IAA-GLUCOPYRANOSIDE STIMULATION OF CORN COLEOPTILES ELONGATION

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ABSTRACT

It has been previously suggested that 1-O-IAGluc growth stimulation occurs as the effect of its hydrolysis into a free IAA. In present experiments castanospermine, a known β -glucosidase inhibitor, was included. 1-O-IAGluc in the presence of castanospermine stimulated growth of corn coleoptiles segments even stronger than free IAA. So, it seems that 1-O-IAGluc itself, is responsible for the observed stimulation of corn coleoptile segments elongation.

KEY WORDS: Bound auxins, IAA-glucopyranoside, corn coleoptiles, castanospermine.

INTRODUCTION

The elongation of oat coleoptile segments, under the influence of indole-3-acetic acid (IAA) is a typical effect of the reaction of auxin on plants. Cell elongation is also stimulated, to a significantly lesser degree, by the conjugates of IAA, the ester and amide linkage of the hormone with other chemical particles. When the activity is higher, as for example with a methyl ester of IAA for growth promotion, or IAA amino acids for tissue culture growth (Hangarter and Good, 1981), it can usually be explained on the basis of rapidity of permeation or resistance to oxidation (Cohen and Bandurski, 1978). In the early 1960's, Zenk identified, in *Colchicum neapolitanum* leaves treated with an excess of auxin, a new conjugate of IAA, 1-O-indole-3-acetyl- β -D-glucopyranoside (1-O-IAGluc), possessing a similarly strong ability to stimulate growth in oat coleoptile segments as did IAA (Zenk, 1961; Keglevic and Pokorny, 1969). At first, it was assumed to be the product of the detoxification of cells treated with an excess of the hormone, yet with time it was successfully identified as a naturally occurring compound in other plants (Klamt, 1961; Keglevic and Pokorny, 1969; Cohen and Bandurski, 1982).

1-O-IAGluc is an acyl alkyl acetal and is chemically distinct from the IAA esters of glucose. The bond energy of hydrolysis of this acyl alkyl acetal is not readily determined, but on the basis of the equilibrium constant of the reaction of 1-O-IAGluc synthesis (Leznicki and Bandurski, 1988), the free energy of this compound hydrolysis is many thousands of calories greater than that of an ester bond. It has been shown also that 1-O-IAGluc is an obligatory intermediate in the formation of IAA inositol esters in the endosperm of *Zea mays* kernels (Kopcewicz et al., 1974; Michalczyk and Bandurski, 1980; Kęsy and Bandurski, 1990).

Since, in earlier works it has been suggested that 1-O-IAGluc growth stimulation occurs exclusively in effect to its hy-

drolysis into a free acid (Zenk, 1961; Keglevic and Pokorny, 1969), in present paper it has been decided to verify this hypothesis. Therefore, the effect of the indole-3-acetyl- β -D-glucopyranoside on corn coleoptile segments growth, in the presence of castanospermine, a known β -glucosidase inhibitor, was tested.

MATERIALS AND METHODS

Reagents

Polyethylene glycol 6000, Tris, PIPES, 2-Mercaptoethanol, PMSF, Indole-3-acetic acid (IAA) (Plant cell tissue tested), castanospermine and DEAE-Sephacel was from Sigma; Sephacryl S-200 from Pharmacia; p-Nitrophenyl glucopyranoside (NPG) from Fluka. 1-O-IAGluc was obtained by chemical synthesis, using the Szmidszt-Jaworska et. al. (1996) method.

Plant Material

Four-day-old corn (*Zea mays* L. var. AMA F-1) seedlings and oat (*Avena sativa* L. var. Comes) seedlings, grown in the dark at 26°C, were used. The corn kernels were soaked for 24 hours in distilled water at a temperature of 26°C and then were sown in mixed perlite and sand (2 : 1). The oat caryopses were soaked for 3 hours and planted in the same conditions.

The isolation of enzymes exhibiting β -glucoside activity

The plant material was homogenized in 50 mM Tris-Cl buffer, pH 8.0, 10 mM 2-mercaptoethanol and 1 mM PMSF, using 100 ml of buffer per 300 g of tissue. The homogenate was filtered through cheese cloth, the pH adjusted to 7.8 using solid Tris, and then centrifuged for 30 minutes at 15,000 g. The obtained supernatant was diluted twofold with distilled water and applied to a DEAE-Sephacel column (3.5 x 20 cm). After rinsing the column with 500 ml of 25 mM

Tris-Cl buffer, pH 7.8, containing 1 mM PMSF, the proteins were eluted with the same solution, containing an addition of NaCl, with a linear gradient in salt concentration from 0 to 1.5 M.

The quantification of the hydrolytic enzymes

β -Glucosidase.

