Heterologous Expression and Characterization of a Cold-Adapted Endo-1,4-β-glucanase Gene from Bellamya chinensis laeta

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Highlights

・ A cold-adapted endo-1,4-β-glucanase from *Bellamya chinensis laeta* (BC-EG70a) was expressed in *Pichia pastoris* GS115.

・ The optimum pH and temperature of rBC-EG70a_mature and rBC-EG70a_CatD were the same (pH 5.5 and 50°C).

・ At lower temperature (30°C), the specific activity of rBC-EG70a_mature toward soluble cellulose was 5 to 20-fold higher than those of *Trichoderma reesei* and *Trichoderma viride* cellulases.

・ rBC-EG70a_CatD demonstrated higher activity than rBC-EG70a_mature in the 10%, 15% and 20% (v/v) ethanol solutions.

・ It was suggested that rBC-EG70a_CatD and rBC-EG70a_mature are able to apply the SSF process.
Cellulose from wood → \textit{saccharification} → fermentation → Bioethanol

- Cold-adapted enzyme: \textit{production efficiency}↑ & \textit{cost}↓
  \[ \text{rBC-EG70a} : \text{5 to 20-fold higher specific activity than Trichoderma cellulases at 30°C} \]

- Pretreatment: 120 °C
- \text{~60 °C} (fungi cellulases)
- 25~30 °C

Simultaneous Saccharification and Fermentation Process
Heterologous Expression and Characterization of a Cold-Adapted Endo-
1,4-β–glucanase Gene from Bellamya chinensis laeta:

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Keywords: Bellamya chinensis; cellulase; cold-adapted enzyme; expression;

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ABSTRACT

An endo-1,4-β-glucanase from *Bellamya chinensis laeta* (BC-EG70a) was expressed in *Pichia pastoris* GS115. The molecular masses of the mature recombinant BC-EG70a enzyme (rBC-EG70a\textsubscript{mature}), the rBC-EG70a catalytic domain (rBC-EG70a\textsubscript{CatD}), and the rBC-EG70a cellulose binding domain (rBC-EG70a\textsubscript{CBD}) were 65 kDa, 50 kDa, and 15 kDa, respectively. While the optimum pH and temperature of rBC-EG70a\textsubscript{mature} and rBC-EG70a\textsubscript{CatD} were pH 5.5 and 50°C, rBC-EG70a\textsubscript{mature} was more stable within a range of pHs and temperatures compared to rBC-EG70a\textsubscript{CatD}. The major hydrolysis products from cellohexaose using rBC-EG70a\textsubscript{mature} and rBC-EG70a\textsubscript{CatD} were cellobiose and cellopentaose during the early stages of the hydrolysis reaction. At lower temperature (30°C), the specific activity of rBC-EG70a\textsubscript{mature} using carboxymethyl cellulose as the substrate was 5 to 20-fold higher than those of *Trichoderma reesei* and *T. viride* cellulases. rBC-EG70a\textsubscript{CatD} demonstrated higher activity than rBC-EG70a\textsubscript{mature} in 10%, 15%, and 20% (v/v) ethanol solutions. rBC-EG70a\textsubscript{CBD} showed specific adsorption toward Avicel, powder cellulose, and phosphoric acid swollen cellulose.
1. **Introduction**

