Enzymatic Synthesis of Sinapoyl-L-Malate from 1-Sinapoylglucose and L-Malate by a Protein Preparation from *Raphanus sativus* Cotyledons

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Protein preparations from cotyledons of *Raphanus sativus* transfer the sinapoyl moiety of 1-sinapoylglucose to L-malate to form sinapoyl-L-malate. To our knowledge this is the first time that this type of reaction has been found to be involved in phenylpropanoid depside formation. Most acylation reactions have so far been demonstrated to depend on acyl-CoAs and only one example is known describing 1-O-acyl sugar derivatives as acyl donors.

Introduction

Aromatic acids in higher plants are involved in a great diversity of esterification reactions and it is well established that their formation requires in most cases a CoA-activated acid [1]. Glucose esters are formed from free acids and UDP-glucose [2–5].

Glucose esters have been proposed as possibly essential intermediates in hydroxycinnamic acid metabolism [6–8]. Schlepphorst and Barz [9] have postulated from a biochemical point of view the formation of an ester from an energy-rich acyl glucose as the acyl donor in a transacylation reaction. This hypothetical reaction has recently been proven by Michalczuk and Bandurski [10], who demonstrated *in vitro* the enzymatic synthesis of IAA-*myo*-inositol by the transfer of the IAA moiety from IAA-glucose to *myo*-inositol, catalyzed by an IAA-transferase. These authors demonstrated also, that the preceding reaction is the enzymatic synthesis of IAA-glucose from IAA and UDP-glucose.

The present report describes the same biochemical mechanism in the phenylpropanoid metabolism of *Raphanus sativus* cotyledons for the formation of sinapoylmalate [11]. During early stages of *Raphanus* germination, the seed constituent sinapine (sinapoylcholine) is rapidly degraded [8], catalyzed by a specific sinapine esterase [12]. The freed sinapic acid is reesterified to 1-sinapoylglucose [13] *via* UDP-glucose [5]. In later stages of *Raphanus* germination, the amount of 1-sinapoylglucose in the cotyledons decreases, whereas sinapoylmalate is formed. Protein, extracted from this tissue, catalyzes the transfer of the sinapoyl moiety from 1-sinapoylglucose to L-malate. Analogous to the biosynthesis of IAA-*myo*-inositol, the activity catalyzing the formation of sinapoylmalate might be classified as 1-sinapoylglucose : L-malate sinapoyltransferase.

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Materials and Methods

Plant material and culture conditions are described elsewhere [8].

Protein preparation was obtained from 6–7 day-old *Raphanus* cotyledons as described in ref. [5]. The enzymatic activity was prepared from 30 to 60% $(NH_4)_2SO_4$ precipitable fraction followed by chromatography on Sephadex G-25. The incubation mixture in experiment *a* contained 20 µl protein solution, 1 µmol malate (L- or D-form), and 0.5 µmol 1-sinapoylglucose in 220 µl potassium phosphate buffer (100 mM, pH 6.5). The formation of sinapoylmalate (at room temperature) was determined by TLC and HPLC. In experiment *b* a glucosyltransferase assay [5], containing sinapic acid and UDP-glucose, was mixed with the protein preparation used in experiment *a*, to which L-malate was added at the same concentration. 1-Sinapoylglucose and authentic sinapoylmalate were isolated from *Raphanus* seedlings as described in ref. [8]. D- and L-malate were purchased from Roth, Karlsruhe.

Chromatography is described in Table I and Fig. 1.

