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Formation of α-D-glucose-1-phosphate by thermophilic α-1,4-D-glucan phosphorylase

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For the production of α-D-glucose-1-phosphate (G-1-P), α-1,4-D-glucan phosphorylase from *Thermus caldophilus* GK24 was partially purified to a specific activity of 13 U mg^{-1} and an enzyme recovery of 15%. The amount of G-1-P reached maximum (18%) when soluble starch was used as substrate, and the smallest substrate for G-1-P formation was maltotriose. The structure of purified G-1-P was confirmed by comparison to ^{13}C-NMR data for an authentic sample. In addition to G-1-P, glucose-6-phosphate (12%) was simultaneously produced when 10 mM maltoheptaose was used as substrate. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 89–93.

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Introduction

α-D-Glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P) are found in all living organisms [3,6] and are commercially valuable compounds with possible applications in the development of a wide range of specialty chemicals and medicines. G-1-P has been used as a starting material for synthesis of glucuronic acid which is used as a calcium complexant and as a substrate for further biosynthetic reactions to yield linear maltooligosaccharides or α, α-trehalose [18]. G-1-P can be used as a cytostatic compound essential for cardiopathic therapy, as an antibiotic, as an immunosuppressive drug, and as a circulatory system therapy element [12,16]. G-6-P has been used as a model compound for ATP regeneration along with glucose and hexokinase [8], but its application area still remains to be developed. Besides the two compounds G-1-P and G-6-P, phosphorylase itself can be used as a biosensor for *ortho*phosphate [7].

Two main routes for the enzymatic formation of G-1-P without consuming ATP are: (1) using sucrose phosphorylase (SP, EC 2.4.1.7) with sucrose as a substrate [10,16]; and (2) using α-1,4-D-glucan phosphorylase (GP, 1,4 α-glucan *ortho*phosphate α-D-glucosyltransferase, EC 2.4.1.1) with starch or maltodextrin as substrates [1,14,17]. From an economical point of view, the production of G-1-P from an inexpensive α-D-glucan such as starch or dextrin is more appealing than that from sucrose. Conventionally, G-6-P has been prepared by phosphoglucomutase-catalyzed reaction from G-1-P, or by the activation of glucokinase at the presence of glucose and ATP [8]. Despite the numerous useful applications, processes for the enzymatic formation of G-1-P and G-6-P have been incompletely documented in the literature.

Regarding enzymatic G-1-P and G-6-P production, thermostable enzymes are more useful for industrial application. There are a number of advantages in using thermostable enzymes at high temperatures: fast reaction rate (short reaction time), less chance of microbial contamination, prolonged stability, and eventually high productivity [4]. Although examinations of the purification of GP have been done [1,17,18], more data are required to apply thermophilic enzymes to practical G-1-P production. In this paper, we report the formation of G-1-P and G-6-P from maltooligosaccharides or soluble starch using partially purified thermostable enzyme mixtures from a thermophilic bacterium, *Thermus caldophilus* GK24. The effect of substrates and *ortho*phosphate concentrations on the formation of G-1-P and G-6-P is also discussed.

Materials and methods

Materials

Glucose and maltooligosaccharides (G2–G7) used as substrates were purchased from Sigma Chemical Co (St Louis, MO, USA). Soluble starch was purchased from Junsei Chemical Co (Tokyo, Japan). DEAE-Sephacel was supplied from Pharmacia Biotech (Hong Kong). Other chemicals used in this study were of analytical grade.

Partial purification of α-1,4-D-glucan phosphorylase

Thermus caldophilus GK24 was obtained from Professor T Ohta at the Department of Agricultural Chemistry, University of Tokyo. *T. caldophilus* cells were grown as described in Ko *et al* [9]. Cells from 10 liters of the medium were harvested at their half-maximum growth level. They were suspended in a 2-fold volume of buffer A (50 mM potassium phosphate buffer, pH 7.0) and then disrupted in an ice bath using an ultrasonic homogenizer (Cole-Parmer Instrument Co, Chicago, IL, USA). Cell debris was removed by centrifugation at 17000 × *g* for 1 h at 4°C and the super-

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natants were used as the cell-free extracts. After addition of ammonium sulfate to a final concentration of 50%, precipitated proteins were collected by centrifugation at $17000 \times g$ for 30 min at 4°C and the precipitate dissolved in buffer A was dialyzed against the same buffer. The dialyzed solution was applied on a DEAE-Sephacel column (2.5×8 cm) equilibrated with buffer A. The proteins were eluted at a flow rate of 1 ml min^{-1} with a step gradient of 0, 0.175 M, and 1 M NaCl in buffer A. Fractions eluted at 0.175 M NaCl were pooled and used without further purification. Fractions from the chromatography step were assayed for phosphorylase activity.

