Isolation of Gibberellins A₅, A₃₂, A₃₂ Acetonide and (+)-Absciscic Acid from *Prunus persica*[†]

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Plant growth regulators in *Prunus persica* were surveyed. Gibberellins A₅, A₃₂, A₃₂ acetonide were isolated from immature seeds, and (+)-absciscic acid from young fruits and water sprouts.

Preceding to this study, twenty one gibberellins had been isolated from higher plants and their structures were characterized. Ogawa¹⁾ and Jackson *et al.*²⁾ reported the occurrence of a polar gibberellin-like substance in immature seeds of *Prunus* species. We surveyed plant growth regulators in *Prunus persica*, and preliminarily reported the isolation of a new gibberellin, gibberellin A₃₂, and its acetonide from the immature seeds.³⁾ We now report the details of the isolation of gibberellins A₅ (1), A₃₂ (3), and A₃₂ acetonide (4) from the immature seeds and (+)-absciscic acid (5) from the young fruits and water sprouts.

The immature seeds (*ca.* 35 kg), which were obtained from the young fruits (*ca.* 1000 kg) plucked during May to June in 1969 and frozen at -20°C, were extracted with methanol. The extract was fractionated into an acidic ethyl acetate (AE), a neutral ethyl acetate (NE), an acidic *n*-butanol (AB) and a neutral butanol (NB) fraction in usual way.⁴⁾ The AE and the AB fraction showed gibberellin activity on rice seedling test. The outline of the purification of the AB fraction is shown in the Fig.

Two gibberellin-like substances were separated by successive adsorption chromatography with charcoal, and partition chromatography

with phosphate buffer and benzene-ethyl acetate mixtures. The polar one was further purified through preparative TLC and preparative PPC to afford 38 mg of needles (tentatively named PG-I). It was found to be an ammonium salt of a new gibberellin, to which gibberellin number A₃₂ was allocated. The less polar one (tentatively named PG-II) was obtained as needles (1 mg) after the successive purification by silica gel adsorption chromatography and preparative TLC. Structural elucidation of PG-I and PG-II, the latter of which was identified as gibberellin A₃₂ acetonide, is described in the succeeding paper.

The isolation of gibberellins in the AE fraction from the seeds obtained in 1969 was unsuccessful. Nevertheless, an amorphous solid (tentatively named PG-III) giving a single spot on TLC was obtained from the seeds harvested in 1972 by successive procedure of countercurrent distribution, charcoal adsorption and silica gel adsorption chromatography and preparative TLC. The yield of PG-III was 0.2 mg from *ca.* 10 kg of the seeds. PG-III was identified as gibberellin A₅ (1) based on chromatographic behaviors (TLC and GC) and comparison of the mass spectrum of the methyl ester of PG-III with that of gibberellin A₅ methyl ester (2).⁵⁾

The AE fraction of extracts of the young seed-removed fruits showed growth inhibition

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FIG. Isolation Procedure of PG-I and PG-II.

on rice seedling test. The inhibitor was purified through countercurrent distribution method, silica gel adsorption and partition chromatography, and preparative TLC to give 1.9 mg of white powder, mp 165~166°C, from *ca.* 100 kg of the seed-removed fruits. The methyl ester of the inhibitor was identical with methyl *cis*, *trans*-(+)-abscisate (6) in all respects, *R_f* values on TLC, retention time on GC, mass spectrum and CD curve. Thus, the inhibitor isolated from the young fruits of *Prunus persica* was identified to be *cis*, *trans*-(+)-abscisic acid (5).⁶⁾ *Cis*, *trans*-(+)-abscisic acid was isolated also from water sprouts in the procedure similar to that described above. It is noteworthy that the content of abscisic acid in water sprouts was very high, namely, 11.0 mg from *ca.* 30 kg of

water sprouts.

