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Characterization of a novel manganese dependent endoglucanase belongs in GH family 5 from *Phanerochaete chrysosporium*

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The cDNA encoding a putative glycoside hydrolase family 5, which has been predicted to be an endoglucanase (*PcEg5A*), was cloned from *Phanerochaete chrysosporium* and expressed in *Pichia pastoris*. PcEg5A contains a carbohydrate-binding domain and two important amino acids, E209 and E319, playing as proton donor and nucleophile in substrate catalytic domain. SDS-PAGE analysis indicated that the recombinant endoglucanase 5A (rPcEg5A) has a molecular size of 43 kDa which corresponds with the theoretical calculation. Optimum pH and temperature were found to be 4.5–6.0, and 50°C–60°C, respectively. Moreover, rPcEg5A exhibited maximal activity in the pH range of 3.0–8.0, whereas over 50% of activity still remained at 20°C and 80°C. rPcEg5A was stable at 60°C for 12 h incubation, indicating that rPcEg5A is a thermostable enzyme. Manganese ion enhanced the enzyme activity by 77%, indicating that rPcEg5A is a metal dependent enzyme. The addition of rPcEg5A to cellobiase (cellobiohydrolase and β-glucosidase) resulted in a 53% increasing saccharification of NaOH-pretreated barley straw, whereas the glucose release was 47% higher than that cellobiase treatment alone. Our study suggested that rPcEg5A is an enzyme with great potential for biomass saccharification.

[**Key words:** Endoglucanase; *Phanerochaete chrysosporium*; Manganese dependent enzyme; Barley straw; Saccharification]

Lignocellulose, which is mainly composed of cellulose, has attracted the interest of many researchers over last the few decades, due to the increase in global energy demand and growing concerns about energy security, rural development, and increasing costs as well as environmental impact of production of nonrenewable, non-degradable chemicals (1,2). Cellulose is a long chain consists of glucose, linked to one another primarily by glycosidic bonds, typically linked by β-1-4 bonds, and it makes up 15–30% of the dry biomass of primary cell walls and up to 40% of the secondary cell walls (1,3). Basically, a complex enzyme involving endoglucanases, cellobiohydrolases, and β-glucosidases acting in a synergistic manner is required to completely break down cellulose (4).

β-1,4-Endoglucanases (E.C. 3.2.1.4) are a group of glycoside hydrolases, which cleave internal linkages of cellulose, yielding short-chain glucose polysaccharides. According to the classification of the carbohydrate-active enzymes database, β-1,4-endoglucanases are divided into 14 families 5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 74, and 124. β-1,4-endoglucanases are widely produced by bacteria, fungi, plants, and animals; however, most of industrial β-1,4-endoglucanases originate from bacterial and fungal sources (5,6).

Lignocellulose degrading fungus *Phanerochaete chrysosporium* is known to produce wide range cellulases including endoglucanases, cellobiohydrolases and β-glucosidases. Its genome sequence and secreted protein prediction in cellulose-grown medium indicated that *P. chrysosporium* produces at least 18 putative endoglucanases of the glycosyl hydrolase family 5 (7,8). However, the function of most of these enzymes is still unknown.

Thermostable enzymes offer several potential advantages in lignocelluloses conversation such as: (i) increased solubility of reactants and products, resulting in higher reaction velocities; (ii) shorter hydrolysis times; (iii) decreased risk of contamination; (iv) facilitated recovery of volatile products; and (v) decreased cost of energy for cooling after thermal pretreatment. Therefore, thermostable enzymes including β-1,4-endoglucanases are attracting the attention of researchers (9). Herein, we report the characterization of a recombinant thermostable β-1,4-endoglucanases from *P. chrysosporium* and its application in biomass saccharification.

MATERIALS AND METHODS

Gene identification and manipulation In order to amplify the putative endoglucanase gene of *P. chrysosporium*, the total mRNA was extracted from mycelia cultured for 7 days. *P. chrysosporium* using the Oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA), then the cDNA library was synthesized using the SMARTer PCR cDNA Synthesis kit (Clontech, Mountain View, CA, USA).

