# Evidence on the Natural Occurrence of Gibberellin $A_1$ in Rhubarb Seeds

YOSH KIMURA

A biologically active substance was isolated in microgram quantity from the immature seeds of the rhubarb by a combination of charcoal adsorption, aluminum oxide column separation, adsorption, and partition thin-layer chromatography. By visualization of the substance on thin-layer plates, comparison of the $R_f$'s in several solvent systems, and comparison of the characteristics of fluorescence and of its development with authentic gibberellins, the substance has been identified as gibberellin $A_1$.

The gibberellins are an important class of plant growth regulators originally discovered as metabolic products of the fungus *Gibberella fujikuroi* (*16*) and, subsequently, demonstrated to be naturally present in the same or related chemical forms in a number of plants (*2*, *5*, *6*, *8*, *9*, *11*, *12*, *15*). Gibberellin $A_1$ has previously been identified in the seeds of *Phaseolus multiflorus* (*8*) and *vulgarus* (*15*), water sprouts of *Citrus unshiu* (*12*), seeds of *Eshenocystis macrocarpa* Greene (*2*), and citrus fruits (*6*). The effectiveness of applied gibberellin $A_3$ in replacing the normal exposure of rhubarb crowns to low temperatures for initiating early growth (*14*) led to a search of the natural gibberellins in this plant.

*Experimental*

**Extraction.** One kilogram of immature rhubarb seeds (*Rheum rhaponticum*, cultivar Victoria) harvested June 17 (frozen for 38 days at −17° C.) was homogenized at high speed in a VirTis 45 in several batches, with a total of 800 ml. of acetone and 1000 ml. of 50% v./v. acetone–water, and allowed to stand overnight at 2° C. The solids were separated by centrifugation, returned to the original container, and extracted twice more with 1000 ml. each of 50% acetone–water. After the removal of acetone from the combined extracts under reduced pressure (at <35° C.), a preliminary separation was made by charcoal adsorption and pH 2 ethyl acetate extraction (*15*). The crude extract obtained after evaporation was taken up in 10 ml. of ethyl acetate.

**Purification.** One milliliter of the crude extract was measured into a 17-mm. i.d. column containing 27 grams of aluminum oxide powder (Matheson Coleman & Bell AX 710) suspended in ethyl acetate, and eluted successively with 50 ml. each of ethyl acetate (I), ethyl acetate–methanol 1 to 1 (II), methanol (III), methanol–water 1 to 1 (IV), and water. Each eluate was evaporated under reduced pressure, the residue suspended in 5 ml. of water, acidified with one drop of 7*N* sulfuric acid to a pH of about 2.0, and extracted three times with 6 ml. of ethyl acetate. The combined extracts of each eluate were evaporated, and the residue was redissolved in 0.5 ml. of ethyl acetate.

Western Washington Research and Extension Center, Washington State University, Puyallup, Wash.

Eluates I to IV each were streaked on a 250-micron thickness 20 × 20 cm. silica gel G plate and developed in ethyl acetate–acetic acid 95 to 5 (*7*), air-dried, and scraped in several fractions, *A* to *E*. Each scraping was washed three times with 10-ml. portions of methanol. The combined methanol extract was evaporated, the residue was suspended in 5 ml. of water and extracted into the ethyl acetate phase from the pH 2 aqueous phase as before, re-evaporated, and the solids were redissolved in 0.5 ml. of ethyl acetate.

Fraction II,C was further subdivided using the same technique into subfractions II,C–1 to 7 by chromatography on Kieselguhr G (KGG) with benzene–propionic acid–water 8:3:5 (*10*). This solvent system, previously reported by MacMillan and Suter as unreliable, gave consistently good results when the plate was equilibrated 4 hours in the presence of both the benzene and the aqueous phases before separation in the benzene phase. The aqueous phase was placed in a filter paper–lined tank (Brinkmann Instruments, standard size No. 041006), the benzene phase in a narrow trough inside the tank, and the plate was suspended

