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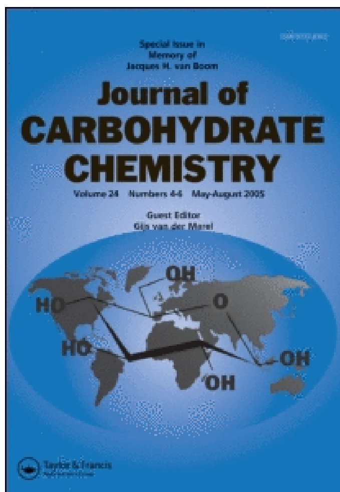

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Efficient Synthesis of β-Primeverosides as Aroma Precursors by Transglycosylation of β-Diglycosidase from *Penicillium multicolor*

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Efficient Synthesis of β-Primeverosides as Aroma Precursors by Transglycosylation of β-Diglycosidase from *Penicillium multicolor*

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The enzyme activity transferring a β-primeverosyl unit was found from culture filtrates of *Penicillium multicolor* IAM7153 and was useful for synthesizing a series of β-primeverosides via a β-primeverosyl transfer reaction in an aqueous-organic biphasic system. With the acceptors benzyl alcohol, 2-phenylethanol, and (*Z*)-3-hexenol, the enzyme induced the transfer products benzyl, 2-phenylethyl, and (*Z*)-3-hexyl β-primeverosides in high yields of 51% to 70% based on the donor added. When geraniol and eugenol were used as acceptors, the corresponding geranyl and eugenyl β-primeverosides were obtained in lower yields of 8% to 12%. The enzyme was an excellent tool for producing naturally occurring β-primeverosides on a mmol scale.

Keywords Enzymatic synthesis, Plant aroma precursors, β-Primeverosides, β-Diglycosidase, *Penicillium multicolor* IAM7153

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INTRODUCTION

Floral aroma is one of the most important factors in determining the quality of brewed tea and various fruits and flowers. Monoterpene alcohols (linalool, geraniol, α-terpineol, etc.) and aromatic alcohols (benzyl alcohol, 2-phenylethanol, etc.) are known to be the major floral aroma constituents of tea.[1] Various tea aroma constituents, such as geraniol,[2] linalool, benzyl alcohol, and 2-phenylethanol,[3] were shown to be present mainly as β-diglycoside precursors such as β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside, Psd) in tea plant leaves used to produce oolong tea. In addition, many naturally occurring compounds, such as anthraquinones[4–9] and (*R*)-lucumin,[10] exist as β-primeverosides in various plants.

Our purpose is to develop an efficient synthetic method of obtaining such β-Psds in sufficient amounts to study the function. Therefore, we searched β-primeverosidase-like enzymes among microbes showing transglycosylation activity so that it transfers the entire primeverose from *p*-nitrophenyl (*p*NP) β-Psd as substrate. If such an enzyme was found, it might make it possible to catalyze direct transfer of primeverosyl unit to various acceptor aromas. No study has been reported regarding transglycosylation of a primeverose unit. We recently reported that a β-Psd hydrolyzing enzyme from *Aspergillus fumigatus* AP-20 cleaved in an endo-manner *p*NP β-Psd into primeverose and *p*-nitrophenol.[11] However, the enzyme showed only a limited transferring activity. Therefore, we searched for another source of β-primeverosidase-like enzyme among microbes already commonly used in traditional food processing. The availability of this enzyme from such sources would be useful not only for the enzymatic synthesis of β-Psds as aroma precursors, but also for its potential applications in biotechnology, for example, in the control of aroma release and the high recovery of aromas in extracts.

In the present study, we performed transglycosylation of a primeverose unit from *p*NP β-Psd to various alcoholic and phenolic aroma compounds by use of the partially purified enzyme powder preparation from *P. multicolor* IAM7153.

RESULTS

A total of 30 types of cultures were grown in a liquid medium and screened for secretion of a β-primeverosidase activity, namely the hydrolysis of *p*NP and eugenyl β-Psds to primeverose and the corresponding aglycones. Each culture supernatant was reacted with *p*NP and eugenyl β-Psds, and the reaction mixture was analyzed by TLC. Hydrolysis of the substrates were detected by the release of primeverose. The β-primeverosidase activity was detected in the supernatant from eight different types of cultures (*Aspergillus oryzae* JMC5560, *Aspergillus niger* IAM2107, *Aspergillus awamori* IFO4033, *Rhizopus oryzae* JCM5560, *Talaromyces emersonii* IFO9747, *Trichoderma reesei* QM9414B, *Penicillium camembertii* FERMP-10452, *Penicillium multicolor* IAM7153), with *P. multicolor* IAM7153 having the highest enzyme activity. The supernatant also showed transglycosylation activity with *p*NP β-Psd and eugenyl β-Psd.