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The putative endoglucanase gene was searched in the *P. chrysosporium* RP78 genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html) and confirmed based on the protein secreted into the cellulose medium (8). A sequence of putative endoglucanase GH5 (Genbank accession number AY682743) was amplified with two specific primers 5′-ATGCTCAAGTACGCGTCCATCG-3′ and 5′-TCTAGACAAGGAAGGTTGGGCTTTACA-3′. The gene was designed as *PcEg5A*. The standard gene manipulation procedure was carried out as described by Huy et al. (10). Gene sequence was confirmed by sequencing of three different colonies, and then aligned on the *P. chrysosporium* RP78 endo-glucanase GH5 template. *PcEg5A* sequence was submitted to the Protein homology/analogy recognition engine V 2.0 for model analysis (11). The best matched gene was selected for clonning and expression in *Pichia pastoris* GS115 (Invitrogen, Carlsbad, CA, USA). The mature region of the gene was amplified using 5′-GAATTCCAGCAGCAACAATGGGGT-3′ and 5′-TCTAGACAAGGAAGGTTGGGCTTTACA-3′ primers and constructed with pPICZαA vector (Invitrogen, Carlsbad, CA, USA) through *Eco*RI and *Xba*I restriction enzyme sites. The constructed *PcEg5A*-pPICZαA was sequenced again using AOX primers (5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCTGACATCC-3′) to avoid expression errors as manufacturer's instructions.

PcEg5A-pPICZαA was linearized with *Pme*I, then transformed into *P. pastoris* GS115 using an electroporation method, as recommended by the manufacturer (Bio-Rad, Hercules, CA, USA). Transformed cells were selected on YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates containing 100 μg ml^{-1} of zeocin and 1 M sorbitol at 30°C, until colonies were observed (2–3 days). The integration of *PcEg5A* into the *P. pastoris* genome was confirmed by PCR using AOX1 primers.

Enzyme expression and purification The recombinant *P. pastoris* was confirmed by PCR using both AOX and intrinsic primers. The expression level of twenty recombinant *P. pastoris* colonies was qualified using dot blot analysis with His-tag antibody. The highest hybridization significant for the transformant was selected for enzyme production in 100 ml YP (1% yeast extract, 2% peptone) medium scale and carried out at 30°C, 200 rpm. Enzyme production was induced by adding 1 ml pure methanol for every day. The third day culture was harvested by centrifuging for 10 min at 2000 rpm, and then filtered using 0.45 μm membranes. Recombinant enzyme (rPcEg5A) was purified following the procedure mentioned in our previous study (10). Protein concentration was estimated by the Bradford method using a Thermo Scientific Protein Assay kit (Rockford, IL, USA), using serum albumin as the standard. The molecular mass of purified enzyme was quantified by SDS-PAGE.

Enzyme determination and biochemical characterization The endoglucanase activity of the recombinant enzyme was quantified using carboxymethylcellulose (CMC). Briefly, 5 μg of the purified enzyme was incubated with 200 μl of 2% CMC in a total of 400 μl reaction solution containing 50 mM sodium acetate buffer, pH 5. The reaction was carried out at 50°C for 15 min. The release of reducing sugars was measured using 3,5-dinitrosalicylic acid (DNSA) (12). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute. The standard curve was generated using glucose as the reducing sugar.

Optimum pH for the recombinant enzyme was investigated by assaying for the enzyme activity at 50°C at different pH values of 3.0–6.0 using 50 mM sodium acetate and of 7.0–8.0 using 50 mM sodium phosphate buffer, while the optimum temperature was examined at temperatures of 30–90°C and at optimal pH. Experiments were carried out using CMC as the substrate, and the amount of reducing sugar released was quantified as described above. Thermostability of the recombinant enzyme was examined at different temperatures (50°C, 60°C, 70°C) and at various time periods; 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h. The retained activity was measured as described above.

The substrate specificity of endo-activity in rPcEg5A was studied using laminarin, birchwood xylan, locust bean gum (Sigma, St. Louis, MO, USA), xyloglucan, glucomannan, barley glucan, arabinoxylan, debranched arabinan (Megazyme, Bray, Co. Wicklow, Ireland), whereas exo-activity was investigated using *p*-nitrophenyl-β-D-glucopyranoside (pNPβG), *p*-nitrophenyl-β-D-xylopyranoside (pNPβX), *p*-nitrophenyl-α-D-xylopyranoside (pNPαX), *p*-nitrophenyl-α-L-arabinofuranoside (pNPαA), *p*-nitrophenyl-α-D-mannopyranoside (pNPαM) and *p*-nitrophenyl-β-D-mannoside (pNPβM) (Sigma). The endo-activity was measured by the release of reducing sugars as described above, whereas the exo-activity was determined by quantifying the amount of *p*-nitrophenol (pNP) released following the procedure described in our previous study (13).

