Enhancing Effect of a Roasting Treatment on the Radical Scavenging Activity of a Marine Brown Alga Polysaccharide (Alginic acid)

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Abstract: We found an effective roasting method that enhances the effects on various radical scavenging activities of polysaccharide (alginic acid) derived from the marine brown alga Lessonia trabeculata. These enhancing effects were observed by a roasting treatment under relatively high temperature conditions (160°C and 180°C), which were measured by (i) a stable radical compound diphenylpicrylhydrazyl (DPPH), (ii) a hydroperoxide generating system of linoleic acid autooxidation, and (iii) an opsonized zymosan (Opz)-induced oxygen radical generating system in human blood neutrophils. Although a significant enhancing effect of the roasting treatment on the radical scavenging activity of the alginic acid itself was not detected under relatively low temperature conditions (100°C and 130°C), the roasting treatment of a mixture of alginic acid and several specific amino acids caused considerable radical scavenging activities under the same roasting conditions. When alginic acid was roasted at relatively high temperatures (160°C or 180°C), the mixture of the alginic acid and specific amino acids exhibited much higher radical scavenging activities than did the alginic acid alone. The significance of this finding is discussed from the viewpoint of healthy food science.

Keywords: alginic acid, amino acid, roasting treatment, radical-scavenging activity.

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Introduction

Oxygen radical-induced oxidative stress leads to damage of genetic DNA, cellular proteins, and membrane lipids in the human body, which causes acute or chronic diseases such as inflammatory diseases, diabetes, cardiovascular diseases and cancer [1, 2]. To prevent the initiation and progression of these diseases, dietary natural antioxidants have attracted much attention as therapeutic agents that can prevent oxidative damage to these important biomolecules in the human body [3, 4].

Various edible algae have been traditionally consumed in Asian countries such as Japan, Korea and China as a source of dietary antioxidants because they have various kinds of antioxidant constituents. Several brown algae, for example, contain significant amounts of the antioxidant phlorotannin [5]. The methanol extract of brown alga, Hijikia fusiformis (Hijiki), has a strong radical scavenging activity, the active principle...
of which was identified as a carotenoid, fucoxanthin [6]. Antioxidant phenolic compounds, including epigallocatechin and catechol, have also been observed in the extracts of specific green algae, Halimeda species [7]. A Mycrosorine-like amino acid in a red alga, Porphyra yezoensis (Susabi-nori), showed a radical-quenching activity against singlet oxygen in a photodynamic action [8].

Alginic acid and fucoidan, typical sulfated polysaccharides derived from brown algae, have various antioxidant or radical scavenging activities [9–14]. In addition to these studies, several investigating groups have developed improved methods that cause an enhancement of the radical scavenging activities of algae-derived polysaccharides by the acid hydrolysis of polysaccharide[15], hydrogen peroxide (H2O2)-induced degradation of polysaccharide[16], and enzymatic digestion of polysaccharide[17,18].

An edible red alga, Porphyra tenera (P. tenera, Asakusa-nori), which is called “Yaki-nori”, is usually dried and roasted at a high temperature. Although it has been believed that the purpose of the roasting treatment of P. tenera is the improvement of its taste and flavor, we found that it has a remarkable enhancing effect on radical-scavenging activity [19]. We also detected that a brown alga, Laminaria japonica (Ma-kanbu), exhibited strong roasting effects on various radical scavenging activities under high temperature conditions [20]. The effect of the roasting treatment of algae-derived polysaccharides on radical scavenging activities, however, has not been elucidated until present.

In the present study, we show the enhancing effects of a roasting treatment on various radical scavenging activities of a brown alga-derived polysaccharide (alginic acid) under specific roasting conditions.

Materials and Methods

Chemicals and reagents

Aluminum chloride, ascorbic acid, potassium iodide, soluble potato starch and 8-amino-5-chloro-7-phenylpyrido [3, 4-d] pyridazine-1, 4-[2H, 3H] dione (L-012) were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Amino acids (glycine, alanine, tryptophan, threonine, phenylalanine, tyrosine, histidine, leucine, isoleucine, methionine, aspartate, glutamate, glutamine, arginine and lysine), diphenylpicrylhydrazyl (DPPH), yeast zymosan, and bovine serum albumin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Linoleic acid and o-phenanthroline were purchased from Kanto Chemicals Co. (Tokyo, Japan). Sodium alginic acid (soluble alginate) prepared from a marine brown alga (Lessonia trabeculata) harvested in Chile was kindly donated by Fuji Chemical Co. (Wakayama City, Japan). Potassium iodide solution was prepared by 2 g of KI in 100 ml of ethanol. Aluminum chloride solution was prepared from 2 g of AlCl3 and 20 μg of o-phenanthroline in 100 ml of ethanol. Starch solution was prepared by 1 g of soluble potato starch and 20 g of NaCl in 10 ml of distilled water.

