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PARTIAL PURIFICATION AND PROPERTIES OF UDP-GLUCOSE: VANILLATE 1-*O*-GLUCOSYL TRANSFERASE FROM OAK LEAVES

GEORG G. GROSS

Universität Ulm, Abteilung Allgemeine Botanik, D-7900 Ulm, West Germany

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Abstract—An enzyme from leaves of red oak (*Quercus rubra*) which catalyses the esterification of various phenolic acids with the glucose moiety of UDPG has been purified *ca* 45-fold. This transferase has a MW of 68 000 and a broad pH optimum of 6.5–7.0. UDP-glucose has been found to act exclusively as the donor molecule. Benzoic acids and, at significantly lower rates, cinnamic acids are utilized as acceptor molecules. The preferred substrates are vanillic acid (K_m = 0.57 mM), veratric acid (0.72 mM), gallic acid (1.11 mM) and *p*-hydroxybenzoic acid (3.45 mM). Reversibility of the reaction has been demonstrated by the enzymatic formation of UDP-glucose from β-glucogallin (1-*O*-galloyl-β-D-glucose) and UDP. In spite of the fact that this new enzyme has to be designated, according to its best substrate, as UDP-glucose: vanillate 1-*O*-glucosyl transferase (EC 2.4.1.—), it is concluded that its physiological role is the formation of β-glucogallin, the putative first intermediate in the biosynthesis of gallotannins.

INTRODUCTION

Several dicotyledonous plant families are characterized by the frequently abundant production of hydrolysable tannins, i.e. polygalloyl derivatives of a central polyol moiety (usually β-D-glucopyranose). The results obtained to date by a multitude of investigations show a fairly detailed picture of the chemistry and taxonomic distribution of these natural products (for recent reviews see [1–6]). Comparatively little data, in contrast, are available on the biosynthesis of these complex phenolic compounds. It is assumed that their biogenesis is initiated by the esterification of gallic acid and glucose, yielding β-glucogallin (1-*O*-galloyl-β-D-glucose). This first intermediate is thought to undergo gradual substitution reactions which finally lead to pentagalloyl-β-D-glucose, the parent compound of gallotannins and the related ellagitannins (cf. [5, 6]).

Such esterification reactions would necessarily depend on the participation of activated intermediates. This requirement could be met by the utilization of energy-rich acyl derivatives, as has been shown for the cinnamoyl-CoA dependent formation of chlorogenic acid and related depsides [7–10]. On the other hand, it is well documented that the energy required in the biosynthesis of glucose esters of various aromatic acids is provided by UDP-glucose [11–17].

With respect to β-glucogallin, it has been reported recently [18] that cell-free extracts from oak leaves catalyse the synthesis of this compound in accordance with the latter reaction type, i.e. by the transfer of the glucose moiety of UDP-glucose to free gallic acid. The purification and characterization of this enzyme are described in this communication in more detail.

RESULTS

Enzyme purification

The glucosyl transferase was extracted from leaves of young *Q. rubra* plants which had been grown in a greenhouse. Serious problems arose from the abundant quantities of highly viscous mucilage present in the crude extracts which made it impossible to employ the usual enzyme purification techniques and caused extensively varying, irreproducible results. We finally discovered that the enzyme could be separated from the disturbing contaminants by adsorption on $CaPO_4$ gel and subsequent elution with K-Pi buffer. Although this procedure resulted in appreciable losses of enzyme activity, it enabled us to purify the enzyme further by ammonium sulphate fractionation, gel-filtration and chromatography on anion-exchange cellulose. The results of a representative purification experiment are summarized in Table 1. Dilute enzyme solutions, particularly after the last purification step, proved to be unstable. The enzyme could be stabilized by immediate concentration and storage at 0–4°; under these conditions, no significant losses of activity were observed over a period of 1–2 weeks.

