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Radioimmunoassay of Gibberellins A_5 and A_{20}

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Polyclonal anti-GA_5-antiserum and anti-GA_{20}-antiserum were prepared by immunizing rabbits with conjugates of *N*-GA_5-β-alanyl BSA and *N*-GA_{20}-β-alanyl BSA. By radioimmunoassay using these antisera, GA_5 and GA_{20} in developing fruits of *Pharbitis nil* Chois were analyzed after several purification steps and the results were compared with those obtained by GC-MS to examine the reliability of the analysis by radioimmunoassay. The analytical results by radioimmunoassay did not agree with those by GC-MS until two-step purifications by HPLC, indicating that samples must be suitably purified prior to radioimmunoassay to obtain reliable results.

Key words: GC-MS — Gibberellin (Radioimmunoassay) — Immunoassay (Gibberellin) — *Pharbitis nil.*

The qualitative and quantitative analysis of gibberellins by GC-MS has been shown to be a very powerful and reliable technique for the study of the roles of gibberellins in plant growth and development in terms of high sensitivity and accuracy. The disadvantage of GC-MS is that it requires rather complicated prepurification and expensive instruments.

Weiler and his coworkers have shown that immunological analyses of gibberellins (Weiler et al. 1981, Atzorn et al. 1983a, b, Eberle et al. 1986) could be as effective and promising as GC-MS in the study of gibberellins. Especially their high sensitivity and convenience in dealing with large numbers of samples without extensive prepurification are considered to be very attractive and advantageous. However, in the case of analysis of gibberellins, such attractive immunological methods may encounter some problems of cross-reactivity, because gibberellins are a large family consisting of at present 72 members with a wide range of polarities and some of them very similar in structure.

We have developed an immunoassay system for GA_5 and GA_{20}, which are structurally very similar, and have compared the analytical data of these gibberellins obtained by RIA and by GC-SIM, in the hope of obtaining information on the scope and limitations of immunoassays for plant hormones with a large number of similar structures.

Materials and Methods

Preparation of immunogens—*N*-Gibberellin-β-alanine: GA_{20} (31.3 mg, 0.094 mmol) and *N*-hydroxysuccinimide (11.9 mg, 0.10 mmol) were dissolved in 200 μl of dry dimethylformamide in

Abbreviations: AE, acidic ethyl acetate; BSA, bovine serum albumin; GC-MS, gas chromatography-mass spectrometry; GC-SIM, gas chromatography-selected ion monitoring; GPC, gel permeation chromatography; RIA, radioimmunoassay; TMSi, trimethylsilyl.

a small glass vial. To this mixture, 0.14 mmol of *N,N'*-dicyclohexylcarbodiimide dissolved in 207 μl of dimethylformamide was added with thorough mixing. The reaction was continued for 8 h at room temperature. After the addition of 40 μl of water to decompose the excess dicyclohexylcarbodiimide, the reaction mixture was centrifuged to remove the dicylcohexylurea. The supernatant was concentrated in vacuo and purified by silicic-acid adsorption chromatography eluted with mixtures of *n*-hexane and ethyl acetate. From the fractions eluted with 50% and 60% ethyl acetate, 38.2 mg (0.089 mmol) of GA_{20}-active ester was obtained. The active ester (38.2 mg) was dissolved in 800 μl of acetonitrile containing 10% water, and 0.2 mmol of β-alanine triethylamine salt dissolved in 200 μl water was added to the active ester solution. The reaction was continued for one week at room temperature. A concentrate obtained by removal of the solvent in vacuo was subjected to silicic-acid column chromatography and the column (20 cm × 1 cm) was eluted with mixtures of ethyl acetate and chloroform containing 3.5% acetic acid. From the 50% ethyl acetate fraction, 28 mg of *N*-GA_{20}-β-alanine was obtained. ^{1}HNMR ($^2H_6$-acetone): 1.05 (18-CH_3), 2.6–2.9 (N-CH_2-CH_2-CO), 3.6–3.8 (5-H, 6-H), 5.0 and 5.3 (17-H_2), 7.9 (NH). In a similar way, 21.4 mg (0.065 mmol) of GA_5 yielded 24.3 mg (0.057 mmol) of GA_5-active ester, which was converted to *N*-GA_5-β-alanine (21.6 mg) in a similar way. ^{1}HNMR ($^2H_6$-acetone): 1.12 (18-CH_3), 2.4–2.7 (N-CH_2-CH_2-CO), 3.4–3.7 (5-H, 6-H), 4.98 and 5.24 (17-H_2), 5.7–6.1 (2-H, 3-H), 7.78 (NH). *N*-Gibberellin-β-alanyl-BSA: A mixture of *N*-GA_{20}-β-alanine (28 mg, 0.07 mmol) and *N*-hydroxylsuccinimde (8.5 mg, 0.074 mmol) was dissolved in 150 μl of dimethylformamide, and 0.075 mmol of dicyclohexylcarbodiimide in 150 μl of dimethylformamide was added to the solution. The reaction was continued for 24 h at room temperature and 30 μl of water was added. The dicyclohexylurea which formed was removed by centrifugation. The supernatant containing the desired active ester of *N*-GA_{20}-β-alanine was immediately used without further purification for the coupling with BSA. Crystalline BSA (39 mg) purchased from Nakarai Kagaku Co., Ltd. (Tokyo) was dissolved in 1 ml of dimethylformamide–water (1 : 2) containing 7.8 μl of triethylamine. To this solution, the supernatant containing the active ester was added at 5°C. The reaction was continued under N_2 at 5°C for 24 h. The reaction mixture was injected into a Sephadex G-10 column (total volume, 150 ml) and eluted with the lower phase of the solvent mixture of *n*-butanol : acetic acid : water (4 : 1 : 5). The eluate from 65 ml to 110 ml was combined and lyophilized. The crude material thus obtained was further purified using a Sephadex G-25 column (total volume, 50 ml) eluted with 5% acetic acid, and 28 mg of the conjugate of *N*-GA_{20}-β-alanine and BSA was obtained after lyophilization of fractions of 20–30 ml elution volume. In a similar way, 72 mg of *N*-GA_5-β-alanyl-BSA was prepared from 21.6 mg of *N*-GA_5-β-alanine and 60.5 mg of BSA.

