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Chitooligosaccharides suppress the level of protein expression and acetylcholinesterase activity induced by $A\beta_{25-35}$ in PC12 cells

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ABSTRACT

Clinical applications of acetylcholinesterase (AChE) inhibitors are widespread in Alzheimer's sufferers in order to activate central cholinergic system and alleviate cognitive deficits by inhibiting the hydrolysis of acetylcholine. In this study, six kinds of chitooligosaccharides (COSs) with different molecular weight and degree of deacetylation were examined for their inhibitory effects against AChE. The 90-COSs exhibited potent AChE inhibitory activities compared to 50-COSs, while 90-MMWCOS (1000–5000 Da) in the 90-COSs showed the highest activity. Cell culture experiment revealed that 90-MMWCOS suppressed the level of AChE protein expression and AChE activity induced by $A\beta_{25-35}$ in PC12 cell lines.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the presence of senile plaques composed of β-amyloid peptide (Aβ), and/or the loss of cognition and memory. The cause of AD is not clearly understood but one of the most consistent and profound changes associated with AD is a deficiency in cholinergic neurotransmission, which leads to the loss of cognition and memory.[1] Based on the cholinergic hypothesis, the loss of cholinergic function is the only evidential finding responsible for cognitive decline. As a result, drug-based clinical strategies are often used to slow down the progression of cognitive deterioration by elevating the transient levels of acetylcholine in the brain through cholinesterase inhibition.[2]

Acetylcholine (ACh) is considered as a neurotransmitter in both the peripheral nervous system (PNS) and central nervous system (CNS), where it functions a neuromodulator. ACh is released to choline and acetate by acetylcholinesterase (AChE), which is an oligomeric enzyme that attaches to the neuromuscular junction, and plays a fundamental role in impulse transmission through the breakdown of the neurotransmitter acetylcholine at neuromuscular junction and brain cholinergic synapses.[3,4] Currently, AChE inhibitors are widely use in AD patients to inhibit the hydrolysis of ACh, thereby activating the central cholinergic system and alleviating cognitive deficits. It has been suggested that AChE plays a pivotal role in the development of AD by means of accelerating Aβ deposition, which appears to be neurotoxicity,[5] and also Aβ causes an increase in AChE expression.[6] It has also been suggested that the expression of AChE in response to Aβ deposition may accelerate amyloid deposition.[7]

Chitooligosaccahrides (COSs) are derivative of chitosan and it can be obtained by either enzymatic or chemical hydrolysis of chitosan. COSs are regarded as physiologically bioactive substances since they possess versatile biological activities such as antitumor,[8] immuno-stimulating,[9] antioxidant,[10] and antimicrobial characteristics.[11] As part of our ongoing investigation on COSs biological activities, we prepared COSs with different degree of deacetylation (DD) and molecular weight (MW) in order to carry out an evaluation of their AChE inhibitory effects. Furthermore, we evaluated their inhibitory effect on level of protein expression and AChE activity induced by $A\beta_{25-35}$ in PC12 cell lines.

Two kinds of COSs (90-COSs and 50-COSs) were prepared from 90% and 50% deacetylated chitosan as described in our previous report, and further fractionated into three kinds of COSs using an ultrafiltration membrane system.[12] 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). The assay for AChE inhibition was performed according to the methods developed by Ellman et al.[13] The percentage of AChE inhibition was calculated using the following equation:

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$$\text{Inhibition } (\%) = (1 - (A_{\text{sample}} - A_{blank})/A_{\text{control}}) * 100$$

where A_{sample} is absorbance of enzyme inhibition reaction at 415 nm, A_{blank} is absorbance of sample at 415 nm and A_{control} is absorbance of enzyme reaction at 415 nm. For cell-based assay, PC12 cell lines were treated with 25 μM $A\beta_{25-35}$ and COSs and incubated for 24 h to stimulate AChE. After incubation cell lysates were prepared in lysis buffer, centrifuged, and then collected the supernatant. AChE activity assay and western blotting analysis of the supernatant were performed.

