

Evaluation and Comparison of the Antioxidative Potency of Various Carbohydrates Using Different Methods

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A detailed analysis of the antioxidative activity of 12 carbohydrates including chondroitin sulfate, fucoidan, agaro-oligosaccharide, 2-deoxy-*scyllo*-inosose (DOI), Gal β 1-4DOI, D-glucuronic acid, chitobiose, D-mannosamine, D-galactosamine, D-glucosamine, heparin, and colominic acid was performed using four established methods: 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, superoxide dismutase (SOD) activity assay, and the deoxyribose method. Ascorbic acid and/or catechin were used as positive standards. In the DPPH radical scavenging activity measurements, fucoidan, DOI, and Gal β 1-4DOI showed remarkable levels of activity, although at lower levels than ascorbic acid. The SOD assay revealed that DOI, Gal β 1-4DOI, and agaro-oligosaccharide had high antioxidant activity, with DOI and Gal β 1-4DOI notably showing almost half of the antioxidative potency of ascorbic acid. Using the deoxyribose method, chitobiose and heparin showed the highest hydroxyl radical scavenging activity, followed by chondroitin sulfate, colominic acid, Gal β 1-4DOI, and D-glucosamine. Given that 11 of the carbohydrates analyzed share a common structure, agaro-oligosaccharide being the exception, we propose from our current results that at least one amino, carboxyl, carbonyl, or sulfonyl group is required, but is not in itself sufficient, for carbohydrates to function as antioxidants.

KEYWORDS: Antioxidative activity; radical scavenging; DPPH; FRAP; SOD; deoxyribose assay; carbohydrate; fucoidan; chondroitin sulfate

INTRODUCTION

Antioxidative materials have recently attracted much attention because of their potential as scavengers of the reactive oxygen species which are known to cause damage to nucleic acids and membrane lipids (1). These damaging events can trigger a variety of diseases, most notably cancer when DNA is affected (2). In addition, the cumulative damage to membrane lipids by reactive oxygen species can cause accelerated aging. Many antioxidative materials have thus been identified for use as food additives or medical supplements as scavengers of reactive oxygen species that will thus negate their damaging effects. However, most of the reported antioxidant materials are water insoluble with the exception of ascorbic acid and several polyphenolic compounds. Water soluble antioxidants such as ascorbic acid are excreted in the urine after the trapping of oxygen radicals from water insoluble antioxidative materials such as vitamin E. Water soluble antioxidants thus play an important role in assisting their water insoluble counterparts in the removal of reactive oxygen species from the body.

Recently, several carbohydrates including fucoidan (3), agaro-oligosaccharides (4, 5), chondroitin sulfate (6), chitobiose (7), and D-glucosamine (8-10), have been reported to possess antioxidative properties. Chitobiose is often used to denote *N*-acetylglucosaminyl- β -(1,4)-*N*-acetylglucosamine, but we use this term herein to define D-glucosaminyl- β -(1,4)-D-glucosamine. Carbohydrate antioxidants are predicted to have significant utility as they are generally water soluble and have low toxicity. Moreover, most of these molecules have been safely consumed as constituents of food products for many years. In this context, much attention has recently been paid to the development of novel carbohydrate additives with potent antioxidative activity. However, most recent reports lack any data that compare the antioxidative potency of these compounds with that of established antioxidants such as ascorbic acid, catechin, or trolox. It is therefore unclear whether any of the carbohydrates so far tested are equivalent or even superior to known antioxidants. The purpose of our present study was to examine this issue by comparing the relative antioxidative potencies of various carbohydrates with well-known antioxidants.

Four generally established methods were employed for this purpose: the measurement of 2-diphenyl-1-picrylhydrazyl

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Figure 1. Enzymatic synthesis of Gal β 1-4DOI by transglycosylation using β -galactosidase from *Aspergillus oryzae*.

(DPPH) radical scavenging activity, which reflects the ability of a compound to scavenge general radical species (11); the ferric reducing antioxidant power (FRAP) assay, which indicates the potency of reduction of the Fe³⁺/tripyridyl-*s*triazine (TPTZ) complex to Fe²⁺ (12); the superoxide dismutase (SOD) assay, which measures the capacity to decrease the levels of superoxide radicals (13); and the deoxyribose assay, which is used to estimate hydroxyl radical scavenging activity (14). Another crucial aim of our present study was to assess the structure/activity relationship of the carbohydrates under analysis.

