Synthesis and Characterization of Amino Acid Modified Chitooligosaccharides

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Summary: Amino acid modified chitooligosaccharides were synthesized by the new synthetic route. The chloroacetyl-chitooliogosaccharide intermediates were prepared under a mild condition via a reaction between chitooligosaccharide (COS) and chloroacetic anhydride. The intermediates were subsequently reacted with a variety of amino acids, e.g., glycine, aspartic acid, alanine, arginine, and serine, under a basic condition, yielding amino acid modified COS products. The degree of chloroacetylation was calculated based on new ^{1}H NMR absorption peaks at 3.80 and 3.94 ppm, corresponding to –NH–CO–CH_2–Cl and –O–CO–CH_2–Cl, respectively. The degrees of chloroacetylation determined were 0.40, 0.44, 0.62, and 0.93 when the mole ratios of chloroacetic anhydride to COS were 0.5, 1, 2, and 4, respectively. The chemical structures of the COS derivatives were also determined using ^{1}H NMR spectroscopy. The biological properties of the derivatives were evaluated. Cytotoxicity of the derivatives was assessed by a direct contact, using L929 cells. An MTT assay was a method of choice to evaluate the efficacy of the derivatives to enhance the proliferation of L929 cells.

Keywords: amino acid; chitooligosaccharide

Introduction

Chitosan is a partially deacetylated polymer of acetyl glycosamine obtained after alkaline deactylation of chitin. It is accepted as a non-toxic, biocompatible and biodegradable polymer which attracts scientific and industrial interest in such fields as biotechnology, pharmaceutics, wastewater treatment, cosmetics, agriculture, food science and textiles.[1] Chitooligosaccharide (COS) is a water-soluble material prepared by either chemical or enzymatic decomposition of chitosan.[2] Some studies on the chloroacetylation of chitosan have been reported previously. Kato and co-workers, for example, reported a procedure where chitosan was treated with chloroacetic anhydride in 4-dimethylaminopyridine (DMAP) used as a catalyst,[3] whereas Jenkins and Hudso studied the heterogeneous chloroacetylation of chitosan powder with either chloroacetic anhydride or chloroacetyl chloride.[4] The aim of this work was to synthesize COS-amino acid conjugates. Chloroacetylation was served as an important synthetic modification of COS, yielding chloroacetyl-chitooligochitosan as an intermediate product, under a mild condition. The intermediate was further reacted with various amino acids. The two-step synthetic route for the preparation of amino acid modified COS is presented in Scheme 1. The products obtained in each step were characterized. The synthetic parameters were simultaneously studied.

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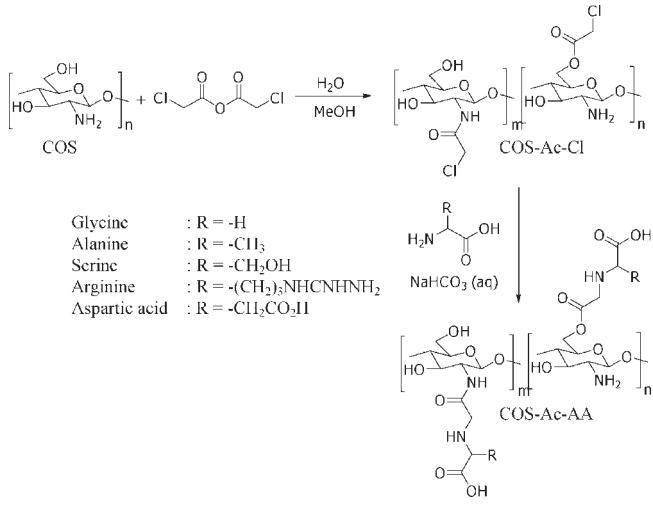
Experimental Part

Materials

Water-soluble chitooligosaccharide (COS) (95.5% degree of deacetylation, $\overline{M}_n = 2524$ g/mol) was directly prepared in our laboratory according to the method described in



Scheme 1.
Synthesis of chitooligosaccharide-acetyl amino acid (COS-Ac-AA).

the literature.[5] Glycine was purchased from Merck. L-arginine monohydrochloride, L-aspartic acid and sodium hydrogen carbonate were purchased from Fluka. L-alanine and L-serine were purchased from Acros. Chloroacetic anhydride was purchased from Aldrich. Reagent grade solvents for reactions and purifications were purchased from Lab Scan. All chemicals were used as received.

Synthesis of Chloroacetyl-Chitooligosaccharide (COS-Ac-Cl)

Chitooligosccharide (0.5 g, 3.0 mmol of GluN) was dissolved in water (20 mL) and methanol (40 mL). Then, chloroacetic anhydride (0.5, 1, 2 and 4 eq mole of COS) was added, and the solution was stirred overnight at ambient temperature. The mixture was concentrated by means of evaporation. The residue was precipitated in acetone, washed with methanol and dried under reduced pressure at room temperature.

