Escherichia coliで発現したThermotoga maritima由来のエンド-β-1,4-グルカナーゼの特性

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著者名	Rahman,Md.M. Bhuiyan,S.H. 韮澤,悟 北岡,本光 林,清
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Characterization of an Endo-β-1,4-glucanase of *Thermotoga maritima* Expressed in *Escherichia coli*

Md. Majibur Rahman, Shakhawat Hossain Bhuiyan, Satoru Nirasawa, Motomitsu Kitaoka and Kiyoshi Hayashi*

Enzyme Laboratory, National Food Research Institute (2–1–12, Kannondai, Tsukuba, Ibaraki 305–8642, Japan)

A gene (TM1751, Swissprot Q9X273, GenBank AAD36816) of *Thermotoga maritima* encoding endo-β-1,4-glucanase of family 5 was cloned and expressed in *Escherichia coli*. The recombinant protein was purified by combination of Ni-NTA and Q-Sepharose FF column chromatography. SDS-PAGE analysis of purified fractions showed a homogeneous protein band with an expected molecular mass of 38 kDa. The specific activity of the enzyme increased about 16-fold while the recovery was 21% after purification. The optimal temperature of the enzyme was found to be 90°C while its optimal pH was 6.6. The enzyme was active over a wide pH range (4–9.5) and stable up to 85°C. Kinetic studies showed that the PNP-β-D-cellotetraoside and PNP-β-D-cellopentaoside had similar K_m values of 0.25 and 0.24 mM respectively. However, maximum catalytic efficiency (k_{cat}/K_m) was observed with PNP-β-D-cellopentaoside. Further analysis of the enzyme hydrolyzate by HPLC suggests that the carbohydrate binding cleft of the enzyme may be composed of four subsites for glucopyranose units. At the initial stage, the hydrolysis of PNP-oligosaccharides (DP up to 5) yielded a range of products with cellobiose, cellotriose and cellotetraose being the major products. CMC hydrolysis also predominantly produced cellobiose and cellotriose as end products. The enzyme hydrolyzed mixed linked β-1,3/1,4 soluble substrates such as barley glucan and lichenan more strongly than CMC. Transglycosylation activity was also found to be taking place with smaller soluble cellooligosaccharides.

Enzymatic hydrolysis of cellulosic compounds is of paramount importance from both fundamental and applied points of view. Cellulose is the most abundant organic material found in nature, accounting for up to 40% of plant biomass. It is an unbranched linear polymer of glucose molecules with β-1,4 linkages. Naturally occurring cellulosic compounds are structurally heterogeneous and have both amorphous and highly ordered crystalline regions. Enzymatic hydrolysis of cellulose requires a consortium of enzymes, including endo-β-glucanases (1,4-β-D-glucan 4-glucohydrolase [EC 3.2.1.4]), exoglucanases (1,4-β-D-glucan cellobiohydrolase [EC 3.2.1.91]), glucan glucohydrolases (1,4-β-D-glucan glucohydrolase [EC 3.2.1.74]) and β-glucosidases (β-D-glucoside glucohydrolase [EC 3.2.1.21]). However, in general, the cellulase enzymes use either exo- or endo-type modes of action.[1] Endoglucanases randomly hydrolyze the internal glycosidic linkages, which results in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration.[2,3] Exoglucanases, on the other hand, hydrolyze cellulose chains by removing cellobiose from either the reducing ends or the nonreducing ends[4] with the release of reducing sugars but little change in polymer length. Glucose is primarily produced by the action of β-glucosidases on cellobiose and by the action of glucohydrolases on cellooligomers.[5,6]

There is a growing interest in the use of thermozymes as biocatalysts.[7,8] The members of the genus *Thermotoga* are important sources of different cellulolytic enzymes.[9] Different species including *Thermotoga elfii*, *T. neapolitana*, *T. ther-*

* Corresponding author (khayashi@nfri.affrc.go.jp).

marum, and *T. maritima* have already been isolated from various geothermally heated areas which possess a number of thermostable carbohydrolases including cellulases and xylanases.[10] Among the *Thermotoga* species, *T. maritima* and *T. neapolitana* represent the most thermophilic organotrophic eubacteria described to date.

