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Characterization of β-glucosidase from Brown Rot Fungus, *Laetiporus sulphureus*[*1]

Jae-Won Lee[*2], Jun-Yeong Park[*3], Ki-Seob Gwak[*2],
Bon-Wook Koo[*2], and In-Gyu Choi[*2†]

ABSTRACT

β-Glucosidase from *Laetiporus sulphureus* among the enzymes related to lignocellulosic biomass degradation to sugars for using alternative bioethanol production was characterized. The highest activity of β-glucosidase was obtained on cellobiose at shaking culture. For the characterization and purification of β-glucosidase culture solution was concentrated and then purified by FPLC using ion exchange and size exclusion column. According to the results of SDS-PAGE, native PAGE and microfluidic system of purified enzyme, protein band was observed at about 132 kDa. Optimal pH and temperature of purified β-glucosidase were 5.0 and 60°C, respectively. In the kinetic properties of β-glucosidase on various substrates such as sophorose, gentiobiose and cellobiose, K_m was 0.81, 1.07 and 1.70 mM, respectively.

Keywords : β-glucosidase, *Laetiporus sulphureus*, lignocellulosic biomass, bioethanol, purification

1. INTRODUCTION

The enzymatic hydrolysis of a lignocellulosic biomass is carried out by secreting cellulase and hemicellulase from various microorganisms that have highly specific activities on a substrate. Enzymes related to enzymatic hydrolysis are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process, namely: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4), which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) cellobiohydrolase (CBH, 1,4-β-D-glucancellobiohydrolase, or EC 3.2.1.91.), which degraded into a low molecule by removing the cellobiose units from the free chain-ends; and (3) β-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose to produce glucose (Yang and Kim 2001). Besides these three major groups of cellulase enzymes, there are also a number of accessory enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase, and glucomannanase (Eriksson *et al.*, 1990).

Among these enzymes, β-glucosidase has been

*1 Received on June 27, 2007; accepted on August 6, 2007.
*2 Dept. of Forest Sciences, College of Agriculture & Life Sciences, Seoul National University, Seoul 151-921, Korea.
*3 Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea.
† Corresponding author : In-Gyu Choi (cingyu@snu.ac.kr)

characterized as a cellobiase responsible for the hydrolysis of cellobiose, which is a major product of cellulose degradation. It is a glycoside hydrolase that catalyzes the hydrolysis of various β-glucosidic linkages with the release of β-D-glucose from the non-reducing end. Therefore, it has been investigated as a terminal enzyme of the cellulose-hydrolyzing system that cleaves cellobiose into two glucose units (Shewale, 1982; Bao *et al.*, 1994).

Additionally, it catalyzes transglycosylation. This occurs through a double-displacement reaction of retaining glycosyl hydrolases, as follows: an enzyme-substrate complex is first formed, and the glycosidic linkage of the substrate is cleaved by the protonation of the leaving aglycone moiety (Withers, 2001). Many researchers have studied the relationship between substrate inhibition and enzyme activity, but this has not been quantitatively established yet (Li *et al.*, 1991; Zechel *et al.*, 2003).

The molecular weights of β-glucosidases from fungi are very broadly ranged from 41 to 170 kDa, and in the case of *Botryodiplodia theobromae*, the fungus has a molecular weight of 350 kDa (Selby and Maitland 1965). On the other hand, the molecular weight of bacteria ranges from 50 to 122 kDa. Recently, the characterization of β-glucosidase from the white rot fungus *Phanerochaete chrysosporium* was reported. The enzyme has a molecular weight of 116 kDa and a highly specific activity on laminaribiose with β-1,3 glycosidic linkage (Igarashi *et al.*, 2003). Until now, however, the characterization of β-glucosidase from wood rot fungi has been rarely studied.

