Enzymatic Synthesis of 1,6-Digalloylglucose from β-Glucogallin by β-Glucogallin: β-Glucogallin 6-0-Galloyltransferase from Oak Leaves

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β-Glucogallin (1-0-galloyl-β-D-glucopyranose), 1,6-Di-0-galloyl-β-D-glucopyranose, Acyltransferase, Gallotannin Biosynthesis, *Quercus robur*

Cell-free extracts from oak *(Quercus robur)* leaves catalyze the transfer of the galloyl-moiety of β-glucogallin (1-0-galloyl-β-D-glucopyranose) specifically to the 6-position of the same compound, yielding 1,6-di-0-galloyl-β-D-glucopyranose, an intermediate of gallotannin biosynthesis. β-Glucogallin thus functions as both donor and acceptor molecule in this reaction. The partial purification and some general properties of this new acyltransferase are reported.

Introduction

In the course of recent studies on the enzymology of gallotannin biosynthesis in oak leaves it has been shown that β-glucogallin, the first intermediate in that pathway [1], is formed from UDP-glucose and free gallic acid under the catalysis of a specific glucosyltransferase [2, 3]. Further experiments revealed that the group-transfer potential of this ester is sufficiently high to permit subsequent transacylation reactions [4]. Besides one enzyme catalyzing an exchange reaction between β-glucogallin and free glucose [4, 5], other enzyme activities have been detected that caused the formation of di- and trigalloylglucose [4]. With respect to digalloylglucose, it became evident that this compound was synthesized by a new reaction mechanism in which β-glucogallin is utilized as both acyl donor and acceptor molecule. Meanwhile, this conclusion has been supported by the isolation of two analogous acyltransferases catalyzing the synthesis of 1,2-disinapoylglucose from 1-sinapoylglucose in radish seedlings [6, 7], and of 3,5-dicaffeoylquinic acid from chlorogenic acid in sweet potato roots [8]. Here, we report on the partial purification and the characterization of the digalloylglucose-synthesizing enzyme from oak leaves, and particularly on the identification of the reaction product and the stoichiometry of the reaction.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

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Material and Methods

Chemicals and isotopes

β-Glucogallin was synthesized from 2,3,4,6-0-tetraacetyl-β-D-glucose and triacetylgalloylchloride (*cf.* [2]). [U-^{14}C-Glucosyl]β-glucogallin was prepared enzymatically [2–4]. 6-Galloylglucose was provided by Dr. H. Schick (Heidelberg). Authentic reference samples of 1,6-digalloylglucose, 1,2,6- and 1,3,6-trigalloylglucose, 1,2,3,6- and 1,2,4,6-tetragalloylglucose, and 1,2,3,4,6-pentagalloylglucose were generous gifts of Prof. E. Haslam (Sheffield) and Dr. G.-I. Nonaka (Fukuoka), respectively.

Enzyme assays

Transferase activities were measured in assay mixtures (25 µl vol.) containing 5 µmol K-phosphate buffer (pH 6.5), 250 nmol [770 Bq (20 nCi)] [^{14}C]β-glucogallin and appropriate amounts of protein. After incubation for 90 min at 30 °C, the reaction was terminated by adding 10 µl 1 N HCl. Radioactivity of the formed digalloylglucose was measured after TLC as described previously ([2–5]; see also Fig. 2). Alternatively, a 25 µl aliquot of the deproteinized sample was applied onto a column (2.5 × 0.9 cm i.d.) of LiChroprep RP-18 (particle size 40–63 µm; Merck, Darmstadt) equilibrated in water. After washing with 4 ml of 20% aq. methanol, digalloylglucose (plus any eventually formed higher substituted derivative) was eluted with 2 ml 100% methanol and its radioactivity determined by liquid scintillation. Also HPLC was employed as a convenient assay method. In this case, the reaction mixtures (labeled substrate omitted) were stopped with 10 µl 1 N methanolic

HCl and chromatographed on LiChrosorb RP-18 (*cf.* Fig. 3). Quantification was done with a computing integrator referring to a standard solution of 1,6-digalloylglucose.