Measurement of total sugar concentration

The total sugar concentration in the polysaccharide preparation was measured by the phenol/H2SO4 method [21] by using glucose as standard sugar.

Roasting experiment of alginic acid

Polysaccharide (alginic acid) was dissolved with distilled water at the concentration of 100 mg/ml in a glass test tube and heated to 100°C, 130°C, 160°C or 180°C for 30 min in an electric oven (Type NE-A251, Panasonic Co., Osaka, Japan). The dried powder of the roasted polysaccharide was then dissolved with distilled water, and its radical scavenging activities were assayed.

Roasting experiment of the mixture of polysaccharide and amino acid

As control experiments, the alginic acid and each amino acid (glycine, alanine, tryptophan, threonine, phenylalanine, tyrosine, histidine, leucine, isoleucine, aspartate, methionine, glutamate, glutamine, arginine and lysine) were dissolved with distilled water at the concentration of 100 mg/ml in glass test tubes, respectively, and the alginic acid solution and each amino acid solution was heated to 100°C, 130°C, 160°C and 180°C for 30 min in an electric oven (Type NE-A251, Panasonic Co., Osaka, Japan). The dried powder of the roasted alginic acid and roasted amino acid was dissolved with distilled water, and their radical scavenging activities were assayed. The same concentrations of alginic acid solution and each amino acid so-
olution described above were mixed in glass test tubes and heated to 100°C, 130°C, 160°C or 180°C for 30 min in the same electric oven. The dried powder of the roasted mixture of alginic acid and each amino acid was then dissolved with distilled water, and the radical scavenging activities of these mixture solutions were assayed as follows.

Assay for radical scavenging activity against the DPPH radical

The radical scavenging activity of alginic acid and the mixture of alginic acid and amino acid were measured by using a diphenylpicrylhydrazyl (DPPH) radical according to a modified previous method [22]. The test solution of alginic acid and the mixture of alginic acid and amino acid (0.2 mL) were mixed with 3.8 mL of 0.2 mM DPPH in ethanol prepared daily, and a control experiment was performed with distilled water alone. The absorbance at 520 nm (At) was measured at 15, 30 and 60 min after the initiation of the assay reaction by a Shimadzu UV-265 spectrophotometer. The background value of absorbance (Ab) derived from the test sample was subtracted from the apparent value of absorbance in the assay reaction. The radical scavenging activity (RSA) was calculated by the following equation: RSA (%) = ((Ao – At) / (Ao – Ab)) × 100%, where Ao is the absorbance at 520 nm at the start of the assay reaction. As a standard radical scavenging substance, ascorbic acid was successively diluted with distilled water, and the DPPH radical scavenging activity of ascorbic acid solution was assayed as described above.

Assay for hydroperoxide generation by linoleic acid autooxidation

The amount of hydroperoxide generated by linoleic acid autooxidation was measured by a modification of the aluminum chloride method [23]. One mL of test solution was mixed with 1 mL of linoleic acid in ethanol and 2 mL of 50 mM phosphate buffer (PB, pH 7.5) in a glass test tube and kept for 2 weeks at 33°C. One mL of distilled water or PB was used as a negative control. After the two week period, the test solution mixture mentioned above (200 μL), 250 μL of 0.2% KI solution in ethanol, 250 μL of 2% aluminum chloride solution, and 1 mL of hexane were mixed in glass test tubes and incubated for 5 min at 37°C under a dark condition, then 250 μL of starch solution and 7.5 mL of 10 mM HCl were added and the solution was shaken vigorously. After the solution mixture in the test tube was centrifuged at 1,500 × g for 5 min, the absorbance of the lower layer was measured at 560 nm by using Shimadzu UV-265 spectrophotometer.

Assay for oxygen radical generation in human blood neutrophils

Isolation of human neutrophils was carried out as follows. Human venous blood obtained from a healthy nonsmoking male donor was recovered in the presence of 3.8% citrate and centrifuged using Monopoly Resolving Medium (Dainihon Pharmatheutical Co. Osaka, Japan) and Krebs Ringer phosphate solution (KRP, pH 7.4). The neutrophils in the cell layer were recovered and washed two times with KRP by centrifugation and kept in ice until use in the experiment. The experimental design of the isolation of the blood human neutrophils and other associated experimental procedures was accepted by the experimental moral and hazard committee of Osaka Prefecture University, and these experiments were carried out according to the rules of this committee. The assay for oxygen radical generation in human neutrophils was carried out by a modification of the previous method [24]. Human neutrophils (1 × 10⁶ cells) were incubated with a 50 mM phosphate buffered saline (PBS pH 7.4) in the presence of 400 μM L-012 (chemiluminescence reagent) at a final volume of 500 μL. After preincubating the cell suspension mixture including the test solution (alginic acid or the mixture of alginic acid and amino acid) at 37°C for 3 min, a reaction was started by the addition of opsonized zymosan (Opz, 1 μg/mL) and it was further incubated for another 30 min. The chemiluminescence (CHL) intensity of the cell suspension mixture was recorded continuously during the incubation at 37°C by a CHL detector apparatus (Type BLR-102, Aloka Co., Tokyo, Japan).

