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Note

Depolymerization of *N*-succinyl-chitosan by hydrochloric acid

Yoshinori Kato,* Hiraku Onishi, Yoshiharu Machida

Department of Drug Delivery Research, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

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Abstract

N-Succinyl-chitosan (**1**) was depolymerized with 7.5 M aqueous HCl at room temperature or 3.3 M aqueous HCl at 40 °C and the molecular weights (MW) of the products were determined by size-exclusion chromatography–multi angle light scattering (SEC–MALS) and their viscometric features were investigated. The intrinsic viscosity ([η]) obtained at the concentration of 0.1–0.3% (w/v) in saline showed a linear relationship between log[η] and log MW, which provided the coefficients in the Mark–Houwink equation. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *N*-Succinyl-chitosan; Molecular weight (MW); Depolymerization; Viscometric feature; Acid treatment

1. Introduction

N-Succinyl-chitosan (**1**) is water-soluble, has very low toxicity[1,2] and is only slightly biodegradable in vivo[3,4] and is thus attractive as a drug carrier showing long-term retention in the body after i.v. administration.[4] A water-soluble macromolecular prodrug of mitomycin C with **1** was shown to possess good antitumor activity after i.v. administration to Sarcoma 180-bearing mice.[5] To improve characteristics of that prodrug, the effects of the molecular weight (MW) of **1** on the pharmacokinetic properties remain to be elucidated. The MW of **1** previously used was around 3×10^5, and its solutions were too viscous. This high MW and high viscosity may influence the transfer of **1** from the blood circulation to the tumor site, where the permeability of blood vessels is enhanced. It was thus considered important to obtain materials of lower MW by acid treatment, as by MW manipulation of chitosan by nitrous acid.[6,7] The present study used hydrochloric acid for depolymerization. For MW analysis, sedimentation equilibrium may be used,[8] but the present study employed size-exclusion chromatography–multi angle light scattering (SEC–MALS), and the viscosity properties of the products were examined.

* Corresponding author. Tel.: +81-3-54985760; fax: +81-3-54985759.

E-mail address: d711@hoshi.ac.jp (Y. Kato).

2. Experimental

Materials.—*N*-Succinyl-chitosan sodium salt (**1**) was generously supplied by Katakura Chikkarin Co., Ltd. (Tokyo, Japan). Deuterium chloride (20%) and sodium 4,4-dimethyl-4-silapentanoate 2,2,3,3-d_4 acid (TSP) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, USA). All other chemicals were of reagent grade and were obtained commercially.

Acid treatment.—To a solution of **1** (50 mg) in water (6 mL) was added 10 M aq HCl solution (6 mL) and the solution was stirred at rt. At selected time intervals (10, 30, 60, and 90 min), 3 mL aliquots of the mixture were sampled, neutralized with NaOH (solid) and dilute aq NaOH (6 and 1 M), and dialyzed against water using seamless cellulose tubing (molecular weight cut-off limit $1.2–1.4 \times 10^4$; Viskase Sales Corp., USA) to remove salts. Finally, the product was obtained as a powder by lyophilization of the dialyzed solution.

Depolymerization was performed under other conditions, as follows: Aq HCl solution (10 M, 6 mL) was added to a solution of **1** (50 mg) in water (6 mL), and stirred at 40 °C. At selected time intervals (5, 10, 20, and 40 min), aliquots (3 mL) were taken and treated in the same way as at rt to afford each product. Similarly, **1** (50 mg) was dissolved in a mixture of aq HCl solution (10 M, 4 mL)–water (8 mL) or in a mixture of aq HCl solution (10 M, 9 mL)–water (3 mL), and stirred at rt or 40 °C. Aliquots were taken at appropriate times, and

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each product was obtained as a powder by the procedure already described. The depolymerized products thus obtained are denoted as **1a** and **1b**, respectively.

Analysis of molecular weight.—The MWs of **1**, **1a**, and **1b** were examined by size-exclusion chromatography–multi angle light scattering (SEC–MALS) using a Shodex DS-4 (Shoko Co., Ltd., Tokyo) apparatus equipped with a Shodex OH-pak SB-805HQ column. The flow rate was 1.0 mL/min, and the temperature of the column was 40 °C. Wyatt Dawn E and Shodex RI-71 systems were used as a multi angle light scattering detector and a differential refractive index detector, respectively. The elution solvent was 0.05 M phosphate buffer, pH 7.0. Products **1**, **1a**, and **1b** were dissolved in 0.05 M phosphate buffer at an appropriate concentration, and aliquots of 200 μL of these samples were applied on the SEC–MALS instrument. The refractive index increment (dn/dc) of **1** was measured in 0.05 M phosphate buffer, pH 7.0, by a Wyatt OPTILAB DSP interferometric refractometer at 690 nm.

