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Identification and Characterization of 1,4- β -D-Glucan Glucohydrolase for the Saccharification of Cellooligomers from *Paenibacillus* sp. HPL-001

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Abstract

The 1,4-β-D-glucan glucohydrolase gene (Ggh) which was isolated from *Paenibacillus* sp. strain HPL-001 (KCTC11365BP) has been cloned and expressed in *Escherichia coli*, and efficiently purified using affinity column chromatography. Recombinant 1,4-β-D-glucan glucohydrolase (719aa, NCBI accession number KJ573391) was highly active at 40°C in pH 6.0 without significant changes to most salts tested, and exhibited K_m 0.893 mg/mL V_{max} 33.33 U/mg on *p*NPG, respectively. All soluble cellooligomers (e.g., cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose) had been virtually hydrolyzed to glucose by this enzyme. When compared with the commercialized-enzymes, the hydrolytic pattern to all substrates was almost same to the Celluclast 1.5L with about 1/3 strength, however hydrolytic pattern of Glucosidase and Almonds was significantly decreased with substrates increasing number of glucose polymer from cellotriose to cellohexaose. About 90% of the initial enzyme activity was maintained even after 10 consecutive recycle by the 11-carbon bridge and aldehyde-functionalized MCF-immobilization. 3-D structure of this enzyme has the ligand of cellobiose and cellulose binding in the center of niche according to the sequence information.

Keywords: 1,4-β-D-glucan glucohydrolase; Cellooligomer; Immobilization; Saccharification

Introduction

The conversion of lignocellulosic biomass to platform chemicals for fermentation and biorefinery typically involves a disruptive pretreatment process followed by enzyme-catalyzed hydrolysis of the cellulose and hemicelluloses to fermentable sugars [1]. The hydrolyzing process from cellulose to glucose is critical to the bio refinery of plant biomass for a wide variety of biotechnological and industrial application alternatives to fossil fuels [2].

The enzymatic hydrolysis involves the synergistic activity of endo-β-1,4-glucanases (EC3.2.1.4, EG), exoglucanases, including both cellobiohydrolases (EC 3.2.1.91, CBH) and β-glucosidases (EC 3.2.1.21, BGL). All three enzyme classes must be present in this system in order to produce glucose. These enzymes cooperate in the following manner: EG act randomly along the chain cellulose depolymerization, producing new attack sites for the CBH act as exo-enzymes, liberating cellobiose as their main product; and β-glucosidases, which are not regarded as legitimate cellulases, play an important role in the process, because it complete the process, which converts the intermediate cellobiose into two glucose molecules by the hydrolysis of β-glucosidic linkages, is a key rate-limiting enzyme in the cellulolytic process [3].

A high level of β-glucosidase is important to avoid the accumulation of cellobiose, which is a strong inhibitor of CBH to increase the productivity of saccharification and get a better product composition. The amount of BGL secreted from *T. reesei* is quite low compared to that of CBH and EG [4]. Usually, BGLs produced by other fungi must be added to a *T. reesei* enzyme mixture to increase the efficiency of the hydrolysis of cellulosic substrates. The competitive product inhibition of cellobiose can be overcome to some extent by addition of a surplus of β-glucosidase activity. The hydrolyzing enzyme mixture can be supplemented with additional soluble or immobilized β-glucosidase produced by another organism.

Recently, an excellent method for cellulose depolymerization using a solid catalyst in an ionic liquid has been reported [5]. The solid catalyst, it was possible to displace the role of EG, decreasing degree of polymerization (DP), over the course of hydrolysis for cellulosic substrates. Conclusively, identification of an enzyme for hydrolysis cellulose after depolymerization using a solid catalyst in an ionic liquid was essential to complete the alternative approach for cellulose saccharification. An enzyme 1,4-β-D-glucan glucohydrolase was identified from direct evolution [6], which showed the successive hydrolysis functions of cello-oligomers as substrates. This enzyme is capable of producing glucose from soluble cello-oligomers without requiring a CBH; this is different from the traditional model for cellulose digestion process of CBH and BGL, what is known about a fungal cellulase system. At this point, we decided to develop an alternative approach to hydrolyze cellulose to glucose with combining the starting step of cellulose depolymerization with solid catalyst in ionic liquid and the final step of glucose producing from soluble cellooligomers with this enzyme 1,4-β-D-glucan glucohydrolase, completely.

