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Bioorganic & Medicinal Chemistry 13 (2005) 6551–6555
Bioorganic & Medicinal Chemistry

Water-soluble chitosan derivatives as a BACE1 inhibitor

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Received 20 June 2005; revised 11 July 2005; accepted 12 July 2005
Available online 9 August 2005

Abstract—BACE1 (the β-site APP-cleaving enzyme) inhibitory activities of water-soluble chitosan derivatives substituted with aminoethyl, dimethylaminoethyl and diethylaminoethyl groups were investigated. AE-chitosan (90%) prepared from 90% deacetylated chitosan showed the strongest BACE1 inhibitory activity than those of other derivatives. The inhibitory pattern was found to be non-competitive by Dixon plot, and the value of the inhibition constant (K_i) was 85 μg/mL.

1. Introduction

The most common cause of dementia in older people, Alzheimer's disease (AD), claims over 20,000 lives each year, a number that is expected to explode in the next few decades as our population ages. AD is a progressive neurodegenerative disorder pathologically characterized by the presence of senile plaques composed of an amyloid Aβ-peptide (Aβ). Production of Aβ, a protein essential for the development of AD, begins with cleavage of amyloid precursor protein (APP). On the basis of the amyloid hypothesis, which states that the neurodegenerative process comprises a series of events triggered by abnormal processing of APP,[1] β- and γ-secretases that mediate the amyloidogenic processing of APP are thought to be prime drug targets in the treatment of AD. The β-secretase generates the N-terminus of Aβ peptides by cleaving APP at Met_{670}/Asp_{671}, while γ-secretase cleaves the C-terminus of the peptides by proteolysis either at Val_{711} or Ala_{713}, with the resultant Aβ peptide being either 40 or 42 amino acid residues in length.[2] The $A\beta_{42}$ peptide is the most abundant one, and plays critical roles in the induction of AD.[3]

Beta-secretase is an aspartic protease and is also known as BACE1 (the β-site APP-cleaving enzyme). This enzyme cleaves an easily accessible site at the luminal side of β-APP, and its activity is the rate-limiting step in Aβ peptide production in vivo.[4] BACE1 activity is present in a majority of cells and tissues of the body.[5] The maximal activity is found in neural tissues and cell lines.[6,7] BACE1 is widely expressed in various tissues and cell lines, but could be present at higher levels in neurons of the brain.

BACE1 is a major target for the screening of inhibitors since it occupies an initial step in the pathological cascade of AD. Thus, inhibition of BACE1 acting in vivo may reduce the production of Aβ peptides and consequently slow or halt the progression of AD.

Chitin is a naturally abundant mucopolysaccharide and is distributed in the shell of crustaceans, in the cuticle of insects, and also in the cell wall of some fungi and microorganisms. It consists of 2-acetamido-2-deoxy-(1–4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) that have intra- and inter-molecular hydrogen bonds and is a water-insoluble material resembling cellulose in its solubility. Chitosan is an N-deacetylated derivative of chitin and consists of 2-amino-2-deoxy-(1–4)-β-D-glucopyranose residues (D-glucosamine units), and is derived from chitin by deacetylation in the presence of alkali, and is rendered water-soluble by forming salts with various acids on the amino group of the D-glucosamine unit. Chitosans have been developed as new physiologically bioactive materials since they possess various biological activities, such as antibacterial activity,[8–11] hypocholesterolemic activity,[12] antitumor activity,[13] immuno-stimulating effects[14] antioxidant activity[15], and antihypertensive activity.[16] However, poor solubility of chitosan is probably the major limiting factor in its utilization. Therefore, chemical modification has been attempted to improve water solubility and bioactivities of chitosan.

Keywords: Water-soluble chitosan; BACE1; Non-competitive; AE-chitosan.
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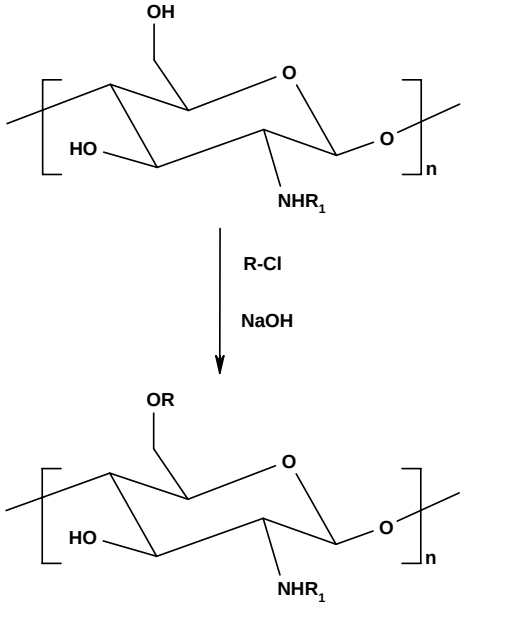
doi:10.1016/j.bmc.2005.07.004

In the present study, BACE1 inhibitory activities of water-soluble chitosan derivatives were investigated. In addition, the inhibition pattern was determined using Dixon plots.

