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TREHALASE ACTIVITY IN PLANT TISSUE CULTURES*

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Key Word Index—Trehalase; activities; inhibition; validamycin; tissue cultures.

Abstract—Trehalose degradation was examined in homogenates of callus and cell suspension cultures. Stable, high trehalase activity was found in three wheat lines, jack pine, and *Selaginella lepidophylla*. Stable, low trehalase activity was found in alfalfa, black Mexican sweet corn, sunflower, and white spruce. Labile, moderate activities were found in two different suspensions of canola. No trehalase activity was found in barley, bromegrass, soybean and black spruce. Trehalose degrading activities (except in canola) were inhibited by using validamycin as a specific trehalase inhibition. These results demonstrate that trehalase activity is not confined to seed or pollen in higher plants nor is it due to fungal contamination.

INTRODUCTION

Trehalose is widespread in the fungi, pteridophytes and bryophytes [1] as well as anhydrobiotic organisms such as brine shrimp cysts and nematodes [2]. However, its presence in the spermatophytes has only been firmly documented in caraway seed [1]. A report of trehalose in sunflower seeds [3] was questioned by Kandler and Hopf [1] who suggested that the reported trehalose came from fungal contamination. Previous reports of trehalose in beech [4] and cabbage [5] could not be verified by Gussin [6]. In spite of the apparent rarity of trehalose in the spermatophytes, there are several reports of trehalase activity (EC 3.2.1.28) in orchids [7] sugar cane [8, 9] and in the pollens of *Lilium* [10], *Camellia*, *Hemerocallis*, *Lathyrus* and *Lycopersicon* [11].

Several of the aforementioned studies on trehalases utilized growth of the plant or pollen tubes as measures of trehalase activity. However, because trehalose can be broken down by free radical reactions, it would seem important to not only show trehalose breakdown but also show trehalase inhibition. Fortunately very specific trehalase inhibitors, the validamycins (1,5,6-trideoxy-3-*O*-β-D-glucopyranosyl-5-(hydroxymethyl)-1-{[4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]-amino}-D-chiro-inositol, validamycin A), are known.

The validamycins (A and B) were discovered in *Streptomyces hygroscopicus* [11, 12]. Additional studies elucidated the structures of validamycin C–F [14] and more recently, validamycin G [15]. The validamycins (and validamines) showed inhibitory activities against trehalases from various sources ranging from (IC_{50}) 10^{-6} M to 10^{-10} M [16, 17]. Validamycins are specific for trehalase with no significant activities against α- and β-glucosidases or pectinase [16]. The inhibitors were almost equally effective against trehalose from porcine, rat, rabbit, baker's yeast, *Mycobacterium* and insect larvae (*Spodoptera litura*) [17]. The most active component, validamycin A, has been formulated for use against *Rhizoctonia solani* (sheath blight) on rice plants [16] and is sold under the name, 'Solacol'.

In order to learn more about the distribution of trehalase in higher plants, we surveyed several plant families for the presence of trehalose degrading activity. Aseptic material from callus or cell suspension cultures were used to avoid possible fungal contamination (because trehalase and trehalose are common components in the fungi). The purposes of this study were to survey several higher plant families for the presence of trehalose degrading activities and to determine the portion of the activity that is due to trehalase.

RESULTS AND DISCUSSION

Validamycin A was isolated from Solacol, a commercial formulation that includes wetting agents (soaps) and copper salts. The inhibitory activity of the preparation (IC_{50} 10^{-8} M, Fig. 1A) was comparable to published values for validamycin A [17].

Selaginella lepidophylla is known to accumulate trehalose and was included in this study as a positive control. Leaf homogenates demonstrated a high level of trehalose degrading activity, >99% of which could be inhibited by validamycin A (Fig. 1B, Table 1). Trehalase activity at 50–75% of *Selaginella* levels was also detected in three wheat callus lines (Fig. 2, Table 1). None of these lines had detectable levels of trehalose yet the degradative activity appeared to be enzymatic as 95% was inhibited by validamycin A (Table 1). Other Poaceae, including corn, bromegrass and barley contained little or no trehalose degrading activity. In Pinaceae there were similar contrasts. Callus cultures of jackpine and white spruce had moderate (15–34% of *Selaginella* levels), activities which could be inhibited by validamycin, whereas no activity was detectable in black spruce. Suspension cultures of soybean did not degrade trehalose. However, alfalfa suspensions contained a low (3% of *Selaginella* levels) but stable activity. Validamycin A at 10^{-4} M

