Purification and Some Properties of a β-Glucosidase from \textit{Flavobacterium johnsonae}

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\textit{Flavobacterium johnsonae} was isolated as a microorganism that produced a β-glucosidase with hydrolytic activity of β-glucosyl ester linkages in steviol glycosides. The enzyme was purified to homogeneity from a cell-free extract by streptomycin treatment, ammonium sulfate fractionation, and column chromatographies on S-Sepharose and phenyl-Toyopearl. The molecular mass of the purified enzyme was about 72 kDa by SDS-PAGE. An isoelectric point of pI 8.8 was estimated by isoelectric focusing. The enzyme was most active at pH 7.0, and was stable between pH 3.0 and 9.0. The optimum temperature was 45°C, and the enzyme was stable below 35°C. The enzyme hyrdrolyzed glucosyl ester linkages at site 19 of rebaudioside A, stevioside, and rubusioside, although it could not degrade β-glucosidic linkages at site 13 of rebaudioside B or steviol. The enzyme acted on aryl β-glucosides such as p-nitrophenyl β-glucoside, phenyl β-glucoside, and salicin, and glucobioses such as sophorose and laminaribiose. The enzyme activity on Rub was inactivated completely by I₃⁻, and reduced by Fe²⁺, Cu²⁺, p-chloromercuric benzoate, and phenylmethylsulfonyl fluoride (residual activity: 67.9–84.8%). The pNPG hydrolysis was also inactivated to almost the same degrees. Kinetic behaviors in the mixed substrate reactions of rebaudioside A and steviol monoside, and of steviol monoglucoyl ester and phenyl β-glucoside suggested the glucosidic and glucosyl ester linkages were hydrolyzed at a single active site of the enzyme.

\textbf{Key words:} β-glucosidase; \textit{Flavobacterium johnsonae}; steviol glycosides; β-glucosyl ester linkage

β-Glucosidase (β-β-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of β-glucosidic linkages of various oligosaccharides and glycosides.¹ Many β-glucosidases have so far been purified from microorganisms, plants, and animals, and their substrate specificities have been examined in detail.¹,³,⁵ In our previous report,⁷ we showed an enzyme from \textit{Clavibacter (C.) michiganense} hydrolyzed not only β-glucosidic linkage but also β-glucosyl ester linkage at the site 19 of steviol glycosides, natural sweet diterpenoids, although the ester bond were scarcely decomposed by the usual β-glucosidase preparations. The purified enzyme acted well on p-nitrophenyl β-glucoside (pNPG), phenyl β-glucoside, and salicin, common substrates for β-glucosidases, and according to such substrate specificity, we concluded the enzyme was a kind of β-glucosidases.

In this paper, we report the purification and characterization of a β-glucosidase from \textit{Flavobacterium (F.) johnsonae}. The enzyme also hydrolyzed both β-glucosidic and β-glucosyl ester linkages. However its substrate specificity, especially that toward steviol glycosides, was different from that of the \textit{Clavibacter} enzyme.⁷ This paper also describes the kinetic behavior of the enzyme to show a single active site is responsible for the hydrolysis of the two linkages.

\textbf{Materials and Methods}

\textbf{Chemicals.} pNPG, phenyl β-glucoside, salicin, cellobioside, sophorose, laminaribiose, and gentiobiose were purchased from Sigma-Aldrich Co., St. Louis, US. Rebaudioside A (ReB), steviol (St), and rubusioside (Rub) were gifts from Maruzen Pharmaceuticals Co., Japan. Rebaudioside B (ReB), steviol bioside (StB), steviol monoside

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Abbreviations: ReB, rebaudioside A; ReB, rebaudioside B; St, stevioside; StB, steviol bioside; Rub, rubusioside; StE, steviol monoglucoyl ester; StM, steviol monoside; Glc, glucose; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pNPG, p-nitrophenyl β-glucoside; pNP, p-nitrophenol; HPLC, high-pressure liquid chromatography; IEF, isoelectric focusing; Phe β-Glc, phenyl β-glucoside; CBB, Coomassie Brilliant Blue R-250
(SteM), and steviol monoglucosyl ester (SteE) were prepared as described previously. S-Sepharose was purchased from Amersham Pharmacia Biotech, Upsala, Sweden. Phenyl-Toyopearl 650M was purchased from Tosoh Co., Japan. All other chemicals were purchased from Nacalai Tesque Inc., Japan and Sigma-Aldrich Co.

