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Complete Saccharification of Cellulose at High Temperature Using Endocellulase and β-Glucosidase from *Pyrococcus* sp.

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We investigated a potential for glucose production from cellulose material using two kinds of hyperthermophilic enzymes, endocellulase (EG) and beta-glucosidase (BGL). Two BGLs, from hyperthermophile *Pyrococcus furiosus* and mesophile *Aspergillus aculeatus*, were compared with *P. horikoshii* endocellulase (EGPh) for complete hydrolysis of cellulose. The combination reactions by each BGL enzyme and EGPh could produce only glucose without the other oligosaccharides from phosphoric acid swollen Avicel (PSA). The combination of both the hyperthermophilic cellulases, BGLPf and EGPh, will be adaptable to a high efficiency system to produce glucose at high temperature.

Keywords: Hyperthermophilic, cellulase, saccharification, *Pyrococcus*

Cellulosic materials are the most abundant biomass on the earth, and are capable to change into bioethanol as the next generation biofuel [5, 8, 10, 20]. The high efficiency process of bioethanol production from the biomass requires complete saccharification of cellulose to obtain fermentable sugar. In nature, a typical cellulolytic microbe can produce three categories of cellulases such as endoglucanase (EG), cellobiohydrolase (CBH), and beta-glucosidase (BGL) to convert cellulose into glucose [2, 21, 22]. EG and CBH can produce cellooligosaccharides or cellobiose. The resulting products serve as the substrate of enzyme BGL to produce glucose. Like natural saccharification, therefore, the cellulase system using these three types of enzymes has been recognized as a general enzymatic complete saccharification of cellulose in its industrial application process.

Trichoderma reesei is well known as a strongly cellulolytic and xylanolytic microorganism. However, complete saccharification of cellulose was not observed by the cellulases from only *T. reesei* because of its low BGL activity. Therefore, BGL from *Aspergillus aculeatus* (BGLAa) was developed and applied to emphasize the cellulase activity of *T. reesei.* [14]. At present, a thermophilic cellulase system remains to be developed for industrial application, because the enzymatic degradation of biomass in high temperature has the obvious advantage of eliminating bacterial contamination and increasing substrate solubility. Recently, a hyperthermophilic endocellulase (EGPh, E.C. 3. 2. 1. 4, family 5) from archaeon *Pyrococcus horikoshii* was identified, the recombinant enzyme of which was successfully expressed using *Escherichia coli* [1, 13, 15, 16]. The EGPh has processive hydrolysis activity to release cellobiose, not generate oligocellosaccharide by random scission, after an initial endo-type attack against cellulose. The hyperthermophilic endo cellulase (EGPf, family 12) was found in archaeon *P. furiosus.* However, the activity of EGPf against Avicel is lower than that of EGPh [4, 13]. On the other hand, hyperthermophilic archaeal BGL has also been isolated from *P. horikoshii* as well as *P. furiosus* [17, 19], but an exocellulase like CBH was not observed in the genome of the related *Pyrococcus* sp. Interestingly, BGL from *P. horikoshii* showed a unique activity against only cellobiose, and not against other cellooligosaccharides [19]. This substrate specificity supports the hypothesis that the saccharification system in *P. horikoshii* may be consisted of EGPh and BGL. This finding was not only a discovery of potential industrial cellulases but also presented a new approach for saccharification using two types of cellulases from the archaea. The production of BGL from *P. horikoshii* (BGLPh) was very poor in *E. coli*, a representative host for bulk production, which might be caused by its membrane-related property. The activity of BGLPh was observed in the presence of detergents [19]. On the other hand, BGL from *P. furiosus* (BGLPf) could be successfully produced by using the *E. coli* expression system and exhibited high activity.

Therefore, we investigated a possibility of cellulose saccharification under hyperthemophilic condition using EGPh and BGLPf. The BGL enzyme (BGLAa, E.C.

