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A synthesis of *O*-diethylaminoethyl chitosan and its binding ability of cholate and deoxycholate anion *in vitro*

Chun Ho Kim[1], Heung Jae Chun[2,*]

[1] Biomaterials Research Center, Korea Institute of Science and Technology, P.O. Box 131, Seoul 130-650, Korea
[2,*] Research Institute of Biomedical Engineering, Catholic Research Institutes of Medical Science, School of Medicine, Catholic University, Seoul 137-701, Korea

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Summary

The aim of this study was to investigate the bile salts adsorption behavior of the diethylaminoethyl (DEAE)-chitosan, *in vitro*. In order to prepare the DEAE-chitosan, where the DEAE groups are specifically introduced onto 6-OH groups of chitosan with minimal by-product, DEAE-chitin was prepared by reacting chitin with *N,N*-diethylaminoethyl chloride, and direct *N*-deacetylation of DEAE-chitin was carried out in 10% of an aqueous sodium hydroxide solution containing sodium borohydride. DEAE chitosan samples were incubated with cholic acid sodium salt and deoxycholic acid sodium salt in a tromethane buffer solution (pH 7.5) for 1 hour, and the bile salts adsorption performances of the samples were examined by HPLC. The modified chitosan samples showed improved bile salts adsorption performances compared to chitosan, and the absorption amount of bile salt to the samples increased with increase in the DEAE groups introduced. In most cases, the adsorption amount of deoxycholate to the samples was greater than that of cholate.

Introduction

The recent introduction of a new class of drugs able to reduce the levels of cholesterol has made a noteworthy contribution in the treatment of hypercholesterolaemia, one of the greatest risk factors in atherosclerotic diseases and cardiovascular complications [1]. Among the ways to reduce the cholesterol level in serum, a method that enhances the excretion rate of bile salts using the absorbent for bile acid has been highlighted [2]. Cholesterol is oxidized to form bile salts by the liver hepatocytes and secreted to the duodenum [3]. The secreted bile salts help to emulsify the large fat particles of food into many minute particles, and aid in the transport and absorption of the digested fat through the intestinal mucosal membrane [3]. Approximately 99% of the secreted bile salts absorbed in the ileum are transported to the liver through a hepatic portal vein and stored in the gall bladder until re-used. Introducing a bile salt absorbent in the ileum may decrease the adsorption rate of bile salts and bile acid-lipid complexes to the intestinal membrane. This results in an increase of cholesterol excretion and in turn lowers the cholesterol level in serum [4]. Since bile salts exist as an anion, bile acid, *in vivo*, the anion exchanging substances can be suitable candidates for bile salt absorbent. Therefore, some anion exchange resins [5], comprised of cholestyramine and/or cholestipol possessing free amine

* Corresponding author

groups have been used to treat hypercholesterolaemia. These anion exchange resins showed a lowering effect of cholesterol levels in the blood by approximately 20%, but they are generally known to exhibit problems in relation to proper dosages [6]. Therefore, other studies have been made to determine the hypocholesteromic effect through bile salt removal by using natural alimentary fibers in order to minimize the undesirable host responses to drugs [7-10].

Chitosan, the fully or partially deactylated chitin, is an attractive material that can be used as a hypocholesteromic agent, not only because chitosan has an improved biocompatibility compared to other synthetic bile salt absorbents, but also because the cationic characteristics of chitosan due to free amine groups in its chain has a high potential to absorb bile acid ionically [11]. In this study, chitosan was selected as the bile salts absorbent, and the diethylaminoethyl groups were introduced to chitosan in order to enhance the cationic properties of chitosan and to provide improved bile acid adsorption performances to chitosan. This paper reports a novel preparation method of DEAE-chitosan, and the bile salts absorption behavior of DEAE-chitosan *in vitro*.