Screening of microorganisms. Screening of microorganisms was done as described previously except that the activities on Rub were checked in place of RebA. Detailed identification of the isolated bacterial strain was done by the National Collection of Industrial and Marine Bacteria, Scotland, UK.

Enzyme activity. (1) Activity on Rub. Reaction mixtures containing 100 µl of 2 mM Rub in 50 mM phosphate buffer (pH 7.0) and 100 µl of enzyme solution appropriately diluted with the same buffer were incubated at 40°C for 10 min. The reaction was stopped by the addition of 150 µl of 0.1 N NaOH. After neutralization with 0.1 N acetic acid solution (150 µl), glucose (Glc) was measured by the glucose oxidase method (Glucose B-Test Kit, Wako Pure Chemical Industries, Ltd.).

(2) Hydrolytic activity on pNPG. Reaction mixtures containing 250 µl of 10 mM pNPG in buffer A and 250 µl of enzyme solution in the same buffer were incubated at 40°C for 10 min. The reaction was stopped by the addition of 500 µl of 0.2 M Na₂CO₃. p-Nitrophenol (pNP) was measured at 405 nm.

One unit of enzyme activity was expressed as the amount of enzyme that liberated 1 µmol of the products (Glc from Rub, and pNP from pNPG) per min under the above reaction conditions.

 Cultures. Cultivation medium was composed of 2.0% peptone, 0.4% yeast extract, 0.1% KH₂PO₄, 0.1% K₂HPO₄, 0.05% NaCl, and 0.05% MgSO₄·7H₂O (pH 6.5). The first pre-cultivation was done at 28°C for 24 h. This culture (0.4 ml) was inoculated to 100 ml of the above medium in 500 ml shaking flasks, and reciprocal shaking cultivation was done at 28°C for 48 h.

Enzyme purification. All procedures were done at 15°C. Protein was monitored by the absorbance at 280 nm.

Step 1 Extraction. Cell was collected from the culture broth (8.8 l) by centrifugation at 7,000 × g for 60 min, and washed twice with 50 mM phosphate buffer (pH 7.0, buffer A). Cells were suspended in 50 ml of buffer A and passed through a French pressure cell press (Ohtake Works Co., Japan) at 15,000 kg/cm². The disrupted cell was removed by centrifugation at 7,000 × g for 60 min.

Step 2 Streptomycin treatment. Streptomycin sulfate solution of 20% (w/v) was added to the crude enzyme solution up to 1.3%, and kept overnight at 15°C. The precipitates were removed by centrifugation at 7,000 × g for 45 min. The supernatant was dialyzed against buffer A.

Step 3 (NH₄)₂SO₄ fractionation. Solid ammonium sulfate was added to the dialyzed solution to 40% saturation. After removing the precipitates, solid ammonium sulfate was added again to the supernatant to 70% saturation. The precipitates were collected by centrifugation at 7,000 × g for 45 min, dissolved in 75 ml of buffer A, and dialyzed against the same buffer.

Step 4 S-Sepharose cation exchange column chromatography. The dialyzed sample was put on a column (2.5 × 18 cm) of S-Sepharose equilibrated with 25 mM phosphate buffer (pH 6.0). The column was washed with the same buffer. The enzyme protein was eluted with a linear gradient of NaCl from 0 to 1 M at a flow rate of 18 ml/h into 6 ml-fractions. The active fractions were collected.

Step 5 Phenyl-Toyopearl hydrophobic chromatography. Solid ammonium sulfate was added to the enzyme solution up to 1.0 M and it was put on a column (2.5 × 16 cm) of phenyl-Toyopearl 650M equilibrated with buffer A containing 1.0 M ammonium sulfate. The enzyme was eluted with a linear gradient of 1.0 M to 0 M ammonium sulfate in buffer A at a flow rate of 18 ml/h into 6.5-ml fractions. The active fractions were collected, concentrated into 5 ml with Centricon-10 (Nihon Millipore Co.), and dialyzed against buffer A.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli, using a 12% acrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 (CBB).

