Oxidative Reaction of Oxindole-3-acetic Acids

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The oxindole-3-acetic acids, oxidative metabolites of indole-3-acetic acid, were isolated from a byproduct of a corn starch manufacturing plant, and were further converted to the 3-hydroxyl derivatives in the presence of metal ion. The mechanical study was followed by a chemical analysis including other byproducts, and suggested the presence of an intermediate that had a radical at the C-3 position of oxindole-3-acetic acids.

Key words: dimerization; oxindole-3-acetic acid; hydroxyl radical; radical reaction

Dietary guidelines recommend the consumption of whole grains to prevent chronic diseases (reviewed in Ref. 1). Recently, Reddy et al. reported that dietary inclusion of the lipid fraction of wheat bran significantly inhibited azoxymethane-induced colon carcinogenesis.2 However, the minor constituents of cereals are poorly understood. From these backgrounds, we are interested in the low-molecular-weight phytochemicals in cereals or related materials.

In our previous study, we isolated and identified phenolic constituents from corn steep liquor, produced by our starch manufacturing plant, with the guidance of antioxidative activity.3 Corn-gluten meal (CGM) is also produced by our manufacturing process as a protein fraction, and has been used for animal feed. In this study, we also detected the five well-known phenolic acids in the extract of CGM by HPLC, which had been isolated from corn-steep liquor. However, we found two unidentified peaks on the spectrum. Structural analyses of these compounds and their derivatives by spectroscopic methods showed that they were oxindoles derived from indole-3-acetic acid (IAA). IAA is known as a plant hormone, and it is also reported as a strong antioxidant against hydroxyl radicals.4 We measured their antioxidative activity against hydroxyl radicals, and also examined the reaction mechanisms of oxindole-3-acetic acids in oxidative conditions.

Materials and Methods

Chemicals. Hydrogen peroxide, L-phenylalanine, and 18-crown-6 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Iron (II) sulfate heptahydrate, Iron (III) chloride hexahydrate, and 2,6-di-tert-butyl-p-cresol were products of Kanto Chemical Co., Inc. (Tokyo, Japan). EDTA was purchased from Katayama Chemical (Osaka, Japan). DL-o-Tyrosine was obtained from Sigma Chemical Co. (St. Louis, MO). (Trimethylsilyl)diazomethane was supplied by Aldrich (Milwaukee, WI). Potassium superoxide (KO2) was purchased from Strem Chemicals (Newburyport, MA).

Instruments. HPLC analysis was accomplished by a 250 mm × 4.6 mm i.d. Wakosil-II SC18 HG column (Wako Pure Chemical) on a Shimadzu CLASS-LC10 series HPLC system equipped with a SPD-M10Avp photodiode array detector (Shimadzu, Kyoto, Japan) at 40°C with a flow rate of 1.0 ml/min. LC-MS was done with the same column connected to a Fisons/VG Platform II mass spectrometer with a positive electrospray interface. Preparative HPLC was done using a Wakosil-II SC18 column (i.d. 20 × 250 mm) with a flow rate of 5.0 ml/min at ambient temperature. The NMR spectra were measured with a Bruker AM-400 spectrometer. High resolution EIMS experiments were done on a JEOL JMS-700 mass spectrometer (Tokyo, Japan). The UV spectra were recorded on a U-2000 spectrophotometer (Hitachi, Tokyo, Japan). The IR spectra were measured with a JASCO 7000S FT/IR spectrometer (Tokyo, Japan).

Purification of oxindole-3-acetic acids from CGM. In the isolating procedures of 1 and 2, we found that an alkaline treatment of CGM extract significantly increased the yield, which might be due to the prevention of emulsification at the EtOAc extraction and

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Abbreviations: CGM, corn-gluten meal; IAA, indole-3-acetic acid; oxIAA, oxindole-3-acetic acid; dioxIAA, 3-hydroxy-oxindole-3-acetic acid
reduced insoluble gummy materials for column chromatography. Also, an alkaline treatment had little affect on the HPLC chromatogram (data not shown), thus we described here the procedures that used an alkaline hydrolysis. CGM (200 g), obtained from our plant (Aichi, Japan), was extracted with 50% aq. EtOH (600 ml) for six hours at ambient temperature. The extract was filtered and the filtrate was then hydrolyzed for 30 min by the addition of 1 N NaOH (100 ml). The reaction mixture was made weakly acidic with 12 N HCl (10 ml), and evaporated in vacuo to remove alcohol. The residual solution was extracted with EtOAc, then the organic phase was washed, dehydrated over anhydrous Na$_2$SO$_4$, and evaporated in vacuo. The EtOAc soluble fraction (0.83 g) was separated by reversed-phase column chromatography (i.d. 20 × 200 mm, Wakogel L-P 50C18; Wako Pure Chemical) eluted with H$_2$O/MeOH/TFA (500:500:1). The fractions were analyzed by HPLC and corresponding fractions were collected (273 mg). Further purification was done with preparative HPLC with a UV detector at wavelength 254 nm using a solvent mixture of H$_2$O/MeOH/TFA (750:250:1), and gave 1 (21.5 mg) and 2 (19.2 mg).

