Biosynthesis of Gallotannins. β-Glucogallin-Dependent Galloylation of 1,6-Digalloylglucose to 1,2,6-Trigalloylglucose

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Z. Naturforsch. **46c**, 389–394 (1991); received February 5, 1991

β-Glucogallin (1-O-galloyl-β-D-glucopyranose), 1,6-Di-O-galloyl-β-D-glucopyranose, 1,2,6-Tri-O-galloyl-β-D-glucopyranose, Galloyltransferase, Gallotannin Biosynthesis, *Rhus typhina* (sumach)

An enzyme from leaves of sumach (*Rhus typhina*) was partially purified that catalyzes the β-glucogallin (1-O-galloylglucose)-dependent galloylation of 1,6-digalloylglucose, thus forming 1,2,6-trigalloylglucose and free glucose. This acyltransferase had a molecular weight of *ca.* 750,000 and a pH optimum at 5.0–5.5. Besides β-glucogallin (K_m = 3.9 mM), also related 1-O-phenylcarboxylglucoses acted as acyl donors. On the other hand, the acceptor substrate, 1,6-digalloylglucose (K_m = 0.9 mM), could only be replaced by 1,6-diprotocatechuoylglucose (relative activity 46%); however, also tri-, tetra-, and pentagalloylglucoses were galloylated. A pronounced stimulation of the enzymatic reaction was observed upon addition of penta- or hexagalloylglucose into the assay mixtures. The systematic name "β-glucogallin: 1,6-di-O-galloylglucose 2-O-galloyltransferase" (EC 2.3.1.–) is proposed for the enzyme.

Introduction

Current investigations on the biosynthesis of 1,2,3,4,6-pentagalloylglucose, the common precursor of gallotannins and the related ellagitannins [1], have shown that this ester is the result of a metabolic sequence that comprises a series of highly position-specific galloylation steps and which begins with β-glucogallin (1-O-galloylglucose) as the first intermediate (for review, *cf.* [2]). This monoester is of particular importance as it functions also as the preferred acyl donor in all these transacylation reactions, though also higher substituted galloylglucoses bearing the energetically indispensable 1-O-acyl ester bond (*e.g.* 1,6-digalloylglucose) can be utilized for this purpose [3]. With respect to 1,2,6-trigalloylglucose, a committed intermediate of the pathway to pentagalloylglucose, it has been found by *in vitro* studies with cell-free extracts from sumach (*Rhus typhina*) leaves that this ester can be formed by two different reactions, either by "disproportionation" of two molecules of 1,6-digalloylglucose [3] or by the transfer of the galloyl moiety of β-glucogallin to the acceptor, 1,6-digalloylglucose [4]. Here, we describe the partial purification and properties of this latter enzyme which was found to differ significantly from the above mentioned β-glucogallin-independent 2-O-galloyltransferase.

Abbreviations: AcN, acetonitrile; HIC hydrophobic interaction chromatography; HPLC, high-performance liquid chromatography; RP, reversed phase.

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0939–5075/91/0500–0389 $ 01.30/0

Experimental

Chemicals

β-Glucogallin was synthesized chemically [5]. Enzymatic procedures were employed for the preparation of other 1-O-phenylcarboxyl-β-D-glucose esters [6] and of [U-^{14}C-glucosyl]β-glucogallin [7]. 6-O-Galloylglucose and 3,6-di-O-galloylglucose were gifts of Dr. H. Schick (University of Heidelberg, Germany). 1,6-Di-O-galloyl-β-D-glucose was isolated from rhubarb (*Rhizoma rhei*) [4] or prepared more conveniently and efficiently with an immobilized acyltransferase [8]. The latter procedure was also employed for the synthesis of 1,6-di-O-protocatechuoyl-β-D-glucose, 1,2-di-O-anisoyl-β-D-glucose, and 1,2-di-O-benzoyl-β-D-glucose. 1,2,6-Tri-O-galloyl-β-D-glucose was kindly provided by Professor G. Nonaka (Kyushu University, Fukuoka, Japan); 1,3,6-tri- and 1,2,4,6-tetra-O-galloyl-β-D-glucose were gifts of Professor E. Haslam (University of Sheffield, England). 1,2,3,6-Tetra-O-galloyl-β-D-glucose was iso-

lated from commercially available tannin (C. Roth GmbH, Karlsruhe, Germany) [9]. 1,2,3,4,6-Penta-O-galloyl-β-D-glucose was synthesized chemically [10]. Hexa- and heptagalloyl-β-D-glucoses were purified from leaves of *R. typhina* [11].

