Occurrence of Castasterone, Brassinolide and Methyl 4-Chloroindole-3-acetate in Immature *Vicia faba* Seeds

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The rice lamina inclination test indicated the presence of brassinosteroid-like active substances in immature *Vicia faba* seeds. Two of these were identified as castasterone and brassinolide by GC/MS and GC/SIM, respectively. Another active principle was identified as methyl 4-chloroindole-3-acetate by GC/MS and HPLC.

Recently, brassinosteroids, new steroidal plant growth regulators, have been demonstrated to have unique physiological roles^{1~5)} in growth regulation, and also to be distributed in a wide range of plants.^{1,6~13)} Among them, immature seeds of leguminous plants are known to contain a variety of brassinosteroids.^{8,13)} Further interest in leguminous plants prompted us to investigate the presence of brassinosteroids in seeds of broad bean (*Vicia faba* L.).

Immature *Vicia faba* seeds (29.5 kg from 82.3 kg of pods) were extracted with methanol and the extract was concentrated *in vacuo* to afford an aqueous residue. The aqueous phase was neutralized and successively extracted with benzene and chloroform. The organic extracts were combined and partitioned between *n*-hexane and 80% methanol. Remarkable activity was detected in the aqueous methanol phase by the rice lamina inclination test,¹⁴⁾ which is the most sensitive bioassay for brassinosteroids.¹⁵⁾ The aqueous methanol fraction was subjected to a silica gel column eluted step-wise with increasing concentrations of methanol in chloroform. A less polar fraction (fraction I) and a more polar

fraction (fraction II) were separated as shown in Fig. 1.

Fraction II was purified by repetitive silica gel chromatography using an acetone-chloroform mixture as the solvent. Eluates with 5% acetone and 15~20% acetone in chloroform were biologically active and, therefore, were combined and then subjected to Sephadex LH-20 chromatography using 70% ethanol as the mobile phase. Activity was found in the elution volume (0.675~0.800, Ve/Vt) which corresponded to that of typical brassinosteroids,¹⁶⁾ as shown in Fig. 2. This active fraction was successively submitted to further purification by preparative TLC, normal phase HPLC and reversed-phase HPLC, and finally purified by HPLC on an Aquasil column with a chloroform-methanol-water system. As shown in Fig. 3, biological activity was detected in the fractions with retention times of 17~18 min and 25~26 min. On the basis of the retention times of authentic brassinosteroids on HPLC and the R_f values of the active fractions on HPTLC (high-performance TLC), the active substances in the main fraction (t_R 17~18 min, R_f 0.37) and in the minor fraction (t_R 25~26 min, R_f 0.29)

FIG. 1. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test with Seedlings of Tongjin-byeo after Silica Gel Adsorption Chromatography of the Extract from *V. faba*.

FIG. 2. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test with Seedlings of Kiho-byeo after Sephadex LH-20 Column Chromatography of Fr. II.

were suggested to be castasterone and brassinolide, respectively.

The main active fraction was analysed by GC/MS after methaneboronation. The retention time (15.35 min) of the main peak observed and its full mass spectrum (Fig. 4) were identical with those of the bismethaneboronate of castasterone.

The minor active fraction was subjected to GC/SIM analysis after conversion to the methaneboronate. Fragment ions at m/z 528 (M^+), 374, 345, 177 and 155 were selected as characteristic ions of brassinolide bismethaneboronate. As shown in Fig. 5, the observed peaks of the fraction were identical with those of authentic brassinolide with respect to the

intensity ratio of these ions and the retention time (17.2 min). Thus, the biologically active brassinosteroids in immature *Vicia faba* seeds were identified as castasterone and brassinolide.

On the other hand, fraction I obtained from the silica gel column was subjected to Sephadex LH-20 column chromatography using the same solvent system as that with fraction II. Activity was found in the elution volume (1.0~1.5, V_e/V_t) shown in Fig. 6. This fraction is clearly different from those of typical brassinosteroids, and it was successively submitted to further purification by normal-phase HPLC and reverse-phase HPLC, and finally subjected to HPLC on an Aquasil column with a chloroform-water system. As shown in Fig. 7, biological activity was found in the fraction with a retention time of 9~10 min. This active fraction was concentrated to give crystals.