Immunization and antiserum preparation—Immunogen (approx. 1 mg) was dissolved in 0.5 ml of phosphate buffer saline (0.01 M phosphate, 0.15 M NaCl, pH 7.4) and mixed with 0.5 ml of Freund's complete adjuvant to form an emulsion of water-in-oil, which was administered to two New Zealand white rabbits initially via the foot pad and then subcutaneously four times at one week intervals. When the titer (final dilution of antiserum binding approx. 30% of tracer in assay mixture) exceeded 30,000 after three booster injections at three-week intervals, blood was collected from the carotid artery, warmed at 37°C for 15 min, and kept in a cold room overnight. The blood cake was removed, and the antiserum was heated at 57°C to decompose complement. Sodium azide was added to 0.1% concentration. The antiserum was stored at −80°C and thawed just before use.

Preparation of tracers—tritiated gibberellin methyl esters—Two milligrams (0.06 mmol) of GA_{20} was dissolved in 100 μl of dimethylformamide and 0.05 mmol of sodium methoxide (0.5 mmol/ml) was added. To this mixture, 0.03 μmol of C^3H_3I (70 Ci/mmol, Amersham) in 1 ml of toluene was added at 0°C, and the reaction was continued in a sealed vial for 4 days at room temperature. After the removal of solvents under N_2 flow, the crude tritiated GA_{20} methyl ester was immediately

purified by preparative TLC developed in *n*-hexane–ethyl acetate (1 : 1). Tritiated GA_5 methyl ester was prepared in a similar way.

Plant material and processing—Immature fruits of *Pharbitis nil* harvested at 2, 4, 8, 16, and 20 days after flowering were used for the analyses. Materials were homogenized in 70% acetone and subjected to a general solvent fractionation procedure to produce an AE fraction (Takahashi et al. 1986). A sample of the AE fraction was methylated and analyzed by RIA. Another sample of the AE fraction was subjected to GPC on a Shodex HF-2001 (50 cm × 20 mm) eluted with tetrahydrofuran at a flow rate of 3.2 ml/min, and the eluate of the retention time of 23–25 min, at which GA_5 and GA_{20} were eluted, was collected. A part of the GA_5/GA_{20} fraction was methylated and analyzed by RIA. The rest of the GA_5/GA_{20} fraction was further purified using a Nucleosil $N(CH_3)_2$ column to separate GA_5 from GA_{20}. Each fraction containing GA_5 or GA_{20} was analyzed by means of anti-GA_5-antiserum and anti-GA_{20}-antiserum.

For GC-SIM, deuterated GA_5 and GA_{20} were added as internal standards to another sample of the AE fraction, which was purified by GPC and then by a nucleosil $N(CH_3)_2$ column to produce GA_5 and GA_{20} fractions (Yamaguchi et al. 1982).

