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UDP GLUCOSE: 4-(β-D-GLUCOPYRANOSYLOXY) BENZOIC ACID GLUCOSYLTRANSFERASE FROM THE POLLEN OF *PINUS DENSIFLORA**

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Key Word Index—*Pinus densiflora*; Pinaceae; Japanese red pine; pollen germination; pollen tube elongation; 4-hydroxybenzoic acid; 4-(β-D-glucopyranosyloxy) benzoic acid; UDPG: 4-(β-D-glucopyranosyloxy) benzoic acid glucosyltransferase.

Abstract—Exogenously supplied 4-hydroxybenzoic acid (HBA) was converted quantitatively to 4-(β-D-glucopyranosyloxy) benzoic acid (HBA glucoside) during 30 hr of germination of the pollen of *Pinus densiflora* (pine pollen). [^{14}C-*carboxyl*]HBA ([^{14}C]-HBA) was also converted to [^{14}C]-HBA glucoside by pine pollen. UDP glucose: 4-(β-D-glucopyranosyloxy) benzoic acid glucosyltransferase from pine pollen was assayed by measuring the rate of formation of [^{14}C]-HBA glucoside from [^{14}C]-HBA and UDPG. The enzyme was partially purified from the pine pollen for the first. The M_r of the enzyme was estimated to be *ca* 33 000 by Sephacryl S-300 gel filtration. The enzyme had a pH optimum at 7.5, and its activity was increased *ca* 50 and 60% by the addition of 3 mM Ca^{2+} and 0.1 mM EDTA, respectively. The K_m values for UDP glucose and 4-hydroxybenzoic acid were 2.4×10^{-4} M and 2.9×10^{-3} M, respectively.

INTRODUCTION

In the course of screening for substances which control the process of germination and tube elongation of the pollen from *Pinus densiflora* Sieb. et Zucc. (pine pollen), we found two endogenous inhibitors, 4-hydroxybenzoic acid (HBA) and 4-(β-D-glucopyranosyloxy) benzoic acid (HBA glucoside) [1]. HBA strongly inhibited germination and tube elongation when sucrose was not added to the medium. In the presence of exogenous sucrose, HBA inhibited germination and tube elongation less strongly. In contrast to these results, HBA glucoside inhibited both processes slightly regardless of the presence or the absence of exogenous sucrose. These results suggest that HBA is converted to a less inhibitory compound, HBA glucoside, during germination of pine pollen. Furthermore, these results suggest that the presence of the glucosyltransferase which catalyses the conversion of HBA to HBA glucoside in the pine pollen.

In this report, we describe the assay and partial purification of UDP glucose: 4-(β-D-glucopyranosyloxy) benzoic acid glucosyltransferase (HBA glucosyltransferase) from pine pollen.

RESULTS AND DISCUSSION

Changes in the amounts of HBA glucoside and HBA during germination

Changes in the amounts of HBA glucoside and HBA in pollen germinated on an agar medium with or without 3% sucrose are shown in Fig. 1. In the presence of exogenous sucrose, the amount of HBA glucoside increased rapidly and showed a 2.4-fold increase after 36 hr. Thereafter the amount of glucoside decreased. In the absence of exogenous sucrose, the amount of HBA glucoside increased more gradually. It showed a 1.9-fold increase after 24 hr of germination, and thereafter decreased. The amount of HBA showed no significant change regardless of the presence or absence of sucrose.

The amounts of HBA glucoside were 952 μg/g fr. wt at 36 hr of germination in the presence of exogenous sucrose, and 734 μg at 24 hr of germination in the absence of exogenous sucrose (Fig. 1).

HBA glucoside has been detected in fruit of anise (730–1080 ppm fr. wt), dill (188 ppm), fennel (106 ppm), coriander (30 ppm), caraway (42 ppm), carrot (65 ppm), celeriac (56 ppm), parsley (165 ppm), staranise (730–840 ppm) [2], cell cultures of *Lithospermum erythrorhizon* (1–1.3 mg/g fr. wt) [3], and aerial parts of *Calea pinnatifida* (not shown) [4]. From these results, the amounts of HBA glucoside in germinating pine pollen seem to be relatively high.

