

# Trehalase in Conidia of *Aspergillus oryzae*

KOKI HORIKOSHI AND YONOSUKE IKEDA

Department of Microbiology, The Institute of Physical and Chemical Research, Komagome, Bunkyo-ku, Tokyo, Japan

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## ABSTRACT

HORIKOSHI, KOKI (The Institute of Physical and Chemical Research, Bunkyo-ku, Tokyo, Japan), AND YONOSUKE IKEDA. Trehalase in conidia of *Aspergillus oryzae*. J. Bacteriol. 91:1883-1887. 1966.—Trehalases (soluble trehalase and coat-bound trehalase) were found in the conidia of *Aspergillus oryzae*, and the total activity of the trehalases increased during the germination process. The soluble trehalase was purified by diethylaminoethyl-cellulose column chromatography; its optimal pH, Michaelis constant, and heat stability were studied. In vitro, the trehalases were competitively inhibited by D-mannitol, which was also contained in the conidia. Since the trehalose content in the conidia decreased at an early stage of germination, it was assumed that trehalase might begin to hydrolyze trehalose after the inhibitory effect of D-mannitol decreased.

The disaccharide trehalose is common in fungus spores, having been found in ascospores of *Neurospora tetrasperma* (9, 14), uredospores of *Puccinia graminis f. tritici* (12), phaeodictyospores of *Pithomyces chartarum* (1), conidia of *Penicillium chrysogenum* (2), and in spores of *Myrothecium verrucaria* (10). The chemistry and biochemistry of trehalose have been reviewed by Birch (3). Trehalase in fungi has been studied by many investigators (4, 5, 10, 11). According to these investigations, trehalose seems to act as a reserve material sustaining endogenous respiration and accelerating germination.

Trehalase is found in dormant and in germinating conidia of *Aspergillus oryzae*, and the total activities increase during the germination process. Trehalase activity is inhibited by D-mannitol, another reserve material. Trehalose is also found in dormant conidia, and the content decreases at an early stage of germination. The biological significance of these observations is discussed in this paper.

## MATERIALS AND METHODS

**Organism and cultivation.** Preparation of conidia, the medium used, and the growth conditions were reported previously (6).

**Chromatography.** Sugars and sugar alcohols were chromatographed on Toyo Roshi no. 51 paper, by use of the following solvent systems: isopropanol-water (4:1) and pyridine-ethyl acetate-water (5:12:4). Ammoniacal silver nitrate was used as a spraying reagent. The  $R_G$  value expresses the rate of movement of the compound compared with that of glucose.

**Trehalase activity.** Unless otherwise stated, trehalase activity was measured in the following manner: 0.1 ml of 0.1 M trehalose, 0.1 ml of the enzyme solution (0.02 to 0.2 units), and 0.3 ml of 0.1 M acetate buffer (pH 4.0) were mixed and incubated at 37 C for 10 min with shaking. The reaction was stopped by boiling for 5 min. Reducing power was determined by the method of Sumner and Somers (13). The course of hydrolysis is a linear function of time under the standard conditions. In experiments where inhibitors were studied, the glucose released was measured by use of the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). One unit of trehalase activity is defined as that amount of enzyme which produces 1  $\mu$ mole of glucose per min under the conditions specified above.

**Conidial coats and cell-free extract.** Conidial coats and cell-free extract were prepared by the methods described previously (7).

**Reagents.** D-Mannitol, D-fructose, D-galactose, L-sorbose,  $\alpha$ -lactose,  $\alpha$ -melibiose, and maltose were obtained from Wako Junyaku Co., Osaka, Japan. Cellobiose,  $\alpha$ , $\alpha$ -trehalose, *p*-nitrophenyl- $\beta$ -D-glucopyranoside, D-sorbitol, meso-inositol, dulcitol, adonitol, D-arabitol, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) were the products of Calbiochem. Glucostat reagent was purchased from Worthington Biochemical Corp., Freehold, N.J.

