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Antioxidant properties of some different molecular weight chitosans

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ABSTRACT

Chitosan, a cationic polysaccharide, is widely employed as dietary supplement and in pharmacological and biomedical applications. Although numerous studies have focused on its applications as pharmaceutical excipients or bioactive reagents, relationships between molecular weight (Mr) and biological properties remain unclear. The focus of this study was on the antioxidant properties of several Mr chitosans. We measured the ability of seven Mr chitosans (CT1; 2.8 kDa, CT2; 17.0 kDa, CT3; 33.5 kDa, CT4; 62.6 kDa, CT5; 87.7 kDa, CT6; 604 kDa, CT7; 931 kDa) to protect plasma protein from oxidation by peroxyl radicals derived from 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). A comparison of the antioxidant action of high Mr chitosans (CT6–CT7) with that of low Mr chitosans (CT1–CT5) showed that low Mr chitosans (CT1–CT5) were more effective in preventing the formation of carbonyl groups in plasma protein exposed to peroxyl radicals. AAPH substantially increases plasma protein carbonyl content via the oxidation of human serum albumin (HSA). We also measured the ability of these chitosans to protect HSA against oxidation by AAPH. Low Mr chitosans (CT1–CT5) were found to effectively prevent the formation of carbonyl groups in HSA, when exposed to peroxyl radicals. Low Mr chitosans were also good scavengers of N-centered radicals, but high Mr chitosans were much less effective. We also found a strong correlation between antioxidant activity and the Mr of chitosans in vitro. These activities were also determined by using the 'TPAC' test. These results suggest that low Mr chitosans (CT1–CT3) may be absorbed well from the gastrointestinal tract and inhibit neutrophil activation and oxidation of serum albumin that is frequently observed in patients plasma undergoing hemodialysis, resulting in a reduction in oxidative stress associated with uremia.

1. Introduction

Natural products have been the starting point for the discovery of many important modern drugs. This has led to chemical and pharmacological investigations and general biological screening programs for pharmacological activity of natural products on a world wide scale.[1] Marine organisms are also the subject of significant interest as potential sources of compounds with beneficial pharmacological activities. A group of molecules that has attracted considerable interest are chitosans, soluble derivatives of chitin. Chitin is a polysaccharide composed of *N*-acetyl glucosamine units and is one of the most abundant naturally occurring polysaccharides on earth and is found in shellfish, clams, krill, oysters, squid, fungi, and insects.[2,3] It is insoluble in water, but after deacetylation and partial hydrolysis is converted to a more soluble lower molecular weight (Mr) chitosan. Chitosan, a linear copolymer of β-(1,4)-poly-2-amino-2-deoxy-D-glucose and β-(1,4)-poly-2-acetamido-2-deoxy-D-glucose, has found a wide range of applications in mining, water treatment, the paper industry, medicine, biotechnology, food and cosmetic industries, and in agriculture.[4,5] The principal property of the various preparations of chitosan responsible for these applications is its solubility, which depends on the Mr, degree of residual acetylation, and any chemical modifications. Soluble chitosans and their derivatives have a range of properties that are useful in biology and medicine, which include ready uptake by cells and the intestines, low toxicity to eukaryotes, ability to stimulate the immune system, hypocholesterolemic and antitumor activity, and other benefits.[6–12] A property of particular interest for this study is the antioxidant properties of chitosan.[13,14] Studies by Xie et al. and Xue et al. showed that the scavenging of hydroxyl radicals by chitosan inhibits the lipid peroxidation of phosphatidylcholine and linoleate liposomes.[13,15] In a previous study, we also showed that the scavenging of hydroxyl radicals by chitosan inhibits the peroxidation of human serum albumin (HSA).[16] Santhosh et al. showed that the administration of chitosan to rats treated with isoniazid or rifampicin prevented hepatotoxic

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lipid oxidation.[17] Similarly, chitosan injections have been reported to inhibit glycerol-induced renal oxidative damage in rats.[18] We also showed that the administration of chitosan to human volunteers prevented HSA oxidation in vivo.[19] Although numerous studies have been reported concerning the antioxidant activities of chitosan, relationships between molecular weight and biological activity have not been extensively reported.

The aim of this study was to examine the possible antioxidant and free radical-scavenging properties of several different Mr chitosans in in vitro studies. We also evaluated the relevance of the Mr of chitosans and their antioxidant activities.

2. Results

2.1. Carbonylation of plasma protein from a normal volunteer in the presence and absence of chitosan

We investigated the antioxidant effects of molecular weight (Mr) for some different chitosans. As shown in Figure 1, oxidized proteins were derived with dinitrophenylhydrazine, separated by SDS–gel electrophoresis, and screened with antibodies against dinitrophenyl groups. HSA was the only major plasma protein that was significantly oxidized in human plasma in the absence of chitosans and, in the group treated with low Mr chitosans (CT1–CT5), the oxidation of HSA was decreased. It is noteworthy that no significant difference was found in the group treated with high Mr chitosans (CT6, CT7). The order of their effectiveness was found to be CT1 > CT2 > CT3 > CT4 > CT5 ≫ CT6 > CT7. In addition, no significant difference was found in the carbonyl contents of the other plasma proteins (transferrin, immunoglobulin, and fibrinogen). These findings show that the decrease in plasma protein carbonyl content as the result of plasma oxidation was largely due to a decrease in the level of oxidized HSA.

