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ORIGINAL ARTICLE

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Characterization and application of recombinant β-glucosidase (BglH) from *Bacillus licheniformis* KCTC 1918

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Abstract β-Glucosidase (β-1,4-D-glucoside glucohydrolase: EC.3.2.1.21) catalyzes the hydrolysis of β-glucosidic bonds between saccharides and aryl or alkyl groups. A gene encoding β-glucosidase from *Bacillus licheniformis* KCTC 1918, an anaerobic spore-forming soil bacterium, was cloned and characterized. The structural gene for the β-glucosidase consists of 1410 bp encoding 469 amino acid residues, and has a molecular weight of 53.4 kDa as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis with 12% separating gel. The enzyme activity was determined against *p*NPG as a substrate. The enzyme was optimally active at pH 6.0 (citrate–phosphate buffer) and 47°C. β-Glucosidase retained 100% of its original activity for 24 h. The activity of the enzyme was stimulated by glycerol and urea and was decreased by Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, and Mn^{2+}. In particular, Cu^{2+} had the strongest negative effect on β-glucosidase activity. The purified β-glucosidase was active against *p*NPG and cellobiose. When the β-glucosidase was tested for cellulose hydrolysis, the supplement of β-glucosidase with cellulose increased the glucose yield from pine wood powder by 139.8%.

Key words Cellulose · Cellulose degradation · β-Glucosidase

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Introduction

β-Glucosidases are hydrolytic enzymes that hydrolyze β-glucosidic linkages such as alkyl-β-glucosides or aryl-β-glucosides, as well as diglucosides and oligosaccharides. For most enzymatic hydrolysis processes, β-1-4-endoglucanase randomly hydrolyzes amorphous regions and β-1-4-exoglucanase hydrolyzes the ends of crystalline cellulose to produce cellobiose, which is hydrolyzed to glucose by β-glucosidase.[1–5] Therefore, β-glucosidase is considered a component of cellulolytic process, despite lacking direct action on cellulose.[6] Furthermore, β-glucosidase regulates the enzymatic hydrolysis of cellobiose, and is often inhibitory to endoglucanase and exoglucanase activities.[7,8] As a result, β-glucosidase is also necessary for rapid and efficient cellulose degradation.

On the basis of substrate specificity, β-glucosidases can be classified into three groups; (1) aryl-β-glucosidases, which have affinity for aryl-β-glucosidases; (2) the cellobiases, which hydrolyze only oligosaccharides; and (3) those hydrolyzing both aryl-β-glucosides and oligosaccharides.[9] On the basis of amino acid similarities, β-glucosidases can be further divided into three families.[10–12] Family 1 (β-glucosidases and phospho-β-glucosidases) and family 9 (β-glucosidases) were purified from bacteria, plant, and mammals, while family 3 β-glucosidases were purified from yeast, molds, and rumen bacteria.[13] The β-glucosidase gene (BglH) of *Bacillus licheniformis* was sequenced. The deduced amino acid sequence resembles that of the family 1 glycosyl hydrolases. Various strains of *Bacillus* species such as *Bacillus circulans*,[9] *Bacillus polymyxa*,[14] and *Bacillus subtilis*[15,16] are known to synthesize β-glucosidase. However, BglH has high thermostability when compared with other Bgl of *Bacillus* strain, and may be suitable for industrial application. The present report describes the characterization of BglH and testing of its potential application.

Materials and methods

Gene cloning

Bacillus licheniformis (KCTC1918) was obtained from the Korea Research Institute of Bioscience and Biotechnology and was maintained on brain heart infusion (BHI) agar. Total genomic DNA was isolated as described in previous methodology using lysozyme and proteinase K.[17] β-Glucosidase gene encoding mature protein was amplified from total genomic DNA by using primer 1 (CTG CAG ATG ACT GAA CAA ACG AAA AAG) with *Pst I* recognition site, and primer 2 (CTC GAG TCA CAA ACT CTC GCC ATT CG) with *Xho I* recognition site. The conditions for polymerase chain reaction (PCR) consisted of 30 cycles of denaturation at 94°C for 5 min, annealing at 52°C for 1 min, and extension at 72°C for 5 min. The resulting PCR product was separated by electrophoresis on 0.7% (w/v) agarose gel and recovered. The recovered structural gene was ligated in previously *Xcm*-digested pBSK vector.