Figure 8 shows the electron impact mass spectrum of the active substance, in which the molecular ion at m/z 223 and base peak at m/z 164 can be observed. The clusters consisting of a 3:1 ratio of m/z 223/225 and m/z 164/166 ions indicate the presence of a chlorine atom in the molecule.¹⁷⁾ The latter was apparently due to a chloro-indole moiety,^{18,19)} indicating that the compound should be a methyl ester of chlorinated indole acetic acid. Because the mass spectrum was similar to the literature data of methyl 4-chloroindole-3-acetate (4-Cl-IAA-Me),^{17,19)} a direct comparison was made

FIG. 3. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test with Seedlings of Koshihikari after HPLC on an Aquasil Column of the Fr. II.

FIG. 4. Mass Spectrum of the Bismethaneboronate of Castasterone Obtained from the HPLC Active Fraction.

FIG. 5. GC/SIM Profiles of Methaneboronate Derivatives of Authentic Brassinolide and the HPLC Active Fraction.

by capillary GC/MS between the active principle and authentic 4-Cl-IAA-Me. The retention times and mass spectra of the com-

pound (11.7 min) and its heptafluorobutyric derivative (8.3 min) were identical with those of authentic 4-Cl-IAA-Me and its heptafluoro-

butyric derivative.

The 400 MHz $^1\text{H-NMR}$ spectrum of the active principle was also indistinguishable from that of the authentic 4-Cl-IAA-Me.

FIG. 6. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test with Seedlings of Sangpung-byeo after Sephadex LH-20 Column Chromatography of Fr. I.

FIG. 7. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test with Seedlings of Koshihikari after HPLC on an Aquasil Column of Fr. I.

FIG. 8. Mass Spectrum of Methyl 4-Chloroindole-3-acetate Isolated from Fr. I.

Thus, the biologically active compound in fraction I was identical with 4-Cl-IAA-Me.

4-Cl-IAA-Me, which was first isolated from immature seed of *Pisum sativum*,^{20,21)} has been detected by Hofinger *et al.*²²⁾ in immature *Vicia faba* seeds. The distribution of 4-Cl-IAA and/or its methyl ester in seeds of various *Viciae*^{23~26)} and *Pinus sylvestris*²⁷⁾ has also been demonstrated.

The present work seems to be the first concerning the isolation of 4-Cl-IAA-Me as an active compound in the rice lamina inclination test. 4-Cl-IAA-Me showed activity at 0.5 ppm equivalent to 0.0001 ppm of brassinolide, but 4-Cl-IAA was almost inactive at 0.5 ppm.

EXPERIMENTAL

Plant material. *Vicia faba* L. was cultivated at Jindo, Chonnam, Korea. The immature seeds (29.5 kg fr. wt. from 82.3 kg fr. wt. of pods) were harvested *ca.* 40 days

after flowering.

Bioassay. The rice lamina inclination test was carried out using the cultivars Tongjin-byeo, Kiho-byeo, Sangpung-byeo or Koshihikari as described by Arima *et al.*²⁸⁾

Extraction. Immature seeds were homogenized and extracted three times with excess MeOH. The combined MeOH extract was reduced to the aqueous phase *in vacuo*, and after adjusting to pH 7, the aqueous solution was successively extracted with benzene and chloroform. The benzene extract (37.86 g) and the chloroform extract (0.53 g) were combined, dissolved in *n*-hexane (1 l) and partitioned twice against 80% MeOH (1 l). The combined 80% MeOH fractions were concentrated to afford an oil (19.45 g).

Silica gel adsorption chromatography. The oil obtained from the 80% MeOH fraction was charged onto a column packed with 200 g of silica gel (Merck, 100~200 mesh). Elution was carried out step-wise with 1.6 l each of CHCl₃ and then CHCl₃-MeOH (95:5; 90:10; 85:15; 80:20). Two biologically active fractions, fraction I (0.49 g) and fraction II (6.07 g), were obtained (Fig. 1).

