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GIBBERELLIC ACID DECOMPOSITION AND ITS LOSS OF BIOLOGICAL ACTIVITY IN AQUEOUS SOLUTIONS

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Key Word Index—*Gibberella fujikuroi*; gibberellins; gibberellic acid (GA_3); iso-GA_3; gibberellenic acid.

Abstract—In addition to gibberellic acid (GA_3), significant amounts of iso-GA_3, an isomer of GA_3, accumulated in cultures media of the fungus *Gibberella fujikuroi*. It has been reported that iso-GA_3 is an intermediate in the decomposition of GA_3 to gibberellenic acid, a dicarboxylic acid lacking the γ-lactone ring. In this work, we studied the decomposition of GA_3 in buffered aqueous solutions at pH 5 and 7, both by spectrophotometry, measuring the appearance of gibberellenic acid at 254 nm, and using a bioassay by measuring the induction of α-amylase in barley endosperm. Results indicate that at both pH values, biological inactivation of GA_3 is more rapid than the appearance of gibberellenic acid, suggesting that inactivation is largely due to conversion to the intermediate, iso-GA_3. Furtheremore, no induction of α-amylase activity in barley endosperm was obtained in bioassays with iso-GA_3.

INTRODUCTION

Gibberellic acid (GA_3) is a commercially important phytohormone which regulates many different plant growth and development processes [1]. Industrially, GA_3 is produced by liquid cultivation of the ascomycetous fungus *Gibberella fujikuroi* [1, 2]. However, solid substrate cultivation (SSC) of the fungus has also been reported as an alternative that results in higher GA_3 yields [3, 4]. We have shown that in both cultivation systems, a significant amount of the total GA_3, measured fluorimetrically [5], is iso-GA_3, the product of GA_3 isomerization [6]. Aqueous solutions of GA_3 decompose to give iso-GA_3, gibberellenic acid, allogibberic, 9-epi-allogibberic and 9,11-didehydroallo-gibberic acid in different proportions depending on reaction time, temperature and pH [7–9]. Recently, it has been reported that GA_3 decomposes in weak alkaline solutions to the corresponding dicarboxylic acid, via iso-GA_3 [10]. Therefore, conditions that slow GA_3 decomposition in both cultivation systems and data pertaining to the biological activity of iso-GA_3 are highly desirable in order to increase GA_3 yields by *G. fujikuroi*.

In the present work, we determined rate constants for GA_3 decomposition, measured by gibberellenic acid appearance and by loss of biological activity in buffered aqueous solutions at pH 5 and 7. The biological activity of iso-GA_3 in the α-amylase bioassay was also determined.

RESULTS AND DISCUSSION

Analysis of GA_3 and iso-GA_3 in cultures of G. fujikuroi

After 6 days of growth on wheat bran–starch medium, *G. fujikuroi* produced 5 g of total GA_3 per kg of solid substrate (initial dry weight), measured fluorimetrically [5]; this is greater than the yield of 0.3 $g\,l^{-1}$ obtained in liquid medium. However, GC-mass spectral analysis using selective ion monitoring (SIM) of the molecular ion from the trimethylsilyl derivative of GA_3 (GA_3-TMSi-TMSi), revealed the presence of two peaks with retention times of 23.4 min and 24.3 min, corresponding to iso-GA_3 and GA_3, respectively (Fig. 1), from the full-scan mass spectra of the respective peaks (see Experimental). Iso-GA_3 was detected in both cultivation systems by GC-mass spectrometry using total ion current (TIC). From the area of the corresponding peaks, it was estimated that in solid and liquid medium, respectively, 12% and 29% of total GA_3, measured fluorimetrically corresponded to iso-GA_3. GC-mass spectral analysis (TIC) of pure GA_3 run under the same experimental conditions showed one peak, indicating that GA_3, at least under these experimental conditions, does not isomerize to iso-GA_3 during gas chromatography analysis. Furthermore, pure GA_3 submitted to the same analysis as the samples, showed no isomerization. From the above

