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TREHALASE AND THE ENZYMES OF TREHALOSE BIOSYNTHESIS IN *LILIUM LONGIFLORUM* POLLEN*†

ARNOLD E. S. GUSSIN‡ and JEFFREY H. MCCORMACK§

Department of the Biological Sciences, Clark Science Center, Smith College, Northampton, Massachusetts 01060, U.S.A.

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Abstract—*Lilium longiflorum* cv. "Ace", contained a trehalase which was not bound to cell structure. The enzyme was active over a broad range of pH, with an optimum at 5·4. It had an energy of activation of 8·2 kcal/mole, a transition temp. of 56°, and was exceptionally heat stable, retaining full activity when incubated at 50° for 30 min in the absence of substrate. The enzyme had a K_m of 1×10^{-3} M for trehalose and was competitively inhibited by phloridzin. The enzymes of trehalose biosynthesis—hexokinase, phosphoglucomutase, UDPG pyrophosphorylase, trehalose-6-P synthetase, and trehalose-6-P phosphatase—were present in pollen; all except hexokinase were fully soluble.

INTRODUCTION

RECENTLY we[1] presented evidence for the existence of a specific trehalase (trehalose 1-glucohydrolase, E.C. 3.2.1.28) in the pollen of several plants, and for the utilization of exogenous trehalose as an energy source for *in vitro* pollen germination. We suggested that the synthesis and breakdown of trehalose might be instrumental in the transport of glucose moieties across the pollen membrane. Our hypothesis was dependent upon the findings of Sacktor[2] and Glasziou and Gayler.[3] The former proposed that trehalose may be involved in the transport of glucose across the kidney and intestine of vertebrates, while the latter authors suggested that trehalose synthesis and breakdown may be part of a system for the transport of hexoses out of the sugar-cane vacuole. According to the view of Glasziou and Gayler,[3] trehalase would be an unbound enzyme and the enzymes of trehalose biosynthesis would be located either in the vacuole or on the vacuolar membrane.

This paper is a report on some properties of *Lilium longiflorum* pollen trehalase and on the presence of all of the enzymes of trehalose biosynthesis in *Lilium*. In addition, evidence is presented which indicates trehalose is probably not involved in the transport of glucose into the pollen grain.

RESULTS AND DISCUSSION

Effect of pH on Trehalase Activity

Trehalase from lily pollen manifests a broad optimum between pH 4·5 and 6·0, with maximal activity at 5·4. Soluble trehalases with pH optima around 5·5 have been found in *Neuro-*

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† A preliminary report of these data was made at the May 1969 Northeastern regional meeting of the American Society of Plant Physiologists.
‡ From 1 September 1969 to 31 August 1970, N.S.F. Science Faculty Fellow, Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22903, U.S.A.
§ Present address: Program of Regulatory Biology, University of Connecticut, Storrs, Connecticut 06268, U.S.A.

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spora,[4] *Dictyostelium*,[5] and various insects.[6,7] Some soluble trehalases exhibit considerably higher pH optima: sugar-cane, 6·2;[3] yeast, 6·9.[8]

Effect of Substrate Concentration on Trehalase Activity

The K_m value is $1{\cdot}0 \times 10^{-3}$ M for trehalose (Fig. 1). Several workers have discovered soluble trehalases with K_m values of approximately 10^{-3} M,[9,10] but values of 10^{-2} M[8,11] and 10^{-4} M[3] are not uncommon. Particulate trehalases also exhibit K_m's which vary over several orders of magnitude.

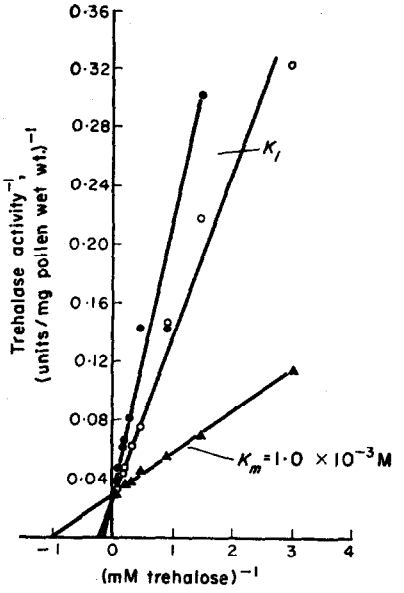

FIG. 1. DOUBLE RECIPROCAL PLOT OF THE EFFECT OF TREHALOSE CONCENTRATION ON POLLEN TREHALASE ACTIVITY.

Reaction conditions are standard. No inhibitor (▲). Inhibition by phloridzin at 2 mM (o) and 5 mM (●) was fully competitive. Values are adjusted for the effects of phloridzin on the standard curve.

