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Antioxidative Activities of Oxindole-3-acetic Acid Derivatives from Supersweet Corn Powder

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The components contributing to the antioxidative activity of supersweet corn powder (SSCP), which is commonly used in corn soup and snacks in Japan, were clarified and the effects investigated. 7-(*O*-β-Glucosyloxy)oxindole-3-acetic acid (GOA) was found to be the component most strongly contributing to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity of the 80% ethanol extract of SSCP, and the presence of its aglycone, 7-hydroxy-oxindole-3-acetic acid (HOA) was confirmed. GOA and HOA respectively contributed 35.1% and 10.5% to the DPPH radical-scavenging activity of the 80% ethanol extract of SSCP. Mice orally administered with HOA at doses of both 500 and 1500 mg/kg showed a significantly lower ($p < 0.05$) level of thiobarbituric acid reactive substances (TBARS) in the plasma than the vehicle-treated control. These results suggest that GOA and HOA were at least partly involved in the antioxidative activity of SSCP *in vitro* and that HOA might have possessed antioxidative activity *in vivo*.

Key words: supersweet corn; antioxidative activity; oxindole-3-acetic acid

Oxidative stress plays an important role in the development of cancer, diabetes, and cardiovascular disease. A large number of food-derived, natural, antioxidants have been identified, and those in foods consumed in daily life may inhibit development of the clinical conditions of such diseases.[1,2] The levels of antioxidants in grains, vegetables, and fruit are high, and the consumption of these foods may be important to prevent oxidative stress-related disorders.[3–6] Grains are the main supply source of antioxidants in daily life. In particular, the antioxidative activity of corn is relatively high,[7] and its consumption methods vary; corn may therefore be a possible source of antioxidative substances.

Dent corn, which is consumed as a grain, comprises the greatest proportion of corn consumption. This is harvested as dry kernels and is employed as a food component, including corn flour for corn bread and cereals, oil, high-fructose corn syrup, and alcohol, as well as feed for domestic animals.[8] On the other hand, supersweet corn comprises the greatest proportion of corn consumed as a vegetable. Immature kernels are harvested in which the water content is high.[9] It has been shown in supersweet corn that starch synthesis is inhibited *via* the mutation of a gene involved in the synthesis of starch from glucose (gene mutation *sh2*: ADP-glucose pyrophosphorylase), increasing the sugar content and contributing to the sweet taste.[10] The sugar content reaches approximately 30% of the dry weight, markedly differing from other corn types. This is the reason why supersweet corn is utilized as a vegetable, and it is also used as a component of processed foods. In particular, supersweet corn powder (SSCP), which is manufactured by heating, drying and crushing supersweet corn, is commonly employed in Japan as a material to add flavor/color to powdered corn soup and snacks.[11]

The antioxidative activities of heated/processed vegetables have been considered to be less potent than those of fresh vegetables; however, studies using corn and tomato have shown that heating decreased the level of L-ascorbic acid, whereas it increased their antioxidative activities.[12,13] We therefore became interested in the antioxidative activity of SSCP manufactured *via* a heating process in comparison with that of commercially available supersweet corn.

Supersweet corn has been reported to contain such antioxidants as lutein, zeaxanthin, tocopherols, ferulic acids, and L-ascorbic acid.[14–16] These substances are thought to contribute to the antioxidative activity of supersweet corn and also of SSCP; however, our preliminary analysis showed that these substances contributed to only part of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity of the SSCP extract; other antioxidants in the SSCP extract were therefore predicted to contribute to its DPPH radical-scavenging activity.

To clarify the components contributing to the antioxidative activity of SSCP, we evaluated its DPPH radical-scavenging activity and identified two oxindole-3-acetic acid derivatives, 7-(*O*-β-glucosyloxy)oxindole-3-acetic acid (GOA, Fig. 1) and its aglycone, 7-hydroxy-oxindole-3-acetic acid (HOA) which has been reported to be isolated as an antioxidant from corn gluten meal.[17] We measured their contents and antioxidative activities in this study.

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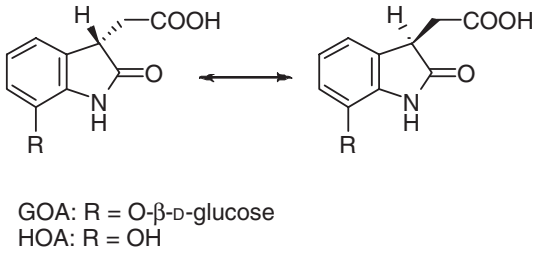
Abbreviations: SSCP, supersweet corn powder; DM, dry matter; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GOA, 7-(*O*-β-glucosyloxy) oxindole-3-acetic acid; HOA, 7-hydroxy-oxindole-3-acetic acid; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde



GOA: R = O-β-D-glucose
HOA: R = OH

Fig. 1. Structures of GOA and HOA.
The two-headed arrow indicates the equilibrium of two stereoisomers.