In this study, we suggested a 1,4-β-D-glucan glucohydrolase gene *Ggh* (PGDβ4, GenBank accession code: ZP_07902992.1) isolated from a *Paenibacillus* sp. HPL-001 (Korean Collection for Type Culture: KCTC11987BP) from an organic-rich, soil sample collected from a rearing farm of the wood-eating, oriental horned beetle, *Allomyrina dichotoma* (Linnaeus) in Okcheon County, Korea (ROK). The enzyme was purified and characterized for its activity and properties. Also, we immobilized this enzyme on aldehyde-, amine-, SH-, epoxy-functionalized meso-structured cellular foam silica (MCF) for practical applications.

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Material and Methods

Gene selection from gDNA library

A strain of *Paenibacillus* sp. HPL-001 was isolated from an organic-rich soil, and obtained the whole genomic DNA from the strain. Size-fractionation was conducted in a 0.5% low-melting-point agarose gel after fragmentation by shearing with a nebulizer (Invitrogen). DNA fragments (around 5 kb) were collected and purified for library construction, and fragments were blunt-end repaired and dephosphorylated, and then ligated into pCB31 plasmid vector (MACROGEN Co., Korea). The packaged library was electro-porated into *E. coli* DH10B cells according to the manufacturer's instructions. *E. coli* transformants were selected on LB agar plates supplemented with kanamycin, a total of 1,536 clones were collected into sixteen 96-well plates containing 200 μl LB broth (Difco) in each well. After incubation for 24 h at 37°C, 25 μl glycerol (Sigma-Aldrich) was added to each well and mixed prior to storage at -80°C. Routine 1,4-β-D-glucan glucohydrolase activities were determined in duplicate using 100 mM 4-nitrophenyl-*p*-n-glucopyranoside (*p*NPG) as substrate. Reactions were performed in 96-well plate filled with 90 μl of 100 mM potassium phosphate buffer pH 7.0, 10 μl of *p*NPG solution and 100 μl of crude enzyme from each library clone [7]. Crude enzyme was harvested from destruction of the library cells with sonification after cultivation for 48 hrs at 37°C. Assays were conducted at 40°C water bath for 10 min. The amount of 4-nitrophenol (*p*NP) released was estimated by absorbance at 400 nm using a *p*NP standard curve. One unit (U) of 1,4-β-D-glucan glucohydrolase activity is defined as one μmol of *p*NP released mg protein^{-1} min^{-1}.

Activities against other 4-nitrophenyl derivatives were measured in the same way while activities on other substrates were determined by the release of reducing sugars.

DNA sequencing and expression of 1,4-β-D-glucan glucohydrolase in *E. coli*

The most glucosidase-active clone (PGDβ4, arbitrary named) was selected from the library screening, and the nucleotide sequence of the insert was determined by automated sequencing under BigDyeTM terminator cycling condition. The reacted product was purified using ethanol precipitation and run with Automatic Sequencer 3,730 × l (Applied Biosystems, Weiterstadt, Germany). Open Reading Frames (ORFs) from the sequence data of the insert in clone PGDβ4 were predicted using the ORF Finder (NCBI), taking ATG, GTG, and TTG as possible start condones. And also, homology searches for resulting seven ORFs were carried out by using the BLAST program in the GenBank database. All seven sets of primers fully-covering each ORF (ORF clone ID: PGDβ4 ORF1 ~ PGDβ4 ORF7) were designed, and amplified to construct transformants. The each PCR product was inserted into pGEM-T Easy plasmid vector and transformed to *E. coli* JM109 (Takara Bio Inc.), and the 1,4-β-D-glucan glucohydrolase activity of each transformant was examined with same method above mentioned. The most 1,4-β-D-glucan glucohydrolase-active subclone inserted with ORF1 was selected and the insert, which was modified with detaching Ribosomal Binding Site (RBS) from ORF1 sequence, was designated as *Ggh* gene. The gene, *Ggh* was inserted into the pIVEX GST fusion vector for the transformation into *E. coli* BL21 (Roche Applied Science) to produce the recombinant fusion protein.