Enzyme preparation

All operations were done at 0–4 °C. Protein concentrations were determined turbidimetrically after precipitation with trichloroacetic acid [12]. Crude enzyme extract from leaves of *R. typhina* was obtained as described recently [3]. This solution was brought to 20% saturation with solid $(NH_4)_2SO_4$, stirred for 10 min, and centrifuged (15 min, 30,000 × *g*). The pH of the clear supernatant was adjusted to 6.5 with 2 N acetic acid and applied to a column (10 × 2.7 cm i.d.) with phenyl-Sepharose CL-4B (Pharmacia, Heidelberg, Germany) equilibrated in 50 mM K-phosphate buffer, pH 6.0, containing 1.25 M $(NH_4)_2SO_4$. The column was washed with this buffer-salt mixture, and then eluted stepwise with 0.625 M $(NH_4)_2SO_4$ in buffer (*ca.* 60 ml), pure buffer without salt (*ca.* 60 ml), and finally with redistilled water (10–15 ml). The enzyme activity, which was eluted in the last eluate, was pooled, concentrated by ultrafiltration (Millipore "Centrifugal Ultrafree" kit, 30,000 MW exclusion limit), and subjected to gel filtration on a Sephacryl S-200 column (40 × 3 cm i.d.; Pharmacia) equilibrated in 50 mM Na-citrate buffer, pH 5.5. The most active fractions were pooled, concentrated, and stored at 0–4 °C; under these conditions, these enzyme was found to be stable for at least two months.

Enzyme assay

Standard assay mixtures (vol. 50 µl) containing 10 µmol Na-citrate buffer, pH 5.5, 1 µmol β-glucogallin, 0.1 µmol 1,6-di-galloylglucose, and 20 µl (20–60 µg) enzyme solution were incubated at 30 °C for 0.5–2 h. After terminating the reaction with 50 µl 2 N HCl (blanks were stopped immediately at 0 h), addition of 100 µl methanol and sonication (1 min) to assure complete dissolution of reaction products, the assays were centrifuged and the clear supernatant removed. Aliquots (50 µl) of this solution were analyzed by RP-HPLC on LiChrospher 100 RP-18 (LiChroCART cartridges, Merck, Darmstadt, Germany; particle size 5 µm, 125 × 4 mm i.d.; flow rate 2 ml/min; detection UV 280 nm). Rapid and efficient separation of substrates and products was achieved by elution with an acetonitrile (AcN, solvent A)/0.05% (v/v) aq. H_3PO_4 (solvent B)-gradient: 0–3.5 min = 4% A, 3.5–4.5 min = 16% A, 4.5–7.5 min = 16–19% A, 7.5–9.5 min = 19–50% A [3]. Quantification was done with a computing integrator (Merck-Hitachi D-2500) referring to external standard solutions. In experiments with other acylglucoses, reaction products were measured at their respective absorption maxima [6, 8].

Determination of the stoichiometry

Enzyme assays were supplemented with [^{14}C-glucosyl]β-glucogallin as substrate and incubated under standard conditions for 1 h at 30 °C (blanks were stopped at 0 h reaction time). Aliquots of the deproteinized samples were subjected to RP-HPLC (see above). Complete separation of all components of interest (*cf.* Table II) was achieved with a methanol (A)/0.05% (v/v) aq. H_3PO_4 (B)-gradient: 0–2 min = 6% A, 2–20 min = 6–60% A (flow rate 2.0 ml/min). Quantification was done by UV photometry at 280 nm (galloylated compounds), and by radioactivity measurements after fractionation of the column eluates in 20 sec intervals and liquid scintillation counting in Bray's solution [14] (glucose and glucose esters).