Brown rot fungi secrete various cellulases and hemicellulases for the carbohydrate hydrolysis of a woody biomass. Therefore, extracellular enzymes from brown rot fungi could be used for enzymatic saccharification. The brown rot fungus *Laetiporus sulphureus* mainly secretes endoglucanase, xylanase, and β-glucosidase during carbohydrate degradation.

In this study, the characterization and detailed kinetic analysis of substrates with various β-glucosidic linkages were performed by using β-glucosidase from *L. sulphureus*.

2. MATERIALS AND METHODS

2.1. Microorganism and Culture Condition

Laetiporus sulphureus was maintained on a potato dextrose agar (PDA) medium at 30°C for 14 days. The Kremer and Wood medium was used for β-glucosidase production (Kremer and Wood, 1992). For the search of the optimal carbon source of β-glucosidase, glucose, cellobiose and avicel were used as carbon sources.

Gentiobiose, sophorose, and p-nitrophenyl-β-D-glucopyranoside (pNPG) that were used for the measurement of enzyme activity were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

2.2. Protein Determination and Enzyme Assays

Protein was determined using the method of Bradford with 1 mg/mℓ of bovine serum albumin (BSA; Sigma Chemical Co., USA) used as the standard (Bradford, 1976). The reaction mixture contained 1 mℓ of Bradford reagent and 0.1 mℓ of sample solution. The protein concentration was determined at 595 nm within 1 h.

β-Glucosidase activity was assayed by monitoring the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (Sigma Chemical Co., USA). The enzyme solution (100 μℓ) was incubated for 5 min at 30°C with 1 mM p-nitrophenyl-β-D-glucopyranoside in a 10 mM sodium acetate buffer (pH 5.0). The enzymatic reaction was stopped by the addition of 100 μℓ of

2 M sodium carbonate. The amount of p-nitrophenol released by β-glucosidase was determined colorimetrically at 420 nm (Igarashi *et al.*, 1997).

2.3. Enzyme Purification

The culture supernatant of *L. sulphureus* grown on cellobiose was spun down (30 min, 1,500 × g), and the culture supernatant was incubated with 5% (w/v) bentonite for 30 min, at room temperature. After the bentonite was removed through centrifugation, the solution was concentrated with a PY-10 ultrafiltration membrane (Amicon, USA).

The concentrated sample was purified by FPLC (Amersham, AKTA Explorer 10) using ion exchange and size exclusion. The sample was put on a Mono Q column (0.5 × 5 cm) as ion exchange chromatography equilibrated with a 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with a linear gradient of 0 to 1 M NaCl in the buffer. Then a Superdex 75 (100 × 300 cm) column was used in the size exclusion chromatography after equilibrating the enzyme with a 20 mM sodium acetate buffer (pH 5.0). The fraction containing enzyme activity was collected and concentrated through ultrafiltration. Then the microfluidic system (Experion Protein A260, BIO-RAD) and SDS-PAGE were used to determine the molecular weight of the purified enzyme.

2.4. Electrophoresis

2.4.1. Native-PAGE

Non-denaturing polyacrylamide gel electrophoresis in a 10% polyacrylamide gel was carried out at pH 8.3 (Kwon *et al.*, 1994). After the electrophoresis, the gel was soaked in a 0.2 M sodium acetate buffer (pH 5.0) for 10 min at room temperature to exchange the buffer system. The gel was then incubated in a 0.2 M sodium acetate buffer containing 0.1% esculin and 0.03% ferric chloride for 5 min at 50°C. During incubation, the black bands corresponding to the β-glucosidase appeared against the background. The reaction was stopped by immersing the gel in a 10% aqueous solution of glucose.

2.4.2. Microfluidic System

To determine the purity of purified β-glucosidase, Experion A 260 (Bio-Rad) was used with the electrophoresis system. Sizing and quantitation calculation of protein was performed automatically. The separated protein on the Experion A 260 chip was displayed using the Experion software.

