

보 문

Novel substrate specificity of a thermostable β -glucosidase from the hyperthermophilic archaeon, *Thermococcus pacificus* P-4

Yun Jae Kim^{1,2}, Jae Eun Lee¹, Hyun Sook Lee^{1,2}, Kae Kyoung Kwon^{1,2}, Sung Gyun Kang^{1,2}, and Jung-Hyun Lee^{1,2*}

¹Korea Institute of Ocean Science and Technology, Ansan 426-744, Republic of Korea

²Department of Marine Biotechnology, University of Science and Technology, Daejeon 305-350, Republic of Korea

초고온 고세균 *Thermococcus pacificus* P-4로부터 내열성 β -glucosidase의 새로운 기질 특이성

김윤재^{1,2} · 이재은¹ · 이현숙^{1,2} · 권개경^{1,2} · 강성균^{1,2} · 이정현^{1,2*}

¹한국해양과학기술원, ²과학기술연합대학원대학교 해양생명공학부

(Received February 4, 2015; Accepted March 13, 2015)

ABSTRACT: Based on the genomic analysis of *Thermococcus pacificus* P-4, we identified a putative GH1 β -glucosidase-encoding gene (Tpa-glu). The gene revealed a 1,464 bp encoding 487 amino acid residues, and the deduced amino acid residues exhibited 77% identity with *Pyrococcus furiosus* β -glucosidase [accession no. NP_577802]. The gene was cloned and expressed in *Escherichia coli* system. The recombinant protein was purified by metal affinity chromatography and characterized. Tpa-Glu showed optimum activity at pH 7.5 and 75°C, and thermostability with a half life of 6 h at 90°C. Tpa-Glu exhibited hydrolyzing activity against various pNP-glycopyranosides, with k_{cat}/K_m values in the order of pNP- β -glucopyranoside, pNP- β -galactopyranoside, pNP- β -mannopyranoside, and pNP- β -xylopyranoside. In addition, the enzyme exhibited exo-hydrolyzing activity toward β -1,3-linked polysaccharide (laminarin) and β -1,3- and β -1,4-linked oligosaccharides. This is the first description of an enzyme from hyperthermophilic archaea that displays exo-hydrolyzing activity toward β -1,3-linked polysaccharides and could be applied in combination with β -1,3-endoglucanase for saccharification of laminarin.

Key words: β -1,3-linked polysaccharide, β -glucosidase, *Thermococcus pacificus* P-4, exo-hydrolyzing activity, laminarinase activity

The hyperthermophilic archaeon, *Thermococcus pacificus* P-4 was isolated from a geothermal vent in Whale Island, New Zealand and grows optimally at 80 to 88°C. The strain can grow on various substrates, such as peptides and starch (Miroshnichenko *et al.*, 1998). Most proteins from these organisms are thermostable and thermoactive, and these features make these proteins attractive due to the biotechnological advantages offered by stabilized biocatalysts (Imanaka and Atomi, 2002; Schiraldi *et al.*, 2002; de Miguel Bouzas *et al.*, 2006; Atomi *et al.*, 2011). The glycosyl hydrolases from hyperthermophilic archaea have recently been the focus of intense research in the fields of biotechnology due to their high

stability and reactivity. Among them, β -glucosidases are attractive targets for the saccharification of cellulosic materials through the hydrolytic activity and synthesis of glycosides with glycosylation activity.

β -Glucosidases are enzymes that catalyze β -1,4-linked disaccharides or glucose-substituted molecules. At present, many β -glucosidases have been isolated and characterized from all domains, including bacteria, archaea, and eukarya (Bhatia *et al.*, 2002). These enzymes were classified by nucleotide sequence identity or substrate specificity. Extremely thermostable β -glucosidases from some hyperthermophilic archaea were isolated and characterized, and it was revealed that these enzymes form a distinct subfamily among the glycosyl hydrolase family 1 by sequence identity and exhibit