Thin layer chromatography The end-hydrolysis forms of CMC, barley β-glucan, and NaOH-pretreated barley straw using rPcEg5A or the combination of rPcEg5A and *Aspergillus niger* cellobiase (Sigma) were analyzed by thin layer chromatography (TLC) using chloroform/acetic acid/H_2O (6:7:1) as a mobile phase and visualized by spraying TLC plates with sulfuric acid/ethanol (5:95, v/v) containing 1 mg/ml of orcinol, followed by baking at 110°C for 5 min. Glucose, glucobiose, glucotriose, and glucotetraose were used as standards.

Saccharification of NaOH-pretreated barley straw rPcEg5A was applied for biomass saccharification using NaOH-pretreated barley straw. Barley straw was treated with 0.3 M NaOH at 150°C for 6 h. NaOH-pretreated barley straw is consisted of 61.52% glucose, 19.34% hemicellulose and 11.79% lignin. Approximately fifty units of rPcEg5A were incubated with 100 g of NaOH-pretreated barley straw in a 5 ml reaction mixture containing 50 mM sodium acetate buffer, pH 4.5. The synergistic effect of rPcEg5A with cellobiohydrolase and β-glucosidase was studied in a combination with fifty units of endoglucanase activity of *A. niger* cellobiase. The reaction was carried out at 50°C for 16 h, and then it was terminated by boiling at 100°C for 5 min. The amount of reducing sugars released was measured as described above.

The hydrolysis products of NaOH-pretreated barley straw with rPcEg5A or cellobiase or the combination of rPcEg5A and cellobiase were quantified by High performance liquid chromatography (HPLC) using Hi-Plex H column and Ultimate 3000 system (Agilent, Santa Clara, USA) according to manufacturer's recommendation. Approximately 20 μl of filtered reaction solution were injected into the system. Water was used as a mobile phase with a maximum retention time of 20 min.

RESULTS AND DISCUSSION

Cloning and expression of PcEg5A For cloning of the putative *PcEg5A*, we designed two specific primers to amplify the coding sequence consists of 1161 bp from the *P. chrysosporium* cDNA library. The results showed a PCR amplicon of the expected size on agarose gel (data not shown). Therefore, we isolated and cloned the PCR amplicon into pGEM T-easy vector, and then confirmed by gene sequencing with three independent colonies. Sequence alignment of PCR amplicon with a gene template revealed 100% similarity to the template sequence (AY682743) (8). Blast search of the amino acid sequence against the NCBI database indicated that *PcEg5A* shares 84%, 74%, and 70% similarity with glycoside hydrolase family 5 from *Phanerochaete carnosa*, hypothetical protein from *Trametes versicolor*, endoglucanase from *Fomitiporia mediterranea*, respectively. The first sixteen amino acids of mature *PcEg5A* were found as secretion signal peptide. *PcEg5A* contains carbohydrate binding module family 1 at the C-terminus, where one potential N-glycosylation site (Asn–Thr–Ser) was found in *PcEg5A* sequence. Protein model analysis indicated that the structure of *PcEg5A* was in accordance with that of endoglucanase family 5 from *Hypocrea jecorina* (14), where two importance amino acids E209 and E319 play as proton donor and nucleophile in substrate catalytic domain, respectively which conserved of both enzymes (Fig. 1).

We expressed *PcEg5A* in the *P. pastoris* heterologous expression system. The expression of recombinant enzyme in *P. pastoris* was evaluated with dot blot analysis, and it showed that all of the recombinant *P. pastoris* clones produced high levels of the recombinant enzyme (data not shown). We purified the rPcEg5A for further enzyme characterization. As shown in Fig. 2, purified rPcEg5A exhibited a single band with molecular mass of around 43 kDa. This band was also observed in the culture medium. Although one *N*-glycosylation site was predicted in *PcEg5A* sequence, it did not affect the protein size. *P. pastoris* have been demonstrated to produce a large amount of the recombinant protein (15,16). Our study showed that the main secreted protein is rPcEg5A with approximately 100 mg per liter.

