A Simple Method for the Preparation of Stable Chitooligomers

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ABSTRACT: The chitosan was degraded by the cheap, efficient, and commercially available enzymes in hydrochloric acid solution. The effect of molar ratio of added HCl to $-NH_2$ of chitosan on the degradation was investigated, and the favorable ratio was between 0.50 and 0.95. The chitooligomer hydrochlorides were directly prepared by this simple method. The chitooligomer hydrochlorides were characterized by thermogravimetric analysis, differential thermal analysis, and Fourier transform infrared. These results show that the obtained chitooligomer hydrochlorides were much more stable than the chitooligomer acetates. This preparation procedure of chitooligomer hydrochlorides was simple and cheap, and helpful for the preservation of chitooligomers. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 956–961, 2009

Key words: chitooligomer hydrochloride; enzymatic degradation; stability

INTRODUCTION

Chitosan is a linear heteropolysaccharide made of β-1.4-linked D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) in various compositions. Chitosan is mostly available in the exoskeletons of shellfish and insects.[1] It is a nontoxic, biodegradable, and biocompatible polysaccharide. Chitosan is widely used in foods, pharmaceutics, and cosmetics.[2–5] Usage of chitosan in diverse areas is directly related to the polymer's molecular weight and degree of deacetylation. Chitooligomers have some additional physiological abilities such as antitumor and enhancing immunity because of their good water-solubility and low molecular weight.[6–9]

Chitooligomers were commonly achieved by enzymatic depolymerization of chitosan in weak acid solution such as acetic acid,[10] lactic acid,[11] and ascorbic acid.[12] The obtained chitooligomer products were actually the salts of chitooligomers with the added weak acids. However, the defect is that chitooligomer products can easily turn brown in the process of preparation, thermal drying, and preservation.[13] These undesirable effects may be minimized by freeze-drying and proper storage conditions in laboratory scale. To be economically feasible and technically acceptable, large-scale production of chitooligomers should be based on simple preparation, separation, and thermal drying.[14]

Glucosamine hydrochloride has been used in various forms for osteoarthritis in the Europe for decades. Glucosamine hydrochloride was much more stable in the process of preparation and preservation, suggesting that chitooligomers might be stable in their hydrochlorides. Chitooligomer hydrochlorides have antimicrobial activity. Although chitooligomer hydrochlorides could be directly prepared by concentrated hydrochloric acid, the yield was very low and the main product was monomer. The yield of chitooligomers from enzymatic hydrolysis was greater than that from strong acidic hydrolysis.[15]

In this article, to obtain much more stable chitooligomers, hydrochloric acid solution was used as solvent to dissolve chitosan. The cheap, efficient, and commercially available enzymes were employed to degrade chitosan, and chitooligomer hydrochlorides were achieved. Moreover, the stability of the products was evaluated.

EXPERIMENTAL

Materials and equipments

Commercial cellulase and hemicellulase were purchased from Wuhan (China). Commercial product

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α-amylase was purchased from Beijing Xing'ao Science and Technology Co. (China). Chitosan (CS0) with degree of deacetylation (DD) of 86% and glucosamine hydrochloride (GAH) were prepared in our laboratory. The UF membranes (OS001C11, OMEGA) with NMWL 5 kDa were purchased from PallFiltron Corporation (USA). Other reagents were of analytical grade.

Characterizations

Weight-average molecular weight (M_w) was measured by gel permeation chromatography (GPC). The GPC equipment consisted of the connected column (TSK G5000-PW and TSK G3000-PW), Spectra system P1000, and RI 150 refractive index detector. 0.2*M* CH_3COOH/0.1*M* CH_3COONa was used as the eluent. The flow rate was maintained at 1.0 mL/min. The standards used to calibrate the column were TOSOH pullulan. All data provided by GPC system were collected and analyzed using Jiangshen Workstation software package.

IR spectra were recorded on Nicolet Impact 380 spectrophotometer with KBr pellets.

