Enzymatic Synthesis of 1-Sinapoylglucose from Free Sinapic Acid and UDP-Glucose by a Cell-Free System from *Raphanus sativus* Seedlings

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Protein extracts from seedlings of *Raphanus sativus* catalyze the transfer of the glucosyl moiety of UDP-glucose to the carboxyl group of phenolic acids. Enzymatic activity was determined spectrophotometrically by measuring the increase in absorbance at 360 nm and/or by the aid of high performance liquid chromatography (HPLC).

From 12 phenolic acids tested as acceptors, sinapic acid was by far the best substrate. The glucosyltransfer to sinapic acid has a pH optimum near 7 and requires as SH group for activity. *p*-Chloromercuribenzoate (PCMB) inhibits activity, which can be restored by the addition of dithiothreitol (DTT). The formation of 1-sinapoylglucose was found to be a reversible reaction, since the addition of UDP results in a breakdown of the ester.

Introduction

Higher plants contain a large number of various hydroxycinnamoyl esters. Most of them might be synthesized via hydroxycinnamoyl-CoA thiolesters as the activated reaction partners [1]. The widely occurring glucose esters [2], however, might be exclusively synthesized from free hydroxycinnamic acids and UDP-glucose [3, 4]. The formation of glucose esters of benzoic acids might follow the same mechanism [5].

Whereas the involvement of UDP-glucose is well established in the formation of phenylpropanoid glycosides, containing hydroxycinnamyl alcohols [6], flavonols [7, 8], or anthocyanidins as aglyca [9], there is a lack of knowledge of the enzymatic reaction of glucose ester formation.

Seedlings of *Raphanus sativus* accumulate a high amount of 1-sinapoylglucose (80–100 nmols per cotyledon) in early stages of germination. The sinapic acid of this ester is derived from sinapoylcholine (sinapine), which is hydrolyzed by a specific sinapine esterase [10].

The present report describes the enzymatic formation of 1-sinapoylglucose *via* uridine diphosphate-D-glucose (UDPG) and free acid by protein extracts from young seedlings of *Raphanus sativus*. This reaction is freely reversible, which might be of physiological significance for the *Raphanus* seedling.

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Materials and Methods

Plant material and *culture conditions* are described elsewhere [10].

Thin-layer chromatography

Phenolic acid derivatives were chromatographed on microcrystalline cellulose (Avicel) in CAW, chloroform – acetic acid – water (3 : 2, water saturated) and were detected under UV with and without NH_3-vapor. Sugar chromatography was carried out on the same layer with BPW, *n*-butanol – pyridine – water (3 : 1 : 1) with an overflow technique: The upper margin of the plates was connected with chromatographic paper, which hung over the back of the plates and allowed development for 15–20 h. Sugar detection was achieved with ammoniacal silver nitrate [11].

High performance liquid chromatography

The liquid chromatograph used was obtained from Spectra Physics (Santa Clara, Cal., USA) and is described elsewhere [12].

The applied column (250 × 4 mm) was prepacked with LiChrosorb RP-8 (5 µm) (Merck, Darmstadt, Germany). Separations were accomplished by gradient elution: in 35 min linear from 1% phosphoric acid (A) to 70% methanol (B) in A + B. The flow-rate was 1 ml/min and detection at 330 or 265 nm was achieved with a Schoeffel SF 770 UV-VIS detector (Kratos Inc., Trappenkamp, Germany).

HPL-radiochromatograms from enzyme assays containing UDP-[^{3}H]glucose were obtained by fractionating eluants into 1 ml samples, which were collected at the detector flow-cell outlet into 5 ml Unisolve I (Zinsser) in scintillation vials. Counting was done with a Tricarb-liquid-spectrometer (Packard).

Substrates

Phenolic acids were purchased from Fluka, Neu-Ulm, Merck, Darmstadt, and Roth, Karlsruhe. Uridine diphosphate-D-glucose (UDPG) disodium salt was obtained from Merck; UDP-D-[6-^{3}H]glucose ammonium salt (3.1 Ci/mmol) from Amersham Buchler, Braunschweig; ADP, CDP, GDP, and UDP from Boehringer, Mannheim.

