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Isolation and Characterization of A β-Primeverosidase-like endo-manner β-Glycosidase from *Aspergillus fumigatus* AP-20

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A novel β-glycosidase-producing microorganism was isolated from soil and identified as *Aspergillus fumigatus* AP-20 based on its taxonomical characteristics. The enzyme was found to be an extracellular protein in the culture of the isolated fungus and was purified 88-fold by fractionation with ammonium sulfate followed by successive column chromatographies on phenyl-Sepharose HP and Mono P HR. The molecular mass was estimated to be 47 kDa by SDS-PAGE and the isoelectric point to be pH 6.0 by isoelectric focusing. The purified enzyme was highly specific for a substrate, *p*-nitrophenyl β-primeveroside (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside), which was cleaved in an endo-manner into primeverose and *p*-nitrophenol.

Key words: β-glycosidase; *Aspergillus fumigatus*; purification; characterization; β-primeverosidase

Aroma precursors of the floral tea aroma like linalool, 2-phenylethanol *etc.*, have been isolated and identified as disaccharide glycosides. Most of them were identified as β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside).[1–7] At the same time β-primeverosidases have been purified from fresh leaves of cvs. Yabukita (*Camellia sinensis* var. *sinensis*) for Japanese green tea, Shuixian (*C. sinensis* var. *sinensis*) for oolong tea, and a cultivar (*C. sinensis* var. *assamica*) for black tea. These enzymes showed high substrate specificity toward β-primeverosides to hydrolyze them into primeverose and the corresponding aglycons.[8–10] Quite recently, Wang *et al*. have demonstrated that β-primeverosides play an important role in tea aroma formation during black tea manufacturing.[11]

However, no β-primeverosidase from a microorganism has been reported. From such an aspect, we tried to search for β-primeverosidase-like enzymes from microbial sources, because the finding of such kinds of enzymes will expand the possible applications in industry, for example, the control of aroma formation or high recovery of aroma extraction. In this study, we describe screening for microorganisms producing β-primeveroside-hydrolyzing enzyme and the purification and characterization of this unique β-primeverosidase-like endo-manner β-glycosidase.

Materials and Methods

Microorganisms. The microbial strain AP-20, isolated from soil on a field in Aichi prefecture in Japan, was used in this study. Strain AP-20 was identified taxonomically as *Aspergillus fumigatus* based on Raper's Manual.[12]

Materials. Eugenyl β-primeveroside was prepared from young leaves of *Camellia sasanqua*.[13] *p*-Nitrophenyl (*p*NP) β-primeveroside and primeverose were enzymatically synthesized by our method.[14] *p*NP gentiobioside (6-*O*-β-D-glucopyranosyl-β-D-glucopyranoside), *p*NP monosaccharide glycosides (*p*NP β-D-gluco- and β-D-xylopyranosides), gentiobiose, sophorose (2-*O*-β-D-glucopyranosyl-β-D-glucopyranose), and laminaribiose (3-*O*-β-D-glucopyranosyl-β-D-glucopyranose) were purchased from Sigma Chemical Co. Cellobiose (4-*O*-β-D-glucopyranosyl-β-D-glucopyranose) was obtained from Wako Pure Chemical Industries, Ltd. All other chemicals were of analytical grade.

Media. Solid medium for primary screening contained 0.5% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.3% (w/v) eugenyl β-primeveroside, and 2% (w/v) Bacto-Agar (Difco Laboratories). The pH of the medium was adjusted to 6.0. The separating solid media was Potato Dextrose Agar (Eiken Chemical Co. Ltd.). Liquid medium base for secondary screening contained

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Abbreviations: *p*NP, *p*-nitrophenyl; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography

0.3% (w/v) $NaNO_3$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) KCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, and 2% (w/v) glucose. The pH of the medium was adjusted to 6.0. For medium P, 0.1 mM *p*NP β-primeveroside was added to the liquid medium of the secondary screening base and for medium X, *p*NP β-D-xylopyranoside was added. For the first cultivation for enzyme production, Dextrose Peptone Broth (Eiken Chemical Co. Ltd.) was used. The cultivation medium for enzyme production contained 2% (w/v) Soyaflour A (Nissin Oil Mills Ltd.), 0.3% (w/v) NaCl, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 3% (w/v) Soluble Starch, and 1% (w/v) Gentose #80 (Nihon Syokuhin Kako Co. Ltd.). The pH of the medium was adjusted to 5.6.