MATERIALS AND METHODS

Materials. Chitobiose was purchased from Seikagaku Biobusiness Co. (Tokyo, Japan). Colominic acid was obtained from Funakoshi Co. (Tokyo, Japan), and fucoidan derived from kelp was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Agarobiose and agaro-oligosaccharide were prepared as described previously (15). Most disaccharides used in this study were synthesized via a condensation (reverse hydrolysis) reaction using the corresponding glycosidase or through a glycosidase assisted regioselective transglycosylation reaction using the corresponding p-nitrophenyl glycoside as a donor. DOI and galactose was also combined by a transglycosylation reaction using β -galactosidase from Aspergillus oryzae or Bacillus circulans. (Figure 1) The structures of the compounds synthesized for this study were confirmed by NMR spectroscopy after HPLC purification. The more common carbohydrates such as D-glucose or N-acetyl-D-glucosamine that could be readily sourced commercially were used without further purification.

Measurement of NMR Spectroscopy. NMR spectra were recorded at 27 °C with a Bruker AVANCE DPX-250 spectrometer in D_2O .

Enzymatic Synthesis of Gal/β1-4DOI. 2-Deoxy-scyllo-inosose (DOI) was prepared from D-glucose as the starting material from cultures of metabolically engineered recombinant Escherichia coli (16). DOI (168 mg, 1 mmol) and p-nitrophenyl β -D-galactopyranoside (303 mg, 1 mmol) were dissolved in 0.1 M phosphate buffer (pH 6.0) containing 20% (v/v) dimethylformamide (DMF). β -Galactosidase from Aspergillus oryzae (3 U) was then added to the solution. The solution was then incubated at 37 °C, and the time course of the reaction was monitored by HPLC using an Asahipak NH2P-50 column (4.5 mm i.d. \times 25 cm, Shimadzu GLC Ltd., Tokyo, Japan) and RI monitor at 25 °C. Elution was with 80% (v/v) acetonitrile at 1 mL/min, and the reaction was stopped after 180 min by heating in a boiling water bath for 3 min. The reaction mixture was then applied to an activated carbon column (2.5 cm i.d. \times 48 cm) and eluted with a gradient of water (1 L) and 10% (v/v) ethanol (1 L). The eluate was collected in 10 mL fractions, each of which was examined by HPLC. Fractions containing Gal β 1-4DOI were pooled and concentrated, yielding 19.7 mg of this product.

¹³C NMR data for the transglycosylation product are listed in **Table 1**. As DOI exists in equilibrium between its keto and hydrate forms in aqueous solution, each carbon in a DOI residue exhibits two signals with different chemical shifts. Both of the C-4 carbon signals (74.7 ppm and 74.4 ppm) for DOI shifted to a lower field (84.4 ppm and 86.0 ppm) following conjugation with D-galactose. Hence, the structure of the product was concluded to be Galβ1-4DOI.

Table 1. ¹³C NMR Chemical Shifts Measured for the Gal-DOI Conjugate^a

	C-1	C-2	C-3	C-4	C-5	C-6
Gal DOI	103.3	71.2	72.5	68.6	75.2	61.0
hydrate form keto form	92.9 206.4	39.9 43.2	67.0 66.7	86.0 84.4	73.0 73.4	75.6 77.3

^a NMR analysis was performed for a D₂O solution of Gal-DOI conjugate using a Bruker AVANCE DPX-250 spectrometer.

Measurement of DPPH Radical Scavenging Activity. A previously described standard procedure was used for the measurement of DPPH radical scavenging activity (17). Briefly, 1 mL of DPPH (100 μ M) in ethanol and 1 mL of each carbohydrate sample (2 mg/ mL) in 100 mM Tris-HCl buffer (pH 7.4) were mixed to adjust the final sample concentration to 1 mg/mL. This reaction mixture was then incubated for 20 min in the dark at room temperature. The absorbance at 515 nm was measured against a blank control (100 mM Tris-HCl buffer instead of carbohydrate solution). Measurements were performed in triplicate over a 60 s period for each sample. The measurements of all the samples were not performed within one day, but it took several days. The DPPH radical scavenging activity, namely, the inhibitory ratio, was calculated using the following equation: scavenging activity (%) = $(1 - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank.

Measurement of the Concentration-Dependent Antioxidative Activity of Carbohydrates. (a) DPPH Radical Scavenging Activity. The radical scavenging activities of 12 carbohydrates at different concentrations (0.5–20 mM) were measured in triplicate using the DPPH procedure as described above. Ascorbic acid and catechin were used as the positive standard.

