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Improvement of ACE inhibitory activity of chitooligosaccharides (COS) by carboxyl modification

Ronghua Huang,[a,b] Eresha Mendis[a] and Se-Kwon Kim[a,*]

[a]*Department of Chemistry, Pukyong National University, Busan 608-737, South Korea*
[b]*Department of Water Chemistry, Wuhan University, Wuhan 430072, PR China*

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Abstract—In the present research, chitooligosaccharides (COS) were carboxylated with $-COCH_2CH_2COO^-$ groups to obtain specific structural features similar to Captopril®. Angiotensin I converting enzyme (ACE) inhibitory activity of carboxylated COS was studied and observed to enhance its activity with increased substitution degree. Further, Lineweaver–Burk plot analysis revealed that inhibition was competitive via obligatory binding site of the enzyme. This was accompanied with substitution of positively charged quarternized amino groups to COS with different substitution degrees, in which negative impact on ACE inhibition was observed.

1. Introduction

Hypertension is a major risk factor for the development of cardiovascular diseases and mortality in Western countries.[1] Angiotensin I converting enzyme (ACE; EC 3.4.15.1), is responsible for hypertension by producing vasoconstrictor angiotensin II and degrading vasodilator, bradykinin. Therefore, inhibition of ACE is considered to be an important therapeutic approach for controlling hypertension. Initial interest in ACE inhibitors began with the discovery of snake venom peptides that could compete well with angiotensin I, the natural substrate of ACE.[2,3] With the model development of catalytic structure of ACE, specific inhibitors that bind more precisely to the enzyme active site were developed.[4] Even though the synthetic inhibitors are remarkably effective as antihypertensive drugs, they often result adverse side effects. Certain functional foods containing ACE inhibitory compounds have exhibited to act as alternative treatment for hypertension.[5] Therefore, during the past decades fundamental studies have opened a new field of study searching for ACE inhibitors from natural bioresources.[6,7]

Chitosan is a biodegradable, non-allergenic deacetylated derivative of chitin, a polysaccharides abundantly found in nature. Numerous studies have demonstrated that chitosan has various biological activities such as antimicrobial, antitumor, and immune enhancing effects.[8] Chitooligosaccharides (COS), partially hydrolyzed products of chitosan are of great interest in pharmaceutical and medicinal applications due to high solubility and non-toxicity. Moreover, recent advances in understanding the structure and properties of chitosan and its derivatives have opened new avenues for its applications.[9] Improvement of structural properties of chitosan for a particular application can be easily brought about by chemical modifications. However, researches on synthesis of COS derivatives and identification of their biological activities have not been reported so often. Therefore, studies aimed for developing new COS derivatives and to test their bioactivities are of interest.[10,11]

Recently, ACE inhibitory activity of COS was identified and brought about a new non-peptidic group of competitive inhibitors.[12] However, properties of COS that would be beneficial to interact with the active site of ACE, are still remain to be elucidated and further studies on this would contribute to the knowledge for the development of safe and novel ACE inhibitors. According to already confirmed interactions of ACE inhibitors such as Captopril® and Lisinopril® with ACE, it can be presumed that specific structural characteristics along with higher negative charge density contribute for a

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* Corresponding author. Tel.: +82 51 620 6375; fax: +82 51 628 8147; e-mail: sknkim@mail.pknu.ac.kr

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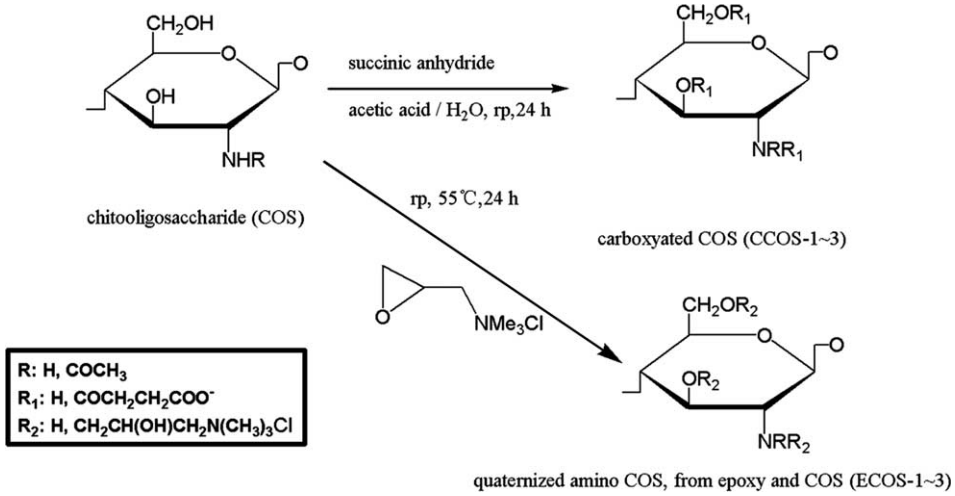