Isolation of microorganisms and fermentation. For the primary screening of microorganisms producing β-primeveroside-hydrolyzing enzyme, soil samples were suspended in sterilized saline and spread on the solid media. After incubation at 30°C for 5 days, the viable microorganisms were transferred to Potato Dextrose Agar slants. The secondary screening was done as follows: the microorganisms obtained by the primary screening were inoculated into 100 μl of liquid media and incubated at 30°C for 30 h. Two hundreds μl of 0.5 M Na_2CO_3 was added to the medium. Microorganisms that were more deeply colored in medium P than medium X by releasing *p*-nitrophenol were selected. Each agar block (5× 5 mm) taken from the Potato Dextrose Agar slant was inoculated into 100 ml of first cultivation medium in a 500 ml-Sakaguchi flask and incubated at 30°C for 24 h on a reciprocal shaker (140 strokes/min). After this cultivation, 1 ml of the culture was inoculated into the second cultivation medium in a 500 ml-Sakaguchi flask and incubated at 30°C for 6 days on a reciprocal shaker (140 strokes/min). The culture filtrates were used as a crude enzyme solution.

Enzyme assay. During purification, the enzyme fractions were measured for hydrolyzing activities toward *p*NP monosaccharide glycosides (*p*NP β-D-glucopyranoside and *p*NP β-D-xylopyranoside) and *p*NP β-primeveroside. The incubation mixture (245 μl) consisted of 45 μl of an enzyme sample solution and 200 μl of 2 mM substrate solution. The buffers of substrate solutions were 20 mM acetate buffer (pH 5.5) for *p*NP β-monosaccharide glycosides and 20 mM acetate buffer (pH 4.0) for *p*NP β-primeveroside. The reaction was started by adding an enzyme sample to a substrate solution at 37°C and stopped by addition of 250 μl of 0.5 M Na_2CO_3. The liberated *p*-nitrophenol was measured spectrophotometrically at 412 nm. One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min under these assay conditions.

The optimum temperature of the enzyme was measured with *p*NP β-primeveroside in 20 mM disodium citrate-HCl buffer (pH 2.5) at various temperatures (30–65°C). The thermal stability of the enzyme was examined from the residual activities after incubation in 20 mM glycine–NaCl–NaOH buffer (pH 8.0) at various temperatures (30–65°C) for 1 h. The optimum pH of the enzyme was measured with *p*NP β-primeveroside in 20 mM disodium citrate-HCl buffer at different pHs (2.0–5.0) at 37°C. The pH stability of the enzyme was examined from the residual activities after incubation in different pHs (3.0–12.0) at 37°C for 1 h. The buffers used were 20 mM citrate buffer (pH 3.0–6.0), 20 mM Tris-malate-NaOH buffer (pH 6.0–8.0), and 20 mM glycine-NaCl-NaOH buffer (pH 8.0–12.0).

Purification of a β-glycosidase. All operations were done in 20 mM potassium phosphate buffer (pH 6.0) at room temperature, unless otherwise stated. The culture was filtered through filter paper to remove mycelium, and the filtrate (8,600 ml) was concentrated to 710 ml by using ultrafiltration AIP-1010 (Asahi Kasei Corporation) with 100% recovery of β-primeverosidase activity. The enzyme solution (200 ml) was centrifuged to remove insolubles. To the supernatant solution (184 ml), solid ammonium sulfate was added up to 50% saturation with stirring and left overnight at 4°C. The resulting precipitates were collected by centrifugation, dissolved in 10 ml of the buffer saturated with 20% ammonium sulfate. The enzyme solution in the buffer containing 20% ammonium sulfate was put onto a HiLoad 16/10 phenyl-Sepharose HP column (16 mm i.d. × 100 mm; Pharmacia Biotech Co. Ltd.) using the FPLC system, equilibrated with the same as shown in Fig. 2. No β-primeverosidase was eluted when the column was further washed with a high salt concentration. After the column was washed with a linear gradient of 20% (20 ml) ~ 0% (180 ml) ammonium sulfate, active enzyme fractions (Fr. 53 ~ 55) were eluted with a buffer solution containing no ammonium sulfate. Active fractions were combined and desalted using by Econo-Pac 10DG columns (Bio-Rad Laboratories) with a 25 mM triethanolamine-iminodiacetic acid (pH 8.3) solution. The desalted solution (7.2 ml) was put on a Mono P HR 5/20 column (5 mm i.d. × 200 mm; Pharmacia Biotech Co. Ltd.) using the FPLC system, equilibrated with 25 mM triethanolamine-iminodiacetic acid (pH 8.3). When the column was eluted with a linear gradient from 0% (12 ml) ~ 100% (72 ml) Polybuffer (pH adjusted to 5.0 with iminodiacetic acid; 3% Polybuffer 96, 7% Polybuffer 74; Pharmacia Biotech Co. Ltd.), active fractions (Fr. 31 ~ 32) were eluted with about 30% Polybuffer. There was a symmetrical peak of the enzyme activity associated with the protein peak, which was almost devoid of other β-glycosidase ac-