Effect of pH and temperature on rPcEg5A activity It is known that PcEg5A is produced by *P. chrysosporium* under biomass cultivation condition; thus, PcEg5A may play an importance role in cellulose saccharification. We aimed to study this enzyme by assessing the recombinant enzyme expression in *P. pastoris* for further application. We examined the effect of temperature in the range from 20°C to 90°C for 10 min. The results showed that the optimal temperature for rPcEg5A ranged from 45°C to 70°C (Fig. 3A). Interestingly, rPcEg5A still exhibited a high activity at a temperature below 45°C or above 70°C, and rPcEg5A retained 66.8% and 43% at 20°C and 90°C of maximal activity, respectively. The optimal pH for rPcEg5A was 4.0. The rPcEg5A activity was dramatically decreased with an increase in pH (Fig. 3B). The thermostability of rPcEg5A was investigated at 50°C, 60°C, and 70°C by incubating for 12 h. As shown in Fig. 3C, rPcEg5A was stable at temperatures of 50°C and 60°C for 12 h incubation

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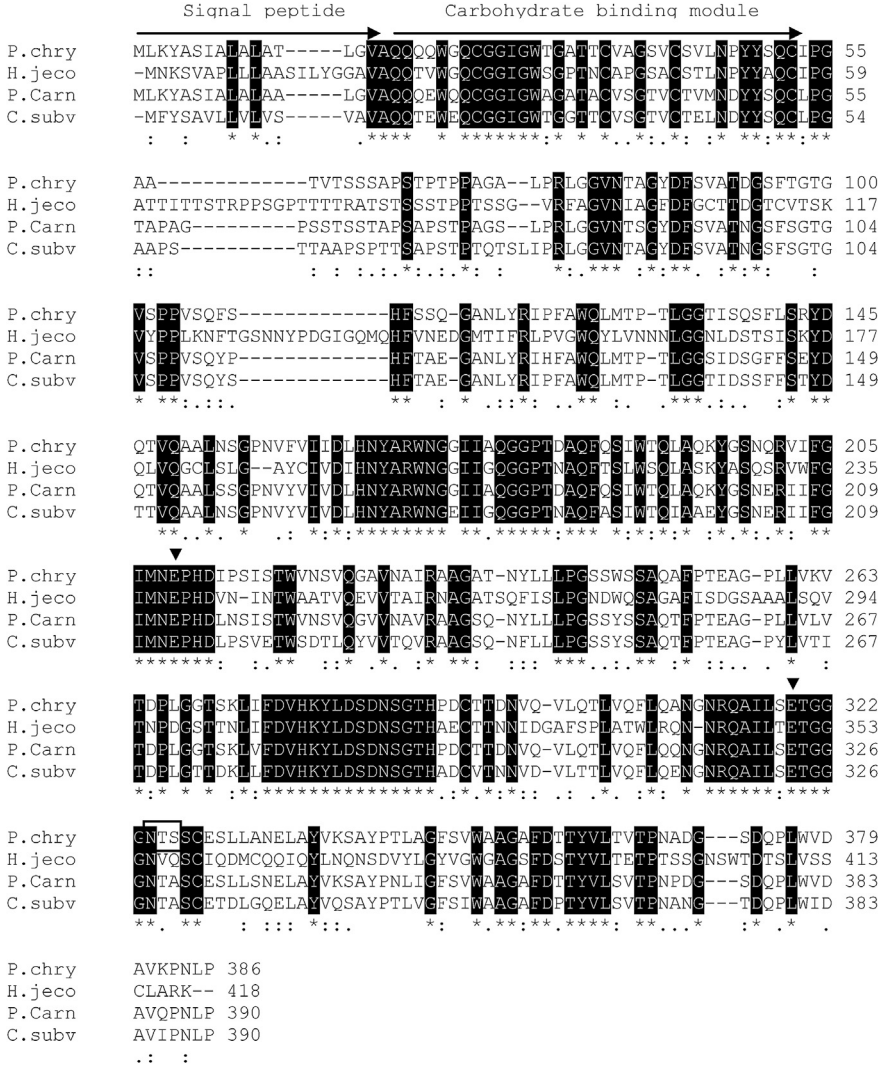