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3.2.1.21, family 3) from *Aspergillus aculeatus* [14], which has the highest activity among mesophilic BGLs, was also examined to compare the enzyme activity and saccharification efficiency. The BGLAa expressed in yeast *Saccharomyces cerevisiae* and purified by using anion-exchange and hydrophobic chromatographies was a gift from Dr. Takashi Kawaguchi of Osaka Prefecture University. EGPh (Gene ID: PH1171) [1] and BGLPf (Gene ID: PF0073) [3] were prepared as follows. The coding genes were expressed in *E. coli* BL21(DE3) cells (Novagen) by the T7 promoter of pET11a (Novagen). Cell cultures were grown at 37°C in Luria Broth with 100 mg/ml ampicillin until OD_{600} reached 0.8, and IPTG was added to a final concentration of 0.1 mM for the protein induction. Both enzymes were purified by ammonium sulfate fractionation after heat treatment (30 min at 80°C) and eluted through a HiTrapQ anion-exchange column. After confirming the purity of the proteins using SDS–PAGE, the protein concentrations of EGPh, BGLPf, and BGLAa were determined from UV absorbance at 280 nm, using 136,270, 128,160, and 133,850 as the molar extinction coefficient calculated from their protein sequences, respectively [9]. Cellobiose, the substrate used for kinetic analysis of the BGLs, was purchased from SEIKAGAKU Co. (Tokyo, Japan). The determination of reducing sugar and glucose released by the enzyme activities was carried out by the modified Somogyi–Nelson method [10] and using a glucose assay kit (Wako Pure Chemical Industries, Osaka, Japan). Phosphoric acid swollen Avicel (PSA) was prepared by the method described previously [12] and used as the cellulose substrate. The enzymatic activity toward *p*-nitrophenyl cellobiose was determined by measuring the released *p*-nitrophenol derived from *p*-nitrophenyl cellobiose (pH 5.5) at 50°C.

To compare the specific activity toward cellobiose, the main product of the EGPh, the activities of both BGLs were examined at the optimum temperature for each enzyme (50°C for BGLAc and 85°C for BGLPf). The optimum specific activity (k_{cat}) of BGLPf showed about 1.8-fold higher value than that of BGLAa, whereas the K_m value of BGLAa was about 3-fold lower than that of BGLPf (Table 1).

When EGPh was applied for the cellulose-saccharification reaction with each BGL using PSA as a substrate, the combination reaction with BGLPf could more efficiently produce the final product "glucose" than that with BGLAa (Fig. 1). This higher productivity by EGPh and BGLPf may be due to the common hyperthermophilic property of the two enzymes and substrate specificities of BGLs. Considering that the values of the kinetic parameters of both enzymes were not significantly distinctive in their optimal temperature, the activities of both the BGLs in each combination reaction might be likely to exhibit a similar pattern, as shown in the glucose productivity by the addition of low amount of each BGL in Fig. 1.

Analysis of the conversion and product for the reaction was performed using EGPh and BGLPf at 50°C and 85°C. In order to examine the complete saccharification, we measured the activity for a long time period (over 10 days). It is difficult to measure the time course of the detailed amount of released reducing sugar and glucose at higher

Table 1. Kinetic parameters of the BGLs on hydrolysis activity toward cellobiose as substrate.

	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m
BGLAa	6.6±0.3	336±6	50.9±5.9
BGLPf	20.3±0.9	594±2	29.3±1.6

The activities of BGLAa and BGLPf were measured in 0.1 M sodium acetate buffer (pH 5.5) at 50°C and 85°C, respectively. The k_{cat} values were determined from the initial velocity measured using the nonlinear least-squares method.