2.5. Optimal pH and Temperature

To determine the optimal pH, β-glucosidase activity was determined at various pH buffer solutions (pH 2.5~3.5, glycine; pH 4.0~5.5, sodium acetate; pH 6.0~8.0, sodium phosphate). The optimal temperature was estimated in the range from 20 to 70°C. The enzyme activity was determined using the previously described method.

2.6. Substrate Specificity and Kinetic Characterization

The kinetic constants K_m and V_{max} on pNPG, cellobiose, sophorose, and gentiobiose were determined at 50°C for the purified enzyme. The activities of β-glucosidase towards various substrates were determined by monitoring the amount of glucose that was released depending on the substrate concentration, using the BMI Glucose-E Test. The concentration of these substrates was 0~5 mM (pNPG) and 0~20 mM (for cellobiose, sophorose, and gentiobiose) in a 10 mM sodium acetate buffer (pH 5.0).

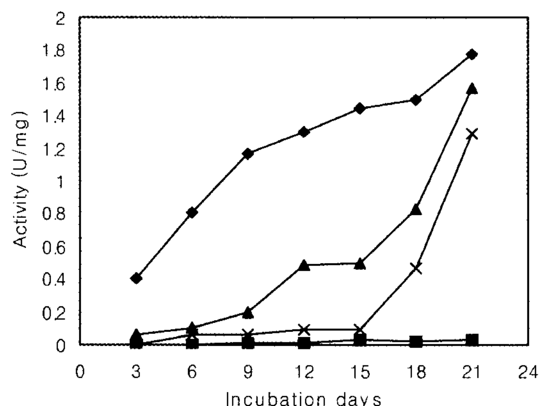

Fig. 1. Change of enzyme activities of *L. sulphureus* using avicel as a carbon source during incubation days (◆: xylanase, ▲: endoglucanase, ×: β-glucosidase, ■: cellobiohydrolase).

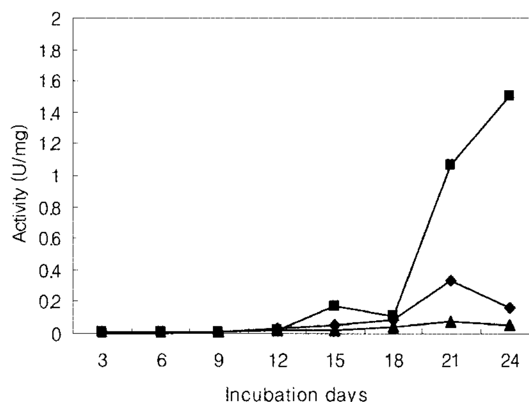

Fig. 2. Change of β-glucosidase activities on various carbon sources (■: cellobiose, ◆: glucose, ▲: avicel).

The data were obtained by the Michaelis-Menten equation. The following equation was used to calculate the kinetic parameters of the substrate:

$$V = V_{max}/(1+K_m/S),$$

where V is the initial velocity, V_{max} the maximum velocity, K_m the Michaelis constant, and S the substrate concentration.

3. RESULTS and DISCUSSION

3.1. Enzyme Assay

The enzyme activities during the cultivation on avicel were shown in Fig. 1. The activities of endoglucanase, β-glucosidase, and xylanase were higher than that of cellobiohydrolase on *L. sulphureus*.

The hydrolytic enzymes from *L. sulphureus* indicated that the fungus showed high cellulase and xylanase activities on the *Eucalyptus grandis* chip. These activities were increased according to the number of incubation days, and the highest activities were shown at 150 days incubation (Machuca and Ferraz 2001). The trend of total cellulase and xylanase in this study coincided with that in the report of Machuca and Ferraz (2001).

To determine the optimal carbon source for β-glucosidase production, various carbon sources (glucose, cellobiose and avicel) were used. The results showed that β-glucosidase activity was the highest in cellobiose (Fig. 2). Cellulase is an inducible enzyme system, and several compounds have been described as its inducers. In this study, cellobiose was a good inducer for the production of β-glucosidase from *L. sulphureus*. The end-product of cellulose hydrolysis, glucose, inhibits enzyme synthesis. It is a regulatory phenomenon that the cell can not consume resource and energy waste in an enzyme that is synthesizing when a rapid carbon source is available (Umile and Kubicek 1986).

