Purification of Tetragalloylglucose 4-O-Galloyltransferase and Preparation of Antibodies against This Key Enzyme in the Biosynthesis of Hydrolyzable Tannins

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Polyclonal Antibodies, Hydrolyzable Tannins, Galloyltransferase, *Quercus robur* L. (*Q. pedunculata*)

The enzyme, β-glucogallin: 1,2,3,6-tetra-O-galloyl-β-D-glucose 4-O-galloyltransferase, which catalyzes the last common step in the biosynthesis of the two subclasses of hydrolyzable tannins, i.e. gallotannins and ellagitannins, was purified 868-fold from leaves of pedunculate oak (*Quercus robur*, syn. *Q. pedunculata*) to apparent homogeneity. Polyclonal antibodies against this pivotal enzyme were raised in rabbits and purified by protein-A chromatography, gel-filtration and affinity complexation. They were found to react specifically with acyltransferase from oak, displaying no cross-reactivity towards analogous enzymes from other plants synthesizing hydrolyzable tannins along the same biogenetic route, e.g. *Rhus typhina* or *Tellima grandiflora*.

Introduction

Enzyme studies have shown that the biosynthesis of gallotannins proceeds by stepwise galloylation of the primary specific metabolite, β-glucogallin (1-O-galloyl-β-D-glucose) (**1**), leading to 1,2,3,4,6-penta-O-galloyl-β-D-glucose (**3**). This polyester is further substituted by additional galloyl residues to form the *meta*-depsidically attached galloyl residues characteristic of complex gallotannins (for recent reviews, see Gross, 1999a, 1999b; Niemetz *et al.*, 1999). Though experimental evidence is still lacking, it is generally assumed that pentagalloylglucose (**3**) is also the immediate precursor of the second branch of hydrolyzable tannins, i.e. ellagitannins, which are the result of oxidative linkages between spatially adjacent galloyl groups of this compound affording hexahydroxydiphenoyl moieties bound to the glucose core (cf. Feldman *et al.*, 1999; Helm *et al.*, 1999). As summarized in Fig. 1, pentagalloylglucose (**3**) thus represents an essential key intermediate in the biosynthesis of both subclasses of hydrolyzable tannins, and this feature must also be attributed to the enzyme, β-glucogallin: 1,2,3,6-tetra-O-galloyl-β-D-glucose 4-O-galloyltransferase, that catalyzes the synthesis of this pivotal metabolite (Cammann *et al.*, 1989).

As a prerequisite for current immunohistochemical investigations on the sites of origin and deposition of hydrolyzable tannins in plant tissues, we were already able to produce an antibody against gallotannins and ellagitannins that was raised against protein-bound pentagalloylglucose as antigen (Kaspar *et al.*, 1998). Here, we report the preparation, purification and characterization of polyclonal antibodies raised against the pentagalloylglucose-synthesizing enzyme, 4-O-galloyltransferase, from oak leaves.

Materials and Methods

Chemicals

Chemical methods were employed for the synthesis of β-glucogallin (**1**) (Gross, 1982) and 1,2,3,4,6-penta-O-galloyl-β-D-glucose (**3**) (Gross, 1983a). For the preparation of 1,2,3,6-tetra-O-galloyl-β-D-glucose (**2**), freshly harvested leaves of staghorn sumac (*Rhus typhina* L.) were dried overnight at 35 °C, frozen in liquid N_2 and ground in an ultracentrifugal mill (Retsch KG, Haan, Germany). The resulting powder (40 g) was extracted with 70% aq. acetone (2 × 400 ml) on a gyrotory-shaker for 3 days. The residue of the freeze-dried extracts was taken up in H_2O and partitioned 10-times against ethyl acetate. The combined organic phases were dried over anhydrous $CaSO_4$, filtered, and evaporated. The solid residue was redissolved in ethanol, filtered (0.2 μm glass-fiber filters) and