Materials and Methods

Chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, Trolox, ferulic acid, and indole-3-acetic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). β-Glucosidase was purchased from Oriental Yeast Co. (Tokyo, Japan), and thiobarbituric acid was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Materials. Corn flour (dent corn), popcorn, supersweet corn, tomato, spinach, pumpkin, onion, potato, barley, wheat, rice, and soybean were purchased from a market for use as materials. The fresh materials, supersweet corn, tomato, spinach, pumpkin, onion and potato, were freeze-dried and crushed. The dry products, corn flour and popcorn, were crushed to prepare dry powder. SSCP was purchased from Knorr Trading Co. (Kawasaki, Japan); SSCP was manufactured from the kernels of supersweet corn which were harvested, crushed, heated, dried, and processed to a powder.[11] This powder was stored at $-20\,^{\circ}$C until needed.

DPPH radical-scavenging activity. Each agricultural product sample was prepared by mixing 1 g of dry powder with 10 ml of 80% ethanol, agitating at 280 rpm for 20 min, and passing through a 0.45-μm filter for use as the test sample.

Stepwise-diluted 37.5 μl of each sample was mixed with 75 μl of a 0.1 M MES (pH 6.0)/10% ethanol solution and 37.5 μl of 400 μM DPPH in ethanol, and the solution was kept at room temperature for 20 min. A DPPH-free reactive solution was simultaneously prepared as a blank, and the absorbance of each solution at 520 nm was measured. The DPPH radical-scavenging activity of each solution was calculated from assay lines of Trolox (0, 12.5, 25, 50, 100, 200, and 400 μM) and is expressed as the Trolox equivalent (Trolox eq.). The activity of each product was determined by using four samples differing in origin. The activity of each fraction was measured by using three samples employing high-performance liquid chromatography (HPLC). The activity of each substance was determined three times.

Phenolic compound content. The polyphenolic content of an extract prepared in the presence of 80% ethanol was measured by the Folin & Ciocalteu method with gallic acid as a standard sample, and is expressed as the gallic acid equivalent (gallic acid eq.). We added 100 μl of the sample, 100 μl of the Folin & Ciocalteu phenol reagent (Wako Pure Chemical Industries, Osaka, Japan), and 200 μl of a saturated sodium carbonate solution to 1.6 ml of purified water, let the solution stand for 30 min, and measured the absorbance at 760 nm. The activity of each product was determined for four samples differing in origin.

Fractionation by HPLC. Five milliliters of an SSCP extract was eluted at 35 °C by HPLC with a COSMOSIL 5C_{18}-MS-II column (ϕ20 × 250 mm, Nacalai Tesque, Kyoto, Japan) to fractionate the eluate. Elution was performed with a linear gradient, using a flow velocity of 10 ml/min. We employed solutions A (0.1% formic acid) and B (99.9% methanol and 0.1% formic acid) with the initial concentration of solution A set at 98%. Its subsequent concentrations 10, 30, 35, 60, and 65 min after the start of elution were 79, 77, 59, 58, and 0%, respectively. This 0% concentration of solution A was continued until 80 min. A linear return to the initial condition (98% of solution A) was performed until 85 min after the start of elution, and the same condition was continued until 100 min. Detection was at an absorbance of 250 nm. The eluate was collected at 8-min intervals by dividing it into 11 fractions. Each fraction was dried, dissolved in 3.0 ml of 80% ethanol, and used as a sample to measure the DPPH radical-scavenging activity.

HPLC-MS analysis. Ten microliters of the SSCP extract was eluted at 30 °C, using HPLC with a COSMOSIL 5C_{18}-MS-II column (ϕ4.6 × 250 mm, Nacalai Tesque). Elution was performed employing a linear gradient, with a flow velocity of 0.3 ml/min. We employed solutions A (0.1% formic acid) and B (70% methanol and 0.1% formic acid), with the initial concentration of solution A set as 100%. Gradient elution was started after 15 min, using respective concentrations of solution A 30, 50, 65, 100, 135, and 160 min after the start of elution of 90, 80, 70, 60, 40, and 0%. This 0% condition was continued until 175 min. Detection was performed with a Micromass Quattro micro API mass spectrometer (Waters Corp., Milford, MA, USA) in the positive-ion electrospray mode. A drying nitrogen gas temperature of 300 °C, drying gas flow rate of 10 l/min, fragmentation voltage of 20 V, and capillary voltage of +4,600 V were used.

Purification of the antioxidative substances. SSCP (36 g) was extracted twice with 90 ml of 80% ethanol, and the liquid extract was dried by using a concentrating centrifuge. The dried extract was dissolved in methanol, and the target fraction was collected by conducting HPLC several times on 30 μl of the extract eluted at room temperature by HPLC with a CAPSELPAK C_{18} UG80 column (ϕ20 × 250 mm, Shiseido Co., Tokyo, Japan). Elution was performed at a flow velocity of 6 ml/min for 30 min, employing 25% methanol and 0.1% formic acid as vehicles. The next sample was then injected. The target fraction was collected, dried by using the concentrating centrifuge, and dissolved in a small volume of methanol. The dried sample was subsequently purified as a single substance by conducting HPLC several times on 40 μl of the fraction eluted at 30 °C, using HPLC with a COSMOSIL 5C_{18}-MS-II column (ϕ4.6 × 250 mm, Nacalai Tesque). Elution was performed by employing a linear gradient with a flow velocity of 1 ml/min. We employed solutions A (15% methanol and 0.1% formic acid) and B (99.9% methanol and 0.1% formic acid) with the initial concentration of solution A set at 100%. Gradient elution was started after 20 min, the concentration of solution A 22 min after the start of elution being 30%. This concentration was held until 32 min, before a linear return to the initial condition (100% of solution A) up to 35 min after the start of elution, and the same concentration held until 50 min. The next sample was then injected. The target fraction was dried in the concentrating centrifuge and then overnight in a desiccator, substituting the atmosphere with nitrogen gas and then storing at −80 °C. We obtained approximately 10 mg of the purified substance.

