Enzymatic Synthesis of Sinapine from 1-O-Sinapoyl-β-D-glucose and Choline by a Cell-Free System from Developing Seeds of Red Radish (*Raphanus sativus* L. var. *sativus*)

Dieter Strack, Wolfgang Knogge, and Brigitte Dahlbender
Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41

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**Introduction**

Numerous investigations have shown so far, that the formation of most hydroxycinnamic acid conjugates is catalyzed by transferases which require either coenzyme A-activated acids [for reviews see ref. 1–3] or, in the biosynthesis of glucose esters, UDP-activated glucose [e.g., 4–6].

It has recently been shown that the formation of a hydroxycinnamic acid ester was catalyzed by a third type of transferase which uses an energy-rich 1-O-acyl glucoside as the acyl donor. This enzyme in phenylpropanoid metabolism of plants, found for the first time in seedlings of red radish, was involved in the formation of O-sinapoylmalate [7, 8].

Relevant tissues in which this class of transferase might also exist are those, for which it was not yet possible to demonstrate acylation using an acyl-thiol ester in the formation of a conjugate in question, and tissues which show transiently accumulating, metabolically active 1-O-acyl glucosides [9–11]. On the other hand we could discuss the possibility that in complex metabolic grids of phenylpropanoid metabolism the aromatic acids are channelled into different pathways by using both, acyl-thiol and acyl-O-glucose esters as the acylation reagents. It was proposed that such multiple mechanisms could explain, for example, the occurrence of a variety of different indoleacetic acid esters in the phytohormone metabolism of corn kernels [12].

Among a large number of hydroxycinnamic acid esters occurring in higher plants [1, 3, 13, 14], members of the Brassicaceae exhibit a characteristic accumulation of the choline ester of sinapic acid (sinapine) [15] in the seeds [16–18]. Sinapine is of considerable interest with respect to its wide distribution, its possible functional role in the general metabolism in these plants [19]; and last not least sinapine called attention to workers in food science [20, 21].

Attempts to elucidate the biosynthetic mechanism of the formation of sinapine led to results which were not unequivocal so far. It has been suggested that sinapic acid would not be directly involved in the pathway of sinapine biosynthesis in embryos of white mustard (*Sinapis alba*) [22, 23]. It was not possible to detect an enzymatic activity in *Sinapis* which uses sinapoyl-CoA as a substrate for the ester formation with choline and it was proposed that the substitution pattern of sinapic acid

Reprint requests to Dr. D. Strack.

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may be formed at the ester level and that the direct precursor of sinapine might be either hydroxylferuloylcholine or sinapoylshikimate [22]. The latter is of some interest. Recently it has been discussed in a study on p-coumaroyl-CoA: shikimate p-coumaroyltransferase [24] that shikimate esters may act as acyl donors in acylation reactions so far unknown.

In this publication we report on a cell-free system from seeds of red radish, which catalyzes the synthesis of sinapine via 1-O-sinapoyl-β-D-glucose. This reaction mechanism is analogous to that in the formation of indoleacetic acid-myoinositol ester [12] and O-sinapoylmalate [7, 8]. The described transacylase activity refers to an enzyme which can be classified as 1-sinapoylglucose: choline sinapoyltransferase (SCT).

Materials and Methods

Plant material and growing conditions

Seeds of red radish (*Raphanus sativus* L. var. *sativus*) were purchased from Zwaan u. Co’s u. Komp., Delfter Marktgärtner-Samenzucht GmbH, Netherlands. Fruit bearing plants were cultivated in the field of the botanical garden of the university of Cologne during spring and summer 1982. Developing fruits (4–8 weeks after pollination) were harvested and immature seeds isolated, which were at different stages of development. Reproducible staging of these seeds was achieved by determination of the chlorophyll content according to the method of Bruinsma [25].

Extraction and quantification of sinapine

Extractions for quantitative determination of sinapine in developing seeds were carried out by treatment of 25 seeds with an Ultra-Turrax homogenizer for approx. 4 min in 4 ml 80% aqu. methanol. The homogenates were allowed to stand for 1 h and then centrifuged at 3000 × g for 15 min. The supernatants were subjected to HPLC-analysis employing the system described in the section on chromatography and in the legend of Fig. 2.