2. Results and discussion

Chitin was prepared from crab shells, and chitosans with a different degree of deacetylation were prepared successfully according to our previous method, and the degree of deacetylation designated as 90% and 50%.[17] To improve the solubility and bioactivity, the amino functionality was successfully grafted onto chitosan to form water-soluble aminoethyl-chitosan (AE-chitosan), dimethylaminoethyl-chitosan (DMAE-chitosan), and diethylaminoethyl-chitosan (DEAE-chitosan). Chitosan derivatives were designated as AE-chitosan (90%), DMAE-chitosan (90%), and DEAE-chitosan (90%) prepared from 90% deacetylated chitosan, and AE-chitosan (50%), DMAE-chitosan (50%), and DEAE-chitosan (50%) prepared from 50% deacetylated chitosan (Scheme 1).

Chitosan was aminoalkylated, and the hydroxyl group was successfully replaced by an aminoalkyl group, while the polymeric structure was maintained. Characterization of water-soluble chitosan derivatives is shown in Table 1. The degree of substitution (DS) of DEAE-chitosan was obtained from the elemental analysis and the peak ratios between anomeric protons and methyl protons of the DEAE groups were determined using the ^{1}H NMR method.[18] Chitosan derivatives were characterized by FT-IR and ^{1}H NMR. In the IR spectra of the substituted DEAE group, the adsorptions at 2965 cm^{-1} due to C–H stretching and at 1000–1150 cm^{-1}due to C–O–C stretching were stronger, supporting the occurrence of substitution (Fig. 1). It was estimated that the C-6 hydroxyl group was possibly replaced by a DEAE group in accordance with the result of Katsura et al.[18] The ^{1}H NMR spectra of DEAE-chitosan in D_2O showed peaks at 1.30 ppm for the methyl, at 3.28 ppm for methylene protons of the DEAE group, and between 1.5 and 1.6 ppm for methyl protons of the protonated DEAE groups (Fig. 2C). In the same manner, a peak was observed between 2.9 and 3.0 ppm for methyl and methylene protons of the DMAE group (Fig. 2B), and a peak at 2.9 ppm for methylene protons of the AE group was also observed (Fig. 2A). AE-chitosan (50%), DMAE-chitosan (50%), and DEAE-chitosan (50%) were characterized in the same manner.



AE-chitosan : R=$(CH_2)_2NH_2$; R_1=H, $COCH_3$

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

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

Scheme 1. Structure of chitosan derivatives.

BACE1 (β-secretase) is a key enzyme that is involved in the production of amyloid β-peptides found in extracellular amyloid plaques of AD. This and other genetic and pathological evidence has led to therapeutic approaches that have focused on the inhibition of BACE1. The BACE1 inhibitory activities of chitosan derivatives are shown in Figures 3 and 4. Among chitosan derivatives that were prepared from 90% deacetylated chitosan, AE-chitosan exhibited inhibitory activity higher than those of DMAE-chitosan and DEAE-chitosan. Chitosan derivatives derived from 50% deacetylated chitosan also exhibited the same inhibitory pattern in the order of AE-chitosan > DMAE-chitosan > DEAE-chitosan. In comparison with AE-chitosan (90%) and AE-chitosan (50%), AE-chitosan (90%) showed inhibitory activity stronger than that of AE-chitosan (50%), and AE-chitosan (90% and 50%) revealed inhibitory activity higher than those of other derivatives. However, DMAE-chitosan and DEAE-chitosan (90%) exhibited little higher or similar inhibitory activity than those of DMAE-chitosan and DEAE-chitosan (50%). These results have suggested that free amino group at the C-2 and C-6 positions plays an important role in BACE1 inhibitory activity; however, free amino group at C-2 is a minor factor according to the above results. In recent studies, peptidic inhibitors are targeted as β-secretase inhibitors. Shuto et al.[19] elucidated that a synthesized octapeptide (Glu-Val-Leu-Pns-Asp-Ala-Glu-Phe) showed the highest activity (IC_{50} value = 0.41 μM) among the tested peptidic inhibitors. In spite of the highest inhibition efficiency, they reported that this octapeptide is needed to reduce the size of molecular weight to overcome metabolic instability. Non-peptidic inhibitors extracted from green tea exhibited an IC_{50} value of 1.6–4.5 μM.[20] Park et al.[21] isolated from the culture broth of *Phellinus linteus* and identified it as hispidin with an IC_{50} value of 4.9 μM. The BACE1 inhibitory activity in the present study was less than those of peptidic inhibitors and non-peptidic inhibitors. Also, further studies on cell culture system are needed. However, it is very meaningful in that this is the first report on chitosan derivatives. In addition, AE-chitosan (90%) was non-competitive with a substrate in the Dixon plots, and inhibition constant (K_i) was 85

