Enzymatic Synthesis of Di-O-Phenylcarboxyl-β-D-glucose Esters by an Acyltransferase from Oak Leaves

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Z. Naturforsch. **45c**, 37–41 (1990); received August 11, 1989

Di-O-Phenylcarboxyl-β-D-glucose Esters, Acyltransferase, Immobilized Enzyme, *Quercus robur*

Leaves from oak *(Quercus robur)* contain an acyltransferase that catalyzes the conversion of β-glucogallin (1-O-galloyl-β-D-glucose) to 1,6-di-O-galloylglucose, an intermediate in the biosynthesis of gallotannins. Substrate specificity studies revealed that this enzyme was also active with several structurally related 1-O-phenylcarboxyl-β-D-glucoses; appreciable reaction rates, however, were observed only in the formation of 1,6-di-O-protocatechuoyl-β-D-glucose. This to date unknown ester, as well as its digalloyl analog, was synthesized using acyltransferase immobilized on phenyl-Sepharose, and characterized by UV and ^{1}H NMR spectroscopy. In addition, the 1,2-di-O-β-D-glucose esters of benzoic and anisic acid were obtained in this investigation.

Introduction

In enzymatic studies on the biosynthesis of gallotannins in higher plants it was discovered that β-glucogallin (1-O-galloyl-β-D-glucose), the first intermediate in this pathway, was utilized as acyl donor in a sequence of consecutive reactions yielding higher galloylated glucose derivatives [1]. Detailed investigations with an enzyme preparation from oak leaves [2] showed that the first product of this series, 1,6-di-O-galloyl-β-D-glucose, was formed in a then rather unusual reaction, *i.e.* by the "disproportionation" (*cf.* [3]) of two molecules β-glucogallin serving as both acyl donor and acceptor under the concomitant release of one molecule glucose – a reaction mechanism that was found to apply also to the formation of 1,2-disinapoylglucose in radish seedlings [3, 4] and of isochlorogenic acid in sweet potato roots [5, 6]. Recently, a variety of differently substituted 1-O-phenylcarboxyl-β-D-glucose esters became available to us [7], allowing studies on the substrate specificity of the galloyltransferase from oak which, as described below, led to the preparation of several new di-O-phenylcarboxylglucose esters.

Abbreviations: AcN, acetonitrile; DMSO, dimethylsulfoxide; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; RP, reversed phase.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0100-0037 $ 01.30/0

Experimental

Chemicals

β-Glucogallin was synthesized chemically (*cf.* [8]), other 1-O-phenylcarboxylglucoses were prepared enzymatically [7]. 1,6-Di-O-galloylglucose was isolated from rhubarb [9].

Enzyme preparation

Acyltransferase used in analytical experiments was purified according to [2], with the modification that the previously employed protamine sulfate step was omitted. Enzyme for preparative purposes was obtained by extracting 240 g of oak leaves, followed by treatment with Dowex 1X4, precipitation with $(NH_4)_2SO_4$, and chromatography on DEAE-cellulose (for experimental details, see [2]). The eluate from this last step was concentrated by ultrafiltration (Millipore "Centrifugal Ultrafree" kit; 30,000 MW exclusion limit) and subjected to gel filtration on a Sephacryl S-300 col-

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umn (40 × 3 cm i.d.; Pharmacia) equilibrated in 50 mM K-phosphate buffer, pH 6.0 (*cf.* Fig. 1). Fractions 32–42, which were largely depleted of a contaminating esterase activity, were combined and immobilized by adsorption on a phenyl-Sepharose CL-4B column (4 × 1.6 cm i.d.; Pharmacia) equilibrated in the same buffer as above, but supplemented with 1 M $(NH_4)_2SO_4$. After carefully washing out unbound protein, in particular any traces of residual esterase, with K-phosphate buffer containing no $(NH_4)_2SO_4$ (*ca.* 10 bed vol.), the column with the hydrophobically attached galloyltransferase was ready for use as an "enzyme reactor" [10].