EXPERIMENTAL

Thin-layers of silica gel GF₂₅₄ with 0.25 mm and 0.5 mm thickness were used for analytical and preparative TLC, respectively. Solvent systems used in this experiment were as follows; (i) chloroform: methanol: acetic acid (50: 20: 3), (ii) ethyl acetate: chloroform: acetic acid (20: 8: 1), (iii) ethyl acetate: chloroform: *n*-hexane: acetic acid (20: 8: 10: 1), (iv) benzene: *n*-butanol: acetic acid (70: 25: 5), (v) *n*-butanol: *n*-propanol: 0.3 N ammonium hydroxide (2: 6: 3), (vi) ethyl acetate: benzene (1: 3). A Hitachi-K53 was used for gas chromatography, a RMU-6L for measurement of mass spectra, and a JASCO J-20 for measurement of optical rotatory dispersion.

1) Isolation of gibberellin A₃₂ (PG-I) and A₃₂ acetoneide (PG-II)

a) *Extraction and fractionation.* Immature seeds

(ca. 35 kg) were taken out by cracking young fruits (ca. 1000 kg) stored at -20°C , and extracted with methanol three times. All the extracts were combined (60 liters) and concentrated to 15 liters under reduced pressure. The concentrate (15 liters) was extracted with ethyl acetate three times (3 liters in each time) at pH 2.5. The ethyl acetate extract was further fractionated into an acidic (AE) and a neutral (NE) fraction in the usual manner.⁴¹ The aqueous residue was successively extracted with *n*-butanol. The *n*-butanol extract was fractionated into an acidic (AB) and a neutral (NB) fraction.⁴¹ The AB fraction was concentrated at pH 6.0 (adjusted with 7 N methanolic ammonia) to give an acidic gum (115 g).

b) Charcoal adsorption chromatography. The gum (115 g) of the AB fraction was dissolved in 500 ml of distilled water and adsorbed on charcoal column (300 g), which was eluted with water-acetone mixtures containing an increasing acetone concentration by 5% steps every three fractions (5 liters a fraction from 5 to 15% and 2 liters a fraction from 20 to 100%). The fractions (30%-2, 35%-1, 2, 3, 40%-1 and 2) exhibiting the biological activity on rice seedling test (Tan-Ginbozu) were combined and concentrated to give a gum (1.42 g).

c) Partition chromatography. Ninety ml of 1 M phosphate buffer (pH 5.5) saturated with ethyl acetate containing 20% *n*-butanol was thoroughly mixed with 20 g of Sephadex G-50. The gel was suspended in the above solvent and packed in a glass column. The gum (1.42 g) obtained in *b)* was dissolved in 5 ml of the buffer, adsorbed in 1.2 g of Sephadex G-50, and placed on the top of the column. The column was eluted with ethyl acetate-*n*-butanol mixture containing an increasing *n*-butanol concentration by 2% steps, started with 20%. Six fractions (100 ml a fraction) were obtained at each concentration. Two different gibberellin-like substances (PG-I, PG-II) were shown to be separately eluted in a fraction (20%-1; PG-II) and in other fractions (22%-4, 5, 6, 24%-1, 2, 3, 4, and 5; PG-I) by TLC (solvent system (i)).

d) Preparative TLC. The fractions (22%-4 to 24%-5, 133 mg) containing PG-I was subjected to preparative TLC (solvent system (i)). The zone of $R_f 0.3 \sim 0.4$ afforded crude PG-I as a gum (56 mg).

e) Preparative PPC. Crude PG-I (56 mg) was developed on Toyo filter paper (No. 51) with $i\text{PrOH} : 5\text{ N NH}_4\text{OH} : \text{H}_2\text{O}$ (8:1:1). Monitored by spraying with aqueous potassium permanganate, three zones ($R_f 0.4 \sim 0.5$, $0.55 \sim 0.6$ and $0.63 \sim 0.7$) were cut off and eluted with methanol. Finally, 38 mg of PG-I (an ammonium salt of gibberellin A₃₂) was obtained as needles from the zone of $R_f 0.63 \sim 0.7$.

