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BIOSYNTHESIS OF 1,2,3,6-TETRA-*O*-GALLOYL-β-D-GLUCOSE

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Key Word Index—*Quercus robur* (*Q. pedunculata*); Fagaceae; pedunculate oak; *Rhus typhina*; Anacardiaceae; staghorn sumac; biosynthesis; hydrolysable tannins; galloyltransferase; β-glucogallin; 1,2,6-tri-*O*-galloyl-β-D-glucose; 1,2,3,6-tetra-*O*-galloyl-β-D-glucose.

Abstract—An enzyme that catalysed the β-glucogallin (1-*O*-galloyl-β-D-glucose)-dependent galloylation of 1,2,6-tri-*O*-galloyl-β-D-glucose to 1,2,3,6-tetra-*O*-galloyl-β-D-glucose was partially purified from leaves of oak (*Quercus robur*). This acyltransferase had a M_r of *ca* 380 000, and pH and temperature optima of 6.0 and 55°, respectively, and was most stable between pH 4.0 and 6.5. In addition to the natural substrates β-glucogallin (donor) and 1,2,6-trigalloylglucose (acceptor), 1,3,6-trigalloylglucose (which is not an intermediate in the biosynthesis of hydrolysable tannins in oak and sumac) was an equally efficient acceptor molecule; in both cases, 1,2,3,6-tetragalloylglucose was the reaction product. Based on the physiological role of this new enzyme, the systematic name 'β-glucogallin: 1,2,6-tri-*O*-galloyl-β-D-glucose 3-*O*-galloyltransferase' (EC 2.3.1.-) is proposed.

INTRODUCTION

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose is regarded as the common precursor of the two classes of hydrolysable tannins, i.e. gallotannins and ellagitannins (cf. e.g. [1]). In an on-going investigation of the biosynthesis of this pivotal intermediate, it was elucidated that this ester is the end product of a sequence of consecutive and highly position-specific galloylation steps. It was shown with enzyme preparations from leaves of oak or sumac that β-glucogallin (1-*O*-galloyl-β-D-glucose), the first metabolite of this pathway, was acylated to 1,6-di-, 1,2,6-tri-, 1,2,3,6-tetra-, and finally to 1,2,3,4,6-pentagalloylglucose (reviewed in refs [2–5]). The individual enzyme activities responsible for these conversions have all been characterized, except for the one catalysing the step from 1,2,6-trigalloylglucose to 1,2,3,6-tetragalloylglucose, primarily because insufficient amounts of the required acceptor substrate were available. Here, we report on the synthesis of this rare trigalloylglucose ester and on its enzymatic conversion to 1,2,3,6-tetragalloylglucose.

RESULTS AND DISCUSSION

Synthesis of 1,2,6-*tri*-O-*galloyl*-β-D-*glucose*

Initial attempts to purify greater quantities of this substrate from natural sources (rhubarb roots, sumac leaves, green acorns of *Quercus robur*, commercial tannin) were unsuccessful because the available starting materials were found to contain only traces of this ester. Later, when sufficient amounts of 1,6-digalloylglucose were easily obtainable by enzymatic synthesis with immobilized 6-*O*-galloyltransferase [6], a very convenient procedure for the enzymatic conversion of this di-ester to 1,2,6-trigalloylglucose was developed. For this purpose, β-glucogallin-dependent 2-*O*-galloyltransferase [7, 8] was extracted from sumac leaves and partially purified by acid-precipitation [7]. The resulting insoluble pellet was simply suspended in buffer, supplemented with substrates and incubated until maximal conversion was reached (*ca* 5 hr). The enzyme was easily collected from the reaction mixture by centrifugation and reused in subsequent incubations with fresh substrates; its stability was usually sufficient for at least four reaction cycles. The reaction product was conveniently isolated by chromatography of the combined supernatants on small columns with octadecyl silica gel from which, after appropriate washing steps, 1,2,6-trigalloylglucose was obtained in 90–95% purity (yield 20%). By this means, *ca* 15 mg of this rare ester could be conveniently synthesized within one week.