**Derivatization of I.** Reaction of 1 (25 mg) with an excess of (trimethylsilyl)diazomethane in MeOH at ambient temperature for 3 hours. The reaction was quenched by the addition of dilute AcOH, and then evaporated in vacuo. Preparative TLC by Merck precoated glass plates (silica gel PF254; 1.0 mm) eluted with a solvent mixture of n-hexane/EtOAc (2:3) gave dimethyl derivative (3, 10 mg).

3: white powder. HR-ELMS $m/z$ (M$^+$): Calcd. for C$_{12}$H$_{13}$NO$_4$: 235.0845. Found: 235.0827. UV $\lambda_{max}$ (EtOH) nm (ε): 249 (8,290), 291.5 (3,480). IR (KBr) $\nu_{max}$ cm$^{-1}$: 3350 (NH), 1700 (CO$\equiv$). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ $H$ 7.57 (1H, brs, NH), 6.98 (1H, dd, $J$ = 7.4, 8.3 Hz, H-5), 6.85 (1H, d, $J$ = 7.4 Hz, H-4), 6.82 (1H, d, $J$ = 8.3 Hz, H-6), 3.87 (3H, s, Ar-OCH$_3$), 3.84 (1H, dd, $J$ = 4.5, 8.2 Hz, H-3), 3.70 (3H, s, COOCH$_3$), 3.08 (1H, dd, $J$ = 4.5, 16.9 Hz, CH$_2$a), 2.81 (1H, dd, $J$ = 8.2, 16.9 Hz, CH$_2$b). $^1$C NMR (100 MHz, DMSO-d$_6$) $\delta c$ 177.9 (s, C-2), 171.5 (s, COOCH$_3$), 143.7 (s, C-7), 130.2 (s, C-7a), 129.4 (s, C-3a), 123.0 (d, C-5), 116.4 (d, C-4), 110.7 (d, C-6), 55.6 (q, Ar-OCH$_3$), 52.0 (q, COOCH$_3$), 42.9 (d, C-3), 34.6 (t, CH$_3$).

**Measurement of scavenging activity against hydroxyl radicals in vitro.** Antioxidative activity against hydroxyl radicals was done with a method of Kaur et al.$^{30}$ with slight modification. In brief, a reaction mixture containing phenylalanine (0.4 mM), FeSO$_4$ (2.0 mM), EDTA (2.0 mM), and H$_2$O$_2$ (6.6 mM) in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) was incubated at 25°C with the presence or absence of samples (0.25 mM) in a 1.5 ml microtube. After 20 min, the reaction was quenched by the addition of 10 mM 2,6-di-tert-butyl-p-cresol dissolved with 2-propanol (50 μl). The o-tyrosine produced was evaluated with HPLC eluted with H$_2$O/MeOH/TFA (900:100:1) at the wavelength of 270 nm.

**Oxidation of oxindole-3-acetic acid (oxIAA) methyl ester by FeCl$_3$.** To a solution of oxIAA methyl ester (4; 100 mg) in MeOH (25 ml) was added a solution of FeCl$_3$ (3 eq) in H$_2$O (25 ml). The reaction mixture was stirred overnight at ambient temperature and then concentrated in vacuo to remove methanol. The resulting solution was diluted with 100 ml H$_2$O and extracted with 70 ml of EtOAc (×3). The organic layer was washed with water and saturated brine, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure. Preparative HPLC equipped with a UV detector at wavelength 210 nm using a solvent mixture of H$_2$O/MeOH/TFA (600:400:1) gave 3-hydroxy-oxindole-3-acetic acid (dioxIAA) methyl ester (5; 22.6 mg) and the 3,3'-dimer (6; 5.9 mg). One fraction was further purified with preparative HPLC using a 25% MeCN as eluent, and 2,4-quinolinolinediol (7; 0.4 mg) was obtained.

**Results and Discussion**

Extraction of CGM has been done by aq. EtOH using several concentrations. Among these solvents used, 50% EtOH extract showed good results in terms of the antioxidative activity on the rabbit erythrocyte membrane$^{30}$ at the same weight concentration (data not shown). CGM was extracted with
HPLC was completed with a Wakosil-II 5C18 HG column (i.d. 4.6 × 250 mm) eluted with the solvent mixture of H2O/MeOH/TFA (750:250:1) at a flow rate of 1.0 ml/min at 40°C. (a) 4-hydroxybenzoic acid, (b) vanillic acid and caffeic acid, (c) p-coumaric acid, (d) ferulic acid. Peak (b) was analyzed under different conditions after preparative HPLC (data not shown).