Purification of recombinant 1,4-β-D-glucan glucohydrolase

The transformed *E. coli* with GST-fused 1,4-β-D-glucan glucohydrolase were grown overnight in 10 ml LB medium containing ampicillin (100 μg/ml) at 37°C and 200 rpm in a shaking incubator. After re-inoculation with 100 ml of new LB medium and grown to 0.6~0.7 at A595, the culture was induced with 1 mM IPTG and incubated at 18°C and 200 rpm for 18 hr longer. The induced bacteria were collected by centrifugation, suspended in 10 ml of ice-cold 1X phosphate-buffered saline (Sigma-Aldrich), repeated triple times. The cells from the final washing process was resuspended in the lysis buffer (pH 7.0, 200 mM Tris-HCl, 10 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA), and treated with sonic disruptor (CosmoBio Co., LTD). After cell disruption, the lysate was centrifuged at 10,000 × g for 20 min at 4°C, the supernatants was eluted through the GST binding resin column (Novagen, Madison WI, USA). The column was previously equilibrated with the washing buffer (pH 7.0, 50 mM Tris-HCl, 15 mM NaCl), then the lysate was applied to the equilibrated column with the flow rate at 10-15 cm/h. After washing the GST resin with 20 bed volumes of cold washing buffer, the fusion protein was eluted and fractionated with 10 bed volumes of freshly made elution buffer (pH 7.0, 20 mM Glutathione, 50 mM Tris-HCl, 15 mM NaCl). Active fractions were pooled and treated with Restriction Protease Factor Xa (Roche Applied Science), and eluted through *p*-aminobenzamidine-agarose column (Sigma-Aldrich) according to the manufacturer's instruction. The elute was precipitated with 70% ammonium sulfate, solubilized in phosphate-buffered saline, and dialyzed to concentrate at 4°C with a dialysis membrane (Spectra/Por CE, MWCO 10,000), then protein content was determined prior to store at -20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide was performed to separate each protein, and the protein fractions mixed with denaturing agent were boiled for 5 min and applied to the gel. Proteins were visualized by Coomassie brilliant blue R 250 staining. The protein concentration was determined with Bradford reagent (Sigma-Aldrich) assay using bovine serum albumin as a standard.

Properties of recombinant 1,4-β-D-glucan glucohydrolase

The enzyme activity, recombinant 1,4-β-D-glucan glucohydrolase, was measured according to the method mentioned above. The amount of 4-nitrophenol (*p*NP) released was estimated by absorbance at 400 nm using a *p*NP standard curve. The optimal temperature and pH condition for the 1,4-β-D-glucan glucohydrolase activity of recombinant protein were examined in 96-well micro plates at various temperatures (ranging from 20 to 70°C) and pH conditions (ranging from pH 2 to 12). The kinetic parameters (Hanes-Woolf constant, Km and maximal reaction velocity, Vmax) were estimated by linear regression from double-reciprocal plots. Effect of metallic ions and other chemicals on the 1,4-β-D-glucan glucohydrolase activity was studied as described above at pH 7 with addition of 1 mM NaCl, LiCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $ZnSO_4$, $FeCl_3$, $CsCl_2$, ethylenediamine tetraacetic acid (EDTA), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), phenylmethane sulphonyl fluoride (PMSF), acetate, and furfural, respectively.

Immobilization of recombinant 1,4-β-D-glucan glucohydrolase

Meso-structured cellular foam silica (MCF) was prepared by the hydrothermal method that has been reported elsewhere. 1.62 g (0.279 mmol) of Pluronic P123 (triblock copolymer, Chemical formula: $HO(CH_2CH_2O)_2O(CH_2CH(CH_3)O)_7O(CH_2CH_2O)_2OH$, Molecular weight: 5,800) was transferred into 100 ml volume of polypropylene bottle and dissolved in a mixture of 33.33 g (1,852 mmol) of de-ionized water containing 0.8 g (13.32 mmol) of acetic acid. After clearly dissolved, the solution was heated up to 60°C and kept it for 1 hr in oil bath. Another solution was prepared with 2.67 g (11 mmol) of sodium

silicate as a silica source in 33.33 g of water. The molar composition of the final solution was Na_2SiO_3: P123: H_2O: acetic acid = 1: 0.025: 336.4: 1.21. The latter solution was dropped into the solution, then aged at 60°C for 1h and reacted at 100°C for 12 h. After that, the bottle was cooled to room temperature.