Substrates

1-Sinapoylglucose (1-O-sinapoyl-β-D-glucose) and 1-feruloylglucose were isolated from 3-d-old red radish seedlings essentially by a procedure described elsewhere [26]. 1-p-Coumaroylglucose came from petals of snapdragon (*Antirrhinum majus*) [27]. These glucose esters were identified by comparison with those, which had been prepared for a previous study [8]. Choline chloride was obtained from Fluka, Neu-Ulm, FRG; other substrates were from Merck, Darmstadt, FRG, Roth, Karlsruhe, FRG, or Serva, Heidelberg, FRG. [methyl-14C]choline chloride (2.24 GBq mmol⁻¹) was supplied by Amersham Buchler, Braunschweig, FRG.

Preparation of crude protein extracts

Fifty seeds were frozen with liquid nitrogen and immediately ground in a mortar together with 0.5 g quartz sand, 0.5 g Dowex 1 X 2 (Cl⁻), 0.1 g dry insoluble polyclar AT, and 7.5 ml 0.17 M potassium phosphate buffer, pH 6.5. Centrifugation at 48000 × g for 15 min and filtration of the supernatant through miracloth and glass wool resulted in 6 ml extract solution, which was finally treated with 0.5 g Dowex 50 WX 1 (H+). The filtrate was used as source of enzymatic activity.

Enzyme assay

The standard reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.5, 10 μl protein extract, 1 mM 1-sinapoyl-glucose, and 10 mM choline chloride in a total volume of 100 μl. After incubation at 30 °C for various lengths of time, the reaction was stopped by transferring the mixture to a freezer (−20 °C) or it was immediately analyzed by HPLC.

Radioactive assay contained [methyl-14C]choline (1.66 · 10⁶ dpm).

Calculations of *Km* and *Vmax* values based on plots according to Lineweaver and Burk.

Protein was measured according to Lowry et al. [28] using bovine serum albumine as the standard.

Identification of reaction products

The identity of the reaction product sinapine was proven by co-chromatography (Table II), by color reaction obtained with Dragendroff’s spray reagent [29], by HPLC analysis of a [14C]choline-containing assay (Fig. 3), and by TLC of choline [19], which was liberated from the reaction product by alkaline hydrolysis (1 N NaOH, room temperature for 30 min). p-Coumaroyl- and feruloylcholine were
identified tentatively by their similar chromatographic behaviour as compared to sinapine, by their characteristic color reaction with Dragendorff’s reagent, and through identification of the choline moiety.

**Chromatography**

Thin layer chromatography (TLC) was performed on cellulose plates as described in Table II. Chromatograms were viewed under UV light (366 nm) and sprayed with Dragendorff’s reagent.

High performance liquid chromatography (HPLC) was carried out with a Spectra-Physics (Santa Clara, Calif., USA) system, connected with a computing integrator (System I, Spectra-Physics), a UV/VIS-detector (Schoeffel Instrument-Corp., Trappenkamp, FRG), and a HPLC radioactivity monitor (LB 503, Berthold, Wildbad, FRG), which was equipped with a 100 μl Cer-activated glass scintillator cell. Injection was done via a Rheodyne rotary valve with a 20 μl loop. The chromatographic column (250 × 4 mm) was prepacked with LiChrosorb RP-8 (5 μm) (Merck, Darmstadt, FRG). Elution system is described in Fig. 2. In general, the detection wavelength was 330 nm. Reaction with p-coumaroylglycerol was determined at 315 nm. Quantitative values were obtained using 1-sinapoylglucose as the standard for UV-detection and [14C]choline as the standard for 14C-detection.

**Results and Discussion**

In the presence of 1-O-sinapoyl-β-D-glucose and free choline, protein extracts of red radish seeds are capable of forming a product which cannot be distinguished from sinapine (O-sinapoyl-choline). Fig. 1 depicts the proposed reaction mechanism for this enzymatically catalyzed sinapine synthesis.