Structure of the purified substance. A 1-mg sample of the purified substance was dissolved in 1 ml of water, and the solution mixed with 18 units of β-glucosidase. This solution was reacted at 30 °C for 2 h, and then mixed with 5 ml of ethanol to stop the reaction. After the precipitated protein had been removed by centrifugation, the supernatant was dried in the concentrating centrifuge. A mixture of a formic acid solution (pH 3) and 1-butanol (1:1) was added to this sample and distributed to the respective layers. The formic acid solution layer containing constitutive sugars was concentrated and dried, and the material compared with an authentic substance by using ^{1}H-NMR and HSQC with an AV-600 spectrometer (Bruker Co., Rheinstetten, Germany). The 1-butanol layer containing the aglycone was dried, purified by HPLC as just described, and analyzed by ^{1}H-NMR, HMBC, NOESY, and H-H COSY with the Bruker AV-600 spectrometer, and by ^{13}C-NMR, TOCSY, and HSQC with a DMX-600 spectrometer (Bruker Co., Rheinstetten, Germany).

Purified substance (GOA). LC/MS (ESI-positive): m/z 370.1 $[M+H]^+$; UV (H_2O): λmax 248, 283 nm.

Aglycone (HOA). LC/MS (ESI-positive): m/z 207.9 $[M+H]^+$; UV (H_2O): λmax 246, 290 nm; ^{1}H-NMR (DMSO-d_6) δ: 2.63 (1H, dd, J = 7.2, 16.7 Hz), 2.86 (1H, dd, J = 4.3, 16.8 Hz), 3.67 (1H, m), 6.86 (1H, m), 6.94 (1H, dd, J = 4.5, 6.8 Hz), 7.10 (1H, dd, J = 3.3, 8.2 Hz), 10.19 (1H, s); ^{13}C-NMR (DMSO-d_6) δ: 34.1 (d), 42.5 (s), 115.7 (d), 117.8 (d), 121.9 (s), 130.5 (s), 132.2 (d), 141.5 (d), 172.2 (s), 178.1 (d).

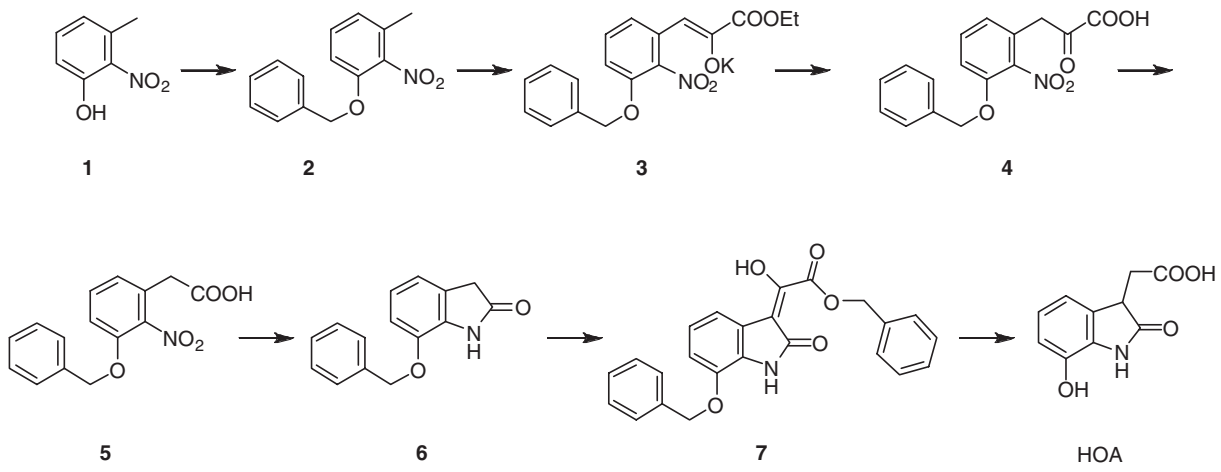


Fig. 2. Chemical Synthesis of HOA.

Quantification of GOA and HOA. One hundred microliters of 500 μM flavone was added to 150 mg of a sample as an internal standard, and the mixture extracted three times with 1 ml of 80% ethanol. After centrifugation, the supernatant was dried in the concentrating centrifuge, dissolved in 500 μl of methanol, and passed through a 0.25-μm filter for use as the sample for analysis. A 10-μl amount of the sample was eluted at 35 °C by using HPLC with a COSMOSIL $5C_{18}$-MS-II column (ϕ4.6 × 250 mm, Nacalai Tesque). Elution was performed with a linear gradient at a flow velocity of 1.0 ml/min. We employed solutions A (0.1% formic acid) and B (99.9% methanol and 0.1% formic acid) with the initial concentration of solution A set at 98%, before its respective concentrations 15, 30, and 35 min after the start of elution being 80, 70, and 0%. This condition was continued up to 45 min. A linear return to the initial condition (98% Solution A) was subsequently made up to 50 min after the start of elution, and the same condition was continued until 60 min. The next sample was then injected, detecting at an absorbance of 250 nm. We calculated the level of each substance from the assay line of the standard solution that was simultaneously analyzed with each sample. The detection limit for GOA was 0.04 μmol/g of dry matter (DM), and that for HOA was 0.03 μmol/g DM when employing this method. Four samples differing in origin were employed in the analysis of corn, while three samples differing in origin were employed when analyzing GOA and HOA in the other products.

