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Purification and Characterization of an Intracellular β-Glucosidase from a *Candida sake* Strain Isolated from Fruit Juices

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Abstract

A yeast strain isolated in the laboratory from fruit juices was studied and classified as *Candida sake*. The strain produces an intracellular β-glucosidase when grown with cellobiose as the carbon source. The enzyme was purified by ion-exchange chromatography and gel filtration. The molecular mass of the purified intracellular β-glucosidase, estimated by gel filtration, was 240 kDa. The tetrameric structure of the β-glucosidase was determined following treatment of the purified enzyme with sodium dodecyl sulfate. The enzyme exhibited optimum activity at 52°C and pH 4.25 with citrate-phosphate buffer. The enzyme was active against soluble glycosides with the (1→4)-β configuration, and from Lineweaver Burk plots, a K_m value of 6.9 mmol/L was found for *p*-nitrophenyl-β-D-glucopyranoside. The β-glucosidase was found to be tolerant to glucose inhibition with a K_i value of 0.2 mol/L.

Index Entries: β-Glucosidase; purification; *Candida sake*.

Introduction

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) constitute a group of well-studied hydrolases that have been isolated from members

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of all three domains of life: Eucarya, Bacteria, and Archaea *(1)*. The principal reaction catalyzed by this class of enzyme is the hydrolytic cleavage of β-glycosidic linkages of low molecular mass glycosides. The physiologic roles postulated for β-glucosidases are extremely diverse: glucoside ceramide catabolism in human tissue; cell wall, pigment, and cyanoglucoside metabolism in plants; and the utilization of oligosaccharide substrates by many fungi and bacteria *(2)*. Interest in industrial use of β-glucosidases for cellulose conversion is based on the fact that cellulose is the most abundant substrate on earth and is very likely to be an important source of energy in the future. The enzymatic saccharification of cellulosic materials to glucose requires the synergistic action of three classes of enzymes: endo-1,4-β-glucanases (EC 3.2.1.4), exo-1,4-β-glucanases (EC 3.2.1.91), and β-1,4-glucosidases. β-Glucosidases were also studied for cassava detoxication *(3,4)* and, more recently, for their potential to release aroma-rich terpenes. Such aroma precursor compounds found in certain fruits (mango, passion fruit, grapes) and bound to glucosides (terpenylglucosides) are more effectively released by enzymatic treatment than by acid hydrolysis *(5,6)*.

In this article, we report on the purification and detailed biochemical study of an intracellular β-glucosidase of *Candida sake* that is expected to possess fundamental functions in the host organism. *C. sake* has been shown to play an important role in the control of major postharvest apple pathogens *(7)*, and its β-glucosidase might be involved in the defense process against pathogens. Moreover, to our knowledge, this study is the first purification and characterization of a β-glucosidase from *Candida sake*.

Materials and Methods

Isolation of Yeast

The yeast strain was isolated in our laboratory after 24 h of incubation at room temperature from fresh fruit juices from the local market. The yeast strain was isolated on YEG medium (0.5% glucose [w/v], 0.5% yeast extract [w/v], 2% agar [w/v]) and identified by the Centraalbureau voor Schimmelcultures (Identification Service, CBS yeast division, Delft, Netherlands) as *C. sake* (Saito & Ota) van Uden & H.R. Buckley.

Culture Conditions

The basal culture medium was "G" medium *(8)* to which carbon sources were added to a final concentration of 0.5% (w/v). The cultures were incubated at 28°C in Erlenmeyer flasks filled to one-tenth of their volume. Growth was monitored by measuring the absorbance at 420 nm. Glassware and equipment were as previously described by Guiraud and Galzy *(8)*. Cultures were incubated at 28°C and shaken (80 oscillations/min, 8-cm amplitude).

Cell Breakage

Cells were centrifuged at 4000*g* for 10 min, washed twice with phosphate buffer (20 mmol/L, pH 7.3), resuspended in this buffer, and ground with glass beads (0.45–0.55 mm diameter) in an M.S.K. apparatus Braun (B. Braun Melsungen AG, Germany) according to the method of Guiraud and Galzy *(8)*. The ground cells suspension was centrifuged at 4000*g* for 10 min. The supernatant (S1) contained the soluble β-glucosidase.