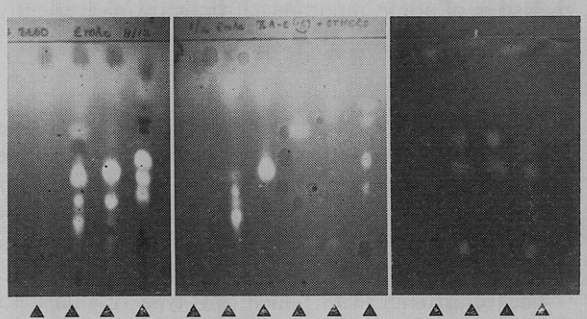

Figure 1. Photographs taken under ultraviolet light of various rhubarb seed fractions on silica gel G (left and center sections) and Kieselguhr G (right section)

Silica gel plates were developed in ethyl acetate–acetic acid 95 to 5 and the Kieselguhr in benzene–propionic acid–water 8:3:5 to the 15-cm. mark. Plates were sprayed with 70% sulfuric acid and heated 30 minutes at 120° C.
*Left* (left to right). Aluminum oxide column eluates I, II, III, and IV
*Center* (left to right). Fractions II, *A*, II, *B*, II, *C*, II, *D* II, *E*, and gibberellin standards (from top down) $A_9$, $A_7$, $A_3$, and $A_8$. Fraction II, *A* is indicated by a nonfluorescent spot at the starting point
*Right* (left to right). Fraction II, *C*, standards, II, *C* + $A_1$ and $A_3$, and standards. The standards are from center down $A_1$, $A_3$, and $A_8$

above the two phases by means of a glass rack. Four hours' equilibration gave $R_f$ values of about 0.1, 0.4, and 0.5 for gibberellins $A_8$, $A_3$, and $A_1$, respectively. The $R_f$ of $A_2$ is roughly 0.6 (*10*). The $R_f$ values of the gibberellins in this system were dependent upon the length of equilibration, the longer times giving smaller $R_f$ values.

**Visualization of Spots** (*10*). Compounds separated by thin-layer chromatography were viewed under long-wave ultraviolet light (366-m$\mu$ peak) after spraying with 70 to 30 sulfuric acid–water mixture, after heating of the sprayed plate for 10 minutes at 120° C., and after 30 minutes' heating.

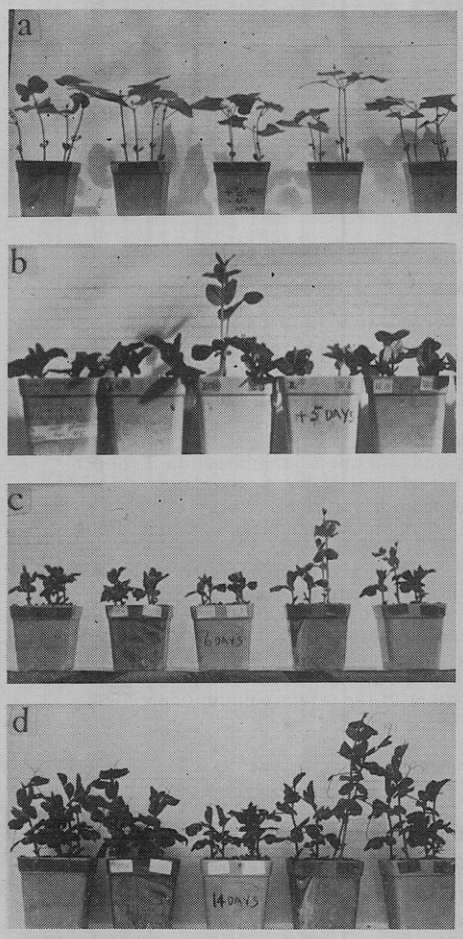


Figure 2. Bioassay of rhubarb seed extracts

*a.* Small California white beans 6 days after treatment. Left to right, 1st pot, all controls; 2nd pot, fractions IV, *A* and IV, *B*; 3rd pot, IV, *C* and II, *A*; 4th pot, II, *B* and II, *C*; 5th pot, II, *D* and II, *E*
*b.* Dwarf peas 5 days after treatment. Left to right, 1st pot, all controls; 2nd pot, II, *A* and II, *B*; 3rd pot, II, *C* and II, *D*; 4th pot, II, *E* and IV, *A*; 5th pot, IV, *B* and IV, *C*
*c.* Dwarf peas 6 days after treatment. Left to right, 1st pot, controls; 2nd pot, III, *A* and III, *B*; 3rd pot, III, *E* and III, *D*; 4th pot, II, *C*–1 and II, *C*–4; 5th pot, II, *C*–5 and II, *C*–7
*d.* Same as above, 14 days after treatment. Fractions III, *E* and III, *D*, center pot, show growth inhibition

