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Chemical modification of chitin and chitosan 1: preparation of partially deacetylated chitin derivatives via a ring-opening reaction with cyclic acid anhydrides in lithium chloride/*N*,*N*-dimethylacetamide

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Abstract

Derivatives of partially *N*-deacetylated chitin (DAC) were prepared via ring-opening reactions with various cyclic acid anhydrides in lithium chloride/*N*,*N*-dimethylacetamide (LiCl/DMAc) system. Some cyclic acid anhydrides such as succinic, maleic, glutaric, and phthalic anhydrides gave successfully water-soluble DAC derivatives. From the enzymatic studies, the glycosyl bond of succinyl and maleoyl DAC-20 (20% DAC) was rapidly degraded by lysozyme or chitinase, though that of phthaloyl DAC-20 was not. The ester linkage of succinyl DAC-20 was stable against lipase for five days at room temperature.

Keywords: Chitin; Chitosan; Chemical modification; Cyclic acid anhydride; Ring-opening reaction

1. Introduction

Chemical modification of chitin is difficult in general, because chitin is a highly crystalline material with a strongly hydrogen-bonded network structure. A number of water-soluble chitin derivatives have been reported. Carboxymethylchitin and dihydroxypropylchitin were prepared through alkali chitin suspension under heterogeneous conditions (Tokura, Nishi, Tsutsumi & Somorin, 1983). Diethylaminoethylchitin was also prepared using an alkali chitin solution under homogeneous conditions (Kurita, Inoue & Koyama, 1989). Chitin sulfate was prepared under heterogeneous conditions in pyridine (Nishimura, Nishi & Tokura, 1986a), and chitin phosphate was prepared under homogeneous conditions in methanesulfonic acid (Nishi, Nishimura, Ebina, Tsutsumi & Tokura, 1984). A number of water-soluble chitosan derivatives have also been previously reported (Kurita & Ishi, 1995). For example, *N*-trimethylchitosan ammonium iodide was prepared under heterogeneous conditions in *N*-methyl-2-pyrrolidone (Domard, Rinaudo & Terrassin, 1986), and disaccharide substituted chitosan was prepared via reductive *N*-alkylation under homogeneous conditions in acidic water and methanol (MeOH) mixed solvent (Yalpani & Hall, 1984). Under heterogeneous conditions, generally, the reactivity of chitin would increase with decreasing degree of crystallinity and the reaction in the amorphous region would proceed more smoothly than that in the crystalline region. Therefore, for the products obtained under heterogeneous conditions it would be difficult to control the degree of substitution (DS), solubility, and other properties. Under alkaline conditions further, some *N*-deacetylation of chitin was unavoidable and the amino group was also substituted. *N*,*N*-dimethylacetamide (DMAc), a polar aprotic solvent, containing lithium chloride (LiCl) allows a range of organic reactions including typical alcohol modification reactions such as esterification and carbamate formation. Chemical modifications of cellulose in the LiCl/DMAc system have been studied by numerous workers (Dawsey, Fornes & Gilbert, 1992; Samaranayake & Glasser, 1993; Suzuki, Kurata & Ikeda, 1992). Further, chlorination (Sakamoto, Tsung & Furuhata, 1994), bromination (Tseng, Lee, Furuhata & Sakamoto, 1995), acylation (Terbojevich, Carraro & Cosani, 1988), and carbamoylation (McCormick & Lichatowich, 1979) of chitin were also studied in the LiCl/DMAc system which gave a homogeneous reaction mixture.

Previously, we had synthesized (1-carboxyethyl)chitosan as an analog of polymeric *N*-acetylmuramic acid (Shigemasa et al., 1995). McCormick and Dawsey reported the preparation of cellulose derivatives via a ring-opening reaction with cyclic reagents in the LiCl/DMAc system (McCormick & Dawsey, 1990). Hirano and coworkers

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have synthesized some *N*-(carboxyacyl)derivatives of chitosan with some cyclic acid anhydrides in aqueous AcOH and MeOH (Hirano & Moriyasu, 1981; Yamaguchi, Arai, Itoh & Hirano, 1981).

Herein we report the preparation and characterization of water-soluble derivatives of partially deacetylated chitin (DAC) via ring-opening reactions with cyclic acid anhydrides in LiCl/DMAc.