FIG. 1. The deduced amino acid sequence alignment of *P. chrysosporium* endoglucanase GH5 A (*PcEg5A*) and other endoglucanases from *Hypocrea jecorina* (PDB: 3QR3), *Phanerochaete carnosa* (XP_007390721), and *Ceriporiopsis subvermispora* (EMD41942) using the ClustalW2 tool. Identical amino acids are written in black letters. The signal peptide and carbohydrate binding module are indicated as line arrows. The E209 and E319 conserved catalytic residues of endoglucanase GH5 are shown as closed inverted triangles. The *N*-glycosylation sites (Asn–Thr–Ser) are shown boxed.

period; however, at a temperature 70°C, rPcEg5A lost almost all its activity after 1 h.

Previous reports indicated that *P. chrysosporium* secretes numerous endoglucanases (7,8,17,18). *P. chrysosporium* β-1,4-endoglucanases, which belong to glycosyl hydrolases families 12 (Cel12A) and 61 (PcGH61D) have been characterized (19,20). However, there is no report on β-1,4-endoglucanases from glycosyl hydrolase family 5 such as PcEg5A. The optimal pH for rPcEg5A was 4.0, which was slightly different from the optimal pH 5.0 for Cell12A and PcGH61D. The optimal temperature for rPcEg5A was higher than that for Cell12A (37°C) but it was in accordance with PcGH61D (50°C). However, it seemed that rPcEg5A displayed higher thermostability than the other enzymes.

We examined the effect of various metal ions on rPcEg5A activity. As shown in Fig. 4, most of the tested metal ions did not decrease the rPcEg5A activity compared with control. Moreover, the rPcEg5A activity was increased up to 77% by manganese ion, indicating that rPcEg5A is a manganese dependent enzyme. It has been reported that *P. chrysosporium* also produced a manganese-dependent endoglucanase family 61 (PcGH61D) (20). However, while PrGh61D is also dependent on copper, our enzyme was slightly inhibited by this ion (Table 1). Ca^{2+} is reported to enhance the activity of endoglucanase GH5 from *Hahella chejuensis* (21) and

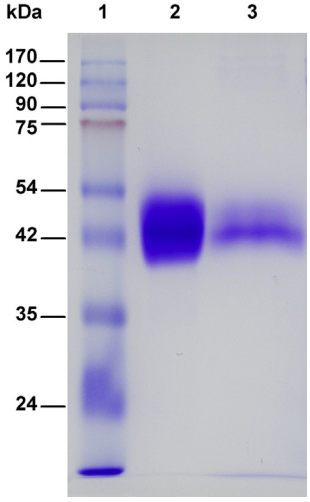

FIG. 2. SDS-PAGE of purified recombinant endoglucanase 5A (rPcEg5A). Lane 1, protein standard molecular maker; lane 2, purified rPcEg5A; lane 3, recombinant *P. pastoris* medium culture. Approximately 10 μg of rPcEg5A was loaded onto the gel while 20 μl of third day recombinant *P. pastoris* cultivation was used for analysis. The gel was run at 80 V for 3 h, and then stained with Coomassie brilliant blue.

