Purification and Characterization of Trehalose Phosphorylase from *Catellatospora ferruginea*

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Trehalase phosphorylase was purified from the cell extracts of *Catellatospora ferruginea*. The enzyme had an apparent molecular weight of 400,000 by gel filtration and 98,000 by SDS-PAGE, suggesting that the enzyme was a tetramer. The enzyme was specific for trehalose in phosphorylation and specific for β-D-glucose 1-phosphate in synthesis. In addition to β-glucose, D-xylose and D-fucose were also possible sugar acceptors during synthesis. Phosphate ions were a key to the activity and stability of the enzyme, controlling the equilibrium of the reversible reaction and the heat stability of the enzyme. The enzyme was strongly inhibited by p-chloromercuribenzoate and pyridoxal phosphate. The enzyme was inactivated by heat or by storage frozen with ammonium chloride and lithium chloride.

Key words: trehalose phosphorylase; *Catellatospora ferruginea*; disaccharide; phosphorylase

Trehalose is a non-reducing disaccharide that protects cells against adverse conditions. It has many potential uses in medicine, the food industry, and so on. Trehalose can be synthesized (a) with trehalose 6-phosphate synthase (EC 2.4.1.15) and trehalose 6-phosphate phosphatase (EC 3.1.3.12), (b) with maltoligosyl trehalose synthase and maltoligosyl trehalose trehalohydrolase, (c) with trehalose phosphorylase (EC 2.4.1.64), and (d) with trehalase (EC 3.2.1.28). Of these enzymes, trehalose phosphorylase catalyzes the reversible reaction of phosphorylase and synthesis of trehalose as follows:

\[
\text{Trehalose} + \text{orthophosphate} = \beta(\alpha)-\text{D-glucose 1-phosphate} + \text{D-glucose}
\]

This enzyme has been found in an alga, *Euglena gracilis*, a basidiomycete, *Flammulina velutipes*, a microsymbiont, *Bradyrhizobium japonicum*, a yeast, *Pichia* sp., and a bacterium, *Micrococcus varians*. The enzymes from *E. gracilis* and *M. varians* have been purified and characterized. The enzyme from *E. gracilis* has been used in the synthesis of trehalose, coupled with maltose phosphorylase.

We earlier found the enzyme in a cell extract of the actinomycete *Catellatospora ferruginea*. This enzyme might be used in the synthesis of trehalose and its derivatives. Here, we purified and characterized the enzyme from this source. The purified trehalose phosphorylase could be used for trehalose synthesis because of its high affinity for substrates in the synthesis and because its equilibrium favored synthesis. The enzyme was unusual because the activity and stability were regulated by cationic and anionic ions.

Materials and Methods

Microorganism and culture conditions. *Catellatospora ferruginea* KY2039 was the source of the enzyme for purification. The medium for cultivation contained 3.0% sucrose, 3.0% corn steep liquor, 0.05% KH₂PO₄, 0.05% MgSO₄-7H₂O, and 0.05% MgHPO₄, pH 7.0. Cultivation was done in a 5-liter jar fermentor containing 2.5 liters of the medium at 30°C for 88 h with aeration (2.5 liters/min) and agitation (300 rpm). The cells were harvested by continuous-flow centrifugation. The enzyme productivity was about 50 munits/ml broth.

Enzyme assays. The phosphorolytic activity of trehalase was assayed by the glucose oxidase-peroxidase method with measurement of the D-glucose released as described previously. This method was used during purification and most steps in the investigation of enzymatic properties. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of D-glucose per min. The synthetic activity of trehalase was assayed by purine-nucleoside phosphorylase-xanthine oxidase method with measurement of the inorganic phosphate released as described previously. In some cases, the formation and disappearance of mono- and disaccharides were assayed by HPLC as described previously.

Protein assay. Protein was measured by the method of Bradford with bovine serum albumin as the standard.