TG and DTA of samples (5.0 mg) in dynamic nitrogen atmosphere were performed by a SDT Q600 Model DSC/TGA from 30 to 600°C at a heating rate of 10°C/min.

The enzymic degradation of chitosan in dilute acetic acid or hydrochloric acid

1.0 g chitosan CS0 was completely dissolved in 50.0 mL 2% (w/v) HAc solution or 0.2–0.4% (w/v) HCl solution. The chitosan solution was placed in water bath at 50°C for 1 h, and the enzyme (The mass of the same enzyme was the same in dilute HAc or HCl) was added to the solution to initiate the reaction. At various intervals, the mixture solution was taken out and boiled for 10 min to denature the enzyme. The product was analyzed by GPC.

Preparation of chitooligomer acetates sample

20.0 g chitosan CS0 was completely dissolved in 400.0 mL 2% (w/v) HAc solution. The chitosan solution was placed in water bath at 50°C for 1 h, then, 1.0 g α-amylase was added to the solution to initiate the reaction. After 6 h, the mixture solution passed out UF membrane of 5 kDa to separate the enzyme. The filtrate was concentrated to about one-fifth at 50–60°C under reduced pressure. The remaining solution was freeze-dried to give the product COS-HAc with M_w of 2.5×10^3.

Preparation of chitooligomer hydrochlorides samples

20.0 g chitosan was completely dissolved in 400.0 mL dilute HCl solution, and the pH of solution was about 5. The chitosan solution was placed in a water bath at 50°C for 1 h, and 1.0 g α-amylase was added to the solution to initiate the reaction. After 6 h, the mixture solution passed UF membrane of 5 kDa to separate the enzyme. The filtrate was adjusted to pH 3 by adding HCl and concentrated to about one-fifth under reduced pressure. Finally, the product precipitated after adding ethanol. The precipitate was washed with ethanol and dried at 60°C for 12 h to get the solid COS-HCl with M_w of 2.3×10^3.

Moisture-absorption capacity test of COS-HCl and COS-HAc

Three fractions of dry COS-HCl with M_w of 2.3×10^3 and three fractions of dry COS-HAc samples ($\sim$ 0.5000 g for each sample, the samples were prepared by α-amylase) were placed in a desiccator containing saturated $(NH_4)_2SO_4$ solution with 81% relative humidity. The weight of these samples was measured at the interval of 12 h. The moisture-absorption capacity of samples was evaluated by the percentage of weight increase of dry sample: R_a (%) $= 100\ (W_n - W_0)/W_0$, where W_0 and W_n were the weight of sample before and after being placed in a desiccator.

Estimation of the browning of COS-HCl and COS-HAc

COS-HCl and COS-HAc samples ($\sim$ 0.5000 g for each sample, these samples were prepared by α-amylase) were placed in a desiccator containing saturated $(NH_4)_2SO_4$ solution at 35°C. These samples were picked out at predetermined time and dissolved in distilled water. The UV absorbance of solution was measured by UV-Vis spectrophotometer at room temperature.

To evaluate the effect of thermal-drying on the browning of chitooligomer, 5% (w/v) COS-HCl and 5% (w/v) COS-HAc solution underwent thermal-drying at 100°C until they gave the solid product. The solid products were again added into distilled water with the final concentration of 0.25%, and the solutions measured the UV-vis spectra.

RESULTS AND DISCUSSION

The enzymatic degradation of chitosan in dilute hydrochloric acid

Samples were taken at intervals during the enzymatic hydrolysis with the aid of dilute hydrochloric acid solution as solvent. Figure 1 shows the gel

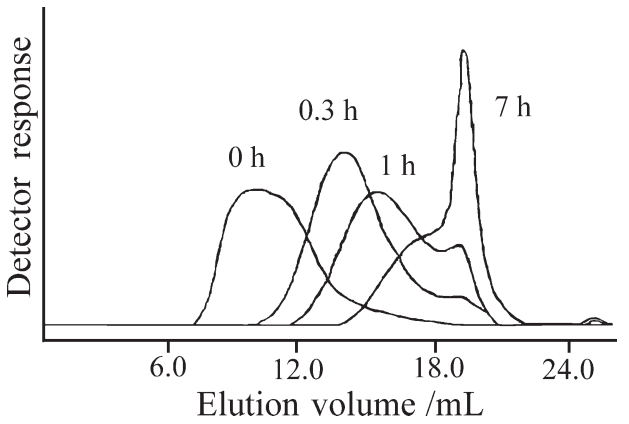

Figure 1 GPC profiles of enzymatic products of chitosan in HCl.

