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Radioimmunoassay for Brassinosteroids and its Use for Comparative Analysis of Brassinosteroids in Stems and Seeds of *Phaseolus vulgaris*

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Abstract. Antiserum against the brassinosteroid (BR), castasterone, was produced by immunizing a rabbit with castasterone-carboxymethoxylamine oxime conjugated with bovine serum albumin (BSA). In a radioimmunoassay (RIA), the antiserum recognized a range of naturally occurring BRs with varying specificities. Detection limits of castasterone and brassinolide were approximately 0.3 pmol. This RIA system was successfully used for analyzing endogenous BRs in seeds and stems of *Phaseolus vulgaris* L., and showed that stems are quite different from seeds in terms of the species and quantity of the endogenous BRs.

Brassinosteroids (BRs) are steroidal plant hormones which are widely distributed in lower and higher plants and elicit a variety of growth effects (Adam and Marquardt 1986, Mandava 1988, Marumo 1987, Yokota and Takahashi 1986). Castasterone (compound **1**) and brassinolide (compound **13**) are most frequently detected in plant extracts and exhibit quite strong biological activity (Yokota and Takahashi 1986), suggesting that these two steroids might be important BRs. Thus, analysis of castasterone (**1**) and brassinolide (**13**) in relation to certain physiological phenomenon could be of great value in knowing functions of BRs which remain unclear.

Detection and quantitation of BRs have been based on bioassays, such as the rice-lamina-bending assay (Wada et al. 1984), the bean internode assay (Grove et al. 1979), and the wheat-leaf-unrolling assay (Wada et al. 1985), gas chromatography—mass spectrometry (GC-MS) or GC-selected ion monitoring (SIM) under either chemical or electron impact ionization (Takatsuto et al. 1982, Yokota et al. 1987b), and high-performance liquid chromatography (HPLC) after derivatization (Gamoh et al. 1989). Although immunoassay techniques to analyze plant hormones have advanced and are readily accessible by plant physiologists (Rivier and Crozier, 1987), there are seemingly no efficient immunological assay systems for BRs. Horgen and coworkers (1984) reported a radioimmunoassay (RIA) of 24-epibrassinolide, which appears to be quite unreliable and impractical.

We have recently produced an antiserum against castasterone (**1**) and found that the antiserum can recognize a variety of BRs in an RIA. This paper reports an RIA using the anti-castasterone antiserum and its utility in analyzing endogenous BRs in plants.

Materials and Methods

Brassinosteroids

Compounds **1**, **13**, **25**, and **26** were kindly supplied by Zennoh Agricultural Technical Center (Tokyo, Japan) and Fuji Chemical Industries Ltd (Takaoka, Japan). Compounds **2–8**, **17**, **18**, **25**, and **26** were generous gifts from Prof. N. Ikekawa, Tokyo Institute of Technology (Tokyo, Japan) and Dr. S. Takatsuto, Joetsu University of Education (Joetsu, Japan). Compounds **9–12**, **15–21**, and **27** were generous gifts from Prof. K. Mori, The University of Tokyo (Tokyo, Japan). Compound **14** was prepared by feeding brassinolide to mung bean shoots (unpublished observations). Compounds **22–24** were natural ones isolated from immature seeds of *Phaseolus vulgaris* L. (Yokota et al., 1987a, 1988).

Radiolabeled Castasterone and Brassinolide

Dolichosterone, **9**, (5 mg) in dioxane was catalytically reduced over platinum using excess tritium gas at Amersham International (Amersham, UK). The product was purified by thin-layer chromatography (TLC) on silica gel in $CHCl_3$-EtOH (6:1) using castasterone as a marker. The radioactive castasterone mixture recovered was dissolved in EtOH, passed through Sep-Pak ODS (Waters Associates, Milford, USA), concentrated by dryness in vacuo, redissolved in $CHCl_3$, and passed through Sep-Pak silica.

The eluate with 6% MeOH in $CHCl_3$ was evaporated to dryness in vacuo, then purified in several portions by HPLC on a 10 × 150 mm column of 5 μm Develosil ODS (Sensyu Pak) using 50% MeCN at a flow rate of 3 ml min^{-1} to yield 1.7 mg of [24, 28-$^3H_2$]castasterone [3.03 × 10^8 Bq; specific activity 8.3 × 10^{13} Bq mol^{-1}; retention time (Rt) 19.0 min] and [24, 28-$^3H_2$]-24-epicastasterone (2.4 × 10^8 Bq; specific activity, not determined; Rt 22.3 min).

Dolicholide (5 mg) was likewise tritiated and purified (HPLC solvent, 45% MeCN, 2.1 ml min^{-1} flow rate) to afford 2.12 mg of [24, 28-$^3H_2$]brassinolide (3.92 × 10^8 Bq; specific activity, 8.4 × 10^{13} Bq mol^{-1}; Rt 19.3 min) and [24, 28-$^3H_2$]-24-epibrassinolide (Rt 22.0 min: the yield was about half that of [^{3}H]brassinolide). The tritiated products were stored at 5°C in benzene-EtOH (9:1). We observed unexpectedly low specific activities in the products (theoretical value should be more than 1.5 × 10^{15} Bq mol^{-1}.

