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Effect of carboxymethylation on antioxidant properties and radical degradation of mannans and glucans

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ABSTRACT

Carboxymethyl derivatives (CM-derivatives) of α,β-mannans from yeasts, *Saccharomyces cerevisiae* β-glucan and dextran (α-glucan) were found to possess strong antioxidant activities against reactive hydroxyl radicals (OH•) compared to underivatized polysaccharides. When CM-derivatives having similar DS (0.41–0.45) were compared, the antioxidant activity decreased in order CM-mannan > CM-β-glucan > CM-dextran. Moreover, the antioxidant activities against OH• increased with increasing degree of substitution (DS) of polysaccharides. The CM-mannan and CM-dextran with the highest DS (0.73 and 1.1, respectively) were the strongest antioxidants and their degradation by OH• decreased with increased carboxymethylation. The scavenging abilities of CM-polysaccharides against stable DPPH radical (DPPH•) were lower than those of original underivatized ones. Also this scavenging property against DPPH• was lower compared to antioxidant effect against OH•.

1. Introduction

Polysaccharides, commonly found in microorganisms, plants and animals, play an important role as free radical scavengers and protect the living organisms against oxidative damage (Wang et al., 2013). The antioxidant activities of glucans prepared from various sources considerably differ. For example, the structurally characterized water-soluble β-(1,3-1,6)-D-glucan from edible mushroom *Entoloma lividoalbum* is relatively strong antioxidant (Prasenjit et al., 2014). Similarly, the β-(1,3-1,4)-D-glucan from barley exhibited strong antioxidant activity against OH• (Kofuji et al., 2012). Microbial β-glucans such as laminarin (β-(1,3-1,6)-D-glucan), lichenan (β-(1,3-1,4)-D-glucan) and curdlan β-(1,3)-D-glucan were shown to be weak antioxidants (Machová & Bystrický, 2013). α-Glucans such as pullulan (α-(1,4-1,6)-D-glucan) and dextrin (α-(1,4-1,6)-D-glucan) did not neutralize OH• produced by Fenton reagent (Kofuji et al., 2012). Similarly, yeast mannans (α-(1,6)-linked mannose backbone branching with α-(1,2-1,3)- and β-(1,2)-linked oligomers) from different species exhibited weak antioxidant effect against OH• (Machová & Bystrický, 2013).

Polysaccharides like cellulose and chitin isolated from the natural sources are water-insoluble. The introduction of functional groups (carboxymethyl, sulfate and phosphate) makes them more soluble in aqueous systems and alters their biological effectiveness. Up to date, there is quite a lot of information concerning the antioxidant abilities of carboxymethyl polysaccharides (CM-polysaccharides). The considerable antioxidant activities on hydroxyl radicals were determined for CM-pachymaran (β-(1,3-1,6)-D-glucan from *Poria cocos*) (Li, Gao, Cao, Cao, & Zhang, 2014) and CM-polysaccharide from *Ganoderma lucidum* (Xu et al., 2009). CM-β-glucan prepared from the cell wall β-glucan of *Saccharomyces cerevisiae* was described as a good antioxidant against OH• (Machová & Bystrický, 2013), it also inhibited lipid peroxidation in liposomes (Babincová, Machová, & Kogan, 1999). Carboxymethylation of chitosan improved its water solubility and consequently antioxidant properties (Upadhyaya, Singh, Agarwal, & Tewari, 2013; Zhao, Huang, Hu, Mao, & Mei, 2011). The other derivatives of *S. cerevisiae* β-glucan, glucan phosphate and sulfated glucan, were found to exhibit strong antioxidant activities (Tsiapali et al., 2001). Two low molecular weight polysaccharides, agar with sulfate groups, and chitosan with amino groups inhibited hydrogen peroxide induced oxidative stress in skin fibroblasts (Chen, Hsu, Tsai, Chen, & Drummen, 2013). CM-polysaccharides have potential for use as natural antioxidants in various applications. For example, CM-chitosan could be used as antioxidant and preservative coating of peach fruit (Elbarbary & Mostafa, 2014) in food industry. Moreover, its ability to bind bile acids may be used in the pharmaceutical industry (Zhao et al., 2011). CM-cellulose together with starch and alginate was employed for preparation of edible film which protected bamboo shoots against free radicals and bacteria (Badwaik, Borah, & Deka, 2014).