Gas chromatography-selected ion monitoring—Samples were dried and trimethylsilylated in a mixture of *N,O*-bistrimethylsilylacetamide : pyridine : trimethylchlorosilane (10 : 20 : 1). For accurate estimation of the endogenous levels of GA_5 and GA_{20}, (1,2,3,6-$^2H_4$)-GA_5 and (17,17,15-$^2H_3$)-GA_{20} were used as internal standards, and the peak area ratios of the molecular ions, m/z 474(d_0)/476 (d_2) for GA_5 and m/z 476(d_0)/479(d_3) for GA_{20}, were used for quantification.

A Hitachi M-80A gas chromatography-mass spectrometer was used under the following conditions: column, 2% OV-1 on Chromosorb W (AW DMCS)(1 m × 3 mm i.d.); column temperature, 200°C; He flow, 45 ml/min; ionization, EI 22 eV.

Radioimmunoassay—RIA was performed by a procedure similar to that describd by Weiler and Wieczorek (1981), with the assay mixture containing 100 μl of diluted antiserum, 100 μl of diluted rabbit or bovine serum as a carrier, tracer (tritiated methyl ester of gibberellin) dissolved in 300 μl of phosphate buffered saline, and 100 μl of sample to be analyzed or standard sample (non-labeled gibberellin methyl ester) in 5% methanol. Samples were methylated with ethereal diazomethane, dissolved in 5% methanol and used for the assays. Assays were done in triplicate for each dilution of samples. Cross-reactivities of a variety of gibberellins were examined according to the method reported by Weiler and Zenk (1976). In RIA recovery of GA_5 and GA_{20} through prepurification was not examined because it is usually over 80% (Yamaguchi et al. 1982) and accurate values could be obtained by GC-SIM, in which deuterated internal standards were used.

Results

Immunogen—The preparation procedures for *N*-GA_{20}-β-alanyl-BSA and a tritiated tracer are shown in Fig. 1. The structures of *N*-GA_5-β-alanine and *N*-GA_{20}-β-alanine were confirmed by ^{1}HNMR. In their spectra, characteristic signals due to GA_5 and GA_{20} were observed together with those of β-alanine, suggesting that neither GA_5 nor GA_{20} was susceptible to structural change during the peptide-bond formation via active esters. The molar ratios of *N*-GA_5-β-alanine and *N*-GA_{20}-β-alanine bonded to BSA were estimated from the molar ratios of β-alanine to alanine, phenylalanine and/or lysine by amino acid analyses of the hydrolyzates of the conjugates. In the case of *N*-GA_5-β-alanyl-BSA, about 10–11 moles of GA_5 were coupled to 1 mole of BSA, and in the case of *N*-GA_{20}-β-alanyl-BSA, 16–17 moles of GA_{20} were coupled to 1 mole of BSA.

Assay parameters—The characteristics of two anti-GA_5-antisera were shown to be very similar, with only a slight difference between their titers. The same tendency was observed for the two anti-GA_{20}-antisera. Table 1 summarizes the general parameters of an anti-GA_5- and an anti-GA_{20}-antiserum. The logit/log plotted standard curves with anti-GA_5- and anti-GA_{20}-antisera are

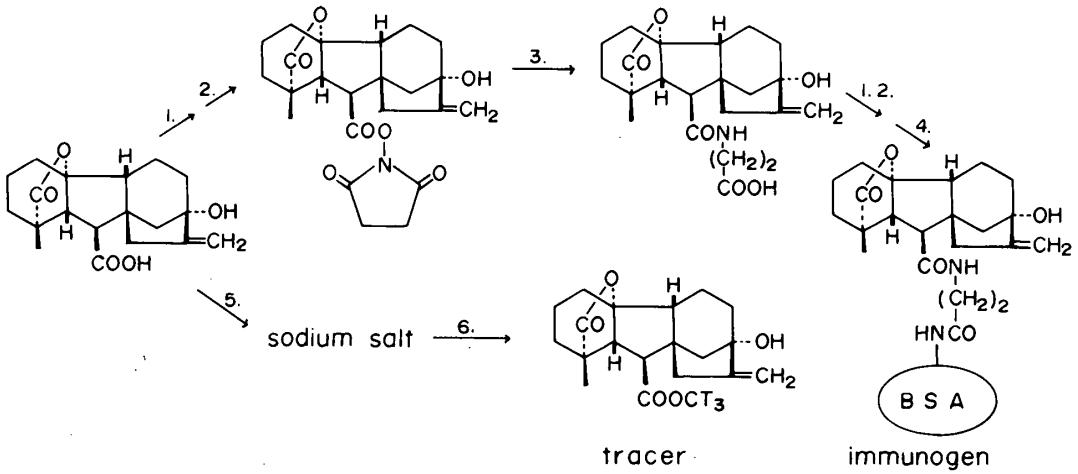

Fig. 1 Preparation of immunogenic GA_{20}-β-alanyl-BSA and tritiated GA_{20} methyl ester. 1. N-hydroxysuccinimide 2. dicyclohexylcarbodiimde 3. β-alanine 4. BSA 5. $NaOCH_3$ 6. C^3H_3I

shown in Fig. 2 and 3, respectively.