In the genomic sequence of *T. maritima*, two known (CelA and CelB) and six putative (TM 0305, TM1048, TM1049, TM1050, TM1751 and TM1752) endoglucanases have been reported.[1] However, two endoglucanases (CelA and CelB) from *T. neapolitana* and two endoglucanases (CelA and CelB) from *T. maritima* have been well characterized.[11,12] The CelA of *T. maritima* which was 58% identical to CelB has been reported to be active against soluble substrates such as mixed-linkage β-glucan and carboxymethylcellulose. The CelA and CelB showed similar substrate specificity and temperature optima but differed in their pH optima and stability.

The endoglucanases from *Thermotoga* sp. investigated so far have displayed a high thermostability making them potentially interesting candidates for biotechnological application. It is evident that the endo-β-1,4-glucanase encoded by the gene TM 1751 has not yet been studied. On the assumption that this enzyme will play a vital role in cellulose breakdown and be thermostable, the TM1751 gene encoding endo-β-1,4-glucanase was cloned and expressed in *Escherichia coli* followed by purification and characterization of the enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. The genomic DNA of *T. maritima* MSB8 was obtained from Professor Dr. Stetter (Lehrstuhl fuer Mickobiologie, Universitaet Regensburg, Universitaetsstrasse 31, D-93053 Regensburg, Germany). Topo-XL TOP 10 [F′ *mcr* A△ (*mrr-hsd*RMS-*mcr*BC) ϕ80 *lac*Z△M15△*lac*X74 *rec*AI *deo*R *ara*D139△(*ara-leu*) 7697*gal*U *gal*K *rps*L (str^R) *end*A1 *nup*G] was used as a host for the gene TM1751 from *T. maritima* (TOPO®XL cloning kit, Invitrogen, USA). *E. coli* BL21 (DE3-gold, RP and RIL) [*hsd* (*clts*857 *ind*1 *sam* 7 *nin*5*lac* UV5-T7 gene 1) was used as a host for the recombinant plasmid harboring the pET-28b vector (Novagen, Madison, USA). Recombinant DNA techniques as described by Sambrook *et al*.[13] were adapted to perform DNA manipulations.

Amplifying the TM1751 gene. The nuleotide sequence of the open reading frame is annotated as ORF TM1751 gene (accession number AAD36816 of the GenBank, Q9X273 of the Swissprot). The enzyme belongs to the Glycoside Hydrolase Family 5. Two synthetic oligonucleotide primers with *Nde*I and *Sac*I restriction sites (underlined) having the nucleotide sequences 5′ CATATGGGTGTTG-ATCCTTTTGAAAGG3′ and 5′ GAGCTCTTAT-TCAATGCTATCTCCTCCTATTA3′ were used for PCR-aided amplification. The expected fragment of 954 base pairs was obtained with the following steps: 98°C for 1 min, 55°C for 1 min and 68°C for 1 min in 25 cycles using DNA polymerase (KOD plus, Toyobo). The amplified DNA fragment of the TM1751 gene was cloned into a pCR-TOPO-XL vector using a pCR-TOPO-cloning kit (Invitrogen, USA). The resulting recombinant plasmid TM1751-TOPO-XL was extracted using a QIA miniprep kit (QIAgen, Germany) and was sequenced. DNA sequencing was performed on an Applied Biosystems 310 Genetic analyzer using a Dye Terminator Cycle sequencing kit (Perkin-Elmer, USA). The sequence data were analyzed with the GENETYX program (Software Development Co., Tokyo, Japan).

