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TEMPO-mediated oxidation of chitin, regenerated chitin and *N*-acetylated chitosan

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Abstract

A commercial chitin, regenerated chitin prepared from chitin solutions in 6.8% NaOH and *N*-acetylated chitosans with degrees of *N*-acetylation (DNAc) of 77–93% were subjected to oxidization in water with NaClO and catalytic amounts of 2,2,6,6-tetramethylpiperidinyloxy radical (TEMPO) and NaBr. When regenerated chitin with DNAc of 87% and *N*-acetylated chitosan with DNAc of 93% were used as starting materials, water-soluble β-1,4-linked poly-*N*-acetylglucosaminuronic acid (chitouronic acid) Na salts with degrees of polymerization of ca. 300 were obtained quantitatively within 70 min. On the other hand, the original chitin and *N*-acetylated chitosan with DNAc of 77% did not give water-soluble products, owing to incomplete oxidation. The high crystallinity of the original chitin brought about low reactivity, and the high C2-amino group content of the *N*-acetylated chitosan with DNAc of 77% led to degradations rather than the selective oxidation at the C6 hydroxyls. The obtained chitouronic acid had low viscosities in water, and clear biodegradability by soil microorganisms.

Keywords: TEMPO; Chitin; Chitosan; Regenerated chitin; Oxidation; Biodegradability

1. Introduction

Catalytic oxidation of chitin using TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical), NaBr and NaClO in water at pH 10–11 gives water-soluble polyuronic acid having a chemical structure like hyaluronic acid (Bragd, van Bekkum, & Besemer, 2004; Isogai & Kato, 1998; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999). When commercial dried α-chitins originating from e.g. crab shell were used as starting materials, however, longer reaction times were required for complete oxidation at the C6 primary hydroxyl groups and correspondingly yields of the water-soluble oxidized products obtained were clearly lower than the theoretical value. Muzzarelli et al. used water-swollen chitins and regenerated chitins prepared from chitin solutions in 5% LiCl/*N,N*-dimethyacetamide (DMAc) as starting materials for the TEMPO-mediated oxidation, which gave water-soluble 6-oxychitins in yields of more than 90%.

Also in the case of cellulose, regenerated and mercerized celluloses can be quantitatively converted to water-soluble and almost pure β-1,4-linked polyglucuronic acid sodium salt, i.e. cellouronic acid, by the TEMPO-mediated oxidation (Isogai & Kato, 1998). On the other hand, only quite small amounts of carboxyl groups were introduced, when native celluloses were used as starting materials (Saito & Isogai, 2004). Probably differences in accessibility among cellulose or chitin samples bring about the different reactivity to the oxidation. Regenerated chitins prepared from LiCl/DMAc solutions have amorphous structures (Focher, Naggi, Torri, Cosani, & Terbojevich, 1992), and regenerated and mercerized celluloses also have lower crystallinities than those of native celluloses (Isogai, Usuda, Kato, Uryu, & Atalla, 1989).

Chitin is soluble in aqueous NaOH solutions by a specific procedure developed by Sannan, Kurita, and Iwakura

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(1975). Therefore, regenerated chitin can be prepared also from chitin/aqueous NaOH solutions, and may be suitable as starting materials for the TEMPO-mediated oxidation to prepare the corresponding polyuronic acid. However, the presence of C2-amino groups, which remain in chitin samples or are formed during the dissolution process in aqueous NaOH may cause depolymerization of the oxidized products, because amino groups are instable to the TEMPO-mediated oxidation. Cleary depolymerized products were obtained, when chitosan was subjected to the oxidation (Isogai & Kato, 1998). Thus, degrees of *N*-acetylation (DNAc) of chitins used as starting materials may influence molecular weights of the TEMPO-oxidized products. In fact, degrees of polymerization of the oxidized products prepared from chitins by Muzzarelli et al. (1999) were less than 50, whereas those of the original chitins must be more than 1000 (Hasegawa, Isogai, & Onabe, 1994). Depolymerization is likely to occur at the anhydro-glucosamine residue having the C2-amino group.

