Carbohydrate Polymers 101 (2014) 511–516

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Cloning and expression of the cold-adapted endo-1,4-β-glucanase gene from *Eisenia fetida*

Mitsuhiro Ueda[a,*], Akihiro Ito[a], Masami Nakazawa[a], Kazutaka Miyatake[a], Minoru Sakaguchi[b], Kuniyo Inouye[c]

[a] *Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan*
[b] *Laboratory of Cell Biology, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan*
[c] *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form 5 September 2013
Accepted 16 September 2013
Available online 25 September 2013

Keywords:
Cloning
Expression
Endo-1,4-β-glucanase
Eisenia fetida
Cold-adapted enzyme

ABSTRACT

Biofuel production from plant-derived lignocellulosic material using fungal cellulases is facing cost-effective challenges related to high temperature requirements. The present study identified a cold-adapted cellulase named endo-1,4-β-glucanase (EF-EG2) from the earthworm *Eisenia fetida*. The gene was cloned in the cold-shock expression vector (pCold I) and functionally expressed in *Escherichia coli* ArcticExpress RT (DE3). The gene consists of 1368 bp encoding 456 amino acid residues. The amino acid sequence shares sequence homology with the endo-1,4-β-glucanases of *Eisenia andrei* (98%), *Pheretima hilgendorfi* (79%), *Perineresis brevicirris* (63%), and *Strongylocentrotus nudus* (58%), which all belong to glycoside hydrolase family 9. Purified recombinant EF-EG2 hydrolyzed soluble cellulose (carboxymethyl cellulose), but not insoluble (powdered cellulose) or crystalline (Avicel) cellulose substrates. Thin-layer chromatography analysis of the reaction products from 1,4-β-linked oligosaccharides of various lengths revealed a cleavage mechanism consistent with endoglucanases (not exoglucanases). The enzyme exhibited significant activity at 10 °C (38% of the activity at optimal 40 °C) and was stable at pH 5.0–9.0, with an optimum pH of 5.5. This new cold-adapted cellulase could potentially improve the cost effectiveness of biofuel production.

1. Introduction

The development of renewable energy sources has become increasingly important to cope with the fast growing world population and the limited crude oil supplies (Schiffer, 2008). While the production of ethanol from corn or cane juice has raised controversy due to its effect on food costs, this study paves the way for the development of more cost-effective processes involving the digestion of plant-derived lignocellulosic wastes, such as pulp and paper (Dashtban, Schraft, & Qin, 2009). Lignocellulose is composed of cellulose, hemicellulose, and lignin. Among them, cellulose is the most abundant organic molecule on earth. Fungi, bacteria, and invertebrates produce enzymes named cellulases that are capable of degrading the linear biopolymers of anhydroglucopyranose connected by 1,4-β-glycosidic bonds into sugars for conversion to bioethanol by fermentation.

Three types of cellulases function in concert to hydrolyze cellulose into sugars. First, 1,4-β-glucan cellobiohydrolase (exoglucanases; EC 3.2.1.91) degrades cellobiosyl units from the ends of the cellulose polymer. Second, endo-1,4-β-glucanase (endo-glucanases; EC 3.2.1.4) randomly degrades internal 1,4-β-glycosidic bonds into cello-oligosaccharides of various lengths. Third, 1,4-β-glucosidase (EC 3.2.1.21) generates glucose from the cleavage of cello-oligosaccharides by a process named saccharification. Traditionally, the lignocellulosic material is pretreated using alkaline and heat to expose the cellulose, which requires a costly cooling step before hydrolysis (Ingram et al., 2011; Kovacs, Macrelli, Szakacs, & Zacchi, 2009). However, the cooling step can be avoided by the use of thermophilic fungal species, such as *Sporotrichum thermophile* and *Thielavia terrestris* (Dashtban et al., 2009). Unfortunately, the activity of most thermophilic organisms is lower than that of mesophilic fungi, which would also interfere with production efficiency.

It is likely that the cold-adapted enzymes have a great potential value for biotechnological aspects. The enzymes have significant advantages at the level of the specific activity, lower stability, and unusual specificity (Gerday et al., 2000). Some of the

Abbreviations: EF-EG2, endo-1,4-β-glucanase from *Eisenia fetida*; CMC, carboxymethyl cellulose; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; GH, glycoside hydrolase; dNTP, deoxynucleotide triphosphate.

* Corresponding author. Tel.: +81 72 254 9468; fax: +81 72 254 9468.
E-mail address: mueda@biochem.osakafu-u.ac.jp (M. Ueda).