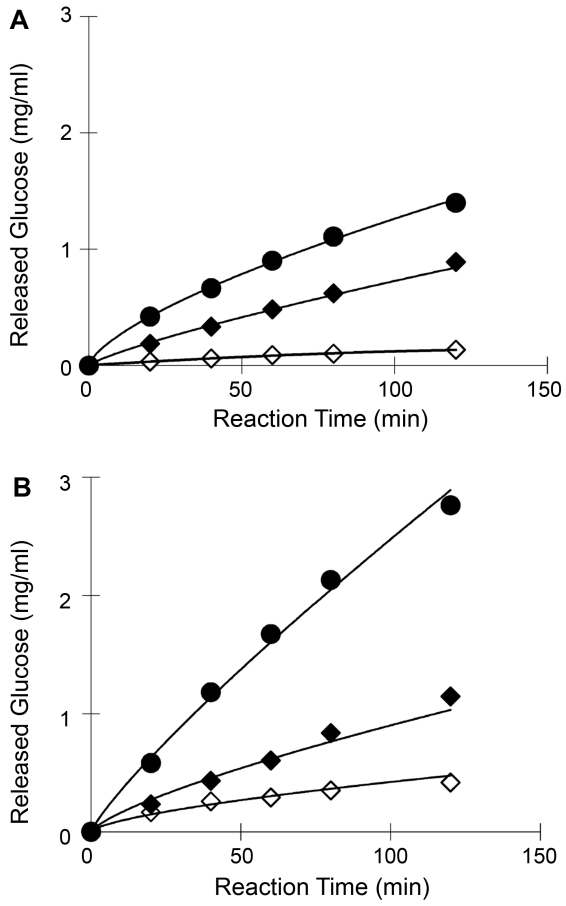

Fig. 1. Effect on efficiency of glucose production from phosphoric acid swollen Avicel (PSA) according to the combination of *P. horihoshii* EGPh and individual BGL (*A. aculeatus* BGLAa or *P. furiosus* BGLPf).
(**A**) PSA hydrolysis by EGPh and BGLAa, and (**B**) EGPh and BGLPf, was performed using 0.5% PSA (0.1 M sodium acetate buffer, pH 5.5) at 50°C and 85°C, respectively. The EGPh used was 2.5 nmol/1 ml of the reaction mixture, and the added BGLs were 0 (◇), 10 (◆), and 50 (●) pmol/ml, respectively. The initial glucose concentration was calibrated to zero.