f) Silica gel adsorption chromatography. The

fraction (20%-1, 154 mg) in *b)* was dissolved in water and extracted with ethyl acetate at pH 3.0. The ethyl acetate fraction (33 mg) was subjected to silica gel (500 mg) adsorption chromatography eluted with benzene-ethyl acetate mixtures. The ethyl acetate concentration was increased by 5% steps in 10 ml fractions, started with benzene containing 20% ethyl acetate. The less polar gibberellin-like substance (PG-II) was eluted in the fractions of 30~40% ethyl acetate in benzene.

g) Preparative TLC. The fractions (4.6 mg) containing PG-II were combined and subjected to preparative TLC (solvent system (ii)). Recrystallization of the solid obtained from the zone of $R_f 0.25 \sim 0.4$ afforded PG-II (1 mg) as needles (mp $150 \sim 151^{\circ}\text{C}$).

2) Isolation of gibberellin A₅ (PG-III)

a) Extraction and fractionation. Immature seeds (ca. 10 kg) were obtained from young fruits (ca. 300 kg) plucked in 1972 in the same manner as described in 1)-a). Extraction and fractionation were also carried out in the same procedure described in 1)-a) to give the AE fraction (12 g).

b) Countercurrent distribution. The AE fraction (12 g) was subjected to 10 transfers countercurrent distribution between ethyl acetate (200 ml) and 1.5 M-phosphate buffer (pH 6.2, 200 ml). Fractions No. 1 (4.29 g),* No. 2 (786 mg) and No. 3 (310 mg) showed the biological activity.

c) Charcoal adsorption chromatography. Succinic acid (2.8 g) was precipitated on treatment of the fraction No. 1 in *b)* with methanol. The residue (1.49 g) obtained by evaporation of methanol was combined with fractions No. 2 and No. 3, and dissolved in warm water (10 ml). The solution was chromatographed on a charcoal column (15 g), which was eluted with water-acetone mixtures containing an increasing acetone concentration by 5% steps in 200 ml fractions. The fractions eluted with 65~80% acetone in water were found to contain a gibberellin-like substance (PG-III) on TLC.

d) Silica gel adsorption chromatography. The fractions (173 mg) containing PG-III was chromatographed on a silica gel (4 g) eluted with benzene-ethyl acetate mixtures. Started from benzene containing 10% ethyl acetate, the concentration of the ethyl acetate was increased by 5% steps in 50 ml. PG-III was eluted with benzene containing 20~30% ethyl acetate. Concentration of these fractions afforded an acidic gum (15 mg).

* In the countercurrent distribution method used throughout this study, fraction No. 1 contains the most polar substances and increasing number of fraction indicates to contain less polar substances.

e) Preparative TLC. The acidic gum (15 mg) obtained in *d)* was purified by preparative TLC (double development, solvent system (iii)) to give 0.2 mg of an amorphous solid showing a single spot on TLC.

f) TLC, GC, and MS. PG-III showed the same *R_f* values in several solvent systems as gibberellin A₅, namely *R_f* 0.60, 0.38, and 0.89 in solvent systems (i), (iii), and (iv), respectively. The methyl ester afforded by treatment of PG-III with ethereal diazomethane also showed the same *R_f* value as that of gibberellin A₅ methyl ester²⁾; *R_f* 0.30 in solvent system (vi). Besides, the retention time on GC and the fragmentation pattern in the MS of the methyl ester of PG-III were identical with those of gibberellin A₅ methyl ester. *T_R* 4.6 min (OV-1, 2% on Chromosorb W, 1 m × 3 mm, N₂ flow 31 ml/min, oven temp. 196°C). MS *m/e*: 344 (M⁺), 312 (M⁺-32), 300 (M⁺-44), 285 (M⁺-59).