Purification of fraction I. Fraction I was subjected to preparative TLC on silica gel plates (Sigma, 10~40 μ m, 1 mm; 20 $\times$ 20 cm, EtOAc-EtOH, 22:3, v/v). The biologically active fraction from TLC (*R_f* 0.3~0.6, 316 mg) was chromatographed on a Sephadex LH-20 column (bed volume, 500 ml; 64 $\times$ 3.15 cm) using 70% EtOH. This eluate was collected in 12.5 ml fractions. The active fraction (elution volume, 500~700 ml; 18.4 mg) was dissolved in CHCl₃ and subjected to HPLC using a μ Bondapak Porasil column (Waters, 10 μ m, 0.8 $\times$ 10 cm); flow rate, 1.5 ml/min; mobile phase, CHCl₃-iso-PrOH (99:1, v/v). The active fraction (*t_R* 4~8 min, 5.0 mg) was subjected to HPLC on a C₁₈ column (Waters, 10 μ m, 0.8 $\times$ 10 cm); flow rate, 2 ml/min; mobile phase, CH₃CN-H₂O (45:55, v/v). The active fraction (*t_R* 6~10 min, 3.5 mg) was subjected to HPLC on a Senshu Pak Aquasil column (1 $\times$ 20 cm); flow rate, 3 ml/min; mobile phase, CHCl₃-H₂O (100:0.1, v/v). Fractions were collected at 1 min intervals. From the fractions of *t_R* 9~10 min, the active substance was isolated as *ca.* 1 mg of crystals; 400 MHz ¹H-NMR (CDCl₃): δ 3.73 (3H, s, O-CH₃), 4.05 (2H, s, allyl CH₂), 7.17 (1H, d, *J* = 2.5 Hz, H-2 of the indole ring) and 7.07 and 7.26 (2H, m and 1H, m, respectively; first-order analyses of these two absorptions for H-5 and H-7 of the indole ring were impossible).

Purification of fraction II. Unless otherwise stated, the purification procedure was the same as that employed for fraction I. Fraction II was subjected to silica gel adsorption chromatography, with 60 g of silica gel. Elution was carried out step-wise with mixture of CHCl₃-acetone

(100:0, 100 ml; 95:5, 400 ml; 90:10, 400 ml; 85:15, 400 ml; 80:20, 400 ml). The obtained active fraction (5~20% acetone eluates, 3.84 g) was chromatographed on a Sephadex LH-20 column (bed volume, 320 ml; 2.7 $\times$ 56 cm), the eluate being collected in 8 ml fractions. The active fraction (elution volume, 216~256 ml; 0.92 g) was subjected to TLC. The active fraction (*R_f* 0.3~0.9, 282 mg) was subjected to HPLC using a Porasil column; flow rate, 2 ml/min; mobile phase, CHCl₃-iso-PrOH (95:5, v/v). The combined active fraction (*t_R* 18~27, 34 mg) was subjected to HPLC on a C₁₈ column. The active fraction (*t_R* 6~10 min, 6.1 mg) was then subjected to HPLC on an Aquasil column. The fractions corresponding to castasterone (*t_R* 16~19 min) and brassinolide (*t_R* 25~26 min) were subjected to HPTLC (Merck, developed by CHCl₃-EtOH, 5:1, v/v) and GC/MS examination.

GC/MS and GC/SIM. The analyses were carried out on a JEOL DX-303 under the following conditions: ionization, EI (70 eV); ionization chamber temp., 180°C; column, fused silica capillary, DB-1 (0.25 mm $\times$ 15 m, 0.25 μ m film thickness); column temp., 150°C for 2 min and then elevated to 290°C at 32°C/min; carrier gas, He 1.1 kg/cm²; splitless mode. Bismethaneboronation was carried out by treating samples with pyridine containing 2 mg/ml of methaneboronic acid at 70°C for 30 min.²⁹⁾

GC/MS for 4-Cl-IAA-Me. A Shimadzu QP 1000 was used under the following conditions: ionization, EI (70 eV); ionization source temp., 250°C; column, FS-WCOT, silicone OV-1 (0.3 μ m $\times$ 25 m); column temp., 140°C for 1 min and then elevated to 260°C at 4°C/min; carrier gas, He 1 ml/min. Heptafluorobutyric derivatives were obtained by treating samples with CH₃CN containing 100 μ l/ml of heptafluorobutyric anhydride at room temp. for 30 min.²⁵⁾

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