Purification of trehalose phosphorylase. Purification was done at 0–5°C unless otherwise specified.

(a) Cell extracts. *C. ferruginea* KY2039 cells (about 200 g wet wt.) were suspended in 1 liter of 200 mm potassium phosphate buffer, pH 6.5, and homogenized in a Dyno-Mill (W. A. Bachofen, Basel, Switzerland). The homogenate was centrifuged at 12,000 × g for 30 min to remove cell debris, and the supernatant was used as the

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cell extract.
(b) \((\text{NH}_4)_2\text{SO}_4\) fractionation. \((\text{NH}_4)_2\text{SO}_4\) was added to the supernatant to 50% saturation with continuous stirring. The precipitate was collected by centrifugation at 12,000 \(\times g\) for 30 min, dissolved in 100 ml of 200 mM potassium phosphate buffer, pH 6.5, and dialyzed against two 2-liter volumes of the same buffer.
(c) Heat treatment. The residue from dialysis was heated at 65°C for 15 min. The resulting precipitate was removed by centrifugation at 12,000 \(\times g\) for 30 min. The supernatant was concentrated to 40 ml on a YM10 membrane (Amicon, Inc., Beverly, MA).
(d) Gel filtration. The concentrate was placed on the surface of a Toyopearl HW65F column (4.5 \(\times\) 50 cm) equilibrated with 200 mM potassium phosphate buffer, pH 6.5, and the proteins were eluted with the same buffer. The enzyme activity was eluted at almost the void volume of the column. The active fractions (96 ml) were pooled, and concentrated to 2 ml on the YM10 membrane.
(e) Resource Q column chromatography. The enzyme solution was put on a column of Resource Q (6 ml) equilibrated with 200 mM potassium phosphate buffer, pH 6.5. The column was washed first with the same buffer, and then the enzyme was eluted with a linear gradient of NaCl from 0 to 0.4 M. The enzyme activity was eluted at about 0.2 to 0.3 M NaCl. The active fractions were combined and dialyzed against 200 mM potassium phosphate buffer, pH 6.5. The enzyme solution was stored at -20°C until use.

**N-Terminal amino acid sequence.** The N-terminal sequence of the purified enzyme was identified with an Applied Biosystems 470A gas-phase sequencer.

**Molecular weight estimation.** Gel filtration was done with a TSK-gel G3000SW column (0.75 \(\times\) 60 cm, Tosoh Corp., Tokyo, Japan) and Superose 6 (HR 10/30, Pharmacia LKB Biotechnology, Uppsala, Sweden) at a flow rate of 0.3 ml/min and elution with 200 mM potassium phosphate buffer, pH 6.5, containing 0.1 M NaCl. The molecular weight of the enzyme was calculated from the mobilities of standard proteins obtained from Oriental Yeast Co. Ltd., Osaka, Japan and Pharmacia.

SDS-PAGE was done by the method of Laemmli with 0.1% SDS and a 12.5% polyacrylamide gel (1 mm thick). The molecular weight of the enzyme was calculated from the relative mobilities of standard proteins (Pharmacia).

**Estimation of isoelectric point.** Isoelectric focusing was done with a Pharmacia-LKB Phastsystem by the procedures recommended by the manufacturer. Precast 4-6.5 gels were used. The pl was estimated with the Pharmacia broad-range calibration kit (pl 3.50 to 8.65). Proteins were stained with Coomassie blue.

**Chemicals.** Trehalose was purchased from Nacalai Tesque (Kyoto, Japan). \(\beta\)-d-Glucose 1-phosphate and \(\alpha\)-d-glucose 1-phosphate were obtained from Sigma Chemical Co., (St. Louis, MO). All other chemicals were of the best analytical grade available.

**Results**

**Purification of Trehalose Phosphorylase**

Procedures for the purification of trehalose phosphorylase are summarized in Table 1. The enzyme was purified 550-fold from the cell extract, with a yield of 15%. The final preparation was almost homogeneous as judged by SDS-PAGE.