In this study, regenerated chitin prepared from chitin/aqueous NaOH solutions and *N*-acetylated chitosans having various degrees of *N*-acetylation were subjected to the TEMPO-mediated oxidation, and the oxidized products obtained were characterized in terms of viscosity in aqueous solutions and biodegradability by an embedding test in soil.

2. Materials and methods

2.1. Materials

Commercial chitin (Wako Pure Chemicals Co. Ltd, Japan) and chitosan (Dainichi Seika Co. Ltd, Japan) were used without any purification treatments. TEMPO, sodium bromide, about 11% sodium hypochlorite solution, and other chemicals and solvents were of laboratory grade (Wako Pure Chemicals Co. Ltd, Japan), and used without further purification.

2.2. Preparation of regenerated chitin

Dissolution of chitin in aqueous NaOH was carried out on the basis of the method reported by Sannan et al. (1975). Chitin (30 g) was soaked and swollen in a 45% NaOH solution (750 g) at room temperature for 2 h. Crashed ice made from distilled water (2520 g) was then added into the chitin suspension, which was cooled with an ice bath. The swollen chitin suspension became a clear solution, as the added ice melted. As soon as chitin was completely dissolved in 6.75% aqueous NaOH, the solution was neutralized with a diluted HCl, where regenerated chitin gel was formed as a precipitate. This regenerated chitin was repeatedly washed with water by centrifugation until no Cl^- ion was detected in the supernatant, and then freeze-dried.

2.3. N-acetylation of chitosan

N-acetylation of chitosan was carried out according to the method reported by Hirano, Ohe, and Ono (1976). Chitosan (30 g) was suspended in water (270 ml) for 1 h, and 20% aqueous acetic acid (300 ml) was added to the suspension to dissolve chitosan. Methanol (1200 ml) was then added to the solution, and a small amount of insoluble chitosan residue (less than 0.1%) was removed from the solution by filtration. The solution was diluted to 1% chitosan with methanol. A desired amount of acetic anhydride (0.8, 0.9 or 1.5 mol to that of amino groups in chitosan) in 500 ml methanol was added to the chitosan solution. The chitosan solution became a gel, as acetic anhydride was added. After stirring for 18 h, the *N*-acetylated chitosan gel was repeatedly washed with water/acetone (1:7 by weight) and finally with water by filtration, and then freeze-dried. The degrees of *N*-acetylation (DNAc) of the products were in the range of 77–93%.

2.4. Oxidation procedure

Chitin, regenerated chitin or *N*-acetylated chitosan (30 g) was suspended in water (1500 ml), and this suspension was kept below 5 °C using an ice bath. TEMPO (0.46 g, 0.02 mol per anhydro-*N*-acetylglucosamine unit) and sodium bromide (6.08 g, 0.4 mol per anhydro-*N*-acetylglucosamine unit) dissolved in 100 ml water at 5 °C were then added to the suspension, whose pH was adjusted to 10.75 with 0.5 M NaOH. A sodium hypochlorite solution (270 g) was gradually added to the suspension for 30 min., and the pH was maintained at 10.75 by continuous addition of 0.5 M NaOH using a pH-stat (automatic titrater, Hiramuma Co. Ltd, Japan). The volume of the 0.5 M NaOH solution added was monitored. When the mol of NaOH added was twice as much as that of anhydro-*N*-acetylglucosamine unit of the chitin sample, ethanol (30 ml) was added to the solution to quench the oxidation. The oxidized product was precipitated by adding ethanol of three times as much as the volume of the solution, and repeatedly washed with water/acetone (1:7 by weight) by filtration until no Cl^- ion was detected in the filtrate. The wet oxidized product was then washed with acetone followed by drying in vacuo at 45 °C for 1 day.

