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Physiological and Biochemical Studies on Germinating Fungal Spores V.[1] Partial Purification and Characterization of Trehalase in Conidia of *Cochliobolus miyabeanus*

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Soluble trehalase (EC 3.2.1.28) was isolated and purified from the conidia of *Cochliobolus miyabeanus* by diethylaminoethyl Sephadex A-50 column chromatography and polyacrylamide gel electrophoresis. The molecular weight of this enzyme was estimated to be 160000 by the gel filtration method. Although the purified trehalase hydrolyzed both trehalose and maltose, this enzyme showed higher activity toward trehalose than maltose (the K_m values were 7.3×10^{-4} M for trehalose and 1.5×10^{-2} M for maltose). The optimal pH and temperature for the enzyme reaction were 4.0 and 37°C respectively. The enzyme became unstable at 50°C. This enzyme was competitively inhibited by fructose-6-phosphate and Na-pyruvate.

Keywords——trehalase; *Cochliobolus miyabeanus*; fungal spore; conidia; trehalose

In the previous paper,[1] we reported that trehalose stored in conidia of *Cochliobolus miyabeanus* was consumed rapidly within 15 min after the commencement of germination and that the trehalase activity which was detectable in the ungerminated conidia increased at that time, and then began to decrease gradually after 30 min of incubation. To clarify the regulatory mechanism of trehalase activity in the germinating conidia, we attempted the partial purification and characterization of the trehalase (EC 3.2.1.28) contained in the conidia.

Materials and Methods

Organism and Germinating Conditions——The harvesting procedure for conidia of *Cochliobolus miyabeanus* and the germinating conditions were as described previously.[1]

Extraction of Crude Trehalase——The conidia (10.0 g) were homogenized with 2 parts of carborundum in 0.01 M Tris-HCl buffer, pH 7.0, using a chilled pestle and mortar and the homogenate was made up to 200 ml with the same buffer and allowed to stand for 12 h. The cell debris was then removed by centrifugation (4000×g, 10 min), and the pH of the supernatant was adjusted to 3.5 by adding 1 M acetic acid. After removal of sediments by centrifugation (10000×g, 10 min), the same volume of chilled acetone was added to the supernatant and the mixture was allowed to stand for 6 h at 0°C. The precipitate was collected by centrifugation (10000×g, 15 min), resuspended in the same buffer (10 ml), and dialyzed against the same buffer (24 h, 4°C). After removal of some insoluble matter, the dialyzate was used as crude enzyme solution.

Enzyme Assay——The assay of glycosidase activity was based on the colorimetric determination of glucose released from the substrate. The reaction mixture consisted of the enzyme solution (0.1 ml), 0.1 M trehalose or an appropriate substrate (0.1 ml) and 0.1 M acetate buffer, pH 4.0, (0.3 ml). After incubation for 10 min at 37°C, glucose released was determined by using the Glucostat reagent (Worthington Biochemical Co.). Enzyme activity (unit) was expressed as μ mol of glucole released/min and the specific activity as units/mg of protein. Protein was determined by the method of Lowry *et al.*[2]

DEAE-Sephadex A-50 Column Chromatography——The crude enzyme solution (23 mg of protein) was applied to a diethylaminoethyl (DEAE)-Sephadex A-50 (acetate form) column (2.5×30 cm) preequilibrated

with 0.01 M acetate buffer, pH 7.0, and eluted with a 0 to 1.0 M linear gradient of NaCl in the same buffer. The eluate was collected in 4 ml fractions.

Preparative Polyacrylamide Gel Electrophoresis——Preparative gel electrophoresis was performed with a preparative disc electrophoretic analyzer (SJ-1050, JB-2 Type, Mitsumi Kagaku Co., Tokyo). A polyacrylamide gel column was composed of 7.5% gel in 0.05 M Tris-glycine buffer, pH 8.9, (column length 2.5 cm) as the electrophoretic separation layer, 3.3% gel in 0.05 M Tris-glycine buffer, pH 6.7, (column length 1.0 cm) as the concentrating layer and the same gel (column length 0.5 cm) as an anticonvectional layer containing the sample (3 mg of protein). For electrophoresis and elution, 0.05 M Tris-glycine buffer, pH 8.3, was used and a constant current of 40 mA (500 V) was applied for 24 h. The eluate was collected in 4 ml fractions.

SDS-Polyacrylamide Gel Electrophoresis——The separating gel was composed of 10% acrylamide in 0.19 M Tris-glycine, pH 8.8, and 0.1% SDS. The upper spacer gel contained 4.4% acrylamide in 0.12 M Tris-glycine buffer, pH 6.8, and 0.1% SDS. Electrophoresis was performed at constant 100 V for 3 to 5 h in 0.05 M Tris-glycine buffer, pH 8.4, and 0.1% SDS. Gel was stained for protein with 0.05% Coomassie brilliant blue in 12.5% trichloroacetic acid.