Chemical synthesis of HOA (Fig. 2). Compounds 2 and 3 were synthesized from compound 1, as described by Cogan *et al.*[18] Under an atmosphere of nitrogen, 145.6 g of benzyl bromide was dripped into a mixture of 1-benzyloxy-3-methyl-2-nitrobenzene (130.4 g), potassium carbonate (147.1 g), and acetonitrile (2,077 ml) at 35–40 °C. The solution was agitated at 75 °C for 2.5 h after dripping, cooled to room temperature, mixed with water, and extracted with ethyl acetate. The organic vehicle layer was then washed and dehydrated with anhydrous magnesium sulfate, and the vehicle was removed to obtain 203.5 g of compound 2. In a nitrogen atmosphere, 131.4 g of diethyl oxalate was added to a mixture of potassium *t*-butoxide (100.9 g), tetrahydrofurane (899 ml), and *t*-butyl methyl ether (2,936 ml). Compound 2 (a *t*-butyl methyl ether solution, 219 g/367 ml) was dripped into this mixed solution on ice, heated and agitated at perfusion temperature for 24 h, and then cooled to 5 °C. The formed crystals were filtered out and then washed and dried under reduced pressure to obtain 258.9 g of compound 3.

Compound 4 was synthesized from compound 3, as described by Beer *et al.*[19] In a nitrogen atmosphere, 787 ml of a 5% sodium hydroxide solution was added to a mixture of compound 3 (109.7 g), ethanol (1,055 ml), and water (714 ml). The mixed solution was agitated at room temperature for 2.5 h and then dried under reduced pressure. This procedure was repeated for one more batch. These dried samples were mixed and extracted with *t*-butyl methyl ether. The aqueous layer was mixed with 1 N hydrochloric acid to adjust the pH value to 3. The solution was subsequently extracted with a mixed chloroform/methanol (3:1) vehicle, added to the foregoing *t*-butyl methyl ether layer, and dehydrated with anhydrous magnesium sulfate. The vehicle was removed to obtain 174.7 g of compound 4.

Compound 5 was synthesized from compound 4, as described by King *et al.*[20] In a nitrogen atmosphere, a mixture of compound 4 (174.7 g) and a 0.05 N sodium hydroxide solution (2,183 ml) was cooled to 0 °C, and a 6% hydrogen peroxide solution was dripped into the mixture. This was then mixed with 1,310 ml of dioxane, placed at room temperature for 1 h, and cooled. The pH value was subsequently adjusted to 3 by adding 1 N hydrochloric acid. The formed crystals were filtered out, washed, and dried under reduced pressure to obtain 138.0 g of compound 5.

HOA was synthesized from compound 5, as described by Lewer *et al.*[21] Under an atmosphere of nitrogen, a mixture of compound 5 (97.4 g), acetic acid (1,770 ml), and zinc (177 g) was agitated at 110 °C for 4 h. After this solution had been filtered, the collected compound was washed in heated acetic acid and then concentrated under reduced pressure. This concentrate was purified by using silica gel column chromatography (hexane/ethyl acetate/chloroform = 3:1:4) to obtain 29.2 g of compound 6. In a nitrogen atmosphere, a tetrahydrofurane solution (27.0 g/221 ml) containing compound 6 was dripped into a tetrahydrofurane suspension (7.39 g/123 ml) containing sodium hydroxide at room temperature, and the mixture agitated for 2 h. Dibenzyl oxalate (33.6 g) was then dripped in, and the mixture agitated overnight. The solution was mixed with 1 N hydrochloric acid to prepare a neutral mixture which was concentrated under reduced pressure and further mixed with 1 N hydrochloric acid until the pH value reached 2. The formed crystals were filtered out and washed to obtain 41.6 g of compound 7. In a nitrogen atmosphere, 16.2 g of 10% palladium-carbon was added to the mixture of compound 7 (41.5 g), high-concentration sulfuric acid (2.0 ml), and acetic acid (808 ml). The solution was agitated at room temperature for 26 h, and then mixed with 1.9 ml of high-concentration sulfuric acid, 758 ml of acetic acid, and 15.1 g of 10% palladium-carbon. The mixture was agitated at room temperature for 4 h, mixed with 31.3 g of sodium acetate, and filtered. The residue was washed in ethyl acetate, concentrated under reduced pressure, mixed with 1 N hydrochloric acid, and then extracted with ethyl acetate. The organic vehicle layer was subsequently dehydrated with anhydrous sodium sulfate, the vehicle was removed, and the residue was washed and dried under reduced pressure to obtain 17.4 g of HOA. This product was dissolved in isopropyl alcohol to remove the trace vehicle, and the solution dried under reduced pressure to obtain 16.2 g of HOA.