Isoelectric focusing (IEF) was done on mini IEF cell (Bio-Rad model III), using carrier Pharmalyte (pH 3.5–10, Pharmacia Co.). Electrophoresis was done for 2 h at 100 V. The enzyme protein was stained with CBB.

High-pressure liquid chromatography. High-pressure liquid chromatography (HPLC) was done out under the following conditions: column, YMC-pak ODS-AQ303 (4.6 × 250 mm; YMC Co., Japan); solvent, 35%/v/v acetonitrile containing 10 mM HCl; flow rate, 1.0 ml/min; temperature, 25°C; pump, Tosoh CCPD; and detector, Shimadzu SPD 2A (at 213 nm).
Results

Enzyme purification, molecular mass, and isoelectric point

Table 1 shows typical results of the purification. The enzyme was purified about 110-fold from the crude extract with a recovery of the activity of about 16%. The purified preparation showed a single protein band on SDS-PAGE (Fig. 1A) and the molecular mass of the enzyme was determined to be about 72 kDa. The isoelectric point of about 8.8 was obtained by IEF method (Fig. 1B).

Effects of pH and temperature

Effects of pH and temperature on the enzyme activity were examined using Rub and pNPG as substrates. Irrespective of the substrates, the enzyme was most active at around pH 7.0 in a 10-min reaction at 40°C. After keeping the enzyme at 15°C for 24 h in buffers of various pHs, the residual activity was measured under the standard assay conditions. The enzyme was stable from pH 3.0 to 9.0. The optimum temperature was found to be 45°C, when the reaction was done at pH 7.0 for 10 min. After keeping the enzyme at various temperatures for 10 min in 50 mM phosphate buffer (pH 7.0), no inactivation was observed below 35°C. Further, when the enzyme was incubate with substrates, it stable up to 40°C.

Effects of metal ions and chemicals

The enzyme was incubated with various metal ions and chemicals in buffer A at 5°C for 24 h. The samples were diluted with buffer A, and the residual activities on pNPG and Rub were measured under standard assay condition. 

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Crude extract</td>
<td>19,200</td>
<td>1,310</td>
<td>0.068</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(2) Streptmycin</td>
<td>12,600</td>
<td>1,180</td>
<td>0.094</td>
<td>90.0</td>
<td>1.4</td>
</tr>
<tr>
<td>(3) (NH₄)₂SO₄</td>
<td>1,880</td>
<td>719</td>
<td>0.382</td>
<td>54.9</td>
<td>5.6</td>
</tr>
<tr>
<td>(4) S-Sepharose</td>
<td>147</td>
<td>301</td>
<td>2.05</td>
<td>23.0</td>
<td>30.1</td>
</tr>
<tr>
<td>(5) Phenyl-Toyopearl</td>
<td>28.0</td>
<td>207</td>
<td>7.39</td>
<td>15.8</td>
<td>110</td>
</tr>
</tbody>
</table>

The activity was measured by the liberation of Glc from Rub. The protein concentration was calculated under the assumption that E₁₀₀₀ at 280 nm was 10.

Fig. 1. SDS-PAGE and IEF of the Purified Flavobacterium β-Glucosidase.

Proteins were stained with CBB. A, SDS-PAGE; B, IEF; lane 1, standard proteins whose sizes (in kDa) are indicated; lane 2 and 3, purified Flavobacterium β-glucosidase; lane 4, isoelectric point marker proteins. The following proteins (Amer sham Pharmacia Biotech) were used as molecular mass markers: phosphorylase b (molecular mass, 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The following standard proteins (Amersham Pharmacia Biotech) were used for the estimation of isoelectric point: trypsinogen (pI 9.30), lectin (pI 8.65, 8.45, and 8.15), horse myoglobin (pI 7.35 and 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase B (pI 5.85), β-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55) and amyloglucosidase (pI 3.50).

phenylmethylsulfonyl fluoride (1.0 mM, residual activity; 67.9%) reduced the activity on Rub. The pNPG hydrolysis was also inactivated to almost the same degree.

HPLC analysis of the hydrolysis products of steviol glycosides

The purified enzyme was reacted with RebA, Ste, and Rub, and the products were analyzed by HPLC (Fig. 2). RebA and Ste were degraded to form RebB...
Fig. 2. HPLC Analysis of the Hydrolysates from RebA, Ste, and Rub.

