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Purification and properties of α-glucose 1-phosphate-forming trehalose phosphorylase from a basidiomycete, *Pleurotus ostreatus*

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Pleurotus ostreatus **produced a high activity of α-glucose 1-phosphate (α-Glc 1-P) forming trehalose phosphorylase in vegetative mycelia and fruit-bodies. The enzyme was purified to homogeneity from the fruit-bodies by a procedure involving ammonium sulfate fractionation, DEAE-cellulose column chromatographies and cellulose phosphate column chromatographies. The enzyme catalyzes both the phosphorolysis of trehalose to produce α-Glc 1-P and glucose, and the synthesis of trehalose. It was not active toward other α- or β-glucosyl disaccharides and polysaccharides. The optimum pH was 7.0 for phosphorolysis and 6.4 for synthesis of trehalose. The Km values for trehalose and Pi in phospholytic reaction were 75 mM and 4.2 mM, respectively. Those for glucose and α-Glc 1-P in synthetic reaction were 505 mM and 38 mM, respectively. The estimated molecular mass by the sedimentation equilibrium method using an ultracentrifuge was 120 kDa. The molecular mass of the subunit (61 kDa) by SDS-polyacrylamide gel electrophoresis suggested that the enzyme was a dimer of two identical subunits. The addition of glycerol higher than 25% into the enzyme solution stabilized its activity. The removal of phosphorus ions from the enzyme solution, by means of dialysis or electrophoresis, caused inactivation of the enzyme, probably by dissociation of the holoenzyme into the subunit proteins.**

Key Words——basidiomycete; α-glucose 1-phosphate formation; *Pleurotus ostreatus*; trehalose; trehalose phosphorylase.

The mycelium and fruit-bodies of mushrooms contain a large amount of trehalose (Mikamo, 1974; Kitamoto and Gruen, 1976; Hammond and Nichols, 1976; Kitamoto et al., 1978; Kitamoto et al., 1982; Yoshida et al., 1986). However, few reports have been published on the role of trehalose in the fruiting of higher basidiomycetes. From a survey of the quantitative distribution of cellular carbohydrates in *Flammulina velutipes* (Curt.: Fr.) Sing., Kitamoto and Gruen (1976) suggested that trehalose is the main carbohydrate translocated into fruit-bodies from the vegetative mycelium and that it is utilized for the growth of fruit-bodies in this fungus.

In many microorganisms, trehalose is hydrolyzed by trehalase to produce two glucose molecules (Elbein, A. D., 1974). It is also subsrate for an α-glucose 1-phosphate forming phosphorylase, which was first found in *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Kitamoto and Mori, 1983). The enzyme from *F. velutipes* catalyzes the phosphorolysis of trehalose to produce α-glucose 1-phosphate (α-Glc 1-P) and glucose and also the synthesis of trehalose by the reverse reaction (Kitamoto et al., 1988). Comparison of the specific activities of the trehalose phosphorylase and the trehalase showed that the action of the former enzyme predominated in the developing fruit-bodies of this fungus. α-Type phosphorylase occurred in the mycelia and fruit-bodies of Agaricales and Aphyllophorales in the Holobasidiomycetidae, and at least one species of Gasteromycetes, but not in Tremellaceae or Auriculariales of the Phragmobasidiomycetidae, Heterobasidiomycetes or Hemibasidiomycetes (Kitamoto et al., 1998). *Pleurotus ostreatus* contained the highest activity among these mushrooms. The enzyme was partially purified from *F. velutipes*, and some of its enzymatic properties were demonstrated (Kitamoto et al., 1988). The purification and characterization of the α-type phosphorylase from *Agaricus bisporus* (Lange) Imbach (Wannet et al., 1998) and *Schizophyllum commune* Fr.: Fr. (Eis and Nidetzky, 1999) were recently reported.

In the present paper, we describe the purification and properties of α-glucose 1-phosphate-forming trehalose phosphorylase from fruit-bodies of *P. ostreatus*.

