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Characterization of a New Antidementia β-Secretase Inhibitory Peptide from *Rubus coreanus*

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Abstract In order to develop a potent antidementia β-secretase inhibitor from phytochemicals, β-secretase inhibitory activities of extracts from many medicinal plants and herbs were determined. Water extracts from *Rubus coreanus* showed the highest β-secretase inhibitory activity of 84.5%. After purification of the β-secretase inhibitor from *R. coreanus* using systematic solvent extraction, ultrafiltration, Sephadex G-10 column chromatography, and reverse-phase high performance liquid chromatography (HPLC), a purified β-secretase inhibitor with IC_{50} inhibitory activity of 6.3×10^3 ng/mL (1.56×10^{-6} M) was obtained with a 0.08% solid yield. The molecular mass of the purified β-secretase inhibitor was estimated to be 576 Da by liquid chromatography-mass spectrometry (LC-MS) and β-secretase inhibitor also is a new tetrapeptide with the sequence Gly-Trp-Trp-Glu. The purified β-secretase inhibitory peptide inhibited β-secretase non-competitively and also show less inhibition on trypsin, however no inhibition on other proteases such as α-secretase, chymotrypsin, and elastase.

Key words: antidementia, β-secretase inhibitory peptide, *Rubus coreanus*

Introduction

Dementia is thought to be caused by β-amyloid precipitation by β-secretase in the brain. The dense plaque, β-amyloid is a 39-43 residue fragment of a larger membrane spanning glycoprotein known as the β-amyloid precursor protein (β-APP) (1). The APP is processed into many different forms through a combination of select cellular protease (α-, β-, and γ-secretase).

β-Secretase is an aspartic protease known as BACE1 (the β-site APP-cleaving enzyme). This enzyme cleaves to 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* (2). β-Secretase has maximal activity at an acidic pH, as agents that disrupt intracellular pH also inhibit β-secretase activity (3,4). Moreover, its active site is located within the lumen of acidic intracellular compartments. β-Secretase is highly sequence-specific (5) and it is also insensitive to pepstatin, an inhibitor of many types of aspartic protease (6).

For β-secretase inhibitor, numerous groups have focused on the identification of inhibitors using high-throughput screening of compound collection and natural product extracts. Some peptidic β-secretase inhibitors and non-peptidomimic derivatives were developed or synthesized (7-10). In order to be a good candidate for therapeutic potential, the Mw of inhibitors should be under 700 Da because efficacy requires penetration of the blood-brain barrier. Thus, the metabolites of plants and microbes which have relatively low Mw and lipophilicity may be good β-secretase inhibitors as drug candidates (7,11,12). Chitosan derivatives from crab shell and latifolin from *Dalbergia sissoo* exhibited weak β-secretase inhibition (13,14). Catechins from green tea (15), ellagic acid, and punicalagin from pomegranate (16), hispidin from mycelial cultures of *Phellinus linteus* (17) and several compounds isolated from *Sanguisorbae radix* (18) have been studied as β-secretase inhibitors. Although many studies regarding the treatment of dementia have been performed, useful antidementia drugs or nutraceuticals with high β-secretase inhibitory activity without side effect have yet to be developed. This study was perfermed to screen a potent β-secretase inhibitor-containing medicinal plant and optimize its extraction condition. Further, this study tries to purify and characterize the β-secretase inhibitor for development of a novel antidementia β-secretase inhibitor leading to utilization in the drug or functional food industries.

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Materials and Methods

Medicinal plants and chemicals One-hundred and five kinds of medicinal plants including *Aralia elata, Caragana sinica, Chaenomeles sinensis, Coriandrum sativum, Eleutherococcus senticosus, Foeniculum vulgare, Forsythia koreana, Lonicera japonica, Lycii cortex Radicis, Lycii folium, Lycii fructus, Menispermum dauricum, Parthenocissus tricuspidata, Phellodendron amurense, Scrophularia takesimensis,* and *Rosa hybrida* were obtained from Research Institute of *Lycii fructus* in Cheongyang, Chungnam and oriental medicinal plant markets of Geumsan, Chungnam, Korea.