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The aim here was to find out if the antioxidant properties of CM-mannans and glucans could be attributed to their structures and if the presence of carboxymethyl groups generally increases antioxidant effectiveness of polysaccharides. Our concern was also the consequence of carboxymethyl derivatization on radical degradation of polysaccharides.

2. Material and methods

2.1. Polysaccharides and their CM-derivatives

The yeast strains *Candida albicans* serotype A (CCY 29-3-32), *Candida dubliniensis* (CCY 29-177-1), *Candida tropicalis* (CCY 29-7-6), and *S. cerevisiae* (CCY 9-84-1) were from the Culture Collection of Yeasts (Institute of Chemistry, Bratislava, Slovakia). Mannans were isolated from the yeast cell wall as mannan-copper complexes (Jones & Stoodley, 1965). All mannans from above mentioned *Candida* species consist of α-(1,6)-linked mannose backbone branching with α-(1,2-1,3)- and β-(1,2)-linked side chains of different length. Mannan from *S. cerevisiae* consists of α-(1,6)-linked mannose backbone branching only with α-(1,2-1,3)-linked oligomers. Dextran from *Leuconostoc* spp. (Mr ~ 70,000) characterized as a branched glucan composed of linear α-(1,6)-linked glucose units and α-(1,3)-link initiated branches was purchased from Sigma Aldrich (Bratislava, Slovakia). β-Glucan was prepared from the cell wall of *S. cerevisiae* (Machová, Kogan, Alföldi, Šoltés, & Šandula, 1995). It is a branched β-(1,3)-glucan containing β-(1,6)-glucosidic interchain linkages. CM-mannans, CM-β-glucans and CM-dextrans were prepared and characterized (Machová, Bystrický, Malovíková, & Bystrický, 2014).

2.2. Determination of antioxidant activity

2.2.1. Hydroxyl radical antioxidant assay

Antioxidant activity ($AO_{OH^\bullet}$) of polysaccharides against $OH^\bullet$ was measured by salicylate test (Smirnoff & Cumbes, 1989). The reaction mixture contained 1.5 mM $FeSO_4$, 6 mM H_2O_2, 20 mM sodium salicylate and 1.6 mg mL^{-1} of polysaccharides. Ascorbic acid was used as a positive control. After incubation at 37 °C for 1 h, absorbance of hydroxylated salicylate complex was measured at 562 nm (UVmini-1240, Shimadzu, Japan). The percentage of antioxidant activity of polysaccharides was calculated as

$$AO_{OH^\bullet} = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100\%$$

where A_0 was the absorbance of the control without the polysaccharide, A_1 was the absorbance in the presence of the polysaccharide, and A_2 was the absorbance without sodium salicylate. The data were expressed as means ± SD ($n = 3$).

2.2.2. DPPH radical-scavenging assay

Antioxidant properties of polysaccharides were tested spectrophotometrically using 2,2-diphenyl-2-picrylhydrazyl radical – $DPPH^\bullet$ (Capek, Machová, & Turjan, 2009). 1 mL of 0.069 mM $DPPH^\bullet$ was dissolved in methanol and added to 1 mL of polysaccharide (1.0 mg mL^{-1}). The reaction mixture was incubated at ambient temperature for 1 h. The absorbance of reduced DPPH was measured at 517 nm (UVmini-1240, Shimadzu, Japan). The percentage of antioxidant activity ($AO_{DPPH^\bullet}$) of a polysaccharide was calculated as

$$AO_{DPPH^\bullet} = \left[\frac{A_0 - A_{polysaccharide}}{A_0}\right] \times 100\%$$

where A_0 was the absorbance of free $DPPH^\bullet$, $A_{polysaccharide}$ was the absorbance of $DPPH^\bullet$ treated with solution of a polysaccharide for 1 h. Ascorbic acid was used as a positive control. The data were expressed as means ± SD ($n = 3$).

2.3. Fe^{2+}-chelating activity assay

Chelating activity of polysaccharides was determined with ferrous ions (Dinis, Madeira, & Almeida, 1994). Briefly, 0.05 ml of 2 mM $FeCl_2$ was added to 0.4 mL of polysaccharide solution at the concentration of 4 mg mL^{-1}. Reaction was initiated by the addition 0.2 mL of 5 mM ferrozine [sodium 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonate] and 3.35 mL of ethanol. The absorbance was measured at 562 nm after 10 min incubation. The metal chelating efficiency was determined by comparing with the chelating activity of ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2). The Fe^{2+} chelating activities were calculated as

$$\text{Chelating activity} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100\%$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the polysaccharide or EDTA. The data were expressed as means ± SD ($n = 3$).

