Relationship Between Molecular Structure and Moisture-Retention Ability of Carboxymethyl Chitin and Chitosan

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ABSTRACT: Carboxymethyl chitins and chitosans (CM-chitins, CM-chitosans) of different substitution sites were prepared under different reaction conditions, and partially depolymerized carboxymethyl chitins of various molecular weights from 24.8×10^4 to 0.26×10^4 were obtained by degrading with chemical reagents. Degree of substitution (DS) was estimated by potentiometric titration. Substitution site was confirmed by infrared and ^{13}C-NMR spectra. Molecular weights were determined with gel permeation chromatography and gel permeation chromatography combined with laser light scattering (GPC-LLS). Moisture-absorption and retention abilities of these compounds were tested in comparison with those of hyaluronic acid (HA). The results reveal that 6-carboxymethyl group in the molecular structure of chitin and chitosan is a main active site responsible for moisture retention. Although carboxymethylation at OH-3 and N position is not essential, they contribute to the ability. Moisture-retention ability is also related to molecular weight; that is, higher molecular weight helps to improve moisture-retention ability. 6-*O*-CM-chitin (chitosan) with a DS above 0.8 and molecular weight higher than 24.8×10^4 has the potential to substitute for HA for use in cosmetics and clinical medicine. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 83: 1233–1241, 2002

Key words: carboxymethyl chitin; carboxymethyl chitosan; degree of substitution; substitution site; molecular weight; moisture absorption; moisture retention

INTRODUCTION

Hyaluronic acid (HA), which belongs to glycosaminoglycans, is unique for its moisture-retention ability and plays a key role in cosmetics and clinical medicine.[1] Although HA is ubiquitous in plants and animals, the total amount is limited, and the price is very high.

Chitin, a natural polymer from marine resources, has attracted more and more attention nowadays, owing to their abundant resources, friendliness to the environment, and potential to substitute for some petrochemicals.[2] As the fundamental chemical structure of chitin is close to that of HA, the moisture-absorption and retention properties of several kinds of chitin derivatives were examined and compared with those of HA by Shuichi et al. in 1989.[3] Carboxymethyl chitosan (CM-chitosan) appears to be more suitable than other derivatives such as hydroxyethyl chitin and carboxypropenoyl chitin for preparing HA-like substances. Muzzarelli et al. also pointed out that 0.25% aqueous solution of *N*-carboxymethylated chitosan (*N*-CM-chitosan) and carboxymethylated chitin (CM-chitin) were comparable to a 20% aqueous solution of propylene glycol in terms of

moisture-retention ability and the viscosities of the solutions containing minor amounts of *N*-CM-chitosan or CM-chitin were higher than those of other moisture-retention agents and almost equal to that of HA.[4]

In recent years, CM-chitin and CM-chitosan are used more and more widely in cosmetics and as soft tissue augmentation in medicine for their excellent moisture-retention ability.[5–6] Systematic research into the moisture-retention properties of CM-chitin and CM-chitosan is very important for further applications. However, published studies on the relationship between moisture-retention ability and molecular structure of CM-chitin and CM-chitosan are scarce. In this work, CM-chitin and CM-chitosan of different substitution sites were successfully prepared under different reaction conditions, and CM-chitins of different molecular weights were obtained by degradation with chemical reagents. The degree of substitution was estimated by potentiometric titration, and the substitution site was determined with the aid of IR and ^{13}C-NMR. Molecular weights were determined by using GPC and gel permeation chromatography combined with laser light scattering (GPC-LLS). Moisture-absorption and retention properties of prepared samples were investigated.

EXPERIMENTAL

Materials

Chitin and chitosan were purchased from Zhejiang Yuhuan Ocean Biochemistry Co., Ltd., in Zhejiang province, and were purified by extracting with methanol at 50°C for 15 h before use.[7] The deacetylation degree (DD) of chitosan determined by elemental analysis[8] was 0.85. Standard pullulans for GPC and glyoxylic acid monohydrate were purchased, respectively, from Showa Denko Co., Ltd. (Japan), and Merk-Schuchardt Co., Ltd. (Germany). All other chemicals were of reagent grade and were used without further purification.

Preparation of CM-Chitins and CM-Chitosans

6-*O*-CM-chitin and *N*-CM-chitosan were prepared according to the methods provided by Tokura et al. and Muzzarelli et al.; monochloroacetic acid and glyoxylic acid were used in those two reactions, respectively.[9–10]

The preparation method of 3,6-*O*-CM-chitin was as follows: chitin (20 g) was swollen in aqueous 46% NaOH (76 mL) by stirring at room temperature for 5 h. To the swollen mixture, several pieces of crushed ice were added to obtain a solution of 8% sodium chitin salt in aqueous 14% NaOH.[11] NaOH was added again to bring the concentration to 42%. Na-chitin solution was then added stepwise into isopropanol solution of ClCH$_2$COOH (300 mL) and stirred at room temperature for 1 h. Ethanol was used to precipitate and wash the product. After dialyzing against deionized water for 5 days, the sample was vacuum dried at room temperature.