Enzyme assays

Standard enzyme assay mixtures (25 µl vol.) containing 5 µmol K-phosphate buffer (pH 6.0), 250 nmol β-glucogallin and appropriate amounts of protein were incubated for 90 min at 30 °C, then stopped with 10 µl 2 N HCl, and centrifuged. A 10 µl aliquot of the supernatant was analyzed by HPLC (Merck LiChrospher RP-18, 5 µm particle size; column 125 × 4 mm; solvent 13% AcN in 0.05% aq. H_3PO_4; flow rate 2 ml/min; detection UV 280 nm). Quantification was done with a computing integrator (Merck-Hitachi D-2500) referring to external standard solutions of 1,6-digalloylglucose. 1,6-Di-protocatechuoylglucose was determined analogously (solvent 15% AcN; UV 265 nm). In assays with other acylglucoses, reaction products were measured at their respective absorption maxima (*cf.* [7]) after separation by HPLC (RP-18) with a linear AcN-gradient (0–50% AcN in 0.05% aq. H_3PO_4 within 25 min).

1,6-Di-O-galloyl-β-D-glucose

β-Glucogallin (100 mg) was dissolved in 50 ml K-phosphate buffer (50 mM, pH 6.0) and pumped (flow rate 6 ml/h) at room temperature through a column containing galloyltransferase immobilized on phenyl-Sepharose (see *Enzyme preparation*). The eluate was pumped through a subsequent column (5 × 2 cm i.d.) with LiChroprep RP-18 (Merck; particle size 40–63 µm) which effectively adsorbed the enzymatically formed digalloylglucose. The second eluate containing the unreacted substrate was recycled into the reservoir which was kept in ice to prevent hydrolysis. After 5 d, when β-glucogallin was used up almost completely, the enzyme reactor was washed with buffer, thus being ready for further experiments. The RP-18 column was washed with water (50 ml) to remove salt and residual substrate, followed by elution of the bound digalloylglucose with methanol. After evaporating the solvent, the solid residue was crystallized from a minimum of hot water, yielding off-white crystals of 1,6-digalloylglucose (41.7 mg; purity 98%). The mother liquor was lyophilized and worked-up by semi-preparative RP-HPLC (conditions *cf. Enzyme assays*), yielding a second crop (20.7 mg) of digalloylglucose. The overall recovery (62.4 mg, 42.8%) thus corresponds to a total conversion rate of at least 86%.

1,6-Di-O-protocatechuoyl-β-D-glucose

The principal reaction conditions were as described for 1,6-digalloylglucose; the RP-18 column was omitted, however, as it was found to bind the substrate partially. Briefly, 25 mg 1-O-protocatechuoyl-β-D-glucose in 25 ml K-phosphate buffer was cycled through the enzyme reactor (flow rate 3 ml/h). After 2 d, the reaction mixture was passed through a RP-18 column (40–63 µm; 1 ml bed vol.). After washing with water (10 ml) and 5% aq. methanol (10 ml), the diester was eluted with 100% methanol; the solvent was evaporated, the residue taken up in water, lyophilized, and dried *in vacuo* (85 °C, 2 mm Hg) for 3 d, affording 10.6 mg (30% yield, corresponding to 60% reaction rate) of 1,6-di-O-protocatechuoyl-β-D-glucose (purity 88%). For analytical purposes, an aliquot of this product was further purified by semi-preparative HPLC on Merck LiChrosorb RP-18 (7 µm particle size; column 25 × 0.7 cm i.d.; flow rate 3 ml/min; detection UV 265 nm). After washing with 5% methanol in 0.05% acetic acid, the diester was eluted with 100% methanol. This fraction was concentrated and rechromatographed on the same column with 37% methanol in 0.05% acetic acid, yielding analytically pure 1,6-di-O-protocatechuoylglucose.

^{1}H NMR (200 MHz, [D$_6$]DMSO): δ (ppm) 3.1–3.7 (H-2, 3, 4, 5), 4.22 (dd, $^2J_{H\text{-}6,6'}$ = 12.3 Hz, $^3J_{H\text{-}5,6}$ = 5.3 Hz, H-6), 4.40 (dd, $^3J_{H\text{-}5,6'}$ = 1.3 Hz, H-6′), 5.52 (d, $^3J_{H\text{-}1,2}$ = 7.5 Hz, H-1), 6.75 (d, $^3J_{arH\text{-}5,6}$ = 7.9 Hz, arH-5), 6.76 (d, $^3J_{arH\text{-}5',6'}$ = 7.9 Hz, arH-5′), 7.2–7.4 (4H, arH-2, 2′, 6, 6′).