Enzyme and Protein Assays

β-Glucosidase activity against *p*-nitrophenyl-β-D-glucopyranoside (GlcβNp) was determined by adding 0.1 mL of enzyme solution to 4.9 mL of citrate-phosphate buffer (0.1 mol/L, pH 6.0) containing GlcβNp (5 mmol/L final concentration) *(9)*. The reaction mixture was incubated at 30°C. Samples (0.5 mL) were taken at regular intervals and added to 1.0 mL of carbonate buffer (0.2 mol/L, pH 10.2). Liberated *p*-nitrophenol in this mixture was assayed by spectrophotometry at 400 nm. The molar extinction coefficient used was 18,300 $mol^{-1} \cdot cm^{-1}$. One β-glucosidase activity unit was defined as the quantity of enzyme required for hydrolysis of one micromole of substrate (GlcβNp) per minute (U/mL) under initial rate conditions.

When the substrate used did not contain *p*-nitrophenol, the β-glucosidase activity was determined by assaying the released glucose. In this case, enzyme solution (0.1 mL) and the substrate (0.2 mol/L final) were added to 2.9 mL of citrate-phosphate buffer (0.1 mol/L, pH 6.0). The reaction mixture was incubated at 30°C. Samples (0.2 mL) were taken at regular intervals and added to 0.4 mL of carbonate buffer (0.2 mol/L, pH 10.2). Liberated glucose was determined using a hexokinase and glucose 6-*P*-dehydrogenase procedure *(10)*.

When detection of β-glucosidase activity was required in a nondenaturating electrophoresis process, the following technique was used: the gel was immersed in a citrate-phosphate buffer, pH 6.0, containing 4-methyl-β-lumbelliferryl-β-D-glucoside (5 mmol/L) for 10 min at 30°C. The plate was observed under ultraviolet light. The fluorescent band that appeared corresponded to the enzyme activity *(11)*.

Protein was determined by the Biuret method on samples containing cell extract *(12)* and in all other cases by the Lowry method *(13)*. Bovine serum albumin (BSA) was used as a standard.

Purification of β-*Glucosidase*

During purification, all procedures were carried out at 4°C.

Sephacryl S-300 Chromatography (Gel Filtration Chromatography)

The Pharmacia HR column (10 × 1000 mm) was equilibrated and further eluted with Tris-HCl, 10% (w/v) glycerol buffer (50 mmol/L, pH 7.0). The elution rate was 17.0 mL/h and the eluent was collected in

1.7-mL fractions. The column was calibrated using molecular weight standards from Pharmacia (669-kDa thyroglobulin, 440-kDa ferritin, 200-kDa β-amylase, 158-kDa aldolase, 66-kDa BSA, 13.7-kDa ribonuclease A, 12.3-kDa cytochrome c).

Q-Sepharose Chromatography (Ion-Exchange Chromatography)

The Pharmacia HR column (26 × 400 mm) was equilibrated with Tris-HCl buffer (50 mmol/L, pH 7.0). Proteins were eluted with a linear gradient of NaCl from 0 to 1 mol/L, at a flow rate of 200 mL/h, and the eluent was collected in 10-mL fractions. Active fractions were pooled and concentrated in an Amicon cell with a PM 10 membrane (W.R. Grace, Amicon division, Danvers, MA).

Polyacrylamide Gel Electrophoresis

For native electrophoresis, precasted slab gels from Daiichi Chemical were used. Electrophoresis was performed at pH 8.4 with Tris (0.025 mol/L) glycine (0.192 mol/L) buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with precasted slab gels. The enzyme and molecular weight standards (Bio-Rad) were denatured in the presence of 5% (v/v) β-mercaptoethanol and 2% (w/v) SDS at 100°C for 10 min. Tris (0.025 mol/L) glycine (0.192 mol/L), pH 8.4, with 0.1% SDS (w/v) was used. Electrophoresis was carried out at room temperature and at a 30-mA constant current on vertical slabs (10 × 8 cm). The protein bands were revealed with Coomassie brilliant blue R-250 or with the Bio-Rad silver staining kit.

Isoelectric focusing (IEF) PAGE was performed on the Phast System of Pharmacia LKB using a precast gel, Phastgel IEF 3-9.

Determination of Molecular Mass

The molecular mass was determined by gel filtration on Sephacryl S-300, as already described and by SDS-PAGE. The molecular mass of the subunit was extrapolated from a plot of log (molecular mass) vs mobility (Bio-Rad electrophoresis calibration kit). For each data set, the results are expressed as the average of two experiments.