General properties of the glucosyl transferase

Under standard assay conditions (see Experimental) and with vanillic acid as substrate, the glucosyl transferase reaction was linear with respect to protein concentration up to at least 7 μg (1.5 pkat) of purified enzyme per assay. Similarly, linearity of the reaction was maintained for at least 40 min under these conditions. The pH dependence of the enzyme was determined with vanillic and gallic acids as substrates. In both cases, a broad optimum at pH 6.5–7.0 was observed (K-Pi or Tris–HCl buffer); half-maximal activities were found at pH 4.7 (NaOAc buffer) and pH 9.0 (glycine–NaOH buffer). These values are in accordance with those obtained previously using crude enzyme preparations [18]. Variation of the incubation temperature revealed a linear increase of the reaction rate between 25° and 40° and an optimum at 42°. From these data, an average activation energy of 8.4 kcal/mol was

Table 1. Purification of UDPG: vanillate glucosyl transferase*

Step	Total protein (mg)	Total activity (nkat)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)
Crude extract	988	4.28	4.3	1	100
Dowex supernatant	606	4.32	7.1	1.7	101
$CaPO_4$-gel eluate	180	1.08	6.0	2.4	25
$(NH_4)_2SO_4$, 35–65% ppt.	74.2	0.71	9.6	2.2	17
Sephadex G-200	12.5	0.66	52.8	12.3	15
DEAE cellulose	2.2	0.43	195.5	45.5	10

*Enzyme activities were determined with gallic acid as substrate.

calculated, which corresponds to a Q_{10} value of 1.6. From gel-filtration experiments with a calibrated Sephadex G-200 column [19], an apparent MW of 68 000 was estimated for the enzyme.

Substrate specificity

It has been reported previously that the enzyme catalyses the transfer of the glucose moiety of UDPG, and a K_m value of 2.3 mM was determined for this co-enzyme [18]. A closer analysis of the donor specificity with purified glucosyl transferase revealed an absolute requirement for UDP-glucose. No reaction occurred when this cofactor was substituted by ADP-, CDP-, GDP- or TDP-glucose. Consistent with this result, the reaction product UDP, in contrast to ADP, strongly inhibited the formation of glucose esters (Table 2). The observed pronounced specificity for UDP-glucose was confirmed in further studies, which showed that also no transfer of the carbohydrate moiety of UDP-galactose, UDP-galacturonic acid, UDP-glucuronic acid, UDP-mannose, UDP-xylose and UDP-*N*-acetylglucosamine was catalysed by the enzyme.

The results from experiments on the acceptor specificity with various benzoic and cinnamic acids as potential substrates are summarized in Table 3. It is evident from these data that benzoic acids represent the preferred substrates. Among these, the highest reaction rates were found, in decreasing order, with vanillate, veratrate, gallate and *p*-hydroxybenzoate. Normal Michaelis–Menten kinetics were observed with these four substrates; their K_m values, as calculated from Lineweaver–Burk plots, parallel the above sequence.

No reaction occurred in the presence of phenylacetate, D(+)-quinate and shikimate.

Table 2. Effect of UDP and ADP on the activity of UDPG: vanillate glucosyl transferase

Nucleoside diphosphate (mM)	Inhibition relative to control (%) in the presence of	
	UDP	ADP
0.1	8	0
1.0	75	9
10.0	100	27

Determinations were conducted under standard assay conditions with vanillic acid as substrate.

Table 3. Acceptor specificity of UDPG glucosyl transferase

Substrate	Relative activity* (%)	K_m (mM)	R_f value of product†
Benzoic acids			
Benzoate	16	—	0.68
Salicylate	0	—	—
p-Hydroxybenzoate	48	3.45	0.66
Anisate	38	—	0.71
Protocatechuate	22	—	0.53
Vanillate	100	0.57	0.63
Veratrate	71	0.72	0.66
Gallate	52	1.11	0.49
3,4,5-Trimethoxybenzoate	7	—	0.60
Cinnamic acids			
Cinnamate	9	—	0.74
o-Coumarate	2	—	0.69
m-Coumarate	14	—	0.68
p-Coumarate	32	—	0.72
Caffeate	4	—	0.51
Ferulate	35	—	0.70
Sinapate	13	—	0.62

*Determinations were conducted under standard assay conditions at 2 mM final concentration of the various acids. Relative activities are compared to the reaction rate observed with vanillate equal to 100%.