2. Experimental

2.1. Materials

Partially DAC (DAC-20, degree of deacetylation (DDA) = 20%) from shrimp shell was purchased from Wako Pure Chemical Industries Co. Ltd. Partially acetylated chitosan (PAC-96, degree of *N*-acetylation = 96%) was prepared from DAC-88 (crab shell, supplied by Sunfive Co. Ltd.) by *N*-acetylation (Hirano & Yamaguchi, 1976). Hen egg-white lysozyme [EC 3.2.1.17] (50 000 units mg^{-1}) and chitinase from *Bacillus sp.* PI-7S (0.04–0.06 units mg^{-1}) were purchased from Seikagaku Kogyo Co. Ltd. Lipase [EC 3.1.1.3] from porcine pancreas (41 units mg^{-1}) was purchased from Sigma chemical Co. Other reagents were purchased from Nacalai Tesque, Inc. and used without further purification unless specifically noted.

2.2. General methods

IR spectra were recorded as KBr pellet on a Shimadzu FT-IR 4200 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 60°C in D_2O on a JEOL JMN-GX270 spectrometer, sodium 3-(trimethylsilyl)propanesulfonate being used as an internal standard. In the evaluation of ^{1}H NMR spectra of various DAC-20 derivatives prepared in this article, the proton number of each signal was estimated on the assumption that the proton number of $-NH(CO)C\mathbf{H}_3$ at δ 1.94–2.04 was 2.4. The DS of the water-insoluble product could be estimated by ^{1}H NMR using 5.5 mol l^{-1} DCl/D_2O as solvent. This solvent was especially useful to dissolve products of lower DS. The DS was defined as follows:

DS = number of substituted group/number of monosaccharide residue (MR).

The molecular weight of chitin derivatives was determined by gel permeation chromatography (GPC) with pullulan as standard on a Shimadzu LC-6A apparatus (column, Asahipak GS-220H, GS-310H, and GS-510H; eluent, 0.1 mol l^{-1} phosphate buffer solution (pH = 7.4); flow rate, 1.0 ml min^{-1}; column temperature, 50°C). The 1.0% (w/v) DAC-20 solution was prepared as follows: DAC-20 (3.0 g) was added to 300 ml of 5% (w/v) LiCl/DMAc solution, and the mixture was stirred at room temperature for 3 h to give a clear solution. The solubility of chitin or DAC in 5% (w/v) LiCl/DMAc would depend on the DDA and the molecular weight of chitin or DAC. Higher DDA of DAC (DAC-50, 88 and 100) or relatively higher molecular weight of chitin did not dissolve in this solvent though the boundary value of the molecular weight of undissolved chitin is unclear in this study.

2.3. Typical procedure

Cyclic acid anhydride (100 mmol) was added to 200 ml of the 1.0% (w/v) DAC-20 (2.0 g, 10 mmol as MR) solution. In the case of succinic and maleic anhydrides, these reaction mixtures became dark brown by the addition of triethylamine (Et_3N, 10.2 g, 10 mmol), and then turned to a gel within 30 min. In contrast, the reaction mixtures of phthalic, tetrahydrophthalic, *cis*-5-norbornyl-*endo*-2,3-dicarboxylic, *cis*-1,2-cyclohexanedicarboxylic, or trimellitic anhydride and DAC-20 were white suspensions or gels. After stirring for 24 h, the reaction mixture (containing a gel) was poured into MeOH (100 ml). The precipitate was filtered, followed by dispersing in 200 ml of water. The product was precipitated from the solution or suspension by adjusting the pH to 1–2 with 3 mol l^{-1} HCl. The precipitate was filtered, washed with MeOH, and dried in vacuo at 60°C to give a protonated product (H type). To obtain a sodium salt of product (Na type), the product was dispersed in 200 ml of water, followed by adding an adequate amount of Na_2HPO_4 to give a clear solution of pH 8–10. The solution was dialyzed against deionized water for three days and lyophilized. In this procedure, further, the absence of C-2 proton (δ 2.8 ppm) having free amino groups would suggest that both the hydroxy group and amino group are substituted with carboxyacyl groups. We, however, could not evaluate the DS of the hydroxy group and the amino group from ^{1}H NMR spectra, independently.