## RESULTS

**Preparation of trehalase from the conidia.** A spore paste [about 5 g (dry weight) of conidia of *A. oryzae*], to which 10 g of alumina (300-mesh) had been added, was ruptured by hand grinding for 30 min with a chilled mortar and pestle. The

resultant paste was suspended in 200 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.0), and was centrifuged at  $10,000 \times g$  for 10 min. To the supernatant solution, 1 M acetic acid was added and the pH was adjusted to 4.0. The precipitate formed by this treatment was removed by centrifugation ( $10,000 \times g$  for 10 min). The supernatant solution was cooled to 0 C, and an equal volume of cold acetone was added. The precipitate collected by centrifugation was dissolved in 10 ml of Tris buffer (pH 7.0), and was dialyzed against 0.01 M phosphate buffer (pH 7.0). The resultant solution adsorbed on a diethylaminoethyl (DEAE)-cellulose column (2.0 by 30 cm), which had been equilibrated with 0.01 M phosphate buffer (pH 7.0). The enzyme was eluted by applying a linear gradient of 0.01 M phosphate to 0.1 M phosphate supplemented with 0.3 M NaCl (pH 7.0). The flow rate through the column was 25 ml/hr, and 5-ml fractions were collected. A typical elution pattern is shown in Fig. 1. Table 1 summarizes the results of the purification of the enzyme. The following properties of the enzyme were determined with the most highly purified sample.

*Kinetics of trehalase from the conidia.* In the following experiments, enzyme solutions with 0.02 to 0.2 units of trehalase activity were used. A linear relationship was observed between the rate of formation of reducing power and the concentration of enzyme solution under these conditions. The optimal pH for the enzyme was about 4 (Fig. 2). The enzyme solutions (pH 7.0 and 4.0) were heated at various temperatures for 5 min, and cooled rapidly. The remaining activity was determined (Fig. 3) by the method described above. The Michaelis constant was calculated by the method of Lineweaver and Burk (8) to be  $2.5 \times 10^{-3}$  M for trehalose. Chromatographic analysis showed that trehalose was hy-

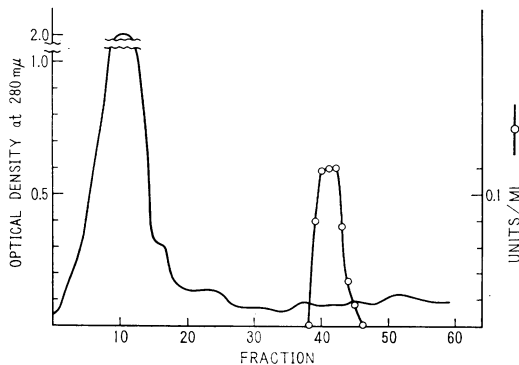


FIG. 1. Gradient elution pattern of trehalase from a DEAE-cellulose column. Trehalase activity (○) is expressed as units in 1 ml of eluate.

TABLE 1. Purification of trehalase from the conidia of *Aspergillus oryzae*

Step	Total vol	Total units	Total protein*	Specific activity (units per mg of protein)
	ml		mg	
Crude extract . . . . .	200	48	750	0.06
Treatment at pH 4 . . . . .	250	42	320	0.13
Acetone . . . . .	10	36	75	0.48
DEAE-cellulose column chromatography fraction no. 42 . . . . .	5	0.6	0.25	2.4

\* Protein was determined spectrophotometrically by measuring absorption at 280 and 260  $\mu$  (15).

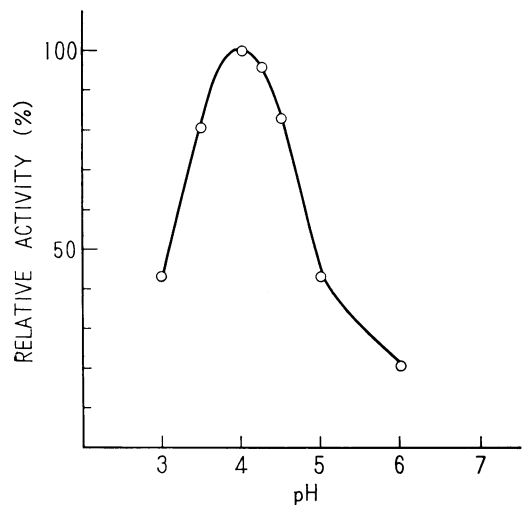


FIG. 2. Effect of pH on enzyme activity. Acetate buffer, 0.1 M (0.3 ml); 0.1 ml of trehalase; and 0.1 ml of 0.1 M trehalose were mixed and incubated at 37 C for 10 min. The glucose released was determined by the method of Sumner and Somers (13). The enzymatic activity at pH 4.0 is expressed as 100%.

drolyzed by the purified enzyme, with glucose being produced.