Experimental

Materials and reagents

Chitin was isolated from crab shell according to the modified Hackman method [12, 13]. The degrees of deacetylation of chitin and chitosan were calculated by the following analysis data: Anal. Calcd for $[(C_8H_{13}NO_5)\cdot 0.5\,0H_2O]_n$: C, 45.28; H, 6.60; N, 6.60 Found C, 44.96; H, 6.50; N, 6.57, and Calcd for $[(C_6H_{11}NO_4)_{0.77}\ (C_8H_{13}\ NO_5)_{0.23}\cdot 0.70H_2O]n$: C, 42.30; H, 7.12; N, 7.64. Found C, 42.07; H, 6.54; N, 7.64. *N,N*-Diethylaminoethyl chloride ·H C l (DEAE-C l ·H C l), and $NaBH_4$ were purchased from Aldrich Chemical Co. Lysozyme from hen egg white(EC 3.2.1.17) supplied by Sigma Chemical Co. and cellulase(*trichoderma reesei*) supplied by Novo Nordisk Co. were used for the hydrolysis of the chitin derivatives. Cholic acid (Fluka Chemie Co.) and deoxy-cholic acid sodium salt (Jassen Co.) for adsorption experiment were used as provided.

Preparation of DEAE-chitin

Powdered chitin (5 g, 100 ~ 140 mesh) was dispersed in 100 mℓ of a 42 % aqueous NaOH solution, which was kept at room temperature for 3 hours inside a vacuum desiccator. The dispersion was stirred vigorously with 250 g of crushed ice made of deionized water at 0 °C to give a clear alkali chitin solution. DEAE- Cl ·HC l (10 mol equiv. DEAE-Cl ·HCl per pyranose unit), dissolved in 100 mℓ of deionized water was added to the above prepared alkali chitin solution by agitating at 0 °C for 2 hrs. The mixture was kept at room temperature for 24 hours, and dialyzed with cellulose tube (cutoff size 3000), and then poured into the excess of acetone. The precipitates (DEAE-chitin) were collected by filtration, and dried [14].

Preparation of DEAE-chitosan

DEAE-chitin of 2.6 g was dissolved into a 10 % of aqueous NaOH (130 mℓ) solution containing 0.2 g of $NaBH_4$, and heated up to 80 °C. After 9 hours of heating, the solution was cooled down to room temperature, neutralized with acetic acid, and concentrated by a dialysis. The concentrate was poured into acetone, and the precipitates (DEAE-chitosan) were collected by filtration, and dried.

Characterization of samples

The changes in chemical structure of the DEAE-chitin and DEAE-chitosan due to the chemical modifications were investigated by FTIR, ^{1}H NMR , and EA. FT-IR spectra were obtained using a Nicolet Magma-IR spectrometer 550, and ^{1}H NMR spectra were obtained by a Varian VX300 using D_2O as a solvent. The elemental analysis of C, H, and N was carried out by the Elemental Analyzer (Foss Heraeus Analysentechnik GmbH). The ionic exchange capacities of the samples were measured according to H. Egawa's method [28]. Briefly, the ionic exchange capacity was calculated as given below:

$$E\ (meq/g) = (0.1N \times X) / (weight\ of\ sample),$$

where, X: the amount of NaOH aq., used titration.

Determination of enzymatic susceptibility

The mixtures, consisting of 1.5 mℓ of 1 wt.% chitin derivatives in 0.05 M citric acid-0.1 M Na_2HPO_4 buffer (pH 5.6) and 0.2 mℓ of enzyme solution (2.5 mg/mℓ; lysozyme or cellulase), were incubated at 40 °C for 15 min. After incubation, the solution was put into a test tube, boiled to cease the enzyme activities, and then the precipitates were removed by centrifugation. The amount of reducing sugars produced in the supernatant were measured by UV spectrophotometer according to the modified Schale's method [15] using *N*-acetyl-D-glucosamine as a reference compound. The enzyme activities equivalent to the amount of the reducing sugar produced by the enzymes were expressed by μmol of N-acetyl-D-glucosamine/min./mg.

