IN VITRO OXIDATION OF INDOLEACETIC ACID BY CRUDE ENZYME FROM RICE HUSK—AN ASPECT OF PREHARVEST SPROUTING*

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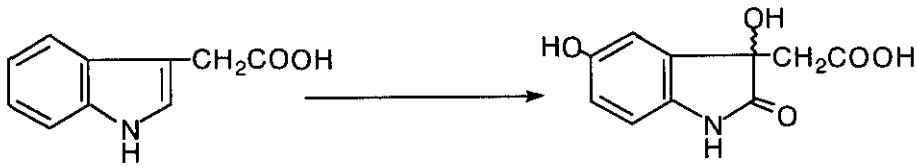
Abstract – Indoleacetic acid was oxidized under oxygen atmosphere by the crude enzyme solution prepared by blending rice husk in buffer solution of pH 7.3. The major oxidation products (**3**, **4**, and **5**), characterized by physical and chemical methods, showed little effects on germination and growth of lettuce seeds while IAA inhibited completely the germination at 5 ppm. This evidence suggests that the amounts of IAA in embryos decreases due to the oxidation by water soluble oxidation enzyme existing in rice husk. The decrease of IAA may be related, in some aspect, to dormancy breaking and also preharvest sprouting of rice seeds. The synthesis and optical resolution of **2** was also achieved.

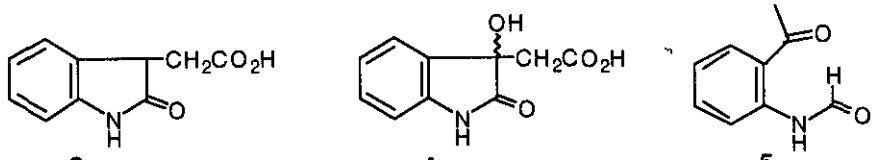
In the course of our search of growth regulators in rice seeds, we have characterized indoleacetic acid (IAA, **1**)[1] and its oxidation product (**2**)[2] from rice bran, mainly consisting of rice embryos. The latter compound showed little activities toward assay of germination and growth inhibition using lettuce seeds while the former exhibited the strong inhibition activities.[3] Recently, Takahashi and Miyoshi observed the large difference of germination of rice seeds between intact and dehusked seeds belonging to japonica groups. Almost all the japonica rice varieties showed less germination by dehusking as compared with that of intact seeds.[4] These findings when associated with our isolation of **1** and **2** from rice bran suggested that the amounts of active IAA in embryos decreases due to the oxidation of IAA by water soluble oxidation enzyme exsisting in rice husk.[5] Oxidation activity may increase by contact with water since pre-harvest sprouting, which is attibuted to the absence of dormancy in japonica rice, is sometimes observed in paddy field where rain often prevails. Based on the above assumption, we examined a possibility of oxidation of IAA by crude enzyme from rice husk.[6]

* Dedicated to Professor Koji Nakanishi on the occasion of his 75th birthday



IAA (1) 2

Rice husk was blended with polyton at 0℃ for 15 min in phosphate buffer solutions of pH 5.6, 6.8 and 7.3, respectively. After centrifuged for an hour, each supernatant solution was examined to find the oxidation activity using IAA as substrate. It was found that the solution of pH 7.3 had an ability to oxidize IAA under oxygen atmosphere, giving three major oxidation products (**3**, **4**, and **5**). No oxidation was observed when the reaction was carried out without oxygen or the enzyme solution was preheated at 90 ℃ for 15 min. The oxidation products were purified by HPLC after esterification of **3** and **4** with CH_2N_2 and the structure was deduced from physical evidence and confirmed as follows. Treatment of IAA with DMSO in the presence of aq HCl afforded **3** in 61% yield.[7] The corresponding methyl ester was converted into dl-**4** in 88% yield by successive reactions of enolate formation with LDA at -78 ℃, followed by oxidation with Davis reagent.[8] Methyl ester of dl-**4** was submitted to esterification with (-)-camphanic chloride to give a diastereomeric mixture of (-)-camphanyl esters, which was separated by silica gel HPLC. Hydrolysis of each diastereomer with aq NH_4OH in acetone furnished (+)- and (-)-**4** possessing $[\alpha]_D^{26}$ +2.6° (*c* 0.2, MeOH) and -2.8°(*c* 0.2, MeOH), respectively. The physical data of **3** and (+)-**4** thus synthesized were indistinguishable with those of the enzyme oxidation products (**3** and **4**). The absolute configuration of (+)-**4** remains undetermined at present. The UV spectrum of **5** was completely different from that of **4**, indicating that **5** possesses the different chromophore. The detailed inspection of NMR spectra including NOESY and C-H COSY techniques suggested that **5** was *N*-(2-acetylphenyl)formamide. The structure was confirmed by oxidation of 3-methylindole following the literature procedure.[9]