Inhibition of Trehalase Activity

The K_i for phloridzin inhibition of sugar transport across the hamster intestine is approximately 10^{-6} M;[12] in order for an enzyme to be directly related to the transport process it would require that the K_i for phloridzin be equal to or less than 10^{-6} M. Semenza and Řihová[13] observed that the K_i for phloridzin inhibition of trehalase activity from the small intestine of the rat was 2×10^{-3} M, a K_i three orders of magnitude greater than the K_i for intestinal sugar transport. Our data (Fig. 1) show that, at a substrate concentration of 16·7

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mM trehalose, the K_i for phloridzin on pollen trehalase at pH 5·4 was $2{\cdot}08 \times 10^{-3}$ M. This K_i value indicates that the trehalose–trehalase system is probably not directly involved in sugar transport in pollen.

Effect of Temperature on the Rate of Trehalose Hydrolysis

The maximal rate of enzyme activity, in the presence of substrate, occurred at approximately 58° (Fig. 2, curve a). The energy of activation, calculated from these data according

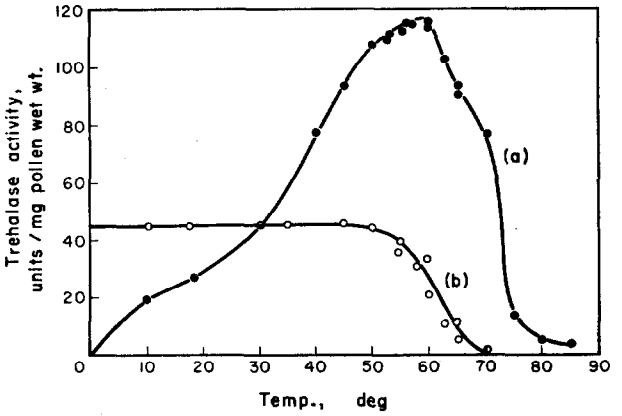

FIG. 2. EFFECT OF TEMPERATURE ON TREHALASE ACTIVITY.

(a) Rate of hydrolysis at different temperatures of incubation. Enzyme was incubated with trehalose for 30 min under standard conditions (●). (b) Stability of the enzyme. Enzyme was maintained in the absence of substrate for 30 min at the indicated temperatures. Residual activity was then assayed at 30° under standard conditions (○).

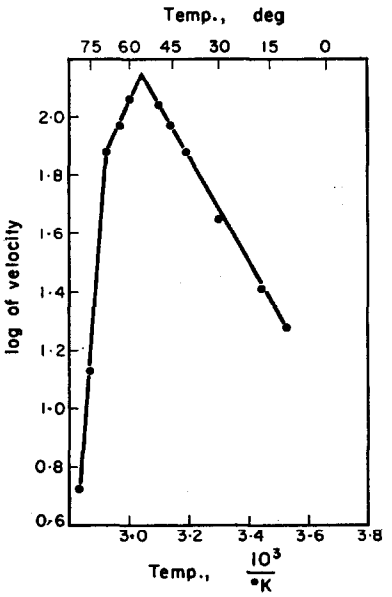

FIG. 3. ARRHENIUS PLOT FOR THE HYDROLYSIS OF TREHALOSE.

Data are taken from curve (a), Fig. 2.

to the Arrhenius equation, was 8·2 kcal/mole between 10° and 50°, with a transition temperature of 56° (Fig. 3). Other investigators have found similar values in various systems.[8,10]

In the absence of substrate, the enzyme maintained full activity until approximately 50°, whereupon activity decreased as a linear function of increasing temperature. Inactivation was complete at 70° (Fig. 2, curve b). Thus pollen trehalase, with or without substrate, is exceptionally heat stable. We are unaware of other trehalases which are so highly heat resistant; yeast trehalase, for example, is destroyed at a temp. above 40°.[8]

Lily trehalase activity was completely stable at −15° for at least 3 weeks, and 95% of its total activity remained at 9°. At 30°, half of the enzyme was denatured in 6 days and 93% in 3 weeks (Fig. 4).

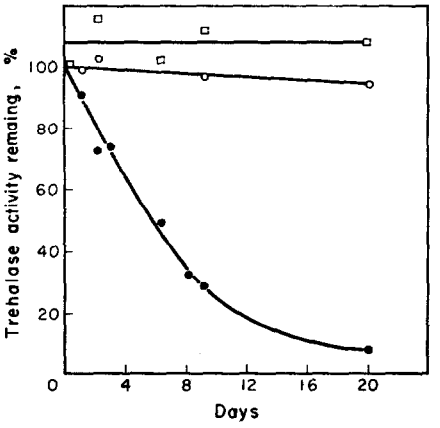

FIG. 4. STABILITY OF POLLEN TREHALASE AS A FUNCTION OF TIME AT THREE TEMPERATURES.
Enzyme was maintained without substrate in 0·1 M NaOAc buffer, pH 5·4, at the indicated temperatures [−15°, (□); 9° (○); 30°, (●)] for various times and assayed at 30° under standard conditions.