Enzyme purification

Recombinant *Escherichia coli* cells were cultivated by using a rotary platform at 37°C in LB broth medium (400 ml). Cultivation was continued overnight, after which *E. coli* cells were harvested by centrifugation (3500 *g*, 20 min, 4°C) and suspended in lysis buffer. The cells were sonicated and any debris removed by centrifugation (3500 *g*, 20 min, 4°C). Purification was accomplished by binding the crude extracted cell lysate with Ni-NTA agarose slurry. The resin was packed into a column and subsequently subjected to washing and elution. For the final purification step, the active fractions were concentrated by ultrafiltration (Amicon Ultra-15) with centrifugation (3500 *g*, 40 min, 4°C). The fractions with β-glucosidase activity eluted as a single protein peak and the purity of the enzyme was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay

β-Glucosidase activity was determined by measuring *p*-nitrophenol release from the *p*NPG.[18] The specific activity was obtained with *p*NPG as substrate at 47°C. The enzyme mixture containing 100 mM *p*NPG in citrate–phosphate buffer (pH 6.0) was incubated with enzyme for 90 min in a total volume of 1 ml. The reaction was stopped by the addition of 1 ml of 1 M Na_2CO_3 (pH 12), while the amount of *p*-nitrophenol release was determined by measuring the absorbance at 405 nm. One unit of β-glucosidase was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under the assay conditions described above.

Characterization of β-glucosidase

SDS-PAGE

SDS-PAGE used 12% separating gels and 5% stacking gels. The samples to be analyzed were treated with sample buffer and boiled for 5 min before application to the gel. Prestained protein marker (New England Biolabs, UK) were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins were stained with Coomassie brilliant blue R 250.

Effect of pH and temperature stability

The optimum temperature for the enzyme was obtained by determining activity on *p*NPG between 32°C and 52°C. The optimum pH was determined by measuring activity between pH 4.0 and 8.0 using citrate–phosphate buffer at 47°C. Thermostability was measured by preincubating the enzyme in citrate–phosphate buffer at pH 6.0 for 0–48 h, while residual activity was measured with *p*NPG. The citrate–phosphate buffer was prepared by adjusting pH at room temperature.

Effect of metal and reagent

The effects of different metal ions and reagents at 1 mM on the purified β-glucosidase were tested by preincubating with Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, glycerol, and urea in pH 6.0 citrate–phosphate buffer at 47°C for 90 min. The residual activity was measured using 100 mM *p*NPG, using the same method used for the enzyme assay. The activity assayed in the absence of metal ions or reagents was recorded as 0%.

Substrate specificity

To analyze β-glucosidase substrate specificity, several β-glucosides, saccharides, and arylglycosides were tested with purified enzyme. The *p*-nitrophenol release was determined under standard enzyme assay conditions.[18] The end-product saccharides were measured by high-performance liquid chromatography (HPLC) with refractive index detection (2414, Waters, USA). Aliquots of the enzyme-treated mixtures (50 μl) were loaded onto a Rezex RPM column (4.6 × 300 mm; Phenomenex, USA) and eluted with deionized water at a flow rate of 0.6 ml/min.

Assay of the mode of cellulose hydrolysis

Enzymatic hydrolysis of wood powder (pine wood, 40–60 mesh) was performed as described previously.[19] Cellulase (Sigma, from *Aspergillus niger*) and β-glucosidase were tested together for additive activity on wood powder. The mode of degradation of the cellulase was examined by observing the released reducing sugars from the hydrolysis

of wood powder. To observe enzymatic hydrolysis of wood powder, cellulase was mixed with β-glucosidase. The released sugars from cellulose were analyzed by HPLC (described above).

Results

Gene cloning

The amino acid sequences were identified for β-glucosidase *Bacillus licheniformis* and other β-glucosidases from *Bacillus* strain with the NCBI database. The amino acids of β-glucosidase (*B. licheniformis*) showed 87%, 78%, 67%, and 32% homology with the β-glucosidases from *Bacillus subtilis* str. 168, *Bacillus circulans* subsp. *alkalophilus*, *Bacillus cereus* E33L, and *Bacillus pumilus*, respectively (Fig. 1). The PCR product of β-glucosidase from *B. licheniformis* had 1410 bp as shown in Fig. 2a.