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http://dx.doi.org/10.1016/j.carbpol.2013.09.057

obvious applications for cold-adapted enzymes include the industries and processes such as detergent additives, textile industry, food industry, and bioremediation. A well known enzymes, such as proteases, lipases, α-amylases, and cellulases, are able to use as additive in detergent. The benefits of cold washing using cold-adapted enzymes were reduced in the energy consumption and in unnecessary wear and tear.

A recent study showed that the earthworm *Eisenia fetida* expresses a cold-adapted carboxymethylcellulase (EF-CMCase25) significantly active at lower temperatures (Ueda et al., 2010). This enzyme forms a complex with β-glucosidase, 1,3-β-glucanase, and β-xylosidase to efficiently hydrolyze intractable cellulosic and hemicellulosic substrates of plant cell walls. Therefore, cold-adapted enzymes could potentially decrease the energy cost of hydrolysis for the bioenergy industry. However, information about cellulase genes and cold-adapted cellulolytic enzymes from *E. fetida* is currently limited. The present study shows that *E. fetida* functionally expresses a cold-adapted endo-1,4-β-glucanase (EF-EG2) that could be developed as a cost-effective industrial biocatalyst.

2. Materials and methods

2.1. Isolation of total RNA and cDNA synthesis

E. fetida earthworms were obtained from Nagane Industry (Sapporo, Japan), and only those at nearly the same age were used in the experiments. The earthworms were washed, kept on wet filter papers, and starved for 24 h at 20 °C. Next, they were freeze-dried and grinded to a fine powder using a mortar and pestle. Total RNA was extracted using Isogen II (Nippon Gene, Japan) according to the manufacturer's instruction. First-strand cDNA was synthesized using an oligo(dt)17 adapter primer (GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT) and Superscript II reverse transcriptase (Invitrogen, USA), according to the manufacturer's instructions.

2.2. Cloning of the EF-EG2 gene

Since *E. fetida* and *Eisenia andrei* are closely related earthworm species, we used the sequence of endo-1,4-β-glucanase from *E. andrei* (EA-EG2; GenBank No. EU315749) to conduct cDNA cloning of EF-EG2. Forward (5′-ATGGCGACACGATTGATGATGCTGCTG-3′) and reverse (5′-TCACTTGCCGTCTCTCAGCTGAATGGC-3′) primers were synthesized on regions corresponding to amino acids 1–9 and 449–456, respectively. The reaction mixture (50 μl Ex Taq buffer) contained *E. fetida* cDNA (100 ng), 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate (dNTP), and 1.25 U EX Taq Hot Start DNA polymerase (Takara Bio, Kyoto, Japan). Thermocycling consisted of 30 cycles at 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 90 s. The resulting 1.4-kb DNA fragment was cloned into the pGEM-T-easy vector (Promega, Fitchburg, WI, USA), and the nucleotide sequence of the amplified fragment was confirmed by sequencing.

2.3. Construction of the expression plasmid

The forward (5′-CGC CAT ATG GGT CAA TAT AAT TAT GAC GAA GTT CTG-3′, *Nde*I site underlined) and reverse (5′-TGC TCT AGA CTT GCC GTC TCT CAG CTG CTG AAT G-3′, *Xba*I site underlined) primers were synthesized on regions corresponding to amino acid residues 21–29 and 449–456 of EF-EG2, respectively. The PCR reaction mixture (50 μl Prime STAR buffer) contained *E. fetida* cDNA, 0.25 μM of each primer, 200 μM of each dNTP, and 1.25 U of Takara Prime STAR DNA polymerase (Takara Bio, Kyoto, Japan). Thermocycling consisted of 25 cycles at 98 °C for 10 s, 60 °C for 5 s, and 72 °C for 90 s. The resulting 1.4-kb DNA fragment was cloned into the pGEM-T-easy vector according to the manufacturer's instructions. The pGEM-T-easy vector containing the DNA fragment was treated with the restriction enzymes *Nde*I and *Xba*I, and the DNA fragments were purified by agarose gel electrophoresis. The DNA fragments and pCold I vector (Takara Bio, Kyoto, Japan) were mixed and ligated with T4 DNA ligase (Nippon Gene, Tokyo, Japan). This expression plasmid coding a matured EF-EG2 was named pCo_EF-EG2.

