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Isolation and Characterization of a β-Primeverosidase-Like Enzyme from *Penicillium multicolor*

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***p*-Nitrophenyl and eugenyl β-primeveroside (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) hydrolytic activity was found in culture filtrate from *Penicillium multicolor* IAM7153, and the enzyme was isolated. The enzyme was purified as a β-primeverosidase-like enzyme by precipitation with ammonium sulfate followed by successive chromatographies on Phenyl Sepharose, Mono Q, and β-galactosylamidine affinity columns. The molecular mass was estimated to be 50 kDa by SDS–PAGE and gel filtration. The purified enzyme was highly specific toward the substrate *p*-nitrophenyl β-primeveroside, which was cleaved in an endo-manner into primeverose and *p*-nitrophenol, but a series of β-primeveroside as aroma precursors were hydrolyzed only slightly as substrates for the enzyme. In analyses of its hydrolytic action and kinetics, the enzyme showed narrow substrate specificity with respect to the aglycon and glycon moieties of the diglycoside. We conclude that the present enzyme is a kind of β-diglycosidase rather than β-primeverosidase.**

Key words: β-primeverosidase-like enzyme; β-diglycosidase; plant aroma precursors; β-primeveroside; *Penicillium multicolor* IAM7153

Various tea-aroma constituents, such as geraniol,[1] benzyl alcohol, 2-phenylethanol,[2] and (*Z*)-3-hexenol have been shown to be present mainly as diglycoside precursors such as β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside, Psd) in tea plant leaves used to produce oolong tea. Such β-Psds are known to be cleaved in an endo-manner into primeverose and aglycones by β-primeverosidase. A β-primeverosidase was purified from crude enzyme extract from fresh leaves of cv. Yabukita (*Camellia sinensis* var. *sinensis*), used for Japanese green tea,[3,4] Shuixian (*C. sinensis* var. *sinensis*), used for oolong tea,[5] and a cultivar (*C. sinensis* var. *assamica*), used for black tea.[6] The purified enzyme showed high substrate specificity toward β-Psds to produce primeverose and aglycones.[7] These results demonstrate that β-primeverosidase plays an important role in the development of tea aroma during black tea, oolong tea, and green tea production.[8–10] The amount of aroma precursors and β-primeverosidase activity both decrease as tea leaves mature.[11] In tea leaves, alcoholic aroma precursors are predominantly conjugated to disaccharides, such as primeverose, rather than monosaccharides.[8]

Recently we reported that a β-Psd hydrolyzing enzyme from *Aspergillus fumigatus* AP-20 cleaved in an endo-manner *p*NP (*p*-nitrophenyl) β-Psd into primeverose and *p*-nitrophenol,[12] but enzymes derived from pathogenic microbes are strictly regulated in their use as food processing aids and it is difficult to overcome the regulatory obstacles. Demonstrating the safety of enzymes from such microbes is expensive and time-consuming, and the enzyme might not be readily accepted by the consumer. Hence we searched for another source showing β-Psd hydrolyzing activity with *p*NP β-Psd among microbes already commonly used in traditional food processing. The availability of such an enzyme from these sources would expand its potential applications in biotechnology, *e.g.*, in the control of aroma release, the high recovery of aromas in extracts, and the enzymatic synthesis of β-Psds as aroma precursors. This paper describes screening for a microorganism producing β-Psd hydrolyzing enzyme, the purification and characterization of a unique β-diglycosidase from *Penicillium multicolor* IAM7153.

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Abbreviations: *p*NP, *p*-nitrophenyl; Psd, primeveroside; Glcd, glucopyranoside; Gald, galactopyranoside; Xyld, xylopyranoside; Lamd, laminaribioside; Gend, gentiobioside; Celd, cellobioside; Xyl, xylopyranose; Glc, glucopyranose

Materials and Methods

General methods. Furcatin was isolated from leaves of *Viburnum furcatum* Blume by a previously published method with slight modifications.[13] *p*NP β-Psd, primeverose, *p*NP β-gentiobioside (Gend), and 2-phenylethyl β-Gend were prepared by our method.[7,14] Sophorose, cellobiose, gentiobiose, *p*NP β-glucopyranoside (Glcd), and *p*NP β-galactopyranoside (Gald) were purchased from Sigma Chemical (St. Louis, MO). Cellooligosaccharides (DP 2–5), Laminarioligosaccharides (DP 2–5), *p*NP β-cellobioside (Celd), and *p*NP β-laminaribioside (Lamd) were purchased from Yaizu Suisankagaku Industry (Shizuoka, Japan). Gentiotriose and gentiotetraose were prepared by previous method.[15] The affinity adsorbents with β-galactosylamidine as the ligand were prepared by our method.[16,17] All other chemicals were obtained from commercial sources.

Media. The formula for the medium used for seed cultures for screening was as follows: Defatted soy meal [Honen, Tokyo, Japan (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 Shokuhin Kako, Tokyo, Japan (3.0%)], KH_2PO_4 (2.0%), $(NH_4)_2SO_4$ (1.0%), and Meast P1G [Asahi Food and Healthcare, Tokyo, Japan (3.1%)]. The medium for enzyme production consisted of Solulys A-ST [Roquette Japan, Tokyo, Japan (3.0%)], Pinedex No. 2 [Matsutani Chemical Industry, Hyogo, Japan (1.0%)], and KH_2PO_4 (0.5%). The pH of the medium was adjusted to 5.5.