Unless otherwise specified, all chemicals and solvents were of analytical grade. The β-secretase (recombinant human BACE1) assay kit was purchased from the PanVera Co. (BACE1; Invitrogen, Madison, WI, USA), and Sephadex G-10 was purchased from Pharmacia Fine Chemicals (Sephadex™ G-10; Amersham Biosciences, Uppsala, Sweden). The Stirred Ultrafiltration® Cell was procured from Millipore Co. (Centriprep; Bedford, MA, USA).

Extraction of β-secretase inhibitor and selection of the potent β-secretase inhibitor-containing medicinal plant Dried medicinal plants (5 g) were pulverized and then added to 100 mL of water and 70% methanol, respectively. After shaking for 12 hr at 30°C, the extracts were centrifugated at 1,000×g for 30 min and filtered with Whatman No. 41 filter paper. Each supernatant was lyophilized for analysis (19). Finally, a potently β-secretase inhibitor-containing medicinal plant was selected after a comparison of the inhibitory activities with the extracts.

Assay of β-secretase inhibitory activity An assay was carried out according to the supplied manual with modifications. A mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of β-secretase (1.0 U/mL), 10 μL of a 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 25°C in darkness. The mixture was placed under 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)}]×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 zero time, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 was the fluorescence of the tested samples at zero time.

All data are the mean of duplicated experiments. To check the quenching effect of the samples, the sample solution was added to the control C, and any reduction in fluorescence by the sample was then investigated. The IC_{50} value was defined as the concentration of the β-secretase inhibitor that is required to inhibit 50% of β-secretase activity (20). All data presented are the mean values of triplicate experiments.

Purification of β-secretase inhibitor The water extract of *R. coreanus*, which was finally selected as a producer of β-secretase inhibitor, was fractionated stepwise with *n*-hexane, chloroform, ethyl acetate, butanol, and water, and the β-secretase inhibitory activities of the fractions were then determined. The active fraction was ultrafiltrated using a 1 kDa cut-off filter (Ultrafiltration Membranes; Millipore Co.) and the β-secretase inhibitory activity of the filtrates was determined. The active fraction was concentrated by lyophilization and then applied to a Sephadex G-10 column (3.0×30 cm) equilibrated with distilled water and eluted with distilled water at a flow rate of 1 mL/min. The fractions with β-secretase inhibitory activity were then applied to a preparative reverse-phase high performance liquid chromatography (HPLC, μ Bondapak C_{18} column) equilibrated with acetonitrile. A linear gradient was carried out with 0.1% trifluoroacetic acid (TFA) in water from 0 to 100%(v/v). The active fractions were collected and lyophilized immediately. The fractions with β-secretase inhibitory activity were then applied to a analytical reverse-phase HPLC (Protein & Peptide C_{18} column; VYDAC, Hesperia, CA, USA) equilibrated with acetonitrile. A linear gradient was carried out with 0.1% TFA in water from 0 to 70%(v/v). The active fractions were collected and lyophilized immediately.

Determination of Mw and amino acid sequence The molecular mass of the purified β-secretase inhibitor was determined using an liquid chromatography/mass spectrometry (LC/MSD, HP 1,100 series; Hewlett-Packard Co., Palo Alto, CA, USA). The amino acid sequence was determined by the method of Edman (21) using an Applied Biosystems 491 A automatic protein sequencer.

Determination of the inhibition pattern on β-secretase To investigate the inhibition pattern on β-secretase, the purified inhibitor was added to each reaction mixture. The β-secretase inhibitory activity of each was measured with different concentrations of the substrate. The inhibition constants (*Ki*) of β-secretase inhibitors were calculated using Dixon plots. The β-secretase substrate divided into 3 group concentrations. The reaction velocity was measured at a fixed concentration of substrate but with a variety of inhibitor concentrations. A graph of the 1/V (min/F.U.) against the inhibitor concentration was plotted (15).