2.2. Effect of oxidation on HSA in the presence and absence of chitosan

The ability of some different Mr chitosans to inhibit the oxidation of HSA by the levels of AAPH-derived peroxyl radicals generated from AAPH was determined. AAPH decomposes as a function of temperature at a constant rate to two carbon-centered radicals, with about half of which react rapidly with dioxygen to form reactive peroxyl radicals. The results show that low Mr chitosans (CT1–CT5) lowered the amount of HSA carbonyl groups generated, compared to an experiment in which no chitosan was present, and that the effect was time-dependent (Fig. 2). Moreover, after 6 and 24 h of incubation, the formation of the carbonyl moieties was strongly inhibited in the presence of Vit C, with an identical degree of protection provided by CT1. Otherwise, no significant difference was found in the group treated with high Mr chitosans (CT6, CT7). These findings also show that low Mr chitosans have a higher antioxidant activity than high Mr chitosans.

2.3. Scavenging activity of chitosan on DPPH and ABTS radicals

Figure 3 shows that the scavenging activity of several Mr chitosans on DPPH radicals was significant and concentration related. The scavenging rate of these chitosans increased with increasing concentration. Moreover, as shown in Figure 3, the IC_{50} values for

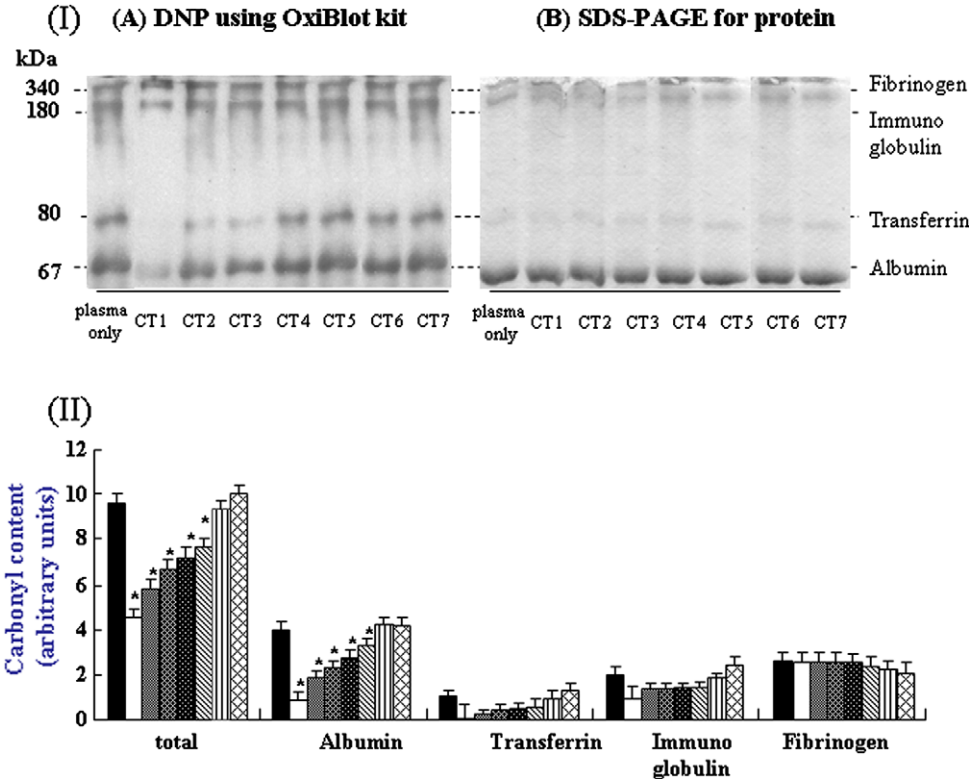

Figure 1. Carbonyl content of major plasma proteins from a normal volunteer with or without several molecular weight chitosans. (I) Plasma samples with or without several chitosans were derived with DNP and subjected to duplicate SDS–PAGE gels. Following electrotransfer, one blot was stained for DNP using OxiBlot kit reagents (A) and the second blot was stained with Coomassie brilliant blue G for protein (B). (II) Carbonyl formation of major plasma proteins (albumin, transferrin, immunoglobulin, and fibrinogen) was determined as the densitometry ratio of the DNP area and the protein area, and is reported as densitometry units. Values are expressed as mean ± SD. Plasma protein only (■), CT1 (□), CT2 (▨), CT3(▩), CT4 (▦), CT5(▧), CT6 (◫), and CT7 (◪). The concentration of the chitosans was 0.5 mg/mL. $^{*}P < 0.05$ as compared with plasma from volunteers without chitosans.

