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ORIGINAL PAPER

Cloning, expression and characterization of a thermostable exo-β-D-glucosaminidase from the hyperthermophilic archaeon *Pyrococcus horikoshii*

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Abstract An exo-*β*-D-glucosaminidase gene (PH0511) was cloned from the hyperthermophilic archaeon, *Pyrococcus horikoshii*, and expressed in *Escherichia coli*. The purified protein showed a strong exo-*β*-D-glucosaminidase activity by TLC analysis. DTT (50 mM) had little effect on its homodimeric structure during SDS-PAGE. The enzyme was optimally active at 90°C (over 20 min) and pH 6. It had a half-life of 9 h at 90°C and is the most thermostable glucosaminidase described up to now. The activity was not inhibited by ethanol, 2-propanol, DMSO, PEG-400, denaturing agents SDS (5%, w/v), urea, guanidine hydrochloride (5 M) and Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, Ni^{2+} (at up to 10 mM).

Keywords Denaturing agents · Exo-*β*-D-glucosaminidase · *Pyrococcus horikoshii* · Thermostability

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Introduction

Chitin is an insoluble polysaccharide of *β*-1,4-linked *N*-acetyl glucosamine (GlcNAc) residues. Chitosan, a deacetylation form of chitin, is a linear polymer of glucosamine (GlcN). Chitosan and its partially hydrolyzed form, chitooligosaccharides, as well as GlcN, have received much attention because of their many applications in biomedical, agricultural and environmental fields (Shahidi et al. 1999). Chitosan oligomers and glucosamine have been prepared by hydrolysis with HCl (Muzzarelli et al. 1980). An alternative mild method is to use chitosanolytic enzymes. Until now, chitosanolytic enzymes, which are divided into endo- and exo-chitosanases, have been reported from many microorganisms (Eom and Lee 2003).

Pyrococcus horikoshii OT3 is an anaerobic hyperthermophilic archaea which grows from 85 to 105°C. By analysis of the completed sequencing genome, at least six glycosidase are present in this organism (Kawarabayasi et al. 1998). However, only one *β*-glycosides, one *β*-mannosidase, and an endoglucanase have been reported (Matsui et al. 2000; Kaper et al. 2002; Ando et al. 2002). In this paper, we report the cloning, expression and characterization of the exo-*β*-D-glucosaminidase (GlcNase) from *Pyrococcus horikoshii* OT3.

Materials and methods

Materials, bacterial strains, plasmid and medium

Pyrococcus horikoshii OT3 genomic DNA was kindly presented by Dr Ikuo Matsui (National Institute of Advanced Industrial Science and Technology of Japan). Restriction enzymes, *Pyrobest* DNA polymerase and DNA ligation kit ver.2.1 were obtained from TakaRa. *Escherichia coli* DH5α and BL21-CodonPlus (DE3)-R-IL were used as the cloning and expression hosts, respectively for plasmid pET15b (Novagen) and were cultivated in LB medium at 37°C. Ampicillin and chloramphenicol were added at 100 μg ml^{-1} and 34 μg ml^{-1}, respectively, when needed.

Cloning of the exo-β-D-glucosaminidase gene

Based on the exo-β-D-glucosaminidase gene (PH0511) sequence (BAA29599, Genbank), two primers [sense (5′-TCCACCTCATATGGTAGGTATGAAAGTTCAACACGATGG-3′) and antisense (5′-TGTTGCGTGTCGACTAAAATTCTATCTCAAAGGTTTCCCTGTCGTGCC-3′)], underlined sequences indicate the *Nde*I site in the sense primer and the *Sal*I site in the antisense primer) were designed to amplify the gene by PCR. The 100 μl reaction mixture contained genomic DNA (10 μg), two primers (0.5 μM each), dNTPs (125 μM), *Pyrobest* buffer and 2.5 units of *Pyrobest* polymerase. It was subjected to 30 cycles of amplification (30 s at 94°C, 30 s at 55°C, 1 min at 72°C). The amplified DNA was digested with *Nde*I and *Sal*I and then cloned into plasmid pET15b as directed by the enzyme and kit manufacturers. The resulting plasmid was designated pET15b-$BglA_{Ph}$. The absence of unintended mutations was confirmed by DNA sequencing.

