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NICOTIANA GLAUCA INVERTASE: CHARACTERIZATION AND EFFECTS OF ENDOGENOUS ALKALOIDS

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Key Word Index—*Nicotiana glauca*; Solanaceae; invertase; β-D-fructofuranosidase fructohydrolase; characterization; alkaloids.

Abstract—An acid-soluble invertase from *Nicotiana glauca* has been studied for the first time. The plant was selected because it is known that the genus *Nicotiana* has vacuolar alkaloids; the same localization of the acid-soluble invertases in higher plants. The enzyme was partially purified and the optimum pH, Km, Mr, activation energy, fructose effect, activation by protein, sugar specificity, lectin activation were determined. The effects on invertase activity of the alkaloids, nicotine, anabasine and nornicotine present in the plant were also studied. These alkaloids produced a cumulative inhibition of the invertase activity. Other substances like TRIS hydrochloride, pyridoxal hydrochloride and pyridoxine hydrochloride also produced inhibition. All these substances have in common the presence of a quaternary nitrogen atom in their structure. The individual effect of these compounds on the invertase activity appears without meaning as regulators of the enzyme, but their general occurrence may have a role in the functional behaviour of the invertase.

INTRODUCTION

Sucrose is an important plant metabolite and is the principal form of transport of the photosynthate in higher plants. Consequently, the enzymes which form or transform sucrose have been the subject of much research. Among these enzymes, sucrose phosphate synthases (EC 2.4.1.14), sucrose synthases (EC 2.4.1.13), and invertases (EC 3.2.1.26) have been the subject of many studies. Invertases have been purified from different sources. Biochemical and molecular biology techniques have been applied to the study of their kinetic and molecular properties, production, localization and regulation [1–5]. Invertases may be classified into three categories: (1) those invertases whose activity is regulated by fructose, (2) those invertases which show a simple competitive inhibition by this sugar and whose inhibition by the reaction products is suppressed by proteins, and (3) those invertases whose classical inhibition by the reaction product is not suppressed by proteins [6]. However, the behaviour of invertases from plants containing significant amounts of secondary compounds has not been studied. In a recent paper, we reported that alkaloids could modify the activity of several plant invertases *in vitro* [7]. These results raise the question as to whether or not these compounds can interfere with the physiological behaviour of the invertases in plants whose vacuoles contain both alkaloids and invertase. Most studies have been carried out on edible plants [8–10], and the present problem has never been studied.

Nicotiana glauca is a wild plant occasionally used as an ornamental and which has external applications as a maturative and an antiinflammatory in the folk medicine in the northwest of Argentine. The genus *Nicotiana* has been reported as containing alkaloids in the vacuole [11], a known localization of the soluble acid invertase [12–14]. This paper reports the results of the first study on the leaf invertase from *N. glauca*, a plant containing the alkaloids anabasine, nicotine and nornicotine [15–17]. The enzyme was partially purified and characterized and the effects of the plant alkaloids, proteins, reaction products, etc. were examined.

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RESULTS

The acid-soluble invertase from *N. glauca* leaves was purified by saline fractionation, gel filtration

Table 1. Purification of the acid-soluble invertase for *N. glauca* leaves

Step	Total protein (mg)	Specific activity (units/mg prot.)	Purity	Yield (%)
Homogenate	1599	0.44	1	100.0
Supernat. of centrifugation	1149	0.58	1.3	93.8
$(NH_4)_2SO_4$ (30–50% sat.)	99	1.53	3.4	21.4
Sephadex G-150	23	5.38	12.2	17.6
ConA Sepharose	1	107.00	240.00	15.0

through Sephadex G-150 and affinity chromatography on ConA Sepharose 6B (Table 1). The invertase preparation used throughout this work showed two protein bands on native analytical PAGE and only one activity band. The binding of the soluble acid invertase of ConA-Sepharose and the elution pattern with α-methylmannoside suggest that this enzyme is a glycoprotein. Attempts at further purification led to inactivation of the invertase. The invertase preparation contained 0.012 mg/ml of protein and about 1.3 units/ml at this purification step. The enzyme preparation was unstable and lost 70% of its activity in a week.