2.5. Analyses

^{13}C NMR, H–H COSY and C–H COSY spectra of chitin and the oxidized products were collected on a JEOL JNM-LA400 using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid Na salt as an internal standard. Chitin (0.05 g) was suspended in a 40% NaOD solution (0.5 g), and this suspension was frozen. D_2O (1.5 ml) was added to the frozen chitin suspension. Defrosting at room temperature and freezing of the chitin suspension at −20 °C was repeated until a clear solution was obtained. The chitin/NaOD/D_2O solution was then subjected to ^{13}C NMR analysis. The oxidized products

soluble in water were, on the other hand, dissolved in D_2O for NMR measurements.

Degrees of *N*-acetylation (DNAc) of chitin, regenerated chitin, chitosan and *N*-acetylated chitosan samples were calculated from their nitrogen and carbon contents determined using an elementary analyzer (FLASH EA1112NSC; Thermo Finnigan Co., USA). Nitrogen/carbon ratios of the oxidized products were also obtained by elementary analysis. X-ray diffraction patterns were collected using a vertical powder diffractometer (Rigaku RU-300, Japan), where Ni filtered Cu K_α radiation ($\lambda = 0.154$ nm) was used. Crystallinity of chitin having the α-chitin structure was calculated from diffraction intensities at 14 and 24° of diffraction angles 2θ due to the base line and the 013 peak, respectively, on the basis of Segal's method (Segal, Creely, Martin, & Conrad, 1959). Molecular weights of oxidized products were determined by high performance size-exclusion chromatography (HPSEC) using 0.1 M NaCl as an eluent at a flow rate of 1.0 ml/min, where TSK gel columns of G6000 and 3000 PWXL were used. Weight and number average molecular weights (M_w and M_n, respectively) were calculated from the chromatograms using pullulan standards. The viscosities of aqueous solutions of the oxidized products were measured at 25 °C using a vibration-type viscometer (Viscomate Model VM-1G, Yamaichi Electronics Co., Ltd, Japan). Biodegradability of chitin, oxidized chitin and others were evaluated by determining CO_2 formed from the samples in a soil chamber containing microorganisms at 35 °C and at a carrier gas flow rate of 40 ml/min (Micro Oxidative Degradation Analyzer, Saita Tekkoujo, Japan).

3. Results and discussion

3.1. Regenerated chitin prepared from aqueous chitin/NaOH solution

Sannan, Kurita, and Iwakura (1976) reported that water-soluble chitins, which had degrees of *N*-acetylation (DNAc) of 45–52%, were prepared by dissolution of chitin in aqueous 10% NaOH followed by standing the solution at 25 °C for 48–77 h, where random deacetylation of chitin gradually proceeded. Thus, deacetylation to some extent is essentially inevitable for chitin during the dissolution process in aqueous NaOH. Fig. 1 shows DNAc of regenerated chitins prepared from chitin solutions (in 10 and 20% NaOH) or swollen chitin gels (in 5 and 45% NaOH) after standing the solutions or gels for 6 or 69 h at room temperature. DNAc decreased from 91 to 87–88% by the alkali treatment for 6 h, although significant decreases in DNAc were observed after standing of the chitin solutions or gels in 10–45% NaOH for 69 h. Because deacetylation proceeds homogeneously on chitin at 10 and 20% NaOH, the higher the NaOH concentration, the more the deacetylation occurs. On the other hand, because chitin is not soluble

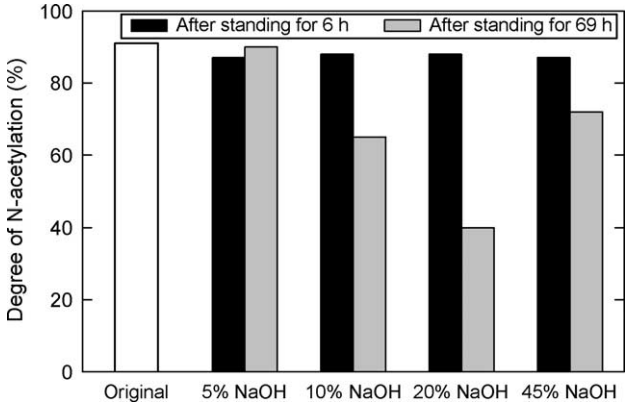

Fig. 1. Degree of *N*-acetylation of the original chitin and regenerated chitins prepared from chitin solutions or swollen chitin gels in 5–45% NaOH, which are stored for 6 or 69 h at room temperature.

