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Characterization of β-Secretase Inhibitory Peptide Purified from Blackfin flounder (*Glyptocephalus stelleri*) Protein Hydrolysate

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Abstract The objective of this study was to purify and characterize the β-secretase inhibitor from enzymatic hydrolysates of blackfin flounder muscle, for development of a novel anti-dementia agent that may be used in the drug or functional food industries. β-secretase inhibitory peptide was purified from various enzymatic hydrolysates of blackfin flounder muscle. Among six enzymatic hydrolysates, the Alcalase hydrolysate revealed highest β-secretase inhibitory activity. Consecutive purification of the blackfin flounder muscle hydrolysate using Sephadex G-25 column chromatography and octadecylsilane C18 reversed phase HPLC techniques were used to isolate a potent β-secretase inhibitory peptide composed of 5 amino acids, Leu-Thr-Gln-Asp-Trp (MW: 526.7 Da). The IC_{50} value of purified β-secretase inhibitory peptide was 126.93 μM. Results of this study suggest that peptides derived from blackfin flounder muscle may be beneficial as anti-dementia compounds in functional foods or as pharmaceuticals.

Keywords : Alzheimer’s disease, β-secretase inhibitory activity, Blackfin flounder muscle, Alcalse, Anti-dementia

Introduction

Alzheimer's disease (AD) is a disease of the brain that causes problems with memory, thinking and behavior. Alzheimer’s disease is not a normal aging or a mental illness. Dementia is a general term for loss of memory and other intellectual abilities critical enough to interfere with daily life. Alzheimer's accounts for an estimated 60-80% of dementia cases. Prevalence of AD in population usually increases with age, especially older than age 65. Korea is among the countries with most rapidly aging population. According to the report released by the Ministry of Health and Welfare [1], with increasing elderly population in Korea, the number of dementia patients are estimated to reach nearly 1 million by 2027. If this trend continues, the number of elderly people suffering from Alzheimer's is expected to surpass 1 million in 2024, one year earlier than the ministry forecast in 2008. In 2050, the number of Alzheimer's patients is expected to reach 2.71 million. Symptoms of the disease include memory loss, especially about recent events, confusion about time and place, poor judgment, trouble of learning new information. The signs start slowly and can vary or worsen over time. A person cannot control the signs. Treatment can help, but it does not cure the disease.

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In the past, many theories were presented, among which the amyloid hypothesis is the most widely accepted. This hypothesis is based on one of two main pathological AD hallmarks, the amyloid plaques formed in the human brain. Their formation results from abnormal production of β-amyloid peptides (Aβ) in neural cells by proteolytic cleavage of the amyloid precursor protein (APP) by β- and ɣ-secretases, wherein β -secretase (beta-site APP cleaving enzyme 1: BACE1) acts as the rate-limiting enzyme. Based on these findings, the amyloid hypothesis states that the specific inhibition of BACE1 will retard or even stop progression of AD, thus pointing to BACE1 as a promising target in AD drug development [2]. In developing drugs for the AD, different approaches have been used in many extensive investigations; however, most drugs currently available serve to modulate the degree of symptoms without affecting fundamental causes [3]. Also these drugs can improve cognitive function of patients with AD temporally, adverse effects and toxicity can occur in case of long-term use. Because of these problems, a trend towards development of natural β-secretase inhibitors isolated from various organisms proteins was developed.

β-secretase is an aspartic protease, a family of protease enzymes that uses an aspartate residue for the catalysis of their substrates. The 501 amino acid sequence of BACE1 has N-terminal signal sequence of 21 amino acids (amino acid 1 – 21), the first part of the protein that exits the ribosome during translation [4]. Together with the pro-peptide domain (amino acids 22 – 45), the N-terminal signal sequence is removed after translation, forming the mature aspartic protease, that starts at amino acid Glu-46. Near its C-terminus, BACE1 contains a single trans-membrane domain (amino acids 455 – 480) characteristic for mammalian aspartic proteases [5]. For the β-secretase inhibitor many groups have focused on the identification of inhibitors using high-throughput screening of compound collections and natural product extracts. The peptidic β-secretase inhibitor, OM99-1 and other aspartic protease inhibitors [6], OM99-2, an eight-residue transition state inhibitor [7], and OM00-3, are more potent eight-residue transition state inhibitor [8]. Non-peptidomimetic derivatives, such as analogues based on the phenyl-piperazine scaffold with various heterocyclic moieties, have been synthesized to optimize BACE1 inhibition [9]. Recently, small-sized synthetic inhibitors containing a tetrazole ring and acidic heterocycle bioisosteres such as KMI-570, KMI-684, KMI-420, and KMI-429 were synthesized [10].