2.4. Degradation by $OH^\bullet$

The original *C. albicans* mannan and CM-mannans with various degrees of substitution (0.30, 0.41 and 0.73) were dissolved in water at 2.5 mg mL^{-1} and exposed to $OH^\bullet$ (Fenton reagent: 1.5 mM $FeSO_4$ + 6 mM H_2O_2) for 30 min, 1.5 h and 3.5 h at ambient temperature. The same procedure was used for dextran and CM-dextrans (DS 0.33, 0.60 and 1.1). The degradation was measured by high performance liquid chromatography (HPLC) analysis using two HEMA-BIO 100 + 300 and 300 + 1000 columns (8 mm × 250 mm) connected in series. The mobile phase used was 0.1 M $NaNO_3$. The carbohydrate content in the eluent was monitored by differential refractometer (Shodex, Japan). A set of dextrans (American Polymer Standard Corporation) was used for the calibration of the HPLC system.

3. Results and discussion

3.1. Antioxidant activities of CM-derivatives against $OH^\bullet$

The antioxidant activities of CM-derivatives were determined by salicylate assay and compared with original (underivatized) polysaccharides. The principle of the salicylate assay is competitive capture of $OH^\bullet$ by both the salicylate and the polysaccharide. The hydroxylation of salicylate by $OH^\bullet$ is accompanied by change of colour. Reactive hydroxyl radicals are generated by the reaction of H_2O_2 with Fe^{2+} (Fenton-type reaction). All CM-derivatives of yeast mannans, β-glucan and dextran were much stronger antioxidants than their respective original underivatized polysaccharides. When the antioxidant activities of CM-derivatives having similar DS (0.41–0.45) were compared, the highest AO against $OH^\bullet$ (49.8%) was found with CM-mannan from *C. dubliniensis* (DS 0.45), the lowest value (34.1%) was found in the case of CM-β-glucan from *S. cerevisiae* (DS 0.43) as is shown in Table 1. Although CM-dextran was prepared using identical amount of sodium monochloroacetate compared to CM-mannans and CM-β-glucan, it was substituted to higher DS (0.60) but exhibited lower AO (46.5%). It is evident that the antioxidant activities of original mannans (ranging from 12.5% – *C. tropicalis* mannan to 10.5% – *Candida glabrata* mannan) and dextran (7.2%) were significantly lower than those of CM-derivatives. Unfortunately it was impossible to compare antioxidant activities of original β-glucan from *S. cerevisiae* and its derivatized form (CM-β-glucan) due to water insolubility of original β-glucan. Hence we

Table 1
Radical quenching and chelating activities of mannans and glucans and their carboxymethyl derivatives.

Yeast	Samples	DS	$AO_{OH^{\bullet}}$ (%)	$AO_{DPPH^{\bullet}}$ (%)	Chelating activity (%)
C. albicans ser A	Mannan		11.2 ± 0.9	4.1 ± 0.8	12.9 ± 0.9
	CM-mannan	0.41	45.1 ± 2.1	2.9 ± 0.6	15.7 ± 1.2
C. tropicalis	Mannan		12.5 ± 2.3	5.1 ± 0.9	10.7 ± 1.8
	CM-mannan	0.44	46.3 ± 1.9	3.7 ± 0.8	14.9 ± 1.9
C. dubliniensis	Mannan		10.8 ± 0.7	3.9 ± 0.7	11.4 ± 1.1
	CM-mannan	0.45	49.8 ± 1.2	3.0 ± 0.6	13.0 ± 1.6
C. glabrata	Mannan		10.5 ± 1.1	5.9 ± 0.8	11.6 ± 1.4
	CM-mannan	0.45	40.8 ± 0.9	4.1 ± 0.5	16.1 ± 2.0
S. cerevisiae	Mannan		11.8 ± 1.1	5.0 ± 0.6	10.9 ± 1.7
	CM-mannan	0.43	45.1 ± 1.4	4.1 ± 0.4	14.5 ± 1.5
S. cerevisiae	CM-glucan	0.43	34.1 ± 1.2	6.6 ± 0.7	11.2 ± 0.7
	Dextran		7.2 ± 0.7	4.2 ± 0.7	11.9 ± 1.2
	CM-dextran	0.60	46.5 ± 2.5	2.9 ± 0.5	13.4 ± 1.6
	Ascorbic acid		100	96.1	
	EDTA				100

can conclude that the presence of carboxymethyl groups on mannan, glucan and dextran noticeably increased the elimination of reactive hydroxyl radicals generated by Fenton reagent.

