Studies on the Germination Inhibitor in Dormant Rice Seed, Isolation of Vanillin from Active Fraction

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Studies on the Germination Inhibitor in Dormant Rice Seed, Isolation of Vanillin from Active Fraction

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The isolation of a germination inhibitor was attemted from rice seed of a tropical variety (Oryza sativa L. var. Peta) in the dormant state. From the active fraction, vanillin (3-methoxy-4-hydroxybenzaldehyde) was isolated. Vanillin itself had little inhibitory activity, but it was suggested to be a probable degradation product of the dormant factor (germination inhibitor) in the rice seed.

The presence of compounds which regulate the dormant state of rice seed has been suggested by one of the anothurs N. Takahashi in the seed of wild and some tropical varieties of rice and it was reported that the germination inhibitors which diffused out from the outer membranes of the seed were located in the hull and the bran. It was also suggested that two germination inhibitor fractions were found in the paperchromatogram at Rf range of 0.0–0.4 and 0.7–0.9. The solvent system was isopropanol-ammonia-water (10:1:1). (1)

We attempted to isolate the germination inhibitors from rice seed in a dormant state. The active principle was found to be extremely labile and the isolation of the active principle has not yet been successful. However we confirmed on a paper chromatography that the Rf of most active band (X) coincided with the band which can be developed with 2,4-dinitrophenylhydrazine suggesting that the active principle might have a ketone or aldehyde functional group, although the possibility of the contamination by an aldehyde or ketone which had a similar Rf with the active principle can not be excluded. By evaporation of the active fraction, this carbonyl compound (X) decreased and it gave a new degradation product (Y) which also gave a yellow spot with 2, 4-dinitrophenylhydrazine. The degradation of (X) to (Y) was accompanied with a decrease of the inhibitory activity. The isolation of carbonyl compound (X) was also unsuccessful. How-

ever the degradation product (Y) was isolated and found to be vanillin. These results suggest the possibility that the active principle may have a vanillin moiety in the molecule and may be readily degragated into vanillin during the isolation procedure.

Experimental

Material

Seeds of the Phillipine variety of rice, Peta, which were known to have strong dormant tendency after ripening, were used. The rice was grown in 1967 and 1968 in the paddy field of the International Rice Research Institute (Los Banous, Laguna, Phillipins) and contributed by courtesy of the director Dr. R.F. Chandler. In 1968, the rice was transplanted on September 3rd, harvested in December and transported by air to Japan. After arriving at our laboratory, all of the seeds (240 Kg) were soaked with methanol.

Bioassay

The assay of the dormant factor was carried out according to Takahashi's method used for the inhibition of germination of lettuce seeds. A non-light requiring variety, Lactuca sativa L. var. Wayahead, was used. A sheet of filter paper containing test substance was placed in a Petri dish of about 3 cm diameter. The paper was moistened with 0.3 ml of water. 50 seeds of lettuce were placed in a dish. These Petri dishes were maintained at 20°C in the dark for 24 hours. The ratio of germination was compared with the blank.

Separation by Solvent Extractions

The active principle was fractionated first by the solvent extraction. In each solvent extraction the biological activity was checked and the active fraction was further fractionated. The scheme of the extraction is shown in Fig. 1. in the following diagram. The methanol extract (300 l) of the rice (240 Kg) was combined and condensed to about 20 l by a flash evaporator. The methanol extract was washed with petroleum ether and the fat was removed without loss of the activity. The solvent was exchanged with water by adding the water and subsequent evaporation of the methanol. The aqueous layer, after complete removal of the methanol, was extracted with petroleum ether and the active principle was extracted in the petroleum ether fraction. The petroleum ether layer was extracted with 2% sodiumbicarbonate (1.5 l) solution. The active principle was moved to this layer. It was wahed with ether and then acidified with acetic acid to pH 3.5. The acidified aqueous layer was extracted with ether. The ether extract (fraction A) showed a strong inhibitory activity i.e. 100% inhibition at 1000 ppm in 0.3 ml water. This fraction was used for further chromatographic study.