Enzyme preparation

Unless otherwise stated, all operations were carried out at 0–4 °C, and all buffers were supplemented with 5 mM 2-mercaptoethanol. Protein concentrations were determined turbidimetrically [9]. Leaves (70 g) from *Q. robur* were washed, frozen in liquid nitrogen and homogenized in a precooled ultracentrifugal mill (Retsch KG, Haan). The frozen powder was mixed with 70 g (wet weight) prewashed insoluble polyvinylpyrrolidone (polyclar AT) and stirred for 30 min with 60 ml 0.1 M borate buffer (pH 7.5) and 100 ml 1 M Tris-HCl buffer (pH 8.0). It was found important to readjust the pH of the brei occasionally with Tris buffer during the initial phases of the extraction procedure. The homogenate was squeezed through muslin and centrifuged (30000 × *g*, 15 min). The supernatant crude extract was stirred for 15 min with 7 g Dowex 1X4 (50–100 mesh, borate form) and filtered through glass wool. A 1% solution of protamine sulfate was added slowly to the filtrate to reach a final ratio of 2 mg protamine per 50 mg protein. After stirring for 15 min, the precipitate was removed by centrifugation and the supernatant was fractionated with solid ammonium sulfate. The 35–50% precipitate was redissolved in a minimum of 50 mM Tris-HCl (pH 7.5), clarified by centrifugation and desalted by gel-filtration on Sephadex G-25 (Pharmacia PD-10 columns). This solution was applied onto a DEAE-cellulose column (Whatman DE-52, 2.5 × 1.5 cm i.d.) equilibrated in the same buffer. After washing with 0.1 M KCl in buffer, the enzyme was eluted with 0.25 M KCl. The combined active fractions were adsorbed on a 1 × 0.9 cm i.d. column of hydroxyapatite (Bio-Rad) equilibrated in 50 mM Tris-HCl (pH 7.5). After washing, the enzyme was eluted with 20 mM K-phosphate buffer (pH 7.5). The active fractions were pooled, concentrated by ultrafiltration through Amicon PM-30 membranes and stored at 0–4 °C.

Identification of reaction product

1,6-Digalloylglucose was isolated from scaled-up enzyme assays (total vol. 3.5 ml) containing 650 μmol K-phosphate buffer (pH 6.5), 100 μmol (34 mg) β-glucogallin and 2.6 ml (6.1 mg protein) of ammonium sulfate-purified and concentrated enzyme. The mixture was incubated and deproteinized as described above. Prepurification was achieved by chromatography on a LiChroprep RP-18 column (6 × 2 cm i.d.). β-Glucogallin and by-products were removed by washing with 20% methanol (60 ml), followed by elution of higher galloylated products with 100% methanol (20 ml). This fraction was concentrated by rotary evaporation, filtered (pore-size 8 μm) and purified by preparative HPLC on LiChrosorb RP-18 (25 × 0.7 cm i.d., particle size 7 μm; Merck, Darmstadt) with 26% methanol in 0.05% acetic acid at a flow rate of 3 ml/min. The 1,6-digalloylglucose containing eluate was concentrated *in vacuo*, lyophilized, and analyzed by HPLC on RP-18 using the acetonitrile-H_3PO_4 gradient given by Haslam and coworkers [1, 10]. Definitive proof of the structure of the reaction product was achieved by the 300 MHz ^1H NMR spectrum. The full glucose proton sequence was determined by spin decoupling experiments. As a consequence of the well known acylation shift the protons 1-H, 6-H and 6′-H (δ = 5.56, 4.40 and 4.18) are deshielded while the other protons absorb in the region δ = 3.2–3.7. The coupling constant $^3J_{1\text{-H},2\text{-H}}$ = 7.0 Hz unequivocally shows the β-configuration at the anomeric C-1.