Lignocellulose is composed of carbohydrate polymers (cellulose and hemicellulose) and an aromatic polymer (lignin). Among these components, cellulose is the most abundant renewable bioprotect on earth. The concepts of bioethanol production from lignocellulosic materials, such as wood and agricultural crops, start with pretreatment of the hemicellulose via thermo-chemical hydrolysis. This is followed by saccharification via enzymatic hydrolysis of the cellulose and yeast-based fermentation of the resulting sugar. Lignocellulosic material is traditionally pretreated using alkaline and heat to expose the cellulose, which requires a costly cooling step before enzymatic hydrolysis with fungal cellulases [1, 2]. After the cooling step, the reaction temperature must be kept above 60°C to maintain enzymatic activity during the saccharification process. This high temperature also prohibits adaptation to a simultaneous saccharification and fermentation (SSF) process, where enzymatic hydrolysis is performed together with fermentation. The benefits of the SSF process include reduced end-product inhibition of enzymatic hydrolysis and investment costs. In addition, SSF is capable of producing highly concentrated ethanol (> 20%). Currently, SSF is important in the corn-based ethanol industry in the USA [3] and is an interesting processing option for bioethanol production from lignocelluloses [4]. However, the enzymatic activity of most thermophilic organisms is greatly reduced at the optimal temperature (25–30°C) for the SSF process. Cold-adapted enzymes have great potential in biotechnological fields. Such enzymes have significant advantages in their specific activity at low and moderate temperatures
These properties are useful in biotechnology to reduce energy costs, lower the enzyme concentration required, obtain high yields from reactions involving thermosensitive components, prevent undesirable chemical transformations, and reduce the loss of volatile compounds [6, 7].

We previously reported that *Bellamya chinensis laeta*, an Asian fresh-water snail called the pond snail, showed endo-1,4-β-glucanase (BC-EG70) activity in cold conditions [8], which enables it to purify water and live in conditions below 25°C. The molecular mass of BC-EG70 was estimated as 70 kDa by SDS-PAGE. The enzyme was most active at pH 5.5 and 50°C, and stable at around pH 10 and 50°C. The enzyme exhibited significant activity at 20°C (30% of the activity at optimal 50°C). The enzyme hydrolyzed cellohexaose into cellobiose, cellotriose, and cellotetraose as main products. The deduced proteins consisted of a signal sequence, cellulose binding domain, linker, and catalytic domain. The amino acid sequence of BC-EG70a shares sequence identity with endo-1,4-β-glucanases of *Haliotis discus hannai* (BAC67186.1, 61%), *Ampullaria crossean* (ABD24275.1, 52%), and *Mizuhopecten yessoensis* (AB513362.1, 51%), which all belong to glycoside hydrolase family 9. The cellulose hydrolyzing enzymes produced by *B. chinensis laeta* may exist in the digestive gland, similar to other molluscs. Among snail and clams, purification and characterization of cellulases from *H. discus hannai* [9], *A. crossean* [10], and *Corbicula japonica* [11] have been reported. The enzymes from these molluscs also exhibited activity in cold conditions (< 25°C). There have been reports of molecular cloning of cellulase cDNA fragments from *B. chinensis laeta* [8], *H. discus hannai* [9], and *A. crossean* [10]. However, no information about the expression
of the cellulolytic enzyme gene from *B. chinensis laeta* is currently available. In this study, we describe the expression of the BC-EG70a gene from *B. chinensis laeta* and characterize the BC-EG70a mature enzyme (BC-EG70a\textsubscript{mature}), its catalytic domain (BC-EG70a\textsubscript{CatD}), and its cellulose binding domain (BC-EG70a\textsubscript{CBD}).
2. Materials and Methods

2.1. Chemicals

Avicel was purchased from Asahikasei Co. (Tokyo, Japan), cellulose powder was purchased from ADVANTEC (Tokyo, Japan), and carboxymethyl cellulose (CMC; MW, 135,000) was obtained from Nacalai Tesque Co. (Kyoto, Japan). Cellulases from *Trichoderma reesei* (cat. no., c8546) and *T. viride* (cat. no., 219466) were purchased from Sigma-Aldrich (St. Louis, USA). Phosphoric acid swollen cellulose (PASC) was prepared according to the method of Honga et al. [12].

2.2. Enzyme assay and protein determination

BC-EG70a activity was measured by determining the amount of reducing sugars released from CMC. The enzyme activity required to form reducing sugars corresponding to 1 μmol of glucose per min of reaction time was regarded as one unit of enzyme activity. The reaction mixture consisted of 0.4% CMC in 0.1 M acetate buffer (pH 5.5) and enzyme for a final volume of 0.3 ml. After incubating the reaction mixture for 20 min at 37°C, the amount of reducing sugars contained in the sample was determined according to the standard method of Somogyi-Nelson [13]. The protein concentration was determined using a Micro BCA protein assay kit (Thermo Fisher Scientific, Waltham, USA) with bovine serum albumin as the standard. The protein concentration of BC-EG70a was
calculated using an absorbance of 280 nm and the protein extinction coefficient, according to the method of Gill and von Hippel [14].

