Synthesis of Mono-, Di- and Trigalloyl-β-D-glucose by β-Glucogallin-Dependent Galloyltransferases from Oak Leaves

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Cell-free extracts from oak (*Quercus robur*) leaves were found to contain at least two β-glucogallin-dependent galloyltransferases. One enzyme catalyzed a rapid exchange of the galloyl-moiety of β-glucogallin with free glucose. A second enzyme fraction was shown to form digalloyl-glucose, utilizing β-glucogallin both as acyl-donor and acceptor molecule; in addition, evidence for the synthesis of trigalloylglucose was obtained. Both enzyme-systems had their pH-optimum at pH 6.0 – 6.5. It is assumed that these newly described galloyltransferases are involved in the biosynthesis of gallotannins.

Introduction

Hydrolyzable tannins are polyphenolic plant products which are characterized by a central polyol moiety (usually β-D-glucose) whose hydroxyl groups are esterified with gallic acid (3,4,5-trihydroxy-benzoic acid). It is assumed that their biosynthesis is initiated by the formation of β-glucogallin (1-O-galloyl-β-D-glucose) which undergoes a series of further substitution reactions leading to penta-galloyl-β-D-glucose, the central intermediate in the biogenesis of gallotannins and the related ellagi-tannins [1, 2]. Such esterification reactions would require, for thermodynamic reasons, the participation of activated intermediates. During the past few years, it has been shown unequivocally that con-jugates of cinnamic acids with a wide variety of hydroxylated compounds are formed *via* cinnamoyl-CoA thioesters (for reviews, see [3, 4]). On the other hand, there is increasing evidence that 1-O-acyl glucose esters appear to be synthesized generally from the free acid and UDP-glucose as the energy-rich component [5 – 12]. Moreover, it has been found that the group-transfer potential of such glucose esters is sufficiently high to permit subse-quent transacylation reactions [11 – 15].

With respect to gallotannins, an enzyme has been isolated recently from oak leaves which catalyzes, in accordance with the latter reaction type, the syn-thesis of β-glucogallin from UDP-glucose and free gallic acid [16, 17]. Here, I report that cell-free extracts from leaves of *Quercus robur* are capable of utilizing this ester for subsequent transacylation reactions by which mono-, di- and trigalloylglucose are formed as further intermediates in the biosyn-thesis of gallotannins.

Experimental

Analytical methods

Analytical TLC of galloylglucoses was carried out on silica-gel plates (SIL N-HR; Macherey-Nagel, Düren) with the solvent system (I) ethyl acetate – ethyl methyl ketone – formic acid – water = 5:3:1:1 (by vol.). The preparative separation of β-glucogallin was carried out on prewashed Schleicher & Schüll 2043 b paper (descending) and the organic phase of a mixture of (II) *n*-butanol – acetic acid – water = 4:1:5 (by vol.) was used as solvent [18]. HPLC-analyses of monogalloylglucoses were carried out on a reversed-phase column as described previously [16] (*cf.* Fig. 2). Gallic acid and mono- to pentagalloylglucose were separated by HPLC on a silica-gel column with a modification of the solvent given in ref. [19] (*cf.* Fig. 3). Radioactivity was recorded with the HPLC-radioactivity monitor LB 503 (Berthold, Wildbad).

Chemicals

[U-14C]Gallic acid was a gift of Prof. N. Amrhein (Bochum). Samples of α-glucogallin, 6-galloylglu-cose, 3,6-digalloylglucose and tetragalloylglucose were provided by Dr. H. Schick (Heidelberg). The synthesis of β-glucogallin from 2,3,4,6-O-tetraacetyl-

