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 trehalase in sunflower seeds [3] was questioned by Kandler and Hopf [1] who suggested that the reported trehalase came from fungal contamination. Previous reports of trehalase in beech [4] and cabbage [5] could not be verified by Gussin [6]. In spite of the apparent rarity of trehalase 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 trehalase can be broken down by free radical reactions, it would seem important to not only show trehalase 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]-aminol)-D-chiro-inositol, validamycin A), are known.

The validamycins (A and B) were discovered in Streptomycetes 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 (IC₅₀) 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 trehalase from porcine, rat, rabbit, baker's yeast, Mycobacterium and insect larvae (Spodoptera litura) [17]. The most active compo-

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Fig. 1. Inhibition of trehalase activity by validamycin A. Validamycin A was isolated from Solaco (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 arabinol and specified concentrations of validamycin A. Aliquots were removed at 0 and 1320 min and dried in vacuo. Trehalase determinations made by GC analysis after silylation with 400 μl TriSiZ.

Table 1. Trehalose degrading activities in cultured plant species

<table>
<thead>
<tr>
<th>Plant source</th>
<th>Activity (μg min⁻¹ g⁻¹ fr. wt)</th>
<th>Stability in homogenate (hr)</th>
<th>Trehalose degraded (mg per 22 hr) No inhibitor</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helianthus annuus</em> line SF111, cell suspension</td>
<td>15.2</td>
<td>&gt;44</td>
<td>16.8 ± 0.5</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td><em>Brassica napus</em> cv. Jet Neuf, Suspension 3% sucrose media</td>
<td>37.1</td>
<td>&lt;5</td>
<td>2.6 ± 0.26</td>
<td>3.68 ± 0.6</td>
</tr>
<tr>
<td><em>Glycine max</em> cv. Mandarin, suspension</td>
<td>24.4</td>
<td>&lt;5</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td><em>Medicago sativa</em> cv. Rangelander, suspension undifferentiated cells</td>
<td>0</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td><em>Picea glauca</em> callus</td>
<td>21.2</td>
<td>&gt;33</td>
<td>47.3 ± 1.1</td>
<td>3.2 ± 0.0</td>
</tr>
<tr>
<td><em>Pinus mariana</em> callus</td>
<td>0</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td><em>Pinus banksiana</em> callus</td>
<td>44.8</td>
<td>&gt;22</td>
<td>9.5 ± 0.8</td>
<td>0.31 ± 0.0</td>
</tr>
<tr>
<td><em>Bromus inermis</em> cv. Manchar, suspension</td>
<td>0</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em> cv. Heartland, suspension</td>
<td>0</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> SWP9302 callus</td>
<td>72.7</td>
<td>&gt;22</td>
<td>42.7 ± 6.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td><em>Zea mays</em> Black Mexican sweet corn suspension</td>
<td>8.22</td>
<td>&gt;22</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td><em>Selaginella lepidophylla</em>, leaves</td>
<td>133.0</td>
<td>&gt;22</td>
<td>71.3 ± 2.5</td>
<td>0.4 ± 0.0</td>
</tr>
</tbody>
</table>

NT: Not tested.

inhibited 90% of this activity. Two suspension cultures of canola showed moderate trehalase degrading activity. In this case the disappearance of trehalase 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 trehalase degradation in these homogenates.
Trehalase activity in plant tissue cultures

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 arabinol (internal standard) and incubated at 22°C. 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 disaccarases. However, the presence of validamycin A did not inhibit the degradation of naturally occurring sucrose in wheat, soybean, canola, jackpines 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 µg min⁻¹ g⁻¹ fr. wt. in SWP9302 and HY320 callus to 14 and 19 µg 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 trehalase 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 trehalase 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 mays 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 H₂O to remove superficial sucrose, frozen in liquid N₂, 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 arabinol (int. standard, Sigma, A-3506) and 7.33 mg trehalose (α-D-glucopyranosy-β-D-glucopyranoside, Pfannstiel Laboratories T-104) in a sterile 1.5 ml centrifuge tube. The homogenate was incubated at 22°C, and 100 µl aliquots taken after 15, 30, 90, 300 and 1320 min. The samples were vacuum-dried at 40°C and silylated (400 µl Tri-Sil 'Z', Pierce) at 80°C 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°C, FID 250°C; temp. program 150°C for 2 min, then 3°C min⁻¹ to 270°C, hold for 8 min at 270°C. A 5 µl aliquot was injected and split 1:20 (see ref. [18] for details). Identities were based on comparisons of Rₘ 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. Ind., Tokyo). Typically 1 ml of Solacol was loaded on the column and eluted with H₂O 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, δ 5.995, [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⁺]¹ ion as 498 (validamycin A: [M⁺]² 497).

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REFERENCES