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SPECIFICITY OF SUGAR CANE TREHALASE

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Key Word Index—*Saccharum officinarum*; Gramineae; sugar cane; trehalase; invertase; growth.

Abstract—An extract containing trehalase and invertase was prepared from apical internodes of sugar cane. The extract hydrolysed three glucosides: maltose, trehalose and sucrose. By reprecipitation with ammonium sulphate, maltase and trehalase activities appear to be due to different enzymes. As was also shown by differential inhibition and activation and by studies on the behaviour of both enzymes during growth, invertase and trehalase activities are attributed to different enzymes whose activities do not overlap. Invertase-free preparations confirm these results. Sucrose is a simple competitive inhibitor of sugar cane trehalase, excluding a regulatory role for this sugar. Sucrose was found at inhibitory levels in the first four apical internodes. A close correlation between sugar cane growth and invertase and trehalase levels was found in the apical internodes. Invertase has the greatest activity during growing, and trehalase reaches a maximum at maturity, prior to the flowering process. The high levels of trehalase in the flower suggest that the enzyme is involved in flowering or in related processes linked to seed formation.

INTRODUCTION

Trehalase activities were reported for alfalfa [1], sugar cane [2, 3], *Lycopersicon pimpinellifolium*, *Hemerocallis minor*, *Galtonia candicans*, *Camellia japonica*, *Lathyrus odoratus* [4], *Lilium longiflorum* [5], *Lycopersicon esculentum* [6], *Selaginella martensii* [7] and a number of yeast, fungi and bacteria. Thus, trehalase appears throughout the vegetable kingdom. However, little is known about this enzyme and nothing about its physiological function in higher plants. The occurrence of trehalose in higher plants is a controversial matter [8], but the presence of the enzymes involved in trehalose biosynthesis in pollen of *Lilium* [5] suggests that trehalose is formed in plants. Trehalase is basically an α-glucosidase and may also attack sucrose, the substrate of invertase, a key enzyme of the metabolism of sucrose in sugar cane [9] and probably an important enzyme in other plants [10]. Higher plant trehalases have not been extensively purified, yet most of the studies on these enzymes and invertases were carried out on crude, or exceptionally, partially purified extracts containing both enzymes. Thus, all available determinations of plant invertases, including our work [11, 12], might include trehalase activity. The available studies on sugar cane trehalase specificity are referred to the glycolytic activities of the extract instead of the trehalase activity [2, 3], and the specificity for sucrose was not established. When the specificity of pollen trehalase from *C. japonica* has been studied by thermal inactivation, 97% of the invertase activity is lost and an increment of 6% of trehalase was found [4]. Consequently *ca* 3% of the invertase activity may be attributed to the trehalase. The α-glucosidase activity from *Lycopersicon esculentum* may account for *ca* 3% of the sucrose hydrolysing ability of the extract [6]. Thus, current knowledge of higher plant trehalase suggests that the enzyme is a relatively unspecific α-glucosidase with a more or less preferential attack on trehalose.

In this paper the specificity of trehalase is examined from three points of view. Firstly, the finding and use of differential effectors for invertase and trehalase: secondly, the separation of both enzymes and thirdly, a study of the physiological variations in the levels of these enzymes during the growth of sugar cane.

RESULTS AND DISCUSSION

The activity of the extract towards sucrose and other sugars is shown in Table 1. Maltose hydrolysis may be ascribed to an α-glucosidase. The ratio between trehalase and maltase activities of some extracts was *ca* 2.8 but after reprecipitation with ammonium sulphate the ratio shifted to 15.3. This relation appears to change during the year. These results showed that trehalase and maltase activities are due to different enzymes, and confirm our previous report about the occurrence of a specific maltase in sugar cane [11]. Ficoll (Pharmacia), a synthetic polymer of sucrose, and

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Table 1. Activities of the sugar cane extract

Substrate	Concentration (mM)	U/g fresh tissue	Relative activity
Sucrose	60	2.40	100
Trehalose	60	1.014	42
Maltose	25	0.026	1.08
Turanose	25	0	0
Methyl-α-D-glucoside	60	0	0
Ficoll	0.05	0	0

Assays were performed at pH 6 with an extract prepared in April. Concentrations of Ficoll were fixed because of its low solubility. Relative activities are given as percentage of sucrose hydrolysis.