Protein purification of β-glucosidase (BglH)

The genomic sequence of *B. licheniformis* was assembled to design primers for subcloning. Its *bglH* gene was transferred into the pRSETA overexpression vector (Invitrogen, USA). The vector was used to transform *Escherichia coli* BL21. The enzyme was purified by using affinity chromatography. To trap the His-tagged protein, Ni-NTA agarose slurry was added to the crude extract and the resin was packed into a column. The enzyme was then eluted with elution buffer. The purity and molecular weight of the enzyme were evaluated by SDS-PAGE as shown in Fig. 2b. The molecular weight of the single protein band was 53.4 kDa.

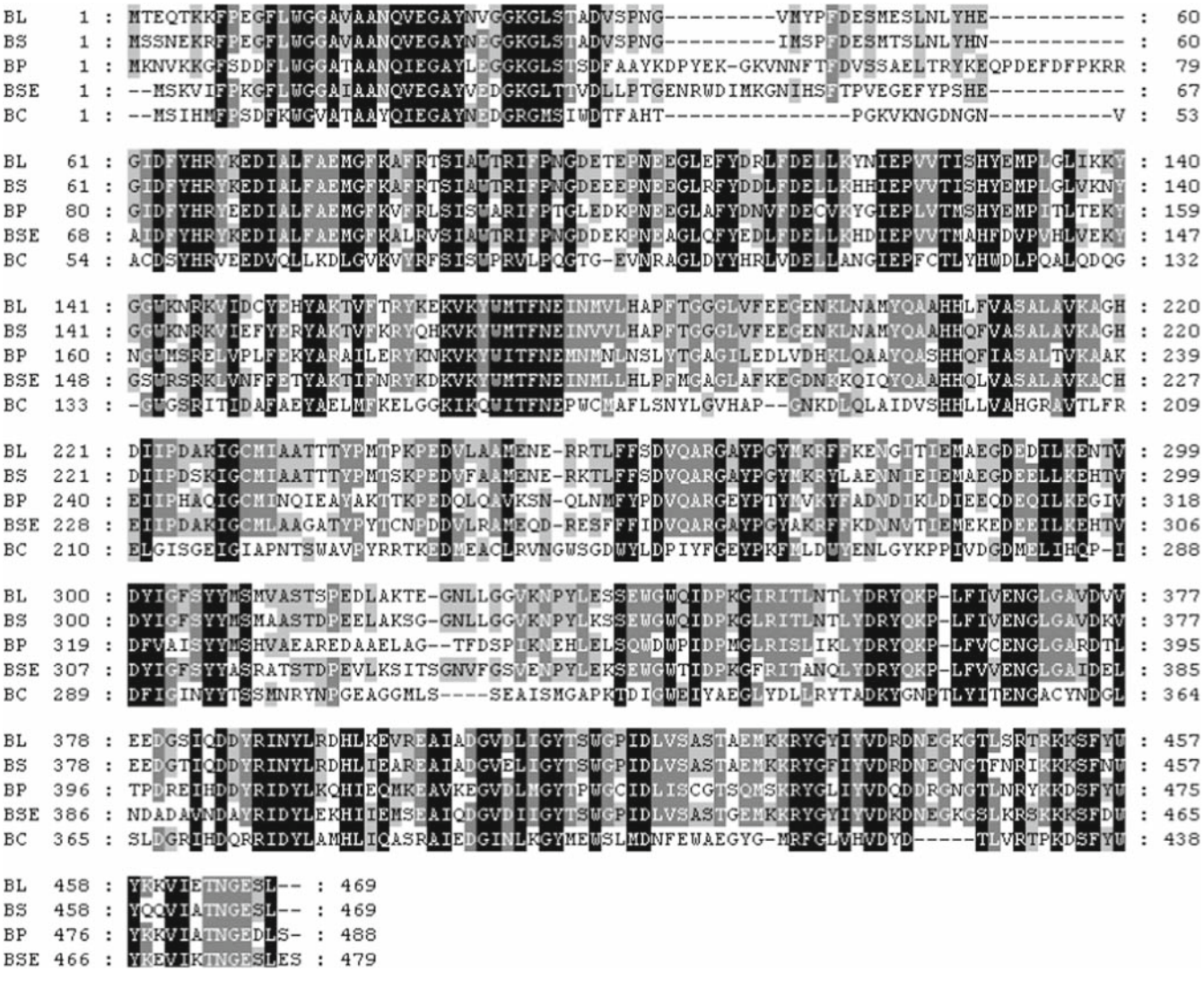

Fig. 1. Homology in amino acid sequences of β-glucosidases from *Bacillus licheniformis*, *Bacillus subtilis* 168, *Bacillus pumilus*, *Bacillus cereus* E33L, and *Bacillus circulan*. BL, *B. licheniformis*; BS, *B. subtilis* 168; BP, *B. pumilus*; BCE, *B. cereus* E33L; BC, *B. circulan*. *Shaded regions* represent regions of conserved residue