Isolation of microorganisms and fermentation. Screening for microorganisms producing the β-Psd hydrolyzing enzyme was carried out with 30 type cultures of strains traditionally used for food processing. Each type culture was streaked on a potato dextrose agar slant and incubated at 27–32 °C for 3–14 d. 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 d 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-Psd hydrolyzing 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 incubation in boiling water for 10 min. Each assay mixture (20 µl) was analyzed by TLC [Silica gel 60 F_{254}, Merck, Frankfurt, Germany; ethyl acetate/acetic acid/H_2O = 3:1:1 (v/v); detected by the orcinol-sulfuric acid method].[18] 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-Psd hydrolyzing enzyme was detected in the supernatant from several microorganisms (*Aspergillus oryzae* JMC5560, *Aspergillus niger* IAM2107, *Aspergillus awamori* IFO4033, *Rhizopus oryzae* JCM5560, *Talaromyces emersonii* IFO9747, *Trichoderma reesei* QM9414B, *Penicillium camembertii* FERMP-10452, and *Penicillium multicolor* IAM7153). *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 was streaked on a Potato Dextrose Agar slant and incubated at 27 °C for 14 d. 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 d on a reciprocal shaker (140 strokes/min). The culture broth from multiple cultures was combined (5-liter total) and filtered through a filter paper to remove the mycelia. The culture filtrate (4-liter) was concentrated to 400 ml with an ultrafiltration module SIP-1010 (Asahi Kasei, Osaka, Japan). The enzyme solution was centrifuged to remove insoluble material. The supernatant was lyophilized to yield crude enzyme powder, which was used for enzyme purification.

Enzyme assay. During purification, the enzyme fractions were assayed for hydrolyzing activities 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 carbonate. The *p*-nitrophenol released was measured photometrically at 405 nm in a microplate reader (Biolumin 960, Amersham Pharmacia Biotech, Sweden). One unit of the enzyme was defined as the amount releasing 1 µmol of *p*-nitrophenol per min. Assays to determine the optimum temperature, the thermal stability, the optimum pH, and the pH stability of the enzyme essentially followed previous methods.[12]

Analytical 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 (ϕ150 × 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. Protein was measured by the method of Lowry *et al.*[19] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done with a PhastSystem (Amersham Pharmacia Biotech, Sweden). "Perfect" protein markers 15–150 kDa (Novagen, Darmstadt, Germany) were used for mass calibration. Proteins were detected with PhastGel Blue R (Coomassie R 350 stain). Gel filtration was done with a HiPrep Sephacryl S-200 HR column (ϕ2.6 × 60 cm) equilibrated with 20 mM sodium acetate

buffer (pH 4.7). The protein was eluted with a same buffer (flow rate, 1.0 ml/min).

Purification of β-Psd hydrolyzing enzyme. 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% ammonium sulfate. The precipitate was dissolved in 15 ml of 20 mM sodium acetate buffer (pH 5.0) containing 30% ammonium sulfate. After 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 (ϕ1.6 × 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 two peaks (F-1 and F-2) showing enzyme activity with assay A. F-2 (tubes 36–42), which showed the same elution profile of the activity as assays A, B, and C, was pooled, concentrated with Amicon PM10 membrane, and lyophilized. This fraction was next applied to a Mono Q R5/5 column (ϕ 0.5 × 5 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed with 50 ml of the same buffer (flow rate, 0.5 ml/min), and eluted with a linear gradient from 0 M (40 ml) to 0.2 M NaCl (40 ml) in the same buffer. The elution pattern showed two fractions (F-1a and F-1b) with assay A. F-1a (tubes 18–21), which showed higher activity than F-1b with assay A, was pooled and concentrated to a small volume. The solution was then applied to a β-galactosylamidine affinity column (2 ml bed volume) equilibrated with 20 mM Tris–HCl buffer (pH 7.0). After the column was washed with the same buffer, proteins were eluted stepwise by lowering the pH of the 20 mM sodium acetate buffer to 5.0, 4.0, and 3.0 (flow rate, 0.5 ml/min). A high level of activity with assay A, associated with the protein peak, was recovered from the column at pH 5.0. More tightly bound proteins, mainly showing activity with assays B and C, were eluted with successive washes with 50 ml of 20 mM sodium acetate buffer (pH 4.0) and 50 ml of 20 mM sodium acetate buffer (pH 3.0), followed by 20 ml of the same buffer containing 1 M NaCl. The fractions eluted at pH 5.0 (tubes 53–61) were pooled, concentrated with Amicon PM10 membrane, and lyophilized to give a purified enzyme. The purity of the enzyme was checked by SDS–PAGE (PhastSystem, Sweden Pharmacia Biotech) and gel filtration. This preparation was used as purified enzyme for this study.

Relative rate of hydrolysis of various substrates by the purified enzyme. The relative rates of hydrolysis catalyzed by the purified enzyme of various kinds of β-mono- and diglycosides and β-linked disaccharides 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-mono and diglycosides, or 80 mU for aroma precursors, 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 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%.