Scheme 1. Synthesis of COS derivatives. Two groups, negatively charged carboxyl groups and positively charged quaternized amino groups were separately introduced to COS.

greater affinity to the enzyme.[13] Therefore, in the present research we introduced negatively charged carboxyl group to COS with structural similarities to Captopril® (see CCOS-1–3 in Scheme 1) and hypothesized that it would increase ACE inhibitory activity. In addition, positively charged quaternized amino groups were also separately introduced to COS and studied their influence on ACE inhibition (see ECOS-1–3 in Scheme 1).

2. Results and discussion

2.1. Structural confirmation of new COS derivatives

Substitution of carboxyl or quaternized amino groups was clearly confirmed by ^{13}C NMR, ^{1}H NMR, and FT-IR spectra of COS derivatives. In comparison to the FT-IR spectrum of COS (Fig. 1A), both symmetric

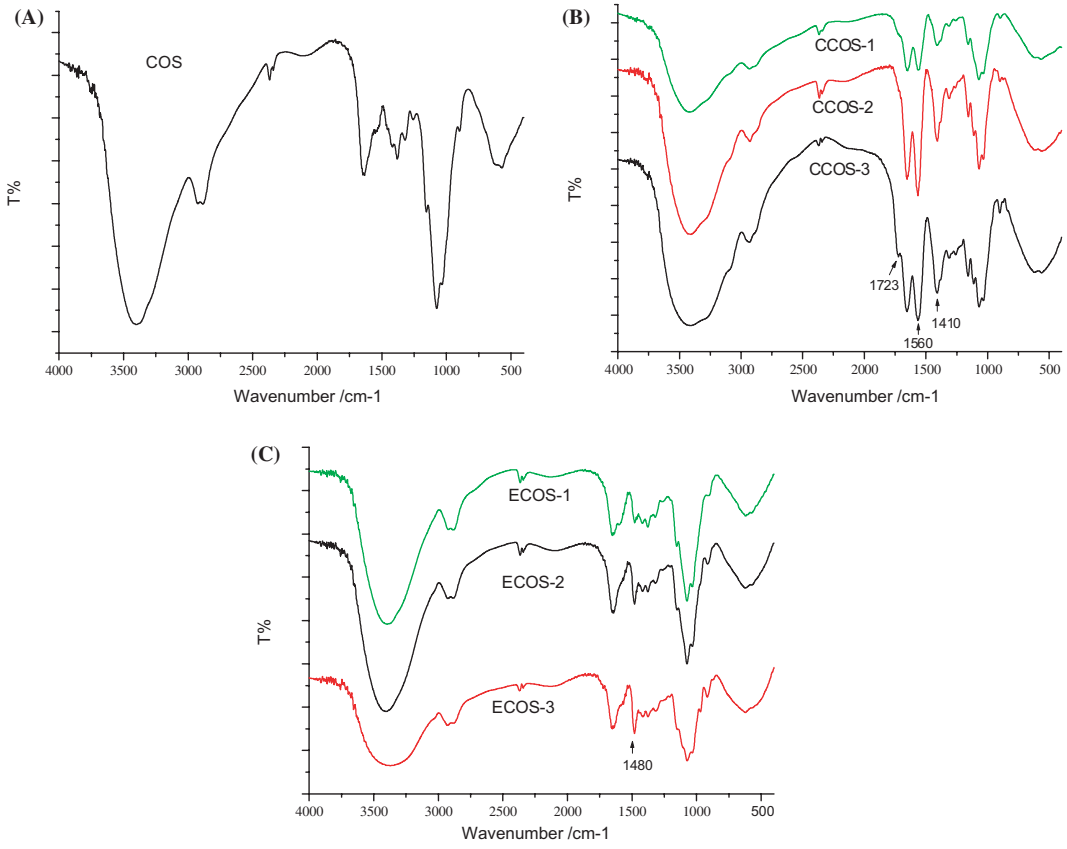


Figure 1. FT-IR spectra of (A) COS and its (B) carboxyl, or (C) quaternized amino derivatives. Derivatives with increasing substitution degree exhibited increased absorption intensities such as 1560, 1410 (COO^-), 1723 (C=O), and 1480 cm^{-1} ($N–CH_3$).