Material and Methods

Material Fruit-bodies of *P. ostreatus* used as a source of

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the enzyme were obtained from a mushroom farm near Tottori, Japan. Fresh material was stored in a deep freezer at -20°C before use.

Preparation of cell extracts Crude cell extracts were prepared by homogenization of the frozen fruit-bodies in 100 mM potassium phosphate buffer containing 30% glycerol and 5 mM EDTA (pH 7.0) in a electric mixer. After removal of the cell debris by centrifugation at 12,000 $\times g$ for 15 min, the supernatant was concentrated to about one-third of the original volume by lyophilization.

Enzyme assay The enzyme activity for phosphorolysis of trehalose was usually measured by a colorimetric method (Kitamoto et al., 1988). The reaction mixture contained 200 mM trehalose, 0.1 mg of bovine serum albumin (BSA), 40 mM potassium phosphate buffer (pH 7.0), and the enzyme in a total volume of 1.0 ml. After incubation of the same mixture at 30°C for 15 min, glucose liberated was measured by the method of Somogyi (1952). The activity was also assayed spectrophotometrically (Kitamoto et al., 1988) in the earlier steps of purification: The assay mixture contained 40 mM potassium phosphate buffer (pH 7.0), 200 mM trehalose, 10 mM glutathione reduced form (GSH), 1.3 mM $MgCl_2$, 0.1 mM α-Glc 1,6-diphosphate, 1.0 mM NADP, 0.16 mM EDTA, 3 units of α-phosphoglucomutase, 3 units of glucose 6-phosphate dehydrogenase, and the enzyme in a total volume of 3 ml. The increase in absorbance at 340 nm was monitored at 30°C with a spectrophotometer.

The reaction mixture for synthesis of trehalose (Kitamoto et al., 1988) contained 100 mM MES (2-(N-morpholino)ethane sulfonic acid) (pH 6.4) and 100 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid) (pH 6.4), 1 M glucose, 100 mM α-Glc 1-P, 0.1 mg BSA, and the enzyme in a total volume of 1.0 ml. After incubation at 30°C for 15 min, the Pi that was liberated was measured by the purine nucleotide phosphorylase method of Machida and Nakanishi (1982).

One unit of enzyme activity is defined as the amount of enzyme needed for formation of 1 μmol of product per min. Specific activity is expressed in units per mg protein. Protein was assayed by the method of Lowry et al. (1951).

Disc gel electrophoresis Electrophoresis in polyacrylamide gel (native PAGE) was performed according to the method of Davis (1964) at 4°C for 2 h at a current of 2 mA per gel in a CD-8 apparatus (Tokyo Kagaku Sangyo Co. Ltd., Tokyo). A mixture of 0.005 M Tris and 0.012 M glycine (pH 9.5) was used as buffer. Gel was stained for protein with 1% Amid Black dissolved in 7% acetic acid, and decolored electrophoretically with 7% acetic acid. Sodium dodecylsulfate (SDS) gel electrophoresis (SDS-PAGE) was performed according to the method of Weber and Osborn (1969). Trehalose phosphorylase and marker proteins were incubated in 10 mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 2% 2-mercaptoethanol for 5 min in a boiling water bath before the electrophoretic run.

Molecular mass determination The molecular mass of the native enzyme was estimated by the sedimentation equilibrium method (Meselson et al., 1957; Yphantis, 1964). About 0.1 ml of trehalose phosphorylase solution in 0.05 M potassium phosphate buffer (pH 7.0) containing 20% glycerol and 5 mM EDTA, and the same buffer solution were taken into each of the double-sector cells of a Hitachi SCP85H equipped with a UV scanner (ABS-7), and the centrifuge was run at 25°C at 11,500 $\times g$ for about 24 h to attain the sedimentation equilibrium. The data obtained was analyzed by using a data processor (Hitachi DA-7). The molecular weight of the