**Properties of Trehalose Phosphorylase**

(a) **N-Terminal amino acid sequence**

Sixteen amino acids at the N-terminus of the purified enzyme were sequenced. Methionine was at the N-terminus, and followed by Ile-Xaa-Glu-Xaa-Ala-Tyr-Pro-Val-Glu-Ala-Trp-His-Val-Pro-Glu.. Similar sequences in the DDBJ database were searched for, but not found.

(b) **Molecular weight**

The apparent molecular weight of the purified enzyme was estimated to be 400,000 by TSK-gel G3000SW and 420,000 by Superose 6 gel filtration. SDS-PAGE of the purified enzyme gave a single protein band with an apparent molecular weight of 98,000. These results suggest that the enzyme was composed of four identical subunits.

(c) **Isoelectric point**

The pl value of the purified enzyme was estimated to be 4.6 by isoelectric focusing.

(d) **Substrate specificity**

The specificity of phosphorolytic cleavage of disaccharides containing trehalose and the related compounds by the purified enzyme was examined in the presence of phosphate. Trehalose (\(d\)-glucose(\(\alpha\), 1-1)\(d\)-glucose) and trehalosamine (\(d\)-glucose(\(\alpha\), 1-1)\(d\)-glucosamine) were cleaved at relative rates with 100% and 5%, respectively. Trehalulose (\(d\)-glucose(\(\alpha\), 1-1)\(d\)-fructose), maltose (\(d\)-glucose(\(\alpha\), 1-4)\(d\)-glucose), laminaribiose (\(d\)-glucose(\(\beta\), 1-3)\(d\)-glucose), cellobiose (\(d\)-glucose(\(\beta\), 1-4)\(d\)-glucose), and sucrose (\(d\)-glucose(\(\alpha\)-\(\beta\), 1-2)\(d\)-fructose) were not cleaved at all. Thus the trehalase phosphorylase had specificity for the \(d\)-glucose configuration and

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<th>Table I. Purification of Trehalose Phosphorylase.</th>
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<td>Cell extract</td>
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its α, 1-1 connected disaccharides during phosphorolysis. The relationship between activity and trehalose concentration was of the Michaelis-Menten type, and the apparent Km for trehalose was 12.5 mM.

The effect of phosphate donors on the phosphorolytic cleavage of trehalose was examined. Orthophosphate, metaphosphate, and polyphosphate gave 100%, 28%, and 26% cleavage at relative rates, respectively. ATP and pyrophosphate did not act as phosphate donors. The relationship between activity and the orthophosphate concentration was of the Michaelis-Menten type, and the apparent Km for phosphate was 6.0 mM.

The phosphorolytic reaction of trehalose was done at various ratios of phosphate and trehalose concentrations. After 20 h of incubation, the trehalose remaining was assayed. The apparent end-point of the phosphorolytic reaction depended on the phosphate-to-trehalose ratio (Fig. 1). An increase in the phosphate concentration changed the extent of phosphorolysis. This finding means that the metabolism of trehalose is regulated by the phosphate concentration.

The specificity of synthesis of disaccharides was examined. First, the effect of the sugar donor was examined with D-glucose as the acceptor. Only when β-glucose 1-phosphate was used, trehalose could be synthesized. α-Glucose 1-phosphate, glucose 6-phosphate, and fructose 1-phosphate were not sugar donors. The apparent Km for β-glucose 1-phosphate was 5.0 mM. Next, the effect of sugar acceptors was examined with β-glucose 1-phosphate as the donor. Fifty-two kinds of monosaccharides and related compounds were tested. In addition to D-glucose, D-xylose and D-fucose also were sugar acceptors, although D-xylose differs at the sixth carbon from the D-glucose configuration and although D-fucose differs at the fourth and sixth carbons. The apparent Km values for D-glucose, D-xylose, and D-fucose were 3.7, 100, and 250 mM, respectively (Fig. 2).