2.3.1. Succinyl DAC-20 (1)

The yield of succinyl DAC-20 of DS = 1.3 was 2.8 g (77%) from 2.0 g of DAC-20; M_n = 170 000. The yield of succinyl DAC-20 of DS = 0.6 was 0.26 g (94%) from 0.2 g of DAC-20; M_n = 170 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, ν_{O-H}. ^{1}H NMR (D_2O): δ 1.94 and 2.00 (br s, 2.4 H, $-NH(CO)C\mathbf{H}_3$), 2.46 (br s, 2.5 H, $-O(C{=}O)C\mathbf{H}_2-$), 2.62 (br s, 2.5 H, $-C\mathbf{H}_2COONa$), 3.6–4.2 (br m, 5.8 H, H-2, H-3, H-4, H-5, and H-6 of MR). ^{13}C NMR (D_2O): δ 24.9 ($-NH(CO)\mathbf{C}H_3$), 32.9 ($-O(C{=}O)\mathbf{C}H_2-$), 34.5 ($-\mathbf{C}H_2COONa$), 57.6 (C-2 of MR), 63 (C-6 of MR unsubstituted), 65.4 (C-6 of MR substituted), 74–75 (C-3 and C-5 of MR), 82.7 (C-4 of MR), 104–105 (C-1 of MR), 177.2 ($-NH(\mathbf{C}O)CH_3$), 178.0 ($-O(\mathbf{C}{=}O)CH_2-$), 183.3 ($-CH_2\mathbf{C}OONa$). Anal. Calc. for $C_{12.8}H_{17.8}O_{8.7}N$: C, 47.32; H, 5.48; N, 4.31. Found: C, 47.15; H, 5.76; N, 4.00.

DS = Proton number of δ2.46 and 2.62/4.

2.3.2. Maleoyl DAC-20 (2)

The yield of maleoyl DAC-20 of DS = 1.3 was 2.14 g from 2.0 g of DAC-20; M_n = 120 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, ν_{O-H}. ^1H NMR (D_2O): δ 2.00 (br s, 2.4 H, –NH(CO)C**H**$_3$, 3.2–4.3 (br m, H-2, H-3, H-4, H-5, and H-6 of MR), 5.88 (br s, 1.3 H, O(C=O)C**H**=), 6.68 (br s, 1.3 H, =C**H**COONa). ^{13}C NMR (D_2O): δ 24.9 (–NH(CO)**C**H$_3$), 57.6 (C-2 of MR), 62.7 (C-6 of MR), 74.8 and 77.0 (C-3 and C-5 of MR), 83 (C-4 of MR), 104.2 (C-1 of MR), 120.1 and 121.0 (–O(C=O)**C**H=), 145.2 and 147.0 (=**C**HCOONa), 168.2 and 169.3 (–O(C=O)CH=), 177.1 (–NH(**C**O)CH$_3$) and =CH**C**OONa).

DS = Proton number of δ5.88 and 6.88/2.

2.3.3. Glutaryl DAC-20 (3)

The glutarylation of DAC-20 with 4-dimethylaminopyridine (DMAP) and Et_3N did not proceed. Using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base, the reaction was successfully proceeded and the mixture colored orange. The yield of glutaryl DAC-20 of DS = 0.8 was 0.63 g (100%) from 0.4 g of DAC-20. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, ν_{O-H}. ^1H NMR (D_2O): δ 1.87 (br m, 1.71 H, –CH$_2$–C**H**$_2$–CH$_2$–), 2.00 (br s, 2.4 H, –NH(CO)C**H**$_3$, 2.2 (br m, 1.5 H, –O(C=O)C**H**$_2$–), 2.44 (br m, 1.5 H, –C**H**$_2$–COONa), 3.5–4.4 (br m, H-2, H-3, H-4, H-5, and H-6 of MR). ^{13}C NMR (D_2O): δ 23.8 (–CH$_2$–**C**H$_2$–)CH$_2$–), 24.9 (–NH(CO)**C**H$_3$), 35.9 (–O(C=O)–**C**H$_2$–), 39.2 (–**C**H$_2$–COONa), 57.7 (C-2 of MR), 62.7 (C-6 of MR unsubstituted), 65.3 (C-6 of MR substituted), 72.5–76.1 (C-3 and C-5 of MR), 83.0 (C-4 of MR), 104.2 (C-1 of MR), 177.2 (–NH(**C**O)CH$_3$), 178.3 (–O(**C**=O)CH$_2$–), 184.7 (–CH$_2$**C**OONa). Anal. Calc. for $C_{11.6}$ $H_{17.6}O_{7.2}N$:C, 48.67; H, 6.15; N, 4.90. Found: C, 48.20; H, 6.07; N, 4.25.

DS = Proton number of δ1.87, 2.2 and 2.44/6.

2.3.4. Phthaloyl DAC-20 (4)

The yield of phthaloyl DAC-20 of DS = 0.6 was 0.61 g (100%) from 0.4 g of DAC-20; M_n = 110 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, $\nu_{O=H}$. ^1H NMR (D_2O): δ 1.78 (br m, 2.4 H, –NH(CO)C**H**$_3$), 3.3–4.3 (br m, H-2, H-3, H-4, H-5, and H-6 of MR), 7.1–8.0 (br m, 2.35 H, –Ph–). ^{13}C NMR (D_2O): δ 24.8 (–NH(CO)**C**H$_3$), 57.4 (C-2 of MR), 65.8 (C-6 of MR), 74–78 (C-3 and C-5 of MR), 83 (C-4 of MR), 102 (C-1 of MR), 124–146 (–Ph–), 177 (–NH(**C**O)CH$_3$), 178.9 (–O(**C**=O)Ph–), 180.0 (–Ph–**C**OONa).