α-methyl-D-glucopyranoside were not attacked by these enzymes.

The specificity of trehalase towards sucrose was first studied by thermal inactivation. The effect of heating on the enzymes is shown in Fig. 1. About 95.3% of invertase was inactivated after 30 min at 47°. According to these results *ca* 4.7% of sucrose hydrolysis may be attributable to trehalase. The data are in agreement with those reported for *C. japonica* [4] and *L. esculentum* [6].

As the aldonolactones are known to be competitive inhibitors for the corresponding glycosidases [13], δ-D-gluconolactone was used as a possible inhibitor of trehalase. δ-Gluconolactone was found to be an inhibitor of trehalase and an activator for invertase (Fig. 2). The complex curve for invertase does not allow conclusions about the specificity of trehalase but these results are new for both enzymes.

A new set of experiments including the comparative study of the inhibition of trehalase and invertase by urea was carried out. In 3 M urea, trehalase activity was inhibited *ca* 20%, and invertase activity was completely lost. Although there is not a detectable attack on sucrose by trehalase, an invertase inhibitor with less effect on trehalase is desirable. Ca^{2+} was a total inhibitor of invertase (Fig. 3), and this inhibition follows a simple exponential law. On the contrary, trehalase is activated up to 15×10^{-2} M Ca^{2+}. Higher concentrations of Ca^{2+} produce a gradual enzyme inhibition. As the inhibition curve of invertase does not show any interference due to trehalase, the conclusion was that sucrose is not a substrate for the trehalase. The effects of other chemicals tested for the

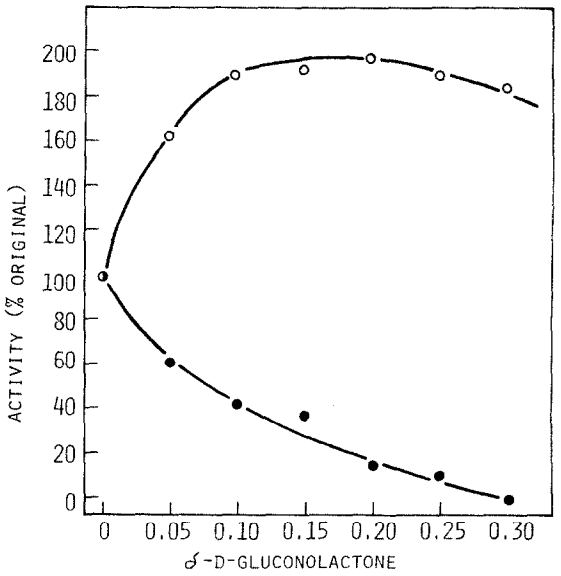

Fig. 2. Effect of increasing concentrations of δ-D-gluconolactone on trehalase and invertase activities. Trehalase (●—●), invertase (○—○).

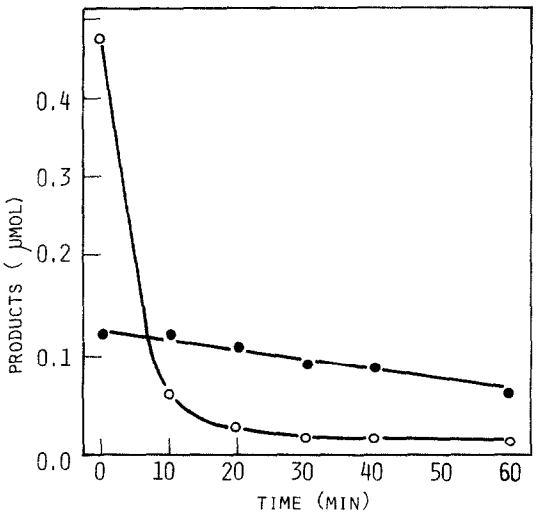

Fig. 1. Thermal stability of the extract. Standard mixtures of 0.09 ml, without substrate, were pre-incubated at 47°, pH 6. At intervals, the tubes were removed and assayed under standard conditions. Trehalase (●—●), invertase (○—○).