and asymmetric stretch absorptions of carboxyl groups (1560 cm^{-1} and 1410 cm^{-1}, respectively, as in Fig. 1B),[14–16] and the bend absorption of $N(CH_3)_3^+$ (1480 cm^{-1} as in Fig. 1C)[17,18] confirmed the successful introduction of new groups. In addition, different substitution degrees of carboxyl and quaternized amino groups were also clearly observed by increased absorption intensities of substituted groups following adjustment of conditions required for higher substitution. However in highly substituted carboxylated COS derivative (CCOS-3), in which the highest succinic anhydride/COS ratio was applied, a new peak was observed at 1723 cm^{-1} and it was assigned to the carbonyl absorption of ester groups.[14,15] This suggested that, even though amide groups were dominantly resulted by the reaction of anhydride and COS, esterification was also possible due to excess anhydride. But for the quaternized amino COS, it was hard to identify the substitution position based only on FT-IR spectra.

As shown in Figure 2, information of ^{13}C NMR spectra of the derivatives was also in agreement with FT-IR spectra and clearly confirmed the substitution of carboxyl and quaternized amino groups to COS. According to these data, the original material, COS clearly exhibited characteristic peaks (for example, δ 102 ppm, C-1; δ 54 ppm, C-2; δ 61 ppm, C-6; δ 174 ppm, carbonyl carbon C=O; δ 22 ppm, CH_3; etc.). Comparing to COS, CCOS-3 presented some new chemical shifts for $COCH_2CH_2COO^-$ group at δ 180 ppm, δ 176 ppm, and δ 32 ppm (assigned to the carbonyl carbon –COO, –CO, and $–CH_2CH_2–$, respectively).[14,17] Moreover, ECOS-3 also presented some characteristic chemical shifts for $–CH_2CH(OH)CH_2N(CH_3)_3^+$ group at δ 54 ppm (NCH_3, NCH_2), δ 64 ppm, and δ 65 ppm (CH_2CH), respectively.[15,19]

Further, ^1H NMR spectra were used to confirm the existence of substituted groups. A new chemical shift that appeared in spectra of CCOS-3 at δ 2.4 ppm was assigned to protons of $–COCH_2CH_2CO–$ groups (Fig. 3). On the other hand, in ^1H NMR spectra of ECOS-3 chemical shifts appeared at δ 3.1 ppm (assigned to protons in quaternized amino groups $–NCH_3$ and $–NCH_2$), δ 3.3 ppm and δ 4.2 ppm (assigned to protons in $–NCH_2CH(OH)^-$ groups).[14,17] Further, ^1H NMR spectra of quaternized amino COS was utilized to analyze the substitution degree of samples. Because differences observed in calculated elemental ratio (C/N wt %) among COS and its derivatives were negligible, it was not suitable to clearly distinguish the substitution degree of quaternized amino groups by elemental analysis data. Therefore in this study, we analyzed substitution degree by comparing the integral calculus of chemical shifts at δ 3.1 ppm to other shifts belong to protons assigned to pyranose unit and quaternized amino groups including H-1–6 and $–NCH_2CH(OH)–$.

Substitution degrees of the derivatives obtained from elemental analysis data or ^1H NMR spectra are presented in Table 1 and these results indicated that, different functional derivatives of COS could be easily obtained by controlling the degree of substitution. Compared to COS, elution volumes of the derivatives, which were also included in Table 1, exhibited slightly higher

