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Different chemically substituted chitooligosaccharides inhibit β-secretase activity

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ABSTRACT

Chitooligosaccharide or COS is a kind of oligosaccharide that is an integral part of the nervous system. COS is synthesized by chemical substitution and derived AE-COS, DEAE-COS and DMAE-COS that are being shown on IC_{50} value. AE-COS and DMAE-COS exhibited four fold less inhibition than DEAE-COS. In addition, non-competitive inhibitor was identified via a Dixon plot and the Ki inhibition constant (100 µg/mL).

We declared, chemical substitution of COS is a water soluble human safe BACE-1 inhibitor.

INTRODUCTION

Alzheimer's disease (AD) is a progressive and ultimately fatal neurodegenerative disorder, afflicting approximately 40 percent of the population over 80 years of age. There are around 30 million people worldwide (1, 2). By 2010, it is estimated that there will be half a million AD sufferers in the United Kingdom (3). Currently, there are approximately 600 million persons aged 60 years and older and this number will reach close to 2 billion in 2050 (4). Mechanistically speaking, AD is characterized by the accumulation of amyloid plaques and neurofibrillary tangles in the brain. The central etiology factor of AD is the amyloid beta (Abeta) peptide-mediated toxic amyloid plaque formation, which is generated by proteolytic processing of the amyloid precursor protein (APP) by beta-secretase (BACE-1) and gamma-secretase. The transmembrane protein, Presenilin 1 (PS1) plays a key role in the gamma-secretase dependent processing of APP to generate the beta-amyloid peptide, which leads to the pathogenesis of Alzheimer's disease (5). A series of dual inhibitors of acetylcholinesterase (AChE) and β-secretase (BACE-1) have been studied to explore effective novel drugs for the treatment of AD (6). Though the treatment of AD is possible through the inhibition of the enzyme, acetylcholinesterase (AChE), preventing beta-amyloidosis by inhibiting the β-secretase enzyme is a prime target for the development of novel drugs (7). Therefore, inhibition of BACE-1, the rate-limiting enzyme in the production of Abeta, is an attractive therapeutic approach and many biopharmaceutical companies are currently working to discover new BACE 1 inhibitors for the treatment of AD (8, 9). At present, several BACE 1 inhibitors are under clinical trial. These include synthetic peptides, chemically modified tetracyclines, bisphosphonates compounds isolated from natural sources. However, most of these drugs are reported to exert side effects such as, musculoskeletal pain in tendons and joints (10).

Chitin is a natural polymer found in the exoskeleton of crustaceans and insects as well as in the cell walls of certain fungi (11). Chitosan, produced by the deacetylation of chitin, is a nontoxic biopolymer with versatile chemical and physical properties, but with poor solubility (12). Chitooligosaccharides (COS) are the hydrolyzed products of chitosan that is to have biocompatibility and biodegradability with greater solubility in water. COS has several bioactive applications in medicine, dentistry, as well as in the pharmaceutical, cosmetic and food industries (13). COS of low molecular weight are highly soluble and non-toxic in nature (14). COS has shown different antimicrobial, anticancer, antioxidant, angiotensin-converting enzyme inhibitory and immune stimulant effects (14, 15-19).

The inhibitory effect of COS on acetylcholine esterase is of significant importance to the development of drugs from marine sources, for application in brain related diseases (20). On the basis of developing AChE inhibitors it became interesting to chemically modify the structural properties of chitosan and COSs in the search for novel treatment for AD and other diseases. In the present study, COS derivatives with different substitution groups such as aminoethyl-COS (AE-COS), diethylaminoethyl (DEAE-COS), and dimethylaminoethyl (DMAE-COS) were studied to evaluate their effectiveness in the inhibition of BACE 1.

MATERIALS AND METHODS

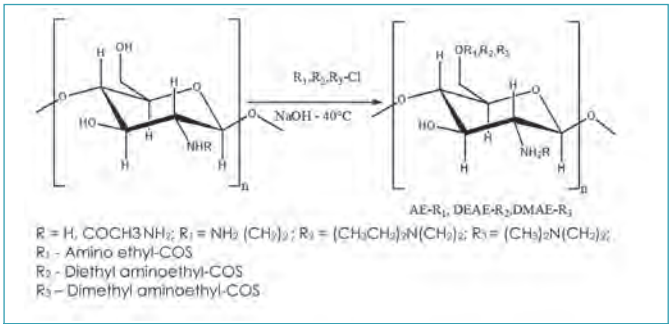
Preparation of COS

Chitooligosaccharides (MW 3-8 kDa) of 90 percent degree of deacetylation (DD) prepared from crab shells were donated by Kitto Life Co. (Seoul, Korea). 2-chloroethylamino hydrochloride (Fluka, Buchs, Switzerland), 2-dimethylamino-ethylchloride hydrochloride and 2-diethyl amino-ethylchloride hydrochloride (Sigma, St. Louis, MO) were used for chemical substitution reaction. In addition, BACE 1 (recombinant human BACE 1) assay kit was purchased from Pan Vera, USA and all other chemicals used were of high-grade.

