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QUARTERLY

Transformation of 1-*O*-(indole-3-acetyl)-β-D-glucose into di-*O*-(indole-3-acetyl)-D-glucose catalysed by enzyme preparations from corn seedlings

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A new enzymatic activity, which catalyses formation *in vitro* of di-*O*-(indole-3-acetyl)-D-glucose from 1-*O*-(indole-3-acetyl)-β-D-glucose has been found in extracts of *Zea mays* seedlings. The structure of di-*O*-(indole-3-acetyl)-D-glucose, not as yet described, has been assigned by GC-MS, ^{1}H NMR and ammonolysis.

Despite the fact that the metabolism of indole-3-acetic acid (IAA), a known plant hormone, is generally not fully elucidated, much progress has been made in determination of metabolic pathways of formation of ester conjugates of this hormone [1]. 1-*O*-(Indole-3-acetyl)-β-D-glucose (1-*O*-IAGlc) plays an important role in these reactions. It has been shown that inositolic esters of IAA, present in corn endosperm, are formed in a two-stage process, in which IAGlc is an intermediate [2]. It is assumed that 1-*O*-IAGlc is also a precursor in IAA amide conjugate synthesis in other plants. 1-*O*-IAGlc occurs naturally in oat coleoptiles [3]. It can be also a product of cell detoxification, after the application of IAA in excess [4]. In aqueous solutions, 1-*O*-IAGlc is subject to non-enzymatic conversion into 4- and 6-*O*-IAGlc [5], which were isolated from corn endosperm. The present report describes the isolation from corn seedlings of the enzymatic activity causing formation of the hitherto unknown IAA ester, di-*O*-(indole-3-acetyl)-D-glucose (di(IAA)Glc).

MATERIALS AND METHODS

Reagents. Castanospermine and DEAE-Sephacel were from Sigma. Silica gel 60 for column chromatography (230–400 mesh) and Silica gel 60 glass plates (0.25 mm thickness) for thin-layer chromatography (TLC) from Merck. 1-*O*-IAGlc was obtained by chemical synthesis, using the method of Szmidt-Jaworska *et al.* [6].

Plant material. Four-day-old corn seedlings (*Zea mays*), grown in the dark at 26°C, were used. The kernels were sown to the mixture of perlite and sand (2:1, v/v).

Enzyme purification. The corn seedlings were stripped of their roots, rinsed with dis-

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Abbreviations: di(IAA)Glc, di-*O*-(indole-3-acetyl)-D-glucose; 1-*O*-IAGlc, 1-*O*-(indole-3-acetyl)-β-D-glucose; IAA, indole-3-acetic acid; PEG, polyethylene glycol 8000; Solvent A, a TLC solvent consisting of butanone/ethyl acetate/ethanol/water (3:5:1:1, by vol.).

tilled water and homogenised in 0.2 M Tris/Cl buffer, pH 8.0. The homogenate was filtered through cheese cloth, and brought to 5% PEG by adding solid PEG. The mixture was centrifuged for 20 min at 6000 × ***g***, and the supernatant was decanted, brought to 30% PEG and left overnight in the refrigerator. The next day, the mixture was centrifuged at 15000 × ***g*** for 30 min, and the supernatant discarded. The pellet was dissolved in 120 ml of 30 mM Tris/Cl buffer (pH 7.8), centrifuged and the supernatant was applied to a 3 cm × 20 cm DEAE-Sephacel column equilibrated with 30 mM Tris/Cl, pH 7.8.

The column was washed with 200 ml of equilibration buffer and the proteins were eluted consecutively with 110 mM, 130 mM, 145 mM, and 165 mM NaCl, in equilibration buffer. The active fractions were pooled and proteins were precipitated at a 75% ammonium sulphate saturation.

Enzymatic activity. The enzymatic activity was tested for the presence of indolic compounds by TLC using the colour reaction [7]. The reaction mixture contained 0.1 M Pipes, pH 6.8, 1 mM 1-*O*-IAGlc and 100 mM castanospermine. The reaction was carried out at 25°C for 30 min, and stopped by placing the test tubes into boiling water for 2 min. TLC plates were developed in a solvent consisting of butanone/ethyl acetate/ethanol/water (3:5:1:1, by vol., Solvent A). Indolic compounds were detected by means of the Ehmann reagent [7] used as a dip, followed by blotting, and drying for 5 min at 100°C.