3) Isolation of *cis, trans*-(+)-abscisic acid from young fruits

a) Extraction and fractionation. Young fruits (ca. 100 kg), from which seeds were removed, were extracted with methanol using a blender. Fractionation of the extract was carried out in the same manner described in 1)-*a)* to give the AE fraction (24 g).

b) Countercurrent distribution. The AE fraction (24 g) was subjected to 12 transfers countercurrent distribution between 1 M-phosphate buffer (pH 5.5, 500 ml) and ethyl acetate (500 ml). Fractions No. 10~No. 13 showed apparent growth inhibition on rice seedling test. These fractions were combined and concentrated to give an acidic gum (4 g).

c) Silica gel adsorption chromatography. The acidic gum in *b)* was chromatographed on a silica gel (20 g) column eluted with benzene-ethyl acetate mixtures containing an increasing ethyl acetate concentration by 5% steps in 300 ml fractions. The inhibitor was eluted in the fractions containing 20~45% ethyl acetate, which afforded a gum (840 mg).

d) Partition chromatography. The gum (840 mg) in *c)* was dissolved in 10 ml of *n*-hexane containing 5% ethyl acetate and subjected to a partition column of silica gel (500 g) pretreated with 400 ml of 1M-phosphate buffer (pH 6.2). The column was eluted with *n*-hexane-ethyl acetate mixtures containing an increasing ethyl acetate concentration by 5% steps every 5 fractions (100 ml in each fraction). The inhibitor was eluted in three fractions (20%-1, 2, 3), which were combined and concentrated to a gum (160 mg).

e) Preparative TLC. The gum (160 mg) in *d)* was purified by preparative TLC (double development, solvent system (iii)). The zone of *R_f* 0.4~0.5 was eluted with ethyl acetate and a crude inhibitor (2.2 mg)

was obtained. Recrystallization from hot benzene afforded pure but amorphous white powder (1.9 mg) showing a single spot on TLC developed with several solvent systems.

f) TLC, GC, and MS. The inhibitor obtained in *e)* showed the same *R_f* values on TLC as *cis,trans*-(+)-abscisic acid, namely *R_f* 0.72 in solvent system (i) and *R_f* 0.90 in (v). The methyl ester obtained by treatment of the inhibitor with ethereal diazomethane showed the same *R_f* value on TLC (0.47 in solvent system (vi)), retention time on GC (7.6 min, QF-1, 2% on chromosorb W, 1 m × 3 mm, N₂ flow 27 ml/min, oven temp. 179°C) and fragmentation pattern in MS (M⁺; *m/e* 278) as those of methyl *cis,trans*-(+)-abscisate. The optical rotatory dispersion of the inhibitor in methanol possesses a positive Cotton curve (289 nm [α] +24000, 246 nm [α] -69000). Thus, the inhibitor was identified to be *cis,trans*-(+)-abscisic acid.

4) Isolation of *cis,trans*-(+)-abscisic acid from water sprouts

a) Extraction and fractionation. Water sprouts (ca. 30 kg fresh weight) was used as material. After removal of leaves, the water sprouts were chopped into 5~10 cm in length and extracted with methanol three times repeatedly. The methanol extract was fractionated in the same manner described in 1)-*a)*.

b) Countercurrent distribution. The AE fraction (41 g) was subjected to 10 transfers countercurrent distribution between 1 M-phosphate buffer of pH 5.8 (1 liter) and ethyl acetate (1 liter). The least polar fraction (No. 11, 12.7 g) showed apparent growth inhibition on rice seedling test.

c) Silica gel adsorption chromatography. The fraction No. 11 (12.7 g) in *b)* was chromatographed on a silica gel column (200 g) eluted with benzene-ethyl acetate mixtures containing an increasing ethyl acetate concentration by 5% steps in 2 liters fractions. The inhibitor was eluted in the fractions containing 20~25% ethyl acetate, which were concentrated to a gum (570 mg). This gum was rechromatographed on a silica gel column (10 g). The column was eluted in a linear gradient elution mode which was conducted with benzene (200 ml)—ethyl acetate (200 ml) mixtures containing 0.5% acetic acid, and each 20 ml eluate was collected as one fraction. The fractions No. 8~No. 10 contained the inhibitor.

d) Preparative TLC. The gum (47 mg) from the fractions No. 8~No. 10 in *c)* was subjected to preparative TLC in the manner described in 3)-*e)*. Finally, 11.0 mg of the inhibitor was obtained as white powder showing a single spot on TLC. This inhibitor was identified as *cis,trans*-(+)-abscisic acid (see 3)-*f)*).

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