*For correspondence. E-mail: jlee@kiost.ac;
Tel.: +82-31-400-6243; Fax: +82-31-406-2495

distinct substrate specificities in comparison with their mesophilic counterparts (Ezaki *et al.*, 1999). However, these compounds also show intrinsic substrate specificity. The β -glucosidase from *Thermococcus kodakaraensis* KOD1 shows bifunctional enzyme activity towards pNP-glucopyranoside and pNP-mannopyranoside (Bhatia *et al.*, 2002). The β -glucosidases from *Pyrococcus furiosus* (Kengen *et al.*, 1993; Bauer *et al.*, 1996; Kado *et al.*, 2011) and *Sulfolobus solfataricus* (Nucci *et al.*, 1993; Moracci *et al.*, 1995; Aguilar *et al.*, 1997) are well studied through biochemical characterizations and three-dimensional structure studies. The β -glucosidase from *P. furiosus* exhibits broad substrate specificities toward various pNP-glycopyranosides with the highest specific activity toward pNP-glucopyranoside, whereas it did not present hydrolyzing activities toward polysaccharides and only very little activity toward β -1,3-glucans such as laminarin (Kengen *et al.*, 1993; Bauer *et al.*, 1996). These features were similar to those of β -glucosidase from *S. solfataricus*. To date, only the β -glucosidase from the mesophilic bacterium *Cellvibrio mixtus* has been reported to hydrolyze laminarin as a substrate (Sakellaris *et al.*, 1997). However, no report has revealed a β -glucosidase from hyperthermophilic archaea that can actively hydrolyze β -1,3-glucan.

In this study, we identified a novel GH1 β -glucosidase-encoding gene (Tpa_orf01364) from *T. pacificus* genome, cloned and expressed of it in the *Escherichia coli* system, and examined the enzymatic properties of the purified recombinant enzyme toward various pNP-glycopyranosides and polysaccharides.

Materials and Methods

Strains and growth conditions

Thermococcus pacificus P-4 (=DSM 10394^T) was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures). The strain was routinely cultured on YPS (yeast extract-peptone-sulfur) medium (Bae *et al.*, 2006) at 85°C. *E. coli* DH5 α and *E. coli* BL21-RosettaTM(DE3) pLysS (Stratagene) strains were used for the cloning and overexpression of the Tpa-glu encoding gene, respectively. *E. coli* strains were cultured in Luria-Bertani (LB) medium with 50 mg/L

kanamycin at 37°C.

Cloning of the Tpa-glu gene

The draft genome sequence of *T. pacificus* was analyzed by Illumina platform (not published). The genomic DNA from *T. pacificus* was prepared using a standard procedure (Robb *et al.*, 1995). The Tpa-glu gene was amplified from the genomic DNA by PCR using the following primers: 5'-TAAGAAGGA GATATACATATGTATAAGTTTCCTAAAGATTT-3' (sense primer, containing an *Nde*I site as underlined); 5'-CTCGAGTGC GGCCGCAAGCTT CCTCCTCACGAGGAA GTCTA-3' (antisense primer, containing a *Hind*III site as underlined). The PCR product was digested with *Nde*I/*Hind*III, ligated into the *Nde*I/*Hind*III-digested pET-24a(+) vector (Novagen Inc.), and transformed into *E. coli* DH5 α cells. Positive transformants were selected using restriction enzyme digestion, and the clones were confirmed by DNA sequencing.

Expression and purification of Tpa-glu

The recombinant plasmid harboring the Tpa-glu gene was retransformed into *E. coli* BL21-RosettaTM(DE3) pLysS cells. The overexpression of the Tpa-glu gene was induced by the addition of IPTG (0.5 mM) to an optical density of 0.5 at OD₆₀₀, and the cells were further incubated for 16 h at 10°C. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, 0.1 M KCl, and 10% glycerol, pH 7.0). The cells were disrupted by sonication, the cellular debris was removed by centrifugation at 20,000 \times g for 1 h, and a crude enzyme sample was prepared by heat treatment at 80°C for 20 min. The supernatant was applied to a column of TALONTM metal affinity resin (BD Biosciences Clontech) washed with 10 mM imidazole (Sigma) in 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M KCl and 10% glycerol. The proteins were eluted in 50 mM Tris-HCl buffer (pH 7.0) containing 300 mM imidazole, 0.1 M KCl and 10% glycerol, and the eluted proteins were desalted with 50 mM sodium phosphate buffer (pH 7.0), using a Centricon YM-10 column (Millipore). The protein concentration was determined through the Bradford assay (Bradford, 1976) and the purity was examined by SDS-PAGE according to the standard procedure.

