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Renin inhibition activity by chitooligosaccharides

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Abstract—Six kinds of chitooligosaccharides (COSs) with different molecular weight (MW) and degree of deacetylation (DD) were prepared using ultrafiltration membrane reactor, and their renin inhibition modes were evaluated. All the COSs showed the renin-inhibitory activities with dose-dependent manner, and 90-COSs had the potent renin-inhibitory activity than that of 50-COSs. Among them, 90-MMWCOS (1000–5000 Da) exhibits the highest activity with IC_{50} value of 0.51 mg/mL and acts as competitive inhibitor with K_i value of 0.28 mg/mL by Lineweaver-Burk and Dixon plots. These results indicated that DD value and MW of COSs are important factors affecting renin-inhibitory activity.

The renin-angiotensin system (RAS) plays a pivotal role in the control of blood pressure and the pathophysiology of cardiovascular diseases such as congestive heart failure and hypertension.[1,2] Renin, also known as angiotensinogenase, is a circulating enzyme secreted from the granular cells of juxtaglomerular apparatus in the kidney. Renin has high substrate specificity, and catalyzes the hydrolysis of only one naturally occurring substrate, angiotensinogen.[3] Renin is a rate-limiting enzyme in RAS and cleaves plasma angiotensinogen to angiotensin I (Ang I), which is further converted by soluble or endothelial cell-associated angiotensin converting enzyme (ACE) to angiotensin II (Ang II), a powerful vasoconstrictor that has been identified as a major factor in hypertension.[4] ACE is also involved in the inactivation of bradykinin, a potent vasodilator. Today, ACE inhibitors are widely developed to prevent Ang II production for cardiovascular diseases and utilized in clinic. However, Ang II can be produced through the hydrolysis of Ang I by chymase in the heart, which can reduce the efficacy of ACE inhibitors. Since renin is a rate-limiting step in the RAS and has a specific substrate as angiotensinogen, renin inhibition is thought to be an attractive target for antihypertension strategy.

Chitosan, a polycationic polymer comprised of mainly glucosamine units, is known for biocompatibility, biodegradability and less toxic nature. It has been developed into physiological bioactive substances that possess various biological activities such as antibacterial activity,[5–8] hypocholesterolemic activity,[9] antitumor activity,[10] immuno-stimulating effect,[11] antioxidant activity[12] and antihypertensive activity.[13] Chitooligosaccharides (COSs) are derivative of chitosan and it can be obtained by either enzymatic or chemical hydrolysis of chitosan. COSs are not only water-soluble but also possess versatile biological activities such as antitumor,[14] immunostimulating,[15] antioxidant[16] and antimicrobial activity.[17] In recent years, ACE inhibitory activity and antihypertensive activity of COSs with different molecular weights have been reported.[18,19] Furthermore, several chitosan derivatives with water-soluble property have been developed for improving inhibitory activity against ACE in order to prevent production of Ang II.[20,21] However, Ang II, which is the main product increasing blood pressure in RAS, can be produced by chymase in the heart, leading to decrease the efficacy of ACE inhibitors. Therefore, more specific inhibitions are needed for the treatment and prevention of hypertension. The objective of this study is to determine the in vitro activity and kinetic study of COSs with different molecular weight and degree of deacetylation against renin, a potential target for the hypertension.

Two kinds of COSs (90-COSs and 50-COSs) were prepared from 90% to 50% deacetylated chitosan as de-

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scribed in our previous report, and further fractionated into three kinds of COSs using an ultrafiltration membrane system.[22] COSs were designated based on their molecular weights as high molecular weight COSs (5000–10,000 Da: 90-HMWCOSs and 50-HMWCOSs), medium molecular weight COSs (1000–5000 Da: 90-MMWCOSs and 50-MMWCOSs) and low molecular weight COSs (below 1000 Da: 90-LMWCOSs and 50-LMWCOSs).

Renin activity was determined as follows using the fluorescence method of Yuan et al.[23] For enzyme assay, the substrate stock solution and renin were each diluted to the appropriate concentration using 50 mM Tris–HCl buffer (pH 8.0 containing 100 mM NaCl). The buffer (control) and/or sample (inhibition) was first mixed with the substrate (5 μM) and incubated at 37 °C for 10 min and then the reaction was initiated by the addition of renin solution (0.1 μg). The time-dependent increase in fluorescence intensity was monitored for 10 min in a GENions® fluorescence microplate reader (Tecan Austria GmbH, Austria). Instrument parameters were as follows: excitation wavelength, 340 nm; emission wavelength, 490 nm; excitation bandwidth, 5 nm; and emission bandwidth 10 nm.

A Lineweaver-Burk and Dixon plots were drawn to estimate the renin inhibitory types of COS. The inhibition constant (K_i) of COS was obtained from the secondary plot of Lineweaver-Burk plot and was directly calculated from Dixon plot. The intercept on the horizontal axis is the value of the K_i.