2.3. Construction of expression plasmid in Pichia pastoris

We used previously reported PCR primers for BC-EG70a [8] (forward: 5′-GGCTGAAGCTGAATTC[CATCATCATCATCATCAT]GTTAACGTCATCATTCAA AACCACTGGGCT-3′, with the EcoRI site underlined and His x 6 boxed; reverse: 5′-GAGTTTTTGTCTAGAAAACGTTGCACGCACATCTGTGTGTTGGT -3′, with the XbaI site underlined). The synthesized region corresponds to amino acid residues 17–586.

Forward (5′-GGCTGAAGCTGAATTC[TATGGCGACGCCCT]-3′, with the EcoRI site underlined) and reverse PCR primers were used to amplify the regions corresponding to amino acid residues 144–586 of the catalytic domain of BC-EG70a. Forward (5′-GGCTGAAGCTGAATTCGTAAACGTCATCATTCAAAAACCACTGGGCT-3′, with the EcoRI site underlined) and reverse (5′-TAGAAAGCTG[GCGGCCGC]TACACCCTCGACAAAAGCCTC-3′, with the NotI site underlined) PCR primers were used to amplify the region corresponding to amino acid residues 17–112 of the cellulose binding domain of BC-EG70a. BC-EG70a cDNA was prepared according to the previously reported method [8].

All PCR reactions (50 μL) were performed using BC-EG70a cDNA [8], 0.25 μM of each primer, 200 μM of each dNTP, and 1.25 U of Takara PrimeSTAR polymerase
Thermocycling involved 30 cycles at 98°C for 10 sec, 55°C for 15 sec, and 72°C for 60–90 sec. The DNA fragments obtained by PCR were cloned into the EcoRI and XbaI or NotI site of the pPICZαA expression vector (Thermo Fisher Scientific). The nucleotides of the amplified fragments were confirmed by sequencing after ligation. These expression plasmids encoding the mature BC-EG70a, the catalytic domain, and the cellulose binding domain were named pPICZαA-BC-EG70a_{mature}, pPICZαA-BC-EG70a_{CatD}, and pPICZαA-BC-EG70a_{CBD}, respectively.

2.4. Expression and purification of recombinant enzymes

Each expression plasmid (pPICZαA-BC-EG70a_{mature}, pPICZαA-BC-EG70a_{CatD}, and pPICZαA-BC-EG70a_{CBD}) was linearized using SacI and transformed into competent _P. pastoris_ GS115 cells by electroporation. Cells were spread on YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 1.5% agar) containing 100 μg/mL Zeocin and incubated at 28°C for 2–4 days. Colonies were picked and spread onto YPDS plates containing 100 μg/mL, 500 μg/mL, 1000 μg/mL, or 2000 μg/mL of Zeocin. Colonies capable of growth in the presence of a high concentration of Zeocin were then selected. The selected colonies were cultured in a 500 mL Erlenmeyer flask containing 25 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM KH₂PO₄-K₂HPO₄ pH 6.0, 1.34% YNB, 4x10⁻⁵% biotin, 1% glycerol) at 28°C. The culture media was centrifuged at 3000 x g for 5 min and the resulting cell pellets were resuspended in BMMY medium (1% yeast extract, 2% peptone, 100 mM KH₂PO₄-K₂HPO₄ pH 6.0, 1.34% YNB, 4x10⁻⁵%...
biotin, 0.5% methanol). The cell suspension was then added to 200 mL of BMMY medium and grown at 17°C for 7 days at 230 rpm, during which 0.5% methanol was added daily.