Purification of 3-O-*galloyltransferase*

In continuation of our preceding studies on the biosynthesis of trigalloylglucose with enzymes from leaves of *Rhus typhina* [7–9], this plant source was also chosen for initial experiments on the formation of tetragalloylglucose. It soon became apparent, however, that this material was unsuitable because the *in vitro* reaction

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Table 1. Partial purification of β-glucogallin: 1,2,6-trigalloylglucose 3-*O*-galloyltransferase

Step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat mg^{-1})	Purification (-fold)	Recovery (%)
Crude extract	250	2.3	0.01	—	100
$(NH_4)_2SO_4$, 30–35% ppt.	38.5	26.4	0.69	69	1148
Phenyl-Sepharose	24.0	19.0	0.79	79	826
DEAE cellulose	4.7	15.0	3.2	320	652
Sephacryl S-200	3.6	13.7	3.8	380	596

product was immediately further acylated to higher substituted derivatives, i.e. pentagalloylglucose and gallotannins (cf. [4]). Such problems were not encountered with cell-free extracts from green acorns of *Q. robur*; in contrast to leaves from this species, no problems due to the presence of viscous mucilage in the extracts (cf. [10, 11]) were encountered. The galloyltransferase from green acorns was partially purified by fractionation with ammonium sulphate which evidently removed highly inhibitory components from the crude extract (Table 1), followed by hydrophobic interaction chromatography on phenyl-Sepharose, anion-exchange chromatography on DEAE-cellulose, and gel-filtration on Sephacryl S-200. Efforts to bind the enzyme on AE- or CM-cellulose or hydroxyapatite were unsuccessful. The results of a representative purification experiment are summarized in Table 1. The dilute enzyme solutions obtained after the last purification step were stabilized by immediate concentration by ultrafiltration and storage at 0–4°; under these conditions, no significant losses of activity were observed over a period of two to three weeks.

General properties of the enzyme

Under standard assay conditions (see Experimental), the galloyltransferase reaction was linear with respect to protein concentration for at least 40 μg (0.15 pkat) of purified enzyme per assay; no reaction occurred with protein previously denatured by heat or acid. Linearity of the reaction was maintained for *ca* 3 hr under these conditions. The enzyme was inactive below pH 2 and above pH 8, with a maximum at pH 6.0 (Na-citrate buffer); half-maximal activities were at pH 4.0 and 7.0. Highest stability of the enzyme, determined after preincubation at 30° for 1 hr, was found between pH 4.5 and 6.5. The temperature optimum of the reaction was at 55°; heat-denaturation was observed at 65–70°. Between 25° and 35°, an average activation energy of 49.3 kJ mol^{-1} was calculated which corresponds to a Q_{10} value of 1.9.

From gel-filtration experiments with a calibrated Sephacryl S-200 column [12], an apparent M_r of 380 000 was estimated for the enzyme. Though this value appears exceedingly high, it is in line with the data reported for related β-glucogallin-dependent galloyltransferases from oak and sumac (M_r 300 000–700 000; reviewed in ref. [5]). Only the β-glucogallin-synthesizing glucosyltransferase [10] and the 1,6-digalloylglucose 'disproportionating' galloyltransferase [9] were exceptions with values of 68 000 and 56 000, respectively, a finding that supports the view that these two enzymes do not belong to the β-glucogallin-dependent main pathway to pentagalloylglucose.

