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RESEARCH NOTE

Molecular Cloning and Anti-fungal Effect of Endo-β-1,3-glucanase from *Thermotoga maritima*

Chang-Bin Woo, Hae-Na Kang, and Soo-Bok Lee

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Abstract A gene encoding an endo-β-1,3-glucanase from *Thermotoga maritima* MSB8 (TmβG) was cloned and expressed in *Escherichia coli*. The purified enzyme produced various β-1,3-glucooligosaccharides from soluble laminarin, and mainly β-1,3-glucooligosaccharides smaller than laminaritetrose from insoluble curdlan. The optimum pH and temperature of the enzyme were 5.0 and 80ºC, respectively. TmβG inhibited the growth of *Candida albicans*, which indicates that the enzyme could potentially be used as an anti-fungal agent to control invasive infections.

Keywords: endo-β-1,3-glucanase, *Thermotoga maritima,* β-1,3-glucans, β-1,3-glucooligosaccharide, anti-fungal

Introduction

β-1,3-Glucan, a polysaccharide of β-(1,3)-linked glucose, is a major constituent of fungal, plant, and marine macroalgae. Laminarin and curdlan are two common β-1,3-glucans. Laminarin is a soluble storage polysaccharide mainly found in brown algae such as *Laminaria* spp. (1). Curdlan is usually a bacterial, water-insoluble linear β-(13) homopolymer of D-glucose (2).

β-1,3-Glucanase can degrade β-1,3-linked glucan, which is major component of fungal and plant cell walls (3). The enzyme may thus help protect against fungal pathogens by hydrolyzing β-1,3-glucan, a major cell wall component, which also functions in cell differentiation (4). β-1,3-Glucanases in bacteria belong to GH 16, members of which degrade β-1,3-glucan polymer as substrate for assimilation. Endo-β-1,3-glucanase can be used in the industrial production of yeast extract (5) and soluble β-1,3-glucan, which is a potential immunomodulator (6). Endo-β-1,3-glucanase can also serve as a biocontrol agent for protecting plants from fungal invasion. Extensive hydrolysis of the β-glucan polymer of the fungal cell wall may play a role in fungal cell lysis, allowing for the control of fungal diseases (7). For example, growth of *Candida* sp. was reported to be prohibited by endo-β-1,3-glucanases from *Pichia anomala* and *Paenibacillus* sp. (8). *C. albicans* is a dimorphic fungus that can switch from a unicellular yeast form to one characterized by mycelial hyphae, thereby enhancing its virulence as a fungal pathogen. It is a widespread pathogenic yeast-like fungus that has increased in importance because it causes disease in immuno-compromised humans (9,10). The growth of *C. albicans* is reportedly inhibited by endo-β-1,3-glucanase (11). Thus, endo-β-1,3-glucanases can be utilized industrially to protect foods and plants from fungal invasion (11,12). Endo-β-1,3-glucanases have also been described in hyper-thermophilic bacteria, including *Rhodothermus marinus* (13), *Thermotoga neapolitana* (14), and others. The β-1,3-glucooligosaccharide pattern in a complete hydrolysis is characteristic for a given enzyme.

In this study, the gene encoding an endo-β-1,3-glucanase from *T. maritima* was cloned and expressed in *Escherichia coli*. The biochemical properties and anti-fungal activity of the purified enzyme against the disease-causing fungus, *C. albicans*, were investigated.

Materials and Methods

Strains, plasmids, and chemicals Genomic DNA of *T. maritima* MSB8 was purchased from the American Type Culture Collection. *E. coli* strain BL 21 (DE3) was used as

Chang-Bin Woo, Hae-Na Kang, Soo-Bok Lee (✉)
Department of Food and Nutrition, Brain Korea 21 Project, Yonsei University, Seoul 120-749, Korea
Tel: +82-2-2123-3124; Fax: +82-2-312-5229
E-mail: soobok@yonsei.ac.kr

a host for gene manipulation and expression of the recombinant protein. Plasmid pGEX-4T-1 vector was used as an expression vector, containing the tac promoter, the glutathione S transferase (GST) tag, and ampicillin resistance gene. The *E. coli* transformants were cultured at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/mL). *C. albicans* were purchased from the Korean Culture Center of Microorganisms (KCCM). All other chemicals used were of reagent grade.