Fluorescence emission spectra were obtained on a photomultiplier-equipped manual Beckman DU spectrophotometer with fluorescence attachment No. 73500 using as an activation source a F4T5/BL lamp with Schott UG-11 filter. Scrapings from sprayed and heated thin-layer plates were placed directly in photometric tubes and suspended in 2 ml. of an 85 to 15 sulfuric acid–water mixture. Readings were made after centrifugation.

Biological assays (*1*) were performed on Grand Rapids lettuce seedlings (*3*), Little Marvel peas (*6*), and small California white beans. Application of extracts to peas and beans were made to the leaves as an aqueous solution of 0.1% Tween 20. For lettuce seedlings, microdiffusion dishes (60 × 30 mm.) were used with water in the outer ring to prevent drying and 1 ml. of aqueous test solution and 10 seedlings in the center.

*Results*

Small amounts (ca. 10 $\mu$l.) of eluates I to IV from the aluminum oxide column were chromatographed on silica gel G using ethyl acetate–acetic acid 95 to 5. Eluates II to IV showed numerous spots under ultraviolet light before and after spraying with 70% sulfuric acid and after heating (Figure 1, left). When bioassayed on white beans, eluate II (methanol–ethyl acetate) showed biological activity in the form of leaf area enlargement, discoloration, and final elongation. Subsequently, the remainder of eluate II was streaked on silica gel G and separated into five fractions, II, *A* to *E*. These fractions were bioassayed on dwarf peas and beans. Fraction II, *C* showed biological activity on both plants (Figure 2, *a* and *b*). Chro-

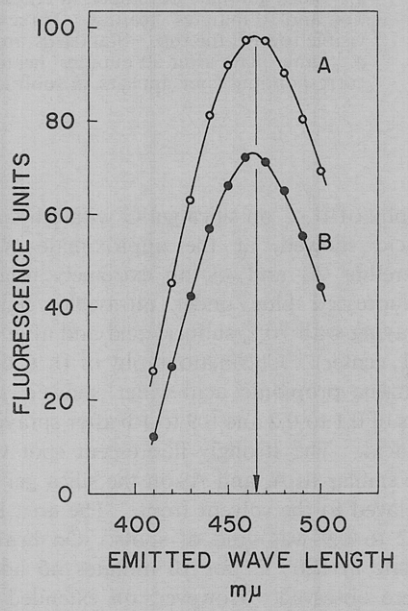


Figure 3. Fluorescence emission spectrum of biologically active substance in fraction II, *C*