Determination of the stoichiometry

Scaled-up enzyme assays with [^{14}C-glucosyl]β-glucogallin as substrate and blanks containing acid-denatured protein were incubated under standard conditions. Aliquots (10 μl) of the deproteinized samples were subjected to reversed-phase HPLC on RP-18. β-Glucogallin, glucose and gallic acid were separated with 7% methanol in 0.05% H_3PO_4, digalloylglucose was determined by chromatography with 26% methanol in 0.05% H_3PO_4; for further details see legend to Fig. 3. Quantification was done by UV-photometry at 280 nm (aromatic compounds) and/or by radioactivity measurements after fractionation of the HPLC-eluates (glucose and glucose esters).

Results

Enzyme purification and stability

The galloyltransferase was isolated from young oak leaves (ca. 2- to 3-months-old) which did not yet contain the viscous mucilage characteristic of older leaves that had occasionally inhibited previous investigations (*cf.* [3, 5]). In the standard purification se-

Table I. Purification of β-glucogallin: β-glucogallin 6-0-galloyltransferase. Enzyme activities were determined by the HPLC-method (*cf.* Materials and Methods and Fig. 3).

Step	Protein [mg]	Total activity [nkat]	Specific activity [pkat/mg]	Purification [x-fold]	Recovery [%]
Crude extract	470	19.0	40.4	1	100
Dowex supernatant	460	15.5	33.7	0.8	82
Protamine supernatant	377	16.6	36.1	0.9	72
Ammonium sulfate, 35–50% ppt.	80.2	11.2	140	3.5	59
DEAE-cellulose	4.3	4.8	1116	28	25
Hydroxyapatite	2.1	2.6	1238	31	14

quence, crude extracts were prepurified by treatment with anion exchange-resin and protamine sulfate, followed by ammonium sulfate precipitation. Further purification was achieved by chromatography on DEAE-cellulose and hydroxyapatite, resulting in an about 30-fold purification and with a recovery of 14% (Table I). The application of gel-filtration was found impractical due to excessive losses of enzyme activity which probably are caused by the inevitable dilution of the enzyme in such experiments. This assumption is supported by studies on the stability of the partially purified enzyme; concentrated solutions, when kept at 2–4 °C for 7 d, exhibited the same residual activity (ca. 50%) as dilute samples stored for 2 d under these conditions. Best stability was observed when the concentrated transferase solution was kept at −20 °C in the presence of 10% glycerol.

In initial experiments, a contaminating esterase activity had been observed that effectively hydrolyzed β-glucogallin (see also ref. [5]). This disturbing enzyme could be eliminated almost completely in the course of the purification procedure. Moreover, crude extracts were often found to catalyze, besides digalloylglucose, the formation of trigalloylglucose and higher substituted derivatives, too. These different galloyltransferases were effectively separated from the enzyme described here by the ultimate two purifications steps.

General properties

Under standard assay conditions, the enzymatic reaction was linear with respect to protein concentration up to about 3 μg (ca. 4 pkat) of hydroxyapatite-purified enzyme, and was also linear with time for 90 min; maximal conversion was reached after about 5 h incubation. The temperature optimum of the reaction was at 30 °C; heat denaturation was observed at 55–60 °C. Surprisingly, a relative enzymatic activity of 25% was found even at 0 °C. Between 0 °C and 20 °C, an average activation energy of 42.2 kJ/mol (10.1 kcal/mol) was calculated which is equivalent to a Q_{10}-value of 1.8. The effect of pH on the stability of the enzyme and on the velocity of the reaction is depicted in Fig. 1. The optimal pH was

