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Angiotensin I Converting Enzyme (ACE) Inhibitory Activity of Hetero-Chitooligosaccharides Prepared from Partially Different Deacetylated Chitosans

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Angiotensin I converting enzyme (ACE) inhibitory activity of hetero-chitooligosaccharides (hetero-COSs) prepared from partially different deacetylated chitosans was investigated. Partially deacetylated chitosans, 90, 75, and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deacetylation with 40% sodium hydroxide solution for durations. In addition, nine kinds of hetero-COSs with relatively high molecular masses (5000−10 000 Da; 90-HMWCOSs, 75-HMWCOSs, and 50-HMWCOSs), medium molecular masses (1000−5000 Da; 90-MMWCOSs, 75-MMWCOSs, and 50-MMWCOSs), and low molecular masses (below 1000 Da; 90-LMWCOSs, 75-LMWCOSs, and 50-LMWCOSs) were prepared using an ultrafiltration membrane bioreactor system. ACE inhibitory activity of hetero-COSs was dependent on the degree of deacetylation of chitosans. 50-MMWCOSs that are COSs hydrolyzed from 50% deacetylated chitosan, the relatively lowest degree of deacetylation, exhibited the highest ACE inhibitory activity, and the IC_{50} value was 1.22 ± 0.13 mg/mL. In addition, the ACE inhibition pattern of the 50-MMWCOSs was investigated by Lineweaver−Burk plots, and the inhibition pattern was found to be competitive.

KEYWORDS: Chitooligosaccharides; ACE; hetero-chitosan; competitive inhibition

INTRODUCTION

Chitosan is a deacetylated polymer of *N*-acetyl glucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. It has a hypocholesterolemic effect (*1*−*3*), an immunomodulating function (*4*), and a hypoglycemic effect (*5*). However, recent studies on chitosan have attracted interest for converting chitosan to its oligosaccharides, because the oligosaccharides are not only water soluble but also possess special functional properties such as antitumor activity (*6*, *7*), immunostimulating effects (*8*, *9*), antimicrobial activity (*10*−*13*), and radical scavenging activity (*14*, *15*).

Hypertension is one of the major independent risk factors for arteriosclerosis, stroke, myocardial infarction, and end stage renal disease. ACE (E. C. 3.4.15.1) is a dipeptidylcarboxypeptidase, which catalyzes the formation of angiotensin II, a strong pressor, from angiotensin I and inactivates bradykinin, which has depressor action. ACE plays an important physiological role in regulating blood pressure (*16*). ACE converts an inactive form of decapeptide, angiotensin I, to a potent vasoconstrictor, octapeptide angiotensin II, and inactivates bradykinin, which has a depressor action.

Since the discovery of an ACE inhibitory peptide in snake venom, many peptides have been identified from the enzymatic hydrolysates of various natural sources such as cheese whey (*17*, *18*), casein (*19*), corn zein (*20*), soy sauce (*21*), soybean (*22*), bovine skin gelatin (*23*), Alaska pollack skin gelatin (*24*, *25*), sardine (*26*), tuna (*27*), and bonito (*28*). In addition, many researchers have attempted the synthesis of ACE inhibitors, and three dozen inhibitors have been tested clinically (*23*, *29*). However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, and skin rashes (*30*). Therefore, the search for natural ACE inhibitors as alternatives to synthetic ones is of great interest among researchers for safe and economical use.

In the present study, ACE inhibitory activity of hetero-COSs prepared from partially different deacetylated chitosans was investigated, and the inhibition pattern was also determinated using COSs with relatively medium molecular masses (1000−5000 Da) prepared from 50% deacetylated chitosan, which exhibits the highest ACE inhibitory activity as an inhibitor.