Over-expression of the gene and purification of the exo-β-D-glucosaminidase

An overnight culture of *E. coli* BL21-CodonPlus (DE3)-RIL harboring pET15b-$BglA_{Ph}$ was diluted 1:100 and grown until the OD_{600} reached 0.5. The culture was induced with 0.5 mM IPTG for 4 h. Cells were harvested by centrifugation (6000*g* for 15 min at 4°C), resuspended in 50 mM Tris/HCl and 50 mM NaCl (pH 8), disrupted by sonication. The disrupted cells were incubated at 85°C for 30 min to obtain heat-stable enzymes, and then centrifuged (14,000*g* for 30 min). The supernatant was loaded on a nickel column (Novagen). The enzyme was eluted with the buffer containing 150 mM imidazole. The resulting elute was dialyzed in 50 mM Tris/HCl buffer (pH 8) and then applied on a HiTrap Q anion exchange column (Amersham). The enzyme was eluted with a linear gradient of NaCl (0–1 M) in the same buffer, and the peak fractions eluting at 0.3 M NaCl were collected. The purified protein was analyzed by SDS-PAGE and gel filtration. Protein concentration was determined by Bradford method. The purified enzyme solution was mixed with same volume of glycerol (50%, v/v) and stored at –20°C. His-tag sequence of the recombinant protein was removed by thrombin (Sigma) to study its probable effect on the enzyme activity.

Enzyme assay

The standard assay for $BglA_{Ph}$ activity was carried out by modifying a spectrofluorimetry method which was devised for *Tk-Dac* (diacetylchitobiose deacetylase from *Thermococcus kodakaraensis*; Tanaka et al. 2004) assay with a fluorogenic substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (GlcNAc-4 MU, Sigma). The reaction was performed at 75°C for 1 h in 100 μl of 100 μM GlcNAc-4 MU, 50 mM HEPES/NaOH (pH 8) with 100 ng Dac_{ph} (diacetylchitobiose deacetylase from *Pyrococcus horikoshii*, Liu et al. 2006) which could deacetylate one acetyl group of GlcNAc and chitin oligosaccharides. The condition was confirmed to be sufficient for complete deacetylation of GlcNAc-4 MU. The protein in the reaction mixture was removed with a microfilter (10 kDa, Millipore). Removal of the Dac_{ph} protein was confirmed by the absence of detectable deacetylation activity in the filtrate. The filtrate was then added to 4 vol 50 mM MES/NaOH (pH 6) with 60 ng purified $BglA_{Ph}$. The mixture was incubated at

80°C for 20 min. After the reaction the mixture was cooled, mixed with same volume of 100 mM glycine/NaOH (pH 11) and the fluorescence (excitation at 350 nm and emission at 440 nm) was measured. One unit of the enzyme activity was defined as the amount of the enzyme which liberated 1 μmol 4 MU per min. Each sample was measured in triplicates and averaged.

The effects of various additives on enzyme activity were determined by pre-incubating the enzyme with various concentrations of additives at room temperature for 30 min, followed by standard assay. The activity of enzyme without any additive was defined as 100% level.

Results

Cloning, expression of the gene and purification of the protein

In the genome of *P. horikoshii*, a gene (PH0511) was initially annotated as a *β*-galactosidase (Kawarabayasi et al. 1998). It is consisted of 2337 bp and encodes a protein of 778 amino acids with a predicted molecular mass of 91.7 kDa. The deduced amino acids sequence has overall sequence identity with its archaeal homologs: putative *β*-galactosidase from *Pyrococcus furiosus* and *Pyrococcus abyssi* (PF0363, 81% and PAB1349, 69%), and exo-*β*-D-glucosaminidase from *Thermococcus kodakaraensis* (TK1754, 61%), but it shows no identity with the only reported sequences of exo-*β*-D-glucosaminidase from eukaryotes (fungi) *Trichoderma reesei* (BAD99604, GenBank) and bacteria *Amycolatopsis orientalis* (AY962188, GenBank).

In order to determine the enzymatic properties of PH0511 product, a plasmid pET15b-BglA_{Ph} was constructed. The recombinant protein was purified to homogeneity as described in Materials and methods. When BglA_{Ph} was not boiled, it migrated as a complex of molecular mass about 180 kDa in SDS-PAGE. After boiling for 10 min in the sample buffer containing reducing agent *β*-mercaptoethanol, only a small fraction of the protein was denatured and appeared at the position about 90 kDa, with another slightly smaller band which was probably a partly denatured subunit (Fig. 1A). Gel filtration showed that the protein was eluted at a peak corresponding to a 180 kD molecular mass (Fig. 1B). This suggests that the active form of BglA_{Ph} is a thermostable homodimer which is resistant to reducing agent in SDS-PAGE.