2.4. Expression and purification of recombinant enzyme

The expression plasmid pCo_EF-EG2 was transformed into *Escherichia coli* ArcticExpress RT (DE3) co-expressing the chaperon system Cpn60/10 from *Oleispira antarctica* (Agilent Technologies, Santa Clara, CA, USA). The transformed bacteria were cultured at 37 °C in 400 ml LB medium containing ampicillin (final concentration 100 μg/mL) till an optical density of 0.4 at 600 nm was reached. Next, 1 mM isopropyl thio-β-D-galactopyranoside (Nakarai Tesque, Kyoto, Japan) was added to induce protein expression at 13 °C overnight. The cultures were harvested by centrifugation (9000 × *g*, 20 min, 4 °C). The cells were resuspended in 40 ml of 20 mM Tris–HCl buffer (pH 7.0) containing protease inhibitor cocktail (catalog No. 03969-21, Nakarai Tesque, Kyoto, Japan), sonicated, and centrifuged (9000 × *g*, 20 min, 4 °C). The supernatant was applied to a Ni-Sepharose 6 FF column (1 cm × 7 cm) (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and equilibrated with 50 mM phosphate buffer (pH 8.0) containing 5 mM imidazol and 300 mM NaCl. EF-EG2 was eluted over 5–300 mM imidazol gradient (60 min; 1 ml/min). The active fractions were desalted on a PD-10 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and then loaded onto a Resource Q column (column volume: 6 ml) (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and equilibrated with 20 mM Tris–HCl (pH 7.0). The enzyme was eluted with linear gradient of NaCl (0–1.0 M) in 20 mM Tris–HCl (pH 7.0) at a flow rate of 1 ml/min. Active fractions were pooled and used as the purified enzyme solution.

2.5. Enzyme and protein assay

The activity of EF-EG2 was detected by the release of reduced sugar from carboxymethyl cellulose (CMC; Nakarai Tesque, Kyoto, Japan), as previously described (Ueda et al., 2010). The assays of the purified enzyme with powdered cellulose and Avicel (Funakoshi, Tokyo, Japan) were conducted using the same procedure as the standard assays. The amount of enzyme activity required to form an amount of reducing sugar corresponding to 1 μmol of glucose per min of reaction time was regarded as one unit of enzyme activity. Protein concentration of EF-EG2 protein was calculated using absorbance at 280 nm and the protein extinction coefficient, according to the method of Gill and von Hippel (1989).

2.6. Effects of pH and temperature on enzyme activity and enzyme stability

The activity of EF-EG2 was measured over a range of pH and temperatures with CMC as the substrate. The buffer systems were 0.1 M sodium acetate (pH 3.5–6.0), 0.1 M sodium phosphate (pH 6.0–8.0), and 0.1 M Tris–HCl (pH 8.0–9.0). The effect of temperature on enzyme activity was examined at 10–60 °C.

The effect of pH on the stability of EF-EG2 activity was examined by incubating the enzyme for 30 min at 37 °C in 0.1 M of the following buffers: sodium acetate (pH 3.5–6.0), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.0–9.0), and glycine–NaOH (pH 9.0–12.0). After the incubation, the remaining activity was measured under the standard assay conditions. To measure thermal stability, the enzyme was incubated in 0.1 M acetate (pH 6.0) for 30 min at various temperatures (10–60 °C). After the incubation, the

remaining activity was measured under the standard assay conditions.

2.7. Substrate specificity

Standard enzyme assays were conducted to test the specificity of EF-EG2 for polymers containing β-glycosidic bonds, such as Avicel, cellulose powder, and CMC. In each case, enzyme activity was measured by the production of reduced sugars.

2.8. Thin-layer chromatography (TLC)

The mode of action of EF-EG2 was investigated by TLC of the reaction products generated by hydrolysis of various 1,4-β-linked oligosaccharides. The reactions were conducted in 0.1 M acetate buffer (pH 5.5), containing 50 μl enzyme (0.47 U/ml) and 1 mg/ml 1,4-β-linked oligosaccharides (cellotriose, cellotetraose, cellopentaose, or cellohexaose). The reactions were conducted at 25 °C, and samples collected after 0–200 min were identified by TLC, as previously described (Kusuda, Nagai, Hur, Ueda, & Terashita, 2003; Ueda et al., 2010).

2.9. Molecular mass and N-terminal amino acid sequence

The molecular mass was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970) using the Precision Plus Protein™ Unstained Standards (Bio-Rad Laboratories, Inc., CA, USA). Protein bands were detected by Coomassie Brillant Blue (CBB) R-250 staining. Protein band pattern analysis was conducted using the ImageJ software.