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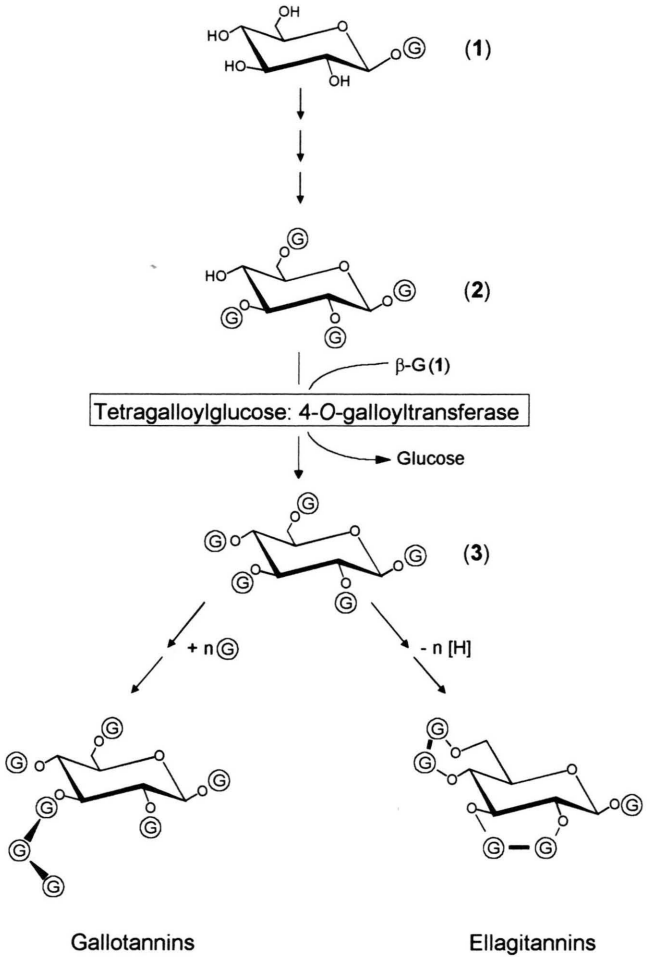

Fig. 1. Key function of the enzyme β-glucogallin: 1,2,3,6-tetra-O-galloyl-β-D-glucose (**2**) 4-O-galloyltransferase in the pathway from β-glucogallin (1-O-galloyl-β-D-glucose, **1**) to gallotannins (example: 3-O-trigalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose) and ellagitannins (example: casuarictin) via 1,2,3,4,6-penta-O-galloyl-β-D-glucose (**3**) as immediate common precursor. Ⓖ, Galloyl (3,4,5-trihydroxybenzoyl) residue; Ⓖ▶Ⓖ, *meta*-digalloyl residue; Ⓖ–Ⓖ, hexahydroxydiphenoyl (HHDP) residue.

chromatographed in 10 g-portions (dissolved in 20 ml ethanol) on Sephadex LH-20 (Pharmacia; column size 40×6 cm i.d.) as described by Nishizawa *et al.* (1980). Rechromatography of the tetragalloylglucose-containing fractions afforded 60–100 mg of ca. 55% pure product which was dried by vacuum evaporation, redissolved in ethanol (10 mg/ml) and subjected in 2.5 mg portions to semi-preparative reversed-phase (RP) HPLC (Merck LiChrosorb RP-18, particle size 5 μm; column 300×20 mm i.d.; solvent A: 0.05% aq. H_3PO_4, solvent B: acetonitrile, gradient: 0–5 min 5–13% B, 5–10 min 13–23% B, 10–23 min 23–50% B; flow rate 16 ml/min; detection UV 280 nm). The tetragalloylglucose-containing fractions were adjusted to pH 6.5 with 0.1 N NaOH, depleted of organic solvent by rotary evaporation and extracted with ethyl acetate, leaving 98% pure 1,2,3,6-tetra-O-galloylglucose (**2**) which was stored after evaporation over P_2O_5 at −20 °C.

Analytical methods

Normal-phase HPLC was used to analyze LH-20 eluates; it was carried out on LiChrosorb Si-60 (Merck CGC-glass cartridges; 150 × 3 mm i.d.; particle size 5 μm) with the solvent *n*-hexane-methanol-tetrahydrofuran-formic acid (56:33:11:1; by vol.) plus 400 mg oxalic acid per liter (modified after Nishizawa *et al.*, 1980). RP-HPLC was performed on LiChrospher RP-18 (Merck steel cartridges; 125 × 4 mm i.d.; particle size 5 μm) with the eluents 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B). Progress of the purifi-

cation of tetragalloylglucose was determined with a gradient of 0–2 min 5% B, 2–4 min 5–15% B, 4–20 min 15–30% B. Enzyme assays were analyzed with a gradient of 0–2 min 3% B, 2–4 min 3–15% B, 4–23 min 15–23% B. Flow rates were generally 1 ml/min. Quantification was done by UV photometry at 280 nm and a computing integrator (Merck-Hitachi D-2500) under reference to external standards.