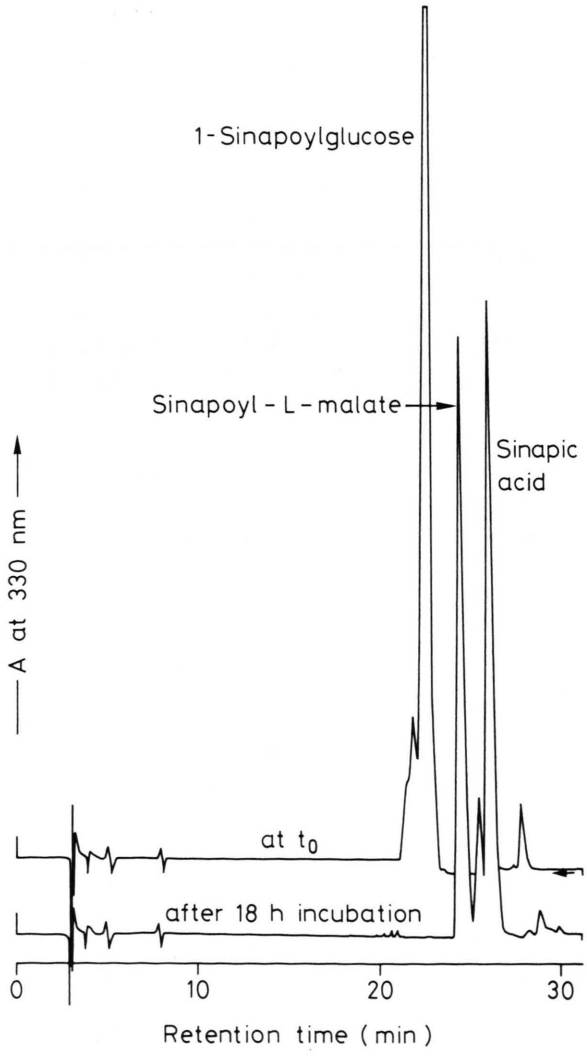
Results and Discussion

When protein preparations from 6–7 day-old *Raphanus* cotyledons were incubated with 1-sinapoylglucose and L-malate, a product was formed which could not be distinguished from authentic sinapoylmalate (Table I). After 18 h of incubation time, ca. 40% of 1-sinapoylglucose was converted to sinapoylmalate, whereas 60% was hydrolyzed to give free sinapic acid. The latter indicates the metabolic activity of 1-sinapoylglucose [9]. Fig. 1 shows HPLC analyses of the assay at t_0 and after 18 h incubation.

Table I. Spectral properties and chromatographic behaviour of sinapoylmalate [11] and a product formed by a *Raphanus* protein preparation, which was incubated with 1-sinapoylglucose and L-malate.

Compound	Spectral analysis λ_{max} in CH_3OH, nm		TLC R_F (× 100) [b]				Polyamide-CC [8] Elutable with			HPLC [c]
	alone	+ NaOH [a]	CAW	BAW	TAW	H_2O	H_2O	CH_3OH	0.025% NH_4OH in CH_3OH	t_R [sec]
Reaction product	324	385	82	79	24	90	–	–	+	1603
Sinapoylmalate	322	384	82	79	24	90	–	–	+	1602

[a] 0.1 ml 0.25 N aqueous NaOH to 0.9 ml methanolic solution of the compound.
[b] Determined on microcrystalline cellulose (Avicel): CAW, chloroform-acetic acid-water (3:2, water saturated); BAW, *n*-butanol-acetic acid-water (6:1:2); TAW, toluene-acetic acid-water (2:1, water saturated).
[c] See Fig. 1.



Protein preparations which were not subjected to $(NH_4)_2SO_4$ fractionation showed ca. 85% hydrolysis.

In assays without protein or containing heat denatured protein no product was formed. D-malate was unable to accept sinapic acid in the sinapoyltransferase reaction.

In an enzyme assay to determine glucosyltransferase activity [5], to which a protein preparation from 6–7 day-old cotyledons and L-malate were added (experiment *b*), we observed the conjugation of ca. 4% sinapic acid with L-malate, obviously *via* the energy-rich acyl glucoside intermediate.

We might deduce from the results described in this paper and in ref. [5] and [12] that in cotyledons of *Raphanus sativus* the following reaction sequence leads to the formation of sinapoyl-L-malate:

(1) sinapoylcholine
→ sinapic acid + choline

(2) sinapic acid + UDP-glucose
→ 1-sinapoylglucose + UDP

(3) 1-sinapoylglucose + L-malate
→ sinapoyl-L-malate + glucose.

Fig. 1. HPLC analyses of an enzyme assay with a protein preparation from *Raphanus* cotyledons, 1-sinapoylglucose, and L-malate at t_0 and after 18 h incubation. The chromatograph used was Spectra Physics (Santa Clara, Cal., USA) and is described in ref. [14]. The applied column (250×4 mm) was prepacked with LiChrosorb RP-8 (5 µm) (Merck, Darmstadt). Separation was accomplished by gradient elution: in 40 min linear from solvent A (0.5% acetic acid in water) to 50% solvent B (acetonitrile) in A+B. The flow-rate was 1 ml/min and detection was achieved at 330 nm with a Schoeffel SF 770 UV-VIS detector (Kratos Instr., Trappenkamp). 20 µl of incubation medium was directly injected without any pretreatment.

The alternative biochemical mechanism for the synthesis of sinapoylmalate, proceeding *via* a sinapoyl-coenzyme-A-ester intermediate, which would be dependent on the reversibility of the *in vivo* formation of 1-sinapoylglucose by a glucosyltransferase [5], seems to be unlikely since all attempts failed so far to demonstrate any sinapoyl CoA-ligase activity.

Experiments are underway to purify the sinapoyltransferase activity for enzyme characterization, and further studies have to show the specificity of the transacylase. It should be of interest to study other systems for their capability of an enzymatically catalyzed acylation by a 1-O-acyl sugar.

Acknowledgement

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