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Partially Purified β-diglycosidase

The supernatant was precipitated with ammonium sulfate at 50% (w/v) saturation and subjected to Phenyl Sepharose column chromatography [HiLoad 16/10 Phenyl Sepharose H.P., sodium acetate buffer (pH 5.5) containing 30% (w/v) ammonium sulfate]. Two fractions of this column (F-1 and F-2) showed enzyme activity with assays A and B. The major fraction (F-1) was used as partially purified enzyme preparation for the synthesis of β-primeverosides as aroma precursors.

Synthesis of Five Different Aroma Precursor β-Psds

β-Psds exist in various kinds of plants,[11–15] but it is not easy to obtain large amounts of the diglycosides from natural sources. Therefore, we attempted the mmol scale synthesis of aroma precursor β-Psds by β-primeverosidase-like enzyme-mediated transglycosylation. Generally, glycosidases have transglycosylation activity as a reverse reaction of hydrolysis. At first, the transglycosylation reaction was carried out on a small scale by using the partially purified enzyme from *P. multicolor* IAM7153 and products resulting from transglycosylation from *p*NP β-Psd donor to various alcoholic aroma acceptors (benzyl alcohol, 2-phenylethanol, (*Z*)-3-hexenol, and geraniol), and phenolic aroma acceptors (eugenol) were analyzed by TLC. A new spot corresponding to a transglycosylation product was detected in each sample on the TLC plate (data not shown). On the basis of these data, benzyl β-Psd (**1**) was synthesized on a preparative scale from *p*NP β-Psd and benzyl alcohol by transglycosylation. The molar ratio of the donor to the acceptor was 1:5. The transfer product **1** was readily obtained in one step by Toyopearl HW-40S column chromatography in a 51% yield, based on the amount of donor added.

The typical elution pattern was as shown in Fig. 1. Similarly, with the acceptors 2-phenylethanol and (*Z*)-3-hexenol, the transfer products 2-phenylethyl (**2**) and (*Z*)-3-hexenyl *β*-Psds (**3**) were obtained in high yields of 70% and 62%, respectively. However, geraniol was a much poorer acceptor [8% yield of geranyl *β*-Psd, (**4**)] than the other aroma alcohols. In this case, the transfer product was isolated in two steps by successive chromatographies on Toyopearl HW-40S and Sep-pak C_{18} columns. In the case of eugenol, the rate of formation of eugenyl *β*-Psd (**5**) was too rapid at the standard concentration of enzyme to be sure of its maximum production. In a separate experiment, compound **5** was hydrolyzed 25-fold faster than the alcoholic aroma *β*-Psds (**1–4**), as mentioned below. Thus, **5** is a much better substrate than **1–4** under hydrolytic conditions. In general, the order of formation of transfer products closely corresponds to that of hydrolytic rate. The present reaction made it possible to synthesize **5** by lowering the enzyme concentration. Therefore, the reaction was performed at an enzyme concentration one-eighth that used for the preparation of *β*-Psd from the alcoholic aromas mentioned above. The desired compound **5** was obtained in a 12% yield based on the amount of donor added. Structures of these synthetic transfer products were elucidated by ^{1}H and ^{13}C NMR (Table 2 and Fig. 5) and FAB-mass analysis and identical to those of the authentic samples reported previously.[2,3,17,18]

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Time Course of the Formation of β-Psd During Transglycosylation

The time courses of **2** synthesis and *p*NP *β*-Psd substrate degradation during transglycosylation are shown as an example of reaction profile in Fig. 2. The samples (10 μL) were taken at intervals during the incubation, inactivated by

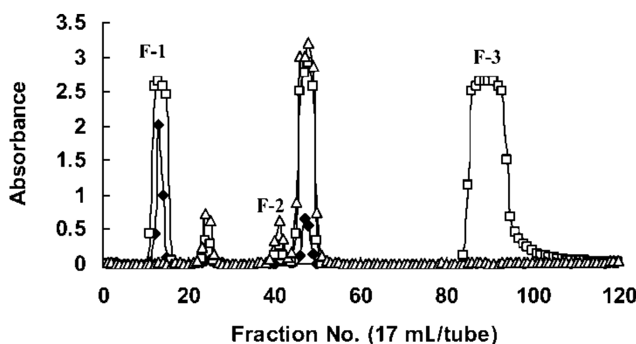

Figure 1: Isolation of transglycosylated products **1** by Toyopearl HW-40S chromatography. The transglycosylation products were extracted with chloroform and applied to a Toyopearl HW-40S column. The fractions corresponding to compound **1** (F-1) were combined and lyophilized. F-2, the fractions corresponding to the absorbance of *p*-nitrophenol; F-3, the fractions corresponding to the absorbance of benzyl alcohol; □, A_{210}; △, A_{300}; ◆, A_{485}.