Cloning and epression of TM1751 gene in E. coli. The TM1751 was excised from the recombinant plasmid TM1751-TOPO-XL using the restriction enzymes *Nde*I/*Sac*I, recovered by ethanol precipitation and ligated to pET-28b vector which had previously been digested with the same pair of restriction enzymes. Ligation was conducted at 16°C overnight using a Ligation kit, High T4 DNA ligase (Toyobo Co., Osaka, Japan). *E. coli* BL21 (RP and RIL) competent cells were transformed by electroporation with ligated plasmid. The positive colonies from the transformed cells grown on Luria-Bertani (LB) medium containing kanamycin (50 μg/mL) were screened by colony PCR using vector-specific primers (T7 promoter and T7 terminator) and used to prepare plasmid

DNA on a small scale. One pET-TM1751 clone was selected and confirmed by sequencing. For expression, the *E.coli*-harboring plasmids pET28b-TM1751 were grown in LB medium containing kanamycin (50 μg/mL) at 37°C. When the turbidity measured at 600 nm reached 0.5, IPTG was added to a final concentration of 0.5 mM. After induction for 5 h, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl buffer (pH 7.5) and disrupted by sonication (Branson sonifier 250D, 5 cycles at 35% maximum output with 15 s on/off). The supernatant obtained by centrifugation (at 12,000 rpm for 20 min) was used as cell extract.

Purification of the recombinant enzyme. After induction, the enzyme was purified first by Ni-NTA affinity chromatography followed by Q-Sepharose FF anion exchange chromatography. For the preparation of Ni-NTA-protein mixture, 40 mL sample was mixed with 4 mL Ni-NTA-agarose resin, kept on ice for 20 min and mixed thoroughly with occasional gentle shaking by hand. Ni-NTA resin-bound target protein was then packed on to the column and unbound protein was washed out with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM imidazole at a flow rate of 1 mL/min in a FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden). The elution of the protein was performed in a 10 CV (Column volume) linear gradient with 250 mM imidazole in Tris-HCl buffer (pH 8.0). The fractions containing endoglucanase activity were pooled together and dialyzed for 1 h against distilled water.

After dialysis, the protein fractions from the Ni-NTA column were subjected to a Q-Sepharose FF column (26/10). The target protein was then eluted from the column in a 20 CV linear gradient with 0.5 M NaCl in MOPS buffer (pH 6.5). The fractions containing endoglucanase activity were pooled together and subjected to enzyme characterization. Protein concentration was estimated by using the extinction coefficient of 99,430 at 280 nm based on the amino acid composition.

Analysis of the purified protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique as described by Laemmli[14] was used to resolve the target protein. Protein samples were boiled for 5 min in the presence of 0.01 M mercaptoethanol containing 1% SDS (w/w) before being applied to the gel (10%). The gel was stained with 1% (w/w) Coomassie Brilliant Blue (R-250) in methanol/acetic acid/water (50:10:40, v/v). 10 kDa protein ladder (LIFE TECHNOLOGIES, GIBCO BRL, Rockville, USA) was used as molecular weight marker. After proper destaining in methanol/acetic acid/water (30:10:60 v/v), the gel was subjected to scanning by Canoscan using Photostudio 2000.

Endo-β-1,4-glucanase Assay. Carboxymethylcellulose (CMC, obtained from Sigma, Lot 30K 0157) was used as substrate for endo-β-1,4-glucanase activity.[9] Acid-swollen cellulose was prepared as reported previously.[15] In a 200 μL reaction mixture, 100 μL of 1% substrate (0.5% final concentration), 50 μL of 0.2 M Tris-HCl buffer (pH 7.5, 50 mM final concentration) and 50 μL of diluted (10–50 times) enzyme were used and incubated at 50°C for 15 min. The reducing sugar released was determined by the method of Somogyi[16] and Nelson.[17] One unit of enzyme corresponds to 1 μmol of reducing power equivalent as glucose produced per minute.