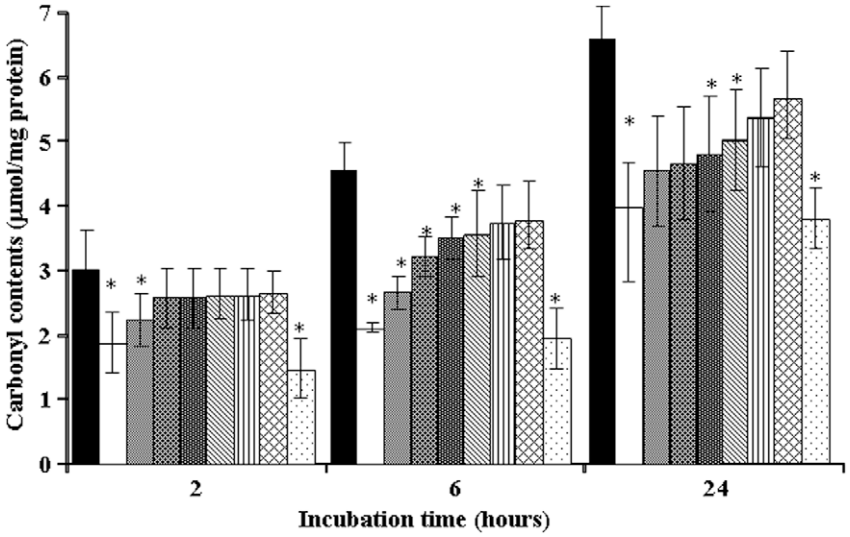

Figure 2. Inhibition of oxidation of human serum albumin by chitosans. Solutions of 20 μM HSA were exposed at 37 °C to 40 mM AAPH for various times and the degree of protein oxidation was measured by carbonyl assays. The bars indicate the levels of oxidized HSA formed after subtraction of control values. Plasma protein only (■), CT1 (□), CT2 (▩), CT3(▩), CT4 (▩), CT5(▧), CT6 (▯) CT7 (▨), and VitC (□). Several Mr chitosans and VitC concentrations were 5 and 0.5 mg/mL, respectively. $^{*}P < 0.05$, compared to HSA in the absence of chitosans.

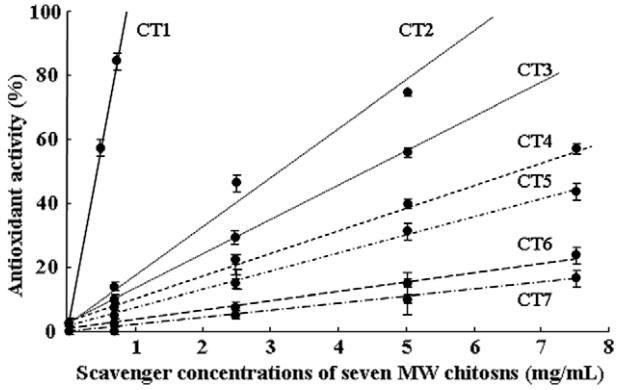

Figure 3. Relative effectiveness of different concentrations of the antioxidants in reducing DPPH radicals. The activities are shown relative to fully reduced DPPH (100%). DPPH radical concentration was measured at 517 nm. CT1 (—), CT2 (·····), CT3 (·····), CT4 (- - - -), CT5 (- · - · -), CT6 (– – –), and CT7 (– · –).

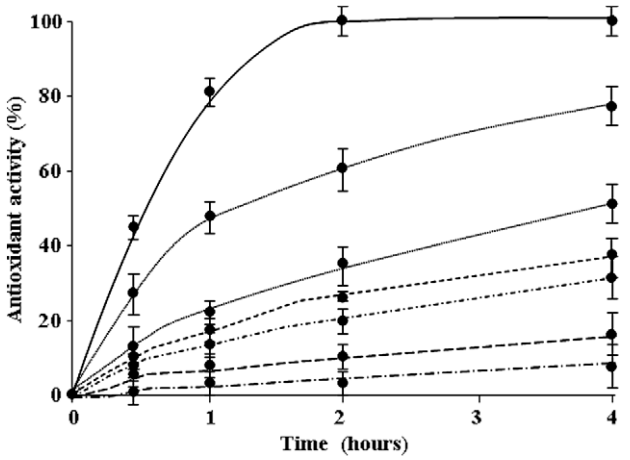

Figure 5. Time course for the reaction of the antioxidants with ABTS radical cations. The concentration of $ABTS^{\cdot+}$ was measured at 734 nm. The scavenger concentrations were 5 mg/mL. CT1 (—), CT2 (·····), CT3 (·····), CT4 (- - - -), CT5 (- · - · -), CT6 (– – –), and CT7 (– · –).

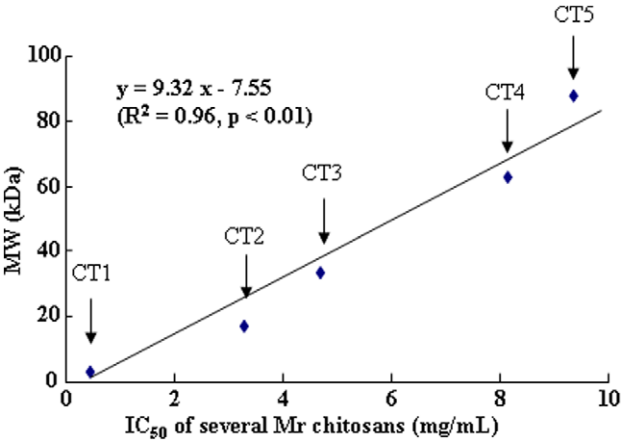

Figure 4. Relationship between Mr and antioxidant activity of low Mr chitosans. The correlation coefficient (r) between low Mr chitosans and antioxidant activity was significant at the indicated p value.