Results

Enzyme purification

In initial experiments on the biosynthesis of 1,2,6-trigalloylglucose [4] in *R. typhina*, the 2-O-galloyltransferase was partially purified by treatment with protamine sulfate and an acid-precipitation step. Though this rather simple procedure resulted in good purification factors and was also effective in diminishing disturbing side reactions it became apparent that it was unsuitable for a detailed investigation because solubility problems with the acid-precipitated proteins made subsequent purification steps impractical by causing irreproducible results and excessive losses of enzyme activity.

The problem was solved by developing a new purification protocol (*cf.* Table I) that comprised $(NH_4)_2SO_4$-fractionation, hydrophobic interaction

Table I. Purification of β-glucogallin: 1,6-digalloylglucose 2-O-galloyltransferase.

Step	Protein [mg]	Total activity[a] [nkat]		Specific activity [pkat/mg]		Purification [x-fold]		Recovery [%]	
		A[b]	B[c]	A	B	A	B	A	B
Crude extract	636	11.4	12.7	17.9	20.0	1.0	1.0	100	100
$(NH_4)_2SO_4$, 20% supernatant	319	9.1	10.2	28.5	32.0	1.6	1.6	78	80
Phenyl-Sepharose	24	1.3	0.3	54.2	12.5	3.0	0.6	11	2
Sephacryl S-200	3.5	0.9	0.2	257.1	57.1	14.4	2.9	8	2

[a] Values are corrected against blanks containing no β-glucogallin to make allowance of the interferring β-glucogallin-independent formation of 1,2,6-trigalloylglucose in the initial purification steps (*cf.* Results).
[b] System A: standard assays were supplemented with 1,2,3,4,6-pentagalloylglucose (0.5 nmol) and hexagalloylglucose (6 nmol).
[c] System B: enzyme activities as measured under standard assay conditions (*cf.* Experimental).

chromatography (HIC) with phenyl-Sepharose, and gel filtration on Sephacryl S-200. The HIC step of this sequence caused considerable losses of enzyme activity, however, it also effected the complete elimination of a contaminating and highly reactive 2-O-galloyltransferase that catalyzed a β-glucogallin-*independent* formation of 1,2,6-trigalloylglucose [3]. This interferring enzyme was eluted from the column with salt-free buffer, while the β-glucogallin-dependent analog could be eluted only with water (*cf.* Experimental).

As described below, the enzyme was strongly stimulated in the presence of penta- or hexagalloylglucose. It is evident from Table I that addition of these compounds remained virtually ineffective with crude extracts that had not yet been depleted of these natural constituents. In contrast, a pronounced increase of catalytic activity in response to these tannins was observed in the case of purified enzyme solutions.

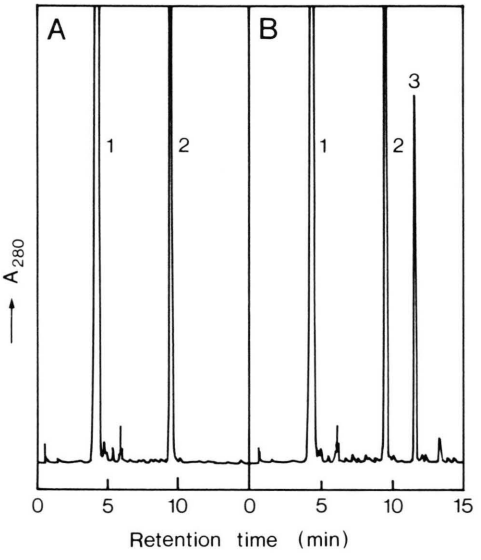

Fig. 1. HPLC-analysis of 2-O-galloyltransferase standard assays after incubation for 0 h (A; control) and 2 h (B). (1) β-Glucogallin; (2) 1,6-digalloylglucose; (3) 1,2,6-trigalloylglucose. Conditions were as given in Experimental, except for the modified solvent system (linear gradient of 0–30% AcN in 0.05% aq. H_3PO_4 within 20 min).