Ex vivo *plasma oxidation.* Fifty or 150 mg/ml of the HOA solution in 50% polyethylene glycol 400, or a control vehicle (50% polyethylene glycol 400) was administered orally to 9-week-old male C57BL/6J mice (n = 6 per group) at a dosage of 10 ml/kg. Blood was collected 120 min after this administration, mixed with heparin, and centrifuged to isolate the plasma. Since a preliminary time-course experiment had given the highest activity at 120 min, the time for blood collection was determined as 120 min. Plasma samples were diluted with physiological saline at a ratio of 10, and mixed with

$CuSO_4$ to prepare a final concentration of 100 μM. Each of these samples was reacted at 37 °C for 240 min. Thiobarbituric acid reactive substances (TBARS) in the reactive solution were quantified, as described by Ohkawa *et al.*,[22] TBARS values being expressed as the malondialdehyde equivalent (MDA eq.). This experiment was approved by the Animal Experiment Ethics Committee of Ajinomoto Co., Ltd.

Hydrolysis of GOA with the brush border membrane fraction. A rat jejunum-derived brush border membrane fraction was extracted by modifying the method described by Kessler *et al.*[23] Briefly, a laparotomy was performed under ether anesthesia on 6- to 8-week-old SD rats to extirpate the jejunum. The inner area of the jejunum was washed in physiological saline. The mucosa was exfoliated by using a slide glass, mixed with a 2 mM Tris–HCl solution (pH 7.0) containing 50 mM D-mannitol, and homogenized. Calcium chloride was added to the homogenate to achieve a final concentration of 10 mM. The solution was allowed to stand at 4 °C for 15 min and then centrifuged (750 × *g* at 4 °C for 30 min). The supernatant was then also centrifuged (9,000 × *g* at 4 °C for 30 min). The precipitate was mixed with 10 mM phosphate-buffered saline (pH 7.0) to prepare a final concentration of 100 mg/ml, and then stored at −80 °C as the brush border membrane fraction. This experiment was approved by the Animal Experiment Ethics Committee of Ajinomoto Co., Ltd.

A 1-μl sample of the brush border membrane fraction was added to 100 μl of 0.2 mM GOA and a 50 mM maleic acid buffer (pH 6.0), and the mixture reacted at 37 °C for 24 h. A 5-μl aliquot of the solution was used as a sample for HPLC according to the "Quantification of GOA and HOA."

Statistical analysis. The data are expressed as the mean ± standard deviation (SD). We used SPSS ver. 13.0J software (SPSS Japan, Tokyo, Japan) for the statistical analysis. Significance was tested by using ANOVA, and we employed Bonferroni's multiple comparison to compare individual values.

Results

DPPH radical-scavenging activity and phenolic compound content

The DPPH radical-scavenging activity of the 80% ethanol extract of SSCP was compared with corn and found to be higher than that of corn flour and popcorn. There was no significant difference between this extract and commercially available supersweet corn (Table 1).

The phenolic compound content of the 80% ethanol extract of SSCP was also compared with corn and found to be higher than that of corn flour and popcorn. There was no significant difference between this extract and supersweet corn (Table 1).

The data on individual samples of corn (n = 16) showed a high correlation between the DPPH radical-scavenging activity and the phenolic compound content, with a Pearson correlation coefficient of 0.956 ($p = 7.89 \times 10^{-9}$).

Identification of the SSCP components with DPPH radical-scavenging activity

We measured the activity of each fraction by using HPLC to identify the components with DPPH radical-scavenging activity contained in SSCP (Fig. 3). Fraction 4 showed the strongest activity (Table 2) and was analyzed by using HPLC-MS. Two peaks were noted, with an ESI-MS value of 370.1 m/z $[M+H]^+$, suggesting that these two peaks were stereoisomers of 7-(*O*-β-glucosyloxy)oxindole-3-acetic acid (GOA, Fig. 1);[24,25] however, there was no sample of this, so

Table 1. DPPH Radical-Scavenging Activities and Phenolic Contents of SSCP and the Other Corn Types

Sample	DPPH activity[A] (μmol Trolox eq./g DM)	Phenolic content[A] (μmol gallic acid eq./g DM)
Corn flour (dent corn)	2.2 ± 0.9a	4.8 ± 1.5a
Popcorn	3.2 ± 1.2a	5.0 ± 2.4a
Supersweet corn	6.7 ± 1.8b	14.4 ± 1.1b
SSCP	7.8 ± 0.7b	16.2 ± 0.9b

[A]Data in the same column with different letters are significantly different at $p < 0.05$.