Statistical analysis of experimental results

The experimental results were expressed as the mean and standard deviation (SD) or the average value of triplicate assays. The statistical comparison between the control and sample-treated experiment was analyzed by Student’s t test. A P value less than 0.05
was considered to be significantly different. The statistical analysis of the experimental results was carried out by the analysis tool pack of Add-in of Excel 2016.

Results

Increase in browning substances generated in alginic acid solutions by roasting treatment

As an interesting finding in the present study, we detected a significant increase in browning substances generated in the polysaccharide solutions by the roasting treatment. As shown in Table 1, when alginic acid was roasted at 100°C or 130°C for 30 min, the absorbance intensity at 420nm corresponding to the browning substances generated in the roasted alginic acid significantly increased as compared with unheated alginic acid at room temperature. Furthermore, when alginic acid was roasted at 160°C or 180°C for 30 min, a remarkable increase in the browning reaction was observed (Table 1).

These experimental results suggest the possibility that the roasting treatment of the polysaccharide (alginic acid) at different temperature conditions (100°C, 130°C, 160°C and 180°C) might promote a polysaccharide-dependent browning reaction (Caramel reaction).

Table 1. Effect of roasting treatment on the browning reaction of alginic acid

| Treatment           | Absorbance intensity at 420 nm (OD/assay tube) |
|---------------------|-----------------------------------------------|
| Room temperature (22°C) | 0.04 ± 0.01 (—)                                  |
| 100 °C (30 min)      | 0.12 ± 0.03 (P<0.05)                            |
| 130 °C (30 min)      | 0.20 ± 0.05 (P<0.01)                            |
| 160 °C (30 min)      | 1.65 ± 0.18 (P<0.0001)                          |
| 180 °C (30 min)      | 2.89 ± 0.16 (P<0.0001)                          |

The results in the table show the mean and SD of absorbance intensity at 420 nm of alginic acid solution in triplicate assays. The statistical differences in the results between the control (room temperature) and different temperature conditions were analyzed by Student’s t test. SD: standard deviation

Effect of roasting treatment on the radical scavenging activity of alginic acid

We then assumed that the browning substances generated by the roasting treatment of polysaccharide (alginic acid) might have potent radical scavenging activities.

First, the radical scavenging activities of alginic acid roasted at different temperatures were measured by using the stable radical compound DPPH. As indicated in Figure 1, although the roasting treatment of alginic acid at 100°C or 130°C did not show a significant increase in radical scavenging activities as compared with unheated alginic acid at room temperature, the roasting treatment at 160°C showed very strong radical scavenging activity (82.5% inhibition), and the roasting treatment at 180°C caused considerable radical scavenging activity (54.0% inhibition), although it was lower than the radical scavenging activity at 160°C (Figure 1).

Secondly, we analyzed the radical scavenging activities of alginic acid roasted under different temperature conditions by using a hydroperoxide generating system in linoleic acid autooxidation. As shown in Figure 2, when alginic acid was roasted at 100°C or 130°C, no significant increase in radical scavenging activity against hydroperoxide generation was detected, as compared with that of unheated alginic acid at room temperature. When alginic acid was roasted at 160°C, a strong radical scavenging activity was observed (71.9% inhibition). The alginic acid roasted at 180°C also exhibited a considerable radical scavenging activity (52.4% inhibition), although it was lower than that at 160°C. These experimental results suggest that alginic acids roasted under higher temperature conditions (160°C or 180°C) have relatively strong radical scavenging activities against hydroperoxide generation in linoleic acid autooxidation.

In addition to the cell-free experiments mentioned above, we analyzed the effects of the roasted polysaccharide (alginic acid) on oxygen radical generation in opsonized zymosan (Opz)-stimulated human blood neutrophils as a typical oxygen radical-producing cell system. As shown in Figure 3, Opz alone strongly enhanced oxygen radical generation in human blood neutrophils, which showed remarkable L-012-dependent chemiluminescence (CHL) generation. When the alginic acid roasted at 160°C or 180°C was added before the Opz stimulation, relatively strong suppressive effects (77.5 and 61.0% inhibition, respectively) on Opz-induced oxygen radical generation were observed (Figure 3), but when the alginic acid was roasted at 100°C or 130°C, no significant increase in radical
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Scavenging activity was detected as compared with unheated alginic acid at room temperature. These experimental results suggest that alginic acid roasted at relatively higher temperatures (160ºC or 180ºC) causes strong radical scavenging activities against oxygen radical generation in Opz-stimulated human blood neutrophils.