β-D-glucose and triacetylgalloyl chloride has been reported previously [16]. [U-^{14}C-glucosyl] and [U-^{14}C-galloyl] β-glucogallin were prepared enzymatically with UDP-glucose: vanillate 1-O-glucosyltransferase [16, 17] in upscaled enzyme assay mixtures. After incubation at 40° for 5 h, the deproteinized reaction mixtures were chromatographed twice on paper (system II). The labeled β-glucogallin was eluted with methanol and gave one radioactive spot after analytical TLC (system I). For the synthesis of 4-O-β-D-glucosidogallic acid and galloyl-CoA, see ref. [20]. Pentagalloylglucose was prepared by reacting triacetylgalloyl chloride with β-D-glucose [21] and subsequent hydrolysis of the protecting acetate groups [22]. The crude product was purified by column-chromatography on Sephadex LH-20 [19], affording pure pentagalloylglucose and, as by products, tri- and tetragalloylglucose [both representing mixtures of at least two isomers as shown by TLC on CEL 300 cellulose (Macherey-Nagel) with solvent (II)].

Enzyme preparation

For the preparation of cell-free extracts, leaves (7.5 g) from approx. 3-months-old *Q. robur* plants grown in the green-house were cut into small pieces, mixed with 7.5 g pre-wet Polyclar AT and ground in a mortar with 25 ml 0.1 M borate-buffer (pH 7.5) supplemented with 3 ml 1 M Tris-HCl (pH 8.0) and 20 mM β-mercaptoethanol. Alternatively, the leaves were frozen in liquid nitrogen, powdered in an ultracentrifugal mill (Retsch KG, Haan) and extracted under stirring with the above additions. The homogenate was centrifuged (15 min, 35000 × g), the supernatant crude extract stirred for 15 min with 0.75 g Dowex 1X4 (100−200 mesh, borate form) and centrifuged again. The supernatant was fractionated with solid ammonium sulfate. The sediments obtained after centrifugation were redissolved in a minimal volume of 0.05 M Tris-HCl (pH 7.5) plus 5 mM β-mercaptoethanol, clarified by centrifugation and desalted by gel-filtration on Sephadex G-25 (Pharmacia PD-10 columns). All operations were done at 0−4°. Protein determinations were made turbidimetrically [23].

Enzyme assays

The galloyl-exchange reaction between β-glucogallin and glucose was measured in assay mixtures

(25 μl vol.) containing 2.5 μmol K-phosphate buffer (pH 6.5), 250 nmol β-glucogallin, 250 nmol (0.1−1 μCi) [U-^{14}C]glucose and appropriate amounts of protein. After incubation at 30° for 15 min, the reaction was stopped with 10 μl 1 N HCl. An aliquot (25 μl) of the deproteinized solution was spotted on silica-gel plates and chromatographed (system I). β-Glucogallin was located under UV-light, the area cut out and the radioactivity determined 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 (56%) and final volume (35 μl) of the assay mixture, using an adaptation of the equation given in ref. [24]. The formation of digalloylglucose was analyzed analogously by incubation of labeled β-glucogallin together with enzyme in 0.1 M K-phosphate buffer (pH 6.5).

Results

By analogy to other metabolic routes, both β-glucogallin and galloyl-CoA represent potential acyl-donors for the galloylation of glucose in the course of gallotannin biosynthesis. In initial orientating experiments on that question, these two compounds were incubated for 3 h at pH 6.5 and 7.5, respectively, together with [U-^{14}C]glucose and an enzyme preparation obtained by a 20−75% ammonium sulfate fractionation step of crude extracts from oak leaves. TLC-analysis of the assay mixtures revealed that no reaction had occurred in the presence of galloyl-CoA (except for a pronounced hydrolysis of the thioester). The samples containing β-glucogallin as substrate which had been incubated at pH 6.5, however, were found to contain two newly synthesized radioactive substances. The main product cochromatographed with β-glucogallin, whereas the R_f-value of the minor by-product was close to that of authentic 3,6-digalloylglucose. Definitely no reaction had occurred in controls lacking β-glucogallin or containing heat-denatured protein.