Substrate specificity

The enzyme showed normal Michelis–Menten saturation curves with both standard substrates. Replots of the data according to Lineweaver–Burk gave K_m values of 0.47 mM for β-glucogallin (V_{max} = 3.4 pkat) and 5.0 mM for 1,2,6-trigalloylglucose (V_{max} = 13.2 pkat). As summarized in Table 2, the enzyme exhibited affinities towards related substrates. 1,6-Digalloylglucose could partially replace the donor β-glucogallin, a fact that was not too surprising since this ester (as well as higher substituted analogues possessing the essential activated 1-*O*-acyl group) has been recognized as a potential galloyl donor [9]. In contrast, an unexpectedly high transacylation rate from β-glucogallin to 1,3,6-trigalloylglucose as acceptor was observed. Though the K_m value of this compound (5.4 mM) was very similar to the 1,2,6-isomer, its remarkably higher V_{max} (169 pkat) caused a *ca* 12-fold higher reactivity. With both acceptors, however, the same product, 1,2,3,6-tetragalloylglucose, was formed. It must be emphasized in this context that 1,3,6-trigalloylglucose is known as a typical constituent of the fruit of the myrobalan tree (*Terminalia chebula*, Combretaceae) [1]

Table 2. Substrate specificity of 3-*O*-galloyltransferase

Donor	Acceptor	Enzyme activity (pkat)*	Relative activity (%)†
None	1,2,6-tri-GG‡	0.2	2
None	1,3,6-tri-GG	0.4	4
β-Glucogallin	1,2,6-tri-GG	8.9	100
β-Glucogallin	1;3,6-tri-GG	106.8	1200
1,6-di-GG	1,2,6-tri-GG	4.3	48
1,6-di-GG	1,3,6-tri-GG	1.7	19

*Determinations were carried out under standard assay conditions (see Experimental).

†Values refer to the reaction rate in the standard assay with β-glucogallin and 1,2,6-trigalloylglucose equal to 100%.

‡GG, Galloylglucose.

but does not occur in *Quercus* or *Rhus*. Accordingly, the galloylation of 1,6-digalloylglucose leads exclusively to 1,2,6-trigalloylglucose as has been shown with cell-free extracts from these two species [7–9].

Reaction product identification

The reaction product isolated from scaled-up standard enzyme assays was analysed by analytical HPLC (see Experimental); it co-chromatographed with authentic 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose and was clearly different from isomeric 1,2,4,6-tetragalloylglucose. Treatment of the reaction product with the fungal esterase tannase [13] revealed a ratio of 3.9 galloyl residues bound per one glucose moiety, thus confirming the expected tetragalloylglucose structure. This method, however, does not discriminate between galloyl groups that are directly attached to the central glucose molecule and those being depsidically bound *via* phenolic hydroxyls of the galloyl residues. The latter must be classified as gallotannins, and it has recently been shown that their biosynthesis also requires *β*-glucogallin as acyl donor [14]. The enzyme reaction product was, therefore, subjected to methanolysis [15]; by this procedure, only depsidically bound galloyl residues are split off as methyl esters from the galloylglucose core, without affecting the aliphatic ester linkages. While controls with a gallotannin standard, the hexagalloylglucose 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloyl-*β*-D-glucose, yielded methyl gallate and 1,2,3,4,6-pentagalloylglucose in the expected ratio of 1:1, no formation of methyl gallate was observed with the enzyme reaction product, as well as with authentic 1,2,3,6-tetragalloylglucose. Both sample and reference remained unaltered by methanolysis, indicating the absence of depside linkages and confirming the proposed structure of the reaction product, i.e. 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose.

CONCLUSIONS

The properties of the enzyme described above reveal it to be a typical acyltransferase that catalyses the position-specific transfer of the galloyl moiety of *β*-glucogallin to the OH-3 of 1,2,6-trigalloylglucose which must be considered as the natural substrate of this enzyme. With the unphysiological 1,3,6-isomer, the OH-2 is substituted. Thus, 1,2,3,6-tetragalloylglucose is produced in each case. Considering both the substrate specificity and the physiological role, the systematic name '*β*-glucogallin: 1,2,6-tri-*O*-galloylglucose 3-*O*-galloyltransferase' (EC 2.3.1.-) appears justified; the reaction catalysed by this new enzyme is depicted in Fig. 1.