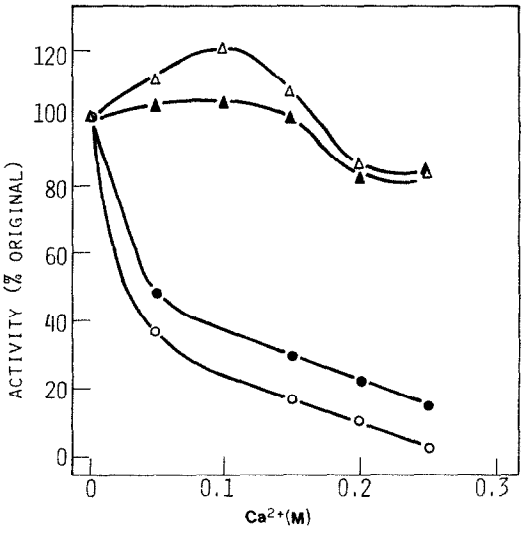

Fig. 3. Effect of increasing concentrations of Ca^{2+} on the extract activities. Invertase and trehalase assays were performed at pH 5.5 and 6. Trehalase pH 5.5 (△—△), trehalase pH 6 (▲—▲), invertase pH 5.5 (●—●), invertase pH 6 (○—○).

Table 2. Effect of some chemicals on the enzyme activities measured in the conditions described in Experimental

Effector	Concentration (mM)	Invertase % Inhibition	Invertase % Activation	Trehalase % Inhibition	Trehalase % Activation
$Mo_7O_{24}^{6-}$	5.5	31	0	31.4	0
Mg^{2+}	50	17	0	0	0
Sr^{2+}	100	100	0	0	8
Ba^{2+}	100	100	0	0	24
Co^{2+}	100	100	0	6	0
Zn^{2+}	10	67	0	45	0
Hg^{2+}	10	100	0	100	0
Mn^{2+}	10	54	0	45	0
NH_4^+	10	25	0	15	0
Cu^{2+}	10	81	0	43	0
BO^{3-}	100	60	0	0	0
NaF	100	0	85	0	0
Tris	10	32	0	31	0
EDTA	11	20	0	6	0
Inositol	10	17	0	0	0
Mannitol	10	36	0	18	0
Sorbitol	10	0	0	0	0
Fructose	10	25	0	0	0
Sucrose	60	—	—	22.4	0

first time as effectors of these enzymes are shown in Table 2. The total inhibition of invertase by Sr^{2+} and Ba^{2+}, concomitant with trehalase activation, suggests that sucrose is not hydrolysed by trehalase. In Table 2 sucrose is seen to be an inhibitor of trehalase. Similar inhibitions by sucrose have been reported for a number of insect [14–16] and mammalian [17] trehalases. The results were obtained using Co^{2+} as an inhibitor for the invertase, but they were confirmed with an invertase-free extract. The kinetics of the trehalase inhibition by sucrose correspond to a simple competitive case. The replot of K_m (apparent) versus sucrose concentration was linear, and K_i extrapolated from the replot was 1.67×10^{-2} M. These results pose a new problem about the physiological role of trehalase. If the enzyme is functional then it must be separated from sucrose, unless sucrose works as a regulator. In connection with this point, Glasziou [2] and Alexander [3] showed that higher levels of trehalase occur in growing tissues, and these tissues have the lowest levels of sucrose. However, the fact that trehalase appears not to have specific sites for sucrose and that sucrose is at inhibitory levels even in growing tissues (Table 3) indicates that sucrose is not a physiological effector for trehalase. Consequently, trehalase and sucrose must be in different compartments. Trehalase may be expected to be in the cytoplasm because sucrose is in high concentrations in the intercellular spaces and vacuoles [9, 18]. The competitive inhibition of trehalase by sucrose suggests that other compounds containing end glucose residues may be inhibitors of the enzyme. In Table 4 is shown the inhibition of trehalase by several α- and β-glucosides, and by free glucose which was previously reported as a non-inhibitory sugar [3].