tivities such as β-glucosidase or β-xylosidase.

Analytical methods. Protein was measured by the method of Lowry *et al.*[15] using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electorophoresis (SDS-PAGE) was done with the PhastSystem (Pharmacia Biotech Co. Ltd.) using a PhastGel Gradient 8–25 (Pharmacia Biotech Co. Ltd.). ''Perfect'' protein markers 15–150 kDa (Novagen) were used for the mass calibration. Proteins were detected with a Pharmacia silver stain. Isoelectric focusing was done on the sucrose density-gradient isoelectric separation using Ampholine pH 3.5–10.0 (Pharmacia Biotech Co. Ltd.) as the carrier ampholyte.

HPLC was done a YMC-packed column ODS-AQ-303 (ODS) (4.6 mm i.d. × 250 mm) on a Shimadzu liquid chromatograph equipped with a Shimadzu SPD-10Avp UV detector. When *p*NP β-glycosides (*p*NP β-primeveroside and *p*NP β-gentiobioside) were used as substrates, elution was done with water/MeOH (80/20, v/v) at a flow rate of 0.8 ml/min at 40°C, and detected at 300 nm. With eugenyl β-glycosides (eugenyl β-primeveroside and eugenyl β-glucoside), it was also done with water/MeOH (55/45, v/v) at 280 nm.

Cleavage ratios and kinetic studies were done as follows. The incubation mixture (0.5 ml) consists of 0.1–30 mM *p*NP or eugenyl β-glycoside in 50 mM acetate buffer (pH 4.0). Reactions were started by adding the enzyme sample at 37°C. Samples (100 μl) were taken at 3-min intervals (3, 6, 9, 12 min) during the incubation, and inactivated by adding 200 μl of 0.1 M trichroloacetic acid. The amount of *p*-nitrophenol or eugenol formed from the initial substrates at an early stage (15% hydrolysis) during incubation with the enzyme was analyzed by HPLC. The amount of each product increased linearly as time passed in the initial stage of the reaction. On the basis of these data, the ratio of enzyme-catalyzed cleavage of each glycosidic linkage was calculated. And the initial velocity (v) of each reaction was obtained directly from the initial slope of time vs product produced plots of the reaction. Six different substrate concentrations (0.1–30 mM) were used for each experiment. The K_m and V_{max} for *p*NP and eugenyl β-primeverosides were calculated from plots of v against substrate concentrations using the Michaelis-Menten equation and the least squares method.[16]

Relative hydrolysis rates of various kinds of β-linked disaccharides and β-glycosides by the β-glycosidase were measured by incubating 1 mM of substrates in 1.0 ml of 25 mM acetate buffer (pH 4.0) with the enzyme. Reactions were started by adding each enzyme sample at 37°C. Samples (200 μl) were taken at intervals (3, 6, 9, 12 min) during the incubation, and inactivated by boiling for 5 min. The amount of hydrolysates formed at an early stage (within 15% hydrolysis) was analyzed by using TC D-glucose (Determination kit for D-glucose, Roche Diagnostics K.K.) for β-linked disaccharides or HPLC for β-glycosides. From these results, relative hydrolysis rates for various kinds of substrates were calculated, taking the reaction velocity for *p*NP β-primeveroside as 100 (%).

Results and Discussion

Screening for microorganisms producing β-primeveroside-hydrolyzing enzyme

In the primary screening for β-primeveroside-hydrolyzing enzyme-producing strains, four positive strains were obtained. The fermentation broth of strain AP-20 showed the highest β-primeveroside-hydrolyzing activity among these strains at the secondary screening. The crude enzyme solution hydrolyzed *p*NP β-primeveroside, which was cleaved in an endo-manner into primeverose and *p*-nitrophenol.