*A.* Gibberellin $A_1$
*B.* Substance from II, *C*

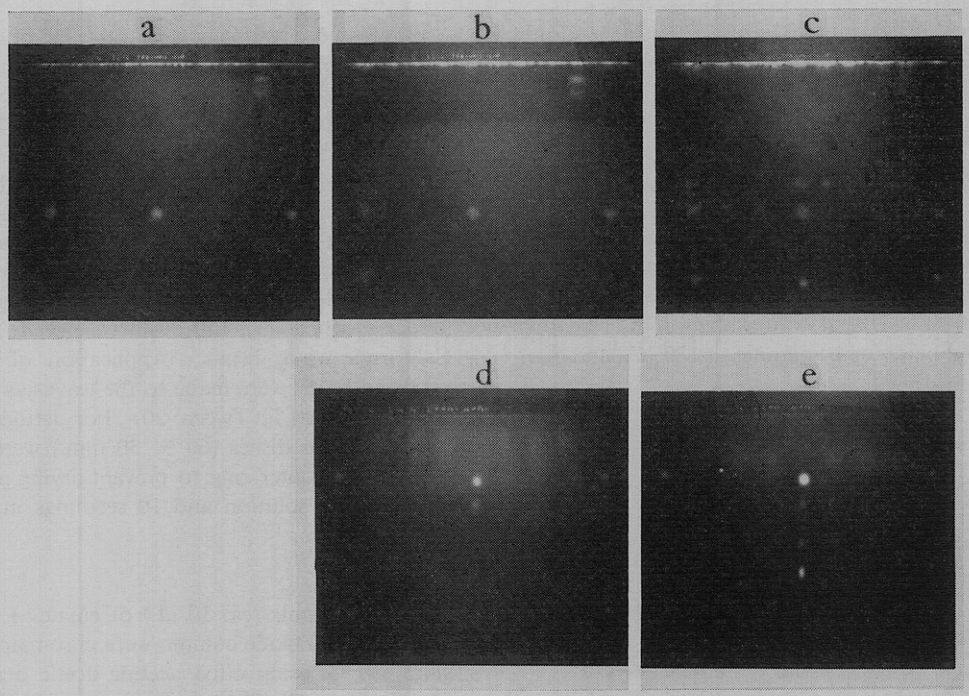


Figure 4. Photographs taken under ultraviolet light showing various stages of fluorescence development of gibberellin standards and of subfractions II, *C*–1 through 7 (1 through 6 in *d*, *e*)

Standards used on Kieselguhr plates were the dihydroxy gibberellin $A_1$ and $A_3$ and the trihydroxy $A_8$. Dihydroxy $A_2$, which would separate above $A_1$ ($R_f$ 0.6), was not available. Standards on silica gel G were $A_8$, $A_1$, $A_7$, and $A_9$ representing gibberellins containing tri-, di-, mono-, and no hydroxy groups

*a*. Kieselguhr plate developed in benzene–propionic acid–water 8:3:5, photographed after spraying with 70% sulfuric acid. A row of gibberellin $A_3$ standards and two spots in subfraction 7 (upper right) are visible. Subfractions 1 to 3 are located between the left and center $A_3$ standards; 4 to 7, between the center and right $A_3$ standards in numerical order from left to right

*b*. Same plate after 10 minutes at 120° C. A row of gibberellin $A_8$ standards is visible along the bottom

*c*. Same plate after 30 minutes' heating. Gibberellin $A_8$ becomes brighter and $A_1$ standards appear above $A_3$, and corresponding spots in the adjacent II, *C* subfractions, 4 and 5

*d*. Silica gel plate developed in ethyl acetate–acetic acid 95 to 5, pictured after sulfuric acid application and 10 minutes' heating. Three columns of standards and impurities from the ethyl acetate are visible toward the top. Standards are, from top to bottom, gibberellin $A_9$, $A_7$, and $A_8$

*e*. Same plate after 30 minutes' heating. Standard gibberellin $A_1$ appears between $A_7$ and $A_8$. A corresponding spot appears in subfractions 4 and 5 to the right of the center column of standards

matography of II, *C* on silica gel G with ethyl acetate–acetic acid showed, at the approximate $R_f$ value of gibberellins $A_1$ and $A_3$, an extremely bright spot which fluoresced blue under ultraviolet light both after spraying with 70% sulfuric acid and after heating (Figure 1, center). Chromatography of II, *C* on KGG with benzene–propionic acid–water showed spots at $R_f$ values of 0.1 to 0.2 and 0.9 to 1.0 after spraying with sulfuric acid. The strongly fluorescent spot with the $R_f$ value similar to $A_1$ and $A_3$ on the silica gel G plate was displaced to the solvent front. The area from $R_f$ value 0.2 to 0.9 was void of spots. On heating the KGG plate at 120° C. for 10 minutes no additional spots were observed. However, on extended heating to 30 minutes a lone blue fluorescent spot appeared at $R_f$ value 0.58 that corresponded exactly with the $R_f$ value of authentic gibberellin $A_1$ on the same plate (Figure 1, right). The fluorescence emission spectra of the suspected $A_1$ and the authentic $A_1$ were similar between 400 and 500 mμ; both peaked at 465 mμ (Figure 3).