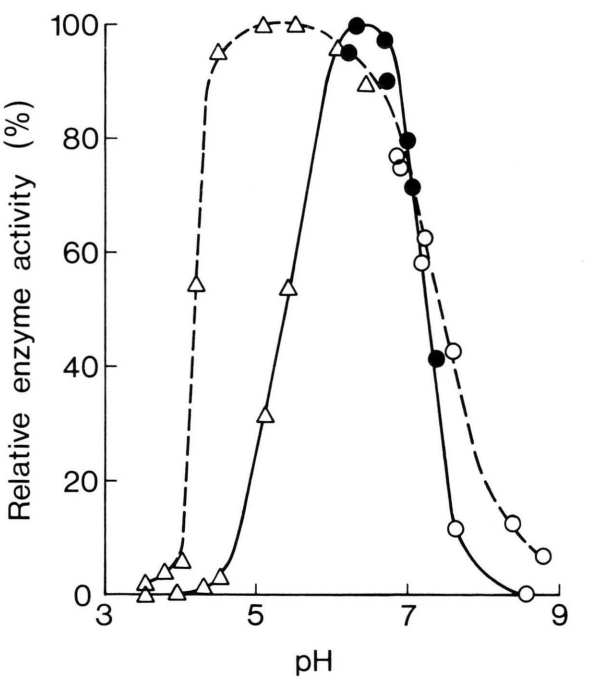

Fig. 1. Effects of pH on activity and stability of the galloyltransferase. The activity of the enzyme was measured under standard assay conditions at the indicated pH-values. To test the stability, the enzyme was exposed for 1 h at 30 °C to the pH-values given with subsequent determination of the residual activity at 6.5. (——): pH Optimum; (– – –): stability. Buffers: (△) sodium acetate; (●) potassium phosphate; (○) Tris-HCl.

6.5; maximal stability was observed between pH 4.5 and 6.0.

The enzyme exhibited normal Michaelis-Menten kinetics with the substrate; saturation, however, was not reached even at 20 mM final concentration, the highest possible amount of β-glucogallin in the standard assay. This rather low affinity of the enzyme towards its substrate is also documented by a K_m-value of 66 mM as calculated from Lineweaver-Burk plots. Definitely no reaction occurred when β-glucogallin was replaced by 6-0-galloylglucose.

No significant effect on the transferase activity was observed when the inorganic ions K^+, Mg^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, Zn^{2+} and NH_4^+ were added to the standard assay in final concentrations of 10^{-4}–10^{-2} M. The thiols 2-mercaptoethanol and dithioerythritol were found inhibitory at concentrations exceeding 50 mM and 20 mM, respectively.

From gel-filtration experiments with a calibrated Sephacryl S-300 column (Pharmacia; *cf.* ref. [11]) an apparent molecular weight of about 400000 dalton was estimated for this enzyme.

Reaction product

Preliminary experiments, based on TLC-analysis of the reaction products obtained after incubation of [^{14}C-glucosyl]β-glucogallin together with enzyme, showed that the substrate was converted to a higher galloylated labeled derivative that cochromatographed with digalloylglucose (Fig. 2), whereas no reaction occurred in controls with heat or acid-denatured protein. More precise information on the nature of the reaction product was gained by reversed-phase HPLC on RP-18 columns. In these experiments, the reaction product cochromatographed with authentic 1,6-digalloylglucose either under isocratic conditions in aqueous methanol (Fig. 3) or using an acetonitrile-phosphoric acid gradient (not shown).