Proteins separated by SDS-PAGE were transferred onto a polyvinylidene fluoride membrane. The membrane was washed extensively with water, stained with 0.25% CBB R-250, 5% aqueous methanol, and 7.5% acetic acid for 5 min, and then de-stained with 90% aqueous methanol for 10 min. A portion of the membrane containing the target protein band was cut out, and the protein was extracted from the membrane. The N-terminal amino acid sequence was determined with an automated protein sequencer, as previously described (Ueda et al., 2008; Ueda, Asano, Nakazawa, Miyatake, & Inouye, 2008).

2.10. Nucleotide sequence accession number

The *E. fetida* EF-EG2 mRNA data reported in the present paper have been submitted to the DDBJ, EMBL, and NCBI databases under the accession number AB679653.

3. Results and discussion

3.1. Cloning and sequence analysis of EF-EG2 gene

The present study shows that endo-1,4-β-glucanase is functionally expressed by the earthworm species *E. fetida*. The length of the EF-EG2 gene was determined to be 1368 bp, encoding for a protein of 456 amino acids. The mRNA sequence of EF-EG2 has been deposited in the GenBank database (AB679653). The amino acid sequence of EF-EG2 shows similarity with endo-1,4-β-glucanases of *E. andrei* (98%; EU31579), *Pheretima hilgendorfi* (79%; AB452993), *Perineresis nuntia brevicirris* (63%; AB558290), *Strongylocentrotus nudus* (58%; AB282677), *Nematostella vectens* (58%; XP_001640312), and *Ampullaria crossen* (54%; FJ716619). The fact that these invertebrate 1,4-β-glucanases all belong to the glycoside hydrolase (GH) family 9, suggests that EF-EG2 may also belong to this enzyme family. In addition, all catalytically important residues of endo-1,4-β-glucanase of the GH family 9 (Nishida et al., 2007) were conserved in EF-EG2 (Asp74, Asp77, His378, Asp422, and Glu431), as shown for *E. andrei* and *P. hilgendorfi* (Fig. 1). The structure of EF-EG2 contains a signal peptide and a catalytic domain, as reported for the endo-1,4-β-glucanases of *P. hilgendolfi, S. nudus, and Teleogryllus emma* (Kim et al., 2008; Nishida et al., 2007; Nozaki, Miura, Tozawa, & Miura, 2009). EF-EG2 exhibited high activity against soluble substrate, but no activity against insoluble (powdered cellulose) or crystalline cellulose (Avicell) substrate containing 1,4-β-glycoside bond. On the other hand, the endo-1,4-β-glucanases of *Ampullaria crossean* and *H. discus* are modular enzymes composed of a signal sequence, a cellulose binding module, a linker, and a catalytic domain (Li, Yin, Ding, & Zhao, 2009; Suzuki, Ojima, & Nishita, 2003). In the case of endo-1,4-β-glucanase of *A. crossean*, the enzyme exhibited hydrolytic activities against both CMC and Avicell. It was suggested that endo-1,4-β-glucanases of GH family 9 require the cellulose binding domain to hydrolyze the insoluble and crystalline cellulose substrates.

3.2. Expression of EF-EG2 in E. coli ArcticExpress

The mature active form of EF-EG2 (catalytic domain of EF-EG2) was successfully expressed in *E. coli* ArcticExpress co-expressing the chaperon system Cpn60/10 of *O. antarctica*. 1,4-β-Glucanase activity of the recombinant crude enzyme solution was determined to be 1.0 U/ml culture. The recombinant enzyme purified in this study was from *E. coli* ArcticExpress RT harboring pCo_EF-EG2. Based on SDS-PAGE analysis, the molecular mass of the purified recombinant enzyme was estimated to be 50 kDa (Fig. 2). Protein band pattern analysis indicated that EF-EG2 accounted for 64% of all bands. Partially purified enzyme contained chaperonine proteins (Cpn60/10), corresponding to the 60 kDa band on the SDS-PAGE. Cpn60/10 could not be removed using the protocol of Joseph and Andreotti (2008). The recombinant enzyme was purified 136-fold, with a recovery of 1.15%. A total of 41.2 μg protein was harvested from the cell-free extract of *E. coli* harboring pCo_EF-EG2 (400 ml culture broth).