The remainder of II, *C* was streaked on a KGG plate and divided into subfractions II, *C*–1 to 7. Bioassay on peas showed that the area of biological activity was in subfractions II, *C*–4 and II, *C*–5 (Figure 2, *c* and *d*), which represented $R_f$ values of 0.40 to 0.66.

**Table I. Lettuce Bioassay of Rhubarb Seed Extract**

| | Length after 3 Days, Mm. ± σ |
|---|---|
| Control | 12.9 ± 1.7 |
| II, *C*–1 | 12.8 ± 1.8 |
| II, *C*–2 | 13.3 ± 1.4 |
| II, *C*–4 | 14.9 ± 1.7 |
| II, *C*–5 | 14.8 ± 2.0 |

Table II. $R_f$ Values of the Biologically Active Subfraction (II, *C*–4) in Various Thin-Layer Chromatographic Solvent Systems

| Layer | Solvent System | $R_f$, II, *C*–4[a] | $R_f$, $A_1$[a] |
|---|---|---|---|
| Kieselguhr-G[b] | Carbon tetrachloride–acetic acid–water (*4*) 8:3:5, lower phase + 20% ethyl acetate | 0.17 | 0.17, 0.16 |
| | Benzene–acetic acid–water (*10*) 8:3:5 | 0.06 | 0.06, 0.06 |
| | Benzene–propionic acid–water (*10*) 8:3:5 | 0.49 | 0.49 |
| Silica gel G | Ethyl acetate–acetic acid (*7*) 95 to 5 | 0.45 | 0.46 |
| | Methyl acetate–2-propanol–ammonia (*13*) 45:35:20 | 0.31 | 0.32, 0.31 |
| | Diisopropyl ether–acetic acid (*10*) 95 to 5 | 0.04 | 0.05, 0.05 |
| | Benzene–1-butanol–acetic acid (*4*) 80:15:5 | 0.10 | 0.10, 0.10 |
| | Benzene–1-butanol–acetic acid (*4*) 70:25:5 | 0.50 | 0.51, 0.50 |
| | Benzene–acetic acid–water (*10*) 8:3:5, lower phase | 0.00 | 0.00, 0.00 |

[a] Visualized under long-wave ultraviolet light after sulfuric acid spraying and 30 minutes' heating at 120° C. No spots were visible after 10 minutes at 120° C.
[b] Kieselguhr plates were equilibrated for 4 hours in the presence of both organic and aqueous phases and developed in the organic phase.

Bioassay on lettuce showed the same subfractions to be biologically active (Table I). Partition chromatography of II, *C*–4 and II, *C*–5 on KGG revealed a spot mostly in II, *C*–4 corresponding to authentic $A_1$ after spraying with sulfuric acid and 30 minutes of heating (Figure 4, *a*, *b*, and *c*). Subsequent chromatography of subfraction II, *C*–4 in several solvent systems (Table II) showed that the biologically active material was chromatographically identical to gibberellin $A_1$.

Another eluate of interest was III, the methanol eluate from aluminum oxide. Fractions *C* and *D* representing $R_f$ values 0.6 to 1.0 both contained substances inhibiting the growth of peas (Figure 2, *c* and *d*).

The amount of $A_1$ in rhubarb seeds was estimated in a separate analysis to be at least 150 μg. per kg. of fresh seeds on the basis of visual chromatographic comparison with known $A_1$ standards.