Assay selectivities—The cross-reactivities of a variety of gibberellins (structures are shown in Fig. 4) were examined. The results are shown in Table 2. Both antisera showed high reactivity with GA_5, GA_9 and GA_{20}, which have a structural similarity in the A ring that carries a γ-lactone but no hydroxyls. Since the reactivities of other gibberellins to both antisera are reasonably low, quantification of GA_5 and GA_{20} by means of these antisera is not expected to be interfered with by the coexistence of other gibberellins, if GA_5, GA_9 and GA_{20} are well separated.

Analyses of GA_5 and GA_{20} in fruits of P. nil—The analytical results of RIA of samples after each purification step and those of GC-SIM after purification with a Nucleosil $N(CH_3)_2$ column are presented in Table 3. Because of the limited amounts of the 2- and 4-day samples, RIA was performed on AE fractions and GA_5/GA_{20} fractions obtained by GPC. In the case of 8-, 16- and 20-day samples, RIA was performed on AE fractions and GA_5/GA_{20} fractions obtained by GPC, and on GA_5 and GA_{20} fractions separated by a Nucleosil $N(CH_3)_2$ column. The amount of GA_5

Table 1 General assay parameters

	Anti-GA_5-antiserum for GA_5	Anti-GA_{20}-antiserum for GA_{20}
K_a (10^9 liter·mol^{-1})	2.5	4.7
Titer	60,000	60,000
Specific activity of tracer (10^{15} Bq·mol^{-1})	2.59	2.59
Amount of tracer per assay (fmol)	77	77
Measuring range (pmol)	0.03–10	0.03–10
Detection limit (fmol)	30	30
Unspecific binding (%)	0.6	1.8
CV (%)	5.2	4.8

K_a, Average affinity constant; CV, average variation coefficient for sample triplicates throughout measuring range; Titer, final dilution of antiserum binding 32% of the tracer added.

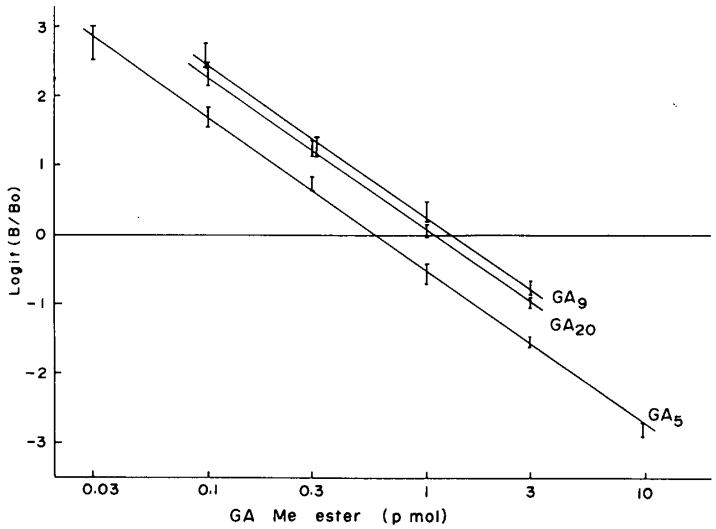

Fig. 2 Logit-log plots of methyl esters of GA_5, GA_9 and GA_{20} with use of anti-GA_5-antiserum and tritiated GA_5 methyl ester as a tracer. The bars represent ± SD of triplicated standards. Logit(B/Bo) = ln[B/Bo/(100-B/Bo)] with B = tracer binding in presence of unlabeled GA; Bo = tracer binding in absence of unlabeled GA.

in the GA_5 fractions separated by a Nucleosil $N(CH_3)_2$ column of 16- and 20-day samples was estimated using anti-GA_5-antiserum and that of GA_{20} in the GA_{20} fractions using anti-GA_{20}-antiserum.