3.3. Properties of recombinant EF-EG2

The functional properties of purified recombinant EF-EG2 were determined by enzyme assays with CMC as the substrate. The optimum pH of EF-EG2 was found to be 5.5 (Fig. 3A, Table 1). Its activity was stable at 60–65% of maximal value at pH 6.0–9.0 (Fig. 3B). Similar optimum pH values have been reported for other types of cellulases, namely EF-CMCase25 (Ueda et al., 2010), *Nasutitermes takasagoensis* (Tokuda, Watanabe, Matsumoto, & Noda, 1997) *Reticulitermes speratus* (Watanabe et al., 1997), *T. emma* (Kim et al., 2008), and *A. crossean* (Li et al., 2009). In contrast, endo-1,4-β-glucanases exhibit optimum pH values ranging from 6.3 to 7.5, namely *Haliotis discus hannai* (Suzuki et al., 2003), and *S. nudus* (Nishida et al., 2007).

The optimal temperature of EF-EG2 was identified as 40 °C (Fig. 3C, Table 1), and the enzyme exhibited significant activity at 10 °C (38% of the activity at optimal 40 °C) (Fig. 3C). These data show that EF-EG2 is a cold-adapted enzyme. The earthworm *E. fetida* expresses other cold-adapted enzymes, namely EF-CMCase25, raw starch-digesting α-amylases, and an anti-plant viral serine protease (Ueda et al., 2008a; Ueda, Asano, et al., 2008; Ueda et al., 2010). In addition, the endo-1,4-β-glucanases from invertabrates are also cold-adapted enzymes (Table 1). The optimal temperatures of cellulase (SnEG54) of *S. nudus* and cellulase (TeEG-I) of *T. emma* were identified as 35–40 °C, and the enzymes exhibited significant activities at 10 °C (30–40% of the activities at optimal temperatures) (Kim et al., 2008; Nishida et al., 2007). Cellulase (AC-EG2) of *A. croccean* also had a significant activity at lower temperature (Li et al., 2009). All these invertebrates are considered psychrophiles. They

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EF-EG2            1 MATRLMMLLGMIALCMSGVAGQYNYDEVLEKSILFYEAERSGDLPANNRIPYRGDSALGDQGNQGQDLTG 70
EA-EG2            1 MATRLMMLLGMIALCMSGVAGQYNYDEVLEKSILFYEAERSGDLPSNNRIPYRGDSALGDQGNQGQDLTG 70
P.hil_Cel         1 --MMLKLLLG-IFVYLGTATGQYNYDEVLSKSILFYEAERSGDLPANNRIDYRGDSALGDRGNGGQDLTG 68

EF-EG2           71 GWYDAGDHVKFGFPMAFATTTLAWGILEFRDGYEAAGQYNLALDSIRWTLNYFLKAHVSDNEFYGQVGDA 140
EA-EG2           71 GWYDAGDHVKFGFPMAFATTTLAWGILEFRDGYEAAGQYNLALDSIRWTLNYFLKAHVSDNEFYGQVGDA 140
P.hil_Cel        69 GWYDAGDHVKFGFPMAFSTTTLAWGILEFRAAYEAAGQYSYALDSIRWPLDYFIKAHVSDNEFYGQVGDG 138

EF-EG2          141 NTDHAYWGRPEDMTMERPAWSISPSAPGSDLAAETAAALAAGYLVFRDSDAAFANNLLAHSRTLYDFALN 210
EA-EG2          141 NTDHAWWGRPEDMTMERPAWSISPSAPGSDLAAETAAALAAGYLVFRDLDAAFANNLLAHSRTLYDFALN 210
P.hil_Cel       139 NADHSYWGRPEDMIMARPAWSITPSAPGADLAAETAAALAAGYLVFRDSDAGYAANLLDHARRLYTFAYN 208

EF-EG2          211 NRGIYSQSISNAAGFYASSAYEDELAWGAAWLYRATEEQEYLDRAYEFGTTTNTAWAYDWNEKIVGYQLL 280
EA-EG2          211 NRGIYSQSISNAAGFYASSAYEDELAWGAAWLYRATEEQEYLDRAYEFGTTTNTAWAYDWNEKIVGYQLL 280
P.hil_Cel       209 NRGIYSQSISNAAQFYSSSSYVDELAWGAAWLYRATNEQTYLNYALEFADTSAISWAYDWNEKIVGYQLL 278

EF-EG2          281 LTTSAGQTDFLPRVENFLRNWFPGGSVQYTPLGLAWLAQWGPNRYAANAAFIALVSAKYNILASESEQFA 350
EA-EG2          281 LTTSAGQTDFLPRVENFLRNWFPGGSVQYTPLGLAWLAQWGPNRYAANAAFIALVSAKYNILASESEQFA 350
P.hil_Cel       279 LFSSAGQTVFQTPVEGYIRSWMPGGSVTYTPQGLAWRQQWGPNRYAANSAFIALVAAKYNILTAEAQNFA 348