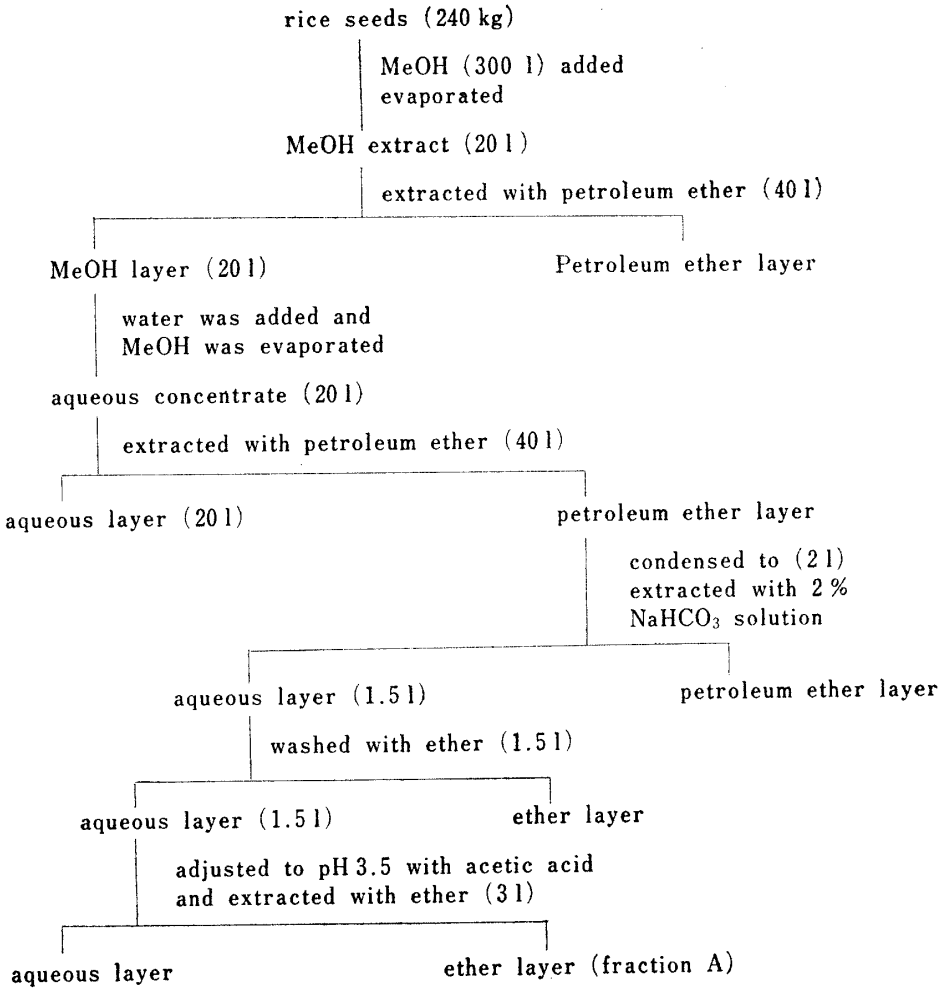

FIG. 1. Separation Diagram.

Paper chromatography

An aliquot of fraction A was chromatographed on Toyo Roshi No. 51A paper with water as the developing solvent. The devloped paper was air dried at room temperature. The strip was cut into 9 pieces as shown in Fig. 2–(A). Each pieces was submitted to the bioassay. The inhibitory activity was observed in the pieces from No. 4 to No. 8 (Rf 0.28–0.88), among them the strongest activity was found in piece No. 6 (Rf. 0.52–0.64). In another paper chromatogram of the same source (Fig. 2–(B)), the paper was treated with 2,4-dinitrophenylhydrazine (a reagent for aldehyde and ketone), the zone corresponding to the No. 6 (c, Rf 0.53–0.61) gave orange color (X).

On both papers two fluorescent bands were obaerved under ultraviolet light and these bands were used as an inner Rf standard for the comparison of the two chromatograms in different runs. Thus the fluorescence were detected at No. 3 and No. 5 on Fig. 2–(A) and at part (a) and part (b) in Fig. 2–(B). The results suggest that the active principle might have a carbonyl group which gave positive

spot with 2,4-dinitrophenylhydrazine although there was still left the possibility of contamination of the carbonyl compound which gave the same Rf in the above chromatography.