*Substrate specificity.* About 10  $\mu$ moles per 0.1 ml of the following sugars was tested as substrates for the purified enzyme: sucrose, cellobiose, maltose, lactose, melibiose, and *p*-nitrophenyl -  $\beta$  - D - glucopyranoside. No detectable amount of reducing sugar was formed from these compounds. The NAD-linked or NADP-linked D-mannitol dehydrogenase activity was also assayed by the method described previously (6). No dehydrogenase activity was detected in the purified enzyme solution.

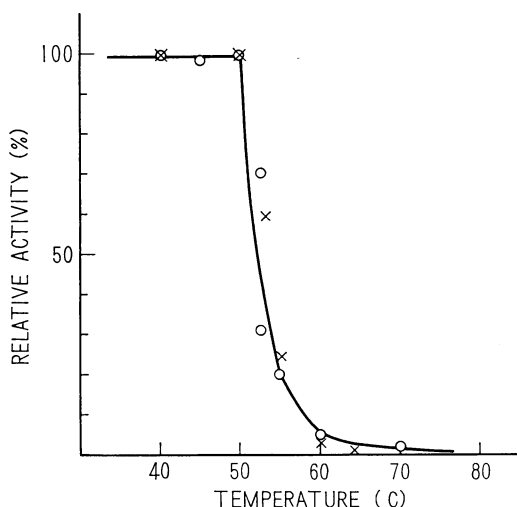


FIG. 3. Heat stability of trehalase. The enzyme solutions (pH 7.0 and 4.0) were heated at various temperatures for 5 min. The residual activity was determined under standard conditions. Symbols: ○, 0.1 M Tris buffer (pH 7.0); ×, 0.1 M acetate buffer (pH 4.0).

**Inhibition of enzyme activity by sugars and sugar alcohols.** Sugars or sugar alcohols (10  $\mu$ moles) were added to the reaction mixture described in Materials and Methods, and the velocities of the reaction were compared with the velocity under the standard conditions. The following sugars and sugar alcohols were tested: D-galactose, D-mannose, L-sorbose, D-fructose, D-mannitol, D-sorbitol, *meso*-inositol, dulcitol, adonitol, and D-arabitol. About 50% of the activity of trehalase was inhibited by D-mannitol. However, the enzymatic activity was not inhibited by the other samples, nor by the addition of 2  $\mu$ moles of glucose to the reaction mixture. To confirm the inhibitory effect of D-mannitol, various amounts of D-mannitol (1 to 400  $\mu$ moles) were added to the reaction mixture (20  $\mu$ moles of trehalose; 0.08 units of trehalase and acetate buffer, pH 4.0; total, 1 ml), and the enzymatic activities were determined under standard conditions (Fig. 4). A typical Lineweaver and Burk plot in the presence of D-mannitol is shown in Fig. 5. D-Mannitol was a competitive inhibitor with a dissociation constant ( $K_i$ ) of about  $5 \times 10^{-3}$  M. The pH of the reaction mixture (pH 3-6) had no effect on the amount of inhibition by D-mannitol.

**Development of trehalase in the germinating conidia.** A 6-g amount of dormant conidia in 6,000 ml of the germination medium was equally divided into six 5-liter Erlenmeyer flasks, and incubated at 30 C on a rotary shaker. At 0, 30, 70, 120, 180, and 240 min of incubation, one flask was removed for analysis. Microscopic ob-

servation revealed that more than 90% of the conidia sprouted germ tubes after 240 min of incubation. The germinating conidia were washed with 0.01 M Tris buffer (pH 7.0) and were disrupted in a Nossal shaker. Details of the procedure were reported in a previous paper (7). Trehalase activity was found in the conidial coats and in the cell-free extracts. As shown in Fig. 6, the soluble activity in the cell-free extract increased after a lag of about 60 min, and the lag of the coat-bound activity was about 100 min. Properties of the coat-bound trehalase were examined; the optimal pH, heat stability, Michaelis constant, substrate specificity, and effect of D-mannitol were the same as those of the purified soluble trehalase.