Reaction mixtures contained 5.0 mm substrate (RebA, A; Ste, B; and Rub, C), the enzyme (0.05 unit/ml) and 50 mm phosphate buffer (pH 7.0), and the mixtures (200 μl) were incubated at 40°C for 3 and 20 h. A portion of the mixtures was taken after 3 and 20 h of reaction, and analyzed by HPLC. Steviol was not detected under our analytical conditions due to its strong adsorption to the HPLC column.

| Table 2. Substrate Specificity of the Purified β-Glucosidase from F. johnsonae |
|---------------------------------|------------------|------------------|-----------------|
| Substrate                      | Concentration (mm) | Specific activity (μmol Glc/min/mg protein) | Relative activity (%) |
| pNPG                            | 1                | 167              | 100             |
| Phenyl β-glucoside             | 1                | 39.8             | 23.8            |
| Salicin                         | 1                | 38.5             | 23.1            |
| Rub                             | 0.5              | 29.0             | 17.4            |
| SteM                            | 0.5              | 14.5             | 8.68            |
| SteE                            | 0.5              | 14.4             | 8.62            |
| SteE                            | 0.5              | 3.59             | 2.15            |
| RebA                            | 0.5              | 0.59             | 0.353           |
| RebB                            | 0.5              | 0                | 0               |
| SteB                            | 0.5              | 0                | 0               |
| Sophorose                       | 5                | 3.98             | 2.38            |
| Laminaribiose                   | 5                | 2.47             | 1.48            |
| Gentioibiose                    | 5                | 0.10             | 0.062           |
| Cellobiose                      | 5                | 0.042            | 0.025           |

The activity on pNPG was measured by the liberation of pNP. The other activities were measured by the liberation of Glc.

and SteB, respectively, and no further hydrolysis was occurred even after a prolonged incubation (20 h). These results indicated the enzyme hydrolyzed only the ester linkage at site 19, but not the glucosidic linkages in the side chains of site 13 of RebB and SteB any more (see Fig. 6). From Rub, both SteE and SteM were formed at an early reaction time (1 h). These intermediate products were hydrolyzed further, indicating the enzyme degraded both glucosyl ester and glucosidic linkages of Rub.

**Substrate specificity**

Table 2 shows relative activities on steviol glycosides, aryl β-glucosides, and β-glucobioses. The activity on pNPG was the highest among the substrates tested. The enzyme also hydrolyzed phenyl β-glucoside (Phe β-Glc), and salicin (saligenin β-glucoside) with almost the same rates (23–24% activity on pNPG).

The enzyme hydrolyzed SteE with a glucosyl ester linkage at site 19 and SteM with a glucosidic linkage at site 13 at an almost same rate. Rub, which has one Glc residue at each site, was the best substrate among the steviol glycosides, and the hydrolysis rate was almost double those for SteM and SteE. RebB and SteB were not hydrolyzed at all as expected from HPLC analysis of the products (Figs. 2A and 2B). The degradation rates of RebA and Ste, therefore, corresponded to those of glucosyl ester linkage at site 19, although they were considerably lower than that
of SteE, a substrate having only ester linkage.

β-Glucobioses were hydrolyzed very slowly. β-1,2-Linked sophorose and β-1,3-linked laminarinobiose were better substrates than the other two glucobioses (β-1,6-linked gentiobiose and β-1,4-linked cellobiose).

Kinetic study on the active site

Kinetic behavior in mixed substrate reactions is often determined to elucidate whether hydrolysis of two different substrates occurs at a single active site or at two separate sites. A single active site is predicted mainly from the following kinetic information: linearity of double reciprocal plots, and the agreement of the experimental and theoretical values of $K_m$ and $V_{max}$ on molar fraction ($f$) of one of substrates.