Gel permeation chromatography (GPC) using polyethylene oxides (PEO) as standard molecular weight markers was used for MW analysis of the depolymerization products of **1**. This GPC was performed with a Shimadzu LC-6AD pump at a flow rate of 0.8 mL/min at rt, with a Shimadzu RID-10A differential refractive index detector, and a column TSK-gel G5000PW (7.5 mm i.d. × 60 cm; Tosoh Co.; Tokyo, Japan). This GPC method is abbreviated as $GPC_{G5000PW}$. The elution solvent was 0.05 M phosphate buffer, pH 7.0. Polyethylene oxides having MWs 9.2×10^5, 2.5×10^5, 10.7×10^4, and 2.4×10^4 (Tosho, Japan) established by absolute MW determination were dissolved in 0.05 M phosphate buffer and applied to $GPC_{G5000PW}$ as standard MW markers. The chitosan samples were dissolved in 0.05 M phosphate buffer at an appropriate concentration. Aliquots (20 μL) of **1** or PEO samples were injected on the $GPC_{G5000PW}$.

Viscometry.—Viscosity features of **1**, **1a**, and **1b** were examined with an Ubbelohde viscometer (Shibata, Japan) according to Japanese Pharmacopoeia XIII. Powder samples of **1**, **1a**, or **1b** were dissolved in saline at various concentrations, and kept at 37 ± 0.1 °C. Each solution (15 mL) was poured into the viscometer fixed in a container set at 37 ± 0.1 °C and the time for the surface of the solution to move between the specified lines of the viscometer was measured in triplicate. The reduced viscosity η_{red} was calculated by the following equation:

$$\eta_{red} = (\eta/\eta_0 - 1)/c \quad (1)$$

where η and η_0 are sample solution viscosity and solvent viscosity, respectively, and c is the concentration of the polymer. The intrinsic viscosity $[\eta]$ was obtained from the intercept when c was near zero.

Table 1
Molecular weight and ratio of M_w/M_n determined by MALS and calculated molecular weight by $GPC_{G5000PW}$ for **1**, **1a**, and **1b**

	SEC–MALS			$GPC_{G5000PW}$
	M_w	M_n	M_w/M_n	MW
1	310,000	150,000	2.1	310,000
1a	70,000	45,000	1.6	73,000
1b	28,000	21,000	1.3	36,000

3. Results and discussion

Analysis by SEC–MALS.—The products, **1a** and **1b**, prepared by treatment of **1** (12.5 mg) at rt for 30 or 90 min with 7.5 M aqueous HCl were obtained in the yields of 7.5 and 7.0 mg, respectively. Dialysis and subsequent lyophilization of **1** (12.5 mg) alone under the same conditions gave 8.6 mg of the powder, indicating loss by dialysis. The lower yield at increased time indicated that the amount of product with MW of less than $1.2–1.4 \times 10^4$ (MW cut-off limit of the dialysis membrane) increases with prolongation of acid treatment. The chemical structure of **1** was characterized previously.[5] The degraded products **1a** and **1b** were analyzed by ^1H NMR spectrometry (D_2O–DCl; pD 6; 90 °C; reference TSP) using a JEOL JNM-LA500 spectrometer (Tokyo, Japan): CH_2 of the succinyl group 2.4–2.7 (multiplet), H-2, H-3, H-4, H-5, H-6 of the sugar unit 3.4–4.0 (multiplet), H-1 of sugar unit (broad) for **1a** and **1b**. Elemental analysis (Yanako Analytical Industrial Co., Japan) of **1a** and **1b** after drying at 80 °C in vacuo in a desiccator with P_2O_5 for 3 h showed C/N (mol/mol) 7.4–7.9, suggesting that the fundamental structure of **1** was retained in the degraded products.

The refractive index increment (dn/dc) was approximately 0.13 mL/g for **1**. In all of the SEC–MALS profiles, the sum of the differential refractive index of each eluted part was close to the differential refractive index of the sample before elution, showing that most of the polymer injected had been eluted from the column. Further, in these SEC–MALS profiles, log MW of the eluted molecules decreased linearly as the elution volume increased (data not shown). The weight-average MWs (M_w values) of **1**, **1**, and **1b** were calculated to be 3.1×10^5, 7.0×10^4, and 2.8×10^4, respectively. The number-average MW (M_n) and the ratio of M_w/M_n were also obtained from the SEC–MALS profiles, as shown in Table 1.