Enzyme assay

The optimal pH and temperature of Tpa-Glu were determined using a *p*-nitrophenyl- glucopyranoside (pNP-glu) as the substrate. The pH for the enzyme was determined to be 80°C at a pH range of 4.0 to 8.5 using the following buffers: 50 mM sodium citrate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 8.0–8.5). The temperature range for the enzyme was determined under the standard condition to be 60–100°C. All of the reaction mixtures (200 µl) containing 0.5 µg of enzyme and 1 mM pNP-glu, were incubated for 10 min. The absorbance of released *p*-nitrophenol was measured at 405 nm using an Asys UVM 340 microplate reader (Biochrom). All of the reactions were performed in triplicate. The thermostability of Tpa-glu was assessed by incubating the enzyme at 75°C and 90°C for various periods, and the remaining activities were measured using a pNP-glu as a substrate.

To determine the kinetic properties of Tpa-Glu against various pNP-glycopyranosides (ϵ of pNP at 405 nm = 8.4 mM⁻¹ · cm⁻¹), the standard reaction mixture contained 50 mM sodium phosphate (pH 7.0), 0.25–5 mM pNP-glycopyranosides, and H₂O in a final volume of 1 ml. The reaction mixture was preheated at 75°C for 10 min before addition of the enzymes (1–3 µg). The reaction was conducted at 70°C for 2 min using a UV-2600 spectrophotometer (Shimadzu) at 405 nm. The initial velocities were obtained directly from the initial slopes of the time course plots. The K_m and k_{cat} values were calculated using the Michaelis-Menten equation. To determine the kinetic properties of Tpa-Glu against cellobiose and laminaribiose, the standard reaction mixture contained 50 mM sodium phosphate (pH 7.0), 0.25–7 mM substrates, and H₂O in a final volume of 200 µl. The reaction mixture was preheated at 75°C for 10 min before addition of the enzymes (2 µg). The reaction was carried out at 75°C for 1–10 min, and the reaction was stopped by the addition of 800 µl of 0.1% dinitrosalicylic acid (DNS). The reaction mixture was incubated at 100°C for 5 min. The absorbance was measured at 540 nm using a UV-2600 spectrophotometer, and then converted into the amount of glucose released using a standard curve.

TLC assay

To confirm the hydrolytic activity toward oligosaccharides

and polysaccharides, we used cellooligosaccharides (2–6 mers), laminarioligosaccharides (2–6 mers), and laminarin. The reaction mixture (100 µl) contained 50 mM sodium phosphate (pH 7.0), 1 mM substrates, and 1 µg enzyme. The products were analyzed by thin-layer chromatography using silica gel 60 F₂₅₄ (Merck) and butanol : acetate : water (2 : 1 : 1). The plate was dried in a hood and stained with 0.03% N-(1-naphthyl)-ethylendiamine and 5% H₂SO₄ in methanol. The plate was then dried in a hood and incubated at 110°C for 10 min.

Nucleotide sequence accession number

The nucleotide sequence of Tpa-*glu* can be accessed through the NCBI Database under the accession number KM583848.

Results and Discussion

The organization of gene cluster and sequence analysis of Tpa-*glu*

The hyperthermophilic archaeon, *T. pacificus* (T_{opt} of 80–88°C) can grow on various substrates, such as peptides and starch (Miroshnichenko *et al.*, 1998). Based on the genomic analysis of the strain, we identified a novel GH1 β-glucosidase-encoding gene located in a novel glycoside hydrolase gene cluster consisting of cellulose-, laminarin-, and agarose-degrading enzymes (Fig. 1). The gene revealed an ORF of 1,464 bp encoding 487 amino acid residues.