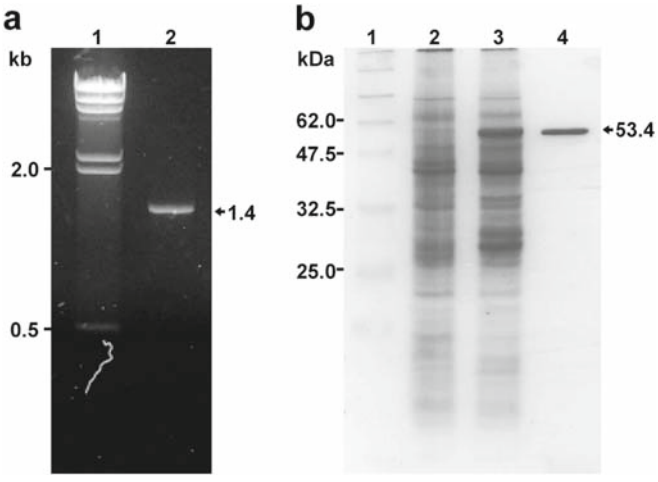

Fig. 2a, b. *β*-Glucosidase DNA and protein size of *B. licheniformis*. **a** DNA electrophoresis of *β*-glucosidase. *Lane 1*, DNA size marker; *lane 2*, cloned DNA (1410 bp). **b** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of a purified *β*-glucosidase fraction. *Lane 1*, protein marker; *lane 2*, negative Control (BL21); *lane 3*, crude extracted *β*-glucosidase; *lane 4*, purified *β*-glucosidase (53.4 kDa)

Effect of pH and temperature on *β*-glucosidase activity

The effects of pH and temperature on *β*-glucosidase were determined over ranges in pH and temperature in citrate–phosphate buffer. Within the tested region of 32°C to 52°C in citrate–phosphate buffer, the optimal activity of the enzyme was observed at 47°C. The optimal pH was pH 6.0 in the same buffer (Fig. 3a). The enzyme activity increased with temperature and peaked at 47°C, and the temperature optimum at pH 6.0 was also 47°C (Fig. 3b). Above 52°C and below than 32°C, the enzyme showed less than 50% of its optimal activity. Under optimal conditions, the enzyme retained 100% of its original activity for 24 h, and retained 53.8% activity after 96 h (Fig. 3c).

Effect of metal ions and chemical reagents on *β*-glucosidase

β-Glucosidase was tested in the presence of different metal ions and compounds, as shown in Fig. 4. Urea and glycerol positively influenced the activity of *β*-glucosidase. However, enzyme activity was decreased by divalent metal ions such as Mg^{2+}, Mn^{2+}, Hg^{2+}, and Ca^{2+}. In particular, the enzyme activity was strongly decreased by Cu^{2+} by as much as 97.6%.

Substrate specificity

The substrate specificity was measured using arylglycosides and saccharides. For arylglycosides, *o*NPG and *p*NPGal were hydrolyzed at 13% and 8.5% of the level of hydrolysis of *p*NPG, respectively. However, the enzyme showed no activity against *β*-xyloside. For the saccharides, cellooligosaccharides were hydrolyzed by BglH, although the enzyme showed no activity against laminarin, lichenan, Avicel, and carboxymethyl cellulose (Table 1).

