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Chitooligosaccharides as a novel β-secretase inhibitor

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Abstract

Nine kinds of hetero-chitooligosaccharides (hetero-COSs) with different degrees of deacetylation and molecular weights were prepared using an ultrafiltration (UF) membrane reactor system. In addition, their sulfated derivatives were also synthesized by a method using trimethylamine-sulfur trioxide to investigate the functional group of COSs on β-secretase inhibitory activity. 90-MMWCOSs-I, which are 90% deacetylated COSs passed through the 5 kDa membrane but not passed through the 3 kDa membrane, exhibited the highest β-secretase inhibitory activity (25–42 μM) based on molecular weight of 3 and 5 kDa. The inhibition pattern of the inhibitor was found to be a non-competitive by Dixon plot, and K_i of 90-MMWCOSs-I was 3.87–6.47 μM. Therefore, the data of this research suggest that 90-MMWCOSs-I is a good candidate target molecule to inhibit β-secretase.
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Keywords: Alzheimer's disease; β-Secretase inhibitor; Chitooligosaccharide (COS)

1. Introduction

Alzheimer's disease (AD) is thought to be caused by the progressive brain accumulation of β-amyloid (Aβ) peptides into fibrillar aggregates and insoluble plaques resulting severe memory loss and neuronal cell death (Selkoe, 2001). The Aβ peptides are generated by endoproteolysis of the β-amyloid precursor protein (β-APP) by two proteolytic enzymes, β- and γ-secretase. 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}, the resultant Aβ peptides is either 40 or 42 amino acid residues in length (Dorrel, 2000). The $Aβ_{42}$ peptide is the most abundant and which play critical roles on the induction of AD (Zohar, Cavallaro, Agata, & Alkon, 2003).

β-Secretase is an aspartic protease and also known as BACE (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 (Vassar et al., 1999). β-Secretase activity is present in the majority of cells and tissues of the body (Haass et al., 1992). The maximal activity is found in neural tissues and cell lines (Seubert et al., 1993; Zhao et al., 1996). β-Secretase is widely expressed in various tissues and cell lines, but would be at higher levels in neurons of the brain.

β-Secretase is a major target for screening of inhibitors since it occupies the initial step in the pathological cascade of AD. Thus, the inhibition of β-secretase acting in vivo may reduce the production of Aβ peptides expecting that it slow or halt the progression of AD.

Recently, two transition-state analog inhibitors of β-secretase were reported on the basis of the model on the cleavage site on the β-secretase of the Swedish mutation (Zhao et al., 1996). The first inhibitor, P10-P4'StatVal, contains Asn at P2, statine group at P1, and Val at P1', and has an IC_{50} value of ~30 nM (Sinha et al., 1999). The other type or reported inhibitors are OM99-1 and OM99-2. OM99-2 has an IC_{50} of ~1.6 nM and is P4-P4′ with AsnLeu at P2-P1, Ala at P1′, and a hydroxyethylene isostere between P1 and P1′ (Ghosh et al., 2001). However, the therapeutic potential of above inhibitors might be restricted by their higher molecular weight (MW, about 1100 Da) and numerous peptide bonds (Ghosh et al., 2001). In order to be

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a good candidate for therapeutic potential, the molecular weights of inhibitors are preferably smaller than 700 Da, so large peptide-base inhibitors are not viable drug candidates. Thus, the peptidic inhibitors and the metabolites of plants and microbes which have relatively low molecular weights and lipophilicity might be good β-secretase inhibitors as drug candidates (Dorrel, 2000).

In previous studies, potent peptidic inhibitors of the β-secretase have already been identified (Ghosh et al., 2001; Vassar et al., 1999; Zohar et al., 2003). However, there was not published report of carbohydrates as β-secretase inhibitors. Chitosan is a deacetylated polymer of *N*-acetyl glucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. It has shown to possess a hypocholesterolemic effect (Jennings, Boleyn, Bridges, Wood, & Anderson, 1988; LeHoux & Grondin, 1993; Maezaki et al., 1993), an immunomodulating function (Lim et al., 1997), and a hypoglycemic effect (Miura, Usami, Tsuura, Ishida, & Seino, 1995). However, recent studies on chitosan have attracted interest for converting chitosan to its oligosaccharides, because the oligosaccharides are not only water soluble but also reported to have special functional properties such as antitumor activity (Jeon & Kim, 2002; Suzuki et al., 1986), immunostimulating effect (Jeon & Kim, 2001), antimicrobial activity (Hadwiger & Beckman, 1980; Kendra, Christian, & Hadwiger, 1989; Park, Je, Byun, Moon, & Kim, 2004), radical scavenging activity (Je, Park, & Kim, 2004).

