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Soo-In YUN[a], Choon-Soo JEONG[a], Dae-Kyun CHUNG[b] & Hye-Seon CHOI[a]
[a] Department of Biological Sciences, University of Ulsan
[b] Institute and Department of Genetic Engineering, Kyung-Hee University
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Purification and Some Properties of a β-Glucosidase from *Trichoderma harzianum* Type C-4

Soo-In YUN, Choon-Soo JEONG, Dae-Kyun CHUNG,[1] and Hye-Seon CHOI[†]

Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea
[1]*Institute and Department of Genetic Engineering, Kyung-Hee University, Suwon, Korea*

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Type C-4 strain of *Trichoderma harzianum* was isolated as a microorganism with high cellulolytic activity. β-Glucosidase is involved in the last step of cellulose saccharification by degrading cellobiose to glucose, and plays an important role in the cellulase enzyme system with a synergic action with endoglucanase and cellobiohydrolase for cellulose degradation. β-Glucosidase from *T. harzianum* type C-4 was purified to homogeneity through Sephacryl S-300, DEAE-Sephadex A-50, and Mono P column chromatographies. It was a single polypeptide with the molecular mass of 75,000 by SDS-PAGE. The enzyme was very active at pH 5.0 and 45°C. No significant inhibition was observed in the presence of metal ions, thiol reagents, or EDTA. The enzyme was stable in the presence of 5% ox gall and digestive enzymes. *p*-Nitrophenyl-β-D-cellobioside worked as a substrate for the enzyme as much as *p*-nitrophenyl-β-glucopyranoside. Glucose and gluconolactone showed competitive inhibition with a K_i of 1 mM and 1.8 μM, respectively, while galactose, mannose, and xylose did not inhibit the enzyme significantly.

Key words: animal feed; cellulolytic activity; β-glucosidase; *Trichoderma harzianum* type C-4

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of the β-glucosidic linkages of aryl and alkyl β-glucosides, β-linked oligosaccharides, and several other oligosaccharides with the release of glucose.[1-6] It plays an important role in the cellulase enzyme system with a synergic action with endoglucanase and cellobiohydrolase for cellulose degradation. β-Glucosidase is involved in the last step of cellulose saccharification by degrading cellobiose to glucose and releasing the former two enzymes from cellobiose inhibition.[7-10] We have found *Trichoderma harzianum* (*T. harzianum*) type C-4 which has high cellulolytic activities and tried to use its particular cellulases for efficient degradation of cellulose for animal feed. Since β-Glucosidase from *T. harzianum* type C-4 was necessary to keep a high cellulolytic activity, we have purified and characterized the enzyme to get practical information on using the enzyme mixture for animal feed.

Materials and Methods

Strains and culture conditions. T. harzianum type C-4 isolated from etiolated leaves in Korea was used as a source of β-glucosidase. The strain was identified by morphological observation and cultural characteristics.[11] Spores at 6×10^7 of the C-4 strain were inoculated in 200 ml of liquid culture medium (Mandel's media[12] containing 0.5% carboxymethylcellulose and 0.5% Avicel) in a 1-l flask and incubated on a rotary shaker (180 rev/min) at 28°C for 7 days.