(b) FRAP Assay. The FRAP assay was performed as described by Benzie et al. (12). FRAP reagent (40 mM TPTZ and 20 mM ferric chloride in 0.3 M acetate buffer; pH 3.6) was freshly prepared on each occasion. Carbohydrates at concentrations of 1-20 mM (in a 30 μ L volume), 900 μ L of FRAP reagents, and 70 μ L of water were mixed and incubated at 37 °C for 4 min. Catechin was used as a positive standard. Complex formation was measured by the decrease in the absorption at 593 nm at room temperature.

(c) SOD Activity. SOD activity was measured using the SOD assay kit-WST (Dojin Chemicals Co., Kumamoto, Japan) according to the manufacturer's instructions. The method involved is based on previous studies of Ukeda et al. (18, 19). Briefly, the mixed solution of carbohydrate sample of various concentrations in water (20 μ L), WST working solution (200 μ L) containing 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium in 50 mM carbonate buffer (pH 10.2), and enzyme working solution (20 μ L) containing xanthine oxidase in the same buffer was incubated at 37 °C for 20 min, and the absorbance at 450 nm was measured. The SOD activity (percentage inhibition rate) was calculated as follows: inhibition rate (%) = (1 - $A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank (dilution buffer instead of carbohydrate solution).

(d) Deoxyribose Method. The deoxyribose assay for hydroxyl radical scavenging activity was performed using catechin as a positive standard. To a mixture of 2-deoxyribose (10 mM, 0.36 mL), Fe³⁺ chloride (10 mM, 0.01 mL), EDTA (1 mM, 0.1 mL), and H₂O₂ (10 mM, 0.1 mL) were added 1 mL of carbohydrate sample (1-20 mM) or 1 mL of mannitol (1.0 µM), 0.33 mL of 50 mM potassium phosphate buffer (pH 7.4), and 1 mL of ascorbic acid (1 mM). The mixture was then incubated for 1 h at 37 °C. A solution of thiobarbituric acid (TBA) in 50 mM NaOH (1 mL, 1% w/v) and trichloroacetic acid (TCA) in aqueous solution (1 mL, 2.8% w/v) was next added, and the mixture was heated for 20 min in a boiling water bath. The amount of chromogen produced was measured at 532 nm. The hydroxyl radical scavenging activity (percentage inhibition rate) was calculated as follows: scavenging activity (%) = $(1 - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank (10 mM potassium phosphate buffer instead of carbohydrate solution).

In all measurements obtained using methods (a)-(d) above, the experiments were performed in triplicate, and the averages of the obtained values were plotted against the carbohydrate concentration.