MATERIALS AND METHODS

Materials. Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). The chitosanase (35 000 U/g protein) derived from *Bacillus* sp. was purchased from Amicosen Co. (Jinju, Korea), and cellulase was donated from Pacific Chemical Co. Ltd. (Seoul, Korea). The UF reactor (Minitan) system and membranes for the fractionations of hetero-COSs, based on molecular mass, were from Millipore Co. (Bedford, MA). ACE (from rabbit lung) and substrate peptide (hippuryl-histidyl-leucine) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade available commercially.

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Preparation of Hetero-COSs. Three kinds of partially deacetylated chitosans, 90, 75, and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deacetylation with 40% (w/v) sodium hydroxide solution for durations according to our previous method (*31*). In addition, hetero-COSs, which are COSs prepared from 90, 75, and 50% deacetylated chitosans, were prepared by hydrolysis of hetero-chitosans in an UF membrane reactor system according to the method of Park et al. (*31*). A 1% solution of hetero-chitosans was prepared by dispersing 100 g of hetero-chitosans in 1 L of distilled water, dissolving it and stirring by adding 550 mL of 1.0 M lactic acid and making up to 10 L with distilled water. The pH was adjusted to be 5.5 with a saturated sodium carbonate solution. The UF membrane reactor system (Millipore Minitan, Millipore Co.) was used for preparation of hetero-COSs. Ninety percent (90%) deacetylated chitosan was hydrolyzed with an endo type chitosanase (35 000 U/g protein) with a substrate to enzyme ratio of 1:1.5 units for 36 h in a batch reactor and then heated at 98 °C for 10 min to inactivate the enzyme. Thereafter, the hydrolysates were separated using an UF membrane reactor system. The UF membranes used in the system were MMCO 10, 5, and 1 kDa, respectively. Seventy-five and 50% deacetylated chitosan were hydrolyzed with a substrate to enzyme ratio of 1:5 units and of 1:10 units by cellulase (CMC 100 000 U/g protein) for 2 h at 43 ± 2 °C in a batch reactor. After then, chitosanase was added in the reactor, hydrolyzed for 36 h at the same temperature, and separated by an UF membrane reactor system. The hetero-COSs were fractionated into nine kinds of COSs with relatively high molecular masses (5000−10 000 Da; 90-HMW-COSs, 75-HMWCOSs, and 50-HMWCOSs), medium molecular masses (1000−5000 Da; 90-MMWCOSs, 75-MMWCOSs, and 50-MMW-COSs), and low molecular masses (below 1000 Da; 90-LMWCOSs, 75-LMWCOS, and 50-LMWCOSs). All COSs recovered were lyophilized on a freezing drier for 5 days.

Assays for ACE Inhibitory Activity. The ACE inhibitory activity assay was performed using a modified version of the method of Cushman and Cheung (*32*). A sample solution (50 μL) with 50 μL of ACE solution (25 milliunits/mL) was preincubated at 37 °C for 10 min and then incubated with 150 μL of substrate at 37 °C for 30 min. The reaction was stopped by the addition of 250 μL of 1.0 M HCl. The resulting hippuric acid was extracted with 500 μL of ethyl acetate. After the solution was centrifuged, the 200 μL upper layer was transferred into a test tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 1 mL of distilled water, and the absorbance was measured at 228 nm using a spectrophotometer. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Determination of the Inhibition Pattern on ACE. 50-MMWCOSs were added to each reaction mixture according to Bush et al. (*33*) with some modification. The enzyme activity was measured with different concentrations of the substrate. The kinetics of ACE in the presence of the inhibitor were determined by the Lineweaver−Burk plots.

Statistical Analysis. All assays were carried out in triplicate, and results are reported as means ± standard deviation. Data were analyzed using a SAS program (version 8.1), and the least significant difference was used to evaluate significance among means.