Enzymatic synthesis and isolation of di(IAA)Glc. In order to obtain a larger amount of the product of the investigated enzymatic conversion of 1-*O*-IAGlc, the reaction was carried out in a 3 ml reaction mixture with substrate at a concentration of 4 mM, for 0.5 to 1 h at 20°C. The reaction progress was estimated by TLC, developed in Solvent A. After termination of the reaction, the mixture was extracted three times with ethyl ether and the combined extracts evaporated to dryness in a stream of nitrogen. The oily residue was chromatographed on Silica gel 60 column (1.5 cm × 20 cm) equilibrated with a solvent consisting of dichloromethane/ethyl acetate/ethanol (8:1:1, by vol.) and on ODS C_{18} reversed phase HPLC column (4.5 mm × 150 mm) equilibrated with 80% methanol solution. In the case of the Silica gel 60 column chromatography, the effluent was tested by TLC developed in dichloromethane/ethyl acetate/methanol (8:1:1, by vol.), using Ehmann's methods [7] for indole detection. In the case of HPLC chromatography, the effluent was tested at 254 nm.

Structure assignment. The chemical structure of the product of the enzymatic reaction tested was verified by GC-MS, ^{1}H NMR (Varian Gemini 200, 200 MHz, in deuterated acetone), as well as by its hydrolysis and ammonolysis products. The GC-MS analysis were carried out on a Varian 3300 gas chromatograph combined with ITD Finnigan Mat mass spectrometer. Silylated samples [8] were chromatographed on a 30 m DB-17 fused silica capillary column. The injector temperature was 300°C. The temperature of column was 120°C for 2 min and then programmed to increase by 10°C/min to 240°C.

Hydrolysis. Hydrolysis of the tested compound was carried out in 1 M NaOH solution during 1 h. Afterwards, the solution was neutralised by adding an appropriate amount of 1 M HCl solution. Samples, after hydrolysis, were tested by TLC and GC-MS.

Ammonolysis. Ammonolysis was carried out in 25% ammonia solution. Samples were dried, then 50 µl of ammonia solution was added and the mixture placed in boiling water for 10 min [9]. Afterwards, samples were analysed by TLC.

RESULTS

Enzyme assay

When 1-*O*-IAGlc conversions catalysed by corn seedling extracts were investigated, formation of a yet unknown indolic compound was observed. It manifested itself as an unidentified spot on TLC plates, with R_F differing from that of known indoles (Fig. 1).

Since the protein fraction isolated from corn seedlings always hydrolysed 1-*O*-IAGlc, it was necessary to inhibit this reaction. For this purpose, castanospermine, a known β-

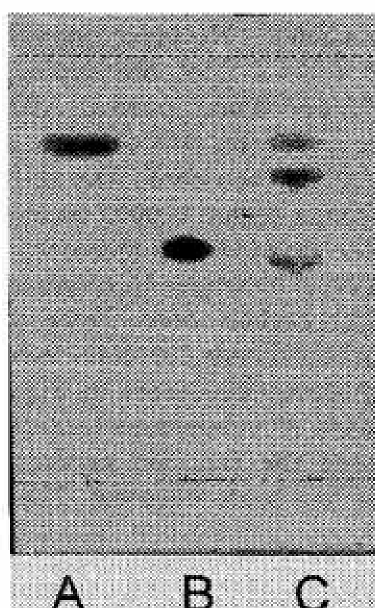

Figure 1. Silica gel 60 TLC chromatogram of the products of 1-*O*-IAGlc conversion, catalysed by corn seedling extracts (lane C).

Lane A, IAA standard; lane B, 1-*O*-IAGlc standard. The developing system consisted of butanone/ethyl acetate/ethanol/water (3:5:1:1, by vol.).

glucosidase inhibitor was used at a concentration of 100 μM [10]. It was also observed that higher concentration of the enzyme caused an increase in the yield of the unidentified product. Likewise, it was observed that this product, on being left in the reaction mixture for a longer period, become converted to free IAA.

Enzyme isolation and purification

After homogenisation and PEG precipitation, proteins were purified immediately by DEAE-Sephacel chromatography. At first, the proteins were applied to the column after being precipitated at 5% PEG, although greater enzymatic activity was later observed when the applied proteins were precipitated at 5–30% PEG (for a typical elution profile see Fig. 2).

The greatest enzyme activity was eluted from the DEAE-Sephacel column at 145 mM NaCl. Attempts at further purification, including rechromatography on DEAE-Sephacel and gel filtration, caused a total loss of activity.