Enhancing effect of roasting treatment of the mixture of alginic acid and amino acid on the radical scavenging activity against the DPPH radical

Maillard reaction is a non-enzymatic browning reaction caused by a heating treatment, involving the reaction of carbonyl compounds (especially saccharides) and amino group-containing substances such as amino acids or proteins [25]. In the present study, as a roasting model experiment for Maillard reaction, we analyzed the effect of a roasting treatment of a mixture of polysaccharide (alginic acid) and each amino acid on the radical scavenging activity against the DPPH radical.

Although alginic acid itself did not show a significant enhancing effect on radical scavenging activity against the DPPH radical by a roasting treatment under relatively low temperature conditions (100ºC or 130ºC, DPPH Radical scavenging activity (% Inhibition)

| Roasting temperature | 100ºC | 130ºC | 160ºC | 180ºC |
|----------------------|-------|-------|-------|-------|
| (Control)            | 0     | 0     | 0     | 0     |
| RT                   | 0     | 0     | 0     | 0     |

Figure 1. Enhancing effect of roasting treatment on the radical scavenging activity of alginic acid against DPPH radical. The radical scavenging activity against DPPH radical was calculated as described in Materials and Methods. The column and bar in the figure show the mean and SD of DPPH radical scavenging activity of roasted alginic acid in triplicate assays. The statistical difference of the results between the control (room temperature) and different roasting temperature conditions was analyzed by Student’s t test. (*: P<0.01, **: P<0.001) SD: standard deviation, DPPH: diphenylpicrylhydrazyl.

Hydroperoxide generation (A560/ assay tube)

| Roasting temperature | 100ºC | 130ºC | 160ºC | 180ºC |
|----------------------|-------|-------|-------|-------|
| Water (Control)      | 0     | 0     | 0     | 0     |
| RT                   | 0     | 0     | 0     | 0     |

Figure 2. Effect of roasting treatment on the radical scavenging activity of alginic acid against hydroperoxide generation from oxidized linoleic acid. The amount of hydroperoxide generated by linoleic acid autooxidation in the absence and presence of roasted alginic acid was measured by the aluminum chloride method, as described in Materials and Methods. The column and bar in the figure show the mean and SD of the radical scavenging activity of roasted alginic acid against hydroperoxide generation in triplicate assays. The statistical difference of the results between the control (water) and alginic acid prepared under different temperature conditions was analyzed by Student’s t test. (*: P<0.01, **: P<0.001).

Chemiluminescence (× K cpm)

| Roasting temperature | 100ºC | 130ºC | 160ºC | 180ºC |
|----------------------|-------|-------|-------|-------|
| PBS (Control)        | 0     | 0     | 0     | 0     |
| Opz alone            | 0     | 0     | 0     | 0     |
| RT                   | 0     | 0     | 0     | 0     |

Figure 3. Effect of roasting treatment on the radical scavenging activity of alginic acid against oxygen radical generation in Opz-stimulated human neutrophils. The oxygen radical generating activity in human blood neutrophils was measured in the absence and presence of opsonized zymosan (Opz: 1 μg/ml). The roasted alginic acid was added and mixed with human blood neutrophils before the addition of Opz. The column and bar in the figure show the mean and SD of L-012-dependent CHL generating activity in human blood neutrophils in triplicate assays. The statistical difference of the results between the positive control (Opz alone) and alginic acid under different temperature conditions was analyzed by Student’s t test. (*: P<0.01, **: P<0.001) ND: not detected, CHL: Chemiluminescence, PBS: Phosphate-buffered saline.
for 30 min) (Figure 1), the mixture of alginic acid and each amino acid was roasted under the same roasting conditions, and the radical scavenging activity of each mixture against the DPPH radical was measured.

When alginic acid and each amino acid were mixed and roasted at 100 ºC for 30 min, glycine and tryptophan caused considerable enhancing effects on DPPH radical scavenging activity (39.4% and 37.6% inhibition, respectively) (underlined data in Table 2). Although arginine showed a moderate enhancing effect (18.7% inhibition), as a control experiment, the roasting of arginine alone in the absence of alginic acid showed significant radical scavenging activity (16.5% inhibition). The mixtures of other amino acids and alginic acid did not show any significant enhancing effect on the radical scavenging activity as compared with the control roasting experiment of each amino acid or alginic acid alone. Thus we concluded that only glycine and tryptophan cause enhancing effects on the scavenging activity against the DPPH radical by the roasting treatment at 100 ºC for 30 min in the presence of alginic acid.