Assay of β-glucosidase activity. The standard assay of β-glucosidase activity was done at 40°C in 150 μl of 50 mM sodium acetate buffer (pH 5.0) containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside (Np-β-Glu) and an appropriate amount of enzyme. The assay reaction was stopped by addition of 50 μl of 1 M Na_2CO_3 after 15 min of incubation. The amount of *p*-nitrophenol generated from Np-β-Glu was measured by the absorbance at 420 nm. One unit (1 U) of enzyme activity is that which releases 1 μmol of Np equivalent per min. For substrate specificity, *o*-nitrophenyl-β-D-glucopyranoside (*o*-Np-β-Glu), *p*-nitrophenyl-α-D-glucopyranoside (Np-α-Glu), *p*-nitrophenyl-β-D-galactopyranoside (Np-β-Gal), *p*-nitrophenyl-β-D-fucopyranoside (Np-β-Fuc), *p*-nitrophenyl-β-L-arabinopyranoside (Np-β-Ara), *p*-nitrophenyl-β-D-glucuronide (Np-β-Glucuronide), *p*-nitrophenyl-β-D-maltoside (Np-β-Mal), *p*-nitrophenyl-β-D-gentiobiose (Np-β-Gen), *p*-nitrophenyl-β-D-xylopyranoside (Np-β-Xyl), *p*-nitrophenyl-β-D-cellobioside (Np-β-Cellobi), *p*-nitrophenyl-β-D-cellotrioside (Np-β-Cellotri), *p*-nitrophenyl-β-D-cellotetraoside (Np-β-Cellotetra), and *p*-nitrophenyl-N-acetyl β-D-glucosamide (Np-β-NacGlm) were added

[†] To whom correspondence should be addressed. Hye-Seon CHOI, Tel: +82-52-259-2357; FAX: +82-52-259-1694; E-mail: hschoi@uou.ulsan.ac.kr

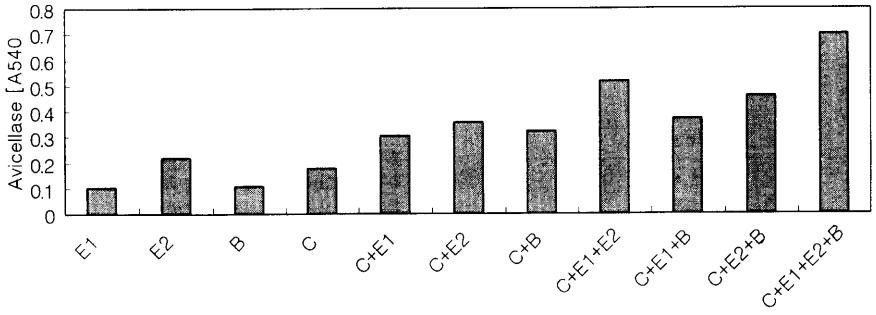

Fig. 1. Avicelase Activity of Different Combinations of Exoglucanase-1, -2, and β-Glucosidase with Endoglucanase-1 and -2.
All enzymes, combination of endoglucanase-1 and -2 (C), exoglucanase-1 (E1), -2 (E2) were purified by the methods of Sul, O. (14) Purification of β-glucosidase (B) was in Materials and Methods. The amount of each enzyme used in this assay was enough to detect the enzyme activity, but not to reach the maximum level. The enzyme activities were measured as an average of 2 sets (each set a triplet).

instead of Np-β-Glu. The effects of ox gall on β-glucosidase were measured after incubation for 60 min with the indicated concentration of ox gall. The effects of proteases on β-glucosidase (ratio, 1:20) were assayed after incubation for the indicated times with trypsin, pepsin, and chymotrypsin. Inhibitory effects of monosaccharides on Np-β-Glu hydrolysis were measured, using 0.047 mM–0.2 mM of Np-β-Glu as a substrate. The K_i was calculated from Dixon plots.

Assay of endoglucanase and exoglucanase activity. The enzyme activities towards carboxymethylcellulose (CMC) and Avicel were measured according to the method of Somogy and Nelson.[13] The reaction mixture was composed of a total of 1 ml of 0.5% CMC or Avicel in 50 mM acetate buffer, pH 5.0, and an appropriate amount of enzyme. After incubation at 40°C for 15 min with CMC or Avicel, the concentration of reducing sugar was measured at 540 nm. The absorbances were used as a relative enzyme activity for comparison of cellulolytic activity.