With the characterization of this earlier proposed enzyme [16], the last gap in our knowledge of the pathway from free gallic acid to 1,2,3,4,6-pentagalloylglucose has been filled. Its general properties closely resemble those of the other enzymes of this biogenetic sequence (cf. [3–5]); in particular, the dual role of *β*-glucogallin as both first intermediate and predominant, though not exclusive [9], acyl donor in the pathway to pentagalloylglucose, and in turn to gallotannins [14], has been corroborated. It should be mentioned finally that the surprisingly pronounced position-specificity of each of the galloylation steps in this sequence, i.e. the series OH-1 > OH-6 > OH-2 > OH-3 > OH-4, finds its counterpart in the chemical esterification of *β*-D-glucose for which an identical order of reactivities has been determined [17, 18].

EXPERIMENTAL

Chemicals. Chemical methods were employed for the synthesis of *β*-glucogallin [19] and 1,2,3,4,6-pentagalloylglucose [20]. 1,6-Digalloylglucose was prepared enzymatically [6]. A reference sample of 1,2,6-trigalloylglucose was kindly provided by Professor G. Nonaka (Kyushu University, Fukuoka, Japan), samples of 1,3,6-tri- and 1,2,4,6-tetragalloylglucose were gifts of Professor E. Haslam (University of Sheffield, U.K.). 1,2,3,6-Tetragalloylglucose was purified from commercially available tannin (Roth GmbH, Karlsruhe, Germany) [16], the gallotannin 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloylglucose was isolated from leaves of *R. typhina* [14]. Tannase was a generous gift of Dr M. Kanaoka (Sankyo Co., Tokyo).

Plant materials. Green acorns of *Quercus robur* L. (*Q. pedunculata* Ehrh.) were collected from trees in the surroundings of the university in July and August; similarly, mature leaves of *Rhus typhina* L. were harvested in September. Both materials were frozen in liquid N_2 and stored at −25° in evacuated plastic bags.

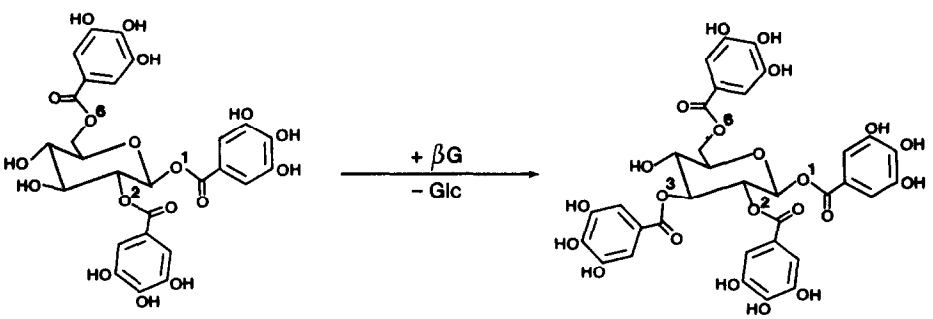

Fig. 1. Reaction catalysed by *β*-glucogallin: 1,2,6-trigalloylglucose 3-*O*-galloyltransferase from *Q. robur*. *β*-G, *β*-Glucogallin (1-*O*-galloyl-*β*-D-glucose).

Analytical methods. Quantitative measurements of the synthesis of 1,2,6-trigalloylglucose and 1,2,3,6-tetragalloylglucose, and of the reaction products after degradation with tannase were done by HPLC on LiChrospher 100 RP-18 (Merck LiChro-CART cartridges, particle size 5 μm, column 125 × 4 mm i.d., flow rate 2 ml min^{-1}, detection UV 280 nm) with a gradient of MeCN (solvent A) and 0.05% aq. H_3PO_4 (solvent B): 0–2 min 4–17% A, 2–13 min 17% A, 13–15 min 17–50% A. In the methanolysis expts, the reaction products were analysed by HPLC on LiChrosorb RP-18 (Merck CGC glass columns, particle size 5 μm, column 180 × 3 mm i.d., flow rate 1 ml min^{-1}) with a linear gradient of 3–33% A in B within 22 min (modified from ref. [21]). Quantification was done with a computing integrator (Merck-Hitachi D-2500) and reference to ext. standards.