Table 2.	DPPH	Radical	Scavenging	Activity o	of Various	Carbohydrates ^a
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	carbohydrate	functional group	activity (%)
positive control	L-ascorbic acid catechin		$\begin{array}{c} 96.4 \pm 0.2 \\ 85.0 \pm 0.2 \end{array}$
monosaccharide	L-arabinose D-fructose D-galactose D-mannose D-glucosamine D-galactosamine D-mannosamine N-acetyl-D-glucosamine methyl-ac-D-glucopyranoside methyl-2-acetamide-2-deoxy- β -D-glucopyranoside	NH ₂ NH ₂ NH ₂ NHAC	$\begin{array}{c} 1.6 \pm 0.4 \\ 5.7 \pm 0.1 \\ 6.5 \pm 0.9 \\ 5.1 \pm 0.2 \\ 8.6 \pm 0.6 \\ 9.4 \pm 1.1 \\ 10.8 \pm 0.3 \\ 6.3 \pm 0.5 \\ 5.9 \pm 0.3 \\ 6.2 \pm 0.6 \end{array}$
	D-glucuronic acid D-galacturonic acid D-glucose-3-sulfate D-glucose-6-sulfate potassium D-gluconate D-glucono-1,5-lactone <i>N</i> -acetylneuraminic acid	-СООН -СООН -SO ₃ H -SO ₃ H -СООН	$\begin{array}{c} 11.0 \pm 0.3 \\ 5.2 \pm 0.1 \\ 5.9 \pm 0.7 \\ 5.7 \pm 0.5 \\ 5.3 \pm 0.2 \\ 6.2 \pm 0.4 \\ 2.0 \pm 0.2 \end{array}$
disaccharide	Gal β 1-3GlcNAc Gal α 1-6Glc Glc β 1-6Glc Gal β 1-6Gal Fuc α 1-3GlcNAc Man α 1-6Man Man α 1-2Man Gal β 1-3Gal β -OMe Gal β 1-4Gal β -OMe agarobiose chitobiose	-NHAC -NHAC	$5.4 \pm 0.3 \\ 4.4 \pm 0.4 \\ 3.3 \pm 0.2 \\ 3.4 \pm 0.2 \\ 6.4 \pm 0.4 \\ 2.1 \pm 0.2 \\ 3.4 \pm 0.5 \\ 2.6 \pm 0.3 \\ 2.2 \pm 0.1 \\ 29.7 \pm 0.8 \\ 9.6 \pm 0.8 \\ \end{array}$
oligosaccharide	Galβ1-6Glcα1-2Frc tri-N-acetyl chitotriose agaro-oligosaccharide	-NHAc	$\begin{array}{c} 2.6 \pm 0.2 \\ 3.7 \pm 0.1 \\ 8.7 \pm 0.3 \end{array}$
polysaccharide	chondroitin sulfate ^b chondroitin sulfate ^c fucoidan heparin sodium salt colominic acid sodium salt	SO₃H SO₃H SO₃H SO₃H COOH	$\begin{array}{c} 35.6 \pm 1.6 \\ 4.3 \pm 0.7 \\ 30.1 \pm 0.9 \\ 3.7 \pm 1.1 \\ 2.1 \pm 0.7 \end{array}$
miscellaneous	2-deoxy- <i>scyllo</i> -inosose (DOI) Galβ1-4DOI	-C=0 -C=0	$\begin{array}{c} 24.8\pm1.1\\ 28.4\pm3.2\end{array}$

^a Activity was measured in triplicate at a final concentration of 1 mg/mL. Experiments were performed in triplicate, and values are means and SE. ^b Chondroitin sulfate purchased from Extrasynthese Co. (Genay, France), but the origin was not disclosed from the manufacturer. ^c Chondroitin sulfate from shark cartilage purchased from Sigma-Aldrich Japan (Tokyo, Japan).

RESULTS AND DISCUSSION

Evaluation of the Antioxidative Activity of Selected Carbohydrates Including Mono-, Di-, Oligo-, and Polysaccharides. To our knowledge, this is the first report that compares the antioxidative activities of a wide range of carbohydrates. As a first screening, DPPH radical scavenging activity was measured for 67 different carbohydrate molecules including mono-, di-, oligo-, and polysaccharides of various structures, most of which were synthesized in our laboratory. In Table 2, only the representative results are presented. In addition to the carbohydrates listed in Table 2, the following carbohydrates were also examined: N-acetyl-D-galactosamine, N-acetyl-Dmannosamine, methyl- β -D-glucopyranoside, methyl- α - and - β -D-galactopyranoside, methyl- α - and - β -D-mannopyranoside, methyl- α - and - β -D-xylopyranoside, methyl- α - and - β -D-rhamnopyranoside, Gal α - and Gal β -1,3Glc, Gal β 1-6Glc, Gal α 1-3Gal (20), Gal α 1-6Gal (20), Gal β 1-4GlcNAc, Gal β 1-6GlcNAc, $Gal\beta 1$ -2Man (21), $Gal\beta 1$ -3Man (21), $Gal\beta 1$ -4Man (21), $Gal\beta 1$ - 6Man (21), Fucα1-6GlcNAc (22), Galβ1-6Galβ-OMe, maltitol, isomaltitol, di-*N*-acetylchitobiose, chitotriose, NeuAcα2-3Galβ1-4GlcNAc, NeuAcα2-6Galβ1-4GlcNAc, Galβ1-4GlcNAcβ1-2Man (23), agaro-oligosaccharide (a mixture of disaccharide– hexasaccharide) (15), Galβ1-6Glcα1-2Frc.