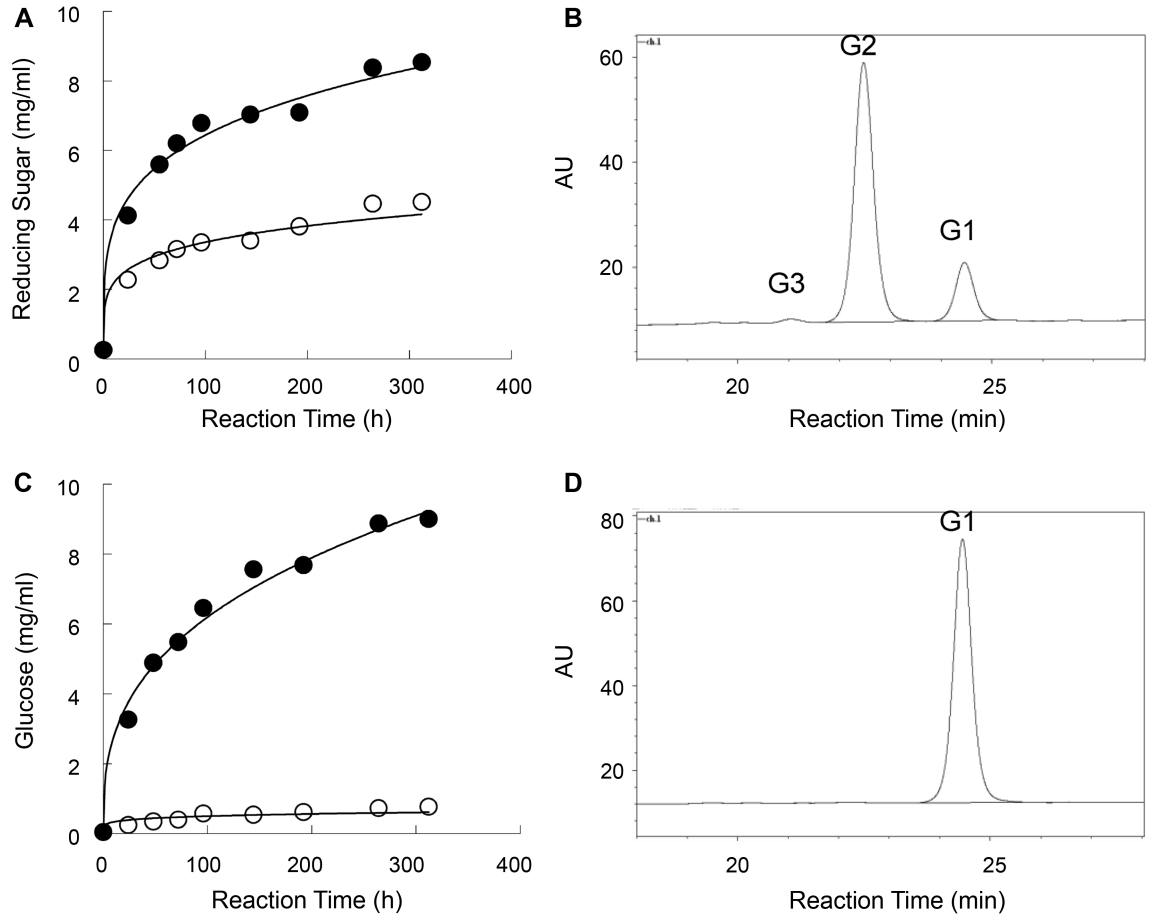

Fig. 2. Degradation of PSA by EGPh and BGLPf.
A and **C**. Hydrolytic analysis by EGPh alone (0.4 nmol) (○), and the EGPh (0.4 nmol) and BGLPf (0.1 nmol) (●) in 1 ml of 1% PSA (0.1 M sodium acetate buffer, pH 5.5). Degradation activities were measured by reducing sugars (**A**) and releasing glucose (**C**). The enzyme–PSA mixtures were reacted in a shaker-incubator at 50°C and 200 rpm. The initial product concentration was calibrated to zero. **B** and **D**. HPLC analysis of the final product by the EGPh alone (**B**), and the EGPh and BGLPf (**D**). The product analysis was performed using a RI detector (RID-6A, Shimadzu, Japan) equipped with a TSK gel G-Oligo-PW column (7.8×300 mm; TOSOH, Japan). Distilled water was used as a mobile phase at the flow rate of 0.7 ml/min.

temperature (85°C). Fig. 2A and 2C show the time course of amount of reducing sugar and glucose from the degradation reaction by EGPh alone, or EGPh and BGLPf, at 50°C. The remarkable difference of the glucose productivity was observed according to the addition of BGL (Fig. 2C). The product analysis was also performed with HPLC using the final reaction mixture by EGPh alone, and EGPh and BGLPf (Fig. 2B and 2D). These results indicate that the main product by EGPh is cellobiose, which is continuously and completely digested by BGLPf. When comparing the amount of reducing sugar from the EGPh alone and the combination reaction, its difference was approximately 2-fold (Fig. 2A). This result suggests that the activity of BGLPf has no remarkable synergy effect by removing of the cellobiose inhibitor under this reaction condition (1% of PSA). Hydrolysis of cellulose (2% of PSA) using EGPh and BGLPf was examined after incubation for 3 days at 85°C. The final product by the reaction of EGPh alone was cellobiose as the main product, and glucose was also detected as a minor product by HPLC analysis (data not shown). Furthermore, the result that some amount of the precipitate cellulose observed in the reaction mixture contained only EGPh indicates that the complete saccharification could not be achieved by EGPh alone. The combination reaction using EGPh and BGLPf could achieve complete saccharification of cellulose, and produced only glucose without the other cellooligosaccharides after the incubation for 3 days at 85°C. The cellobiose released from the EGPh-catalyzed reaction behaved as an inhibitor when presenting in the reaction mixture. Cellobiose exhibited 9.08 mM of K_i value against EGPh using *p*-nitrophenyl cellobiose as the assay substrate of EGPh. The non-complete saccharification of cellulose by EGPh seems to be due to product-feedback inhibition by cellobiose. It seems that cellobiose stops the enzyme (EGPh) reaction and BGL removes the inhibitor, cellobiose. Some BGLs exhibit hydrolysis activity that digests glycosidic linkages of oligosaccharides, as well as transglycosylation activity that transfers the released saccharide moieties onto

the compound containing hydroxyl groups [6, 7]. This transglycosylation reaction allows to produce non-fermentable oligosaccharides. Our results revealed that the EGPh and BGLPf mixture was a successful enzyme combination in the process of converting cellulose into glucose completely at extremely high temperature.

To achieve information about the efficient depolymerization mechanism of cellulosic materials, "omics" analysis for wood-degrading organisms was studied focusing on cellulase-relative enzymes [18, 23, 24]. In the case of wood-decaying basidiomycetes, unexpectedly, the genome of brown-rot fungi lacks cellulose-binding domains and the CBH gene, unlike white-rot fungi with both endo- and exo-acting cellulases [18]. We have no information about the thermophilic CBH from thermophiles. Among other cellulolytic microbes, the absence of CBH was also exhibited in the cellulolytic gliding bacterium *Cytophaga hutchinsonii* [24] and metagenomic analysis of microbiota in the hindgut paunch of a wood-feeding termite [23]. Interestingly, the principle endocellulase from these microbes was family 5 cellulases lacking the cellulose-binding domain, which was in common with that of *Pyrococcus* sp. These facts provide a potential possibility of a cellulase system using EG and BGL for efficient complete breakdown of cellulose material.

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