Blackfin flounder (*Glyptocephalus stelleri*), commonly known as Korean flounder and arrow toothed, range from the southern and eastern coastal waters of Korea, through Japan, the Sea of Okhotsk, and the North Pacific including the Bering Sea. Off Korea, they are found mainly at depths of 70-700 m [11]. There are many studies of the biology and ecology of blackfin flounder in Korean waters which have been included research on growth [12] and reproduction [13]. But, no studies have reported bioactive materials of blackfin flounder muscle hydrolysates. Therefore, this study investigated β-secretase inhibitory activity peptide of enzymatically prepared blackfin flounder muscle protein hydrolysate. Protein solubilisation from raw fish materials has been conducted mostly by biological methods, especially by enzymatic hydrolysis. Autolytic process, that depends only on the action of the natural digestive enzymes occurring in the fish, is economically interesting [14]. Many commercial proteases from plant, animal or microbial sources have been reported as the potential proteinases for hydrolysing fish proteins [15,16,17]. Bioactive peptides are inactive within the sequence of their parent protein and can be released by enzymatic hydrolysis either during gastrointestinal digestion or during food processing. They usually contain 2 – 20 amino acid residues per molecule, but in some cases may consist of more than 20 amino acids. Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract. Depending on se-

quence of amino acids, these peptides can exhibit diverse activities, including opiate-like, mineral binding, immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic, and antihypertensive actions [18,19,20]. Bioactive peptides can be liberated by gastrointestinal digestion through proteolytic enzymes or during the fermentation process [21]. Results of this study suggests that the β-secretase inhibitory peptide derived from blackfin flounder muscle could be a potential candidate to develop nutraceuticals and pharmaceuticals.

Materials and Methods

Materials

Blackfin flounder (*Glyptocephalus stelleri*) was donated by Department of Seaport and Traditional Fish Market of Jumunjin (Gangneung, Korea); the muscle was washed to remove remaining skins and bones. The blackfin flounder muscle was immediately frozen and stored at -80°C until used. Various commercial enzymes, such as α-chymotrypsin, papain, pepsin and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Alcalase (from *Bacillus licheniformis*) and Neutrase (*Bacillus amyloliquefaciens*) were purchased from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). β-secretase (from human) and MCA-EVKMDARFK-(DNP)-NH_2 (β-secretase substrate I) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used in this study were reagent grade chemicals.

Preparation of blackfin flounder muscle protein hydrolysates

For production of β-secretase inhibitory activity peptide from blackfin flounder muscle protein, enzymatic hydrolysis was conducted using various commercial enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin and trypsin) at an enzyme/substrate ratio of 1/100 (w/w) for 6 h, under optimum pH and temperature conditions (Table 1). Next, mixtures were allowed to cool room temperature, by placing in 50 mL graduated centrifuge tubes, and centrifuged at 6,000 rpm for 10 min. The supernatant was collected after conducted by glass filter and lyophilized hydrolysates and they were stored at -80°C until use. Yield of hydrolysate from blackfin flounder muscle was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{weight of the blackfin flounder muscle hydrolysates}}{\text{weight of the blackfin flounder muscle}} \times 100$$

Measurement of β-secretase inhibitory activity

β-secretase inhibitory activity was measured by following Johnston's method [22] using a commercially available fluorogenic substrate, MCA-EVKMDAEFK-(DNP)-NH_2 (Sigma-Aldrich, MO, USA). This substrate corresponds to the wild-type APP sequence, derivatized at its N–terminus with a fluorescent 7-methoxycoumarin-4-yl acetyl (MCA) group, and on its C–terminal lysine residue with a 2,4-dinitrophenyl (DNP) group. In the intact peptide, fluorescence of the MCA group is abolished by internal quenching from the DNP group. Upon cleavage by β-secretase (Sigma-Aldrich, MO, USA), MCA fluorescence can be detected.

Table 1. Optimum hydrolysis conditions of blackfin flounder muscle hydrolysates obtained by various enzymes.

Enzyme	Buffer	pH	Temperature (°C)
Alcalase	50mM Na_2HPO_4-NaH_2PO_4	7.0	50
α-chymotrypsin	50mM Na_2HPO_4-NaH_2PO_4	7.0	37
Neutrase	50mM Na_2HPO_4-NaH_2PO_4	7.0	50
Papain	50mM Na_2HPO_4-NaH_2PO_4	7.0	37
Pepsin	20mM HCl	2.0	37
Trypsin	50mM Na_2HPO_4-NaH_2PO_4	7.0	37