Chemicals

All chemicals were analytical grade. GlcβNp, *o*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-α-L-rhamnopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-β-D-xylopyranoside, arbutin, methyl-β-D-glucopyranoside, cellobiose, gentiobiose, laminaribiose, amygdalin, linamarin, and prunassin were all purchased from the Sigma. Esculine, α-trehalose, and salicin were obtained from Fluka Biochemika. Q-Sepharose Fast Flow and Sephacryl S-300 were from Pharmacia LKB Biotechnology.

Results and Discussion

Location of β-*Glucosidase Activity*

The location of the β-glucosidase activity was investigated after 24 h of growth on "G" medium buffered with tartrate phosphate (0.1 mol/L, pH 5.5). The carbon source was cellobiose 0.5% (w/v). The main activity was detected in the supernatant of the disrupted cells following centrifugation (10,000*g* for 10 min) (S1) and ultracentrifugation (180,000*g* for 90 min) (S2) steps. However, a small amount of β-glucosidase activity was also detected in the culture medium supernatant and in the whole cells, suggesting the possible presence of several β-glucosidases. The endocellular enzyme was considered for further characterization.

Purification of β-*Glucosidase*

The S1 supernatant fluid of the disrupted cells following centrifugation (10,000*g* for 10 min) was ultracentrifuged (135,000*g*, 90 min, 4°C) and the supernatant (S2) was concentrated in an Amicon cell with a PM 10 membrane and chromatographed on a Sephacryl S-300 column (Fig. 1A). The enzyme was found in fractions 22–34. Fractions 23–34 were pooled and fractionated on a Q-sepharose column (Fig. 1B). β-Glucosidase activity eluted as a single peak in fractions 20–30 at 0.14 mol/L of NaCl. Fractions 21–27 were pooled, concentrated in an Amicon cell with a PM 10 membrane, and chromatographed again on a Sephacryl S-300 column (Fig. 1C). The enzyme was found in fractions 26–40. Fractions 29–35 were pooled to give the purified β-glucosidase preparation. Table 1 summarizes the data for the purification steps. The specific activity of the purified enzyme was 140.3. The purity of the β-glucosidase preparation was confirmed by native and SDS-PAGE, and one single band was detected in both cases. No enzyme isoforms were detected during any stage of the purification.

Physical and Chemical Properties of β-*Glucosidase*

The properties of the enzyme were determined using the purified extract. The molecular mass of the β-glucosidase was estimated to be 240 kDa by Sephacryl S-300 chromatography. This value is close to that previously obtained for other β-glucosidases, which were 220 kDa for *Candida cacaoi (14)* and 230 kDa for *Aspergillus niger (15)*. Molecular masses for β-glucosidases from yeast range from 48 kDa for *Candida guillermondi (16)* to 560 kDa for *Arxula adenivorans (17)*. The denaturated enzyme showed one major band (molecular mass of 60 kDa) on SDS-PAGE. The β-glucosidase seems to be composed of four subunits. β-Glucosidases purified from Fungi, Yeast, Eubacteria, and Archaea were reported to have monomeric (*Clostridium thermocellum [18]*), dimeric (*Brevibacteruim* sp. R312 *[19]*), or tetrameric (*Candida entomophila [20]*) structures. The β-glucosidase of *Lactobacillus casei* is the only β-glucosidase that has been described with a hexameric structure *(21)*.