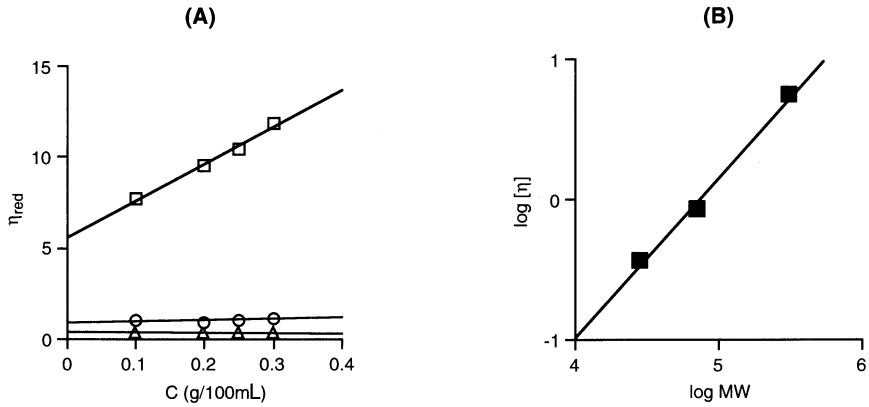

Fig. 1. Relationships of **1** concentration (C) vs. reduced viscosity (η_{red}) (A) and log MW vs. log intrinsic viscosity (log[η]) (B). In (A), open squares, circles and triangles represent **1**, **1a**, and **1b**, respectively, and each point represents mean $\pm$ S.D. ($n = 3$). The standard deviations were less than each symbol.

Viscosity characteristics.—Fig. 1(A) depicts the relationship between concentration and reduced viscosity (η_{red}) for **1**, **1a**, and **1b** in saline at 37 °C. For each plot, a straight line was fitted to the mean values of η_{red} by the least-squares technique. Each intrinsic viscosity ([η]) was obtained from the intercept of the line at $c = 0$; although the viscosities were not checked at concentrations below 0.1% (w/v). The logarithmic plot of the obtained [η] values versus log MW showed a linear relationship, as shown in Fig. 1(B). Simple application to the Mark–Houwink equation, $[\eta] = k \times MW^a$,[9,10] gave the a and k values of 1.2 and 2.7×10^{-4} (mL/g), respectively. For more accurate evaluation of the coefficients of the Mark–Houwink equation, viscosity data at further lower concentration might be required.

GPC analysis of products obtained after acid treatment.—The products obtained acid treatment under various conditions were investigated by GPC with a G5000PW column, $GPC_{G5000PW}$, using standard MW markers. The relationship between MW and elution volume (V_e) of the elution peak of PEO (MW range; 9.2×10^5–2.4×10^4) is illustrated in Fig. 2. The calibration equation for MW versus V_e was given by curve fitting as follows:

$$V_e = -3.7 \times \log MW + 35 \quad (2)$$

when r (correlation coefficient) was taken as 1.00. For **1**, **1a**, and **1b**, each elution profile in $GPC_{G5000PW}$ was similar to that in SEC–MALS already stated, and the relationship point of the V_e of the peak versus log MW was located almost on the PEO calibration line. Since these chitosan samples possessed a relatively narrow and nearly symmetrical single-lobe MW distribution on SEC–MALS (data not shown), it is suggested that Eq. (2) should be applicable for calculation of MW of **1**. The calculated MWs from the peaks of **1**, **1a**, and **1b** are shown in Table 1. The elution profiles of the products obtained after treatment under various conditions are shown in Figs. 3 and 4. At room temperature, the products obtained by using 3.3 M aqueous HCl showed little shift of the elution peak with time of treatment. It is suggested that **1** resists acid hydrolysis at a 10 M aqueous HCl solution–water ratio of less than 1 at room temperature. With 7.5 M aqueous HCl, the elution profile was shifted toward small molecules with increasing treatment time. At 40 °C, the elution profiles were quickly shifted in favor of small molecules after reaction for 5 min, except with 3.3 M aqueous HCl. Therefore, at 40 °C, the depolymerization of **1** was suggested to proceed rapidly, indicating that the MW of **1** may be difficult to control under these conditions except in 3.3 M aqueous HCl. Although only products **1a** and **1b** were investigated structurally, it appears that the fundamental structure of **1** is retained in all of the degraded products. Fig. 3 suggests that manipulation of the MW of **1** can be performed at room temperature under 7.5 M aqueous HCl. The

