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Acetylcholinesterase inhibitory activity of novel chitooligosaccharide derivatives

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ABSTRACT

In this study, three kinds of chitooligosaccharides (COS) with different substitution groups, including aminoethyl (AE), dimethylaminoethyl (DMEM) and diethylaminoethyl (DEAE) groups, were evaluated for their inhibitory activities against cholinesterase (acetylcholinesterase and butyrylcholinesterase). These COS derivatives were synthesized and their structures were characterized as AE-COS, DMAE-COS and DEAE-COS, respectively; the structures were elucidated via spectroscopic techniques (^{1}H NMR). Of these, DMAE- and DEAE-COS were synthesized for the first time in this work. These COS derivatives evidenced potent acetylcholinesterase (AChE) inhibitory activities with IC_{50} values of 56.5 ± 0.26, 24.1 ± 0.39 and 9.2 ± 0.33 μg/ml, respectively; however, these compounds exhibited no activity against butyrylcholinesterase. These were further analyzed for their inhibitory pattern on AChE via Lineweaver–Burk plots. AE-COS showed non-competition type inhibition, and DMAE-COS and DEAE-COS exhibited competition type inhibition against AChE. In this study, we suggested that COS derivatives have potential as AChE inhibitors for preventing Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disorder that is characterized by several symptoms including memory loss, disorientation, cognitive dysfunction, personality changes and behavioral disturbances (Adams, Crai, & Parsons, 1984; Aisen & Davis, 1997; Jann, 1998). In neuropathological studies, AD is associated with a decline in cholinergic function in the basal forebrain and cortex (Davies & Maloney, 1976; Whitehouse et al., 1982). As deficiencies in cholinergic neurotransmission are considered to perform an essential function in the diminution of the learning and memory of AD patients, it has also been reported that the activation of the cholinergic function via the inhibition of cholinesterase may prove a clinically effective method for the treatment of AD (Millard & Broomfield, 1995).

Cholinesterases (ChEs) including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are key enzymes that perform pivotal functions in cholinergic transmission via the hydrolysis of the neurotransmitter acetylcholine (Massoulie, Pezzementi, Bom, Krejci, & Vallette, 1993).

AChE, which consists of multiple subunits, is a membrane-bound enzyme and present in the brain, muscles and cholinergic neurons. In the mammalian brain, the majority of the AChE exists in the membrane-bound G4 form, but its levels decline as the neurons degenerate (Weinstock & Groner 2008). It performs functions in the regulation of several physiological reactions via the hydrolysis of the neurotransmitter acetylcholine in the cholinergic synapses (Milatovic & Dettbarn, 1996; Schetinger et al., 2000). BChE is expressed in the neuroglia and detected in the intestine, liver, kidney, heart, lung and serum. It performs a major role in the metabolism of ester-containing compounds (Ecobicon & Corneau, 1973). It can also hydrolyze acetylcholine, and its levels do not decline, or may even exacerbate AD (Mesulam, Guillozet, Shaw, & Quinn, 2002). In the normal brain, AChE predominates but BChE activity rises, whereas AChE activity remains unchanged or diminished in the brains of AD patients (Greig et al., 2001). Therefore, a drug that inhibits both AChE and BChE may be preferable to selective AChE or BChE inhibitors. Over the past few years, synthetic ChEs inhibitors including tacrine, donepezil, rivastigmine and galanthamine have been employed for clinical treatments. However, the use of these drugs is restricted owing to their insufficient activity and their side-effects, which include hepatotoxicity and gastrointestinal disturbances (Melzer, 1998; Schulz, 2003; Small et al., 1997). Because of the adverse side-effects of existing ChEs inhibitors, the development of non-toxic ChEs inhibitors as alternatives to existing drugs is of substantial interest in the treatment of AD.

Chitooligosaccharides (COS) are chitosan derivatives (polycationic polymers comprised principally of glucosamine units) and can be generated via either chemical or enzymatic hydrolysis of

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chitosan (Dou et al., 2007). Recently, COS have been the subject of increased attention in terms of their pharmaceutical and medicinal applications, due to their non-toxic and high solubility properties as well as their positive physiological effects which include antimicrobial, anticancer, antioxidant, angiotensin-converting enzyme inhibitory and immune-stimulant effects (Je, Park, & Kim, 2004; Jeon & Kim, 2002; Jeon, Park, & Kim, 2001; Kim, Ngo, & Rajapakse, 2006; Ngo, Qian, Je, Kim, & Kim, 2008; Tokoro et al., 1988).