PCR cloning and expression of endo-β-1,3-glucanase The gene for the endo-β-1,3-glucanase from *T. maritima* MSB8 (TmβG, Q9WXN1) was amplified using the genomic DNA as a template with PCR using i-StarTaq DNA Polymerase (iNtRON Biotechnology, Seongnam, Korea). Oligonucleotide primers for the 5- and 3-flanking ends of the gene were 5-ATGAAAAGGGGATCCATGATGAGC AGGCTGGTT-3 (forward) and 5-CACCGGGGAGTCGA CTCATTGAGGGCTCACCGT-3 (reverse), and contained BamHI and SalI restriction sites, respectively (underline). The 1.9 kb PCR-amplified DNA fragment was subsequently digested with BamHI and SalI, and thereafter ligated into the expression vector pGEX4T1 to construct pGEX4T1-TmβG. The final construct was transformed into competent *E. coli* BL21(DE3).

Purification and assay of the recombinant enzyme TmβG was purified according to a previously-described procedure (15). The recombinant GST-fusion enzyme in the supernatant after centrifugation (7,000×*g*,10 min, 4°C) was purified by Glutathione Sepharose 4 Fast Flow (FF) affinity column chromatography (GE Healthcare Bio-Sciences, Sweden), in which the protein was eluted with 50 mM Tris-HCl (pH 8.0) containing 40 mM reduced glutathione. The eluted fractions were pooled and treated with thrombin protease (40 U/mg protein) for 24 h at 25°C. After dialysis, the GST portion of the fusion protein was removed through the Glutathione Sepharose 4 FF column, and the eluted solution was applied to a Q-Sepharose FF column (Hi-Load 16/10) equilibrated with 20 mM Tris-HCl (pH 7.5). This solution was then eluted with a linear NaCl gradient (0.0-1.0 M) in the same buffer to separate the GST and the thrombin protease from free TmβG and dialyzed.

TmβG activity was determined in sodium acetate buffer (pH 5.0) at room temperature by measuring hydrolysis of laminarin (1%) using the copper-bicinchoninate method at 560 nm (16). The reaction was stopped by adding 0.1 N HCl. The hydrolytic reactions catalyzed by TmβG were analyzed TLC and high performance anion exchange chromatography (HPAEC). TLC was carried out according to a previously-described procedure on Whatman K5F silica gel plates (Whatman, Kent, UK) with the solvent system of ethyl acetate:acetic acid:water (3/2/1, v/v/v) (17). HPAEC was also employed to analyze the reaction product on a Dionex CarboPac PA1 column (250×4 mm; Sunnyvale, CA, USA) with a gradient of 120 mM sodium acetate in 150 mM NaOH (solvent A) and 600 mM sodium acetate in 150 mM NaOH at a flow rate of 1.0 mL/min.

Effects of pH, temperature, and chemicals on enzyme activity To determine the optimal pH of the enzyme, the activity was measured in the following 50 mM buffers at room temperature: sodium acetate (pH 2.0 to 5.0), sodium citrate (pH 5.0 to 6.0), sodium phosphate (pH 6.5 and 7.0), Tris-HCl (pH 8.0 to 9.0), and glycine-NaOH (pH 10.0 to 11.0). The optimal temperature of the enzyme was investigated in a range of 25 to 90°C in 50 mM sodium acetate buffer (pH 5.0). The effects of metal ions ($CaCl_2$, $CuCl_2$, $MgCl_2$, and $MnCl_2$) and organic solvents [dimethylsulfoxide (DMSO), methanol, and ethanol] on the enzyme activity were also examined. The enzyme was added and mixed in the absence and presence of 10 mM cations or a 10% (v/v) organic solvent in 50 mM sodium acetate buffer (pH 5.0), and immediately after an appropriate aliquot was taken, the relative activity was measured at room temperature.