Changes in the amounts of HBA glucoside and HBA in pollen germinated in a liquid medium containing 3% sucrose and 3 mM HBA are shown in Fig. 2. The amount of HBA decreased markedly and was undetectable after 20 hr of germination, whereas the amount of HBA glucoside increased rapidly to reach a maximum value at 30 hr which was nearly equimolar with the amount of HBA added to the medium.

Conversion of [^{14}C]-*HBA to* [^{14}C]-*HBA glucoside*

To determine the fate of exogenously supplied [^{14}C]-HBA during germination, methanol extracts from pollen

*Part XXXIV in the series 'Biochemical studies on pollen'. For part XXXIII see *Agric. Biol. Chem.* (1987) **51**, 1553.

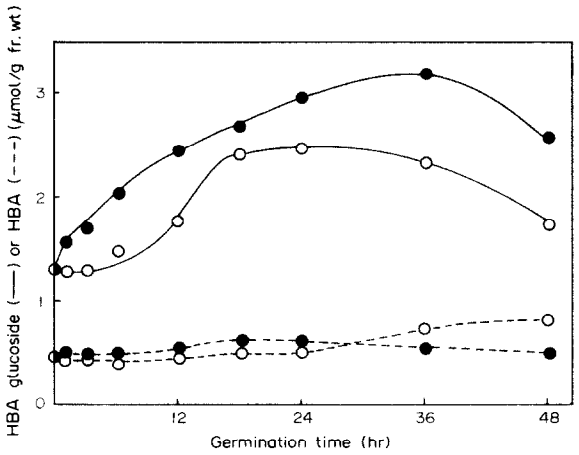

Fig. 1. Changes in the amounts of HBA and HBA glucoside during germination of pine pollen on an agar medium with or without addition of sucrose. Pine pollen was germinated at 30° on a 3% agar medium (○) or 3% agar medium containing 3% sucrose (●). TCA extracts or water extracts from the germinated pollen were analysed by HPLC.

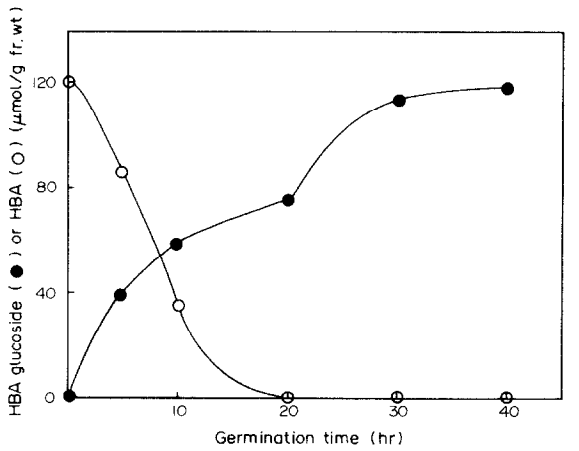

Fig. 2. Changes in the amounts of HBA and HBA glucoside during germination of pine pollen in a liquid medium containing sucrose and HBA. Pine pollen was germinated at 30° in a liquid medium containing 3% sucrose and 3 mM HBA. MeOH extracts from the germinated pollen were analysed by HPLC.

germinated in a liquid medium containing 0.1 mM [^{14}C]-HBA (0.2 μCi) were subjected to a HPLC, and the radioactivity in the eluate measured in a liquid scintillation spectrometer. [^{14}C]-HBA glucoside and [^{14}C]-HBA were eluted at 5 and 11 min, respectively. The peak of [^{14}C]-HBA decreased with increasing time of germination (6–12 hr), while a peak of [^{14}C]-HBA glucoside increased with increasing time of germination (Fig. 3 A–C). Confirmation of the conversion of [^{14}C]-HBA to [^{14}C]-HBA glucoside by pollen grains was provided by TLC autoradiography of the methanol extracts of pollen germinated in the presence of [^{14}C]-HBA. These results pointed to the presence of a glucosyltransferase which