White precipitated product was filtered with water, ethanol. The final product was obtained by heat treatment at 550°C for 6 hrs. For the amine-group grafting on MCF, as-prepared MCF sample (SBET = 780m^2/g) was degassed for 6 h at 150°C under vacuum of about 10^{-3} torr. 1.0 g of the MCF sample was suspended in 80 ml of toluene. 1.11 g (5 mmol) of (3-aminopropyl) triethoxylsilane was slowly dropped to the solution. After mixing for 5 min, the mixture was heated to 110°C and maintained at that temperature for 24 h under reflux condition. Final product of MCF-C_3-NH_2 was recovered by filtration with water and ethanol. In case of aldehyde grafting on MCF, 0.5 g amine functionalized MCF was added to 50 ml water and then 0.4 g (2 mmol) of glutaraldehyde (50 wt% in water) was injected to the mixture. After stirring at room temperature for 5 min, the mixture was heated to 60°C and maintained at that temperature for 24 h. The final product of MCF-C3-RC(=O)H was filtered with water and ethanol. Final products were aldehyde(3)-MCF, aldehyde(3)-SiO2, and aldehyde(11)-MCF. A proper degree of silylation was confirmed by FTIR spectra with Thermo Nicolet (US) 6700 FTIR spectrometer (data were not shown). Each sample was degassed for 6 h at 150°C under vacuum of about 10^{-3} torr in the degas port of the adsorption apparatus before being analyzed. Surface areas were calculated by the Brunauer-Emmett-Teller (BET) method. The pore size was calculated using Barrett-Joyner-Hatenda (BJH) model (data were not shown). Enzyme immobilization was conducted at pH 7, MCF or alkyl MCF (10 mg) was added to 300 µl buffer solution (50 mM of potassium phosphate, pH 7.0) containing 1,4-β-D-glucan glucohydrolase (0.5 mg/ml) in a 15-ml micro tube. The micro tube was shaken at 60 strokes per minute using a shaking incubator model SI-300 overnight at 4°C. The suspension was centrifuged at 4,000 rpm for 2 min and then the supernatant was taken from each micro tube. The solid (the MCF or alkyl-MCF with immobilized enzyme) was washed twice with 4 ml of buffer, centrifuged and the liquid removed. Repeated batch reaction was conducted with this enzyme immobilized on MCF using the same reaction mixture as for batch mode reaction of 1,4-β-D-glucan glucohydrolase activity assay after wash enzyme and filtering with membrane filter (ø 100 µm).

Analysis of hydrolytic products

Hydrolysis products were measured as substrates of cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose with 50 µg of purified 1,4-β-D-glucan glucohydrolase in 2 ml vial under constant temperature of 40°C, with a stirring rate of 200 rpm in a shaking incubator for time-based reaction. Each portion of 10 µl, cello-oligomer standard, and enzyme blank was analyzed after clean up with boiling 5 min and filtering with 0.2 µm syringe filter. Hydrolytic activity of 1,4-β-D-glucan glucohydrolase was also compared with commercialized-enzymes, Glucosidase (Lucigen), Almonds (Sigma), and Celluclast 1.5L (Novozyme).

Results and Discussion

Identification and expression of 1,4-β-D-glucan glucohydrolase in *E. coli*

Based on the screening of the gene library clones for their ability to exhibit the best 1,4-β-D-glucan glucohydrolase activity toward substrate 4-nitrophenyl-*p*-n-glucopyranoside (*p*NPG), the most glucosidase-active clone was selected and analyzed Open Reading Frames (ORF) from the sequence data. Seven putative 1,4-β-D-glucan glucohydrolase-encoding genes (ORF 1-7) were detected by sequence similarity search with using the ORF Finder at the National Center for Biotechnology Information (NCBI) from the BLAST software (Table 1). All seven sets of genes were cloned into pIVEX GST fusion vector and transformed to *E. coli* BL21 (Roche Applied Science). Among seven genes a bacterial recombinant carrying ORF1 was selected as the most active plastid by measuring the amount of 4-nitrophenol (*p*NP) released in the glucohydrolase activity assay by absorbance at 400 nm using a *p*NP standard curve and designated as 1,4-β-D-glucan glucohydrolase. The gene sequence was submitted to GenBank, and assigned as Accession Number JF573391.

Purification and characterization of recombinant 1,4-β-D-glucan glucohydrolase

The final preparation of 1,4-β-D-glucan glucohydrolase gave a major single band on SDS-PAGE (Figure 1), with a molecular weight (MW) of 79 kDa, which appeared between the 66 and 97.4 kDa MW marker (Figure 1, lane 2). Also, the GST-fused *Ggh* protein of MW 105 kDa was observed between the 97.4 and 116 kDa MW markers (Figure 1, lane 1).

The optimal pH, showing maximal activity, was measured at a pH of 6.0, which was considered 100% activity, and retaining about 60-70% of its activity at a pH range of 5-7 under citrate buffer solution and potassium phosphate buffer solution, respectively (Figure 2A). The optimal temperature for this enzyme activity appeared to be 40°C (Figure 2B). This enzyme was stable for 40 min at 40°C; however, the activity was sharply decreased at 50°C after incubation for 10 minutes (Figure 3A). Most salts, such as NaCl, LiCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, and $CsCl_2$, did not significantly change the enzyme activity at 1 mM, however, $ZnSO_4$ inhibited 60% of the control (Figure 3B). Kinetic analysis of this enzyme with 4-nitrophenyl-β-D-glucopyranoside (*p*NPG) was performed at 40°C in pH 6.0. The *Km* value of 0.893 mg *p*NPG/ml and *V*max of 33.333 was estimated by means of Hanes-Woolf equation (data were not shown) Hans-Woolf analysis of this enzyme kinetics with 4-nitrophenyl-p-n-glucopyranoside (*p*NPG) as substrate.