The deduced amino acid sequences of Tpa-*glu* showed 77% identity with the β-glucosidase from *P. furiosus* (Bauer *et al.*, 1996), 54% identity with the β-glycosidase from *S. solfataricus* (Cubellis *et al.*, 1990), 40% identity with the β-glycosidase from *T. kodakaraensis* (Ezaki *et al.*, 1999), and 33% identity with the β-glucosidase from *P. horikoshii* (Matsui *et al.*, 2000). We analyzed the phylogenetic tree among the family 1 glycosyl hydrolases from hyperthermophilic archaea. As shown in Fig. 2A, the tree has three branches. One branch includes the β-glucosidase from *P. horikoshii*, which is known to be a membrane protein and approximately 13% shorter than the others in polypeptide length (Matsui *et al.*, 2000). The second branch includes the β-glycosidase from *T. kodakaraensis* and β-mannosidase from *P. furiosus*. According to previously

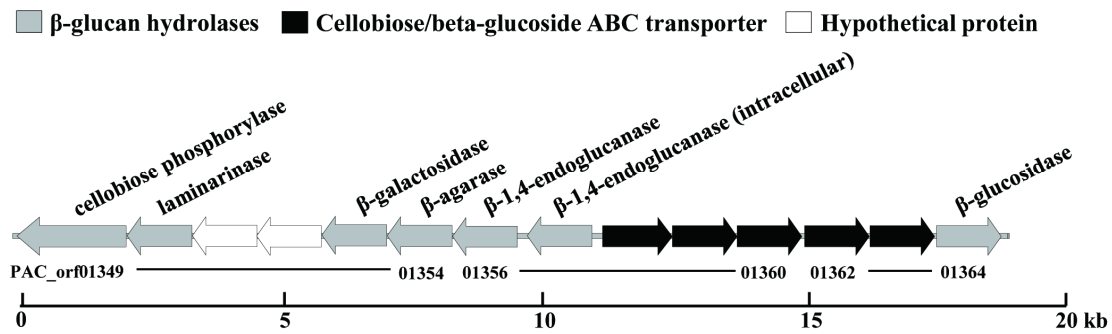


Fig. 1. Novel β -glycosyl hydrolase gene cluster in *T. pacificus*.

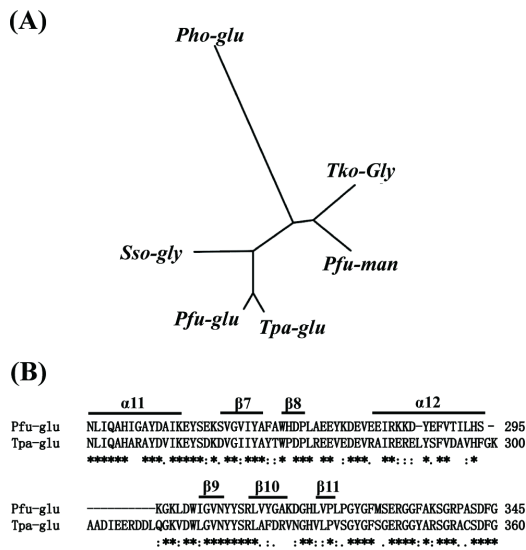


Fig. 2. Phylogenetic relationships of various family 1 glycosyl hydrolases from hyperthermophilic archaea (A) and alignments of Tpa-glu and Pfu-glu (B). The abbreviations are as follows: Tpa-glu, β -glucosidase from *T. pacificus*; Pfu-glu, β -glucosidase from *P. furiosus*; Sso-gly, β -glycosidase from *S. solfataricus*; Pho-glu, β -glucosidase from *P. horikoshii*; Tko-gly, β -glycosidase from *T. kodakaraensis*; Pfu-man, β -mannosidase from *P. furiosus*.

reported results, the β -glucosidase from *T. kodakaraensis* exhibits bifunctional β -glucosidase and β -mannosidase activity, which is related to a conserved region (Ezaki *et al.*, 1999). Tpa-glu was located in the third branch, which includes β -glucosidase from *P. furiosus* and β -glycosidase from *S. solfataricus*.

When pair-wise structure-based alignment with the β -glucosidase from *P. furiosus*, Tpa-glu showed an insertion sequence of 12 amino residues (299 to 310) in the loop region between α -helix 12 and β -strand 9 of the β -glucosidase from *P. furiosus* (Fig. 2B). This extended loop was also observed in the

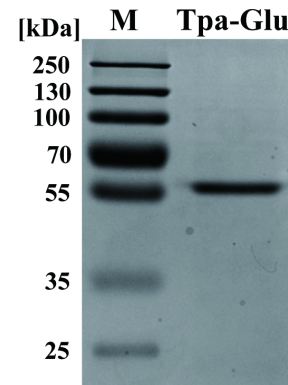


Fig. 3. SDS-PAGE analysis of Tpa-Glu. M, molecular weight marker.