Preparation of the cell-free extract

To 50 seedlings 0.5 to 1.0 g insoluble Polyclar AT (Serva, Heidelberg) was added and treated with an Ultra Turrax homogenizer in an ice bath for 2 min in 10 ml potassium phosphate buffer (100 mM, pH 7.0), containing 10 mM dithiothreitol (DTT) (Serva, Heidelberg). The suspension was centrifuged at 3,000 × *g* for 15 min, the filtered supernatant was centrifuged at 38,000 × *g* for 30 min, and was finally subjected to a Sephadex G-25 centrifugation.

Enzyme assay and determination of activity

100 µl protein extract was mixed with 100 µl phenolic acid solution (in buffer) and 2.7 ml potassium phosphate buffer (100 mM, pH 7.0, 10 mM DTT) and preincubated for 30 min at 30 °C. The reaction was started by the addition of 10 µl UDPG solution (in water). Substrates were in a final concentration of 1 mM each. Radioactive assays contained in addition 3 µCi UDP-[6-^{3}H]glucose.

Enzyme activity was determined spectrophotometrically by measuring the increase in absorbance at 360 nm (PMQ II, Zeiss) and/or by high performance liquid (radio)chromatography (Fig. 1 and 2). The absorbance of the produced 1-sinapoylglucose in the reaction mixture followed the Lambert-Beer law, which was determined by HPLC.

Product identification

Identification of 1-sinapoylglucose was achieved by TLC [13], HPLC [14], mild alkaline hydrolysis (1 N NaOH, room temperature for 30 min) and by analysis of the sugar [13].

Tentative identification of glucose esters of other phenolic acids were done by HPLC and mild alkaline hydrolysis.

Results

Identification of reaction products

In the presence of UDP-glucose protein extracts of *Raphanus sativus* cotyledons are capable to form glucose esters with various phenolic acids (Table I). In each assay only one product was found (TLC, HPLC), exhibiting high sensitivity towards alkaline treatment.

When the enzyme assay contained sinapic acid, the product formed showed TLC and HPLC identity with 1-sinapoylglucose, which was isolated from *Raphanus sativus* seedlings [13]. The sugar released by alkali could not be distinguished from authentic glucose. Incubations containing in addition UDP-[^{3}H]glucose gave 1-sinapoylglucose with radioactivity found exclusively in the glucose moiety. HPLC analysis of this assay gave the expected simultaneous elution of the UV absorbing 1-sinapoylglucose and its radioactivity (Fig. 1). HPLC analyses of incubations, which contained other phenolic acids (Table I), gave the same results. When these samples were treated with 1 N NaOH for 30 min at room temperature the UV absorbing- and the ^{3}H-product

Table I. Specificity of the transfer of the glucosylmoiety of UDP-glucose to the carboxyl group of different phenolic compounds. Activities were determined by calculating the amount of label incorporation from UDP-[^{3}H]glucose into each substrate, obtained by HPLC (see fig. 1).

Substrate (1 mM)	% Relative activity (compared to sinapic acid = 100)
Sinapic acid	100
p-Coumaric acid	40
Ferulic acid	38
Caffeic acid	38
3,5-Dimethoxycinnamic acid	33
Cinnamic acid	16
Benzoic acid	39
Vanillic acid	36
Syringic acid	35
Anthranilic acid	31
4-Hydroxybenzoic acid	31
3,5-Dihydroxybenzoic acid	5

