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Molecular investigation of a novel thermostable glucan phosphorylase from *Thermoanaerobacter tengcongensis*

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Abstract

The *glgP* gene in *Thermoanaerobacter tengcongensis* encodes a putative α-glucan phosphorylase (Tte-GlgP), which is much smaller than its homologs in most other bacteria and is grouped into a less-recognized family. To characterize this novel enzyme, it was overexpressed in *Escherichia coli* and purified to apparent homogeneity. This purified Tte-GlgP, with expected molecular size (∼64 kDa) and isoelectric point (∼pI 6.2), exhibited a wide substrate spectrum in transformation of a variety of glucans such as soluble starch, maltodextrins and glycogen into glucose-1-phosphate. Western blot analysis indicated that the production of Tte-GlgP in *T. tengcongensis* was repressed by glucose but induced by maltose slightly, suggesting that it was involved in the carbohydrate metabolism in this thermophile. The Tte-GlgP was optimally active at 60 °C, and was thermostable with 90% residual activity after treating at 60 °C for 6 h, thus the overexpressed Tte-GlgP might also be a good candidate for structure–function analysis in its family.
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1. Introduction

α-Glucan phosphorylases (GP or GlgP, EC 2.4.1.1) are widely distributed in microorganisms, plants and animals. They catalyze the reversible phosphorolysis of α-glucan substrates such as maltodextrin, starch and/or glycogen into glucose-1-phosphate (G-1-P) [1–4], and play a central role in the mobilization of the storage carbohydrates [1,2]. Several kinds of the GlgPs from *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus salivarius*, and *Corynebacterium callunae* have been reported [2], among which the starch phosphorylase from *C. callunae* was extensively investigated [3–8]. Although the *glgP*-like genes are detected in many thermophilic microorganisms, to our knowledge only the α-glucan phosphorylases from a thermophilic bacterium *Thermus thermophilus* [9,10] and the maltodextrin phosphorylase (MalP) from a hyperthermophilic archaeon *Thermococcus litoralis* [11] have been investigated in some respects.

Thermoanaerobacter tengcongensis MB4[T] is isolated from a hot spring in China and grows optimally at 75 °C. Its com-

plete genome sequence (GenBank No. NC003869) has been determined in 2002 [12]. A putative glucan phosphorylase gene, *glgP* (TTE1805), was detected through screening of the genome sequence data. It is noteworthy that the Tte-GlgP in *T. tengcongensis* is only about 60 kDa, which is much smaller than most other bacterial GlgPs (∼90 kDa) [3]. Therefore, it would be interesting to investigate its function in this thermophile. In addition, G-1-P produced by glucan phosphorolysis has many potential applications. For example, it can be used as an antibiotic or immunosuppressive drug for medical purposes [13], or can be used as a starting material for the synthesis of trehalose [14,15]. Trehalose is a non-reducing disaccharide of glucose which has been used as an active stabilizer in food storage, pharmaceutical preparations, and cosmetic industry [16,17]. We have found previously that the trehalose phosphorylase from the *T. tengcongensis* can actively catalyze trehalose synthesis from G-1-P and glucose [18]. Investigation of G-1-P production through glucan phosphorolysis may help to understand this trehalose synthesis pathway and to establish an alternative two-step procedure for production of trehalose from cheap glucan substrates such as starch.

In this paper, we report the molecular characterization of this novel glucan phosphorylase (Tte-GlgP) from *T. tengcongensis*.

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2. Materials and methods

2.1. Bacterial strains, plasmids and media

T. tengcongensis MB4[T] [19] was used as DNA source for cloning the glucan phosphorylase gene glgP. E. coli BL21DE3 (pLysS) (Novagen) was used as a host for gene expression. The expression vector utilized in this study was pET23b (Novagen). The T. tengcongensis cells were grown in the modified MB medium at 75 °C [19]. The E. coli strains were cultured at 37 °C in Luria-Bertani (LB) medium, and ampicillin was added to a final concentration of 100 μg/mL when necessary.

2.2. Gene cloning and overexpression

The glgP gene (TTE1805 in the GenBank NC003869) was amplified by PCR with primers 5′-ACGGATCCTATGGAAAACAAAAAACT-3′ and 5′-TAGTCGACTTTGGTATACATGAGGTC-3′ (the underlines indicate the BamHI and SalI site, respectively). The PCR products were digested by BamHI and SalI and cloned into pET23b at the same sites, resulting in the plasmid pGlgP. The inserted glgP gene was confirmed by DNA sequencing.