EF-EG2          351 RSQIHYMLGDAGRSYVVGFGNNPPQQPHHRSSSCPDQPAECDWDEFNQPGPNYQILYGALVGGPDQNDQF 420
EA-EG2          351 RSQIHYMLGDAGRSYVVGFGNNPPQQPHHRSSSCPDQPAECDWDEFNQPGPNYQILYGALVGGPDQNDQF 420
P.hil_Cel       349 RSQIHYMLGDTGKSFVVGFGNNPPQQPHHRSSSCPDQPNPCDWDEYNNPGPNYQILYGALVGGPDQNDNY 418

EF-EG2          421 EDLRSDYIRNEVANDYNAGFQGAVAALRAIQLRDGK 456
EA-EG2          421 EDLRSDYIRNEVANDYNAGFQGAVAALRAIQLRDGK 456
P.hil_Cel       419 NDARSDYISNEVACDYNAGFQGAVAGLRALTL---- 450
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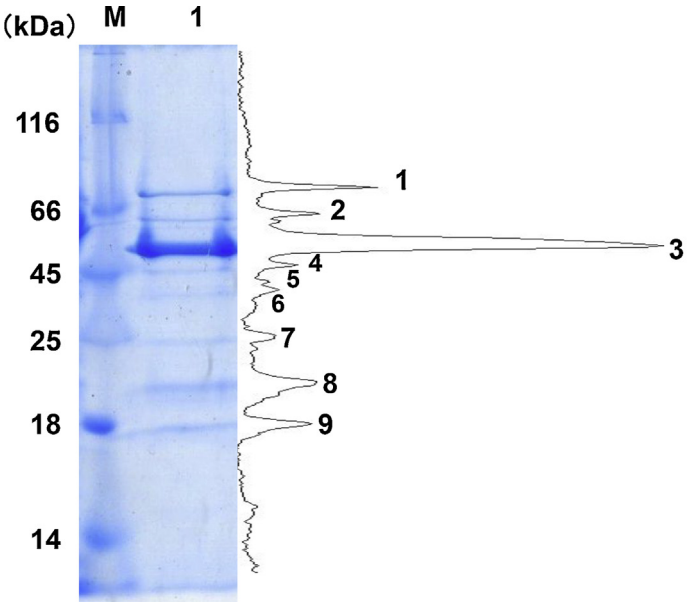
Fig. 1. Sequence alignment of earthworm 1,4-β-glucanases from *Eisenia fetida* (EF-EG2, AB679653), *Eisenia andrei* (EA-EG2, EU315749), and *P. hilgendorfi* (*P. hil*_Cel, AB452993). Red letters indicate the catalytic amino acids. All sequences are numbered from Met-1 of the peptide (For interpretation of the references to color in this text, the reader is referred to the web version of the article.).

grow vigorously in low temperature environments, and their baseline metabolism may be maintained at low temperatures with little or no change in enzymatic activities.

The enzymatic activity of purified recombinant EF-EG2 was 10.8 units/mg protein with CMC as the substrate. This value was higher than that of *E. fetida* EF-CMCase25 (7.25 units/mg protein) (Ueda et al., 2010). In contrast, EF-EG2 was unable to hydrolyze insoluble (powdered cellulose) or crystalline (Avicel) cellulose substrates containing 1,4-β-glycosidic bonds. The inability of EF-EG2 to digest Avicel is shared by the endo-1,4-β-glucanase (Cel5M) of psychrophilic deep-sea bacteria *Pseudomonas* (Garsoux, Lamotte, Gerday, & Feller, 2004). In contrast, Avicel is a substrate of cellobiohydrolase in *Cellulosilyticum ruminicola* (Cai et al., 2010) (ACZ98592.1). Cellobiohydrolase from *C. ruminicola* has cellulose binding domain in the multi-domain structure, but EF-EG2 and Cel5M do not have cellulose binding domain. Generally, the cellulose binding domains have been enhanced the hydrolytic activity against the insoluble and crystalline cellulose substrates. These studies confirm that EF-EG2 expresses only an endo-1,4-β-glucanase activity against soluble cellulose substrates.

To clarify the mode of action of purified recombinant EF-EG2, enzymatic assays were conducted with 1,4-β-linked



Band No.	Area
1	505
2 (Cpn60)	151
3 (EF-EG2)	4001
4	117
5	45
6	78
7	184
8	672
9	501
Total	6254

Fig. 2. SDS-PAGE analysis of partially purified recombinant EF-EG2. M, Precision Plus Protein™ Unstained Standards (Bio-Rad Laboratories, Inc., CA, USA); 1, Purified 1,4-β-glucanase. Protein band pattern analyzed by ImageJ (http://rsb.info.nih.gov/ij/). The table indicates the relative intensity of each band, with the identity of EF-EG2 and Cpn60.