*Discussion*

Gibberellins containing tri-, di-, mono-, and no hydroxy substituents separate at $R_f$ values of approximately 0.4, 0.5, 0.7, and 0.8, respectively, when the silica gel G plate is developed in ethyl acetate–acetic acid. Thus, fraction II, *C* from the silica gel G plate, corresponding to $R_f$ values 0.38 to 0.54, is an area associated with dihydroxygibberellins ($A_1$, $A_2$, $A_3$) and trihydroxygibberellin ($A_8$). Therefore, the biologically active substance in fraction II, *C* could be one of three gibberellins, $A_1$, $A_2$, or $A_3$ (gibberellin $A_8$ being biologically inactive). These can easily be differentiated (*5*, *10*) in the absence of an overlapping spot as follows: Gibberellin $A_3$ becomes fluorescent blue-green under ultraviolet light immediately upon spraying with 70% sulfuric acid. $A_2$ is not visible after spraying with the sulfuric acid but becomes a fluorescent purple upon 4 to 8 minutes of heating. $A_1$ becomes a fluorescent blue only upon heating of the sulfuric acid–sprayed plate for 30 to 40 minutes at 120° C. However, since none of the descriptions matched the spot on silica gel G, an overlapping spot was suspected. Subsequent chromatography of fraction II, *C* on KGG with benzene–acetic acid–water, confirmed this by revealing a spot with the characteristics of $A_1$ (Figure 1, right). The inductive heating period (*5*) is another distinguishing feature of the gibberellins. Among the gibberellins $A_1$ to $A_9$, $A_1$ requires the longest period of inductive heating for the development of fluorescence.

The KGG plate in the benzene–propionic acid–water system (*10*) was especially useful in the separation of $A_1$ because of an exceptionally good resolution of the dihydroxygibberellins, $A_1$, $A_2$, and $A_3$, and the trihydroxygibberellin, $A_8$. Others through $A_9$ are on or close to the solvent front.

The existence of gibberellin $A_1$ in the seeds of the rhubarb is not indisputable, for there is no substitute for isolation of a substance in crystalline form and characterization of the crystals. However, the available evidence clearly points to two possibilities, the existence of $A_1$ in rhubarb seeds or the existence of a very similar acid compound with the same chromatographic, fluorescence, fluorescence development, and biological properties of $A_1$. The latter possibility seems remote.

*Acknowledgment*

The author thanks Lynda K. Solomon for her able technical assistance; D. R. Tompkins and G. Ekuan, Horticulture Department, W.W.R.E.C., for a supply of the immature rhubarb seeds; and D. Broadbent, Pharmaceuticals Division, Imperial Chemical Industries, Ltd., for a supply of gibberellins $A_1$, $A_4$, $A_5$, $A_7$, $A_8$, and $A_9$.

*Literature Cited*


(1) Brian, P. W., Hemming, H. G., Lowe, D., *Ann. Botany (London)* [N. S.] **28,** 369 (1964).
(2) Elson, G. W., Jones, D. F., MacMillan, J., Suter, P. J., *Phytochemistry* **3,** 93 (1964).
(3) Frankland, B., Wareing, P, F., *Nature* **185,** 255 (1960).
(4) Ikekawa, N., Kagawa, T., Sumiki, Y., *Proc. Japan Acad.* **39,** 507 (1963).
(5) Jones, D. F., MacMillan, J., Radley, M., *Phytochemistry* **2,** 307 (1963).
(6) Khalifah, R. A., Lewis, L. N., Coggins, C. W., Jr., *Plant Physiol.* **40,** 441 (1965).
(7) Kimura, Y., J. AGR. FOOD CHEM. **13,** 260 (1965).
(8) MacMillan, J., Seaton, J. C., Suter, P. J., *Tetrahedron* **11,** 60 (1960).
(9) *Ibid.*, **18,** 349 (1962).
(10) MacMillan, J., Suter, P. J., *Nature* **197,** 790 1963).
(11) Radley, M. E., *Ibid.*, **210,** 969 (1966).
(12) Sumiki, Y., Kawarada, A., in "4th International Conference on Plant Growth Regulation," R. M. Klein, Ed., p. 483, Iowa State University Press, Ames, Iowa, 1959.
(13) Stahl, E., in "Thin Layer Chromatography," E. Stahl, Ed., p. 293, Springer-Verlag, Berlin, 1965.
(14) Tompkins, D. R., *Proc. Am. Soc. Hort. Sci.* **87,** 371 (1965).
(15) West, C. A., Phinney, B. O., *J. Am. Chem. Soc.* **81,** 2424 (1959).
(16) Yabuta, R., Sumiki, Y., *J. Agr. Chem. Soc. Japan* **14,** 1526 (1938).



*Received for review January 9, 1967. Accepted March 28, 1967. Scientific paper No. 2913, Washington State University, College of Agriculture Research Center, Pullman, Wash., Project 9037.*