Synthesis of Immunogen

[^{3}H]Castasterone (38.27 mg, 82.5 μmol; 1097 Bq) was mixed with carboxymethoxylamine hydrochloride (27.0 mg, 248 μmol) in dry pyridine (0.68 ml). After an overnight reaction at 50°C, the mixture was diluted with water, adjusted to pH 3~4 with 1 N HCl and extracted three times with $CHCl_3$. The combined extracts were evaporated to dryness without heating, yielding a carboxymethoxylamine oxime of castasterone as a white solid (42 mg, 78 μmol), which was shown to be homogenous on TLC (R_f 0.25 in solvent; $CHCl_3$-MeOH-AcOH, 5:1:0.2): ^{1}H-nuclear magnetic resonance (NMR) in $CDCl_3$-CD_3OD, δ_{TMS} 0.69 (18-CH_3), 0.76 (19-CH_3), 4.53 (carboxymethoxyl H_2); mass spectrum (MS) (70 eV), m/z as 462, 448, 362. The oxime and *N*-hydroxysuccinimide (13.4 mg, 117 μmol) were dissolved in 150 μl dimethylformamide and mixed with *N,N'*-dicyclohexylcarbodiimide (24 mg, 117 μmol) in dimethylformamide (150 μl). After stirring for 3 h at room temperature, the reaction mixture was filtered through glass wool and evaporated to dryness in vacuo to afford an active ester of castasterone-carboxymethoxylamine oxime as a crude solid (78 mg). The R_f using the same solvent as above was 0.60: ^{1}H-NMR (100 MHz) in $CDCl_3$-CD_3OD, δ_{TMS} 2.83 (-$COCH_2CH_2CO$-) and 4.90 (carboxymethoxyl CH_2). The active ester was dissolved in 0.4 ml dimethylformamide and filtered through glass wool. The filtrate was added dropwise at 4°C to a solution of bovine serum albumin (BSA) (89.2 mg, 1.3 μmol) in a 1:2 mixture of dimethylformamide and water (2 ml) containing 15.6 μl triethylamine. After stirring for 2 days at 4°C, the mixture was evaporated to dryness, dissolved in water (10 ml), dialyzed overnight against water (150 ml) containing 10% dimethylformamide, and then against pure water (1 L) for 4 days. The dialysate was lyophilized to yield the castasterone-BSA conjugate (approximately 100 mg, 380 Bq) as a fluffy powder. The number of castasterone residues per one mole of conjugate was calculated to be around 22, based on the radioactivity.

Immunization

The conjugate (approximately 1 mg) was dissolved in 0.6 ml phosphate-buffered saline (PBS) (150 mM NaCl, 24 mM K_2HPO_4, and 6 mM NaH_2PO_4), pH 7.4 and emulsified with 0.6 ml Freund's complete adjuvant (DIFCO). The emulsion (approximately 0.5 ml) was injected into a New Zealand white rabbit for each immunization treatment. For the first 2 weeks, the emulsion was injected once a week subcutaneously into the back and foot pad. For the next 2 weeks, the emulsion was injected once a week only into the back. Booster injections were injected into the hindquarter muscle and back (subcutaneously) 5, 9, and 13 weeks after the immunization started. Sera were collected from the ear vein 3, 6, 7, 10, 11, and 14 weeks after the immunization started and stored in several portions at −75°C. The sera were tested for titers (dilutions of the antisera binding 50% of the tracer) by RIA at 10- to 1.6 × 10^4-fold dilutions in PBS. Throughout this study, the serum harvested after the third booster injection was used in RIA.

Radioimmunoprecipitation Assay

A test sample was dissolved by sonication in PBS containing 5% MeOH, and 100 μl aliquots were transferred into three Pyrex assay tubes (10 × 57 mm). In the case of partially purified plant extracts, they were dissolved in MeOH, and 5-μl aliquots were transferred into assay tubes which contained 95 μl PBS. One hundred microliters of sevenfold diluted bovine serum and 300 μl PBS containing 5 μl MeOH solution of [^{3}H]-castasterone (160 Bq) were added to the assay tubes, followed by vigorous mixing. One hundred microliters of antiserum diluted with PBS was then added to the tube, vigorously mixed, allowed to stand at room temperature for 1 h (4°C, overnight for titer determination). Seven hundred fifty microliters of saturated ammonium sulfate (pH 7.4) was then added. The mixture was allowed to stand at room temperature for 15 min (4°C, 30 min for titer determination), then centrifuged (2500 *g*) at 20°C for 20 min (4°C, 30 min for titer determination). The precipitate obtained by decantation was mixed with 750 μl half-saturated ammonium sulfate, then centrifuged as above. The precipitate was dissolved in 0.3 ml water by vigorous mixing for 5 min and mixed with 1.5 ml scintillation cocktail (ACS-II, Amersham) prior to radioactivity measurement by an LSC-700 (Aloka, Tokyo, Japan).