Taxonomic characterization

Culture tests were done by the procedures of Raper[11] by using Czapeck-Dox agar (CA) and Malt extract (MEA) agar as test media. Morphological examinations of the organism grown on CA and MEA at 25°C for 7 days were done by slide-culture methods with an optical microscope. The culture and morphological characteristics are shown in Table 1. This strain formed macroscopic colonies 73–75 mm in diameter at 37°C on CA after 3 days. Growth was observed at 45°C on CA. Metulae and ascospores were not formed. Conidial heads were strongly columnar, not nodding, appearance, and its color was dull green to grayish green. Conidia were globose and their surfaces were echinulate. Conidiophores was mostly 500 μm or less in length. The vesicles and phialides were shown in Fig. 1. Based on these cultural and morphological characteristics, this strain was identified as *Aspergillus fumigatus*.

Purification and characterization of the β-glycosidase

An enzyme hydrolyzing *p*NP β-primeveroside from *A. fumigatus* AP-20 was purified by ammonium sulfate preciptation followed by successive column chromatographies using phenyl-Sepharose HP and Mono P HR. An elution profile showed a separation of β-primeverosidase activity from the β-glycosidase one on phenyl-Sepharose HP (Fig. 2). The active fraction was further purified by Mono P HR. A summary of the purification is presented in Table 2. The final recovery of the enzyme activity was 40% and the specific activity increased 88-fold after the first filtration. The purified enzyme gave a single band corresponding to a molecular mass of about 47 kDa on SDS-PAGE (Fig. 3). The isoelectric point was pH 6.0 by isoelectric focusing. The enzyme had a

Table 1. Characterization of the Strain AP-20 Identified as *Aspergillus fumigatus*

		Growth medium	
A.	Culture characteristics (after 7 days at 27°C)	Czapek-Dox agar	Malt extract agar
	Growth rate (diameter, mm)	48–50	78–80
	Surface texture	Velutinous to powdery	Velutinous to powdery
	Conidiogenesis	Dull green to grayish green, light to moderate	Dull green to grayish green, abundant
	Soluble pigment	None	None
	Reverse	Light yellowish brown to brown	Colorless to yellowish white
B.	Morphological characteristics		
	Conidial heads	Strongly columnar, 48–128 μm in length by 16–52 μm in diameter, dull green to grayish green	
	Conidiophores	Smooth, 125–800 μm (mostly less than 500 μm) in length. Based in length by 5–10 μm in diameter, arising directly from submerged hyphae	
	Vesicles	Flask shaped, 10–25 μm in diameter	
	Metulae	None	
	Phialides	5.6–12 by 2.4–3.2 μm	
	Conidia	Green in the mass, echinulate, globose to subglobose, 2.6–3.6 μm in diameter	
	Ascospores	None	

Table 2. Purification of the β-Glycosidase from *Aspergillus fumigatus* AP-20

Purification step	Total activity (u)	Total protein (mg)	Specific activity (u/mg)	Purification fold	Recovery (%)
Ultrafiltration	45.2	828	0.05	1	100
50% $(NH_4)_2SO_4$ ppt	41.2	199.8	0.20	4	91
Phenyl Sepharose	23.3	8.0	2.9	58	52
Mono P	18.2	4.1	4.4	88	40

The enzyme activities were measured with *p*NP β-primeveroside.

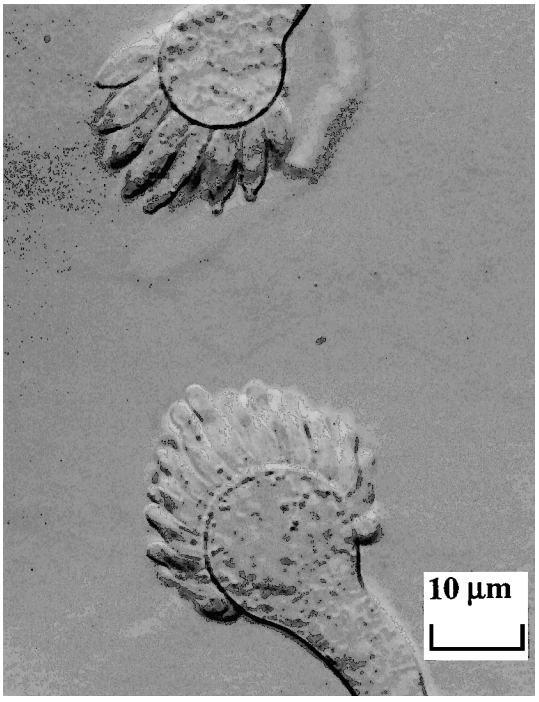

Fig. 1. Light Microphotograph of *Aspergillus fumigatus* AP-20, Grown on MEA Medium for 7 Days at 25°C.

broad pH optimality in the range of pH 2.5–3.0 and was stable from pH 7.0–8.0. It had its optimum activity at 55°C and showed near-optimum activities (>90%) from 50 to 60°C.