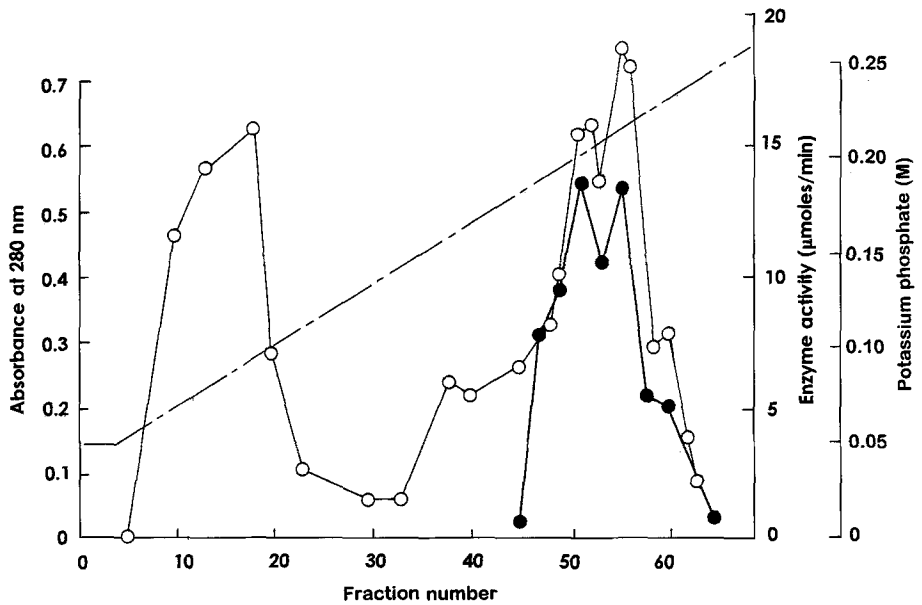

Fig. 1. Elution pattern of trehalose phosphorylase from 2nd cellulose phosphate column chromatography.
●: Enzyme activity, ○: Proteins measured as absorbance at 280 nm.

Table 1. Summary of the purification of trehalose phosphorylase from *P. ostreatus*.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude extract	660	520.3	6,841	0.076	—	100
Fractionation with 37–55% saturation of $(NH_4)_2SO_4$	114	404.1	1,781	0.227	3.0	77.7
1st DEAE-cellulose chromatography	47.7	373.2	176.8	2.11	27.8	71.7
2nd DEAE-cellulose chromatography	15.9	124.0	27.6	4.49	59.1	23.8
1st cellulose phosphate chromatography	51.2	40.0	4.6	8.73	114.9	7.7
2nd cellulose phosphate chromatography	35.2	20.0	1.8	11.4	150.0	3.8

denatured protein was estimated after treating the native enzyme with urea. The enzyme was dialyzed against 20% glycerol aqueous solution by centrifugation with Centriflo CF-25 (Amicon Co., USA), and urea and 2-mercaptoethanol were added concentrations of 6 M and 2 mM, respectively. After incubation at 60°C overnight, the solution was applied to the sedimentation equilibrium analysis.

Chemicals α-Phosphoglucomutase, glucose 6-phosphate dehydrogenase, and NADP were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.. Trehalose was the product of Aldrich Chemical Co. Inc. DEAE-Cellulose (DE52) and Cellulose phosphate (P-11) were the products of Whatman International Co.. A molecular weight marker kit was purchased from BHD Chemicals Ltd., Poole, England. All other chemicals were obtained from commercial sources.

Results

Purification of trehalose phosphorylase Trehalose phosphorylase was purified from 250 g fresh weight of fruit-bodies. A potassium phosphate buffer (0.05 M, pH 7.0) containing 25% glycerol and 2 mM EDTA was used unless otherwise described.