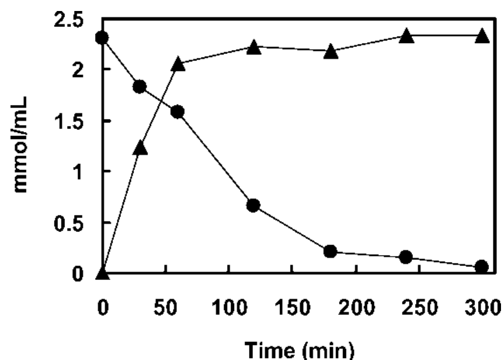

Figure 2: The time course of the transglycosylation reaction. As the synthesis of **2** proceeded, the amount of *p*NP *β*-Psd decreased. ●, *p*NP *β*-Psd; ▲, compound 2.

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heating in a boiling water bath for 5 min, and then diluted with water (190 μL) for analysis by HPLC. With 2-phenylethanol as an acceptor, compound **2** was formed in appreciable amounts. The maximal concentration of **2** was reached in 50 min and then decreased slightly during the subsequent reaction. The same trend was also observed for the formation of **1, 3**, and **4** (data not shown).

Hydrolytic Activities on Various Substrates

In a separate experiment, hydrolytic activities on a series of *β*-Psds and related compounds were analyzed by the partially purified enzyme. Because hydrolytic activity generally has a close relationship with transglycosylation activity, the partially purified enzyme was shown to hydrolyze *p*NP *β*-Psd into primeverose and *p*-nitrophenol. Based on this result, five synthetic *β*-Psds (**1–5**), three *p*NP *β*-glucobiosides, and *p*NP *β*-Glcd were tested as substrates for the enzyme (Table 1). In this experiment, the amounts of *p*-nitrophenol or aglycones formed from initial substrates were analyzed at an early stage of incubation with the enzyme (up to 15% to 20% hydrolysis). The best substrate for the enzyme was the artificial substrate *p*NP *β*-Psd; five other *β*-Psds were not readily hydrolyzed. The relative rates of attack on these *β*-Psds were compared with that on *p*NP *β*-Psd. The relative rate of hydrolysis of *β*-Psds having alcoholic aglycones (**1–4**) was 0.1% (1,000-fold slower than *p*NP *β*-Psd). Of the naturally occurring aroma precursors, compound **5** was the most rapidly hydrolyzed, although with 1/40 activity of *p*NP *β*-Psd. It is notable that the alcoholic aroma precursors were very poor substrates. The relative rate of hydrolysis of *p*NP *β*-Gend was 2.5%, but two other *p*NP *β*-Lamd and *p*NP *β*-Celd did not act as substrates. No detectable hydrolysis of reducing primeverose, other *β*-linked glucobioses, and glucooligosaccharides was observed.

Table 1: Relative hydrolytic rates of the partially purified β-diglycosidase on various kinds of β-glycosides and reducing saccharide substrates. Hydrolytic rate on *p*NP β-Psd was set at 100%.

Substrate	Rate of hydrolysis (%)
*p*NP β-Psd[a]	100
*p*NP β-Gend[a]	2.5
*p*NP β-Lamd[a]	ND[c]
*p*NP β-Celd[a]	ND
*p*NP β-Glcd[a]	15
Eugenyl β-Psd[b] (**5**)	2.5
Benzyl β-Psd[b] (**1**)	0.1
2-Phenylethyl β-Psd[b] (**2**)	0.1
(*Z*)-3-Hexenyl β-Psd[b] (**3**)	0.1
Geranyl β-Psd[b] (**4**)	0.1
Gentiooligosaccharide	ND
Laminarioligosaccharide	ND
Cellooligosaccharide	ND
Sophorose	ND
Primeverose	ND

[a]Release of *p*-nitrophenol was detected photometrically at 405 nm.
[b]Release of each aroma compound was detected by HPLC as the hydrolytic product.
[c]No hydrolytic products were detected.

