Synthesis of β-glucogallin from UDP-glucose and gallic acid by an enzyme preparation from oak leaves

G.G. Gross

Universität Ulm, Abteilung Allgemeine Botanik, 7900 Ulm, FRG

Received 26 July 1982

Cell-free extracts from oak leaves were found to catalyze the esterification of ^{14}C-labeled gallic acid and UDP-glucose. The product of the enzymatic reaction was identified as β-glucogallin by means of TLC and HPLC. The reaction proceded linearly with respect to time and protein concentration and had a pH-optimum at 7.0. Normal Michaelis–Menten kinetics were observed for the substrates gallic acid (K_M = 1.1 mM) and UDP-glucose (2.3 mM). The new enzyme described here can be classified as UDP-glucose: gallate glucosyltransferase, and it is assumed that it catalyzes the first step in the biosynthesis of gallotannins.

β-glucogallin (1-galloyl-β-D-glucose) *Gallic acid* *Uridine-5'-diphosphoglucose* *Esterification* *Cell-free extracts* Quercus robur

1. INTRODUCTION

Gallotannins are phenolic secondary plant products characterized by a central polyol moiety (usually β-D-glucose) whose hydroxyl groups are esterified with gallic acid. The latter residues are substituted with additional galloyl groups linked together in the form of *meta*-depsides. In contrast to the rather detailed knowledge of the chemistry and natural distribution of these hydrolyzable tannins (reviews [1–3]), little is yet known concerning their biosynthesis. It is assumed that esterification of glucose and gallic acid yields β-glucogallin (1-galloyl-β-D-glucose) as the primary intermediate. Further substitution reactions should lead to pentagalloyl-β-D-glucose, the parent compound of the different classes of gallotannins (cf. [3]). For thermodynamic reasons, the participation of activated intermediates has to be postulated in such esterification reactions. This has been proven, for instance, by enzymatic studies on the formation of chlorogenic acid and related depsides [4–7], and it has been found that cinnamoyl-CoA thioesters were utilized as the required carboxyl-activated reagents. On the other hand, the necessary group-transfer potential can also be provided by an energy-rich derivative of the alcoholic moiety, as has been shown for the synthesis of various 1-cinnamoyl glucosides from UDP-glucose and the free acids [8–12].

Here, I report that preparations from oak leaves catalyze, by analogy to the latter reaction-type, the transfer of the glucose-moiety of UDP-glucose to gallic acid yielding β-glucogallin, the proposed primary reaction product in the biosynthesis of gallotannins.

2. MATERIALS AND METHODS

Glucosidogallic acid (4-*O*-β-D-glucopyranosyl-gallic acid) was synthesized and characterized as in [13,14]. For the synthesis of β-glucogallin, α-acetobromoglucose (Serva, Heidelberg) was recrystallized from ether [15] and converted to tetra-*O*-acetyl-D-glucose with silver carbonate [16]. Tri-acetylgallic acid [17] was converted to the corresponding acid chloride with PCl_5 [18] and subsequently esterified with tetra-acetylglucose [19]. The protecting groups of the resulting hepta-acetylgalloylglucose were finally removed by treatment with sodium methylate [20] yielding analytically pure β-glucogallin (m.p. 210°C, FD-MS: M^+ 332 *m/e*). α-Glucogallin and 6-galloylglucose were provided by Dr H. Schick (Heidelberg). [U-^{14}C]Gallic acid

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

was a gift of Professor N. Amrhein (Bochum).

For the preparation of cell-free extracts, young leaves (7.5 g) from oak (*Quercus robur L.*) were cut into small pieces, mixed with 7.5 g pre-wet Polyclar AT and ground in a mortar in 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. The homogenate was squeezed through muslin and the filtrate centrifuged (20 min, 35 000 × *g*). The clear supernatant was fractionated with solid ammonium sulfate; the pellet precipitating between 35–65% saturation was resuspended in 0.05 M $KHPO_4^-$ buffer (pH 7.0) containing 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°C. Protein determinations were made as in [21].

Enzyme activities were assayed in reaction mixtures (final vol. 50 μl) containing 5 μmol Tris–HCl (pH 7.0), 150 nmol gallic acid, 150 nmol (0.05 μCi) UDP-D-[U-^{14}C]glucose and 0.2–0.5 mg protein. After incubation for 30 min at 30°C, the reaction was stopped by the addition of 1 μmol β-glucogallin and 10 μl 1 N HCl. An aliquot (25 μl) of the deproteinized solution was spotted on silica-gel coated plastic-sheets (SIL N-HR, Macherey-Nagel, Düren) and chromatographed (ethyl acetate–ethylmethyl ketone–formic acid–water, 5:3:1:1, by vol.). β-Glucogallin was located under UV-light, the area cut out, and the radioactivity was determined by liquid-scintillation in Liquifluor (NEN).