AChE inhibitory activities of COSs with various molecular weights produced from different degree of deacetylation were investigated using Ellman assay,[13] and their results were depicted in Figures 1 and 2. Three COSs, 90-HMWCOS, 90-MMWCOS, and 90-LMWCOS, showed different AChE inhibitory activities with dose-dependent, and percentage inhibitions were reached to 70.77%, 82.98%, and 55.03% at the concentration of 4.0 mg/mL, respectively (Fig. 1). Among them, 90-MMWCOS showed the strongest AChE inhibitory activity compared to 90-HMWCOS and 90-LMWCOS. We also tested 50-COSs for its AChE inhibitory activity because degree of deacetylation is a major factor affecting for bioactivity. Figure 2 showed that 50-HMWCOS, 50-MMWCOS and 50-LMWCOS possessed weak AChE inhibitory activities compared to the same molecular weight range of 90-COSs. At the concentration of 4.0 mg/mL, 50-HMWCOS, 50-MMWCOS, and 50-LMWCOS recorded 64.67%, 57.28%, and 42.18% AChE inhibitory activities, respectively. According to these results, the AChE inhibitory activities were increased with increasing degree of deacetylation on COSs. However, it was not clear to what extent this was related to different molecular weights. So, we could be concluded that major factor affecting the AChE inhibitory activity of COSs was degree of deacetylation. The IC_{50} values of COSs were calculated by the non-linear regression method and were shown in Table 1. They confirm that 90-MMWCOS has the highest potency against AChE. To investigate this further, we used 90-MMWCOS in order to assess level of protein expression and AChE activity induced by $A\beta_{25-35}$ in PC12 cell lines. After treatment of PC12 cell lines with 25 μM $A\beta_{25-35}$ and 90-MMWCOS, further incubation was carried out for 24 h to stimulate AChE. Measurement of cellular AChE activity showed that AChE activity significantly increased compared to non-treatment group (data not shown). As shown in Figure 3A, the level of AChE protein expression markedly decreased with increasing concentration of 90-MMWCOS. We also investigated cellular AChE inhibition activity in PC12 cell lines using Ellman assay, the results of which showed that AChE inhibition activity was increased with increasing concentration of 90-MMWCOS (Fig. 3B). These results suggested that 90-MMWCOS effectively suppresses AChE activity and the level of AChE protein expression.

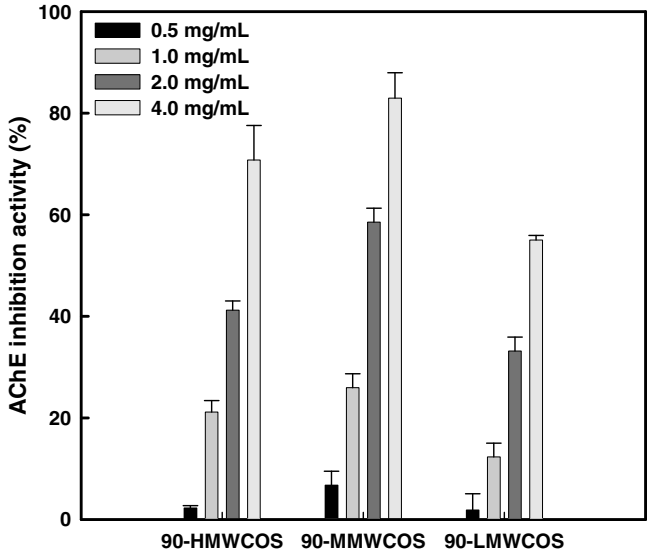

Figure 1. AChE inhibitory activities of 90-COSs. Results represent means ± SE of three independent experiments.

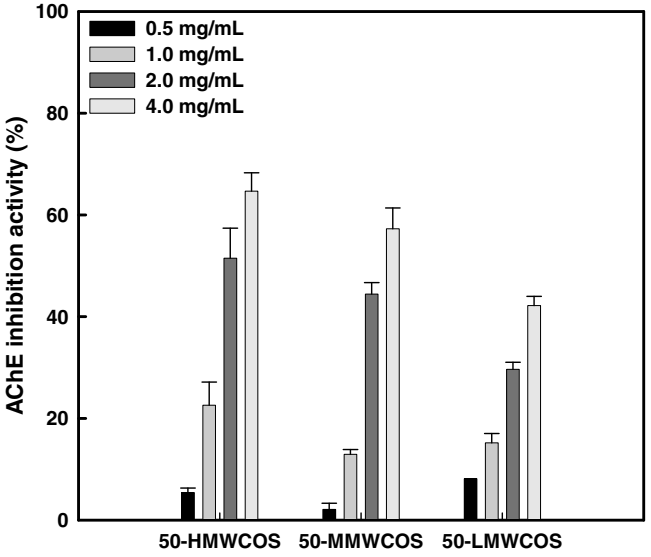

Figure 2. AChE inhibitory activities of 50-COSs. Results represent means ± SE of three independent experiments.