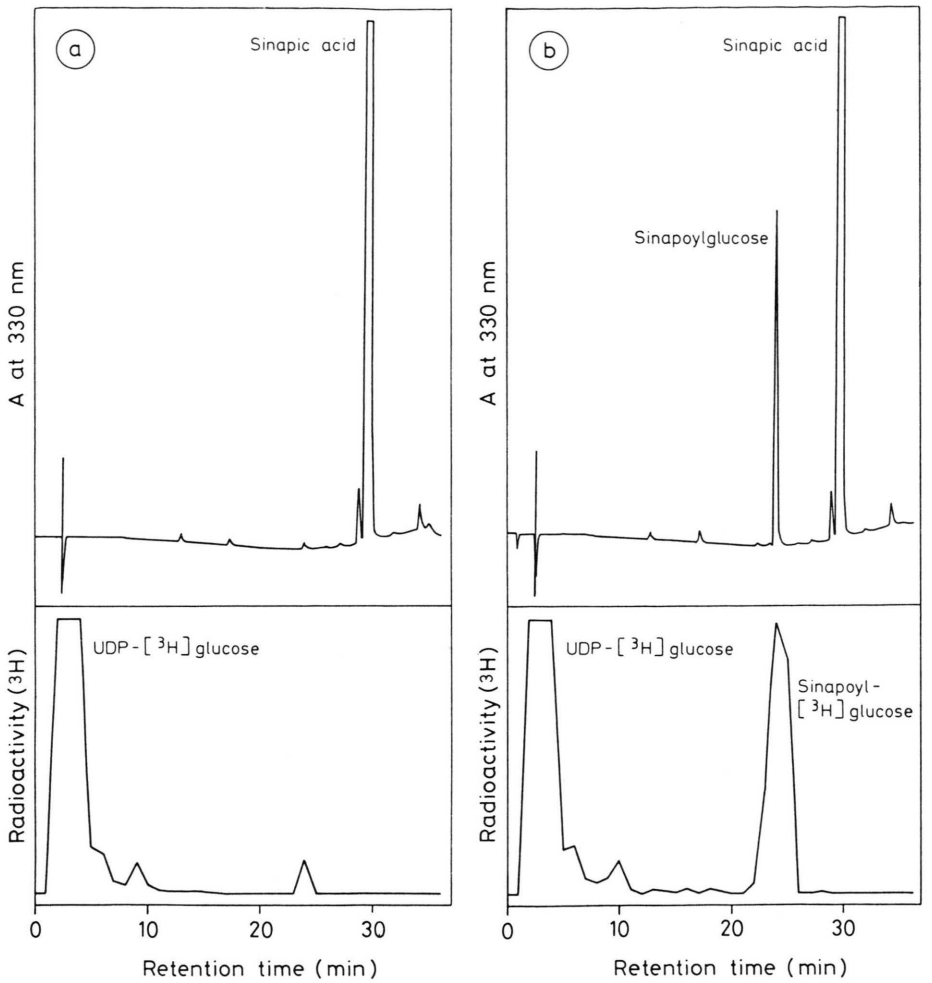

Fig. 1. HPLC analyses of a protein extract from *Raphanus sativus* cotyledons, incubated with sinapic acid and UDP-glucose and UDP-[^{3}H]glucose, (a) at t_0 and (b) after 3 h incubation. 20 µl of the incubation medium was directly injected without any pretreatment.

peaks were no longer detectable. The identity of the products *p*-coumaroyl-, feruloyl-, and caffeoylglucose was further substantiated by the HPLC analysis described in ref. [14].

Characterization of the enzymatic formation of 1-sinapoylglucose

Extraction and assay buffers with molarities between 50 and 100 mM potassium phosphate of pH values around 7.0 gave maximal activities. The addition of DTT or 2-mercaptoethanol markedly increased the rate of sinapoylglucose synthesis. The optimal concentration of 10 mM DTT stimulated activity by a factor of three, compared with assays without DTT. 72 and 62% of this activity was obtained with 1 mM and 100 mM DTT, respectively. PCMB at 10^{-3} M gave 100% inhibition, but activity could be restored up to 50% by the addition of 10 mM DTT. The influence of 10^{-4} M PCMB, which inhibited to 24%, could be neutralized by 10 mM DTT.

The amount of 1-sinapoylglucose formed from sinapic acid and UDP-glucose was found to be proportional to added enzyme – 25 to 150 µl in assays of 3 ml were tested – and to time up to 15 min. After 3 h around 10% of the incubated free sinapic acid was converted to 1-sinapoylglucose.

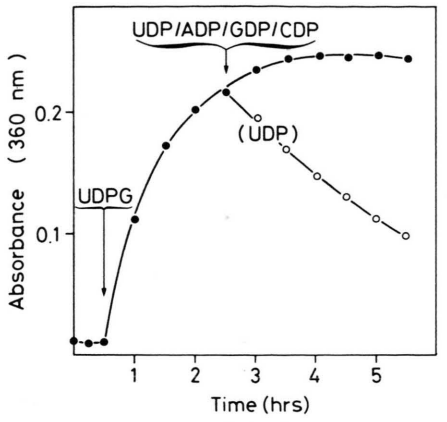

Fig. 2. Time-course of the formation of 1-sinapoylglucose by a protein extract from *Raphanus sativus* cotyledons, incubated with sinapic acid and UDP-glucose. The formation of 1-sinapoylglucose is measured by the increase in absorbance at 360 nm. Arrows indicate the time where UDPG and free nucleotides in 4 independent experiments were added.