The lyophilized cell extracts was thawed at 0°C and fractionated by ammonium sulfate precipitation between 37 and 55% saturation with 0.1 M potassium phosphate buffer (pH 7.0) saturated with ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in the buffer and dialyzed against the same buffer overnight. The enzyme was put on the first DEAE-cellulose column (DE52: 3.3×30 cm) equilibrated with 0.025 M potassium phosphate buffer (pH 7.0) containing 20% glycerol and 5 mM EDTA. The elution was done with a linear gradient from 0.025 to 0.5 M of the same buffer. Glycerol was added to the active fractions to make 25%, and the solution was concentrated with Centriflo CF-25 (Amicon Corp., U.S.A.). The enzyme solution was applied to the second DEAE-cellulose column (2.2×30 cm) and treated by the same procedures as in the first chromatography. The concentrated enzyme solution was loaded on the first cellulose phosphate column (P-11: 1.6×27 cm) equilibrated with the same buffer as was used for the DEAE-cellulose chromatographies (Fig. 1). A linear gradient elution from 0.05 to 0.5 M potassium phosphate concentration was done, the active fractions were pooled, and then concentrated with Centriflo CF-25 after adding glycerol to make 25% concentration.

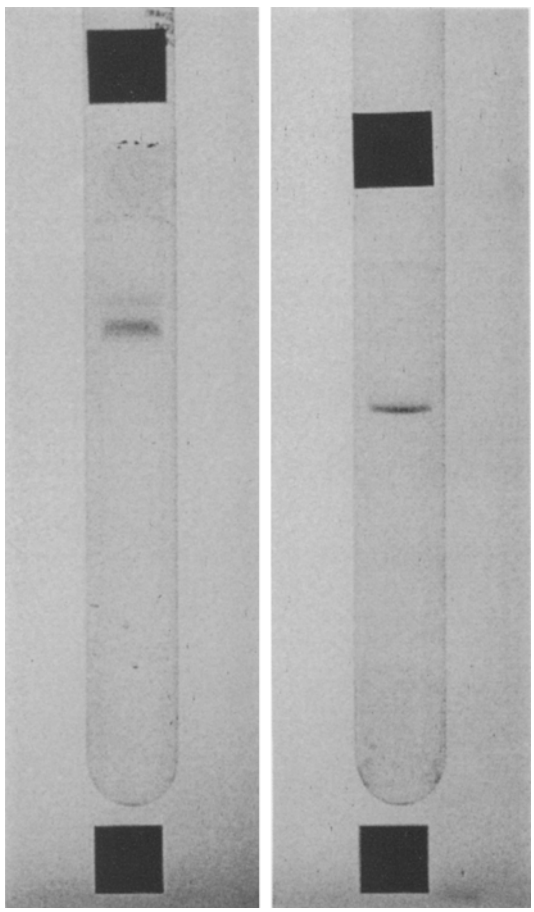

Fig. 2. Native (left) and SDS-PAGE (right) of purified trehalose phosphorylase.

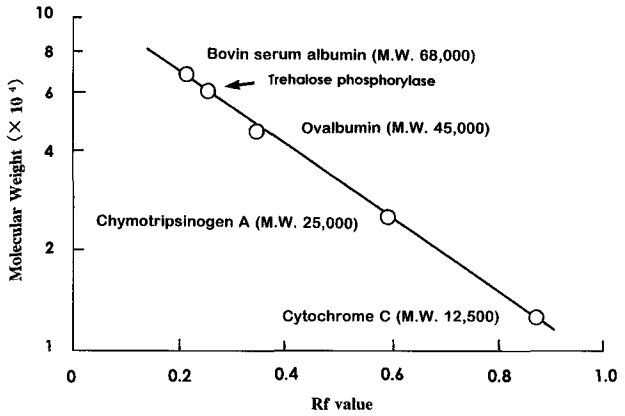

Fig. 3. Estimation of the molecular mass of the subunit of trehalose phosphorylase by SDS-PAGE.

The enzyme solution was further chromatographed on the second cellulose phosphate column (1.6×20 cm). The activity peak fractions from the column were combined and glycerol was added to 25% in the enzyme solution.

The purification procedures are summarized in Table 1. Trehalose phosphorylase was purified about 150-fold, giving preparations with the specific activity of 10.6 units·mg^{-1}.