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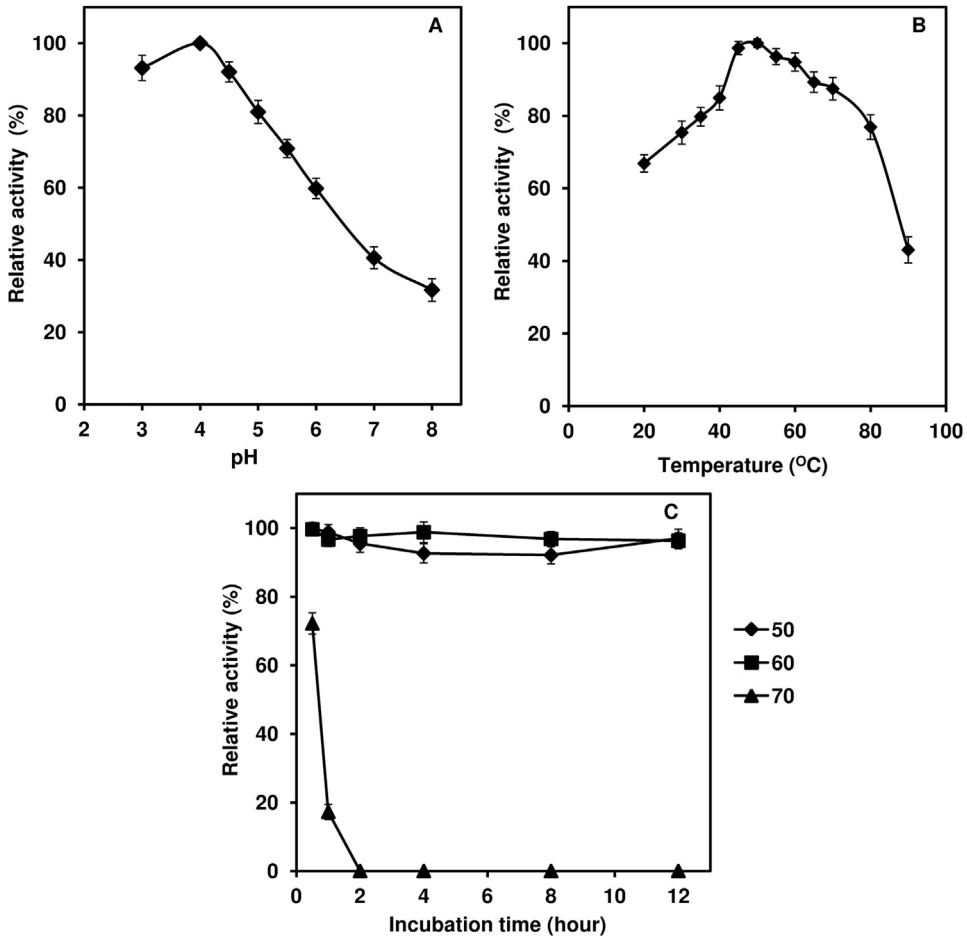

FIG. 3. Effect of pH (A) and temperature (B) on recombinant endoglucanase 5A (rPcEg5A), and thermostability (C) of rPcEg5A. Reaction was carried out using 5 μg of the purified enzyme in 400 μl of 50 mM sodium acetate or sodium phosphate buffer containing 1% CMC as substrate. The release of reducing sugars was determined as described in Materials and methods. Data represent the means of three experiments, and error bars represent means ± standard errors.

Saccharophagus sp. Myt-1 (22), but this ion did not increase the rPcEg5A activity. The 3D crystal structure of cel5A from *H. jecorina* indicated cel5A contains magnesium ion ligand. However, our enzyme was not enhanced by these ions.

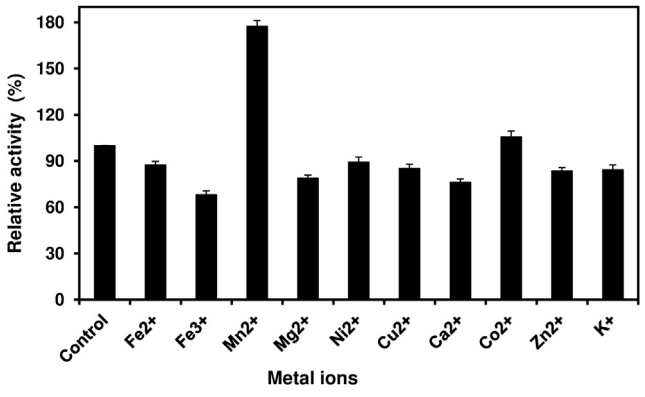

FIG. 4. Effect of metal ions on recombinant endoglucanase 5A (rPcEg5A) activity. Approximately 5 μg of purified rPcEg5A was incubated with 20 mM of each ion in 200 μl of 10 mM sodium acetate buffer, pH 4 for 1 h at 45°C, and then 200 μl of 2% CMC preincubated at 45°C was added to the reaction. The reaction was carried out for 10 min. The residual activity was measured using DNSA as described in Materials and methods. Data represent the means of three experiments, and error bars represent means ± standard errors.