β -glucosidases from other hyperthermophilic archaea and exhibited variable size and sequence (data not shown). However, the function of this loop region is unclear.

Expression and characterization of Tpa-glu

Tpa-glu in pET-24a(+) was expressed well in *E. coli* and was purified using TALONTM metal affinity chromatography. The purified Tpa-Glu revealed a major protein band with a molecular mass of 58 kDa (Fig. 3). The effect of the pH on hydrolyzing activity of Tpa-Glu was examined in the pH range of 4.0 to 8.5. The optimum activity occurred at pH 7.5 in an assay using pNP-glu as a substrate (Fig. 4A). The effect of temperature on the hydrolyzing activity was also determined in the range of 60 to 100°C, and the optimum polymerase activity of Tpa-Glu was found to occur at 75°C (Fig. 4B). The thermostability of Tpa-Glu was tested by measuring the decrease in hydrolyzing activity after pre-incubation at 75°C and 90°C. The half-life ($t_{1/2}$) of the enzyme at 75°C and 90°C was 16.0 h and 6.0 h, respectively (Fig. 4C).

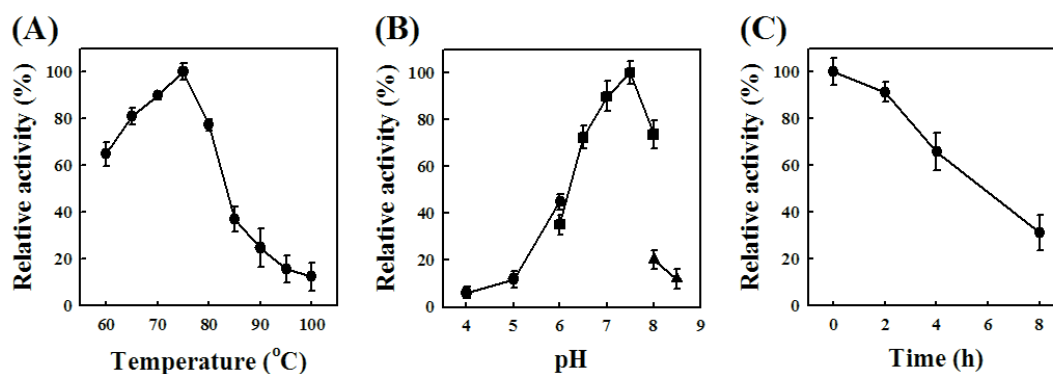


Fig. 4. Effects of temperature (A) and pH (B) on the activity of Tpa-Glu and measurement of the thermostability of Tpa-Glu (C). The activity assays were performed under standard conditions with the following buffers (each at 50 mM): sodium citrate, pH 4.0–6.0 (●); sodium phosphate, pH 6.0–8.0 (■); Tris-HCl, pH 8.0–8.5 (▲). Tpa-glu was pre-incubated at 90°C, and the remaining activities were assayed at 75°C.

Table 1. Kinetic constants for Tpa-Glu

Substrates	K_m (mM)	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$ ($s^{-1} mM^{-1}$)
pNP- β -glucopyranoside	0.58	64	110.34
pNP- β -galactopyranoside	10.36	58	5.62
pNP- β -mannopyranoside	5.06	6	1.18
pNP- β -xylopyranoside	6.47	1.18	0.18
Cellobiose	22.48	89	3.96
Laminaribiose	2.61	41	15.71

Kinetic properties of Tpa-Glu

The hydrolyzing activity of Tpa-Glu toward various pNP-glycopyranosides, such as pNP- β -glucopyranoside (pNP-glu), pNP- β -galactopyranoside (pNP-gal), pNP- β -mannopyranoside (pNP-man), and pNP- β -xylopyranoside (pNP-xyl), was tested. Tpa-Glu exhibited hydrolyzing activity against various pNP-glycopyranosides and showed the highest affinity with pNP-glu, with a K_m value of 0.58 mM. The highest catalytic efficiency (k_{cat}/K_m value) of Tpa-Glu was also observed for pNP-glu as the substrate, followed by pNP-gal, pNP-man, and pNP-xyl (Table 1). Additionally, the hydrolyzing activity of Tpa-Glu toward β -disaccharides, including cellobiose and laminaribiose, was examined. Tpa-Glu showed highest affinity with laminaribiose, with a K_m value of 2.61 mM, which is lower than the value of 22.48 mM found in the case of cellobiose. The k_{cat}/K_m value of Tpa-Glu against laminaribiose was 3.97-fold higher than that of cellobiose and 7.02-fold lower than that of pNP-glu (Table 1). The characteristics of these broad substrate specificities of Tpa-Glu were similar to those of previously characterized and reported β -glucosidases