The 1H NMR spectra of the compounds had similar and specific signals between 4–2 ppm, which suggested an asymmetric methine proton and an adjacent methylene moiety. Further spectral analysis found 2 as oxIAA (Fig. 2). The chemical shift on the 1H NMR spectrum was very consistent with the literature, even though they did not describe the vicinal couplings of the methylene protons. However, the structure was confirmed using a synthetic method. The 1H NMR spectrum of 1 suggested that it had the same oxIAA skeleton. The LC-MS analysis (m/z = 208 [M + H]+) showed that 1 was 16 mass units heavier than 2. From the 3H multiplet aromatic proton signals on the 1H NMR spectrum, we considered that 1 had an additional oxygen atom as a hydroxyl moiety on the 4–7 position of 2. However the position could not be identified from the 1H NMR spectrum because of the multiplet aromatic signals (data not shown). We derivatized 1 with (trimethylsilyl)diazomethane, and found two methoxyl signals (δ 3.87, 3.70) on the 1H NMR spectrum (CDCl3). Fortunately this reaction distributed the three protons of the aromatic ring on the 1H NMR spectrum. The coupling constants (J = 7.4, 8.3 Hz) of aromatic regions suggested that 1 was a 4- or 7-hydroxy derivative of 2. From the HMBC experiment of 3, we found the cross-peak between a double doublet aromatic methine signal (δ 6.98) and a quaternary carbon (δ 129.4), to which the methylene signals (δ 3.08, 2.81) of the side chain had correlation. We concluded that 1 was 7-hydroxy-oxindole-3-acetic acid (Fig. 2), which had been detected as a metabolite of IAA, even though the three aromatic protons were described as singlet in the literature and we recorded them as multiplet on the 1H NMR spectrum in acetone-d6. 1 and 2 were reported as metabolites of IAA in Zea mays and would be endogenous compounds, but they did not have optical rotation even in an isolating procedure without alkaline hydrolysis.

In the most recent studies, the antioxidative activity of indole compounds have been described. We examined the scavenging activity of oxindol-3-acetic acids by measuring the hydroxyl radical-mediated o-tirosine formation from phenylalanine. Both 1 and 2 inhibited the reaction, but the oxidation at C-2 decreased the inhibitory activity of IAA (Fig. 3). The antioxidative mechanisms of indole compounds were also studied, and the reaction often occurred at the C-3 position via the 2,3-double bond. However oxindoles lost the conjugated double bond, and as we were interested in the reactions of 2 in oxidative conditions, we then examined the oxidation of oxIAA methyl ester (4) to avoid the reaction derived from the free carboxylic acid. Iron (III) chloride is often used as an oxidative...
reagent in vitro, and we previously used the agent for preparing the dehydroaminpinic acid dilactone.15) The oxidation of 4 by FeCl₃ produced a dioxIAA methyl ester (5).6) Also, two peaks were detected as minor constituents on the HPLC analysis. One product that had a similar UV spectrum to the starting material, lost the methine proton at C-3 on ¹H NMR, and gave a quaternary carbon (51.8 ppm) that had a HMBC correlation between NH and the asymmetric methylene protons. With the mass spectrum, we identified the structure as the 3,3'-dimer of oxindole (6), even though the stereochemistry was not yet established.

Another product was identified as 2,4-quinolinediol (7) from spectral analysis and comparison with the authentic sample (Scheme 1). A formation of 6 suggested the presence of an intermediate which had a radical at C-3, however hydroxy radical scavengers, mannitol nor 2-propanol, did not inhibit the formation of 5 nor 6 (data not shown). The hydroxylation by FeCl₃ was also observed in 2 itself, even though the unidentified minor products increased, whereas the methyl ester of IAA was stable under these conditions.

In the previous study, a hydroxylation at C-3 of oxindole proceeded with CuCl₂, and this reaction produced byproducts that produced via a dioxetane intermediate.16) However, oxidation of 4 with a superoxide anion radical generated by KO₂ gave 2,4-quinolinediol (7) predominantly. A small amount of dioxIAA ester was produced, but 6 was not detected (data not shown). This result suggested that the dioxetane intermediate would prefer a dicarbonyl formation, which produced 7 as illustrated in Scheme 2, and might not be concerned in the hydroxyl reaction.

3-Hydroxy-oxindoles have been detected as the metabolites of indoles, not only of plants but also animals,17,18) and the oxidative modification of indoles would be an important step in their biological activity.19–21) However, the metabolic pathway was not fully established. We could not yet identify the actual radical species for the hydroxylation and the precise mechanism of the formation of C-3 radicals of oxindoles. However, our results suggested that the oxindoles are important intermediates for the metabolism of indoles in oxidative conditions.

Cereal prolamins represented by wheat gliadin and maize zein are known as antioxidative materials,22,23) although the identities of the active compounds have been ambiguous. Wang et al.24) suggested that the antioxidative activity of "zein" is derived from cooperative action of tocopherols and other phenolic compounds, not from the antioxidant functions of the proteins themselves. Our results suggested that the low-molecular-weight phenolics would play a part of the antioxidative activity of CGM, even though the participation of "zein protein" itself was ambiguous.

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References

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