General properties

Some fundamental characteristics of the 2-O-galloyltransferase (*e.g.* substrate requirements, time and protein dependence, reaction product identification), have already been reported in a preliminary communication [4]. Whereas these data were obtained with acid-precipitated enzyme, the following studies were performed with enzyme that had been prepared as described above. Acyltransferase thus purified was found to catalyze the efficient conversion of 1,6-digalloylglucose to 1,2,6-trigalloylglucose without significant accumulation of by-products (Fig. 1). The temperature optimum of the reaction was at 50 °C, heat denaturation was observed at *ca.* 60 °C. In accordance with the cold tolerance of related enzymes [3, 9, 10], also here some activity (*ca.* 7%) was exhibited by the enzyme even at 0 °C. Between 10 and 30 °C, a Q_{10} value of 1.8 was calculated that corresponds to an average activation energy of 42.7 kJ/mol (10.2 kcal/mol). The effect of pH on the stability of the enzyme and on the velocity of the reaction is

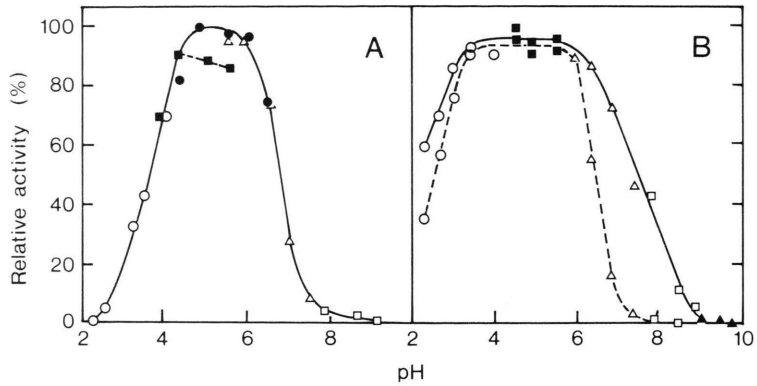

Fig. 2. Effect of pH on activity (A) and stability (B) of 2-O-galloyltransferase. Enzyme activity was measured under standard assay conditions at the indicated pH values (incubation time 30 min). For the stability experiments, the enzyme was exposed for 2 h (——) or 12 h (– – –) to the pH values given, with subsequent determination of the residual activity under standard conditions at pH 5.5. Buffers: (○) Na-citrate/HCl; (■) Na-acetate; (●) citric acid/NaOH; (△) K-phosphate; (□) Tris-HCl; (▲) glycine/HCl.

depicted in Fig. 2. The optimal pH was at 5.0–5.5; maximal stability was found at pH 3.4–5.8.

Normal Michaelis-Menten saturation curves were observed with both substrates; from Lineweaver-Burk plots, K_m values of 3.9 mM and 0.9 mM were calculated for β-glucogallin and 1,6-digalloylglucose, respectively.

From gel filtration experiments with a calibrated Sephacryl S-200 column [5], a remarkably high molecular weight of *ca.* 750,000 was estimated for the enzyme.

Surprisingly, it was found that addition of 1,2,3,4,6-pentagalloylglucose or hexagalloylglucose, *i.e.* compounds that are known as extremely potent protein denaturing and/or enzyme inhibiting agents (*cf.*, *e.g.* [1, 16, 17], caused a pronounced stimulation of enzyme activity. As depicted in Fig. 3, the presence of only 15 μM pentagalloylglucose in standard assays (containing 20 mM β-glucogallin and 2 mM 1,6-digalloylglucose) was sufficient to accelerate the enzyme reaction rate about 3-fold.