For overexpression of the Tte-GlgP in E. coli, a colony of E. coli BL21DE3 (pLysS) harbouring the plasmid pGlgP was selected and grown overnight in 3 mL LB containing ampicillin. Two millilitres of the overnight cultures were inoculated into 200 mL LB medium and cultured at the same condition. When $OD_{600\,nm}$ of this culture reached around 0.6, IPTG was added to 0.5 mM and cultivation was continued for an additional 3 h. Cells were then harvested by centrifugation at $6000 \times g$ for 10 min, washed with 50 mM potassium phosphate buffer (pH 7.4) and subjected to the following purification procedures.

2.3. Purification of the His-tagged Tte-GlgP

Cell pellets were resuspended in 8 mL binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9). After sonication, the cell lysates were centrifuged at $14,000 \times g$ for 20 min. The supernatants were filtrated though a 0.45 μm membrane. Further purification was operated by nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography with the His-Bind Kits (Novagen) according to the manufacture's instruction. Five millilitres of the purified Tte-GlgP was dialyzed overnight at 4 °C against 500 mL 25 mM potassium phosphate buffer (pH 7.4), and then further dialyzed twice for 3 h each in freshly prepared buffer with 20% glycerol. The purified Tte-GlgP was examined by SDS-PAGE analysis and the protein concentrations were measured by using the BCA[TM] protein assay kit (PIERCE).

2.4. Western blot analysis

To assess the expression profile of Tte-GlgP in T. tengcongensis via Western blotting assay, the antibodies against Tte-GlgP were obtained by immunizing BALB/c mice with the purified Tte-GlgP. The T. tengcongensis cells from fresh culture were treated for 4 h at 75 °C, respectively, with different concentration of glucose (0.5–1.5%) or maltose (0.5–1.5%) in fresh medium. Protein extracts were then separated by SDS-PAGE and transferred to PVDF membranes for Western blot analysis as previously described [20]. Briefly, the blots were incubated with mouse anti-Tte-GlgP antibody at 4 °C for 3 h, and then washed and incubated with alkaline phosphatase labeled goat anti-mouse IgG secondary antibody for 1 h at room temperature, followed by washing and incubation with staining solution including AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5) 10 mL, NBT 35 μL (76 mg/mL, Promega) and BCIP 25 μL (50 mg/mL, Promega) for 5 min at room temperature.

2.5. Determination of isoelectric point (pI)

The p*I* of the recombinant Tte-GlgP was determined by a capillary isoelectric focusing-whole column imaging detection method using cIEF 3–10 kit (Beckman). The separation column used is 27 cm × 50 μm (L.D.) uncoated fused-silica capillary. Analyses were performed with a P/ACE 5500 system (Beckman). The p*I* markers used were 2.75, 5.10 and 9.45, respectively.

2.6. Determination of substrate spectrum by electrospray ionization mass spectrometry (ESI-MS) analysis

The ESI-MS analysis [21] was used to determine the substrate spectrum of Tte-GlgP in this study. The reaction mixture was made as the following: 50 mM potassium phosphate buffer (pH 8.0) 60 μL, 0.01% mercaptoethanol 1.2 μL, 1% substrate (including soluble starch, glycogen, maltodextrin, maltoheptaose, maltopentaose, or maltotriose, respectively) 30 μL, Tte-GlgP (500 μg/mL) 2 μL, ddH₂O was added until the total volume was 120 μL. The reaction mixtures were incubated at 60 °C for 6 h, boiled at 100 °C for 5 min to stop the reaction, cooled on ice, and then filtrated through 0.22 μm membranes, respectively. Twenty microlitres of the reaction mixture was taken for ESI-MS analysis to measure the production of glucose-1-phosphate, while the reaction mixture without Tte-GlgP was used as the control. The EMI-MS was performed as the following: LCQ Deca XP[plus] ESI-ion trap (IT) mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) in negative mode was used to detect the G-1-P. The capillary temperature and the spray voltage were kept at 275 °C and 5.5 kV, and the sample solution containing 35% acetonitrile and 0.2% triethyamine was constantly infused into the ion source at 5 μL/min by attached syringe pump. The data processing was performed using Qual Browser software incorporated into Thermo Finnigan Xcalibur software package.