in 45% NaOH but turns to only swollen gel, DNAc of the chitin treated with 45% NaOH for 69 h is higher than that for 20% NaOH. Anyway, these results show that deacetylation can be controlled to minimum levels by reduction of the laps of time from the complete dissolution of chitin in aqueous NaOH to the regeneration treatment, and that regenerated chitin prepared from aqueous NaOH solutions can be used as starting materials for the TEMPO-mediated oxidation to prepare the corresponding polyuronic acid.

3.2. TEMPO-mediated oxidation of chitin, regenerated chitin and N-acetylated chitosans

The values of DNAc and crystallinity of the original chitin, regenerated chitin and *N*-acetylated chitosans used as starting materials for the TEMPO-mediated oxidation are listed in Table 1. The original chitin required a long reaction time (400 min) for the stoichiometric consumption of 0.5 M NaOH corresponding to the oxidation of the C6 primary hydroxyls. At this NaOH consumption, chitin was not

Table 1
Effects of starting materials on TEMPO-mediated oxidation

	DNAc[a] (%)	Crystallinity (%)	Oxidation time[b] (min)	Yield (%)	Solubility[c]
Chitin	91	84	400	75	Insoluble
Regenerated chitin	87	71	70	95	Soluble
N-acetylated chitosan A	93	56	50	98	Soluble
N-acetylated chitosan B	81	56	90	95	Soluble
N-acetylated chitosan C	77	56	190	50	Soluble

[a] Degree of *N*-acetylation.
[b] Time required for the stoichiometiric consumption of 0.5 M NaOH corresponding to the oxidation of C6 primary hydroxyls of the starting material.
[c] Solubility of the starting material in the oxidation medium after the stoichiometric consumption of 0.5 M NaOH.

completely dissolved in the reaction medium, and the yield of the product was only 75%. Even after the added amount of 0.5 M NaOH was increased to 1.5 times as much as the stoichiometric value, complete dissolution of chitin in the reaction medium was not achieved. This low reactivity of the original chitin must be due to its high crystallinity and the corresponding low accessibility to the heterogeneous TEMPO-mediated oxidation.

On the other hand, regenerated chitin and *N*-acetylated chitosans with DNAc of more than 81% gave water-soluble oxidized products within 90 min, and the yields were more than 95%. *N*-acetylated chitosans with DNAc of more than 81% were oxidized in shorter time than for regenerated chitin, because the formers had lower crystallinities. Thus, the reactivity of chitin to the TEMPO-mediated oxidation is primarily governed by crystallinity of the starting chitin samples. Furthermore, in the case of regenerated chitin and *N*-acetylated chitosans with DNAc of more than 81%, the consumption of 0.5 M NaOH proceeded stoichiometrically, suggesting that the reagents were primarily consumed for oxidation of the C6 primary hydroxyls of chitin with less side reactions. When *N*-acetylated chitosan with DNAc of 77% was used, however, the yield of the oxidized product was only 50%. Probably, the reagents are consumed for depolymerization, which begins at the C2-amino groups. Moreover, even though complete dissolution of the starting material was achieved by the oxidation, the oxidized product after isolation and drying was no longer soluble in water; some side reactions must occur on the *N*-acetylated chitosan with lower DNAc during the oxidation.