Optimum pH and stability. In order to find out the optimum pH of the enzyme, a range of buffers of differing pH were used. Citrate (pH 2.18–4.21), acetate (pH 3.76–5.73), MES (2-[*N*-Morpholino]ethanesulfonic acid, pH 5.15–7.13), MOPS (3-[*N*-Morpholino]propanesulfonic acid, pH 6.22–8.20), phosphate (pH 6.22–8.22), HEPES (*N*-[2-Hydroxyethyl]piperazine-*N*′-2 ethanesulfonic acid, pH 6.54–8.54), Tris (pH 7.20–9.00), CHES (3-[Cyclohexylamino]-1-ethanesulfonic acid, pH 8.17–10.14) and CAPS (Cyclohexylaminopropanesulfonic acid, pH 9.44–11.39) were used. For the enzyme stability study, the enzyme plus buffers were preincubated at 80°C for 30 min and then assayed at 30°C for 10 min.

Optimum temperature and stability. For the determination of optimum temperature, the enzyme-substrate-buffer mixture was incubated at different temperatures ranging from 0–100°C and reducing sugar released was then determined by the Nelson-Somogyi method. For temperature stability, the enzyme and MES buffer (pH 6.6) were preincubated

at different temperatures for 30 min before being subjected to assay temperature (50°C) for 10 min.

Kinetics with p-nitrophenyl (PNP) oligosaccharides. Kinetics were performed following the changes in rates due to the release of *p*-nitrophenol. A spectrophotometer (Beckman DU 650) fitted with a circulating water bath (EYELA DIGITAL UNI ACE UA-100) which maintained the cells (cuvettes) at 30°C was used for this experiment. The rates of enzyme-catalyzed hydrolysis were determined by incubating different concentrations of PNP-oligosaccharides (DP up to 5) with suitably diluted enzyme in 50 mM MOPS buffer (pH 6.5). The reactions were initiated by the addition of the enzymes and monitoring the absorbance at 405 nm. After confirming that the initial rate of hydrolysis is linear with time, the obtained values are subjected to kinetic parameter analysis.

Analysis of products of PNP-oligosaccharide hydrolysis. Composition of cellooligosaccharide in the enzyme hydrolyzate of PNP derivative has been analyzed by HPLC (Dionex LC20) equipped with pulsed amperometric detector. Thin-layer chromatography performed on silica gel plates (Merck Silica Gel 60F 254; E, Merck, Darmstadt, Germany) was used to analyze the reaction products of *p*-nitrophenyloligosaccharide conjugates (DP up to 5) incubated with the enzyme in 50 mM MOPS buffer (pH 6.5) at 30°C. After incubation, the reaction mixtures were spotted onto the silica gel plate, dried and developed thrice in a chloroform–acetic acid–water (6:7:1, v/v)[18] solvent system. After drying, the plate was dipped into a methanol–sulfuric (95:5, v/v) solution. The product spots were then visualized by heating them in an oven for few minutes.

RESULTS

PCR amplification and cloning.

The endo-β-1,4-glucanase gene (TM1751, Swissprot Q9X273, GenBank AAD36816) was amplified by using specific forward and reverse primers. Based on the primer design, *Nde*I and *Sac*I restriction sites were incorporated at the 5′ and 3′ ends of TM1751 open reading frame, respectively. The PCR product of *Nde*I/*Sac*I was used for subcloning into TOPO-XL followed by cloning into pET-28b expression vector. Subsequent transformation and colony PCR showed that *E. coli* BL21 (RIL) strain possessed the desired gene TM1751 which was confirmed by DNA sequencing.