UV (0.1 M K-phosphate buffer, pH 7.0): $\lambda_{max\,1}$ = 265 nm (ε = 18.7 [cm²/μmol]); $\lambda_{max\,2}$ = 300 nm (ε = 12.1).

1,2-Di-O-benzoyl-β-D-glucose

^{1}H NMR (200 MHz, [D$_6$]DMSO): δ (ppm) 3.75 (t, H-3), 5.10 (dd, H-2), 5.96 (d, *J* = 8 Hz, H-1), 7.45–7.95 (10 arH).

1,2-Di-O-anisoyl-β-D-glucose

^{1}H NMR (200 MHz, [D$_6$]DMSO): δ (ppm) 3.80 (s, OCH$_3$), 3.82 (s, OCH$_3$′), 5.05 (t, H-2), 5.24, 5.43 (d, d, O<u>H</u>-3, O<u>H</u>-4), 5.90 (d, *J* = 7 Hz, H-1), 6.95–7.05 (4H, arH-3, 3′, 5, 5′), 7.80–7.90 (4H, arH-2, 2′, 6, 6′).

Results and Discussion

Substrate specificity studies

Earlier investigations on the properties of a digalloylglucose-forming acyltransferase from oak leaves [2] provided evidence that this enzyme also exhibited some affinity toward 1-O-benzoylglucose, the only compound related to the standard substrate, β-glucogallin, being available to us at that time. The recent preparation and characterization of several 1-O-phenylcarboxylesters of D-glucose [7] allowed detailed substrate specificity studies. Determination of the relative activities of these compounds, as well as of the more reliable V_{max}/K_m-ratios, showed that only 1-O-protocatechuoyl-β-D-glucose, *i.e.* the ester structurally most closely related to the natural substrate β-glucogallin, was converted to the diacyl derivative with significant reaction rates (Table I). Only traces of enzymatic activity, or none, were observed with the glucose esters of *p*-hydroxybenzoic, veratric, and syringic acid.

Inconclusive results were obtained in experiments with the 1-O-glucose esters of benzoic, anisic, and vanillic acid. With these potential substrates, two products were formed as indicated by double peaks upon analytical HPLC. The comparatively good reaction rates observed with the benzoyl and the anisoyl ester permitted the isolation of the respective major reaction product from scaled-up assay mixtures. ^{1}H NMR studies with these compounds revealed that they were to date unknown 1,2-di-O-acylglucoses *(cf. Experimental)*. It is still unclear whether this unexpected result must be attributed to a diverging product specificity of the enzyme with these particular substrates, or to an artificial, non-enzymatic acyl migration (*cf., e.g.,* [11–13]).

Summarizing these substrate specificity studies, the tentatively proposed systematic name of the enzyme, β-glucogallin: β-glucogallin 6-O-galloyltransferase (EC 2.3.1.90) according to the best substrate [2], has been fully corroborated.

Preparative work with immobilized enzyme

On the basis of the substrate specificity studies discussed above, it appeared promising to prepare larger quantities of 1,6-digalloyl- and 1,6-diprotocatechuoylglucose as substrates for current investigations on the biosynthesis of trigalloylglucose [9]. An indispensable prerequisite for the intended synthesis of these esters in long-term incubation experiments was the necessity of depleting the enzyme solution of a contaminating esterase that

Table I. Specificity of the galloyltransferase toward differently substituted 1-O-benzoylglucoses.

Substrate	Relative activity [%][a]	V_{max} [nmol/min]	K_m [mM]	V_{max}/K_m [× 10^3]
1-O-Galloylglucose	100	3.3	66.7	49.5
1-O-Protocatechuoylglucose	58	1.4	44.4	31.5
1-O-*p*-Hydroxybenzoylglucose	8	0.04	2.5	16.0
1-O-Veratroylglucose	7	0.13	50.0	2.6
1-O-Syringoylglucose	0	–	–	–

[a] Rate relative to galloylglucose (100%) as determined under standard assay conditions. For further details see Experimental section.