The predicted active sites of this enzyme through the 3D-strcuture which is obtained from the hierarchical protein structure modeling approach [8], based on secondary-structure enhanced Profile-Profile threading Alignment (PPA) and the iterative implementation of the Threading ASSEmbly Refinement (TASSER) program shows binding site residues for cellulose and glucose of D88, F132, R146, K186, H187, R198, M230, F233, D265, W266, S405, M468 residues (Figure 4).

Enzyme immobilization

Many enzymes secreted by microorganisms are available on a large scale and there is no effect on their cost if they are used only once in a process. In addition, many more enzymes are such that they affect the cost and could not be economical if not reused. Therefore, reuse of enzymes led to the development of immobilization techniques. It involves the conversion of water soluble enzyme protein into a solid form of catalyst by several methods.

This enzyme was immobilized to aldehyde(3)-MCF, aldehyde(3)-SiO2, and aldehyde(11)-functionalized mesostructured cellular foam with mesopores. Activity of this enzymes immobilized on the aldehyde(3)-MCF and aldehyde(3)-SiO_2 were maintained until recycle number of 3 (R3), however, decreased after R4 with same pattern regardless of immobilizing temperature at 4°C. After recycle number of 10, this enzyme activity was decreased to 30-40% of the beginning

ORF clone ID	Length		Blast P result	Identities (%)	Ref
PGD4 β4-ORF1	bp	aa	glycoside hydrolase family 3 domain protein [*Paenibacillus* vortex V453] (721aa)	68	ZP_07902992.1
	2160	719			
PGD4 β4-ORF2	2616	871	hypothetical protein GYMC10_5946 [*Geobacillus* sp. Y412MC10] (1125aa)	64	YP_003245958.1
PGD4 β4-ORF3	1476	491	putative beta-glucosidase [*Mycobacterium tuberculosis* K85] (296aa)	49	ZP_05770822.1
PGD4 β4-ORF4	501	166	RagB, SusD and hypothetical protein [*Bacteroides ovatus* SD CMC 3f] (578aa)	40	ZP_06616036.1
PGD4 β4-ORF5	1233	410	hypothetical protein PBAL39_16576 [*Pedobacter* sp. BAL39] (607aa)	42	ZP_01885563.1
PGD4 β4-ORF6	762	253	set domain containing protein [*Grosmannia clavigera* kw1407] (1619aa)	34	EFW99026.1
PGD4 β4-ORF7	678	225	ABC-type sugar transport system, periplasmic component [*Roseburia intestinalis* XB6B4] (580aa)	47	CBL11917.1

Table 1: BlastP analysis results and schematic representation of each ORFs for β-glucosidase production clone in GenBank

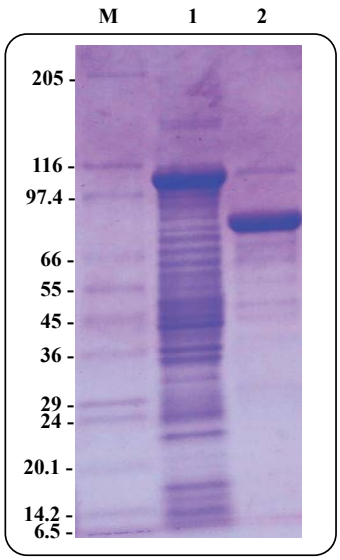

Figure 1: SDS PAGE analysis of the purified 1,4-β-D-glucan glucohydrolase (MW 79 kDa, lane 2) from cell lysate (lane 1) over-expressed with GST-fused 1,4-β-D-glucan glucohydrolase in E. coli BL21 (105 kDa, fused with GST 26 kDa and 1,4-β-D-glucan glucohydrolase 79 kDa), and molecular weight markers (lane M).

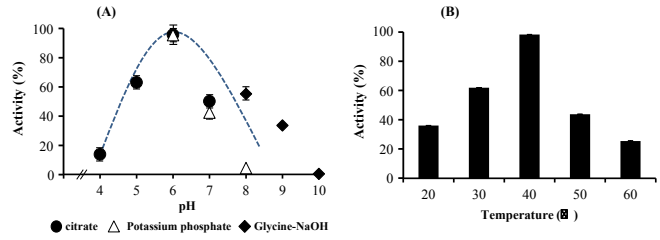

Figure 2: Optimal pH (A) and temperature (B) of 1,4-β-D-glucan glucohydrolase.

activity. Among them, about 90% of the initial enzyme activity was maintained even after 10 consecutive recycles by the aldehyde(11)-functionalized MCF-immobilized enzyme (Figure 5). The advantages of using immobilized enzymes are : (i) reuse (ii) continuous use (iii) less labor intensive (iv) saving in capital cost (v) minimum reaction time (vi) less chance of contamination in products, (vii) more stability (viii) improved process control and (ix) high enzyme : substrate ratio.