Protein concentrations were determined colorimetrically according to Bradford (1976), using bovine serum albumin as standard; very dilute solutions were measured by UV photometry (Kalckar, 1947). Standard enzyme assay mixtures were as reported previously (Cammann *et al.*, 1989); the amount of enzymatically formed pentagalloylglucose was analyzed by RP–HPLC as described above.

Polyacrylamide-gel electrophoresis

Anodic discontinuous native polyacrylamide-gel electrophoresis (PAGE) was carried out with 4% stacking gels (pH 6.8) and 7% separating gels (pH 8.8) according to Holtzhauer (1992); optimal separations were achieved by supplementing the sample buffer with 10% Tween 20. For denaturing PAGE on 4% stacking gels and 12.5% separating gels (15% for antibody separations), all solutions were supplemented with 0.1% SDS. Protein bands were detected on the gels by silver staining (Blum *et al.*, 1987). Galloyltransferase activities were determined after non-denaturing PAGE by cutting gel-lanes into 2 mm-segments which were homogenized and extracted with 20 µl 0.1 M phosphate buffer, pH 6.5; after addition of substrates, the eluates were incubated and analyzed under standard enzyme assay conditions (see above).

Dot blot assays

The reactivity of antibody preparations was tested by dot blotting (Hawkes *et al.*, 1982; Hawkes, 1986) in a Schleicher & Schuell 'Minifold Dot Blotter' apparatus.Visualization of the spots was done by peroxidase staining with avidin-horseradish peroxidase conjugate (Sigma ExtrAvidin kit) and diaminobenzidine. This method was also effective in assaying the affinity of antibodies towards various antigens, starting the reaction cascade by application of antigen to the membrane in the dot blot apparatus and blocking with phosphate-buffered saline (PBS)/Tween prior to the above sequence.

Results and Discussion

Purification of antigen, 4-O-galloyltransferase

The enzyme, 1,2,3,6-tetra-O-galloyl-β-D-glucose 4-O-galloyltransferase, a key catalyst in the biosynthesis of hydrolyzable tannins that had been discovered by Camann *et al.* (1989) but was only partially purified in this earlier investigation, was extracted from oak leaves as described previously (Camann *et al.*, 1989), depleted of contaminating phenolics by treatment with Amberlite XAD-4 resin and fractionated by ammonium sulfate precipitation. After this step it was apparent that abundant amounts of highly viscous mucilage in the extracts caused serious problems in the progress of the purification protocol which could be solved, by analogy to earlier experience (Gross, 1983b), by separating the enzyme from these disturbing contaminants by adsorption on calcium phosphate gel (prepared according to Keilin and Hartree, 1938; 1.2 mg gel/mg protein) and subsequent elution with K-phosphate buffer (0.1 M, pH 7.0). The eluate was further purified by gel-filtration on a 30 × 2.8 cm i.d. column of Sephacryl S-300 (Pharmacia) in 50 mM tris(hydroxymethyl)-

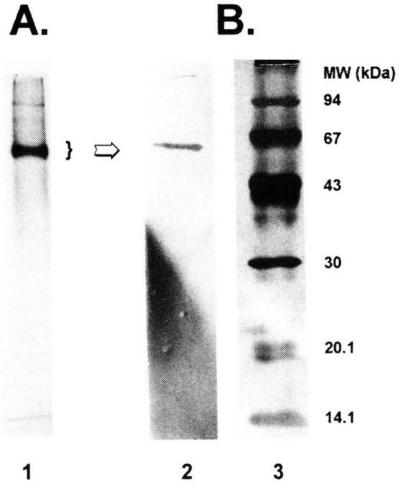

Fig. 2. Two-dimensional PAGE of 4-O-galloyltransferase. **A**: native PAGE of purified enzyme (lane 1). **B**: rechromatography under denaturing conditions (SDS-PAGE) of protein eluted from A (lane 2); lane 3, molecular weight marker proteins.