and β -glycosidases from hyperthermophilic archaea (Sakellaris *et al.*, 1997; Schiraldi *et al.*, 2002; de Miguel Bouzas *et al.*, 2006).

Substrate specificity of Tpa-Glu

The hydrolytic activity of Tpa-Glu toward various polysaccharides including avicel, cellulose, mannan, laminarin, and xylan, was tested. Interestingly, Tpa-Glu exhibits strong exo-hydrolyzing activity toward laminarin among polysaccharides, however, unable to hydrolyze avicel, cellulose, mannan, and xylan (data not shown). The β -glucosidase from *P. furiosus* displays very little activity toward laminarin. According to previously reported results, the β -glucosidase from the mesophilic bacterium, *Cellobivrio mixtus* was found to hydrolyze laminarin as a substrate (Sakellaris *et al.*, 1997). This substrate specificity of Tpa-Glu was different that of β -glucosidases or β -glycosidases from hyperthermophilic archaea (Cubellis *et al.*, 1990; Bauer *et al.*, 1996; Matsui *et al.*, 2000).

To address this question, we further tested the hydrolytic activity of Tpa-Glu toward β -1,3- and β -1,4-linked oligosaccharides and laminarin through TLC analysis. Tpa-Glu exhibited hydrolyzing activity toward all of the tested oligosaccharides and released monomeric glucose as the final end-products (Fig. 5A and B). The results suggest that Tpa-Glu is an exo-type hydrolase. Additionally, Tpa-Glu showed substrate specificity with higher efficiency against β -1,3-linked oligosaccharides than β -1,4-linked oligosaccharides, and these results are consistent with the kinetics data. Furthermore, as shown in Fig. 6, Tpa-Glu showed efficient

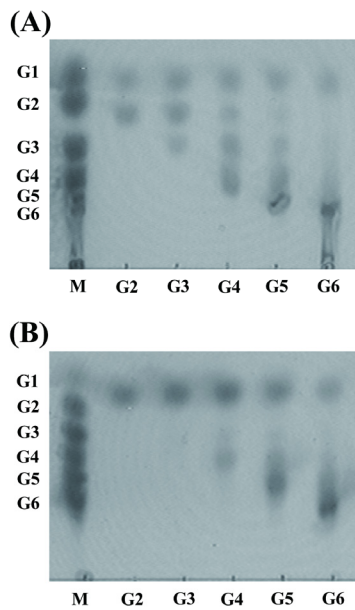


Fig. 5. The hydrolytic activities of Tpa-Glu toward β -1,4- (A) and β -1,3-linked (B) oligosaccharides were measured by thin-layer chromatography. The reaction was performed in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM substrates and 1 μ g of enzyme at 75°C for 4 h. The products were analyzed by thin-layer chromatography. Each substrate is indicated on the bottom line. The abbreviations are as follows: M, size marker; G1, glucose; G2, glucose dimer; G3, glucose trimer; G4, glucose tetramer; G5, glucose pentamer; G6, glucose hexamer.

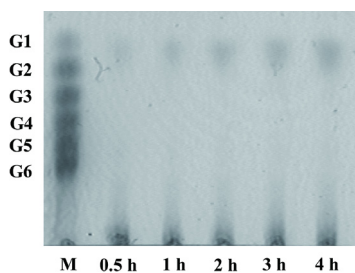


Fig. 6. The hydrolytic activities of Tpa-Glu toward laminarin were measured by thin-layer chromatography. The reaction was performed in 50 mM sodium phosphate buffer (pH 7.0) containing 0.25% laminarin and 1 μ g of enzyme at 75°C for 0.5, 1, 2, 3, and 4 h. The products were analyzed by thin-layer chromatography. The reaction time is indicated on the bottom line. The abbreviations are as follows: M, size marker; G1, glucose; G2, glucose dimer; G3, glucose trimer; G4, glucose tetramer; G5, glucose pentamer; G6, glucose hexamer.