Results and Discussion

Selection of the β-secretase inhibitor-containing medicinal plant To select a potent β-secretase inhibitor-containing medicinal plant, water and methanol extracts from medicinal plants were investigated for their β-secretase inhibitory activities (Table 1). Generally, water extracts were showed more high β-secretase inhibitory activity than those of methanol extracts. Water extracts of *R. coreanus* was showed the highest β-secretase inhibitory activity at 84.5%. Finally, *R. coreanus* was selected as a novel β-secretase inhibitor containing plant.

It is known that unripened fruit of *R. coreanus* Miquel were contained several bioactive compounds such as electron donating ability (antioxidant activity) (22,23), antimicrobial compounds against *B. cereus* (22), xanthin oxidase inhibitor (24), galli acid, 2,3-(S)-HHDP-D-glucopyranose and sanguin (25), elastase inhibitor (26), and antimutagenic activity (27) etc. This is the first report that edible, medicinal *R. coreanus* has β-secretase inhibitory activity. Therefore, it will be very useful in functional or medicinal food industries.

Meanwhile, the effects of the temperature and time on the extraction of β-secretase inhibitor were investigated. The β-secretase inhibitor was maximally extracted when *R. coreanus* was treated by water at 40°C for 12 hr (data not shown).

Purification of a β-secretase inhibitor from *R. coreanus* To investigate physico-chemical properties of the β-secretase inhibitor and further to elucidate structure-function relationship, the β-secretase inhibitor from the water extracts of *R. coreanus* was purified as described in the Materials and Methods section.

Among the fractions from the systematic solvent extraction, the water fraction showed the highest β-secretase inhibitory activity of 94.5% (IC_{50}; 7.9×10^4 ng/mL). The β-secretase inhibitory activity of the filtrates from 1 kDa cut-off ultrafiltration of the active water fraction was 3.7×10^4 ng/mL of IC_{50}. After Sephadex G-10 column chromatography, the active fraction showed IC_{50} 1.47×10^4 ng/mL of β-secretase inhibitory activity. The active

Table 1. β-Secretase inhibitory activities of various extracts from medicinal plants (Unit: %)