Subsequent experiments showed that an enzyme fraction precipitating at 55−70% ammonium sulfate concentration catalyzed the almost exclusive formation of β-glucogallin as indicated by a ratio of the putative di- to monogalloylglucose of 0.02 (Fig. 1. B). A less soluble fraction (40−55% ammonium sulfate), in contrast, was found to produce under the
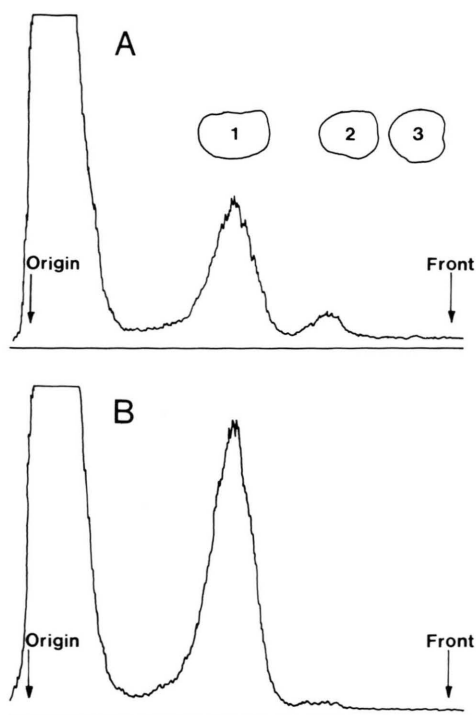
Fig. 1. TLC-analysis (*cf.* Experimental) of reaction products formed upon incubation of β-glucogallin and [U-14C]glucose with (A) 40–55% and (B) 55–70% ammonium sulfate fractionated enzyme. Reference compounds: (1) β-glucogallin; (2) 3,6-digalloylglucose; (3) gallic acid. Radioactivity was recorded with the thin-layer scanner II (Berthold, Wildbad).

demonstrated unequivocally that radioactive β-glucogallin had been formed from [U-14C]glucose and unlabeled β-glucogallin, obviously as the product of an enzymatic exchange reaction (Fig. 2). This result was confirmed by acidolysis experiments under conditions which are known to hydrolyze selectively 1-O-benzoylglucose, but not the corresponding 6-isomer [25, 26]. It was found that both the product investigated here and authentic [U-14C-glucosyl]β-glucogallin were cleaved quantitatively, yielding gallic acid and labeled glucose.

Concerning the minor metabolite which had been tentatively identified as a digalloylglucose, it could be clearly shown by reversed-phase HPLC (using a linear gradient of 0–70% methanol in 0.2% acetic
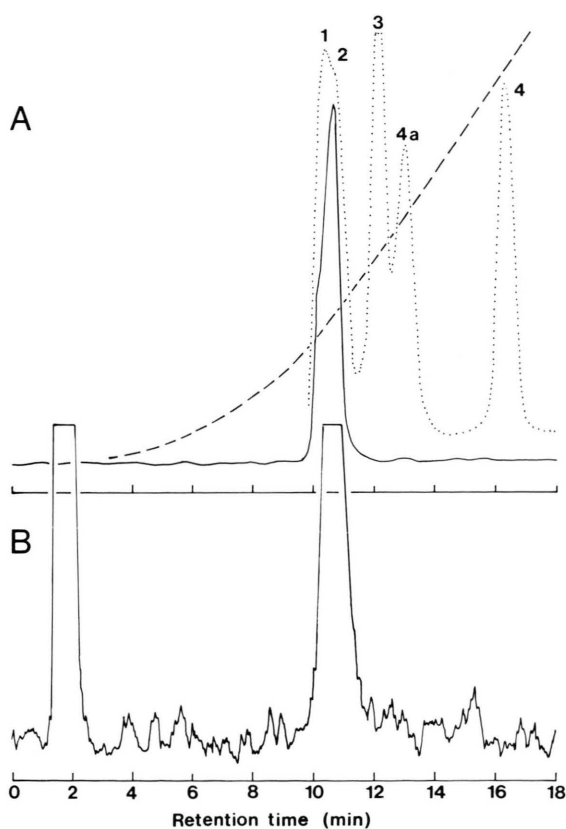