Demonstration of Trehalase and the Enzymes of Trehalose Biosynthesis

Table 1 lists the five enzymes of trehalose biosynthesis[14] and their activities in lily pollen. Phosphoglucomutase, UDP-glucose pyrophosphorylase, and trehalose-6-P synthetase were completely soluble. Trehalose-6-P phosphatase, was probably 100% soluble, although a wash of the pellet was not done. Hexokinase, on the other hand, was only 25% soluble. Karpatkin[15] showed that 50% of the hexokinase from frog skeletal muscle was soluble and Sacktor[2] demonstrated that 25% of the enzyme from the rabbit renal cortex was soluble. Trehalase activity, as previously reported[1] and as shown in the table, was entirely soluble.

Suggested Roles for the Trehalose–Trehalase System in Pollen

Trehalase and the enzymes of trehalose biosynthesis in pollen are soluble, and hence, as suggested by Glasziou and Gayler[3] (see Introduction), could mediate hexose transport out of the grain. We are unaware of a reason for glucose to be actively transported out of the grain. Perhaps there is intra-pollen transport, for example in the movement of glucose into the generative cell.

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TABLE 1. ACTIVITIES OF THE ENZYMES OF TREHALOSE BIOSYNTHESIS AND OF TREHALASE IN *Lilium longiflorum**

Enzyme	Fraction	Activity (mμmoles/min/mg pollen)	Total recovered activity (%)
Hexokinase	Total homogenate	14·4	—
	Pellet	8·0	73·4
	Wash	0·8	7·3
	Supernatant	2·1	19·3
Phosphoglucomutase	Total homogenate	70·3	—
	Pellet	0·0	0·0
	Wash	0·5	0·7
	Supernatant	72·2	99·3
UDPG pyrophosphorylase	Total homogenate	82·7	—
	Pellet	0·0	0·0
	Wash	6·1	10·3
	Supernatant	53·3	89·7
Trehalose-6-P synthetase	Total homogenate	23·5	—
	Pellet	0·3	1·4
	Wash	1·2	6·1
	Supernatant	18·4	92·4
Trehalose-6-P phosphatase†	Total homogenate	5·1	—
	Pellet	0·7	17·5
	Supernatant	3·3	82·5
Trehalase	Total homogenate	45·7	—
	Pellet	0·0	0·0
	Wash	1·2	2·7
	Supernatant	43·1	97·3

* Enzymes were assayed as described in Experimental.
† A wash was not done.

A second role for this system in pollen arises from the recent experiments of Van Handel.[16] He found that the mosquito, which feeds principally on nectar, a mixture of glucose and fructose, converted the fructose to trehalose. Free glucose was not an intermediate product between fructose and trehalose. Van Handel suggested that "fructose is phosphorylated and the resulting fructose phosphate is converted to glucose phosphate and uridine diphosphate glucose, which then enters known pathways to trehalose biosynthesis". A similar system may be present in pollen and in other plant tissues. Pollen has an active β-fructofuranosidase,[17] which would cleave sucrose into its constituents. The fructose produced could then be converted into the readily metabolizable glucose via the trehalose–trehalase system.

Work in this laboratory is continuing in an attempt to determine if either of the above hypotheses is valid.

EXPERIMENTAL

Botanical Material

Mature pollen of *Lilium longiflorum*, cv. "Ace", was collected and handled as previously described.[1]

Reagents

Trehalose-6-P (barium salt) was a gift of Dr. Donald L. Macdonald, Oregon State University. It was treated with a cation exchange resin prior to use. Enzymes and biochemicals were otherwise obtained from commercial sources. Other chemicals were as previously described.[1]

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Assays

The enzymes of trehalose biosynthesis were assayed according to the following method: hexokinase,[2] phosphoglucomutase and UDP-glucose pyrophosphorylase,[18] trehalose-6-P phosphatase,[14] and trehalose-6-P synthetase in the direction of UDP formation.[19]

The standard trehalase assay was essentially identical to that previously employed[1] except that enzyme was heated in the presence of substrate[1] for 30 min at 50° prior to dialysis, and that a 0·1 ml aliquot of dialyzed enzyme in 0·1 M NaOAc buffer, pH 5·4, was added to 0·1 ml of 0·12 M trehalose in the same buffer. Units of trehalase were expressed as mμmoles of glucose liberated from trehalose per min under the conditions of the assay. Enzyme activity was linear with time and enzyme concentration.

Preparation of extracts. Desiccated pollen (usually 25 mg/ml) (previously dried over $CaCl_2$ for 8–10 hr) was ground in an all-glass homogenizer at 0° until the grains were disrupted. The homogenate was dialyzed against 0·33% NaCl (w/v) for 16 hr and centrifuged at 105,000 *g* for 15 min. The resulting pellet was washed with the homogenization medium called for by the specific assay procedure being followed. The total homogenate, supernatant, and washings were assayed in order to determine the concentration and the localization of the various enzymes.

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