In the previous study, COS derivatives with different molecular weights and degrees of deacetylation have been reported to suppress the level of protein expression and AChE activity induced by $A\beta_{25-35}$ in PC12 cells (Lee, Park, Kim, Ahn, & Je, 2009). On the basis of these data, we have focused on the ChEs-inhibitory activities of COS derivatives.

In this study, we synthesized novel COS derivatives with different substitution groups and evaluated their cholinesterase inhibitory activities.

2. Experiment

2.1. Materials

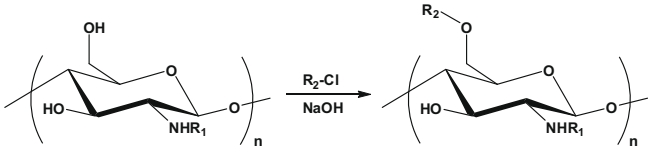
Chitooligosaccharides (MW 800∼3000 Da, degree of deacetylation, DD, 90%) prepared from crab shells were donated by Kitto Life Co. (Seoul, Korea). 2-chloroethylamino hydrochloride was purchased from Fluka (Buchs, Switzerland). 2-dimethylamino-ethylchloride hydrochloride, 2-diethylamino-ethylchloride hydrochloride, electric-eel AChE (EC 3. 1. 1. 7), horse-serum BChE (EC 3. 1. 1. 8), acetylthiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5, 5′-dithiobis [2-nitrobenzoic acid] (DTNB) and eserine were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were high-grade.

2.2. Synthesis of COS derivatives

COS derivatives were synthesized in accordance with the method developed in our previous study (Je & Kim, 2006), and the method is described in Scheme 1. COS (0.4 g) was added to each 3 M aqueous substitution solution (20 ml, AE-COS; 2-chlorethylamino hydrochloride, DMAE-COS; 2-dimethylamino-ethylchloride hydrochloride, DEAE-COS; 2-diethylamino-ethylchloride hydrochloride) with stirring at 40 °C. 3M of NaOH (20 ml) was added to the reaction mixture in a dropwise fashion, and stirred continuously for 48 h. After reaction, the solution was filtered with filter paper. The reaction solution was acidified with 0.1 N HCl and dialyzed against water for 2 days. The COS derivatives were freeze-dried and obtained as AE-COS (0.334 g), DMAE-COS (0.342 g) and DEAE-COS (0.345 g), respectively.

2.3. Characterization of COS derivatives

COS derivatives were characterized by ^{1}H NMR spectra. ^{1}H NMR spectra were determined on a JNM ECP-400 spectrometer (JEOL, Japan), using D_2O with tetramethylsilane (TMS) as an internal standard.



AE-COS : R_1=H, $COCH_3$; R_2=$(CH_2)_2NH_2$

DMAE-COS : R_1=H, $COCH_3$; R_2=$(CH_2)_2N(CH_3)_2$

DEAE-COS : R_1=H, $COCH_3$; R_2=$(CH_2)_2N(CH_2CH_3)_2$

Scheme 1. Synthesis of COS derivatives.

2.4. In vitro cholinesterase (ChEs) inhibitory activity assay

The assay for ChEs inhibition was conducted using the spectrophotometric methods developed by Ellman, Courtney, Andres, & Featherstone (1961). Acetylthiocholine iodide (ACh) and butyrylthiocholine chloride (BCh) were employed as substrates to assay the inhibitions of AChE and BChE, respectively. The reaction mixture contained: 140 μl of 100 mM sodium phosphate buffer (pH 8.0), 20 μl of test sample solution, and 20 μl of either AChE (0.36 U/ml) or BChE (0.36 U/ml) solution, which were mixed and incubated for 15 min at room temperature. The reactions were then initiated via the addition of 10 μl of DTNB (0.5 mM) and 10 μl of either ACh (0.6 mM) or BCh (0.6 mM), respectively. The hydrolysis of ACh or BCh was monitored by the following formation of yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min, which resulted from the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of either ACh or BCh, respectively. Test samples and eserine, which was used as a positive control, were dissolved in 10% ethanol. All reactions were conducted in triplicate in a 96-well microplate (SPL Life Science, Korea), using a GENios® microplate reader (Tecan Austria GmbH, Austria). The inhibitory activity of AChE was calculated as follows:

$$\text{Inhibition } \% = [\{1 - (E - S)/E\} \times 100] \quad (1)$$

where S is the initial reaction rate with the test sample and E is the initial control reaction rate. The inhibitory effects of the test compounds were expressed in IC_{50} values (the concentration necessary to inhibit 50% of the enzyme activity).

