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Characterization and Rooting Ability of Indole-3-Butyric Acid Conjugates Formed during Rooting of Mung Bean Cuttings[1]

Zeev Wiesman, Joseph Riov*, and Ephraim Epstein

Department of Horticulture, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel (Z.W., J.R.); *and Agricultural Research Organization, ARO, The Volcani Center, Bet Dagan 50250, Israel* (E.E.)

ABSTRACT

Indole-3-butyric acid (IBA) is rapidly metabolized by mung bean cuttings during rooting. Twenty-four hours after application, less than 20% of the applied IBA remained in the free form and its level decreased continuously in the later stages of rooting. Indole-3-butyrylaspartic acid (IBAsp) and at least two high molecular weight conjugates were the major metabolites in IBA-treated cuttings. In the latter conjugates, at least part of the IBA moiety is attached to a high molecular weight constituent in an amide linkage. IBAsp level peaked 24 hours after application of IBA to the cuttings and then declined. The level of the high molecular weight conjugates increased continuously throughout the rooting process. The conjugates were active in inducing rooting of cuttings, with IBAsp being superior to free IBA. It is suggested that IBA conjugates, and particularly IBAsp, serve as the source of auxin during the later stages of rooting.

The ability of auxins to stimulate adventitious root formation is well documented (11–13, 17). IBA[2] is the most widely used auxin for rooting of cuttings (11, 13). It is believed that the high rooting ability of IBA results from its high stability in the plant (7, 11). However, we have recently demonstrated that IBA is metabolized very rapidly during rooting of mung bean cuttings, at a rate similar to that of IAA which possesses a low rooting capability (18). Similar results were obtained with several other species (our unpublished data). These observations led us to suggest that IBA conjugates, formed following application of IBA, are playing an important role in rooting by slowly releasing free IBA (18). This hypothesis is supported by the observations demonstrating that auxin conjugates have a biological activity in various plant systems (8, 10).

There are only a few studies on IBA metabolism in plants. Andreae and Good (1) reported that plants treated with IBA accumulated substances which were identified tentatively as indolebutyramide and IBAsp. Chromatographic data provided by Fawcett *et al.* (7) also indicated the formation of IBAsp from exogenous IBA. We report here that IBA is conjugated during rooting of mung bean cuttings to IBAsp and to at least two high mol wt conjugates. All these conjugates were active in inducing adventitious root formation in mung bean cuttings.

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[2] Abbreviations: IBA, indole-3-butyric acid; IBAsp, indole-3-butyrylaspartic acid; IAAsp, indole-3-acetylaspartic acid; m/z, mass to charge ratio; PVPP, polyvinylpolypyrrolidone; TMS, trimethyl silyl.

MATERIALS AND METHODS

Plant Material

Seeds of mung bean (*Vigna radiata* L.) were imbibed in aerated water for 24 h and germinated and grown as previously described (18). For rooting studies, cuttings were made from 7-d-old seedlings. A cutting consisted of a terminal bud, two primary leaves, epicotyl, and 4 cm of the hypocotyl. Four cuttings were placed in a 25 mL vial containing 15 mL distilled water or test solutions. Solutions were renewed every 24 h. Rooting was performed under the environmental conditions specified above. Number of visible roots was determined 7 d after the cuttings were made. Results are reported as means of four vials.

IBA Metabolism

Four cuttings were treated with 100 μM IBA to which 50.5 kBq [5-^{3}H]IBA (1.11 TBq mmol^{-1}, Dimona, Israel) were added. After 24 h of IBA treatment, the cuttings were transferred to distilled water. At various periods after the initiation of treatment, hypocotyls were excised and extracted as previously described (18). Aliquots were analyzed for IBA and IBA metabolites by either TLC or HPLC following the procedures of Wiesman *et al.* (18).

GC-MS

GC-MS was performed on a Finigan MAT 4600 Mass Spectrometer. Capillary GC was carried out on a 15 m × 0.32 mm i.d. DB-5 column (J & W Scientific, Folsom, CA) with a film thickness of 0.25 μm. The helium flow rate was 1.2 mL min^{-1} and the injector temperature was 250°C. For methylated IBAsp, a temperature program of 150 to 200°C at 5°C min^{-1} followed by an increase of 2°C min^{-1} from 200 to 260°C was started upon injection. For TMS-IBA, the temperature was programmed from 130 to 220°C at 4°C min^{-1}. Mass spectra were recorded at an ionizing voltage of 70 eV with a source temperature of 270°C.

