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PURIFICATION AND PROPERTIES OF 1-(HYDROXYCINNAMOYL)-GLUCOSE: 1-(HYDROXYCINNAMOYL)-GLUCOSE HYDROXYCINNAMOYLTRANSFERASE FROM RADISH SEEDLINGS

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Key Word Index—*Raphanus sativus*; Brassicaceae; radish; hydroxycinnamic acid; phenylpropanoid metabolism; 1-*O*-acyl glucoside; acyltransferase.

Abstract—A hydroxycinnamoyltransferase (EC 2.3.1.—) which catalyses *in vivo* the formation of 1,2-di-*O*-sinapoyl-β-D-glucose has been purified 240-fold from cotyledons of 5-day-old dark-grown seedlings of radish (*Raphanus sativus* L. var. *sativus* cv. Saxa). The enzyme catalysing a reaction between two identical substrate molecules used 1-*O*-(hydroxycinnamoyl)-β-D-glucose both as acyl donor and acceptor molecule and exhibited a strict specificity of transfer to the C-2 hydroxyl group of the acceptor. It had an apparent M_r of 55 000 and showed greatest activity at pH 8.0 (50% at pH 7.0 and 9.0) and 42°. Apparent energy of activation was found to be 62 kJ/mol. There was no requirement for divalent cations or thiols. The Michaelis curve did not show a rectangular hyperbola but was slightly sigmoid. A linear curve was obtained on replotting the Michaelis curve according to Hanes, using squared values of substrate concentrations. The $S_{0.5}$ for 1-sinapoyl-, 1-feruloyl- and 1-(*p*-coumaroyl)-glucose were 0.42, 0.40 and 0.58 mM, respectively, and the ratios of the $V_{max}/S_{0.5}$ values were 100:92:45.

INTRODUCTION

When grown in the dark, seedlings of radish (*Raphanus sativus* L. var. *sativus*) accumulate 1,2-di-*O*-sinapoyl-β-D-glucopyranose in their cotyledons [1]. Recently we described the enzymatic synthesis of this di-ester from 1-*O*-sinapoyl-β-D-glucose by a protein preparation from cotyledons of radish grown in the dark [2]. This activity can be classified as 1-(hydroxycinnamoyl)-glucose: 1-(hydroxycinnamoyl)-glucose hydroxycinnamoyltransferase (CGT), catalysing *in vivo* the synthesis of the di-sinapoylglucose.

$$2 \times \text{1-Sinapoylglucose} \xrightarrow{\text{CGT}} \text{1,2-Di-sinapoylglucose} + \text{Glucose}$$

Such a di-ester formation in phenylpropanoid metabolism has also been demonstrated to occur in the biosynthesis of di-galloylglucose [3] and a similar reaction has been shown to be realized in the formation of dicaffeoylquinic acid [4]. From a thermodynamic point of view this type of enzyme-catalysed transacylation represents an interesting mechanism of ester formation in plant metabolism and might be more widespread than known to date.

We have now partially purified the CGT from radish cotyledons and investigated some of its physical and kinetic properties.

RESULTS AND DISCUSSION

Occurrence of enzyme activity

Cotyledons of radish exhibit activities of 1-*O*-acyl-glucose dependent acyltransferases, 1-sinapoylglucose: L-malate sinapoyltransferase (SMT) [5] and 1-(hydroxycinnamoyl)-glucose: 1-(hydroxycinnamoyl)-glucose hydroxycinnamoyltransferase (CGT) [2; this paper], showing contrary developments depending on light conditions. Light-grown seedlings showed high SMT and low CGT activities, while dark-grown seedlings showed low SMT and high CGT activities. These differential enzyme activities have been reflected by the light-dependent accumulations of their products, *O*-sinapoyl-L-malate and 1,2-di-*O*-sinapoyl-β-D-glucose [1]. Concomitantly with the accumulation of disinapoylglucose, CGT activity increased until day 6 in dark-grown seedlings and reached *ca* 6 pkat/cotyledon pair. In contrast, light-grown seedlings reached only 1 pkat/cotyledon pair. Figure 1 illustrates the CGT activity determined by HPLC. In general we observed high activities of this enzyme, when the trans-esterification of 1-sinapoylglucose to sinapoyl-L-malate, 'normally' proceeding in the radish cotyledons, was suppressed.