**Purification of recombinant BC-EG70a<sub>mature</sub> (rBC-EG70a<sub>mature</sub>):** After cultivation, the culture media was centrifuged at 8200 x g for 10 min at 4°C before the supernatant was recovered. The rBC-EG70a<sub>mature</sub> solution was concentrated using an ultrafiltration membrane (30 kDa MWCO; Pall Co., NY, USA). The concentrated enzyme solution was then loaded onto a HisTrap FF column (1 mL column volume; GE Healthcare, Little Chalfont, Buckinghamshire, UK) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.5 M NaCl. The enzyme was eluted using a linear gradient of imidazole (0–500 mM) in 20 mM Tris-HCl buffer (pH 7.0) containing 0.5 M NaCl. The active fractions after HisTrap FF column chromatography then underwent buffer exchange to 20 mM Tris-HCl buffer (pH 7.0) using a Vivaspin 20 concentrator (30 kDa MWCO; Sartorius Stedim Biotech, Tokyo, Japan) according to the Vivaspin manual (https://www.sartorius.com/mediafile/Appi_Vivaspin-20_Diafiltration-cups_SL-4076-e.pdf).

**Purification of recombinant BC-EG70a<sub>Ca<sub>th</sub></sub> (rBC-EG70a<sub>Ca<sub>th</sub></sub>):** After cultivation, the culture media was centrifuged and the supernatant recovered as described above. The BC-EG70a<sub>Ca<sub>th</sub></sub> solution was concentrated using an ultrafiltration membrane (10 kDa MWCO; Pall Co.). The concentrated enzyme solution was purified using the same method as above for rBC-EG70a<sub>mature</sub> except for using a Vivaspin 20 concentrator (10 kDa MWCO; Sartorius Stedim Biotech).
Purification of recombinant BC-EG70aCBD (rBC-EG70aCBD): After cultivation, the culture media was centrifuged and the supernatant recovered as described above. The rBC-EG70aCatD solution was concentrated using an ultrafiltration membrane (10 kDa MWCO; Pall Co.). The concentrated protein solution was purified using the same method as above for rBC-EG70amature except for using a Vivaspin 20 concentrator (10 kDa MWCO; Sartorius Stedim Biotech).

2.5. Effects of pH and temperature on enzymatic activity

Purified BC-EG70amature and BC-EG70aCatD enzyme solutions (0.12 U/mL) were used to determine the optimum pH and temperature for enzyme activity. Enzyme activity was measured using the standard assay method with 0.4% CMC as the substrate at various pHs and temperatures. The buffer systems used were 0.1 M sodium acetate buffer (pH 3.5–6.0), 0.1 M KH2PO4-K2HPO4 buffer (pH 6.0–8.0), 0.1 M Tris-HCl buffer (pH 8.0–9.0), and 0.1 M glycine-NaOH buffer (pH 9.0–11.0). The effect of temperature on enzyme activity was examined between 10°C and 70°C.

2.6. Effects of pH and temperature on enzyme stability

The effects of pH on enzyme stability were determined by incubating the BC-EG70amature and BC-EG70aCatD (0.12 U/mL) for 30 min at 37°C in the following 0.1 M buffers: sodium acetate buffer (pH 3.0–6.0), KH2PO4-K2HPO4 buffer (pH 6.0–8.0), Tris-
HCl (pH 8.0–9.0), and glycine-NaOH buffer (pH 9.0–11.0). To measure thermal stability, the purified enzymes were incubated in 20 mM Tris-HCl buffer (pH 7.0) for 30 min at various temperatures ranging between 10°C and 70°C. After incubation, the remaining activity was measured under standard assay conditions.

2.7. Molecular mass

The molecular mass was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli [15], with the Unstained Protein Molecular Weight Marker (Biorad Co., CA, USA). Protein bands were detected by staining with Coomassie Brilliant Blue R-250.

2.8. Substrate specificity

The substrate specificity of purified rBC-EG70amature and rBC-EG70aCatD were tested using polymers containing β-glycosidic bonds, such as Avicel, powdered cellulose, and CMC. In each case, enzyme activity was measured by calculating the production of reducing sugars. The average value of triplicate measurements was used as each activity value.