6-*O*-CM-chitin and 3,6-*O*-CM-chitin were *N*-deacetylated by refluxing in the solution of 40% NaOH and isopropanol (1 : 1) for 5 h at 60°C to give 6-*O*-CM-chitosan and 3,6-*O*-CM-chitosan.[12]

Degradation of 6-*O*-CM-Chitin

6-*O*-CM-Chitin power (5 g) was suspended in 250 mL deionized water. After stirring at 75°C for 1 h, hydroperoxide of a desired volume was added for predetermined time to yield 6-*O*-CM-chitins of various molecular weights. To get samples of very low molecular weight, concentrated HCl was also adopted, taking reference to the method provided by Muzzarelli et al.[13]

Characterization

DS of each sample was estimated from potentiometric titration.[14] Samples were dissolved in 0.1 mol/L hydrochloric acid (50 mL) in the presence of 0.1 mol/L sodium chloride and titrated with 0.1 mol/L sodium hydroxide. The alkalimetric curves were recorded on a DELTA-320-S pH meter.

IR spectra were taken with KBr pellets on a Shimadzu 8210 FTIR spectrometer. ^{13}C-NMR spectra were recorded on a Mercury 300 spectrometer, and chemical shifts were given by taking methanol as reference for ^{13}C-NMR in D$_2$O at 323 K.

The intrinsic viscosity [η] of CM-chitin and CM-chitosan of different substitution sites in 0.1 mol/L NaCl aqueous solution at 30°C was measured by using a Ubbelodhe viscometer, and the viscosity-average molecular weight (M_η) of the samples was calculated according to the Mark–Houwink equation for CM-chitin in 0.1 mol/L NaCl at 30°C:[15]

$$[\eta] = 7.92 \times 10^{-4} M_\eta^{1.0} \quad \text{(ml/g)} \qquad (1)$$

GPC-LLS measurements were also performed with a DAWN-DSP multi-angle laser photometer combined with a P100 pump (Thermo Separation Products) equipped with a TSK G6000PWXL column (7.8 × 300 mm) and a differential refractive index detector (RI-150) at 30°C. The eluent was a 0.1 mol/L NaCl aqueous solution with a flow rate of 0.8 mL/min. All solutions were filtered first with a sand filter and then with a 0.45-μm filter (Whattman, U.K.). Astra software was utilized for the data acquisition and analysis. The weight-average molecular weight (M_w) was determined with Berry fit method.

An HPLC instrument (Shimadzu) equipped with TSK G3000 PWXL column (7.8 × 300 mm), LC-6A pump, RI-150 differential refractometer, and Jiangshe Workstation was used for GPC analysis of weight-average molecular weight (M_w) of the degraded 6-*O*-CM-chitin samples. Each sample was dissolved in 0.1 mol/L NaCl aqueous solution, which was the eluent. The flow rate was 1.0 mL/min. The weight-average molecular weight (M_w) was calculated by the following equation:

$$\text{Lg}(M_w) = -0.8540V_e + 10.0382 \quad (2)$$

Moisture Absorption and Retention Test

The moisture-absorption and retention ability of the samples was determined according to the method reported by Shuichi et al.[3] Prior to the moisture-absorption testing, the samples were dried over P_2O_5 *in vacuo* for 24 h. The water-absorption ability was evaluated by the percentage of weight increase of dry sample (R_a):

$$R_a(\%) = 100 \times (W_n - W_0)/W_0 \quad (3)$$

where W_0 and W_n are the weights of sample before and after putting in the saturated $(NH_4)_2SO_4$ desiccator (81% relative humidity) and the saturated K_2CO_3 desiccator (43% relative humidity) at 20°C after 48 h of the test.

In the moisture-retention test, wet samples were prepared by adding 10% water to dry samples. The moisture-retention ability was evaluated by the percentage of residual water of wet sample (R_h):

$$R_h(\%) = 100 \times (H_n/H_0) \quad (4)$$

where H_0 and H_n were the weights of water in the sample before and after putting in the saturated

K_2CO_3 desiccator (RH 43%) and the silica gel at 20°C after 48 h of the test.