Enzyme purification

The CGT activity was purified 240-fold with a recovery of 20% by means of molecular exclusion and ion exchange chromatography. Table 1 summarizes the purification steps of the protein preparation and Fig. 2 shows ion exchange column chromatography of the enriched

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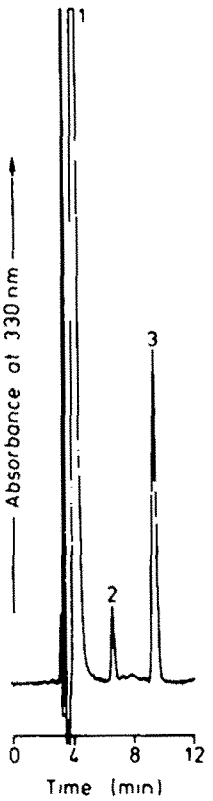

Fig. 1. HPLC analysis of a standard assay of the partially purified CGT. 1, 1-Sinapoylglucose; 2, sinapic acid; 3, di-sinapoylglucose. 20 µl were injected onto the column (RP-8, 250 × 4 mm) and developed isocratically with 70% solvent B (1% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1% H_3PO_4 in H_2O) at a flow rate of 1 ml/min. Detection was at 0.1 absorbance unit full scale (330 nm).

CGT activity. The M_r of the CGT determined by molecular exclusion on Ultrogel AcA 44 was near 55 000 (Fig. 3). Slab gel SDS-PAGE showed 1 major (CGT) and a few very minor protein bands after staining with Coomassie Brilliant Blue R250. The mobility of the enzyme as compared to reference proteins also indicated that the M_r was near 55 000. The partially purified CGT showed no loss of the initial activity when stored at −20° in the presence of BSA for at least 1 year.

Contaminating high esterase activities towards 1-sinapoylglucose observed in crude protein extracts [2] were eliminated during the purification procedure. However, compared with the maximal rate of di-ester formation, 10% of the sinapic acid was liberated (Fig. 1). This may be attributed to the CGT-'hydrolase activity'. Since only the 1,2-di-ester was formed there is a strict specificity of acyl transfer to the C-2 hydroxyl group of the acceptor-glucose molecule.

Properties of enzyme activity

General properties. The formation of di-sinapoylglucose was positively correlated with protein, and was linear with time up to 4 hr. Greatest enzyme activity was found at pH 8.0 in HEPES buffer showing 50% of activity near pH 7.0 and 9.0 (Fig. 4). Maximal initial reaction velocities were obtained at 42° and the apparent activation energy was found to be at 62 kJ/mol. The Arrhenius plot showed no discontinuity.

Divalent cations at concentrations above 5 mM inhibited the enzyme activity. In the presence of Mg^{2+}, Ca^{2+} and Co^{2+} (10 mM each), 65%, 45% and 0% of the control activities, respectively, were observed. There was also an inhibition when thiols such as DTT or 2-mercaptoethanol, were included showing almost total inhibition at 1 mM.

Kinetics and substrate specificity. Figure 5 shows the effect of 1-sinapoylglucose concentration on CGT activity resulting in a slightly sigmoid curve. Sigmoidal concentration dependence is not an inherent property of bi-substrate reactions with identical molecules as seen with squalene synthetase [6] or fructosyltransferase [7]. Therefore we tested the CGT activity for possible cooperativity and random mechanism. Hill plots gave the following data: V_{max} = 4.9 nkat/mg protein; $S_{0.5}$ = 0.51 mM; ^nH = 1.64. Using these data, a recalculation of the graph in Fig. 4 gave a significantly incongruent curve. Assuming however a random mechanism of CGT activity, sigmoid Michaelis and parabolic Lineweaver Burk curves could be expected [8, 9]. Plotting S^2 *vs.* v and S^2 *vs.* S^2/v (Hanes plot) [10], rectangular hyperbolic and linear curves, respectively, were obtained. The CGT catalysed reaction was found to be irreversible. This and the fact that we could not distinguish between donor and acceptor molecules prevented more detailed investigations of the enzymatic reaction mechanism as has been possible in studies on adenylate kinase [11, 12]. Summarizing our kinetic results, a random bi-bi mechanism of CGT activity is likely.