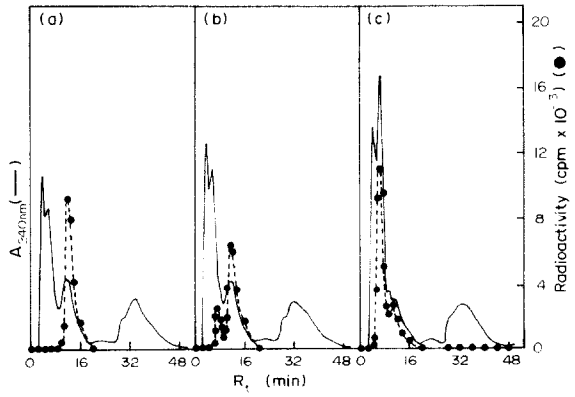

Fig. 3. HPLC of [^{14}C]-HBA and [^{14}C]-HBA glucoside during germination of pine pollen. Pine pollen was germinated for the following times at 30° in a liquid medium containing 0.1 mM [^{14}C]-HBA (0.2 μ Ci): (A) 0 hr; (B) 6 Hr; (C) 12 hr. MeOH extracts from the germinated pollen were analysed by HPLC.

catalyses the conversion of HBA to HBA glucoside in pine pollen.

Purification and characterization of HBA glucosyltransferase

HBA glucosyltransferase was partially purified from pine pollen by ammonium sulphate fractionation followed by chromatography on DEAE-cellulose and Sephacryl S-300. In both chromatographic systems a single peak of HBA glucosyltransferase activity was observed. It was purified *ca* nine-fold with a yield of 5%. Further purification of the enzyme is in progress.

Although UDP glucose:quercetin glucosyltransferase (*Zea mays*) [5–7], UDP glucose: flavonol 3-*O*-glucosyltransferase (*Alnus glutinosa*, *Corylus avellana*, *Quercus robur*, *Tulipa* cv. Apeldoorn, *Narcissus pseudonarcissus*, and *Secale cereale*) [8], UDP glucose: D-glucose-6-phosphate 1-α-D-glucosyltransferase (*Lilium longiflorum*) [9], and UDP glucose: D-fructose 2-α-D-glucosyltransferase (abbreviation: sucrose synthase, *Camellia japonica*, *Camellia sinensis*, *Camellia sasanqua*, *Erythrina indica*, *Lilium auratum*, and *Pinus thunbergii*) [10] are found in the pollen, this is the first report of the presence of UDP glucose: 4-(β-D-glucopyranosyloxy) benzoic acid glucosyltransferase in pollen. The M_r of HBA glucosyltransferase was estimated to be *ca* 33 000 by Sephacryl S-300 gel filtration.

Maximum enzyme activity was observed at pH 7.5 using 0.1 M Tris–malate–NaOH buffer (pH 5.5–8.5). The maximum activities of UDP glucose: quercetin glucosyltransferase [6] and sucrose synthase [10] are observed at pH 8.0–8.5. HBA glucosyltransferase activity was increased *ca* 50 and 60% by the addition of 3 mM Ca^{2+} and 0.1 mM EDTA, respectively. EDTA increased the enzyme activity presumably due to the removal of heavy metal ions. Ca^{2+} may replace heavy metal ions associated with the enzyme. The increase of the activity by the addition of Ca^{2+} is also reported for glucosyltransferase from various pollen [5, 10]. In contrast to glucosyltransferase from pollen [6, 10], the enzyme from pine pollen was not stimulated by the addition of Mg^{2+}.

The K_m values for UDP glucose and HBA were calculated from Lineweaver–Burk plots. The plots were linear, and the K_m values for UDP glucose and HBA were 2.4×10^{-4} M and 2.9×10^{-3} M, respectively. The K_i values for 3-hydroxybenzoic acid, gallic acid, and vanillic acid were 0.4×10^{-3} M, 0.7×10^{-3} M, and 1.3×10^{-3} M, respectively. These compounds show a competitive inhibition, and although they have higher affinities for the enzyme than HBA as judged from their K_i values they are unlikely to be natural acceptors as they occur at very low levels in pine pollen (unpublished results). The K_i values for 2-hydroxybenzoic acid and 4-coumaric acid were 3.1×10^{-3} M and 5.0×10^{-3} M, respectively. Thus these compounds appear to have a lower affinity than HBA for the enzyme. Quercetin was not a competitive inhibitor of the enzyme, and is also unlikely to be the glucose acceptor.

Yazaki *et al.* isolated HBA (one of the precursors in shikonin biosynthesis) and its glucoside (HBA glucoside) from cell cultures of *Lithospermum erythrorhizon*. Interestingly, the glucoside was accumulated by the cells producing no shikonin but it decreased rapidly when the cells were transferred to 'production medium' to induce shikonin synthesis [3]. From these results, they suggest that the precursor HBA is stored in the form of a glucoside when the cells are not synthesizing shikonin.