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DISCUSSION

β-Psds occur quite widely in plants,[18] but it is very difficult to obtain large amounts of the diglycosides from natural sources. A partially purified enzyme preparation of *P. multicolor* IAM7153 was directly used for the synthesis of a series of β-Psds without further purification. An enzymatic synthesis method, involving β-diglycosidase-mediated transglycosylation, facilitated large-scale preparation of β-Psds by as short a pathway as possible. A series of β-Psd aroma precursors were synthesized by transglycosylation from *p*NP β-Psd (donor) to various aromas (acceptors). Reactions were carried out in an aqueous-organic biphasic system. The transfer products **1, 2**, and **3** were readily synthesized on an mmol scale in high yields of 51%, 70%, and 62% based on the amount of donor added, respectively, and were conveniently isolated by chromatography on a column of Toyopearl HW-40S. In contrast, when geraniol and eugenol were used as acceptors, the yields of **4** and **5** were very low (8% to 12% based on the amount of donor added). The product yield might be greatly influenced by the extent of substrate migration from the organic phase to the aqueous phase in the biphasic system. Indeed, benzyl alcohol, 2-phenyhlethanol, and (*Z*)-3-hexenol have a solubility of 2% to 4% in water and can gradually move into the aqueous layer to undergo

transglycosylation. In contrast, geraniol and eugenol have only a poor solubility in water and thus are less likely to be acted on by the enzyme. The transglycosylation reaction in the biphasic system is schematically shown in Fig. 3.

After formation of a primeverosyl-enzyme complex, two reactions are possible: (i) hydrolysis and (ii) synthesis. The efficiency of glycosidase-catalyzed transglycosylation is generally low because of an equilibrium shift favorable to hydrolysis.[19] However, the biphasic system has several characteristics that may explain the high product concentrations obtained in the present study.[20] The β-Psd concentration that can be attained is controlled by the equilibrium of the system. As an example, we monitored a transglycosylation reaction from *p*NP β-Psd to 2-phenylethanol, following the time course of **2** production and substrate consumption in the biphasic system, by analyzing the corresponding spots on TLC plates, as in Fig. 4.

We found that the product and the substrate were present in both the aqueous and organic phases (2-phenylethanol), indicating that some portion of the transglycosylation product in the aqueous phase migrates to the organic phase. As a consequence of this product removal, the equilibrium shifts toward synthesis and the synthesis reaction comes to be more favored. Furthermore, once **2** production reached its maximum, its concentration then varied little during the subsequent reaction (Fig. 4). Consequently, aromas such as benzyl alcohol, 2-phenylethanol, and (*Z*)-3-hexenol serve as suitable acceptors for transglycosylation and the target β-Psds accumulate in the biphasic system. Once the β-Psd formed, not much was hydrolyzed because it is a poor substrate for the enzyme. In a separate experiment, the relative rate of hydrolysis of **1, 2**, and **3** compared with *p*NP β-Psd (100) was

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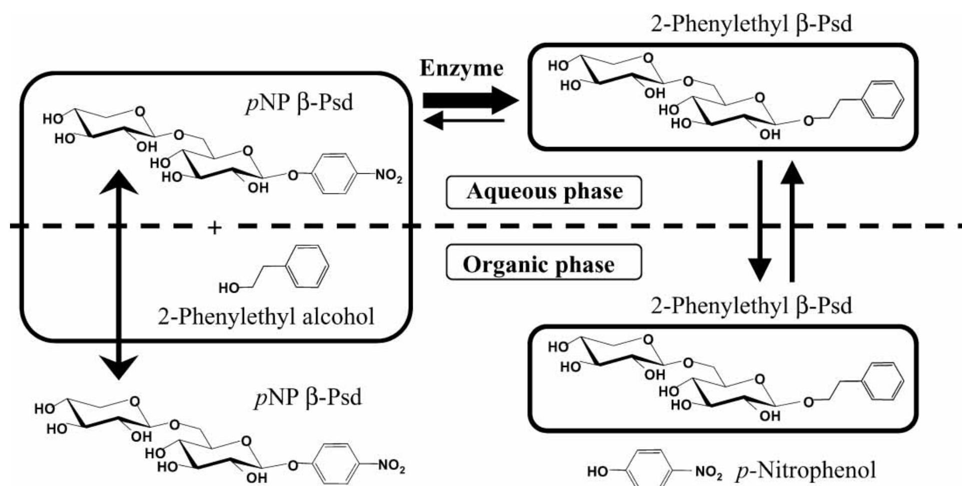

Figure 3: Scheme of the transglycosylation reaction in the aqueous-organic biphasic system. The synthesis of **2** is shown as a representative example.