2.5. Kinetic analysis

Kinetic parameters, including the inhibition constant (K_i), Michaelis constant (K_m) and maximal velocity (V_{max}), were determined via the Lineweaver–Burk plot method at increasing concentrations of substrate (0.6, 1.2 and 1.8 mM) and inhibitors.

3. Results and discussion

3.1. Synthesis of COS derivatives

In the present study, we attempted to estimate the ChEs inhibitory activities of COS derivatives. We synthesized the COS derivatives with different substitution groups. The synthesis of COS derivatives was accomplished via the displacement of functional groups of COS. The hydroxyl groups of pyranose ring structure in COS evidenced strong reactivity in chemical reaction (Katsura, Isogai, Onabe, & Usuda, 1992). In particular, the hydroxyl group at the C-6 of the pyranose ring evidenced the highest reactivity and was replaced with aminoethyl (AE), dimethylaminoethyl (DMAE) and diethylaminoethyl (DEAE) groups, respectively. Their chemical structures were determined as AE-COS, DMAE-COS and DEAE-COS, respectively (Scheme 1). The subsistence of the substituted group was identified using the ^{1}H NMR spectra and is shown in Fig. 1. An inspection of the ^{1}H NMR spectra of AE-COS demonstrated the presence of three functional proton signals for acetyl residue, $-CH_2N$ and pyranose unit at δ 2.0, 2.8 and 2.9–3.6, respectively (Ngo et al., 2008). Additionally, DMAE-COS showed peaks for the methyl and methylene proton group at between δ 2.9 and 3.0. In the same fashion, DEAE-COS displayed signals for methyl and methylene protons at δ 1.3 and 3.3, as well as methyl protons of the protonated DEAE group at between δ 1.5 and 1.6. Of these COS derivatives, DMAE- and DEAE-COS were synthesized for the first time in this study.

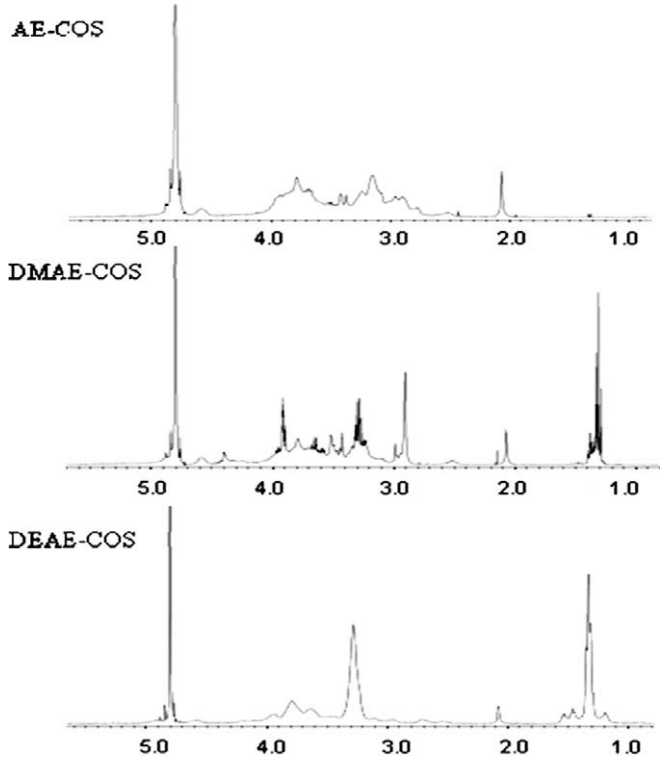


Fig. 1. ^{1}H NMR spectrum (400 MHz) of COS derivatives in D_2O.