Standard curve and cross-reactivity were determined using 6 × 10^3-fold diluted antiserum (final dilution, 1:3.6 × 10^4), which can bind about 30–50% of added [^{3}H]castasterone. The cross-reactivities were defined as the relative amounts of test compounds required to reduce [^{3}H]castasterone binding by 50%, where the amount of nonlabeled castasterone was taken as 100.

Plant Material

Phaseolus vulgaris L. cv Kentucky Wonder seeds were planted at the end of April and grown under field conditions. Stems were obtained from 31-day-old shoots by cutting just above the cotyledonary node. All the leaves were removed. Seeds were harvested at the early immature stage.

Extraction and Purification of BRs in Stems and Seeds of Phaseolus vulgaris

Stems (1580 g fresh weight) were homogenized in pure MeOH. The MeOH was evaporated and the aqueous residue was acidified to pH 3, then partitioned with EtOAc (500 ml, three times). The combined EtOAc phases were washed with aqueous $NaHCO_3$ (500 ml, three times), evaporated to dryness in vacuo, then partitioned between hexane (100 ml) and 80% aqueous

MeOH (100 ml). The hexane phase was partitioned further (two times) against 80% MeOH. The aqueous MeOH phases were combined and evaporated to dryness prior to chromatography on a column of silica gel (11 g) eluted with 100-ml mixtures of MeOH and $CHCl_3$ with increasing stepwise the concentration of MeOH. Eluates with 4–20% MeOH in $CHCl_3$ were combined and chromatographed on a column of Sephadex LH-20 (26.6 × 900 mm) using a 4:1 mixture of MeOH-$CHCl_3$ as the mobile phase at a flow rate of 30 ml h^{-1}. Fractions 33 ~ 39 were combined and subjected to HPLC on a 8 × 250 mm column of 5 μm Develosil ODS (Sensyu Pak). Elution was done at 40°C at a flow rate of 2 ml min^{-1} with 45% MeCN for 40 min, then with 80% MeCN for 25 min.

Extraction and partitioning for immature seeds (100 g fresh weight) were carried out at one-twelfth the amounts used for stems. Further purification was carried out using the same chromatographic conditions as for stems, except that the scale of silica gel chromatography was one sixth, and the eluates with 3–20% MeOH in $CHCl_3$ were combined prior to LH-20 chromatography.

Quantitation of Castasterone in Shoots and Seeds by SIM Using Internal Standard

The MeOH extract of seeds (56.3 g fresh weight) was mixed with [26, 27-$^2H_6$]CS (300 ng) kindly supplied from Dr. S. Takatsuto (Takatsuto and Ikekawa 1986) and was processed as stated above to afford a neutral ethyl acetate fraction. This was chromatographed on silica gel eluted with 3% then 10% MeOH in $CHCl_3$. The latter eluate was purified on a Sephadex LH-20 column (see above). Fractions 35–39 were collected and purified on a 6 × 200 mm column of 5 μm Develosil ODS (Sensyu Pak) eluted at a flow rate of 1.5 ml min^{-1} with 45% MeCN (oven temperature, 40°C). A broad zone (14.33–18.33 min) was col-