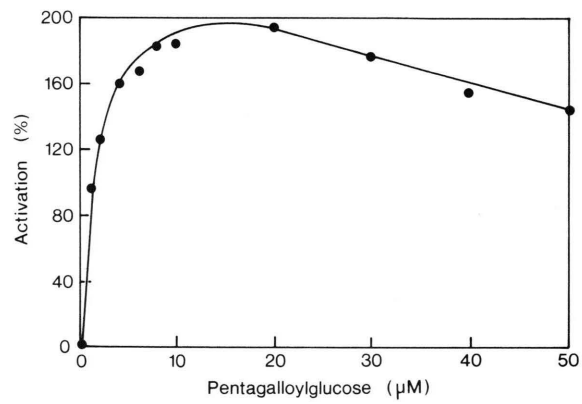

Fig. 3. Activation of 2-O-galloyltransferase by 1,2,3,4,6-pentagalloylglucose. Standard assay reaction mixtures containing 5.3 μg protein were supplemented with pentagalloylglucose as indicated; reaction time was 2.5 h.

Stoichiometry

According to the mechanism proposed for the 2-O-galloyltransferase reaction [4], 1 mol each of β-glucogallin and 1,6-digalloylglucose are required for the formation of 1 mol 1,2,6-trigalloylglucose, and 1 mol glucose is concomitantly liberated in this process (*cf.* Fig. 4). Experimental proof of this assumption was complicated, however, by the presence of a contaminating enzyme that catalyzed the synthesis of 1 mol 1,6-digalloylglucose from 2 mol of the substrate β-glucogallin, releasing 1 mol glucose [13]. For the quantification of this counter-directed reaction, the assays were supple-

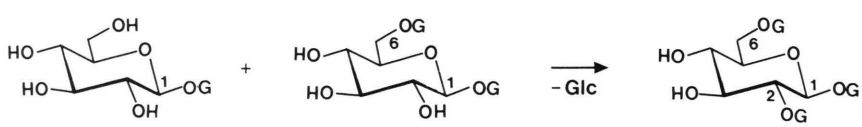

Fig. 4. Reaction equation for the formation of 1,2,6-trigalloyl-δ-D-glucose by β-glucogallin; 1,6-digalloylglucose 2-O-galloyltransferase from *R. typhina*. G, galloyl residue; Glc, glucose.

Table II. Stoichiometry of the 2-O-galloyltransferase catalyzed formation of 1,6-digalloylglucose. For technical details, see Experimental.

Component assayed	Gross reaction rate [nmol/h]	Corrected reaction rate[c] [nmol/h]	Molar ratio[d]
β-Glucogallin, consumed	−8.6	−2.6	−1.1
1,6-Digalloylglucose, consumed	+0.8[b]	−2.2	−0.9
1,2,6-Trigalloylglucose, formed	+2.4	+2.4	+1.0
Glucose, formed	+5.7	+2.7	+1.1
[^{14}C]1,6-Digalloylglucose, formed[a]	+3.0	0	–

[a] Formed from [^{14}C-glucosyl]β-glucogallin by β-glucogallin: β-glucogallin 6-O-galloyltransferase [13] present in the enzyme preparation.
[b] The observed apparent increase of 1,6-digalloylglucose, instead of the expected decrease, is due to a contaminating 6-O-galloyltransferase producing this substrate in excess (*cf.* footnote [a]) over its consumption.
[c] Corrections are based on the assumption that 1 mol 1,6-digalloylglucose (determined as [^{14}C]digalloylglucose) is formed from 2 mol β-glucogallin under the concomitant release of 1 mol glucose.
[d] Values refer to the reaction rate determined for 1,2,6-trigalloylglucose.

mented with [^{14}C-glucosyl]β-glucogallin; the radioactivity found in 1,6-digalloylglucose could thus serve as a measure of this interferring reaction, allowing to correct the determined concentrations of β-glucogallin, glucose, and 1,6-digalloylglucose. Under these conditions, and as summarized in Table II, it is evident that the calculated molar ratios of substrate consumption and product formation are well balanced and fully consistent with the proposed reaction equation.