The results of Yazaki *et al.* [3], and our early demonstration that HBA-glucoside is less inhibitory than HBA to germination and tube elongation of pine pollen [1], suggest that the conversion of HBA to HBA-glucoside catalysed by pine pollen HBA glucosyltransferase may be important for the detoxification of HBA inhibition. As no significant change of the enzyme activity was observed during pine pollen germination (unpublished results), there may be mechanisms which control the activity during the process.

EXPERIMENTAL

Materials. Pine pollen was collected from anthers just before dehiscence and stored at −20° as described in refs [11, 12]. The following materials were from the sources indicated in parentheses: methylbenzimidazole-2-yl-carbamate (MBC) (Nihon Soda), chloramphenicol (Sigma), UDP glucose (UDPG) (Yamasa Shoyu), 4-hydroxy[*carboxyl*-^{14}C]benzoic acid ([^{14}C]-HBA, 50 mCi/mmol, CEA, France), DEAE-cellulose (Whatman), Sephacryl S-300 (Pharmacia). The other reagents used were of analytical grade.

Germination of pollen. Pollen was germinated at 30° for different periods as indicated in Figs 1–3, on a 3% agar medium or in a liquid medium in the presence or absence of sucrose, HBA, and [^{14}C]-HBA. To these media 50 ppm chloramphenicol (200 ppm in a liquid medium) and 10 ppm MBC were added as antibiotics.

Measurement of HBA glucoside and HBA. (i) *TCA extraction.* 250 mg of pollen germinated on an agar medium was suspended in 10 ml of 5% TCA and stirred on ice for 1 hr. The suspension was filtered through filter paper (No. 2, Advantic, Tokyo), and the filtrate was extracted (× 5) with H_2O satd Et_2O to remove TCA. The filtrate was concd to near dryness at 45° *in vacuo*, and dissolved in 1 ml of H_2O. After centrifugation at 10000 *g* for 10 min, the supernatant was subjected to HPLC. This extraction method was used to measure the amount of HBA glucoside during germination (Fig. 1).

(ii) *Water extraction.* 250 mg of pollen germinated on an agar medium was suspended in 2 ml of H_2O, and homogenized with a glass homogenizer. The homogenate was centrifuged at 10000 *g* for 10 min, and the supernatant heated on a boiling water bath for 1 min to ppt. proteins. The supernatant obtained by centrifugation at 10000 *g* for 10 min was concd to near dryness at 50° *in vacuo*, and dissolved in 1 ml of H_2O. After centrifugation at 10000 *g* for 10 min, the supernatant was subjected to HPLC. This extraction method was used to measure the amount of HBA during germination (Fig. 1).

(iii) MeOH *extraction.* 25 mg of pollen germinated in 1 ml of liquid medium was heated on a boiling water bath for 20 min. After cooling, 1 ml of MeOH was added to the liquid medium. The mixture was shaken in cold H_2O for 30 min and filtered through filter paper (No. 2, Advantic, Tokyo). The germinated pollen was washed with 2 ml of 50% MeOH and suspended in 2 ml of 75% MeOH and shaken in cold H_2O for 30 min, and filtered. Finally, the germinated pollen was treated with 2 ml of 99.6% MeOH as described above. The filtrates were combined and concd to 2 ml *in vacuo*, and then subjected to HPLC. This extraction method was used to measure the changes in the amounts of HBA glucoside and HBA during germination (Figs 2, 3).

TLC and autoradiography. MeOH extracts from the pollen germinated at 30° in a liquid medium containing 0.1 mM [^{14}C]-HBA (0.2 μCi) were subject to TLC on cellulose [Eastman chromagram sheet (8 × 8 cm)] in the following solvent, *iso*-PrOH–0.05 N NH_4OH (4:1). Formation of [^{14}C]-HBA glucoside from [^{14}C]-HBA was detected by autoradiography using an X-ray film (Fuji, Tokyo).