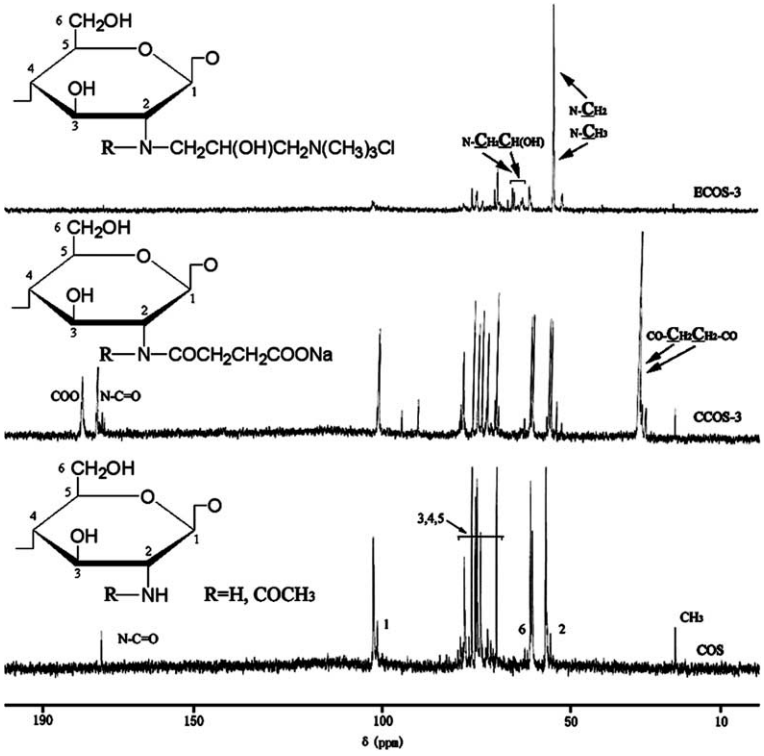


Figure 2. ^{13}C NMR spectra of carboxyl or quaternized amino derivatives of COS.

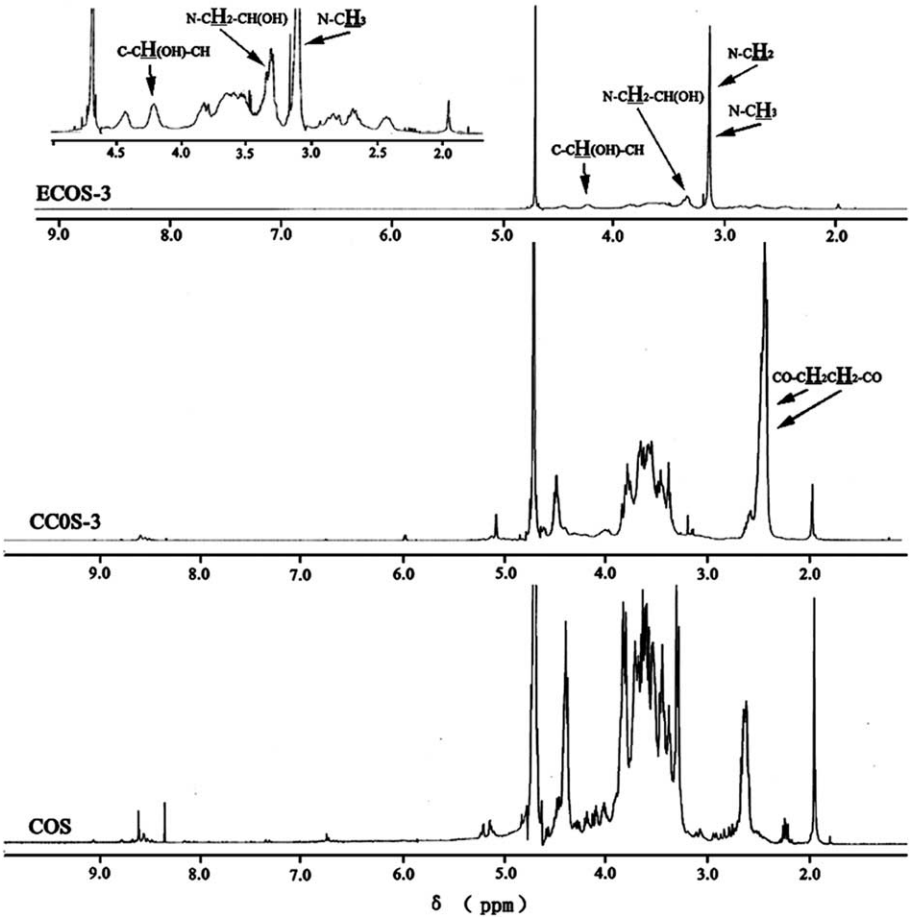


Figure 3. ^{1}H NMR spectra of ECOS-3, CCOS-3, and COS. Chemical modifications to COS was confirmed by peaks at δ 2.5 ppm (for –CH_2CH_2–, CCOS-3) and δ 3.1, 3.2, and 4.3 ppm (for quaternized amino groups, ECOS-3). The latter was used for substitution degree calculation.