hydrolytic activity against laminarin and also produced monomeric glucose as the major end-products depending on the reaction time. The characteristics of Tpa-Glu, which hydrolyzes the polysaccharide laminarin, were distinct from those of previously reported β -glucosidases or β -glycosidases from hyperthermophilic archaea. Although Tpa-Glu is very

similar to the β -glucosidase of *P. furiosus* in terms of primary structure, it is unclear why Tpa-Glu has unique substrate specificity. It was postulated that the loop region of Tpa-Glu may be important for the substrate specificity of the enzyme. As mentioned above, the structure of the loop region was located in *A-C* dimers of the tetrameric structure of β -glucosidase from *P. furiosus* (Kado *et al.*, 2011). Moreover, in the β -glucosidase from *S. solfataricus*, it was reported to be involved in ion-pair networks with connecting sequences from the second and fifth β α units, and the network occurs at the tetrameric interface between the carboxy termini and involves 16 individual ion-pair interactions (Aguilar *et al.*, 1997). We predicted that the substrate specificity of Tpa-Glu was associated with the ionic interaction between the loop region and the fourth barrel helix at the tetrameric interface. To understand the characteristics of the substrate specificity of Tpa-Glu, additional investigations, such as structural and/or mutational analyses of protein, are required.

In this paper, we described the cloning and characterization of a gene encoding a thermostable β -glucosidase from *T. pacificus*. The purified Tpa-Glu showed hydrolyzing activity toward not only various pNP-glycopyranosides and oligosaccharides but also laminarin. The feature of the exo-hydrolyzing activity of Tpa-Glu toward laminarin suggests that it may be applied in combination with β -1,3-endoglucanase for the saccharification of brown algae containing laminarin as a cell wall component.

적 요

Thermococcus pacificus P-4의 유전체 서열 분석을 통하여 예측되는 GH1 β -glucosidase를 암호화하는 유전자를 동정하였다. 그 유전자는 487 아미노산들을 암호화하는 1,464 bp 나타내었으며, 그 아미노산 서열은 *Pyrococcus furiosus* β -glucosidase와 77% 상동성을 나타내었다. 그 유전자는 *Escherichia coli* 시스템 내에서 복제 및 발현하였다. 재조합된 단백질은 금속 친화 크로마토그래피를 통하여 정제하고 특성을 분석하였다. 정제된 단백질(Tpa-Glu)은 pH 7.5와 75°C에서 최적활성을 나타내었으며, 열안정성은 90°C에서 약 6시간의 반감기를 보였다. Tpa-Glu는 pNP- β -glucopyranoside, pNP- β -galactopyranoside, pNP- β -mannopyranoside, 그리고 pNP- β -xylopyranoside 순으로 우수한 k_{cat}/K_m 값을 나타내었다. 또한, Tpa-Glu는 β -

1,3-linked polysaccharide (laminarin) 그리고 β -1,3-와 β -1,4-linked oligosaccharides에 대하여 exo-hydrolyzing 활성을 보였다. 본 연구는 초고온 고세균으로부터 β -glucosidase가 exo-hydrolyzing 활성을 처음 확인한 것으로 이 효소는 laminarin의 당화공정에 β -1,3-endoglucanase와 함께 적용할 수 있을 것으로 기대된다.

Acknowledgements

This work was supported by the KIOST In-house Program (PE99314), the Development of Biohydrogen Production Technology using Hyperthermophilic Archaea Program of the Ministry of Ocean and Fisheries of the Republic of Korea.

