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Trehalase: A New Pollen Enzyme

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Abstract. Pollen from 5 plant species (*Lycopersicon pimpinellifolium* Mill., *Hermerocallis minor* Mill., *Galtonia candicans* Decne., *Camellia japonica* L., and *Lathyrus odoratus* L.) representing 4 families germinated well in media containing trehalose as the sole carbon source. Data are presented indicating that pollen metabolized this disaccharide for germination and subsequent pollen-tube growth; the sugar was not merely an osmoregulator. An inhibitor of trehalase activity depressed germination in trehalose but not in sucrose. Phloridzin dihydrate, an inhibitor of glucose transport, depressed germination in both disaccharides.

Biochemical tests demonstrated that a pollen extract was capable of hydrolyzing trehalose to its constituent glucose monomers. Heat inactivation experiments confirmed the presence of a distinct trehalase having a rigid specificity for its substrate. By this method, trehalase activity was completely distinguishable from the activities of other α- and β-glucosidases and β-galactosidases. Localization data indicated that the enzyme diffused from intact grains and was probably soluble. The presence of its substrate could not be demonstrated in pollen or in stigmatic or stylar tissues.

Many enzymes are located in the pollen of flowering plants (see 19 for a tabulation). We now report on the existence of a new pollen enzyme, trehalase (trehalose 1-glucohydrolase, EC 3.2.1.28), whose substrate, trehalose (1-α-D-glucopyranosyl-α-D-glucopyranoside), could not be demonstrated in pollen or in stigmatic or stylar tissues. Trehalase has an ubiquitous occurrence (20), often being present where there is no trehalose substrate. The enzyme has recently been detected in extracts of roots, leaves, and stalk tissue from sugar cane (12).

Trehalose occurs widely in invertebrate phyla, in fungi, in many algae, and in occasional lower vascular plants (*Selaginella lepidophylla* and *Botrychium lunaria*) (3, 20, 29). It is especially common in the dormant stages of several organisms (6) and in organisms that are resistant to storage and desiccation (29). Trehalose has never been identified in vertebrates and until 1967 had never been identified in flowering plants. In that year Oesch and Meier (22) isolated trehalose from the cambial sap of the beech, *Fagus silvatica*.

Materials and Methods

Botanical Material. Mature pollen was collected from flowers of the following plants grown in the Smith College Botanical Gardens: the currant tomato, *Lycopersicon pimpinellifolium* Mill., line 206; the dwarf yellow day-lily, *Hemerocallis minor* Mill., the summer hyacinth, *Galtonia candicans* Decne., the camellia, *Camellia japonica* L., variety single pink; and the sweet pea, *Lathyrus odoratus* L. All grains were binucleate (4). Stigmatic and stylar tissues were obtained from the same plants.

Germination Studies. Mature pollen (fresh or dried over $CaCl_2$ for 8–10 hr) was tested for percent germination and pollen-tube length. The germination medium was either an aqueous solution of the substance to be tested (most often a carbohydrate) and borate, or Brewbaker and Kwack medium (5) modified so that sucrose was replaced with other sugars or non-nutritive osmoregulators. The pH of all media was maintained at values between 6.0 and 7.0; no difference in results occurred in this range.

In early experiments with tomato pollen germination was effected by a hanging drop technique. In all other experiments pollen (50–150 or more

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grains) was added to 0.01 ml drops of medium on glass slides. Each slide, with 3 to 5 identical preparations, was placed on moist filter paper in Petri dishes and incubated at room temperature for 2 hr, at which time percent germination was estimated. Pollen-tube length was determined for the same grains by inspection. Data were gathered from several identical preparations and averaged for presentation.