DS = Proton number of δ7.1–8.0/4.

2.3.5. Tetrahydrophthaloyl DAC-20 (5)

The yield of tetrahydrophthaloyl DAC-20 of DS = 1.2 was 1.28 g (78%) from 0.8 g of DAC-20; M_n = 110 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, $\nu_{O=H}$. ^1H NMR (D_2O): δ 1.93 and 2.01 (br m, 2.4 H, –NH(CO)C**H**$_3$), 2.37 (br, 4.5 H, –C**H**$_2$–), 3.08 (br s, 1.0 H, –O(C=O)–C**H** <), 3.4–4.3 (br m, H-2, H-3, H-4, H-5, and H-6 of MR and >**C**H–COONa), 5.74 (br s, 2.4 H, –C**H**=C**H**–). ^{13}C NMR (D_2O): δ 25.0 (–NH(CO)**C**H$_3$), 28.6 and 29.2 (–CH$_2$–), 42.8 (–O(C=O)–**C**H<), 44.8 (>CH–COONa), 127.6 and 129.3 (–**C**H=**C**H–), 177.2 (–NH(CO)CH$_3$), 179.1 (–O(**C**=O)–), 184.1 (–**C**OONa).

DS = Proton number of δ5.74/2.

2.3.6. 2-Carboxy-4-norbornylcarbonyl DAC-20 (6)

The yield of 2-carboxy-4-norbornylcarbonyl DAC-20 of DS = 0.9 was 1.06 g (72%) from 0.8 g of DAC-20; M_n = 190 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, ν_{O-H}. ^1H NMR (D_2O): δ 1.30 (br s, 1.73 H, –C**H**$_2$–), 2.01 (br s 2.4 H, –NH(CO)C**H**$_3$), 3.08 (br s, 2.29 H, >C**H**–), 3.26 (br s, –O(C=O)–C**H** < and > C**H**–COONa), 3.4–4.4 (br m, H-2, H-3, H-4, H-5, and H-6 of MR), 6.21 (br s, 1.77 H, –C**H**=C**H**–). ^{13}C NMR (D_2O): δ 24.9 (–NH(CO)**C**H$_3$), 49.3 (>**C**H–), 51.5 (–O(C=O)–**C**H<), 54.2 (>**C**H–COONa), 137.8 (–**C**H=**C**H–), 182.8 (–O(**C**=O)–), 184.8 (–**C**OONa).

DS = Proton number of δ6.21/2.

2.3.7. 2-Carboxycyclohexylcarbonyl DAC-20 (7)

The yield of 2-carboxycyclohexylcarbonyl DAC-20 of DS = 1.2 was 1.6 g (64%) from 1.2 g of DAC-20; M_n = 50 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, ν_{O-H}. ^1H NMR (D_2O): δ 1.42 and 1.71 (br s, 8.3 H, –C**H**$_2$–), 2.02 (br s, 4.8 H, –NH(CO)C**H**$_3$), 2.74 (br m, 2.33 H, > C**H**–), 3.2–4.2 (br m, 6 H, H-2, H-3, H-4, H-5, and H-6 of MR). In this case, the total proton number of H-2, H-3, H-4, H-5, and H-6 at δ 3.2-4.2 was assumed 6 as a base signal, because the signal of –NH(CO)C**H**$_3$) at δ 2.02 was partially overlapped with two neighboring signals at δ 1.71 and 2.74. ^{13}C NMR (D_2O): δ 24.9 (–NH(CO)**C**H$_3$), 26.4 and 28–31 (–CH$_2$–), 45.9 (–O(C=O)–**C**H<), 47.7 (>**C**H–COONa), 57.7 (C-2 of MR), 62.5 (C-6 of MR), 74–78 (C-3 and C-5 of MR), 82.9 (C-4 of MR), 104.2 (C-1 of MR), 177.2 (–NH(**C**O)CH$_3$), 179.5 (–O(**C**=O)–), 184.5 (–**C**OONa).

DS = Proton number of δ2.74/2.