Isolation and Identification of IBAsp

One hundred g of hypocotyls excised from cuttings treated with IBA for 24 h were extracted in 80% methanol with a

Polytron homogenizer. The extract was filtered and then centrifuged at 10,000*g* for 10 min. The supernatant was reduced to an aqueous solution on a rotary evaporator at 45°C. The aqueous solution was adjusted to pH 2.5 and extracted three times with ethyl acetate. The ethyl acetate fraction containing the IBAsp was reduced to dryness. The gum obtained was dissolved in a small volume of 5% acetic acid in water and chromatographed on a PVPP column (1.8 × 20 cm) equilibrated with the above solution. The column was washed with the same solution and IBAsp was eluted with 20 mM K-phosphate buffer (pH 7). Fractions containing IBAsp were pooled and the pH was adjusted to 2.5. IBAsp was adsorbed onto a Sep-Pak C_{18} cartridge and eluted with methanol. IBAsp was further purified by HPLC using a semipreparative column (7 × 250 mm) packed with 7 μ Li-Chrosorb RP-18 (Merck, Darmstadt). The solvents were water (A) and methanol (B) containing 1% acetic acid. The solvent program was: 0 to 10 min, 20% B; 10 to 20 min, 20 to 40% B; 20 to 36 min, 40 to 60% B. The flow rate was 3 mL min^{-1}. Fractions of 3 mL were collected and those containing radioactivity were pooled and reduced to dryness. Analysis by TLC revealed one major radioactive spot which gave a blue coloring characteristic to indoles with Ehmann's reagent (5). IBAsp was methylated with diazomethane (15) for GC-MS analysis.

Isolation of High Mol Wt Conjugates of IBA

High mol wt conjugates of IBA remaining in the aqueous fraction after removing IBAsp by ethyl acetate were chromatographed on PVPP column as described above. The conjugates were eluted with 5% acetic acid. Fractions containing radioactivity were passed through a Sep-Pak C_{18} cartridge and the adsorbed conjugates were eluted with methanol. Further purification was achieved by a semipreparative HPLC as above. Fractions containing radioactivity were evaporated to dryness and dissolved in 50 mM K-phosphate buffer (pH 7). The conjugates were chromatographed on a Sephadex G-25 column (1.8 × 25 cm) equilibrated with the above buffer. Elution was performed with the same buffer and 4 mL fractions were collected. Aliquots were taken from each fraction for radioactivity determination. The column was calibrated with naringin (mol wt = 580) and vitamin B_{12} (mol wt = 1355).

Hydrolysis of High Mol Wt Conjugates

Aliquots of each of the two conjugates obtained by Sephadex G-25 chromatography were placed in a small test tube and evaporated to dryness. The residue was resuspended in 200 μL of fresh 7 N NaOH. The test tubes were flushed with N_2, sealed and incubated at 100°C for 3 h. After hydrolysis, the solutions were diluted with water and brought to pH 2.5 with HCl. The free auxin was adsorbed onto a Sep-Pak C_{18} cartridge and eluted with methanol. The IBA liberated by hydrolysis was analyzed by HPLC. The IBA collected from HPLC was silylated (6) and its identity was confirmed by GC-MS.

Synthesis of IBAsp

Synthesis of IBAsp was based on the procedure for synthesis of IAAsp (3, 16). Five mg of L-aspartic acid *bis-t*-butyl ester HCl (Sigma Chemical Co.) and 2 mL acetonitrile solution containing 2 mg IBA were added to a vial equipped with a teflon-line screw cap. After chilling on ice to 0°C, 6.2 mg of dicyclohexylcarbodiimide (Aldrich Chemical Co.) were added. After 8 h at 0 to 4°C, the mixture was transferred to a larger vial containing 2 mL 2 N NaOH and heated to 60°C. Fourteen h later, the mixture was chilled to 0°C, acidified to pH 2.5, passed through a Sep-Pak C_{18} cartridge and eluted with methanol. The purity of IBAsp was checked by TLC and its identity was verified after methylation by combined GC-MS.