Table 1. Elemental analysis and elution volume of COS and its derivatives

Sample	Elemental analysis (wt %)			Substitution degree	Elution volume (min)
	C%	N%	H%		
COS	43.39	7.825	6.815	0.2346[a]	16.67
ECOS-1	43.81	7.829	6.780	0.2624[b]	17.60
ECOS-2	45.89	7.798	7.644	0.5387[b]	16.48
ECOS-3	44.91	7.880	8.260	0.7674[b]	17.25
CCOS-1	43.20	6.351	5.630	0.3666[c]	15.90
CCOS-2	39.87	5.020	4.250	0.6992[c]	14.74
CCOS-3	39.77	4.610	4.924	0.8989[c]	14.90

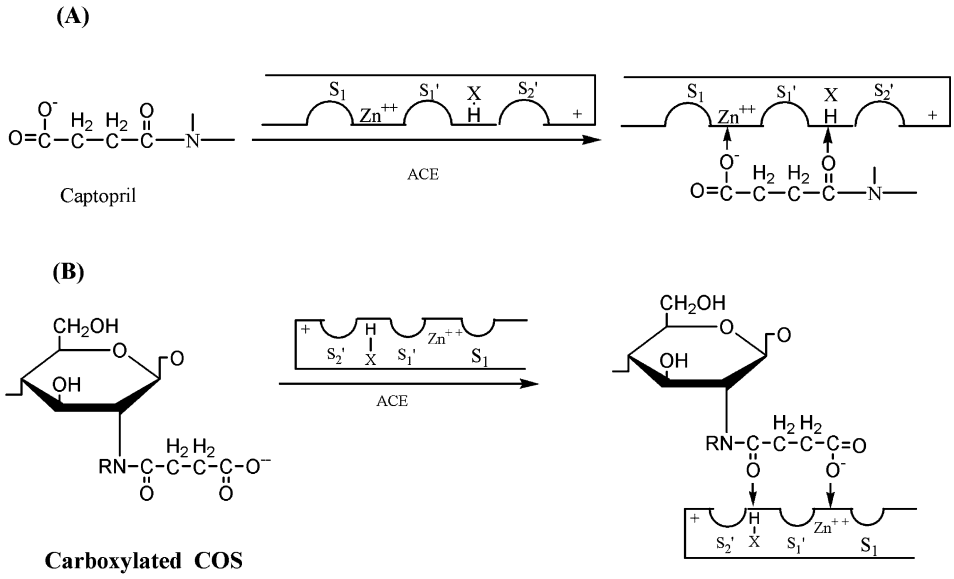
[a] Acetylation degree of COS calculated from C/N (wt %) ratio.
[b] Substitution degree of $–CH_2CH(OH)CH_2N(CH_3)_3$ groups calculated by comparing the integral calculus of chemical shifts at δ 3.2 ppm to other ones belong to pryanose unit (δ 3.2–4.6 ppm) as mentioned above.
[c] Substitution degree of carboxyl derivatives were calculated from C/N (wt %) ratio according to elemental analysis data.

values for quaternized amino derivatives and little lower values for carboxylated derivatives. These regular differences were presumed due to the configuration changes of negatively or positively charged groups linked to glucosamino units.[14,17]

2.2. ACE inhibitory activities of *N*-carboxylated COS with different substitution degrees

Only a few non-peptidic ACE inhibitors have been reported so far and Park et al.[12] recently discussed the effectiveness of COS for ACE inhibition. Authors have further stated that COS could inhibit ACE in competitive manner. According to Ondetti and Cushman,[20] Captopril®, one of the most effective synthetic ACE inhibitors compete with the natural substrate of the enzyme for several binding sites in the catalytic domain (Scheme 2A). These interactions confirmed that positively charged N-terminal of ACE attracts negatively charged inhibitors to its specific binding sites and the chemical group $^{-}OOC–CH_2CH_2–CO–N=$ is highly beneficial for ACE inhibition.