Synthesis of COS derivatives

COS derivatives were synthesized based on a previous protocol (19, 20) and described in Scheme 1. COS (500 mg) was dissolved in 20 ml (each) of 3 M aqueous substitution solution: 2-chlorethylamino hydrochloride, 2-diethylamino-ethylchloride hydrochloride and 2-dimethylamino-ethylchloride hydrochloride at 40°C, which yielded AE-COS, DEAE-COS and DMAE-COS respectively. In addition, 20 ml NaOH (3M) was added drop wise to the reaction mixture and stirred for 48 h continuously.

This solution was filtered, acidified (0.1 N HCl) and dialyzed against water for 2 days. The derivatives (AE-COS, DEAE-COS and DMAE-COS) were freeze-dried and stored until used in the subsequent experiment with BACE enzyme (Figure 1).



Scheme 1. Synthesis of AE, DEAE and DMAE-Chitooligosaccharide.

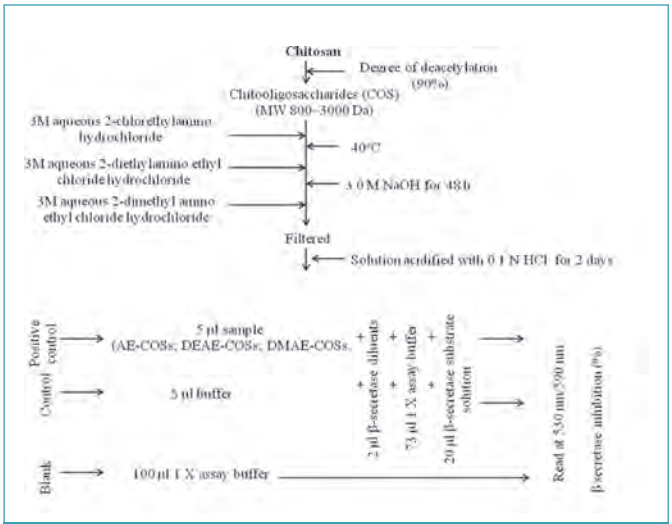


Figure 1. Schematic diagram representation of chitooligosaccharides preparation and β-secretase inhibition assay.

Enzyme assays

BACE1 assay was carried out according to the manual supplied with slight modifications (21). Briefly, a mixture of 10 µL of an assay buffer (50 mM sodium acetate, pH 4.5), 10 µL of BACE1 (1.0 U/mL), 10 µL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 µL of a sample dissolved in the assay buffer was incubated for 60 min at 25°C under dark conditions. The mixture was allowed for excitation at 530 nm and the emitted light at 590 nm was collected. The inhibition ratio was obtained by the following equation: Inhibition (percent) = [1- {(S-S_0 / C-C_0)}] x 100, where C was the fluorescence of a control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of the control at time zero, S was the fluorescence of the test samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 was the fluorescence of the tested samples at time zero. All data are the means of duplicated experiments. To check the quenching effect of the samples, the sample was added to the reaction mixture C, and any reduction in fluorescence by the sample was then calculated.

Dixon plot analysis

Dixon plot analysis was performed as follows: BACE 1 substrate was divided into three concentration groups. The reaction velocity was measured at a fixed substrate concentration with different inhibitors. A graph of the 1/V (min/F.U) against the inhibitor concentration was plotted. A graphical method was determined, the type of enzyme inhibition and constant enzyme inhibitor complex (*Ki*). In addition, the enzyme rate (v) was determined at two or more substrate concentrations, and over a range of inhibitor concentrations (I). In a plot of 1/v against I, data for each substrate concentration fell on straight lines that intersected at 1 - *Ki* and the presentation of enzyme kinetic data by which a Michaelis constant, *Km* (Michaelis kinetics) on an inhibitor constant, *Ki* was determined.