2.3.8. 2,4-Dicarboxybenzoyl DAC-20 (8)

The yield of 2,4-dicarboxybenzoyl DAC-20 of DS = 1.0 was 1.29 g (73%) from 0.8 g of DAC-20; M_n = 110 000. IR (cm^{-1}): 1740, $\nu_{C=O}$; 3500, ν_{O-H}. ^1H NMR (D_2O): δ 1.92 and 2.04 (br m, 2.4 H, –NH(CO)C**H**$_3$), 3.4–4.2 (br m, H-2, H-3, H-4, H-5, and H-6 of MR), 7.4–8.5 (br m, 3.0 H, –Ph–). ^{13}C NMR (D_2O): δ 24.8 (–NH(CO)**C**H$_3$), 57.7 (C-2 of MR), 104.0 (C-1 of MR), 129–146 (–Ph–), 176.4 (–O(**C**=O)–Ph–), 177.3 (–NH(**C**O)CH$_3$), 179.1 (–Ph–**C**OONa).

DS = Proton number of δ7.4–8.5/3.

Table 1
Preparation of various DAC-20 derivatives. (Solvent 5% (w/v) LiCl in DMAc, 10 ml per 100 mg of DAC-20; temperature, 25°C; time, 24 h)

Entry	DAC-20 (mg)	Anhydride[a]	Equiv	Base[b]	Equiv	Product Yield[c] (%)	DS[d]	
1	600	Succinic	10	DMAP	10	88	1.1	1
2	2000	Maleic	10	Et_3N	10	60	1.3	2
3	400	Glutaric	6	DBU	5	100	0.8	3
4	100	Glutaric	10	DMAP	10	90	0	
5	400	Glutaric	6	Et_3N	5	90	0	
6	400	Phthalic	6	Et_3N	5	100	0.6	4
7	800	THP	6	DMAP	5	78	1.2	5
8	800	Nolb	10	Et_3N	10	72	0.9	6
9	1200	Cycl	6	Et_3N	5	64	1.2	7
10	800	Trimellitic	6	Et_3N	5	73	1.0	8

[a] Equiv, mol equivalent of anhydride per MR of DAC-20; THP, tetrahydrophthalic; Norb, *cis*-5-norbornyl-*endo*-2,3-dicarboxylic; Cycl, *cis*-1,2-cyclohexanedicarboxylic anhydride.
[b] DMAP: 4-dimethylaminopyridine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene.
[c] Yield(%) = ((weight of product) × (M_W of MR of DAC-20) × 100)/((weight of DAC-20) × (M_W of MR of product)).
[d] Determined by ^{1}H NMR; DDA of those products was ca. 20%.

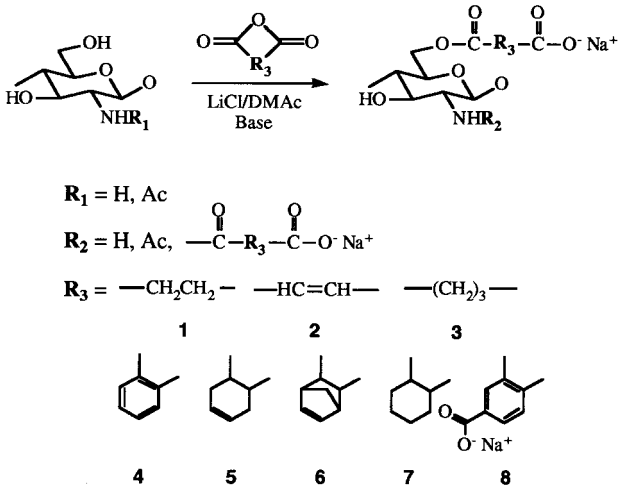
2.4. Enzymatic degradation

Enzymatic hydrolysis of DAC-20 derivatives was performed according to the method described previously (Shigemasa, Saito, Sashiwa & Saimoto, 1994). A typical procedure is as follows: the hydrolytic reaction of DAC derivatives [substrate] = 9.1 mg ml^{-1}, [enzyme] = 0.33 mg ml^{-1} (lysozyme, 16 500 units ml^{-1}; chitinase, 0.083 units ml^{-1}) was started by adding an enzyme solution (0.1 ml) to 1.0 ml of distilled water (pH 7) containing 1% (w/v) DAC derivatives at 37°C, followed by measuring the viscosity of the solution with a Visconic ELD rotational viscometer (Tokyo Keiki Co. Ltd) at the prescribed time.