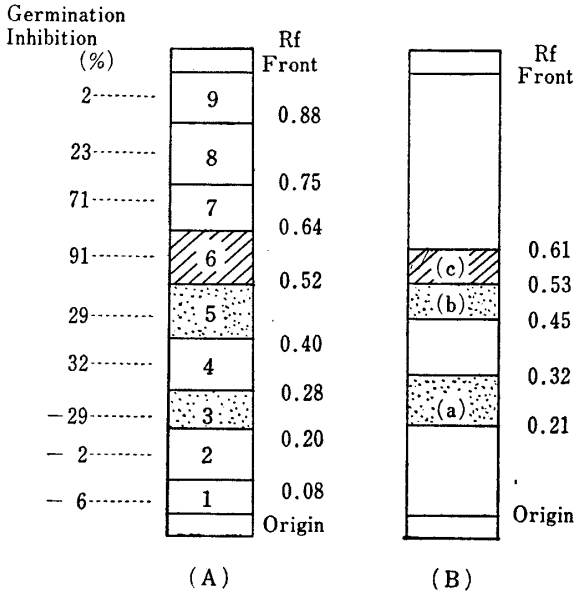

FIG. 2. Paper chromatography of fraction A.
solvent; Water
3, 5, (a), (b),; fluorescence under ultraviolet ray
(c); orange color with 2,4-dinitrophenylhydrazine

The degradation of 2, 4-dinitrophenylhydrazine positive factor

On the silica gel thin layer chromatography (T L C) by the developing solvent; benzene, ethylacetate (85:15), fraction A gave one spot, spot (X), under both ultraviolet ray and 2, 4-dinitrophenylhydrazine. However, after the evaporation of ether and acetic acid under reduced pressure by a rotary evaporater at below 40°C, fraction A gave two spots (X and Y), and the activity of germination inhibition reduced from 100% to 82% (concentration: 1,000 ppm). After 2 days, the methanol solution of fraction A revealed only the (Y) spot. The (X) spot had disappeared, suggesting that the (X) had degradated into (Y). (X; Rf 0.26, Y; Rf 0.46)

Isolation of 2,4-dinitrophenylhydrazine active compound (Y) from fraction A.

Fraction A was evaporated. The residue (86 mg) was dissolved into benzene-ethyl acetate mixture and chromatographed by silica gel column. A column of 100 g of Kieselgel (0.05–0.2 mm) diam. 4.5 cm, length 13 cm was used. The column was eluted successively with 100 ~200 ml each of benzene-ethylacetate (85:15), (70:30), (60:40), ethyl acetate and finally with methanol. The color reaction with 2, 4-dinitrophenylhydrazine was positive in the fractions of benzene-

ethyl acetate (60:40) and ethyl acetate. The TLC of the fractions gave spot (Y) and spot (X) was not detected. This suggests that spot (X) had completely degradated into (Y) during the evaporation procedure. The fractions of (Y) were evaporated and again purified by a preparative thin layer chromatography, thickness 0.5 mm, 20×20 cm of Kieselgel FG 254 activated at 110°C for 1 hour. The 2, 4-dinitrophenylhydrazine positive band which also gave a dark shadow under UV light at Rf 0.34–0.40 developed with benzene-ethyl acetate mixture (85:15) was scrapped off and eluted with methanol. About 3.9 mg of oily substance was obtained in the chromatographically pure state. The physical and spectrometric data of the substance are shown in Table 1. It was identified as vanillin (3-methoxy-4-hydroxybenzaldehyde) and the IR, UV, NMR and Mass data were identical with those of the authentic sample.

TABLE 1. *Physical constant of the compound (Y)*

Chemical shift (τ)	number of H	
0.15	1	-CHO
2-3	3	aromatic H
3.5-4	1	phenolic H
6.03	3	$-OCH_3$

Mass. M/e=152 NMR in $CDCl_3$
UV. max. 231.5 nm, 279 nm and 310 nm in EtOH.
IR. OH (3480 cm^{-1}), -CHO (1700 cm^{-1}).

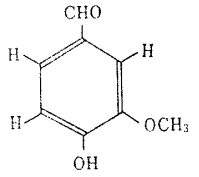

FIG. 3. The structure of vanillin (3-methoxy-4-hydroxybenzaldehyde)

The bioassay of the compound (Y).

The 2, 4-dinitrophenylhydrazine positive compound (Y) was dissolved in water and its germination inhibition was checked by bioassay. The biological activity showed a marked decrease compared with fraction A and the percent of inhibition at 1000 ppm was 45%. The authentic vanillin showed a 47% inhibition at 1000 ppm.

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Reference

1) N. Takahashi. *The Report of the Institute for Agricultural Research. Tohoku University*, **19**. 1–14 (1968)