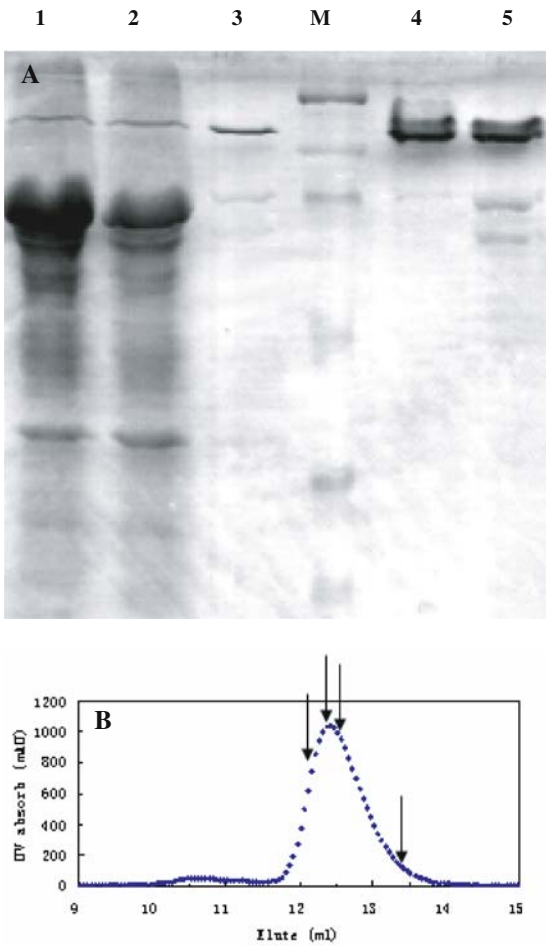

Fig. 1 (**A**) SDS-PAGE (12% polyacrylamide gel) of BglA_{Ph}. Lanes: 1. Crude cell extract; 2. Soluble fraction; 3. Supernatant after heat-treatment for 30 min at 85°C; M. protein markers (from top to bottom: 201, 120, 100, 56, 38, 30 kDa); 4. The purified BglA_{Ph} was not boiled; 5. BglA_{Ph} was boiled for 10 min in SDS-PAGE sample buffer containing 50 mM Tris/HCl (pH 6.8), SDS (2%, w/v), bromophenol blue (0.1%, w/v), glycerol (10%, v/v), and reducing agent *β*-mercaptoethanol (1%, w/v). (**B**) Gel filtration of BglA_{Ph}. The arrows indicated the eluted positions of BglA_{Ph} and protein markers (from left to the right): catalase (232 kDa, 12.25 ml), BglA_{Ph} (12.41 ml), aldolase (158 kDa, 12.51 ml), and albumin (67 kDa, 13.38 ml)

Substrate specificity and mode of action

The enzyme showed no activity towards lactose, $GlcNAc_2$, $GlcNAc_3$, cellobiose and cellotriose, as well as colloidal chitin, cellulose, lichenan, laminarin and xylan, but distinct activity were observed towards chitosan and chitooligosaccharides (data not shown). The product towards $GlcN_2$ and $GlcN_3$ was one GlcN monomer shorter oligosaccharide of the substrate (Fig. 2). Because the GlcNAc group is from the non-reducing end of fluorogenic substrate GlcNAc-4MU, the results above suggest that $BglA_{Ph}$ is an exo-type enzyme which specifically hydrolyzes the non-reducing terminal glycosidic bond of chitooligosaccharides or chitosan.

Temperature and pH optima, thermostability and pH stability

The enzyme was optimally active at 90°C (over 20 min) and pH 6. It showed more than half of the maximum activity at 70–100°C and pHs between 5.5–7 (Fig. 3A, B). After incubation for 12 h at pH 6 or 7, the residual activity was greater than 60%, but most (>85%) was lost at pH 4, 8 or 9 (Fig. 3D). The enzyme was highly thermostable, with a half-life about 9 h at its optimal temperature (90°C) (Fig. 3C). Removal of the His-tag sequence had no effect on the activities of the recombinant enzyme (data not shown).

Effect of various additives

No influence on the activity was observed with Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, Ni^{2+} (1, 5, 10 mM each) and the chelating reagent EDTA, indicating that it is not a metalloenzyme. However, Cu^{2+}, Zn^{2+}, Fe^{2+} at 10 mM inhibited the enzyme [0, <5% and <40% of the original activity (69 U mg^{-1}), respectively].

The enzyme was relatively stable (>90%) in the reducing agent, DTT (50 mM), and denaturing agents SDS (5%, w/v), guanidine hydrochloride (5 M) and urea (5 M). It also appeared to be stable (>85%) in organic solvents (100% v/v) ethanol, 2-propanol and PEG-400, more than 65% activity was maintained in presence of DMSO. However, the serine modifying reagent PMSF (50 mM) caused a drastic loss of the enzyme activity (<1%), suggesting that serine residues may be involved in catalysis of the enzyme.