(1) Kinetic behavior in the reaction of RebA and SteM. The enzyme did not hydrolyze glucosidic linkages in the saccharide chain at 13 site of RebA (Fig. 2A, Table 2). Therefore, the hydrolysis rates of RebA corresponded to those of the glucosyl ester linkage. Lineweaver-Burk plots for the reactions of RebA and SteM (Fig. 3) were linear at several molar fractions of RebA ($f=[\text{RebA}]/([\text{RebA}]+[\text{SteM}])$. Figure 4 shows the dependence of the experimental and theoretical values of $K_m$ and $V_{max}$ on $f$. The experimental values were in good agreement with the theoretical ones. These kinetic behaviors indicated that the hydrolysis of the two linkage types occurred at a single active site.
(2) Kinetic behavior in the reaction of SteE and Phe β-Glc. Lineweaver-Burk plots for a mixed reaction of SteE and Phe β-Glc also gave a straight line at several molar fractions of SteE \((f = \frac{[\text{SteE}]}{([\text{SteE}] + [\text{Phe β-Glc}])})\), data not shown. Figure 5 shows the dependence of the experimental and theoretical values of \(K_m\) and \(V_{max}\) on \(f\). The \(K_m\) and \(V_{max}\) changed continuously with \(f\), and we observed no maximum and no minimum. The experimental values were in good agreement with the theoretical ones. These results suggested that only one active site was responsible for the hydrolysis of the two substrates.

**Discussion**

In our previous paper, we reported purification and characterization of a β-glucosidase from *C. michiganensis* that hydrolyzed β-glucosyl ester linkages of the steviol glycosides. The *F. johnsoniae* enzyme was also obtained during the screening for such enzymes. However, the two enzymes were different in some properties. For example, the new enzyme had slightly larger molecular weight (72 kDa) than the *Clavibacter* enzyme (65 kDa). Isoelectric point of the former was 8.8, but that of the latter was 4.6. Optimum pH (pH 7.0) and stability pH range (pH 3–9) of the *Flavobacterium* enzyme were slightly acidic compared to those of the *Clavibacter* enzyme (pH 7.5 and pH 6–10, respectively). A large difference was found in the specificity toward steviol glycosides (Fig. 6). First, the *Clavibacter* enzyme could not hydrolyze the β-glucosidic linkage at site 13 of Rub and SteM. On the contrary, the *Flavobacterium* enzyme degraded Rub to form steviol via SteM or SteE, indicating the enzyme hydrolyzed a glucosidic linkage when only one Glc residue was present at site 13 of steviol glycosides. The second difference existed in the hydrolyzability of the saccharide chains at site 13 of RebB and SteB, where three and two Glc residues were bound with steviol through β-glucosidic linkages, respectively. The *Clavibacter* enzyme hydrolyzed these linkages in RebA and Ste to form SteM. On the other hand, the *Flavobacterium* enzyme did not degrade RebB and SteB. Considering the enzyme preferred smaller glucosides such as Rub, SteM and SteE, the bulky side chains at site 13 may inhibit the binding with the enzyme.

The low activities on β-glucobioses were similar between the two enzymes. This shows that both enzymes had much higher specificity on phenolic β-glucosides than on β-glucanogalactosides. The natural substrates for the enzymes seemed to be phenolic glucosides, for example, salicin and arbutin, or glucosides with glucosyl ester linkages such as steviol glycosides, but not the oligosaccharides derived from naturally abundant glucose polymers such as cellulose and laminaran.
different active sites, each specific for one linkage. Kinetic approach had often been used to settle such questions of multiple activities of several glycosidases; for instance, Rhizopus delemar glucoamylase in the hydrolysis of substrates with α-1,4- and α-1,6-glucosidic linkages, and Thai rosewood β-glucosidase in the hydrolysis of pNPG and p-nitrophenyl β-fucoside. The kinetic behavior of the Clavibacter β-glucosidase was difficult to identify because of the strong substrate inhibition phenomena. However such inhibition was not apparent in the Flavobacterium enzyme. Thus, this enzyme was used for the kinetic analysis. We used two sets of mixed substrates, RebA with SteM and SteE with Phe β-Glc, where RebA and SteE were substrates with ester linkage, and SteM and Phe β-Glc were those with glucosidic linkage. The following kinetic characteristics were obtained for the enzyme; linearity of the plots of 1/v versus 1/s (Fig. 3), and the agreement of the experimental and theoretical values of K_m and V_max on f (Figs. 4 and 5). These predicted that the Flavobacterium enzyme hydrolyzed β-glucosyl ester and β-glucosidic linkages at the same active site. Other biochemical approaches such as chemical modification of the catalytic amino acid residues may also be useful to confirm the single active site responsible for the two linkage types.

References