3.2. ChEs inhibitory activities of COS derivatives

ChEs inhibitors have been well-characterized in clinical studies as the most promising AD therapeutic agents. In particular, AChE inhibitors increase the endogenous levels of acetylcholine and cholinergic neurotransmission in the brains of Alzheimer's type dementia patients (Yu, Utsuki, Brossi, & Greig, 1999).

The inhibitory activities of synthesized COS derivatives against ChEs were evaluated (Table 1). Among the COS derivatives, DEAE-COS evidenced the most potent AChE inhibitory activity with IC_{50} values of 9.2 ± 0.33 μg/ml. AE- and DMAE-COS evidenced marginal inhibitory activity toward AChE with IC_{50} values of 24.1 ± 0.39 and 56.5 ± 0.26 μg/ml, respectively. However, COS-derivatives evidenced no activity toward BChE (data not shown). Therefore, these COS derivatives exert selective inhibitory effects against AChE. Among the COS derivatives, DEAE COS, which is the most hydrophobic, showed strongest AChE inhibitory activity and this indicate that there is a hydrophobic interaction between DEAE group and AChE.

Eserine, also known as (−)-physostigmine, is a parasympathomimetic and a reversible cholinesterase inhibitor isolated from the Calabar bean, which is used to treat AD; esterine was utilized herein as a positive control. However, its side effects, which include depression and overdose, can induce cholinergic syndrome (Brenner, 2000).

Table 1
Cholinesterase inhibitory activities of COS derivatives.

Samples	IC_{50} (μg/ml)[a]
AE-COS	56.5 ± 0.26
DMAE-COS	24.1 ± 0.39
DEAE-COS	9.2 ± 0.33
Eerine[b]	0.0089 ± 0.00005

[a] Each value represents the mean ± SD of three determinations.
[b] Eerine was used as a positive control.

3.3. Determination of the inhibition pattern on AChE

The inhibitory kinetics of COS derivatives toward AChE were analyzed via Lineweaver–Burk plotting (Figs. 2–4). The lines, which were obtained from two different concentrations of AE-COS, intersect on the horizontal axis (Fig. 2). Moreover, the K_i value of AE-COS was estimated to be 0.26 mM at 40 μg/ml and 0.37 mM

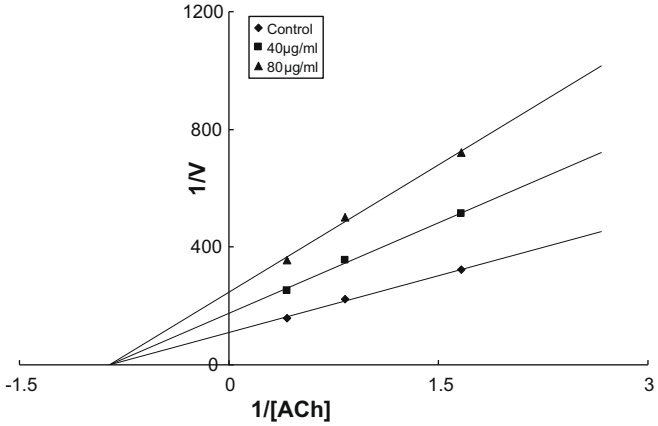


Fig. 2. Lineweaver–Burk plot of AChE in the presence of AE-COS.

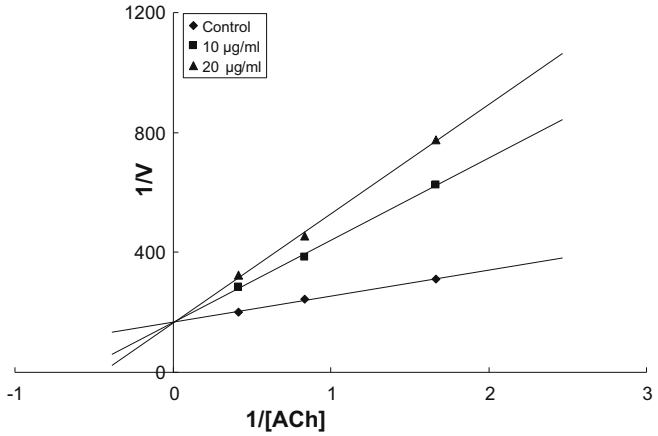


Fig. 3. Lineweaver–Burk plot of AChE in the presence of DMAE-COS.