Table 2. DPPH Radical-Scavenging Activity of Each Fraction from the SSCP Extract after HPLC

Fraction	DPPH activity[A] (μmol Trolox eq./g DM[B])	Proportion (%)[C]
1	0.52 ± 0.06a	4.4
2	0.64 ± 0.02ab	5.4
3	0.55 ± 0.01a	4.7
4	3.94 ± 0.05e	33.5
5	0.72 ± 0.09bc	6.1
6	1.11 ± 0.04d	9.5
7	0.73 ± 0.04bc	6.2
8	0.57 ± 0.03a	4.8
9	1.01 ± 0.03d	8.6
10	1.14 ± 0.03d	9.6
11	0.84 ± 0.07c	7.1
Total	11.77	100.0

[A]Data in the same column with different letters are significantly different at $p < 0.05$; [B]per SSCP dry weight of pre-fractionation; [C]activity × 100/total activity

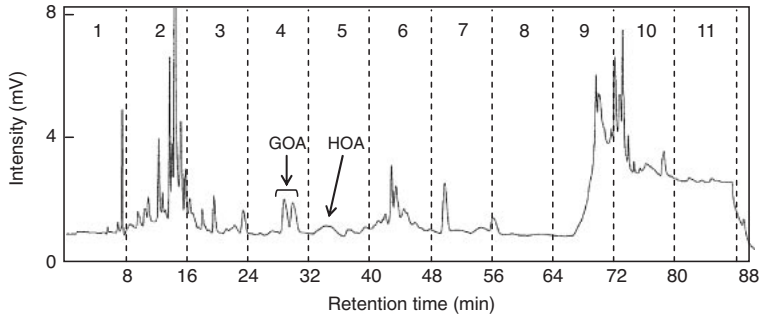
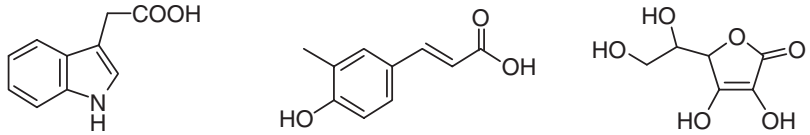


Fig. 3. HPLC Trace of the SSCP Extract.

The 80% ethanol extract of SSCP was eluted by reversed-phase HPLC according to the conditions described in the Materials and Methods section. The eluate was collected at 8-min intervals and divided into 11 fractions. The peaks with retention times of 28.5–29.5 min and 34.0 min are GOA and HOA, respectively.



Indole-3-acetic acid Ferulic acid L-Ascorbic acid

Fig. 4. Structures of the Antioxidants Assayed in This Study.

Table 3. Contents of GOA and HOA in Corn

Sample	Content (μmol/g DM)	
	GOA	HOA[A]
Corn flour (dent corn)	ND	0.12 ± 0.02a
Popcorn	ND	0.08 ± 0.02a
Supersweet corn	3.03 ± 1.20	0.21 ± 0.14a
SSCP	3.20 ± 0.30	0.43 ± 0.04b

[A]Data in the same column with different letters are significantly different at $p < 0.05$.

Table 4. DPPH Radical-Scavenging Activities of GOA and HOA

Substance	DPPH activity (mol Trolox eq./mol)[A]
GOA	0.86 ± 0.04b
HOA	1.91 ± 0.12d
Indole-3-acetic acid	0.35 ± 0.03a
Ferulic acid	1.11 ± 0.04c
L-Ascorbic acid	1.20 ± 0.01c

[A]Data in the same column with different letters are significantly different at $p < 0.05$.

its presence was confirmed by purification. The NMR spectrum of the purified substance was complicated, so it was cleaved by β-glucosidase to a sugar moiety and an aglycone. We confirmed that the sugar moiety of the purified substance was glucose by comparing with the ^{1}H-NMR and HSQC data for the authentic substance, and that the aglycone was 7-hydroxy-oxindole-3-acetic acid (HOA) by ^{1}H-NMR, HMBC, NOESY, H-H COSY, ^{13}C-NMR, TOCSY, and HSQC data. The purified substance was finally confirmed to be GOA (Fig. 1). It was also confirmed that its aglycone, HOA, was contained in fraction 5 by using HPLC-MS/MS (Fig. 3).

Quantification of GOA and HOA

The GOA and HOA contents of corn (Table 3) and other agricultural products were measured by using purified GOA and HOA as standard samples. GOA was detected in SSCP and supersweet corn at similar levels among the corn types, while HOA was detected in all corn types investigated and was highest in SSCP. On the other hand, the GOA and HOA levels in such other agricultural products as tomato, spinach, pumpkin, onion, potato, barley, wheat, rice, and soybean were below the limits for detection.

DPPH radical-scavenging activities of GOA and HOA

We compared the DPPH radical-scavenging activities of GOA and HOA with those of other antioxidants (Fig. 4, Table 4). HOA showed the highest DPPH radical-scavenging activity, followed by L-ascorbic acid, ferulic acid, GOA, and indole-3-acetic acid.

Table 5. Contribution Rates of GOA and HOA to the DPPH Radical-Scavenging Activities of Supersweet Corn and SSCP

Substance	Contribution rate (%)	
	Supersweet corn	SSCP
GOA	39.1	35.1
HOA	5.9	10.5
Total	45.0	45.6

Table 6. Effect of Orally Administered HOA on the Susceptibility to Plasma Oxidation *ex Vivo*

	Dose (mg/kg)	TBARS[A] (μM MDA eq.)
Vehicle	—	275 ± 29a
HOA	500	198 ± 19b
	1,500	176 ± 25b

[A]Data in the same column with different letters are significantly different at $p < 0.05$.

