

## The Preparation and Characterization of Low Molecular and Water Soluble Free-amine Chitosan

Mi-Kyeong Jang, Young-Il Jeong,<sup>†</sup> Chong-Su Cho,<sup>‡</sup> Suk-Hyang Yang,<sup>§</sup> Young-Eui Kang,<sup>§</sup> and Jae-Woon Nah<sup>\*</sup>

*Department of Polymer Science and Engineering, Suncheon National University, Jeonnam 540-742, Korea*

*<sup>†</sup>The Research Institute of Medical Science Chonnam National University, Gwangju 501-746, Korea*

*<sup>‡</sup>School of Biological Resources and Materials Engineering, Seoul National University, Suwon 441-744, Korea*

*<sup>§</sup>Department of Clothing and Textiles, Suncheon National University, Jeonnam 540-742, Korea*

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Chitosan has the amine group in their C-2 position. Because of this, chitosan has specific properties, such as chelation applied to areas of health care, water quality improvement, pharmaceuticals,<sup>1</sup> affinity for protein,<sup>2,3</sup> inhibition of tumor cells,<sup>4</sup> and inhibition of microbial cell growth.<sup>5</sup> Recently, many investigators have focused gene carrier development on genetic disease therapy. Among these biomaterials for gene carriers, chitosan has a unique potential as a useful DNA carrier<sup>6</sup> due to the strong positive charge of the amine group at the C-2 position. However, one of severe disadvantages of traditional chitosan is its poor solubility in water or organic solvents due to its crystalline structure<sup>7</sup> and then its actual use in bioindustry is limited. Since chitosan is only dissolved in water containing acetic acid and its use as solvent is considered another limitations for actual use due to that bioactive agents such as peptide or protein drug, genetic material, and anticancer drugs may affect by acetic acid.

One of the processes to produce water-soluble chitosan is enzymatic<sup>8</sup> method chitosan oligosaccharide from high molecular weight chitosan (HMWC). In this process the various amine salts were produced by solvents such as lactic acid, acetic acid, hydrochloric acid, etc., used to dissolve the HMWC. In this process various kind of salts by solvents can attach at the C-2 amine group, resulting that lost their positive charge although water-solubility is increased. Therefore, water-soluble chitosan having free-amine group is necessary to develop for its application in the field of pharmaceutical or food industry. This article describes the preparation of water-soluble chitosan having free-amine group as removal the amine salts derived by enzymatic process and its degree of deacetylation and molecular weight (Mw) were calculated by Kina titration and Viscometer, respectively. The characters of low molecular and water soluble free-amine chitosan (LM-WSFC) were identified by spectroscopic analysis.

### Experimental Section

**Materials.** Chitosan with a molecular weight of 22 kDa

and the degree of deacetylation (DDA) of 72% was supplied from KITTOLIFE Co., Seoul, Korea. Triethylamine (TEA) with 99% purity purchased from Aldrich Chemical Co. and used without further purification. The water filter which is composed of activated carbon and ion exchange resin was purchased from the Brita Products Co. Various solvents as a reagent grade were used without further purification and double distilled water was used at all of the experiments.

**Preparation of LM-WSFC.** Five gram of chitosan oligosaccharide prepared by enzymatic process was dissolved in 10 mL of phosphate buffered saline (PBS, pH 7.0) to remove the amine salt attached in C-2 position of glucose amine units. TEA 5.2 mL was slowly dropped in chitosan solution and this solution stirred for 2 h at the room temperature. After reaction, acetone was added and this mixture was harvested by centrifuge (supra 30 K, 15,000 × g, 20 minutes, 4 °C). To obtain the pure product, this process was repeated four times and then dried on the air. This product was treated with 0.001 N-HCl aqueous solutions, again for 2 h. After 2 h, acetone was added to this solution following above procedure repeated 5 times and then dried on the air. To remove the residue HCl solution and impurity, the product was dissolved in double distilled water following pass through the column. The resulting aqueous solution was lyophilized and then water-soluble chitosan having free-amine was obtained.

**The DDA and Mw measurement of LM-WSFC.** The DDA of LM-WSFC was determined by Kina titration method<sup>9</sup> and calculated by following equation.