Scientific names	Water extracts	MeOH extracts	Scientific names	Water extracts	MeOH extracts
Acanthopanax sessiliflorum	9.4	67.7	*Limonium tetragonum*	ND	26.7
Achyranthes japonica	ND[1)]	32.9	*Liriope platyphylla*	ND	ND
Adenophora triphylla	ND	ND	*Lonicerae flos*	4.2	4.7
Adonis amurensis	29.8	ND	*Lycium chinense* (fruit)	16.2	38.2
Agastache rugosa	46.0	ND	*Lycium chinense* (root)	37.8	ND
Akebia quinata	59.7	64	*Magnolia officinalis*	ND	ND
Alisma orientale	ND	ND	*Magnolia ovobata*	ND	24.9
Anemarrhena asphodeloides	12.7	23.7	*Morus alba* (bark)	18.6	2.6
Angelica dahurica	ND	39.6	*Morus alba* (leaf)	ND	ND
Angelica gigas	36.9	ND	*Morus alba* (stem)	ND	ND
Aralia continentalis	60.0	46.1	*Myristica fragrans*	70.2	48.2
Araxacum platycarpum	37.3	64.6	*Ostericum koreanum*	5.4	39.5
Arctium lappa	ND	ND	*Paeonia lactiflora*	10.5	ND
Artemisia princeps	66.2	73.5	*Panax ginseng*	49.6	49.3
Astragalus membranaceus	16.3	ND	*Phellodendron amurense*	26.2	8.9
Atractylodes lancea	ND	ND	*Phellodendron amurense* (bark)	5.9	ND
Atractylodes macrocephala koidz	ND	ND	*Phllostachys nigra*	20.3	ND
Aucklandia lappa	ND	ND	*Phragmites communis*	24.7	13.2
Benincasa hispida	57.5	ND	*Pinellia ternata*	4.6	ND
Bombyx mori	47.2	7.6	*Pinus koraiensis*	27.5	ND
Caesalpina sappan	ND	ND	*Plantago asiatica*	15.3	12.8
Carthamus tinctorius	21.1	60	*Polygala tenuifolia*	32.5	ND
Cassia obtusifolia	60.7	31.0	*Polygonatum sibiricum*	15.6	ND
Cervus elaphus	62.1	16.7	*Poncirus trifoliata Rafinesque*	67.3	59.3
Cervus elaphus (young)	32.3	ND	*Populus davidiana*	ND	ND
Cinnamomum cassia (fruit)	49.1	51.8	*Poria cocos*	57.5	19.2
Cinnamomum cassia (stem)	ND	ND	*Poria cocos*	49.8	22.7
Cistanche deserticola	5.4	22.4	*Prunus armeniaca*	16.6	ND
Cnidium officinale	18.4	ND	*Prunus mume*	41.4	60.9
Cocicis Semen	67.1	56.2	*Pueraria lobata* (root)	ND	41.4
Coix lacryma-jobi	36.1	ND	*Pueraria lobata* (flower)	68.4	69.9
Coptidis rhizoma	ND	17.8	*Rehmannia glutinosa*	ND	ND
Coptis chinensis	11.2	40.1	*Rehmannia glutinosa* (dried)	12.6	ND
Cordyceps sinensis	35.6	11.9	*Rosa rugosa*	21.4	ND
Cornus officinalis	ND	ND	*Rubus coreanus*	84.5	63.2
Crataegus pinnatifida	ND	ND	*Salviae miltiorrhiza*	31.4	50.2
Cuscuta chinensis	28.5	ND	*Schisandra chinensis*	46.1	ND
Eisenia bicyclis	ND	ND	*Schizonepeta tenuifolia*	17.4	28.8
Ephedra sinica	62.2	15.6	*Scrophularia buergeriana*	64.6	64.7
Epimedium koreanum	29.6	ND	*Scutellaria baicalensis*	27.0	ND
Eucommia ulmoides	ND	ND	*Sesamum indicum*	ND	12.4
Euonymus alatus	48.1	5.4	*Sesamum indicum* (hugjima)	ND	55.2
Fraxinus rhynchophylla	ND	ND	*Smilax china*	32.2	ND
Gallus domesticus	26.9	1.8	*Spirodela polyrhiza*	6.7	21.9
Ganoderma lucidum	ND	16.9	*Swertia japonica*	29.5	41.2
Gardenia jasminoides	ND	ND	*Syzygium aromaticum*	70.3	5.1
Glechoma hederacea	69.8	78.2	*Torilis japonica*	20.5	10.2
Glycine max (black)	ND	ND	*Trichosanthes kirilowii*	43.5	8.7
Glycine max (white)	48.6	ND	*Viscum album*	37.2	ND
Glycyrrhiza uralensis	60.3	69.0	*Zea mays*	ND	18.5
Hovenia dulcis	32.8	ND	*Zingiber officinale*	28.7	ND
Kalopanax pictus	77.1	66.5	*Zizyphus jujuba*	5.7	ND
Ligusticum acutilobum	60.3	60.5			

[1)]ND: Not detected.

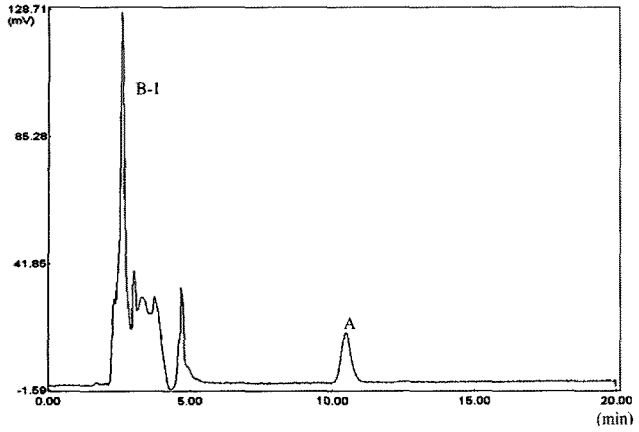

Fig. 1. Reverse-phase HPLC chromatogram of H1H2. Flow rate, 0.8 mL/min; UV absorbance, 215 nm. A, purified β-secretase inhibitor; B-1, buffer pick.