The contribution rates of GOA and HOA to the DPPH radical-scavenging activity of the 80% ethanol extract of SSCP and supersweet corn were calculated from their contents (Table 3) and activities (Table 4). Table 5 shows that the respective contribution rates of GOA to the DPPH radical-scavenging activity of the 80% ethanol extract of SSCP and supersweet corn were 35.1 and 39.1%, both figures being higher than those of HOA.

Chemical synthesis of HOA

Although it was extremely difficult to obtain the required quantity of GOA for animal experiments by both purification and chemical synthesis, it was possible to chemically synthesize HOA as shown in Fig. 2 to investigate its effect by animal experiments. The structure of chemically synthesized HOA was confirmed from its ^{1}H-NMR data, and its purity was estimated to be 95.9% by using HPLC.

Ex vivo *plasma oxidation*

We investigated whether chemically synthesized HOA absorbed *via* an oral route had any effects. A dose of 500 or 1,500 mg/kg of HOA orally administered to mice resulted in the plasma TBARS level being significantly lower than the control value in vehicle-treated mice (Table 6).

Hydrolysis of GOA with the brush border membrane fraction

We investigated the possibility that GOA might be hydrolyzed into HOA in the animal intestine. GOA incubated with the rat brush border membrane fraction *in vitro* resulted in GOA being converted to HOA (Fig. 5).

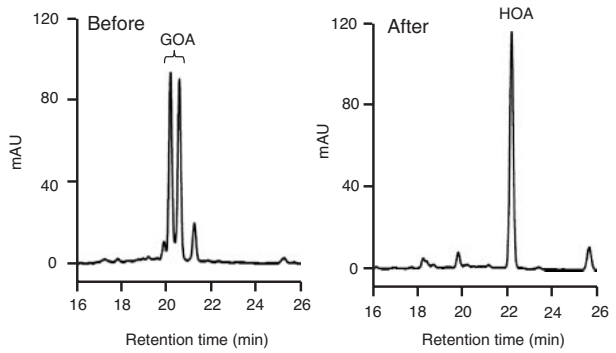


Fig. 5. Conversion of GOA to HOA by Rat Brush Border Membranes.

A 100-µl sample of the 0.2 mM GOA solution was incubated with 1 µl of the rat brush border membrane fraction at 37 °C for 24 h. Ten-microliter aliquots before and after incubation were eluted by reversed-phase HPLC according to the conditions described in the Materials and Methods section.

Discussion

We identified in this study GOA and HOA, an aglycone of GOA, as antioxidative components of SSCP. The GOA content of SSCP and of commercially available supersweet corn was high among the corn types, and neither GOA nor HOA was contained in the nine other agricultural products measured in this study. HOA decreased the blood TBARS level when orally administered to mice, indicating its *in vivo* effect. The presence of GOA and HOA in corn and the antioxidative effect of HOA *in vitro* have been reported;[17,24,25] however, no study has examined the antioxidative effect of GOA, a quantitative determination of GOA and HOA in agricultural products other than corn, or the *ex vivo* effect of HOA. This study is the first to report these aspects.

The DPPH radical-scavenging activity of an SSCP extract prepared in the presence of 80% ethanol was higher than that of corn flour or popcorn, and was similar to that of supersweet corn (Table 1). Furthermore, there was a high correlation between the DPPH radical-scavenging activity and the phenolic compound content, suggesting that SSCP had similar or more potent antioxidative activity to that of other types of corn, despite the heating process, and that phenolic compounds may have contributed to this activity. Processing is generally thought to reduce the antioxidative activity of foods. However, Dewanto *et al.*[13] have indicated that the antioxidative activity of sweet corn treated at 100 °C for 25 min was increased by 36%, and that the activity of free ferulic acid was elevated by approximately 125%. They speculated that these increases in antioxidative activity might have been associated with the heating-related release of antioxidative phenolic compounds from the tissues. Heating may therefore not reduce the antioxidative activity of sweet corn, but rather may increase it. The HOA content of SSCP was higher than that of supersweet corn (Table 3), which might have been due to the heating-related release of HOA from the tissues. We did not investigate changes in the antioxidative activity, HOA content and other phenolic compounds before and after SSCP manufacture in this study. As the SSCP production process consists of crushing, heating with a steamer at approximately 80 °C for 30 min or more, and drying in a drum drier,[11] the antioxidative activity may be increased during the manufacturing process. Measurement of the antioxidative activities and quantification of GOA, HOA and the phenolic compounds should be performed in the future before and after SSCP manufacture.

We measured the DPPH radical-scavenging activities of HPLC fractions of the 80% ethanol extract of SSCP, and identified GOA in a fraction showing the highest activity. GOA was present as two peaks on the HPLC trace; however, even when one was isolated and employed for HPLC, two similar peaks were detected (data not shown), suggesting the presence of stereoisomers in GOA, showing equilibrium in a solution from the formation of a double bond between the 2nd and 3rd carbon by keto-enol tautomerization. In respect of the structure of GOA, two diastereomers for the asymmetric carbon at the 3rd position exist due to the presence of an asymmetric carbon at the 1st position of β-D-glucose connected to the 7th position (Fig. 1).