Action of the purified enzyme on β-diglycosides. Incubations with substrates (20 mM) were carried out at 40 °C in 0.5 ml of 20 mM acetate buffer (pH 5.5). Reactions were initiated by the addition of the enzyme (8 or 80 mU with assay A). Samples (50 µl) were taken at 5-min intervals (0, 5, 10, 15, and 20 min) during incubation, and inactivated by adding 50 µl of 1 M sodium carbonate. The amount of each hydrolysate formed from the initial substrates by an early stage of incubation with the enzyme (up to 15–20% hydrolysis) was determined by HPLC. The amount of each product increased linearly with time in the initial stage of the reaction. Based on these data, the frequency of the purified enzyme-catalyzed cleavage of glycosidic linkages was determined.

Kinetic parameters of the purified enzyme. The amount of *p*-nitrophenol or eugenol liberated from the initial substrates (*p*NP β-Glcd, *p*NP β-Gend, *p*NP β-Psd, and eugenyl β-Psd) was determined by spectrophotometry (405 nm) for *p*-nitrophenol and by HPLC for eugenol. The initial rates of the enzymatic reaction were derived from kinetic curves of product accumulation, as described above. The Michaelis–Menten parameters were determined by $1/v$–$1/[S]$ plots and the least-squares method. Five different substrate concentrations (0.1–40 mM) were used for each experiment.

Results

A total of 30 type cultures were grown in a liquid medium and screened for secretion of a β-Psd hydrolyzing activity with *p*NP β-Psd, 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 was detected by the release of primeverose. β-Psd hydrolyzing activity was detected in the supernatant from eight different type cultures; *P. multicolor* IAM7153 had the highest enzyme activity. The super-

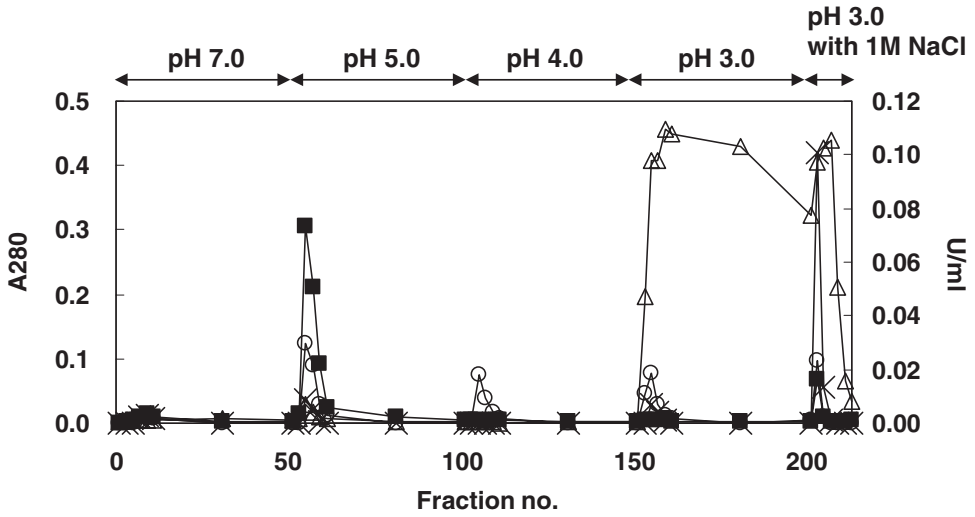

Fig. 1. Separation of the β-Psd Hydrolyzing Enzyme on a Column of β-Galactosylamidine Affinity Resin.

After Mono Q column chromatography, the sample was chromatographed on a β-galactosylamidine affinity column (see "Materials and Methods"). The pH of the buffer was changed stepwise: tubes 0–50, elution with 20 mM Tris–HCl buffer (pH 7.0); tubes 51–100, elution with the same buffer at pH 5.0; tubes 101–150, elution with the same buffer at pH 4.0; tubes 151–200, elution with the same buffer at pH 3.0. Tubes containing β-primeverosidase activity (tubes 51–61) were collected and lyophilized. ■, β-primeverosidase activity with assay A; ○, β-glucosidase activity with assay B; △, β-galactosidase activity with assay C; ×, A_{280}.

natant of the culture filtrate was lyophilized to give a crude enzyme powder.

Purification and characteristics of β-Psd hydrolyzing enzyme

The crude enzyme preparation from *P. multicolor* IAM7153 was precipitated with ammonium sulfate at 50% saturation and subjected to Phenyl Sepharose column chromatography [HiLoad 16/10 Phenyl Sepharose H. P., sodium acetate buffer (pH 5.5) containing 30% 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 then applied to a Mono Q HR 5/5 column [sodium acetate buffer (pH 5.0)] and fractionated into two peaks (F-1a and F-1b), with assay A activity overlapping protein absorbance. The major fraction (F-1a) was finally loaded onto a β-galactosylamidine affinity column, as shown in Fig. 1. After the column was washed with 20 mM Tris–HCl at pH 7.0, the adsorbed *p*NP β-Psd hydrolyzing activity was eluted stepwise with 20 mM sodium acetate buffer at pHs 5.0, 4.0, and 3.0. Most of the enzyme activity adsorbed to the β-galactosylamidine affinity resin was eluted at pH 5.0 with a very small amount of β-Glcd activity. β-Galactosidase, which was more tightly bound to the β-galactosylamidine affinity resin, was eluted below pH 3.0. The fractions eluted at pH 5.0 were combined and lyophilized to give a purified enzyme powder. The purification is summarized in Table 1. The enzyme was purified 20-fold based on assay A, with a specific activity of 1.5 U/mg and 1.0% yield. The β-Psd hydrolyzing enzyme eluted at pH 5.0 gave a single protein band corresponding to 50 kDa on SDS–PAGE (Fig. 2) and gel filtration using a column of Hi Prep Sephacryl S-200 HR (data not shown). This indicates that the enzyme does not possess a subunit structure. The enzyme had a broad pH optimum in the range of pH 4.5–5.5 and was stable at all pHs tested. The optimal temperature was 55 °C, with near-optimum activities observed from 50 to 60 °C.