RESULTS AND DISCUSSION

DS and Substitution Site

Preparations of 6-*O*-CM-chitin, 6-*O*-CM-chitosan, and *N*-CM-chitosan were already reported by Tokura et al., Park et al., and Muzzarelli et al.,[9–10,16] but the preparation of 3,6-*O*-CM-chitin was not reported in detail. Because O-3 is engaged by hydrogen bonds, attack at O-6 seems to be particularly favorable.[4] Thus, a homogeneously dispersed condition is needed to prepare CM-chitins whose C-6 and C-3 positions are modified almost randomly. Taking reference to the method described by Hirano, 1996,[11] we prepared alkaline chitin solution successfully before carboxymethylation of chitins. In this reaction, the alkaline concentration was also one of the main factors to regulate the degree of substitution, the same as the preparation of 6-*O*-CM-chitin.[9]

CM-chitins and CM-chitosans of different prepared substitution sites are all white, free-flowing powder, and well soluble in water. Their degree of substitutions (DS) and molecular weight are summarized in Table I.

Because of the intramolecular electrostatic attraction between —COO^- and —NH_3^+ groups, 6-*O*-CM-chitosan, 3,6-*O*-CM-chitisan, and *N*-CM-chitosan showed characteristic ampholytic polyelectrolyte behavior in aqueous solution during the process of potentiometric titration, precipitating over the pH range 2.0–6.5. Although 6-*O*-CM-chitin was insoluble in water in the pH range 2.3–3.6, which was much narrower than CM-chitosans, 3,6-CM-chitin was completely soluble in all pH ranges.

The IR spectra for 6-*O*-CM-chitin, 3,6-*O*-CM-chitin, and *N*-CM-chitosan insolubilized at pH 1 are shown in Figure 1. The characteristic absorptions due to —COOH groups at 1737 cm^{-1} were observed obviously in the IR spectra of all carboxymethyl chitins (chitosan).[15] The stretching bands of —CH_2— at 2930 cm^{-1} increase evidently. The absorption bands at 1653, 1560, 1320, and 1379 cm^{-1} in the spectra of 6-*O*-CM-chitin and 3,6-*O*-CM-chitin assigned to amides I, II, III, and —CH_3 vibration bends do not appear in the spectrum of *N*-CM-chitosan, because of a high degree of deacetylation.[17] In the spectrum of 6-*O*-CM-chitin, the C—O stretching band at 1030

Table I   Degree of Substitution (DS) and Molecular Weight (*M*) of CM-Chitins and CM-Chitosans of Different Substitution Sites

Samples	DS	$M_\eta \times 10^{-4}$	$M_w \times 10^{-4}$	d[a]	Insoluble pH Range
		Molecular Weight			
6-O-CM-chitin	0.86	24.3	24.8	2.1	2.3–3.6
6-O-CM-chitosan	0.82	21.6	23.3	1.0	2.2–6.3
3,6-O-CM-chitin	0.60	23.7	24.1	1.0	Soluble in all pH ranges
3,6-O-CM-chitosan	0.65	12.1	16.1	1.1	2.1–6.5
N-CM-chitosan	0.50	5.6	6.9	1.0	2.5–6.6

[a] d, Polydispersity.