By means of the Hanes plot we determined some kinetic parameters. The $S_{0.5}$ values for 1-sinapoyl-, 1-feruloyl-

Table 1. Purification of CGT from *Raphanus sativus* cotyledons

Purification step	Total protein (mg)	Total activity* (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification (-fold)
Crude extract	569	8.92	0.0157	100	
$(NH_4)_2SO_4$, heat	119	3.06	0.0257	34	1.6
Ultrogel AcA 44	14.5	3.15	0.217	35	13.8
CM-Sepharose	0.472	1.78	3.77	20	240

*At 1 mM 1-sinapoylglucose.

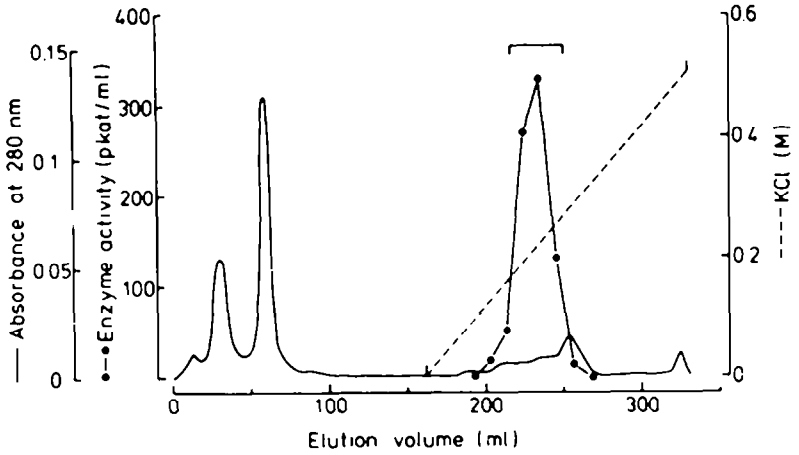

Fig. 2. Chromatography of CGT on a CM-Sepharose column (1.6 × 16.7 cm) using 170 ml KPi buffer (pH 7) and a linear KCl gradient; 9.6-ml fraction volume; pooled fractions of CGT activities indicated by the bar.

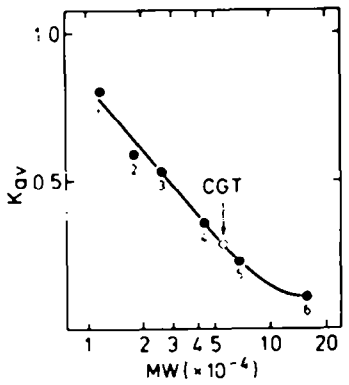

Fig. 3. Determination of M_r of CGT by means of filtration through an Ultrogel AcA-44 column (1.6 × 95 cm). Markers: 1, cytochrome C; 2, myoglobin; 3, chymotrypsinogen; 4, ovalbumin; 5, bovine serum albumin; 6, aldolase.

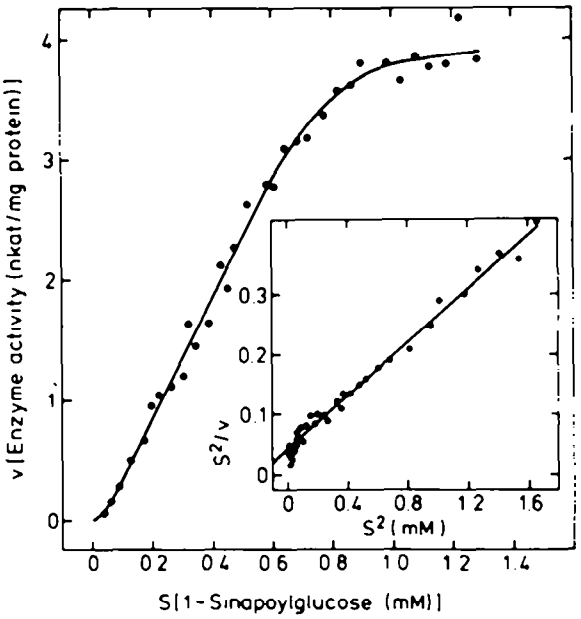

Fig. 5. Effect of 1-sinapoylglucose concentration on CGT activity. Inset shows replotted data according to Hanes [10] using squared values of substrate concentrations.