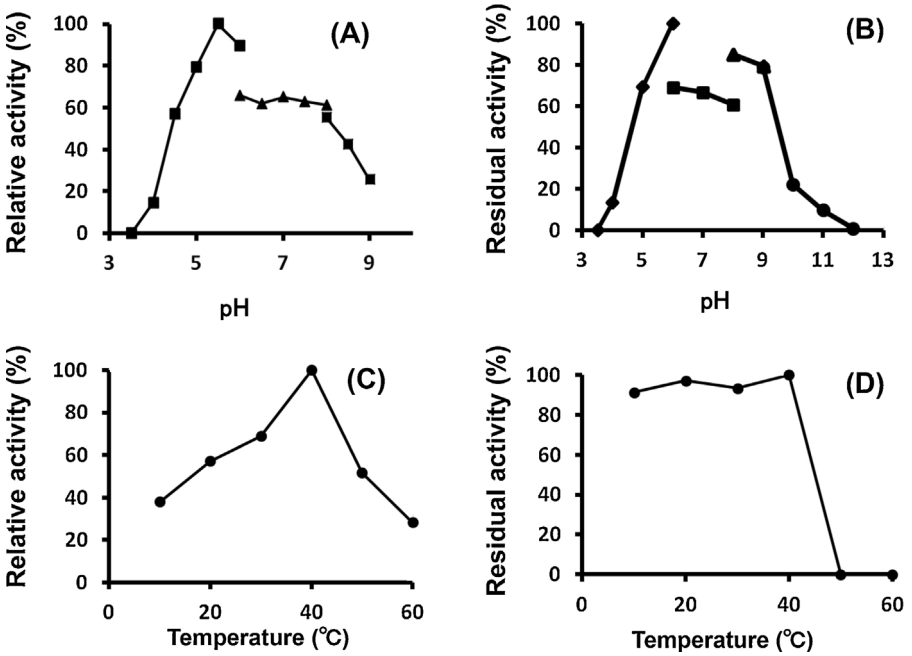


Fig. 3. Functional properties of purified recombinant EF-EG2. All reactions were conducted on purified enzyme with CMC as the substrate. (A) Effect of pH on enzyme activity at 37 °C in 0.1 M of the following buffers: ■, sodium acetate (pH 3.5–6.5); ▲, sodium phosphate (pH 6.0–8.0); ■, Tris–HCl (pH 8.0–9.0). (B) Effect of pH on enzyme stability. Assays conducted at 37 °C (pH 5.5) after a 30-min incubation in 0.1 M of the following buffers: ♦, sodium acetate (pH 3.5–6.0); ■, sodium phosphate (pH 6.0–8.0); ▲, 0.1 M Tris–HCl (8.0–9.0); •, glycine–NaOH buffer (pH 9.0–12.0). (C) Effect of temperature on enzyme activity measured at 10–60 °C. (D) Effect of temperature on enzyme stability. Assays conducted at 37 °C after a 30-min incubation in 50 mM acetate buffer (pH 6.0) at 10–60 °C. The data represent the mean of duplicate measurements. The standard deviations were within the symbols.

oligosaccharides of various lengths (cellotriose, cellotetraose, cellopentaose, or cellohexaose), and buffer aliquots collected over time were analyzed by TLC (Fig. 4). The major hydrolysis products from cellohexaose and cellopentaose were cellotriose and cellobiose. The major hydrolysis product from cellotetraose was cellobiose. In contrast, cellotriose was not degraded. The hydrolysis pattern and products generated by EF-EG2 were similar to those reported for endo-1,4-β-glucanases from *S. nudus* and *Mytilus*

