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ISOLATION OF GIBBERELLIC ACID PRODUCED BY *FUSARIUM MONILIFORME*

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ABSTRACT.—A new method for gibberellic acid isolation from the cultural medium of *Fusarium moniliforme* strain 3211 was developed. The method was based on acid-base extraction of gibberellins from an H_2O phase containing ammonium ions. Under these conditions gibberellic acid (GA_3) and GA_1 were separated. The other gibberellins were isolated by addition of MeOH to the residual H_2O phase and extraction with EtOAc. This method allows the preparation of GA_3 with purity above 75%, practically free of GA_4, GA_7, GA_9, GA_{13}, and GA_3-isolactone.

The gibberellins (GA) are known as plant growth regulators with a hormonal functions. Their main producer is the mold *Fusarium moniliforme* Sheldon [*Gibberella fujikuroi* (Sawada) Wollenweber] (1). This mold produces a complex containing several gibberellins including some with different biological functions (GA_1, GA_3, GA_4, GA_7, GA_{13}) and the biologically inactive GA_3-isolactone. The isolation of the individual gibberellins has been carried out by cc on Si gel (2) and Sephadex LH 20 (3), hplc (4,5), and preparative tlc (6).

Attempts at purification of gibberellins by acid-base extraction have led to isolation of a mixture of GA_3, GA_1, GA_4, GA_7, and GA_3-isolactone (7). In a previous work, we reported the separation of some gibberellins (GA_1, GA_3, GA_4, GA_7, and GA_9) contained in the culture filtrate of *F. moniliforme* as a mixture by tlc, gc, and hplc. In this report the gibberellins were separated by absorption on Amberlite XAD-2 (8).

The purpose of our investigations was to find a method for both the separation of the main gibberellins contained in the cultural filtrate of *F. moniliforme* 3211 and preparation of pure GA_3 on a large scale.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—*F. moniliforme* strain 3211 was isolated in our laboratory and deposited in the collection of the Institute of Microbiology. Large scale fermentations were carried out in a 100-liter tank fermentor under conditions described previously (9). The concentration of the main gibberellins in the cultural medium and crude preparations was determined by tlc combined with fluorescent densitometry and gc (9).

ISOLATION OF GA_3 AND GA_1.—The filtrate of the fermentation broth (50 liters) was acidified to pH 2.0–2.5 with 18% (w/v) HCl and extracted twice with 50 liters of EtOAc. The combined organic layers were concentrated to $^1/_5$ of their volume under reduced pressure at 35° and re-extracted with 1N NH_4OH (2×10 liters). These extracts were acidified with HCl as described above and extracted again with EtOAc (2×10 liters). The upper organic phases were dried over anhydrous Na_2SO_4 and concentrated to $^1/_{50}$ of their initial volume. The concentrate was stored at 8° for crystallization. The white crystals were filtered and rinsed with cold EtOAc. The filtrate was twice concentrated and crystallized as described above. The combined crystals designated as Fraction I were obtained with a yield of approximately 55–60% of the original GA_3 content (22–24 g).

PREPARATION OF OTHER GIBBERELLINS.—MeOH (10% v/v) was added to the residual H_2O phase (pH 2.0–2.5) and then was extracted twice with 10 liters of EtOAc. The combined EtOAc extracts were filtered through a layer of activated charcoal and Na_2SO_4 (1:1). The sorbent was rinsed with EtOAc ($^1/_4$ from its initial volume). The gibberellins were precipitated from the combined EtOAc solutions by addition of petroleum ether (bp 70–100°), to complete sedimentation. The sediment was filtered and rinsed with EtOAc-petroleum ether (1:1). This preparation was designated as Fraction II. Fraction II constituted approximately 45–50% of the GA_9, GA_4, GA_7, GA_{13}, GA_3-isolactone content (16–17 g) as determined in the initial cultural medium.

IDENTIFICATION OF GIBBERELLINS.—Tlc of the gibberellins was performed using silica plates

(Merck) with $CHCl_3$-EtOAc-MeOH-HOAc (20:20:2:0.2) as the developing solvent. The gibberellin spots were visualized by spraying with H_2SO_4-EtOH (1:1). For identification of GA_9 and GA_1, the plates were heated for 5 and 20 min, respectively, at 120°. R_f values of the spots were measured by fluorescent densitometry at 345 nm. The fluorescent spectra of the gibberellins were scanned in situ on the plates using a Shimadzu CS 930 tlc scanner.

The gibberellins were derivatized to methyl esters by reaction with CH_2N_2, followed by treatment with *N,O*-bis-trimethylsilylacetamide-oxalic acid in order to obtain methylester TMSi ethers. The derivatives were applied to a JEOL gc-ms apparatus. The conditions for gc were as described elsewhere (10). Mass spectra were scanned by the electron impact method at 70 eV.