The activity of β -glucosidase was measured spectrophotometrically as an increase in extinction at a wavelength of 410 nm, caused by an increase in p-nitrophenol concentration, derived from the hydrolysis of p-nitrophenol glucoside /NPG/ (Duerksen and Halvorson, 1958). The reaction mixture, 1.2 ml in volume, contained 0.1 M K-phosphate buffer, pH 6.8, 1 mM NPG, and appropriate amount of enzyme. The amount of enzyme added was determined, so that in 30 minutes time, at a temperature of 25°C, the increase in extinction, at 410 nm, would not exceed 0.3 units.

1-O-IAGluc Hydrolysis.

The determination of the hydrolytic activity of 1-O-IAGluc, depended on the estimation of the amount of glucose released through hydrolysis (Kęsy and Bandurski, 1990). The amount of glucose was measured by the enzymatic method using the Sigma Glucose HK No. 16 UV diagnostic set. The enzymatic hydrolysis reaction was carried out in a test tube with a reaction mixture formed by adding three solutions: (1) 100 μ l of 300 mM PIPES, pH 6.8; (2) 100 μ l of 1.5 mM 1-O-IAGluc in aqueous solution; (3) 100 μ l of enzyme solution. The protein concentration was selected so that the amount of glucose produced by the reaction in 30 minutes time did not exceed 50 nmoles. The reaction was carried out at a temperature of 25°C for 30 minutes, after which it was stopped by placing the test tubes into boiling water for 2 minutes. After cooling, 0.7 ml of glucose reagent (prepared using Sigma Diagnostics Procedure No. 16 UV) was added, and after five minutes the increase in A_{340} was measured. The amount of glucose produced was estimated from the standard curve.

The inhibition of enzyme activity.

The inhibiting effect of castanospermine on hydrolytic activity was tested by adding this substance to the reaction mixture, at a concentration of 10-100 mM, and comparing it to the hydrolytic activity of a sample not containing the inhibitor.

The protein determination

Protein concentration was measured by the Bradford method (1976), using BSA as a standard.

The effect of 1-O-IAGluc and IAA on coleoptile segments elongation

Using a specially constructed guillotine, five millimetres shoot segments, three mm removed from the tip, were cut from corn or oat coleoptiles and placed in distilled water for an hour (Bonner and Audus, 1959). Then the segments were placed in Petri dishes containing solutions whose effect on growth were to be tested. Every solution contained 20 mM PIPES, pH 6.8 and 2% (w/v) sucrose. Castanospermine (5×10^{-4} M), IAA (5×10^{-5} M) 1-O-IAGluc (5×10^{-5} M) as well as combination of IAA (5×10^{-5} M) / castanospermine (5×10^{-4} M) and 1-O-IAGluc (5×10^{-5} M) / castanospermine (5×10^{-4} M) were additionally included to incubation solutions. In the case of testing the influence of 1-O-IAGluc and IAA, in the presence of castanospermine, the coleoptile segments were priorly placed in a solution containing only PIPES, castanospermine and sucrose.

About 20 coleoptile segments were placed in each dish which were then set on a mechanical shaker. Experiments were carried out in the dark, at a temperature of 25°C. All operations requiring light were carried out under safe green light. The length of the coleoptile segments in fourfold magnification was measured, using the photographic projector.

RESULTS

The hydrolysis of indole-3-acetyl glucopyranoside by β -glucosidase from corn seedlings

Plant material homogenization and protein isolation.

After homogenizing the tissue the solution's pH decreased. Therefore, immediately after homogenization, the pH of the homogenate was brought to 7.8 and centrifuged at a 15,000 g. The clear, slightly yellow supernatant was applied to a DE-AE-Sephacel column and rinsed with elution buffer. The proteins eluted from the column were tested with regards to the hydrolytic activity in relation to 1-O-IAGluc and NPG. Proteins which were not adsorbed onto the column did not exhibit such activity. Proteins exhibiting hydrolytic activity due to 1-O-IAGluc and NPG were washed from the column at NaCl concentrations of 60-210 mM. There was also a little activity concerned with proteins washed out at 210-500 mM NaCl (results not shown).

The inhibition of hydrolytic activity.

The activity of protein fractions, isolated from corn tissue, catalysing the hydrolysis of 1-O-IAGluc and NPG, were tested in regards to its sensitivity to castanospermine (a known β -glucosidase inhibitor) inhibition (Saul et al., 1983). The activity of all studied fractions was inhibited by castanospermine. Over 98% of the inhibition of the enzymes activity tested was obtained at a 50 μ M concentration of castanospermine. A typical course of the dependence of 1-O-IAGluc and NPG enzymatic hydrolysis activity inhibition on the inhibitor concentration is shown in Fig. 1.