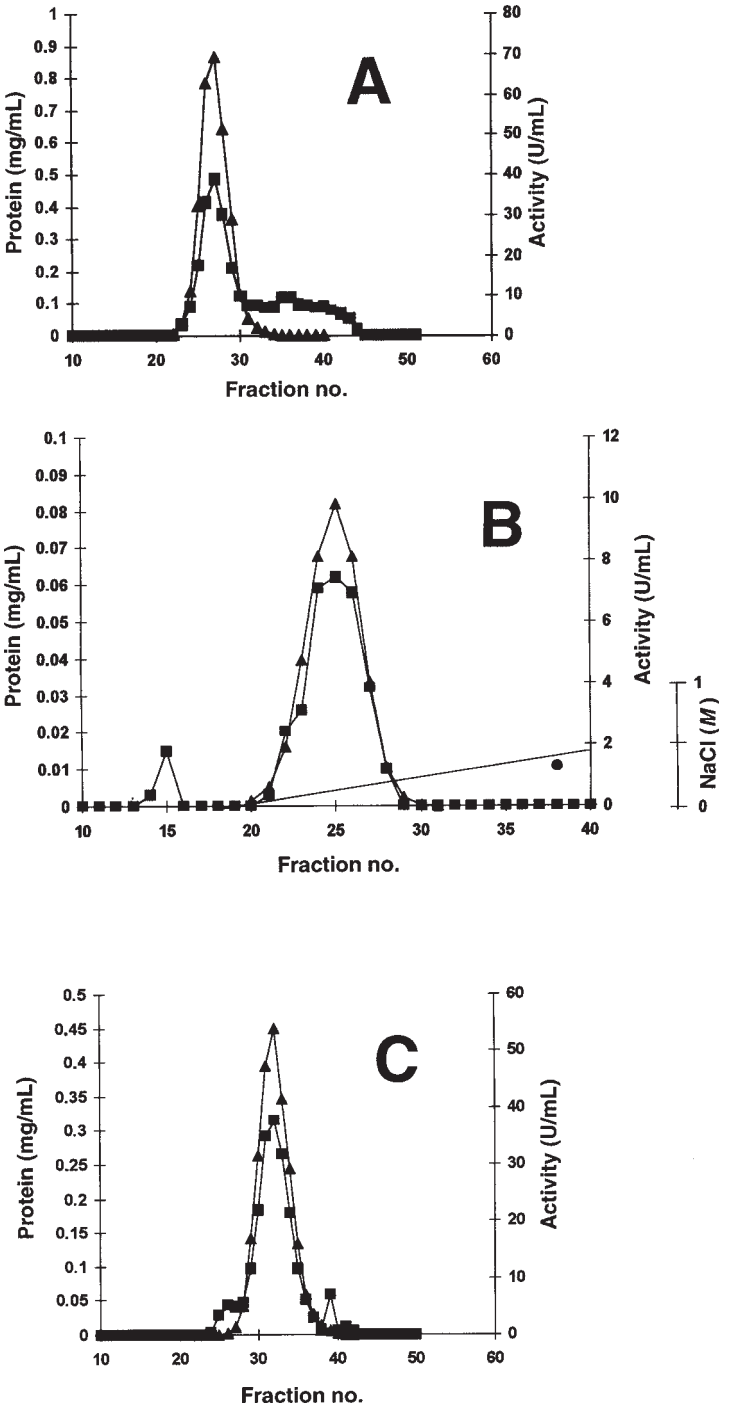

Fig. 1. Purification of the β-glucosidase of *C. sake*. **(A)** Elution of the β-glucosidase activity from a Sephacryl S-300 gel filtration column. **(B)** Fractions 23–34 were collected and rechromatographed using a Q-Sepharose Fast Flow chromatography column. **(C)** Resulting peak fractions containing β-glucosidase activity were further purified by gel filtration chromatography on a Sephacryl S-300 column. β-Glucosidase activity was determined at 30°C, using 5 mmol/L of GlcβNp as the substrate. Experimental procedures are described in Materials and Methods. ■, protein; ▲, activity on GlcβNp; ●, NaCl concentration (0–1 mol/L).

Table 1
Purification of *C. sake* β-Glucosidase[a]

Purification step	Protein (mg)	Specific activity (U/mg protein)[b]	Yield (%)	Purification factor[c]
S2	135.3	3.95	100.0	1.0
Sephacryl S-300	30.4	12.6	72.1	3.2
Q-Sepharose	2.4	112.4	50.6	28.5
Sephacryl S-300	1.8	140.3	46.3	35.5

[a]β-Glucosidase activity was determined at 30°C, using 5 mmol/L of GlcβNp as the substrate.
[b]Expressed as micromoles of GlcβNp/(hydrolyzed minutes^{-1} · milligrams^{-1} of protein).
[c]The purification factor is defined as the ratio of the specific activity to that of S2.

The isoelectric point (p*I*) was estimated to be 5.05. It is quite similar to those found for the yeast β-glucosidases of *Candida pelliculosa* (p*I* 4.9) *(22)* and *Hanseniaspora vineae* (p*I* 4.8) *(23)*. β-Glucosidases have generally acidic p*I*s *(24)* between 3.5 and 5.5; however, β-glucosidases have also been described with basic p*I* values like that of *Trichoderma reesei* QM9414 with a p*I* of 8.7 *(25)*.