Preparation of crosslinked DEAE-chitosan beads

Chitosan and DEAE-chitosan were ground to be the powder of a 100~140 mesh. The powder was immersed in 1 wt.% glutaraldehyde solution for 3 hours in order to induce the surface crosslinking, and dried at 50 °C. The crosslinked powder was immersed to 0.05 M $NaBH_4$ solution for 48 hours, and immersed to 0.1 N HCl for 24 hours, respectively. The beads of salt type were prepared after filtering and drying.

Measurement of adsorption amount and binding constant

Tris hydroxymethyl aminomethane buffer (tromethane buffer) was prepared [16] by dissolving 50 ml of 0.05 M 2-amino-2-hydroxymethyl-1,3-propanediol to 40 ml of 0.05 M HCl solutions, followed by adding 100 ml of double distilled water at pH 7.5. A bile acid solution was prepared by dissolving 8 mM of cholic acid sodium salt (cholate) and deoxycholic acid sodium salt (deoxycholate) in a tromethane buffer solution at pH 7.5. Crosslinked chitosan and DEAE-chitosan of 50 mg was put into the 20 ml of bile acid solution and shaken at 37 °C, respectively. After a given period of incubation, 100 μl of supernatants were taken, and the amount of the bile acid remaining in the supernatant was analyzed.

Chitosan and DEAE-chitosan (10mg, respectively) dissolved in 4 ml of bile acid salt solution with desired concentrations were stirred in a water bath at 37 °C for 1 hour, and dialyzed. The filtrates were taken and analyzed by a dilution within the calibration limit. High Performance Liquid Chromatography (Spectra Physics SP8100 Liquid Chromatograph) was used to determine the adsorption amount from the difference between the concentration of the initial bile acid solution and the filtrate; the steady phase was S10 ODS

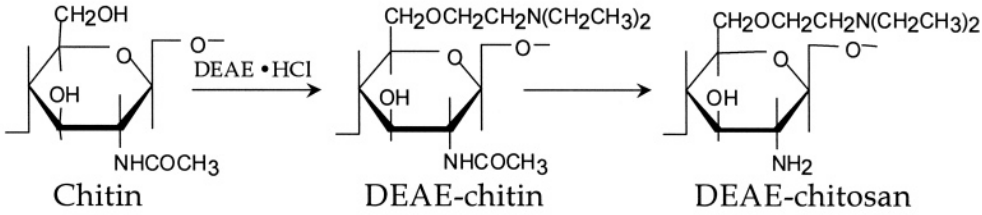

Fig. 1. Schematic representation for the preparation of DEAE-chitosan.

(0.46X 30 cm) column (Phase Sep. Co.), and the mobile phase was 77:23(vol. %) mixture of 0.1M MeOH/acetic acid. The flow (1ml/min) was detected by the RI detector (SP6040 Differential Refractometer Electronics Unit) at Range 4, where the column temperature was 35 °C. The binding constants were measured by plotting the adsorption data according to the Langmuir adsorption equation [26].

Results and discussions

Preparation of chitin derivatives

A pure white colored chitin was obtained from a crab shell according to a modified Hackman method, and DEAE-chitin was synthesized by introducing DEAE groups on the C-6 position of acetylglucosamine unit of chitin, as represented in Fig. 1.

Fig. 2 shows the IR spectra of chitin and its derivatives. Although there are no remarkable differences between the IR-spectrum of chitin (2-a) and that of DEAE-chitin (2-b), the intensities of C-H stretching bands of DEAE-chitin at 2900 cm^{-1} became somewhat stronger than those of chitin. In particular, two crystalline peaks at 1640 cm^{-1} and 1630 cm^{-1} due to a hydrogen bond [17] of α -chitin became single band. This strongly suggests that the deformation of the strong hydrogen bond in the chitin backbone is made due to the introduction of the DEAE groups to chitin. In addition, the absorption bands of amide I and II for DEAE chitin, however, seemed to become weaker to a small extent than those of chitin. That is, there existed a partial deacetylation during the substitution reaction in strong alkaline solutions.