CT1, CT2, CT3, CT4, CT5, CT6, CT7 were 0.87, 3.28, 4.68, 8.15, 9.34, 15.9, 20.6 mg/mL, respectively. Further, the results shown in Figure 4A show an inverse relationship between antioxidant activity and the Mr of chitosans. These results suggest that the scavenging activities of low Mr chitosans for DPPH radicals were more pronounced than that of high Mr chitosans. In the case of the $ABTS^{\cdot+}$, the reduction in the presence of low Mr chitosan (CT1–CT5; 2.5 mg/mL) was over 60% in 24 h (Fig. 5). Otherwise, high Mr chitosans (CT6, CT7; 2.5 mg/mL) were much less effective. Overall, these results demonstrate the general ability of low Mr chitosans to scavenge oxygen- and nitrogen-centered radicals and suggest that their antioxidant potential shown in other systems may be due, at least in part, to this property.

2.4. Reducing power of chitosans using the TPAC test

Figure 6 shows data related to the reducing power of several Mr chitosans. The reducing power of low Mr chitosans correlated well with increasing concentrations but, the change in reducing power

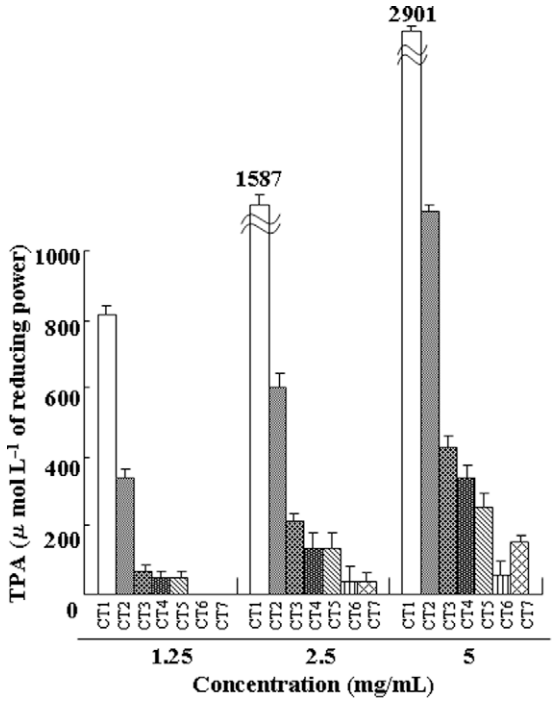

Figure 6. Reducing power of different Mr chitosans in a TPAC test. CT1 (□), CT2 (▩), CT3 (▩), CT4 (▩), CT5 (▨), CT6 (▥), and CT7 (▢). The values of 2.5 and 5 mg/mL of CT 1 were 1587 and 2901 μmol/mL, respectively.

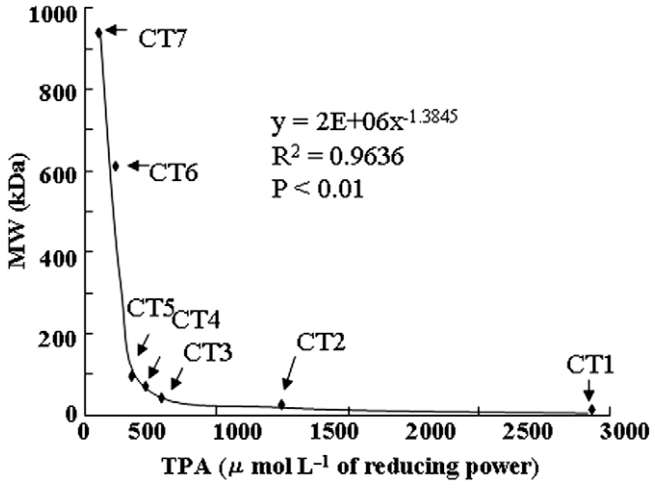

Figure 7. Relationship between Mr and reducing power of several chitosans. The correlation coefficient (r) between the Mr of chitosans and reducing power was significant at the indicated p value.

for high Mr chitosans was not obvious. These results indicate that low Mr chitosans have a higher reducing power. Moreover, good negative correlations were found between changes in reducing power in all Mr chitosans and changes in Mr (Fig. 7). These results suggest that the reducing power of low Mr chitosans is clearly more pronounced than that of high Mr chitosans.

3. Discussion

The intestinal absorption of various Mr chitosans by oral administration was directly related to its Mr. The amount of chitosan absorbed decreases with increasing Mr, as reported by Chae et al.[20] As a bioactive material, chitosan has several of the above discussed bioactivities such as antitumor, cholesterol-lowering, immunostimulating, antidiabetic, and antimicrobial effect. The cholesterol-lowering effect of chitosan is one of its most intensively studied bioactivity. It is generally accepted that the origin of the cholesterol lowering effect of chitosan is due to unique ability to bind lipid and bile acids.[21–23] The binding resulted in an increased elimination of fat in the stool, reduced bile acid recycling, and the induction of hepatic synthesis of new bile acid constituents from cholesterol.[21,24] These effects may contribute to a reduction of cholesterolemia. Considered with these biological events, the absorption of chitosan into the systemic circulation could be regarded as an unbeneficial event. However, the systemic circulation of chitosan may contribute to other bioactivities, such as antimicrobial, antitumor, immuno-stimulating, antidiabetic, and antioxidant effects. In particular, the antioxidant properties of chitosan derivatives have attracted considerable attention.[15,25]