GA_9, GA_4, GA_7, GA_1, and GA_{13} standards were kindly supplied by Dr. N. Takahashi (Japan). GA_3 with purity of 80% was purchased from Fluka (Switzerland). An Et_2O solution of CH_2N_2 was obtained by the reaction with Diazald (Merck) and KOH. The other chemicals were of an analytical grade purity.

RESULTS AND DISCUSSION

The recommended method for isolation of gibberellins from the cultural medium of *F. moniliforme* has been extraction with EtOAc (at pH 2.0) followed by re-extraction with $NaHCO_3$ solution (1). We modified this method by replacing $NaHCO_3$ with NH_4OH. The reason for that was our observation that GA_3 and GA_1 could be separated from the other gibberellins by an extraction with EtOAc from the H_2O phase containing ammonium ions. Under these conditions GA_3 and a portion of GA_1 were extracted into the organic phase. The acidification and extraction of the gibberellins should be done immediately in order to avoid isomerization of GA_3 to GA_3-isolactone. GA_3 and GA_1 crystallized easily from the concentrated EtOAc solutions with a purity of the final product of 84.60% and a yield of 55–60% of the original GA_3 content. Fraction I contained as a main component GA_3 (73.2%) and GA_1 (11.4%) according to gc data compared to standards (Table 1). The repeated crystallization led to an increase in the purity of Fraction I to 97.0% (85.0% GA_3 and 12.0% GA_1).

Gc-ms analysis of the gibberellins contained in Fraction I showed the following results: the mass spectra of peaks 1 and 2 were characterized by molecular ions at *m/z* 506 and 504, respectively, and $[M-15]^+$, $[M-31]^+$, and $[M-60]^+$ ions. The base peaks of the ms at *m/z* 75 and 73 were associated with TMSi ether groups. Ions with *m/z* 209 and 207 were very characteristic for GA_1 and GA_3 (11). The results of tlc and gc suggested the presence mainly of GA_3 and GA_1 in Fraction I.

The H_2O phase, after extraction of GA_3 and GA_1, contained mainly unpolar gibberellins. We observed that decreasing the polarity by addition of MeOH to a 10% concentration increased gibberellin solubility in organic solvents. The extracts in EtOAc under these conditions contained a lot of impurities, and further purification by filtration through a mixture of activited charcoal and Na_2SO_4 and

TABLE 1. Concentration of the Gibberellins in Fractions I and II Determined by Gc as Methyl Ester TMSi Ethers.[a]

Gibberellins	Fraction I (%)	Fraction II (%)
GA_9	—	1.74
GA_4	0.45	11.64
GA_7	—	11.40
GA_{13}	1.75	11.07
GA_3-isolactone	1.60	13.41
GA_1	11.40	1.40
GA_3	73.20	4.83

[a] GA_{25} was not determined quantitatively because of the absence of a suitable standard.

sedimentation with petroleum ether gave a more reliable product (Fraction II). GA_4, GA_7, and GA_9 were identified in this fraction by their characteristic fluorescence on the tlc plates (reaction with H_2SO_4) and gc (as methyl ester TMSi ethers) compared with standards. Gc-ms analysis provided the following data.

Two main peaks on the chromatogram with Rt 17.8 and 18.4 min were identified as GA_4 and GA_7 by their molecular ions at *m/z* 418 and 416, respectively. The mass spectra of these peaks were characterized with ions $[M-134/135]^+$ and $[M-193/194]^+$, typical of GA_4 and GA_7.

The peak with Rt 13.9 min was identified as GA_9-methyl ester by its molecular ion at *m/z* 330 and loss of two methoxycarbonyl groups, $[M-31/32]^+$ and $[M-59/60]^+$.

One of the unknown peaks in the chromatogram was identified as a GA_{25}-trimethyl ester because of the intensive ion with *m/z* 312 $[M-92]^+$; loss of 60 and 32 mass units from the molecular ion, and ions with *m/z* 284 and 225, associated with splitting of two or three methoxycarbonyl groups. In the mass spectra of GA_9 and GA_{25} we did not find the peaks of TMSi ether groups, which showed the absence of hydroxyl groups.

The peak with Rt 19.6 min was identified as GA_{13}, a very intensive peak with *m/z* 129, characteristic of 2-hydroxygibberellins ($C_6H_{13}OSi$). We did not observe the peak of the molecular ion (theoretically 492), but it is indicated by the peaks of $[M-32]^+$, $[M-160]^+$, and $[M-120]^+$ ions. The fragments with *m/z* 310, 282, 251, and 160 have been shown as characteristic of GA_{13} (11).

The peak with Rt 20.6 min had a molecular ion at *m/z* 504. The fragmentation pattern of this peak was very similar to GA_3. These data suggest it to be a GA_3-isolactone.

The data from these experiments demonstrate a methodology for the specific enrichment of GA_3 (with minor contamination of GA_1) from other gibberellins. We hope that this new method could find application as a part of the fermentation technology for preparation of plant hormones.

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