Our results concerning the antioxidant activities against $OH^{\bullet}$ of original polysaccharides are in agreement with previously published results–mannans from various yeast species, as well as some glucans (dextran, pullulan, laminarin, lichenan, curdlan), exhibited very low antioxidant activity against $OH^{\bullet}$ (Machová & Bystrický, 2013).

3.2. Influence of degree of substitution on antioxidant activities of CM-derivatives

CM-polysaccharides with various degrees of carboxymethylation prepared from *C. albicans* mannan, *S. cerevisiae* β-glucan, and dextran were tested as antioxidants against $OH^{\bullet}$. It has been shown that AO of CM-polysaccharides depend on the extent of carboxymethylation. Consequently AO increased with increasing DS (Fig. 1). However, the 50% antioxidant activity of CM-derivatives was achieved with different polysaccharides at various degrees of substitution: CM-mannan (DS 0.47), CM-β-glucan (DS 0.56) and CM-dextran (DS 0.66). Thus, the antioxidant activity decreased in order CM-mannan > CM-β-glucan > CM-dextran.

Entirely different results of antioxidant activity against $OH^{\bullet}$ were obtained for carboxymethyl derivatives of chitosans with various DS (Zhao et al., 2011). The authors supposed that the antioxidant activity against $OH^{\bullet}$ of polysaccharides was directly related to the number of active hydroxyl groups in molecules. As the DS increased, the number of hydroxyl groups capable to eliminate $OH^{\bullet}$ decreased and thus the scavenging ability of CM-chitosans with a higher DS was lower than those of CM-chitosans with lower DS.

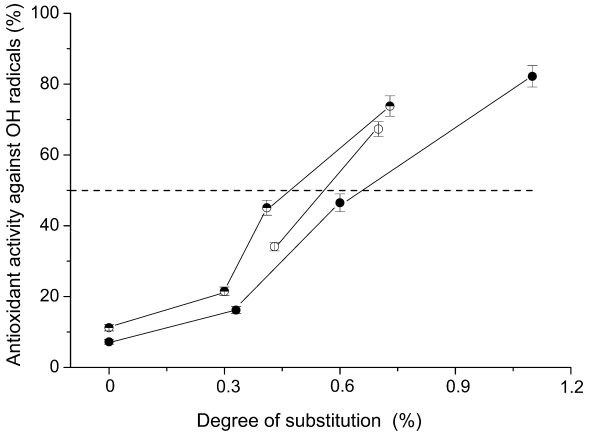

Fig. 1. Correlation of antioxidant capacities of CM-mannan from *C. albicans*, CM-β-glucan from *S. cerevisiae* and CM-dextran versus their degrees of substitution.

3.3. Chelating activity

Since Fenton-type reaction requires Fe^{2+} ions for generation of $OH^{\bullet}$ from hydrogen peroxide, the large chelation of Fe^{2+} ions by CM-polysaccharide could negatively influence the production of $OH^{\bullet}$. All carboxymetylated polysaccharides (CM-mannans, CM-β-glucan and CM-dextran) exhibited higher Fe^{2+}-chelating activity than the original polysaccharides (Table 1). The chelation range was from 11.2% (CM-β-glucan of *S. cerevisiae*, DS 0.43) to 16.1% (CM-mannan of *C. glabrata*, DS 0.45). It appears that neither polysaccharides nor their CM-derivatives noticeably influenced the generation of $OH^{\bullet}$ from H_2O_2 by Fe^{2+}.

3.4. Radical scavenging activity of CM-derivatives against $DPPH^{\bullet}$

This assay is routinely used for monitoring of the radical scavenging activity of various compounds. Reduction of stable free radical of DPPH with an antioxidant is accompanied by colour change resulting from reduction of $DPPH^{\bullet}$ to $DPPH_2$. All CM-mannans exhibited lower scavenging activities (2.9–4.1%) against $DPPH^{\bullet}$ compared to their underivatized counterparts (3.9–5.9%) as shown in Table 1. The scavenging activities of CM-derivatives against $DPPH^{\bullet}$ were considerably lower compared to antioxidant activity against $OH^{\bullet}$.