In these measurements, the final carbohydrate concentration in each case was adjusted to 1 mg/mL. Most carbohydrates in Table 2 showed 2-7% scavenging activity. D-Glucosamine, which showed $8.6 \pm 0.6\%$ scavenging activity in our measurement, was reported to be active (8). For the next stage of our evaluation, a DPPH radical scavenging activity of 10% was tentatively used as a benchmark for selection. According to this criterion, chondroitin sulfate, fucoidan, agarobiose, 2-deoxyscyllo-inosose (DOI), and Gal β 1-4DOI were judged to have high antioxidative potency, since they showed nearly 30% scavenging activity. In contrast, D-galactosamine, D-mannosamine, Dglucosamine, chitobiose, and D-glucuronic acid were deemed to have only mild antioxidative potency as they showed about 10% activity. Chondroitin sulfate purchased from Extrasynthese Co. (Genay, France) showed high antioxidative activity (chondroitin sulfate^b in **Table 2**), but the origin was not disclosed from the manufacturer. In contrast, chondroitin sulfate from shark cartilage (Sigma-Aldrich Japan, Tokyo, Japan, chondroitin sulfate^c in **Table 2**) showed much lower antioxidative activity. The results observed here are quite surprising. We purified the chondroitin sulfate from Extrasynthese Co., by HPLC connected with anion exchange column (Mono Q) followed by gel chromatography column (Superdex Peptide). The ¹H NMR spectrum of the purified chondroitin sulfate from Extrasynthese Co. was quite analogous to that of chondroitin sulfate from shark cartilage (data not shown). We are now analyzing the structure and origin of the chondroitin sulfate from Extrasynthese Co. Therefore the purified chondroitin sulfate from Extrasynthese Co. was used hereafter, though the origin was not clear. Although agarobiose showed almost equal activity with fucoidan or chondroitin sulfate, it was not easy to prepare large quantities of pure agarobiose, therefore agaro-oligosaccharide was used in our subsequent experiments.

Heparin and colominic acid were included in our panel as with the exception of agarobiose, all of the sugars showing antioxidative activity contain at least one amino, carboxyl, carbonyl, or sulfonyl group. From this point of view, we speculated that, based upon their structure, heparin and colominic acid would likely show potent antioxidative activity. Although D-glucose-3-sulfate and D-glucose-6-sulfate may fit the above criteria, these monosaccharides were not included, since oligo- or polysaccharide seems to have higher antioxidative activity than monosaccharide due to the cluster effect. Hence, a total of 12 carbohydrates were selected for further detailed analysis of their antioxidative properties using a DPPH radical scavenging activity assay, FRAP assay, superoxide radical scavenging activity assay, and hydroxyl radical scavenging activity assay (deoxyribose method). For each of these assays, ascorbic acid and/or catechin was used as the positive standard.

DPPH Radical Scavenging Activity. The DPPH radical scavenging activity of each compound in our carbohydrate panel at various concentrations was measured. The most striking antioxidant activities measured using this method are shown in **Figure 2**. In **Table 2**, the concentration of all samples was normalized to 1 mg/mL. However, for the comparison of the structure–activity relationship including polysaccharides, we assessed the antioxidative activity levels based on the concentration of mono- or disaccharide repeats. Therefore, the concentration



Figure 2. DPPH radical scavenging activity of various carbohydrates. Experiments were performed in triplicate, and values shown are the means \pm SE.

tion (mM) of each polysaccharide solution was roughly calculated using the molecular weights of the repeat mono- or disaccharide units. Similar to the findings of our preliminary analysis, chondroitin sulfate and Gal β 1-4DOI showed high activity. The activity of DOI was almost equivalent to that of fucoidan, and Gal β 1-4DOI showed higher activity than DOI, possibly due to the lower stability of DOI during the assay. A 1 mg/mL concentration of fucoidan and chondroitin sulfate corresponds to approximately 4.1 mM and 2.2 mM, respectively. As shown in **Figure 2**, the DPPH radical scavenging activity of fucoidan and chondroitin sulfate of the above concentration was measured as 24% and 28%, respectively. These values are just a little lower than the values in **Table 2** (30.1% and 35.6%, respectively). These differences can be estimated within experimental error.

The DPPH radical scavenging activity of each of the carbohydrates we tested was lower than that of either ascorbic acid or catechin. Agaro-oligosaccharide, chondroitin sulfate, and chitobiose showed only mild activity in the DPPH radical scavenging assay. The activities of the remaining carbohydrates in this assay are not shown as they were very marginal in each case.