Enzyme activities were analyzed by high performance liquid chromatography (HPLC) (Fig. 2). After 4 h of incubation, approx. 20% of the applied 1-sinapoylglucose was transacylated to sinapine. As shown in Table I, this product only was formed in the presence of native protein, free choline, and 1-sinapoylglucose. Co-chromatography of the reaction with authentic sinapine [30] and reaction with Dragendorff’s reagent (appearance of orange color) showed identity (Table II). Incubations containing [14C]choline gave sinapine with radioactivity found

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**Table I. Requirements for sinapine formation.** Assays, containing 65 μmol potassium phosphate buffer (pH 6.5) in a total volume of 650 μl, were kept at 30 °C for 2 h.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>nmol Sinapine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 20 μl Enzyme + 650 nmol 1-sinapoylglucose + 6.5 μmol choline</td>
<td>22.4</td>
</tr>
<tr>
<td>2. 20 μl Enzyme + 650 nmol sinapic acid + 6.5 μmol CDP-choline</td>
<td>0</td>
</tr>
<tr>
<td>3. 20 μl Enzyme + 650 nmol 1-sinapoylglucose</td>
<td>0</td>
</tr>
<tr>
<td>4. 20 μl Heat denatured enzyme + 650 nmol 1-sinapoylglucose + 6.5 μmol choline</td>
<td>0</td>
</tr>
<tr>
<td>5. 650 nmol 1-sinapoylglucose + 6.5 μmol choline</td>
<td>0</td>
</tr>
</tbody>
</table>

Extr. was kept at 80 °C for 3 min.

**Table II. Co-chromatographic behaviour of sinapine and a product formed by a crude protein preparation, which was incubated with 1-sinapoylglucose and choline or [14C]choline.**

<table>
<thead>
<tr>
<th>TLC</th>
<th>HPLC b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAW</td>
<td>BAW</td>
</tr>
<tr>
<td>64</td>
<td>50</td>
</tr>
</tbody>
</table>

a Determined on microcrystalline cellulose (Avicel): CAW, chloroform-acetic acid-water (3:2, water saturated); BAW, n-butanol-acetic acid-water (6:1:2). Sinapine was detected under UV366 nm with and without NH3 vapour and under day light after spraying with Dragendorff’s reagent.

b See Figs. 2 and 3.

c Corrected for delayed detection (UV/VIS- and radioactivity monitors were connected in series).
Fig. 2. High performance liquid chromatographic (HPLC) analysis of a SCT assay (a) at t₀ and (b) after 2 h of incubation. Twenty µl of a standard assay were injected onto the column (RP-8), developed linearly within 30 min from 20 to 80% solvent B (1% phosphoric acid, 25% acetic acid, 50% acetonitrile, and 0.05% Li-dodecylsulphate in water) in solvent A (1% phosphoric acid and 0.05% Li-dodecylsulphate in water) at a flow-rate of 0.8 ml min⁻¹.

exclusively in the choline moiety. Radio [¹⁴C]HPLC gave a radioactive product peak (Fig. 3), which eluted simultaneously with the corresponding UV-absorbing product peak (Fig. 2), identical with sinapine.

A wide range of proportionality with respect to incubation time and protein concentration (Fig. 4) was found in determination of enzymatic activities using HPLC. This permits easy measurements of this transacylase activity. Twenty % loss of enzymatic activity was observed when storing the crude extract at −20 °C for 1 week.

The pH optimum of this reaction was found to be about 7.0 in 0.08 M potassium phosphate buffer. Fifty % of the maximal activity was retained near pH 5.3 and 9.0 (glycine-NaOH). TRIS(Tris(hydroxymethyl)-amino) methane-HCl buffer should not be used for the enzyme assay. When we applied this buffer (0.05 M, pH 8.5), besides a much lower yield of sinapine we observed the appearance of a second product, which possibly could be the result of a sinapoyltransfer to TRIS. This product was not formed when we reduced its concentration to 10 mM in potassium phosphate buffer (0.08 M, pH 7.0) in a separate assay. This tentative result could be interesting in connection with questions on the mechanism of the enzyme-acceptor binding, because a part of the TRIS-molecule strikingly resembles the structure of choline.

Variation of the concentration of substrates showed that the enzyme activity follows Michaelis-Menten kinetics. The apparent Kₘ values for 1-sinapoylglucose (at 10 mM choline) and choline (at 1 mM 1-sinapoylglucose) were found to be 0.30 and 7.64 mM, respectively.