Substrate specificity of rPcEg5A Substrate specificity study showed that rPcEg5A exhibited high activity toward CMC, barley glucan, and glucomannan of 694 U mg^{-1}, 1443.5 U mg^{-1}, and 622.4 U mg^{-1}, respectively. On the other hand, rPcEg5A did not hydrolyze hemicellulose substrates such as birchwood xylan, arabinoxylan, and arabinan, while rPcEg5A displayed low activity on larminarin and xyloglucan. We also did not observe any *exo* activity on *p*-nitrophenyl-β-glucopyranoside. Our study indicated that rPcEg5A hydrolyzes most of the β-1,4-glucose linkage substrates, suggesting that rPcEg5A acts by cleaving the β-1,4-glucose linkage. The K_m and V_{max} for rPcEg5A on CMC were 3.7 μmol and 20.8 μmol min^{-1} ml^{-1}, respectively. While *P. chrysosporium*

TABLE 1. Substrate specific activity of recombinant endoglucanase 5 (rPcEg5A).

Substrate	Specific activity (U/mg)
Carboxymethylcellulose	694 ± 1.2
β-Glucan	1443.5 ± 3.7
Glucomannan	622.4 ± 2.2
Larminarin	<1.2
Xyloglucan	<11.7 ± 0.15
Birchwood xylan	nd
Arabinan	nd
Arabinoxylan	nd
p-Nitrophenyl-β-glucopyranoside	nd

nd, not detectable.
Data represent the means of three experiments, and average data were recorded.

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Cel12A displayed activity on xylan and mannan, our enzyme rPcEg5A did not hydrolyze these substrates (19). On the other hand, PcGH61D which oxidizes cellulose, produces very less amount of reducing sugars; rPcEg5A acts strongly on cellulose substrates (19), suggesting that rPcEg5A may be the main endoglucanase on *P. chrysosporium* cellulosic degrading enzyme system.

Thin layer chromatography showed that rPcEg5A hydrolyzed β-barley glucan to cellotriose indicating that rPcEg5A is an endoglucanase (Fig. 5). However, the final hydrolysis product of barley β-glucan and NaOH-pretreated barley straw was long-chain oligoglucose which did not separate on LTC plate. Cellobiase which mainly contains cellobiohydrolases and β-glucosidases, catalyzed the conversion of CMC, barley β-glucan and NaOH-pretreated barley straw to glucose units. The combination of rPcEg5A and cellobiase increased the amount of glucose produced.

Synergistic action with cellobiase To evaluate the applicability of rPcEg5A in biomass saccharification, we examined NaOH-pretreated barley straw saccharification with rPcEg5A or the combination of rPcEg5A with cellobiase. The results showed that rPcEg5A produced 2.24 mg of reducing sugar, whereas the commercial cellobiase released 10.4 mg of reducing sugar per 100 mg of substrate. As expected, the combination of rPcEg5A and cellobiase enhanced the release of reducing sugars up to 15.87 mg. This result showed that the combination of rPcEg5A and cellobiase increased 53% total reducing sugars than that after cellobiase treatment alone. HPLC analysis indicated that rPcEg5A released cellobiose as major product while cellobiase produced glucose, xylose, and arabinose from NaOH-pretreated barley straw. The combination of rPcEg5A and cellobiase increased both glucose and xylose sugars. The amount of glucose release by converting peak square indicated that the combination of