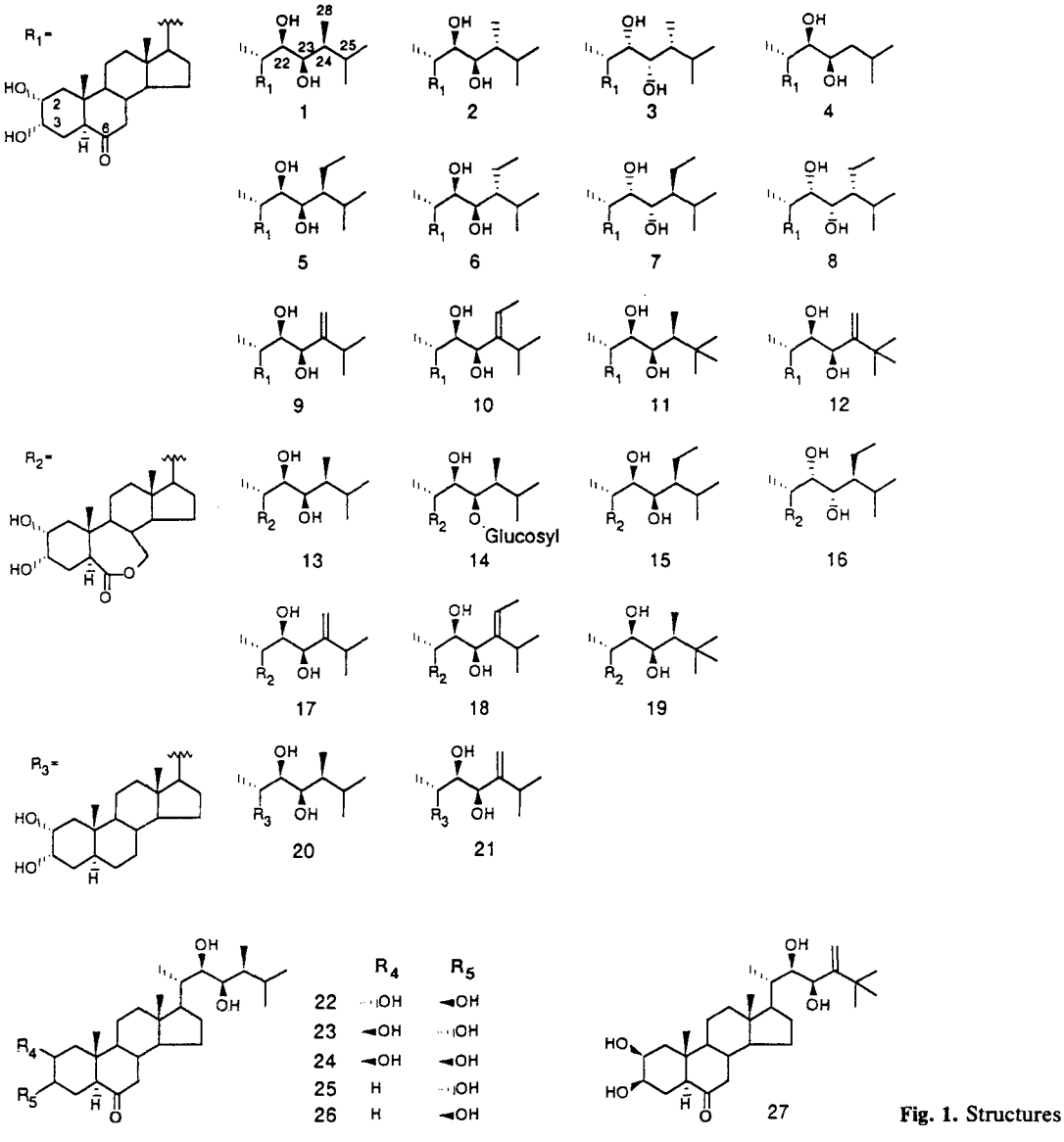

Fig. 1. Structures

lected for SIM quantitation of castasterone (Rt 16.8 min). In the case of stems (480 g fresh weight), deuterated castasterone (300 ng) was added to the neutral ethyl acetate faction.

GC-MS and GC-SIM

Samples were converted to bismethaneboronates by the procedure of Takatsuto and co-workers (1982). Capillary GC-MS and GC-SIM were similar to those previously reported (Yokota et al. 1987b) with the exception of oven temperature which was 175°C for the first 2 min, elevated to 275°C at a rate of 32°C min^{-1}, then to 290°C at a rate of 2°C min^{-1}, and with the final temperature held at 290°C. In SIM (post acceleration, −10 kV), the amounts of castasterone in samples were calculated from the ratio of the molecular ion area to that of the deuterium internal standard.

Bioassay

The rice-lamina-inclination assay was carried out according to the procedure described by Arima and co-workers (1984).

Results and Discussion

Brassinolide and castasterone carry vicinal hydroxyl groups in both the A ring and side chain. This is also common in most other naturally occurring BRs (Yokota and Takahashi 1986). Thus, antibodies which recognize both vicinal hydroxyl groups will be useful for a general assay of BRs. To this end, an immunogenic protein carrier was linked to the 6-position of castasterone with the oxime structure as a spacer in the hope that the raised antibodies would recognize the vicinal hydroxyls situated distant from the coupling site.

Radioimmunoassay System for BRs

Titers of antisera raised against the conjugate leveled off at 1:3400 shortly after the first booster injection. In immunoprecipitation reactions, the unspecific binding was found to be as high as 17% of the added tracer, [^{3}H]castasterone. This was due to either salting out of the tracer or absorption to the assay tube wall following the addition of ammonium sulfate, because such an effect occurred with or without the addition of both antiserum and bovine serum. The "binding" could not be reduced by using other tracers, such as [^{3}H]brassinolide or a sodium borohydride reduction product of [^{3}H]-castasterone, or by using plastic or silicone-coated glass tubes instead of glass tubes (data not shown). However, the "binding" could be diminished to 12% by conducting the precipitation reaction rather quickly at room temperature, and this procedure was employed in further assays. Figure 2 shows