cm^{-1} corresponding to the primary hydroxyl group disappears, whereas at 1121 cm^{-1}, a strong absorption of the asymmetric stretching vibration of C—O—C is evident, verifying a high carboxy-methylation of OH-6. The secondary amide vibration band at 1500–1540 cm^{-1} appears in the spectrum of *N*-CM-chitosan, conforming the modification of —NH$_2$.
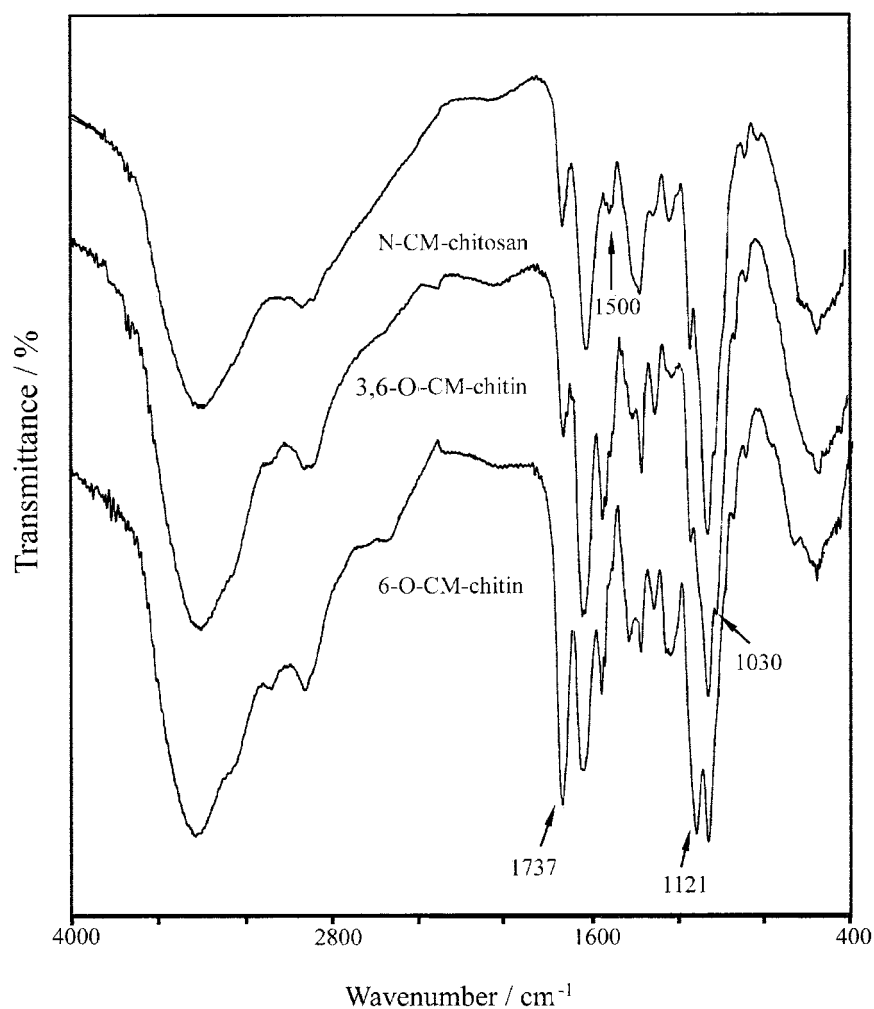
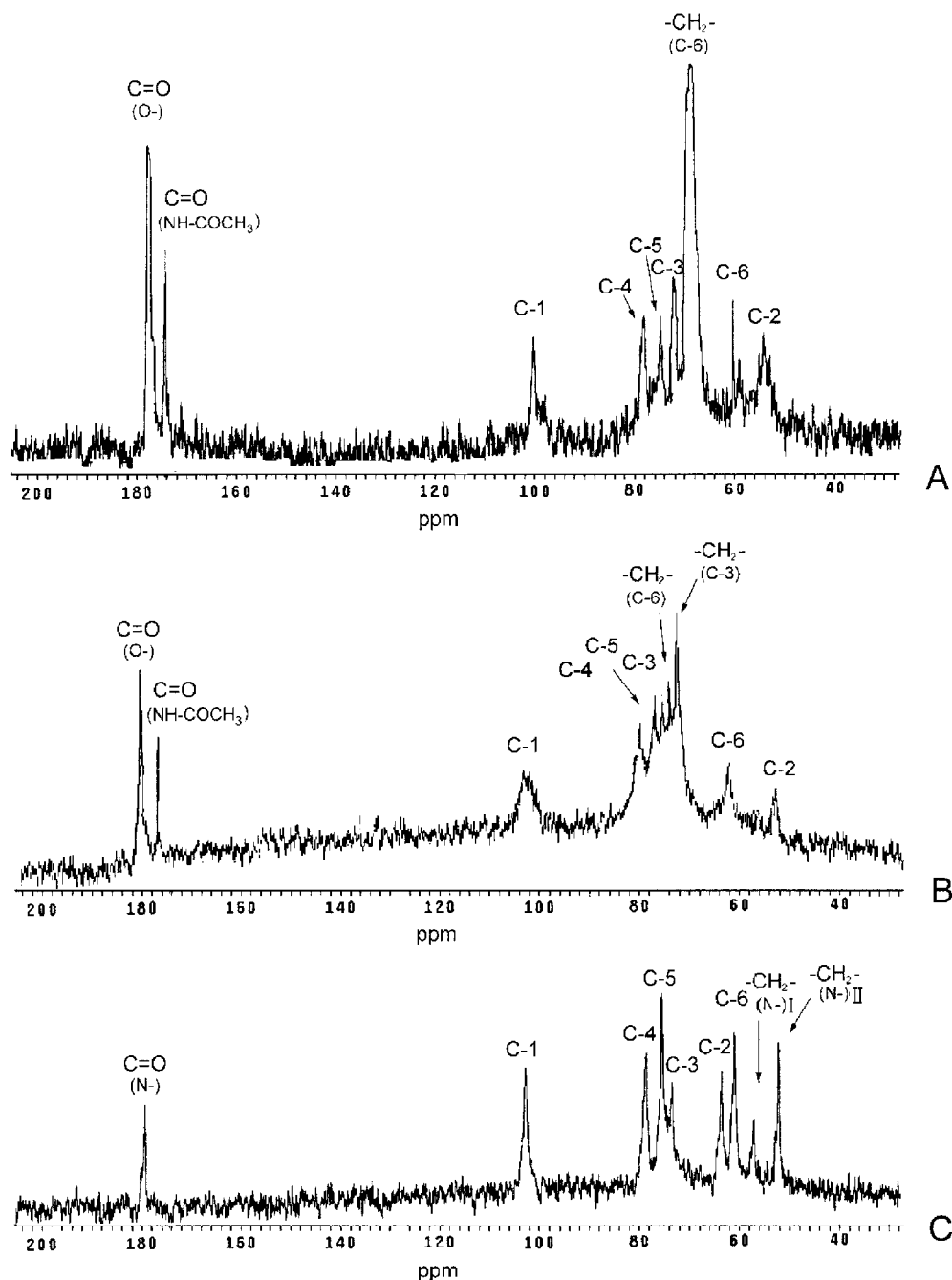**Figure 1**   IR spectra of 6-*O*-CM-chitin, 3,6-*O*-CM-chitin, and *N*-CM-chitosan.

