Synthesis of Water-soluble Oxidized Chitosan Derivatives and Their Biological Activity

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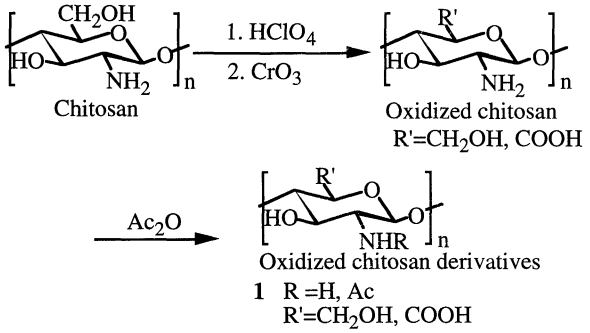
(Received August 3, 1999; CL-990682)

Oxidized chitosan derivatives were prepared by CrO_3 oxidation of chitosan followed by *N*-acetylation, and their effect on canine polymorphonuclear cells was evaluated. The water-solubility of oxidized chitosan derivatives (DS: 0.10-0.28) in the range of pH 1-13 was controlled by the degree of *N*-acetylation (DA), and the oxidized chitosan derivatives of DA 76 and 83% were soluble in water at the neutral pH range. Effect of oxidized chitosan derivatives on canine polymorphonuclear cells (PMNs) was estimated by the reported chemiluminescence (CL) technique, and the peak count of CL induced by zymosan after the treatment of PMNs with oxidized chitosan derivatives was lower than that induced by only zymosan.

Chitin, a polysaccharide made up of β-(1 → 4)-linked *N*-acetylglucosamine units, is widely distributed in nature as a component of bacterial cell walls and exoskeletons of crustaceans and insects. Chitosan is the ideally fully *N*-deacetylated product of chitin. In recent years, both chitin and chitosan have received growing attention as biologically active substances. For example, we have reported that chitin and chitosan showed excellent effects on wound healing in many small or large animal practices.[1-3]



Scheme 1.

As chitin and chitosan are not soluble in neutral water, there have been many attempts to improve their water-solubility by the transformation to oxidized derivatives.[4-6] However, has been unclear the relationship between water-solubility and degree of *N*-acetylation (DA) of oxidized chitosan derivatives which were selectively oxidized at the C-6 positions. Herein we report the effect of DA on the water-solubility of the regioselectively oxidized chitosan derivatives (Scheme 1).

According to the CrO_3 oxidation procedure,[5] chitosan (1.0 g, 6.2 mmol as hexosamine residue; degree of *N*-deacetylation: 97%; supplied by KOYO chemical Co. Ltd.) was first transformed into chitosan acetate which was mixed with acetic acid (200 ml) and then with 60 wt% aqueous perchloric acid (2.4 ml, 14 mmol). To the suspension of chitosan perchlorate thus prepared was added a mixture of chromium trioxide (1.5 g, 15 mmol), water (1.2 ml), and acetic acid (12 ml). The mixture was stirred for 1.0 h at 25 °C, and the excess oxidant was decomposed by the addition of methanol (6 ml). The precipitate formed was filtered, washed with methanol, and dissolved in 4 wt% aqueous sodium hydroxide solution (50 ml). This solution was dialyzed against deionized water and concentrated to give oxidized chitosan[5] (0.85 g, degree of oxidation (DS)[7] 0.10, Entry 1 in Table 1), whose ^{13}C NMR spectra showed a typical COOH signal at 177 ppm. There were not ^{13}C NMR signals corresponding to the ketone or aldehyde groups. Next, oxidized chitosan (100 mg) was dissolved aqueous NaOH (1 mol dm^{-3} 25 ml) and then *N*-acetylated with Ac_2O (0.5 and 1.0 equivalent (Entry 2 and 3 in Table 1, respectively) relative to the amino group in oxidized chitosan). Oxidized chitosan derivative of 83% DA (Entry 4 in Table 1) was obtained by the repeated *N*-acetylation of oxidized chitosan derivative of Entry 3. Degree of substitution of oxidized chitosan **1** was estimated by the ^1H NMR analysis.