Table 1. Purification of the Enzyme

Purification step	Protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification factor (fold)	Yield (%)
Crude enzyme[a]	1500	0.07	111	1.0	100
30–50% ammonium sulfate	179	0.17	30	2.3	27
Phenyl Sepharose	20	0.54	11	7.3	9.9
Mono Q	3.0	0.91	2.7	12	2.5
β-Galactosylamidine	1.1	1.5	1.6	20	1.4

[a]Lyophilized powder of a culture broth was used as starting material.

Hydrolytic activities on various substrates

The purified enzyme was found to hydrolyze *p*NP β-Psd into primeverose and *p*-nitrophenol. Based on this result, a series of naturally occurring β-diglycosides, *p*NP β-diglycosides, and related compounds were tested as substrates for the enzyme (Table 2). 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 15–20% hydrolysis). The best substrate for the enzyme was the artificial substrate *p*NP β-Psd; five other β-Psds and furcatin (*p*-allylphenyl β-acuminoside) 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 eugenyl β-Psd (2.5%) was greater than that

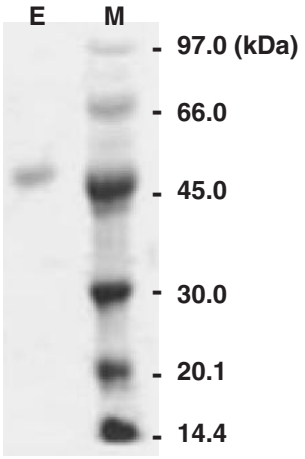

Fig. 2. SDS–PAGE of the Purified Enzyme from *P. multicolor* IAM7153.

SDS–PAGE was performed with a 12.5% polyacrylamide gel (PhastGel Homogeneous 12.5) and the protein visualized by Coomassie Brilliant Blue staining. The migration of markers (15–150 kDa) is shown on the right. E, the purified enzyme; M, size marker.

Table 2. Relative Hydrolytic Rates of the Purified Enzyme 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 (%)	
	Purified enzyme	Tea β-primeverosidase[a]
*p*NP β-Psd[b]	100	100
*p*NP β-Gend[b]	2.5	—[c]
*p*NP β-Lamd[b]	ND[d]	—
*p*NP β-Celd[b]	ND	—
*p*NP β-Glcd[b]	15	0.4
Eugenyl β-Psd[e]	2.5	—
Benzyl β-Psd[e]	0.1	—
2-Phenylethyl β-Psd[e]	0.1	185
(*Z*)-3-Hexenyl β-Psd[e]	0.1	—
Geranyl β-Psd[e]	0.1	—
Furcatin[e]	1.0	—
Gentiooligo.	ND	—
Laminarioligo.	ND	—
Cellooligo.	ND	—
Sophorose	ND	—
Primeverose	ND	—

[a]Data are cited from the report by Ma *et al.*[7]
[b]Release of *p*-nitrophenol was detected photometrically at 405 nm.
[c]Not tested in the cited report.
[d]No hydrolytic products were detected.
[e]Release of each aroma compound was detected by HPLC as the hydrolytic product.

of furcatin (1.0%), despite the structural similarity of their respective aglycones. The relative rate of hydrolysis of β-Psds having alcoholic aglycones (benzyl, 2-phenylethyl, (*Z*)-3-hexenyl, and geranyl) was 0.1% (1,000-fold slower than *p*NP β-Psd). Of the naturally occurring aroma precursors, eugenyl β-Psd was the most rapidly hydrolyzed, although with 1/40 activity relative to *p*NP β-Psd. It is notable that the alcoholic aroma precursors were very poor substrates. No detectable hydrolysis of primeverose, other β-linked glucobioses, or glucooligosaccharides (DP 2–5) was observed. The rate of hydrolysis of *p*NP β-Gend, in which Xyl is replaced with Glc, was only 1/50 that of *p*NP β-Psd. *p*NP β-Glcd still acted as a substrate despite the absence of another sugar residue at the terminal position. 2-Phenylethyl β-Gend, *p*NP β-Lamd, and *p*NP β-Celd did not act as substrates. The substrate specificity of the *P. multicolor* enzyme was also compared to that of β-primeverosidase from tea leaves.[7] The best substrate for the tea enzyme was 2-phenylethyl β-Psd, and *p*NP β-Psd was also hydrolyzed well. *p*NP β-Glcd was only a poor substrate for the tea enzyme. Therefore, the substrate specificity of *P. multicolor* enzyme appears to differ appreciably from that of tea β-primeverosidase.