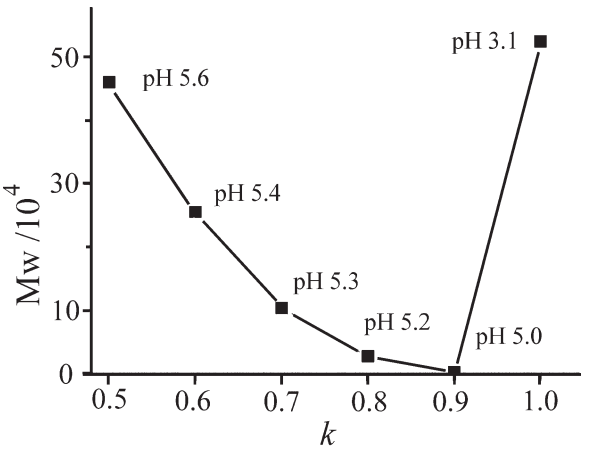

Figure 3 Effect of molar ratio k of added HCl and $-NH_2$ of chitosan on the degradation.

permeation chromatography (GPC) profiles of crude chitosan and its degraded products by the hemicellulase. The shift toward higher elution volume was a consequence of the reduction in average molecular weight. Obviously, the extent of degradation was increased by prolonging the duration.

HAc (2%) was the commonly used solvent for the enzymatic degradation of chitosan. Chitosan could be dissolved in hydrochloric acid solution, but HCl was rarely used as solvent for the enzymatic degradation of chitosan. Thus, the efficiency of degradation in the two solvents was compared with each other, as shown in Figure 2. Three commercially available enzymes were employed to degrade chitosan, respectively. The M_w of chitosan also decreased dramatically with the enzymes in the presence of HCl like HAc. Therefore, dilute HCl solution could be used as solvent for the enzymatic degradation of chitosan to prepare chitooligomers.

The pH of the solution was critical to the enzymatic degradation of chitosan in HCl solution. Figure 3 shows the effect of molar ratio k of the added HCl and $-NH_2$ of chitosan on the degradation by hemicellulase at 50°C for 4 h. When k was below 0.5, it was difficult for chitosan particles to dissolve and degrade. When k was more than 0.95, chitosan was easily dissolved in the dilute HCl solution. When k was 0.9, the pH of the solution was around 5, the optimum pH of hemicellulase. If the ratio was above 1.0, meaning the pH of the solution was below 3.1, the pH not suitable for the enzyme action on chitosan.

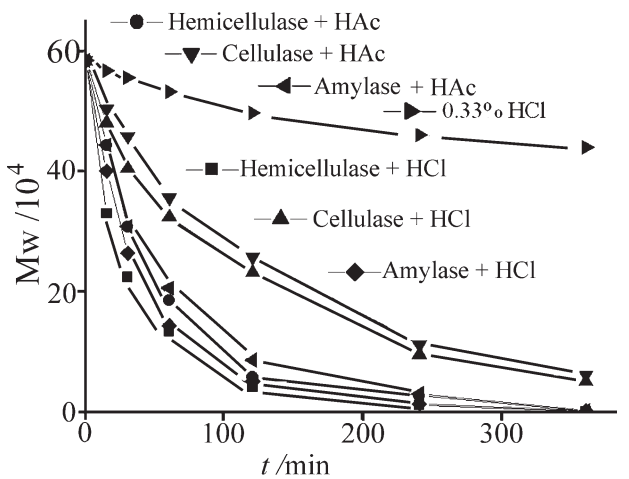

Figure 2 Degradation of chitosan by the enzymes.