Analysis of hydrolytic products

The degradation profile of cellobiose by this enzyme, monitored through the HPLC analysis, revealed that the product was glucose only with the pattern of glucose increasing and cellobiose decreasing during the reaction time (Figure 6).

However, this enzyme might hydrolysis substrates longer than cellobiose, such as cellotriose, cellotetraose, cellopentaose, and cellohexaose, by this enzyme was monitored through the HPLC analysis, which revealed that the major hydrolysis product was glucose with smaller amounts of cellobiose. The amount of glucose was continuously increased during the entire reaction time and from the all substrates. At the beginning of reaction, substrate was hydrolyzed to glucose by the only this enzyme without cellobiohydrolase or exocellulase. This enzyme released detectable levels of glucose within 10 min, regardless of the oligosaccharide substrates. This enzyme was able to release glucose units progressively from each substrate. When incubated with cellotriose, both glucose and cellobiose were released within 10 min. As the incubation progressed, the cellobiose was hydrolyzed subsequently to glucose. The cellotriose was subsequently hydrolyzed to cellobiose, and the cellobiose to glucose, so that, after 360 min, virtually 82.0, 82.0, and 84.1% of the cellotetraose, cellopentaose, and cellohexaose had been reduced to glucose as the same manner, respectively. HPLC result shows that increasing in glucose and decreasing in all of these substrates via intermediates (Table 2).

Traditionally, three enzyme classes must be present in the cellulose hydrolysis system in order to produce glucose. According to the standard endo-, exo-synergy model, EG act randomly along the chain, producing new attack sites for the CBH act as exo-enzymes, liberating cellobiose as their main product; and β-glucosidases converts the intermediate cellobiose into two glucose molecules by the hydrolysis of β-glucosidic linkages [1-3]. The purified enzyme in this study showed not only successive hydrolysis functions of cellobiose but also soluble small cellooligomers as substrates. As the enzyme described here had a higher affinity for cellodextrin substrates longer than cellobiose than for cellobiose, it is suggested that it is a 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) [9-11].

When compared with the commercialized-enzymes and this enzyme, after 20 min reaction time, the hydrolytic pattern to all substrates was almost same to the Celluclast 1.5L (Novozyme) with about 1/3 strength, however hydrolytic pattern of Glucosidase (Lucigen) and Almonds (Sigma) was significantly decreased with substrates increasing number of glucose polymer from cellotriose to cellohexaose (Figure 7). The average activity of this enzyme for total substrate shows 1/2 of Glucosidase (Lucigen), 2 times of Almonds

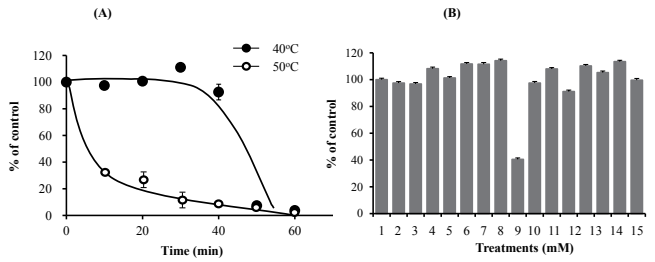

Figure 3: Thermo stability (40 and 50oC) of 1,4-β-D-glucan glucohydrolase (A) and effect of metallic ions
and other chemicals on the 1,4-β-D-glucan glucohydrolase (B).
Treatment 1, NaCl; 2, LiCl; 3, KCl; 4, NH4Cl; 5, CaCl2; 6, MgCl2; 7, MnCl2; 8, CuSO4; 9, ZnSO4;
10, FeCl3; 11, ethylenediaminetetra acetic acid ; 12, 2-mercaptoethanol; 13, dithiolthreitol; 14,
phenylmethanesulphonylfluoride; 15, Sodium dodecyl sulfate