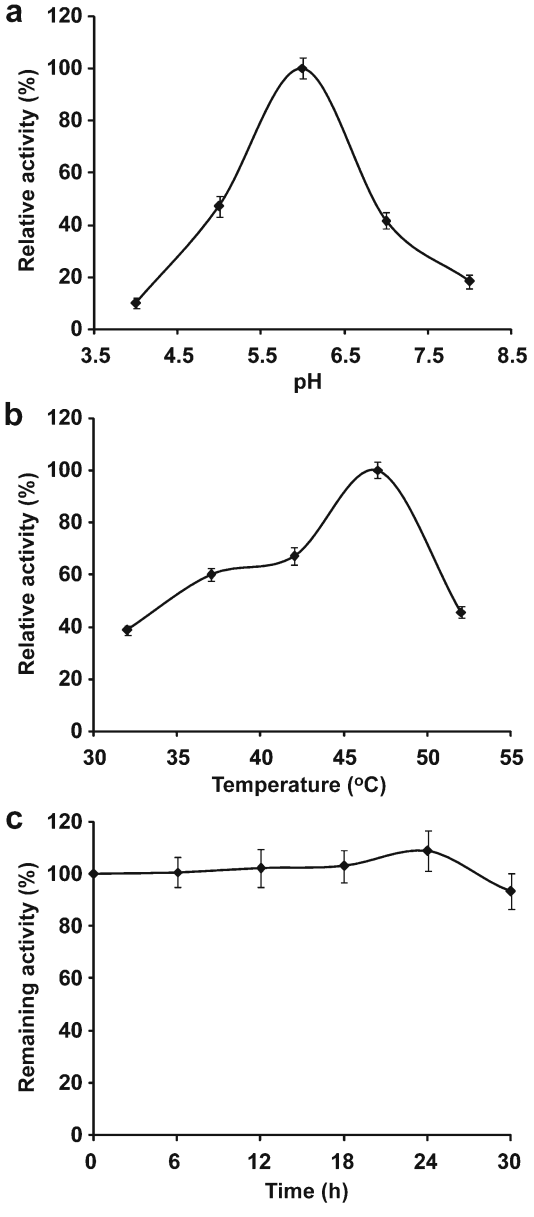

Fig. 3a–c. Effects of **a** pH, **b** temperature, and **c** treatment time on *β*-glucosidase activity. The buffer used was citrate–phosphate buffer. The values shown represent averages from triplicate experiments

Supplementation effect of *β*-glucosidase on cellulose hydrolysis

The activity of the cellulase preparation on pine wood powder was determined, together with *β*-glucosidase activity. Supplementation of cellulase with *β*-glucosidase resulted in significant improvement in wood powder hydrolysis, as determined by HPLC (Fig. 5). Wood powder hydrolyzed with cellulase only gave cellobiose, glucose, and xylose as the main end products. After *β*-glucosidase was added to the enzyme cocktail, the yields of glucose and xylose increased by 139.8% and 126.1%, respectively. This reaction of *β*-glucosidase is important for effective cellulose hydrolysis, because cellobiose is a strong inhibitor of endocellulase activity.

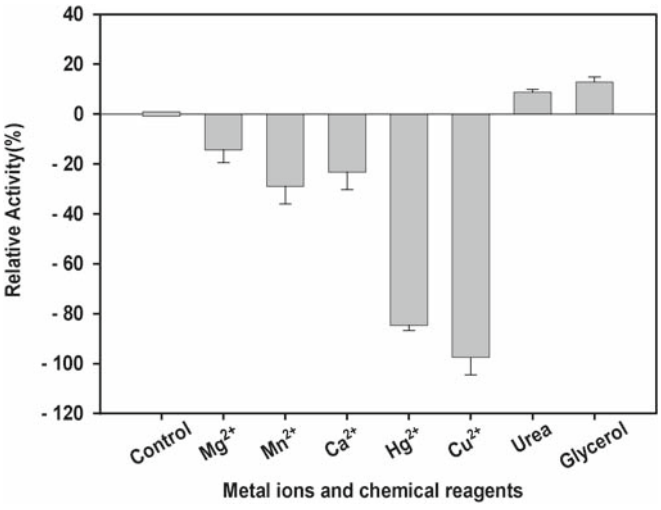

Fig. 4. Effects of metal cations, glycerol, and urea on the activity of purified β-glucosidase from *B. licheniformis* (activities expressed relative to the original activity). The values shown represent averages from triplicate experiments

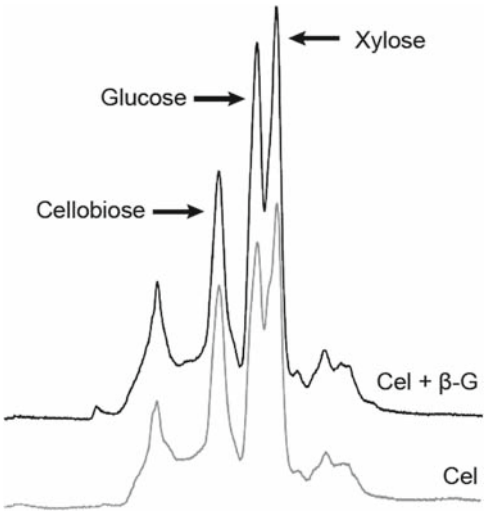

Fig. 5. Hydrolysis of pine wood powder by cellulase with and without β-glucosidase. *Cel*, cellulase; *β-G*, β-glucosidase