In the analyses of GA_5/GA_{20} by RIA of the AE fractions, the estimated values were higher than the sum of GA_5 and GA_{20} obtained by GC-SIM except for the case of the 20-day sample, in particular, the amounts of immunoreactive GA_5 were very high in the 16-day sample even after GPC. After purification by a Nucleosil $N(CH_3)_2$ column, however, the amounts of both GA_5 and GA_{20} in the 16- and 20-day samples estimated by RIA showed reasonable coincidence with those obtaned by GC-SIM. To discover the reason for this overestimation, the 8-day and 16-day samples were reanalyzed after a simplified purification for RIA in which solvent fractionation was

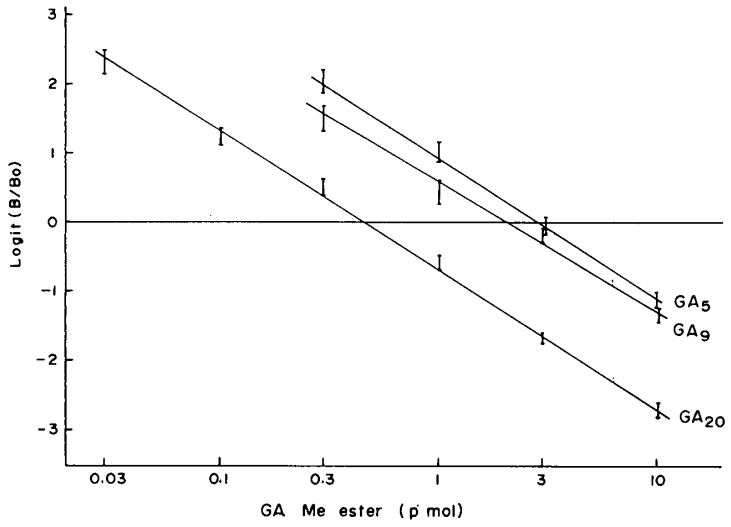

Fig. 3 Logit-log plots of methyl esters of GA_{20}, GA_5 and GA_9 with use of anti-GA_{20}-antiserum and tritiated GA_{20}-methyl ester as a tracer.

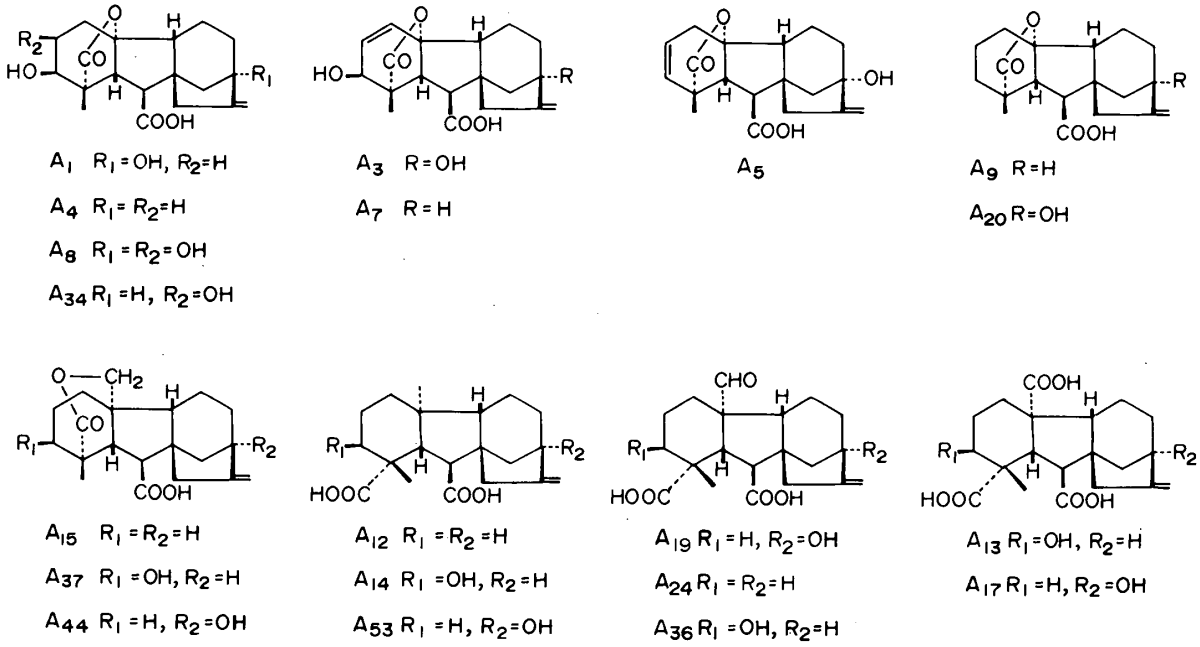

Fig. 4 Structures of GAs whose cross-reactivities were tested.