3.5. Degradation of polysaccharides by $OH^{\bullet}$

The question whether the oxidation of polysaccharides was accompanied by their degradation was addressed by acquisition of their HPLC profiles before and after addition of $OH^{\bullet}$. The influence of carboxymethylation on degradation of CM-polysaccharides was studied and results were compared with underivatized polysaccharides. The course of underivatized mannan and dextran degradation by $OH^{\bullet}$ was monitored during 3.5 h and the considerable radical degradation of both polysaccharides was evident (Fig. 2). In the case of mannan and longer radical reaction time, we noticed decreased polydispersity of the sample. The retention times of original CM-polysaccharides and their degradation products were significantly influenced by the degree of carboxymethylation (DS). With increasing DS, the peaks were shifted to the lower retention times. The degradation of CM-mannans (DS 0.3; 0.41 and 0.73) as well as CM-dextrans (DS 0.33; 0.6; and 1.1) by $OH^{\bullet}$ decreased with increasing DS of CM-derivatives. Although CM-mannan and CM-dextran with the highest DS (0.73 and 1.1, respectively) exhibited the highest

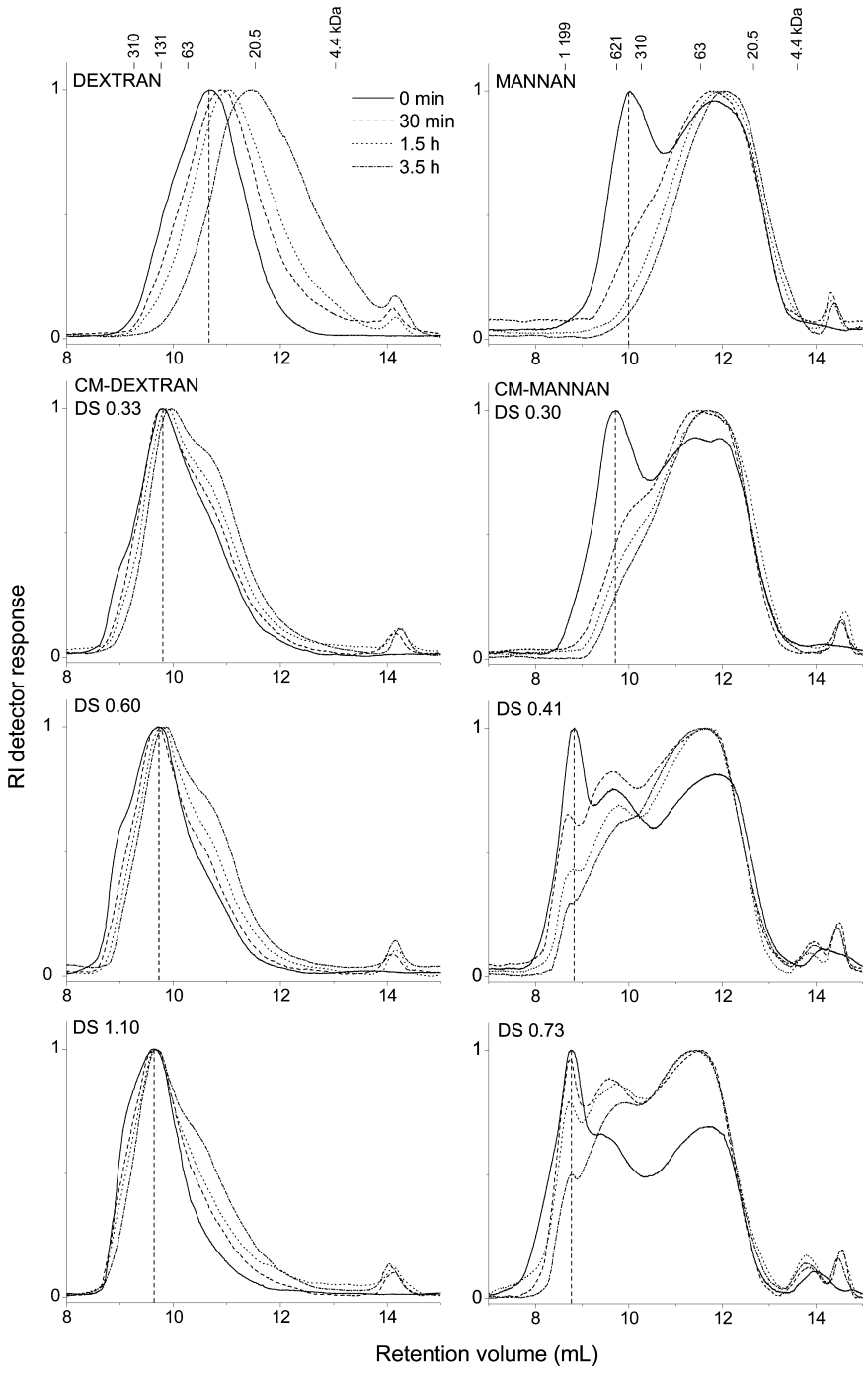

Fig. 2. HPLC degradation profiles of underivatized *C. albicans* mannan and dextran and their CM-derivatives with various degrees of substitution. (Molecular mass of dextran standards (Mp) are indicated on top; Vertical dashed lines represent position of maxima of original polysaccharide.)

antioxidant activities, they considerably resisted radical degradation (Table 1, Fig. 2).

3.6. Mechanism of radical reactions

The mechanism of $OH^{\bullet}$ elimination by a polysaccharide is so-called hydrogen atom transfer (HAT) reaction. It has been well studied and reported (Blanksby & Ellison, 2003). Published bond energies as well as calculated enthalpy and Gibbs free energy (Hernandez-Marin & Martínez, 2012) revealed that removal of the proton by $OH^{\bullet}$ occurs preferentially on carbon-bonded H atoms on the pyranose ring (rather than from hydroxyl groups). Model studies suggest that proton removal from carbons C1, C4 and C5 leads to hydrolysis of the glycosidic bonds (Schuchmann & von Sonntag, 1978) and consequently to degradation of polysaccharides. Situation evidently changes after introduction of the carboxymethyl groups. Our observation, as mentioned in the previous discussion, was that antioxidant effectiveness is improved by carboxymethyl derivatization. The consequence of this is that the accompanied degradation of selected carboxymethylated polysaccharide chains is then less pronounced than with original polysaccharides. That amounts to the fact that reactive hydroxyl radicals are preferentially eliminated by the reaction with carboxymethyl moieties, rather than removal of pyranose