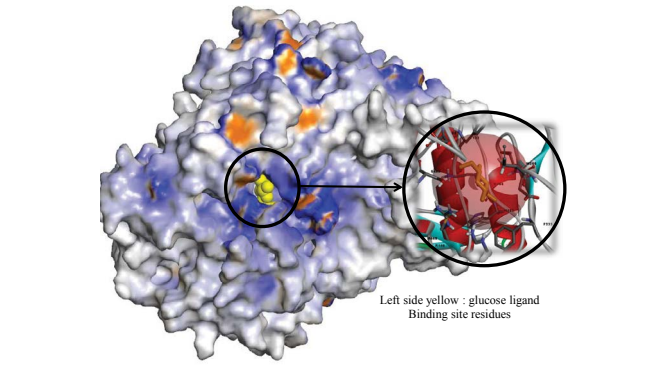

Figure 4: 1,4-β-D-glucan glucohydrolase homology model structure and active sites. Left side yellow : glucose ligand, Binding site residues : D88, F132, R146, K186, H187, R198, M230, F233, D265, W266, S405, M468. Nucleotide and deduced amino acid sequences were analyzed with CLC Free Workbench, Ver. 3.2.1 (CLC bio A/S, www.clcbio.com). Related sequences were obtained from database searches (SwissPort and GenBank). The biomolecular 3D structure of 1,4-β-D-glucan glucohydrolase was predicted with a deduced amino acid sequence as a homology model structure [8].

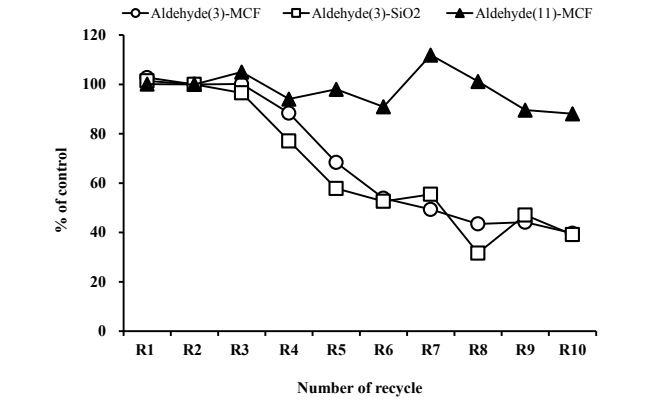

Figure 5: Enzyme activity of 1,4-β-D-glucan glucohydrolase according to the recycle number after surface functionalized MCF-immobilization.

(Sigma), and 1/3 of Cellulclast 1.5L (Novozyme).

Although classified as a glucohydrolase, the enzyme purified from *G. natalis* was similar in size to β-glucosidases found in other arthropods, such as the larvae of the moth *Erinnyis ello* [12] and the rice weevil *Sitophilus oryzae* [13]. The β-glucosidase from *S. oryzae*, like the glucohydrolase from *G. natalis*, was active towards both cellobiose and 4-methylumbelliferyl β-D-glucopyranoside. It is therefore possible that the enzyme found in *S. oryzae* is similar to that from *G. natalis* and, therefore, might also be a glucohydrolase. Glucohydrolase activity has been noted in other insects, such as the larvae of the cardinal beetle *Pyrochroa coccinea* [14] and the termite *Coptotermes lacteus* [15], but was typically considered to be a minor component of cellulose digestion [16]. These enzymes have therefore largely been ignored when discussing cellulolytic systems in invertebrates.

The mechanical disruption of cellulose during pretreatment increases the efficiency of enzymatic breakdown by increasing the surface area of cellulose exposed to the enzymes within the digestive juice and might further reduce the need for a cellobiohydrolase by improving access to glucose residues normally buried within crystalline regions of cellulose fibers. This is in contrast to fungal and bacterial systems, which lack a mechanical mill, relying instead on the presence of a cellobiohydrolase and its synergistic activity with the endo-β-1,4-glucanase to digest crystalline cellulose efficiently [17].

Cellulose solubility decreases drastically with increasing DP due to intermolecular hydrogen bonds. Cellodextrins with DP from 2-6 are soluble in water [18,19], while cellodextrins from 7-13 or longer are somewhat soluble in hot water [8]. A glucan of DP = 30 already represents the polymer "cellulose" in its structure and properties [19]. The DP of cellulosic substrates varies greatly, from 100 to 15,000, depending on substrate origin and preparation.

The change in DP over the course of hydrolysis for cellulosic substrates is determined by the relative proportion of exo-and endo-acting activities and cellulose properties. Exoglucanases act on chain ends, and thus decrease DP only incrementally. Endoglucanases act on interior portions of the chain and thus rapidly decrease DP [20,21]. Exoglucanase has been found to have a marked preference for substrates with lower DP [5], as would be expected given the greater availability of chain ends with decreasing DP. It is well known that endoglucanase activity leads to an increase in chain ends without resulting in appreciable solubilization [22]. We know of no indication in the literature that the rate of chain end creation by endoglucanase is impacted by substrate DP.