Purification of β-glucosidase. A 10-l culture filtrate of *T. harzianum* type C-4 was used as a starting material. This culture filtrate was centrifuged for 20 min at 8,000 × *g*, solid ammonium sulfate was added to the supernatant up to 70% saturation, and the precipitate was collected by centrifugation at 10,000 × *g* for 40 min. The sediments were dissolved in 50 mM sodium acetate buffer (pH 5.0) and put on a Sephacryl S-300 column (3 × 100 cm) previously equilibrated with the same buffer. The active eluates were concentrated by ultrafiltration using an Amicon PM 10 membrane and put on an anion exchanger of DEAE-Sephadex A-50 (Pharmacia, Sweden) column (2.5 × 30 cm) equilibrated with 50 mM sodium actate buffer (pH 5.0). The column was eluted with the same buffer and the fractions of β-Glucosidase appeared before a salt gradient. The pooled preparation was concentrated and equilibrated with 25 mM ethanolamine, pH 9.4, by ultrafiltration. The enzyme was put on a Mono P (Pharmacia; H/R, 10/10), chromatofocusing column previously equilibrated with the former buffer. The active fraction was eluted with Polybuffer 96 and concentrated by ultrafiltration.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was done with 12% acrylamide, and protein bands were detected by Coomasie Blue staining.

Results

Synergism with other cellulases

T. harzianum type C-4 was selected from etiolated leaves taken from soil in Korea as a high cellulase producing organism. It contains four endoglucanases, two cellobiohydrolases, and a β-glucosidase. Hydrolytic action of β-glucosidase with other cellulases for cellulose degradation was measured to get information on the physiological role of the enzyme. Four endoglucanases and two cellobiohydrolases were obtained by the method of O. Sul.[14] The maximum level of cellulolytic activity was obtained by combination of two endoglucanases (data not shown), and all three exocellulases including two types of cellohydrolases and β-glucosidase were necessary for the maximum cellulolytic activity (Fig. 1). It indicated that the high cellulolytic activity of *T. harzianum* type C-4 could be partly caused by high β-glucosidase activity, which plays a role of synergism with the other cellulases for cellulolytic activity.

Enzyme purification and properties

β-Glucosidase was purified through gel filtration, anion exchanger, and chromatofocusing column chromatographies as shown in Table 1. It was purified 24.7-fold with a 6% recovery. The molecular weight of the purified enzyme was 76,000 and 75,000 by gel filtration Sephadex G-150 and SDS-PAGE (Fig. 2), respectively, suggesting that the enzyme is a monomer. The pI of the enzyme was estimated to be 8.7 by chromatofocusing column chromatography.

Table 1. Purification of β-Glucosidase from C-4 Strain of *Trichoderma* sp.

Purification	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture filtrate	71.3	2524	35.4	100	1
Dialysis	43.5	1978	45.5	78	1.3
Sephacryl S-300	40.0	1964	49.1	78	1.4
DEAE-Sephadex A-50	7.2	989	137.4	39	3.9
Mono P	0.18	157	872.2	6	24.7

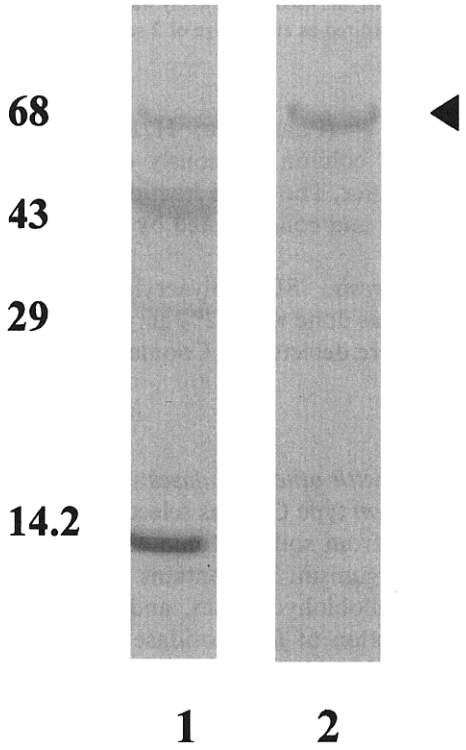

Fig. 2. Estimation of Molecular Weight of β-Glucosidase by SDS-PAGE.