3 **4** **5**

The preliminary bioassay experiments using lettuce seeds showed that oxidation products (**3**, **4**, and **5**) have little effect on germination and growth at 5 and 50 ppm while IAA inhibited completely the germination at 5 ppm. The rice seeds[10] were germinated in crude enzyme and preheated enzyme solutions and growth of roots were compared. The growth in preheated enzyme solution was 20% less than that in enzyme solution. This difference may be explained by considering that oxidation of IAA intrinsically existing in rice seeds occurred in crude enzyme solution but not in the preheated enzyme solution.

In paddy field where rain often prevails, the amounts of IAA in embryos may decrease due to the oxidation by water soluble oxidation enzyme existing in rice husk and the decrease of IAA in embryo is related, in some aspect, to dormancy breaking and also preharvest sprouting of rice seeds. The experiments using wheat seeds are currently investigated.

EXPERIMENTALS

Preparation of 2-Oxindole-3-acetic Acid (3). To a DMSO solution (348 μL) of IAA (92 mg) was added aq 12 N HCl (132 μL) and the mixture was kept for 4 h at rt. aq $NaHCO_3$ was added to arrange pH 6-7 and the volatile materials were removed in vacuo. After excess CH_2N_2 in MeOH was added at 0 ℃, volatile materials were removed in vacuo and the residue was passed through a silica gel column to give lactam (**3**) (65 mg, 61%) as amorphous white powder. δ_C 179.0 (s), 171.6 (s), 141.5 (s), 128.7 (s), 128.4 (d), 124.1 (d), 122.5 (d), 109.8 (d), 52.1 (q), 42.3 (d), and 34.6 (t). HRMS Found: m/z 205.0734. Calcd for $C_{11}H_{11}NO_3$: M, 205.0738.

Preparation of 2,3-Dioxindole-3-acetic Acid (4). To a LDA solution, freshly prepared from diisopropylamine (174 μL) in THF (5 mL) and 1.6 M BuLi in hexane (768 μL), was added THF (7 mL) solution of methyl ester of lactam (**3**) (210 mg, 1.02 mmol) at -80 ℃. After stirring for 2 h at the same temperature, Davis reagent (535 mg, 2.05 mmol) in THF (7 mL) was added and the temperature was gradually raised to 0 ℃. After stirring for 2 h, MeOH was added and volatile materials were removed in vacuo. Silica gel column chromatography of the residue afforded **4** (Me ester) (159 mg, 69%) and recovered Me ester of **3** (41 mg). **4** (Me ester) yellow powder. δ_C 178.4 (s), 170.9 (s), 140.6 (s), 130.2 (d), 129.6 (s), 124.2 (d), 123.2 (d), 110.6 (d), 73.8 (s), 52.1 (q), and 40.9 (t). HRMS Found: m/z 221.0685. Calcd for $C_{11}H_{11}NO_4$: M, 221.0688.

Resolution of (-)-Camphanyl Ester of dl-4. After a mixture of dl-**4** (Me ester) (36 mg, 0.16 mmol), pyridine (129 μL), S-(-)-camphanic chloride (58 mg, 0.38 mmol) in CH_2Cl_2 (4 mL) was stirred at rt overnight, MeOH was added and the volatile materials were removed in vacuo after 2 h. The residue was passed through a silica gel column with $CHCl_3$-AcOEt 10:1 and then 1:1 to give camphanyl ester (48 mg, 75%). Each diastereomer was separated by HPLC using Nova-Pak HR SILICA 7.8 x 300 mm with hex-AcOEt 2:1; Flow rate 2.0 mL/min. Retention time was 22 and 23 min, respectively. HRMS of each diastereomer: Found m/z 401.1488 and 401.1470. Calcd for $C_{21}H_{23}NO_7$: M, 401.1474. Each diastereomer (6.8 and 8.8 mg) was stirred at rt overnight in 70% aq acetone (1 mL) containing 32% aq NH_4OH (150 μL). Volatile materials were removed and excess CH_2N_2 in MeOH was added. Column chromatography using HP-20 with H_2O-MeOH 1:0 and then 1:1afforded a diastereomer of **4** (Me ester) (3.0 mg, $[\alpha]_D^{26}$ -2.8° (c 0.2, MeOH) and its antipode (4.0 mg, $[\alpha]_D^{26}$ +2.6°(c 0.2, MeOH), respectively. The enzyme oxidation product (**4**) (Me ester) showed $[\alpha]_D^{27}$ +5.2°(c 1.0, MeOH).