directly carbon-bonded-hydrogens. One can speculate that carboxymethyl groups bring additional methylene hydrogens and they are removed much easier by reactive hydroxyl radicals. That is supported by an ESR study of irradiated carboxymethyl cellulose where the most probable radical sites were located on the carbon atom of the carboxymethyl groups (Saiki et al., 2011).

Similarly in our DPPH test we measured the scavenging effectiveness of stable free radicals by CM-derivatized mannans and glucans and compared them to underivatized counterparts. It turned out that original, underivatized mannans and glucans had higher AO's than CM-derivatized counterparts. This is similar to previous observations with carboxymethyl chitosan, where the scavenging effect of DPPH• decreased with the increasing degree of carboxymethylation (Sun, Yao, Zhou, & Mao, 2008). The explanation for this is based on very different mechanism. The scavenging of DPPH• is dependent on hydrogen-donating ability of the hydroxyl groups rather than pyranose directly carbon-bonded-hydrogens as is the case with OH•. Specifically, OH group substitution by CM's causes less hydroxyl hydrogens to be present on the pyranose ring that could react with DPPH•. This is the current explanation of low antioxidant scavenging effectiveness of CM-polysaccharides in DPPH test.

However, the results show generally very effective enhancement of antioxidant effectiveness on reactive OH• by carboxymethylation of polysaccharides.

4. Conclusion

From our results it is evident that carboxymethylation expressly increased antioxidant effect of original polysaccharides, mannan and dextran, by enhanced elimination of reactive hydroxyl radicals. The CM-mannan and CM-dextran with the highest degree of substitution were the strongest antioxidants. Moreover the degradation of CM-mannans and CM-dextrans by hydroxyl radicals decreased with increasing degree of substitution of CM-derivatives. Although CM-mannan and CM-dextran with the highest DS exhibited the highest antioxidant activities, they considerably resisted hydroxyl radical degradation.

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