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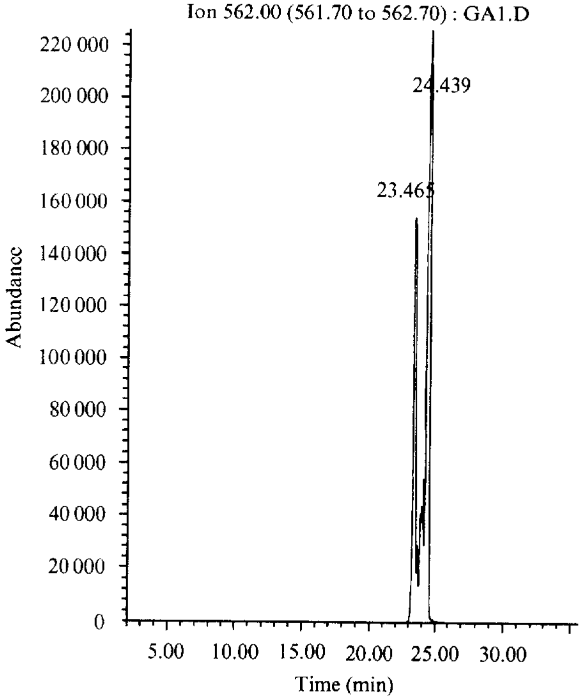

Fig. 1. GC-mass spectral analysis of an extract from solid substrate cultivation of *Gibberella fujikuroi*. The analysis was carried out by single-ion monitoring (SIM) at m/z 562.

results, we conclude that iso-GA_3 is produced in significant amounts during the culture of *G. fujikuroi* in liquid and solid medium. As cultures of *G. fujikuroi* produce a high yield of iso-GA_3, it was important to determine its biological acivity and which conditions could prevent its formation from GA_3.

Kinetics of GA_3 decomposition

Iso-GA_3 is not a product of *G. fujikuroi* fermentation, but is produced by isomerization of GA_3 in weak alkaline conditions [10, 11]. However, there is little information on the biological activity of iso-GA_3. Most biologically active giberellins have the C_{19} (20-norgibberellane) skeleton and possess a C-19/C-10 γ-lactone ring, whereas iso-GA3 has a C-19/C-2 γ-lactone ring [9]. Moreover, it has been suggested that biological inactivation of gibberellins is related to loss of the lactone ring [2]. Figure 2 shows the kinetics of GA_3 decomposition at pH 5 (A) and 7 (B) at 30 ± 1°, measured spectrophotometrically at 254 nm. At this wavelength, gibberellenic acid absorbs strongly ($\varepsilon = 21\,900\ M^{-1}$), whereas GA_3 does not and iso-GA_3 absorbs only weakly [12]. Hence, at this wavelength, it is possible to follow gibberellenic acid appearance. Experimental data points fit first-order rate equations with rate constants of 8.9×10^{-3} and $1.2 \times 10^{-2}\ hr^{-1}$ for decomposition at pH 5 and 7, respectively, calculated by a nonlinear regression program. The half-lives were 77.8 and 57.8 hr, respectively.

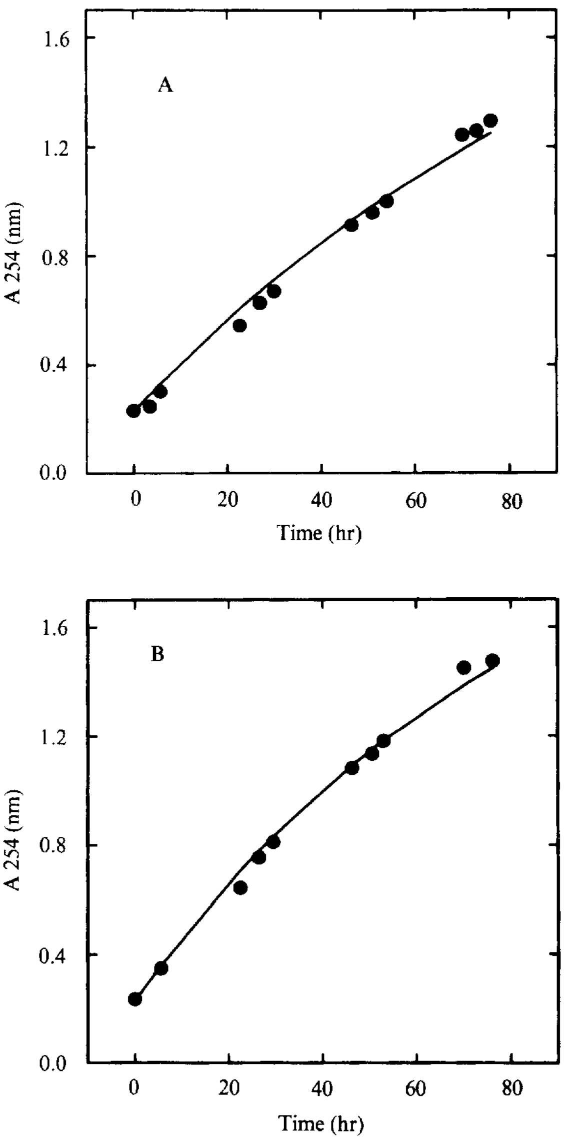

Fig. 2. Kinetics of the decomposition of gibberellic (GA_3) to gibberellenic acid in 0.1 mM citrate buffer pH 5.0 (A) and 0.1 mM phosphate buffer at pH 7.0 (B) at 30 ± 1°. The curves represent the fit of the experimental points to a first-order rate equation. The parameters, rate constants and infinite time were calculated by a nonlinear regression program.