In the present study, β-secretase inhibitory activity of hetero-chitooligosaccharides (hetero-COSs) prepared from partially different deacetylated chitosans was investigated, and the inhibition pattern was also determined using COSs with molecular weights between 3000 and 5000 Da prepared from 90% deacetylated chitosan, which exhibited the highest β-secretase inhibitory activity as an inhibitor determined by using Dixon plots.

2. Materials and methods

2.1. Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). The chitosanase (35,000 U/g protein) derived from *Bacillus* sp. was purchased from Amicosen Co. (Jinju, Korea), and cellulose was donated by Pacific Chemical Co. (Seoul, Korea). An ultrafiltration (UF) membrane reactor system (Minitan™) for production of hetero-COSs was from Millipore Co. (Bedford, MA, USA).

Fluorogenic substrate FS-1, NH2-Arg-Glu (EDANS)-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys(DABCYL)-Arg-COOH (MW, 2005.0 kDa), was synthesized at SynPep (Dublin, CA) and FS-2 (MCA) Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys(DNP) (MW, 1533.6 kDa) was synthesized at the Molecular Biology Resource Center, University of Oklahoma Health Sciences Center, using an Applied Biosystems Peptide Synthesizer 430A (Foster, CA). In addition, β-secretase (198 U/mg-protein) was purchased from the Molecular Biology Resource Center, University of Oklahoma Health Sciences Center. All other reagents were the highest grade commercially available.

2.2. Preparation of hetero-COSs and synthesis of sulfated hetero-COSs

Three kinds of partially deacetylated chitosans, 90, 75 and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deactylation with 40% (w/v) sodium hydroxide solution for different durations based on the method of Park, Je, and Kim (2004), and hetero-COSs, which are COSs prepared from 90, 75, and 50% deacetylated chitosans, were prepared by hydrolysis of hetero-chitosans in an UF membrane reactor system according to the method of Park, Lee, and Kim (2004). The sulfated COSs were synthesized according to the method of Park, Je, Jung, Ahn, and Kim (2004), and dialyzed exhaustively against distilled water using an electronic dialyzer (Micro Acilyzer G3, Asahi Chemical Industry Co.(Tokyo, Japan). The dialyzer membrane used was Aciplex Cartridge (AC-230-400).

2.3. Assay for β-secretase inhibitory activity

The inhibition assay of β-secretase was performed by the method of Ermolieff et al. (2000). Assay for β-secretase inhibitory activity was performed in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C with 10% dimethyl sulfoxide (DMSO) using substrate 300 μM FS-1. The reaction was initiated with 1780 μl buffer, 180 μl DMSO and 20 μl enzyme in cubic cell for 10 min. And then 20 μl substrate was added to it placed in Fluorescence spectrophotometer (Perkin–Elimer LS50B, Beaconsfield Bucks, UK). For kinetic assays, initial velocity and steady state were strictly maintained. Substrate concentrations in the range 0.6–6.0 μM were used. The increase of fluorescence intensity produced during substrate hydrolysis was studied in a continuous assay using Fluorescence spectrophotometer. All data were measured as mean of triplicate. An excitation wavelength of 350 nm and an emission wavelength of 490 nm were used to monitor the hydrolysis of substrate FS-1. The IC_{50} value was defined as a concentration of the β-secretase inhibitor that is required to inhibit 50% of the inhibitory activity. In addition, inhibition constants (K_i) of β-secretase inhibitors were calculated by Dixon plots.

3. Results and discussion

Recently, chitosan and its oligomers have reported to possess various bioactive activities, and their properties are presumed to be dependent on their degree of deacetylation

and molecular weights. However, there is no information on bioactive properties for both degree of deacetylation and their molecular weights. Therefore, nine different kinds of hetero-COSs were prepared by an UF membrane reactor system to investigate a biological activity based on their degree of deacetylation and molecular weights. The deacetylated chitosans of 90, 75, and 50% were hydrolyzed and fractionated by passing them through three UF membranes of molecular weight cut-off (MWCO) 10, 5, and 1 kDa, respectively. The hetero-COSs were named 90-HMWCOSs, 75-HMWCOSs, and 50-HMWCOSs, that are 90, 75, and 50% deacetylated COSs passed through the MWCO 10 kDa membrane but not passed through the 5 kDa membrane, 90-MMWCOSs, 75-MMWCOSs, and 50-MMWCOSs, that are 90, 75, and 50% deacetylated COSs passed through the 5 kDa membrane but not passed through the 1 kDa membrane, and 90-LMWCOSws, 75-LMWCOSs, and 50-LMWCOSs, that are 90, 75, and 50% deacetylated COSs passed through the 1 kDa membrane, respectively.