The initial rate of enzymatic activity was maximal at 40 °C; 25 °C gave only 50% of the maximal activity and at 45 °C 80% was observed.

The enzymatic activity had no requirement for the metal ions Mg^{2+} or Ca^{2+}, and EDTA (Titriplex II) showed no influence on activity.

The enzymatic formation of 1-sinapoylglucose is stopped and the ester split to more than 50%, when UDP (1 mM) is added to an ongoing reaction. ADP, CDP, or GDP showed no such effect (Fig. 2).

Substrate specificity

Of the phenolic acids tested for enzymatic incorporation into a glucose ester, the protein preparation exhibited high specificity towards sinapic acid (Table I). Other naturally occurring phenolic acids were esterified to about 30 to 40%, compared to sinapic acid.

Localization of the activity with sinapic acid in the seedling organs showed that 90% was found in cotyledons. Mixing experiments excluded possible inhibition factors.

Maximal glucosyltransfer activity with sinapic acid was found in cotyledons of 2 day-old seedlings. At this germination stage sinapoylglucose is accumulated at its highest rate [13]. The dry seed showed 32% and 4 day-old seedlings 24% activity, compared to the maximally extractable one. Mixing experiments excluded possible inhibitory effects.

Discussion

In the metabolism of plant phenolic acid esters, the central role of cinnamoyl-CoA derivatives is well established [1]. However, in a few studies the involvement of UDP-glucose in the formation of the most common phenolic glucose esters [3–5] has been demonstrated. The present report is in agreement with the results of these authors and supports the assumption that this reaction might be of general occurrence for biosyntheses of phenolic glucose esters.

From the fact that a wide variety of plants is able to form glucose esters from fed free phenolic acids [15], even plants which do not contain these esters naturally, one might assume, that this biochemical mechanism were an unspecific detoxification reaction.

The present paper does not exclude this possibility for the described enzymatic formation of 1-sinapoylglucose with *Raphanus*, since all applied phenolic acids were found to be acceptors of the glucosyl moiety of UDP-glucose in the formation of glucose esters (Table I). However, I would like to point out, that sinapic acid, which is the naturally occurring main phenolic compound in *Raphanus*, was by far the best acceptor. In this connection it is interesting to note, that Corner and Swain [3] found exclusively 1-sinapoylglucose, when incubating the common free hydroxycinnamic acids and UDPG with an acetone powder from *Brassica oleracea*, which is related to *Raphanus*. The possible specificity of the *Raphanus* transferase reaction for sinapic acid is substantiated by the fact, that highest cell-free glucosyltransfer to sinapic acid was found in those stages of germination, in which highest accumulation of sinapoylglucose can be observed [13, 16].

At present we cannot decide whether contaminating glucosyltransferase activities with different substrate specificities are responsible for the observed capability to transfer glucose to the carboxyl group of different phenolic acids or whether the various acids esterified by the same enzyme. It might be possible that in *Raphanus* cotyledons there is a base level of an unspecific transferase activity which is superimposed by a specific UDPG: sinapic acid glucosyltransferase, with the function to catalyze the reesterification of sinapoylcholine to sinapoylglucose. The possible involvement of a second major

sinapoyl derivative [16] in this reesterification reaction is proven to be unlikely [17].

The results presented here suggest that the glucosyltransferase reaction with sinapic acid is freely reversible *in vitro* and it might be that UDPG is formed from 1-sinapoylglucose and UDP. Surprisingly also a free reversibility of a UDPG: flavonol 3-O-glucosyltransferase reaction is described by Sutter and Grisebach [18]. A free reversibility of the UDPG: sinapic acid glucosyltransferase activity – if occurring *in situ* – might be of physiological significance for *Raphanus* in two ways. Firstly it could provide the cotyledon with large amounts of UDPG. Secondly this reversibility of the sinapoylglucose formation may be of importance regarding the availability of free sinapic acid. The acid is needed in a following reesterification reaction resulting in the accumulation of sinapoylmalate [13, 17]. Since all attempts failed to demonstrate at an appropriate germination stage an esterase activity acting on sinapolyglucose, the release of free sinapic acid from this compound could be entirely dependent on th reversibility of the reaction described in this paper. However, it cannot be excluded that the free form of sinapic acid is bypassed by a direct transacylation to form sinapoylmalate [19].

Experiments are underway to clarify the points discussed above.

Acknowledgement

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