To our knowledge, neither GOA nor HOA has been found in any vegetable/grain other than corn, and they may be specific to corn. Furthermore, GOA was only found in SSCP and supersweet corn, and may be specific to supersweet corn cultivars (Table 3) which have abnormal accumulations of glucose and sucrose compared with other corn types;[10] it is thus possible that supersweet corn cultivars have greater ability to add glucose to HOA. However, a survey should be conducted to clarify species/cultivar-related differences in the levels of these substances, considering the tissue distribution of these substances and the degree of kernel maturation, in addition to quantifying these substances in the part that is eaten.

It has been reported that GOA was synthesized from indole-3-acetic acid, which is known to be a plant hormone and also an antioxidant,[26] through HOA in the kernels of supersweet corn after seed germination. GOA and HOA have been suggested to be inactive metabolites of indole-3-acetic acid;[25] however, the antioxidative effects of these substances were demonstrated here and may protect seeds against oxidative stress, rather than simply being inactive metabolites of indole-3-acetic acid.

Nonhebel *et al.*[24] have reported that the GOA level in the kernels of supersweet corn after seed germination was in the range of 10.6–14.3 nmol/g of fresh matter. The GOA content was calculated as 42.4–57.2 nmol/g of DM when estimating the water content of kernels after seed germination as 75%. The GOA level in SSCP and supersweet corn in our study, which were harvested as immature kernels, may have been 50–80 times higher than that in germinated kernels of supersweet corn (Table 3). Since immature kernels are in an anabolic state and germinated kernels are in a catabolic state, the difference in GOA content between the two types may reflect differences in the physiological state.

The contribution rate of GOA to the DPPH radical-scavenging activity of the 80% ethanol extract of SSCP was 35.1% (Table 5). This is consistent with the activity of fraction 4 by HPLC (33.5%, Table 2). GOA may be the major component of the 80% ethanol extract of SSCP that is involved in its DPPH radical-scavenging

activity. Similarly, the contribution rate of GOA to the DPPH radical-scavenging activity of supersweet corn was 39.1%, suggesting GOA also to be the major component of the 80% ethanol extract of supersweet corn in its DPPH radical-scavenging activity. Although the DPPH radical-scavenging activities of most HPLC fractions were lower than the fraction of GOA (Table 2), the individual components cumulatively contributing to the activity have not been elucidated. Further components must be specified to clarify the components contributing to the DPPH radical-scavenging activity of the SSCP and supersweet corn extracts.

The antioxidative activity of GOA was identified for the first time in this study; however, that of HOA has previously been reported by Niwa *et al.*[17] They isolated HOA from corn gluten meal, a by-product obtained from dent corn in the industrial starch manufacturing process, and indicated the hydroxyl radical-scavenging activity of HOA. Furthermore, they reported the order of the hydroxyl radical-scavenging activity to be indole-3-acetic acid = HOA > oxindole-3-acetic acid (a substance without a hydroxyl group compared with HOA). We found in our study that the order of the DPPH radical-scavenging activity was HOA > GOA > indole-3-acetic acid (Table 4). DPPH radical-scavenging activity is mainly based on the ability of an antioxidant to transfer one electron to reduce the DPPH radical, whereas hydroxyl radical-scavenging activity is based on the ability of an antioxidant to donate a hydrogen atom to quench the hydroxyl radical.[27] It was therefore suggested that the difference in activity between these two parameters might be due to a difference in the assay mechanism. The high activity of GOA and HOA in DPPH radical-scavenging activity compared with indole-3-acetic acid might be attributable to the easier transfer of an electron from GOA or HOA to the DPPH radical than from indole-3-acetic acid due to the loss in aromatic stability of the pyrrole moiety by the addition of the carbonyl group at the 2nd position to the indole ring. Since phenolic compounds are known to have antioxidative activity by their hydroxyl group,[28] the hydroxyl group at the 7th position of HOA is thought to be related to its activity; therefore, the lower activity of GOA in DPPH radical scavenging than that of HOA might be attributable to masking of the hydroxyl group at the 7th position by the addition of glucose. On the other hand, a radical produced by the abstraction of a hydrogen atom from the NH group of indole-3-acetic acid is highly resonance stable, since the unpaired electron may be present not only on the nitrogen but can be delocalized across the indole moiety.[29] Thus indole-3-acetic acid can readily form a resonance-stable radical which might account for its higher antioxidative activity in hydroxyl radical scavenging than that of oxindole-3-acetic acid. The higher activity of HOA in hydroxyl radical scavenging than that of oxindole-3-acetic acid might be attributable to the presence of a hydroxyl group at the 7th position, as with the DPPH radical-scavenging activity; however, details about this remain to be clarified.

This study has shown that HOA orally administered to mice exhibited an antioxidative effect in blood in addition to its antioxidative activity *in vitro* (Table 6); HOA may therefore be effective for oxidative stress-related conditions. Furthermore, GOA was converted to HOA in the presence of a rat brush border membrane-derived extract (Fig. 5); GOA may therefore be absorbed *in vivo* by its conversion to HOA with higher activity. GOA may also exhibit an antioxidative effect when orally administered to mice. Further investigation is required to clarify GOA/HOA absorption in animals and the *in vivo* activity of GOA.

We identified in this study GOA and HOA as antioxidants of SSCP and supersweet corn. We also showed that HOA exhibited an antioxidative effect when orally administered. The effects of GOA and HOA on *in vivo* oxidation-related conditions should be investigated in the future.

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