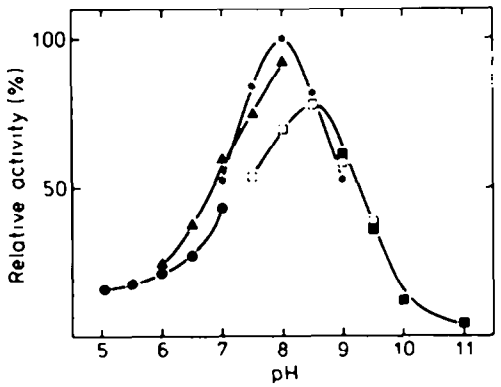

Fig. 4. Effect of pH on CGT activity. (●), MES; (▲), KPi; (○), HEPES; (□), TRICINE; (■), glycine.

and 1-(*p*-coumaroyl)-glucose were 0.42, 0.40 and 0.58 mM, respectively, and the ratios of the $V_{max}/S_{0.5}$ values were found to be 100:92:45. In contrast to this broad specificity towards C_6–C_3 acid esters the enzyme exhibited no activity with 1-*O*-acyl glucosides of C_6–C_1 acids, such as 1-benzoyl- or 1-galloylglucose tested. We propose to classify the enzyme as 1-(hydroxycinnamoyl)-glucose: 1-(hydroxycinnamoyl)-glucose hydroxycinnamoyltransferase (CGT; EC 2.3.1.—).

EXPERIMENTAL

Plant material. Seeds of radish (*Raphanus sativus* L. var. *sativus* cv. Saxa) were purchased from Zwaan & Co Samenzucht GmbH.

Kleve, West Germany, and seedlings grown in a defined soil (type T, Balster, Frôndenberg, West Germany, mixed 1:1 with peat) in a phytotron under fluorescent light (*ca* 10000 lx) with a 14 hr day, or in darkness at 22° and 70% relative humidity.

Substrates. 1-Sinapoylglucose (1-*O*-sinapoyl-β-D-glucopyranose) [13] and 1-feruloylglucose were isolated from 3-day-old radish seedlings; 1-(*p*-coumaroyl)-glucose was from petals of *Antirrhinum majus* [14]. 1,2-Di-*O*-sinapoyl-β-D-glucopyranose came from dark-grown radish seedlings [1]. For extraction and chromatography procedures, see refs [1, 15]. 1-Benzoylglucose and 1-galloylglucose were gifts from Profs. W. Barz (Münster, West Germany) and G. G. Gross (Ulm, West Germany), respectively.

Enzyme preparation. Step i: cotyledons (*ca* 140 g) from 5-day-old dark-grown radish seedlings were frozen with liquid N_2 and ground in a pre-cooled mortar in the presence of 10 g insoluble Polyclar AT and 600 ml KPi buffer (100 mM, pH 7). The homogenate was poured into a pre-cooled beaker, allowed to stand for 40 min with continuous stirring, then passed through Miracloth, and the filtrate centrifuged at 14000 *g* for 40 min. *Step ii*: solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 35% satn. The ppt was removed by centrifugation (20 min at 14000 *g*) and the supernatant was raised to 65% satn. The pptd protein was collected by centrifugation (20 min at 48000 *g*). The protein was dissolved in 34 ml KPi buffer (100 mM, pH 7) and the soln dialysed against the same buffer. *Step iii*: the dialysed protein soln was centrifuged (20 min at 48000 *g*) and the clear supernatant was heated to 60° for 10 min. Denaturated protein was removed by centrifugation (20 min at 48000 *g*) and the clear supernatant, enclosed in a dialysis bag, was concd to 7 ml by treatment with dry Sephadex G-200. *Step iv*: the soln was applied to an Ultrogel AcA-44 column (95 × 1.6 cm) and the protein eluted with KPi buffer (100 mM, pH 7) at a flow rate of 9.6 ml/hr. Three-ml fractions were collected, and those containing high enzyme activities were combined and dialysed against citric acid buffer (50 mM, pH 5). *Step v*: the dialysed enzyme was chromatographed on a CM-Sepharose column (16.7 × 1.6 cm). The column was washed with 170 ml citric acid buffer (50 mM, pH 5) before the following linear gradient was applied: 150 ml 0–500 mM KCl in citric acid buffer at a flow rate of 30 ml/hr. Fractions (9.6 ml) were collected and those containing high enzyme activities pooled. The protein was concd by $(NH_4)_2SO_4$ pptn (70% satn) and after centrifugation at 48000 *g* for 20 min dissolved in 5 ml KPi buffer (100 mM, pH 7). The soln was dialysed, bovine serum albumin added (3 mg/ml) and stored at −20°.