Mode of hydrolysis of chromogenic β-diglycosides

The mode of action of the purified enzyme on three different β-diglycosides was further examined. From HPLC chromatograms of the digests of each substrate, the amounts of the products were calculated from their peak areas. The amount of each product increased linearly during the initial stage of incubation as the enzyme reaction proceeded (data not shown). Based on the product analysis, the frequency of enzyme-catalyzed cleavage of each of the glycosidic linkages in the three substrates was determined (Fig. 3). *p*NP β-Psd was hydrolyzed exclusively, and *p*NP β-Gend predominantly, at the glucosidic bond nearest the *p*NP group (bond 1). Eugenyl β-Psd was hydrolyzed to form aglycon and β-Glcd in a ratio of roughly 0.8:0.2. The stereochemistry of the hydrolysis catalyzed by the purified enzyme was analyzed by ^{1}H-NMR (Fig. 4), essentially as described by Wong *et al.*[20] ^{1}H-NMR spectra of a reaction mixture

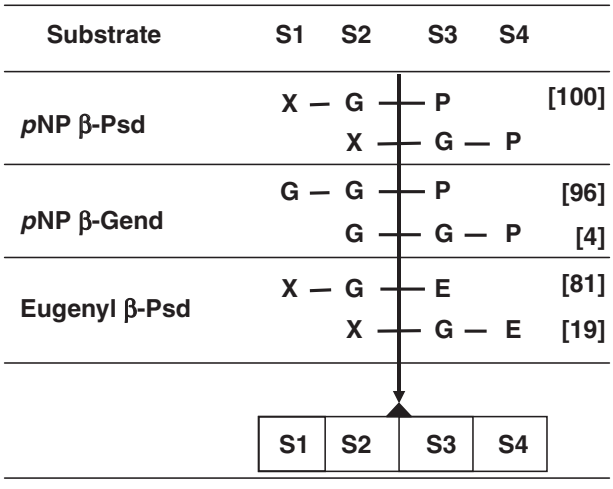

Fig. 3. Proposed Structure of the Cleavage Site of the Purified Enzyme.

The arrow shows the cleavage site of the glycosidic linkage of each substrate. Values in parenthesis indicate the frequency of enzyme-catalyzed cleavage of each glycosidic linkage. *p*NP β-Psd was hydrolyzed exclusively to the primeverose unit by the enzyme. X, xylose; G, glucose; P, *p*-nitrophenol; E, eugenol.

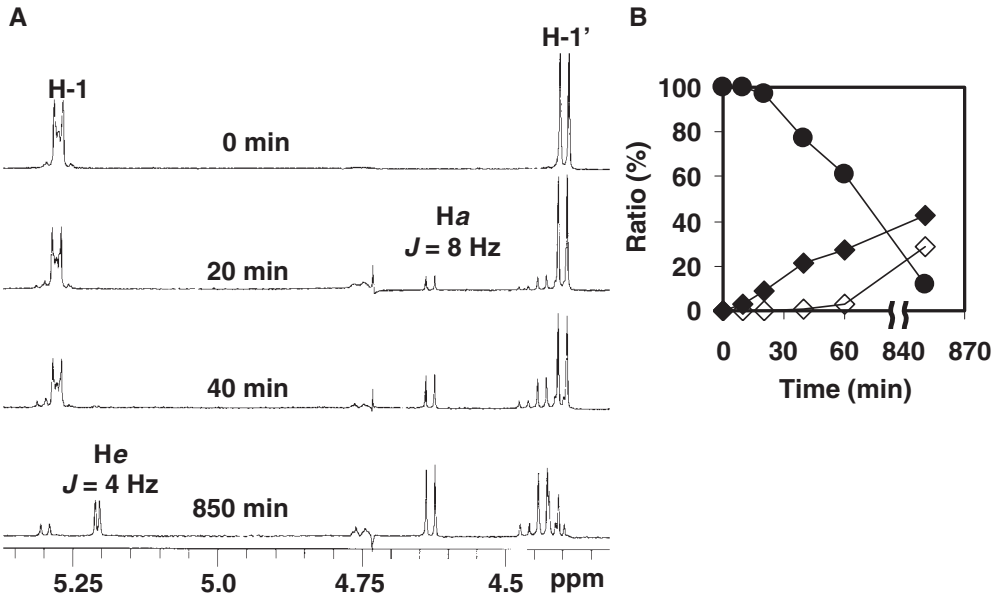

Fig. 4. Time Course of the Hydrolysis of *p*NP β-Psd by the Purified Enzyme from *P. multicolor* IAM7153.

Reaction samples were analyzed by ^{1}H-NMR to study the stereochemistry of the enzymatic hydrolysis by the purified enzyme. A, ^{1}H-NMR spectra recorded at 0, 20, 40, and 850 min after the enzyme reaction. B, Generation of H_a and H_e after hydrolysis of *p*NP β-Psd by the purified enzyme. The relative amounts of H_a and H_e were calculated from the intensity of each signal. H-1 and H-1′, resonances of *p*NP β-Psd; H_a and H_e, resonances of primeverose. ●, H-1 resonance of *p*NP β-Psd; ◆, H_a resonance of primeverose; ◇, H_e resonance of primeverose.

Table 3. Kinetic Paramerters of the Purified Enzyme from *P. multicolor* IAM7153

The parameters of Michaelis–Menten-type kinetics were evaluated by $1/v$–$1/[S]$ plots and the least square method.