Table 2 Cross-reactivities of Anti-GA_5-antiserum and Anti-GA_{20}-antiserum

GAs-Me	Cross-reactivity (%)	
	Anti-GA_5-antiserum	Anti-GA_{20}-antiserum
GA_1	1.90	0.2
GA_3	0.89	0.05
GA_4	0.25	0.09
GA_5	100.0	17.5
GA_7	0.11	0.04
GA_8	0.45	0.12
GA_9	40.0	22.7
GA_{12}	<0.01	—
GA_{13}	<0.01	<0.04
GA_{14}	<0.01	<0.04
GA_{15}	0.16	<0.04
GA_{17}	<0.01	<0.04
GA_{19}	0.45	—
GA_{20}	47.8	100.0
GA_{24}	0.2	0.25
GA_{34}	0.08	<0.04
GA_{36}	0.03	<0.04
GA_{37}	<0.01	<0.04
GA_{44}	<0.2	<0.2
GA_{53}	<0.01	<0.04

—, not determined.

omitted. RIA using anti-GA_5-antiserum was performed on the fractions obtained by GPC. Clear immunoreactivity was found in the 23–25 min zone of retention time corresponding to the GA_5/GA_{20} fraction of both samples, and overestimation was noticed again as in the analysis of the GA_5/GA_{20} fractions obtained by GPC from the AE fractions of the same 8- and 16-day samples. The GA_5/GA_{20} fractions were subjected to purification by Nucleosil $N(CH_3)_2$ column, and each fraction over the retention time range of 0–30 min was analyzed by RIA using anti-GA_5-antiserum. Reanalysis using anti-GA_{20}-antiserum was impossible due to insufficient amounts of samples.

As shown in the histogram of the 8-day sample in Fig. 5, a prominent peak was observed in the GA_{20} fraction (retention time 17–20 min) and two small peaks at retention times 1–2 min and 14–16 min, while there was no clear peak in the GA_5 fraction (retention time 21–24 min). In the histogram of the 16-day sample, two prominent peaks were observed in the GA_{20} fraction and the

Table 3 Quantification of GA_5 and GA_{20} (ng/g fr wt) in developing fruits of *P. nil* by RIA and GC-SIM after some purification steps

Samples	Analyzed fraction (purification step)		Quantification by RIA		Quantification by GC-SIM	
			GA_5 equiv. [a]	GA_{20} equiv. [b]	GA_5	GA_{20}
2 days	AE fr.		21.8± 1.4	7.6± 1.1	nd	nd
	GA_5/GA_{20} fr.	(GPC)	8.3± 0.2	8.2± 0.2	nd	nd
	GA_5 fr.	(NMe_2)	nd	nd	0.6±0.3	—
	GA_{20} fr.	(NMe_2)	nd	nd	—	1.5± 0.9
4 days	AE fr.		26.9± 5.9	9.7± 1.3	nd	nd
	GA_5/GA_{20} fr.	(GPC)	8.6± 0.4	8.1± 1.0	nd	nd
	GA_5 fr.	(NMe_2)	nd	nd	0.4±0.2	—
	GA_{20} fr.	(NMe_2)	nd	nd	—	1.6± 0.2
8 days	AE fr.		35.0± 2.7	21.2± 1.3	nd	nd
	GA_5/GA_{20} fr.	(GPC)	12.4± 0.3 15 [c]	20.5± 2.4	nd	nd
	GA_5 fr.	(NMe_2)	1.5 [c]	nd	1.5±0.2	—
	GA_{20} fr.	(NMe_2)	nd	(43) [d]	—	13.1± 1.7
16 days	AE fr.		527.9±42.3	104.2±14.2	nd	nd
	GA_5/GA_{20} fr.	(GPC)	92.7± 6.4 88 [c]	15.1± 2.8	nd	nd
	GA_5 fr.	(NMe_2)	21.4± 3.9 26 [c]	nd	21.8±2.6	—
	GA_{20} fr.	(NMe_2)	nd	21.9± 3.9 (56) [d]	—	26.0± 1.9
20 days	AE fr.		681.0±75.2	973.8±53.6	nd	nd
	GA_5/GA_{20} fr.	(GPC)	399.7±42.6	808.6±39.2	nd	nd
	GA_5 fr.	(NMe_2)	54.4±20.0	nd	61.9±1.5	—
	GA_{20} fr.	(NMe_2)	nd	731.6± 7.1	—	972 ±39

AE, acidic ethyl acetate; GPC, gel permeation chromatography; NMe_2, Nucleosil $N(CH_3)_2$; nd, not determined.

[a] Immunoreactive GA_5 quantified by use of anti-GA_5-antiserum and (^{3}H)-GA_5-Me.

[b] Immunoreactive GA_{20} quantified by use of anti-GA_{20}-antiserum and (^{3}H)-GA_{20}-Me except for ( ) [d].

[c] Approximate value calculated from Fig. 5.

[d] Approximate value calculated from Fig. 5 and cross-reactivity of GA_{20} to anti-GA_5-antiserum.