3.2. Purification of β-glucosidase

An extracellular β-glucosidase was purified from *L. sulphureus* in cellobiose as a carbon source through two steps of column chromatography. In the ion exchange column, the major

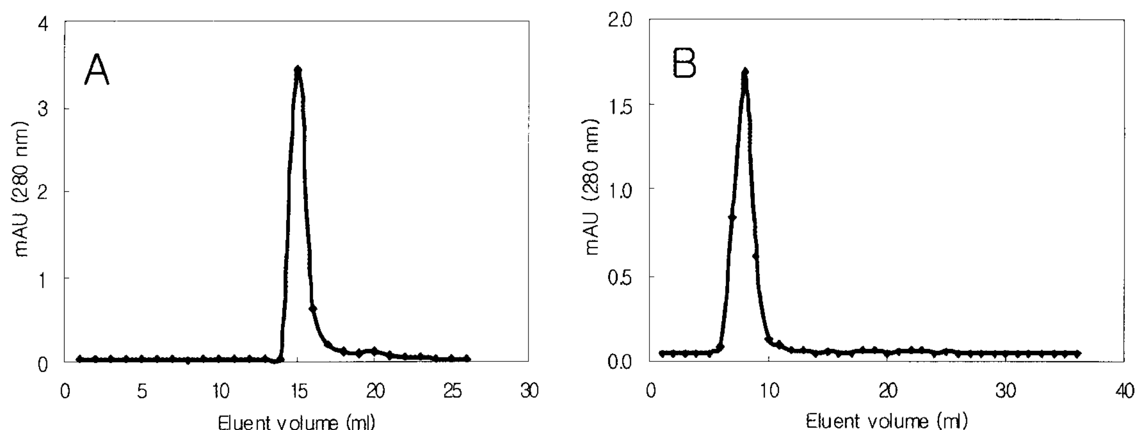

Fig. 3. Purification of β-glucosidase from *L. sulphureus*. Subsequent chromatography steps on Mono Q (A) and Superdex 75 (B) columns. Profiles corresponded to optical density at 280 nm.

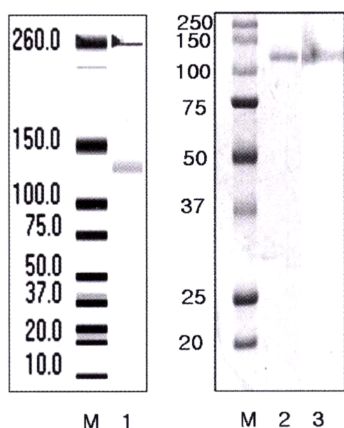

Fig. 4. Determination of molecular weight of purified β-glucosidase from *L. sulphureus* by microfluidic system, SDS-PAGE and native PAGE (M: molecular marker, 1: microfluidic system, 2: purified β-glucosidase, 3: native PAGE of purified β-glucosidase).

peak with β-glucosidase activity was detected between 14 and 16 mℓ of the elution volume (Fig. 3(A)). At the second step, the major peak was detected between 6 and 10 mℓ of the elution volume (Fig. 3(B)). The detection of β-glucosidase activity within a short time after the sample injection on size exclusion column implied that it had a high molecular weight.

As shown in Fig. 4, the purified β-glucosidase gave a single band with a molecular weight of about 130 kDa on SDS-PAGE. Cellulases, cellobiohydrolase, and most β-glucosidases from fungi and bacteria are glycosylated. Especially, β-glucosidase was glycosylated from 0.03 to 50% of its molecular weight. The β-glucosidase of *Taloromyces emersonii* with 135 kDa was glycosylated, except for the 57.6 kDa enzyme (Coughlan, 1988). β-Glucosidase purified from *P. chrysosporium* had molecular weight of 116 kDa and high glycosylation (Kawai *et al.* 2004). Therefore, β-glucosidase from *L. sulphureus* expected to be glycosylated and to maintain its thermal stability, and had resistance against proteolysis for enzymes.