Biochemical Demonstration of Trehalase Activity. Mature pollen from freshly opened flowers was collected and dried. It was used immediately or stored at 4° for not more than 1 week. Pollen (10 mg) was mixed with 1 ml of 1 % NaCl (w/v), pH 6.4 (18), and enzymes were allowed to diffuse from the grains for periods ranging from 1 to 24 hr at room temperature. After the diffusion period the mixture was centrifuged for 5 min at 1500*g*; the resulting supernatant was decanted and filtered through Whatman No. 1 filter paper. The filtrate was dialyzed in the cold against 0.33 % NaCl (w/v) for at least 4 hr to remove endogenous glucose and other small molecules that had diffused from the grains. (High levels of endogenous glucose both inhibit trehalase activity and mask the expression of activity. See below.) The dialyzate was assayed directly, or in some cases after concentration with polyacrylamide gel pellets. The assay was a modification of the method of Dahlqvist (8). A 0.1 ml aliquot of the dialyzate was added to 0.1 ml of 0.12 M trehalose in 0.1 M sodium-potassium phosphate buffer, pH 6.0 (2). The reaction mixture was incubated for periods of up to 2 hr at 30° with shaking and the reaction stopped by boiling for 3 min. Glucose oxidase reagent (3 ml) was then added. (This reagent was prepared by adding in order, with mixing, the following reagents prepared according to Dahlqvist (8): *o*-dianisidine, 1 ml; Triton X-100, 1 ml; peroxidase, 0.5 ml.) A second incubation was carried out at 37° for 40 min, at which time color development was complete. Units of trehalase were expressed as mμmoles of glucose liberated from the trehalose per min under the conditions of the assay. Glucose was read at 420 mμ. Enzyme activity was linear with time.

In some initial experiments tomato pollen was homogenized with glass beads, the protein precipitated with ammonium sulfate, and the precipitate assayed for trehalase activity according to the method of Gussin and Wyatt (14).

In order to ascertain that pollen trehalase was specific for trehalose and not a non-specific α-glucosidase, grains were homogenized in 0.1 M sodium-potassium phosphate buffer, pH 6.0, in the cold, and centrifuged in the Spinco Model L Ultracentrifuge at 105,000*g* for 10 min. The resulting supernatant was treated in 1 of 3 ways: dialyzed against 0.2 % NaCl (w/v), concentrated, and assayed; treated with an equal amount of 0.24 M trehalose in phosphate buffer at 50°[6] for 10 to 30 min, chilled, dialyzed, concentrated, and assayed; or treated with trehalose at 4°, dialyzed, concentrated, and assayed. In all cases the final concentration of the carbohydrates serving as substrates for the treated supernatants was 0.12 M.

Localization of Trehalase Activity in Pollen. In order to determine if trehalase could leach from the cell during germination and/or pollen-tube initiation (10), mature grains of *C. japonica* were placed in Brewbaker and Kwack medium (5) with 10 % glucose (w/v) and incubated for 30 min at 4° or at room temperature. Tube initiation occurred within this period at the latter temperature but not at the former. After the incubation period, media were centrifuged at 105,000*g* for 5 min and the supernatant was decanted. The pellet was washed twice with 1 % NaCl (w/v) and taken up in pH 6.0 phosphate buffer. The supernatant and the reconstituted pellet were dialyzed overnight against 0.1 % NaCl (w/v), concentrated, and assayed.

Experiments designed to determine if *C. japonica* pollen trehalase was soluble or particulate were carried out. Pollen (100 mg) was ground in an all-glass homogenizer at 0° in 2 ml of water, 1 % NaCl (w/v), or in phosphate buffer (*cf.* 10). The homogenate, in which at least 80 % of the grains had been broken, was centrifuged at 105,000*g* for 15 min. The resulting pellet was washed 3 times in homogenization medium and reconstituted to 2 ml. The washed pellet and supernatants were assayed as described. The location of β-fructofuranosidase activity was determined in the same way.

Chromatographic Determination of Trehalose. Carbohydrates were extracted from 1 g of plant tissues (anthers and/or pistils) by the method of Stewart, Richtmyer, and Hudson (25), and by the method of Wyatt and Kalf (30) for the preparation of extracts for quantitative chromatography. All plant extracts were deionized prior to spotting and recovery of known carbohydrates from deionizing columns was quantitative. Separation of isolated sugars was accomplished essentially by the method of De Stefanis and Ponte (9), except that Eastman Chromatographic Sheets (No. 6061) were used. By this method 10 μg of trehalose were separable from glucose, fructose and sucrose.

Reagents. Glucose oxidase was the purified product of Worthington Biochemicals. Peroxidase and *o*-dianisidine were purchased from Nutritional Biochemicals. Trehalose (hydrate) and α-methyl-D-mannoside were products of Mann Research Laboratories. Phloridzin dihydrate was purchased from Calbiochem. Triton X-100 was a gift of Rohm and Haas. Other reagents were purchased commercially and used without further purification.