Results

Purification of Trehalase

Trehalase activity was detected only in the supernatant of conidial homogenate, not in the cell debris fraction, and its specific activity was increased 12.5-fold by the elimination of sediments at pH 3.5 followed by precipitation in 50% acetone solution. When the crude enzyme solution was passed through a column of DEAE–Sephadex A-50 and eluted with a linear gradient of NaCl in 0.01 M acetate buffer, pH 7.0, the protein was separated into 2 peaks. The 2nd peak fraction, which was eluted with 0.18 M NaCl, showed high activities of trehalase together with maltase and β-fructo-furanosidase. By this step, the trehalase activity was increased 53-fold, but since some glycosidase activities other than trehalase coexisted in this fraction, further purification was performed. By preparative gel electrophoresis, the protein of this fraction was separated into 5 peaks and the trehalase activity was found in the first peak fraction. All of the β-fructofuranosidase activity was present in the 2nd peak and the trehalase was completely separated from the β-fructofuranosidase. Nevertheless, low maltase activity was detected in the trehalase fraction. By this step, the specific activity was increased 137-fold from the initial homogenate, and this trehalase fraction gave a single band on SDS-polyacrylamide gel electrophoresis. Table I summarizes the purification procedure.

Table I. Summary of Steps involved in the Purification of Trehalase from the Conidia of *Cochliobolus miyabeanus*

Purification step	Total vol. (ml)	Total units	Total protein (mg)	Specific activity U/mg of protein	Purification (Fold)
Crude extract	200	31.3	326	0.096	1.0
Treatment at pH 3.5	250	28.2	137.5	0.206	2.1
Acetone treatment	10	27.0	22.5	1.200	12.5
DEAE[a]-Sephadex A-50 (Fract. No. 32—36)	4	15.0	2.97	5.069	52.8
Preparative polyacrylamide gel electrophoresis (Fract. No. 9—11)	4	9.63	0.73	13.19	137.4

a) The fractions from tubes 32—36 were pooled and concentrated, then subjected to preparative polyacrylamide gel electrophoresis.

Enzyme Properties

Effects of pH and Temperature——As shown in Fig. 1, almost all the enzyme activity was lost after 5 min of incubation at 50°C, and the highest enzyme activity was obtained at pH 4.0 and at 37°C. Therefore the enzyme assays were usually performed under the latter conditions.

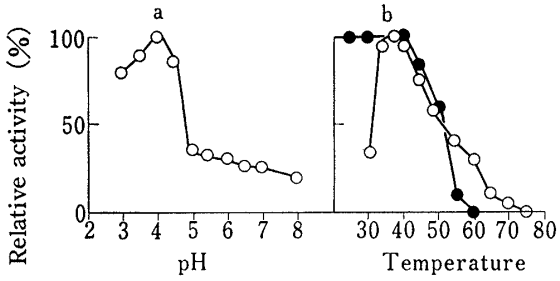

Fig. 1. Effects of pH (a) and Temperature (b) on the Purified Trehalase

Assays were carried out under at various levels of pH or temperature (○) or under the standard conditions described in the text, but with enzyme samples maintained for 5 min at selected temperatures (●).

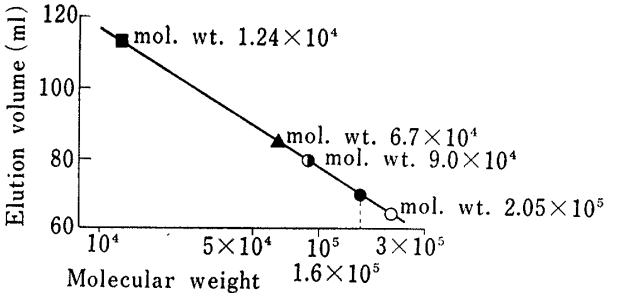

Fig. 2. Estimation of the Molecular Weight of Trehalase by the Gel Filtration Method

Trehalase (●) and cytochrome C (■, mol. wt. 12400), bovine serum albumin (▲, mol. wt. 67000), bovine γ-globulin (◐, mol. wt. 90000) and human γ-globulin (○, mol. wt. 205000) were charged on a Sephadex G-200 column (2.5×25 cm), and their elution volumes were determined.

Molecular Weight——Molecular weight determination was carried out by the method of Andrews[3] using a Sephadex G-200 column (2.5×25 cm). Cytochrome C (mol. wt. 12400), bovine serum albumin (mol. wt. 67000), bovine γ-globulin (mol. wt. 90000) and human γ-globulin (mol. wt. 205000) were used as standards. As shown in Fig. 2, the molecular weight of the enzyme was estimated to be 160000.