2.9. High-performance liquid chromatography (HPLC) analysis of hydrolysis products from cello-oligosaccharides
To identify the cleavage patterns of the hydrolysis products of the purified enzymes, the enzyme reactions were performed using a final concentration of 2.5 mM cello-oligosaccharide substrates (C2-6; Megazyme Inc., Chicago, USA) in 20 mM sodium acetate buffer (pH 5.0), and several microliters of the enzyme solution (0.1–0.2 μM). After the enzyme reaction was carried out at 37°C for an appropriate period, a portion of the reaction mixture was withdrawn and boiled at 100°C for 5 min to terminate the enzymatic reaction. The solution was mixed with 100% acetonitrile (final concentration, 70%). The resultant solution was applied to HPLC using a Sugar-D column (4.6 x 250 mm; Nacalai Tesque Co.). The elution was conducted with 70% acetonitrile at a flow rate of 1.0 ml/min, and the substrates and products were monitored by RI (Jasco Co., Tokyo, Japan). From the peak area obtained by HPLC, oligosaccharide concentrations at each reaction time were calculated using a standard curve obtained with an authentic saccharide solution.

2.10. rBC-EG70aCBD binding assay

Binding assay mixtures containing rBC-EG70aCBD (A_{280} = 0.36) and 10 mg of insoluble substrate in 0.5 ml of 20 mM acetate buffer (pH 5.0) were incubated at 4°C for 4 h with occasional mixing. Each mixture was centrifuged at 4°C for 20 min at 15,000 g to separate the supernatant and substrate with bound protein. The supernatant containing free protein was collected and the protein concentration was determined at an absorbance
of 280 nm. The amount of bound protein was calculated from the difference between the initial protein concentration and the free protein concentration after binding.

2.11. Effects of ethanol concentration on enzyme activity

Purified BC-EG70\textsubscript{amature} and BC-EG70\textsubscript{acatD} (0.12 U/mL) enzyme solutions were used to evaluate the effect of ethanol concentration on enzyme activity. Enzyme activities were measured at standard assay conditions with 0.4% CMC as the substrate with various concentrations of ethanol (approximately 20% v/v).
3. Results and Discussion

3.1. Expression of BC-EG70a\textsubscript{mature} and BC-EG70a\textsubscript{CatD} in \textit{P. pastoris} and characterization of the recombinant enzymes

The recombinant enzymes, rBC-EG70a\textsubscript{mature} and rBC-EG70a\textsubscript{CatD}, were expressed in \textit{P. pastoris} GS115 harboring pPICZ\textalpha{}A-BC-EG70a\textsubscript{mature} and pPICZ\textalpha{}A-BC-EG70a\textsubscript{CatD}, respectively, and successfully expressed as active forms. Based on SDS-PAGE analysis, the molecular masses of purified rBC-EG70a\textsubscript{mature} and rBC-EG70a\textsubscript{CatD} were estimated to be 65 kDa and 50 kDa, respectively (Fig. 1A, 1B). The molecular mass of the mature enzyme was similar to that of cellulase (66 kDa) from \textit{H. discus hannai} \cite{9} and endo-1,4-\beta-glucanase (65 kDa) from \textit{A. crossean} \cite{10}. In our previous paper, the molecular mass of truncated BC-EG70 was found to be 50 kDa \cite{8}, which is almost the same as that of rBC-EG70a\textsubscript{CatD}. Yin et al. also reported that the carbohydrate binding module (CBM) of EG65b from \textit{A. crossean} is cleaved off during the process of enzyme secretion \cite{10}. The calculated molecular mass of BC-EG70a in the expression plasmid pPICZ\textalpha{}A-BC-EG70a\textsubscript{mature} is approximately 65 kDa, which is similar to the molecular mass of BC-EG70a calculated from the amino acid sequence. Furthermore, BC-EG70a does not contain the consensus sequence for N-glycosylation (Asn-X-Ser/Thr, where X is any amino acid except Pro), suggesting a lack of N-glycosylated sugar chains.