Substrate	K_m (mM)	V_{max} (μmol·min^{-1}·mg protein^{-1})	V_{max}/K_m	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}·mM^{-1})
*p*NP β-Glcd[a]	47	1.8	0.04	1.5	0.03
*p*NP β-Gend[a]	21	0.31	0.02	0.26	0.01
Eugenyl β-Psd[b]	3.8	0.26	0.07	0.21	0.06
*p*NP β-Psd[a]	12	12	0.99	9.9	0.82

[a]Release of *p*-nitrophenol was detected photometrically at 405 nm.
[b]Release of eugenol was detected by HPLC as the hydrolytic product.

containing *p*NP β-Psd and the enzyme were measured at the time intervals as shown in Fig. 4A. The β-anomer (H_a, δ 4.63, $J = 8.1$ Hz) of primeverose was formed first and increased with time during the early phase of the reaction. The α-anomer (H_e, δ 5.21, $J = 3.6$ Hz) of primeverose appeared later as a consequence of mutarotation. Therefore, the *P. multicolor* enzyme is a retaining glycosidase, as has been observed for other members of the β-glycosidase family 1.[21,22]

Kinetic parameters for chromogenic diglycoside substrates

The kinetic parameters of the hydrolysis of three β-diglycosides and β-Glcd were determined by colorimetric analysis of *p*-nitrophenol or by HPLC analysis of eugenol liberated from the substrate by the action of the *P. multicolor* enzyme (Table 3). The affinity of the enzyme for *p*NP β-Psd ($K_m = 12$ mM) was 3.2-fold higher than that for eugenyl β-Psd ($K_m = 3.8$ mM), 3.9-fold lower than that for *p*NP β-Glcd ($K_m = 47$ mM), and 1.8-fold lower than that for *p*NP β-Gend ($K_m = 21$ mM). The specificity constant of *p*NP β-Psd ($k_{cat}/K_m = 0.82\ s^{-1}\ mM^{-1}$) was 13.7-fold higher than that of eugenyl β-Psd ($k_{cat}/K_m = 0.06\ s^{-1}\ mM^{-1}$), 27.3-fold higher than that of *p*NP β-Glcd ($k_{cat}/K_m = 0.03\ s^{-1}\ mM^{-1}$), and 82-fold higher than that of *p*NP β-Gend ($k_{cat}/K_m = 0.01\ s^{-1}\ mM^{-1}$). These results indicate that the synthetic diglycoside *p*NP β-Psd is the best substrate for hydrolysis by the enzyme. Eugenyl β-Psd and *p*NP β-Gend are not always good substrates for the enzyme. The enzyme also hydrolyzes *p*NP β-Glcd, a monosaccharide-glycoside, despite the absence of a Xyl residue at the terminal position such as *p*NP β-Psd. Because β-D-glucopyranosides of aroma compounds are present in tea leaves and fruits, this characteristic of the enzyme might be useful for improving the aroma quality of tea drinks and fruit juices.

Discussion

The supernatant of culture filtrate from *P. multicolor* IAM7153 showed the highest β-Psd hydrolyzing activity

of the other culture filtrates of the 30 different type cultures tested. Hence the supernatant was followed by a purification procedure, in order to clarify the entity of the β-Psd hydrolyzing enzyme. The crude enzyme preparation was precipitated with ammonium sulfate precipitation, followed by successive column chromatographies (Table 1). In the final step, β-galactosylamidine affinity chromatography was highly effective for separating the β-Psd hydrolyzing enzyme from contaminants. After the column was washed with 20 mM Tris–HCl buffer at pH 7.0, the enzyme remained on the adsorbent, suggesting that the active site of the enzyme was bound to the positively-charged amidine base of the affinity ligand. Most of the β-Psd hydrolyzing enzyme with a small amount of β-glucosidase activity were easily eluted by lowering the pH to 5.0 (Fig. 1). Under these conditions, contaminating β-galactosidase remained on the column, and then eluted below pH 3.0 (Fig. 1). The purity of the resulting enzyme was judged by SDS–PAGE and gel filtration.

The hydrolytic action of the purified enzyme on various β-Psds, including aroma precursors and related compounds, was investigated to clarify the substrate specificity of the enzyme with respect to the glycon and aglycon moieties. *p*NP β-Psd, which is not a natural substrate, was overwhelmingly the best substrate among the β-glycosides listed in Table 2. Six naturally occurring aroma precursor β-Psds and furcatin were hydrolyzed only to a limited extent. Among these, the four alcoholic aroma precursor β-Psds synthesized as above were hydrolyzed 10 to 25-fold slower than the other two phenolic aroma eugenyl β-Psd and furcatin. Furthermore, three positional isomers of *p*NP β-glucobiosides were compared with *p*NP β-Psd to define the substrate specificity of the enzyme with respect to the glycon moiety. The hydrolysis rate of *p*NP β-Gend was 1/50 that of *p*NP β-Psd. *p*NP β-Lamd and *p*NP β-Celd did not act as substrates. No detectable hydrolysis of primeverose, other β-linked glucobioses, or glucooligosaccharides (DP 2–5) was observed. On the other hand, we recently reported that a β-Psd hydrolyzing enzyme from *A. fumigatus* has broad substrate specificity with respect to β-diglycosides and β-linked disaccharides. *p*NP β-Gend acts as a fairly good substrate for the enzyme (a rate of 41/100 relative to *p*NP β-Psd), whereas primeverose and β-linked glucobioses act only slightly as substrates. This suggests that the substrate specificity of β-Psd hydrolyzing enzyme from *A. fumigatus* differs somewhat from that of the present *P. multicolor* enzyme. Ma *et al.*[7] has reported that tea β-primeverosidase shows a very narrow substrate specificity with respect to the glycon moiety, and a strong preference for the β-primeverosyl moiety (Table 2). 2-Phenylethyl β-Psd is a fairly good substrate for the tea enzyme (185/100 relative to *p*NP β-Psd), whereas *p*NP β-Glcd is a poor substrate (0.4/100 relative to *p*NP β-Psd). Tea β-primeverosidase recognizes aroma precursors more effectively than the *P. multicolor* enzyme. From these results, the present enzyme was shown to be highly specific for only one substrate, *p*NP β-Psd.