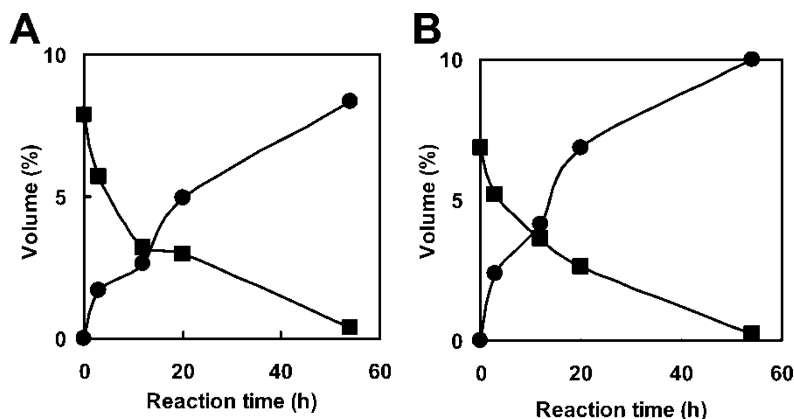

Figure 4: The time course of **2** production and *p*NP β-Psd consumption in the aqueous-organic biphasic system. The synthesis of **2** and the consumption of *p*NP β-Psd in the water phase (A) and the organic phase (B) were monitored by TLC analysis. The relative intensity of spots was scanned densitometrically. ■, *p*NP β-Psd; ●, compound **2**.

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0.1, a 1,000-fold difference (Table 1). It might be meaningless as the hydrolytic substrate for the enzyme. This is a reasonable explanation for the high yield of the transglycosylation product β-Psds. From these results, the present enzyme might be a kind of β-diglycosidase rather than β-primeverosidase, because it acts only to a limited extent toward naturally occurring series of β-Psds. The characteristics of the enzyme will be reported in detail in the near future.

CONCLUSION

By screening for microorganisms producing any β-Psd-hydrolyzing enzyme, we found a *P. multicolor* IAM7153 strain that produces a unique β-diglycosidase that hydrolyzes β-Psd in an endo-manner into primeverose and aglycon. Since the enzyme had a strong transglycosylation activity with *p*NP β-Psd, the synthesis of a series of β-Psds as aroma precursors has been achieved by β-diglycosidase-mediated transglycosylation in an aqueous-organic biphasic system. This enzyme is an excellent tool for producing β-Psds on an mmol scale. This synthetic method has great potential for mass production of useful glycomaterials.

EXPERIMENTAL

General Methods

p-Nitrophenyl (*p*NP) β-Psd, primeverose, *p*NP β-gentiobioside (Gend), and 2-phenylethyl β-Gend were prepared by our method.[21,22] *p*NP

β-Glucopyranoside (Glcd) and *p*NP β-galactopyranoside (Gald) were purchased from Sigma Chemical Co. *p*NP β-Cellobioside (Celd) and *p*NP β-laminaribioside (Lamd) were purchased from Yaizu Suisankagaku Industry Co., Ltd. All other chemicals were obtained from commercial sources.

Hydrolytic Activities

Hydrolytic activities were assayed toward *p*NP β-Psd, *p*NP β-Glcd, and *p*NP β-Gald (assays A, B, and C, respectively). A mixture containing 80 μL of each substrate (2 mM) in 20 mM sodium acetate buffer, pH 5.5, and 20 μL of enzyme solution was incubated in a 96-well microplate for 30 min at 40°C. The reaction was stopped by adding 100 μL of 1 M sodium bicarbonate. The released *p*-nitrophenol was measured photometrically at 405 nm in a microplate reader (Biolumin 960, Amersham Pharmacia Biotech). One unit of the enzyme was defined as the amount releasing 1 μmol of *p*-nitrophenol per minute.

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Analytic Methods

TLC analysis was carried out on a silica gel 60 F_{254} plate using a solvent system of ethyl acetate/acetic acid/water (3 : 1 : 1, v/v). Sugars on the plate were visualized by heating at 120°C for 10 min after spraying with 20% sulfuric acid-methanol solution. HPLC analysis was performed using a Mightysil RP-18(H) column (ϕ 150 × 4.6 mm) on a JASCO LCS-905 HPLC System station with an ultraviolet detector (absorbance at 210 nm). Elution was performed with H_2O at a flow rate of 0.6 mL/min at 65°C. FAB-mass analysis was carried out in the positive ion mode using a JEOL JMS DX-303HF mass spectrometer coupled with the JEOL DA-800 mass data system. An accelerating voltage of 10 kV and mass resolution of 1,000 was employed. A sample of 1 μL in H_2O or CH_3OH was loaded with 1 μL of glycerol as a matrix. ^1H and ^{13}C NMR spectra of each sample in D_2O were recorded on a JEOL JNM-EX 270 spectrometer at 30°C. Chemical shifts were expressed in δ relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an external standard.

Media

The formula for the medium used for seed cultures for screening is as follows: defatted soy meal [Honen Co. Ltd. (2.0%)], glucose (3.0%), KH_2PO_4 (0.5%), $(NH_4)_2SO_4$ (0.4%), and dry yeast powder (0.3%). The medium for the main cultures used for screening consisted of Gentose 80# [Nihon Shokuhinn Kako Co., Ltd., Japan (3.0%)], KH_2PO_4 (2.0%), $(NH_4)_2SO_4$ (1.0%), and Meast P1G [Asahi Food & Healthcare, Ltd. (3.1%)]. The medium for enzyme

production consisted of Solulys A-ST [Roquette (3.0%)], Pinedex No.2 [Matsutani Chemical Industry Co. Ltd. (1.0%)], and KH_2PO_4 (0.5%). The pH of the medium was adjusted to 5.5.