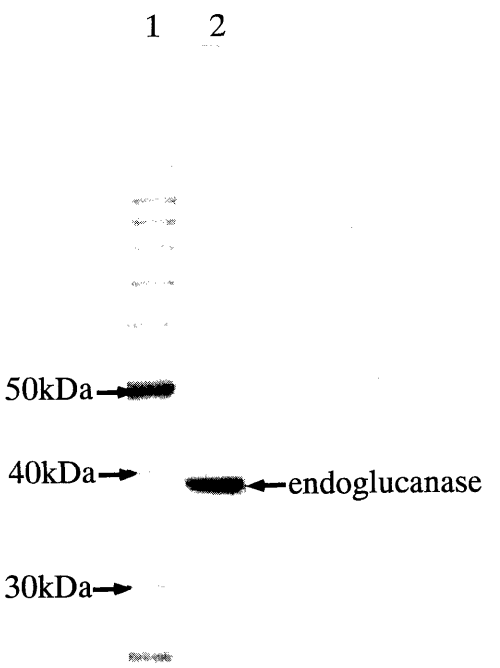

Fig. 1. SDS-PAGE of the purified recombinant protein. Lane 1, marker; lane 2, purified protein.

Table 1. Purification of endo-β-1,4-glucanase of *T. maritima* expressed in *E. coli*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Cell extract	212	1687	7.9	1.0	100
Ni-NTA	22.6	1236	54.5	6.8	73
Q-Sepharose FF	2.71	343	126.7	15.9	20

Expression and purification.

The *Nde*I and *Sac*I restriction sites lying at 5′ and 3′ ends of the open reading frame of TM 1751, respectively, allowed the entire open reading frame to be inserted within the multiclone site of plasmid pET-28b, thereby creating plasmid pET-TM1751. The *Nde*I site was positioned in such way that N-terminal His-tag was attached during

translation of the enzyme. It was found that after 5 h of IPTG induction that strain BL21-RIL (pET-TM1751) expressed the desired protein with an apparent molecular mass of 38 kDa. After extraction of the protein by sonication, the supernatant was collected by centrifugation as cell extract. The recombinant protein was then purified by Ni-NTA which facilitated the purification to a great extent. Further purification to homogeneity was carried out by using a Q-Sepharose FF column. SDS-PAGE showed a homogenous protein with an expected molecular mass of 38 kDa (Fig. 1). Table 1 summarizes the purification process which indicated that after purification by Q-Sepharose FF, the specific activity of the enzyme increased about 16-fold while the recovery was around 21%. The Ni-NTA column also contributed a great deal to the purification having specific activity increased by 7-fold with a yield over 73%.

Characterization of the purified enzyme.

The pH and temperature optima as well as the stability of the purified enzyme were studied at different pH and temperatures. The pH activity profile as depicted in Figs. 2a and 2b showed an optimal pH of 6.6, while the enzyme was stable from pH 4–9.5 at 80°C for 30 min. From the temperature study, it was found that the optimum temperature for maximal enzyme activity was 90°C (Fig. 3). The activity showed a sharp decline at 95°C. The enzyme was however, stable at temperatures up to 85°C (Fig. 3).

Substrate specificity.

The purified enzyme had the highest specific activity toward mixed linked β-1,4/1,3 soluble substrate barley glucan followed by lichenan. Very little or almost no activity has been observed with insoluble cellulose substrates such as avicel and acid swollen cellulose. Activities against laminarin (β-1,3 linkage only), birchwood xylan and oatspelt xylan were also negligible (Table 2).

Kinetic parameters.

Different PNP-oligosaccharides were used for kinetic studies at 30°C. The results as shown in Table 3 indicate that the enzyme had low affinity towards PNP-β-D-cellobioside (K_m 16.7 mM). It was found that with increasing chain length, the affinity increased and the lowest K_m of 0.24 mM was observed with PNP-β-D-cellopentaoside. An