Meanwhile, DEAE-chitosan was already synthesized and reported elsewhere [18, 19]. The reaction method proposed in a previous study was rather complex resulting in a low degree of substitution. Because chitosan was used as a starting material, the free amine groups of chitosan had to be protected with aldehyde in order to introduce DEAE groups onto C(6)-OH of chitosan selectively. In this study, however, DEAE-chitosan was directly prepared by deacetylation of DEAE-chitin to simplify the reaction process and to obtain the DEAE chitosan samples of the improved substitution degree. In general, chitin was treated in 50 % of NaOH aq. solution for 3 hours at 100 °C to obtain chitosan resulting in the decreased substitution efficiency of DEAE-chitin [11]. But, in this study, deacetylation was conducted under milder conditions to minimize the cleavage of the DEAE groups on C(6) position of chitin; DEAE-chitin was *N*-deacetylated by heating in 10 % of aq. sodium hydroxide solution containing sodium borohydride for 9 hours at 80 °C to produce DEAE-chitosan. FTIR spectrum of DEAE-chitosan confirms that the diethylaminoethyl groups are specifically introduced onto 6-OH group of chitosan with this procedure. Fig. 2 (c) shows that the amide II band in DEAE-chitosan appeared at 1570

cm^{-1} was shifted to 1554 cm^{-1}. Moreover, the intensity at 1640 cm^{-1} due to amide I of DEAE-chitosan also decreased compared to DEAE-chitin.

The C/N ratio and the viscosity of the samples that provide the information for the reaction conditions were summarized in Table 1. According to the C/N ratio, when the sodium borohydride was used in the deacetylation of DEAE-chitin, the content of DEAE group in DEAE-chitosan increases, while the content of DEAE group in DEAE-chitosan decreases when the deacetylation was conducted by thiophenol or sodium hydroxide only. However, the viscosity data shows that thiophenol decreases the loss of molecular weight of DEAE-chitin to a minimum. Therefore, the data indicate that the direct deacetylation of DEAE-chitin provides the easy introduction of DEAE groups onto C(6) position of chitosan without side reaction and other treatments. Additionally, it might be thought that the ether-formed modified chitosan derivative, with a proper molecular weight and with the degree of substitution, could be obtained by the control of the contents of the deacetylation agents.

The ^{1}H NMR spectra of DEAE-chitin and its derivatives are shown in Fig. 3. The characteristic peaks of DEAE-chitin were observed at 1.05 ppm for the methyl in the DEAE groups, at 2.64 ppm for the methylene, at 2.05 ppm for the methyl protons in the N-acetyl group, and at 3.4-4.0 ppm for the proton of pyranose ring. The peaks at 2.05 ppm due to acetyl protons for DEAE-chitosan also appeared.

The degree of substitution of DEAE-chitin and DEAE-chitosan were estimated by ^{1}H NMR spectra together with elemental composition and titration, as summarized in Table 2. For DEAE-chitin, the degree of substitution of DEAE groups was found to be 0.81. After deacetylation of DEAE-chitin, the degree of deacetylation and the substitution of DEAE-chitosan were 0.73 and 0.67, respectively. The results indicate that sodium borohydride used for deacetylation affects DEAE groups of DEAE-chitin in minimum, resulting DEAE-chitosan of high degree of substitution.

Enzyme activities of chitin derivatives

The activities of cellulase and lysozyme to DEAE-chitin and DEAE-chitosan are

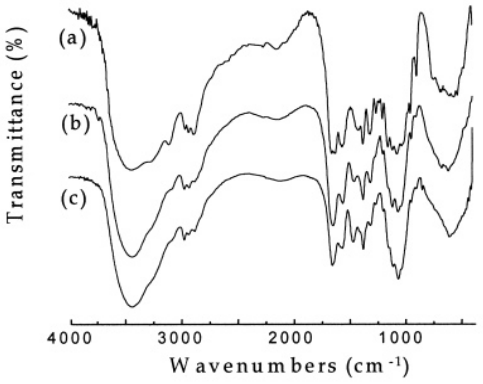

Fig. 2. FT-IR spectra of (a) Chitin, (b) DEAE-chitin, and (c) DEAE-chitosan.