The β-secretase inhibitory activity of nine different kinds of hetero-COSs was shown in Fig. 1. Among different deacetylated COSs, 90% deacetylated COSs showed approximately three times higher β-secretase inhibitory activity than those of 75 and 50% deacetylated COSs in all of the molecular weight distributions. In addition, 90-MMWCOSs exhibited the highest inhibitory activity compared with that of the other hetero-COSs. These results indicated that β-secretase inhibitory activity of hetero-COSs was different on degree of deacetylation compared with molecular weight distributions of COSs.

In addition, 90-COS su lfates were synthesized from 90-COSs that exhibited the highest inhibitory activity, to investigate the effect of functional group of COSs on β-secretase inhibitory activity. In our previous study, sulfate groups were identified at the positions of C-2, C-3 and C-6 (Park et al., 2004). The inhibitory activities of 90-COSs and 90-COS sulfates in all of the molecular weight distributions are shown in Fig. 2. The 90-COS sulfates showed a lower inhibitory activity compared with that of 90-COSs. Above results indicated that the deacetylation and sulfation at C-2 position of COSs have an effect on β-secretase inhibitory activity. Further amine group at C-2 position was shown to be beneficial for the β-secretase inhibitory activity. Also the 90-MMWCOSs were fractionated into 90-MMWCOSs-I, that are 90-COSs passed through the 5 kDa membrane but not passed through the 3 kDa membrane, and 90-MMWCOSs-II, that are 90-COSs passed through the 3 kDa membrane but not passed through the 1 kDa membrane by UF membrane reactor system with MWCO 1 and 3 kDa membranes. β-Secretase inhibitory activity of *N*-acetylglucosamine, D-glucosamine, 90-MMWCOSs-I, and 90-MMWCOSs-II are shown in Fig. 3. *N*-acetylglucosamine and D-glucosamine were

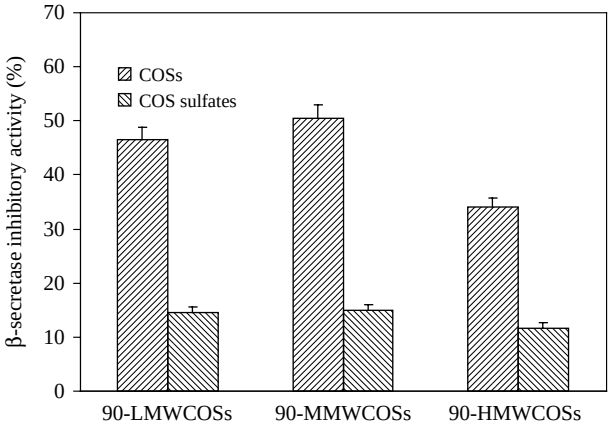

Fig. 2. Comparison of β-secretase inhibitory activity of chitooligosaccharides and chitooligosaccharide sulfates. Molecular weight distributions of each 90% deacetylated COSs is shown 90-LMWCOSs, below 1 kDa; 90-MMWCOSs, 1–5 kDa; 90-HMWCOSs, 5–10 kDa.

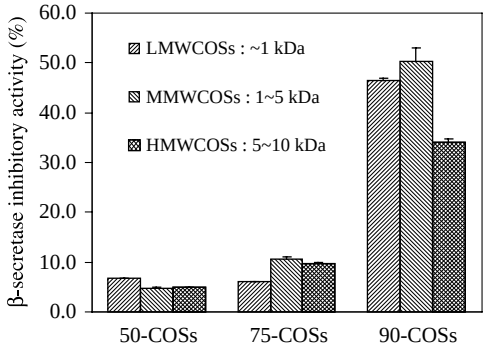

Fig. 1. β-Secretase inhibitory activity of the chitooligosaccharides fractionated by the molecular weight distribution and the degree of deacetylation. 50, 75, and 90% deacetylated chitooligosaccharides is shown 50-COS, 75-COS, 90-COS, respectively. Molecular weight distributions of each COSs is shown LMWCOSs, below 1 kDa; MMWCOSs, 1–5 kDa; HMWCOSs, 5–10 kDa.