Anti-fungal activities of TmβG *C. albicans* (KCCM 11282) was cultivated overnight in 5mL liquid medium containing 0.3% yeast extract, 0.3% malt extract, 1% dextrose, and 0.5% peptone at 37°C. After centrifugation (7,000×*g*, 10 min, 4°C), the cell pellets were harvested and resuspended in 1mL sterile distilled water (DW) at 0.5 optical density (OD). The diluted fungus suspension and the purified enzyme were incubated at various concentration of the enzyme ranging from 0.1-0.5 mg/mL in 50 mM sodium citrate buffer (pH 5.0) at 30°C (3 h), and the mixture was then spread out on an agar plate. The plate was incubated at 30°C for 20 h.

Results and Discussion

Biochemical properties of endo-β-1,3-glucanase (TmβG) The open reading frame (1.9 kb) of TmβG from *T. maritima* MSB8 encodes a single polypeptide of 642 amino acid residues. The TmβG gene was expressed in *E. coli* as a glutathione S-transferase (GST)-tagged protein with a molecular weight of approximately 99 kDa. The GST tag (26 kDa) was completely released by thrombin treatment. TmβG produced broad but distinct hydrolyzed product profiles when β-glucans such as laminarin and curdlan were used as substrates. When TmβG was reacted with laminarin, hydrolyzed products in the size range from glucose to β-1,3-glucooligosaccharides with degree of polymerization (DP)8 were disproportionately represented

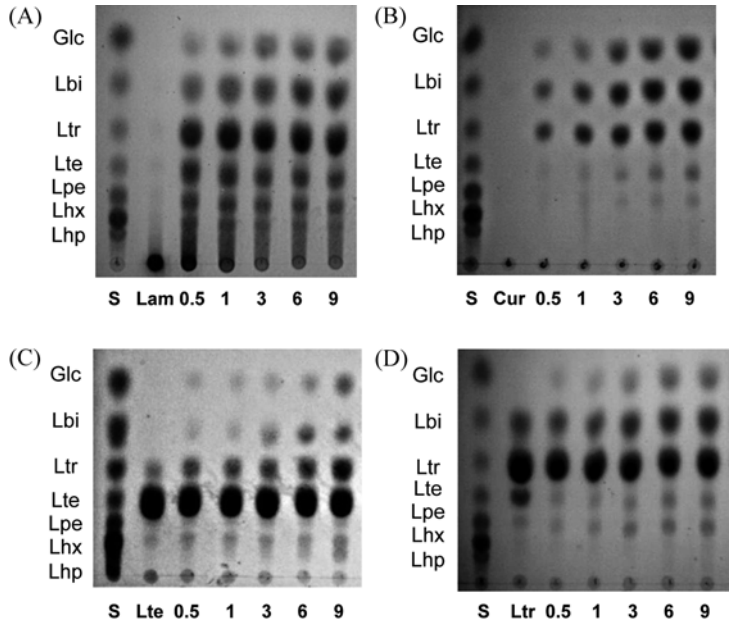

Fig. 1. TLC analysis of *Thermotoga maritima* MSB8 (TmβG) reaction with β-glucans at 50°C. Reactions of TmβG with 1% laminarin (A), with 1% curdlan (B), with 1% laminaritetraose (C), and with 1% laminaritriose. The reactions were carried out with TmβG at 0.1 mg/mL and β-glucans in 50 mM sodium citrate buffer (pH 5.0). Lane S, standard β-1,3-glucooligosaccharides; lanes 0.5, 1, 3, 6, 9, reaction times of TmβG; Cur, curdlan; Lam, laminarin; Lte, laminaritetraose; Ltr, laminaritriose