Loss of GA_3 biological activity

α-Amylase is synthesized *de novo* in response to gibberellic acid in the aleurone layers of barley grain. This enzyme induction depends on mRNA and protein synthesis [13] and has been employed widely as a bioassay for measuring biologically active gibberellins [2]. α-Amylase activity from barley endosperm is related linearly to the log of GA_3 concentration over a wide concentration range. The response depends also on incubation time and temperature. In this study, incubation conditions (16 hr at 20 ± 1°) were chosen to minimize GA_3 decomposition. Under these experimental condi-

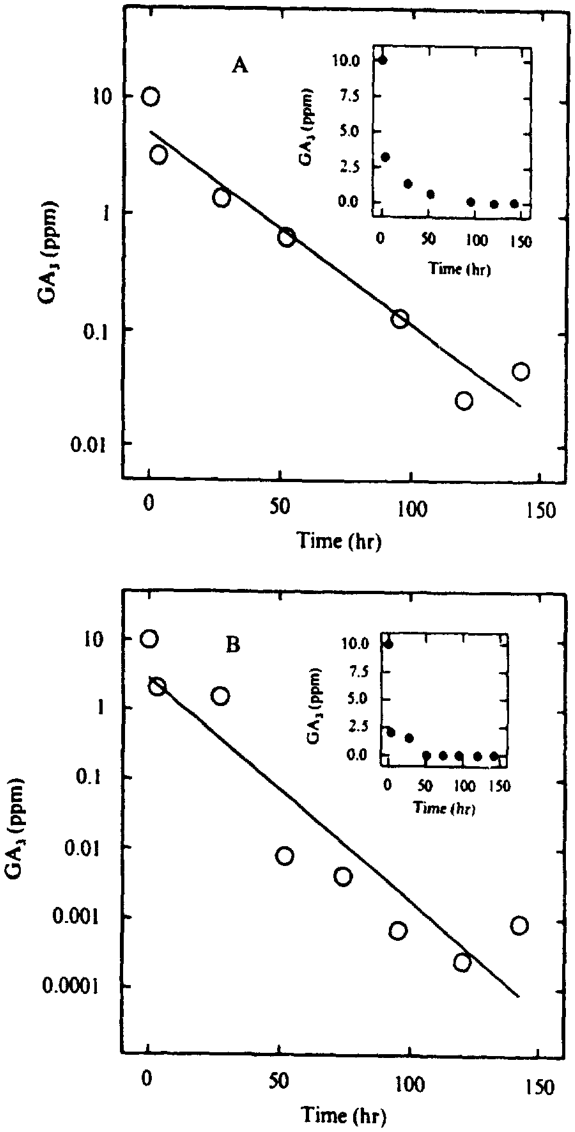

Fig. 3. Exponential decrease of GA_3 biological activity (measured by α-amylase bioassay) with time at pH 5.0 (A, inset) and 7.0 (B, inset). The curves in the semilog plots represent the fit of the experimental points to a first-order rate equation.

Table 1. Rate constants for GA_3 transformation into gibberellenic acid and for its biological inactivation in aqueous buffered solutions at 30 ± 1°

Reaction	pH	k (hr^{-1})	$t_{0.5}$ (hr)
Decomposition of GA_3	5.0	0.0089	77.8
into gibberellenic acid	7.0	0.012	57.8
Biological inactivation	5.0	0.016	43.3
of GA_3 (α-amylase bioassay)	7.0	0.032	21.6

tions, α-amylase activity of nontreated seeds was about 16 U, while seeds treated with 1 ppm of GA_3 gave around 50 U. Solutions of GA_3 (10 ppm) were incubated at 30 ± 1° for different times under the same experimental conditions as for the chemical decomposition experiments (see above). Results show that, at both pH values, biological inactivation of GA_3 decreased exponentially with time (Fig. 3A, B inset). The experimental points fit first-order rate equations (Fig. 3A, B) with rate constants of 0.016 and 0.032 hr^{-1} at pH 5.0 and 7.0, respectively. A comparison of these rate constants with those for gibberellenic acid appearance (Table 1) indicates that, at both pH values, biological inactivation of GA_3 occurs faster than its transformation to gibberellenic acid, suggesting that the first step in GA_3 transformation to gibberellenic acid, its isomerization to iso-GA_3 [10], results in loss of biological activity. Furthermore, bioassays carried out with iso-GA_3, obtained from GA_3 decomposition in 0.1 mM NaOH (Fig. 4), showed that iso-GA_3 and gibberellenic acid do not induce α-amylase activity in barley endosperm (Table 2).