Table 1. Characterization of chitosan derivatives by chemical modification

Derivatives	Elemental analysis (wt%)			Degree of substitution	Color of resultant	Solubility
	C	N	H			
AE-chitosan (50%)	46.08	15.66	8.29	0.92	Yellow	Easily soluble
DMAE-chitosan (50%)	44.00	8.991	7.541	0.69	White	Easily soluble
DEAE-chitosan (50%)	42.52	9.437	10.05	0.63	White	Easily soluble
AE-chitosan (90%)	49.82	13.74	6.888	0.88	Yellow	Easily soluble
DMAE-chitosan (90%)	47.49	8.894	7.482	0.75	White	Easily soluble
DEAE-chitosan (90%)	45.79	9.726	9.710	0.67	White	Easily soluble

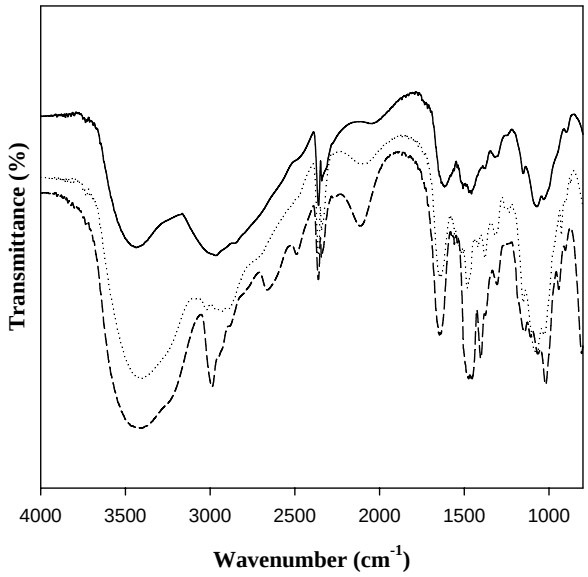


Figure 1. FT-IR spectra of chitosan derivatives prepared from 90% deacetylated chitosan. (–), AE-chitosan; (···), DMAE-chitosan; (--), and DEAE-chitosan.

μg/mL (Fig. 5). Thus, it strongly suggests that AE-chitosan (90%) might bind to either another regulatory site or to the subsite of β-secretase. The immune response is very active in AD and may contribute to the disease rather than helping. The brain's immune cells respond to the plaques and tangles, and attempt to clean up this debris. This is a natural response; however, plaques and tangles are very difficult to dissolve.[22] In the process of trying to digest the material within plaques and tangles, microglia also release pro-inflammatory proteins and free radicals, which cause secondary damage.[23] The chitosan derivatives prepared in this study, inhibited not only BACE1 but also suppressed reactive oxygen species (data not published). These derivatives are expected to be used in the prevention and might be useful for the development of novel non-peptidic inhibitors.