2.5. Ester linkage cleavage of DAC-20 derivative by lipase

To evaluate the biodegradable property of the functional group of DAC-20 derivatives, the cleavage of ester linkage of succinyl DAC-20 by lipase was estimated by ^{1}H NMR analysis. As the two signals of methylene proton of succinyl group (−O(C=O)−C**H**$_2$−C**H**$_2$−COONa, δ 2.49 and 2.62) were changed to singlet and shifted upfield (disodium succinate (NaOOC−C**H**$_2$−C**H**$_2$−COONa), δ 2.41) by the hydrolysis, the degradability of succinyl group could be estimated by ^{1}H NMR analysis as the following equation: Cleavage (%) = Peak area of disodium succinate (δ 2.41) × 100/{Peak area of succinyl group (δ 2.49 and 2.62) + disodium succinate (δ 2.41)}.

To 1 ml of lipase (1 mg) in D_2O solution (pD = 7), was added 9 mg of succinyl DAC-20 (DS = 1.3, M_n = 170 000) and the solution was stood for five days at 37°C. After this, the solution was analyzed as described earlier. Although lipase is known to degrade some esters (Benzonana & Esposito, 1971), lipase had little effect on the succinyl DAC-20 lipase under the aforementioned conditions.



Scheme 1.

3. Results and discussion

The preparation of various DAC-20 derivatives via ring-opening reaction with cyclic acid anhydride are shown in Table 1 and Scheme 1. Under these reaction conditions, the reaction proceeded smoothly and gave water-soluble products. In all cases, the solution was colorless and transparent before adding the base. Each reaction mixture had a characteristic color which was also observed when the corresponding anhydride and base without DAC-20 were mixed in organic solvent such as DMAc or chloroform ($CHCl_3$).

3.1. Succinylation of DAC

Table 2 shows the effect of the p*K*a of the base on the succinylation of DAC-20. The products using pyridine as a base or without a base showed low DS even at 110°C. The succinylation of DAC-20 proceeded successfully with 4-dimethylaminopyridine (DMAP: p*K*a = 9.7). Fig. 1 shows

Table 2
Effect of pK_a of base on succinylation of DAC-20.(Solvent: 5% w/v LiCl in DMAc, 10 ml per 100 mg of DAC-20; succinic anhydride, 10 equivalent per MR of DAC-20; time, 2 h)

Entry	DAC-20 (mg)	Base[a]	(pK_a)	Temperature (°C)	Yield[b] (%)	DS[c]
1	200	—	—	110	95	0.1
2	200	Pyridine	5.3	110	90	0.2
3	200	DMAP	9.7	110	97	0.6
4	600	DMAP	9.7	25	90	0.6

[a] 10 equivalent per MR; DMAP: 4-dimethylaminopyridine.
[b] Yield (%) = (weight of product) × ((M_W of MR of DAC-20) × 100)/((weight of DAC-20) × [($M_W$of MR of DAC-20) × (1-DS) + M_W of MR of succinyl DAC-20 × DS]).
[c] Determined by ^{1}H NMR; DDA of those products was ca. 20%.

the time-courses of succinylation of DAC-20 and 96% *N*-acetylated chitosan (PAC-96). Under the reaction condition, the DS of succinyl group increased with increasing reaction time and reached 1.1–1.3 within 20 h. The effects of the amount of succinic anhydride on the succinylation of DAC-20 and PAC-96 are shown in Fig. 2. The low amount of succinic anhydride (below two equivalent) gave water-insoluble product whose DS was below 0.2. The products above 0.4 of DS are water soluble.

3.2. Solubility of DAC-20 derivatives

Almost all DAC-20 derivatives (H and Na Type) did not dissolve in organic solvents such as dimethylsulfoxide (DMSO), *N*,*N*-dimethylacetamide (DMAc), pyridine, $CHCl_3$, ethanol (EtOH), and acetone. Only the H type of phthaloyl DAC-20 dissolved in DMSO. The Na form of DAC-20 derivatives dissolves or partially dissolves in water, though the H form of these derivatives was insoluble. The effects of pH on the water solubility of succinyl DAC-20 and phthaloyl DAC-20 are shown in Table 3. Both succinyl DAC-20 (DS = 0.6 and 1.3) were dissolved at the pH range from 4.5 to 11.0. A white precipitate appeared at the pH below 4.5 and above 11. The precipitate obtained below pH 4.5 redissolved at pH 7 by addition of 0.1 mol l^{-1} NaOH.