Enzyme properties

The enzyme, purified on DEAE-Sephacel could be stored in a refrigerator for three weeks in 3.2 M ammonium sulphate. Centrifugation of the homogenate at 15000 × *g* caused a loss of enzymatic activity, which was probably linked with protein sedimentation. This may indicate that the tested enzyme is a membrane protein.

Enzymatic synthesis, purification and structural analysis of the 1-O-IAGlc conversion product

Synthesis and purification

The enzymatic synthesis of the tested compound was carried out as described above. The yield of the tested reaction product was greater when a higher concentration of protein was applied to the reaction mixture.

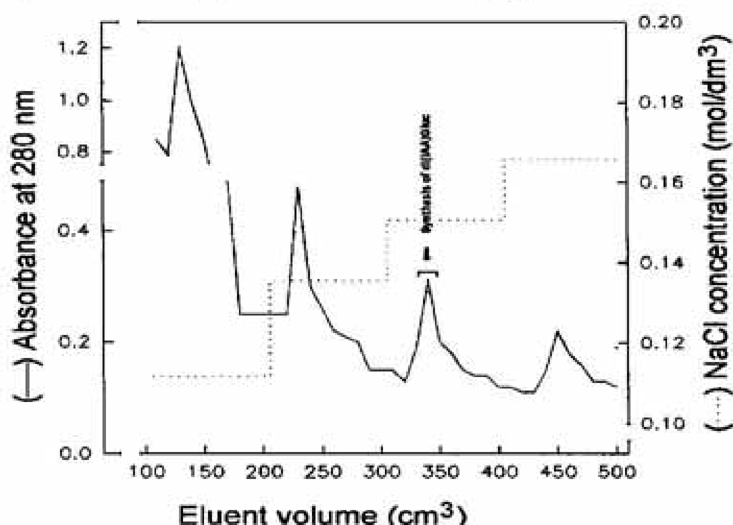

Figure 2. Elution profile of the proteins isolated from corn seedlings purified by DEAE-Sephacel column.

The proteins which catalysed transformation of 1-*O*-IAGlc to di(IAA)Glc were eluted at 145 mM NaCl.

Routinely, about 2 mg of protein was used per 1 ml of the reaction mixture. After stopping the enzymatic reaction and extraction with ethyl ether the obtained substances were chromatographed on a Silica gel column. The elution system used easily separated the indolic compounds present in the mixture. Fractions containing the tested substance were eluted between 70 and 100 ml, and consisted of only one indolic compound, as stated by TLC developed in Solvent A. However, ^{1}H NMR and GC-MS analysis indicated the presence of yet another compound, dioctyl phthalate (not shown). In order to remove this compound the mixture was additionally purified by HPLC.