Figure 2   ^{13}C-NMR spectra of 6-*O*-CM-chitin (A), 3,6-*O*-CM-chitin (B), and *N*-CM-chitosan (C).

NMR method is the most effective technique concurrently to determine the substitution site of chitosan derivatives.[18] Figure 2 depicts the ^{13}C-NMR spectra of carboxymethyl chitins (chitosan) of different substitution sites and chemical shifts of ^{13}C signals are given in Table II. ^{13}C-NMR spectra of 6-*O*-CM-chitin and 3,6-CM-chitin are almost the same; the substitution site just gives

characteristic signals between 69.0 and 72.0 ppm. In the spectrum of 6-*O*-CM-chitosan, only one chemical shift is observed for —O—*CH_2COO^- substituented on C-6, whereas in the spectrum of 3,6-*O*-CM-chitosan, there are two chemical shifts at 71.0 and 72.0 ppm corresponding to —O—*CH_2COO^- subsitituented on C-6 and C-3, respectively.[19] ^{13}C-NMR spectra of *O*-CM-chitin

Table II   Chemical Shifts of ^{13}C-NMR Signals of CM-Chitin and CM-Chitosan of Different Substitution Sites

Samples	C=O (O-)	C=O (N-)	C-1	C-4, C-5, C-3	—CH₂— (C-6)	—CH₂— (C-3)	C-6	C-2	—CH₂— (N-)
6-O-CM-chitin	178.33	174.97	101.43	73.70–79.55	70.93	—	61.93	58.75	—
3,6-O-CM-chitin	180.47	175.68	102.61	76.15–80.34	74.72	73.01	62.65	58.02	—
N-CM-chitosan	—	179.30	102.70	73.33–78.47	—	—	60.88	63.46	I 57.03
									II 52.09

and *N*-CM-chitosan were compared and their signals attributed to the *N*-carboxymethyl substituent (—NH—CH₂*COO⁻,

-N-*CH₂COO⁻ and -N-*CH₂ COO⁻)
|
(I)   *CH₂COO⁻ (II)
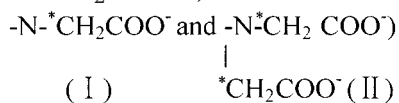
are at 179.3, 57.0, and 52.1 ppm. The spectrum of *N*-CM-chitosan indicates the presence of two monomeric units corresponding to the formation of two species during the reaction of —NH₂ with the very reactive glyoxylic acid, forming the monocarboxylated secondary amine (I) and the tertiary dicarboxylated amine (II) resulting from the reaction of (I) with excess glyoxylic acid.[20] The two forms are distinguishable on the spectra [Fig. 3(C)]. No chemical shifts are observed at 64.0–73.0 ppm, and the chemical shift corresponding to NH—*COCH₃ disappears because of a high degree of deacetylation in the spectrum of *N*-CM-

chitosan. Compared to spectrum of *N*-CM-chitosan, the intensity of C-6 peak in spectra of O-CM-chitosan decreases remarkably.

Molecular Weight

Two methods were applied to investigate the molecular weight of CM-chitins and CM-chitosans of different substitution sites. Viscosity-average molecular weight obtained from viscometry, weight-average molecular weight, and polydispersity obtained from GPC-LLS are shown in Table I. The M_η and M_w estimated from viscometry and GPC-LLS quite agree with each other; the molecular weight obtained by GPC-LLS is a little higher. Almost all samples prepared have a narrow polydispersity. *N*-deacetylation in the solution of 40% NaOH and isopropanol (1 : 1) resulted in degradation of 3,6-O-CM-chitin from 24.1×10^4 to 16.1×10^4, but had little effect on the molecular weight of 6-O-CM-chitin. Further research will be carried out about this interesting phenomenon.

