Synthesis of a New Chitin Derivative, (1-Carboxyethyl)chitosan

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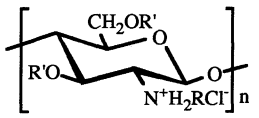
A new chitin derivative, (1-carboxyethyl)chitosan, was synthesized from chitin and chitosan by the reaction with 2-chloropropionic acid, and its activation effect on canine polymorphonuclear cells (PMNs) was evaluated. The degree of substitution was increased by the repetition of the reaction, but the simultaneous *N*-deacetylation proceeded. *N*-Selective 1-carboxyethylation of chitosan was achieved by reductive *N*-alkylation with 2-oxopropionic acid and $NaBH_4$.

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. Commonly, slightly *N*-deacetylated chitins have been also regarded as "chitin" (**1**). Chitosan and partially deacetylated chitins have various degree of *N*-deacetylation (DDA). In recent years, both chitin and chitosan have been received some attention as biologically active substances. For example, we have reported that a single or several treatments using chitin and chitosan agents gave good wound healing with formation of granulated tissue.[1-3]

Some of polysaccharides existed in the bacterial cell wall (e.g. BCG-CWS) show immunoadjuvant activities. *N*-Acetylmuramic acid, one of the major component of these polysaccharides, has an *N*-acetyl-D-glucosamine skeleton, whose 3-*O*-position is substituted with a 1-carboxyethyl group. In addition, various polysaccharides having the 1-carboxyethyl substituent have been isolated from bacterial cell walls.[4-6] However, there have been few reports on the synthesis of analogs of these polysaccharides from chitin. Herein we report the first synthesis of (1-carboxyethyl)-chitosan (**2**) as an analog of polymeric *N*-acetylmuramic acid and its effect on canine polymorphonuclear cells (PMNs).[7]

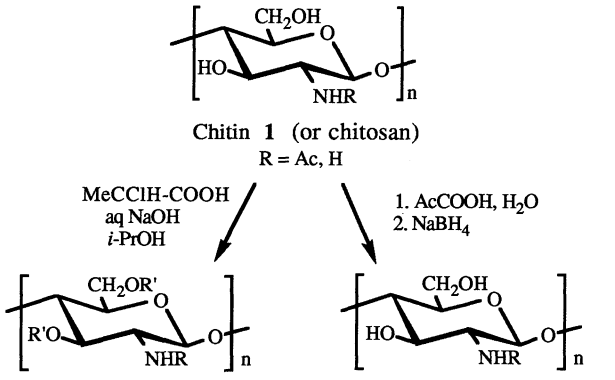
Although the reaction of chitin (**1**) with 2-chloropropionic acid was expected to introduce the 1-carboxyethyl group at 3-*O*- and 6-*O*-positions of chitin, not only 3-*O*- and 6-*O*-carboxyethylation but also *N*-carboxyethylation proceeded as shown in Scheme 1 because of simultaneous *N*-deacetylation under the following reaction conditions.

According to the carboxymethylation procedure,[8] partially deacetylated chitin (1.0 g, 5.1 mmol as hexosamine residue; DDA: 15%; powder from squid pen supplied by Sunfive Co. Ltd.) was suspended in a freshly prepared 45% aqueous sodium hydroxide solution (50 ml) containing sodium lauryl sulfate (0.1 g) at 4 °C. After stirring at 4 °C for 1 h, the slurry was kept in a freezer at -20 °C overnight. The frozen alkali-chitin was suspended in 2-propanol (125 ml) at room temperature, and 2-chloropropionic acid (33 g, 0.30 mol, 60 equiv relative to the hexosamine residue) was added with mechanical stirring. The mixture was stirred at room temperature for 72 h and then poured into methanol (600 ml). The precipitate formed was filtered, washed with methanol, and dissolved in water (50 ml). The pH was adjusted to 10 with concd HCl. This solution was dialyzed against deionized water and concentrated to give Na-type (1-carboxyethyl)-chitosan (0.9 g, 71% yield), which was dissolved in aq HCl (0.5 mol dm^{-3}, 34 ml). The resulting viscous solution was poured into acetone (450 ml). The precipitate formed was separated by centrifugation (3,000 rpm) and was dried to yield H-type (1-carboxyethyl)chitosan (**2**).