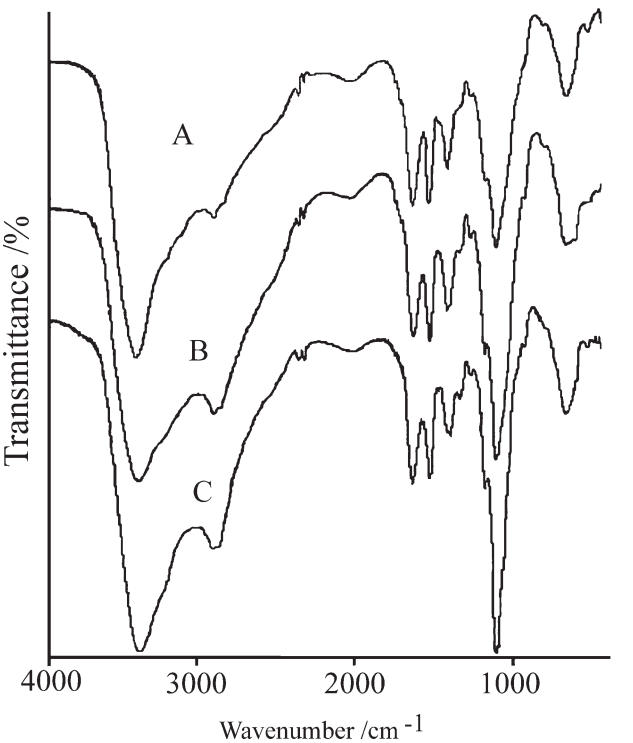

Figure 4 IR spectra of chitooligomer hydrochlorides prepared by cellulase (A) hemicellulase (B) and α-amylase (C).

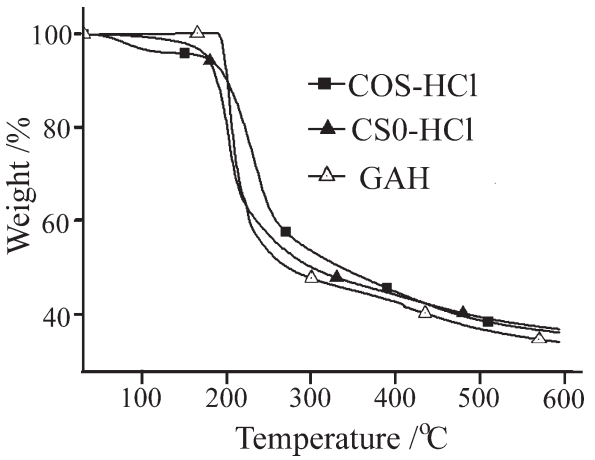

Figure 5 TG curves of COS-HCl, CS0-HCl, and GAH.

FTIR spectra

Figure 4 shows the IR spectra of three COS-HCl samples from enzymatic degradation of chitosan in dilute HCl solution. The characteristic peaks of $-NH_3^+$ vibration deformation appear at 1622 and 1513 cm^{-1}, which are almost the same as that of hydrochloride salt of chitopentaose $(GlcN)_5$ and the reported chitooligomer hydrochlorides.[16]

Thermal analysis

Figure 5 shows the TG curves of chitosan hydrochloride, chitooligomer hydrochloride, and glucosamine hydrochloride. The degradation of chitosan should be composed of a set of concurrent and consecutive reactions. When the temperature rose, all samples were substantially decomposed. The pyrolysis of GAH was at around 205°C, as can be seen in Figure 6. The decomposition of ammonium salt such as GAH had the endothermic effect. However, the disintegration of intramolecular interaction and the partial breaking of the molecular structure of COS had the exothermic effect.[17] Thus, the area of the endothermic peak increased with the decrease of M_W of the sample.