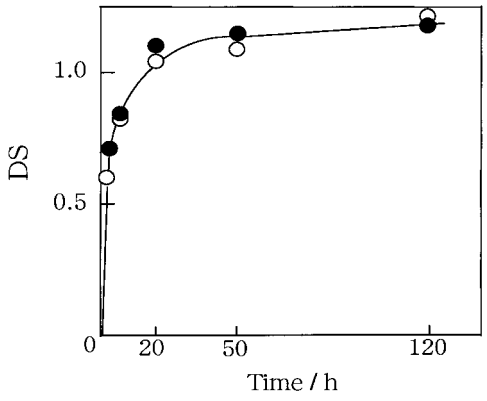


Fig. 1. Time-courses of succinylation of acetylated chitosan derivatives (ACDs). ACD, 600 mg; 5%(w/v) LiCl in DMAc, 60 ml; succinic anhydride, 10 equiv; DMAP, 10 equiv; temp, 25°C; White circle, DAC-20; Black circle, PAC-96.

The precipitate obtained above pH 11, however, did not redissolve at pH 7 on addition of 0.1 mol l^{-1} HCl. In the case of phthaloyl DAC-20, a similar phenomena were observed and the derivative was soluble between pH 3 and 11. The precipitate obtained above pH 11 was recovered at a yield of 83% and the DS changed from 1.3 to 0.06. These results suggest that the hydrolysis of the ester linkage readily occurred above pH 11. The insolubility of these DAC-20 derivatives at the lower pH would be caused by the protonation of the carboxylic group.

3.3. Enzymatic degradation

Fig. 3 shows the change of viscosity of succinyl DAC-20 solution (pH 7) in the presence of lysozyme. In Fig. 3, η_0 and η_t are the initial viscosity (cp) and the viscosity (cp) at the prescribed time, respectively. Within 5 min after adding lysozyme, the relative viscosity decreased rapidly and reached equilibrium. The rate of decrease increased with increasing the amounts of lysozyme. In the absence of lysozyme, the viscosity was almost constant (94%). The viscosity of chitosan (Hiroi, Kawahata & Fujita, 1981), chitin (Tokura & Nishi, 1995), and *O*-(carboxymethyl)chitin (CM-chitin) (Inoue, Kaneko & Tokura, 1982) is proportional to their molecular weight. The rapid decrease of the viscosity suggests that lysozyme hydrolyzes the glycoside linkage of succinyl DAC-20 molecule.

The viscosity changes of DAC-20 derivative solutions in the presence of lysozyme or chitinase are summarized in Table 4. The initial viscosity of the solution and that after 15 min of hydrolysis are represented as η_0 and η_{15}, respectively. The viscosity of succinyl DAC-20 solution and maleoyl DAC-20 solution decreased from 7.2 and 6.2 cP to 1.2 cp for 15 min of hydrolysis at 37°C in the presence of lysozyme (16 500 unit ml^{-1}). The viscosity of succinyl and maleoyl DAC-20 solutions also decreased in the presence of chitinase (0.013 unit ml^{-1}). However, the phthaloyl DAC-20 solution did not show the remarkable viscosity decrease on lysozyme and chitinase addition under the same reaction conditions. We have previously shown that the enzymatic degradation of *N*-acetylated chitosan derivatives (ACDs) by lysozyme and chitinase was

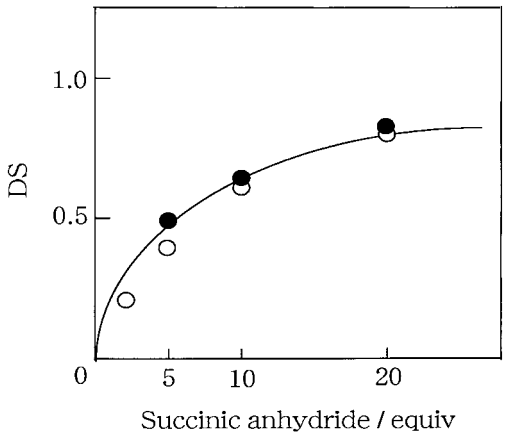


Fig. 2. Effect of succinic anhydride amount on succinylation of acetylated chitosan derivatives (ACDs). ACD, 100–260 mg; 5%(w/v) LiCl in DMAc, 10–26 ml; DMAP, 10 equiv; time, 2 h; temp, 25°C; White circle, DAC-20; Black circle, PAC-96.