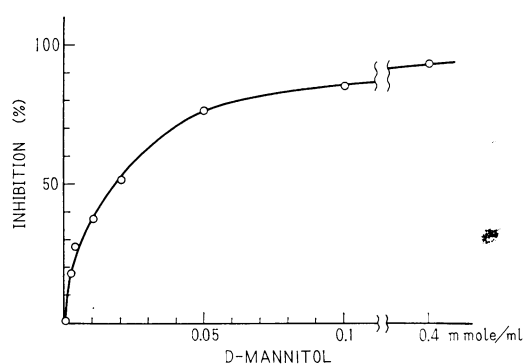


FIG. 4. Effect of D-mannitol on trehalase activity. Trehalose (20  $\mu$ moles), 0.08 units of the enzyme, and D-mannitol (1 to 400  $\mu$ moles) were mixed with 0.1 M acetate buffer (pH 4.0) in a total volume of 1 ml. The mixtures were incubated at 37 C for 10 min, and the enzymatic activities were determined under the standard conditions.

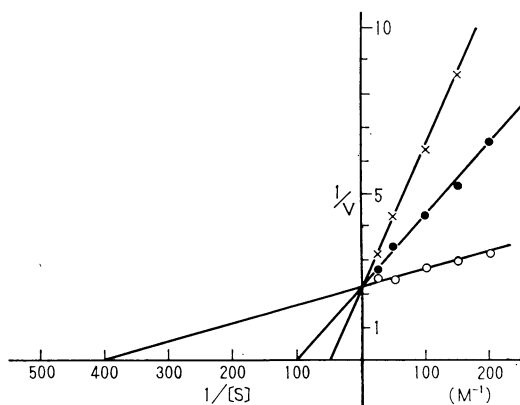


FIG. 5. Lineweaver-Burk plot of the effect of D-mannitol concentration on trehalase. Symbols: ○, without D-mannitol; ●, 20  $\mu$ moles/ml of D-mannitol; ×, 40  $\mu$ moles/ml of D-mannitol.

**Fate of trehalose during germination of conidia.** The germination medium (500 ml) which had been inoculated with 500 mg of conidia was placed in a 5-liter Erlenmeyer flask and incubated at 30 C on a rotary shaker. A 100-ml amount of the broth was withdrawn from the culture after 0, 20, 40, 60, and 90 min. Conidia were collected by centrifugation, and were suspended in 5 ml of hot ethyl alcohol for 30 min with shaking to extract trehalose. This procedure was repeated three times. The consecutive extracts were combined and were concentrated in vacuo. The extracts thus prepared were chromatographed on Toyo Roshi no. 51 papers by use of the ethyl acetate-pyridine-water solvent system. Typical  $R_G$  values of compounds in the extracts are shown in Table 2. The compound which had a typical  $R_G$  value for trehalose was eluted with water; it was nonreducing and reacted weakly with silver nitrate. After hydrolysis (1 N HCl, 100 C, 120 min), this was replaced by a rapidly reacting spot with an  $R_G$  value characteristic of glucose. In addition, this compound was completely hydrolyzed with the purified trehalase described above, and only glucose was detected. Therefore, the compound was identified as trehalose. The content of trehalose in the germinating conidia was determined by the anthrone method after the compound was eluted from the papers. As shown in Fig. 7, the content of trehalose decreases at an early stage of germination. These experiments revealed that the nonreduc-

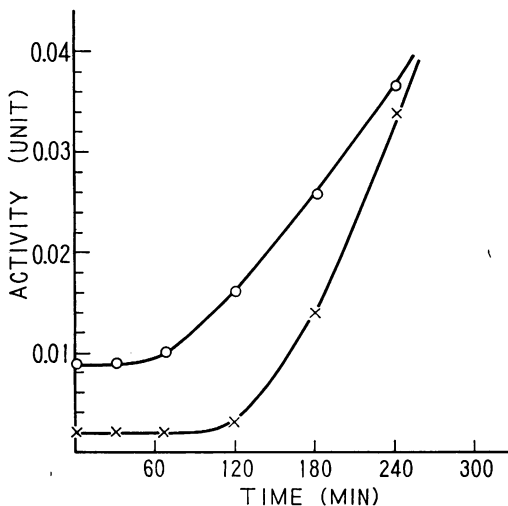


FIG. 6. Activity of trehalase in germinating conidia of *Aspergillus oryzae*. Symbols: O, activity of soluble trehalase per conidia in 1 ml of germination medium; X, activity of coat-bound trehalase per conidia in 1 ml of germination medium.