Purity and molecular mass determination The homogeneity of the purified enzyme was determined by native PAGE as well as SDS-PAGE (Fig. 2). The native PAGE revealed two protein bands on the column. When the gel was sliced into sections 1 mm thick and the enzyme activity of each section was measured, activity was found only in one of the samples containing a faint protein band. However, the SDS-PAGE gave a single band at a molecular mass of 61 kDa (Fig. 3). Thus, it was concluded that purified trehalose phosphorylase was homogeneous. Further, the predominant protein band could have been produced by the dissociation of the holoenzyme protein during electrophoresis.

The molecular mass of the native enzyme protein was estimated to be about 120 kDa by the sedimentation equilibrium method applied in 20% glycerol solution. The molecular mass of enzyme protein treated with 6 M urea was estimated to be about 59 kDa. The native enzyme appeared to be a dimer with two identical subunits, because the SDS-PAGE of the enzyme gave a single band.

Reaction products The product of the phospholytic reaction with trehalose was isolated. The preparative mixture was composed of 200 mM trehalose, 0.1 mg bovine serum albumin, 40 mM potassium phosphate buffer (pH 7.0) and 40 μl (0.68 units for phospholysis of trehalose) of the purified enzyme in a total volume of 2.0 ml. The reaction was carried out at 30°C for 8 h by successive feeding of 5-μl portions of the enzyme solution at intervals of 1 h. By this reaction equimolecular amounts (33.8 μmol) of α-Glc 1-P and glucose were formed. After addition of 100 μl of 1 M magnesium acetate solution the reaction mixture and adjustment of the pH to 8.4 with 7% ammonia, the resulting precipitate was removed by centrifugation. The supernatant was treated with 150 μl of 1 M barium acetate solution, then 3 vol. of ethanol was added, and the mixture was left to stand overnight. The precipitate formed was collected by centrifugation and dissolved in 4 ml of water. The solution was then put on Dowex 1×8 column. The sugar phosphate was eluted with 1 M KOH, and the eluate was neutralized to pH 7.0 with 6 M HCl. The product thus obtained showed the same Rf value on the paper chromatography as authentic α-Glc 1-P (Aldrich, U.S.A.). The sugar phosphate reacted with α-phosphoglucomutase (Sigma Chemicals Co.: from rabbit muscle, which cannot react with α-Glc 1-P), and formation of α-Glc 6-P was detected in the coupling reaction with glucose 6-phosphate dehydrogenase.

To identify the enzymatically obtained trehalose, the sugar product was prepared and isolated as follows. The preparative reaction mixture was composed of 100 mM MES and 100 mM HEPES (pH 6.3), 400 mM glu-

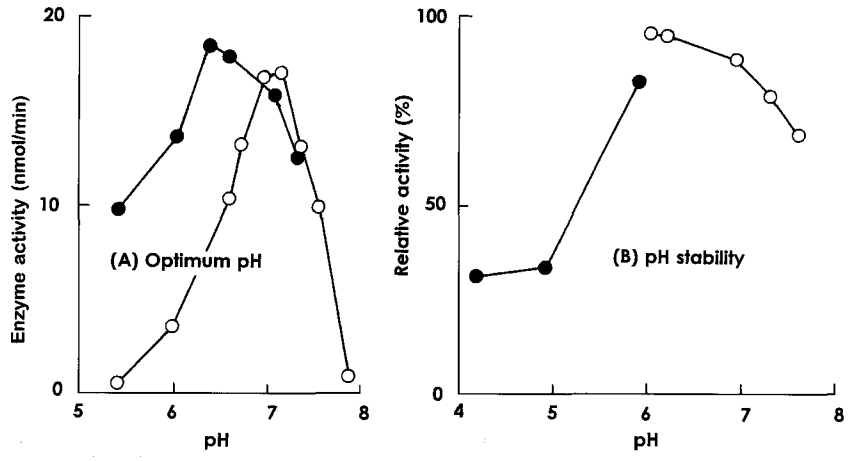

Fig. 4. Optimal pH (A) and pH stability (B) of trehalose phosphorylase.
(A) ○: phosphorolysis, ●: synthesis. (B) ●: 50 mM sodium acetate buffer, ○: 40 mM potassium phosphate buffer.

