Antioxidative activity of the buckwheat polyphenol rutin in combination with ovalbumin

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Abstract. Buckwheat flour is well known for its highly antioxidative ingredient, rutin. We have undertaken to examine alterations in the characteristics of rutin treated with various proteins. In this study, the radical scavenging activities of a rutin-ovalbumin complex were examined. Dissolved rutin hydrate and ovalbumin were combined and boiled in water for 10 min. In the resulting rutin-ovalbumin complex, a new high molecular weight peak was detected using gel permeation chromatography analysis, and an existing high molecular weight area of ovalbumin was observed to be increased by the addition of rutin. This suggested that ovalbumin molecules produce a complex through their interaction with rutin. Alkaline luminol chemiluminescence and electron spin resonance analysis revealed the formation of a rutin-ovalbumin complex that markedly enhanced the peroxyl, but not the hydroxyl, radical scavenging activity of rutin. Rutin also demonstrated antioxidative activity against hydroxyl radicals in a DNA protection assay. We therefore conclude that, compared with ovalbumin or rutin alone, the rutin-ovalbumin complex has improved antioxidative activities in the form of enhanced peroxyl radical scavenging activity and DNA protection from apurinic/apyrimidinic site formation caused by hydroxyl radicals.

Introduction

Active oxygen species generated in the body cause damage to DNA and the lipid membrane structure of cells and play a role in the process of aging and the development of cancer (1). It is therefore important to control the amount of excess active oxygen in our body. Antioxidative components found in food have been shown to be effective as scavengers of active oxygen (2). We have been studying the antioxidative activity of rutin, a flavonoid of buckwheat. Buckwheat and/or tartary buckwheat flours are used to make 'Soba', a typical Japanese thin noodle. Adding egg whites to buckwheat flour during 'Soba' processing makes it easier to form the dough.

Research has indicated that rutin and quercetin may be metabolized by albumin-mediated transport in the body (3-5). Papadopoulou *et al* reported a binding affinity between flavonoids and bovine serum albumin (BSA), ranking in the order of quercetin > rutin > epicatechin = catechin (4). This suggests the possibility of a chemical interaction between rutin and ovalbumin (OVA) during the boiling of 'Soba', which may influence the antioxidative activity of either substance. We previously reported that heating rutin with water-soluble whey protein isolate (WPI), a milk ingredient, results in the alteration of its antioxidative activity (6). Formation of a rutin-WPI complex enhanced the peroxyl and hydroxyl radical scavenging activities of rutin or WPI. We also studied the effects of the interation of another flavonoid, tannin, and WPI on the physical properties of gelatin gel (7), pH alteration on the gelation by rutin in gelatin solution (8) and the alteration of rheological properties of buckwheat noodles for different content ratios of rutin and wheat gluten (9).

In this study, the interaction between rutin and OVA heated together was first investigated using gel permeation chromatography (GPC). Next, chemiluminescence and electron spin resonance (ESR) were respectively used to determine whether or not this interaction enhanced the peroxyl or the hydroxyl radical scavenging activity of the combined substances in comparison with the activity of rutin or OVA alone. The antioxidative activity of rutin was then further examined using a new method that detects the level of DNA protection from the apurinic/apyrimidinic (AP) site formation caused by hydroxyl radicals. The results suggest the possibility of the formation of a rutin-OVA complex with improved antioxidative function.

Materials and methods

Chemicals. 2,2'-Azo*bis*(2-amidinopropane)dihydrochloride (AAPH), sodium tetraborate decahydrate (borax), hydrochloric acid (HCl) and β-carotene were obtained from Wako Pure Chemical, Ltd. (Osaka, Japan). Luminol and cytochrome *c* from horse heart were obtained from Nacalai Tesque

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(Kyoto, Japan). Hydrogen peroxide (H_2O_2) was from Santoku Chemical Co., Ltd. (Tokyo, Japan). Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was from Sigma-Aldrich Japan (Tokyo, Japan). The spin trapping reagent 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Tris (hydroxymethyl) aminomethane and deoxyribonucleic acid (DNA) sodium salt from salmon testes, rutin hydrate, ovalbumin (OVA; from chicken egg white, grade V) and astaxanthin were from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt dihydrate (EDTA-2Na) and a DNA damage quantification kit were from Dojindo Laboratories (Kumamoto, Japan). The kit contained an aldehyde reactive probe (ARP; N'-amino-oxymethylcarbonylhydrazino-D-biotin), horseradish peroxidase (HRP)-streptavidin, ARP-DNA standard, DNA binding buffer including TMBZ (3,3',5,5'-tetramethylbenzidine) and phosphate-buffered saline (PBST; with 0.05% Tween-20, pH 7.4).