Isolation of Microorganisms and Fermentation

The screening for microorganisms producing the β-Psd-hydrolyzing enzyme was carried out with type cultures of strains traditionally used for food processing. Each type culture was streaked on a potato dextrose agar slant and incubated at 27 to 32°C for 3 to 14 days. Agar blocks (5 × 5 mm) were taken from the potato dextrose agar slant, inoculated into the screening medium in a glass test tube, and incubated at 27°C for 8 days on a reciprocal shaker (140 strokes/min). The culture was then centrifuged at 10,000 rpm for 10 min and the supernatant was used to determine β-primeverosidase activity. The supernatant (100 μL) was mixed with 100 μL of eugenyl β-Psd (10 mg/mL in 20 mM acetate buffer) or *p*NP β-Psd (5 mg/mL in 20 mM acetate buffer) and incubated at 37°C for 96 h. The reaction was stopped by incubating in boiling water for 10 min. Each assay mixture (20 μL) was analyzed by TLC (Silica gel 60 F_{254}, Merck; ethyl acetate/acetic acid/ $H_2O = 3:1:1$ (v/v) and detected by the orcinol-sulfuric acid method [23]. Spots of reaction products were compared with those of authentic samples [primeverose, *p*NP β-Psd, eugenyl β-Psd, glucose (Glc), and xylose (Xyl)]. A β-primeverosidase-like enzyme was detected in the supernatant from several microorganisms. *P. multicolor* IAM7153 (Institute of Molecular and Cellular Biosciences The University of Tokyo) had the highest enzyme production of all the microorganisms tested. The fungus were streaked on a potato dextrose agar slant and incubated at 27°C for 14 days. An agar block (5 × 5 mm) taken from the potato dextrose agar slant was inoculated into the production media in 500 mL shakers and incubated at 27°C for 8 days on a reciprocal shaker (140 strokes/min). The culture broth from multiple cultures was combined (5 L total) and filtered through a filter paper to remove the mycelia. The culture filtrate (4 L) was concentrated to 400 mL with an ultrafiltration module SIP-1010 (Asahi Kasei Corporation). The enzyme solution was centrifuged to remove insoluble material. The supernatant was lyophilized to yield crude enzyme powder (1.5 g), giving 0.1 U/mg protein of specific activity.

Partially Purified β-diglycosidase

The crude enzyme powder (1.5 g) mentioned above was dissolved in 15 mL of 20 mM sodium acetate buffer (pH 5.0). Proteins were precipitated in 50% (w/v) ammonium sulfate. The precipitate was dissolved in 15 mL of 20 mM sodium acetate buffer (pH 5.0) containing 30% ammonium sulfate. After

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centrifugation, the supernatant was desalted and concentrated with Amicon PM10 membrane. The lyophilized powder (178 mg) was dissolved in 20 mM sodium acetate containing 30% ammonium sulfate (pH 5.0) and applied to a HiLoad 16/10 Phenyl Sepharose H.P. column (ϕ 1.6 × 10 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.5) containing 30% ammonium sulfate. The column was washed with 350 mL of the equilibration buffer (flow rate, 2 mL/min) and eluted with a linear gradient of ammonium sulfate from 30% to 0% in 480 mL of the same buffer, followed by 150 mL of 20 mM sodium acetate buffer (pH 5.0). The elution pattern showed the enzyme mixture to be fractionated into F-1 (tubes 41–52) and F-2 (tubes 55–60) showing enzyme activity with assay A. F-1 fraction, which showed the same elution profile of the activity with assays A, B, and C, was pooled, concentrated with Amicon PM10 membrane, and lyophilized to yield partially purified enzyme powder (20 mg) giving 0.5 U/mg protein of specific activity, which was used for the enzyme synthesis.