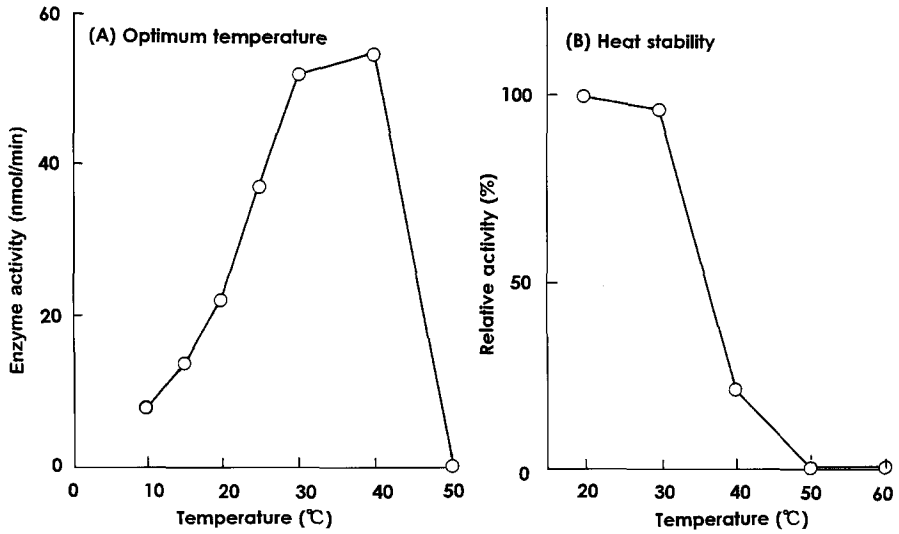

Fig. 5. Optimal temperature (A) and heat stability (B) of trehalose phosphorylase. The enzyme activity is that of the phosphorolytic reaction.

cose, 100 mM α-Glc 1-P and 80 μl (1.37 units for phosphorolysis of trehalose) of the purified enzyme in a total volume of 1.0 ml. The reaction was carried out by successive feeding of 10-μl portions of the enzyme solution as described above. By this reaction a total of 14 μmol of Pi was liberated. The reaction mixture was then put on a charcoal column (2.0 × 6.0 cm), which was washed with water until no reducing power was detected in the eluate. The column was then developed with 10% ethanol and the eluate was evaporated in vacuo. The product thus obtained did not reduce the alkaline copper reagent but showed a positive reaction with the anthrone reagent. The trimethylsilyl derivative of the product was prepared according to the procedure of Sweeley et al. (1963), and analyzed by use of a gas chromatograph (Hitachi, model 163) equipped with SE-30 column, the temperature of which was programmed to rise from 150°C to 250°C at the rate at 3°C·min^{-1}. The retention time of the resulting trimethylsilyl derivative was found to coincide with that of authentic α-trehalose.

pH characteristics The optimal pH for phosphorolysis was 7.0 and that for synthesis was 6.4 (Fig. 4A). The pH stability of the enzyme for the phosphorolytic reaction was measured after pre-incubation for 3 min at 30°C in one of two buffers containing 50 mM phosphate. The enzyme was stable between pH 6.0 and 7.0 (Fig. 4B).

Temperature characteristics The optimal temperature for the phosphorolysis of trehalose was 34°C (Fig. 5A), as was that for the synthetic reaction (data not shown). The temperature stability of the phosphorolytic reaction of the enzyme was measured after incubating the enzyme for 3 min at different temperatures before carrying out the reaction. The enzyme was stable below 30°C, and decreased to 21% of its initial activity after incubating at 40°C (Fig. 4B).