3. RESULTS AND DISCUSSION

Chromatographic analysis of enzyme reaction mixtures containing gallic acid and UDP-[^{14}C]glucose revealed the formation of a product that co-chromatographed with β-glucogallin (fig.1A). The same compound was detected after incubation of radioactive gallic acid and unlabeled UDP-glucose (fig.1B). In both cases, there was no evidence for the formation of glucosidogallic acid, suggesting that the enzyme preparation specifically catalyzed the synthesis of a monogalloyl-glucose ester, most likely the common β-D-glucogallin. This assumption was further strengthend after TLC on cellulose (MN 300, Macherey-Nagel) in 6% acetic acid where the radioactive product again coincided

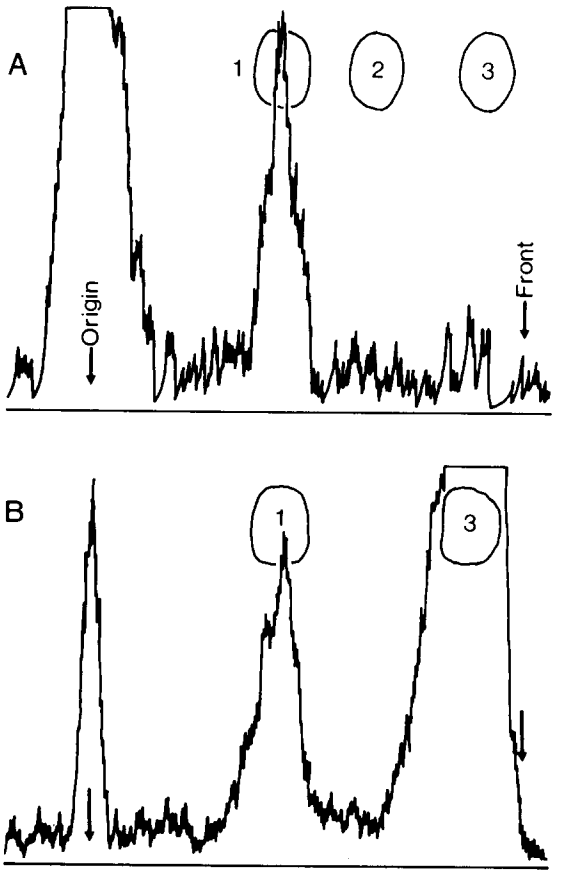

Fig.1. Identification of β-glucogallin by TLC (section 2) in a complete reaction mixture, as given in table 1. Labeled substrates were: (A) UDP-D-[U-^{14}C]glucose; (B) [U-^{14}C]gallic acid. Reference compounds: (1) β-glucogallin; (2) glucosidogallic acid; (3) gallic acid. Radioactivity was recorded with the thin-layer scanner II (Berthold, Wildbad).

with β-glucogallin (R_f 0.72; gallic acid R_f 0.47). With this system, it was also possible to separate the product from 6-galloyl-glucose (R_f 0.57). The reaction mixture was finally analyzed by HPLC (fig.2). The radioactive substance co-eluted exclusively with authentic β-glucogallin and proved also to differ from isomeric α-glucogallin. Summarizing these data, it is highly probable that β-glucogallin represents the product of the enzymatic reaction. Analogous cinnamoyl derivatives were found to be esterified at the C_1 of glucose [9–12], except for one enzyme catalyzing the formation of the 1-glucosyl ester and corresponding glucoside [9].

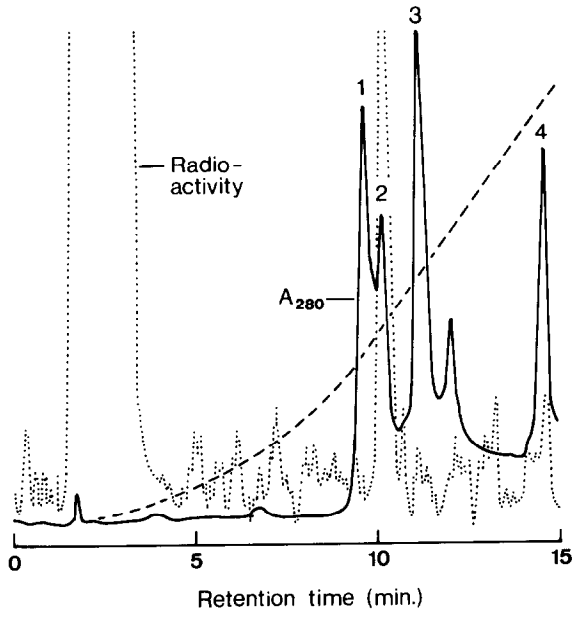

Fig.2. Identification of β-[^{14}C]glucogallin by HPLC. A 50 μl aliquot of deproteinized reaction mixture (with UDP-[U-^{14}C]glucose as labeled substrate) was injected directly onto a μBondapak C_{18}-column (3.9 mm i.d. × 30 cm; Waters, Milford). Separation was carried out with a concave gradient (15 min) of 0–20% methanol in 0.2% acetic acid; flow rate 2 ml/min. Radioactivity was recorded with the HPLC-radioactivity monitor LB 503 (Berthold, Wildbad). Reference compounds: (1) gallic acid; (2) β-glucogallin; (3) α-glucogallin; (4) 6-galloyl-glucose.