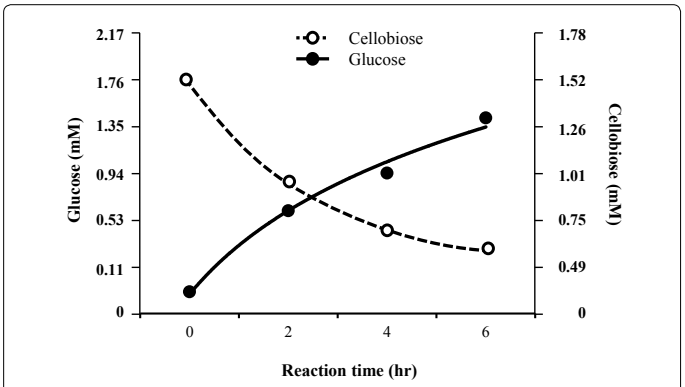

Figure 6: HPLC analysis results of the substrate cellobiose and product glucose by 1,4-β-D-glucan glucohydrolase with the time course of the reaction. HPLC (Younglin Instrument Acme 9000, Korea) analysis was conducted under conditions of Shodex Asahipak column NH2P-50 4E, fluent of water/CH3CN (1:3), flow rate of 1 ml/min., detector of RI (refractive index), column temperature of 30oC.

Reaction (min)	Cellobiose		Cellotriose		Cellotetraose		Cellopentose		Cellohexose	
	Glu (mmol)	Con (%)	Glu (mmol)	Con (%)	Glu (mmol)	Con (%)	Glu (mmol)	Con (%)	Glu (mmol)	Con (%)
0	0	0	0	0	0	0	0	0	0	0
60	0.61	44.4	0.92	55.8	1.06	60.2	1.20	50.7	1.25	59.0
120	0.74	57.6	1.04	63.9	1.20	68.0	1.50	67.0	1.89	78.2
180	0.82	64.7	1.09	70.2	1.38	75.1	1.63	75.0	1.99	82.9
360	0.98	79.8	1.22	79.6	1.50	82.0	1.79	82.0	2.13	84.1

*Glu: Glucose; Con: Conversion

Table 2: Enzymatic glucose producing efficiency of a new 1,4-β-D-glucan glucohydrolase PGDβ4 according to the different glucose number of substrates

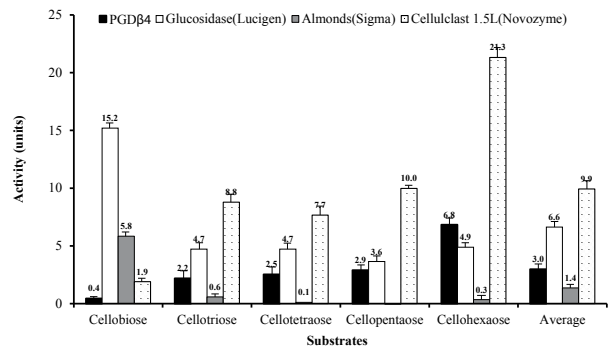

Figure 7: Comparison of hydrolytic activity with 1,4-β-D-glucan glucohydrolase and commercialized-enzymes using different number of glucose polymer substrates.

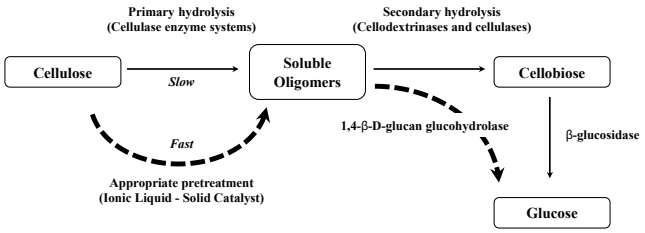

Figure 8: Proposed scheme of a single enzymatic saccharification system for biorefinery.

At this point, if the depolymerization of cellulose proceeds progressively by chemical hydrolysis instead of endo-β-1,4-glucanases, resulting in the formation of soluble oligosaccharides, cellulose fragments ideally suited for further processing by glucohydrolase single enzymatic hydrolysis can easily be isolated.

Conclusion

We identified a new 1,4-β-D-glucan glucohydrolase gene from common bacteria *Paenibacillus* sp. non-extremophile [6], which was cloned and expressed in *Escherichia coli* and efficiently purified using affinity column chromatography, which showed the successive hydrolysis functions of cello-oligomers virtually reduced to glucose. It is suggested that the single enzymatic saccharification system for bio refinery using this enzyme might be expected, if it will be connected to appropriate pretreatment technology, instead of the traditional concept using a three-enzyme system for cellulose hydrolysis (Figure 8). We think this paper will be of interest to those who involved in biorefinery process as well as in enzymatic hydrolysis.

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