Table 1. Substrate specificity of purified β-glucosidase from *Bacillus licheniformis*

Substrate	Relative initial rate of hydrolysis (%)[a]
Arylglycosides[b]	
p-Nitrophenyl-β-D-glucopyranoside	100.0
o-Nitrophenyl-β-D-galactopyranoside	13.0
p-Nitrophenyl-β-D-galactopyranoside	8.5
p-Nitrophenyl-β-D-xylopyranoside	0.0
Saccharides[c]	
Cellobiose	100.0
Cellotriose	140.6
Cellotetraose	150.0
Cellopentaose	137.5
Lichenan	0.0
Laminarin	0.0
Avicel	0.0
Carboxymethyl cellulose	0.0

[a] Activities against arylglycosides and saccharides were determined by measuring the release of *p*-nitrophenol and *o*-nitrophenol, and glucose, respectively. The relative initial rate of hydrolysis of arylglycoside and saccharide are expressed as a percentage of that obtained for *p*-nitrophenyl-β-D-glucopyranoside and cellobiose. Values shown represent averages from triplicate experiments with each substrate
[b] Substrate concentration: 5 mM
[c] Substrate concentration: 0.05%

Discussion

Many cellulolytic enzymes have been isolated and characterized from bacteria or fungus. However, our knowledge of cellulase and hemicellulase from *Bacillus licheniformis* is still in the developmental stage. In other studies, Archana and Satyanarayana[20] reported the production of xylanase, while Abel et al.[21] elucidated the crystal structure of 1,3-1,4-β-glucanase. In this study, β-glucosidase was purified and characterized in detail. Enzyme characterization revealed a molecular weight of 53.4 kDa and an optimal pH of 6.0. As hydrolysis substrates, BglH showed activity against cellooligosaccharides and arylglycosides such as *o*NPG, *p*NPGal, and *p*NPG, but showed no activity against laminarin and lichenan. On the basis of this result, BglH belongs to both the aryl-β-glucosidase and cellobiase, but did not reveal properties of 1,3-β-glucosidase (EC3.2.1.58).[22] In particular, we focused on the optimal temperature and the enzyme half-life. The optimal pH and temperature were pH 6.0 and 47°C with a half-life of 96 h. According to previous reports, half-lives of *Bacillus* β-glucosidases are all shorter. About 80% of the activity of β-glucosidases from *Bacillus circulans* and *Bacillus subtilis* remained after incubating the enzyme at its optimal temperature for 15 min and 2 h, respectively.[9,15] Among the *Bacillus* β-glucosidases, the unique property of BglH of *B. licheniformis* is its long half-life. Enzyme inhibition by metal cations usually suggest the presence of at least one sulfhydryl group, such as that of cysteine, in the active site, where oxidation by the cations destabilizes the conformational folding of the enzyme[23,24] or leads to the formation of disulfide bonds at an irregular position on the protein.[25] The inhibitory effect of Cu^{2+} metal ion on β-glucosidase activity is a common feature.[26,27] Similar effects were also observed in β-glucosidases purified from *Bacillus* strains.[28,29] However, in a study of β-glucosidase from *Neurospora crassa*, the presence of Cu^{2+} promoted enzyme activity by 168%–213%.[30]

This study examined the ability of a mixture cellulase and purified β-glucosidase to hydrolyze cellulose. The ability of cellulase to hydrolyze cellulose was significantly increased with β-glucosidase. β-Glucosidase improved cellulose hydrolysis by reducing end-product inhibition by cellobiose. It appears that the level of endogenous β-glucosidase is an important factor in determining the ability of a cellulase to hydrolyze cellulose materials.[31,32] This thermostable β-glucosidase with a half-life of 96 h is a suitable and unique candidate for bioconversion of cellulosic materials on an

industrial scale. Further analysis of β-glucosidase with other endocellulases might provide a new solution toward better cellulose hydrolysis. Also previously, when the enzyme cocktail treated glycosides, lignans fragments were separated from the substrates as the reason.[33] This is the first report to date to describe the purification and characterization of β-glucosidase from *B. licheniformis*.

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