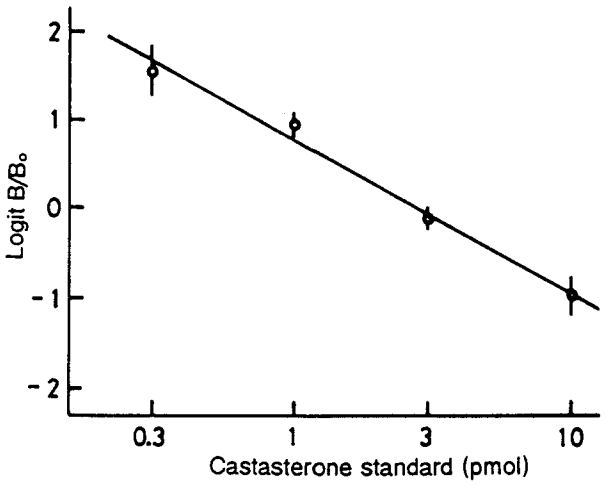

Fig. 2. Standard curve for castasterone RIA using anti-castasterone antiserum. Bars indicate SE (N = 6). B, binding of tracer to antibody in the presence of compound; B°, binding of tracer in the absence of compound. $\log Y = -1.70 \log X + 0.76$, $r = -0.985$.

Table 1. Detection limits (pmol) of castasterone and brassinolide in RIA, bioassay, and SIM.

Compound	RIA	Bioassay	SIM
Castasterone	0.3	2	0.2
Brassinolide	0.3	0.5	1

that standard curve where logit B/B° is plotted against the logarithm of the amounts of castasterone. The linearity in the standard curve is observed between 0.3 and 1 pmol of castasterone. Because of the high background due to the "binding" effect, the least detectable amount of castasterone was not lower than 0.3 pmol. However, the RIA of castasterone was much more sensitive than the bioassay (Wada et al. 1984) and compared with SIM where castasterone was analyzed as a bismethaneboronate on a capillary column (Table 1). Brassinolide was as sensitive to the antiserum as castasterone (see below) and the sensitivity was nearly equivalent to the bioassay (Wada et al. 1984) but much higher than SIM (Table 1).

Cross-Reactivity

Table 2 shows cross-reactivities of 27 compounds, including the naturally occurring and synthetic compounds. Specificities of the antiserum in the recognition of various functional groups are summarized in Table 3.

Table 2. Percentage cross-reactivities of natural and synthetic BRs for anti-castasterone antiserum.

Functionality in rings A and B	BRs[a]	Cross-reactivity
2α, 3α-Diol, 6-ketone	**1** Castasterone[b]	100
	2 24-Epicastasterone[b]	13
	3 22,23-Diepicastasterone	<0.001
	4 28-Norcastasterone (brassinone)[b]	27
	5 28-Methylcastasterone (24-ethylbrassinone)[b]	36
	6 24-epi-28-Methylcastasterone	2.5
	7 22,23-diepi-28-Methylcastasterone	<0.001
	8 22,23,24-triepi-28-Methylcastasterone	<0.001
	9 Dolichosterone[b]	0.8
	10 28-Methyldolichosterone (homodolichosterone)[b]	0.6
	11 25-Methylcastasterone	8
	12 25-Methyldolichosterone[b]	1.3
2α, 3α-Diol, lactone	**13** Brassinolide[b]	105
	14 23-*O*-β-D-Glucopyranosylbrassinolide	<0.001
	15 28-Methylbrassinolide (homobrassinolide)	31
	16 22,23-diepi-28-Methylbrassinolide	<0.001
	17 Dolicholide[b]	1.0
	18 28-Methyldolicholide (homodolicholide)[b]	0.03
	19 25-Methylbrassinolide	22
2α, 3α-Diol, 6-deoxo	**20** 6-Deoxocastasterone[b]	0.3
	21 6-Deoxodolichosterone[b]	0.3
Isomeric hydroxyl, 6-ketone	**22** 3-Epicastasterone[b]	129
	23 2-Epicastasterone[b]	36
	24 2,3-Diepicastasterone[b]	40
	25 Typhasterol[b]	29
	26 Teasterone[b]	45
	27 2,3-diepi-25-Methyldolichosterone[b]	1.6

[a] Nomenclature is based on the names used in the original articles. Otherwise, original names are written in parentheses. The proposal of B. Mandava (1988) for nomenclature of BRs was ruled out because of its improperness.
[b] Naturally occurring BRs.

Table 3. Summary of recognition of functional groups of BRs by anti-castasterone antiserum.