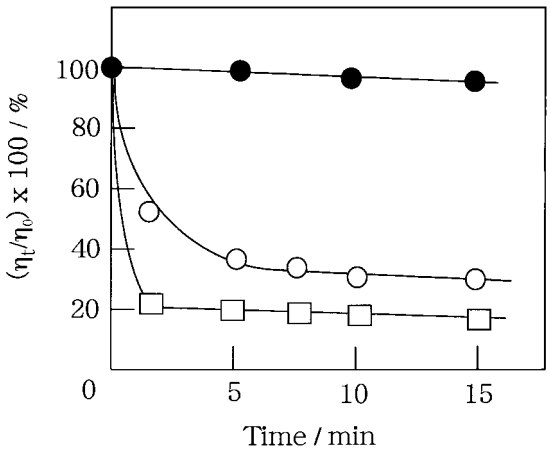


Fig. 3. Lysozyme degradation of succinyl DAC-20. [succinyl DAC-20], 9.1 mg/ml; solvent, H_2O (pH = 7); temp, 37°C; visconic ELD rotational viscometer. Succinyl DAC-20: DS = 1.3, DDA = 20%, Mn = 170 000; White circle, [lysozyme] = 5000 units ml^{-1} (0.10 mg ml^{-1}); White square, [lysozyme] = 16 500 units ml^{-1} (0.33 mg ml^{-1}); Black circle, without lysozyme.

Table 3
Effect of pH on water solubility of succinyl DAC-20 and phthaloyl DAC-20. (DAC-20 derivatives were dissolved in H_2O(2 mg ml^{-1}) and the pH of the solution was adjusted with 0.1 M HCl and 0.1 M NaOH. White bar, soluble; black bar, insoluble)

DAC-20 Derivative	DS	M_n	pH 1 3 5 7 9 11 13
Succinyl DAC-20	0.6	170 000	
Succinyl DAC-20	1.3	170 000	
Phthaloyl DAC-20	1.3	110 000	

Table 4
Change of viscosity of DAC-20 derivative solution in the presence of enzymes. (Enzyme = 0.33 mg ml^{-1}; time, 15 min; temperature, 37°C; solvent, H_2O (pH = 7); VISCONIC ELD rotational viscometer)

Entry	DAC-20[a] Derivative	M_n	mg ml^{-1}	η_0/cP	Enzyme	Units ml^{-1}	Viscosity change[b] (%) η_{15}/cP	
1	Succinyl DAC-20	170 000	9.1	7.2	None	—	6.8	6
2			9.1		Lysozyme	16 500	1.2	84
3			9.1		Chitinase	0.013	3.6	50
4	Maleoyl DAC-20	120 000	27.3	6.2	None	—	6.0	4
5			27.3		Lysozyme	16 500	1.2	81
6			27.3		Chitinase	0.013	2.0	67
7	Phthaloyl DAC-20	110 000	9.1	4.9	Lysozyme	16 500	4.2	14
8			27.3	30.0	None	—	27.9	7
9			27.3		Lysozyme	16 500	26.7	11
10			27.3		Chitinase	0.013	26.4	12

[a] DS = 1.3, DDA ≈ 20%.

[b] Viscosity change (%) = $(\eta_0 - \eta_{15}/\eta_0) \times 100$; η_{15}, viscosity (cP) of reaction mixture at 15 min after adding enzyme; η_0, viscosity (cP) of DAC-20 derivative solution (9.1 or 27.3 mg ml^{-1}) in the absence of enzyme.

evaluated by the determination of the reducing groups under heterogeneous conditions and by measuring the viscosity of the solution (Shigemasa et al., 1994). DAC-70 gave a smooth decrease in viscosity on lysozyme addition (Sashiwa, Saimoto, Shigemasa, Ogawa & Tokura, 1990), but ACD (DDA = 10–20%), and DAC-70 were unaffected by lysozyme under the heterogeneous conditions although chitin was degraded by chitinase (Shigemasa et al., 1994). Hence, it is clear that the solubility of the substrate is an important factor in determining the degradative action of lysozyme on chitin derivatives.

Hamaguchi and Funatsu reported the lysozyme-catalyzed hydrolysis of glycolchitin measured by viscosity decrease (Hamaguchi & Funatsu, 1959). Nishimura et al. reported that there was a remarkable enhancement of accessibility of CM-chitin to lysozyme upon modification at *O*-6 of *N*-acetyglucosamine residue, though the accessibility was decreased by further substitution at O-3 (Tokura et al., 1996; Nishimura, Nishi & Tokura, 1986b). Hirano et al. reported that *O*-carboxymethyl-*N*-acylchitosan (acyl: acetyl, propionyl, butanoyl, pentanoyl, and hexanoyl) was hydrolyzed by both lysozyme and chitinase and the highest hydrolysis was observed by the *N*-acetyl group (Hirano, Hayashi & Hirochi, 1992). Further, the effect of the *N*-substitution of chitosan and DDA on the hydrolysis by chitinase and lysozyme have also been reported (Hirano & Yagi, 1980; and Hirano, Tsuchida & Nagao, 1989. Although the enzymatic hydrolyses of various chitin derivatives have been reported as described earlier, the correlation between the substituted functional group and hydrolytic activity is still not clear.

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