Fig. 2 shows X-ray diffraction patterns of chitin, regenerated chitin and *N*-acetylated chitosan together with the oxidized products prepared thereof. Both the original and regenerated chitins have the clear crystal structure of α-chitin, although the latter has lower crystallinity. *N*-acetylated chitosans have diffraction patterns similar to that of α-chitin, although their crystallinities are lower and peaks are broader. The diffraction peak due to 020 appeared at $2\theta = 8°$ for *N*-acetylated chitosans, whereas that for the original and regenerated chitins at $2\theta = 9°$. Probably water molecules are incorporated in the 020 planes of the *N*-acetylated chitosan crystals. The oxidized product prepared from the original chitin still had clear diffraction peaks due to α-chitin, suggesting that the *N*-acetylglucosamine residues remained in the oxidized product because of incomplete oxidation. The oxidized products prepared from regenerated chitin and *N*-acetylated chitosans had amorphous structures.

^{13}C NMR spectra of the original chitin and the oxidized products prepared from *N*-acetylated chitosans with DNAc of 77 and 98% are shown in Fig. 3. When *N*-acetylated chitosan with DNAc of 77% was used as the starting material, the resonance signal due to C6 of chitin was still detected at 63.2 ppm in the NMR spectrum. On the other hand, regenerated chitin and *N*-acetylated chitosans with DNAc of more than 81% gave ^{13}C NMR spectra having

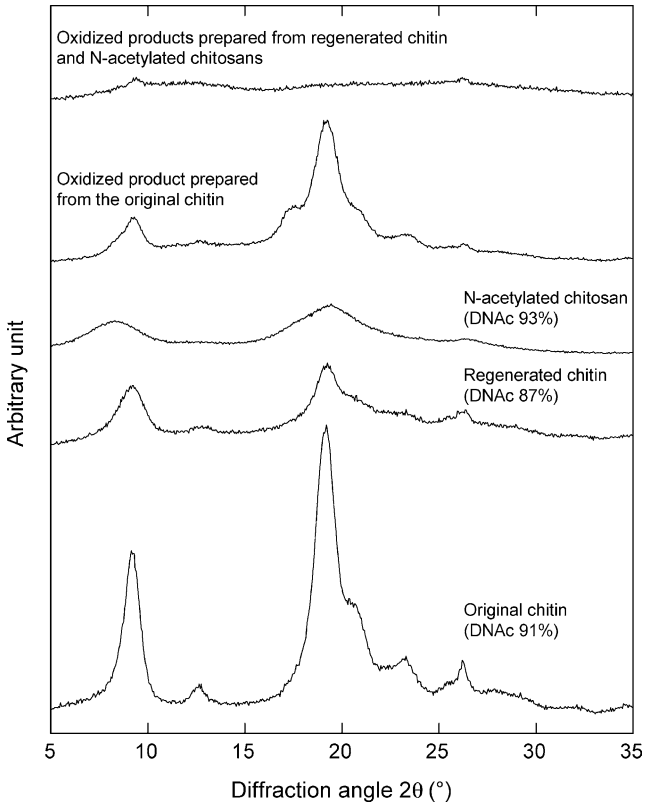

Fig. 2. X-ray diffraction patterns of the original chitin, regenerated chitin and *N*-acetylated chitosan, and TEMPO-oxidized products prepared thereof.

no signal due to the C6 primary hydroxyls by the TEMPO-mediated oxidation, and the C6 carboxyls appeared in turn at 176.9 ppm. These ^{13}C NMR results show that selective and complete oxidation of the C6 primary hydroxyls of chitin was, therefore, achieved by using regenerated chitin and *N*-acetylated chitosans with DNAc of more than 81% as starting materials. Thus, water-soluble β-1,4-linked poly-*N*-acetylglucosaminuronic acid, i.e. chitouronic acid Na salt, having mostly homogeneous chemical structure can be obtained in good yields. Signal assignment of C2, C3, C4 and C5 of chitouronic acid in Fig. 3 was carried out on the basis of H–H COSY and C–H COSY spectra of chitouronic acid.