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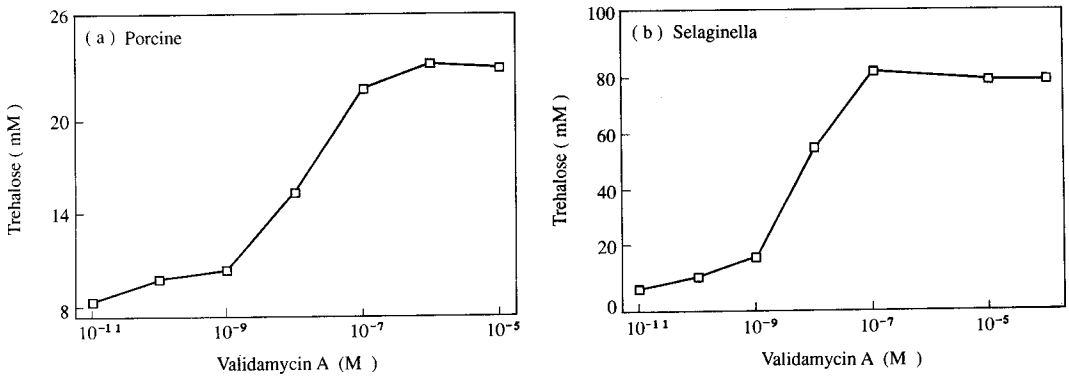

Fig. 1. Inhibition of trehalase activity by validamycin A. Validamycin A was isolated from Solacol by QAE-Sephadex ion exchange chromatography. The inhibitor was tested on porcine trehalase (0.1 unit) and on *Selaginella lepidophylla* homogenates (0.2 g) suspended in 800 μl 0.2 M potassium phosphate buffer (pH 5.8) containing 7.33 mg trehalose, 3.7 mg arabitol and specified concentrations of validamyacin A. Aliquots were removed at 0 and 1320 min and dried *in vacuo*. Trehalose determinations made by GC analysis after silylation with 400 μl TriSilZ.

Table 1. Trehalose degrading activities in cultured plant species

Plant source	Activity ($\mu g\ min^{-1}\ g^{-1}$ fr. wt)	Stability in homogenate (hr)	Trehalose degraded (mg per 22 hr) No inhibitor	Inhibitor
Helianthus annuus				
line SFH11, cell suspension	15.2	>44	16.8±0.5	0.6±0.0
Brassica napus				
cv. Jet Neuf, Suspension				
3% sucrose media	37.1	<5	2.6±0.26	3.68±0.6
13% sucrose media	24.4	<5	N.T.	N.T.
Glycine max				
cv. Mandarin, suspension	0	N.T.	N.T.	N.T.
Medicago sativa				
cv. Rangelander, suspension				
undifferentiated cells	0	N.T.	N.T.	N.T.
differentiated cells	3.7	>44	4.2±0.8	0.4±0.0
Picea glauca callus	21.2	>33	47.3±1.1	3.2±0.0
Picea mariana callus	0	N.T.	N.T.	3.2±0.0
Pinus banksiana callus	44.8	>22	9.5±0.8	0.31±0.0
Bromus inermis				
cv. Manchar, suspension	0	N.T.	N.T.	N.T.
Hordeum vulgare				
cv. Heartland, suspension	0	N.T.	N.T.	N.T.
Triticum aestivum				
SWP9302 callus	72.7	>22	42.7±6.0	0.2±0.0
Star callus	99.2	>22	48.8±1.2	2.0±0.9
HY320 callus	78.5	>22	58.2±0.8	0.8±0.1
Zea mays				
Black Mexican sweet corn suspension	8.22	>22	N.T.	N.T.
Selaginella lepidophylla, leaves	133.0	>22	71.3±2.5	0.4±0.0

NT: Not tested.

inhibited 90% of this activity. Two suspension cultures of canola showed moderate trehalose degrading activity. In this case the disappearance of trehalose from the incubation mixture could not be attributed to trehalase as the amount of degradation was unaffected by validamycin A. The lability of activity (<5 hr) suggests that it may be due to free radical activity in the homogenate. Sunflower suspensions were found to contain a low level of activity. Validamycin A completely inhibited trehalose degradation in these homogenates.

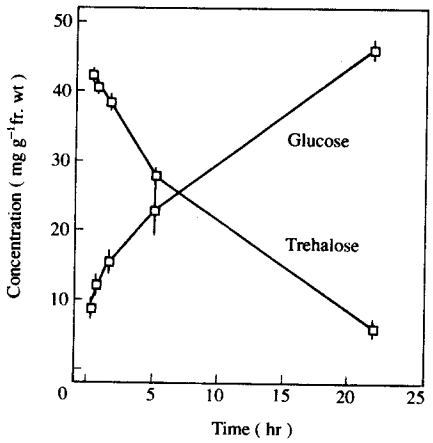

Fig. 2. Trehalose breakdown in HY320 spring wheat callus. Homogenized wheat callus (0.2 g) was suspended in 800 μl 0.2 M potassium phosphate buffer (pH 5.8) containing 7.33 mg trehalose, 3.7 mg arabitol (internal standard) and incubated at 22°. Aliquots (100 μl) were withdrawn at specified intervals and dried *in vacuo*. Trehalose and glucose were quantified by GC analysis after silylation with 400 μl TriSil Z.