[6] Unpublished experiments from this laboratory have indicated that flight muscle trehalase of the house fly, *Musca domestica*, is stable for 30 min at 53° in the presence of substrate.

Results and Discussion

Germination Studies. In experiments (tables I and II) where trehalose was the sole carbon source in the incubation medium, the percent germination and pollen-tube length relative to grain diameter was substantially above controls incubated in the absence of a carbohydrate. Maximal germination in trehalose (80 %) and maximal pollen-tube length (30 diameters) occurred in *L. pimpinellifolium* (table II). We used several substances to determine if germination in trehalose was a non-specific osmotic phenomenon. One of them, mannitol, is metabolized by some species of flowering plants, but not by *Lycopersicon esculentum* (27), and in those species metabolizing it, mannitol can be used as an osmoregulator for short term experiments (27).

Table I. *Germination of* Camellia japonica *and* Galtonia candicans *Pollen in Brewbaker and Kwack Medium*

Germination was carried out for 2 hr at room temperature. In all cases more than 500 grains were counted in 5 individual drops. Results are averaged for presentation. Carbohydrates employed in *C. japonica* germination were 10 % (w/v), while those employed in *G. candicans* were 8 % (w/v). Pentaerythritol was a 6 % (w/v) solution. Inhibitors, I_1 and I_2, were 20 mM α-methyl-D-mannoside and 2 mM phloridzin dihydrate, respectively.

Carbohydrate	*C. japonica* Germination	*C. japonica* Tube-length	*G. candicans* Germination	*G. candicans* Tube-length
	%	× *grain diam*	%	× *grain diam*
None	38	10	0	0
Glucose	66	21	61	16
Sucrose	70	26	34	13
Sucrose + I_1	...	...	39	12
Sucrose + I_2	...	...	18	3
Trehalose	60	20	25	8
Trehalose + I_1	...	...	5	2
Trehalose + I_2	...	...	3	1
Fructose	47	3	39	1
Mannose	33	3	4	2
Melibiose[1]	25	6	...	...
Mannitol	40	2	...	...
Pentaerythritol	40	3	...	...

[1] Germination was difficult to score because the pollen-tubes coiled around the grain.

Our data in table II showed that there was no germination in mannitol; in *C. japonica* (table I) mannitol and the control were similar except that pollen-tube lengths decreased in the polyol. Pentaerythritol has been used in germination studies by Dickinson because it does not interfere with the determination of carbohydrates (10); it caused 70 % germination and pollen-tube lengths of 1.0 to 1.2 mm (11). In our experiments pentaerythritol apparently served only as an osmoregulator as the percent germination was similar both to mannitol and the control (table I). Pollen-tube lengths in this osmoregulator were appreciably below those of the controls. Mannose and melibiose also effected pollen germination approximating the controls (table I). Here, tube-lengths were very low, varying from 2 to 6 diameters. Germination in fructose varied with respect to species but in no case did extensive tube elongation occur.

Table II. *Germination of* Lycopersicon pimpinellifolium *Pollen in Media Supplemented With 1.62 mM* H_3BO_3 *and Carbohydrates*

In these experiments 2 slides for each test were prepared by the hanging drop technique and germination was scored at the end of 2 hr. I_1 is 20 mM α-methyl-D-mannoside

Expt.	Carbohydrate	Concn	Germination	Tube-length
		% (*w/v*)	%	× *grain diam*
1	Sucrose	2.5	20	10
	Trehalose	2.5	50	15
2	Fructose	2.5	0	...
	Glucose	2.5	10	...
	Maltose	2.5	45	...
3	Sucrose	17.0	90	20
	Sucrose + I_1	17.0	90	15
	Trehalose	3.0	80	30
	Trehalose + I_1	3.0	18	5
4	Mannitol[1]	12.5	0	...
	Mannitol	6.3	0	...

[1] Germination media contained 0.97 mM H_3BO_3

The specific nature of germination in trehalose was substantiated by inhibitor studies (tables I and II). Germination in sucrose and trehalose is depressed by about 85 % in the presence of phloridzin, an inhibitor of glucose transport. Sactor (24) showed that phloridzin also inhibits UDPG pyrophosphorylase, a vital enzyme in trehalose biosynthesis. An inhibitor of trehalase activity in insects, α-methyl-D-mannoside (14), decreased germination and pollen-tube lengths where trehalose was the carbon source, but not where sucrose was the source (table I).