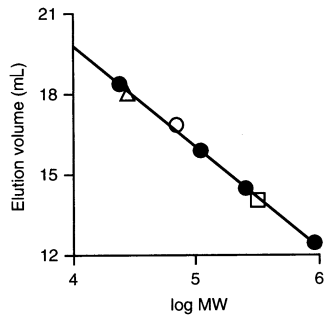

Fig. 2. Relationship between molecular weight and elution volume of the peak top of the sample in $GPC_{G5000PW}$. The MW obtained by SEC–MALS (M_w) was used for **1** (□), **1a** (○), and **1b** (△). Closed circles (●) represent PEO standards. The calibration equation for PEO described a straight line, and is explained in the text.

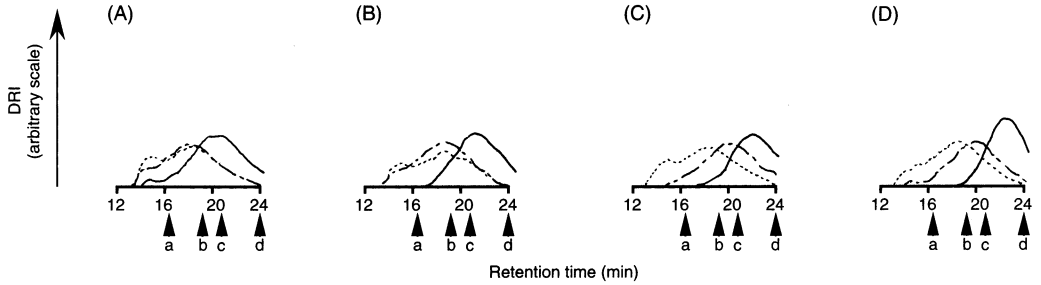

Fig. 3. Elution profiles of the products obtained by acid treatment at room temperature for 10 (A), 30 (B), 60 (C), and 90 min (D) in $GPC_{G5000PW}$. ···, 3.3 M aqueous HCl; –·–, 5 M aqueous HCl; —, 7.5 M aqueous HCl. The elution peaks of the MW makers (PEO) are indicated under the horizontal axis by arrows; a (MW 9.2×10^5), b (MW 2.5×10^5), c (MW 10.7×10^4) and d (MW 2.4×10^4). DRI (vertical axis) represents differential refractive index.

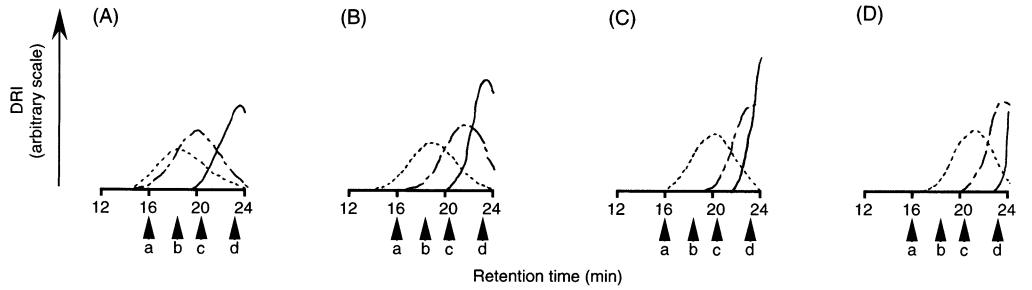

Fig. 4. Elution profiles of the products obtained by acid treatment at 40 °C for 5 (A), 10 (B), 20 (C), and 40 min (D) in $GPC_{G5000PW}$. ···, 3.3 M aqueous HCl; –·–, 5 M aqueous HCl; —, 7.5 M aqueous HCl. The elution peaks of the MW makers (PEO) are indicated under the horizontal axis by arrows; a (MW 9.2×10^5), b (MW 2.5×10^5), c (MW 10.7×10^4) and d (MW 2.4×10^4). DRI (vertical axis) represents differential refractive index.

elution profiles shown in Fig. 4 suggest that treatment with 3.3 M aqueous HCl might be adequate for MW manipulation of **1** at 40 °C; however, confirmation of the structural integrity of the product is needed for evaluation of acid treatment at 40 °C.

Thus *N*-succinyl-chitosan of moderate or low MW can be produced at room temperature with a 7.5 M aqueous HCl, and such treatment is also less expensive than the enzymatic method reported previously.[11]

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