Two recent studies have shown that chitosan preparations have the ability to protect proteins from radicals generated from the Fe^{2+}/H_2O_2 system; in one, D-glucosamine inhibited the oxidation of bovine serum albumin, while in another, chitooligosaccharides and carboxylated chitosan reduced the formation of protein carbonyl groups in RAW264.7 cells.[11,12] It therefore appears likely that low Mr chitosans might also protect HSA from oxidation by ROS and thus function as a potential antioxidant in vascular tissues. In a recent study, we measured the ability of a 2800 Da low Mr chitosan preparation to protect HSA from peroxyl radicals[16] and also showed that the administration of a chitosan supplement to human volunteers prevented HSA oxidation in vivo.[19] However, the antioxidant properties of chitosans with different molecular weights which can be absorbed from the intestinal tract, were not available. Accordingly, the objective of this study was to assess the antioxidant properties of chitosans with different molecular weights. We also examined the relationships between plasma proteins and several molecular weight chitosans in detail.

In the present study, using a Western blot immunoassay, we show that the oxidation of HSA accounts for nearly all of the excess plasma protein oxidation in an AAPH incubation with AAPH alone or with several Mr chitosans, and that low Mr chitosans (CT1–CT5) decrease the extent of oxidation of HSA (Fig. 1). The susceptibility of isolated albumins to oxidation by ROS has generally been demonstrated in several studies.[26] There is one report of the presence of short-lived protein hydroperoxides in plasma that had been subjected to gamma ray irradiation,[27] while the greater stability of carbonyl residues has allowed their detection in albumin samples from diabetic children and rats, and in uremic patients who are undergoing dialysis.[28,29] We have also recently found that the carbonylation of several residues in HSA of patients with uremia resulted in conformational changes, an increase in the ability of albumin to activate neutrophils and a decrease in radical scavenging capacity.[30] This is in agreement with the observation that the levels of oxidized HSA correlated well with the level of renal dysfunction among HD patients.[31] Overall, these observations suggest that the level of oxidized HSA can serve as a measure of the extent of oxidative stress in human blood plasma. Therefore, the ability of chitosan to inhibit the generation of HSA oxidation by hydroxyl radicals was measured. The results show that HSA carbonyl groups are clearly formed, after subtraction of the readings given by the unoxidized HSA, and that the extent of carbonylation increases with the time of exposure to peroxyl radicals (Fig. 2). After 6- and 24-h incubation, the formation of the carbonyl moieties was significantly inhibited in the presence of low Mr chitosans (CT1–CT5), with an identical degree of protection provided by a chitosan concentration of 5 mg/mL. The presence of similar w/v concentrations of high Mr chitosans (CT6, CT7) had no inhibiting effect on carbonyl formation at any time of the incubation after a 6- and 24-h exposure. These results suggest that the antioxidant activity of low Mr chitosans was more pronounced than that of high Mr

chitosans. Furthermore, inhibiting the formation and reactions of HSA radicals may prevent much of the biological damage associated with a range of diseases caused or aggravated by oxidative stress.

The ability of the several Mr chitosans to scavenge radicals other than the peroxyl radical was tested by using stable N-centered DPPH and ABTS radicals. DPPH reduction by chitosan sulfates with different sulfur contents and molecular weights was reported in one study, while another indicated a relationship between molecular weight and radical-scavenging effectiveness, using various preparations of chitosans.[20,32] The relative abilities of low Mr chitosans to react with DPPH radicals is consistent with the results of experiments with peroxyl radicals (Fig. 3, Table 2). Furthermore, we were also able to estimate the relationships between antioxidant activity and the Mr of low Mr chitosans (Fig. 4, $p < 0.01$; $r^2 = 0.96$). These results suggest that the scavenging activities of low Mr chitosans for the DPPH radical were more pronounced than that for high Mr chitosans. These results may not only estimate the antioxidant capacities of several Mr chitosans, but also reflect pharmaceutical applications for chitosans. All of the potential scavengers also reacted with the $ABTS^{\cdot+}$ with widely different effectiveness, ranging from low to high Mr chitosans (Fig. 5). This is the first finding that unequivocally demonstrates the ability of chitosan to reduce the $ABTS^{\cdot+}$ radical. Figure 6 depicts the reducing power of several Mr chitosans, as evidenced by the use of the TPAC test. The reducing power of each Mr chitosan correlated well with decreasing Mr, and the reducing power of high Mr chitosans was not significantly obvious ($p < 0.01$; $r^2 = 0.96$; Fig. 7).