Table 2. Summary for the purification of β-secretase inhibitor from *Rubus coreanus*

Purification steps	β-Secretase inhibitory activity (IC_{50}: ng/mL)	Solid yield (%)
Water extract	8.7×10^4	100
Systematic solvent extracts	7.9×10^4	72.0
Ultrafiltration	6.5×10^4	25.2
Sephadex G-10 chromatography	1.47×10^4	12.9
RP-HPLC	6.3×10^3	0.08

fractions were collected and performed in preparative reverse phase HPLC using μ Bondapak C_{18} column following repeated RP-HPLC. One peak showing β-secretase inhibitory activity (IC_{50}; 6.3×10^3 ng/mL, 1.56×10^{-6} M) was obtained with 0.08% solid yield (Fig. 1) (Table 2). This β-secretase inhibitory activity of the purified β-secretase inhibitor was similar to those of β-secretase inhibitory peptide (IC_{50}; 7.5×10^3 ng/mL, 2.59×10^{-6} M) from *Saccharomyces cerevisiae* K-7 in previous paper (28), non-peptide inhibitors from green tea such as epigallocatechin gallate (1.6×10^{-6} M), epicatechin gallate (4.5×10^{-6} M), gallocatechin gallate (1.8×10^{-6} M) (15), ellagic acid (IC_{50}; 3.9×10^{-6} M) from pomegranate husk (16), tellimagrandin II (IC_{50}; 3.1×10^{-6} M), and 1,2,3,4,6-pentagalloyl-glccopyranoside (IC_{50}; 3.76×10^{-6} M) from *S. radix* (18) and chitosan derivatives (13). However, the purified β-secretase inhibitor was shown weaker β-secretase inhibitory activities than that of punicalagin (IC_{50}; 4.1×10^{-7} M) from pomegranate (16). Although the β-secretase inhibitory activity of the purified β-secretase inhibitor from *R. coreanus* was similar or slightly lower than these values of these natural β-secretase inhibitors, the β-secretase inhibitor from *R. coreanus* is considered to be a good candidate as an antidementia drug or in functional foods as it is extracted from edible oriental medicinal plant. Future studies are required to elucidate characteristics of non-peptidic β-secretase inhibitor in ethyl acetate extracts or butanol extracts of *R. coreanus*.

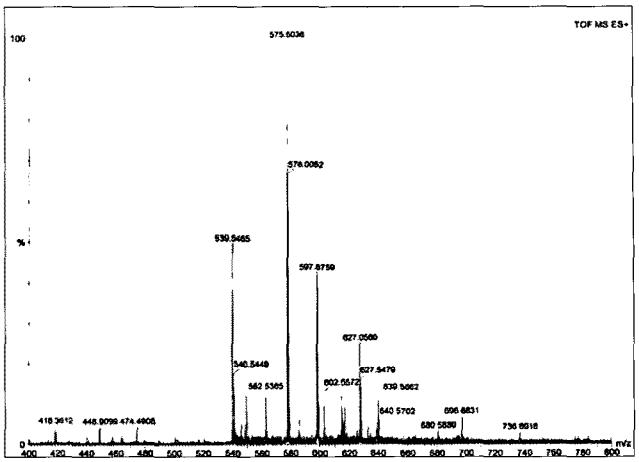

Fig. 2. The peptide sequence determined by tandem LC-MS analysis of the β-secretase inhibitor from *Rubus coreanus*.

