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Characterization of a glucan phosphorylase from the thermophilic archaeon *Sulfolobus tokodaii* strain 7

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Abstract

A glucan phosphorylase from the hyperthermoacidophilic crenarchaeaon *Sulfolobus tokodaii* strain 7 (stGP) was characterized. The enzyme displayed maximum activity at 75 °C and pH 6.0 and was highly stable at 95 °C. The enzyme showed distinct substrate specificity, with maltose being the minimum primer for glucan synthesis and maltotriose being the minimum substrate for degradation. Interestingly, the sequences of GPs from Crenarchaeota clustered all together and formed a distinct lineage. Compared with previously characterized GPs, stGP (56 kDa) was much smaller. Structure modeling revealed that stGP was more compact than other GPs and it seemed that stGP was more primitive and adaptable to high temperature. Taken together, stGP and its homologues in Crenarchaeota probably constitute a novel group of GPs.

Keywords: Glucan phosphorylase; Crenarchaeota; *Sulfolobus tokodaii*; Thermostability; Glucan

1. Introduction

Glucan phosphorylases (GP, EC 2.4.1.1) catalyze reversible degradation of α-1, 4-glucan into glucose-1-phosphate (G-1-P), which provides readily useable energy *in vivo* for the cells. Glucan phosphorylases are ubiquitous in animals, plants, and microorganisms. The GPs analyzed so far are similar in size and the amino acid sequences are highly conserved. They share similar catalytic mechanism [1]: all GPs require pyridoxal 5′-phosphate for enzymatic activity. However, the regulation mechanisms and natural substrates of the enzymes are varied [2]. The substrates of GPs include glycogen, starch, and dextrin.

GPs are expected to be useful in a broad range of industrial applications, including the conversion of starch to G-1-P or the synthesis of amylose, maltooligosaccharides, and other engineered glucose polymers [3–6]. In previous reports, GPs of *Escherichia coli* [7,8], yeast [9], potato [10], and rabbit muscle [11,12] have been studied in detail regarding to structural and functional relationship of the enzymes. Biochemical properties of some mesophilic and thermophilic bacterial GPs have been investigated [13–17]. However, in archaea, only maltodextrin phosphorylase (MalP) from the euryarchaeon *Thermococcous litoralis* has been characterized up to now [18], although glycogen has been identified in cells of many thermoacidophilic archaea including *Sulfolobus*, *Termoproteus*, and *Thermococcous* [19] and glycogen phosphorylase activities have been detected in the cell extracts of *Thermoproteus tenax* [20] and *Methanococcus maripaludis* [21], little is known about the enzymatic properties of GPs from thermophilic Crenarchaeota, another major subdomain of Archaea.

Sulfolobus tokodaii strain 7 was originally isolated from an acidic hot spring in Beppu, Kyushu, Japan in the early 1980s [22,23]. It is an aerobic thermoacidophile growing optimally at pH 2–3 and 75–80 °C. Herein, we report the cloning and expression of the GP gene from *S. tokodaii* strain 7 and the biochemical characterization of the enzyme. Particularly, the thermostability and substrate specificity were investigated. The results show that GPs from thermophilic Crenarchaeota are potentially useful for the synthesis of various expensive glucose polymers in industry.

Abbreviations: GP, glucan phosphorylase; stGP, GP from *Sulfolobus tokodaii*; G-1-P, glucose-1-phosphate; GlgP, glycogen phosphorylase; MalP, maltodextrin phosphorylase; PLP, pyridoxal phosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; TLC, thin-layer chromatography.

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2. Materials and methods

2.1. Strains and chemicals

S. tokodaii strain 7 was purchased from Japan Microbes Collections and cultured in Allen's mineral medium modified by Brock et al. [24]. The genomic DNA was extracted based on the method reported [25]. Restriction enzymes, Pyrobest DNA polymerase, and the DNA ligation kit (ver.2.1) were obtained from Takara. Maltose, maltotriose, and maltotetraose (with the purity of 98%, 96%, and 96% respectively) and other reagents were purchased from Sigma. Plasmid pET15b (Novagen) was modified with *Nde*I being changed to *Nco*I in order to facilitate protein purification. *E. coli* strains DH5α and BL21(DE3)-CodonPlus-RIL were used as the cloning and expression hosts.