RESULTS

Isolation and Identification of IBA Metabolites

Analysis by HPLC of a crude extract of mung bean cuttings treated with [^{3}H]IBA for 48 h revealed two major peaks, one at R_t = 8 min and the other at R_t = 43 min which coeluted with authentic IBAsp (Fig. 1). The two radioactive peaks from HPLC were further analyzed by TLC and stained for indoles with Ehmann's reagent. The fraction corresponding to R_t = 8 min gave one radioactive Ehmann positive spot at R_F = 0.03. The second peak (R_t = 43 min) also gave one major radioactive Ehmann positive spot at R_F = 0.56 which cochromatographed with authentic IBAsp. The identity of the putative IBAsp was confirmed by GC-MS (Fig. 2).

The identity of the metabolite(s) corresponding to R_t = 8 min was further investigated. In view of several reports on the presence of high mol wt conjugates of IAA in plant tissues (2, and literature cited therein), we explored the possibility that IBA also forms similar conjugate(s). Chromatography of a partially purified fraction on Sephadex G-25 revealed two major radioactive peaks (Fig. 3). Based on the elution profiles of various standards, the mol wt of these conjugates is estimated to be between 580 and 1355. Each radioactive fraction

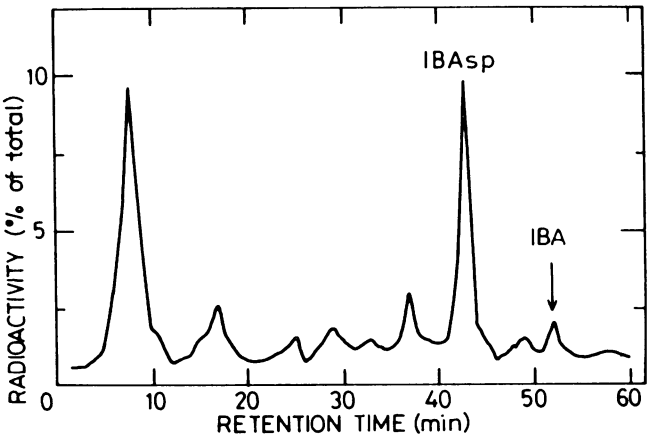

Figure 1. Reverse-phase HPLC profile of [^{3}H]IBA metabolites extracted from mung bean cuttings 48 h after the initiation of IBA treatment. Retention time of IBA and IBAsp are designated by an arrow.

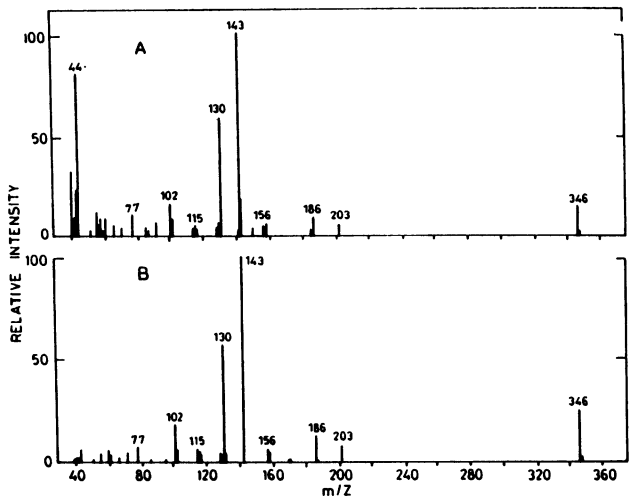

Figure 2. The 70 eV electron impact mass spectra of (A) a methylated sample of putative IBAsp isolated from mung bean cuttings treated with IBA, and (B) authentic *bis*-methyl-IBAsp. Ions characteristic of *bis*-methyl-IBAsp are m/z 346(m^+), 186, 143 (base peak), and 130.

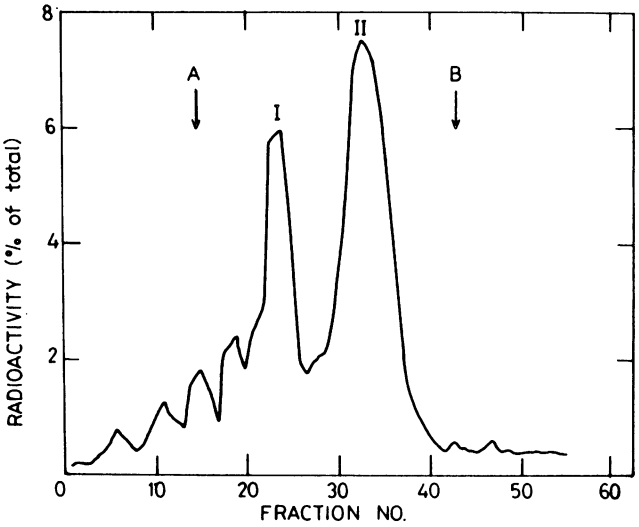

Figure 3. Sephadex G-25 chromatography of a partially purified fraction of H_2O-extractable IBA conjugates of mung bean cuttings. Extraction was performed 24 h after the initiation of IBA treatment. The column was calibrated with (A) vitamin B_{12} (mol wt = 1355), and (B) naringin (mol wt = 580). I and II represent the two major high mol wt conjugates.