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Enzymatic Synthesis of Five Different Aroma Precursor β-Psds

Benzyl β-Psd (1)

A mixture containing 0.21 g (0.5 mmol) of *p*NP β-Psd, 2.9 g (2.4 mmol) of benzyl alcohol, 2.1 mL of water, and 1 mL of partially purified enzyme from *P. multicolor* IAM7153 (2.8 U/mL of *p*NP β-Psd hydrolytic activity with assay A) in 100 mM sodium acetate buffer (pH 5.5) was incubated at 40°C for 3.5 h with vigorous shaking. The reaction was terminated by heating in a boiling water bath for 5 min. Three ml each of water and chloroform were added to the reaction mixture and the pH was adjusted to 4.0 with 0.1 N HCl. After the aqueous phase was separated with a separatory funnel, it was evaporated to dryness and dissolved in 3 mL of water. The solution was loaded onto a column of Toyopearl HW-40S (ϕ 4.5 × 85 cm) equilibrated with 25% (v/v) methanol. The column was eluted with the same solvent. The absorbance of the eluate was monitored at 485 nm (carbohydrate content, determined by the phenol-sulfuric acid method), 210 nm (double bond), and 300 nm (*p*-nitrophenol group). The fractions (tubes 10–16) containing the transfer product were collected and concentrated to dryness followed by crystallization from EtOH and gave compound **1** (99 mg) with a yield of 51% based on the amount of donor *p*NP β-Psd added. Compound **1** had: mp 194°C (EtOH), m/z 403 $(M+H)^+$ and 425 $(M+Na)^+$. ^{1}H NMR data (in D_2O): δ 4.62 (1H, d, J 7.6 Hz, H-1″), 4.69 (1H, d, J 7.9 Hz, H-1′), 7.60-7.63 (5H, m, phenyl). ^{13}C NMR data in D_2O are summarized in Table 2 and Fig. 5.

In a similar manner, 2-phenylethyl β-Psd (**2**, 140 mg) was obtained as crystalline in a yield of 70% based on the amount of donor added. Compound

Table 2: ^{13}C NMR data of aroma precursor β-Psds synthesized by partially purified β-diglycosidase.

	Benzyl β-Psd (1)	**2-Phenylethyl β-Psd (2)**	**(*Z*)-3-Hexenyl β-Psd (3)**	**Geranyl β-Psd (4)**	**Eugenyl β-Psd (5)**
C-1	138.4	140.3	64.2	51.7	146.4
C-2	130.4	130.6	28.3	121.8	151.6
C-3	130.4	130.3	126.0	146.3	116.1
C-4	130.2	128.2	136.2	41.8	139.2
C-5	130.4	130.3	21.4	28.2	124.0
C-6	130.4	130.6	14.8	127.0	119.4
C-7	73.2	36.8	-	135.8	41.8
C-8	-	50.5	-	20.0	140.9
C-9	-	-	-	27.9	118.5
C-10	-	-	-	19.0	58.7
C-1′	103.0	103.9	106.4	103.4	103.4
C-2′	74.7	72.5	73.0	75.8	75.6
C-3′	77.3	77.2	78.5	78.7	78.4
C-4′	70.9	71.0	72.2	72.1	72.0
C-5′	76.6	76.5	77.8	77.7	78.2
C-6′	70.3	70.2	71.4	68.4	70.9
C-1″	105.3	105.2	106.9	105.9	106.0
C-2″	74.8	74.6	75.8	75.8	75.8
C-3″	77.3	77.2	78.5	79.7	78.3
C-4″	70.9	70.8	72.2	71.2	72.0
C-5″	66.8	66.8	68.0	68.0	67.9

Chemical shifts are shown in parts per million downfield from internal TPS.

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2 had: mp 182°C (MeOH), m/z 417 $(M+H)^+$ and 439 $(M+Na)^+$, ^{1}H NMR data (in D_2O): δ 2.96 (2H, t, H-7), 4.30 (1H, d, J 7.6 Hz, H-1″), 4.57 (1H, d, J 8.3 Hz, H-1′), 7.29–7.38 (5H, m, phenyl). (*Z*)-3-Hexenyl β-Psd (**3**, 235 mg) was prepared as lyophilized powder in a yield of 62% based on the amount of donor added. Compound **3** had: m/z 395 $(M+H)^+$ and 417 $(M+Na)^+$, ^{1}H NMR data (in D_2O): δ 0.97 (3H, t, H-6), 2.10 (2H, m, H-5), 2.42 (2H, m, H-2), 4.47 (1H, d, J 7.6 Hz, H-1″), 4.49 (1H, d, J 7.9 Hz, H-1′), 5.49 (1H, m, H-4), 5.60 (1H, m, H-3). ^{13}C NMR data in D_2O are summarized in Table 2.

Geranyl β-Psd (4)

A mixture containing 0.83 g (1.9 mmol) of *p*NP β-Psd, 3.7 g (2.4 mmol) of geraniol, 0.6 mL of water, and 1 mL of partilly purified enzyme (0.6 U/mL with assay A) in 100 mM sodium acetate buffer (pH 5.5) was incubated at 40°C for 3.5 h with vigorous shaking. After termination by heating in a boiling water bath for 5 min, the reaction mixture was treated as in the water extraction described above. The aqueous extract was loaded onto a column of Toyopearl HW-40S treated as described above. The fractions (tubes 62–70) containing the transfer product were combined and concentrated.