Sample preparation. Rutin hydrate (100 ppm) and 3% (w/v) OVA were combined in a flask in a total volume of 10 ml and boiled in water for 10 min. The sample was then quickly cooled with ice water. Rutin solution (100 ppm) and 3% OVA solution as the control were separately but similarly prepared, and defined as the original solutions (100%).

Chromatographic analysis. The HPLC system consisting of an L-6200 pump, an L-4200 UV detector (Hitachi High-Technologies Co., Tokyo, Japan) and a Shodex Asahipak GS-620 column (7.5x30 mm) with a Shodex Asahipak GS-1G short guard column (Showa Denko K.K., Tokyo, Japan) was used for the assay. The mobile phase was 100 mM phosphate buffer (pH 6.8) with a flow rate of 1.0 ml/min, and the eluate was monitored by absorption at 220 nm. The 10-fold diluted samples were injected into the HPLC column after filtration with a membrane filter (0.45 μm). Experimental data was analyzed by the Chromato Integrator D-2500 (Hitachi High-Technologies Co.). The calibration curve was constructed by plotting the various molecular weight pullulans (Shodex Standard P-82; Showa Denko K.K.). A rutin concentration of 500 ppm was used in the chromatographic experiment.

Chemiluminescence analysis. Chemiluminescence analysis was performed as previously described in detail (6). In brief, phosphate buffer was used to prepare a 40 mM AAPH solution and to dilute the samples. The AAPH solution (0.2 ml) was added to 0.2 ml of each diluted sample, then the samples were incubated at 37˚C for 2 min. Immediately after incubation, 0.2 ml luminol solution was added, and the chemiluminescence intensity was detected by a photon counter (Lumitester C-100; Kikkoman Co., Tokyo, Japan). One relative light unit (RLU) represents 43.48 photons/sec.

Electron spin resonance analysis. ESR was performed as previously described (6). In brief, hydroxyl radicals were generated by the DMPO-supplemented Fenton's reaction with or without examination of the samples (rutin, OVA and rutin-OVA complex). The addition of 8.8 mM H_2O_2 (50 μl) to the above reaction mixture (320 μl) was used to initiate the Fenton's reaction, which occurs as in the chemical equation: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$. After 1 min of hydroxyl radical generation, spin adduct DMPO-OH$\cdot$ was measured using the ESR spectrometer (JES-FR30; JEOL Ltd., Tokyo, Japan). ESR measurement conditions were as follows: output, 4 mW (9.4 GHz); magnetic field, 342.790±5 mT; modulation amplitude, 0.079 mT; time constant, 0.1 sec; sweeping time, 1 min; and amplification ratio, 32-125.

Calculation of the IC_{50} value of peroxyl or hydroxyl radical scavenging. IC_{50} values were defined as the concentrations of the individual rutin, OVA and rutin-OVA complex samples that reduced the control RLU value (chemiluminescence intensity generated by peroxyl radical) or the control peak height ratio of ESR (generation of hydroxyl radical) by half. The antioxidative value was calculated by the formula: (log Io/I) x 100, where Io is the RLU value or ESR peak height ratio of the control, and I is the RLU value or ESR peak height ratio of the samples. Thus, the IC_{50} values were the concentration of samples at Io/I =1/2, assessed using the antioxidative results of RLU or ESR obtained from the experiments (10).

Preparation of the protected DNA samples. Salmon testes DNA was prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA-2Na, pH 7.5) at a concentration of 1 mg/ml. Hydroxyl radical damaged or protected DNA samples were prepared according to a previously described method (11). In brief, 300 μl of DNA solution was mixed with 50 μl of 1.0 mM $FeSO_4$ together with or without 250 μl of the antioxidant samples, and immediately incubated at 37˚C for 5 min. Fenton's reaction was initiated by adding 50 μl of 8.8 mM H_2O_2 to the mixture (negative control, H_2O instead of H_2O_2). The mixture was incubated at 37˚C for another 15 min and was cooled down on ice.