2.7. Determination of the enzyme activity by a modified coupled-enzyme method

The coupled-enzyme method for determination of the Tte-GlgP's activity [22,23] was established with minor modification. Briefly, the reaction mixture (120 μL) was prepared as the above section. After 30 min of incubation at 37 °C, the mixture was heated at 100 °C for 5 min and then cooled on ice, following by adding 228 μL of the assay reagent containing 200 mM Tris–HCl (pH 7.4), 10 mM MgCl₂, 0.5 mM NADP, 30 μM glucose-1,6-bisphosphate (Sigma Prod., No. G-6893), and 1.25 U/mL glucose-6-phosphate dehydrogenase (Sigma Prod., No. G-7750). The $A_{i\,340\,nm}$ was determined immediately, and then 12 μL of 1.25 U/mL phosphoglucomutase (Sigma Prod., No. P-3397) was added and the mixture was incubated at 37 °C and the $A_{f\,340\,nm}$ was measured 1 h later. The amount of glucose-1-phosphate was calculated based on the A_{f-i} value ($A_{f-i} = A_{f\,340\,nm} - A_{i\,340\,nm}$) and a standard curve established at the same time.

2.8. Determination of the optimum pH and temperature, and thermostability

For determination of the optimum pH of Tte-GlgP, fifty mM potassium phosphate buffer with pH ranging from 5.0 to 9.0 were used to prepare reaction mixture, and 0.25% soluble starch was used as substrate. The reaction time was 30 min and the other procedure was the same as the modified coupled-enzyme method. For determination of the optimum temperature, the reactions were performed from 37 to 80 °C, respectively. For determination of the thermostability, the Tte-GlgP protein was pretreated at 50, 60, 70 and 80 °C for 0–21 h, and then subjected to the standard activity assay. Relative activities were calculated by comparison of the G-1-P production.

3. Results and discussion

3.1. Phylogenetic analysis of the Tte-GlgP

The glgP gene of T. tengcongensis encodes a 540 amino acid polypeptide (∼60 kDa), which is much smaller than most other bacterial glucan phosphorylases. Phylogenetic analysis of 28 known GlgPs (including maltodextrin phosphorylase, MalP) from bacteria and archaea revealed two phylogenetic groups, named family I and II (Fig. 1A). Interestingly, most glucan phosphorylases from bacteria were about 90 kDa and were grouped into the family I, while the small-size Tte-GlgP from T. tengcongensis was grouped into family II with some

Fig. 1. (A) Phylogenetic relationship of the microbial glucan phosphorylases (GlgP and MalP), which are grouped into two families (I and II). Multiple-sequence alignments were obtained by using ClustalW. The phylogenetic tree was established by using the neighbor-joining method. The accession number for each protein and the amino acid numbers were indicated. The source organisms are as follows: Bli, *Bacillus licheniformis*; Blo, *Bifidobacterium longum*; Bsu, *Bacillus subtilis*; Cac, *Clostridium acetobutylicum*; Cca, *Corynebacterium callunae*; Cdi, *Corynebacterium diphtheriae*; Cpe, *Clostridium perfringens*; Eco, *Escherichia coli*; Hmo, *Heliobacillus mobilis*; Lac, *Lactobacillus acidophilus*; Lla, *Lactococcus lactis*; Lpl, *Lactobacillus plantarum*; Lsa, *Lactobacillus salivarius*; Pen,

archaeal GlgPs (Fig. 1A). The Tte-GlgP shared high sequence similarity (>40% in most cases) with the GlgPs in the family II, including those from thermophilic archaea *Pyrococcus furiosus* (Pfu-GlgP), *Sulfolobus acidocaldarius* (Sac-GlgP) and *Thermococcus* (Tzi-GlgP and Tli-GlgP), and Tth-GlgP from the thermophilic bacterium *T. thermophilus* (Fig. 1B). In contrast, the sequence similarity of the GlgPs between the two families is rather low (usually less than 20). It seems only the amino acids in the active sites and pyridoxal-phosphate (PLP) binding site [24] are conserved in both families, e.g. such sites presented in glucan phosphorylases of *E. coli* (Eco-GlgP) and *C. callunae* (Cca-GlgP) were also found in the family II (Fig. 1B). However, their amino acid sequences in other region are much different between these two families.