Substrate Specificity——The activity of the purified enzyme was tested against the following substrates which contain different glycosidic linkages: isomaltose, amylose, nigeran, cellobiose, gentiobiose, laminarin, sucrose, melibiose, lactose, *p*-nitrophenyl-α-maltoside and *p*-nitrophenyl-β-glucoside. No detectable amount of glucose was formed from these compounds.

Determination of Michaelis Constants——The enzyme activities were examined against increasing concentrations of trehalose or maltose under standard conditions. and the K_m values were calculated by the method of Lineweaver and Burk.[4] By plotting $1/v$ *versus* $1/[S]$, the K_m values were estimated to be 7.3×10^{-4} M for trehalose and 1.5×10^{-2} M for maltose (Fig. 3). From these K_m values, it is clear that this enzyme has higher activity toward trehalose than maltose.

Inhibition of Trehalase Activity——The effects of additional compounds (100 μmol/ml) on the trehalase activity were examined. Phosphoglyceraldehyde did not affect the trehalase activity and glucose-6-phosphate increased it to more than 150% of the control level. Fructose-6-phosphate, Na-pyruvate or D-mannitol decreased the activity to 50%, 60% or 80%, respectively, of the control and the enzyme activity recovered to the original level upon dialysis against distilled water. The effects of different concentrations of fructose-6-phosphate on the trehalase activity were examined by plotting $1/v$ *versus* $1/[S]$ (Fig. 3). These results indicate that this compound is a competitive inhibitor with respect to trehalose (K_i: 2.5×10^{-2} M), and competitive inhibition was also observed with Na-pyruvate (K_i: 4.0×10^{-2} M). (data not shown).

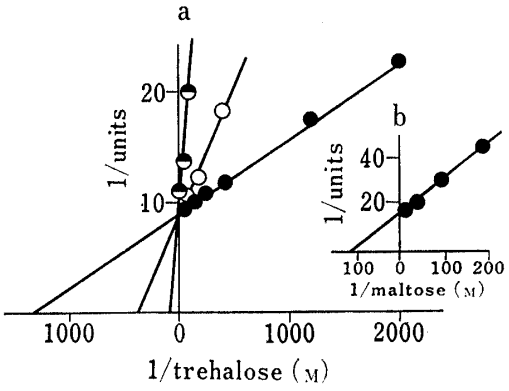

Fig. 3. Lineweaver and Burk Plots of the Dependence of Glucose Release on the Concentration of Trehalose (a) or Maltose (b) and of the Effect of Fructose-6-phosphate Concentration on Trehalase Activity (a)

Symbols: ●, without fructose-6-phosphate; ○, with 60 μ mol/ml of fructose-6-phosphate; ◓, with 120 μ mol/ml of fructose-6-phosphate.

Discussion

Although Horikoshi[5] reported that soluble trehalase and coat-bound trehalase were present in the conidia of *Aspergillus oryzae*, in the case of *Cochlibolus miyabeanus*, trehalase activity was detected only in the supernatant of the conidial homogenate (soluble trhalase), not in the cell debris (coat-bound trehalase). The purification of this soluble trehalase was achieved by subjecting the crude enzyme solution to anion exchange column chromatography followed by preparative polyacrylamide gel electrophoresis. The specific activity of the final purified enzyme obtained by these procedures was increased 137-fold over that of the crude extract and the enzyme protein gave a single band on SDS-polyacrylamide gel electrophoresis. This enzyme showed no activity against any of the substrates examined except trehalose and maltose, and its affinity for trehalose was higher than that for the maltose. The K_m value of this enzyme is similar to that of the enzyme isolated from *Neurospora classa* (5.7×10^{-4} M)[6] or baker's yeast (4.1×10^{-4} M)[7] and is much lower than that of the enzyme found in conidia of *A. oryzae* (2.5×10^{-3} M)[5] or *Streptomyces hydroscopicus* (6.73×10^{-3} M).[8] The pH optimum of this enzyme (pH 4.0) is similar to that of the enzyme of *A. oryzae*. However, the two enzymes differ in their heat stability. This enzyme was unstable at 50°C, but the enzyme of *A. oryzae* was stable at 50°C. In addition, the trehalase from conidia of *A. oryzae* was strongly inhibited by D-mannitol (K_i: 5.3×10^{-3} M),[5] whereas the present enzyme was scarcely inhibited by D-mannitol. Fructose-6-phosphate or Na-pyruvate, which may be a metabolite of trehalose, showed a low level of competitive inhibition against this enzyme (K_i: 2.5 or 4.0×10^{-2} M, respectively). The trehalase activity increased rapidly on the commencement of conidial germination, and 30 min later began to decrease gradually.[1] Further work is required to determine whether the decrease of trehalase activity is caused by the accumulation of trehalose metabolites (fructose-6-phosphate, Na-pyruvate or other unidentified compounds) in the germinating conidia or not.

References and Notes

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