was hydrolyzed by 7 N NaOH and the hydrolyzate was analyzed by HPLC (Fig. 4). About 50% of the radioactivity of each fraction appeared at R_t corresponding to free IBA. The identity of putative IBA was confirmed by GC-MS analysis (Fig. 5). One N NaOH did not release any measurable amount of free IBA.

Kinetics of Changes in [^{3}H]IBA and its Metabolites

IBA was rapidly metabolized and after 24 h only about 20% of the IBA taken up by the cuttings remained in the free form (Fig. 6). The level of free IBA continue to decline to

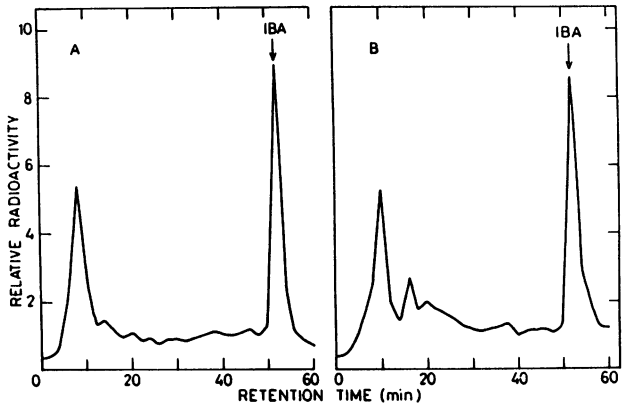

Figure 4. Reverse-phase HPLC profiles of the high mol wt conjugates of IBA after hydrolysis with 7 N NaOH. (A) indicates conjugate I, and (B) indicates conjugates II eluted from Sephadex G-25 column (Fig. 3). Retention time of IBA is designated by an arrow.

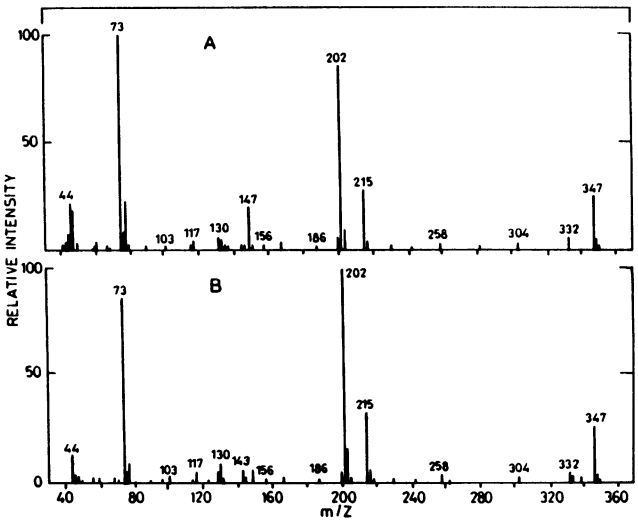

Figure 5. The 70 eV electron impact mass spectra of (A) silylated IBA released by hydrolysis with 7 N NaOH from the high mol wt conjugate II, and (B) authentic TMS-IBA. Ions characteristic of TMS-IBA are m/z 347 (m^+), 332, 304, 258, 215, 202 (base peak), and 130.

only about 3% after 96 h. Levels of IBAsp and the high mol wt conjugates increased following the decrease in [^{3}H]IBA. IBAsp peaked after 24 h and then declined gradually. The level of the high mol wt conjugates increased continuously throughout the entire experimental period. When the experiment was terminated after 96 h, these peptides were the major conjugates accumulated in the cuttings.

Biological Activity of IBA Conjugates

IBA significantly induced adventitious root formation in mung bean cuttings (Table I). IBAsp and the two high mol wt conjugates also showed high rooting capability. IBAsp was the most active compound, inducing the formation of 57.1 roots per cutting compared to 39.2 induced by IBA.