The water-solubility of oxidized chitosan derivatives (DS: 0.10-0.28) in the range of pH 1-13 was controlled by the degree of *N*-acetylation. The oxidized chitosans of lower DA values (Entries 1 and 2 in Table 1) have large amount of the amino group and were insoluble in the pH 3 to 8 region. When DA values increased from 38% to 76% (Entries 2 and 3 in Table 1), the ratio of the amino group to the carboxy group decreased and the oxidized chitosan derivative became soluble in the neutral pH range. Finally, the further *N*-acetylated oxidized chitosan derivative which has 17% of the amino group (Entry 4 in Table 1), was water soluble in the all pH range tested. These phenomena mean that *N*-acetylation inhibited the aggregation caused by the interaction between the amino group and the carboxy group on the polymer chains.

As to exemplify biological activity of oxidized chitosan derivatives as a biomedical material, their effect on canine polymorphonuclear cells (PMNs) was estimated by the reported chemiluminescence (CL) technique.[8,9]

Although the peak count of CL induced by zymosan after the treatment of PMNs with carboxymethylchitosan derivatives

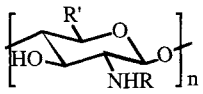
Table 1. Water solubility of oxidized chitosan derivatives [a]

Entry	Oxidized chitosan derivative				Soluble pH range
	Yield/%	DA[e]/%	DS[e]	$\overline{Mw}$[b]	
1	88[c]	8	0.10	64,000	1~3 and 8.0~13
2	83[c]	38	0.28	49,000	1~3 and 8.0~13
3	96[c]	76	0.26	64,000	1~3 and 5.0~13
4	80[d]	83	0.26	67,000	1~13

[a]Sample (10 mg/ml) was dissolved in aq HCl (1 mol dm^{-3}), and then pH of the mixture was adjusted from 1 to 13 by addition of aq NaOH (1 mol dm^{-3}). [b]Determined by GPC. [c]Yield from the starting chitosan. [d]Yield from oxidized chitosan derivative (DA: 76%). [e]Determined by ^1H NMR analysis.

(**2**) was higher than that induced by only zymosan (Entries 1 and 2 in Table 2), the peak count of CL induced by zymosan after the treatment of PMNs with oxidized chitosan derivatives **1** was lower than that induced by only zymosan (Entries 3 and 4 in Table 2). When the sample concentration increased from 5 mg/ml to 10 mg/ml, this tendency was clearly emphasized. The cytotoxicity of **1** was examined by the dye-exclusion test (trypan blue staining) and the cytotoxicity was not observed. These phenomena suggest that inflammatory cells such as PMNs are activated by **2,** but are deactivated by **1**.

Because the $\overline{Mw}$, DS, and DA values of chitosan derivatives don't have any corelationship with the relative intensity shown in Table 2, the different effect on the CL response might be attributed to the different way of carboxylation (carboxymethylation: Entries 1 and 2; oxidation of the C-6 position: Entries 3 and 4). Therefore, these results suggest that newly synthesized chitosan derivatives **1** are expected as promising biomaterials which don't induce inflammatory reaction *in vivo*.



Carboxymethylchitosan derivatives

2 R =H, Ac
R'=CH_2OH, CH_2OCH_2COOH

Table 2. Effect of carboxy and carboxymethyl groups of chitosan derivatives on canine PMNs (whole Blood) chemiluminescence response[a]

Entry		Sample			Relative intensity[b]
		DS	DA/%	$\overline{Mw}$	
1	**2**	0.96[c]	41	83,000	139 ±19 (861 ± 19)[e]
2	**2**	0.36[c]	69	49,000	101 ± 4 (199 ± 4)[e]
3	**1**	0.26[d]	76	64,000	85 ± 7 (62 ± 11)[e]
4	**1**	0.26[d]	83	67,000	83 ± 4 (72 ± 5)[e]

[a]Zymosan (10 mg/ml): 50 µl; sample (5 mg/ml): 50 µl; luminol (0.07 mg/ml): 20 µl; canine whole blood: 100 µl. [b]Relative intensity (%)={(peak count/1000 cells) of (sample + zymosan)}/{(peak count/1000 cells) of zymosan} x 100. [c]Determined by elemental analysis and ^{1}H NMR analysis. [d]Determined by ^{1}H NMR analysis. [e]Sample (10 mg/ml): 50 µl.

This work was partially supported by the Ministry of Education, Science, Sports and Culture of Japan (Grant-in-Aid for Scientific Research on Priority Areas (A) No. 09240103).

References and Notes

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10 Each sample in Table 2 showed a single peak in the GPC analysis ($\overline{Mw}$ / $\overline{Mn}$ = 1.3-2.3).