†TLC, Si gel (Sil N-HR; Macherey-Nagel), solvent: EtOAc–MeCOEt–formic acid–H_2O (5:3:1:1).

Reaction product

It has been reported previously [18] that the reaction product from UDP-glucose and gallic acid does not cochromatograph with 4-*O*-β-D-glucosidogallic acid, indicating that the enzyme does not catalyse the synthesis of phenolic glucosides but specifically forms glucose esters. This conclusion has been supported by recent substrate-specificity studies (see above), which clearly show that the enzyme exhibits considerable affinity also towards acids lacking free OH groups.

Concerning the question of the position of the glucose moiety which is esterified, evidence based on TLC and HPLC analyses has been presented [18] that this reaction occurs at the 1-hydroxyl function and thus is in accordance with the specificity of related phenolic acid glucosyl transferases [11, 14–17]. This interpretation was con-

firmed by studies with benzoyl-[U-^{14}C]glucose isolated chromatographically from a scaled-up enzyme assay mixture. It was found that this compound was cleaved nearly quantitatively, yielding benzoic acid and labelled glucose, under conditions which selectively hydrolyse the energy-rich linkage of 1-*O*-benzoylglucose [20, 21].

Further proof of the enzymatic formation of 1-*O*-acyl glucose esters arose from studies on the reversibility of the glucosyl transferase reaction. For this purpose, chemically synthesized β-glucogallin, UDP and enzyme were incubated in Tris–HCl buffer (pH 7) at 35° for 7 hr. Enzymatic analysis of the deproteinized reaction mixture with UDPG-dehydrogenase [22] revealed that *ca* 20% of the substrates had been converted to UDP-glucose under these conditions, whereas no reaction occurred in the absence of enzyme.

DISCUSSION

The properties of the enzyme described herein reveal it to be a typical glucosyl transferase which specifically catalyses the freely reversible formation of 1-*O*-acyl esters of β-D-glucose in the presence of UDP-glucose and various phenolic acids. Substrate-specificity studies indicate that this enzyme utilizes benzoic acids as the preferred acceptor molecules. According to the best substrate among these, the systematic name UDP-glucose: vanillate 1-*O*-glucosyl transferase (EC 2.4.1.—) is proposed.

Similar enzymes have been isolated from a variety of plants. 1-*O*-Anthraniloyl glucose was formed with extracts from *Lens esculentum* [23]. 1-*O*-Sinapoyl glucose was produced with enzyme preparations from *Brassica oleracea* [11] or *Raphanus sativus* [15, 16]. Other reports deal with the esterification of indolylacetic acid [14] and of various cinnamic (particularly hydroxycinnamic) and benzoic acids [11–13, 15, 17].

Comparison of the properties of the above enzymes, particularly of their pH optima and substrate specificities (as far as detailed data have been reported), reveals significant differences with the glucosyl transferase described here. It would appear that the physiological role of this newly described enzyme is to provide β-glucogallin, the presumed first intermediate in the biogenesis of gallotannins. The fact that this enzyme has a higher affinity towards vanillate and veratrate than towards the substrate gallate with its extremely troublesome 3,4,5-trihydroxy structure is not necessarily contradictory to this assumption if one considers the frequently observed unspecificity of plant enzymes.