During the investigation of molecular weight, we found that when molecular weight was lower than 3.0×10^4, the value was difficult to estimate by viscometry. When molecular weight was below 1.0×10^4, the value estimated by GPC-LLS was much larger due to the effect of the foreign substances. Thus, a Shimadzu GPC system was used for analysis of the weight-average molecular weight of degraded 6-O-CM-chitin. Pullulan standards with a molecular range from 0.27×10^4 to 1.2×10^4 were used. The weight-average molecular weight of degraded 6-O-CM-chitin samples are demonstrated in Table III, together with reaction conditions. The molecular weight of samples studied varies from 0.26×10^4 to 24.8×10^4. All samples obtained by degradation are white free-flowing powders. However, when exposed to moisture for some time, the color of the samples with molecular weight lower than 1.0×10^4 changed from white to brown.
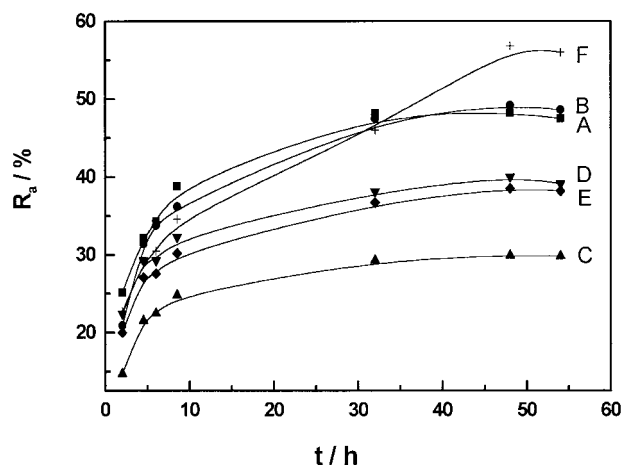

Figure 3   Moisture-absorption ability of dry samples of carboxymethyl chitins, chitosans of different substitution sites, and HA at 81% relative humidity at 20°C. A: 6-O-CM-chitin; B: 6-O-CM-chitosan; C: 3.6-O-CM-chitin; D: 3.6-O-CM-chitosan; E: *N*-CM-chitosan; F: HA.

Table III   Reaction Conditions and Molecular Weights of Degraded 6-*O*-CM-Chitin

Samples	Reagents	Concentration	Volume (mL)	Time (h)	T (°C)	$M_w \times 10^{-4}$
CMC-1	Untreated					24.8
CMC-2	H_2O_2	30%	Several drops	0.5	75	18.3
CMC-3	H_2O_2	30%	0.5	3	75	14.2
CMC-4	H_2O_2	30%	2.0	4	75	8.1
CMC-5	H_2O_2	30%	5.0	4	75	3.1
CMC-6	H_2O_2	30%	10.0	4	75	1.0
CMC-7	HCl	9.0 mol/L	25.0	10	45	0.68
CMC-8	HCl	11.5 mol/L	25.0	10	45	0.26

[a] CMC, 6-O-CM-chitin.
Concentration of H_2O_2 was 30% (v/v).

Moisture-Absorption and Retention Property

The moisture-absorption and retention properties of dry and wet CM-chitin and CM-chitosan samples of different substitution sites were examined and compared with those of HA. Figure 3 demonstrates that the moisture-absorption properties of carboxymethyl chitins and chitosans are quite similar to those of HA; the weight of moisture absorbed increased rapidly in the first hours, slowed down in the later stage, then became constant 10–20 h later, in accordance with the result given by Shuichi et al.[3] The moisture-absorption ability is in the following sequence: HA > 6-*O*-CM-chitosan ~ 6-*O*-CM-chitin > 3,6-*O*-CM-chitosan > *N*-CM-chitosan > 3,6-CM-chitin (RH 81%); 6-*O*-CM-chitin > 6-*O*-CM-chitosan > 3,6-*O*-CM-chitosan > 3,6-CM-chitin > HA > *N*-CM-chitosan (RH 43%). When RH is 81%, moisture-absorption abilities of 6-*O*-CM-chitin and 6-*O*-chitosan are a little less than that of HA, but when RH is 43%, all carboxymethyl chitin and chitosan samples tested have higher moisture-absorption abilities than HA, except for *N*-CM-chitosan.