Functionality	Order of immunoreactivity
Hydroxyl in ring A	2α, 3β-diol > 2α, 3α-diol > 2β, 3α-ol, 2β, 3β-diol, 3β-OH, 3α-ol
Carbonyl in ring B	ketone (6-oxo) = lactone (6-oxo-7-oxa) >>> 6-deoxo
Hydroxyl in side chain	22*R*, 23*R*-diol >>> 22*S*, 23*S*-diol
Alkyl at C24	24*S*-Me > 24*S*-Et > 24-H_2 > 24*R*-Me > 24*R*-Et > 24-methylene, 24-ethylidene
Alkyl at C25	25-Me_2 > 25-Me_3

The antiserum equally recognized both 6-ketone (6-oxo) and lactone (6-oxo-7-oxa) groups (**1, 13**), but did not respond to BRs without the 6-oxo group (**20, 21**). Unexpectedly, the antibodies were found to have a strong affinity to the 6-oxo group, which is situated a short distance from the site of conjugation.

The affinity to the side-chain functionalities was quite selective. Thus, the antibodies recognized 22*R*, 23*R*-vicinal hydroxyls but not 22*S*, 23*S*-vicinal hydroxyls (**1** vs. **3**, **5** vs. **7**, **15** vs. **16**). Glucosylation of the 23*R*-hydroxyl nullified the immunoreactivity (**14**). The antibodies could accept saturated alkyl substituents at C24 with varied affinities. As expected, the affinity to BRs with 24*S*-methyl (**1, 13**) was highest. Replacement of 24*S*-methyl by 24*S*-

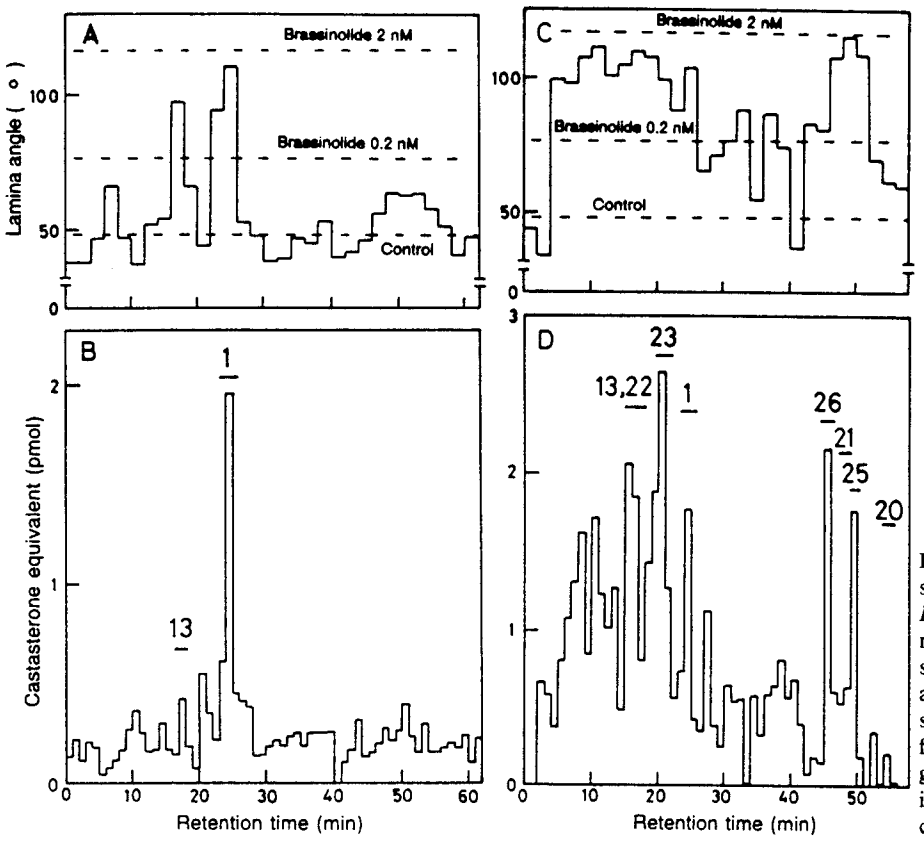

Fig. 3. Reversed-phase HPLC of shoot and seed extracts of *Phaseolus vulgaris* as monitored by rice-lamina-inclination assay [(A) stem; (C) seed] and RIA with anti-castasterone antiserum [(B) stem; (D) seed]. Dosages in fresh-weight tissue equivalents: 150 g in **A**; 2 g in **B**; 30 g in **C**; and 0.5 g in **D**. Bars indicate elution zones of compounds.

ethyl (**1** vs. **5**, **13** vs. **15**) or protons (**1** vs **4**) reduced binding to 30%. The change of the stereochemistry at C24 from *S* to *R* reduced the binding more distinctly, to 9% (**1** vs. **2**) and 7% (**5** vs. **6**). Of interest is that the presence of electron-rich substituents (methylene or ethylidene) at C24 drastically reduced affinity by about 100-fold relative to **1** and **13**. Introduction of a methyl at C25 (namely, tertiary butyl group at the side-chain terminal) also reduced affinity to 10–20% relative to **1** and **13**. The change in immunoreactivity with alkyl substituents at C24 or C25 seems to be due to the direct effects of these alkyl substituents rather than via a secondary conformational change of 22*R*, 23*R*-diol. This was substantiated by ^{1}H-NMR of **1**, **13**, **15** (Yokota et al. 1982), **4** (unpublished observations), **9**, **17**, **18** (Baba et al. 1983), and **12** (Kim et al. 1987) in chloroform, where the coupling constants between 22-H and 23-H are in a similar range (8–9 Hz) and further couplings are quite small or nondetectable. However, the effect of *R*-alkyl at C24 on immunoreactivity might be mediated partly through a conformational change of the 22*R*, 23*R*-diol, which was evidenced from the fact that protons attached to C22 and C23 in **2** were observed as triplet (J = 5, 5 Hz) and double doublet (J = 1.5, 5 Hz).