Among the objectives of our germination studies was the determination of the borate concentrations required. The values given in the tables were those we found to be optimal. For the sweet pea which is not tabulated, maximal germination (50 %) and pollen-tube growth (15 diameters) occurred at 0.97 mM H_3BO_3.

Biochemical Demonstration of Trehalase Activity. One of the many functions postulated for borate in germination and pollen-tube elongation is that of enzyme activation (17). We tested the effects of various concentrations of H_3BO_3 (0–16.2 mM) on trehalase from the housefly, *Musca domestica,* and could obtain no activation in dialyzed or undialyzed preparations. In fact, in the former case, there was an approximate inhibition of 18 % with 1.62 mM borate.

Two plant species were assayed for trehalase activity (table III). In *H. minor* there were about 13 units of trehalase activity, while in *G. candicans* there were approximately 16. Enzyme activity in both species was a linear function of the time of incubation. Trehalase activity in *C. japonica* was inhibited by about 10 % by 20 mM α-methyl-D-mannoside.

Table III. *Biochemical Assay of Pollen Trehalase in* Hemerocallis minor *and* Galtonia candicans

Trehalase was allowed to diffuse from 10 mg of pollen in 1 ml of 1 % NaCl (w/v), pH 6.4, for 24 hr at 4°. The preparation was dialyzed against 0.33 % NaCl (w/v) for 5 hr and assayed as in Materials and Methods.

Expt.	Species	Preincubation time	Glucose liberated	Units of trehalase activity
		min	*mμmole*	*mμmole glucose/min*
A	*H. minor*	30	42	14.0
B		120	144	12.0
A	*G. candicans*	30	48	16.0
B		120	200	16.7

Table IV. *Trehalase Specificity in* Camellia japonica

Specificity was evaluated in terms of differential response of 105,000*g* supernatants to heat inactivation. Experiment 1: Supernatant from 3.0 mg of pollen was assayed for disaccharidase activity as in Materials and Methods. Experiment 2: Supernatant from 30 mg of pollen was incubated with an equal volume of 0.24 M trehalose for 10 min at 50°, dialyzed, and assayed. Experiment 3: Supernatant from 3.0 mg of pollen was divided into 2 portions, to both of which were added an equal amount of 0.24 M trehalose. 3a was incubated for 30 min at 4° and 3b was incubated for 30 min at 50°. Both preparations were then dialyzed and assayed. All carbohydrates used in the assays were 0.12 M. None of the substrates had an affect on the glucose standard curve.

Expt.	Carbohydrate	Units of disaccharidase activity
		mμmoles glucose/min
1	Trehalose	96.0
2		105.1
1	Maltose	27.0
2		3.3
1	Turanose	30.8
2		2.7
1	Melibiose	3.7
2		2.1
1	Sucrose	140.3[1]
2		4.8
1	Lactose	28.4
2		16.1
3a	Lactose	15.8
3b		13.6
3a	Cellobiose	92.5
3b		9.6
3a	Raffinose[2]	9.4
3b		6.7

[1] This value represents only 25 % of the total β-fructofuranosidase activity.

[2] Units are in terms of trisaccharidase activity.

In every case where it has been studied, trehalase has been found to be a highly specific α-glucosidase (4, 16). Because this is the first report of a pollen trehalase, however, we did specificity studies according to the elegant heat inactivation procedure of Dahlqvist (7). This method permitted the activities of the α-glucosidases, β-glucosidases, and β-galactosidases of *C. japonica* to be separated from each other (table IV). As shown in the table, pollen trehalase when heated in the presence of trehalose for 10 min at 50° (experiments 2 and 3b), was activated by 6 %, while with the same treatment β-fructofuranosidase lost 97 % of its original activity. Other glucosidase activities were distinguished from trehalase activity in this manner.