Table I. Purification of β-glucogallin: 1,2,3,6-tetragalloylglucose 4-O-galloyltransferase from oak leaves.

Step	Protein [mg]	Total activity [pkat]	Specific activity [pkat/mg]	Purification (x-fold)	Recovery (%)
Crude extract	432	1.19	2.8	1	100
Amberlite XAD-4 filtrate	416	1.36	3.3	1.2	114
Ammonium sulfate, 35–55% ppt.	79	0.82	10.4	3.7	69
Sephadex G-25 gel-filtration	62	0.78	12.6	4.5	66
$CaPO_4$-gel eluate	49	0.68	13.9	5.0	57
Sephacryl S-300 gel-filtration	34	0.63	18.5	6.6	53
Sephacryl S-300 rechromatography	25	0.58	23.2	8.3	49
Hydroxyapatite	2.5	0.52	208	74	44
DEAE cellulose	0.14	0.34	2429	868	29

aminomethane (Tris)-HCl buffer, pH 7.5. The most active fractions were combined and rechromatographed under identical conditions, followed by applying the enzyme to a 30 × 4 cm i.d. column with hydroxyapatite (Bio-Rad) equilibrated in 50 mM Tris-HCl, pH 7.0. After washing out unbound protein, galloyltransferase was eluted by a linear gradient of 0–0.5 M K-phosphate buffer, pH 7.0, and finally purified by ion-exchange chromatography on a 15 × 8 mm i.d. column of DEAE cellulose (Pharmacia Fast Flow) in 50 mM Tris-HCl, pH 7.0. The column was developed by a step-gradient of NaCl in buffer affording elution of the enzyme at 0.3 M NaCl. Before using the purified protein as immunogen, it was dialyzed against 3 × 5 l H_2O for 3 d, freeze-dried and stored at −80 °C. To obtain sufficient amounts of antigen for the immunization experiments, a total of 2 kg leaf material had to be worked up by the above protocol.

The results of a representative purification experiment are summarized in Table I. Native PAGE revealed that the enzyme had been purified to apparent homogeneity (Fig. 2A). Elution of this protein band and subsequent electrophoresis under denaturing conditions by SDS-PAGE afforded a single protein for which a MW of 65 kDa was determined by reference to marker proteins (Fig. 2B). Molecular weight determinations of the transferase by gel-filtration on calibrated Sephacryl S-300 columns (Andrews, 1965) resulted in a MW of 260 kDa, a value that was fully consistent with earlier results obtained with Sephadex G-200 (Camann *et al.*, 1989) and from which it was concluded that the native enzyme represented a tetramer being composed of four identical subunits.

Preparation and specificity of antigalloyltransferase serum

The immunization procedure was performed by Biotrend (Cologne) with two rabbits (age 3 months). Per boost, lyophilized immunogen (200 μg) was dissolved in 0.3 ml PBS, mixed with 1 ml Freund's complete adjuvant and injected into a rabbit. After 6 injections over 3 months, blood was collected from the animals after a total immunization period of 4 months. Crude antisera were prepurified by fractionation with neutralized saturated ammonium sulfate solution (Harlow and Lane, 1988). The 15–50% precipitate was resuspended in PBS and further purified with protein A-sepharose 4-CLB (Pharmacia) according to Ey *et al.* (1978). Active fractions, as determined by dot blotting (cf. experimental section), were further purified by gel-filtration on Sephacryl S-300 (column size 40 × 1 cm i.d.; Harlow and Lane, 1988) and by a final affinity complexation step (Olmsted, 1981) in which PAGE-separated pure antigen was blotted on nylon membranes, immobilized (Kyhse-Andersen, 1984) and incubated with antiserum for 12 h at 4 °C. The resulting specific antigen-antibody complex was located by staining (Sigma ExtrAvidin kit; peroxidase/diaminobenzidine staining) of a reference lane. The corresponding area of the preparative lane was cut out and washed, followed by cleavage of the antigen-antibody complex with 0.1 M glycine-HCl buffer, pH 2.5. The released pure immunoglobulin was immediately neutralized with 0.01 N NaOH, concentrated by ultrafiltration, supplemented with 0.02% thimerosal (sodium ethylmercurithiosalicylate; Sigma) as preservative and stored at 4 °C.