Properties

The Mr of the invertase was found to be 89 100 by gel filtration [18]. The activation energy was calculated from an Arrhenius plot to be about 8400 cal/mol and the optimum pH was about pH 3.8. Thus, sucrose is the substrate of choice for invertase. Other glycosides or oligosaccharides such as α- and β-methylglucoside, melibiose, melezitose, maltose, turanose, cellobiose, lactose, α- and β-methylgalactoside, β-phenylgalactoside, o-nitrophenylgalactoside, inulin or levan were not hydrolyzed by this enzyme. Fructose, one of the reaction products, was shown to be an inhibitor of the invertase from *N. glauca* (Fig. 1). According to the plot and replot of slopes against fructose concentration, fructose is a classical competitive inhibitor of this invertase. The enzyme is activated by BSA and by ovalbumin (not shown). The activation reaches up to 30% of the original activity and suppresses the inhibition by fructose. Lectins specific for α-mannose or α-glucose also activate the invertase. However, this is not true for lectins specific for N-acetylglucosamine oligomers, N-acetylgalactosamine or galactose (Table 2).

Effect of the plant alkaloids

Nicotine, nornicotine and anabasine were found to be inhibitors of the enzyme (Fig. 2). When the invertase is exposed to the simultaneous action of the three alkaloids an increase of the individual inhibitory effect

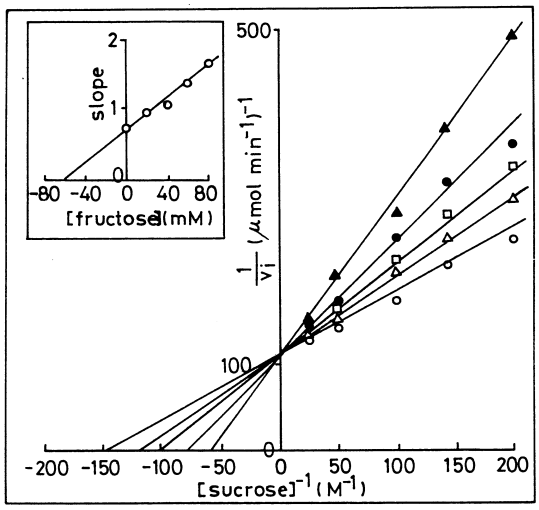

Fig. 1. Lineweaver-Burk plot and replot of the inhibition of *N. glauca* acid-soluble invertase by 0 mM (○), 20 mM (△), 40 mM (□), 60 mM (●) and 80 Mm (▲) fructose.

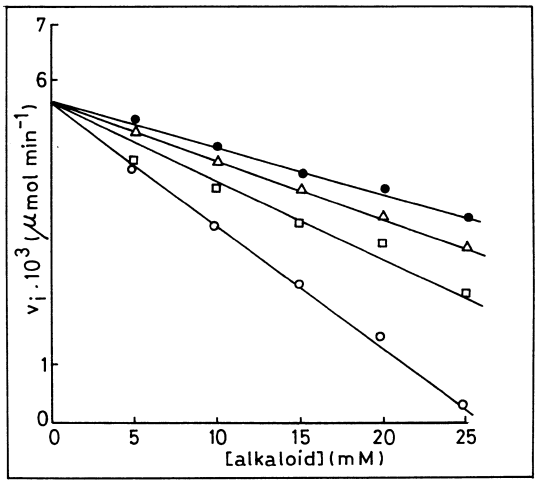

Fig. 2. Inhibition of *N. glauca* invertase activity by the endogenous alkaloids nornicotine (△), anabasine (●), nicotine (□) and nornicotine, anabasine and nicotine together (○).

Table 2. Effects of lectins on the invertase activity of *N. glauca*

Lectin	Specificity	Reference*	Activation (%)
Cyphomandra betacea	GlcNAc (β-1,4GlcNAc)$_{1-4}$	[19]	—
Solanum tuberosum	GlcNAc (β-1,4GlcNAc)$_{1-4}$	[20]	—
Ricinus communis	Gal; GalNAc	[21]	—
Lens culinaris	α-Man; α-Glc	[22]	26
ConA	α-Man; α-Glc	[23]	25

*References related to lectin specificity.