Enzymatic synthesis of α-D-glucose-1-phosphate and glucose-6-phosphate

A reaction mixture (1 ml) containing 0.1 ml of 100 mM maltoheptaose (G7) solution and 0.9 ml of the enzyme solution in 50 mM potassium phosphate buffer (pH 7.0) pooled from DEAE-Sephacel chromatography was incubated at 70°C. Aliquots (0.1 ml) were taken from the reaction mixture over a period of 12 h, and boiled for 15 min to terminate the reaction. Denatured enzymes were removed by centrifugation at $17000 \times g$ for 15 min at 4°C. The formation of products was monitored by high-performance anion-exchange chromatography (HPAEC).

HPAEC analysis

HPAEC was carried out with a Dionex series 4500i chromatography system with a pulsed amperometric detector (Model PAD-II, Dionex Corp, Sunnyvale, CA, USA). For analytical purposes, a Carbopac PA-1 column (4 × 250 mm) was used. A sample (25 μl) was injected and eluted with a gradient of sodium acetate (0–2 min, 100 mM; 2–14 min, increasing from 100 to 200 mM; 14–19 min, 200 mM; 19–21 min, increasing from 200 to 250 mM; and 21–31 min, increasing from 250 to 500 mM) in 100 mM NaOH with a flow rate of 1 ml min^{-1}. Formation yields (%) were calculated from peak areas of HPAEC chromatograms of reaction mixture using authentic samples of G-1-P and G-6-P.

Assay

A 90-μl aliquot of an enzyme solution dissolved in buffer A was mixed with 10 μl of 100 mM substrate solution and incubated at 70°C for 1 h, and the reaction was terminated by immersing the reaction tubes in boiling water for 15 min. Released products were measured by HPAEC. One unit (U) of activity is defined as the amount of enzyme which produces 1 nmol of G-1-P per min. The amount of protein was measured by the method of Bradford with bovine serum albumin as the standard [2].

Preparative-scale production and purification of α-D-glucose-1-phosphate

A reaction mixture (20 ml) containing 2 ml of 100 mM G7 solution and 18 ml of the enzyme solution pooled from DEAE-Sephacel chromatography was incubated at 70°C for 5 h and then boiled for 15 min to terminate the reaction. The denatured enzyme was removed by centrifugation, and the supernatant was lyophilized. The pellet was dissolved in 3 ml of distilled water and an aliquot was injected onto a Carbopac PA-1 column (9×250 mm) and eluted with 200 mM NaOH and 200 mM NaOAc from 0 to 30 min with a flow rate of 2.5 ml min^{-1}. The eluate for each peak was neutralized with 3 N H_2SO_4 through an anion micromembrane suppressor, then collected and lyophilized. This step was repeated several times.

Nuclear magnetic resonance spectroscopy

NMR spectra were obtained on a JEOL JNM-LA 400 spectrometer with ^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz. Samples were exchanged at least twice in D_2O before obtaining the spectra. Chemical shifts are given in ppm using sodium 2,2-dimethyl-2-silapentatne-5-sulphonate (DSS) as the internal standard.

Results

Partial purification of the enzyme

The α-1,4-D-glucan phosphorylase (GP) from *T. caldophilus* GK24 was partially purified by ammonium sulfate precipitation and anion-exchange chromatography steps. The partially purified enzyme had a specific activity of 13 U mg^{-1} and was recovered at a final recovery yield of 15% (Table 1). The specific activity of the purified enzyme is comparable to the values reported for GP from other bacteria such as *T. thermophilus* (18.1 U mg^{-1}) [1], *E. coli* (18.6 U mg^{-1}) [18] and *Corynebacterium callunae* (33.5 U mg^{-1}) [17].

Simultaneous formation of α-D-glucose-1-phosphate and glucose-6-phosphate

Maltoheptaose (G7,10 mM) was incubated with the enzyme from *T. caldophilus* GK24 and the reaction mixture was analyzed by HPAEC as shown in Figure 1. Both G-1-P and G-6-P started to form after 1 h along with maltooligosaccharides and glucose, indicating the existence of both GP and phosphoglucomutase involved in phosphorolysis and phosphoryl shift, respectively. The formation of G-6-P was identified by comparison of its retention time in HPAEC chromatograms with that of an authentic sample. As time progressed, maltooligosaccharides appeared to be gradually hydrolyzed to glucose which eluted at the retention time of 1.93 min while the amount of both G-1-P and G-6-P increased, especially G-6-P. This process continued up to 12 h, while most maltooligosaccharides were hydrolyzed to glucose after 12 h. At this point the amount of G-1-P decreased slightly from its maximum amount (the yield was 18% based on 10 mM maltoheptaose), but the G-6-P peak increased significantly (the maximum yield was 12%). Therefore, it is suggested that G-1-P was converted into G-6-P by the crude enzyme preparation.