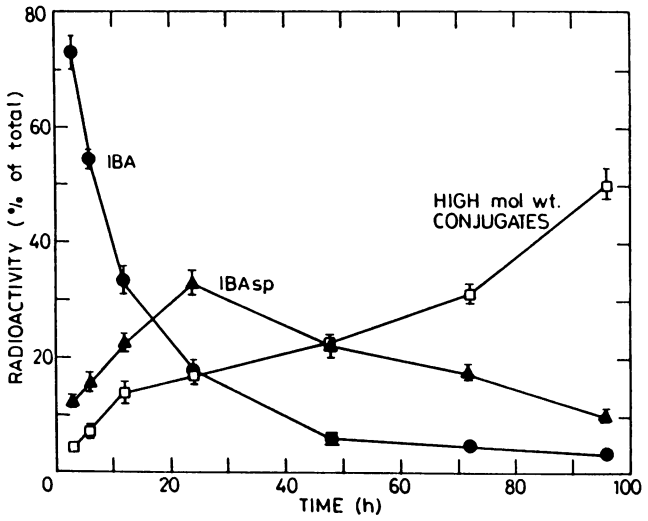

Figure 6. Percentage of IBA, IBAsp, and the high mol wt conjugates of IBA in hypocotyls of mung bean cuttings during adventitious root formation. Data are means of three replicates ± SE.

Table I. *Effect of IBA Conjugates on Rooting of Mung Bean Cuttings*

The concentration of IBA and IBAsp was 100 μM. The concentration of the high mol wt conjugates was 100 μM in terms of free IBA. Different letters indicate significant differences between treatments (P = 0.05) by Duncan's multiple range test.

Treatment	No. Root/Cutting
H_2O	8.5 C
IBA	39.2 B
IBAsp	57.1 A
Conjugate I[a]	29.3 B
Conjugate II	42.5 B

[a] Conjugates I and II refer to the first and second major peaks of the Sephadex G-25 chromatography of the high mol wt conjugates (Fig. 3).

DISCUSSION

The requirement for auxin for rooting of cuttings is well documented (9, 11, 12). Recent studies on IAA (14) and IBA (18) metabolism during rooting of mung bean cuttings indicate that both auxins are metabolized very quickly. Therefore, it has been suggested that the conjugates of both auxins serve as the source of the required auxin during most of the rooting process. In the present study we investigated the nature of IBA conjugates formed during rooting and their biological activity.

Two types of conjugates were detected, IBAsp and high mol wt conjugates. Chromatographic evidence for formation of IBAsp from applied IBA was reported before (1), but this is the first unequivocal identification of this conjugate. Our data also demonstrate the formation of at least two high mol wt conjugates following application of IBA. Hydrolysis of these conjugates with 1 N NaOH did not release any detectable free IBA, whereas 7 N NaOH released considerable amounts of free IBA (Fig. 4). The hydrolysis data indicate that in the high mol wt conjugates, at least part of the IBA moiety is attached to a high mol wt constituent in an amide linkage. Based on Sephadex G-25 chromatography (Fig. 3) the mol wt of these compounds is estimated to be between 580 and 1355. Recently, Bialek and Cohen (2) also reported the occurrence of an IAA-peptide of approximately 3 kD in *Phaseolus vulgaris*.

Formation of IBAsp during rooting peaked 24 h after application of IBA and then gradually decreased (Fig. 6). Formation of the high mol wt conjugates, on the other hand, increased continuously throughout the rooting process. Based on these observations, we propose that IBAsp is the major source of free IBA in the later stages of the rooting process when high auxin activity is required (9, 11, 17). This hypothesis is supported by the observations that IBAsp has a high rooting ability when applied exogenously (Table I). The above hypothesis does not rule out the possibility that the high mol wt conjugates also serve as a source of free IBA since they also possess rooting activity. The continuous accumulation of the high mol wt conjugates during rooting suggests that they are the major storage form of auxin in mung bean, as previously proposed for the 3 kD IAA-peptide in *P. vulgaris* seeds (2).

The present and previous observations (14, 18) demonstrated the importance of auxin conjugates formed endogenously after application of auxin in rooting of cuttings. However, it is still not clear why IAA is much less effective than IBA in inducing rooting. The major IAA conjugate formed endogenously during rooting from applied IAA is IAAsp and its level changes in a similar pattern to that of IBAsp (14). In addition, IAAsp is very active in inducing rooting in this system when applied exogenously (Z Wiesman, J Riov, E Epstein, unpublished data). Further research is warranted to clarify this problem.

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