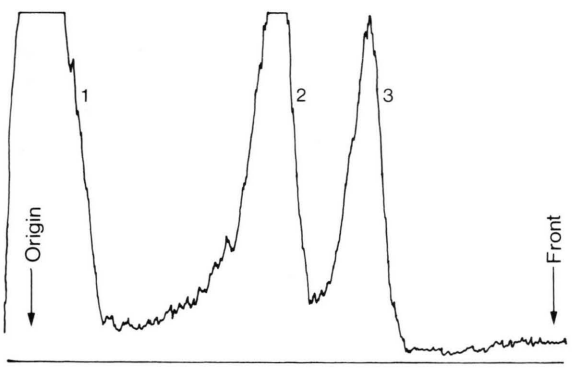

Fig. 2. TLC analysis of reaction products formed upon incubation of [^{14}C-glucosyl]β-glucogallin with ammonium sulfate-purified galloyltransferase. (1) Glucose; (2) β-glucogallin; (3) digalloylglucose. Chromatography was carried out on silica-gel plates (SIL N-HR; Macherey-Nagel, Düren) with the solvent ethyl acetate–ethyl methyl ketone–formic acid–water = 5:3:1:1 (by vol.). Radioactivity was recorded with the thin-layer scanner II (Berthold, Wildbad).

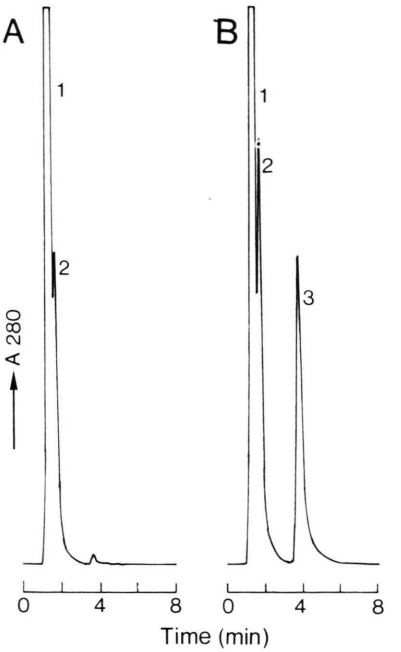

Fig. 3. HPLC-analysis of galloyltransferase assays incubated for 0 min (A; control) and 90 min (B). (1) β-Glucogallin; (2) gallic acid; (3) 1,6-digalloylglucose. Aliquots (10 µl) of deproteinized standard assay mixtures were injected onto a LiChrosorb RP-18 column (Merck CGC glass cartridge, particle size 5 µm, 180×3 mm i.d.) and developed isocratically with 26% methanol in 0.05% H_3PO_4 at a flow rate of 1 ml/min.

Final proof of the structure of the reaction product was achieved after working up scaled-up enzyme assays by preparative HPLC. About 3.5 mg of analytically pure digalloylglucose were thus isolated and subjected to ^{1}H-NMR-analysis in [D_6]dimethylsulfoxide as solvent (*cf.* experimental section). The recorded signals were unequivocally consistent with those expected for 1,6-di-0-galloyl-β-D-glucopyranose, and this result is in full accord with recent proposals for the biosynthesis of gallotannins (*cf.* 1, 12]).

In this context, it should be mentioned that also a second fraction was obtained in the above experi-

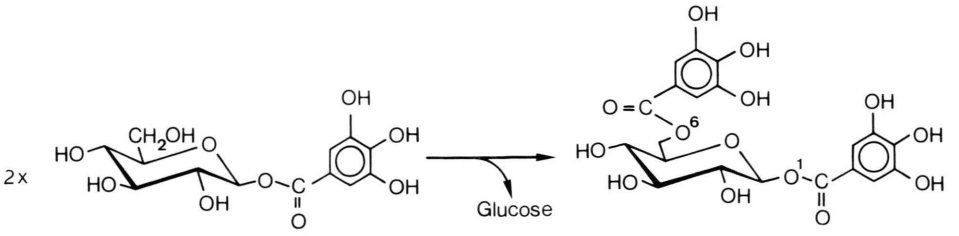

Fig. 4. Reaction equation for the formation of 1,6-di-0-galloyl-β-D-glucose catalyzed by β-glucogallin: β-glucogallin 6-0-galloyltransferase.

ment which was tentatively shown, by means of HPLC and cochromatography with authentic references, to contain a high portion of 1,2,6-trigalloylglucose together with minor amounts of 1,2,3,6-tetra- and 1,2,3,4,6-pentagalloylglucose. These substances, which obviously represent additional enzyme reaction products, are regarded as further natural gallotannin precursors [1, 12].