The behaviour of invertase and trehalase during the year, the variations of total rainfall, mean solar radiation, and maximal and minimal temperatures are shown in Fig. 4. The sharp differences between the seasonal patterns of both enzymes suggest that their activities are not mutually related. Very different regulatory mechanisms must operate in each case, as supported by correlation studies of invertase, trehalase and growth (Table 5). During the period of maximum growth there are high invertase levels and a low stable activity of trehalase. However, invertase activity declines in the ripening period and trehalase reaches a maximum. Other environmental factors such as total

Table 3. Sugar concentrations in the apical internodes of sugar cane

Internode	Glucose (mM)	Fructose (mM)	Sucrose (mM)
1, 2 and 3	7.0±0.8	20.1±4.8	63.7±6.9
4 and 5	10.8±5.2	33.4±10.7	110.3±11.8

Table 4. *Inhibition of trehalase by several natural glycosides and related compounds*

Inhibitor	Trehalose (mM)	Inhibition (%)
Glucose, 20 mM	60	5
4 mM	4	14
20 mM	6	25
40 mM	4	32
Glucosamine, 5 mM	60	32
Sucrose, 60 mM	60	22
Methyl-α-D-glucoside, 60 mM	60	9
60 mM	30	18
Amygdalin, 60 mM	60	70
Esculin, 5 mM	60	25

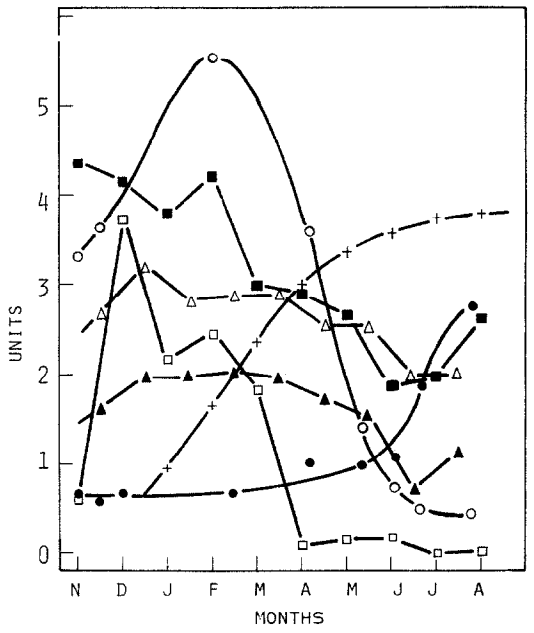

Fig. 4. Seasonal patterns of the levels of invertase, U/g fresh tissues (units × 1) ○—○; trehalase U/g fresh tissues (units × 1) ●—●; sugar cane growth, cm (units × 0.02) +—+; mean solar radiation, cal/cm²/day (units × 0.01) ■—■; total rainfall, mm (units × 0.01) □—□; maximal temperature (monthly means), degrees (units × 0.1) △—△; and minimal temperature (monthly means), degrees (units × 0.1) ▲—▲.

rainfall, mean solar radiation and maximal and minimal temperatures also give good correlations with both enzymes (Table 5). These correlations may correspond to factors interrelated through sugar cane growth. Flowering of sugar cane began in August and the levels of trehalase and invertase in flower extracts were *ca* 4.1 and 8.07 U/g of fresh tissue, respectively. There is an increase of *ca* 7-fold of the trehalase content in November, and 1.4-fold the content found in July. The fact that trehalase activity increased prior to the expansion of the flower, and the higher levels found in the flower suggest that the enzyme is involved in the flowering process. The occurrence of trehalase in the pollen of *L. longiflorum* [5] supports this interpretation. Thus, trehalase may be physiologically involved in flowering or in related processes, such as fertilization or seed formation. However, the primary problem in the interpretation of the physiological role of trehalase is the apparent absence of trehalose in higher plants, but the sugar, if present, must be in very low amounts. Previous reports for the K_m values of sugar cane trehalase were 6.3×10^{-4} [3] and 1.0×10^{-4} M [2]. Determinations of this constant with the invertase-free preparations gave a value of $(6.15 \pm 0.33) \times 10^{-4}$ M. If trehalase, a non-regulatory enzyme, is functional, trehalose concentration may be expected to be in the order of the K_m, and consequently it is not surprising that trehalose is masked by the higher concentrations of sucrose or other sugars in the plant tissue. Detection of plant trehalose must require a more sophisticated method than simple paper or thin layer chromatography.

EXPERIMENTAL

Plant material. Sugar cane, CV Tuc 68-19, cultivated in the field at the Estación Experimental Agrícola de Tucumán, was used throughout this work.