Protein concentrations were determined turbidimetrically after pptn with CCl_3CO_2H [22], using BSA as standard. Very dilute solns were measured by UV photometry [23].

Hydrolysis with tannase was done in reaction mixtures (16 μl vol.) with 10 nmol (8 μg) 1,2,3,6-tetragalloylglucose and 5 U tannase in 0.05 M K-Pi buffer (pH 6.0) at 37° for 1 hr (cf. [13]). For the methanolysis expts, the compound to be analysed (20 μg) was dissolved in 20 μl MeOH–1 M NaOAc buffer, pH 5.5, (9:1) and incubated at 60° for 5 hr [15].

Synthesis of 1,2,6-*tri*-O-*galloyl*-β-D-*glucose.* The enzyme 2-*O*-galloyltransferase was extracted from frozen leaves of *R. typhina* (80 g) and partially purified as described previously [7], with the exception that the protein soln was brought to pH 5.5 in the final acid pptn step and that the resulting pellet was resuspended in 30 ml 1 M citrate buffer (pH 5.0). This turbid suspension was supplemented with 250 mg β-glucogallin and 15 mg 1,6-digalloylglucose (final vol. 45 ml) and incubated at 40° for 5 hr. The reaction was terminated by centrifugation (23 000 *g*, 15 min) and the supernatant kept at 2–4°. The pellet was resuspended as above and employed for further incubations; one enzyme preparation was usually sufficiently stable to permit 4 consecutive incubations. The pellet after the last cycle was washed twice with citrate buffer. The combined supernatants were chromatographed on small columns with silica gel (Merck LiChroprep RP-18, particle size 40–63 μm, column 6 × 2 cm i.d., equilibrated in H_2O, flow rate 3.5 ml min^{-1}, detection UV 254). After washing with H_2O, 10% and 20% aq. MeOH (affording gallic acid, β-glucogallin, 6-galloylglucose, 1,6-digalloylglucose and unidentified by-products), 1,2,6-trigalloylglucose was eluted with 25% aq. MeOH. This fraction was concd at 40° and lyophilized, yielding *ca* 16 mg (25 μmol) of 90–95% pure material from 60 mg 1,6-digalloylglucose (yield 20%).

Enzyme purification. Frozen green acorns of *Q. robur* (100 g) were wrapped in cloth and broken with a hammer; the fragments were homogenized and extracted as described recently [9], but with the addition of 20 mM ascorbic acid as antioxidant. The crude extract was fractionated with solid $(NH_4)_2SO_4$; the 30–55% ppt. was redissolved in 50 mM Tris–HCl, pH 7.5, and applied on a column (6.5 × 2 cm i.d.) of phenyl-Sepharose CL-4B (Pharmacia) equilibrated in 50 mM K-Pi buffer, pH 6.0. After carefully washing with buffer, the enzyme was in part eluted with H_2O (*ca* 20 ml) while the rest could be eluted only with 1 N urea (*ca* 14 ml). After desalting this second fraction by gel-filtration on PD-10 columns (Pharmacia), the two eluates were combined and chromatographed on a column (2.5 × 2 cm i.d.) of DEAE-52 cellulose (Serva, Heidelberg, Germany) equilibrated in 50 mM Tris–HCl, pH 7.5. After washing with buffer, followed by 0.1 M KCl in buffer, the enzyme was eluted with 0.2 M KCl. This fraction was concentrated by ultrafiltration (Millipore 'Centrifugal Ultrafree' kit, 30 000 MW exclusion limit) and subjected to gel-filtration on a Sephacryl S-200 column (40 × 3.2 cm i.d., Pharmacia) in 50 mM citrate buffer, pH 5.0. The most active fractions were pooled, concentrated and stored at 0–4°.