Localization of Trehalase Activity in Pollen. Enzymes from *G. candicans* diffused into 1 % NaCl (w/v). After 12 hr at 4°, 82 % of the trehalase activity remained in the grain, whereas at 36 hr 41 % remained (table V). In a similar experiment *C. japonica* pollen was incubated in Brewbaker and Kwack medium with 10 % glucose (w/v) at 4° or at 25° for 30 min. At the latter temperature pollen-tube growth had already been initiated; at the former it had not. At both temperatures, however, approximately 74 % of the trehalase activity remained in the grain.

Homogenization of *C. japonica* pollen was carried out in 3 different media (table V). In each case 90 % of the enzyme activity was extracted. With subsequent washing of the pellets the release of enzyme from the grain approached 100 %. Other soluble trehalases are known (29). For example, Glasziou and Gayler (12) discovered a soluble trehalase in sugar cane. Insects contain a soluble intestinal trehalase, and simultaneously a particulate one (14). Both forms of the enzyme are specific for trehalose. We also extracted β-fructofuranosidase from pollen by homogenization procedures identical to those cited above. Twenty-five percent of the enzyme was soluble and the rest, even after several washes, remained bound to cell structure. This agrees with Dickinson's work on *Lilium longiflorum* where β-fructofuranosidase was found to be a bound external enzyme (10).

Absence of Trehalose in Pollen. The carbohydrate chromatography procedure (9) finally adopted provided reliable results. We obtained excellent resolution, with spots of desirable geometry, with standard and experimental sugars, alone or in mixtures. Representative R_G values were obtained: sucrose, 0.86; fructose, 1.11; trehalose, 0.67. Still, trehalose could not be isolated from pollen or from stigmatic or stylar tissues. This may not be surprising as some higher plants can metabolize sugars which are not endogenous to them or even naturally occurring (21, 27), and trehalase has often been

Table V. *Localization of Trehalase Activity in the Pollen of* Galtonia candicans *and* Camellia japonica

Trehalase was extracted from grains by 2 methods: Diffusion (D) or homogenization (H) followed by differential centrifugation. In the diffusion experiments grains were incubated at 4° in 1 % NaCl (w/v), or at 4° and 25° in Brewbaker and Kwack medium supplemented with 10 % glucose (w/v) (B & K). In the latter medium germination had occurred in 0.5 hr at 25° but not at 4°. Homogenization experiments were carried out in 1 % NaCl (w/v), sodium-potassium phosphate buffer (0.1 M, pH 6.0), or deionized water.

Species	Extraction conditions			Recovered trehalase activity				
	Method	Medium	Temp	Time	Pellet	Supernate	Wash number 1	2	3
	D or H		*deg*	*hr*	%	%	%	%	%
G. candicans	D	NaCl	4	12.0	82	18	...	...	...
	D	NaCl	4	36.0	41	59	...	...	...
C. japonica	D	B&K	25	0.5	72	28	...	...	...
	D	B&K	4	0.5	76	24	...	...	...
	H	buffer	0	...	3	97	...	...	...
	H	water	0	...	1.5	83	15	0.5	0
	H	NaCl	0	...	2.5	94.5	2.0	1.0	0

found to exist without trehalose (7, 13, 15, 16, 24, 26, 28).

Suggested Role of Trehalase in Pollen. Sactor (24) has proposed that trehalase may be involved in the transport of glucose moieties across the kidney and intestine of vertebrates. In line with this hypothesis, Glasziou and Gayler (12) have suggested that trehalose synthesis and breakdown may be part of a system for transport of hexose out of the sugar cane vacuole. Such a system may also be operable in pollen. Glucose could penetrate the outer pollen membrane, be biosynthesized into trehalose, pass through the membrane as trehalose, enter into the cytoplasm as trehalose, and be immediately hydrolyzed by the soluble trehalase. Arnold (1) has suggested that trehalose has many of the characteristics of a desirable translocate. It could be the protected glucose derivative or "mobile molecule" which, according to Reinhold and Eshhar (23), may be involved in glucose transport. It seems unlikely that sucrose could be the translocate as there is an active β-fructofuranosidase in the pollen wall (10). Finally, in preliminary experiments, we have identified in pollen, 2 of the 5 enzymes of trehalose biosynthesis (trehalose-6-P synthetase and trehalose-6-P phosphatase). Currently, we are continuing our investigation of the occurrence of the enzymes of trehalose biosynthesis and we are searching for a transient trehalose in pollen and in stigmatic and stylar tissues.

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