4 R = -CMeHCOOH, Ac, or H
R' = -CMeHCOOH or H

^1H NMR (D_2O) studies revealed DDA and degree of *N*- and *O*-carboxyethylation (designated as DS(N) and DS(O)). Under the acidic conditions (DCl in D_2O, pD 1), protonated form **4** would be a major component and two signals corresponding to methyl protons of the 1-carboxyethyl group were observed at 1.45 and 1.62 ppm. When pD was changed to 13 by adding NaOD in D_2O, the signal at 1.62 ppm shifted to 1.19 ppm. This result was explained by both deprotonation of the ammonium groups in **4** and transformation of the carboxyl group into sodium carboxylate, and this signal was assigned as methyl protons of the *N*-(1-carboxyethyl) group. In contrast, when the pD rose by adding NaOD in D_2O, the signal at 1.45 ppm was slightly shifted to 1.33 ppm owing to the formation of sodium carboxylate. Therefore, this signal would correspond to methyl protons of the *O*-(1-carboxyethyl) group. These findings enabled us to evaluate DS(N) to be 0.1



Scheme 1.

and DS(O) 0.8. DDA was estimated to be 60% by comparing the peak area of the signal of *N*-acetyl group (2.05 ppm) with that of C3-, C4-, C5-, and C6-protons of chitosan skeleton (3.4 ~ 4.1 ppm).

In order to improve the reaction conditions, the effect of reaction temperature and time was investigated, but the DS range of the products remained between 0 and 0.9. Therefore the effect of repetition of the reaction procedure on the DS value was examined to obtain highly 1-carboxyethylated products. As shown in Table 1, DS of the products increased with an increase in the repeating times of 1-carboxyethylation. Although yields became low, repetition of the procedure was effective to control the DS of 1-carboxyethyl group from 0.5 to 2.0. Table 1 showed also that DDA did increase with repeating the reaction.

Table 1. Effect of Repetition of 1-Carboxyethylation Reaction on the Degree of Substitution (DS)[a]

Time	Yield	DS			DDA
×48 h	%	N	O	Total	%
1	97	0.1	0.4	0.5	60
2	72	0.1	0.8	0.9	70
3	65	0.2	0.9	1.1	90
4	41	0.1	1.4	1.5	90
5	44	0.4	1.6	2.0	90

[a]The reaction was done as described in the text.

Under the alkaline conditions, however, it might be difficult to obtain the selectively *N*- or *O*-substituted products. In 1982, Muzzarelli and his co-workers reported selective *N*-carboxymethylation of chitosan with glyoxylic acid.[9] Modifying the Muzzarelli's method, we attempted regioselective 1-carboxyethylation of the amino group in chitosan (DDA: 85%) by the Schiff base formation with 2-oxopropionic acid followed by $NaBH_4$ reduction (Scheme 1). Because ^1H NMR analysis of the product in D_2O containing DCl (pD 1) showed only the signal assigned as methyl protons of the *N*-substituted 1-carboxyethyl group at 1.64 ppm besides the signals attributed to the protons of chitosan skeleton, selective 1-carboxyethylation of the amino group was confirmed. But the DS(N) (0.1) and yield (14%) of *N*-(1-carboxyethyl)chitosan (**3**) were low. Further studies on improvement of the DS and yield are in progress.

As shown in Table 2, 1-carboxyethylated products having low DS (Entries 1 and 2) were soluble in water in all pH range tested, while ones having both high DS and high DDA were insoluble around neutral pH (Entries 3 and 4). Generally, the solubility of protein in water is minimum around the isoelectric point, and precipitate is formed. Analogously precipitate formation of **2** might be caused around the pH of the isoelectric point by the electronic interaction between widely distributed amino and carboxyl groups in **2**.

As to exemplify biological activity of **2** as a biomedical material, its effect on canine PMNs was estimated by the reported chemiluminescence technique.[10] Among the samples tested, peak count of chemiluminescence induced by a mixture of zymosan[10] and (1-carboxyethyl)chitosan (**2**)

Table 2. Water Solubility of 1-Carboxyethyl Derivatives[a]

Entry	Sample			Soluble pH range
	DS	DDA/%	$\overline{Mn}$[b]	
1	0.3	30	27000	1 ~ 12
2	0.5	80	9000	1 ~ 12
3	1.0	80	36000	1 ~ 4.8 and 6.4 ~ 12
4	1.3	100	16000	1 ~ 3.5 and 8.0 ~ 12

[a]Samples (10 mg/ml) were dissolved in aq HCl (1 mol dm^{-3}), and then pH of the mixture was controlled from 1 to 12 by addition of aq NaOH (1 mol dm^{-3}). [b]Determined by GPC.

showing DS 0.5 and DDA 60% was several times greater than that induced by only zymosan. This result means that **2** might activate canine PMNs, which play an immunologically important role in medical matter such as wound healing and prevention of infection.

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