Enzyme preparation. The first 4 internodes of the apex of sugar cane (*ca* 7 stalks, 400 g) were cut into small pieces and homogenized in 200 ml 10 mM Na-Pi buffer pH 7.2, 1 mM mercaptoethanol, 5 μM $MnSO_4$, 0.5% Na_2SO_3. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 15 000 rpm for 10 min. Solid $(NH_4)_2SO_4$ was

Table 5. Correlation between enzyme levels, growth and environmental factors

Data	Regression equation	r	Sy·x
Invertase			
Growth	$y = 0.072 + 0.124x - +0.0006637x^2$	0.978	0.420
Maximal temperature	$y = -8.793 + 0.456x$	0.885	0.878
Minimal temperature	$y = 3.026 - 0.614x + 0.0358x^2$	0.953	0.570
Rainfall	$y = 1.147 + 0.035x - +0.00007829x^2$	0.884	0.877
Mean solar radiation	$y = -7.387 + 0.0517x - +0.00005477x^2$	0.835	1.033
Data	**Regression equation**	r	Sy·x
Trehalase			
Growth	$y = 2.924(x - 190)^{-0.324}$	0.980	0.126
Maximal temperature	$y = 7.372 - 15.790y + 3.569y^2$	0.948	0.195
Minimal temperature	$y = 3.002 - 0.122x$	−0.880	0.291
Rainfall	$y = 1.545x^{-0.163}$	0.971	0.218
Mean solar radiation	$x' = x \cos 270° + y \sin 270°$ $y' = -x \sin 270° + y \cos 270°$ $y = 412.094/x'^2 + 654.747/x' + 440.815$	0.988	0.095

r = the regression coefficient, calculated according to Spiegel [22].
Sy·x = sample standard deviation from regression.

added to the supernatant and a fraction which precipitated between 25 and 50% satn was collected by centrifugation. The pellet was resuspended in 1 ml 10 mM Na-Pi buffer pH 7.2, 1 mM mercaptoethanol and was dialysed against the same buffer. The pH of the dialysate was adjusted to pH 4 with N HCl and was immediately centrifuged. The sediment was discarded. The supernatant was adjusted to pH 6 with N NaOH and was applied to a column of Bio-Gel P6 (1.9× 20 cm) equilibrated with 10 mM Na-Pi buffer pH 6 and 1 mM mercaptoethanol. Trehalase (35–123 U/ml) and 29–140 U/ml of invertase were obtained in the course of the year. The same procedure was followed with flowers.

Invertase-free preparations. The dialysed enzyme was adjusted to pH 3.5 with N HCl and was then centrifuged. The sediment was discarded and the supernatant allowed to stand for 14 hr. The supernatant was adjusted to pH 6 with N NaOH and percolated through Bio-Gel P6 in the conditions described for *Enzyme preparation.* The extract contained *ca* 1 mg/ml of protein and between 69 and 355 U/ml of trehalase.

Assay of trehalase. The reaction mixture consisted of 25 μl 0.2 M NaOAc buffer pH 6, 10 μl 0.6 M trehalose, 15 μl enzyme prepn and H_2O in a final vol. of 100 μl. Reaction time was 30 min (unless otherwise stated) at 37°. The reactions were stopped by the alkaline-copper reagent of ref. [19] and the reducing power was determined by the arsenomolybdate method [20].

Assay of invertase. The reaction medium (100 μl) containing 25 μl 0.2 M NaOAc buffer pH 6, 10 μl 0.6 M sucrose, 10 μl of enzyme and 55 μl of H_2O was incubated at 37° for 15 min. Determinations of reducing power were made as stated in *Assay of trehalase.*

Enzyme units. To avoid fractionary numbers the enzyme unit (U) was defined as the amount of enzyme which released 0.01 μmol of product in 1 min in the conditions established in *Enzyme assay.*

Tissue sugars. The tissues were weighed, submerged in boiling H_2O for 2 min, and then homogenized in a chilled mortar and pestle with washed sea sand. The mixture was filtered through gauze. H_2O extraction was repeated × 3 and the extracts pooled. The combined extracts were centrifuged at 15 000 rpm for 10 min and kept at −10°. The glucose was measured by using the glucose oxidase method. Fructose and sucrose were measured by the method of ref. [21]. H_2O content was determined by heating the tissues at 80° to constant w.

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