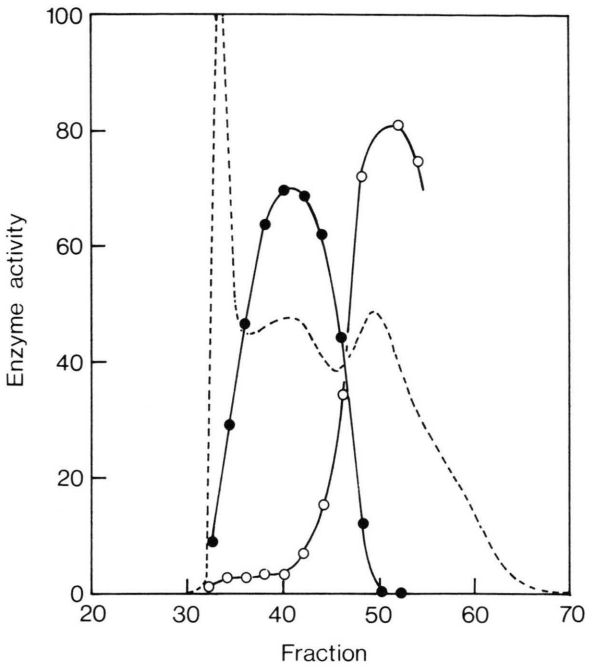

Fig. 1. Gel filtration of galloyltransferase on Sephacryl S-300. (– – –) Protein elution profile monitored by UV_{280} absorption. Enzyme activities of galloyltransferase (●) and of a β-glucogallin-hydrolyzing esterase (○) are expressed as percent conversion of substrate after incubation for 12 h under standard assay conditions. Fraction vol. = 2.3 ml. For further details, see *Experimental.*

effectively hydrolyzed the substrate. For this purpose, the pre-purified enzyme *(cf. Experimental)* was subjected to gel filtration on Sephacryl S-300 which considerably diminished the proportion of the interfering enzyme activity (Fig. 1). Residual traces of esterase were eliminated by subsequent hydrophobic interaction chromatography on phenyl-Sepharose *(cf. Experimental)*.

It was observed in these latter experiments that the galloyltransferase was retained on the matrix even after prolonged washing with salt-free buffer. Considering previous suggestions (*e.g.* [10, 14]), it thus appeared promising to use the easily immobilized enzyme as catalyst in an "enzyme reactor". Without added preservatives, the bound enzyme was active for at least three weeks ($t/2$ = 10 d); during this time, it was employed continuously for the facile and efficient synthesis of several batches of digalloyl- and diprotocatechuoylglucose, reaching overall conversions of 60–90% by simply cycling a buffered substrate solution through the column *(cf. Experimental)*. The effectiveness of this method is illustrated by the fact that it allowed the convenient preparation and purification of 60 mg of analytically pure 1,6-digalloylglucose within one week, thus avoiding the laborious, time consuming isolation of this rare natural product from kg-quantities of plant material [9, 15].

1,6-Di-O-protocatechuoyl-β-D-glucose

As described in detail in the Experimental section, the use of immobilized acyltransferase proved to be an excellent tool for the synthesis of 1,6-di-O-protocatechuoylglucose. The proposed structure of this new compound was confirmed by the ^{1}H NMR spectrum *(cf. Experimental)* where the signals for the glucose protons H-1, H-6, and H-6′ are shifted to higher δ-values in response to acylation, in contrast to the unchanged signals for the other protons. The coupling constant $^3J_{H\text{-}1,2}$ = 7.5 Hz proves retention of the β-configuration at the anomeric C-1.

The diester was further characterized by the UV absorption spectrum *(cf. Experimental)*; it had the same maxima (265, 300 nm) as that of the parent monoester [7] whereas the corresponding ε-values were roughly twice as high due to double acylation.

On grounds of earlier negative experience with related monoacylglucoses containing varying contents of bound water [7], no attempts were made to determine the melting point and the C,H-ratio of diprotocatechuoylglucose.

In conclusion, this communication presents a further example for the value of enzymes, and particularly of immobilized enzymes, in the convenient small-scale preparation of delicate compounds. By this means, quantities sufficient for biochemical investigations are easily accessible, without any necessity of working with suitable protective groups required in most chemical syntheses.

Acknowledgements

We are indebted to Miss U. Semler for valuable technical assistance, and to Prof. G.-I. Nonaka (Fukuoka) for a sample of 1,6-digalloylglucose. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.

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