Progress of the purification sequence was monitored by SDS-PAGE, indicating that the product consisted of only those two immunoglobulin subunits, i.e. a 50 kDa heavy chain and a light chain of 26 kDa, which together constitute native IgGs of 75 kDa. In the above gel-filtration experiments, in contrast, a MW of 150 kDa had been determined for the native antibody on calibrated columns, a value that corresponds to the dimeric form of IgGs.

The antigalloyltransferase serum was found to react specifically with the antigen, 4-O-galloyltransferase, as shown in an immuno-blot experiment in which a partially purified enzyme sample was separated by gel-electrophoresis and transferred on a nitrocellulose membrane. Treatment with antibody, followed by addition of secondary antibody (goat anti-rabbit) and peroxidase/diaminobenzidine staining revealed the formation of only one band that coincided with the enzyme, indicating that the serum showed no cross-reactivities and was sufficiently specific for immunohistochemical applications. The specificity of the antibody was further proven by dot blot assays with cell-free extracts from different plants. Significant color reaction was observed only with extracts from oak (*Q. robur*) leaves, while extracts from staghorn sumac (*Rhus typhina*), known to produce high concentrations of gallotannins via a metabolic pathway identical to that in oak, gave only a very weak reaction. No cross-reactivity was observed with extracts from leaves of *Tellima grandiflora*, a rich source of ellagitannins whose biosynthesis also requires pentagalloylglucose as specific precursor, and from spinach (*Spinacia oleracea*) used as control because this vegetable is known to be devoid of tannins.

The value of the antitransferase serum was further documented in a different set of experiments in which the apoplasmatic compartment of oak leaves, i.e. cell-walls and intercellular space, was analyzed for the occurrence of 4-O-galloyltransferase. For this purpose, young leaves were immersed in water and infiltrated under slight vacuum (200 mbar, 30 min). After slowly releasing the vacuum to avoid cell rupture, the leaves were dried by blotting on paper and centrifuged (3000×*g*, 30 min) (G. Hrazdina, unpublished method). As depicted in Fig. 3, extracts obtained by this means displayed maximal enzyme activity in leaves collected from 2- and 3-month-old greenhouse-grown plants. This pattern was not only paralleled by the formation of enzyme reaction product, pentagalloylglucose (data not shown), but also by the reactivity of antigalloyltransferase serum with these extracts, as determined by the color development in immuno dot blots as a sensitive and rapid assay system.

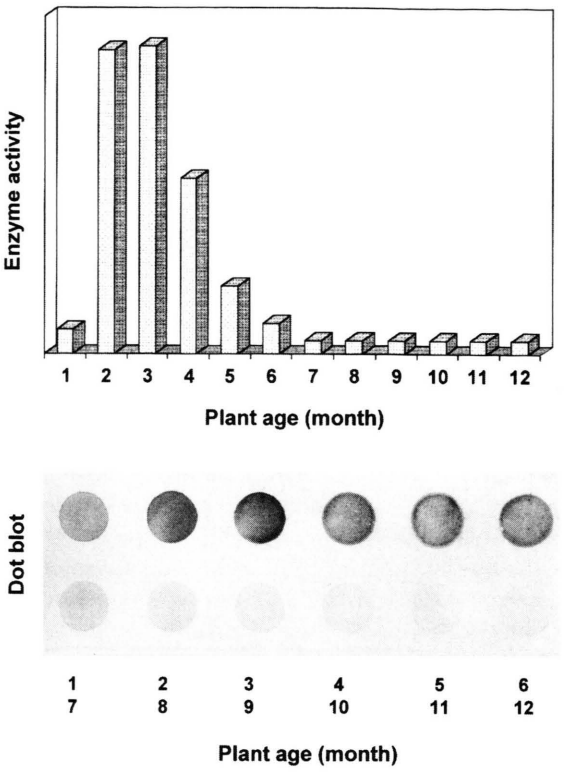

Fig. 3. Activity of 4-O-galloyltransferase in extracts from intercellular space and cellwalls of upper leaves from greenhouse-grown oak plants, as determined by standard enzyme assays (upper panel) and immuno dot blot tests with antigalloyltransferase serum (lower panel).

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