In this study, carboxyl groups were bonded to the nitrogen atom at C-2 position of the pyranose units as shown in Scheme 1. Formation of amide groups in carboxylated derivatives paved a way to obtain much similar structural features to Captopril® (Scheme 2B). Thus, it could interact with ACE by binding to the obligatory active site, which is active in converting angiotensin I into II, and subsequently enhancing the ACE inhibition. Considering these structural similarities and charged



Scheme 2. Hypothesized interaction between carboxylated COS and obligatory site of ACE (B) according to that of Captoril® (A).

nature it could be presumed that CCOS-1–3 should be more effective than COS to compete with the substrates for enzyme's obligatory binding site. Results obtained from ACE inhibitory studies of COS and carboxylated COS with different substitution degrees clearly demonstrated a greater inhibition with a high substitution degree (Fig. 4). All three derivatives with increasing substitution degree exhibited higher inhibition potencies than that of COS and the highest substitution degree was responsible for the highest percentage inhibition. For an example, at 5 mg/mL, ACE inhibitory activity of COS was 53.4%, while at the same concentration 94.3% inhibition was observed for CCOS-3. In addition, a marked dose-dependent inhibition was observed for all groups tested.

According to structural similarities it can be hypothesized that, carboxyl COS derivatives interact with ACE via specific obligatory active sites. To identify the inhibitory mechanism, Lineweaver–Burk plot analysis was carried out utilizing two concentrations of inhibitor. Both plots had exactly similar intercept confirming a competitive inhibitory mechanism (Fig. 5). Therefore, this confirmed our hypothesis that CCOS-3 inhibited ACE activity by specifically binding to the active site of ACE and by competing with its natural substrate. Improvement in ACE inhibitory activity of CCOS compared to COS might be due to these electrostatic interactions between positively charged sites of enzyme and negatively charged carboxyl groups similar to Captopril®.

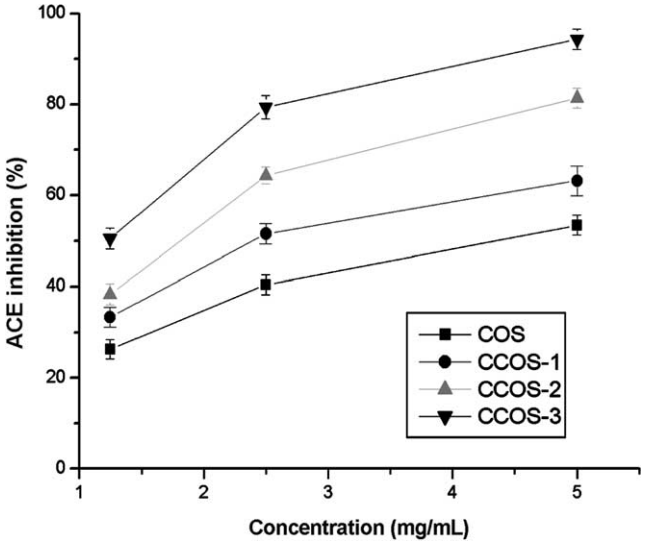


Figure 4. ACE inhibitory activities of *N*-carboxylated COS with different substitution degrees.

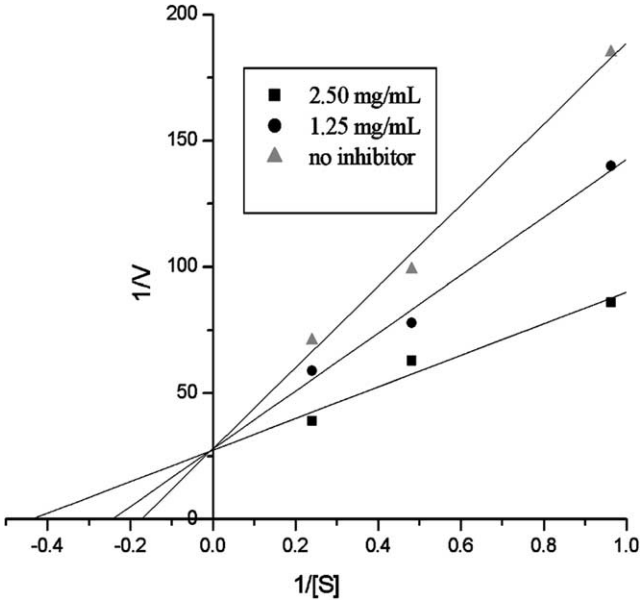


Figure 5. Lineweaver–Burk plot for the determination of inhibitory mode of ACE by CCOS-3. ACE inhibitory activity was determined in the presence (1.25 and 2.5 mg/mL) or absence of CCOS-3 as described in the text using HHL as the enzyme substrate.