The presence of trehalase activity in the (positive) plant species (other than *Selaginella*) could not be due to fungal or bacterial contamination because the test lines had been maintained in sterile culture for months or years. In addition, the assay itself was carried out under aseptic conditions. It is possible that the trehalase activity might arise from other disaccharases. However, the presence of validamycin A did not inhibit the degradation of naturally occurring sucrose in wheat, soybean, canola, jackpine or *Selaginella* (data not shown). Thus, it is unlikely that the breakdown of trehalose was due to residual activity residing in a 'sucrase' enzyme. It was interesting to note that the wheat callus lines had activities comparable to that of *Selaginella*. This activity seems too high to be fortuitous.

Our experiments do not indicate a physiological role for trehalase; however several suggestions were found in the literature. Trehalase may be used to metabolize trehalose produced by fungal infections. Shoot axes from freshly germinated embryos appear to be a reasonable system to test this hypothesis. These tissues are often exposed to fungi and might therefore be expected to have developed defensive mechanisms such as high trehalase activity. Our tests indicate that this is not the case. Trehalase activities diminished from 73 and 78 $\mu g\,min^{-1}\,g^{-1}$ fr. wt in SWP9302 and HY320 callus to 14 and 19 $\mu g\,min^{-1}\,g^{-1}$ fr. wt in axes. Assuming the activity is not inducible, this observation argues against an antifungal role for trehalase. A second possibility was suggested by Glasziou and Gaylor [9] who proposed that enzymes involved in trehalose synthesis and degradation might mediate hexose transport. Gussin and McCormack [10] further speculated that such transport may provide for specific transport of glucose into the germ cell. It has also been suggested that trehalose synthesis and degradation could provide an alternate route for the conversion of fructose to glucose [10]. This report did not directly address these questions; however naturally occurring trehalose was not detected in the plant materials in this study. Thus, if trehalose is formed as an intermediate in hexose transport or metabolism, its synthesis and degradation are very tightly coupled.

MATERIALS AND METHODS

Plant material. Callus was used from three spring wheat (*Triticum aestivum* L.) lines, SPW9302, Star, and HY320; black spruce (*Picea mariana* (Mill.)) B.S.P., white spruce (*Picea glauca* (Moench)) Voss, and jack pine (*Pinus banksiana* Lamb.). Cell suspension cultures were used for alfalfa (*Medicago sativa* L.), canola (*Brassica napus* L.), spring barley (*Hordeum vulgare* L.), black Mexican sweet corn (*Zea mavs* L.), bromegrass (*Bromus inermis* Leysser), soybean [*Glycine max* (L.) Merr.] and sunflower (*Helianthus annuus* L.). Fresh leaves were collected from greenhouse-grown resurrection plants [*Selaginella lepidophylla* (Hook. & Grev.) Spring, Carolina Biological Supply].

Callus or cells from suspension cultures were washed twice in deionized, distilled H_2O to remove superficial sucrose, frozen in liquid N_2, and then ground to a fine powder in a chilled mortar and pestle. For trehalase assay, 0.2 g fr. wt of the ground powder was suspended in 800 μl of 0.2 M K-Pi buffer, pH 5.8, containing 3.70 mg of arabitol (int. standard, Sigma, A-3506) and 7.33 mg trehalose (α-D-glucopyranosy-α-D-glucopyranoside, Pfanstiehl Laboratories T-104) in a sterile 1.5 ml centrifuge tube. The homogenate was incubated at 22°, and 100 μl aliquots taken after 15, 30, 90, 300 and 1320 min. The samples were vaccum-dried at 40° and silylated (400 μl Tri-Sil 'Z', Pierce) at 80° for 1 hr.

Quantitative analysis of trehalose breakdown. The silylated aliquots were analysed by GC on 7% cyanopropylphenyl silicone (0.15 μm coating, 15 m, 0.25 mm i.d. capillary column) with carrier gas He, 30 cm sec^{-1}; injector 250°, FID 250°; temp. program 150° for 2 min, then 3° min^{-1} to 270°, hold for 8 min at 270°. A 5 μl aliquot was injected and split 1:20 (see ref. [18] for details). Identifications were based on comparisons of R_ts with standards and GC-MS analysis.

Inhibition of trehalase activity. The trehalase inhibitor, validamycin A, was isolated by ion exchange chromatography [QAE-Sephadex A-25 (Pharmacia), bed vol. 10 ml, equilibration buffer 0.2 mM Na-Pi pH 7] from a 3% agricultural formulation, Solacol (Takeda Chem. Indust., Tokyo). Typically 1 ml of Solacol was loaded on the column and eluted with H_2O in 7 (1 ml) fractions. Each fraction was analysed gravimetrically for yield and by ^1H NMR for composition. The validamycins contain a vinyl proton (H-2', δ5.995, [15]) that resonates downfield from most other sugar and alkane protons. This provided a convenient way to check the purity of validamycin A preparations. An essentially pure preparation representing nearly 100% of the theoretical yield was obtained in fraction 4. Direct probe of this fraction using FABMS revealed the $[M]^+$ ion as 498 (validamycin A: $[M]^+$ 497).

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