The large differences in the radical-scavenging effectiveness of the compounds used here show that the chemistry underlying the reactions is determined by the size of the molecule. In this study, scavenging activity of low Mr chitosans (CT1–CT5) for peroxy radicals was higher than that of high Mr chitosans. The scavenging activities of low Mr chitosan for the DPPH and $ABTS^{\cdot+}$ radicals were also more pronounced than that of high Mr chitosan. A more plausible mechanism for the antioxidant action of Mr chitosans in vivo is their ability to scavenge secondary peroxyl radicals. Peroxyl radicals are products of fast reactions between dioxygen and C-centered radicals generated by the primary ROS in cell components such as proteins, lipids, and DNA, and are believed to play a major role in the production of biological damage induced by the primary ROS.[33] In this study we tested the ability of chitosan to protect HSA from damage by peroxyl radicals that were generated directly by the decomposition of AAPH. Further, this result can be attributed to the effects of intra-molecular hydrogen bonding in the chitosan molecule. Chitosans show considerable hydrogen bonding on N_2–O_6 and O_3–O_5. High Mr chitosans have compact structures, thus making the overall effect of their intramolecular hydrogen bonds stronger. The strong effect of intramolecular hydrogen bonding decreases the reactivity of hydroxyl and amino groups. On the contrary, low molecular weight chitosan has a more compact structure, thus making the overall effect of intra-molecular hydrogen bonding less effective. At present, no information is available concerning the sites of attack by peroxyl or N-centered radicals on Mr chitosans. However, even without this knowledge, the results of this study suggest that soluble, bioavailable, non-toxic chitosans have the ability to protect living organisms from damage by biologically significant peroxyl radicals with an efficiency similar to that of ascorbate, an efficient antioxidant that is found in human blood serum and other tissues.[34,35] In the future, the mechanism controlling the above observations clearly requires additional study.

In recent studies, several Mr chitosans were used as dietary supplement, all over the world.[36,37] High Mr chitosans such as CT6, 7 having most of these properties would be expected in the absorption of certain lipids and bile acid. On the other hand, the major predicted properties of low Mr chitosans would be to absorb such substances, but also to confer some new properties, including antimicrobial, antitumor, immuno-stimulating, antidiabetic, and antioxidant effects. In fact, we also showed that the administration of low Mr chitosan (Mr = 20,000), as well as CT2, to human volunteers prevented HSA oxidation in vivo.[19] Therefore, the appropriate chitosan should be used and the molecular weight must be taken into consideration, when equivalent amounts of chitosan are used.

These results show that low Mr chitosans (CT1–CT5) have impressive antioxidant properties, especially antioxidant activity, the ability to scavenge hydroxyl radicals and to reduce cupric ions. In addition, high Mr chitosan was much less effective in terms of antioxidant properties. On the basis of the results obtained, low molecular weight chitosans (<30 kDa) with presumed antioxidant properties may be used as a source of antioxidants, as a possible food supplement or ingredient or in the pharmaceutical industry.

4. Experimental

4.1. Chemicals

Several molecular weight (Mr) chitosans (chitosan soluble (CT1), chitosan 5 (CT4), chitosan 10 (CT5), chitosan 100 (CT6), chitosan 50 (CT7), degree of deacetylation >90%), high grade fatty acid free human serum albumin, ascorbic acid (Vitamin C), and AAPH (2,2′-azobis (2-amidinopropane) hydrochloride were purchased from Wako (Tokyo, Japan). Other Mr chitosans (CT2, CT3, degree of deacetylation >90%) were obtained from Dainichiseika Color & Chemicals Mfg. Co., Ltd (Tokyo, Japan). The 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were supplied by Nacalai Tesque (Kyoto, Japan). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals used herein were also of the highest grade commercially available, and all solutions were prepared using deionized and distilled water.

4.2. Human plasma

Human plasma was obtained from healthy volunteers. All participants gave informed consent, consistent with NIH guidelines.

Table 1
Characterization of seven different molecular weight chitosans

Samples[a]	Mr[b] (kDa)
CT1	2.8
CT2	17.0
CT3	33.5
CT4	62.6
CT5	87.7
CT6	604
CT7	931

[a] All samples were obtained from Wako Co. Ltd and Dainichiseika Co. Ltd (Japan).
[b] Calculated from intrinsic viscosity.

Table 2
Scavenging of DPPH and $ABTS^{\cdot+}$ radicals

Antioxidant	DPPH IC_{50} (mg/mL)	$ABTS^{\cdot+}$ IC_{50}[a] (h)
CT1	0.87	0.21
CT2	3.28	0.59
CT3	4.68	2.76
CT4	8.15	6.85
CT5	9.34	10.5
CT6	>10	>24
CT7	>10	>24

[a] Relative radical trapping ability was calculated using 0.5 mM DPPH.

4.3. Determination of molecular weight (Mr) of chitosans

To measure the average viscosity Mr, intrinsic viscosity was measured with a viscometer, and the viscosity average Mr of chitosans was calculated using the Mark–Houwink–Sakurada equation with the reported Mark–Housink's constants (Table 1).[38]

4.4. Individual plasma carbonyl contents measurement

Human plasma, with or without several Mr chitosans, was oxidized by exposure to AAPH (20 mM) in 67 mM sodium phosphate buffer (pH 7.4, 37 °C), as described by Niki.[39] After incubation for 6 hr, the extent of oxidation of individual plasma proteins was determined by western blot analysis, as described by Shacter et al.[40] Plasma protein was diluted to 2 mg/mL of total plasma protein with phosphate-buffered saline (PBS) and derived with anti-2,4 dinitrophenylhydrazine (DNP) using an OxyBlot Kit (Serologicals Corporation, Norcross, GA, USA). Samples were diluted to 1 mg/mL of total protein by the addition of an equal volume of nonreducing sample buffer, and 15 μL samples was electrophoresed on duplicate SDS–PAGE gels (12.5%). Following electrotransfer to a PVDF membrane, one blot was stained for DNP using the OxyBlot Kit reagents. The second blot was stained with Coomassie brilliant blue G for proteins. The bands were visualized with chemiluminescent chemicals and captured on film at 10 min. Each western blot included samples from both HD patients and healthy controls. These data were recorded as DNP area/protein area, and are reported as densitometry units. The mean for each subject group was calculated from each blot.