(Fig. 1A). When the enzyme was reacted with curdlan, hydrolyzed products mainly ranged in size from glucose to laminaritriose (Fig. 1B). When laminaritriose and laminaritetrose were used as substrates, transglycosylation activity was detected as well as hydrolysis, as evidenced by the production of β-1,3-glucooligosaccharide products with DP>3 or 4, such as laminaritetrose, laminaripentaose, and laminarihexaose (Fig. 1C, 1D). The β-1,4-glucans carboxyl methyl cellulose (CMC) and cellobiose, and the α-1,4-glucan amylose could not be hydrolyzed by the enzyme to any appreciable extent. Only β-1,3-glucans like laminarin were efficiently degraded. This resembles the behavior of other hyperthermophilic endo-β-1,3-glucanases (laminarinases) from *Pyrococcus furiosus* and *T. petrophila* (18,19). The optimum temperature and pH of TmβG were determined to be approximately 80°C and pH 5.0, respectively (Fig. 2). The enzyme was thermostable, as expected of an enzyme that originated from a hyperthermophilic bacterium. In addition, the β-1,3-glucanase activity of the enzyme was increased approximately 1.5-2-fold by the presence (10 mM) of Ca^{2+}, Mg^{2+}, or Na^{+} ions. It has been reported that the enzyme contains Ca^{2+} binding sites and that these play a role in its thermoactivity (18). The enzyme's activity was not substantially affected by Cu^{2+}or Zn^{2+}, but was significantly decreased by Co^{2+} and Mn^{2+}. The enzyme's activity in the presence of 10% of the organic solvents methanol, ethanol, and DMSO was 52, 34, and 16% of its activity in their absence, respectively.

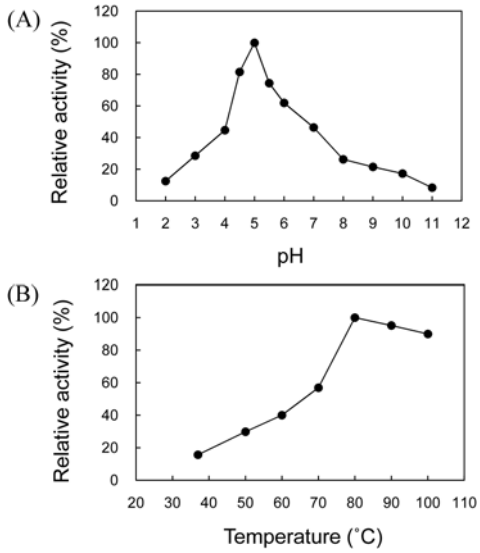

Fig. 2. Effects of pH and temperature on the relative activity of TmβG (A) Optimum pH (B) Optimum temperature. The hydrolysis of laminarin (1%) was determined at 50°C. The enzyme (1 mg/mL) was pre-incubated in various pHs for 90 min at 25°C.

Anti-fungal activities of endo-β-1,3-glucanase Endo-β-1,3-glucanase can hydrolyze and degrade the β-1,3-polysaccharides of fungal cell walls, inhibiting the growth of pathogenic fungi (3). We observed that the growth of *C. albicans* was substantially inhibited by TmβG in a concentration-dependent manner (Fig. 3). Approximately

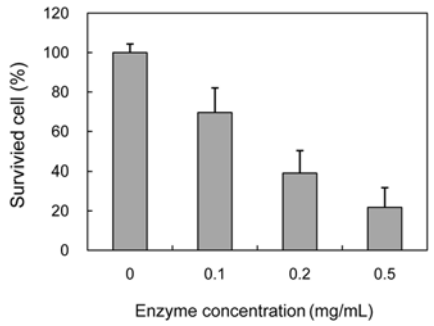

Fig. 3. The growth inhibition of *C. albicans* by various concentrations of TmβG. The enzyme reaction was incubated in 50 mM sodium citrate buffer (pH 5.0) at 30°C.

60% of the growth of *C. albicans* was inhibited by TmβG at levels higher than –200 μg/mL (–2.7 μM). Recently, endo-β-1,3-glucanase from *Paenibacillus* sp. was reported to inhibit growth at approximately 0.3-1 μM (11). TmβG was clearly able to inhibit the growth of *C. albicans*, which pose serious health threat (12). This suggests that TmβG could potentially be used as a new thermostable anti-fungal agent with an improved safety profile.

In conclusion, TmβG can degrade β-1,3-glucan to produce various β-1,3-glucooligosaccharides from soluble laminarin and insoluble curdlan. TmβG also significantly inhibited the growth of *C. albicans*. Thus, TmβG is a thermostable anti-fungal β-1,3-glucanase with potential for use in food preservation.

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Disclosure The authors declare no conflict of interest.

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