ARP labeling of DNA and determination of the number of AP sites in DNA. DNA labeling with ARP (aldehyde reactive probe; N'-aminooxymethylcarbonylhydrazino-D-biotin) was as described previously (12,13). In brief, 10 μl of the above-prepared DNA (100 μg/ml) was mixed with 10 μl of ARP solution and biotinylated using the DNA damage quantification kit. For purification of the DNA samples, the Nanosep 30K device (molecular weight, 30,000; Pall Corp., NY, USA) was used. The ARP-labeled DNA was prepared in 400 μl of TE buffer, then placed in the wells of a 96-well microplate (#650061, Greiner Bio-One, Germany) and examined using the HRP-streptavidin and TMBZ provided in the kit. The absorbance at 650 nm was determined using a microplate reader (Model 680; Bio-Rad Laboratories, Tokyo, Japan). Dual samples were examined for each experiment. For calibration, 60 μl each of standard solutions (0, 2.5, 5, 10, 20 and 40 AP sites per $1x10^5$ bp, respectively) were applied as a duplicate series in 96-well plates.

Calculation of the ratio of DNA protection. The ratio of DNA protection (%) was defined as: [(A-B) - (C-B)] x 100/(A-B) = (A-C) x 100/(A-B), where A is the number of AP sites per 10^5 bp after exposure to hydroxyl radicals in Fenton's reaction, B is the number of AP sites per 10^5 bp without exposure, and C is the number of AP sites per 10^5 bp after examination with antioxidants.

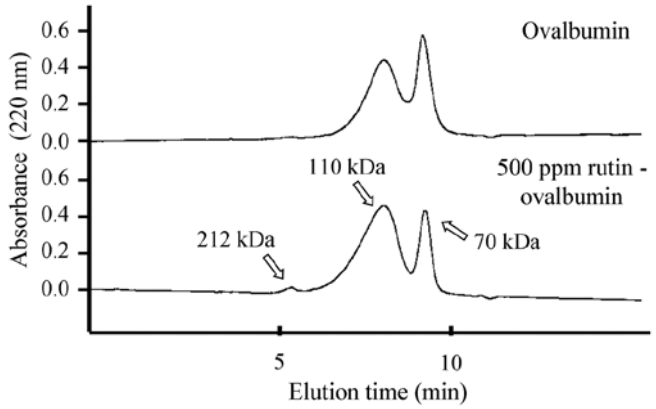

Figure 1. Molecular weight distribution in the OVA control (top) and in OVA with 500 ppm rutin (bottom) determined by gel permeation chromatography.

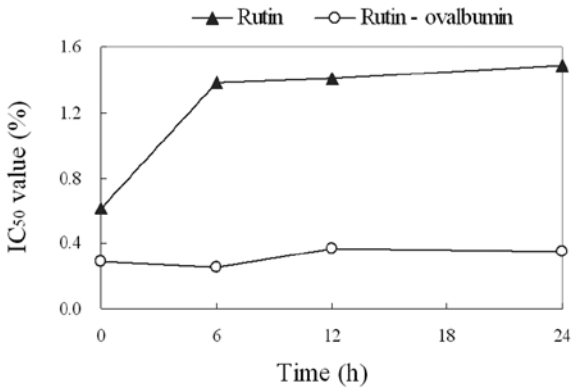

Figure 3. Changes in the peroxyl radical scavenging activity of rutin and the rutin-OVA complex at various intervals after sample preparation. ▲, 100 ppm rutin; ○, OVA-rutin complex.

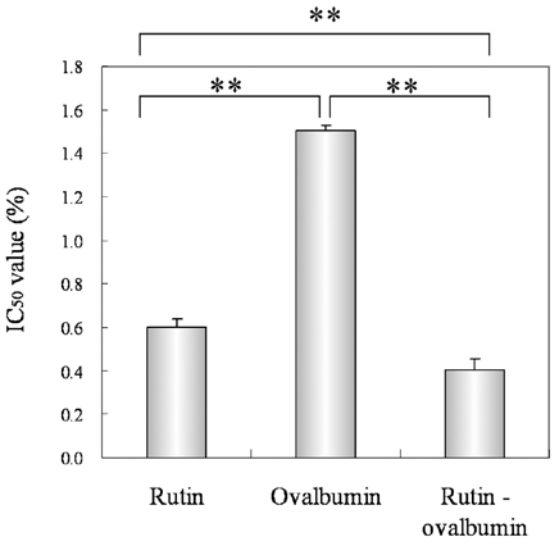

Figure 2. IC_{50} values (%) of the peroxyl radical scavenging activities of rutin, OVA and the rutin-OVA complex measured using chemiluminescence. $^{**}p<0.01$; n=3-8.

