mechanism of sinapine formation. Double reciprocal plots of the sinapoyltransferase reaction at four fixed concentrations of 1-sinapoylglucose and choline chloride, respectively, gave no indication that the K_m's for the acceptor and the donor are dependent on the second substrate.

Decrease of enzyme activity during radish seedling development. In a previous publication on the development of SCT activity in radish seeds [4] it was shown that the extractable enzyme activity correlates well with the *in vivo* accumulation of sinapine. Dark-green immature seeds which rapidly accumulate sinapine show highest SCT activities reaching values around 36 pkat seed^{-1}. During the subsequent processes of seed ripening the activity decreases, but 20% of the maximal activity was still found in the mature seeds, *i.e.* ca. 7 pkat [4]. In the present work we found ca. 12 pkat seed^{-1}. This SCT activity rapidly decreased during seed germination and early stages of seedling development: 80–90% decrease within 48 h, reaching trace activities at day 4 of development.

Summary and Concluding Remarks

This report describes some characteristics of the partially purified 1-sinapoylglucose:choline sinapoyltransferase (SCT) from radish and mustard seeds.

Table II. Substrate (donor) specificity of SCT from radish and mustard seeds. V_{max} = nkat (mg protein)$^{-1}$.

Substrate	K_m [mM]		V_{max}		V_{max}/K_m	
	Radish	Mustard	Radish	Mustard	Radish	Mustard
1-SinGlc[a]	0.48	0.71	8.2	4.9	12.8	6.9
1-FerGlc	0.79	0.9	1.9	1.3	2.4	1.4
1-CoumGlc	7.99	1.1	9	2.1	1.1	2
6-SinGlc	0	0	0	0	–	–
3-SinFru	0	0	0	0	–	–
1-BenzGlc	0	0	0	0	–	–
1-GallGlc	0	0	0	0	–	–

[a] Glc = glucose; Sin = sinapoyl; Fer = feruloyl; Coum = *p*-coumaroyl; Fru = fructose; Benz = benzoyl; Gall = galloyl.

Both enzyme preparations showed pronounced specificities for the formation of sinapine. However, the enzyme from radish accepted 1-feruloylglucose, while that from mustard accepted 1-*p*-coumaroylglucose as the second best donors. Furthermore the mustard enzyme was less affected by EDTA or SDS, indicating a higher stability compared to the radish enzyme. Since both enzymes were not purified to homogeneity, the observed difference in the donor specificity can only carefully be interpreted with regard to differences in the properties of the same enzyme. It might be possible that activities of some minor analogous transferase activities were present in these protein preparations. As pointed out earlier [5] members of the Brassicaceae show a variety of choline esters of phenolic acids besides the family-specific sinapine, *e.g.* feruloyl-, isoferuloyl and hydroxybenzoylcholines, and it would be of interest to study their biosynthesis in the related plants to see if, as in sinapine formation, 1-0-acyl glucosides are used as activated substrates.

In summary this paper and previous reports [4, 5] indicate that the SCT activity can be ascribed to a "sinapine synthase", regulating the accumulation of sinapine in the seeds of members of the Brassicaceae [5].

The ester formation described herein represents a fundamental mechanism of ester formation in higher plants. To date this 1-0-acyl-glucose dependent transferase has shown to be involved in the formation of sinapine ([4, 5], this paper), sinapoyl-L-malate [16, 6], disinapoylglucose [17], digalloylglucose [18], indoleacetyl-*myo*-inositol [19], *p*-coumaroylquinic acid [20], and chlorogenic acid [21]. The widely occurring phenolic glucose esters can (i) accumulate as endproducts in the metabolism of phenolics (*e.g.* most likely in petals of *Antirrhinum majus* and a variety of other plants listed in ref. [8]), (ii) accumulate transiently (*e.g.* in seedlings of *Raphanus sativus*) [6] or (iii) are present at trace amounts (*e.g.* in ripening seeds of *Raphanus sativus*) [4] possibly subject to rapid turnover. So phenolic glucose esters can be essential intermediates in plant metabolism as activated forms of phenolic acids.

[1] O. E. Schultz and R. Gmelin, Z. Naturforsch. **7b**, 500 (1952).
[2] O. E. Schultz and R. Gmelin, Z. Naturforsch. **8b**, 151 (1953).
[3] E. Kerber and G. Buchloh, Angew. Bot. **54**, 47 (1980).
[4] D. Strack, W. Knogge, and B. Dahlbender, Z. Naturforsch. **38c**, 21 (1983).
[5] J. Regenbrecht and D. Strack, Phytochemistry **24**, 407 (1985).
[6] D. Strack, Planta **155**, 31 (1982).
[7] M. Linscheid, D. Wendisch, and D. Strack, Z. Naturforsch. **35c**, 907 (1980).
[8] J. B. Harborne and J. J. Corner, Biochem. J. **81**, 242 (1961).
[9] D. Strack, Z. Pflanzenphysiol. **84**, 139 (1977).
[10] D. Strack, B. Dahlbender, L. Grotjahn, and V. Wray, Phytochemistry **23**, 657 (1984).
[11] M. M. Bradford, Anal. Biochem. **72**, 248 (1976).
[12] U. K. Laemmli, Nature **227**, 680 (1970).

[13] U. K. Laemmli and M. Favre, J. Molec. Biol. **80**, 575 (1973).
[14] H. Lineweaver and D. Burk, J. Amer. Chem. Soc. **56**, 658 (1934).
[15] R. Schlepphorst and W. Barz, Planta Med. **36**, 333 (1979).
[16] N. Tkotz and D. Strack, Z. Naturforsch. **35c**, 835 (1980).
[17] B. Dahlbender and D. Strack, J. Plant Physiol. **116**, 375 (1984).
[18] G. G. Gross, Z. Naturforsch. **38c**, 519 (1983).
[19] L. Michalczuk and R. S. Bandurski, Biochem. Biophys. Res. Commun. **93**, 588 (1980).
[20] M. Kojima and R. J. A. Villegas, Agric. Biol. Chem. **48**, 2397 (1984).
[21] R. J. A. Villegas and M. Kojima, Agric. Biol. Chem. **49**, 263 (1985).