Next, when the mixture of alginic acid and each amino acid was roasted at 130 ºC for 30 min, glycine and lysine caused strong enhancing effects on the radical scavenging activity (88.2% and 64.5% inhibition, respectively), and tryptophan, aspartate, asparagine, glutamine and serine also showed considerable enhancing effects (32.5–49.0% inhibition) (underlined data in Table 2). The mixture of alginic acid and alanine, glutamate, histidine, methionine or threonine showed weak but significant increases in radical scavenging activity (11.0–24.4% inhibition) compared with that of alginic acid alone (underlined data in Table 2). As a control experiment, phenylalanine and arginine exhibited weak but significant radical scavenging activity in the absence of alginic acid. Other amino acids did not show significant increases in the radical scavenging activity against the DPPH as compared with that of alginic acid alone.

Under the room temperature condition, none of the tested amino acids showed any significant enhancing effects on the DPPH radical scavenging activity in the presence of alginic acid.

These experimental results suggest that the roasting treatment of the mixture of alginic acid and specific amino acids exhibited much higher scavenging activities against the DPPH radical than the alginic acid alone under different roasting conditions (100 ºC, 130 ºC, 160 ºC and 180 ºC).

Enhancing effect of roasting treatment of the mixture of alginic acid and amino acid on the radical scavenging activity against hydroperoxide generation in linoleic acid autooxidation

We analyzed the radical scavenging activity of the mixture of alginic acid and each amino acid roasted under different temperature conditions by using a hydroperoxide generating system in linoleic acid autooxidation. When the mixture of alginic acid and a specific amino acid (glycine or tryptophan) were mixed and roasted at 100 ºC for 30 min, they caused significant enhancing effects on radical scavenging activity against hydroperoxide generation (28.1 and 26.0% inhibition, respectively) (underlined data in Table 3). Other amino acids, however, did not show significant increases as compared with the control experiment, while in a control experiment the roasting treatment of
Table 2. Enhancing effect of roasting treatment on DPPH radical scavenging activity of the mixture of alginic acid and each amino acid

| Treatment          | Radical scavenging activity (% Inhibition) 100°C | 130°C | 160°C | 180°C |
|--------------------|-------------------------------------------------|-------|-------|-------|
| Alginic acid alone | 1.0                                             | 4.7   | 83.1  | 45.3  |
| Alginic acid + amino acid |                                  |       |       |       |
| glycine            | 39.4 (0.8)                                      | 88.2 (1.5) | 76.8 (1.3) | 86.7 (2.5) |
| alanine            | 12.0 (0.5)                                      | 11.0 (1.2) | 77.5 (0.9) | 69.9 (1.8) |
| valine             | 0.8 (0.6)                                       | 1.9 (0.3) | 81.1 (1.5) | 66.7 (1.2) |
| leucine            | 1.4 (0.2)                                       | 1.5 (0.6) | 53.1 (1.1) | 81.4 (0.8) |
| isoleucine         | 4.2 (3.5)                                       | 6.3 (5.7) | 86.0 (4.1) | 77.8 (6.4) |
| phenylalanine      | 6.0 (4.9)                                       | 12.4 (11.8) | 82.9 (6.8) | 59.3 (5.1) |
| tyrosine           | 1.5 (1.0)                                       | 5.4 (0.7) | 55.2 (1.5) | 75.6 (1.7) |
| tryptophan         | 37.6 (6.9)                                      | 32.5 (8.4) | 88.7 (10.5) | 86.0 (18.6) |
| aspartate          | 2.4 (1.1)                                       | 49.0 (1.5) | 99.6 (5.3) | 81.7 (5.9) |
| asparagine         | 1.3 (0.8)                                       | 46.1 (0.9) | 81.2 (4.9) | 74.1 (30.5) |
| glutamate          | 2.4 (1.5)                                       | 24.4 (1.2) | 79.2 (8.6) | 76.9 (16.3) |
| glutamine          | 0.8 (0.2)                                       | 42.6 (7.3) | 95.0 (20.7) | 82.5 (33.7) |
| arginine           | 18.7 (16.5)                                     | 11.2 (10.6) | 75.6 (9.2) | 43.8 (21.0) |
| histidine          | 3.5 (1.1)                                       | 14.5 (1.7) | 81.0 (2.8) | 72.2 (5.8) |
| lysine             | 7.4 (6.8)                                       | 64.5 (5.9) | 98.5 (6.3) | 90.3 (8.4) |
| methionine         | 5.7 (0.3)                                       | 15.3 (2.2) | 95.3 (7.5) | 79.9 (6.3) |
| serine             | 2.4 (1.0)                                       | 37.6 (1.8) | 68.5 (11.7) | 48.2 (26.4) |
| threonine          | 4.6 (4.2)                                       | 20.3 (7.4) | 98.8 (6.8) | 72.6 (5.6) |

The results in the table show the average of DPPH radical scavenging activity of the roasted mixture of alginic acid and each amino acid in triplicate assays. The values in the parentheses show the average of radical scavenging activity by each amino acid alone in triplicate assays. The underlined data show significant enhancing effects on the radical scavenging activity as compared with the control values.

arginine alone showed a significant radical scavenging activity in the absence of alginic acid (Table 3).