Preparation of *N*-(2-acetylphenyl)formamide (5). After a mixture of 3-methylindole (50 mg, 0.38 mmol) and $CuCl_2$ (51 mg, 0.38 mmol) in CH_3CN (5 mL) was stirred for 4 h under oxygen atmosphere, volatile materials were removed in vacuo and extracted with ether. The usual workup and silica gel chromatography with hex-AcOEt 10:1 and then 6:1 gave **5** (23 mg, 37%) as yellow powder. UV (EtOH) 320, 259, and 234 nm; δ_H 2.82 (3H, s), 7.16 (1H, dd, J=7.3, 8.1 Hz), 7.58 (1H, ddd, J=1.7, 7.3, and 8.5 Hz), 7.93 (1H, dd, J=1.7, 8.1 Hz), 8.50 (1H, s), 8.75 (1H, d, J=8.5 Hz), and 11.63 (1H, br s). δ_C 203.3 (s), 159.9 (d), 139.8 (s), 135.2 (d), 131.7 (d), 123.0 (d), 121.9 (s), 121.6 (d), and 28.6 (q). HRMS Found: m/z 163.0641.

Calcd for $C_9H_9NO_2$; M, 163.0633.

Preparation of 5-Hydroxydioxindoleacetic Acid (2). To an aqueous tBuOH solution (51mL, tBuOH:H_2O = 50:1) of 5-benzoyloxyindoleacetic acid (Me ester) (113 mg, 0.37 mmol) was portionwisely added NBS (78 mg, 0.44 mmol) at rt. After stirring for 4 h, the reaction mixture was extracted with AcOEt. The usual workup and silica gel chromatography with $CHCl_3$-AcOEt 10:1 gave 5-benzoyloxy-2-oxindoleacetic acid (Me ester) (69 mg, 58%) as yellow powder. δ_C 171.5 (s), 168.3 (s), 165.5 (s), 146.4 (s), 139.4 (s), 133.6 (d), 130.2 (d) x 2, 129.9 (s), 129.4 (s), 128.6 (d) x 2, 121.6 (d), 118.5 (d), 116.2 (d), 52.2 (q), 42.8 (d) and 34.5 (t). HRMS: Found m/z 325.0959. Calcd for $C_{18}H_{15}NO_5$; M, 325.0950. To a LDA solution, freshly prepared from diisopropylamine (68 μL, 0.52 mmol) in THF (5 mL) and 1.6 M BuLi in hexane (290 μL, 0.48 mmol), was added THF (5 mL) solution of methyl ester of 5-benzoyloxylactam (130 mg, 0.40 mmol) at -80 ℃. After stirring for 2 h at the same temperature, Davis reagent (208 mg, 0.80 mmol) in THF (5 mL) was added and the temperature was gradually raised to 0 ℃. After stirring for 2 h, MeOH was added and the volatile materials were removed in vacuo. Silica gel column chromatography of the residue with $CHCl_3$-AcOEt 10:0 and then 1:1 afforded **2** (Me ester) (40 mg, 42%) and benzoate of **2** (Me ester) (55 mg, 40%). Each diastereomer of the benzoate was resolved using HPLC (YMC CHIRAL NEA(R); 4.6 x 250 mm) with H_2O-CH_3CN 8:2; Flow rate 0.9 mL/min, to give enantiomer possessing $[\alpha]_D^{23}$ -13.7° (c 0.5, MeOH) and +12.8° (c, 0.5, MeOH), respectively. Hydrolysis of each enantiomer with aq NH_4OH afforded **2** (Me ester) with $[\alpha]_D^{26}$ -2.3° (c 0.2, MeOH) and +2.1° (c 0.2, MeOH), respectively. The natural product isolated from rice bran showed $[\alpha]_D^{26}$ -2.7° (c 1.0, MeOH).

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