The denaturation of the enzyme was monitored by measuring its activity against GlcβNp at 30°C, following incubation at several temperatures for various time periods. The calculated inactivation energy determined from Arrhenius plots was 247.7 kJ/mol. This value is similar to those generally reported for enzymes, in which the inactivation energy lies between 150 and 400 kJ/mol. These values are high but can be explained by the necessity of simultaneously breaking up a large number of bonds. The enzyme was not heat resistant, with rapid inactivation observed at 47°C and above. No activity was detected after 4 min at 55°C.

Kinetic Properties of Purified β-*Glucosidase*

The influence of pH on enzymatic activity was studied (Fig. 2A). The optimum pH was 4.25 in citrate-phosphate buffer (0.1 mol/L). The influence of temperature on activity was studied with citrate-phosphate buffer (0.1 mol/L, pH 4.25) (Fig. 2B). Activity measurements were performed at several temperatures. The activity plot for GlcβNp gives an optimal temperature of 52°C. The thermal activation energy determined from the Arrhenius plots is 17.1 kJ/mol. Both pH and temperature optima were similar to those reported for other purified yeast β-glucosidases *(14,26)*.

The effect of various cations was tested (Table 2). The enzyme was inhibited by Hg^{2+} and Fe^{2+}. The action of some effectors such as EDTA (10 mmol/L), a chelating agent, allowed us to conclude that the intracellular β-glucosidase of *C. sake* is not dependent on a metallic cofactor at its active site. The inhibition by *N*-bromosuccinimide, at a concentration of 10 mmol/L, indicates that a tryptophan residue of the enzyme molecule is important in its catalytic action. *p*-Chloromercuribenzoate (PCMB), a well-

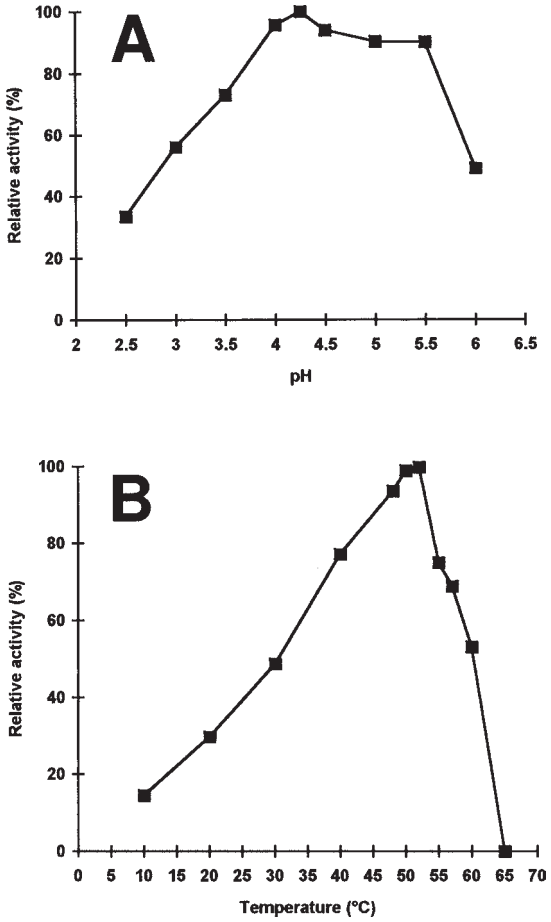

Fig. 2. Effect of pH **(A)** and temperature **(B)** on the β-glucosidase of *C. sake*. Purified β-glucosidase was incubated at the indicated pH (citrate-phosphate buffer for the pH ranges 2.5–6.0) at 30°C (A) and at the indicated temperature at pH 4.25 (B). β-Glucosidase activity was determined at 30°C, using 5 mmol/L of GlcβNp as the substrate. Experimental procedures are described in Materials and Methods.

known sulfhydryl group (SH) blocking agent, had a moderately inhibitory effect on enzyme activity. This result, in combination with the observed sensitivity toward Hg^{2+}, may indicate the presence of important sulfydryl groups. Iodine (5 mmol/L), an agent reacting with tyrosine, strongly inhibited the catalytic activity.