Substrate specificity

The affinity of the enzyme toward a variety of 1-O-phenylcarboxylglucoses structurally related to the natural acyl donor, β-glucogallin, was determined under standard assay conditions. As summarized in Table III, β-glucogallin and its dihydroxy analog, protocatechuoylglucose, exhibited excellent reactivities, and also the corresponding *p*-hydroxy derivative had > 50% activity. Also several other 1-O-acylglucoses were highly reactive donors, however, these compounds cannot be regarded as natural constituents related to the biosynthesis of gallotannins in *Rhus*. In accordance with earlier reports [3, 7, 13], 6-O-galloylglucose was absolutely inactive thus documenting again the essential role of the energy-rich 1-O-acyl bond.

It was further found that tri-O-protocatechuoylglucose was formed upon incubation of 1-O-protocatechuoylglucose together with the 1,6-di-O-glucose ester (46% relative activity), whereas no reaction occurred in analogous assays containing the 1-O- and 1,2-di-O-acyl derivatives of anisic and benzoic acid, respectively, as well as in experiments with β-glucogallin and 3,6-di-O-galloylglucose.

Table III. Donor specificity of 2-O-galloyltransferase toward mono-acyl-β-D-glucopyranoses.

Substrate	Relative activity[a] [%]	K_m [mM]
1-O-Benzoylglucose	100	4.4
1-O-Anisoylglucose	98	2.1
1-O-Galloylglucose (β-glucogallin)	98	3.9
1-O-Protocatechuoylglucose	93	4.0
1-O-*p*-Hydroxybenzoylglucose	54	–
1-O-Veratroylglucose	51	5.0
1-O-Vanilloylglucose	28	2.3
1-O-Syringoylglucose	0	–
6-O-Galloylglucose	0	–

[a] Rate relative to benzoylglucose (100%) as determined under standard assay conditions, with 1,6-digalloylglucose as acceptor.

Finally, it was observed that β-glucogallin could be utilized also as donor for the acylation of tris- to pentakis-substituted galloylglucoses. It appears, however, that the resulting reaction products were not intermediates of the pathway to pentagalloylglucose but represented by-products characterized by depsidically bound galloyl residues (*cf.* [11]).

Discussion

The properties of the enzyme described here reveal it to be a typical acyltransferase that catalyzes the highly position-specific transfer of the galloyl moiety of β-glucogallin to the 2-OH group of 1,6-digalloylglucose, thus forming the 1,2,6-triester and leaving free glucose. Considering the substrate specificity and the physiological role of this enzyme, the previously [4] proposed systematic name "β-glucogallin: 1,6-di-O-galloylglucose 2-O-galloyltransferase" (EC 2.3.1-) appears justified.

It is interesting to note that leaves of *R. typhina* were recently found to contain a related 2-O-galloyltransferase that likewise produced 1,2,6-trigalloylglucose from the 1,6-di-ester [3]. However, this enzyme differs significantly from the one described here as it did not require β-glucogallin as acyl donor, and also its molecular weight of 56,000 was considerably lower. Moreover, this β-glucogallin-independent acyltransferase was drastically inhibited by the addition of pentagalloylglucose [3], in clear contrast to the enzyme described here where penta- and hexagalloylglucose were found to act as potent activators. The mechanism of this sort of feed-back activation is still unclear. Considering the high molecular weight of the enzyme, allosteric effects would appear feasible. However, in contrast to the requirements for the operation of such a regulatory mechanism, there was virtually no evidence for any sigmoidality of the saturation curves obtained with both substrates. At present it remains an open question to what extent specific interactions occur between the enzyme and these tannins. It should be emphasized in this context that evidence has been reported for specific protein-tannin interactions which were thought to reflect physiological functions [18]. To any event, such ideas fit to observations in our laboratory that all tannin-synthesizing enzymes so far investigated appear remarkably resistant to the deleterious effects going out from the products formed under their own catalysis.

Acknowledgements

We thank Mrs. U. Denzel for skillful technical assistance, and Professor E. Haslam (Sheffield), Professor G. Nonaka (Fukuoka) and Dr. H. Schick (Heidelberg) for generous gifts of galloylglucoses. Financial support by the Deutsche Forschungsgemeinschaft (Bonn) and the Fonds der Chemischen Industrie (Frankfurt a.M.) is gratefully acknowledged.

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