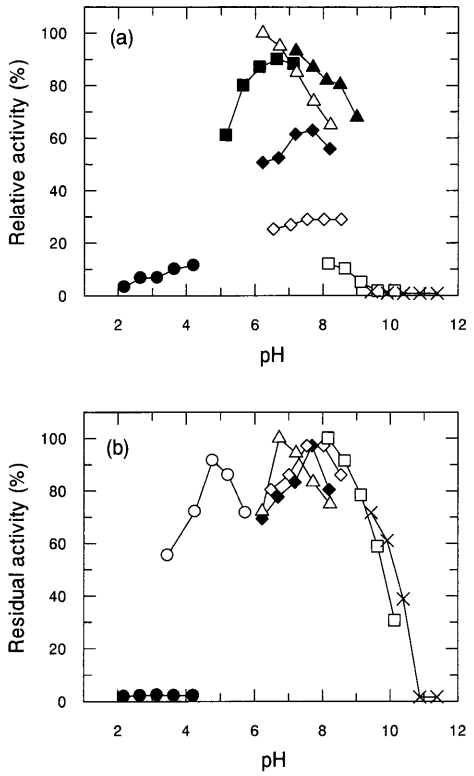

Fig. 2. Optimum pH (a) and pH stability (b) of the enzyme.

●, citrate; ○, acetate; ■, MES; ◆, MOPS; △, phosphate; ◇, HEPES; ▲, tris-HCl; □, CHES; ×, CAPS.

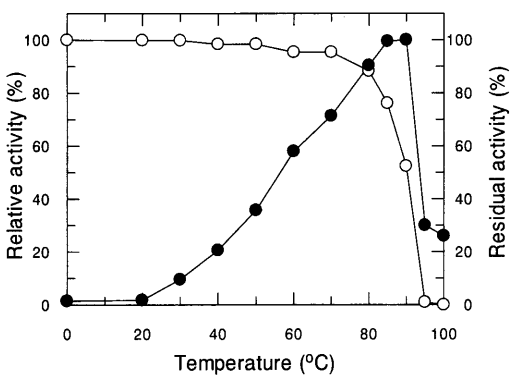

Fig. 3. Optimum temperature and temperature stability of the enzyme.

●, optimum temperature; ○, temperature stability.

Table 2. Substrate specificity of endo-β-1,4-glucanase of *T. maritima* expressed in *E. coli*.

Substrate	Backbone linkage(s)	Specific activity (U/mg)	Relative activity (%)
Barley glucan	β-1,3/1,4	150	100
Lichenan	β-1,3/1,4	105	70
CMC	β-1,4 only	80	53
Avicel	β-1,4 only	<1.5	<1.0
Acid swollen cellulose	β-1,4 only	<0.05	<0.03
Birchwood xylan	β-1,4 only	<0.08	<0.05
Oatspelt xylan	β-1,4 only	<0.08	<0.05
Laminarin	β-1,3 only	<0.44	<0.29

Table 3. Kinetic parameters for the release of PNP from PNP-oligosaccharides.

Substrates	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
PNP-β-D-cellobioside	16.7±0.59	1.78±0.01	0.11
PNP-β-D-cellotrioside	0.85±0.02	1.38±0.01	1.62
PNP-β-D-cellotetraoside	0.25±0.02	1.24±0.04	4.96
PNP-β-D-cellopentaoside	0.24±0.01	2.09±0.03	8.71

almost identical K_m value was obtained with PNP-β-D-cellotetraoside. Further analysis revealed the highest catalytic efficiency k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$) of 8.71 with PNP-β-D-cellotepentaoside followed by PNP-β-D-celloetetraoside, which had a value of 4.96.

Hydrolysis of cellooligosaccharides and mode of action of the enzyme.

Analysis of the hydrolysis products of PNP-cellooligosaccharides (DP up to 5) by HPLC showed that the enzyme preferentially hydrolyzed oligomers with higher degrees of polymerization. In the early stage of the hydrolysis of PNP-G5 and PNP-G4, the fourth glycosidic bond from the non-reducing end was preferentially cleaved by this enzyme (Fig. 4).

On extended hydrolysis, a small amount of glucose was detected in all substrates except for PNP-G, which was not cleaved at all (Fig. 5). A transglycosylation activity was observed with PNP-G2. CMC hydrolysis, however, produced a range of products including cellobiose and cellotriose.