In comparison with the GlgPs in family I, the features of GlgPs in family II is much less revealed. To our knowledge, only the Tth-GlgP from *T. thermophilus* [9] and the Tli-MalP from *T. litoralis* [11] have been characterized in some biochemical features. Both Tth-GlgP (819 aa) and Tli-MalP (831 aa) are phosphorylases with normal molecular sizes (∼90 kDa,), while Tte-GlgP lacks large parts in both the N- and C-termini in comparing with its homologues (Fig. 1). In addition, Tte-GlgP contains all of the conserved active sites and a signature sequence corresponding to the PLP-cofactor binding site (EAS-GTSGMKAAMN, Fig. 1B), thus it may still be active and hence provide a novel natural model to characterize the basic features of the GlgPs in the family II.

3.2. Overexpression and purification of Tte-GlgP

To characterize the Tte-GlgP, the *glgP* gene was cloned into pET23b and overexpressed in *E. coli*. The high-level expression of Tte-GlgP was confirmed by SDS-PAGE analysis (Fig. 2A). The overexpressed His-tagged Tte-GlgP was a soluble protein, thus was easily purified to apparent homogeneity through a Ni-NTA metal affinity chromatography (Fig. 2A, lane 4). To eliminate any contamination of the Eco-GlgP or Eco-MalP from the recombinant host, the cellular proteins were treated at 70 °C for 30 min before purification. The expected molecular size and calculated p*I* of the recombinant Tte-GlgP is ∼64 kDa and ∼6.2, respectively, which were confirmed by SDS-PAGE (Fig. 2A) and isoelectric point assay (Fig. 2B). These results suggested that the Tte-GlgP was successfully expressed in *E. coli* and the recombinant enzyme was likely well folded.

3.3. Expression profile of Tte-GlgP in T. tengcongensis

To investigate if the *glgP* gene was actively expressed in *T. tengcongensis*, the purified Tte-GlgP from the *E. coli* engineering cells was used to generate the specific antibody in mice to det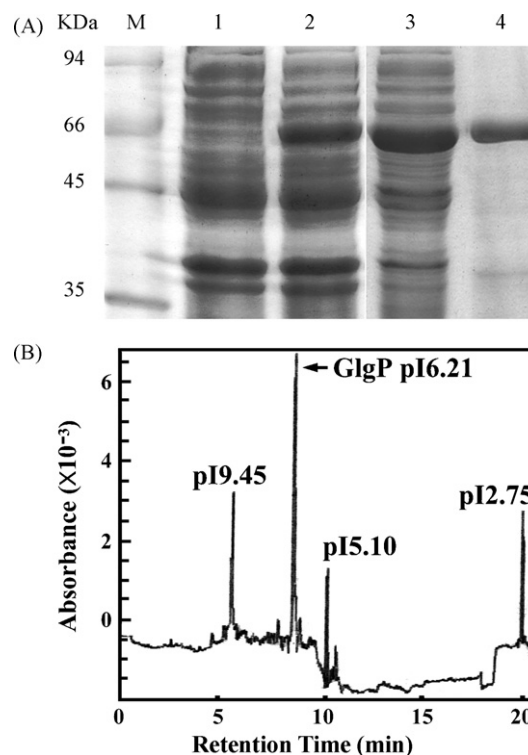ect the wild-type Tte-GlgP in *T. tengcongensis*. The production of Tte-GlgP was clearly observed in *T. tengcongensis* when

Fig. 2. Overexpression, purification and p*I* determination of Tte-GlgP. (A) Overexpression and purification of Tte-GlgP. Lane M, protein molecular weight markers; lane 1, total proteins from strain BL21DE3 (pLysS)/pET23b; lanes 2 and 3, total proteins from strain BL21DE3 (pLysS)/pGlgP; lane 4, purified Tte-GlgP. (B) Determination of p*I* of Tte-GlgP by CIEF 3-10 kit (Beckman).
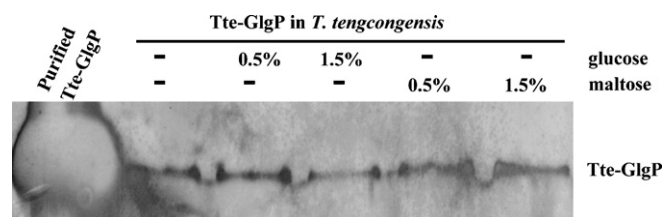