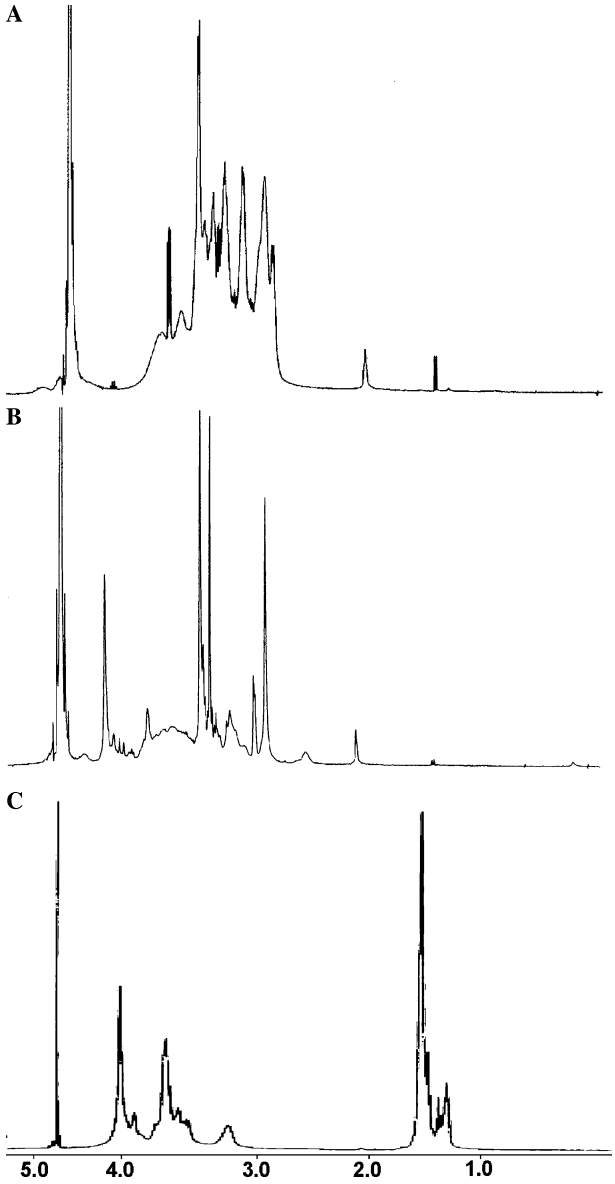


Figure 2. ^{1}H NMR spectra of water-soluble chitosan derivatives prepared from 90% deacetylated chitosan. (A) AE-chitosan, (B) DMAE-chitosan, and (C) DEAE-chitosan

3. Conclusion

In the present study, water-soluble chitosan derivatives were prepared by chemical modification, and their BACE1 inhibitory activities were evaluated. The results showed that AE-chitosan (90%) prepared from 90% deacetylated chitosan exhibited the highest BACE1 inhibitory activity. In addition, the BACE1 inhibition pattern of AE-chitosan was found to be non-competitive, and the inhibition constant (K_i) was 85 μg/mL. This result suggested that the amino group plays an important role in BACE1 inhibitory activity.

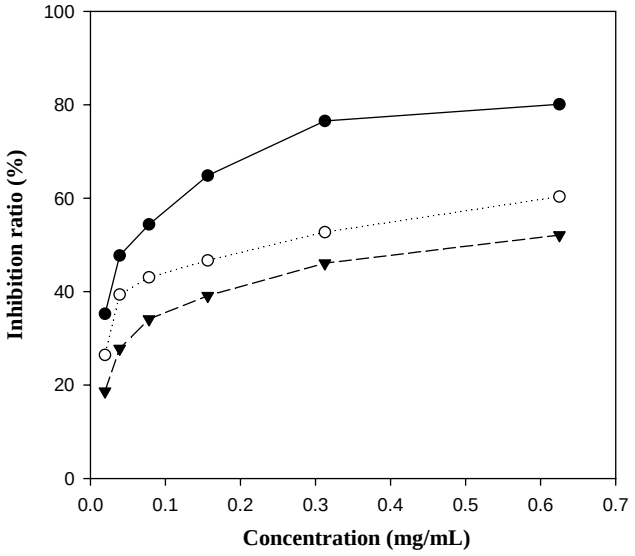


Figure 3. Concentration-dependent inhibition of BACE1 by chitosan derivatives prepared from 90% deacetylated chitosan. (-●-), AE-chitosan; (-○-), DMAE-chitosan; and (-▼-), DEAE-chitosan.

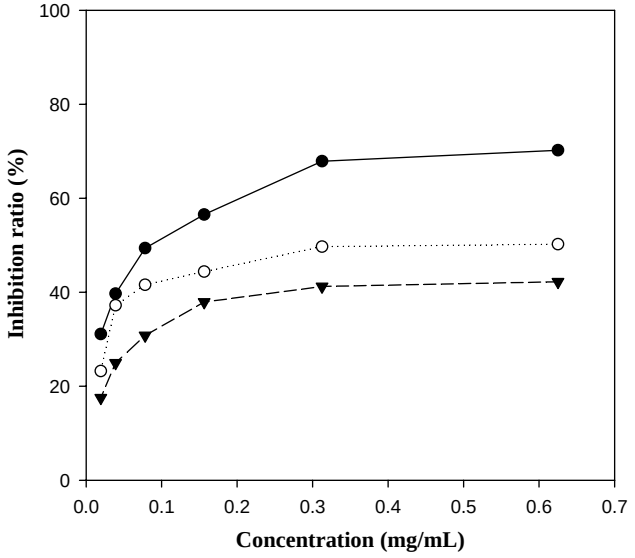


Figure 4. Concentration-dependent inhibition of BACE1 by chitosan derivatives prepared from 50% deacetylated chitosan. (-●-), AE-chitosan; (-○-), DMAE-chitosan; and (-▼-), DEAE-chitosan.