(e) Effects of pH
For examination of the pH optimum for the trehalose phosphorylase reaction, the reaction was done under standard conditions except that the pH of the reaction mixture was changed. The phosphorolytic reaction was the greatest at pH 6.5, and the synthetic reaction was the greatest at that pH, as well. In an examination of the pH-stability of the enzyme, it was left in 50 mM potassium phosphate buffer at various pHs for 15 min at 55°C, and the phosphorolytic activity remaining was assayed as usual. Between pH 6.0 and 7.0, activity was not lost. At pH 5.5 and pH 7.5, some 50% and 40% activity, respectively, was lost.

(f) Effects of temperature
The apparent optimum temperature for the phosphorolytic reaction for 10 min was about 45°C. The thermal stability of the enzyme was completely dependent on the phosphate concentration of the enzyme solution (Fig. 3). In 800 mM phosphate buffer, the enzyme was stable when heated at 65°C for 60 min. In 10 mM phosphate buffer, the enzyme was completely inactivated under the same conditions.

(g) Effects of monovalent cations
The effects of monovalent cations on the activity and stability of trehalose phosphorylase was investigated. The monovalent cations tested (each at 100 mM) had no effects on the enzyme activity (data not shown).

Of the monovalent cations tested (each at 1 M), only LiCl and NH₄Cl decreased the heat stability of the enzyme; the effects were concentration-dependent (Fig. 4). NaCl, KCl, RbCl, and CsCl did not affect heat stability. Freezing of the enzyme in the presence of NaCl, LiCl, or NH₄Cl inactivated it, and effects depended on the concentration (Fig. 5). The presence of 1 M KCl, RbCl, and
Trehalose Phosphorylase from *Catellatospora ferruginea*

Fig. 3. Effects of Phosphate Concentration on the Heat Stability of Trehalose Phosphorylase.

The enzyme was heated at 65°C for 15 to 60 min in 10 to 800 mM potassium phosphate buffer, pH 6.5. The phosphorolytic activity remaining was measured in the usual way. Potassium phosphate buffer concentrations: ◆, 10 mM; ◼, 100 mM; ▲, 200 mM; ◼, 400 mM; and ●, 800 mM.

Fig. 4. Effects of Monovalent Cations on the Heat Stability of Trehalose Phosphorylase.

The enzyme was heated at 65°C for 15 min in 200 mM potassium phosphate buffer, pH 6.5, containing 0.2 to 1.0 M LiCl (●), NaCl (◆), or NH₄Cl (◼). The phosphorolytic activity remaining was measured in the usual way.

CsCl did not inactivate the enzyme (data not shown). These monovalent cations did not inhibit activity when the enzyme was kept at 4°C instead of being frozen.

(h) Effects of inhibitors

The enzyme was incubated in 50 mM potassium phosphate buffer, pH 6.5, containing one of various possible inhibitors at 25°C for 15 min. Then the reaction was started by the addition of trehalose. Of the eight metal salts tested (each at 1.0 mM), only CuSO₄ had any inhibitory effect (40% inhibition), but other metal ions showed no appreciable effects. Three chelating agents (EDTA, α,α'-dipyridyl, and 8-hydroxyquinoline: each at 1.0 mM) did not inhibit enzyme activity at all. Of four thiol-blocking reagents (each at 1.0 mM) tested, p-chloromercuribenzoate, a mercaptide-forming reagent, caused 48% inhibition, but alkylating reagents (iodacetate, iodoacetamido, and N-ethylmaleimido) did not inhibit enzyme activity. Pyridoxal phosphate was a potent inhibitor (13% inhibition at 0.01 mM and 52% inhibition at 0.1 mM). Pyridoxal, pyridoxamine, and pyridoxine did not inhibit the enzyme activity at all.