It will be interesting to examine by what mechanisms the additional galloyl residues are attached to β-glucogallin in the subsequent steps leading to gallotannins. One possibility would be the participation of carboxyl-activated gallic acid derivatives, for example the recently synthesized galloyl-CoA [24]. As an alternative, advantage could be taken from the fact that β-glucogallin itself represents an energy-rich compound whose group-transfer potential could be used in transacylation reactions, as has been shown recently for 1-*O*-indolylacetyl glucose [14] and 1-*O*-sinapoyl glucose [25, 26]. In any event, the glucosyl transferase described here provides a tool for the convenient preparation of radioactively labelled β-glucogallin which should facilitate further studies on these questions.

EXPERIMENTAL

Chemicals. β-Glucogallin was synthesized as described previously [18]. [U-^{14}C]Gallic acid was a gift from Prof. N. Amrhein (Bochum). UDP-D-[U-^{14}C]glucose was purchased from Amersham. $CaPO_4$-gel was prepared according to ref. [27].

Enzyme purification. Unless otherwise stated, all operations were carried out at 0–4° and all buffers were supplemented with 5 mM 2-mercaptoethanol. Leaves (80 g) from 3- to 4-month-old *Q. rubra* plants grown in a greenhouse were frozen in liquid N_2 and homogenized in an ultracentrifugal mill (Retsch KG, Haan). The frozen powder was mixed with 80 g prewashed PVP and extracted with mechanical stirring for 30 min with a mixture of 100 ml 0.1 M borate buffer (pH 7.5) and 150 ml 1 M Tris–HCl buffer (pH 8) plus 20 mM 2-mercaptoethanol. After centrifugation (35 000 *g*, 20 min), the supernatant was stirred for 15 min with 8 g Dowex 1 × 4 (100–200 mesh, borate form) and centrifuged. $CaPO_4$-gel (5 mg/mg protein) was added dropwise to the supernatant. After stirring for 15 min, the gel was collected by centrifugation (6000 *g*, 5 min) and eluted with 3 × 60 ml 0.1 M K-Pi buffer, pH 7.5. The eluate was fractionated with solid $(NH_4)_2SO_4$; the 35–65% ppt. was redissolved in a minimal vol. of 50 mM Tris–HCl buffer, pH 7.5 and clarified by centrifugation. This soln was fractionated on a Sephadex G-200 column (2.5 × 40 cm) equilibrated in the same buffer. The most active fractions were combined and applied to a column of DEAE-cellulose (Whatman DE-52, 0.9 × 3 cm) in the same buffer. After washing with 0.05 M KCl in buffer, the enzyme was eluted with 0.17 M KCl. The active fractions were pooled, concd by ultrafiltration through Amicon PM-10 membranes and stored at 0–4°.

Protein determinations. These were made turbidimetrically by precipitation with trichloroacetic acid [28]; very dilute solns were measured by UV-photometry [29].

Enzyme assay. Enzyme activities were measured in standard assay mixtures (25 μl vol.) containing 2.5 μmol Tris–HCl buffer (pH 7), 75 nmol gallic acid, 75 nmol (0.025 μCi) UDP-D-[U-^{14}C]glucose and appropriate amounts of protein. When UDPG was substituted by other cofactors, [U-^{14}C]gallic acid was used as labelled substrate. After incubation for 30 min (or appropriately reduced periods of time when using very active enzyme preparations) at 30°, the reaction was stopped by the addition of 0.5 μmol (10 μl) β-glucogallin as carrier and 5 μl 1 M HCl. An aliquot (25 μl) of the deproteinized soln was spotted on Si-gel coated plastic-sheets (Sil N-NR, Macherey-Nagel, Düren) and chromatographed (cf. Table 3). β-Glucogallin was located under UV-light; the position of products formed from other substrates was determined with the thin-layer scanner II (Berthold, Wildbad). The respective areas were cut out and their radioactivity was measured by liquid scintillation in a toluene-based PPO–POPOP scintillator (Liquifluor, NEN). Reaction rates were calculated from the counted cpm after correction for counting efficiency and the final vol. (40 μl) of the assay mixture.

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