Note

Occurrence of Endogenous Pollen Growth Inhibitors, 4-Hydroxybenzoic Acid and 4-(β -D-Glucopyranosyloxy)benzoic Acid, in the Pollen of *Pinus densiflora*[†]

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Various pollen growth inhibitors have been found in the pollen of some plants.¹⁾ However, their functions have not been elucidated extensively. 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., we found endogenous inhibitors which are distinct from known inhibitors in pine pollen, such as benzoic acid,²⁾ and dehydrochorismic acid.³⁾

In this paper we describe the procedures for the purification and identification of the inhibitors, 4-hydroxybenzoic acid and 4-(β -D-glucopyranosyloxy)benzoic acid. We refer also to the functions of these compounds in the pollen.

Two hundred grams of pine pollen were extracted with 3 liters of 5% perchloric acid. The extract was neutralized with KOH and the resulting precipitate was removed by centrifugation. The supernatant fraction was applied to a Dowex-1 $\times$ 2 column (3.0 $\times$ 30 cm, formate type). After the column was washed with water, a phenolic glycoside was eluted with 4N formic acid. The eluate was evaporated to dryness and dissolved in water. The dissolved sample was applied to a Dowex-50 $\times$ 8 column (2.0 $\times$ 30 cm, H type) to remove some impurities. The fractions which did not absorb to the column were evaporated to dryness and dissolved in 2 ml of water. The sample was applied to a Sephadex G-10 column (1.8 $\times$ 30 cm) and developed with water. The glycoside was eluted at the fractions near the void volume (16 ml). Then an acidic substance tentatively termed *x*-acid, which was not eluted from the column with water, was eluted with 0.1 M NH_4HCO_3 and evaporated to dryness. The glycoside was recrystallized from water. Twenty mg of the glycoside and a few mg of *x*-acid were obtained from 200 g of the pollen.

x-Acid and the glycoside were identified by high performance liquid chromatography (HPLC): ca. 20 nmol of authentic 4-hydroxybenzoic acid and/or *x*-acid were applied to the HPLC equipped with a column (4 $\times$ 150 mm) containing Hitachi #3013-N gel which was equilibrated with a buffer solution containing 6% CH_3CN , 0.3 M NH_4Cl , 0.05 M KH_2PO_4 , and 0.05 M K_2HPO_4 (pH 6.8). The column was developed with the same buffer solution. Authentic 4-hydroxybenzoic acid and *x*-acid were eluted at the same position (6 min) as a single peak. The glycoside was eluted at 1.3 min as a single peak, while the hydrolysate of the glycoside (hydrolyzed with 1 N HCl for 1 hr at 100°C) was eluted at 6 min. The mixtures of the hydrolysate and authentic 4-hydroxybenzoic acid gave a single peak at 6 min on the HPLC. The sugar obtained from the glycoside was identified as D-glucose with thin layer chromatography. These results indicate that *x*-acid is 4-hydroxybenzoic acid, and that this acid is the aglycone of the glycoside (glucoside). This indication was further confirmed as follows.

The field desorption mass spectrum of the glycoside showed the molecular ion peak at m/z 300 accompanied by two intense fragment ion peaks at m/z 163 and 137. The former fragment peak corresponds to the glucosyl residue of the glucoside and the latter to 4-hydroxybenzoic acid. The $^1\text{H-NMR}$ spectrum (d_4 methanol) of the glucoside indicates the existence of *p*-substituted benzene: δ 7.18 (double, $J=9.03$ Hz) and 8.02 (double, $J=9.03$ Hz). The $^{13}\text{C-NMR}$ spectrum of the glucoside demonstrates that the glucoside is 4-(β -D-glucopyranosyloxy)benzoic acid: $^{13}\text{C-NMR}$ $\delta_{\text{TMS}}^{\text{CD}_3\text{OD}}$: 169.6, 162.8, 132.7, 125.7, 117.2, 101.7, 78.3, 78.0, 74.8, 71.3, and 62.5. The first five peaks can be assigned to the carbons of 4-hydroxybenzoic acid, COOH , C_4 , $\text{C}_2(\text{C}_6)$, C_1 , and $\text{C}_3(\text{C}_5)$, respectively,⁴⁾ and the last six peaks to those of β -D-glucopyranose,⁵⁾ C_1 , C_3 , C_5 , C_2 , C_4 , and C_6 , respectively. Since glucose was liberated from the glucoside by β -glucosidase [EC 3.2.1.21] from sweet almonds, the configuration of the glucopyranose in the glucoside was confirmed to be the β -form.

Since benzoic acid²⁾ had been known as an endogenous inhibitor of the germination of pine pollen, and crude *x*-acid (4-hydroxybenzoic acid) and its glucoside also inhibited that process (unpublished data), we tested the effect of 4-hydroxybenzoic acid and 4-(β -D-glucopyranosyloxy)benzoic acid purified from the pollen on the germination and tube elongation of the pollen. Twenty-five mg of pine pollen were incubated by shaking at 30°C for 24 hr in water in the presence or absence of sucrose, 4-hydroxybenzoic acid, and 4-(β -D-glucopyranosyloxy)benzoic acid. Chloramphenicol (200 ppm) and methylbenzimidazole-2-yl-carbamate (10 ppm) were added to the medium as germicides. As shown in Table I, when sucrose was not added to the medium, 4-hydroxybenzoic acid strongly inhibited germination and

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TABLE I. EFFECTS OF 4-HYDROXYBENZOIC ACID AND 4-(β -D-GLUCOPYRANOSYLOXY)BENZOIC ACID ON THE GERMINATION AND TUBE ELONGATION OF PINE POLLEN

	Conc. (mm)	Sucrose-free medium		3% sucrose medium	
		Germination (%)*	Tube length (μ m)**	Germination (%)*	Tube length (μ m)**
4-Hydroxybenzoic acid	0	85.4	113.6 $\pm$ 16.4	88.6	87.6 $\pm$ 10.3
	1.0	69.5	97.8 $\pm$ 11.3	80.3	75.3 $\pm$ 7.9
	2.0	59.2	70.9 $\pm$ 8.6	71.9	66.9 $\pm$ 9.5
	3.0	13.2	29.8 $\pm$ 8.3	55.3	47.1 $\pm$ 8.3
4-(β -D-Glucopyranosyloxy)benzoic acid	1.0	77.5	100.1 $\pm$ 12.3	81.5	77.8 $\pm$ 8.3
	2.0	74.5	91.3 $\pm$ 10.9	78.0	70.2 $\pm$ 7.9
	3.0	76.5	86.3 $\pm$ 10.3	68.4	57.7 $\pm$ 8.0

* Each value represents the average percentage of germination of 1,000 pollen grains.

** Each value represents the average pollen tube length of 100 pollen tubes with standard deviation.

tube elongation. These inhibitions were relieved when the pollen was transferred to a medium which did not contain 4-hydroxybenzoic acid (data not shown). In the presence of exogenous sucrose, 4-hydroxybenzoic acid inhibited germination and tube elongation less strongly. In contrast to these results, 4-(β -D-glucopyranosyloxy)benzoic acid inhibited both processes slightly regardless of the presence or the absence of exogenous sucrose. These results suggest that 4-hydroxybenzoic acid, which strongly inhibits germination, is converted to a less inhibitory compound, 4-(β -D-glucopyranosyloxy)benzoic acid, during the process of the germination of the pollen.

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