Table 4. Quantitation of castasterone and brassinolide after purification by reversed-phase HPLC.[a]

	Castasterone		Brassinolide
	Stem	Seed	Stem
RIA	1.3	3.5	0.20
Bioassay	1.1	3.3	0.09
SIM[b]	3.0	10.8	Not analyzed

[a] The contents are expressed in pmol g fresh weight tissue^{-1}.
[b] Data corrected with a deuterated internal standard.

BRs without C2α hydroxyl were recognized with somewhat reduced affinity (**25**, **26**), indicating that C2α hydroxyl is recognizable by antibodies but is not a prerequisite for the recognition. As seen in compounds **23** and **24**, introduction of a C2β hydroxyl in typhasterol (**25**) and teasterone (**26**) did not change affinities, suggesting that the recognition of C2β hydroxyl by the antibodies is marginal. When a 3α hydroxyl was compared with a 3β hydroxyl (**1** vs. **22**, **23** vs. **24**), the antibodies tended to recognize the 3β-hydroxyl to a greater extent. Importance of the 3-hydroxyl in recognition by antibodies remains unknown because BRs without 3-

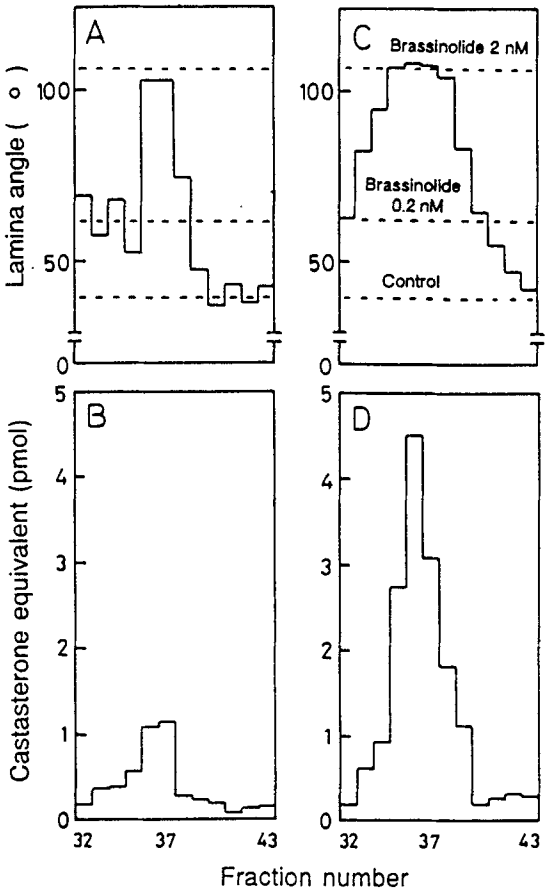

Fig. 4. Sephadex LH-20 chromatographies of the seed and shoot extracts of *Phaseolus vulgaris* as monitored by rice-lamina-inclination assay [(A) stem; (C) seed] and RIA with anti-castasterone antiserum [(B) stem; (D) seed]. Dosages in fresh-weight tissue equivalents: 50 g in A; 1 g in B; 5 g in C; and 0.125 g in D.

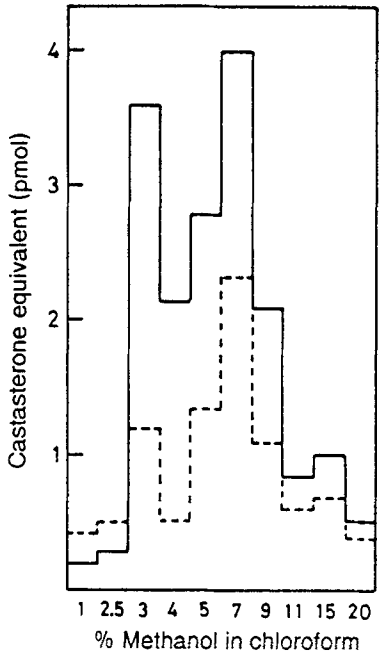

Fig. 5. Silica gel chromatography of the seed extract of *Phaseolus vulgaris* as monitored by RIA with anti-castasterone antiserum. Estimates were obtained for different dosages of 0.125 (——) and 0.0625 (– – –) g fresh-weight tissue equivalents.

hydroxyl are not available. It will be concluded that antibodies are not sensitive to the presence (2-hydroxyl) or the stereochemistry of the hydroxyl groups in the A ring.