Enzyme assay. Enzyme activities were measured in standard assay mixtures (25 μl vol.) containing 5 μmol citrate buffer (pH 5.0), 50 nmol β-glucogallin, 50 nmol 1,2,6-trigalloylglucose and suitable amounts of enzyme (2–13 μg protein). After incubation at 30° for 2–3 hr, depending on the enzyme activity, the reaction was stopped by adding 25 μl 2 M HCl and the denatured protein was removed by centrifugation. To achieve complete desorption of reaction product, 25 μl MeOH was added and the pellet was resuspended by sonication. After centrifuging again, aliquots (25 μl) of the combined supernatants were analysed by HPLC.

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REFERENCES

1. Haslam, E. (1989) *Plant Polyphenols. Vegetable Tannins Revisited.* Cambridge University Press, Cambridge.
2. Gross, G. G. (1989) in *Plant Cell Wall Polymers: Biogenesis and Biodegradation* (Lewis, N. G. and Paice, M. G., eds), ACS Symp. Ser. Vol. **399**, p. 108. Washington, DC.
3. Gross, G. G. (1992) in *Plant Polyphenols: Synthesis Properties, Significance* (Hemingway, R. B. and Laks. P. E., eds), p. 43. Plenum Press, New York.
4. Gross, G. G. (1992) in *Phenolic Metabolism in Plants* (Stafford, H. A., ed.), *Rec. Adv. Phytochem.* **26**, 297.
5. Gross, G. G. (1993) in *Methods in Plant Biochemistry.* Vol. 9. *Enzymes of Secondary Metabolism* (Lea, P. J., ed.), p. 25. Academic Press, London.
6. Gross, G. G., Denzel, K. and Schilling, G. (1990) *Z. Naturforsch.* **45c**, 37.
7. Denzel, K., Schilling, G. and Gross, G. G. (1988) *Planta* **176**, 135.

8. Gross, G. G. and Denzel, K. (1991) *Z. Naturforsch.* **46c**, 389.
9. Denzel, K. and Gross, G. G. (1991) *Planta* **184**, 285.
10. Gross, G. G. (1983) *Phytochemistry* **22**, 2179.
11. Gross, G. G., Schmidt, S. W. and Denzel, K. (1986) *J. Plant Physiol.* **126**, 1273.
12. Andrews, P. (1965) *Biochem. J.* **96**, 595.
13. Nonaka, G., Nishioka, I., Nagasawa, T. and Oura, H. (1981) *Chem. Pharm. Bull.* **29**, 2862.
14. Hofmann, A. S. and Gross, G. G. (1990) *Arch. Biochem. Biophys.* **283**, 530.
15. Nishizawa, M., Yamagishi, T., Nonaka, G. and Nishioka, I. (1982) *J. Chem Soc., Perkin Trans. I* 2963.
16. Cammann, J., Denzel, K., Schilling, G. and Gross, G. G. (1989) *Arch. Biochem. Biophys.* **273**, 58.
17. Williams, J. M. and Richardson, A. C. (1967) *Tetrahedron* **23**, 1369.
18. Reinefeld, E. and Ahrens, D. (1971) *Liebigs Ann. Chem.* **747**, 39.
19. Gross, G. G. (1982) *FEBS Letters* **148**, 67.
20. Gross, G. G. (1983) *Z. Naturforsch.* **38c**, 519.
21. Haddock, E. A., Gupta, R. K., Al-Shafi, S. M. K., Layden, K., Haslam, E. and Magnolato, D. (1982) *Phytochemistry* **21**, 1049.
22. Kunitz, M. (1952) *J. Gen. Physiol.* **35**, 423.
23. Kalckar, H. M. (1974) *J. Biol. Chem.* **167**, 461.