Synthesis of Chitooligochitosan-Acetyl Amino Acid (COS-Ac-AA)

COS-Ac-Cl (0.25 g, 0.53 mmol of chloroacetyl) was dissolved in water (2 mL). Amino acid (0.31 mmol) was dissolved in aq. $NaHCO_3$ solution (0.2 M, 3 mL). Both solutions were mixed and stirred for 5 hr at room temperature. Then, the reaction mixture was neutralized at pH 7 by 2.5% v/v HCl (aq) and precipitated with acetone (90 mL). The resulting precipitate was repeatedly washed with methanol and then dried under reduced pressure.

Characterization

Chemical structure analysis and degree of substitution determination: The chemical structures of COS-Ac-Cl and COS-Ac-AA were confirmed by ^{1}H and ^{13}C NMR spectroscopy (Bruker DPX-300 spectrometer), whereas the degree of chloroacetylation (DC) was determined by ^{1}H NMR spectroscopy.

Cytotoxicity and cell proliferation assessment: The assessment was conducted using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, L929 cells were seeded at a density of 1×10^3 cells/well in 96-well plates and incubated at 37 °C for 48 hr. Afterward, the culture medium was removed from the wells, and the tested samples dissolved in a medium at different concentrations were placed into the wells and re-incubated for 48 hr. Then, MTT was added into all wells, and the whole plates were incubated at 37 °C for 4 hr. After

Table 1.
Degree of chloroacetylation (DC) of COS carried out under various conditions.

Run	Mole ratio		% Recovery	DC
	COS	$(ClCH_2CO)_2O$		
1	2	1	71	0.40
2	1	1	65	0.44
3	1	2	70	0.62
4	1	4	71	0.93

incubation, the medium and MTT were removed, and the obtained purple formazan products were subsequently dissolved in dimethyl sulfoxide (DMSO) and glycine buffer for the measurement of optical density values, subsequently converted to the percentage viability of cells after being in contact with the samples, using a Microplate Reader at 570 nm.

Results and Discussion

COS-Ac-Cl, which was later used as a starting material for the nucleophilic substitution by amino acid, was prepared by using various mole ratios of chloroacetic anhydride to COS (Table 1). The structural change of COS due to the chloroacetylation was monitored by both ^1H and ^{13}C NMR spectroscopy. Compared with the ^1H NMR spectrum of COS (Figure 1(a)), the spectrum of COS-Ac-Cl (Figure 1(b)) displayed the new signals at 3.80 and 3.94 ppm that were assigned to *N*-chloroacetyl (–NH–CO–CH_2–Cl) and *O*-chloroacetyl (–O–CO–CH_2–Cl), respectively. From the spectrum, the *N*-substitution appeared more predominant than the O-substitution as the intensity of the peak at 3.80 ppm was stronger than that of the peak at 3.94 ppm. In accordance with the ^1H NMR result, the ^{13}C NMR spectrum of COS-Ac-Cl (Figure 2(b)) showed the new absorption peaks of –CO–CH_2–Cl at 45.0 and 46.6 ppm and –CO–CH_2–Cl at 173.1 and 177.7 ppm. The degree of chloroacetylation (DC) was calculated accordingly from the ^1H NMR spectra and reported in Table 1.

The nucleophilic substitution of COS-Ac-Cl (DC = 0.40), synthesized by using mole ratio of COS to $(ClCH_2CO)_2O$ = 2:1), with various amino acids, such as glycine, alanine, serine, arginine and aspartic acid, was performed. The chemical structures of the synthesized derivatives were confirmed by ^1H NMR spectroscopy. The overlaid spectra shown in Figure 3(a–e) revealed the important signal at 3.45 ppm, corresponding to –CO–CH_2–NH–CH(R)–COOH in the COS-Ac-AA samples. It was too complicated to determine the degree of substitution due to the peak overlapping. Moreover, as observed from the spectra, there existed some unreacted chloroacetyl groups at 3.80 and 3.94 ppm and unremoved free amino acids.

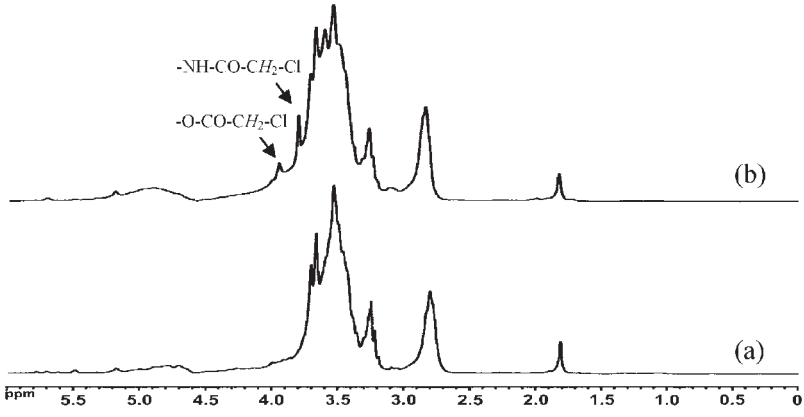


Figure 1.
The ^1H NMR spectra of (a) COS and (b) COS-Ac-Cl in D_2O.