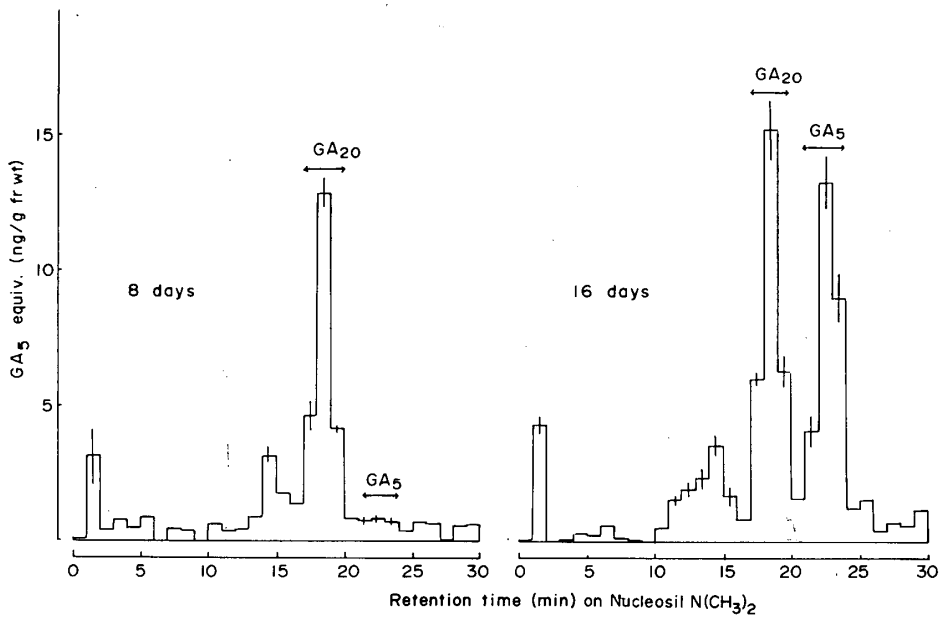

Fig. 5 Histgrams of GA contents (ng GA_5-equivalent/g fr wt of fruits) determined by RIA using anti-GA_5-antiserum in the fractions obtained by developing the GA_5/GA_{20} fractions from GPC of 8 days and 16 days samples with Nucleosil $N(CH_3)_2$ column.

GA_5 fraction, with two minor peaks at retention times 1–2 and 6–16 min. The amounts of GA_5 in both the 8- and 16-day samples showed reasonable coincidence with those obtained by GC-SIM, while the amounts of GA_{20} were still overestimated a little when calculated from the cross-reactivity (47.8%) of GA_{20} to anti-GA_5-antiserum.

Discussion

Immunogens of GA_5-β-alanyl-BSA and GA_{20}-β-alanyl-BSA were prepared by an active ester method using *N*-hydroxysuccinimide. Molar ratios of gibberellins coupled to BSA were reasonably high (>10). ^{1}HNMR analyses of their intermediates showed that GA_5 and GA_{20} were stable during peptide-bond formation using the active-ester method. The stability of gibberellins in the active-ester method and the preparation of highly purified intermediates are advantageous and important in terms of specificity of antisera raised against the immunogens, because the purity of the hapten is expected to have a great influence on the cross-reactivity of the antisera. Insertion of a spacer of β-alanine also gave the advantage of high coupling ratios of haptens to BSA by reducing the steric hindrance between GA moiety and BSA molecule. The high coupling ratios reflected higher values for the titers of the antisera than those previosuly reported (Weiler et al. 1981, Atzorn et al. 1983a).

Cross-reactivity is the most important factor in determining the value of antisera. The insertion of a spacer of an appropriate length like β-alanine is presumed to enhance the specificity of antisera in recognizing a hapten. As shown in Table 2, both anti-GA_5-antiserum and anti-GA_{20}-antiserum exhibited high specificity. Both antisera showed high reactivity to GA_5, GA_9 and GA_{20}. These gibberellins have a structural resemblance in carrying a γ-lactone and no hydroxyl groups in the A-ring, although GA_5 has an olefinic double bond between C-1 and C-2, whereas GA_9 and GA_{20} do not. Cross-reactivities of all tested C_{20} gibberellins including GA_{15} and GA_{44}, which have a δ-lactone instead of a γ-lactone in GA_9 and GA_{20}, were negligible. Cross-reactivities

of C_{19} gibberellins carrying hydroxyl group(s) in the A-ring are also almost negligible except for one: GA_1 showed 1.9% cross-reactivity to anti-GA_5-antiserum. Comparison of the cross-reactivities of various gibberellins suggests that recognition of the 13-hydroxyl group is weak in these antisera. They specifically recognize the structure of an A ring with a γ-lactone and no hydroxyls.