Fig. 3. Determination of the Tte-GlgP expression by Western blot analysis. The cells of *T. tengcongensis* were induced by different amounts (0.5 and 1.5%) of glucose and maltose, respectively, and equal amounts of protein extracts from *T. tengcongensis* were subjected for SDS-PAGE.

cultured in the modified MB medium (Fig. 3). Interestingly, the production of Tte-GlgP was repressed by 1.5%-glucose treatment in comparison with a similar treatment of 0.5% glucose, as revealed by measuring the protein level of Tte-GlgP with Western blotting assay (Fig. 3, lane 4). It was also indicated that Tte-GlgP was likely slightly induced by maltose (Fig. 3, lane 6). This expression profile of Tte-GlgP resembles many other bacterial phosphorylases but is distinct from Tth-GlgP, which seems

Pseudomonas entomophila; Pfl, *Pseudomonas fluorescens*; Pfu, *Pyrococcus furiosus*; Sac, *Sulfolobus acidocaldarius*; Sen, *Salmonella enterica*; Smu, *Streptococcus mutans*; Spn, *Streptococcus pneumoniae*; Sth, *Streptococcus thermophilus*; Tli, *Thermococcus litoralis*; Tte, *Thermoanaerobacter tengcongensis*; Tth, *Thermus thermophilu*s; Tzi, *Thermococcus zilligii*; Vch, *Vibrio cholerae*. (B) The amino acid sequence alignment of glucan phosphorylases in family II. The identical amino acids are indicated by black background. The deduced active sites are indicated by solid lines, and the putative pyridoxal-phosphate cofactor binding site is indicated by dashed line. The asterisks indicate the conserved amino acids also found both in Eco-GlgP and Cca-GlgP.

neither to be inducible by maltose nor repressed by glucose (9, 10). These results also suggested that Tte-GlgP may be involved in the carbohydrate metabolism in *T. tengcongensis* to adapt the environments. It is understandable, as the *glgP* (TTE1805) is the only glucan phosphorylase gene in this bacterium. It is located immediately downstream of a glucose-6-phosphate isomerase gene (GenBank NC003869), also suggesting its involvement in carbohydrate metabolism.
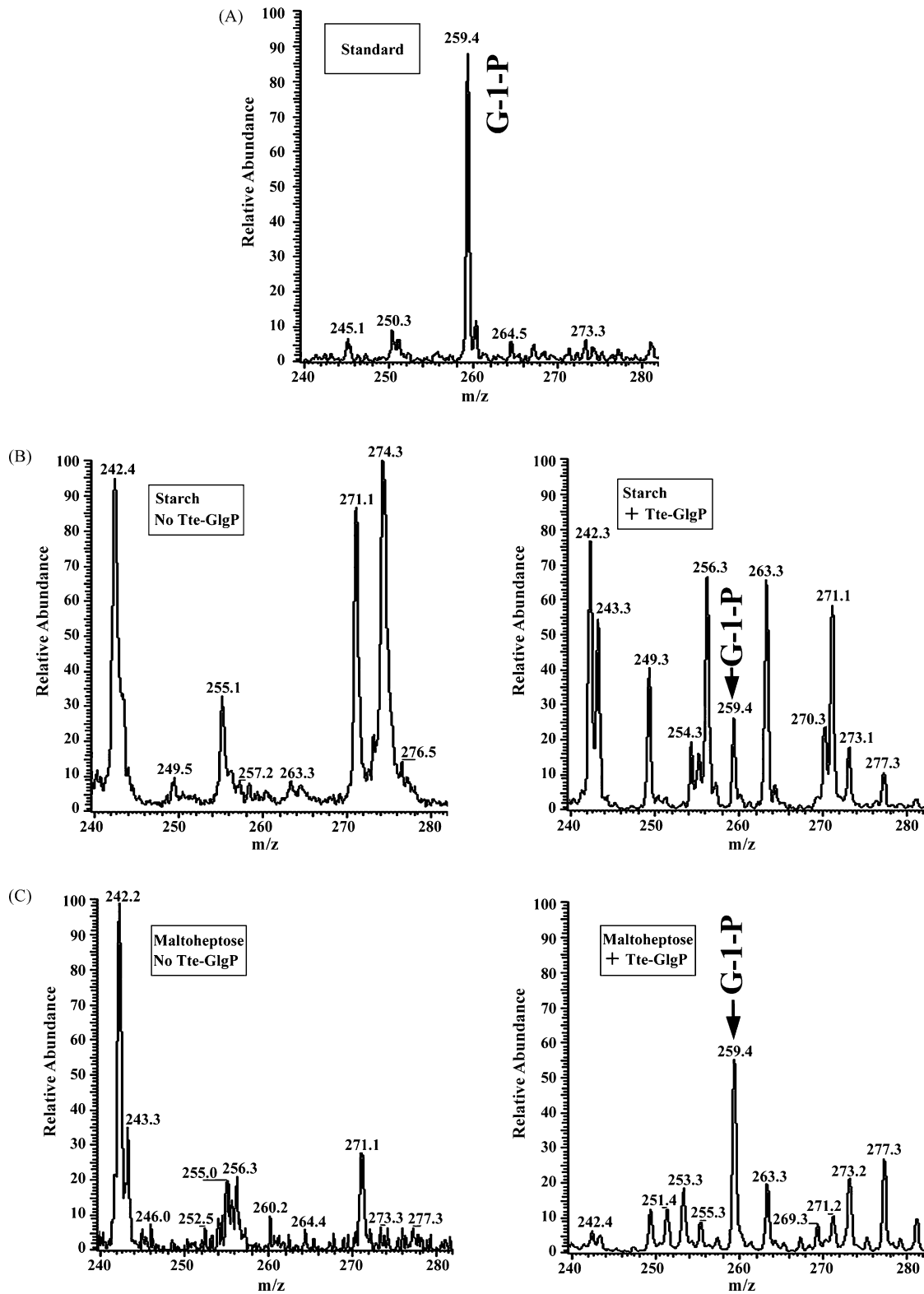