Results and Discussion

In the results of gel permeation chromatography (GPC), OVA alone displayed two large peaks at 70 and 110 kDa (Fig. 1; top). By contrast, the OVA sample boiled with rutin displayed a 212-kDa peak as a new polymer (Fig. 1; bottom). The relative size of the 70-kDa peak was not altered by the addition of rutin; however, the 110-kDa peak became higher and broader towards the high molecular range of the chromatography compared to the OVA alone (Fig. 1; top). This observation suggests that the addition of rutin induced a shift in the molecular weight of OVA, from 70 or 110 kDa to a larger one. We previously reported on the increase of the high molecular weight distribution of a WPI-gelatin complex by the addition of tannin to the solution (7). These results suggest that flavonoids may have the ability to promote an increase in the molecular weight of various proteins. Manach *et al* (3) found that the high affinity of quercetin (aglycon of rutin) to albumin may be caused by its structure, composed of numerous phenolic groups with hydrophobic interaction sites. They concluded that the phenolic groups of quercetin may provide a multiplicity of potential binding sites for the hydrogen bond. The authors also reported the bathochromic effect of albumin by the albumin/quercetin molar ratio, i.e., the absorption wavelength of human albumin shifted from 380 to 434 nm when the albumin/quercetin molar ratio reached 20. We found a similar absorption wavelength shift when rutin was treated with OVA (data not shown). These observations strongly suggest an intimate chemical interaction between OVA and rutin. Doronicheva *et al* demonstrated, using chemiluminescence, that the binding of flavonoids to the protein region of catalase contributed to an improvement in the activity of the enzyme (14). However, there are no reports on the alteration of the antioxidative activity of the rutin-OVA complex during the heating process. If the antioxidative activity of rutin is enhanced by interaction with OVA, the function of 'Soba' (a Japanese buckwheat noodle) may be improved by the addition of egg whites during the cooking process.

Fig. 2 shows the IC_{50} values of the peroxyl radical scavenging activities of 100 ppm rutin, 3% OVA and the rutin-OVA complex, respectively. The IC_{50} values are the final concentrations of the samples that reduced the intensity of chemiluminescence caused by the peroxyl radical by half (6). Thus, in this assay, low IC_{50} values indicated high antioxidative activity. The IC_{50} value of the rutin-OVA complex (0.43±0.05%) was lower than that of rutin alone (0.60±0.04%) or OVA alone (1.51±0.02%). These results indicate that the rutin-OVA complex acquired additional antioxidative activity for peroxyl radical scavenging.

Buckwheat foods are usually consumed after a period of preservation (i.e., there is a period of several days between production and consumption). Thus, the time decay of the peroxyl radical scavenging activity of rutin and rutin-OVA was examined. The IC_{50} value of rutin was 0.61% at the start time, and markedly increased by up to 1.38% within 6 h (Fig. 3). After 24 h, the IC_{50} value of rutin reached 1.49% due to the gradual decay of its scavenging activity. On the other hand, no alteration in the IC_{50} value of rutin-OVA was observed during a period of 24 h; thus, the effect of time decay on the activity of the complex may be negligible. The IC_{50} value of OVA demonstrated a similar tendency. Thus, the depression of the antioxidative activity of rutin is suppressed by its interac-

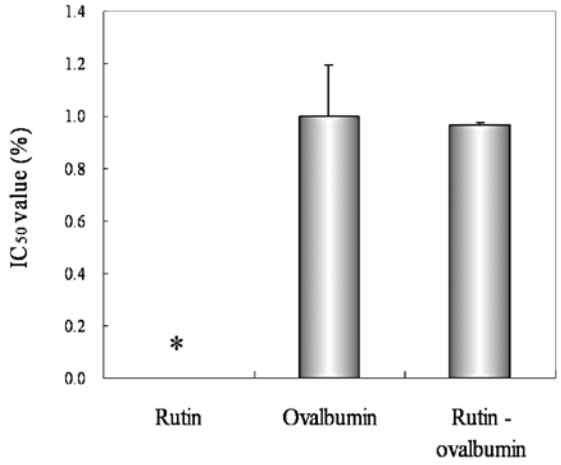

Figure 4. IC_{50} values (%) of the hydroxyl radical scavenging activities of rutin, OVA and the rutin-OVA complex measured using electron spin resonance. *IC_{50} value of rutin for hydroxyl radical scavenging activity >100%; n=3-8.