$$\text{DDA} = \frac{(X/161)}{(X/161) + (Y/203)} \times 100 \quad (1)$$

$$X = (1/400) \times (1/1000) \times F \times 161 \times V$$

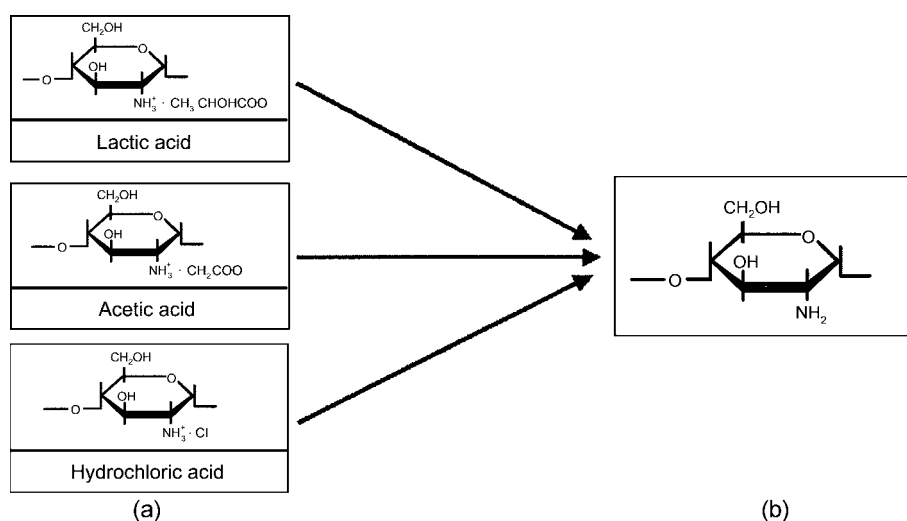
$$Y = 0.5 \times (1/100) X$$

V : the consumption amount of 1/400 (2.5 mN) PVSK solution (mL)

F : the factor of 1/400 (2.5 mN) PVSK

Also, relative viscosity was measured using viscometer (Viscotek, Y501C, U.S.A) to calculate the average molecular weight. From the relative viscosity result, the Mark-

<sup>\*</sup>Corresponding author. Phone & Fax: +82-61-750-3566, E-mail: jwnah@sunchon.ac.kr



**Scheme 1.** (a) The salts predicted by enzymatic method and (b) the pure chitosan removed the salts by this method.

Houwink Equation (MHE) (2) below was used to evaluate the  $M_w$  of LM-WSFC:

$$[\eta] = KM_{vis}^a \quad (2)$$

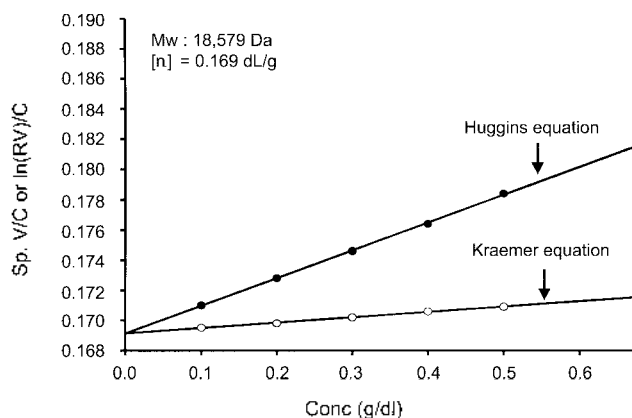
$$M_{vis} = ([\eta]/K)^{1/a}$$

**The Identification of LM-WSFC Structure.** FT-IR and  $^{13}\text{C}$ -NMR spectrophotometer was measured for identification the structure of chitosan, respectively. To measure FT-IR spectrophotometer (Shimadzu, FT-IR 8700), LM-WSFC 3 mg was mechanically blended with 300 mg of KBr for 10 minutes. The KBr disc obtained from a 200 mg aliquot of the mixed powder was desiccated for 12 h at 60 °C under reduced pressure.<sup>10</sup> Also, for measurement the  $^{13}\text{C}$ -NMR spectrometer (Bruker, DRX-500MHz) LM-WSFC was dissolved in  $\text{D}_2\text{O}$ .

## Results and Discussion

LM-WSFC with free amine group was successfully prepared to use as a bioactive material in the pharmaceutical. Scheme 1 presents the type of amine salts (a) and pure free amine (b) removed by this method. The chitosan with amine salts is toxic in the body and have to remove for the pharmaceutical application.

**The DDA and  $M_w$  of LM-WSFC.** By Kina titration method, DDA of LM-WSFC was determined with the average value as repeat 3 times the consumption amount of 1/400 (2.5 mN) PVSK solution (mL). LM-WSFC prepared in this study has about 93% DDA that was higher than that of control chitosan having 72% DDA. This result indicated that free amine groups of LM-WSFC were increased through removal of the impurity or salts attached in amine group of the C-2 position of the glucose amine unit. The relative viscosity was measured to determine the average molecular weight of LM-WSFC. The intrinsic viscosity vs. the concentration of LM-WSFC was plotted, and the intrinsic viscosity of LM-WSFC obtained by fitting the Huggins equation and Kraemer equations (Figure 1). From this result,  $M_w$  of LM-WSFC



**Figure 1.** The Intrinsic Viscosity of LM-WSFC in 0.1 M AcOH-0.2 M NaCl 25.0 ± 0.01 °C as a Function of Concentration.

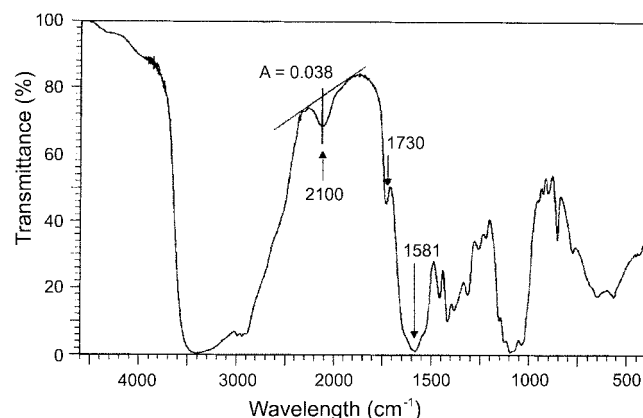
was about 18,579 Da.