From the kinetic parameters and bond cleavage frequency for the hydrolysis of three β-diglycosides, we propose a subsite structure for the enzyme, that matches the shape of *p*NP β-Psd and can accommodate a chain of three residues to fit into the active site (Fig. 3). As a matter of convenience, it is assumed that the active site of the enzyme consists of four subsites (S1, S2, S3, and S4), and that the glycosidic bonds of the substrates are split between S2 and S3. The present enzyme did not act on primeverose or other β-linked glucobioses as substrates. These results show that the disaccharide must have an aglycon moiety to accommodate at S3 and allow for binding to the active site. The enzyme is also strict for binding the aglycon group, because the catalytic efficiencies of eugenyl and 2-phenylethyl β-Psds were dramatically lower than that of *p*NP β-Psd (Table 3). The enzyme is also strict for binding at S1, since the catalytic efficiency of *p*NP β-Gend, in which a Xyl residue is replaced with Glc, decreased remarkably as compared to that of *p*NP β-Psd (Table 3). *p*NP β-Glcd, in which a Xyl residue is absent at the terminal position, still acted as a substrate, but primeverose and β-linked glucobioses did not. This suggests that the *p*-nitophenyl group of *p*NP β-Glcd is predominantly bound to S3 and gives some affinity to the subsite. Thus a structural change to any part of *p*NP β-Psd can affect interaction with the active site of the enzyme. We concluded that *p*NP β-Psd binds best, among the substrates tested, to the subsites geometrically complementary to three residues, S1, S2, and S3. Systematic trends in kinetic data obtained after changes in the substrate structure were helpful in revealing the structural requirements for binding and catalytic specificity. We found that the enzyme was able to hydrolyze *p*NP β-Psd to liberate a primeverose unit and an aglycon, and that the hydrolysis was specific to the primeverose-aglycon β-glycosidic linkage and not the inter-glycosidic bond between Xyl and Glc. The enzyme was shown to catalyze hydrolysis of the glycosyl bond with retention of the anomeric configuration, a trait consistent with the β-primeverosidase in the glycosyl hydrolase family 1.[21,22] In analyses of the hydrolytic action and kinetics, the present enzyme showed narrow substrate specificity with respect to the aglycon and glycon moieties of the diglycoside. The fungal enzyme was highly specific for *p*NP β-Psd rather than for eugenyl β-Psd. On the other hand, we also confirmed that the purified enzyme induced a β-primeverosyl transfer reaction from *p*NP β-Psd as donor to aromas as acceptors to produce a series of β-Psds, which were only slightly hydrolyzed by the present enzyme, mentioned above. In this case, it is notable that aromas such as benzyl alcohol, 2-phenylethanol, and (*Z*)-3-hexenol serve as suitable acceptors for transglycosylation. The efficient synthesis of β-Psds and its synthetic mechanism has been reported recently.[23]

In conclusion, by screening for microorganisms producing any β-Psd hydrolyzing enzymes, we found a *P. multicolor* IAM7153 strain that produces a unique β-primeverosidase-like enzyme that hydrolyzes β-Psd in an endo-manner into primeverose and aglycon. The enzyme showed much narrower substrate specificity with respect to the aglycon moiety than tea β-primeverosidase. We conclude that the present *P. multicolor* enzyme is a kind of β-diglycosidase rather than β-primeverosidase from tea leaves, because naturally occurring aroma precursors such as benzyl, 2-phenylethyl, and geranyl β-Psds are hydrolyzed only slightly as substrates for the enzyme.