RESULTS AND DISCUSSION

Inhibitory Activity of Hetero-COSs on ACE. ACE (dipeptidylcarboxypeptidase) plays an important physiological role in regulating blood pressure. ACE converts an inactive form of decapeptide, angiotensin I, to a potent vasoconstrictor, octapeptide angiotensin II, and inactivates bradykinin, which has a depressor action. Following the discovery of captopril, hundreds of potential ACE inhibitors have been synthesized and at least three dozen have been tested clinically. More than a dozen ACE inhibitors including alacepril, benazepril, captopril, cilazapril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, tandolapril, and zofenopril (*29*, *34*) have been used extensively in the treatment of essential hypertension and heart failure in humans; most ACE inhibitors have been studied from proteins and peptides. However, there is little information about carbohydrate as an ACE inhibitor. **Figure 1** shows ACE inhibitory activity of hetero-COSs with relatively high molecular masses (5000−10 000 Da) prepared from 90, 75, and 50% deacetylated chitosan, 90-HMWCOSs, 75-HMWCOSs, and 50-HMWCOSs. The ACE inhibitory activity was dependent on dose and degree of deacetylation. Among COSs with relatively high molecular masses, 50-HMWCOS exhibited the greatest ACE inhibitory activity. **Figure 2** provides ACE inhibitory activity by COSs with relatively medium molecular masses (1000−5000 Da), 90-, 75-, and 50-MMWCOSs. Their ACE inhibitory activity was also dependent on dose and degree of deacetylation; 50-MMWCOSs showed the highest activity against cleavage of the dipeptide by ACE among three kinds of COSs with relatively medium molecular masses. **Figure 3** presents the ACE inhibitory activity of COSs with relatively

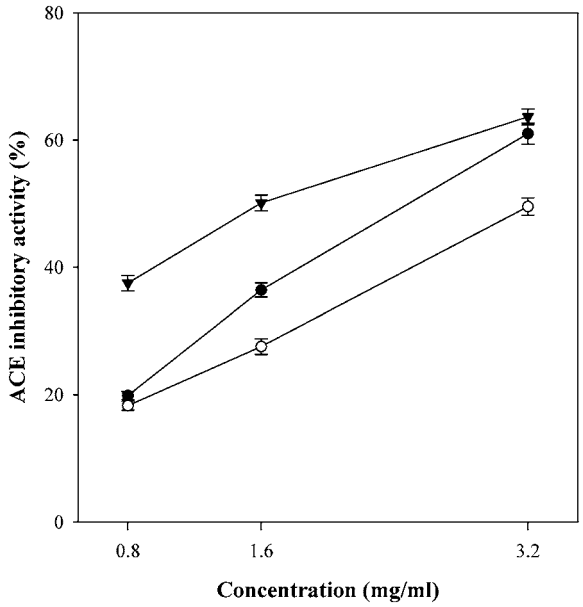

Figure 1. ACE inhibitory activity of COSs with relatively higher molecular masses prepared from 90, 75, and 50% deacetylated chitosan. ●, 90-HMWCOSs; ○, 75-HMWCOSs; ▼, 50-HMWCOSs.

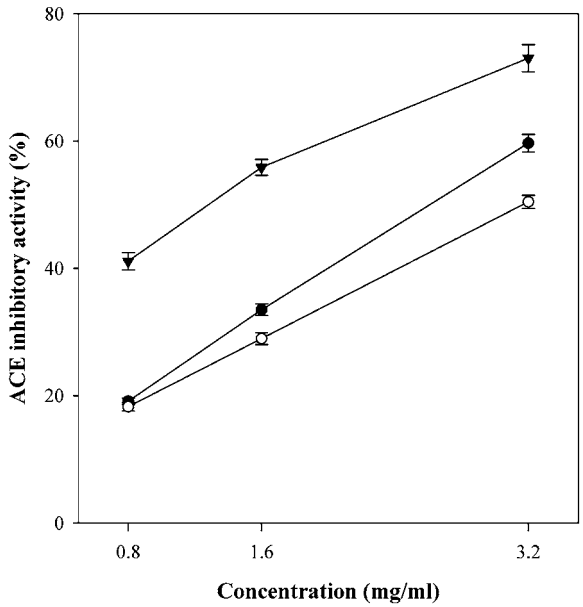

Figure 2. ACE inhibitory activity of COSs with relatively medium molecular masses prepared from 90, 75, and 50% deacetylated chitosan. ●, 90-MMWCOSs; ○, 75-MMWCOSs; ▼, 50-MMWCOSs.