Discussion

Five kinds of disaccharide phosphorylases have been found to date: sucrose phosphorylase, maltose phosphorylase, cellulobiase phosphorylase, laminariase phosphorylase, and trehalose phosphorylase. These phosphorylases each have different molecular and catalytic properties (Table II). Sucrose phosphorylase is unusual in that its reaction does not involve Walden inversion, and proceeds by a ping-pong mechanism. The other four phosphorylases bring about Walden inversion with the exception of trehalose phosphorylase from certain source, and they catalyze the reactions by sequential mechanisms.

The trehalose phosphorylases reported so far can be classified into two categories depending on the inversion of the anomeric configuration in phosphorolysis. The enzymes from *E. gracilis* and *M. visians* form β-D-glucose 1-phosphate and D-glucose in the phosphorolysis of trehalose. The enzymes from *F. velutipes* and a *Pichia sp.* form α-D-glucose 1-phosphate and D-glucose. Trehalose phosphorylase from *C. ferruginea* was in the first category. Generally speaking, this category seems to be characteristic of prokaryotes, with the exception of *E. gracilis*, and the second category seems to be characteristic of eukaryotes. Both reaction mechanisms co-exist only in the trehalose phosphorylases among five kinds of disaccharide phosphorylases.
Trehalose phosphorylase has a high molecular weight. The size of subunits is not very different in four of the phosphorylases; the exception is sucrose phosphorylase.\(^6\) Maltose,\(^7\)\(^8\)\(^9\) cellobiose,\(^10\)\(^11\)\(^12\)\(^13\) and laminaribiose\(^14\)\(^15\)\(^16\)\(^17\) phosphorylases are all dimers, but trehalose phosphorylases from \textit{M. varians}\(^18\)\(^19\)\(^20\)\(^21\) and \textit{C. ferruginea} have more subunits.

Another difference is in the specificity for sugar acceptors in the synthetic reaction. Trehalose phosphorylase used few sugars as sugar acceptors. In contrast, maltose phosphorylases\(^19\)\(^24\)\(^25\) and cellobiose phosphorylase\(^21\)\(^26\) can use various sugars as acceptors. In specificity for sugar donors, all of these disaccharide phosphorylases have a strict requirement for \(\alpha\)-or \(\beta\)-d-glucose 1-phosphate. One reason may be the limited kinds of sugar phosphates available. More kinds might be prepared and tested as donors in the synthetic reaction.

The four kinds of trehalose phosphorylases cannot be readily compared, because except for the enzyme of \textit{M. varians}, they have not been characterized in detail. Especially, properties of \(\alpha\)-d-glucose 1-phosphate-producing trehalose phosphorylases need to be investigated in detail. In its \(K_m\) in the synthesis reaction, the \textit{C. ferruginea} enzyme had higher affinity to both sugar donor and acceptor than the three other trehalose phosphorylases that had lower affinity to the sugar donor, acceptor, or both. These findings suggest that the enzyme from \textit{C. ferruginea} is most suitable for the production of trehalose.

Another characteristic of trehalose phosphorylase from \textit{C. ferruginea} is its regulation by the phosphate concentration. In particular, phosphate was important for the activity and stability of the enzyme. The equilibrium of the reversible reaction was dependent on the ratio of phosphate to trehalose, although it was in favor of the synthesis of trehalose. However, we could not detect trehalose in the cells of \textit{C. ferruginea}, so the physiological role of this enzyme is unknown.

The stability of trehalose phosphorylase from \textit{C. ferruginea} is regulated by certain monovalent cations. The mechanism of enzyme inactivation was not identified, but it seemed to be based on a change in the molecular structure, including dissociation into monomer subunits. Further, the \(N\)-terminal amino acid sequence of \textit{C. ferruginea} enzyme was not similar to sequences of the phosphorylases and glycosylases reported to date. The cloning and sequencing of this enzyme gene are now under way.

### References

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