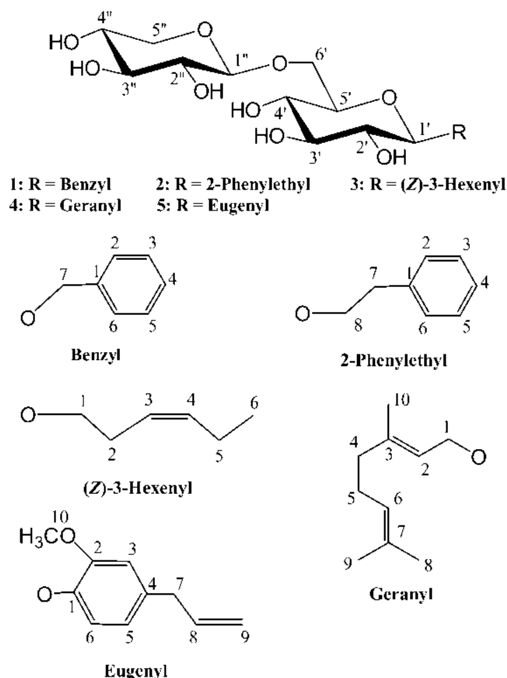

Figure 5: Structures of benzyl β-Psd (**1**), 2-phenylethyl β-Psd (**2**), (Z)-3-hexenyl β-Psd (**3**), geranyl β-Psd (**4**), and eugenyl β-Psd (**5**).

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The crude products were separated by a column of Sep-pak C_{18} Cartridges Vac 35 cc equilibrated with water. The fractions (tubes 10–62) were combined, concentrated, and lyophilized to afford 70 mg of **4** in a yield of 8% based on the amount of donor added. Compound **4** had: m/z 449 $(M+H)^+$ and 471 $(M+Na)^+$, ^{1}H NMR data (in D_2O): δ 1.63 (3H, s, H-8), 1.69 (3H, s, H-9), 1.70 (3H, s, H-10), 2.15 (4H, m, H-4, 5), 4.34 (2H, d, J 7.9 Hz, H-1), 4.52 (1H, d, J 7.6 Hz, H-1″), 4.60 (1H, d, J 7.9 Hz, H-1′), 5.20 (1H, t, H-6), 5.39 (1H, t, H-2). ^{13}C NMR data in D_2O are summarized in Table 2.

Eugenyl β-Psd (5)

A mixture containing 0.2 g (0.08 M) of *p*NP β-Psd, 3.9 g (0.2 M) of eugenol, 1.1 mL of water, and 0.3 mL of partially purified enzyme (0.2 U/mL with assay A) in 100 mM sodium acetate buffer (pH 5.5) was incubated at 40°C for 3.5 h with vigorous shaking. After termination by heating in a boiling water bath for 5 min, the reaction mixture was treated as in the water extraction described above. The water extract was loaded onto a column (ϕ 4.5 × 85 cm) of Toyopearl HW-40S treated as described above. The fractions (tubes 21–33) were combined, concentrated, and lyophilized to afford 26 mg of **5** (12% yield based on the amount of donor added). Compound **5** had: m/z 459 $(M+H)^+$ and 481

(M + Na)$^+$, ^{1}H NMR data (in D_2O): δ 3.86 (3H, s, H-10), 4.37 (1H, d, J 7.9 Hz, H-1″), 5.05 (1H, d, J 7.3 Hz, H-1′), 5.99 (1H, m, H-8), 6.82 (1H, d, H-5), 6.95 (1H, s, H-3), 7.12 (1H, d, H-6). ^{13}C NMR data in D_2O are summarized in Table 2.

The structural data for these synthetic products **1–5** were identical to those reported previously.[2,3,17,18]

Relative Rate of Hydrolysis of Various Substrates by the Partially Purified Enzyme

The relative rates of hydrolysis catalyzed by the partially purified enzyme from *P. multicolor* IAM7153 of a series of β-Psds and related compounds were investigated by incubating a mixture (0.5 mL) containing 20 mM substrate in 20 mM acetate buffer pH 5.5 with 8 mU of enzyme for *p*NP β-glycosides, or 80 mU for aroma precursors (**1–5**), with assay A at 40°C. Samples (50 μL) were taken at 5-min intervals (0, 5, 10, 15, 20, and 25 min) and inactivated by adding 50 μL of 1 M sodium carbonate or by boiling for 5 min. The amount of hydrolysates formed at an early stage of the incubation (up to 15% hydrolysis) was analyzed by spectrophotometry (405 nm) for *p*-nitrophenol, or by HPLC for aroma components, and a determination kit. From these results, the relative rate of hydrolysis for each substrate was calculated with the rate of *p*NP β-Psd hydrolysis taken as 100%.

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