2.2. Cloning of GP gene from S. tokodaii strain 7

Based on nucleotide sequence of the putative GP gene (ST0893), sense primer 5′GCACAGGTTATCT**CCATGG**GGAAACACTT3′ and antisense primer 5′GTCAGAGCTGTA**GTCGAC**CTTATAAAGCGTATT 3′ were designed (the *Nco*I and *Sal*I sites were indicated in bold). The amplified fragments were digested with *Nco*I and *Sal*I, and ligated into *Nco*I/*Sal*I-digested pET15b. The resultant recombinant plasmid, designated pET15b/stGP, was sequenced to ensure that no unintended mutation had occurred.

2.3. Site-directed mutagenesis

The site-directed mutation was introduced by the PCR-based overlap extension method. The PCR products containing the mutations were digested and ligated into pET15b. After sequence confirmation, the recombinant plasmids were transformed into *E. coli* BL21(DE3)-CodonPlus-RIL cells for expression.

2.4. Expression and purification of the wild-type and mutant stGPs

About 1% of overnight culture of *E. coli* BL21(DE3)-CodonPlus-RIL harboring the recombinant plasmid was inoculated into Luria Bertani medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The cells were cultured at 37 °C with shaking. When the OD_{600} reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After induction for further 4 h, the cells were harvested by centrifugation, suspended in 100 mM Tris–HCl (pH 8.0), and disrupted by sonication. The crude extract was incubated at 75 °C for 30 min to denature *E. coli* proteins and centrifuged (10,000 × *g*) for 15 min. The supernatant was filtered using a 0.22 μm pore-sized membrane filter and loaded onto a nickel column (Novogen). The column was eluted with 80 mM Tris–HCl (pH 7.9) containing 150 mM imidazole. The purified enzyme was analyzed by SDS-PAGE.

2.5. Activity assay of stGP

The enzymatic activity of stGP was determined according to the method as described previously with minor modifications [26]. For the synthesis assay, a reaction mixture (250 μl) containing 50 mM MES-NaOH (pH 6.2), 0.4% soluble starch, 10 mM G-1-P and an appropriate amount of enzyme was incubated at 37 °C for 10 min. The reaction was terminated by adding 1 ml of 250 mM glycine-HCl (pH 3.0) followed by rapid cooling in icy water. Ammonium molybdate (125 μl, 1%) in 25 mM H_2SO_4 and 125 μl of ascorbic acid (1%) in 0.05% $KHSO_4$ were then added to the mixture. After incubation at 37 °C for 30 min, the absorbance at wavelength of 700 nm was measured and the enzymatic activity was calculated. One unit of enzymatic activity was defined as the amount of the enzyme that produced 1 μmol of phosphate per minute.

For the phosphorolysis assay, the reaction mixture (1 ml) containing 50 mM MES-NaOH (pH 6.2), 0.5% soluble starch, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 2.5 μM glucose-1,6-diphosphate, 0.015% NADP, 0.5 U of glucose-6-phosphate dehydrogenase, 0.5 U of phosphoglucomutase and enzyme was incubated at 37 °C for 30 min. The enzymatic activity was determined by the production of NADPH in the reaction mixture which was calculated according to the change of absorbance at 340 nm. One unit represents the amount of enzyme, which catalyzes the production of 1 μmol of G-1-P per minute.

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

The effect of temperatures on the enzymatic activity of stGP in the synthetic direction was measured at pH 6.2 using starch as the substrate. To determine the thermostability of stGP, the enzyme in 50 mM MES-NaOH (pH 6.2) was pre-incubated for different lengths of time at various temperatures ranging from 75 °C to 95 °C. The residual activity was determined as described above.

The optimum pH of stGP was determined at 75 °C using the synthesis assay. The buffers used were as follows: 50 mM acetate sodium (pH 3.5, 4.0, 4.5, 5.0), 50 mM MES-NaOH (pH 5.5, 6.0, 6.5), 50 mM HEPES-NaOH (pH 6.5, 7.0, 7.5), and 50 mM Tris–HCl (pH 7.5, 8.0, 8.8).

2.7. Substrate specificity

The kinetic parameters of the synthetic and phosphorolytic activities of stGP towards different substrates were determined by incubating the purified enzyme and five different concentrations of substrates (glucose, maltose, maltotriose, maltotetraose and starch; 0.5 mM to 10 mM) in 50 mM MES-NaOH buffer (pH 6.2) at 37 °C. In the synthetic direction, the final concentration of the enzyme in the reaction mixture is 0.26 μM, and the amount of released phosphate was measured as described above in the synthesis assay. In the phosphorolytic direction, the reaction mixture containing 0.24 μM enzyme and different concentrations of substrates was incubated at 37 °C for 30 min. The level of NADPH produced was monitored at 340 nm as described in the phospho-