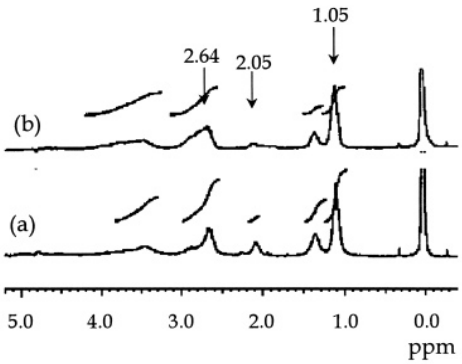

Fig. 3. ^{1}H NMR spectra of (a) DEAE-chitin, and (b) DEAE-chitosan.

Table 1. Effect of oxygen scavenger on the *N*-deacetylation of DEAE-chitin.

Samples	Slurry composition [1]	C(%)	H(%)	N(%)	C/N(%)	Viscosity in cPs [2]
1	-	41.20	7.30	6.94	5.94	2.8
2	Thiophenol 0.1ml	39.80	7.08	6.71	5.93	52.5
3	Sodiumborohydride 0.1g	36.15	6.81	6.27	5.76	13.5
4	Thiophenol 0.1ml + Sodiumborohydride 0.1g	33.00	6.34	5.69	5.80	27.5

1) 10wt% aq. NaOH 50ml : DEAE-chitin 1g at 80°C for 9h.

2) SI unit : 1Ps = 0.1 Pa ·s, Viscosity determined at 20°C, shear rate of 70.0s^{-1}.

Table 2. Elemental analysis and degree of substitution of DEAE-chitin derivatives.

Samples	C(%)	H(%)	N(%)	C/N(%)	Degree of substitution [1]	
					D.A.(%)[2]	D.S.(%)[3]
DEAE-chitin	51.61	8.97	8.72	5.92	0.41	0.81
DEAE-chitosan	48.46	8.52	8.49	5.71	0.73	0.67

1) Relative areas of 1H NMR signals, 2) Degree of deacetylation, 3) Degree of substitution of DEAE groups

Table 3. The susceptibility of lysozyme and cellulase on DEAE-chitin derivatives.

Samples	Reducing sugar value (μmol/min/mg.)	
	Lysozyme	Cellulase
DEAE-chitin	1.395	0.993
DEAE-chitosan	1.041	0.941

summarized in Table 3. The susceptibility of chitin derivatives to lysozyme was higher than that of cellulase. Since lysozyme has a substrate selectivity to *N*-acetyl-D-glucosamine [20], lysozyme activities increased in the order of DEAE-chitin and DEAE-chitosan, depending on the extent of *N*-acetyl-D-glucosamine groups of the samples. On the other hand, cellulase shows the enhanced enzyme activity to chitosan, because it hydrolyzes β-1,4 glycosidic linkages of chitosan [21].

Adsorption behaviors

The monomolecular adsorption of adsorbate molecules from solution, at constant temperature, onto adsorbent may be described by the following Langmuir type equation(26):

$$x/m=(k_1k_2C_{eq})/(1+k_1C_{eq}) \quad \text{(Eq. 1)}$$

$$C_{eq}\, x/m=1/(k_1k_2)+C_{eq}/\, k_2 \quad \text{(Eq. 2)}$$

C_{eq} : the concentration of adsorbate remaining in solution at equilibrium; x: the amount of adsorbate bound to the adsorbent, and m: the amount of adsorbent employed. The constant k_1 may be defined as the adsorption coefficient or affinity constant, which is related to the magnitude of the forces involved in the binding process. The Langmuir-capacity constant, k_2, indicates the apparent maximum amount of adsorbate that can be adsorbed per unit weight of adsorbent. Eq. 2 suggests that a plot of C_{eq} x/m cersus C_{eq}, on rectilinear coordinates should yield a straight line from which one can obtain the constants k_1 and k_2.