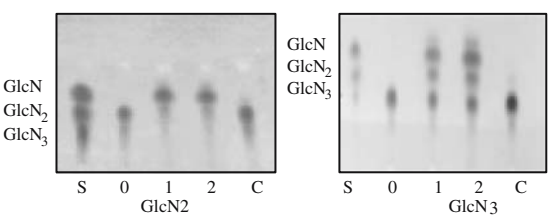

Fig. 2 TLC analysis of the reaction products towards $GlcN_2$ and $GlcN_3$. The oligosaccharide standards were indicated as S. Reaction mixtures (100 μl) containing 8 mM substrate in 50 mM MES/NaoH (pH 6) were incubated with 60 ng purified $BglA_{Ph}$ at 80 °C and analyzed after reaction for 0, 1, 2 h as shown in lanes 0, 1, and 2, respectively. Samples without enzyme were indicated as C

Discussion

An exo-β-D-glucosaminidase from *P. horikoshii* was expressed in *Escherichia coli* and purified to homogeneity. It was optimally active at 90°C and pH 6. The half-life of the enzyme at 90°C was 9 h. To our knowledge, it is the most thermophilic and first reported thermostable GlcNase by now. The enzyme seems to be a homodimer of 90 kDa subunit (resistant to heat and reducing agent in SDS-PAGE) which is consistent with the proposed mechanism that oligomerization is a major factor contributing to the thermostability of hyperthermophilic enzymes (Vieille and Zeikus 2001). Furthermore, a modified sensitive spectrofluorimetry method to assay exo-β-D-glucosaminidase was introduced.

The enzyme was not inhibited by some metal ions and additives such as organic solvents, urea and guanidine hydrochloride. Metal ions are not required for its activity. The exceptional inhibition by Fe^{2+} is similar to that of thermostable chitinase from *Thermococcus chitonophagus* (Andronopoulou and Vorgias 2003). Exo-β-D-glucosaminidase from *Aspergillus fumigatus* is also inhibited by Cu^{2+} (Jung et al. 2006), implying they may share the same catalytic mechanism. Compared with the method by HCl hydrolysis, degradation of chitin-related sugars by thermophillic glucosaminidase could reduce the possibility of unexpected products, avoid contamination and

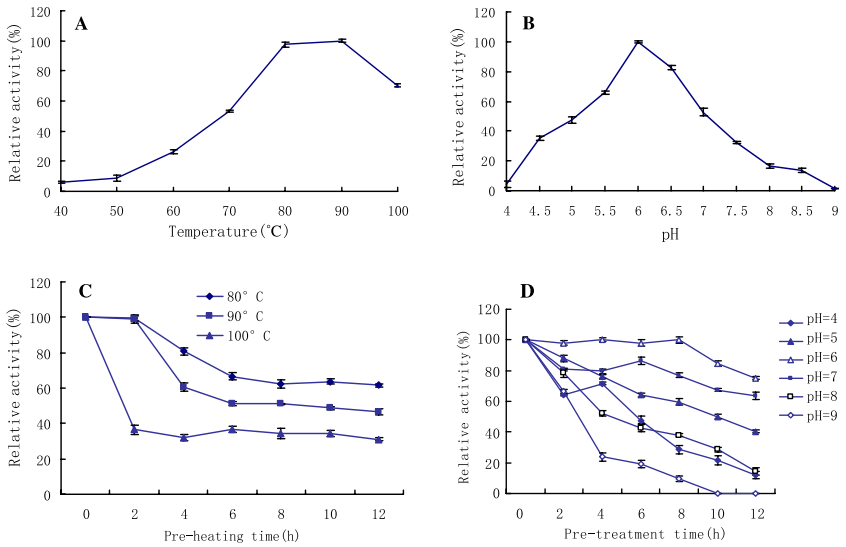

Fig. 3 Temperature (**A**) and pH (**B**) optima, thermostability (**C**) and pH stability (**D**). (**A**) The optimal temperature was determined by running the standard assay at various temperatures. The highest activity was defined as 100% level (71 U mg^{-1}). (**B**) The optimal pH was determined by running the standard assay using 50 mM sodium acetate (pH 4–5.5), 50 mM MES/NaOH (pH 5.5–7), and 50 mM Tris/HCl (pH 7–9). The highest activity was defined as 100% level (69 U mg^{-1}). (**C**) The enzyme was preheated in 50 mM MES/NaOH (pH 6) at 80°C, 90°C, 100°C or (**D**) pre-incubated at various pH values at room temperature for up to 12 h. Samples were taken at various times and the residual activities were measured according to the standard assay and expressed as the percentage of the initial activity (69 U mg^{-1})

improve the rate of reaction (Muzzarelli et al. 1980; Egorova and Antranikian 2005). Therefore, the enzyme may be exploited to be useful in biotechnological applications, such as degradation or structural analysis of chitosan or partly acetylated chitooligosaccharides and peptidoglycan with glucosamine residues (Nanjo et al. 1990).

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