BRs recognized by the antiserum always carry 22*R*, 23*R*-diol and 6-carbonyl, while BRs which lack one of the two groups are never recognized. This indicates that the two regions binding to the above two epitopes are co-existent in the same molecule of the antibody.

In summary, the anti-castasterone antiserum in RIA showed a broad range of cross-reactivities in an RIA, although quite low reactivity was found for 24-methylene and 24-ethylidene BRs, 6-deoxo BRs, and 22*S*, 23*S*-diol BRs. BRs with 24-methylene and 24-ethylidene, however, have been known to be distributed in a limited number of plants, namely, seeds of *Phaseolus vulgaris* (Yokota et al. 1987a, 1987b, 1988) and *Dolichos lablab* (Baba et al. 1983) and rice shoot (Abe et al. 1984). 6-Deoxo BRs, among which 6-deoxocastasterone has been known to be contained in a wide range of plants, seem unimportant because they have little biological activity and hence are considered to be end products in the biosynthesis of BRs (Yokota and Takahashi 1986). BRs with 22*S*, 23*S*-diol are not naturally occurring. Thus, an RIA using the anti-castasterone antiserum seems to be applicable for a broad range of plant extracts.

Brassinosteroids in Seeds and Stems of Phaseolus vulgaris

Figure 3 (A and B) shows the distribution of BRs after reversed-phase HPLC of the stem extract as determined by bioassay and RIA. The bioassay using 150 g fresh-weight-equivalent extracts revealed two distinct peaks of biological activity, which were assignable to castasterone (1) and brassinolide (13), on the basis of the HPLC retention times. GC-MS of the combined fractions 23–26 after methaneboronation confirmed the presence of castasterone [Rt, 13.28 min; mass spectrum (relative abundance), m/z 512 (M^+, 41%), 155 (100%). Brassinolide could not

be identified by GC-MS for the combined fractions 17 and 18 because of the low amount. The RIA for the same extracts, with the dosages as low as 2 g fresh-weight-equivalents, showed a prominent peak of castasterone as well as several small peaks, one of which was ascribable to brassinolide. As shown in Table 4, the estimates of castasterone in the stems as determined by RIA and bioassay, as well as those of brassinolide, are in good agreement. The estimates were 2.5-fold lower than determined by SIM using a deuterated internal standard because of the loss during purification procedures.

Immature seeds have been shown to contain more than 30 BRs (Yokota et al. 1987b). Some of these BRs have been identified as **1, 9, 12, 13, 17, 20–27,** and others (Kim et al. 1987, Yokota et al. 1987a, 1988). Analysis of the extract by bioassay after reversed-phase HPLC (Fig. 3C) could not distinguish and quantify BRs at the dosages of 30 g fresh-weight-equivalent extracts, except for castasterone. The bioassay-based estimate of castasterone was about 3.3 pmol g fresh weight tissue^{-1}. In contrast, RIA could make visible several sharp peaks of BRs at the dosage of 0.5 g fresh-weight-equivalent extracts (Fig. 3D). One of the reasons for this is that the antiserum does not recognize biologically active BRs with the 24-methylene group (compounds **9, 12, 17, 27**). The peaks ascribable to castasterone (**1**), typhasterol (**25**), and teasterone (**26**) were nearly completely isolated, enabling us to quantitate them. The estimates for castasterone, teasterone, and typhasterol were corrected using their cross-reactivities, 3.5, 10, 12 pmol g fresh weight tissue^{-1}, respectively. The estimates of castasterone obtained by bioassay and RIA were in good agreement (Table 4). Thus, it was revealed through RIA (or in combination with the bioassay) that the stems are quite different from the seeds in qualitative and quantitative aspects of endogenous BRs.

Sephadex LH-20 chromatography is quite useful for purifying BRs in crude plant extracts. Figure 4 compares the histograms of RIA and bioassay obtained after Sephadex LH-20 chromatography of the extracts of stems and seeds. Since BRs behave as a group with little separation in the chromatography, essentially identical elution profiles were obtained both in RIA and bioassay. The amounts of extracts required for RIA were found to be much smaller than for the bioassay. Thus, RIA seems to be a more effective method than bioassay in determining BRs after Sephadex LH-20 chromatography.

The parallelism between dosages of plant extracts and immunoreactivity is important for quantitative estimation. Rather crude fractions obtained after silica gel chromatography of seeds were subjected to RIA at two different concentrations. As shown in Figure 5, the estimates were nearly proportional to the dosages of the extract, suggesting that RIA might be useful for rough estimates of BRs in even crude extracts, such that interfering compounds are not present in the extract.

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