Table 2. ACE inhibitory activities of quaternized amino COS with different substitution degrees

Sample	ACE inhibitory activity (%)		
	1.5 mg/mL	2.5 mg/mL	5 mg/mL
COS	26 ± 1.25	40 ± 1.89	53 ± 1.56
ECOS-1	21 ± 0.86	26 ± 0.99	28 ± 0.90
ECOS-2	10 ± 1.10	10 ± 0.95	9 ± 0.88
ECOS-3	Nd[a]	Nd[a]	Nd[a]

[a] Nd, not detected any ACE inhibitory activity.

In contrast, ACE inhibitory activity was markedly decreased following strongly positive charge quaternized amino group substituted to COS (Table 2). This inhibitory activity was also dose dependent, but increment in substitution dramatically decreased ACE inhibitory activity. At all concentrations of COS, inhibitory activity was relatively higher than the quaternized amino derivatives. Even though, low substituted quaternized amino derivatives (ECOS-1 and ECOS-2) possessed some inhibitory activities, the highest substituted derivative (ECOS-3) did not exhibit any activity at all tested concentrations. In previous research our research group found that deacetylation degree of COS influenced on ACE inhibitory activity and the optimal activity was observed at the lowest deacetylation degree (50%) in which the least free amino groups was contained.[12] Therefore, it could be said that the free amino groups was not beneficial to ACE inhibitory activity. It can be expected that the free amino groups get protonized and become positively charged at pH of assay medium (pH 8.3).[16] Because active obligatory site of ACE is positive charged, protonization of amino groups is not preferable for the inhibition.[20] This might be the reason for why free amino groups affected the inhibition of ACE negatively. Once electron-withdrawing acetyl groups are introduced, the electron density at nitrogen atoms decreases and become less attractive to hydrogen, resulting poorer protonization. As a result, attachment to ACE become easier and the inhibitory activity is expected to increase. In this research we introduced strongly positive charge quaternized amino groups to COS, and as hypothesized ACE inhibitory activity was greatly decreased.

3. Conclusions

In this study we found a facile way to modify the structure of COS and thereby to improve its ACE inhibitory activity. For this purpose, synthesis of carboxylated COS was carried out under mild conditions in which no possibilities for adverse influences on structural changes occurred. Therefore, substitution of $^{-}$OOC–CH_2CH_2–CO–N= was predominantly under control. Carboxyl groups were identified to be beneficial for ACE inhibition by enhancing the binding ability of COS to the obligatory active site of the enzyme. In addition, we found the negative influence of quaternized amino groups on the inhibitory activity. Furthermore, results of this research exemplify the possibilities of improving bioactivities of COS for their new potential applications.

4. Experimental

4.1. Materials and instruments

COS was kindly donated by Kitto. Life Co. (Seoul, Korea) with an acetylation degree of 27.92% (measured by elemental analysis) and molecular weight range 6.0–7.0×10^3 (measured by MALDI-TOF mass spectrometry).[21] 2,3-Epoxypropyl trimethylammonium chloride was prepared from 2,3-epoxypropyl chloride and trimethyl amine aqueous solution.[19] All commercial reagents including succinic anhydride, ACE from rabbit lung and its substrate (hippuryl-histidyl-leucine) were obtained from Sigma Chemical Co. (St. Louis, MO).

COS and its derivatives were desalted and purified using a Micro Acilyzer G3 (Asashi Kasei Corp., Japan) equipped with a 500 Da molecular weight cut-off dialysis membrane. Infrared spectra were recorded as clear signals on KBr plates with a Spectrum 2000 FT-IR spectrophotometer (Perkin Elmer, USA). Proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR) spectra were recorded in D_2O on a JNM-ECP-400 (400 MHz) spectrometer (JEOL Japan) and chemical shifts were expressed as parts per million (ppm) relative to tetramethylsilane as an internal standard. Elemental analysis (C, N, and H) was performed using an elementar analysesysteme (Elementar Vario, EL, USA) and were within ±0.4% of theoretical values. Accurate molecular masses of chitooligosaccharides were determined by MALDI-TOF mass spectrometry on a Voyager DE-PRO mass spectrometer (Applied Biosystems, USA) at a laser power of 3000 kW/cm^2 using 2,5-dihydroxybenzoic acid as the matrix. Average molecular weights of derivatives relatively to COS were measured by gel permeation chromatography (GPC) incorporated with a TSP P100 instrument. For this purpose, Bio-Gel P-3000 column (25 °C) was used and sodium acetate buffer (0.1 mol/L, pH 5.4) was applied to elute the sample at a flow rate of 1.0 mL/min. Sample concentrations were monitored by a refractive index detector (Sulfodex RI-71) and molecular weights were estimated by standard Pullulan® molecular weight markers (5.9×10^3, 2.28×10^4, and 4.73×10^4 Da).