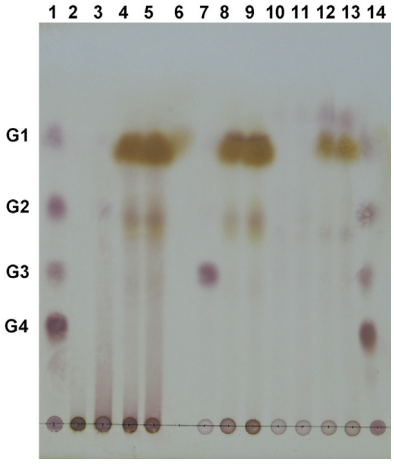

FIG. 5. Thin layer chromatogram of sugars produced during hydrolysis by recombinant endoglucanase 5A (rPcEg5A). Lane 1 and 14 represent glucose (G1), glucobiose (G2), glucotriose (G3) and glucotetraose (G4); lanes 2, 6, and 10 represent CMC, barley β-glucan, and NaOH-pretreated barley straw, respectively; lanes 3, 7, and 11 represent CMC, barley β-glucan, and NaOH-pretreated barley straw degradation by rPcEg5A, respectively; lanes 4, 8, and 12 represent CMC, barley β-glucan, and NaOH-pretreated barley straw degradation by cellobiase, respectively; lanes 5, 9, and 13 represent CMC, barley β-glucan, and NaOH-pretreated barley straw degradation by rPcEg5A and cellobiase, respectively. One percent of CMC and barley β-glucan were incubated with 5 units of each enzyme or a combination in 400 μl of sodium acetate buffer, pH 4.5, 50°C for 10 min, whereas hundred milligrams of NaOH-pretreated barley straw were hydrolyzed using 50 units of each enzyme or a combination in 5 ml of sodium acetate buffer, pH 4.5, 50°C for 16 h. The reaction was terminated by heating at 100°C for 5 min, after which 1 μl of reaction products were taken and subjected to TLC silica gel. The hydrolysis products were separated and visualized on TLC silica gel.

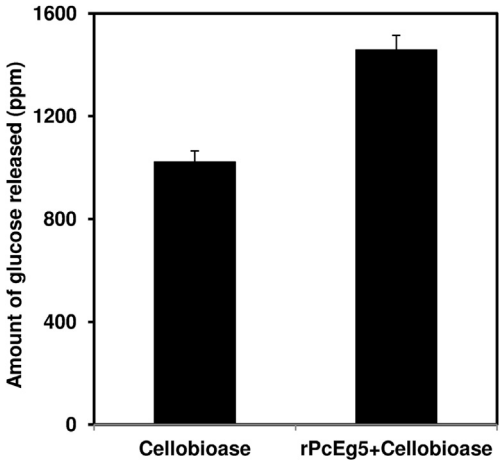

FIG. 6. Amount of glucose released from NaOH-pretreated barley straw using recombinant endoglucanase 5A (rPcEg5A) or cellobiase or a combination of rPcEg5A and cellobiase. The hydrolysis of NaOH-pretreated barley straw was subjected to HPLC for sugar analysis. The peak retention time and area were recorded and compared with standard glucose, and then it was converted into parts per million (ppm). Data represent the means of three experiments, and error bars represent means ± standard errors.

rPcEg5A and cellobiase increased 47% than that after cellobiase treatment alone (Fig. 6).

rPcEg5A is an endo-type enzyme, which cleaves the internal β-1,4-linkage in the cellulose chain; therefore, endoglucanases such as rPcEg5A typically produce less reducing sugars but release more short chain celluloses. On the other hand, cellobiase is a complex enzyme, which includes cellobiohydrolases and β-glucosidases, and it is produced from *A. niger* and catalyzes the short gluco-oligomers to cellobiose and glucose. Thus, cellobiases are very good for glucose production; however, these enzymes may not act on long glucose chains of cellulose, resulting in their limited use in biomass saccharification. Therefore, the addition of an endoglucanase such as rPcEg5A is needed to enhance biomass saccharification. The combination of rPcEg5A and cellobiase increased the release of total reducing sugars by 53% and that of glucose by 47% comparing with cellobiase treatment alone; thus demonstrating the great potential of rPcEg5A for use in the biomass saccharification.

Herein, we reported the characterization of a novel putative endoglucanase GH 5 from *P. chrysosporium*. rPcEg5A displayed high specific activity not only toward CMC but also in natural substrates such as barley glucan and glucomannan than other reported endoglucanases from *P. chrysosporium* (20,23), suggesting rPcEg5A plays an importance role on cellulose utilization of this fungus. Although, the sequence comparison revealed high similar to *H. jecorina* cel5A but rPcEg5A did not depend on of magnesium but highly depended manganese ion, indicating that rPcEg5 mechanism may be differed. rPcEg5 hydrolyzed the β-1,4-glucose linkage, resulting an increase the release of glucose in synergistic with commercial cellobiase (cellobiohydrolases and β-glucosidases), promising a further applicable of this enzyme in biomass saccharification purpose.

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