Renin-inhibitory activities of chitooligosaccharides with various molecular weights produced from different degree of deacetylation were investigated using the fluorescence assay, and their results are depicted in Figures 1 and 2. Three COSs, 90-HMWCOS, 90-MMWCOS and 90-LMWCOS, showed different renin-inhibitory activities with dose-dependent manner, and percentage inhibitions were reached to 75.06%, 98.43% and 90.61% at the concentration of 1.6 mg/mL, respectively (Fig. 1). Among them, 90-MMWCOS showed the strongest renin-inhibitory activity than that of 90-HMWCOS and 90-LMWCOS. On the other side, three COSs, 50-HMWCOS, 50-MMWCOS and 50-LMWCOS, possessed weaker renin-inhibitory activities than that of 90-COSs. At the concentration of 6.4 mg/mL, 50-HMWCOS, 50-MMWCOS and 50-LMWCOS showed lower renin-inhibitory activities than 50%. The renin-inhibitory activity was in the order of 50-MMWCOS > 50-HMWCOS > 50-LMWCOS and the activity was dose-dependent. According to these results, the renin-inhibitory activities were increased with increasing degree of deacetylation at COS, but factor affecting renin-inhibitory activities regarding their molecular weight was not clear. So, we could conclude that major factor affecting renin-inhibitory activity of COSs was degree of deacetylation. The IC_{50} values of 90-COSs were calculated by the non-linear regression method and are shown in Table 1. They confirm that 90-MMWCOS has the highest potency against renin.

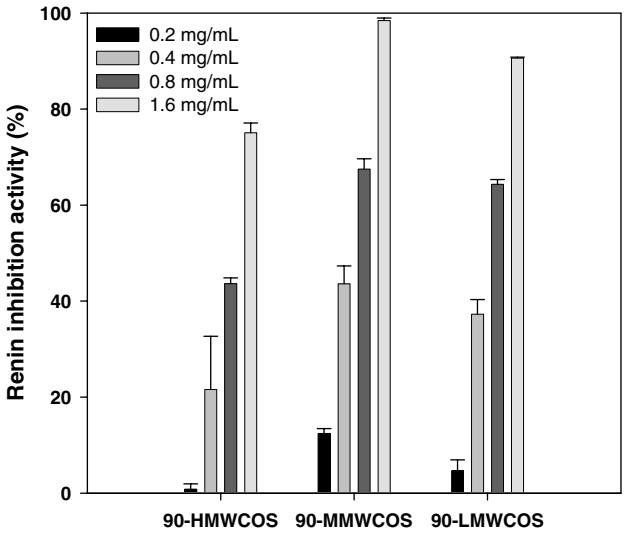

Figure 1. Human recombinant renin-inhibitory activities of 90-COSs. Results are means ± SE of three independent experiments. The reaction mixture contained 5 μM substrate, 20 μL Tris–HCl buffer, and 0.1 μg of renin. Excitation wavelength 340 nm; emission wavelength, 490 nm; excitation bandwidth 5 nm; emission bandwidth, 10 nm.

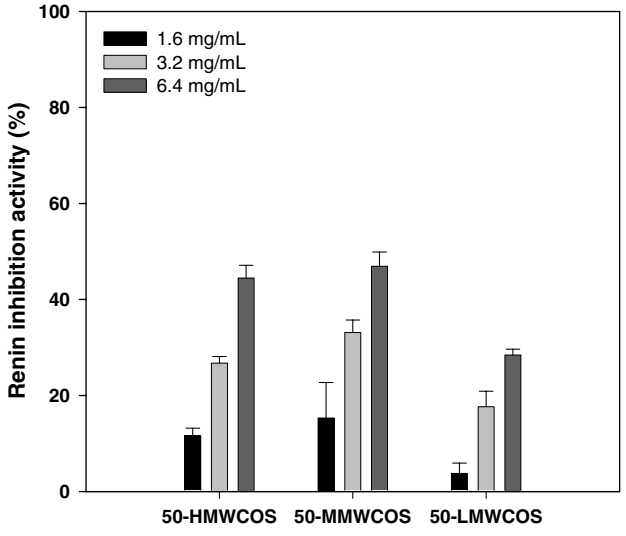

Figure 2. Human recombinant renin-inhibitory activities of 50-COSs. Results are means ± SE of three independent experiments. The reaction mixture contained 5 μM substrate, 20 μL Tris–HCl buffer, and 0.1 μg of renin. Excitation wavelength 340 nm; emission wavelength, 490 nm; excitation bandwidth 5 nm; emission bandwidth, 10 nm.

Several renin inhibitors by chemically synthesized compounds were reported with lower IC_{50} values (high potent) than that of chitooligosaccharides,[24,25] and renin inhibitors with similar IC_{50} values were also reported from naturally occurring compound analogs.[23] In this study, we used the fluorogenic substrate for renin inhibition assay, however, angiotensinogen, natural substrate, is used in some cases as the substrate for renin inhibition assay. So, direct comparison of IC_{50} values is difficult.