When the mixture of alginic acid and each amino acid was roasted at 130°C for 30 min, glycine and lysine caused strong enhancing effects on the radical scavenging activity against hydroperoxide generation (79.0% and 58.3% inhibition, respectively), and tryptophan, aspartate, asparagine, glutamine and serine showed considerable enhancing effects (30.4–42.9% inhibition) (underlined data in Table 3). Alanine, glutamate, histidine, methionine and threonine showed weak but significant enhancing effects (10.6–21.8% inhibition) (underlined data in Table 3), while, as a control experiment, the roasting treatment of phenylalanine or arginine showed weak but significant radical scavenging activity against hydroperoxide generation in the absence of alginic acid.

When roasted at 160°C for 30 min, alginic acid alone showed a relatively strong radical scavenging activity against hydroperoxide generation (Figure 2 and Table 3). When alginic acid was mixed with each amino acid and they were roasted at 160°C, some specific amino acids (aspartate, glutamine, lysine, methionine and threonine) caused much stronger radical scavenging activities than that of alginic acid alone (90.3–98.8% inhibition) (underlined data in Table 3). Other amino acids (aspartate, glutamine, lysine, methionine and threonine) caused much stronger radical scavenging activities than that of alginic acid alone (90.3–98.8% inhibition) (underlined data in Table 3).
acids, however, did not show significant increases in radical scavenging activities against hydroperoxide generation as compared with alginic acid alone.

When roasted at 180°C, alginic acid alone showed a considerable radical scavenging activity against hydroperoxide generation (Figure 2 and Table 3). When alginic acid was mixed with each amino acid and they were roasted at 180°C, many amino acids (glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, aspartate, histidine, lysine, methionine and threonine) caused much stronger radical scavenging activities against hydroperoxide generation than alginic acid alone (underlined data in Table 3). Under the room temperature condition, none of the tested amino acids showed any significant enhancing effects on the radical scavenging activity against hydroperoxide generation from oxidized linoleic acid in the presence of alginic acid.

These experimental results suggest that the roasting treatment of the mixture of alginic acid and specific amino acids exhibited much higher radical scavenging activities against hydroperoxide generation in linoleic acid autoxidation than the alginic acid alone under different temperature roasting conditions.

Enhancing effect of roasting treatment of the mixture of alginic acid and amino acid on the radical scavenging activity against oxygen radical generation in Opz-stimulated human blood neutrophils

We analyzed the radical scavenging activity of the mixture of alginic acid and each amino acid roasted under different temperature conditions by using the oxygen radical generating system in Opz-stimulated human blood neutrophils. As shown in Table 4, when the mixture of alginic acid and specific amino acids (glycine and tryptophan) were mixed and roasted at 100°C, they caused considerable enhancing effects on radical scavenging activity against oxygen radical generation in human neutrophils (32.6 and 35.0% inhibition, respectively) (underlined data in Table 4). As a control experiment, the roasting treatment of arginine alone in the absence of alginic acid showed weak but significant radical scavenging activity in the absence of alginic acid.

When roasted at 160°C for 30 min, alginic acid alone showed a relatively strong radical scavenging activity against oxygen radical generation in Opz-stimulated human blood neutrophils (Figure 3 and Table 4). When alginic acid was mixed with each amino acid and they were roasted at the same temperature, some specific amino acids (aspartate, lysine and threonine) caused much stronger radical scavenging activities than that of alginic acid alone (89.6–98.7% inhibition) (underlined data in Table 4). Other amino acids did not show significant increases in the radical scavenging activity compared with that of alginic acid alone.

When roasted at 180°C, alginic acid alone showed a significant radical scavenging activity against oxygen radical generation in Opz-stimulated human blood neutrophils (Figure 3 and Table 4). When alginic acid was mixed with each amino acid and they were roasted at 180°C, many amino acids (glycine, alanine, valine, leucine, isoleucine, tyrosine, tryptophan, aspartate, asparagine, glutamate, lysine, histidine and methionine) caused much stronger radical scavenging activities than that of alginic acid alone (89.6–98.7% inhibition) (underlined data in Table 4). Under the room temperature condition, none of the tested amino acids showed any significant enhancing effects on the radical scavenging activity against oxygen radical generation in Opz-stimulated human blood neutrophils in the presence of alginic acid.

These experimental results suggest that the roasting treatment of the mixture of alginic acid and specific amino acids exhibited much higher radical scavenging activities against oxygen radical generation in Opz-stimulated human blood neutrophils than alginic acid alone under different temperature roasting conditions.
Roasting Effect on Radical Scavenging Activity of Alginate

Discussion

In the present study, we found enhancing effects of specific roasting treatments on various radical scavenging activities of a brown alga polysaccharide (alginic acid). Namely, strong enhancing effects were observed by roasting treatments under relatively high temperature conditions (160°C and 180°C), which were measured by (i) a stable radical compound diphenylpicrylhydrazyl (DPPH), (ii) a hydroperoxide generating system of linoleic acid autooxidation, and (iii) an opsonized zymosan (Opz)-induced oxygen radical generating system in human blood neutrophils (Figure 1, 2 and 3).