Figure 4 shows the result of the moisture-retention test in the saturated K_2CO_3 desiccator. Weight of residual moisture in the wet samples increased with time and became constant 10–20 h later when RH was 43%. That means all the samples tested have good moisture-retention ability. While in the silica-gel desiccator, all samples released water slowly, as well as HA. The moisture-retention ability follows the sequence of 6-*O*-CM-chitin > 6-*O*-CM-chitosan > 3,6-*O*-CM-chitosan > 3,6-CM-chitin > HA > *N*-CM-chitosan (RH 43%); 6-*O*-CM-chitin > HA > 6-*O*-CM-chitosan > 3,6-*O*-CM-chitosan > *N*-CM-chitosan > 3,6-CM-chitin (silica gel). In accordance with the result obtained from moisture-absorption test, 6-*O*-CM-chitin has better moisture-retention ability than HA.

Introduction of carboxymethyl group is a convenient and effective method to entitle chitin (chitosan) high water-retention ability. It is considered that 6-carboxymethyl group in the molecular structure of chitin and chitosan is a main active site. Carboxymethylation at OH-3 and N position also contribute to the moisture-absorption ability. A high degree of deacetylation helps to improve moisture-absorption ability in some way, but not essentially.

As shown in Figure 5, when RH was 81%, 6-*O*-CM-chitins with molecular weight lower than 3.0×10^{-4} demonstrated very high moisture-absorption ability. The weight of water absorbed increased with time in the whole process of the test, similar to some micromolecular moisture-reten-
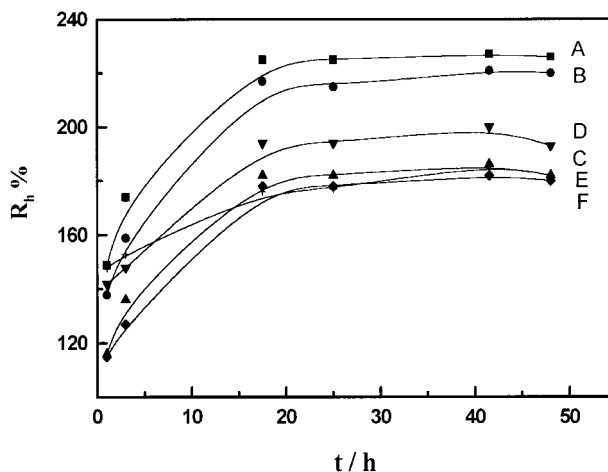

Figure 4   Moisture-retention ability of wet samples of carboxymethyl chitins, chitosans of different substitution sites, and HA at 43% relative humidity at 20°C.

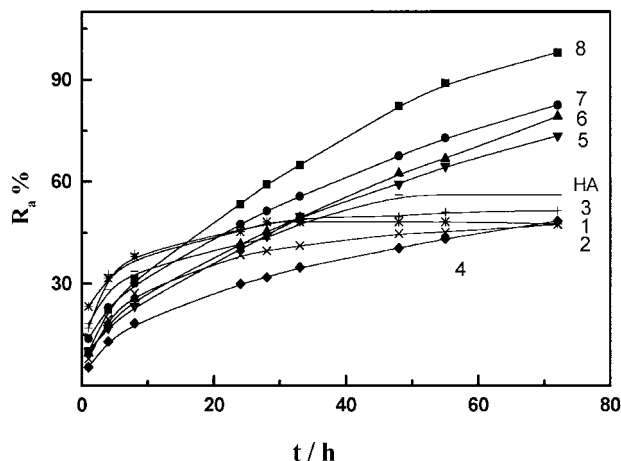**Figure 5** Moisture-absorption ability of dry samples of degraded 6-*O*-CM-chitin and HA at 81% relative humidity at 20°C.

Figure 6 Moisture-retention ability of wet samples of degraded 6-*O*-CM-chitin and HA at 43% relative humidity at 20°C.

tion agents such as sodium lactate (LAC). However, when molecular weight rises to 8.1×10^{-4}, moisture-absorption properties became similar to those of HA gradually, reached constant within 20 h. When RH was 43%, almost all samples absorbed moisture rapidly in the first 8 h, but they released water slowly in the latter hours in varying degrees and became constant after 20 h of the test. Although samples 7 and 8, which have the molecular weight higher than 18.0×10^{-4}, seem stable, their moisture-absorption curves are almost same with that of HA.

All wet samples of degraded 6-*O*-CM-chitin released water slowly in silica gel in the first 24 h, then reached constant gradually. In the saturated K_2CO_3 solution desiccator, as shown in Figure 6, only samples with molecular weight higher than 14.2×10^{-4} have moisture-absorption abilities; samples with lower molecular weight released water slowly in the test, but they all reached constant after 20 h of the test.