Stabilization of enzyme activity A preliminary experiment showed that the trehalose phosphorylase activity rapidly decreased after extracting the enzyme from fungal material with 0.1 M potassium phosphate buffer (pH 7.0). Therefore, the effects of various reagents on the stability of enzyme activity were examined. Glycerol (higher than 25%) had a stabilizing effect, but the SH reagents, Pi and EDTA showed no practical effect against the gradual decrease in enzyme activity, even when the enzyme was stored at in a freezer at below -20°C. However, the storage at below -20°C of cell extracts in 0.1 M potassium phosphate buffer (pH 7.0) containing 25% glycerol rather increased the total enzyme activity. The reduction or removal of potassium ions from the enzyme solution by dialysis, using a Visking tube or by electrophoresis, caused inactivation of the enzyme, probably by the dissociation of holoenzyme into subunit proteins.

Substrate specificity and Michaelis constants Phosphorolytic activity of the enzyme was specific toward α-1,1-disaccharide, trehalose, but inert toward other bonding types of disaccharides and polysaccharides such as maltose, cellobiose, isomaltose, laminaribiose, sucrose, glycogen, laminarin, and starch. Glucose and α-Glc 1-P were substrates for the synthetic reaction, but fructose, mannose, xylose, and β-Glc 1-P were not.

Michaelis constants of trehalose phosphorylase are shown in Table 2. The apparent Km values for trehalose and Pi in the phosphorolytic reaction at pH 7.0 were 75 mM and 4.2 mM, respectively. Those for glucose and α-Glc 1-P in the synthetic reaction at pH 6.3 were 505 mM and 38 mM, respectively. It seems that the enzyme was active for the phosphorolysis of trehalose to form α-Glc 1-P and glucose *in vivo* as well as *in vitro*.

Equilibrium constants Table 3 shows the apparent equilibrium constants of the trehalose reactions at pH 7.0 and 6.4, for the phosphorolysis and the synthesis of trehalose, respectively. The initial concentrations of trehalose, Pi, glucose and α-Glc 1-P of the reaction mixtures used for the determinations of the constants at pH 7.0 or 6.4 were adjusted near the concentrations of the end points of the reaction at either pH 7.0 or 6.4, respective-

Table 2. Michaelis constants for different substrates of trehalose phosphorylase*.

Substate	Km (mM)
Trehalose	75
Pi	4.2
α-Gluc 1-P	38
Glucose	505

* Michaelis constants were determined at pHs 7.0 and 6.3 for phosphorolytic and synthetic reactions, respectively, under conditions described in Materials and Methods.

ly. In this experiment, it was necessary for the reaction to reach equilibrium within a relatively short time, because the enzyme was not stable in the reaction mixture with a phosphate ion concentration lower than 0.04 M. The ratio [trehalose] [Pi] to [α-Glc 1-P] [glucose] at pH 7.0 was 10.5, and the value at pH 6.4 was increased to 14.9.

Table 3. Equilibrium constants for trehalose phosphorylase.

Reactants	Amounts (mmoles) Initial	Final	K value*1
pH 7.0			
Trehalose	196.26	196.43	
Pi	41.66	41.88	10.47
α-Gluc 1-P	28.73	30.80	
Glucose	28.00	25.53	
pH 6.4			
Trehalose	196.26	195.45	
Pi	41.49	40.50	14.93
α-Gluc 1-P	22.07	23.99	
Glucose	22.00	22.12	

*1 α-Gluc 1-P + Glucose ⇄ Trehalose + Pi

$$K = \frac{[\text{Trehalose}]\,[\text{Pi}]}{[\alpha\text{-Gluc 1-P}]\,[\text{Glu}]}$$

Discussion

Many microorganisms contain trehalase, which catalyzes the hydrolytic breakdown of trehalose into two molecules of glucose (Elbein, 1974). In addition, trehalose phosphorylase catalyzes the phosphorolysis of α,α-trehalose into α-Glc 1-P and glucose in *P. ostreatus* and *F. velutipes* (Kitamoto et al., 1983; 1988). This enzyme catalyzes only the phosphorolysis of trehalose to form α-Glc 1-P and glucose and the synthesis of the disaccharide by the reverse reaction; it is not active on β-Glc 1-P or other α- or β-glucosyl disaccharides and polysaccharides. On the other hand, Belocopitow and Maréchal (1970) reported phosphorolytic cleavage of trehalose into β-Glc 1-P and glucose by a phosphorylase from a protist, *Euglena gracilis* Klebs. However, β-type trehalose phosphorylase was not detected in Homobasidiomycetes, Heterobasidiomycetes or Hemibasidiomycetes (Kitamoto et al., 1998).