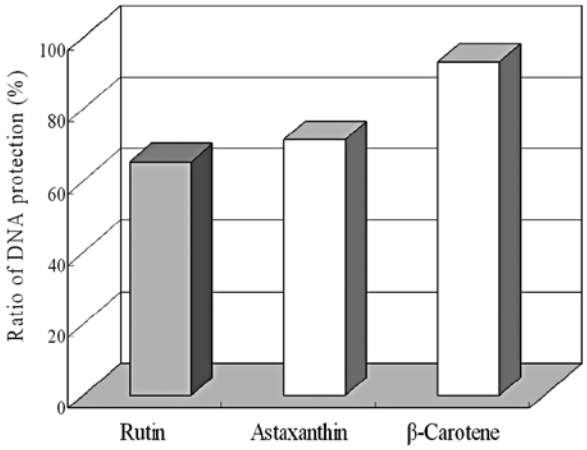

Figure 6. Ratio of DNA protection (%) against hydroxyl radical damage in the Fenton's reaction solution.

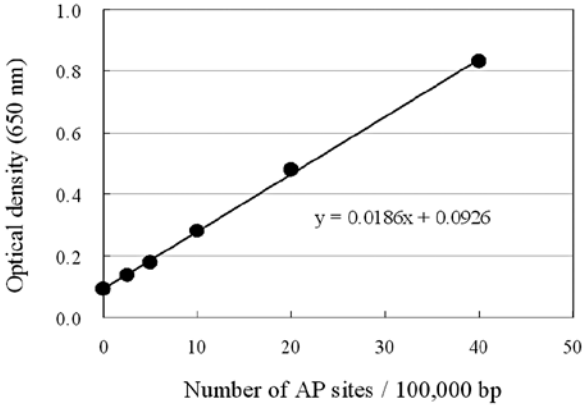

Figure 5. Calibration curve using the ARP-DNA standard. Vertical and horizontal axes indicate the optical density at 650 nm and the number of AP sites per 10^5 bp, respectively.

tion with OVA. This suggests that the antioxidative activity of rutin is controllable, and that the stability of the functional improvement may be maintained by the addition of ovalbumin (or egg whites) to buckwheat foods.

The IC_{50} values of the hydroxyl radical scavenging activities of 100 ppm rutin, 3% OVA and the rutin-OVA complex are shown in Fig. 4. For the hydroxyl radical scavenging, the IC_{50} value of 100 ppm rutin was >100%, indicating no effect at this concentration. The IC_{50} values of OVA and the rutin-OVA complex were 1.00±0.20 and 0.97±0.01%, respectively. We previously demonstrated that a similar combination of rutin with WPI had synergistic effects on hydroxyl radical scavenging activity, leading the rutin-WPI complex to have accelerated radical-reducing activity (6). In contrast, the hydroxyl radical scavenging activities of the rutin-OVA complex improved very slightly compared to those of the OVA alone. The hydroxyl radical scavenging activity of rutin and vitamin B_2 was also found to be low upon ESR analysis with CYPMPO (5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline-N-oxide) (15). The hydroxyl radical scavenging activity of the rutin-protein complex may be influenced by the activity of the protein itself or by the protein species combined.

Chen *et al* reported that rutin and quercetin from Ginkgo protected cerebellar granule cells from apoptosis caused by oxidative damage induced by hydroxyl radicals (16). We also studied the protection of DNA from the damage induced by hydroxyl radicals. AP sites are the major types of damage generated by hydroxyl radicals in double-stranded DNA. The biotinylated reagent ARP specifically reacts with the aldehyde of AP sites; thus, HRP-conjugated streptavidin can detect AP sites by the colorimetric reaction (13). This method has been adopted to detect the antioxidative activity of food or food-derived materials (11,17). The number of AP sites on DNA damaged by hydroxyl radicals was obtained from the calibration curve using AP site standard DNA (Fig. 5). The estimated DNA protection ratio of rutin was compared with that of astaxanthin and β-carotene (Fig. 6). Rutin (65.1%) showed a similar level of DNA protection from damage by the hydroxyl radical as astaxanthin (71.2%), but was greatly inferior to carotene (93.1%), used as a positive control. We also determined the preliminary protection result of the rutin-OVA complex to be approximately 80% (data not shown).

These results indicate the high antioxidative activity of rutin, not only against peroxyl radicals, but also against hydroxyl radicals, using different detection methods. This antioxidative activity was improved by the chemical interaction of rutin with various proteins; in particular, the rutin-OVA complex may have improved DNA protection activity. The heating of rutin with OVA promoted complex formation, suggesting the possibility of the functional improvement of rutin during food processing, such as noodle making. Further research is necessary to determine possible improvements to the function of 'Soba'.

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