4.5. Effect of oxidation on HSA in the presence and absence of chitosan

HSA (20 μM), with or without several Mr chitosans, was oxidized by exposure to AAPH (20 mM) in 67 mM sodium phosphate buffer (pH 7.4, 37 °C), as described by Niki.[39] After incubation for 0, 2, 6, and 24 h, the protein carbonyl content was determined using the method of Levine et al.[41] The carbonyl groups were derived with DNPH, producing dinitrophenylhydrazone products, which were quantified by spectrophotometric analysis using a molar absorption coefficient of $2.20 \times 10^4\ M^{-1}\ cm^{-1}$.

4.6. Scavenging activity of chitosan on DPPH and ABTS radicals

The radical scavenging activities of different concentrations of chitosan were tested in an ethanolic solution (10 ml of ethanol, 10 ml of 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 5.5), and 5 ml of 0.5 mM DPPH. Radical scavenging was estimated from the decrease in the absorbance of DPPH radicals at 517 nm.[42] ABTS was dissolved in water to produce a 7.0 mM stock solution and the radical cation ($ABTS^{\cdot+}$) was produced by reacting 2.0 mM of ABTS with 2.45 mM of potassium persulfate and allowing the mixture to stand in the dark at room temperature overnight. For measurements of the decrease in $ABTS^{\cdot+}$ radicals, an aliquot of the solution of Mr chitosan antioxidant was rapidly added to 75 μL of stock $ABTS^{\cdot+}$ solution and the mixture was diluted with water to a final volume of 2.0 mL. The reaction of any antioxidant present with the $ABTS^{\cdot+}$ was estimated from the decrease of its absorbance at 734 nm.[43]

4.7. Reducing power by using the TPAC test

The evaluation of antioxidant power in several Mr chitosans was determined using the 'TPAC' test (Cosmo Bio Co., Ltd, Tokyo, Japan). This assay evaluated Cu^+ levels derived by reduction of Cu^{2+} by the action of antioxidants present in the sample. The stable complex between Cu^+ and bathocuproine was assayed at 490 nm, with a sensitivity of 22 μmol L^{-1} of reducing power. The assay was found to be linear from 1 to 2000 μmol L^{-1} of uric acid ($r = 0.99$, $p < 0.01$). Both within-run and between-run assay variability, tested by repeatedly assaying five samples, was consistently lower than 5%.

4.8. Statistics

Statistical significance was evaluated by the 2-tailed paired Student's *t*-test for comparison between 2 mean values and by ANOVA followed by Newman–Keuls test for comparison among >2 mean values. For all analyses, values of $p < 0.05$ were regarded as statistically significant. Results are reported as the mean ± SEM.