α-Type trehalose phosphorylase is remarkably unstable, which makes it difficult to purify or estimate its weight described, as is the β-type trehalose phosphorylase by Maréchal and Belocopitow (1972). It might be practically impossible to apply any gel filtration chromatographies because of the high viscosity of the enzyme solution with addition of an essential stabilizer, at least 25% of glycerol. The electrophoretic studies showed that the holoenzyme could be dissociated to inactive subunit protein during development in tris-HCl buffer. Inactivation also occurred on the dialysis of the enzyme against a potassium phosphate buffer, the concentration of which was as low as 0.02 M. On the other hand, when the enzyme was subjected to SDS gel electrophoresis, only a single band was produced. These results suggest that the active form of trehalose phosphorylase (120 kDa) is composed of two identical subunits, the molecular mass of which was estimated to be 61 kDa by the SDS method. On the other hand, Wannet et al. (1998) reported that *A. bisporus* had also 61-kDa of subunits, but the active form had a molecular mass of 240 kDa, consisting of four identical subunits. The active enzyme from *S. commune* was estimated to be a monomeric 61-kDa protein (Eis and nidetzky, 1999).

The *Pleurotus* enzyme had Michaelis constants of 75 mM and 4.2 mM for trehalose and Pi, respectively, in the phosphorolytic reaction. These Km values were almost the same as the 75 mM and 5.0 mM for the enzyme from *F. velutipes* (Kitamoto et al., 1988), and 61 mM and 4.7 mM for the *A. bisporus* enzyme (Wannet et al., 1998). The values for trehalose were more than two times higher than the 33 mM of the β-type trehalose phosphorylase from *E. gracilis* (Maréchal and Belocopitow, 1972). Although the Michaelis constant of the *Pleurotus* enzyme was uncommonly higher than those of the other glycan phosphorylases, i.e., glycogen phosphorylases (Graves, D. J. and Wang, J. H. 1972) or maltose phosphorylases (Fitting and Doudorff, 1952; Kamogawa et al., 1973) for various microorganisms, the enzyme activity was still sufficient to produce α-Glc 1-P and glucose in fruit-bodies, because probably the intracellular concentration of trehalose exceeded 30 mM in this mushroom (Kitamoto et al., 1982; Kitamoto and Mori, 1983).

It was also examined whether the formation of trehalose occurred in the reversible trehalose phosphorylase reaction. However, the Km values for α-Glc 1-P and glucose in the synthetic reaction were 38 mM and 505 mM, respectively. The rate of trehalose formation was only 7% of that of trehalose degradation. Moreover, the trehalose content in the vegetative mycelium was about 5%, but the endogenous glucose did not exceed 1.5% (Kitamoto et al., 1982; Yoshida et al., 1986). Therefore, it is possible that this enzyme essentially functions as a catalyst only in the breakdown of trehalose *in vivo* as well as *in vitro*. In *P. ostreatus* and *F. velutipes*, fruit-bodies showed higher specific activities of trehalose phosphorylase than did the mycelium (Kitamoto et al., 1998). The biosynthetic pathway of trehalose has not

yet been demonstrated in basidiomycotina. However, it is believed that the first step may be the synthesis of trehalose 6-phosphate (Tre 6-P) from α-Glc 1-P and uridine diphosphate glucose (UDPG) as the substrates, and that the subsequent step involves hydrolysis to release inorganic phosphate by a specific phosphatase (Cabib and Leloir, 1968).

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