**Analysis of FT-IR spectrum.** In the case of high molecular weight chitosan (HMWC), the intensity of the amide I and amide II bands at 1655 and 1560  $\text{cm}^{-1}$  decreases due to the deacetylation of chitin and the absorption band of the amine group formed newly increases at 1590  $\text{cm}^{-1}$ . However, it was identified that the FT-IR spectrum of the low molecular weight chitosan (LMWC) prepared by acidic degradation is noticeably split at 1614 and 1510  $\text{cm}^{-1}$ .<sup>11</sup> This is considered that the peak by amide I group (C=O) is showed the strong absorption band due to the weakness of the hydrogen bond. Table 1 shows the characteristic peak of the chitin and chitosan FT-IR spectrum.

The spectrum of control chitosan and LM-WSFC prepared in this work was compared to identify the synthesis of LM-WSFC. At the spectrum of control chitosan (Figure 2), the absorption band by carboxyl group derived from lactic acid at 1730  $\text{cm}^{-1}$  and the impurity formed in the enzyme degradation process at 2100  $\text{cm}^{-1}$  was identified. However, at the spectrum of LM-WSFC prepared in this experiment the absorption band at 1730  $\text{cm}^{-1}$  disappeared and the strong

**Table 1.** The peak analysis of  $^{13}\text{C}$ -NMR spectrum

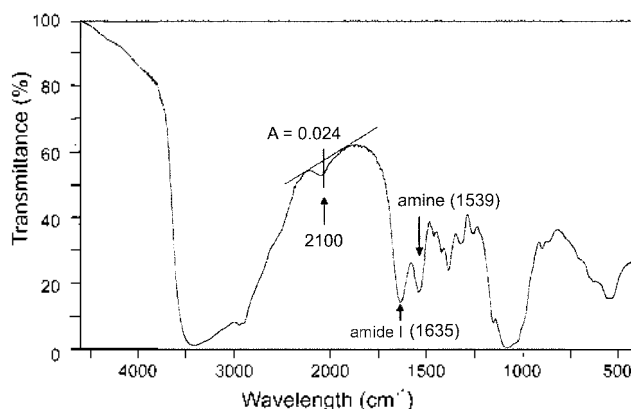
| $^{13}\text{C}$ -NMR spectra |                     |
|------------------------------|---------------------|
| Mw                           | 18,579 Da           |
| DDA                          | 93%                 |
| Chitosan unit                | 107 ea (17,278 Da)  |
| Chitin unit                  | 6 ea (1,301 Da)     |
| Theoretical value            | 113 : 6 (18.83 : 1) |
| Empirical value              | 17.795 : 1          |

**Figure 2.** The FT-IR Spectrum of Control Chitosan.

peak by the impurity at  $2100\text{ cm}^{-1}$  was decreased significantly (Figure 3). Therefore, the pure LM-WSFC removed the impurity and salts was identified through the FT-IR spectrum.

**The analysis of  $^{13}\text{C}$ -NMR spectrum.** The structural characters control chitosan and LM-WSFC was performed from  $^{13}\text{C}$ -NMR. These techniques allow us to determine quantitatively the area ratio of the proton and carbon. At the  $^{13}\text{C}$ -NMR spectrum of it, the absorption band derived from lactic acid presented at 20 ppm and the identification of chitosan peak difficult because of the impurity.

But, LM-WSFC prepared by this experiment process was obtained the  $^{13}\text{C}$ -NMR spectrum. At the  $^{13}\text{C}$ -NMR spectrum the carboxyl group by lactic acid disappeared and the clear peak was obtained. The carbon of the C-1 position at 20 ppm, carbon of the C-3, 4, 5 position at 79-70 ppm, and carbon of the C-2, 6 position at 55, 60 ppm was identified

**Figure 3.** The FT-IR Spectrum of LM-WSFC.

clearly. Table 1 numerically presents the area ratio against the empirical and theoretical value of the carbon number at  $^{13}\text{C}$ -NMR spectrum of LM-WSFC. Considering that  $M_w$  and DDA of LM-WSFC are 18,579 Da, 93% respectively, the area ratio of the carbon number of chitin and chitosan unit also very close to the area ratio assigned at  $^{13}\text{C}$ -NMR spectrum. Thus, it was identified that the LM-WSFC prepared in this manner is pure water-soluble chitosan, having a free amine group with the salts removed.

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