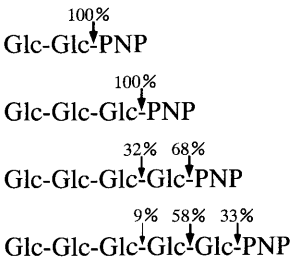

Fig. 4. Relative rate of hydrolysis of different substrates by the enzyme.

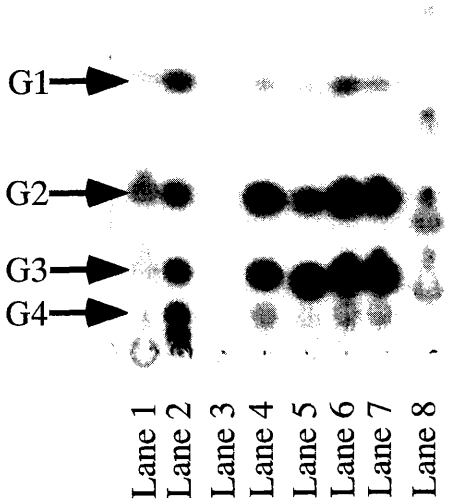

Fig. 5. TLC of hydrolysis products of different substrates.

Lane 1, CMC; lane 2, standard (G1-G5); lane 3, PNPG; lane 4, PNP-G2; lane 5, PNP-G3; lane 6, PNP-G4; lane 7, PNP-G5; lane 8, PNP-standard.

DISCUSSION

An endo-β-1,4-glucanase (TM1751, Swissprot Q9X273, GenBank AAD36816) of *T. maritima* has been purified and characterized after cloning and expression in *E. coli*. Amino acid sequence homology analysis by BLAST search at NCBI showed that TM1751 endoglucanase had only 31 and 20% identities with two known endoglucanases of TMCelA and TMCelB,[12] respectively. However, it had 38, 17, 22, 35 and 31% identities with putative TM0305, TM1048, TM1049, TM 1050 and TM1752 endoglucanases of the same strain, respectively.

The two genes of TM1751 and TM1752 code the Family 5 glycosidase. The gene of TM1752 overlaps with 5 base pair at the end of the TM 1751 gene. A non-coding region of 62 base pairs was found between the gene of TM1750 and TM 1751 and the two candidates for the consensus sequence for the promoter were found at 2 and 14 base pairs upstream of the initiation codon of the TM1751 gene. These results suggest that the two genes of TM1751 and TM1752 may be translated simultaneously. No signal peptide region was found in either the TM1751 or TM1752 endoglucanase.

Initial purification by Ni-NTA proved to be very effective in binding the enzyme selectively. Further purification by Q-Sepharose FF resulted in a homogeneous protein. The 38 kDa protein as resolved in SDS-PAGE was found to correspond well with the value (37,384 Da) deduced from nucleotide sequence of the gene TM1751. Two 29 kDa endoglucanases (CelA) and one 30 kDa endoglucanase (CelB) have been reported in *T. maritima*[12] and *T. neapolitana*.[11] Therefore, it is revealed that the 38 kDa endoglucanase has not yet been studied in *T. maritima* and this is to our knowledge the first report on the purification and characterization of this protein.