Stoichiometry

According to the mechanism proposed for the galloyltransferase reaction [4], 2 mol β-glucogallin are required for the formation of 1 mol digalloylglucose, and 1 mol glucose is concomitantly liberated in this process (*cf.* Fig. 4). The situation is complicated, however, by the existence of a contaminating esterase producing gallic acid and additional glucose from the substrate. Assuming that gallic acid is exclusively formed by this latter process, the amount of this compound can serve to correct the measured concentration of β-glucogallin and glucose. Under these conditions, and as summarized in Table II, it is evident that the observed molar ratios of substrate consumption and product formation are fully consistent with the expected reaction equation.

Discussion

The properties of the enzyme described here reveal it to be a typical acyltransferase that catalyzes the transfer of the galloyl moiety of β-glucogallin (1-0-galloyl-β-D-glucopyranose) specifically to the 6-0H position of a second molecule β-glucogallin, thus forming 1 mol of 1,6-di-0-galloyl-β-D-glucopyranose under the simultaneous release of 1 mol glucose (*cf.* Fig. 4). Consequently, the substrate β-glucogallin exerts a dual role as both acyl donor and acceptor molecule. Since no reaction occurred when isomeric 6-galloylglucose was assayed as a potential substrate it appears that the higher level of free energy characterizing the 1-0-ester is a necessary prerequisite for such a conversion. Unfortunately, no other acylglucoses were available to us for substrate specificity studies, except for a sample of 1-0-benzoylglucose that was active under standard assay conditions indicating that the affinity of this acyltransferase is not restricted to the galloyl ester. We are presently concerned with the synthesis and characterization of various glucose esters of aromatic acids as a prerequisite for studies on these aspects.

Summarizing the data available to date, the acyltransferase described here represents a new enzyme

Table II. Stoichiometry of the galloyltransferase-catalyzed formation of 1,6-digalloylglucose from β-glucogallin. Data (mean of two determinations) are corrected against suitable blanks. Experimental details are described in Materials and Methods.

Component assayed	Gross reaction rate [nmol]	Corrected reaction rate[a] [nmol]	Molar ratio
β-Glucogallin, consumed	20.8	18.2	2.0
1,6-Digalloylglucose, formed	9.1	9.1	1.0
Glucose, formed	12.7	10.1	1.1
Gallic acid, formed	2.6	0	–

[a] It is assumed that gallic acid is produced by hydrolysis of the substrate, β-glucogallin, liberating concomitantly an equal amount of glucose.

for which the systematic name β-glucogallin: β-glucogallin 6-0-galloyltransferase (EC 2.3.1.-) is tentatively proposed. To our knowledge, there have only two analogous enzymes been found in cell-free extracts from *Raphanus* [6, 7] and *Ipomoea* [8]; their characteristics, however, differ significantly from those of the transferase from oak leaves.

Concerning the physiological role of this enzyme it is conceivable that it synthesizes 1,6-digalloylglucose as an intermediate in the biogenesis of gallotannins. It is important in this context to mention that cell-free extracts from oak leaves appear to contain also different acyltransferase activities obviously catalyzing the synthesis of 1,2,6-tri-, 1,2,3,6-tetra- and 1,2,3,4,6-pentagalloylglucose by similar mechanisms. Quite recently, substantial amounts of the latter two compounds have been isolated also from oak tissue cultures [13]. Two challenging questions arise from these results, namely first, does the above sequence of increasingly galloylated glucoses represent, at least in oak, the natural biogenetic route to pentagalloylglucose, the parent compound of gallotannins and ellagitannins? And second, is it legal to assume that the reaction mechanism described here, *i.e.* transfer of galloyl residues with β-glucogallin as donor molecule, does represent the general mode how the individual steps along this pathway are catalyzed *in vivo?* It is hoped that current investigations in our laboratory will contribute to the clarification of these aspects.

Acknowledgements

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