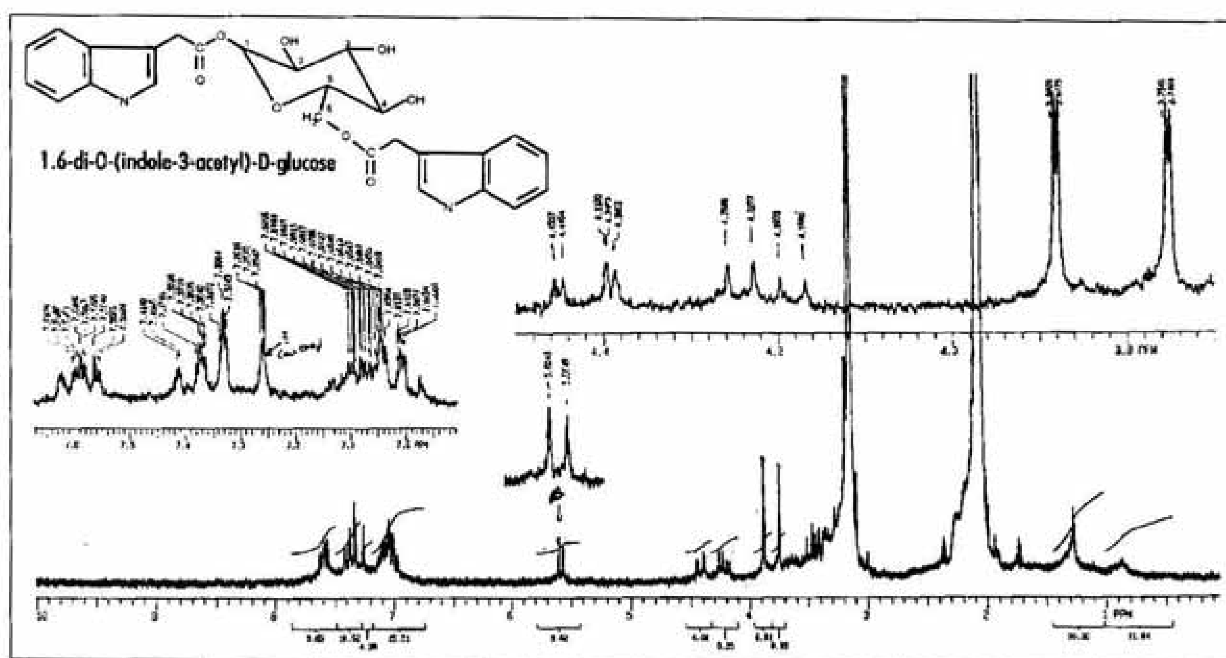

Figure 3. ^{1}H NMR spectrum of the product of 1-*O*-IAGlc conversion catalysed by enzymes isolated from corn seedlings.

For the interpretation see results.

Structural analysis

The ^{1}H NMR spectrum of the HPLC purified compound is shown in Fig. 3. Two doublets at 3.7 and 3.8 ppm indicate the presence of two indolic residues in a molecule of the tested compound. A doublet at 5.6 ppm has a coupling constant (J = 7.96 Hz) similar to that of the proton of the anomeric carbon of β-glucoside substituted by indole-3-acetic acid (not shown). However, signals in the region of 4.1–4.4 ppm are typical for the signals of A and B protons in an ABX system. In glucose molecules this system exists only at their sixth carbon atom. The shift of these signals, from a region typical for the protons of a glucose ring (3.0–4.0 ppm), is caused by substitution of hydrogen in a hydroxyl group at the sixth carbon atom. Thus, the tested substance is probably 1,6-di-*O*-(indole-3-acetyl)-β-D-glucose (see insert in Fig. 3). Also, the presence of indole-3-acetamide, besides IAA, among the reaction products of ammonolysis has been observed. The indole-3-acetamide is formed by the ammonolysis of 1-*O*-IAGlc [9].

Since the tested compound is unstable and decomposes during storage, it was not possible to collect it in a greater amount sufficient to obtain a ^{13}C NMR spectrum, which would have helped to determine more accurately its structure. Likewise, this instability of the substance, did not allow for a univocal mass spectrum interpretation. On the chromatography of silylated samples, a few peaks were obtained, amongst which only that of IAA was identifiable (not shown).

As a result of hydrolysis of the tested compound, glucose (confirmed by GC-MS — not shown), as well as IAA (proved by TLC and GC-MS) have been found in the post-reaction mixture.

DISCUSSION

IAA glucoside esters commonly occur in plants, although the role of these conjugates has not been entirely explained. It is held that they may be either a storage or a transport form. The possibility, that they might play an entirely different role, has not been excluded either. The enzymatic conversion of 1-*O*-IAGlc leads to the formation of di-*O*-(indole-3-acetyl)-D-glucose, an unknown IAA ester. The reaction is catalysed by protein fractions eluted from DEAE-Sephacel at an NaCl concentration of 145 mM. The only substrate used in this reaction was 1-*O*-IAGlc. It was observed, however, that the reaction carried out in the presence of a higher protein concentration gave a better yield, which may be explained by the presence of some co-factor in protein extracts. It seems possible that, because of the need for this co-factor which is lost during the purification process, it was not possible to achieve further enzyme purification by rechromatography on DEAE-Sephacel and gel filtration. Di-*O*-(indole-3-acetyl)-*myo*-inositol esters, compounds with a similar structure have been found earlier in corn endosperm by Ehmann & Bandurski [11].

On the basis of the research performed, it is difficult to predict whether conversion of 1-*O*-IAGlc to di(IAA)Glc occurs naturally in plant cells; it is difficult as well to determine whether this is a single or multi-step reaction. Investigation of this conversion was difficult due to the instability of the enzyme, and to hydrolysis of 1-*O*-IAGlc in extracts. Although nothing can be said on the role of di-(IAA)Glc, yet attention should be paid to the fact that compounds of this type — double glucose esters — occur both in plant and in animal tissues and play an important regulatory role (e.g. glucose-1,6-diphosphate and, especially, fructose-2,6-diphosphate).

Further research on the correct structure, biosynthesis and role of di-*O*-(indole-3-acetyl)-D-glucose, will be dependent upon its chemical synthesis.

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