References

1. Nagle, D. G.; Zhou, Y. D. *Curr. Pharm. Des.* **2006**, *12*, 2673–2678.
2. Cardenas, G.; Orlando, P.; Edelio, T. *Int. J. Biol. Macromol.* **2001**, *28*, 167–174.
3. Shahidi, F.; Abuzaytoun, R. *Adv. Food. Nutr. Res.* **2005**, *49*, 93–135.
4. Chandy, T.; Sharma, C. P. *Biomater. Artif. Cells Artif. Organs* **1990**, *18*, 1–24.
5. Khor, E.; Lim, L. Y. *Biomaterials* **2003**, *24*, 2339–2349.
6. Suzuki, K.; Okawa, Y.; Suzuki, S.; Suzuki, M. *Microbiol. Immunol.* **1987**, *31*, 375–379.
7. Seo, W. G.; Pae, H. O.; Kim, N. Y.; Oh, G. S.; Park, I. S.; Kim, Y. H.; Kim, Y. M.; Lee, Y. H.; Jun, C. D.; Chung, H. T. *Cancer Lett.* **2000**, *159*, 189–195.
8. Harish Prashanth, K. V.; Tharanathan, R. N. *Biochim. Biophys. Acta* **2005**, *1722*, 22–29.
9. Kim, S. K.; Rajapakse, N. *Carbohydr. Polym.* **2005**, *62*, 357–368.
10. Harish Prashanth, K. V.; Dharmesh, S. M.; Jagannatha Rao, K. S.; Tharanathan, R. N. *Carbohydr. Res.* **2007**, *342*, 190–195.
11. Rajapakse, N.; Kim, M. M.; Mendis, E.; Kim, S. K. *Bioorg. Med. Chem.* **2007**, *15*, 997–1003.
12. Yan, Y.; Wanshun, L.; Baoqin, H.; Changhong, W.; Chenwei, F.; Bing, L.; Liehuan, C. *Int. Immunopharmacol.* **2007**, *7*, 29–35.
13. Xue, C.; Yu, G.; Hirata, T.; Terao, J.; Lin, H. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 206–209.
14. Chiang, M. T.; Yao, H. T.; Chen, H. C. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 965–971.
15. Xie, W.; Xu, P.; Liu, Q. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1699–1701.
16. Anraku, M.; Kabashima, M.; Namura, H.; Maruyama, T.; Otagiri, M.; Gebicki, J. M.; Furutani, N.; Tomida, H. *Int. J. Biol. Macromol.* **2008**, *43*, 159–164.
17. Santhosh, S.; Sini, T. K.; Anandan, R.; Mathew, P. T. *Toxicology* **2006**, *219*, 53–59.
18. Yoon, H. J.; Moon, M. E.; Park, H. S.; Kim, H. W.; Im, S. Y.; Lee, J. H.; Kim, Y. H. *Food Chem. Toxicol.* **2008**, *46*, 710–716.
19. Anraku, M.; Fujii, T.; Furutani, N.; Kadowaki, D.; Maruyama, T.; Otagiri, M.; Gebicki, J. M.; Tomida, H. *Food Chem. Toxicol.* **2009**, *47*, 104–109.
20. Chae, S. Y.; Son, S.; Lee, M.; Jang, M. K.; Nah, J. W. *J. Controlled Release* **2005**, *109*, 330–344.
21. Ranaldi, G.; Marigliano, I.; Vespignani, I.; Perozzi, G.; Sambuy, Y. *J. Nutr. Biochem.* **2002**, *13*, 157–167.
22. Ormrod, D. J.; Holmes, C. C.; Miller, T. E. *Atherosclerosis* **1998**, *138*, 329–334.
23. Gallaher, C. M.; Munion, J.; Hesslink, R., Jr.; Wise, J.; Gallaher, D. D. *J. Nutr.* **2000**, *130*, 2753–2759.
24. Sugano, M.; Fujikawa, T.; Hiratsuji, Y.; Nakashima, K.; Fukuda, N.; Hesegawa, Y. *Am. J. Clin. Nutr.* **1980**, *33*, 787–793.
25. Xing, R.; Yu, H.; Liu, S.; Zhang, W.; Zhang, Q.; Li, Z., et al *Bioorg. Med. Chem.* **2005**, *13*, 1387–1392.
26. Pacifici, R. E.; Davies, K. J. *Methods Enzymol.* **1990**, *186*, 485–502.
27. Gebicki, J. M.; Collins, J.; Gay, C.; Duggan, S.; Gieseg, S. *Redox Rep.* **2000**, *5*, 55–56.
28. Anraku, M.; Kitamura, K.; Shinohara, A.; Adachi, M.; Suenaga, A.; Maruyama, T.; Miyanaka, K.; Miyoshi, T.; Shiraishi, N.; Nonoguchi, H.; Otagiri, M.; Tomita, K. *Kidney Int.* **2004**, *66*, 841–848.
29. Cakatay, U.; Kayali, R. *Clin. Biochem.* **2006**, *39*, 907–912.
30. Terawaki, H.; Yoshimura, K.; Hasegawa, T.; Matsuyama, Y.; Negawa, T.; Yamada, K.; Matsushima, M.; Nakayama, M.; Hosoya, T.; Era, S. *Kidney Int* **2004**, *66*, 1988–1993.
31. Mera, K.; Anraku, M.; Kitamura, K.; Nakajou, K.; Maruyama, T.; Otagiri, M. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 1322–1328.
32. Park, P. J.; Je, J. Y.; Kim, S. K. J. *Agric. Food Chem.* **2003**, *51*, 4624–4627.
33. Willson, R. Organic Peroxy Free Radicals as Ultimate Agents in Oxygen Toxicity. In *Oxidative Stress*; Sies, H., Ed.; Academic Press: London, 1985; p 41.
34. Frei, B.; Stocker, R.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9748–9752.
35. Buettner, G. R. *Arch. Biochem. Biophys.* **1993**, *300*, 535–543.
36. Gades, M. D.; Stern, J. S. *J. Am. Diet. Assoc.* **2005**, *105*, 72–77.
37. Kaats, G. R.; Michalek, J. E.; Preuss, H. G. *J. Am. Coll. Nutr.* **2006**, *25*, 389–394.

38. Knaul, J. Z.; Kasaai, M. R.; Bui, V. T.; Creber, K. A. M. *Can. J. Chem.* **1998**, *76*, 1699–1706.
39. Niki, E. *Methods Enzymol.* **1990**, *186*, 100–108.
40. Schacter, E. Y.; Williams, J. A.; Stadtman, E. R.; Levine, R. L. Determination of Carbonyl Groups on Oxidized Proteins. In *Free Radicals—A Practical Approach*; Punchard, N. A., Kelly, F. J., Eds.; IRL Press: Oxford, 1996; pp 159–170.
41. Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. *Methods Enzymol.* **1990**, 186, 464–478.
42. Kogure, K.; Goto, S.; Abe, K.; Ohiwa, C.; Akasu, M.; Terada, H. *Biochim. Biophys. Acta* **1990**, *1426*, 133–142.
43. Leelarungrayub, N.; Rattanapanone, V.; Chanarat, N.; Gebicki, J. M. *Nutrition* **2006**, *22*, 266–274.