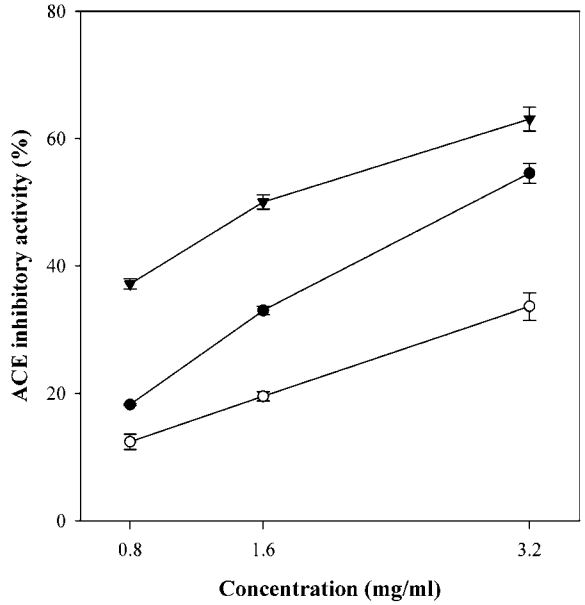

Figure 3. ACE inhibitory activity of COSs with relatively low molecular masses prepared from 90, 75, and 50% deacetylated chitosan. ●, 90-MMWCOSs; ○, 75-MMWCOSs; ▼, 50-MMWCOSs.

Table 1. ACE Inhibitory Activity of Hetero-COSs with Different Degrees of Deacetylation and Molecular Masses

sample	IC_{50} (mg/mL)
90-HMWCOS	2.48 ± 0.35^{b}
90-MMWCOS	2.49 ± 0.21^{b}
90-LMWCOS	2.87 ± 0.37^{b}
75-HMWCOS	3.19 ± 0.36^{c}
75-MMWCOS	3.14 ± 0.23^{c}
75-LMWCOS	>3.2
50-HMWCOS	1.59 ± 0.31^{a}
50-MMWCOS	1.22 ± 0.13^{a}
50-LMWCOS	1.61 ± 0.28^{a}

low molecular masses (below 1000 Da), 90-, 75-, and 50-LMWCOSs. 50-LMWCOSs, COSs with the lowest degree of deacetylation among three kinds of COSs with relatively low molecular masses prepared from chitosans with different degrees of deacetylation, exhibited the greatest ACE inhibitory activity. All nine kinds of hetero-COSs exhibited ACE inhibitory activity, and there were no significant differences in different molecular masses of hetero-COSs. However, their ACE inhibitory activity was dependent on degree of deacetylation of chitosans, and 50-MMWCOSs exhibited the highest ACE inhibitory activity (**Table 1**). The IC_{50} of 50-MMWCOSs was 1.22 ± 0.13 mg/mL.

Many ACE inhibitors have recently been isolated from various protein hydrolysates (*23*−*25*, *35*). The binding model for interactions between the substrate and the active site of ACE was proposed by Ondetti and Cushman (*36*). The C-terminal tripeptide residues may interact with three subsites at the active site of ACE, and it was reported that ACE appears to prefer substrates or competitive inhibitors that contain hydrophobic amino acid residues at three positions of the C terminus (*37*). However, the mechanism of action of COSs as ACE inhibitors was not known.

We have previously reported (*23*) that we could not observe any toxic effects of the COS in three groups of Sprague−Dawley rats given orally 500, 1000, and 2000 mg/kg/day for 28 days, based on the various aspects of death rate, body weight change, general symptoms, food consumption, urinalysis, hematology, blood biochemistry, relative organ weights, and histopathological findings. Therefore, chitosan oligosaccharides were stable, and they may have potential. In this study, the effect of hetero-COSs as an ACE inhibitor in vivo was however investigated. Further research for effects in vivo and the mechanism of action of COSs as an ACE inhibitor is necessary.