Fig. 4. Detection of G-1-P production from glucan phosphorolysis by ESI-MS. (A) Standard G-1-P in ddH$_2$O; (B) starch as substrate, with (right panel) or without (left panel) purified Tte-GlgP in the reaction mixture; (C) maltoheptaose as substrate, with (right panel) or without (left panel) purified Tte-GlgP in the reaction mixture.

3.4. Determination of the substrate spectrum and the enzyme activity of Tte-GlgP

Since the ESI-MS analysis is one of the most direct methods to determine the production of G-1-P [21], it was used to determine the substrate spectrum of Tte-GlgP. When soluble starch and maltoheptaose were, respectively, used as the substrates, the production of G-1-P was clearly revealed in the ESI-MS chromatogram (Fig. 4). When glycogen, maltodextrin and maltotriose were subjected to the same reaction, the production of G-1-P was also clearly detected (data not shown). These results suggested the Tte-GlgP had a relative wide substrate spectrum, which is different from its homologous protein Tli-MalP that only prefers to digest short maltodextrins (4–7 glucose units) (11).

To evaluate the relative activity of Tte-GlgP towards these substrates, a modified coupled-enzyme method [22,23] was used to measure the production of G-1-P in these reactions. When 0.25% soluble starch, glycogen, maltodextrin, maltoheptaose, maltopentaose, and maltotriose were used as substrates, respectively, it was established that the transformation efficiency, measured by the production of G-1-P (μM), was relatively higher for maltooligosaccharides (maltoheptaose and maltopentaose, with production of 86.83 and 85.79 μM of G-1-P, respectively), maltodextrin (82.9 μM G-1-P) and soluble starch (69.68 μM G-1-P), but lower for glycogen and maltotriose (45.81 and 43.60 μM of G-1-P, respectively) at the same reaction condition. These results were consistent with those of ESI-MS analysis. Both results suggested that the Tte-GlgP could transform a wide variety of glucans into G-1-P, with maltooligosaccharides, maltodextrin and starch but not glycogen as the optimum substrates. This is slightly different from Tth-GlgP, which degrades maltooligosaccharide and glycogen with a similar rate (9).

It should be noted that with the present reaction conditions (see Section 2), the enzyme activity of Tte-GlgP in transformation of starch into G-1-P is still low, only about 0.3–0.5% of glucose units in the soluble starch was transformed into G-1-P within 1 h. We have changed the reaction conditions by including PLP and different metal ions, and the activity can not be significantly increased (data not shown), indicating that Tte-GlgP is a low-activity phosphorylase. It seems to be reasonable, as the growth environment (a hot spring) of *T. tengcongensis* usually contains relative low concentration of carbohydrates.