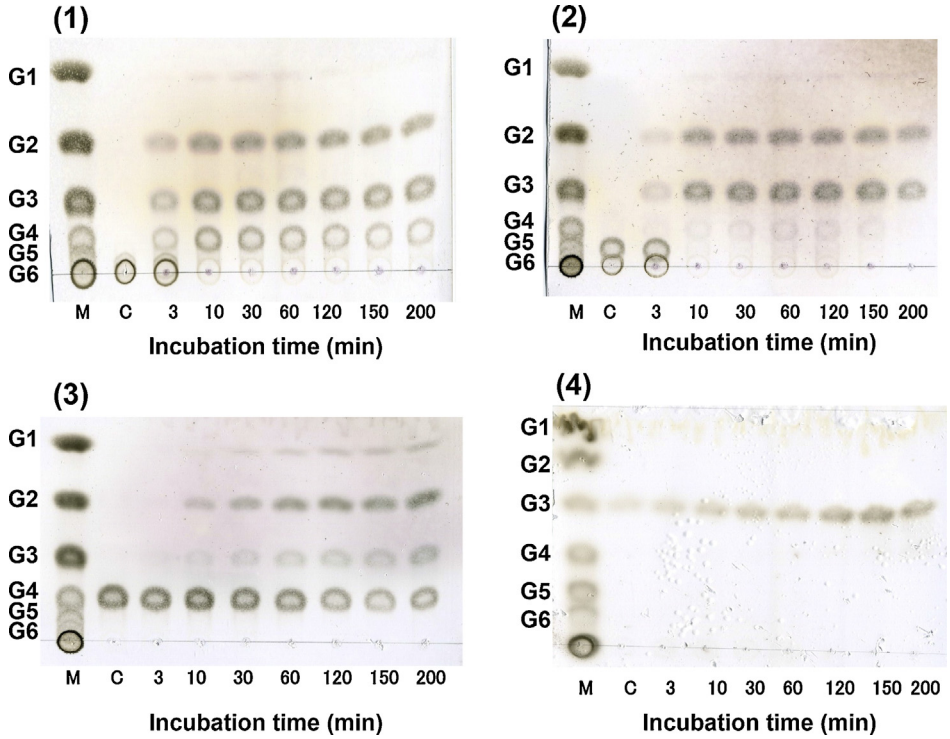


Fig. 4. Mode of action of purified recombinant EF-EG2 analyzed by TLC. Separation of the reaction products from enzyme reactions conducted with (1) cellohexaose, (2) cellopentaose, (3) cellotetraose, or (4) cellotriose, as described in Section 2. Standards: G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose. C (negative control): incubation of each oligosaccharide in 0.1 M acetate buffer (pH 5.5) in the absence of EF-EG2.

Table 1
Properties of GH family 9 endo-1,4-beta-glucanases from invertebrates and insects.

Origin (enzyme name)	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	pH stability	Thermostability (°C)	References
E. foetida (EF-EG2)*	50	5.5	40	5.0–9.0	~40	This study
E. foetida (EF-CMCase25)*	25	5	40	7.0–9.0	~40	Ueda et al. (2010)
Ampullaria crossean (AC-EG2)*	65	5.5–6.5	50–55	4.5–5.5	~50	Li et al. (2009)
Haliotis discus hannai	66	6.3	38	ND	~30	Suzuki et al. (2003)
Nasutitermes takasagoensis (NtEG)	47	6	65	5.5–9.0	~60	Tokuda et al. (1997)
Pheretima hilgendorfi (phhEG)	51	ND	ND	ND	ND	Nozaki et al. (2009)
Reticulitermes speratus (YEG1)	42	6	50	ND	~40	Watanabe et al. (1997)
Reticulitermes speratus (YEG2)	41	6	50	ND	~40	Watanabe et al. (1997)
Strongylocentrotus nudus (SnEG54)*	54	6.5	35	ND	ND	Nishida et al. (2007)
Teleogryllus emma (TeEG-I)*	47	5	40	ND	ND	Kim et al. (2008)

ND: not determined.
* Cold-adapted enzyme and/or cold active enzyme.

edulis (Nishida et al., 2007; Xu, Hellman, Ersson, & Jason, 2000). From these results, this enzyme was considered to be an endo-type 1,4-β-glucanase.

4. Conclusion

This study demonstrates that *E. fetida* expresses a cold-adapted endo-1,4-β-glucanase. Currently, the best scenario envisioned for cost-effective biofuel production involves an alkaline/heat pretreatment of the plant-derived lignocellulosic material to expose the cellulose, followed by high-temperature degradation of the cellulose using thermophilic fungi as a source of endo-1,4-β-glucanase to avoid the costly cooling step. The identification of cold-adapted cellulases, like EF-EG2, constitutes an important step in the development of a low-temperature industrial process that would reduce costly energy consumption, protect thermolabile substrates, decrease the rate of nonspecific chemical reactions and reduce the risks of infection by microorganisms.

Acknowledgments

This work was financially supported by Grants-in-Aid for Scientific Research in Japan. This work was also financially supported by the Iijima Memorial Foundation for the Promotion of Food Science and Technology and Sekisui Chemical Innovations inspired by Nature Research Support Program.

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