As shown in Table 1, the extent of the browning reaction of algic acid in the roasting treatment was proportional to the increase in the roasting temperatures (100°C, 130°C, 160°C and 180°C). Although the roasting treatment of algic acid at 100°C or 130°C showed significant increases in the browning reaction, it did not show significant increases in radical scavenging activities under the same temperature roasting conditions (Table 1 and Figure 1, 2, 3). The maximum browning reaction was observed when algic acid was roasted at 180°C, but the radical scavenging activity at that temperature significantly decreased as compared with the maximum radical scavenging activity at 160°C (Table 1 and Figure 1, 2, 3). These experimental results suggest that the enhancing effect on the radical scavenging activity of the roasted algic acid does not correlate directly with the extent of the roasting-induced browning reaction of polysaccharides (Caramel reaction).

The enhancing effect of the roasting treatment on the radical scavenging activity of algic acid may be due to other reasons besides the browning reaction. For example, previous reports have shown that low-molecular-weight fractions of algae-derived polysaccharides prepared by acid hydrolysis [15], radical process degradation [16], and enzymatic hydrolysis [17, 18] had strong radical scavenging activities. Although a detailed physicochemical analysis of the roasted algic acid was not performed in the present study, if degradative reactions occur in algic acid by a roasting treatment under high temperature conditions, active low-molecular-weight polysaccharide fractions may be generated, and they might cause strong radical scavenging activities. A detailed analysis of this problem should be considered in a future study.

We also showed that the roasting treatment of the mixture of algic acid and specific amino acids exhibited much higher radical scavenging activities than algic acid alone under different temperature roasting conditions (100°C, 130°C, 160°C and 180°C) (underlined data in Table 2, 3 and 4).

In the present study, we found enhancing effects of specific roasting treatments on various radical scavenging activities of a brown alga polysaccharide (algic acid). Namely, strong enhancing effects were observed by roasting treatments under relatively high temperature conditions (160°C and 180°C), which were measured by (i) a stable radical compound diphenylpicrylhydrazyl (DPPH), (ii) a hydroperoxide generating system of linoleic acid autooxidation, and (iii) an opsonized zymosan (Opz)-induced oxygen radical generating system in human blood neutrophils (Figure 1, 2 and 3).

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Maillard reaction is non-enzymatic browning reaction caused by a heating treatment, which involves the reaction of carbonyl compounds (especially saccharides) with amino acids. This reaction occurs through the formation of Amadori products and subsequent degradation products, which may contain reactive species that contribute to the antioxidant properties of the roasted mixture. Further investigation is needed to understand the mechanism of the enhancement of radical scavenging activity by roasting treatments and the role of specific amino acids in this process.
charides) and amino group-containing substances such as amino acids and proteins. Most previous in vitro studies of Maillard reaction were carried out by reducing monosaccharide (glucose, fructose or galactose) or reducing disaccharide (maltose or lactose), and a simple amino acid (glycine) or basic amino acid (lysine, arginine or histidine). Among these Maillard reaction products, some active compounds showed strong radical scavenging activities together with browning reactions [25, 26], but detailed studies of the enhancing effect of the roasting treatment of the brown alga-derived polysaccharide with amino acid on radical scavenging activity have not been carried out until present.

In the present study, we analyzed the relationship between the enhancing effect on the radical scavenging activity and the browning reaction in the roasting treatment of the mixture of alginic acid and amino acids. When alginic acid and effective amino acids (glycine, tryptophan) were mixed and roasted at 100°C for 30 min, they caused considerable enhancing effects on radical scavenging activities (underlined data in Table 2, 3 and 4), but they did not show significant increases in the browning reaction. When the mixture of alginic acid and an effective amino acid (glycine, lysine, aspartic acid, asparagine, glutamine or serine) was roasted at 130°C for 30 min, they showed considerable enhancing effects on the radical scavenging activity (underlined data in Table 2, 3 and 4), but they did not exhibit a significant increase in the browning reaction.

In contrast, when the mixture of alginic acid and an effective amino acid (aspartate, lysine, threonine, methionine or glutamine) was roasted at 160°C for 30 min, aspartate, lysine and threonine showed increases in the browning reaction compared with that of alginic acid alone, but methionine did not show a significant increase.

When the mixture of alginic acid and 16 effective amino acids that had enhancing radical scavenging activity (glycine, alanine, valine, leucine, isoleucine, phenylalanine, asparagine, tyrosine, tryptophan, aspartate, glutamate, methionine, lysine, histidine and threonine) were roasted at 180°C for 30 min, glycine, glutamate and threonine showed significant increases in browning reaction compared with alginic acid alone, but the other 13 effective amino acids did not.