Fig. 2. Identification of [14C]β-glucogallin by reversed-phase HPLC. (A) UV-absorption at 280 nm; (——) reaction mixture; (…) reference compounds: (1) gallic acid; (2) β-glucogallin; (3) α-glucogallin; (4) 6-galloylglucose; (4a) impurity of (4). (B) Radioactivity. Separation conditions: µBondapak C_{18}, particle size 10 µm, 4 mm i.d. × 30 cm (Waters, Milford); gradient (15 min) 0–20% methanol in 0.2% acetic acid; flow rate 2 ml/min.

same conditions only about 1/3 of the amount of β-glucogallin (in spite of an approx. 5-fold higher protein concentration) but was characterized by the formation of significant quantities of the presumed digalloylglucose (ratio of di- to monogalloylglucose = 0.13; Fig. 1. A). The most likely interpretation of these data is that oak leaves contain two different β-glucogallin-dependent galloyltransferases, one catalyzing the synthesis of a monogalloylglucose in the presence of free glucose, and a second enzyme producing digalloylglucose from two molecules of β-glucogallin.

The nature of the two reaction products was studied in more detail, mainly by the application of HPLC-analyses. For this purpose, the deproteinized samples were evaporated at 30–40° under reduced pressure and the solid residue was taken up in methanol. Reversed-phase chromatography, which allows the separation of monogalloylglucoses,

acid) that this substance was not identical with 4-O-β-D-glucosidogallic acid, a possible product whose R_f-value in TLC-system (I) is of the same order as that of digalloylglucose. The correctness of the above assumption was proven by HPLC on silica-gel. As depicted in Fig. 3A, the assay mixtures contained a compound which clearly cochromatographed with authentic digalloylglucose. Moreover, this sample was found to contain also a low, but nevertheless significant portion of trigalloylglucose which had been overlooked in previous TLC-analyses. Determination of the radioactivity of
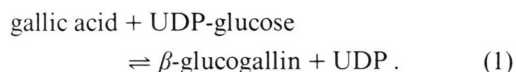

Fig. 3. Identification of [^{14}C]mono-, di- and trigalloylglucose as reaction products by HPLC on silica-gel. (A) UV-absorption at 280 nm; (——) reaction mixture; (...) reference compounds: (1) gallic acid; (2) β-glucogallin; (3)–(6) di-, tri-, tetra-, pentagalloylglucose. (B) Radioactivity; note that the heights of peaks (a)–(c) are not comparable due to different sample concentrations and ratemeter sensitivities (cf. text). Separation conditions: μPorasil, particle size 10 μm, 4 mm i.d. × 30 cm (Waters, Milford); solvent: n-hexane – methanol – tetrahydrofurane – formic acid = 63:27:9:1 (by vol.) plus 320 mg oxalic acid per 1000 ml; flow rate 2 ml/min.

these substances was complicated by the fact that unreacted glucose eluted between gallic acid and β-glucogallin, and its high radioactivity-response interferred with the comparatively low signals of the subsequent products. The problem was solved by preparative HPLC. The eluates containing mono-, di- and trigalloylglucose, respectively, were collected separately, concentrated by rotary-evaporation and rechromatographed. By this means, as depicted in Fig. 3B, it could be demonstrated unequivocally that all three compounds were radioactive, indicating that they had been formed by galloylation of labeled glucose.

The above reported formation of labeled digalloylglucose depended on the preceding synthesis of radioactive β-glucogallin in the presence of [^{14}C]glucose. That this intermediate serves both as acyl-donor and as acceptor-molecule without the addition of further cofactors was demonstrated in experiments with β-glucogallin being labeled either in the glucosyl or in the galloyl-moiety. In both cases, radioactive digalloylglucose was formed as shown by TLC, whereas no reaction occurred with heat-denatured protein.