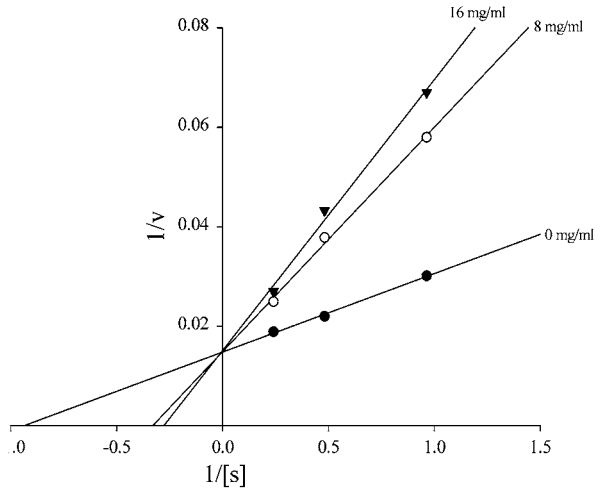

Figure 4. Lineweaver−Burk plots of ACE inhibitory activity in the presence of 50-MMWCOSs.

Determination of the Inhibition Pattern on ACE. ACE inhibition pattern of the 50-MMWCOSs was investigated by Lineweaver−Burk plots and was found to be competitive (**Figure 4**). It means that the ACE inhibitor of COSs binds competitively with the substrate at the active site of ACE. Among the naturally occurring peptides with ACE inhibitory activity, the most potent and specific inhibitors were several peptides with similar structures that have been isolated from the venom of the South American pit viper *Bothrops jararaca* and Japanese pit viper *Agkistrodon halys blomhoffii* (*38*, *39*). The mechanism of ACE inhibitory activity by polysaccharides is not clear.

In conclusion, partially deacetylated chitosans, 90, 75, and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deacetylation with 40% sodium hydroxide solution for durations. In addition, nine kinds of hetero-COSs with relatively high molecular masses (5000−10 000 Da; 90-HMWCOSs, 75-HMWCOSs, and 50-HMWCOSs), medium molecular masses (1000−5000 Da; 90-MMWCOSs, 75-MMWCOSs, and 50-MMWCOSs), and low molecular masses (below 1000 Da; 90-LMWCOSs, 75-LMWCOSs, and 50-LMWCOSs) were prepared using an UF membrane bioreactor system. All nine kinds of hetero-COSs exhibited ACE inhibitory activity, but there were no significant differences in different molecular masses of hetero-COSs. ACE inhibitory activity of hetero-COSs was dependent on the degree of deacetylation of chitosans. 50-MMWCOSs that are COSs hydrolyzed from 50% deacetylated chitosan, the relatively lowest degree of deacetylation, exhibited the highest ACE inhibitory activity, and the IC_{50} value was 1.22 ± 0.13 mg/mL. In addition, the ACE inhibition pattern of the 50-MMWCOSs was investigated by Lineweaver−Burk plots and was found to be competitive.

ABBREVIATIONS USED

ACE, angiotensin I converting enzyme; COSs, chitooligosaccharides; MW, molecular weight; MMCO, molecular mass cut offs; UF, ultrafiltration membrane; 50, 75, and 90-HMWCOSs, chitooligosaccharides with relatively high molecular masses

(5000−10 000 Da) prepared from 50, 75, and 90% deacetylated chitosan; 50, 75, and 90-MMWCOSs, chitooligosaccharides with relatively medium molecular masses (1000−5000 Da) prepared from 50, 75, and 90% deacetylated chitosan; 50, 75, and 90-LMWCOSs, chitooligosaccharides with relatively low molecular masses (below 1000 Da) prepared from 50, 75, and 90% deacetylated chitosan.

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