Table 1. IC_{50} values of chitooligosaccharides against human recombinant renin

	Chitooligosaccharides		
	90-HMWCOS	90-MMWCOS	90-LMWCOS
IC_{50} (mg/mL)	0.94	0.51	0.59

The kinetic behavior of the fluorogenic substrate catalyzed by human recombinant renin with 90-MMWCOS, the most potent of chitooligosaccharides, was studied. Determination of the inhibition type is important to understand the mechanism of enzyme action and the inhibitor-binding site. Renin inhibition pattern of 90-MMWCOS was analyzed by Lineweaver-Burk and Dixon plots without and with 90-MMWCOS (at three concentrations of 0.4, 0.6 and 0.8 mg/mL). The inhibition kinetics analyzed by Lineweaver-Burk plots indicating that 90-MMWCOS acts as competitive inhibitor, which means that 90-MMWCOS can bind competitively with substrate at the active site (Fig. 3). Therefore, 90-MMWCOS formed enzyme–inhibitor complexes during the enzyme reaction to reduce the efficiency of catalysis. The K_i value was calculated by the secondary plot of Lineweaver-Burk, with the slopes of each line in the Lineweaver-Burk plot being plotted against different concentrations of 90-MMWCOS, and the K_i value is the intercept on the x-axis. The obtained K_i value was determined to be 0.28 mg/mL. We also analyzed the kinetic behavior using Dixon plots, and the results showed that 90-MMWCOS acts as competitive inhibitor (Fig. 4). The K_i value was directly measured from Dixon plot as an intercept on the x-axis. Crystal structures of human renin revealed that the enzyme is composed mainly of two β-sheet domains with the cleft between two domains. The active site cleft of human renin became so apparent that the S3 and S1 pockets form a contiguous and large hydrophobic cavity.[26] So, main binding sites of 90-MMWCOS toward renin are not S3–S1 pockets, however, 90-MMWCOS could be formed by the hydrogen bonding and/or the electrostatic interaction between the positively charged amide group at C-2 position and the carboxylates of the catalytic residues. These are reasons for 90-COSs having the potent renin inhibition activity compared to 50-COSs. However, more studies on relationship between molecular size and inhibition activity are needed.

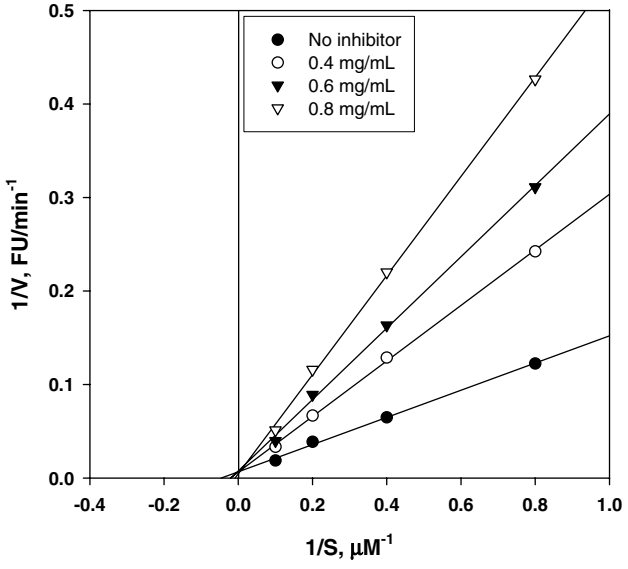

Figure 3. Lineweaver-Burk plot for the inhibition of human recombinant renin by 90-MMWCOS. The inhibitory potency of 90-MMWCOS for human recombinant renin was measured by using fluorogenic peptide substrate. The reactions were performed in the absence of inhibitor or in the presence of 90-MMWCOS as indicated.

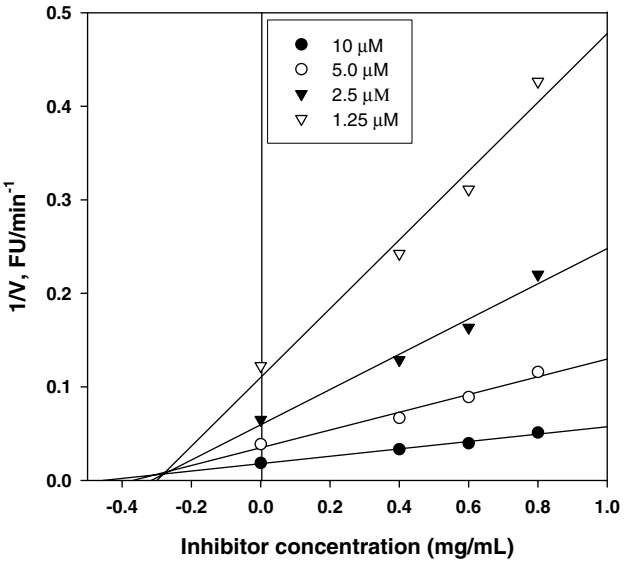

Figure 4. Dixon plot analysis for the inhibition of human recombinant renin by 90-MMWCOS. The inhibitory potency of 90-MMWCOS for human recombinant renin was measured by using fluorogenic peptide substrate at the concentrations indicated.

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