Molecular weights of the oxidized products prepared from regenerated chitin and *N*-acetylated chitosans are summarized in Table 2. The oxidized product prepared from *N*-acetylated chitosan with DNAc of 77% had a clearly bimodal HPSEC pattern, and correspondingly had a large M_w/M_n value of 472. Furthermore, this product is not completely soluble in water. Probably some cross-linkages due to ionic bonds between carboxyl groups introduced and the residual C2-amino groups were formed among molecules in water, thus resulting in such anomalously large M_w with the bimodal M_w distribution.

On the other hand, the oxidized products prepared from regenerated chitin and *N*-acetylated chitosan with DNAc of

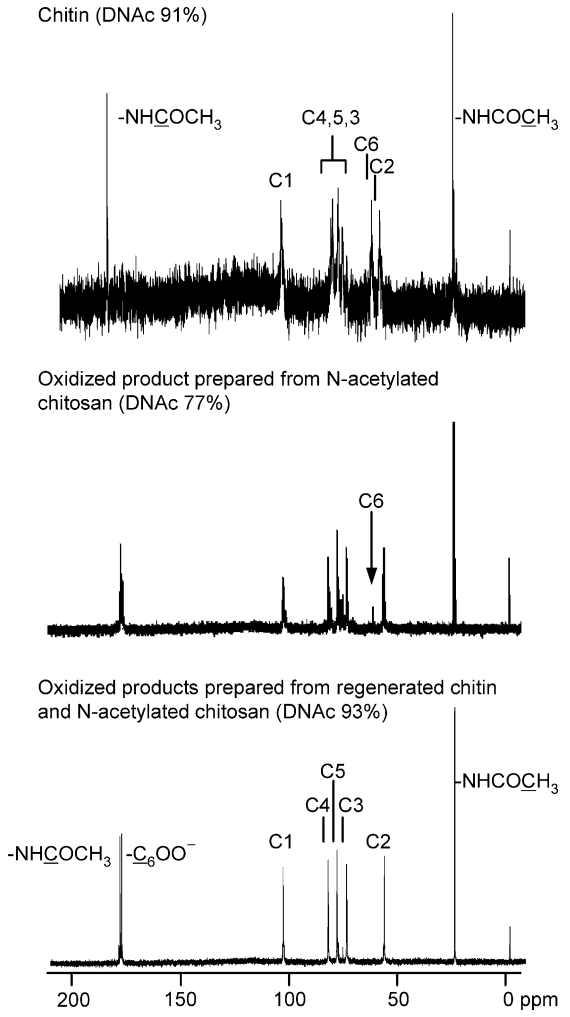

Fig. 3. ^{13}C NMR spectra of the original chitin dissolved in 10% NaOD/D_2O and the oxidized products prepared from *N*-acetylated chitosans with degrees of acetylation of 77 and 93%.

93% were completely soluble in water, and had M_w of more than 7×10^4, which corresponds to approximately degree of polymerization (DP) 300. These DP values suggest that some depolymerization occurs on the polysaccharide chains during the oxidation, as reported for cellulose (Isogai & Kato, 1998).

Table 2
Properties of oxidized products prepared from regenerated chitin and *N*-acetylated chitosans by TEMPO-mediated oxidation

	Water solubility[a]	M_w	M_n	M_w/M_n	N/C[b]
Regenerated chitin	Soluble	7.6×10^4	2.9×10^4	2.6	0.142
N-acetylated chitosan A	Soluble	7.1×10^4	3.0×10^4	2.4	0.142
N-acetylated chitosan B	Mostly soluble	2.6×10^4	1.4×10^4	1.9	0.141
N-acetylated chitosan C	Not completely soluble	3.2×10^6	6.7×10^3	472	0.141

[a] Evaluated visually.
[b] Ratio of nitrogen content/carbon content of the oxidized product.