Figures 7 and 8 show the effects of molecular weight on moisture-absorption and retention ability of 6-*O*-CM-chitins after 48 h of the test. When RH was 81%, as molecular weight increases, the moisture-absorption ability decreases and gradually levels off when molecular weight is higher than 8.1×10^{-4}. Although when RH was 43%, moisture-absorption ability increases until molecular weight reaches 14.2×10^{-4}, then levels off regardless of molecular weight. It is apparent in Figure 8 that moisture-retention ability is influenced by the molecular weight. Samples with molecular weight lower than 3.1×10^{-4} have little
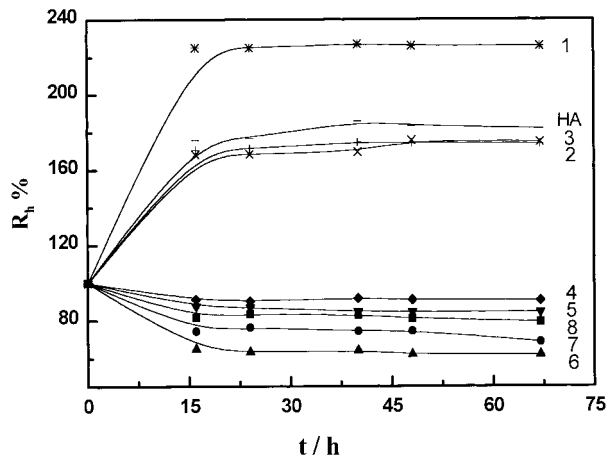
water retention ability, and increasing the molecular weight helps to improve moisture-retention ability.

It is clear that molecular weight plays an important role in moisture-absorption and retention ability. 6-*O*-CM-chitin with molecular weight above 14.2×10^{-4} forms networks easily, which helps to improve moisture-retention ability, because they prevent water from going out. However, in high humidity circumstances (RH 81%), carboxymethyl chitins with low molecular weight dissolved in water so rapidly that they became solutions after 24 h of the test. In such processes, they absorbed a large amount of water; thus, they have a very high moisture-absorption ability.
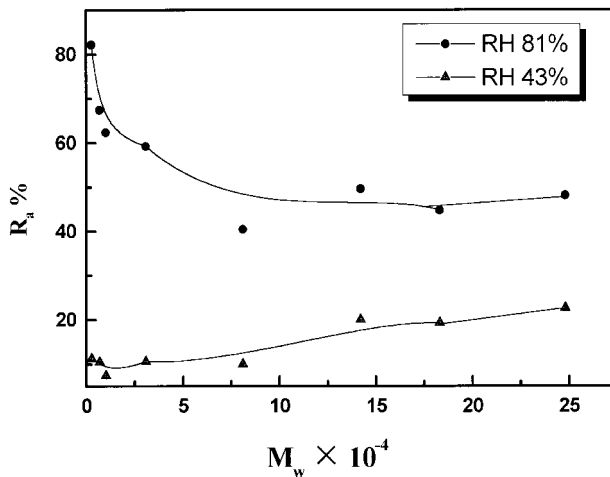

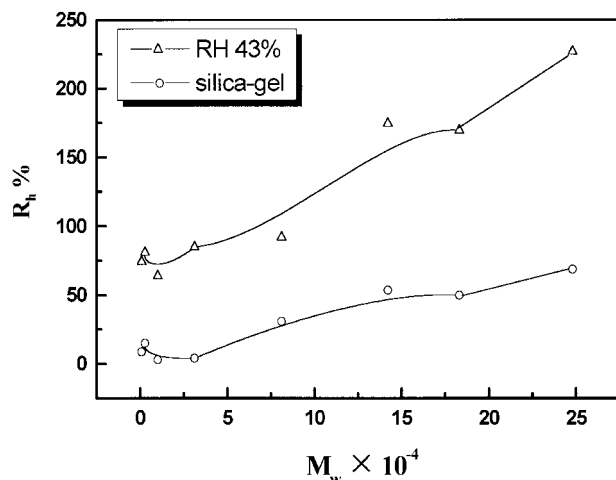**Figure 7** Effects of M_w of 6-*O*-CM-chitin on moisture-absorption ability.

Figure 8 Effects of M_w of 6-*O*-CM-chitin on moisture-retention ability.

CONCLUSION

By the moisture-absorption and retention test, it is considered that the 6-carboxymethyl group in the molecular structure of chitin and chitosan is a main active site for moisture absorption and retention; carboxymethylation at OH-3 and N position also contribute to the moisture-absorption ability. Molecular weight is another important factor to regulate moisture-absorption and retention ability; higher molecular weight results in better moisture-retention ability. 6-*O*-CM-chitins and 6-*O*-CM-chitosans with a degree of substitution higher than 0.8 and molecular weight higher than above 24.8×10^4 have the potential to substitute for HA to use in cosmetics and in clinical medicine.

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