4. Experimental

4.1. Materials

Chitin prepared from crab shells was donated by Kitto Life (Seoul, Korea). For the preparation of chitosan derivatives, 2-chloroethylamino hydrochloride was purchased from Fluka, while 2-(dimethylamino)ethylchloride hydrochloride and 2-(diethylamino)ethylchloride hydrochloride were obtained from Sigma Chemical (St. Louis, MO). BACE1 (recombinant human BACE1) assay kit was purchased from Pan Vera, USA. All other reagents were of the highest grade available commercially.

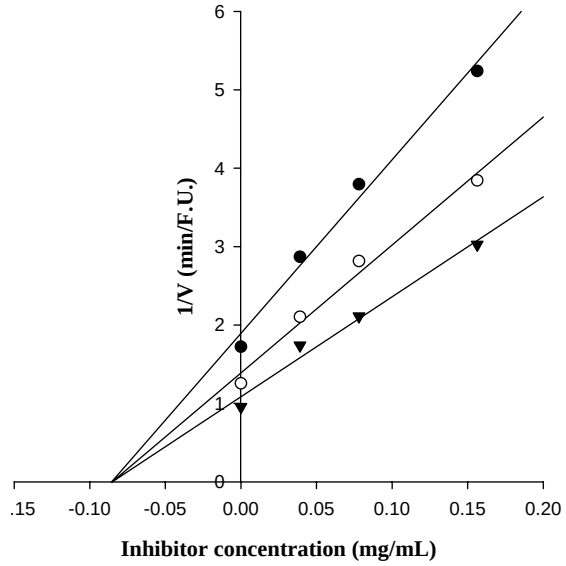


Figure 5. Determination of inhibition pattern of AE-chitosan (90%) on BACE1 using Dixon plots. Substrate concentration was (-●-), 375 nM; (-○-), 563 nM; and (-▼-), 750 nM.

4.2. Preparation of chitosans with a different degree of deacetylation

Partially acetylated chitosans were prepared from crab chitin by N-deacetylation with 40% (w/w) sodium hydroxide solution at 100 °C for various times.[17] The average molecular weights of the chitosan were 1.4×10^5 to 3.1×10^5, as determined by viscosimetry.[22] After the reaction, chitosan samples were washed thoroughly with distilled water and freeze-dried.

4.3. Synthesis of water-soluble chitosan derivatives

Water-soluble chitosan derivatives were synthesized, as shown in Scheme 1. Chitosan was aminoalkylated using the method of Clifford and Naoyuki.[24] Aqueous 3.0 M (15 mL) aminoalkyl hydrochloride was added to chitosan (0.30 g) with stirring at 65 °C. NaOH of 3.0 M (15 mL) was added to the reaction mixture dropwise and continuously stirred for 18 h. Subsequently, the reaction mixture was acidified with HCl and dialyzed against water for 2 day. The product was freeze-dried to give the aminoderivatized chitosan.

4.4. Instrumental analyses

Synthesized derivatives were fully characterized by FT-IR, ^{1}H NMR, and elemental analysis. ^{1}H NMR measurements were performed on a JEOL JNM ECP-400 NMR spectrometer under a static magnetic field of 400 MHz and chemical shift values are given in δ (ppm). IR spectra were obtained on a Perkin Elmer Spectrum GX spectrometer and elemental analysis of chitin derivatives to measure the degree of substitution was performed on Elementar Vario EL elemental analyzer.

4.5. BACE1 inhibitory assay

The assay was carried out according to the supplied manual with modifications. Briefly, the mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL BACE1 (1.0 U/mL), 10 μL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of sample dissolved in the assay buffer was incubated for 60 min at room temperature. 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 $(\%) = [1 - \{(S - S_0)/(C - C_0)\} \times 100$, where C is the fluorescence of a control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 is the fluorescence of control at zero time, S is the fluorescence of tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 is the fluorescence of the tested sample at zero time. All data are the means of duplicate experiments.

4.6. Dixon plot analysis

Experiments for Dixon plot analysis were performed as follows: BACE1 substrate divided into three group concentrations. The reaction velocity is measured at a fixed concentration of substrate but at a variety of inhibitor concentrations. A graph of the $1/V$ (min/F.U.) against inhibitor concentration is plotted.

Acknowledgments

This research was supported by a Grant (P-2004-02) from Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea.

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