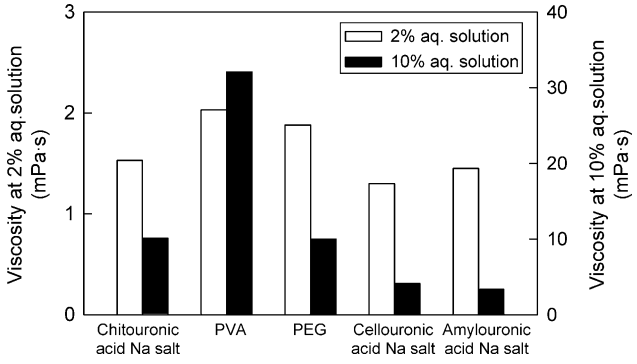

Fig. 4. Viscosities of 2 and 10% aqueous solutions of chitouronic acid Na salt (M_w 7.3×10^4), polyvinylalcohol (PVA: M_w 3.3×10^4), polyethyeleglycol (PEG: M_w 3.9×10^4), cellouronic acid Na salt (M_w 5.3×10^4) and amylouronic acid Na salt (M_w 5.6×10^4).

3.3. Characterization of 6-oxichitin (chitouronic acid)

Viscosities of aqueous chitouronic acid Na salt solutions were lower than those of polyvinylalcohol (PVA) and polyethyleneglycol (PEG) with similar molecular weights (Fig. 4). Correspondingly, concentration of aqueous solutions of chitouronic acid Na can increase up to 30% at 25 °C, and this is one of the characteristic points of chitouronic acid. Cellouronic and amylouronic acid Na salts, which were prepared from regenerated cellulose and water-soluble starch, respectively, by the TEMPO-mediated oxidation (Isogai & Kato, 1998; Kato, Matsuo, & Isogai, 2003), had viscosities similar to those of chitouronic acid.

Fig. 5 shows biodegradability of chitouronic acid, chitin, chitosan and CMC. Chitosan with DNAc of 0% and carboxylmethylcellulose (CMC) had quite low degrees of biodegradability in this test, and similar results were reported also by Kato et al. (2002) and Yomota, Komuro, and Kimura (1990). Degree of biodegradation of chitouronic acid was close to that of chitin with DNAc of 91%,

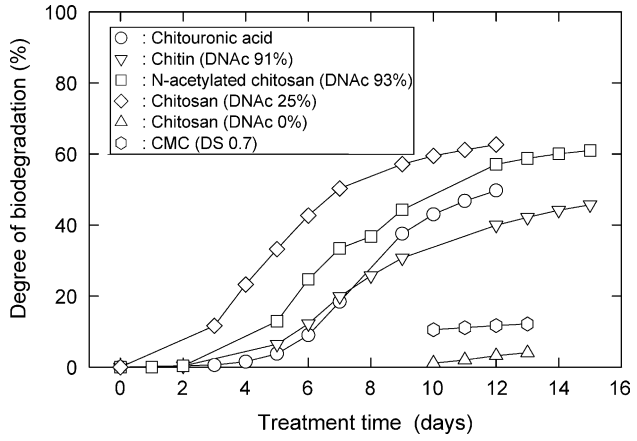

Fig. 5. Biodegradability of chitouronic acid Na salt, chitin, chitosan and carboxymethylcellulose Na salt (CMC).

but lower than those of chitosan with DNAc of 25% and *N*-acetylated chitosan with DNAc of 93%. Thus, chitouronic acid has sufficient biodegradability.

4. Conclusion

Regenerated chitin prepared from chitin solutions in aqueous NaOH and *N*-acetylated chitosan with DNAc of 93% give water-soluble chitouronic acid with DP of ca. 300 quantitatively within 70 min by the TEMPO-mediated oxidation. On the other hand, the original chitin and *N*-acetylated chitosan with DNAc lower than 77% are unsuitable starting materials for the TEMPO-oxidation, because of high crystallinity for the former and high C2-amino group content for the latter. Chitouronic acids prepared from regenerated chitin and *N*-acetylated chitosan with DNAc of 93% have amorphous crystal structures, and give aqueous solutions with low viscosities. Moreover, chitouronic acid has clear biodegradability.

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