3.5. Optimum pH and temperature, and thermostability of Tte-GlgP

To determine of optimum reaction condition for Tte-GlgP with soluble starch as the substrate, 50 mM potassium phosphate buffers with pH ranging from 5.0 to 9.0 were first used to prepare the reaction mixtures to detect the optimum pH for this enzyme. The reactions were performed, respectively, at 50, 60 and 80 °C for 30 min, and the highest activity for Tte-GlgP was achieved at pH 8.0 in all these assays, suggesting that the optimum pH for Tte-GlgP is pH 8.0 (Fig. 5A). Then with this optimum pH (8.0), the reactions were performed at different temperature from 37
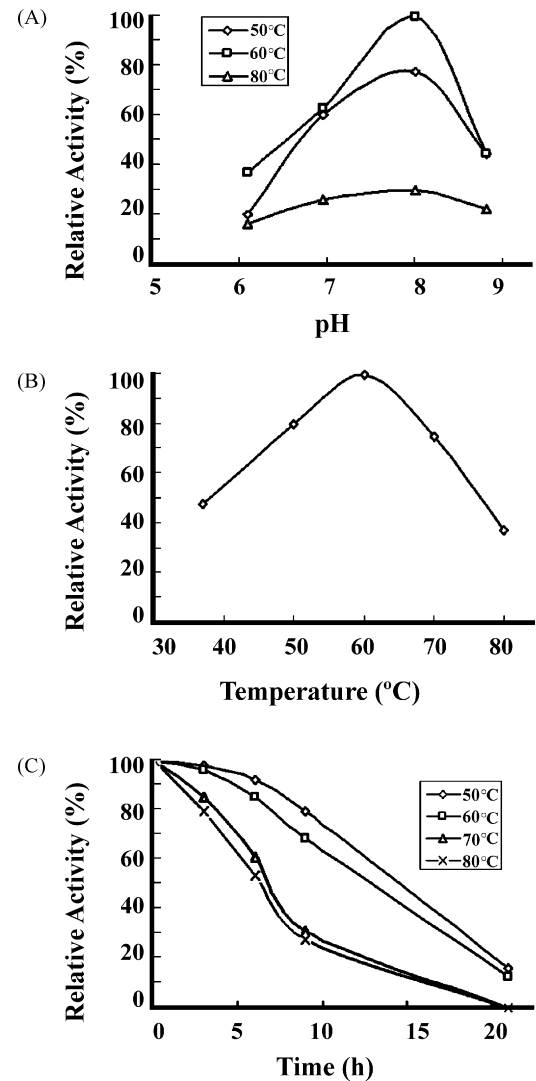

Fig. 5. pH and temperature effects on Tte-GlgP. The enzyme activity of Tte-GlgP was measured by coupled-enzyme method and each sample repeated for three times. (A) Determination of the optimum pH of Tte-GlgP at 50, 60, 80 °C, respectively. (B) Determination of the optimum temperature of Tte-GlgP at pH 8.0. (C) Determination of the thermostability of Tte-GlgP. Tte-GlgP was incubated at 50–80 °C from 0 to 21 h, and then the residual activity was measured.

to 80 °C for 30 min to detect the optimum temperature, which was revealed to be 60 °C (Fig. 5B).

To determine the thermostability of Tte-GlgP, the Tte-GlgP protein was incubated at different temperature from 50 to 80 °C for 0–21 h, and the residual activity was measured by the modified coupled-enzyme method (0.25% soluble starch was used as substrate). Results indicated that there were still 90 and 64% residual activities after treatment for 6 h at 60 and 70 °C, respectively (Fig. 5C). Thus, the Tte-GlgP is indeed a thermostable glucan phosphorylase as expected, distinct from those mesophilic counterparts, e.g. the Eco-GlgP or Eco-MalP, which usually lose the phosphorylase activity at 70 °C within a few minutes.

In conclusion, we have characterized a novel thermostable glucan phosphorylase (Tte-GlgP) from *T. tengcongensis* in many aspects including the gene expression profile, the protein

overproduction and purification, as well as its biochemical and biophysical features. It was suggested that the characteristics of Tte-GlgP, such as the low-level enzyme activity, wide substrate spectrum and highly thermostability, might be well adapted to its living environment. In addition, the Tte-GlgP seems to be a good natural model of the core domains of a novel glucan phosphorylase family where many archaeal homologs belong to (Fig. 1). The overexpressed Tte-GlgP thus might be a good candidate for structure–function analysis in this family.

Acknowledgements

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