The molecular weight standards consisted of bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), and lysozyme (14,200) in lane 1. The active fraction after mono P column chromatography was put on lane 2. The molecular weight of β-glucosidase was calculated to be 75,000.

The optimum pH of the purified β-glucosidase in sodium citrate at 40°C was 5.0. The enzyme was stable from pH 4.5–6.5 for 1 h at room temperature, showing a drastic decrease of activity in higher pH. The optimum temperature of the enzyme was at 45°C. The enzyme was stable below 55°C and lost 64% of its activity after 15 min incubation at 60°C. The effects of various metal ions and chemicals on β-glucosidase were measured. The enzyme was inhibited by 50% of its activity by 1% SDS. No significant inhibition was observed in the presence of Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, DTT, 2-mercaptoethanol, urea, or EDTA. Among tested metal ions, Mn^{2+} and Zn^{2+} activated the enzyme 1.7-fold and 1.1-fold, respectively. Since extracellular β-glucosidase was intended for components of cattle feed, the enzyme activity was measured in the presence of ox gall and it was stable with up to 5% ox gall (data not shown). No decrease of enzyme activity was observed with digestive enzymes, pepsin, trypsin, or chymotrypsin for 1 h of incubation at 37°C (data not shown).

Table 2. Substrate Specificities of β-Glucosidase

Compound	K_m (mM)	V_m (μmol/min/mg)	V_m/K_m
p-nitrophenyl β-glucopyranoside	0.20	187.0	935.0
o-nitrophenyl β-glucopyranoside	0.80	14.0	17.5
p-nitrophenyl β-cellotetraside	0.65	27.4	42.2
p-nitrophenyl β-cellotrioside	0.51	30.7	60.2
p-nitrophenyl β-cellobioside	0.19	172.0	915.0
p-nitrophenyl β-gentiobiose	0.12	11.0	91.7
p-nitrophenyl α-glucopyranoside	0.40	2.4	6.0
p-nitrophenyl β-galactopyranoside	0.22	1.8	8.2
p-nitrophenyl β-fucopyranoside	0.33	5.9	17.9
p-nitrophenyl β-xylopyranoside	4.0	1.5	0.4

Table 3. Inhibition Pattern and Inhibition Constant of Various Monosaccharides on pNPG Hydrolysis by *Trichoderma* C-4 β-Glucosidase

Compound	Inhibition pattern	Inhibition constant (K_i)**
Gluconolactone	Competitive	1.8 uM*
Glucose	Competitive	1.4 mM*
Galactose	Noncompetitive	160 mM†
Mannose	Noncompetitive	41 mM*
Xylose	Noncompetitive	163 mM†

* The K_i value was calculated from a Dixon plot.
† The K_i value was calculated from a replot of a Lineweaver-Burk plot.
** Each value was mearsured as the average of 2 or 3 different sets.

Substrate specificity

The purified β-glucosidase was tested for its substrate specificity. Table 2 shows the K_m, V_{max}, and substrate specificity of the enzyme towards several aryl-glycosides, disaccharides, and some oligosaccharides. The enzyme hydrolyzed Np-β-Cellobi as much as Np-β-Glu. The substrate specificity was in the order of Np-β-Glu, Np-β-Cellobi, Np-β-Gen, Np-β-Fuc, Np-β-Gal, and Np-β-Xyl. It also hydrolyzed an α-linkage monosaccharide, but with much less efficiency. The substrate specificities to the oligosaccharides decreased with the increase in subunit number. Among the tested compounds, Np-β-Ara, Np-β-NacGlm, Np-β-glucuronide, and Np-β-Mal did not act as substrates. The K_m and V_{max} of the enzyme for Np-β-Glu and Np-β-Cellobi were 0.20 mM and 187 μmol/min/mg and 0.19 mM and 172 μmol/min/mg, respectively. Inhibition was measured with several monosaccharides (Table 3). Glucose and gluconolactone showed the competitive inhibition with a K_i of 1 mM and 1.8 μM, respectively, using Np-

β-Glu as a substrate (data not shown). However, galcactose, mannose, and xylose did not inhibit the enzyme significantly with K_i values of 160 mM, 41 mM, and 163 mM, respectively.