Corn coleoptile segment growth stimulation due to 1-O-IAGluc

The effect of 1-O-IAGluc on the growth of 4 day-old corn seedling coleoptile segments is shown in Fig. 2A. In order to limit the ability of 1-O-IAGluc enzymatic hydrolysis, and consequently in order to test if growth stimulation is induced by 1-O-IAGluc or eventually free IAA, obtained from the hydrolysis of 1-O-IAGluc, assessing the influence of these substances on coleoptile growth was carried out in the presence of castanospermine. As can be concluded from Fig. 2A, 1-O-IAGluc stimulated corn coleoptile segment growth. Stimulation was even stronger than in the case of free IAA. Castanospermine exerted a certain influence on coleoptile growth, which manifested itself in the slight inhibition of the growth of coleoptile segments (without and with IAA and 1-O-IAGluc), but still growth stimulation due to 1-O-IAGluc in the presence of castanospermine was stronger than effect of free IAA without castanospermine.

The stimulation of growth, brought about by 1-O-IAGluc, was also observed in the case of oat coleoptile segments. The effect was more pronounced than in the case of corn coleoptile elongation. Castanospermine also slightly inhibited oat coleoptile growth. Likewise, in oat coleoptiles, growth stimulation by 1-O-IAGluc in the presence of castanospermine was greater than that stimulated by free IAA (Fig. 2B). In addition, 1-O-IAGluc caused strong bending in tested oat coleoptiles.

Fig. 1. The effect of castanospermine on the hydrolytic activity of proteins isolated from four-day-old corn seedlings. Protein fractions washed from a DEAE column with NaCl at a concentration of 120-150 mM were used for testing.

Fig 2. The effect of IAA and 1-O-IAGluc on corn (A) and oat (B) coleoptile segment growth. The IAA and 1-O-IAGluc concentration was $5 \cdot 10^{-5}$ M, the castanospermine concentration was $5 \cdot 10^{-4}$ M. Results represent the averages $\pm$ SD (n=15).

DISCUSSION

The coleoptile segment elongation is a very sensitive test used in many laboratories as a method of free IAA concentration quantification. It can be thought, by this, that a reaction of growth is caused solely by free auxin. Meanwhile, Jakubowska et al. (1994) demonstrated, however, that the glucose conjugates of IAA likewise stimulated coleoptile growth. The results of our studies also show that indole-3-acetic acid glucoside causes coleoptile growth stimulation. This stimulation is as strong as that caused by free IAA. Keglevic and Pokorny (1969) demonstrated a similar effect of 1-O-IAGluc on oat coleoptile growth. The stimulating effect of this compound Keglevic and Pokorny (1969) explain by its ability to hydrolyse into free IAA, which is made in plant cells by the action of β -glucosidases.

This paper demonstrates that 1-O-IAGluc, as a β -glucoside, is hydrolysed by β -glucosidases isolated from corn seedlings equally as well as p-nitrophenol glucoside, a synthetic substrate used to indicate this enzyme. However, it is difficult to exclude the hydrolysis of 1-O-IAGluc caused by other specific hydrolytic enzymes. The presence of a 1-O-IAGluc hydrolysing enzyme in corn endosperm which did not hydrolyse saligenin, other β -glucosidase substrate, was shown by Kowalczyk and Bandurski (1990). Yet the hydrolysis of 1-O-IAGluc catalysed by corn seedlings homogenates, can be effectively inhibited by castanospermine, a known β -glucosidase inhibitor (Fig. 1). Thus, the use of this inhibitor in incubative solutions should lessen growth stimulation caused by 1-O-IAGluc in corn coleoptile fragments, if free IAA is responsible for that effect. The obtained results do not confirm such an assumption (Fig. 2). Despite applying castanospermine the effect of 1-O-IAGluc on growth was evident. Therefore, it seems, that 1-O-IAGluc is responsible for the observed growth effect. It seems possible that 1-O-IAGluc could directly stimulate growth processes, for example through protein glucosylation, as suggested Bandurski et al. (1991). This hypothesis has to be experimentally verified.

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STYMULACJA WZROSTU KOLEOPTYLI KUKURYDZY
PRZEZ GLUKOZYD KWASU INDOLILOOCTOWEGO

STRESZCZENIE

Istniały poprzednio sugestie, że stymulacja wzrostu poprzez glukozyd kwasu indolilooctowego (1-O-IAGluc) powstaje w wyniku jego hydrolizy do wolnego IAA. W obecnych doświadczeniach zastosowano więc kastanosperminę, znany inhibitor β -glukozydaz. 1-O-IAGluc w obecności kastanosperminy stymulował wzrost segmentów koleoptyli kukurydzy nawet silniej niż wolny IAA. Tak więc wydaje się, że to glukozyd kwasu indolilooctowego, a nie wolny IAA, jest odpowiedzialny za obserwowaną stymulację wzrostu koleoptyli kukurydzy.

SŁOWA KLUCZOWE: Auksyny związane, glukozyd-IAA, koleoptyle kukurydzy, kastanospermina.