Studies on the acceptor specificity showed that the enzyme only could transfer sinapic acid to the hydroxyl group of choline. Ethanolamine, myo-inosi-
tol, citric, L-malic, L-tartaric, quinic, or shikimic acid, each tested at 10 mM, were not accepted. The tentative result that TRIS possibly can be accepted (see above) will be further investigated in connection with an intended study on the characterization of a partially purified transacylase.

In comparison to some other hydroxycinnamoyl-glucose esters tested, there was a high donor specificity towards 1-sinapoylglucose (Table III). This is indicated by the ratios of the relative reaction velocities for 1-sinapoyl-, 1-feruloyl-, and 1-\(p\)-coumarylglycoside which were found to be 100:41:13, respectively, at 1 mM concentration.

Changes of extractable SCT activity during the development of red radish seeds correlated well with the \textit{in situ} accumulation of sinapine (Figs. 5 and 6). This compound is accumulated in the cotyledons during the growth phase of the developing embryo. This is in accordance with results on white mustard (\textit{Sinapis alba}) [22, 23]. The final sinapine concentration is reached, when the seed development passes over to the actual ripening process in which chlorophyll degradation and loss of water occur. Highest SCT activity, approx. 36 pkat seed\(^{-1}\) and 129 \(\mu\)kat kg\(^{-1}\) protein, could be extracted at stage III. Enzymatic activity already can be detected between stages I and II, at which sinapine occurs in traces. The mature dry seed still exhibits 20\% of the activity obtained from stage III. This observation not only is interesting with respect to the possibility of an easy screening of this new enzyme in members of the Brassicaceae, but also in connection with our earlier results on red radish. It was found [31] that the dry seed contains a considerable activity of UDP-glucose: sinapic acid glucosyltransferase (GT), which might indicate an involve-

![Fig. 3. High performance liquid chromatographic (HPLC) analysis of a SCT assay, containing \(^{14}\)Ccholine, by monitoring radioactivity using a Cer-activated glass scintillator cell: (a) without 1-sinapoylglucose, (b) with 1-sinapoylglucose, both after 14 h of incubation (ratemeter-range = 500 cpm). Chromatographic conditions were as described in Fig. 2.](image-url)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Sinapoylglucose</td>
<td>100</td>
</tr>
<tr>
<td>1-Feruloylglucose</td>
<td>41</td>
</tr>
<tr>
<td>1-(p)-Coumarylglycoside</td>
<td>13</td>
</tr>
</tbody>
</table>

\(^{a}\) An average molar absorption coefficient of \(20 \times 10^{6}\) mol\(^{-1}\) cm\(^{-1}\) was used.

\(^{b}\) Activities were compared with the amount of choline ester formed with 1-sinapoylglucose (100 = 9.1 nmol \(\cdot 100 \mu\)l\(^{-1}\) \(\cdot 2\) h\(^{-1}\)).
Fig. 4. (a) Time course and (b) effect of added crude extract on the formation of sinapine in a standard SCT assay.

Fig. 5. Change in chlorophyll content and accumulation of sinapine in developing red radish seeds. Stages: I, first stages of embryo greening, seed coat colorless and transparent; II, embryo entirely green, seed coat still colorless; III, embryo and seed coat dark-green; IV, first stages of the beginning of browning; V, seed coat light-brown, embryo still green; VI, mature dry seed, embryo light yellow, seed coat dark-brown.

Fig. 6. Change of extractable SCT activities during some stages of seed development of red radish. For description of developmental stages see Fig. 5. Activity-values, obtained by mixed protein preparations, were strictly additive.

In conclusion we propose the following pathway leading to the formation of sinapine in seeds of red radish:

1. Sinapic acid + UDP-glucose $\xrightarrow{\text{SCT}}$ 1-sinapoylglucose + UDP

(2) 1-Sinapoylglucose + choline $\xrightarrow{\text{SCT}}$ sinapine + glucose

Experiments are underway to show the distribution of SCT activity in different plants, and in a joining communication we will report on a screening study on the occurrence of this transacylase activity in seeds of the Brassicaceae.

The proof of a possible central role of this type of transacylase in the metabolism of plant tissues must await further enzymatic studies.

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