The isomerizaation of GA_3 in weak alkaline conditions to the C-19/C-2 γ-lactone iso-GA_3 has been reported [10, 11]. The Δ'-double bond and the 3β-hydroxy group appear to be essential for the lactone rearrangement [14] and a 2β,3β-epoxy-19-carboxylate has been postulated as an intermediate in this rearrangement [14]. Moreover, those authors [14] claimed that this isomerization does not occur in aqueous solution, buffered at pH 5.2, 7.0 and 8.0. However, they used an aqueous solution containing 10% dioxan, stopped the reaction after 12 hr and analysed the products by ^1H NMR spectroscopy. Probably, in their case, the short reaction time, the use of a solvent mixture and the technique used for analysing the products did not allow GA_3 isomerization to be detected.

From the above results we conclude that GA_3 isomerizes to iso-GA_3 in aqueous solutions at neutral pH values and that this isomerization results in the loss of GA_3 biological activity.

EXPERIMENTAL

Inoculum preparation. G. fujikuroi ATCC-12616, a gibberellic acid hyperproducing strain was used throughout this study. Inocula were prepd in a liquid culture medium, containing (g l^{-1}): glucose (80), ammonium tartrate (1.84) potassium phosphate (5), magnesium sulphate (0.45) and 10 ml of a nutrient salts solns.

Cultivation system. Solid substrate cultures were run in Raimbault type columns [15], containing 6 g of organic support, essentially a wheat bran–starch based medium, as described in [3]. Columns were incubated in a water bath at 28°, and a constant saturated air flow was passed through the fermenting substrate at a rate of 4 l g^{-1} hr^{-1}.

Gibberellin extraction. Acidified water (3 × 2 ml) was added to 2 g of wet-fermented matter, pressed in a manual laboratory press, and then centrifuged. For GC-MS analysis, 50 μl of 2 N Na_2CO_3 were added to 0.5 ml supernatant (pH 7–8), and the latter was partitioned against 3 × 500 μl EtOAc. The remaining water phase was acidified to pH 2.5 with HCl and extracted with EtOAc (3 × 500 μl). The organic phases were combined and concentrated to dryness in a stream of N_2.

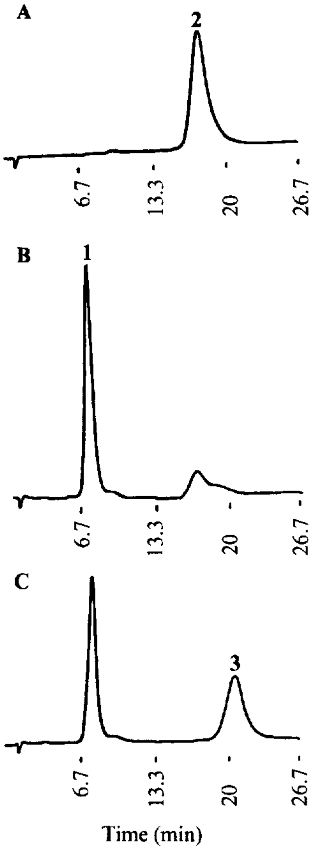

Fig. 4. HPLC chromatograms for the decomposition of GA_3 in 0.1 mM NaOH at 30 ± 1°, taken at 0 (A), 1 (B) and 6 hr (C). Peak (1) corresponds to iso-GA_3 (t_r = 7.4 min), peak (2) to GA_3 (t_r = 17.7 min) and peak (3) to gibberellenic acid (t_r = 20.5 min).

Analysis of GA_3 and iso-GA_3. Samples were analysed by GC-MS using a Hewlett Packard 5980 gas chromatograph coupled to a 5872 mass spectrometer. Samples in MSTFA (1 μl) were injected into a HP-5 fused silica WCOT capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) at an oven temp. of 60° maintained for 1 min. then increased at a rate of 20° min^{-1} to 240° and then at 4° min^{-1} to 300°. The He inlet pressure was 15 psi and injector, interface and MS source temps were 220, 280 and 200°, respectively. Mass spectra were acquired at 70 eV and total ion current (TIC) chromatograms were obtained. GA_3 and iso-GA_3 were identified as TMSi-TMSi derivates by full mass spectra. GA_3 TMSi-TMSi m/z (rel. int.) 562 $[M]^+$ (100), 547 (8.2), 445 (13.2), 427 (4.1), 297 (37.7), 208 (7.8). iso-GA_3 TMSi-TMSi m/z (rel. int.) 562 $[M]^+$ (100), 547 (25), 533 (14.6), 517 (16.4), 472 (12.8), 446 (17.6), 445 (45.7), 428 (35.4), 427 (64), 399 (21.3), 281 (7.4). Samples and standards were analysed by selective ion monitoring (SIM). The ion at m/z 562, which is the molecular ion for GA_3 and iso-GA_3-TMSi-TMSi [16], was monitored. Two peaks were present in the chromatograms of the samples analysed with retention times of 23.4 and 24.3 min corresponding to iso-GA_3 and GA_3-TMSi-TMSi, respectively.