The zymogram technique, using esculin, was used for the detection of β-glucosidase. The active band corresponded to about 130 kDa by native PAGE. Some zymogram techniques have been developed for the detection of β-glucosidase in polyacrylamide gel (Coughlan, 1988; Gabriel and Gersten 1992). This was not a good method, however, for the determination of the exact position of an enzyme because the position of β-glucosidase was located as a yellow band of p-nitrophenyl by incubating gel in a

Table 1. Purification of β-glucosidase from *L. sulphureus* when cellobiose was used as a carbon source

Purification step	Total volume (mℓ)	Total protein (mg)	Volume activity (U/mℓ)	Total activity (U)	Specific activity (U/mg)
Crude filtrate	1000	45.12	0.24	241	5.34
Ultrafiltration	50	10.42	3.15	157.60	15.12
Mono Q (ion exchange)	20	1.77	1.20	40.52	22.86
Superdex 75 (size exclusion)	40	0.56	1.01	24.02	40.71

Table 2. Concentration and purity of purified β-glucosidase by microfluidic system

Peak number	Mig. Time (sec)	Mol. Wt (kDa)	Concentration (ng/μℓ)	% Total
1	31.01	23.08	5.72	1.79
2	31.64	25.40	1.62	0.51
3	31.99	26.83	2.11	0.66
4	33.20	31.95	2.89	0.90
5	33.59	33.59	3.45	1.08
6	34.47	37.31	5.30	1.66
7	38.89	59.51	6.87	2.15
8	43.32	87.27	2.76	0.86
9	45.02	99.79	8.07	2.52
10	45.70	105.35	3.31	1.04
11	49.01	132.46	267.94	83.81
12	54.31	182.21	1.45	0.45
13	54.65	185.68	3.55	1.11
14	55.77	197.08	1.16	0.36
15	59.03	230.27	1.89	0.59
16	60.15	241.67	1.62	0.51

p-nitrophenyl-β-D-glucoside solution. In this study, the location of β-glucosidase with a high resolution was confirmed using esculin and ferric chloride.

The β-glucosidase activity from the supernatant of *L. sulphureus*, according to the purification steps, has been summarized on Table 1. The activity yield in the first step was 65.39%, and the pooled β-glucosidase fractions were concentrated and applied in the second step. Finally, the enzyme was purified 7.62-fold to the specific activity of 40.71 U/mg, with a yield of about 59.28%.

To determine the degree of purity for purified β-glucosidase, the distribution of the molecular weight was estimated using the microfluidic system. The equivalent of 83.81% of the total protein was 132.46 kDa (Table 2). Many bands appeared in the purified protein, but the percentages of these proteins were very low (below 2.52%). The purification of β-glucosidase was successfully performed through the purification step.

3.3. Characterization of β-glucosidase

Depending on the pH and temperature, the results of β-glucosidase activity were shown in