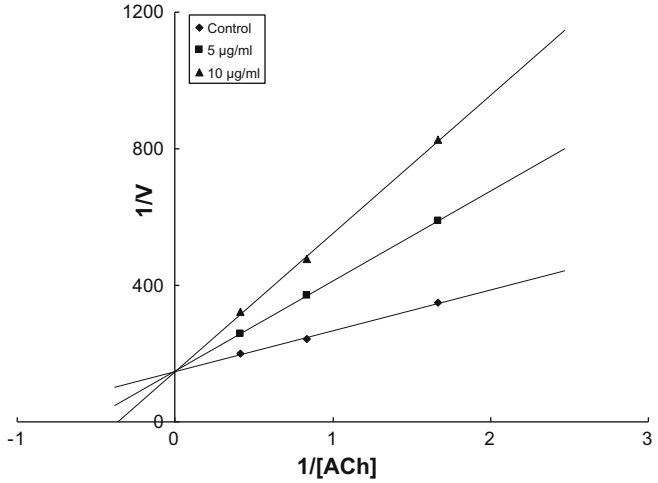


Fig. 4. Lineweaver–Burk plot of AChE in the presence of DEAE-COS.

Table 2
Kinetic parameters of AChE in the presence of COS derivatives.

Compounds (μg/ml)		K_m (mM)	V_{max} (ΔOD_{412}/min)	K_i (mM)
AE-COS	40	1.2	0.0058	0.26
	80	1.2	0.0042	0.37
DMAE-COS	10	3.4	0.011	9.2
	40	5.9	0.011	1.5
DEAE-COS	5	1.7	0.0068	4.4
	10	2.7	0.0068	4.2

at 80 μg/ml, and evidenced the same K_m value of 1.2 mM and V_{max} values of 0.0058 and 0.0042 ΔOD_{412}/min at 40 and 80 μg/ml, respectively (Table 2). As the substrate concentration varies, the V_{max} values of AChE were reduced in a dose-dependent manner, without altering the binding affinity of the catalyst for the substrate. Therefore, AE-COS was determined as a non-competitive AChE inhibitor. In this inhibition mode, the inhibitor induced an alteration in the structure and shape of the enzyme, and the modified enzyme was no longer capable of binding correctly with the substrate (Higa, 1981). The lines, which were obtained from the two different concentrations of DMAE- and DEAE-COS, intersect on the vertical axis (Figs. 3 and 4). The K_i values of DMAE- and DEAE-COS were estimated to be 9.2 mM at 10 μg/ml and 1.5 mM at 40 μg/ml, and 4.4 mM at 5 μg/ml and 4.2 mM at 10 μg/ml, respectively. Their K_m values were 3.4 mM at 10 μg/ml and 5.9 mM at 40 μg/ml, and 1.7 mM at 5 μg/ml and 2.7 mM at 10 μg/ml, respectively. Moreover, their V_{max} values were 0.011 ΔOD_{412}/min at 10 and 40 μg/ml, and 0.0068 ΔOD_{412}/min at 5 and 10 μg/ml, respectively (Table 2). As the substrate concentration varied, the V_{max} value of the reaction was not changed, whereas the binding affinity of the substrate to the binding site (K_m value) was reduced in a dose-dependent manner. Therefore, DMAE- and DEAE-COS were identified as competitive AChE inhibitors. In this inhibition mode, the inhibitor binds to the same active site as the enzyme substrate, and this may be a non-metabolizable reaction (Higa, 1981).

4. Conclusions

In this study, we synthesized a known and two novel COS derivatives with different functionality, and evaluated their AChE inhibitory activities. Of the synthesized COS derivatives, DEAE-COS evidenced the highest levels of AChE inhibitory activity, followed by DMAE-COS and AE-COS, respectively. In addition, the AChE inhibition pattern of AE-COS was shown to be non-competitive, and those of DMAE- and DEAE-COS were shown to be competitive. However, these COS derivatives evidenced no activity toward BChE. On the basis of our results, these COS derivatives exhibited selective inhibitory activities against AChE, and the hydrophobicity of COS performs a critical function in AChE inhibitory activity. Our present findings indicated that COS derivatives might be a beneficial material in the prevention or treatment of Alzheimer's disease. Furthermore, more exact mechanism studies, particularly those investigating how these compounds inhibit AChE activity in cellular systems, will be required in the future.

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