The purified enzyme showed high activity towards CMC suggesting that the enzyme is an endo-β-1,4-glucanase as CMC is conventionally used as an indicator for endo-β-1,4-glucanase.[19] True exoglucanases however, exhibit very low level of CMCase activity. The CelA and CelB of *T. neapolitana* and cellI (CelA) of *T. maritima* have also been characterized as endoglucanases based on their high affinity for CMC and the production of cellobiose and cellotriose from cellohexose.[11] Different products of CMC hydrolysis such as cellobiose, cellotriose and cellotetraose lend further support to the notion that the enzyme may be endoglucanase. The CMCase activity as observed in this study are consistent with that of *Trichoderma reesei*,[20] *Bacillus circulans*,[18] *T. neapolitana*[11] and *T. maritima* celA.[12] In apparent contradiction with the above observation, it also hydrolyzed PNP-β-D-cellobioside which is often used as a model substrate for exo-β-glucanases although with less catalytic efficiency. However, differentiation between exo- and endo-glucanases based on small soluble substrates does not appear to be a very useful method.[21] In addition, the enzyme produced G2 and G3 from G4 and CMC, suggesting that it is not a CBH (Cellobiohydrolase) as according to the enzyme nomenclature; CBH must produce only G2 in the hydrolysate of G4 and cellulose.[18] Therefore, classification of TM 1751 as endoglucanase remains valid. The enzyme was found to hydrolyze mixed-linked β-1,3/1,4 soluble substrates more actively than substrates with β-1,4 linkage only. This endoglucanase does not seem to require β-1,3 bonds to be present to effectively cleave mixed linkage β-glucans. The inability of the enzyme to efficiently hydrolyze insoluble cellulose, and yet its ability to degrade soluble cellulose oligosaccharides and mixed-linkage β-glucans, indicates that it likely attacks the amorphous regions within the insoluble cellulose.[21] Similar substrate specificities have been observed with CelA and CelB of *T. maritima*[22] and Eg1A of *Pyrococcus furiosus*.[22]

Characterization of the recombinant endoglucanase showed that the optimum pH was 6.6 and the enzyme was stable over a wide pH range. The optimal temperature of the enzyme was found to be 90°C while the activity remained stable up to 85°C, suggesting the thermal stability of this enzyme. These results are similar to the values reported for CelA and CelB of *T. maritima*.[12] The major cause of this thermostability may be due to the presence of ionic bonds, salt bridges and hydrogen bonds between charged amino acids in an extremely thermophilic organism.[23]

PNP-celloligosaccharides (DP up to 5) initial degradation rates increased as DP increased from cellobiose to cellopentaose. Judging from the product analysis of the enzyme hydrolyzate of the early stage (less than 20% hydrolysis of the substrates), the enzyme is preferencially hydrolyzing the fourth glycosidic bond from the noreducing end. It is suggesting that the enzyme possesses at least four subsites for glucopyranose units on the nonreducing end from the scissile glycosidic bond.[11,22,24] The transglycosylation activity as observed in this study resembles that of family 12 endoglucanase

of *P. furiosus*.[22] However, further studies in elucidating the detailed mechanism of transglycosylation reactions are in progress.

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*Escherichia coli*で発現した*Thermotoga maritima*由来のエンド-β-1,4-グルカナーゼの特性

Md. マジブル ラーマン, シャカワットホッサン ブイヤン, 韮澤 悟, 北岡本光, 林 清

独立行政法人食品総合研究所酵素機能研究室
（305–8642 茨城県つくば市観音台 2–1–12）

Thermotoga maritima 由来のエンド-β-1,4-グルカナーゼ遺伝子（TM1751, Swissprot Q9X273, GenBank AAD36816）を，大腸菌で発現し，各種カラムクロマトグラフィーにより収率21%で電気泳動的に単一バンドとなるまで精製し，得られた精製酵素の特性を明らかにした．本酵素の分子量は38 kDa，至適反応温度は90℃であり，至適反応pHは6.6であった．本酵素は，85℃ではpH 4–9.5の広範囲で安定であった．PNP-β-D-セロテトラオシド，PNP-β-D-セロペンタオシドに対するK_m値は，それぞれ0.25，0.24 mMであり，触媒活性（k_{cat}/K_m）は，PNP-β-D-セロテトラオシドで最大であることから，本酵素には四つのサブサイトが存在するものと推定された．PNP-β-D-セロオリゴ糖の加水分化物をTLC分析したところ，セロビオース，セロトリオース主産物であり，PNP-β-D-セロビオシドからはセロトリオースが生成することから，本酵素は糖転移作用を有することが明らかとなった．本酵素は，カルボキシメチルセルロースよりも，β-1,3/1,4結合を含むバーレイグルカン，リケナンをよく加水分解した．