Standard enzyme assay and activity determination. The standard reaction mixture contained 1 mM 1-sinapoylglucose, 40 mM HEPES buffer (pH 8) and 10 μl protein soln (20-fold diluted from the stored soln) in a total vol. of 100 μl. This mixture was incubated at 30° for 1 hr and the reaction stopped by transferring to a freezer (−20°). Enzyme activities were determined by HPLC as described previously [2] (Fig. 1). 1,2-Di-sinapoylglucose isolated from dark-grown radish seedlings [1] was used as the standard for quantification. Identification of 1,2-di-sinapoylglucose as reaction product was described elsewhere [2]. Other di-esters formed were tentatively identified by TLC and by chromatographic behaviour in HPLC relative to that of the applied substrates and the native product 1,2-di-*O*-sinapoyl-β-D-glucopyranose.

Protein estimation. Protein contents were determined by the method of ref. [16] using BSA as standard.

Gel electrophoresis. Sodium dodecyl sulphate-gel electrophoresis (slab gel SDS-PAGE) was performed according to ref. [17].

M_r *determination.* The M_r of the enzyme was determined on an Ultrogel AcA-44 column (see above) equilibrated with 0.1 M KPi buffer (pH 7) and by slab gel SDS-PAGE [17] using the following reference proteins (Serva, Heidelberg, West Germany) as standards [18]: cytochrome *c* (12300), myoglobin (17800), chymotrypsinogen (25000), ovalbumin (45000), bovine serum albumin (67000) and aldolase (160000). $K_{av} = (V_e - V_0)/(V_t - V_0)$. V_0 was estimated using Blue Dextran 2000 (Pharmacia) and V_t using DNP-alanine (Serva).

pH *Optimum.* The following buffer systems (each 50 μS equivalent to 0.1 M HEPES buffer, pH 8) were used to determine the pH optimum of CGT activities: MES, 2-(*N*-morpholino)ethane sulphonic acid (pH 5–7); KPi (pH 6–8); HEPES, *N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulphonic acid (pH 7–9); TRICINE, *N*-tris-(hydroxymethyl)-methylglycine (pH 7.5–9.5); glycine (pH 9–11).

Energy of activation. Initial velocities of enzyme-catalysed reactions were determined at different temps (4–70°). The apparent energy of activation was determined from the linear low temp. section of the Arrhenius plot.

Kinetic properties. Apparent $S_{0.5}$ and V_{max} values were graphically estimated according to ref. [10] using squared values of substrate concns.

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REFERENCES

1. Strack, D., Dahlbender, B., Grotjahn, L. and Wray, V. (1984) *Phytochemistry* **23**, 657.
2. Dahlbender, B. and Strack, D. (1984) *J. Plant Physiol.* **116**, 375.
3. Gross, G. G. (1983) *Z. Naturforsch. Teil C* **38**, 519.
4. Kojima, M. and Kondo, T. (1985) *Agric. Biol. Chem.* **49**, 2467.
5. Strack, D. (1982) *Planta* **155**, 31.
6. Beytia, E., Quershi, A. A. and Porter, J. W. (1973) *J. Biol. Chem.* **248**, 1856.
7. Singh, R. and Bhatia, I. S. (1971) *Phytochemistry* **10**, 495.
8. Dixon, M. and Webb, E. C. (1979) *Enzymes*, 3rd edn. Longmans, London.
9. Noda, L. (1962) in *The Enzymes* (Boyer, P. D., Lardy, H. and Myrbäck, K., eds) Vol. 6, pp. 139–149. Academic Press, New York.
10. Hanes, C. S. (1932) *Biochem. J.* **26**, 1406.
11. Rhoads, D. G. and Lowenstein, J. M. (1968) *J. Biol. Chem.* **243**, 3963.
12. Thuma, E., Schirmer, R. H. and Schirmer, J. (1972) *Biochim. Biophys. Acta* **268**, 81.
13. Linscheid, M., Wendisch, D. and Strack, D. (1980) *Z. Naturforsch. Teil C* **35**, 907.
14. Harborne, J. B. and Corner, J. J. (1961) *Biochem. J.* **81**, 242.
15. Strack, D. (1977) *Z. Pflanzenphysiol.* **84**, 139.
16. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
17. Neville, D. M., Jr. and Glossmann, H. (1974) in *Methods in Enzymology*, Vol. 32, pp. 92–102, Academic Press, London.
18. Andrews, P. (1965) *Biochem. J.* **96**, 595.