FRAP Assay. DOI and Gal β 1-4DOI showed relatively high reducing power compared with other carbohydrates. However, none of the other carbohydrates showed appreciable antioxidative activity by FRAP assay when compared with catechin (data not shown). Ruperez et al. reported that a sulfated polysaccharide fraction extracted from the edible marine brown seaweed Fucus vesiculosus showed high potential to be antioxidant by the FRAP assay (24). The fraction they used for the FRAP assay consisted of fucoidan with an average molecular weight of 1.6×10^6 Da. Our result on fucoidan was not so high as catechin. We used a commercial fucoidan of the same species (Fucus vesiculosus) without further purification; therefore the molecular weight and composition were unknown. The difference between both fucoidan samples is not clear, but the number or position of sulfate group may play an important role in antioxidant activity measured by the FRAP method.

SOD Assay. Many of the carbohydrates examined showed high antioxidative activity when analyzed by the SOD assay, but the activities of agaro-oligosaccharide, DOI, and Gal β 1-4DOI were the most notable in this regard. Only the results for these three carbohydrates are therefore presented in **Figure 3**. As shown from these measurements, the SOD activities of DOI and Gal β 1-4DOI were found to be high at 20% and 22%, respectively, of the superoxide scavenging activity at 0.1 mM. Ascorbic acid shows 39% activity at the same concentration. Moreover, DOI and Gal β 1-4DOI showed about 90% scavenging activity at 0.5 mM, while ascorbic acid showed the same activity



Figure 3. SOD activities of various carbohydrates. Experiments were performed in triplicate, and values shown are the means \pm SE.

at 0.2 mM. The SOD scavenging potencies of DOI and Gal β 1-4DOI were supposed as about half that of ascorbic acid.

As can be seen from the results shown in **Figure 3**, agarooligosaccharide also showed remarkably high activity in the SOD assay, although this was lower than either DOI or $Gal\beta$ 1-4DOI.

In the previous report of Xing et al. (8), D-glucosamine exhibited 80% scavenging activity at a concentration of 0.8 mg/ mL, which corresponds to 4.5 mM under our experimental conditions. However, D-glucosamine at a 5 mM concentration showed only about 10% superoxide scavenging activity. Chen et al. (7) have also reported previously that D-glucosamine does not show scavenging activity toward superoxide radicals, though chitobiose displayed such activity. These discrepancies are difficult to explain at present as Xing et al. did not include a positive control in their analysis.

Deoxyribose Method. The hydroxyl radical scavenging activity results for our carbohydrate panel using the deoxyribose method are shown in **Figure 4**. Chitobiose and Gal β 1-4DOI at 1 mM concentration showed high hydroxyl radical scavenging activity whereas chondroitin sulfate, D-glucosamine, and colominic acid showed medium levels of activity. Although heparin and colominic acid did not show high DPPH scavenging activity in our preliminary analysis (Table 1), these two polysaccharides showed this potency when assayed using the deoxyribose method. Both methods measure hydroxyl radical scavenging activity. A plausible explanation for this finding is that these carbohydrates efficiently target different radicals, i.e. the DPPH method measures the ability to scavenge a phenolic radical whereas the deoxyribose method measures the scavenging potency toward aliphatic hydroxyl radicals. The mechanisms by which phenolic and hydroxyl radicals are neutralized may also be different. Our contention that polysaccharides containing





Figure 4. Scavenging activity of various carbohydrates toward hydroxy radicals measured by the deoxyribose method. Experiments were performed in triplicate, and values shown are the means \pm SE.

carboxyl or sulfonyl group may have superoxide and hydroxyl radical scavenging activities was thus supported by these data.

Although the experimental system used herein is different from that employed in the previous study of Chen et al. (7), our findings for chitobiose are in accordance with the data from this previous study in that this compound showed higher hydroxyl radical scavenging activity than D-glucosamine. In the report of Xing et al. (8), the hydroxyl radical scavenging activity of a 3.2 mg/mL D -glucosamine solution, which corresponds to an 18 mM concentration, was 55%. The hydroxyl radical scavenging activity of chitobiose at an 18 mM concentration was approximately 43% in our analysis (**Figure 4**). The difference in this case may fall within the experimental error.

The antioxidative activity of DOI could not be measured using the deoxyribose method because the color of the solution became dark brown soon after the start of the assay. The procedure for the deoxyribose method includes the heating of the sample solution at 95 °C for 20 min, and DOI is likely to be heat labile. In contrast, Gal β 1-4DOI showed higher activity in this assay, possibly due to its greater stability at high temperature, but these levels were still below those of catechin.