RESULTS AND DISCUSSION

Chitooligosaccharide derivatives have bioactive properties, and their pro these properties differ depending on the chemical substitution (20). In the present study, we calculated the BACE 1 inhibition by the differently chemically substituted AE-COS, DEAE-COS and DMAE-COS derivatives.

The COS derivatives were synthesized through the displacement of the functional groups. In addition, the hydroxyl groups of the pyranose ring were strongly reactive in their chemical reaction (22). From the above reactions, we have clearly seen that the hydroxyl group at the C-6 of the pyranose ring showed the highest reactivity and was replaced the AE, DEAE and DMAE groups, respectively (Scheme 1). The substituted group was identified by ^{1}H NMR spectra (20). 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 2.9-3.6, respectively (18). Additionally, DMAE-COS showed peaks for the methyl and methylene proton groups at positions 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 between δ 1.5 and 1.6, respectively (Figure 2).

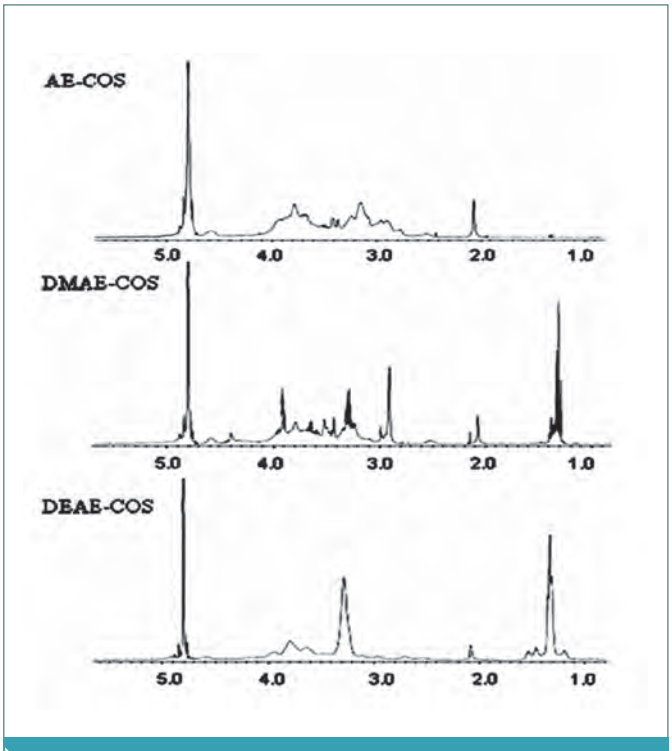


Figure 2. 1H NMR spectrum (400 MHz) of COS derivatives in D2O.

Marine drugs are a potential tool in the search for a cure for Alzheimer's disease and could be rectify the blood brain barrier and the developed plasma membrane (23). Three COSs (AE-COSs, DEAE-COSs, DMAE-COSs) were shown in their different potential for BACE 1 inhibition with dose dependent

and percentage inhibition. The percentage inhibition could reach 69.2, 75.24, and 60.24 percent at the concentration of 500 µg/mL respectively (Figure 3). Among them, DEAE-COSs showed the strongest inhibitory potential compared to AE-COSs, and DMAE-COSs. We have reported that COS with antioxidant properties were synthesized from chitosan by hydrolysis in a dose-dependent manner (24). The importance of this approach is underscored by findings that human mutations at the P1 and P2 BACE 1 cleavage subsides result in early-onset familial AD (25). We also tested concentrations of the COS derivatives at above 500 µg/mL and found that these higher concentrations significantly decreased the BACE 1 inhibition effectiveness.

Figure 2 shows that AE-COSs and DMAE-COSs possess weak BACE 1 inhibition compared to DEAE-COSs. At the concentration of 625 µg/mL AE-COSs, DMAE-COSs, and DEAE-COSs recorded 43.6, 61.5, and 38.2 percent inhibition of BACE 1 (data not shown). The IC_{50} values were calculated by non-linear regression and show the IC_{50} value of DEAE-COSs (4.32 µg/mL) is 4 times less inhibitory than AE-COSs (17.4 µg/mL) and DMAE-COSs (12.9 µg/mL) (Figure 3).