The functional properties of the purified enzymes were determined by enzymatic assays that used CMC as the substrate. The effects of pH and temperature on the recombinant enzymes in the present study were very similar to those of the native enzyme.
The optimum pH of the rBC-EG70mature and rBC-EG70CatD enzymes was noted at pH 5.5 (Fig. 2A). The activity of the mature enzyme was stable at pH 5.0–9.0 (Fig. 3B), while the activity of rBC-EG70CatD was stable at pH 5.0–8.0 (Fig. 3B). Similar optimum pH values have been reported for other endo-1,4-β-glucanases from *Eisenia fetida* [15], *Nasutitermes takasagoensis* [17], *Reticulitermes speratus* [18], *Teleogryllus emma* [19], and *A. crossean* [20]. The optimum pH of endo-1,4-β-glucanase from *H. discus* was found to be pH 6.3 [9].

The optimal temperature for rBC-EG70mature and rBC-EG70CatD was observed at 50°C (Fig. 2C), and the enzymes exhibited significant activity even at 20°C (30–50% of the activity observed at the optimal of 50°C). The mature enzyme was stable up to 50°C, while rBC-EG70CatD became unstable above 40°C (Fig. 2D). Cellulases from *A. crossean* [20], *E. fetida* [16], *Strongylocentrotus nudus* [21], and *T. emma* [19] also exhibited significant activity at lower temperatures. In addition, the endo-1,4-β-glucanases from invertebrates are cold-adapted enzymes, as demonstrated by their ability to perform in low-temperature environments, and their baseline metabolism can be maintained at low temperatures without significant decrease in activity.

Delignification of wood samples promotes solubility as in the case of hemicellulose, but delignified cellulose remains insoluble [22]. To hydrolyze delignified cellulose using cellulase, pretreatment such as acid, NaOH, and oxidation are efficient [23]. In particular, cellulose pretreated with acid was well solubilized in water, and significantly increased the production of reducing sugars during the hydrolysis process using an enzymatic cocktail of cellulases and hemicellulases [23]. The optimum operating temperature of the
SSF process is around 30°C. We compared the specific activities toward soluble cellulose of rBC-EG70\textsubscript{mature}, as well as \textit{T. reesei} and \textit{T. viride} cellulases, at this temperature. The specific activity of rBC-EG70\textsubscript{mature} (5.39 U/mg protein) was found to be 5 to 20-fold higher than those of \textit{T. reesei} (1.05 U/mg protein) and \textit{T. viride} (0.27 U/mg protein) cellulases at 30°C (Fig. 4). If the solubilized cellulose is applied during the saccharification process at lower temperature, cellulase from \textit{B. chinensis laeta} is more suitable than those of fungi.

We previously crystalized the cold-adapted Ef-EG2 from \textit{E. fetida} [24] and reported that the highly negatively-charged surface of Ef-EG2 contributes to its cold adaptation. The increase in the negative surface charge has also been described for cold-adapted cellulase belonging to glycoside hydrolase (GH) family 5 from the Antarctic bacterium \textit{Pseudoalteromonas haloplanktis} [25]. To understand the cold-adapted mechanism of BC-EG70\textsubscript{a}, we intend to clarify the crystal structure in the near future.

### 3.2. Substrate Specificity

The enzyme activity of rBC-EG70\textsubscript{mature} was 6.8 U/mg protein with CMC as the substrate. rBC-EG70\textsubscript{mature} was also active against insoluble (PASC) and crystalline (Avicel) cellulose. This is the first report of Avicel hydrolysis among the GH family 9. In contrast, rBC-EG70\textsubscript{CatD} was not active against powdered cellulose and Avicel, and the native BC-EG70 did not show activity against Avicel [8], where the purified native enzyme is thought to lack the cellulose binding domain (CBD). The activity of rBC-
EG70a<sub>mature</sub> toward PASC was 2-fold higher than that of rBC-EG70a<sub>catD</sub> (data not shown). The CBD is considered to play an important role in the hydrolysis of insoluble cellulose.