These experimental results suggest that the enhancing effect of the roasting treatment on the radical scavenging activity of the mixture of alginic acid and effective amino acids is not directly related to the extent of the browning reaction of the mixture of alginic acid and amino acid.

As another reason besides the browning reaction for the strong radical scavenging activities of the mixture of alginic acid and specific amino acids by the roasting treatment, the following possibility can be considered. In the roasting treatment of the mixture of alginic acid and specific amino acids, alginic acid may be degraded to generate low-molecular-weight saccharide fractions, and they might react with specific amino acids to synthesize the active radical scavenging substances. Possibly, among these active radical scavenging substances, some of these compounds might cause an increase in the browning reaction, but others might not cause a significant browning reaction.

Several Maillard reaction products of polysaccharide and amino acid by heat treatment exhibit harmful effects on the human body. For example, toxic acrylamides generated from the mixture of another polysaccharide (starch) and amino acid (aspartic acid) by heat treatment cause acute and chronic diseases such as neurological disease and cancer [27, 28]. Toxic acrylamides are not generated by the roasting treatment of the mixture of alginic acid and amino acid, however, since alginic acid is composed of the copolymers of D-mannuronate and L-gluronate and it does not contain glucose polymer (starch).

Another interesting finding associated with the present results is that several brown algae themselves show similar enhancing effects of roasting treatment on their radical scavenging activities, but they needed different roasting conditions to get better roasting effects. A Japanese brown alga, Laminaria japonica (Ma-konbu), for example, exhibited strong roasting effects on the radical scavenging activity at 180°C and 200°C, but showed a weak roasting effect at 150°C [20]. Similar results were observed in a roasting experiment of another brown alga, Hijikia fusiformis (Hijiki) (unpublished data).

Although the reason for the different effective roasting conditions between purified polysaccharide and brown algae themselves is unclear at present, some
possible reasons can be considered. One possibility is that there may be non-polysaccharide substances in brown algae themselves that cause the roasting-dependent enhancing effect on the radical scavenging activity. Other experimental results suggest that the important factor for the roasting-induced enhancement of the radical scavenging activity in brown algae is the increase in the non-polysaccharide antioxidant substances such as polyphenols and tannins in the extract of roasted *Laminaria japonica* [24]. Namely, the effective extractability of these non-polysaccharide components such as polyphenols and tannins from the roasted algae seems to be dependent on different roasting temperature conditions.

Another interesting finding in the present study is that the roasted alginic acid or the roasted mixture of alginic acid and specific amino acids caused strong suppressive effects on oxygen radical generation in typical inflammatory cells, human blood neutrophils induced by an inflammatory reagent (opsonized zymosan) (Figure 3 and Table 4). The inflammatory activation of neutrophils is generally associated with the causation and progression of various inflammatory reactions and allergic diseases such as allergic asthma [29], atopic dermatitis and anaphylaxis reaction [30]. It is possible that the dietary intake of roasted alginic acid or the roasted mixture of alginic acid and a specific amino acid may cause suppressive effects on these inflammatory reactions and allergic diseases, although the effects of roasted alginic acid or the roasted mixture of alginic acid and specific amino acids on the functions of other immunocompetent cells such as lymphocytes or macrophages should be analyzed in a future study.

Other researchers have reported a hypolipidemic effect of a specific polysaccharide (fucoidan) derived from brown alga [31]. Namely, they showed that the administration of brown alga-derived polysaccharide preparation reduced the concentrations of serum cholesterol, triglyceride (TG) and low density lipoprotein (LDL), and increased high density lipoprotein (HDL) and the enzyme activities of lipoprotein lipase (LPL) and lecithin cholesterol acyltransferase (LCAT) in hyperlipidemic model rats. The progression of cardiovascular diseases such as atherosclerosis is generally associated with arterial thrombotic and platelet aggregation activities. A previous study showed that different molecular weight fractions from the radical-treated brown alga polysaccharide showed anticoagulant, antithrombotic and antiplatelet activities, and caused a decrease of whole blood viscosity and hematocrit value in a model rat experiment [32]. If similar degradative reactions occurred in the polysaccharide by the roasting treatment in the present study, different molecular weight active fractions might have been generated in the roasted alginic acid. The effects of roasted alginic acid and roasted mixture of alginic acid and amino acid on chronic diseases such as cardiovascular diseases should be analyzed in future studies.

The experimental results in the present study suggest the possible significance of the roasting treatment of alginic acid itself or the mixture of alginic acid and specific amino acids from the viewpoint of their radical scavenging activities and related beneficial health effects. These findings may suggest a new dietary method of alginic acid and amino acid for the prevention of various allergic or chronic diseases in the human body.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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