Influence of substrates and phosphate concentration

The degradability of several substrates by GP from *T. caldophilus* GK24 was determined in terms of the degree of conversion of each substrate into G-1-P measured by HPAEC. GP from *T. caldophilus* GK24 accepted a wide range of substrates, and maltose was not used as substrate (Table 2). Maltotriose (G3) was the smallest substrate for GP, unlike other phosphorylases whose smallest substrates are much larger than G3 [15,18]. The amount of G-1-P

Table 1 Partial purification of α-1,4-D-glucan phosphorylase from *Thermus caldophilus* GK24

Step	Total protein (mg)	Total activity		Specific activity (U mg^{-1})	Purification factor (-fold)
		(U)	% yield		
Cell-free extract	2458	7602	100	3.1	1
Ammonium sulfate precipitation	1058	4647	61	4.4	1.4
DEAE-Sephacel chromatography	85	1122	15	13	4.2

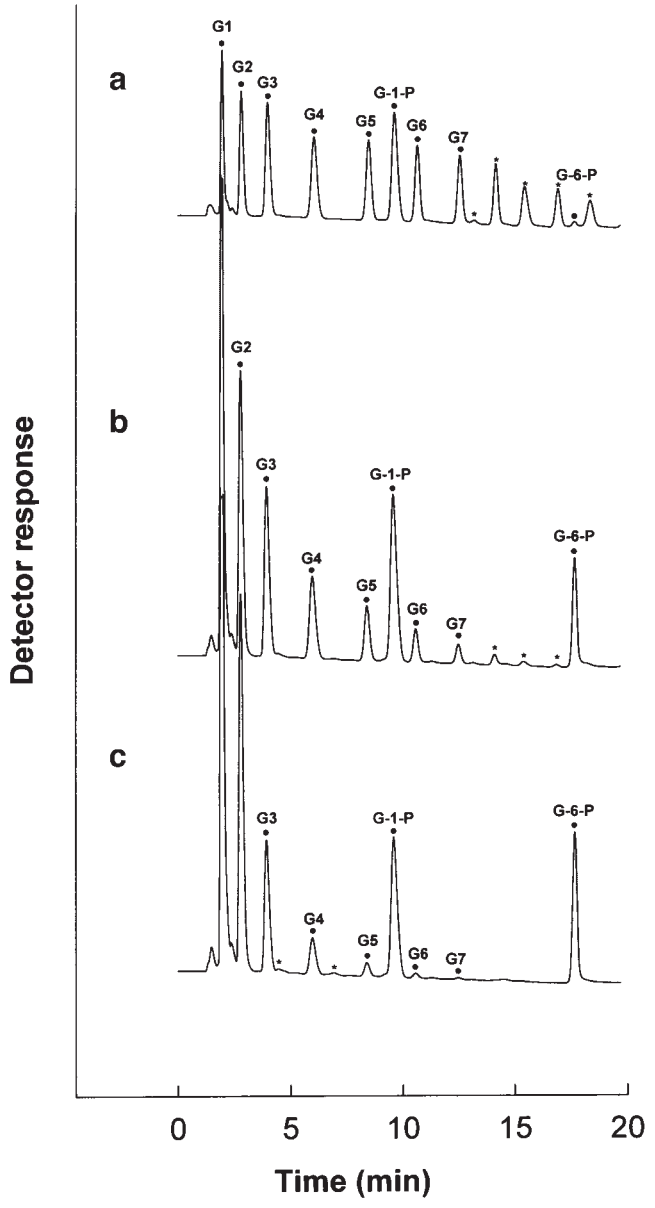

Figure 1 HPAEC chromatograms of reaction mixture after (a) 1 h, (b) 6 h, and (c) 12 h of reaction. *Indicates unidentified peaks. Analysis conditions are described in Materials and methods.

Table 2 Effect of substrate size on G-1-P formation

Substrates	Relative activity (%)[a]
Glucose	0
Maltose (G2)	0
Maltotriose (G3)	8
Maltotetraose (G4)	13
Maltopentaose (G5)	33
Maltohexaose (G6)	41
Maltoheptaose (G7)	64
Soluble starch	100

[a]Relative activity is calculated from the peak area of G-1-P produced from an HPAEC chromatogram.