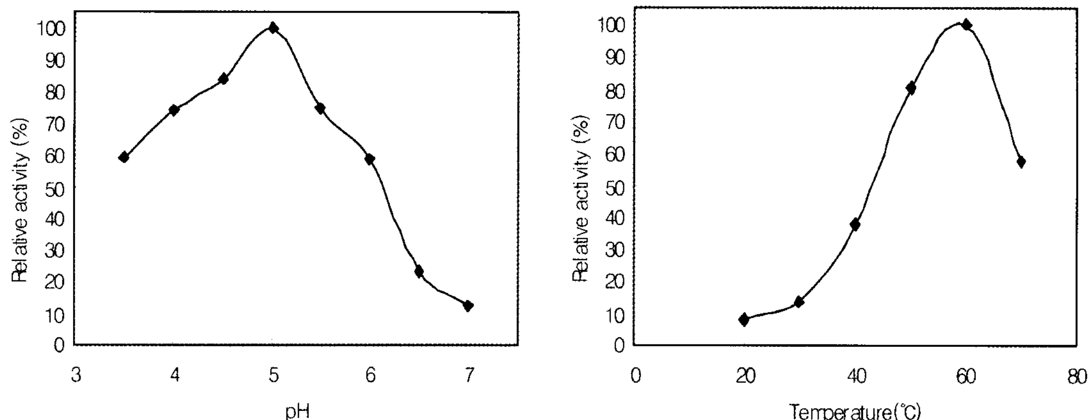

Fig. 5. Activity profile of purified β-glucosidase from *L. sulphureus* depending on optimal pH and temperature.

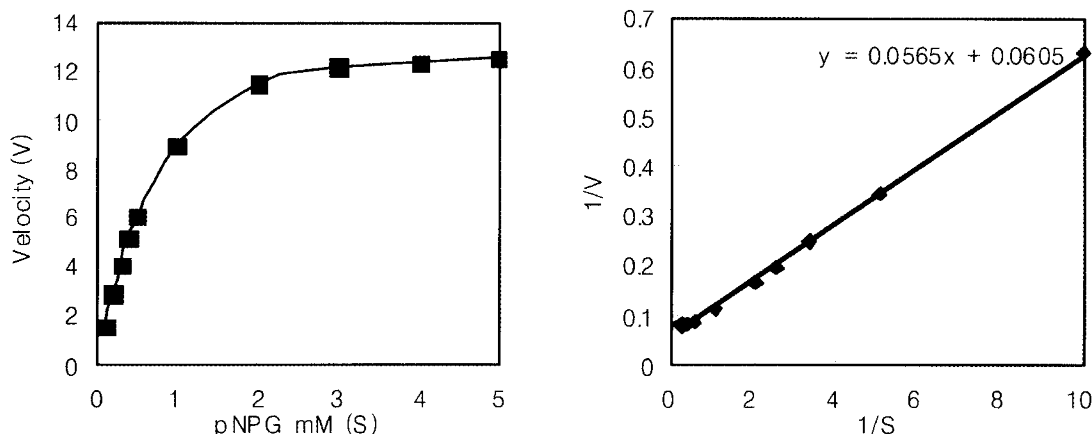

Fig. 6. Hydrolysis effect on different pNPG concentrations by purified β-glucosidase from *L. sulphureus*.

Fig. 5. The optimal pH was 5.0. The β-glucosidase activity increased from 20°C to 60°C, and dramatically decreased at over 60°C. The optimal temperature was 60°C with highest activity. At broad pH ranges, the relative activity was retained at over 50%. The optimal pH range of β-glucosidase from fungi was between 4.5 and 5.5. The optimal temperature also had a broad range, between 40 and 70°C.

Two types of substrates (pNPG and cellobiose) on various concentrations were used to define the optimal conditions for measuring the activity of the enzyme. p-Nitrophenol was measured spectrometrically, and glucose from cellobiose was assayed using the BMI glucose E-kit. The kinetic parameters of pNPG were calculated using the Michaelis-Menten equation. As shown in Fig. 6, the kinetic constants were estimated to be $K_m = 8.27$ and $V_{max} = 16.53$ nmol/min/μg. The effect of the various substrates on the rate of glucose formation was shown in Fig. 7. The substrate specificity of β-glucosidase for three glucosyl-β-glucosidic linkages was examined, and the obtained kinetic parameters have been summarized on Table 3. The results indicated that β-glucosidase could