HPLC. (i) The TCA extracts or water extracts described above were subjected to HPLC (Toyo Soda, HLC-803C) on a Hitachi 3013-N gel column (4 × 150 mm) which was equilibrated with a buffer soln containing 6% MeCN, 0.3 M NH_4Cl, 0.05 M KH_2PO_4, and 0.05 M K_2HPO_4, pH 6.8 (buffer A), for HBA analysis or a buffer soln containing 6% MeCN, 0.06 M NH_4Cl, 0.01 M KH_2PO_4, and 0.01 M K_2HPO_4, pH 6.8 (buffer B), for HBA glucoside analysis. The column was developed with buffer A to measure HBA or with buffer B to measure HBA glucoside, at a flow rate of 1 ml/min at 60°. The elution was monitored at 240 nm.

(ii) The MeOH extracts described above were subjected to HPLC on a LiChrosorb RP-8 column (4 × 250 mm) which was equilibrated and developed with MeOH–0.2% HCl (1:9) at a flow rate of 1 ml/min at 40°. The elution was monitored as described above, and the radioactivity of each fraction was measured in a liquid scintillation spectrometer (Fig. 3).

Preparation of HBA glucosyltransferase from pine pollen. 50 g of pollen germinated for 24 hr in 2 l of liquid medium containing 3% sucrose and 3 mM HBA was filtered through filter paper (No. 2, Advantic, Tokyo) and washed with H_2O. The pollen was suspended in 500 ml of 0.1 M KH_2PO_4–NaOH buffer, pH 7.5, containing 10 mM 2-mercaptoethanol, 0.3 mM EDTA, and 10% glycerol (buffer C) and disrupted twice with a French Press (Otake Seisakusho, Japan) at *ca* 1500 kg/cm^2. The homogenate was centrifuged at 15000 *g* for 20 min. To the extracts, 500 mg of protamine sulphate dissolved in 10 ml of buffer C was added and the mixture stirred for 1 hr. After centrifugation (15000 *g* for 20 min), $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation. The ppt. obtained was collected by centrifugation and dissolved in 200 ml of buffer C. The enzyme soln was put on a DEAE-cellulose column (2.7 × 28 cm) equilibrated with 0.01 M KH_2PO_4–NaOH buffer, pH 7.5, containing 10 mM 2-mercaptoethanol, 0.3 mM EDTA, 1 mM phenylmethylsulphonylfluoride, and 10% glycerol (buffer D). The column was washed with 200 ml of buffer D and eluted with a linear gradient of KCl from 0 to 700 mM in buffer D. The flow rate was about 1.3 ml/min and 5 ml-fractions were collected. The HBA glucosyltransferase frac-

tions (No. 128–140) were pooled. The pooled fractions were put on a Sephacryl S-300 column (1.9 × 70 cm) equilibrated with buffer D. The column was developed with the same buffer at a flow rate of 12 ml/hr and 3.7 ml-fractions were collected. The HBA glucosyltransferase fractions (No. 32–37) were pooled and used in the following experiments.

Enzyme assay. HBA glucosyltransferase was assayed by measuring the formation of [^{14}C]-HBA glucoside from [^{14}C]-HBA and UDPG. In a total vol. of 100 μl, the reaction mixture contained 100 mM Tris–malate–NaOH buffer (pH 7.5), 3 mM $CaCl_2$, 0.1 mM EDTA, 4 mM 2-mercaptoethanol, 2 mM UDPG, 0.01 mM [^{14}C]-HBA (0.05 μCi), and a suitable amount of enzyme preparation. The mixture was incubated at 30° for 20 min, and then the reaction was stopped by addition of 20 μl of 1 M HCl. To the mixture, 2 ml of H_2O satd Et_2O was added to remove free [^{14}C]-HBA, and the mixture was shaken and centrifuged at 3000 rpm for 1 min. After removal of the Et_2O phase, the aq. phase containing [^{14}C]-HBA glucoside was treated three times with Et_2O as described above. 100 μl of the aq. phase was put on a glass filter, dried and the radioactivity measured in a liquid scintillation spectrometer. The HBA glucosyltransferase activity is expressed as pmol [^{14}C]-HBA glucoside formed/20 min.

Protein was measured by the method of ref. [13], using bovine serum albumin as a standard.

Estimation of M_r. The 60% $(NH_4)_2SO_4$ satd fraction described above was put on a Sephacryl S-300 column (0.5 × 84 cm) equilibrated with buffer D. The column was developed with the same buffer at a flow rate of 12 ml/hr and 250 μl fractions were collected. *Cytochrome c* (M_r, 12 500), albumin (45 000), and ferritin (450 000) were used as the standard proteins.

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