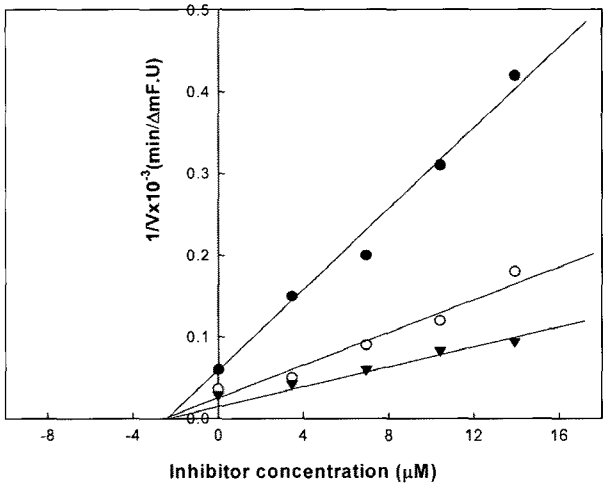

Fig. 3. Dixon plot for determining inhibitor constants of the purified *Rubus coreanus* inhibitor against β-secretase with substrate concentration. ●, 375 nM; ○, 750 nM; ▼, 1125 nM.

Amino acid sequence and Mw of the β-secretase inhibitor The amino acid sequence of the purified β-secretase inhibitor was found to be Gly-Trp-Trp-Glu via a tandem LC-MS analysis (Fig. 2) and its molecular mass was estimated to be 576 Da by LC-MS analysis. Because its Mw was smaller than those of *S. cerevisiae* K-7 (697 Da) (28) and the others (11-13,16), it was considered suitable for absorption in the intestine.

Determination of β-secretase inhibition pattern The inhibition pattern of the purified β-secretase inhibitor against β-secretase was found to be non-competitive at the active site of β-secretase from determination according to a Dixon plot (Fig. 3). The inhibition constant (*Ki*) was presented in 2.2×10^{-6} M. Thus, it strongly suggests that the purified β-secretase inhibitor could bind either to the β-secretase substrate or to another regulatory site.

There are many various β-secretase inhibitors from phytochemical, which it have different *Ki* values. Park *et al.* (17) reported the inhibition of hispidin was non-

Table 3. Inhibitory activity of the purified β-secretase inhibitor against α-secretase and serine proteases

Enzymes[1)]	IC_{50} (M)
α-Secretase (TACE)	$>7.2\times10^{-2}$
Trypsin	2.51×10^{-2}
Chymotrypsin	$>7.2\times10^{-2}$
Elastase	$>7.2\times10^{-2}$

competitive with a substrate in the Dixon plot and its *Ki* values was 8.4×10^{-6} M. Jeon *et al.* (15) was also reported the *Ki* value of gallocatechin gallate, epigallocatechin gallate, and epicatechin gallate from green tea were 1.7×10^{-7}, 2.1×10^{-7}, and 5.3×10^{-6} M, respectively. Furthermore, *Ki* values of tellimagrandin II and 1,2,3,4,6-penta-galloylglccopyranoside from *S. radix* were 6.84×10^{-6} and 5.13×10^{-6} M, respectively (18). The *Ki* value of punicalagin from *Punica granatum* was 2.4×10^{-5} M (16) and water-soluble aminoethyl-chitosan was showed 85 μg/mL (13). The *Ki* values of 8 phenolics compounds from plant phenolic compounds in Medicinal Molecules Bank (MEDMOB) were also 0.823×10^{-6}-13.103×10^{-6} M (29).

Enzyme specificity and heat stability In order to test the enzyme specificity, the inhibitory activity of the purified β-secretase inhibitor on α-secretase (TACE) which is involved in the normal amyloidogenic process and other proteases were determined (Table 3). The purified β-secretase inhibitor showed weak inhibitory activity against TACE (>72.0 μM) and also did not show significant inhibitory activity against other serine protease such as chymotrypsin and elastase except trypsin. Thus, the purified β-secretase inhibitor appeared to be relatively specific inhibitor on β-secretase, as is the case of other natural inhibitors (15).

In addition, the heat stability of the purified β-secretase inhibitor from *R. coreanus* was investigated. The purified β-secretase inhibitor was considerably stable at 70°C for 30 min, showing 87% residual activity. Treatment at 50°C for 30 min and 90°C for 30 min on the purified β-secretase inhibitor resulted in 92 and 81% of residual inhibitory activity, respectively (data not shown). Therefore, this heat stable β-secretase inhibitor, as shown in this study, should be very useful in either the drug or functional food industries.

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