Comparison of the data obtained by RIA to those obtained by the established method of GC-SIM in the analysis of endogenous GA_5 and GA_{20} in developing fruits of *P. nil* indicates that reliable data were obtained by RIA only after samples were purified by a Nucleosil $N(CH_3)_2$ column. The AE fractions and GA_5/GA_{20} fractions obtained by GPC always gave values larger than the real contents. Eight- and 16-day samples were reanalyzed using anti-GA_5-antiserum without solvent fractionation. The results after GPC without solvent fractionation were in good agreement with those after GPC of AE fractions, though overestimation was observed in both cases (Table 3). The histograms in Fig. 5 indicate the occurrence of immunoreactive components other than GA_5 and GA_{20} in the GA_5/GA_{20} fractions obtained by GPC, which partially explains the overestimation after GPC. The immunoreactivity at 11–16 min may have been caused by some unidentifed species of gibberellin. The peak at 1–2 min may have been due to compounds other than gibberellins, because the latter were eluted later than the 4-min retention time under the chromatographic conditions. The immunoreactivity in an earlier eluate than that of gibberellins from the Nucleosil $N(CH_3)_2$ column was recognized in the analyses of the metabolite of *Sphaceloma manihoticola*, GA_4, using a monoclonal anti-GA_4-antibody (Eberle et al. 1986) and of GA_{19} in *P. nil* using an anti-GA_{24}-antibody (Kurogochi et al. 1986). Thus, the immunoreactivity unspecific to gibberellins does not seem to be specific to either the antibodies or the samples used in this study. It may have been caused by some sort of compound with a surfactant nature which dissociates antibody-tracer complexes and exhibits a false positive immunoreactivity.

The amounts of GA_5 estimated by RIA after samples were purified by a Nucleosil $N(CH_3)_2$ column were in good agreement with the data obtained by GC-SIM. The amounts of GA_{20} calculated from the values obtained by the RIA using anti-GA_5-antiserum and its cross-reactivity to GA_{20} (47.8%) were approximately 43 ng/g fr wt in the 8-day sample and 56 ng/g fr wt in the 16-day sample. Even after purification by a Nucleosil $N(CH_3)_2$ column, these amounts were 2–3 times larger than those of GA_{20} fractions (approx. 13 ng/g fr wt in the 8-day sample and approx. 26 ng/g fr wt in the 16 day sample) determined by RIA using anti-GA_{20}-antiserum or by GC-SIM after purification by a Nucleosil $N(CH_3)_2$ column of the corresponding AE fractions. This disagreement may be due to unspecified errors in the experimental procedure. Although it is difficult to explain the disagreement reasonably at this stage, we believe anti-GA_5-antiserum can be used, after adequate purification, for rough estimation of GA_{20} through its cross-reactivity, because the extent of the overestimation is not very great after purification using a Nucleosil $N(CH_3)_2$.

The above experiment indicates that a suitable purification of samples is necessary prior to RIA, because the estimation of gibberellins in crude samples by direct RIA may result in overestimation which cannot be explained only by the cross-reactivities of antisera among gibberellins. When overestimation was observed, the parallelism between the titration curves (logit/log plots) of a sample and the standard was often distorted, but not always. To make the quantification of gibberellins by RIA reliable, it is necessary to analyze gibberellin fractions obtained by purifying representative samples in at least two different modes, for example, TLC, a reversed-phase HPLC, etc., and confirm that the two sets of data are reasonably close. Although the extent of the overestimation may vary depending on the nature of both antisera and impurities in the samples, prepurification by GPC and subsequent HPLC on a Nucleosil $N(CH_3)_2$ column without solvent fractionation seems sufficient to perform reliable RIA of gibberellins, since the Nucleosil $N(CH_3)_2$ column is effective for removing nonspecific immunoreactive compounds.

In the discussion of the contents of GA_5 and GA_{20} in developing fruits of *P. nil*, the values determined after purification by a Nucleosil $N(CH_3)_2$ column can be taken as reliable data. The content of GA_{20} in the fruit was relatively low 2 days and 4 days after flowering; it seemed to start increasing slowly 4–8 days and rapidly 16–20 days after flowering. In contrast, the content of GA_5 was still low in the 8-day sample, suggesting that the increase of GA_5 followed that of GA_{20}. Kamiya et al. (1984) have demonstrated that GA_5 is derived from GA_{20} using a cell-free system prepared from *Phaseolus vulgaris*. The results that the increase of GA_5 followed that of GA_{20} in *P. nil* suggests that an enzyme system similar to that in *Phaseolus vulgaris* may also function in *P. nil*.

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