Discussion

The β-glucosidase has been purified from a culture filtrate of *T. harzianum* type C-4, which has a high cellulolytic activity. The enzyme was purified to homogeneity with a 6% recovery and a specific activity of 450 U/mg protein. The low recovery could be caused by dialysis using cellulose membranes after salting-out of the enzyme in a large volume of cell-free culture filtrate. The size of the purified β-glucosidase was 76,000 and 75,000 from size-fracionated gel filtration and SDS-PAGE, respectively suggesting that the enzyme to be a monomer. This property is shared with *Kluyveromyces fragilis*[15] (a single 94,000 chain polypeptide) and *Acremonium persicinum*[4] (a single 128,000 chain), but it was different from the β-glucosidase of *Pichia etchellsii*,[3] *Candida pelliculosa*,[16] *Candida wickerhamii*,[17] *Saccharomyces cerevisiae*,[18] *Saccharomycopsis fibuligera*,[19] and *Trichosporon adenovorans*,[20] which had high molecular weights and oligomeric natures. A glucose-tolerant β-glucosidase from *Aspergillus niger* was a dimer, but had a lower molecular mass.[6] The pI of the purified enzyme is 8.7, which is similar to that of *Flavobacterium johnsonae*,[21] while that of other sources was in the acidic range (*Aspergillus kawachii*, 4.5;[22] *Aspergillus niger*, 3.2;[6] *Aspergillus aculeatus*, 3.5;[23] and *Acremonium persicinum*, 4.3[4]).

The optimum pH (5.0) was in a similar range (*Aspergillus niger*, 5.0;[6] *Acremonium persicinum*, 5.5[4]). This enzyme was not stable in the broad pH range of 2.0–9.0, but the pH (4.5–6.5) and the thermal stability (up to 55°C for 15 min at pH 5.0) was within the range of most fungal β-glucosidases. The enzyme was unaffected by metal ions, chelating agents, and thiol reagents, suggesting that thiol groups and metal ion are not essential for catalytic activity. Taken together, the enzyme was relatively stable to heat, pH change, and metal ions, indicating that it could be processed for use in cattle feed without losing much activity. Stability in the presence of ox gall and digestive enzymes would result in high concentration of active β-glucosidase under these working conditions.

Among the tested substrates, Np-β-Cellobi worked as a substrate as well as Np-β-Glu. Enari *et al.* classified β-glucosidases into three groups by substrate specificity: 1, aryl-β-glucosidases with a high affinity for aryl β-glucoside; 2, cellobiases which only hydrolyze oligosaccharides; and 3, β-glucosidases that worked with both types of substrates.[24] β-glucosidase from C-4 were classified into group 3. according to above classification. High hydrolytic activity for cellobiose, cellotriose, and cellotetraside makes this enzyme suitable for the degradation of cellulose. The enzyme of *Aspergillus niger*[25] also showed faster hydrolysis of cellobiose than Np-β-Glu. Since the enzyme hydrolyzes α-linkages even with less efficiency, it appears not to have a strict requirement for configuration of the terminal, nonreducing β-glucosides for the substrates.

β-Glucosidase from *T. harzianum* type C-4 was inhibited by glucose like other microbial enzymes[26,27] with a K_i of 1 mM. This product inhibition could cause some limitation for industrial usage. Inhibition constants (K_i value of glucose) were in the range of 0.6–14 mM for most microbial enzymes. However, some showed high glucose tolerance with a K_i value of 300–1400 mM.[6,28,29]

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