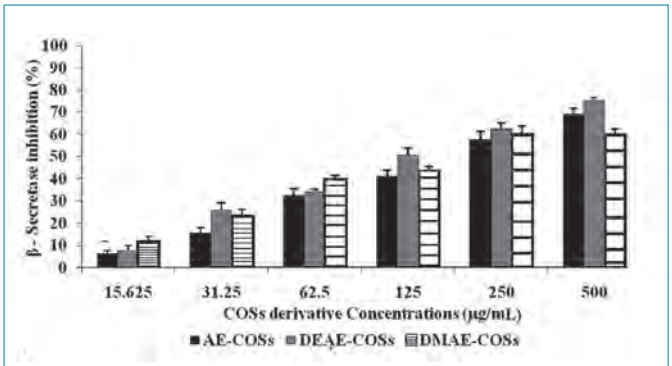


Figure 3. Concentration-dependent inhibition of β-secretase by chemical modification compounds (AE-COSs, DEAE-COSs, DMAE-COSs).

Chemically substituted compounds inhibited BACE 1 in a dose-dependent manner and were non-competitive with the substrate in the Dixon plots (Figure 3).

The chemical modification chitooligosaccharides kinetic behaviour of the chemically modified COSs was determined by Dixon plot analysis under the fluorogenic substrate.

In addition, the type of inhibition pattern is important to for understanding the mechanism of enzyme action and the inhibitor-binding site. DEAE-COSs inhibition pattern was analyzed by Dixon plots (at three concentrations: 125 µg/mL; 250 µg/mL; 500 µg/mL). Therefore, DEAE-COSs formed the inhibitor complexes during the enzyme reaction to reduce the efficiency of catalysis. The Ki value was calculated by the secondary plot of Line weaver-Burk, with the slopes of each line in the Lineweaver-Burk plot being plotted against different concentrations of DEAE-COSs, and the Ki value was determined to be 100 µg/mL. We also analyzed the kinetic behaviour using Dixon plots, and the results showed that DEAE-COSs acts as a competitive inhibitor (Figure 4). Thus, the isolated compounds appeared to be relatively specific inhibitors of BACE 1 as is the case of the other natural inhibitors we had isolated (25-26).

The Ki value was directly measured for Dixon plot as an intercept on the x-axis. DEAE-COSs 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.

There are reasons for DEAE-COSs having the more potent BACE 1 inhibition activity compared to AE-COSs and DMAE-COSs. However, more studies on the relationship between molecular size and inhibition activity are needed.

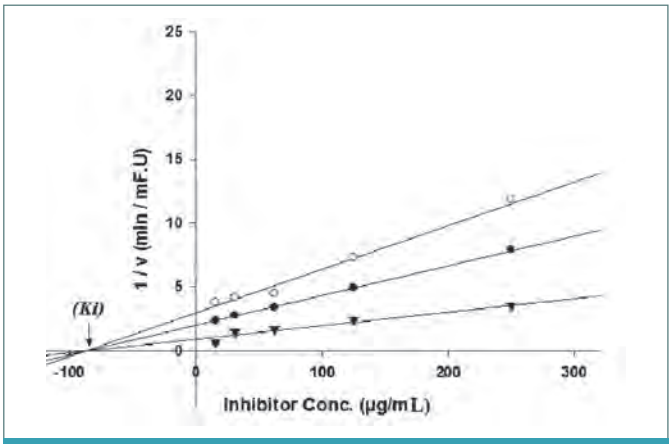


Figure 4. Determination of inhibition pattern of DEAE-Chitooligosaccharides on BACE 1 using Dixon plots. Substrate concentration was --o-- 125 µg/mL; --●-- 250 µg/mL; --▼-- 500 µg/mL.

CONCLUSIONS

In this study, we synthesized novel COS derivatives with different chemical substitution groups and evaluated their BACE 1 inhibitory activities. The results showed that DEAE-COSs prepared from chitooligosaccharides (MW 3-8 kDa) exhibited the highest BACE 1 inhibitory activity. In addition, the BACE 1 inhibition pattern of DEAE-COSs was found to be non-competitive, and the inhibition constant (Ki) was 100 µg/mL. This result suggests that the amino group plays an important role in BACE 1 inhibitory activity. In addition, the β-secretase inhibition is the most promising strategy for modifying the course of AD, and many companies have long been attempting to develop β-secretase inhibitors for this purpose. To develop medicines with sufficient clinical efficacy, the preclinical data must allow accurate prediction of the clinically effective dose. The progress in the knowledge base for β-secretase inhibitors appears to have reached a stage at which the ultimate emergence of β-secretase inhibitor drugs is probable.

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