Relative hydrolysis rates of β-linked disaccharides and β-glycosides by the β-glycosidase

The relative hydrolysis rate of the purified enzyme on *p*NP β-gentiobioside compared to *p*NP β-primeveroside at 100 was 41, while that of eugenyl β-primeveroside was 1.8, a 55-fold difference (Table 3). Replacement of the eugenyl group by *p*NP greatly decreased the hydrolysis rate. β-Glucobioses and primeverose acted slightly as substrates. These results show that primeverose and aglycon units are essential for the hydrolytic action of the enzyme.

Kinetic studies and cleavage ratio

Kinetic parameters for the hydrolysis of three kinds of disaccharide β-glycosides are listed in Table 4. *p*NP β-primeveroside had a K_m of 2.88 mM and K_m/V_{max} of 2.8 and was the best substrate. The K_m/V_{max} of *p*NP β-gentiobioside is 68% of that of *p*NP β-primeveroside and it still acts as a fairly good substrate. On the contrary, eugenyl β-primeveroside acted only slightly as a substrate (K_m/V_{max}: 1.5% of that of *p*NP β-primeveroside). This suggests that the chemical property of the aglycon moiety at the terminal position is strictly required for the hydrolytic action.

The action modes of the enzyme on three kinds of disaccharide β-glycosides were further examined.

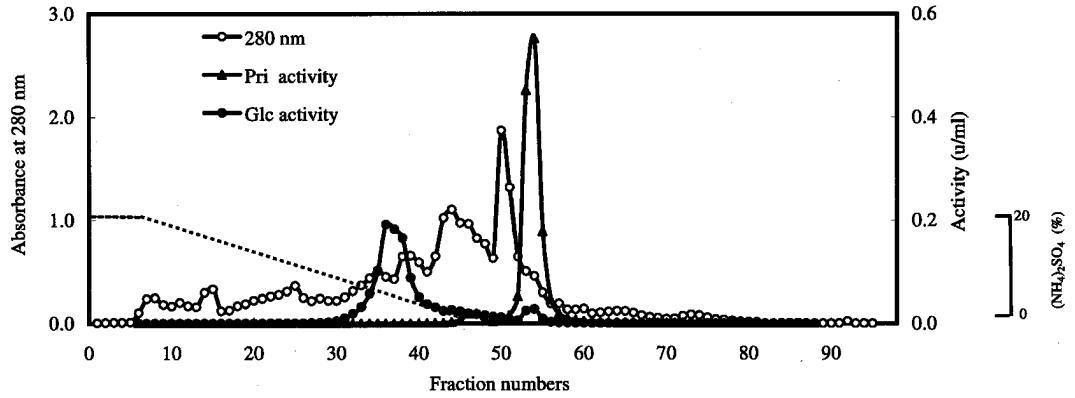

Fig. 2. FPLC Elution Profile of the β-Glycosidase Fraction on a Phenyl-Sepharose Column from *Aspergillus fumigatus* AP-20.
Column, HiLoad 16/10 phenyl-Sepharose HP; elution, 20 mM potassium phosphate buffer (pH 6.0); ammonium sulfate gradient elution; flow rate, 2 ml/min. The β-primeveroside (Pri) and β-glucosidase (Glc) activities were measured using *p*NP β-primeveroside and *p*NP β-glucoside, respectively.

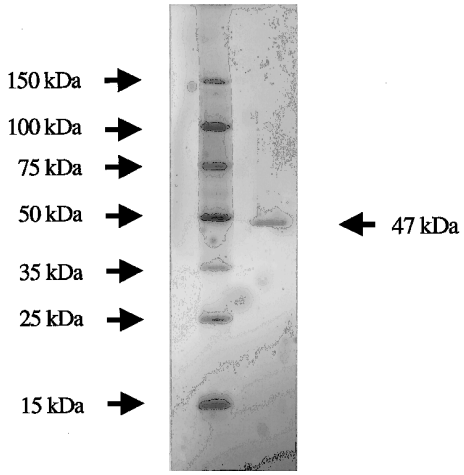

Fig. 3. SDS-PAGE of the β-Glycosidase Purified from *Aspergillus fumigatus* AP-20.
The gel was stained with silver. Lane 1, molecular mass marker; Lane 2, purified enzyme protein.