Table 1
IC_{50} values of chitooligosaccharides against AChE

Chitooligosaccharides	IC_{50} (mg/mL)
90-HMWCOS	2.59
90-MMWCOS	1.67
90-LMWCOS	3.52
50-HMWCOS	1.98
50-MMWCOS	2.93
50-LMWCOS	>4.00

The efficacy of cholinergic therapies in AD supports the cholinergic hypothesis and validates this neurotransmitter system as a therapeutic target.[14] As a result, numerous reversible AChE inhibitors such as tacrine, donepezil, rivastigamine, galanthamine, huperzine, and other drugs currently being used in clinical trials.[15] However, some of these chemically synthesized inhibitors appear to be giving rise to severe side-effects such as nausea, vomiting, bradycardia, anorexia, and sweating.[16] As a result, research on naturally occurring products without side-effects has become a priority. Chitosan and its derivatives as COSs are also naturally occurring carbohydrates from crab shell. Due to their biocompatibility and bioactivity, much attention has been paid to its biomedical applications. The present study indicates that COSs, especially 90-MMWCOS, effectively inhibited direct AChE activity (Fig. 1). Crystal structures of AChE/inhibitor complexes revealed that the active site composed of a catalytic triad and a binding site of the quaternary amino group at the bottom of a deep narrow gorge. Furthermore, an aromatic midgorge recognition site and a peripheral anionic site (PAS) at the lip of the gorge were also discovered.[17] So, 90-MMWCOS could be formed the hydrogen bonding and/or the electrostatic interaction between the positively charged amid group at C-2 position and a PAS at the lip of the gorge. These are reasons why high degree of deacetylation COSs possessed potent AChE inhibition activity compared to low degree of deacetylation COSs. However, the role of molecular weight factor in AChE inhibition was not clear.

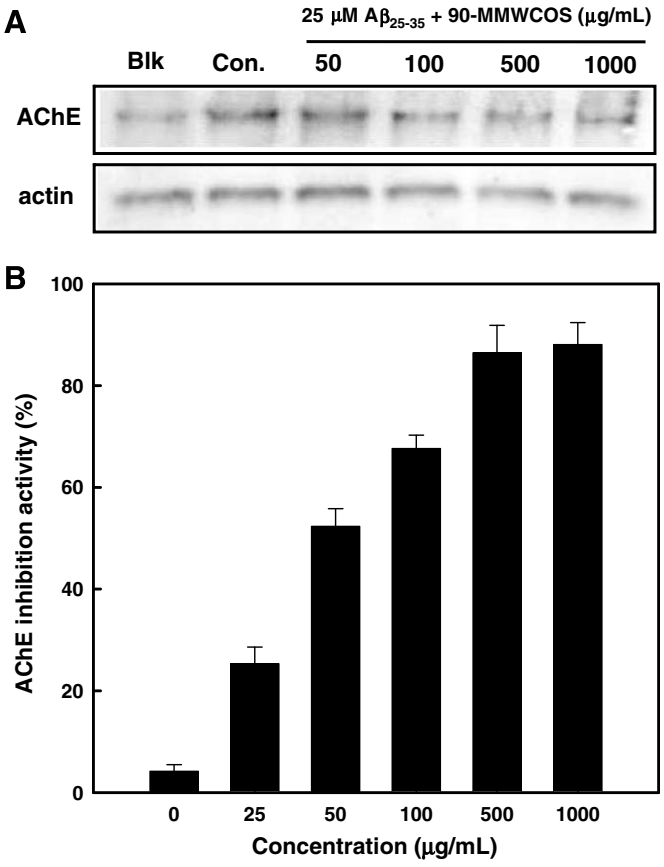

Figure 3. (A) Western blotting analysis of AChE protein expression in PC12 cell lines treated with 25 μM Aβ and 90-MMWCOS. (B) Cellular AChE inhibitory activity of 90-MMWCOS. Results are means ± SE of three independent experiments.

In the AD brain, AChE is associated predominantly with the amyloid core of mature senile plaques composed of Aβ, and the endothelial lining cerebral blood vessels.[18] Furthermore, Aβ causes an increase in AChE expression, and AChE activity is stronger where senile plaques areas in the brain.[6,19] Aβ stimulates AChE expression suggests that it may be possible to boost cholinergic function in the brain by inhibiting Aβ-induced AChE expression.[6] In this study, we treated $A\beta_{25-35}$ in PC12 cell lines in order to induce AChE expression, and investigated the effect of 90-MMWCOS on the level of AChE expression and activity. The result reveals that 90-MMWCOS effectively suppress the level of AChE protein expression induced by $A\beta_{25-35}$ in PC12 cell lines and also cellular AChE activity was decreased with increasing concentration of 90-MMWCOS in PC12 cell lines (Fig. 3). The efficacy of 90-MMWCOS in cellular and non-cellular AChE inhibition was different as shown in Figures. Therefore, more exact mechanism studies, how 90-MMWCOS inhibits AChE activity induced by $A\beta_{25-35}$ in cellular system, are needed.

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