### 3.3. Hydrolysis Products

To clarify the mode of action of purified rBC-EG70a<sub>mature</sub> and rBC-EG70a<sub>catD</sub>, enzymatic assays were conducted with 1,4-β-linked oligosaccharides of various lengths, namely cellobiose (C<sub>2</sub>), cellotetraose (C<sub>4</sub>), cellopentaose (C<sub>5</sub>), or cellohexaose (C<sub>6</sub>), and aliquots collected over time were analyzed by HPLC (Fig. 3). The major hydrolysis products from C<sub>6</sub> were cellobiose (C<sub>2</sub>) and C<sub>4</sub> during the early stages of the hydrolysis reaction, suggesting that C<sub>4</sub> is further degraded to C<sub>2</sub> (Fig. 3D, 3H). The major hydrolysis products from C<sub>5</sub> were C<sub>2</sub> and C<sub>3</sub>, and the minor products were glucose (C<sub>1</sub>) and C<sub>4</sub> (Fig. 3C, 3F). The major hydrolysis product from C<sub>4</sub> was C<sub>2</sub>, and the minor products were C<sub>3</sub> and C<sub>1</sub> (Fig. 3B, 3G). The major hydrolysis products from C<sub>3</sub> were C<sub>1</sub> and C<sub>2</sub> (Fig. 3A, 3E). From these results, this enzyme can be considered an endo-1,4-β-glucanase.

During cello-oligosaccharide hydrolysis, similar products were obtained from rBC-EG70a<sub>mature</sub> and rBC-EG70a<sub>catD</sub>. When C<sub>6</sub>, C<sub>5</sub>, C<sub>4</sub>, and C<sub>3</sub> were digested with rBC-EG70a<sub>mature</sub> and rBC-EG70a<sub>catD</sub>, C<sub>6</sub> (mature, 3.15 mM/min/mg protein; catD, 3.02 mM/min/mg protein) showed higher specific activity than C<sub>5</sub> (mature, 1.9 mM/min/mg protein; catD, 1.96 mM/min/mg protein), C<sub>4</sub> (mature, 9.65x10<sup>-1</sup> mM/min/mg protein; catD, 4.73x10<sup>-1</sup> mM/min/mg protein), and C<sub>3</sub> (mature, 2.25x10<sup>-3</sup> mM/min/mg protein; catD, 2.0x10<sup>-3</sup> mM/min/mg protein) during the early stages of hydrolysis (Fig. 3).
was almost no difference in the specific activities of rBC-EG70a\textsubscript{mature} and rBC-EG70a\textsubscript{catD}, suggesting that rBC-EG70a requires at least six or more subsites for efficient hydrolysis. For comparison, it was reported that EG1 from \textit{T. reesei} QM9414 possesses four subsites [26] and the endo-β-1,4-glucanase (CMCax) from \textit{Acetobacter xylinum} possesses five subsites [27].

3.4. \textit{Effect of ethanol concentration on enzyme activity}

rBC-EG70a\textsubscript{catD} demonstrated higher activity than rBC-EG70a\textsubscript{mature} in the 10%, 15% and 20% (v/v) ethanol solutions (Fig. 5), suggesting that rBC-EG70a\textsubscript{catD} has a more stable structure than rBC-EG70a\textsubscript{mature}. Cellulases from \textit{T. reesei} [28] and \textit{Fusarium oxysporum} [29] have also been reported to demonstrate moderate activity inhibition by ethanol. These results indicate that rBC-EG70a\textsubscript{CatD} and rBC-EG70a\textsubscript{mature} are also applicable to the SSF process.

3.5. \textit{Adsorption specificity of rBC-EG70a\textsubscript{CBD}}

The adsorption specificity of rBC-EG70a\textsubscript{CBD} was determined based on enzyme adsorption to PASC, Avicel, and powdered cellulose, but not to chitin, starch, and curdlan. This is consistent with the previously reported adsorption ability of the CBM from \textit{A. crossean} against Avicel and PASC [30]. Thus, we could confirm the specificity of rBC-EG70a\textsubscript{CBD} to cellulose.
Conflict of interest

The authors declare that there are no conflicts of interest.