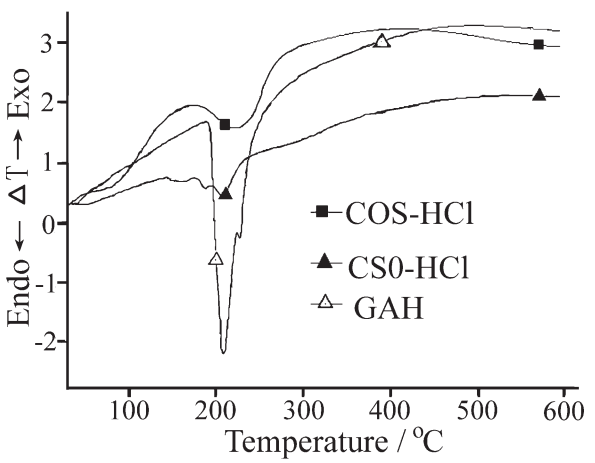

Figure 6 DTA curves of COS-HCl, CS0-HCl, and GAH.

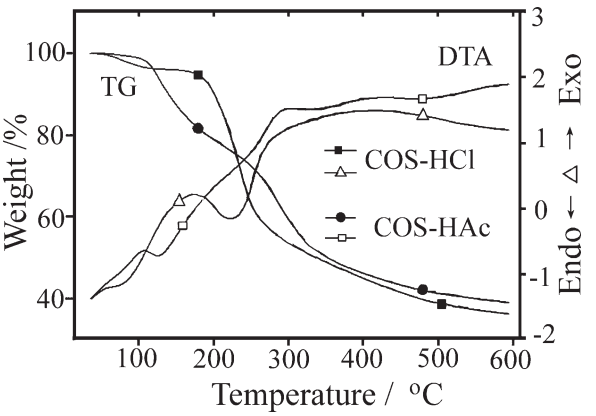

Figure 7 TG and DTA curves of COS-HCl and COS-HAc.

Figure 7 shows the TG and DTA curves of COS-HCl and COS-HAc. The sample COS-HCl had a weight loss before 120°C as a result of absorbed moisture.[18] It is easier to lose the absorbed water of COS-HCl than the absorbed water of COS-HAc. The temperature of initial pyrolysis for COS-HCl was at 170°C, while COS-HAc was at 110°C. COS-HCl was much more stable than COS-HAc. In conclusion, COS-HCl was stable during the process of thermal-drying.

Moisture-absorption capacity

The relative humidity was critical to the browning of chitooligomers, as studied in our previous article.[19] Moisture greatly activated the browning of chitooligomers. Figure 8 depicts the moisture-absorption curves of COS-HCl and COS-HAc at 81% relative humidity. The moisture-absorption capacity of COS-HCl was remarkably weaker than that of COS-HAc during the tested period. Thus, COS-HCl

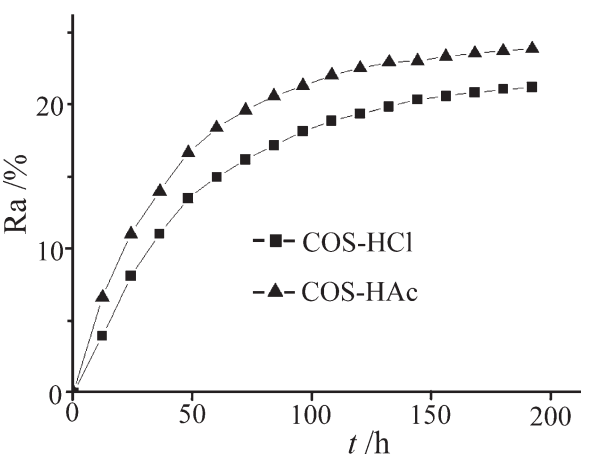

Figure 8 Moisture-absorption capacity of COS-HCl and COS-HAc.

absorbs less water than COS-HAc under the same conditions. The moisture-absorption capacity of COS-HAc would decrease sharply after the sample becomes brown.[19] In brief, the browning of COS-HCl might be slower than that of COS-HAc given same moisture.