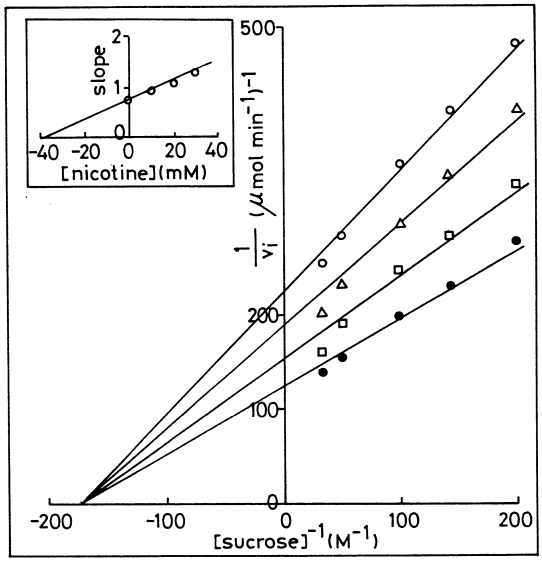

Fig. 3. Lineweaver-Burk plot and replot of the inhibition of *N. glauca* invertase by 0 mM (●), 10 mM (□), 20 mM (△) and 30 mM (○) nicotine.

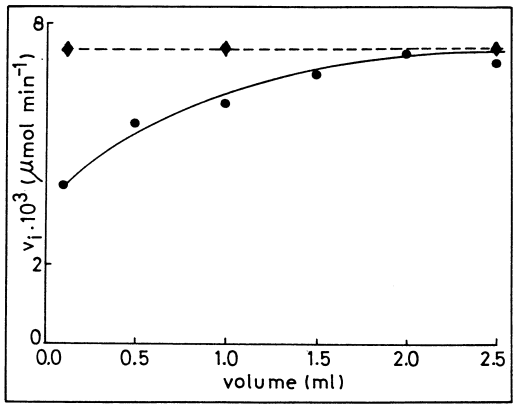

Fig. 4. Recovery of the acid-soluble invertase activity by dilution of the anabasine concentration in the reaction mixture (●); control of enzymic activity without alkaloid (◆).

is obtained (Fig. 2). Nicotine and anabasine were non-competitive inhibitors with a Ki of 37 (Fig. 3) and 56 mM, respectively. The invertase-inhibitor complex was dissociable as shown by the increase of enzyme activity with increasing reaction volume (Fig. 4).

Table 3 shows the effect of several products on invertase activity. Pyridoxal hydrochloride, pyridoxine hydrochloride, TRIS hydrochloride, MES monohydrate and urea produced inhibitory effects on invertase activity. These products, excepting urea, have a quaternary nitrogen atom in the molecule and a connection probably exists between this structure and the inhibition of invertase activity. Mixtures of nicotine plus pyridoxal hydrochloride, nicotine plus urea and nicotine plus TRIS hydrochloride exhibited roughly quantitatively additive effects.

DISCUSSION

The acid invertase from *N. glauca* is an unstable enzyme losing its activity within a week after preparation. The enzyme can hydrolyze oligosaccharides, such as sucrose, raffinose and stachyose, having in common a terminal non blocked fructose moiety. Other substrates with a terminal fructose moiety such as levan or inulin were not hydrolyzed by the enzyme. Sucrose has the lowest Km, and is, therefore, the preferred substrate of the enzyme. Melezitose, another sucrose derived oligosaccharide, with the fructose moiety blocked by glucose, was not hydrolyzed by this invertase. The enzyme specificity and the competitive kinetics of fructose inhibition show that the enzyme is a β-fructofuranosidase. Considering the kinetics of fructose inhibition and the protein effect on the inhibition by fructose, this invertase belongs to the second invertase category [6]. In common with most higher plant invertases, this invertase is activated by proteins and lectins.

Alkaloids are effectors of higher plant invertases [7]. The presence of alkaloids in the vacuoles of the genus *Nicotiana* has been reported [11] and higher plant invertases have the same localization [12–14]. Our results show that the alkaloids nicotine, nornicotine and anabasine are inhibitors of the invertase activity of *N. glauca* and that their individual inhibitory effects are cumulative. Other products, such as TRIS hydrochloride, also produce inhibition. The

Table 3. Effects of endogenous alkaloids and of some chemicals on the activity of the acid-soluble invertase from *N. glauca*

Effector	Concentration (mM)	Inhibition (%)
$(NH_4)_6Mo_7O_{24}$	5	53
Nicotine	10	18
Pyridoxine hydrochloride	10	23
Pyridoxal hydrochloride	10	41
TRIS hydrochloride	10	18
MES monohydrate	10	23
Urea	10	22
Nicotine + pyridoxal hydrochloride	10 + 10	62
Nicotine + urea	10 + 10	35
Nicotine + TRIS hydrochloride	10 + 10	25

effect may be associated to the presence, at least, of a quaternary ammonium group in the molecule.