Further studies revealed that the β-glucogallin-glucose exchange reaction had a pH-optimum at pH 6.0–6.5 (K-phosphate buffer). Half-maximal activities were observed at pH 5.0 and 7.2, and no reaction occurred below pH 4.2 and above pH 8.0. The reaction proceeded linearly with time for approx. 15 min and was proportional to enzyme concentration up to approx. 0.6 mg protein per assay. Substrate saturation was achieved at approx. 10 mM concentration. The pH-optimum of the conversion of β-glucogallin to digalloylglucose was about the same as that of the exchange reaction.

Discussion

It has previously [16, 17] been shown that oak leaves are capable of synthesizing β-glucogallin, the presumed first intermediate in the biosynthesis of gallotannins, according to the following equation:

$$\text{gallic acid} + \text{UDP-glucose}$$
$$\rightleftharpoons \beta\text{-glucogallin} + \text{UDP} . \qquad (1)$$

The results reported in this communication clearly show that this glucose ester, by analogy to other recently detected enzyme systems [11–15], has the potential to serve as acyl-donor in subsequent
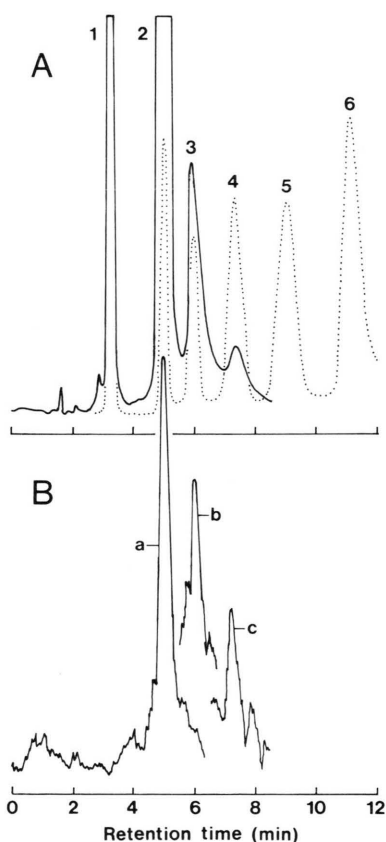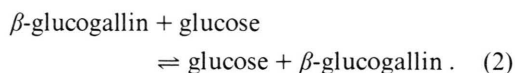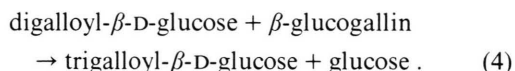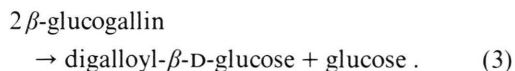
galloyl-transfer reactions. The data point to the existence of at least two different enzymes. The first one has been shown to catalyze a rapid galloyl-exchange reaction (Eq. (2)):

$$\beta\text{-glucogallin} + \text{glucose}$$
$$\rightleftharpoons \text{glucose} + \beta\text{-glucogallin}. \quad (2)$$

The physiological significance of this process, particularly with respect to the biogenesis of gallotannins, is difficult to evaluate at present, in quite contrast to the second reaction-type forming di- and trigalloylglucose, and which can tentatively be written as:

$$2\,\beta\text{-glucogallin}$$
$$\rightarrow \text{digalloyl-}\beta\text{-D-glucose} + \text{glucose}. \quad (3)$$

$$\text{digalloyl-}\beta\text{-D-glucose} + \beta\text{-glucogallin}$$
$$\rightarrow \text{trigalloyl-}\beta\text{-D-glucose} + \text{glucose}. \quad (4)$$

The exact substitution pattern of these two products has still to be elucidated. Concerning the digalloyl-glucose, TLC revealed that the enzymatically formed compound had an R_f-value which was close to, but not exactly at, the position of 3,6-digalloyl-glucose (the only authentic reference compound available to us at present), indicating the non-identity of these two substances. Considering recent proposals for the biosynthesis of gallotannins [1, 2] it is reasonable to assume that 1,6-O-digalloyl-β-D-glucose is produced in this galloyltransferase reaction, followed by the subsequent formation of 1,2,6-O-trigalloyl-β-D-glucose. It is hoped that a detailed characterization of these newly described enzymes and of the products formed under their catalysis will clarify these questions.

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