Comparison of the Antioxidant Activities of Selected Carbohydrates. The results shown in Table 2 confirmed that the activity of fucoidan was greater than that of the other carbohydrates based on the measurements from the concentration dependent DPPH method (Figure 2). However the absolute antioxidant activity of fucoidan was found to be much lower than that of ascorbic acid or catechin. The DPPH radical scavenging activities of DOI and Gal β 1-4DOI were almost equivalent to that of fucoidan as demonstrated in Figure 2, indicating that the absolute antioxidant activities of these carbohydrates were also far lower than that of ascorbic acid. In contrast, agaro-oligosaccharide as well as DOI and Gal β 1-4DOI showed high SOD activity, which was at almost half the level of ascorbic acid. Ascorbic acid is known to be heat labile, and this has hindered its wide use as a food additive. To overcome this problem, derivatives of this compound have been developed to increase its stability at higher temperatures in vitro (25). In contrast to ascorbic acid, Gal β 1-4DOI and agaro-oligosaccharide are heat stable. Agaro-oligosaccharide in particular is quite safe for human consumption as it is derived from agarose, a component of seaweed. Hence, agaro-oligosaccharide may be a viable alternative to ascorbic acid as an additive in food products that require heating during their manufacture. Another possible candidate is Gal β 1-4DOI since it is also heat stable and possesses both high SOD and hydroxyl radical scavenging activity. High hydroxyl radical scavenging activity was also observed for chitobiose and heparin sodium salt, both of which could be potentially used as food additives since these sugars have prebiotic characteristics and can increase the levels of intestinal microflora.

In summary, each of the aforementioned carbohydrates has potential for use as a food additive to serve a different purpose. Although none of the carbohydrates examined in the present study exceeded the antioxidative activity of catechin and ascorbic acid, carbohydrates are of great potential as antioxidant constituents of food since they are safe for use as an additive and stable during heating. For example, to control for superoxide anions, agaro-oligosaccharide or Gal β 1-4DOI could be used, whereas to diminish hydroxyl radicals, heparin sodium salt or chitobiose could be employed.

Relationships between the Structure and Antioxidative Activities of Carbohydrates. This is the first report to deal with the relationship between carbohydrate structure and antioxidative activity. It was of considerable interest to evaluate the functional groups in carbohydrates that showed antioxidative activity. Normal hexoses and their glycosides do not show any scavenging activity, though hexoses have an aldehyde group. We reveal from our present experiments that, with the exception of agaro-oligosaccharide, all of the tested carbohydrates that showed antioxidative activity possessed either an amino, carboxyl, carbonyl, or sulfonyl group.

As shown in **Table 2**, chondroitin sulfates of different origins (chondroitin sulfate^{*b*} and chondroitin sulfate^{*c*}) showed large difference in antioxidative activity. To make the matter worse, the origin of the sample from Extrasynthese Co. was not clear. The ¹H NMR spectra of both samples were very close to each other, meaning that a small difference in structure must have caused the difference of the antioxidative activity.

The fact that D-glucosamine, D-galactosamine, and D-mannosamine all showed activity suggests that the orientation of the hydroxyl groups is not an important determinant of these properties, but the amino group adjacent to the aldehyde may be an important common partial structure in the case of these three sugars. In contrast, D-glucuronic acid and D-galacturonic acid showed quite different antioxidant properties, though the structural difference between these two sugars is only in terms of the orientation of the hydroxyl group at the 4-position. Potassium D-gluconate, N-acetylneuraminic acid, D-glucose-3sulfate, and D-glucose-6-sulfate did not show antioxidant activity, although they possessed carboxyl or sulfonyl groups. This indicated that possession of these functional groups is required but not sufficient to confer antioxidative activity, and that the position or number of those groups may thus be the determining factor. It is noteworthy also in this regard that agarooligosaccharide has no such functional groups. It may be the case therefore that the unstable five membered bicyclic structure

of agaro-oligosaccharide may be an origin of the radical scavenging activity.

The purpose of our present study was to elucidate the functional groups necessary on carbohydrates to confer antioxidative properties. We were not able to precisely determine this in our present analyses, but our data do show that at least one amino, carboxyl, carbonyl, or sulfonyl group is required, but is not in itself sufficient, for carbohydrates to function as antioxidants.

ABBREVIATIONS USED

ASA, ascorbic acid; DMF, dimethylformamide; DOI, 2-deoxyscyllo-inosose; DPPH, 2-diphenyl-1-picrylhydrazyl; FRAP, ferric reduction antioxidant power; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TPTZ, tripyridyl-s-triazine.

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