From the HPLC chromatograms of the digest of each substrate, the amounts of the products were calculated from their peak areas. The amounts of each product increased linearly with time in the initial stage of the reaction (data not shown). Based on the product analysis, the ratio of the enzyme-catalyzed cleavage of glycosidic linkages in the three substrates were different from each other. The number of susceptible bonds varied with the structual differences of the substrates. *p*NP β-primeveroside was hydrolyzed predominantly in an endo-manner into primeverose and *p*-nitrophenol. The action pattern on *p*NP β-gentiobioside was somewhat different from that of *p*NP β-primeveroside and the cleavage occurred at both the first and second bonds from the *p*NP group in the ratio of 0.62:0.38. Eugenyl β-primeveroside was hydrolyzed at the bond between Xyl and Glu with the enzyme, although it was hydrolyzed slowly. Taking the kinetic data into account, this indicates that the

Table 3. Relative Hydrolysis Rates on Various Kinds of β-Linked Disaccharides and β-Glycosides by the β-Glycosidase

Substrate	Relative rate of hydrolysis (%)
p-Nitrophenyl β-primeveroside	100
p-Nitrophenyl β-gentiobioside	41
Eugenyl β-primeveroside	1.8
Sophorose (Glcβ1-2Glc)	0.5
Laminaribiose (Glcβ1-3Glc)	1.6
Cellobiose (Glcβ1-4Glc)	0.6
Gentiobiose (Glcβ1-6Glc)	5.1
Primeverose (Xylβ1-6Glc)	0.2

eugenyl group affected the position of glycosides to be hydrolyzed against the action of the enzyme.

From these results, we suppose a subsite structure so that *p*NP β-primeveroside has a matching shape, which can accommodate a chain of three residues to fit into the active site. 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 glycosodic bonds of the substrates are split between S2 and S3 (Fig. 4). Reducing disaccharides, primeverose and other β-linked glucobioses, act only slightly as substrates. It shows that disaccharide glycoside structure is at least needed for the binding to the active site. A comparison of the catalytic efficiency for *p*NP β-primeveroside with that for eugenyl β-primeveroside shows that the eugenyl group did prohibit reaction of the enzyme. Subsite 3 was strict for binding the aglycon group. Replacement of *p*NP β-gentiobioside by *p*NP β-primeveroside does appreciabily influence the enzyme action, although the former substrate acts as a fairly good substrate. This may explain the flexibility of subsite 1 for binding the glucosyl group at the nonreducing end.

In conclusion, in the screening of microorganisms producing any β-primeveroside-hydrolyzing enzyme, we found a filamentous fungus *A. fumigatus* producing a novel β-glycosidase which hydrolyzes not only

Table 4. Kinetic Parameters of the β-Glycosidase

Substrate	K_m (mM)	V_{max} (μmol/min/mg protein)	V_{max}/K_m
p-Nitrophenyl β-primeveroside	2.9	8.1	2.8
p-Nitrophenyl β-gentiobioside	6.3	12	1.9
Eugenyl β-primeveroside	6.3	0.26	0.041

Substrate	S_1	S_2	S_3	S_4	Ratio
p-Nitrophenyl β-primeveroside	X	G	P		0.96
		X	G	P	0.04
p-Nitrophenyl β-gentiobioside	G	G	P		0.62
		G	G	P	0.38
Eugenyl β-primeveroside		X	G	E	0.91
	X	G	E		0.09
	S_1	S_2	S_3	S_4	

Fig. 4. Schematic Representation of Substrate Binding to Subsites and the Action of the β-Glycosidase on the Substrates.
X, xylosyl residue; G, glucosyl residue; P, *p*-nitrophenyl residue; E, eugenyl residue; ▲, the catalytic site.

β-primeverosides but also β-gentiobiosides in an endo-manner into disaccharide and aglycon. As a result, *p*NP β-primeveroside was very suitable as a synthetic substrate for analytical use of β-primeverosidase-like endo-manner β-glycosidase. Further investigations on the substrate specificities are now in progress.

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