4.2. Purification of COS and its derivatives

COS and its derivatives (10 g) were dissolved in distilled water (250 mL) and dialyzed using a Micro Acylizer equipped with an AC-110 dialysis membrane module. Solutions were dialyzed until a zero electric current and a constant conductance obtained. Then the solutions were concentrated and lyophilized. After 24–36 h, purified oligosaccharides were obtained as a fluffy, yellow solid.

4.3. Synthesis of carboxylated COS

Carboxylation reaction was carried out according to Ronghua et al.[14] with minor modifications. Briefly, COS (6.50 g, ~0.04 mol of glucosamine unit) was dissolved in 50 mL of 10% acetic acid aqueous solution and 15 mL of methanol was added while stirring. A

determined quantity of succinic anhydride (2.2, 4.4, or 6.6 g, about 0.022, 0.044, or 0.066 mol, respectively), was dissolved in acetone (20–50 mL, dependent on the usage of anhydride) adding drop by drop at room temperature for 1 h. The mixture was stirred for 4 h and pH was adjusted to 9.0–10.0 with sodium carbonate. Subsequently, the solution was purified with Micro Acilyzer and lyophilized according to the same stated procedure to obtain fluffy, yellow, light solid product. The samples were labeled as CCOS-1, CCOS-2, and CCOS-3 in increasing sequence of anhydride/COS mole ratio.

4.4. Synthesis of quaternized amino COS

Quaternization of COS was carried out according to Ronghua et al.[17] Briefly, COS (10.00 g, ~0.06 mol glucosamine unit) was dissolved in 50 mL of distilled water and determined quantities of 2,3-epoxypropyl trimethylammonium chloride (3.3, 10.0, and 2×20.0 g, about 0.022, 0.066, and 2×0.132 mol, respectively), were added with different mole ratios. The mixture was reacted at 40 °C for 24 h while stirring. Subsequently, the solution was purified using a Micro Acilyzer and lyophilized for 24–48 h to give quaternized amino COS as fluffy, yellow, and light solids. The samples were labeled as ECOS-1, ECOS-2, and ECOS-3 in increasing sequence of epoxy/COS mole ratio.

4.5. ACE inhibitory assay

ACE inhibitory activity was determined spectrometrically following the procedure of Cushman and Cheung[22] using hippuryl-L-histidyl leucine (HHL) as the substrate. Appropriate solutions of HHL, inhibitor, and ACE were obtained by dissolving them in 50 mM sodium borate buffer (pH 8.3) and a mixture containing 50 μL of inhibitor and 50 μL of ACE solution (25 mU/mL) was preincubated for 10 min at 37 °C. For ACE activity determination, 150 μL of 4.15 mM HHL was added to the mixture followed by incubation for 30 min at 37 °C and the reaction was terminated by addition of 250 μL of 1 N HCl. The hippuric acid formed by the action of ACE was extracted into 500 μL of ethyl acetate. After centrifugation at $800 \times g$ for 10 min, 200 μL of supernatant was separated from the upper layer and subjected to evaporation at room temperature in a vacuum for 2 h. The residue was dissolved in 1.0 mL of distilled water and absorbance was measured against at 228 nm against distilled water. Concentration dependent activities of inhibitors were studied and the concentration required to inhibit 50% of ACE activity was defined as the IC_{50} value.

4.6. ACE inhibitory pattern of carboxylated COS

To clarify the inhibitory mechanism of carboxylated COS derivative (COS-3) on ACE, Lineweaver–Burk plots were plotted with two different concentrations of the inhibitor and HHL at varying incubation times following the above procedure.[23]

4.7. Statistics

Results from ACE inhibitory assay were presented as means of triplicates and Student's *t*-test was used to determine the level of significance.

Acknowledgements

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