Figure 4 shows the linearity observed with all the adsorbate anions under investigation,

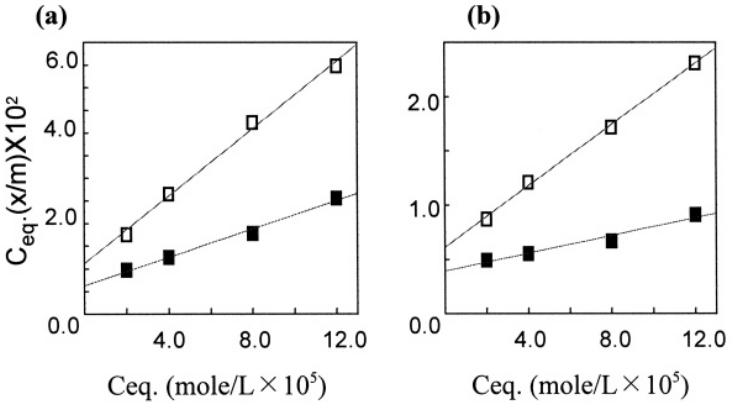

Fig. 4. Langmuir adsorption isotherms for the binding of (a) cholate and (b) deoxycholate anion to chitosan and DEAE-chitosan at pH7.5 and 37℃: (□) Chitosan, (■)DEAE-chitosan.

and demonstrate the adherence of the binding process to the Langmuir type adsorption isotherm (Eq. 2). The adsorption constants k_1 and k_2, obtained from the intercept and slope values of these figures, with the ion exchangeability are reported in Table 4. DEAE-chitosan adsorbed more amount of the cholate than the crosslinked chitosan. This can be attributed the fact that the increase in the amount of DEAE groups increases the ionic exchange capacity of chitosan, resulting the enhanced adsorption of the cholate anions.

Chitosan and DEAE-chitosan showed similar adsorption tendency to cholate and deoxycholate. But the adsorbed amount of deoxycholate to the samples remarkably increased compared to those of cholate. This kind of adsorption behavior that the deoxycholate with 2 hydroxyl groups was adsorbed more than the cholate with 3 hydroxyl groups is similar to those of commercial bile salt adsorbents [6,24,25]. Even though, the primary mode of interaction of bile-salt anions to chitosan is most probably electrostatic in nature, the secondary binding forces may play another role in binding bile-salt anions. These forces, being non-electrostatic in nature, would involve interactions between the hydrophobic regions of the adsorbate and adsorbent molecules. The results obtained in this investigation are consistent with this hypothesis in that the more hydrophobic di-hydroxyl anions are more strongly held by the DEAE matrix of the anion-exchange resin than more polar tri-hydroxyl counterparts [27].

Table 4. Langmuir adsorption constants for the binding of bile salt anions to chitosan and DEAE-chitosan.

Samples (Ion exchangeability)	Bile salts	K_1 (l./moles of bile salt X 10^{-4})	K_2 (Moles of adsorbate bound per mole equiv. of resin)
Chitosan (4.3meq/g)	Cholate	2.977	0.902
	Deoxycholate	4.301	1.646
DEAE-chitosan (6.4meq/g)	Cholate	3.961	1.611
	Deoxycholate	9.596	2.556

Conclusions

1. In order to obtain the polycationic adsorbents of bile acid salt, DEAE-chitosan that DEAE groups selectively introduced onto the C(6)-OH of chitosan was synthesized by *N*-deacetylation of DEAE-chitosan. The structural changes in the samples were confirmed by using EA, FT-IR, and ^{1}H NMR.
2. *N*-deacetylation of DEAE-chitin using sodium hydroxide together with sodium borohydride was found to be more effective in the substitution ratio than with thiophenol alone. However, the molecular weight loss due to *N*-deacetylation could be prohibited by using thiophenol.
3. The DEAE-chitosan prepared in this study showed the enhanced adsorption performance of bile salts compared to chitosan. The adsorption amount of bile salts increased with increase in the DEAE groups introduced. For chitosan and DEAE-chitosan, the adsorption amount of deoxycholate was higher than that of cholate, which are ascribed to the ionic interaction between the bile salt and amine groups in chitosan and DEAE-chitosan, as well as the hydrophobic surface effect of cyclophenanothrene existing in bile acid salts.

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