References

- Aguilar, C.F., Sanderson, I., Moracci, M., Ciaramella, M., Nucci, R., Rossi, M., and Pearl, L.H. 1997. Crystal structure of the beta-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: resilience as a key factor in thermostability. *J. Mol. Biol.* **271**, 789–802.
- Atomi, H., Sato, T., and Kanai, T. 2011. Application of hyperthermophiles and their enzymes. *Curr. Opin. Biotechnol.* **22**, 618–626.
- Bae, S.S., Kim, Y.J., Yang, S.H., Lim, J.K., Jeon, J.H., Lee, H.S., Kang, S.G., Kim, S.J., and Lee, J.H. 2006. *Thermococcus onnurineus* sp. nov., a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent area at the PACMANUS field. *J. Microbiol. Biotechnol.* **16**, 1826–1831.
- Bauer, M.W., Bylina, E.J., Swanson, R.V., and Kelly, R.M. 1996. Comparison of a beta-glucosidase and a beta-mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Purification, characterization, gene cloning, and sequence analysis. *J. Biol. Chem.* **271**, 23749–23755.
- Bhatia, Y., Mishra, S., and Bisaria, V.S. 2002. Microbial β -glucosidases: cloning, properties, and applications. *Crit. Rev. Biotechnol.* **22**, 375–407.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Cubellis, M.V., Rozzo, C., Montecucchi, P., and Rossi, M. 1990. Isolation and sequencing of a new beta-galactosidase-encoding archaeobacterial gene. *Gene* **94**, 89–94.
- de Miguel Bouzas, T., Barros-Velázquez, J., and Villa, T.G. 2006. Industrial applications of hyperthermophilic enzymes: a review. *Protein Pept. Lett.* **13**, 645–651.
- Ezaki, S., Miyaoku, K., Nishi, K., Tanaka, T., Fujiwara, S., Takagi, M., Atomi, H., and Imanaka, T. 1999. Gene analysis and enzymatic properties of thermostable beta-glycosidase from *Pyrococcus kodakaraensis* KOD1. *J. Biosci. Bioeng.* **88**, 130–135.
- Imanaka, T. and Atomi, H. 2002. Catalyzing “hot” reactions: enzymes from hyperthermophilic archaea. *Chem. Rec.* **2**, 149–163.
- Kado, Y., Inoue, T., and Ishikawa, K. 2011. Structure of hyperthermophilic β -glucosidase from *Pyrococcus furiosus*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **67**, 1473–1479.
- Kengen, S.W., Luesink, E.J., Stams, A.J., and Zehnder, A.J. 1993. Purification and characterization of an extremely thermostable β -glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem.* **213**, 305–312.
- Matsui, I., Sakai, Y., Matsui, E., Kikuchi, H., Kawarabayasi, Y., and Honda, K. 2000. Novel substrate specificity of a membrane-bound β -glycosidase from the hyperthermophilic archaeon *Pyrococcus horikoshii*. *FEBS Lett.* **467**, 195–200.
- Miroshnichenko, M.L., Gongadze, G.M., Rainey, F.A., Kostyukova, A.S., Lysenko, A.M., Chernykh, N.A., and Bonch-Osmolovskaya, E.A. 1998. *Thermococcus gorgonarius* sp. nov. and *Thermococcus pacificus* sp. nov.: heterotrophic extremely thermophilic archaea from New Zealand submarine hot vents. *Int. J. Syst. Bacteriol.* **24**, 23–29.
- Moracci, M., Nucci, R., Febbraio, F., Vaccaro, C., Vespa, N., La Cara, F., and Rossi, M. 1995. Expression and extensive characterization of a β -glycosidase from the extreme thermoacidophilic archaeon *Sulfolobus solfataricus* in *Escherichia coli*: authenticity of the recombinant enzyme. *Enzyme Microb. Technol.* **17**, 992–997.
- Nucci, R., Moracci, M., Vaccaro, C., Vespa, N., and Rossi, M. 1993. Exo-glucosidase activity and substrate specificity of the β -glycosidase isolated from the extreme thermophile *Sulfolobus solfataricus*. *Biotechnol. Appl. Biochem.* **17**, 239–250.
- Robb, F.T., Place, A.R., Sowers, K.R., Schreier, H.J., DasSarma, S., and Fleischmann, E.M. 1995. *Archaea: A Laboratory Manual*, pp. 3–29. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sakellaris, H., Manners, J.M., and Pemberton, J.M. 1997. A gene encoding an exo- β -glucosidase from *Cellvibrio mixtus*. *Curr. Microbiol.* **35**, 228–232.
- Schiraldi, C., Giuliano, M., and De Rosa, M. 2002. Perspectives on biotechnological applications of archaea. *Archaea* **1**, 75–86.