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References

1) Guo, W., Sakata, K., Watanabe, N., Nakajima, R., Yagi, A., Ina, K., and Luo, S., Geranyl 6-*O*-β-xylopyranosyl-β-D-glucopyranoside isolated as aroma precursor from tea leaves for oolong tea. *Phytochemistry*, **33**, 1373–1375 (1993).
2) Guo, W., Hosoi, R., Sakata, K., Watanabe, N., Yagi, A., Ina, K., and Luo, S., (*S*)-Linalyl, 2-phenylethyl, and benzyl disaccharide glycosides isolated as aroma precursors from oolong tea leaves. *Biosci. Biotechnol. Biochem.*, **58**, 1532–1534 (1994).
3) Guo, W., Yamauchi, K., Watanabe, N., Usui, T., Luo, S., and Sakata, K., A primeverosidase as a main glycosidase concerned with the alcoholic aroma formation in tea leaves. *Biosci. Biotechnol. Biochem.*, **59**, 962–964 (1995).
4) Guo, W., Ogawa, K., Yamauchi, K., Watanabe, N., Usui, T., Luo, S., and Sakata, K., Isolation and characterization of a β-primeverosidase concerned with alcoholic aroma formation in tea leaves. *Biosci. Biotechnol. Biochem.*, **60**, 1810–1814 (1996).
5) Ogawa, K., Ijima, Y., Guo, W., Watanabe, N., Usui, T., Dong, S., Tong, Q., and Sakata, K., Purification of a β-primeverosidase concerned with alcoholic aroma formation in tea leaves (cv. Shuixian) to be precessed to oolong tea. *J. Agric. Food Chem.*, **45**, 877–882 (1997).
6) Ijima, Y., Ogawa, K., Watanabe, N., Usui, T., Ohnishi-Kameyama, M., Nagata, T., and Sakata, K., Characterization of β-primeverosidase, being concerned with alcoholic aroma formation in tea leaves to be processed into black tea, and preliminary observation on its substrate specificity. *J. Agric. Food Chem.*, **46**, 1712–1718 (1998).
7) Ma, S. J., Mizutani, M., Hiratake, J., Hayashi, K., Yagi, K., Watanabe, N., and Sakata, K., Substrate specificity of β-primeverosidase, a key enzyme in aroma formation during oolong tea and black tea manufacturing. *Biosci. Biotechnol. Biochem.*, **65**, 2719–2729 (2001).
8) Mizutani, M., and Sakata, K., β-Primeverosidase and its contribution to formation of floral tea aromas during oolong and black tea manufacturing. *Foods Food Ingredients J.* (in Japanese), **208**, 991–1003 (2003).
9) Sakata, K., β-Primeverosidase relationship with floral tea aroma formation during processing of oolong tea and black tea. In "Caffeinated Beverages," American Chemical Society, Washington, DC, pp. 327–336 (2000).
10) Sakata, K., Watanabe, N., and Usui, T., Molecular basis of alcoholic aroma formation during tea processing. 3rd International Conference on Food Science and Technology, 93–105 (1999).
11) Ogawa, K., Moon, J. H., Guo, W., Yagi, A., Watanabe, N., and Sakata, K., A study on tea aroma formation mechanism: alcoholic aroma precursor amounts and glycosidase activity in parts of the tea plant. *Z. Naturforsch.*, **50c**, 493–498 (1995).
12) Yamamoto, S., Okada, M., Usui, T., and Sakata, K., Isolation and characterization of a β-primeverosidase-like endo-manner β-glycosidase from *Aspergillus fumigatus* AP-20. *Biosci. Biotechnol. Biochem.*, **66**, 801–807 (2002).
13) Imaseki, H., and Yamamoto, T., A furcatin hydrolyzing glycosidase of *Viburnum furcatum* blume. *Arch. Biochem. Biophys.*, **92**, 467–474 (1961).
14) Murata, T., Shimada, M., Watanabe, N., Sakata, K., and Usui, T., Practical enzymatic synthesis of primeverose and its glycoside. *J. Appl. Glycosci.*, **46**, 431–437 (1999).
15) Nanjo, F., Usui, T., and Suzuki, T., Mode of action of an exo-β-(1→3)-D-glucanase on the laminaran from *Eisenia bicyclis*. *Agric. Biol. Chem.*, **48**, 1523–1532 (1984).
16) Inoue, K., Hiratake, J., Mizutani, M., Takada, M., Yamamoto, M., and Sakata, K., β-Glycosylamidine as a ligand for affinity chromatography tailored to the glycon substrate specificity of β-glycosidases. *Carbohyd. Res.*, **338**, 1477–1490 (2003).
17) Hiratake, J., and Sakata, K., Glycosylamidines as potent selective and easily accessible glycosidase inhibitors and their application to affinity chromatography. *Methods Enzymol.*, **363**, 421–443 (2003).
18) Svennerholm, L. J., The quantitative estimation of cerebrosides in nervous tissue. *J. Neurochem.*, **1**, 42–53 (1956).
19) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
20) Wong, A. W., He, S., Grubb, J. H., Sly, W. S., and Withers, S. G., Identification of Glu-540 as the catalytic nucleophile of human β-glucuronidase using electrospray mass spectrometry. *J. Biol. Chem.*, **273**, 34057–34062 (1998).
21) Mizutani, M., Nakanishi, H., Ema, J. I., Ma, S. J., Noguchi, E., Inohara-Ochiai, M., Fukuchi-Mizutani, M., Nakao, M., and Sakata, K., Cloning of β-primeverosidase from tea leaves, a key enzyme in tea aroma formation. *Plant Physiol.*, **130**, 2164–2176 (2002).
22) Ahn, O. Y., Mizutani, M., Saino, H., and Sakata, K., Furcatin hydrolase from *Viburnum furcatum* blume is a novel disaccharide-specific acuminosidase in glycosyl hydrolase family 1. *J. Biol. Chem.*, **279**, 23405–23414 (2004).
23) Tsuruhami, K., Mori, S., Sakata, K., Amarume, S., Saruwatari, S., Murata, T., and Usui, T., Efficient synthesis of β-primeverosides as aroma precursors by transglycosylation of β-diglycosidase from *Penicillium multicolor*. *J. Carbohydr. Chem.*, **24**, 849–863 (2005).