Table 1

Cofactor requirement for β-glucogallin synthesis

	β-Glucogallin formed (nmol)	
	(A) From UDP-[U-^{14}C]glucose	(B) From [U-^{14}C]-gallic acid
Complete	2.1	2.2
– gallic acid	0.19	–
– UDP-glucose	–	0
– dithiothreitol	2.4	2.7
+ heat-denatured enzyme	0	0

The complete reaction mixture contained in a final volume of 50 μl: 5 μmol Tris–HCl (pH 7.0), 50 nmol gallic acid (= 0.075 μCi in expt. B), 100 nmol UDP-glucose (= 0.05 μCi in expt. A), 200 nmol dithiothreitol and 0.24 mg protein. The assays were incubated at 30°C for 1 h

Formation of β-glucogallin depends on the presence of active enzyme, gallic acid and UDP-glucose (table 1). Dithiothreitol at 4 mM final conc. was slightly inhibitory and therefore omitted in subsequent experiments. Further studies revealed that the rate of the enzymatic reaction was proportional to time and protein concentration, respectively. The optimal pH was 7.0 (Tris–HCl or $KHPO_4^-$ buffer); half-maximal activities were observed at pH ~ 5.5 (Na-acetate buffer) and pH 8.5 (glycine–NaOH buffer). The enzyme exhibited normal Michaelis–Menten kinetics with both substrates. Saturation was achieved with 3 mM gallic acid ($K_M = 1.1 \times 10^{-3}$ M) and with 4 mM UDP-glucose ($K_M = 2.3 \times 10^{-3}$ M).

This is the first report of an in vitro system catalyzing the formation of β-glucogallin. From the above data, the enzyme can be classified as UDP-glucose; gallate glucosyltransferase. We are currently investigating whether this reaction represents the initial step in the biosynthesis of gallotannins, and particularly whether galloyl-CoA [14] can be utilized in the subsequent steps of this pathway as energy-rich intermediate.

ACKNOWLEDGEMENTS

I am indebted to Mrs M. Altmann for skillful technical assistance, to Professor N. Amrhein (Bochum) for a generous gift of radioactive gallic acid, to Mr K. Russ for his aid in the HPLC-separations, to Dr H. Schick (Heidelberg) for providing various galloyl-glucose reference compounds, to Dr G. Schmidtberg (Ulm) for MS-analyses, and particularly to Professor M.H. Zenk (Munich) for helpful suggestions.

REFERENCES

[1] Haslam, E. (1979) in: Biochemistry of Plant Phenolics (Swain, T. et al. eds) Rec. Adv. Phytochem. vol. 12, pp. 475–523, Plenum, New York.

[2] Haslam, E. (1981) in: The Biochemistry of Plants, vol. 7, Secondary Plant Products (Conn, E.E. ed) pp. 527–556, Academic Press, New York.

[3] Haddock, E.A., Gupta, R.K., Al-Shafi, S.M.K., Layden, K., Haslam, E. and Magnolato, D. (1982) Phytochemistry 21, 1049–1062.
[4] Stöckigt, J. and Zenk, M.H. (1974) FEBS Lett. 42, 131–134.
[5] Ulbrich, B. and Zenk, M.H. (1980) Phytochemistry 19, 1625–1629.
[6] Rhodes, M.J.C. and Wooltorton, L.S.C. (1976) Phytochemistry 15, 947–951.
[7] Rhodes, M.J.C., Wooltorton, L.S.C. and Lourenço, E.J. (1979) Phytochemistry 18, 1125–1129.
[8] Macheix, J.J. (1977) CR Acad. Sci. Ser. D 284, 33–36.
[9] Fleuriet, A., Macheix, J.J., Suen, R. and Ibrahim, R.K. (1980) Z. Naturforsch. 35c, 967–972.
[10] Strack, D. (1980) Z. Naturforsch. 35c, 204–208.
[11] Nurmann, G. and Strack, D. (1981) Z. Pflanzenphysiol. 102, 11–17.
[12] Nagels, L., Molderez, M. and Parmentier, F. (1981) Phytochemistry 20, 965–967.
[13] Fischer, E. and Strauss, H. (1912) Ber. Deutsch. Chem. Ges. 45, 3773–3779.
[14] Gross, G.G. (1982) Z. Naturforsch. c, in press.
[15] Koenigs, W. and Knorr, E. (1901) Ber. Deutsch. Chem. Ges. 34, 957–981.
[16] Fischer, E. and Hess, K. (1912) Ber. Deutsch. Chem. Ges. 45, 912–915.
[17] Lesser, R. and Gad, G. (1926) Ber. Deutsch. Chem. Ges. 59, 233–236.
[18] Fischer, E., Bergmann, M. and Lipschitz, W. (1918) Ber. Deutsch. Chem. Ges. 51, 45–79.
[19] Fischer, E. and Bergmann, M. (1918) Ber. Deutsch. Chem. Ges. 51, 1760–1804.
[20] Schmidt, O.Th. and Reuss, H. (1961) Liebigs Ann. Chem. 649, 137–148.
[21] Kunitz, M. (1952) J. Gen. Physiol. 35, 423–450.