α-Amylase bioassay. Seeds of barley (*Hordeum vulgare* L. cv. Cruzat) were cut longitudinally, the halves containing the embryo were discarded and the remaining halves containing the endosperm were surface-sterilized by incubating for 10 min at 20 ± 1° in 2.0% NaOCl and rinsed with distilled H_2O (5 ×). All subsequent manipulations were performed under aseptic conditions. Half-seeds were imbibed in distilled H_2O for 24 hr, separated in groups of 10, incubated either in solns of increasing concns of GA_3 ranging between 10^{-4} to 2 ppm (calibration curves) or in 10 ppm GA_3 solns incubated for different times at 20 ± 1° (samples soln) for kinetic studies. After incubation, 3 ml of 5 mM $CaCl_2$ soln was added (supernatant solns). Seeds were ground with mortar and pestle after adding 5 mM $CaCl_2$ (5 ml) (extract solns). The extracts were centrifuged at 2000 *g* for 15 min and the supernatant filtered through Whatman #1 filter paper. α-Amylase activity was assayed using 0.2 ml of the filtered extract diluted to 1 ml with distilled H_2O. The reaction was started by adding 1 ml of soln A containing 50 mM sodium acetate, pH 4.8, 0.03% soluble starch, 60 mM NaCl and 20 mM $CaCl_2$. The reaction was stopped by adding 1 ml of iodine reagent KI : I_2 0.6% : 0.06% (w/v). The amylase activity is expressed as the reduction in absorbance at 620 nm after 5 min incubation at 20 ± 1°, when the reaction is in the linear phase. One unit of α-amylase activity was defined as 1 μg min^{-1} of starch consumed by 0.2 ml of the extracts.

Kinetics of GA_3 inactivation. Solns of 10 ppm GA_3 in 0.1 mM sodium acetate at pH 5.0 or phosphate pH 7.0 were incubated at 30 ± 1°. After different incubation

Table 2. α-Amylase activity of barley endosperm induced by GA_3, iso-GA_3 and gibberellenic acid

Concentration of test compounds (ppm)	α-Amylase activity induced by		
	GA_3	iso-GA_3	Gibberellenic acid
1×10^{-4}	27.6 ± 1.2	29.0 ± 1.9	27.5 ± 2.2
1×10^{-3}	35.2 ± 1.4	18.5 ± 0.4	29.3 ± 1.0
1×10^{-2}	38.4 ± 1.7	37.0 ± 1.3	20.6 ± 0.9
1×10^{-1}	49.2 ± 0.5	35.3 ± 2.3	35.0 ± 4.5
1.0	50.5 ± 0.5	20.7 ± 0.3	29.9 ± 0.6
2.0	52.3 ± 3.5	28.7 ± 1.0	–

Values are means (n = 3) ± s.e.

times GA_3 activities were bioassayed by the α-amylase test. Units of α-amylase activities were converted to concentration of GA_3 in ppm by means of a calibration curve.

Kinetics of GA_3 decomposition. Solns of 0.1 mM GA_3 were prepared in 0.1 mM sodium acetate or phosphate at pH 5.0 and 7.0, respectively. The reactions were monitored at 254 nm in a Shimadzu 240 UV-vis spectrophotometer using 1 ml quartz cell thermostated at $30 \pm 1°$. Rate constants and infinite time were calculated using a nonlinear regression program.

Preparation of iso-GA_3. GA_3 (50 mg) was dissolved in 5 ml of a 0.1 mM NaOH soln and incubated at $30 \pm 1°$. The isomerization of GA_3 into iso-GA_3 was followed by HPLC as described previously [10]. Once all GA_3 was transformed into iso-GA_3 the reaction mixt. was acidified to pH 2.5 with HCl and extracted immediately with 3×3 ml of EtOAc. The peak corresponding to iso-GA_3 (Fig. 4) was analysed by GC-MS.

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