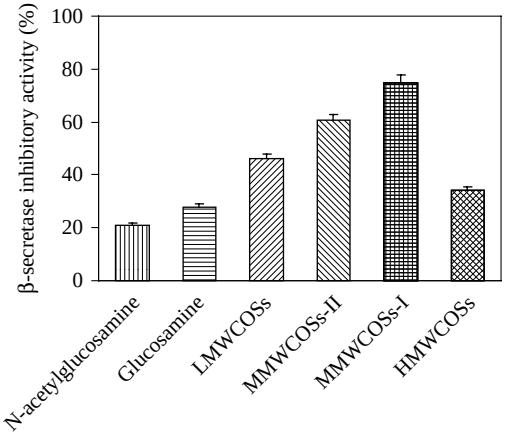

Fig. 3. β-Secretase inhibitory activity of *N*-acetylgulcosamine, glucosamine and 90% deacetylated chitooligosaccharides fractionated by molecular weight distributions. LMWCOSs, below 1 kDa; MMWCOSs-I, 3–5 kDa; MMWCOSs-II, 1–3 kDa; HMWCOSs, 5–10 kDa.

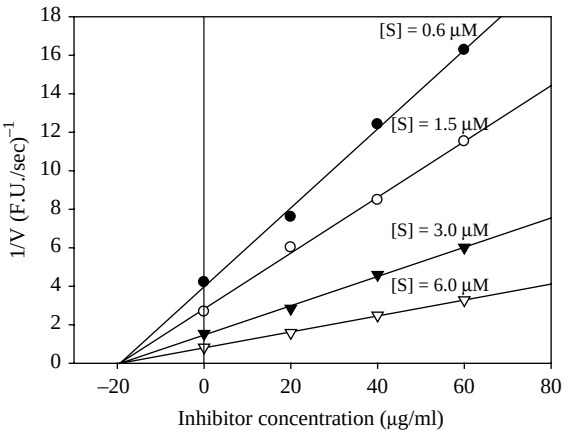

Fig. 4. Dixon plot for determining inhibitor constants of 90-MMWCOSs-II against β-secretase with substrate concentration.

exhibited lower inhibitory activities as much as 55 and 45% compared with that of 90-MMWCOSs-I, respectively. However, the inhibitory activity of D-glucosamine was higher than that of *N*-acetylglucosamine with *N*-acetyl group at C-2 position. The β-secretase inhibitory activity of 90-MMWCOSs-I was approximately 20% higher than that of 90-MMWCOSs-II. We further characterized the inhibitory concentration of 90-MMWCOSs-I, and the IC_{50} value was determined to be 25–42 μM based on molecular weights of 3 and 5 kDa. In recent studies, peptidic inhibitors are targeted as β-secretase inhibitors. Shuto et al. (2003) 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 the octapeptide is needed to reduce the size of molecular weight to overcome the metabolic instability. Kimura et al. (2004) recently reported the synthesis of a small-sized and highly potent β-secretase inhibitor KMI-370 (IC_{50} value = 3.4 nM) using octapeptide as a lead compound.

Non-peptidic inhibitors extracted from green tea exhibited the IC_{50} values of 1.6–4.5 μM (Jeon, Bae, Seong, & Song, 2003). The inhibitory activity of this present study of the 90-MMWCOSs-I was less than that of peptidic inhibitors. However, this is the first report on the target inhibitors using COSs as non-peptidic inhibitors. β-Secretase inhibition pattern against 90-MMWCOSs-I was found to be non-competitive at the active site of β-secretase determined using Dixon plot (Fig. 4). Thus, it strongly suggests that 90-MMWCOSs-I might be bind to either another regulatory site or subsite of β-secretase. As summarized in Table 1, the inhibition constant (K_i) of the 90-MMWCOSs-I was about 3.87–6.47 μM calculated by Dixon plot.

Table 1
Inhibitor constants (K_i) of 90-MMWCOSs-II (MW 3–5 kDa) calculated by Dixon plot

Substrate (μM)	Linear formula of plot	*R*	K_i (M)
0.6	$y=0.205x+3.970$	0.996	3.87×10^{-6}–6.46×10^{-6}
1.5	$y=0.145x+2.823$	0.997	3.89×10^{-6}–6.45×10^{-6}
3.0	$y=0.0760+1.470$	0.997	3.87×10^{-6}–6.45×10^{-6}
6.0	$y=0.0414+0.803$	0.999	3.88×10^{-6}–6.47×10^{-6}

In conclusion, β-secretase inhibitory activity of COSs was dependent on relatively high degree of deacetylation, and the 90-MMWCOSs-I showed the higher β-secretase inhibitory activity. Therefore, 90-MMWCOSs-I is a good candidate target molecules as a β-secretase inhibitor.

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