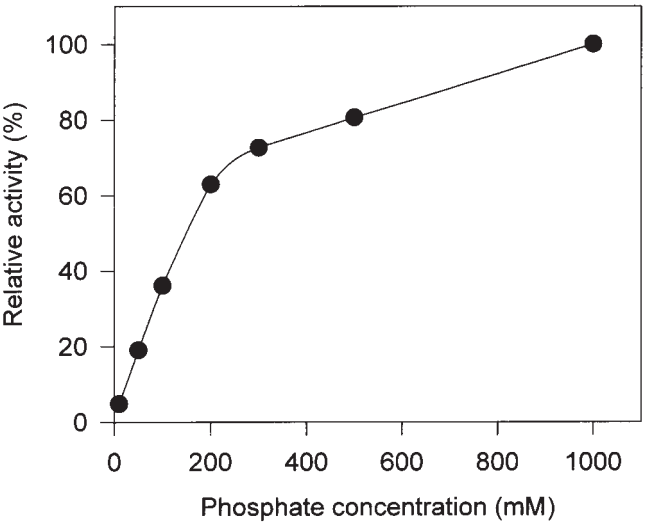

Figure 2 Influence of phosphate ion concentration on relative activity (%) for G-1-P formation. Potassium phosphate was used as phosphate ion source.

reached maximum when starch was the substrate revealing that GP preferred a longer substrate like soluble starch. The effect of the concentration of *ortho*phosphate on the reaction with G7 confirmed that the initial concentration of inorganic phosphate determines the equilibrium concentration of G-1-P (Figure 2). High initial phosphate concentrations of up to 1 M were used without a negative effect. Mg^{2+} or Fe^{3+} (2 mM each) had a weak activating effect on G-1-P formation and Mn^{2+} (2 mM) inhibited GP activity by more than 50% (data not shown).

Structural analysis of α-D-glucose-1-phosphate

To confirm the structure of the product, G-1-P produced was isolated as described in Materials and methods. The purity of G-1-P in fraction was 89.9% as calculated from the peak area on the HPAEC chromatogram. The structure of G-1-P was identified by comparison to ^{13}C-NMR data [13] of the authentic G-1-P. All the NMR data were assigned by ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY.

Discussion

At first, we tried to set up a simple purification scheme for obtaining considerable activity needed for G-1-P and G-6-P formation. Until now, G-6-P has been treated as a useless by-product and other investigators devoted efforts to eliminate phosphoglucomutase (G-1-P ↔ G-6-P) activity solely for G-1-P formation [1,18]. However we view the use of partially purified enzyme advantageous especially for simultaneous formation of G-1-P and G-6-P provided that separation of the products is facile. As for enzyme stability, this enzyme solution has shown no decrease in GP activity for 6 months when stored at 4°C. To increase enzyme stability, immobilization techniques may be considered in down-stream processing of the enzyme [5]. During the enzymatic degradation of maltoheptaose, the feasibility of the simultaneous formation of G-1-P and G-6-P by partially purified GP is shown (Figure 1). In order to verify the viability of this simultaneous formation method of G-1-P and G-6-P, a scheme to economically separate the two products needs to be established. Using conventional carboncelite column chromatography, the two products were eluted at the same time when 30% (v/v) ethanol was used as eluant (data not shown). As an alternative, ion exchange chromatography (cation and/or anion) could be used for separation of these products.

Judging by the amount of G-1-P produced, GP degraded both starch and maltooligosaccharides although the preferred substrate was starch. This substrate specificity is similar to that of *Corynebacterium callunae* and *Thermus thermophilus* [1,17]. In contrast to all known bacterial phosphorylases, the smallest substrate for G-1-P formation was G3 in our case (Table 2). We could not confirm whether G3 was the final product after the reaction because of other contaminating activities of glucoamylase and amylomaltase enzyme present in the reaction mixture (data not shown). Nonetheless, these contaminating enzymes may have contributed to purification of the products by converting other maltooligosaccharides to glucose (see Figure 1c). However, contaminating phosphatase activity should be removed to avoid dephosphorylation of G-1-P and G-6-P. Further purification to remove these hydrolytic enzymes is under way.

In phosphorolysis either inorganic phosphate or the glucan substrate may be the limiting component determining the maximum concentration of the product G-1-P. In the presence of a molar excess of glucan (maltooligosaccharides or starch), the yield of G-1-P was limited mainly by the initial concentration of inorganic phosphate. Therefore, a ratio of substrate to *ortho*phosphate must be established to optimize the reaction system before scale-up of this process.

According to HPAEC chromatograms (see Figure 1c), 18% conversion from maltoheptaose to G-1-P was attained in phosphate-limiting condition (50 mM phosphate and 10 mM maltoheptaose). This value is comparable to that reached by using *Corynebacterium callunae* phosphorylase under a phosphate-limiting condition [17]. The degrees of conversion to G-1-P increase under a glucan-limited condition. If α-glucans are to be used as a substrate, a pretreatment using pullulanase should be applied to give a high yield because of α-1,6-glycosidic branches present in the molecules [11,18]. To our knowledge, there has been no report on the conversion yield of G-6-P from maltodextrins or maltooligosaccharides.

In conclusion, this thermostable enzyme from *T. caldophilus* GK24 can be used as an alternative enzyme source for G-1-P formation from inexpensive substrates. In addition, if phosphoglucomutase is present, G-6-P could be produced simultaneously.

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