Glucose inhibition was studied using GlcβNp as substrate. A competitive type of inhibition was observed. However, the enzyme was found to be tolerant to glucose inhibition with a *Ki* of 0.2 mol/L (36 mg/mL). This value is quite high compared with those usually found for microbial β-glucosidases between 3 and 10 mmol/L *(19,27,28)* and indicated a lower inhibition by glucose, the product of the reaction catalyzed by the enzyme. Such glucose-tolerant β-glucosidases have already been described from

Table 2
Action of Metal Ions and Several Chemical Compounds on Intracellular β-Glucosidase of *C. sake*[a]

Compound	Concentration (m*M*)	Residual activity (%)
None	0	100
$PbCl_2$	10	102.6
$HgCl_2$	10	67.9
$FeCl_2$	10	85.6
$CuCl_2$	10	92.1
AgCl	10	93
$MnCl_2$	10	95
$MgCl_2$	10	97.3
NaCl	10	98.1
$ZnCl_2$	10	105.4
$CoCl_2$	10	100.1
EDTA	10	95.9
Dithiothreitol	10	104
PCMB	10	89.9
N-bromosuccinimide	1	0
Iodine	1	30.7

[a]Activity is expressed as a percentage of the activity level in the absence of metal ions or chemical compounds.

A. niger (K_i of 543 m*M [29]*) and *Candida peltata* (K_i of 1400 m*M [30]*) and are interesting if we consider the potential use of β-glucosidases in a cellulose hydrolysis process. Glucose inhibition of β-glucosidases is a problem that must be overcome if the enzymatic hydrolysis of cellulose is performed as an industrial process.

The action of the purified β-glucosidase was tested over a large number of substrates with α and β configurations. The results summarized in Table 3 show that the β-glucosidase is active against (1→4)-β and (1→4)-α linkage configurations (alkyl-glucosides, aryl-glucosides) and against cellobiose, which is a diglucoside with a (1→4)-β configuration. From Lineweaver Burk plots, K_m values of 6.9 and 44.6 mmol/L were found for GlcβNp and cellobiose, respectively. However the β-glucosidase was not active against diglucosides with (1→4)-α configurations like maltose. The cyanoglucosides linamarin and prunassin were also tested. The β-glucosidase was able to hydrolyze prunassin but not linamarin, the main cyanogenic glycoside found in cassava roots. Thus, the β-glucosidase of *C. sake* is not suitable for a cassava detoxication process. The substrate specificity of the *C. sake* enzyme, in conjunction with the kinetic parameters, justifies its classification as a β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21).

β-Glucosidases have been described for their ability to confer resistance to plants against phytopathogens *(1)*. This ability is relative to the

Table 3
Substrate Specificity of Purified β-Glucosidase of *C. sake*[a]

Substrate	Configuration of glycoside linkage	Concentration (mmol/L)	Specific activity (U/mg) at pH 6.0
p-Nitrophenyl-β-D-glucopyranoside[b]	(1→4)-β	5	6.4
o-Nitrophenyl-β-D-glucopyranoside	(1→4)-β	10	2.8
p-Nitrophenyl-α-D-glucopyranoside	(1→4)-α	10	0.3
Salicin	(1→4)-β	20	26.1
Arbutin	(1→4)-β	20	38.2
Esculin	(1→6)-β	5	32.4
Prunassin	(1→4)-β	5	4.35
Cellobiose[c]	(1→4)-β	5	1.7
p-Nitrophenyl-β-D-xylopyranoside[d]	(1→4)-β	10	1.3
p-Nitrophenyl-β-D-galactopyranoside	(1→4)-α	5	4.8

[a]Depending on the type of substrate, activity was determined by measuring the release of either *p*-nitrophenol (400 nm) or glucose (glucose oxidase method), at 30°C, as described in Materials and Methods. No activity was detected against *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-L-rhamnopyranoside, methyl-β-D-glucopyranoside, gentiobiose, lactose, saccharose, maltose, maltotriose, amygdalin, α-trehalose, and linamarin.
[b]Aryl and alkyl-D-glucosides.
[c]Diglucosides.
[d]Others.

substrate specificity of β-glucosidase. For example, in the fire blight disease of pear and apple trees, hydrolysis of arbutin (3-hydroxyphenyl-β-D-glucoside) by β-glucosidase of plant origin results in the release of hydroxyphenol, which is toxic to the invading pathogens. If we consider the properties of the β-glucosidase of *C. sake*, and especially its high activity against the arbutin substrate, the described enzyme might be involved in the biological role demonstrated for the yeast strain exhibiting antagonistic activity against postharvest apple pathogens *(7)*. To elucidate the role of the strain, measurements of the level of β-glucosidase activity on postharvest fruits at different stages must be investigated further to demonstrate that the β-glucosidase activity of *C. sake* has an important role in defense against pathogens.

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