EXPERIMENTAL

Plant material

Young leaves of *Nicotiana glauca* were used. This plant is cultivated as ornamental at the Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentine.

Chemicals

All chemicals used were of analytical grade.

Enzyme preparation

N. glauca leaves (200 g) were cut into small pieces and homogenized in 200 ml 50 mM NaPi buffer, pH 7, containing 1 mM 2-mercaptoethanol. The homogenate was filtered through two layers of gauze, and centrifuged at 27 100 g for 15 min. The supernatant was 30% saturated with solid $(NH_4)_2SO_4$, then centrifuged at 27 000 g for 15 min and the precipitate discarded. The supernatant was saturated up to 55% with solid $(NH_4)_2SO_4$, centrifuged and the supernatant discarded. The pellet was resuspended in 2 ml 10 mM NaOAc buffer, pH 5.2, containing 1 mM 2-mercaptoethanol (buffer A) and was dialyzed against the same buffer, with two changes of buffer, for 3 h. The dialyzed extract was filtered through a Sephadex G-150 column (2.5×40 cm), equilibrated and eluted with buffer A. Fractions with invertase activity were pooled. Aliquots of 2 ml were further purified by affinity chromatography on a ConA-Sepharose column (1.8×0.6 cm), equilibrated with buffer A. The column was washed with buffer A and then the invertase was eluted with 0.1 M α-methylmannoside in buffer A. Fractions (2 ml) with invertase activity were pooled, exhaustively dialyzed against buffer A, and kept at $-20°$ until use.

Invertase assay

The incubation mixture consisted of 40 μl 0.2 M NaOAc buffer, pH 3.8, 5 μl of the invertase preparation, 10 μl 0.6 M sucrose, and distilled water in a final vol of 0.1 ml. The mixture was incubated at 37° for the selected time and the reactions were stopped with the alkaline reagent of Somogyi [24]. The increase of reducing sugars was determined by the method of Nelson [25].

Molecular weight distribution

Mr was determined by the method of Andrews [18] using a Sigma kit (MW-GF-200) and a 2.5×40 cm column of Sephadex G-150 equilibrated and eluted with buffer A.

Effect of alkaloids

Sols (0.1 M) of anabasine, nicotine and nornicotine were prepared in 0.1 M NaOAc buffer, pH 3.8. The pH of the sols was controlled and adjusted to 3.8 when necessary. The reaction mixture consisted to 40 μl 0.2 M NaOAc buffer, pH 3.8, 10 μl 0.05 to 0.3 M sucrose, 10 to 30 μl of alkaloid soln and distilled water in a final vol of 100 μl. Reactions were run at 37° and were stopped as described in the invertase assay. Using alkaloid sols adjusted at pH 3.8, the pH of the reaction mixtures remained constant during the incubation.

Effect of dilution

The invertase-alkaloid complexes were diluted by increasing the vol of the reaction mixtures (0.1–2.5 ml) with buffered sucrose at constant pH and substrate concentration. Controls of enzyme activity without alkaloid were made at different vols.

Enzyme units

One enzyme unit was defined as the amount of the enzyme able to produce 1 μmol of product/min at 37° and at pH 3.8.

Protein determinations

Proteins were determined according to the Lowry method [26] using BSA as standard and by absorption at 280 nm in the column fractions.

Protein purity

Vertical gel slab electrophoresis of proteins was performed by standard techniques to determine purity. The run was made at 70 volts for 2 h, and at 90 volts for 1 h. Proteins and invertase activity were localized on the gel by $AgNO_3$ [27] and by 2,3,5-triphenyltetrazolium chloride methods [28], respectively.

Effect of the reaction products

The reaction mixtures contained 10 μl 0.05 to 0.4 M sucrose, 20 μl enzyme preparation, 40 μl 0.2 M NaOAc buffer, pH 3.8, 10 μl 0.2 to 0.8 M fructose, and water to 100 μl. Incubations were performed at 37° for 30 min. Glucose was determined by the glucose oxidase method [29].

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