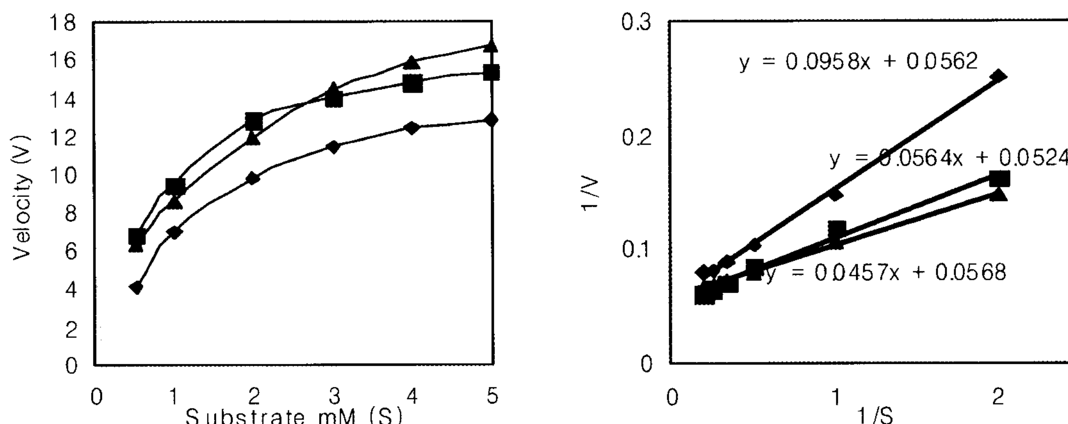

Fig. 7. Hydrolysis effect on different substrate concentrations by purified β-glucosidase from *L. sulphureus* (■: sophorose ▲: gentiobiose ◆: cellobiose).

Table 3. Kinetic parameters of β-glucosidase from *L. sulphureus* for various glucosyl-β-glucosides

Substrate	Linkage	K_m	V_{max}
Sophorose	β-1,2	0.81	17.61
Cellobiose	β-1,4	1.70	17.79
Gentiobiose	β-1,6	1.08	19.08

hydrolyze all three glucosyl-β-glucosidic linkages. Among these substrates, the hydrolysis of sophorose (1,2-β-glucosidic linkage) showed the lowest K_m. Among the three substrates, β-glucosidase had the highest hydrolysis efficiency for sophorose.

The transglycosylation reaction of β-glucosidase has been reported by many researchers (Li and Calza 1991; Zechel *et al.*, 2003). β-Glucosidase from fungi hydrolyze cellobiose to glucose. Then, glucose is transglycosylated disaccharides with various glucosidic linkages such as sophorose (1,2), cellobiose (1,4), laminaribiose (1,3) and gentiobiose (1,6) by enzyme (Li and Calza, 1991; Zechel *et al.*, 2003). Depending on microorganisms, the enzyme has difference of hydrolysis rate on various glucosidic linked substrates. β-Glucosidase from *P. chrysosporium* selectively hydrolyzed laminaribios with a β-1,3 glucosidic linkage among the various substrates (Kawai *et al.*, 2004). They established that substrate transglycosylation occured simultaneously with hydrolysis.

4. CONCLUSION

β-Glucosidase from *L. sulphureus* among the enzymes related to the enzymatic saccharification of a lignocellulosic biomass was characterized in this study. The optimal carbon source for β-glucosidase production was found to be cellobiose. The molecular weight of a purified protein band was observed at about 132 kDa through the microfluidic system, native PAGE and SDS-PAGE. The N-glycosylation of β-glucosidase with a high molecular weight could be predicted through the reported molecular weight of other microorganisms. The optimal pH and temperature of purified β-glucosidase were 5.0 and 60°C, respectively. As for the kinetic properties of β-glucosidase, the K_m value of pNPG on the concentration was 8.27 mM. When sophorose, gentiobiose, and cellobiose with various glucosyl-β-glucosidic linkages were used as substrates, K_m was 0.81, 1.07, and 1.70 mM, respectively. These results suggested

that β-glucosidase from *L. sulphureus* could hydrolyze various glucosyl-β-glucosidic linkages, and has potential for transglycosylation.

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