The shelf stability of COS powder

After COS-HCl powder and COS-HAc powder were stored at 35°C for 6 days, the color of COS-HAc powder had significant change, whereas that of COS-HCl powder had not changed during that period. These samples were dissolved in distilled water with the final concentration of 0.25% (w/v). Figure 9 is the UV-vis spectra of these solutions. The absorbance peak at 278 nm became sharper with increase in storage time, which indicated that COS-HAc was browning. By contrast, there was no significant change for COS-HCl at 278 nm. Although both COS-HCl and COS-HAc contained a large amount of reducing sugar groups, COS-HCl seemed to be more stable than COS-HAc. The Maillard reactions occurred[20] and formed colored compounds even during preparation of COS-HAc, so the solution of initial COS-HAc sample also exhibited significant absorbance at 278 nm.

The stability of COS solution

5% COS-HCl and 5% COS-HAc solution were heated at 100°C for 70 min to give the dry product. The dried COS-HCl solid was easily dissolved in distilled water, but 80% of the dried COS-HAc sample was not soluble in distilled water and 2% HAc. The effect of thermal-drying on the browning of COS-HCl and COS-HAc was shown in Figure 10. The concentration of the dissolved samples was

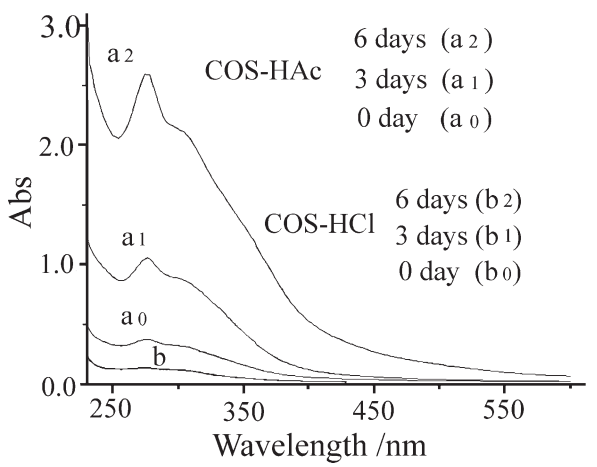

Figure 9 Effect of storage time on the browning of COS-HCl.

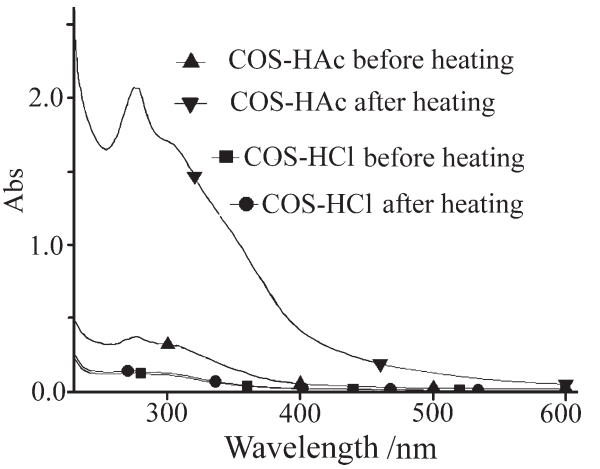

Figure 10 Effect of thermal-drying on the browning of COS-HCl and COS-HAc.

0.25% except that of the thermal-dried COS-HAc containing insoluble substances. After thermal-drying, the soluble substances in the dried COS-HAc sample had strong absorbance at 278 nm even at much lower concentration than 0.25%. There was no significant change for COS-HCl after thermal-drying. The results indicate that solid COS-HCl product could be achieved by thermal-drying, and the liquid COS-HCl product could be preserved on shelf for a long time even at a high temperature.

CONCLUSIONS

Chitosan could be degraded efficiently by these enzymes in dilute hydrochloric acid as in HAc solution. The favorable ratio of added HCl to $-NH_2$ of chitosan for this degradation was between 0.50 and 0.95. The obtained chitooligomer hydrochlorides were much more stable than chitooligomer acetates. This preparation procedure of chitooligomer hydrochlorides was simple and cheap, and helpful for the preservation of chitooligomers. The method is feasible for industrial production of commercial chitooligomers.

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