TABLE 2.  $R_G$  values of compounds soluble in hot ethyl alcohol\*

Compound	$R_G$ value	
	Standard	Extract from conidia
Glycerol.....	1.9	1.9
Erythritol.....	1.5	1.5
Arabitol.....	1.25	1.20
Mannitol.....	0.92	0.95
Trehalase.....	0.54	0.54

\* Sugar and sugar alcohols were extracted from dormant conidia with hot ethyl alcohol, and were chromatographed on Toyo Roshi no. 51 paper by use of an ethyl acetate-pyridine-water (12:5:4, v/v) solvent system.

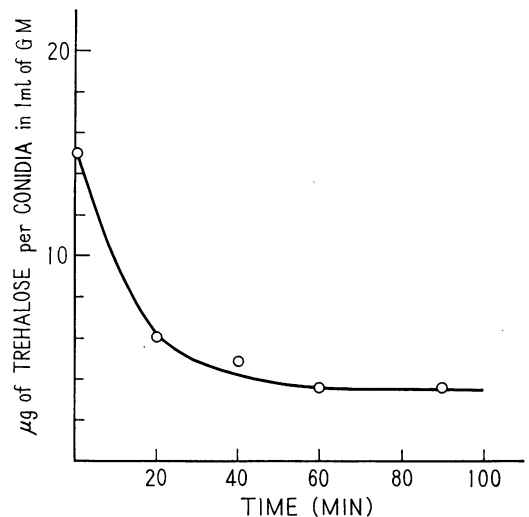


FIG. 7. Fate of trehalose during germination. The fraction of germinating conidia soluble in hot ethyl alcohol was chromatographed on Toyo Roshi no. 51 paper by use of an ethyl acetate-pyridine-water solvent system. Trehalose was eluted from paper and determined by the anthrone method. GM = germination medium.

ing disaccharide trehalose might be the substance which serves as the energy supply for the conidia after being converted to glucose.

#### DISCUSSION

Trehalase in the conidia of *A. oryzae* is different from that of *N. crassa* (4). The Michaelis constants of the trehalases in *N. crassa* are reported to be  $5.7 \times 10^{-4}$  and  $2.7 \times 10^{-4}$  M (4), whereas that in *A. oryzae* is  $2.5 \times 10^{-3}$  M. In addition, they differ in their pH optima and heat stability:

the trehalase in conidia of *A. oryzae* is more active at an acidic pH and is unstable at 60 C.

The inhibition of trehalase activity by D-mannitol is competitive, and the enzymatic activity is completely restored after removal of D-mannitol by dialysis. Total activity of trehalase increases during germination. It is not known whether the increment is due to de novo synthesis of new protein or to activation of a latent enzyme. At any rate, the observation that a large amount of activity is found in the conidial coat is interesting. Similar results were observed for  $\beta$ -glucosidase in germinating conidia of *A. oryzae* (7).

Trehalose contained in the dormant conidia may be used as the substrate of trehalase. It is still questionable whether the trehalose can be utilized as the source of energy in the presence of D-mannitol, which is also contained in the dormant conidia, because D-mannitol inhibits trehalase activity. The inhibition has been demonstrated only in an in vitro system, but, if it also occurs in vivo, trehalase may not be able to act in the dormant conidia. Large amounts of D-mannitol are contained in dormant conidia (6): the molar ratio between D-mannitol and trehalose is about 10:1. Under these conditions, the trehalase should be strongly inhibited by D-mannitol. It is presumed, therefore, that trehalase begins to hydrolyze trehalose only after the concentration of D-mannitol decreases. It was reported by Horikoshi et al. (6) that D-mannitol decreases at an early stage of germination; however, the conclusion will not be made until the time relationship between the consumption of D-mannitol and that of trehalose is established.

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