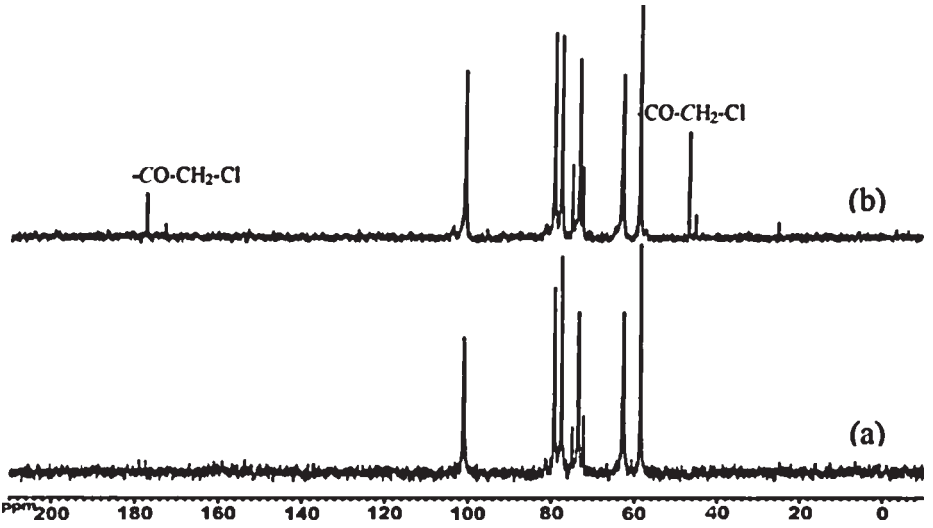


Figure 2.
The ^{13}C NMR spectra of (a) COS and (b) COS-Ac-Cl in D_2O.

The MTT results revealed that the samples were non-cytotoxic when used in the concentration range of 0.025–0.4 mg/mL. In addition, among the tested samples (the starting COS and its amino acid modified samples) there was no significant difference in % cell viability observed.

Conclusion

Amino acids, such as glycine, alanine, serine, arginine and aspartic acid, were successfully incorporated into chitooligosaccharide via a two-step process. Firstly, the intermediate products, COS-Ac-Cl,

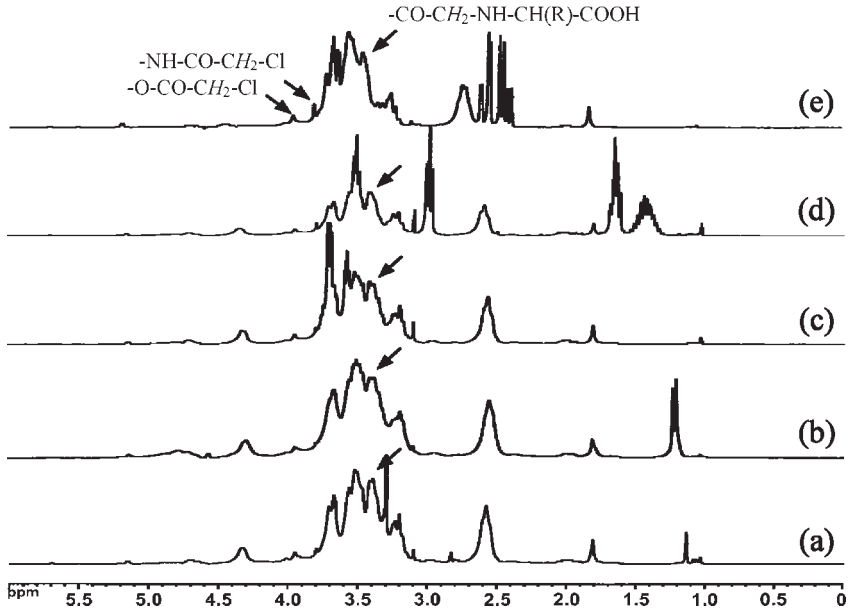


Figure 3.
The ^{1}H NMR spectra of (a) COS-Ac-Gly, (b) COS-Ac-Ala, (c) COS-Ac-Ser, (d) COS-Ac-Arg, and (e) COS-Ac-Asp in D_2O.

were synthesized by the chloroacetylation of COS with chloroacetic anhydride. Secondly, the intermediates were further reacted with such amino acids under a mild alkaline condition. Although the synthesized COS derivatives appeared noncytotoxic, they were unable to enhance the cell proliferation of the L929 cells, compared with their parent, COS.

[1] N. V. Majeti, K. Ravi, *Reactive & Functional Polymers* **2000**, *46*, 1.
[2] K. Suzuki, T. Mikani, Y. Okawa, A. Tokoro, *Carbohydrate Research* **1986**, *151*, 403.
[3] K. Kato, A. Utani, N. Suzaki, M. Mochizuki, M. Yamada, N. Nishi, H. Matsuura, H. Shinkai, M. Nomiza, *Biochemistry* **2002**, *41*, 10747.
[4] D. W. Jenkins, S. M. Hudson, *Journal of Polymer Science. Part A, Polymer Chemistry* **2001**, *39*, 4174.
[5] T. Kristoffer, M. V. Kjell, E. C. Bjorn, S. Olav, *Carbohydrate Research* **2001**, *33*, 137.