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References


Legends

Figure 1. SDS-PAGE analysis of (A) purified rBC-EG70a_mature, (B) rBC-EG70a_catD, and (C) purified rBC-EG70a_CBD. M: Precision Plus Protein All Blue Standard (Biorad, USA), 1: culture supernatant, 2: culture supernatant concentrated using a MinimateTM TFF Capsule (Pall Co., NY, USA) and 3: purified enzyme and CBD solutions.

Figure 2. Functional properties of purified rBC-EG70a_mature and rBC-EG70a_catD. All reactions were conducted using the purified enzyme and CMC as the substrate. Solid lines represent purified rBC-EG70a_mature and dotted lines represent purified rBC-EG70a_catD. (A) Effects of pH on enzyme activity at 37°C in the following 0.1 M buffers: sodium acetate (pH 3.5–6.0), KH₂PO₄-K₂HPO₄ (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 9.0–11.0). (B) Effects of pH on enzyme stability. Assays were conducted at 37°C (pH 5.5) after a 30 min incubation in the following 0.1 M buffers: sodium acetate (pH 3.5–6.0), KH₂PO₄-K₂HPO₄ (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 9.0–11.0). (C) Effects of temperatures ranging from 10–70°C on enzyme activity. (D) Effects of temperature on enzyme stability. Assays were conducted at 37°C after 30 min incubation in 0.1 M Tris-HCl buffer (pH 7.0) at 20–70°C. Each data point represents the average of triplicate measurements. Error bars show standard deviation of the measurements.
Figure 3. High-performance Liquid Chromatography (HPLC) analysis of hydrolysis products from cello-oligosaccharides (C3 to C6) by (A–D) rBC-EG70ₐₘₐₜₐₜ and (E–H) rBC-EG70ₐₖₐₜₜ. The enzymatic degradation products of (A) and (E): cellotriose; (B) and (F): cellotetraose; (C) and (G): cellopentaose; and (D) and (H): cellohexaose were detected by HPLC. Lines: glucose (C1, dark blue); cellobiose (C2, red); cellotriose (C3, yellowish green); cellotetraose (C4, purple); cellopentaose (C5, light blue), and cellohexaose (C6, orange).

Figure 4. Comparison of optimum temperatures of rBC-EG70ₐₘₐₜₐₜ, Trichoderma reesei, and T. viride. Enzyme activity was measured at temperatures ranging from 10–70°C using CMC as the substrate. The blue, red, and yellowish green lines show the relative activity of rBC-EG70ₐₘₐₜₐₜ, T. reesei, and T. viride, respectively. Each data point represents the average of triplicate measurements. Error bars show standard deviation of the measurements.

Figure 5. Effects of ethanol concentration on enzyme activity. Enzyme activity was measured using the standard assay method with 0.4% CMC as the substrate at various concentrations of ethanol (approximately 20% v/v). The dotted line represents rBC-EG70ₐₘₐₜₐₜ, the solid line represents rBC-EG70ₐₖₐₜₜ, and the black dotted line shows 100%. Each data point represents the average of triplicate measurements. Error bars show standard deviation of the measurements.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Adsorption ratio (%)</th>
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<tbody>
<tr>
<td>Avicel</td>
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</tr>
<tr>
<td>Powdered cellulose</td>
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<tr>
<td>PASC</td>
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<td>Raw starch</td>
<td>ND</td>
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<td>curdlan</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND : Not detected

Each data represents the average of triplicate measurements.
Fig. 1  Ueda M. et al.

(A) rBC-EG70a_{mature}

(B) rBC-EG70a_{catD}

(C) rBC-EG70a_{CBD}
Fig. 2 Ueda M. et al.
rBC-EG70a\textsubscript{mature}: (A)-(D)

Fig. 3 Ueda M. et al.
Fig. 3 Ueda M. et al.
Fig. 4  Ueda M. et al.
Fig. 5 Ueda M. et al.