Assays were conducted in 96-well black plates using the Infinite® 200Pro multimode microplate reader (Tecan, Männedorf, Switzerland). β-secretase and β-secretase substrate were incubated in assay buffer (50 mM sodium acetate, pH 4.5) with the final volume of 100 μL. Progress of hydrolysis of β-secretase substrate I was followed at 37°C for 30 min by measuring the accompanying increase in fluorescence. Readings (excitation 320 nm, emission 450 nm) were taken in every 60 s. Inhibition (%) was then obtained by the following equation:

$$\text{Inhibition (\%)} = [1-\{(S-So)/(C-Co)\}] \times 100$$

Wherein C is the fluorescence of a control (enzyme, assay buffer, and substrate) after 60 min of incubation, Co is the fluorescence of the control at zero time, S is the fluorescence of the test sample (enzyme, sample solution, and substrate) after 60 min of incubation, and So is the fluorescence of the test sample at zero time. All data are expressed as the means of triplicate experiments. The IC_{50} value is the concentration of blackfin flounder muscle or peptide yielding 50% inhibition of β-secretase activity.

Purification of β-secretase inhibitory peptide

The blackfin flounder muscle hydrolysate revealing β-secretase inhibitory activity was dissolved in distilled water and loaded onto a Sephadex G-25 gel filtration column (ø 2.5 × 70 cm), previously equilibrated with distilled water. Separated fractions were monitored at 215 nm, collected at a volume of 7.5 mL and measured for β-secretase inhibitory activity. The β-secretase inhibitory fraction was dissolved in distilled water and separated using a Grom-sil 120 ODS-5 ST column (ø 10.0 × 250 mm, 5 μm, Grom™, Germany) by reversed-phase high performance liquid chromatography (RP-HPLC, Agilent Technologies, USA), with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The elution peaks were monitored at 280 nm and purified by RP-HPLC on C18 analytical column (ø 4.6 × 250 mm, 5 μm, Waters, Milford, MA, USA) using an acetonitrile gradient of 0-15% at a flow rate of 0.5 ml/min for 50 min. Elution peaks were monitored at 280 nm on diode array detector. Finally, the fraction with the β-secretase inhibitory activity was collected and lyophilised; this was followed by the identification of the amino acid sequence.

Amino acid sequencing of purified peptide

To identify the molecular weight and amino acid sequence of purified peptide, all MS/MS experiments were conducted by nano-electrospray ionization (ESI) on a quadrupole time-of-flight (Q-TOF) tandem mass spectrometer (AB Sciex Instruments, CA 94404, USA). The peptide solution was desalted using Capcell Pak C18 UG120 V (ø 4.6 × 250 mm, 5 μm, Shiseido, Tokyo, Japan). The purified peptide dissolved in methanol/water (1:1, v/v) was infused into the ESI source and molecular weight was determined by the doubly charged $(M + 2H)^{2+}$ state in the mass spectrum. Following molecular weight determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem MS analysis.

Statistical analysis

Data were analyzed for statistical significance using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test with SPSS software (version 14). All values obtained from three different experiments were expressed as mean value ± S.E.M.

Results and Discussion

β-secretase inhibitory activity of blackfin flounder muscle hydrolysate

The blackfin flounder muscle protein hydrolysates were prepared by hydrolysis using commercial proteases including Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin. The hydrolysis yields were 58.45%, 55.65%, 54.14% and 52.14% for Neutrase, α-chymotrypsin, papain and Alcalase, respectively (Table 2). Among the six hydrolysates, the Alcalase hydrolysate exhibited the greatest β-secretase inhibitory activity relative to the other hydrolysates. In

Table 2. The yields of various hydrolysates from blackfin flounder muscle.

Hydrolysates	Yields (%)
Alcalase	52.14
α-chymotrypsin	55.65
Neutrase	58.45
Papain	54.14
Pepsin	49.56
Trypsin	53.05

terms of the β-secretase inhibitory activation (Fig. 1), the highest IC_{50} value was exhibited by the Alcalase hydrolysate at 1.79 mg/mL. Alcalase is an alkaline protease that has been used not only for production of protein hydrolysates with better functional and nutritional characteristics than the original proteins, also but for the generation of bioactive peptides [23]. Several studies have reported that Alcalase hydrolysates derived from food proteins showed potent bioactivities such as antioxidant [23], antihypertensive [24,25,26] and hypocholesterolemic [27]. These bioactivities are attributed to the ability of Alcalase to produce various bioactive peptides due to its endo-peptidase properties. The enzymatic hydrolysis is one of the primary approaches for the effective release of bioactive peptides from protein sources, and is widely used to improve and upgrade functional and nutritional properties of proteins [28].

Purification of β-secretase inhibitory peptide

To purify β-secretase inhibitory peptide from Alcalase hydrolysate of blackfin flounder muscle hydrolysate required the use of different chromatographic techniques. As shown in Fig. 2, demonstrated chromatographic profiles were obtained during different purification steps of

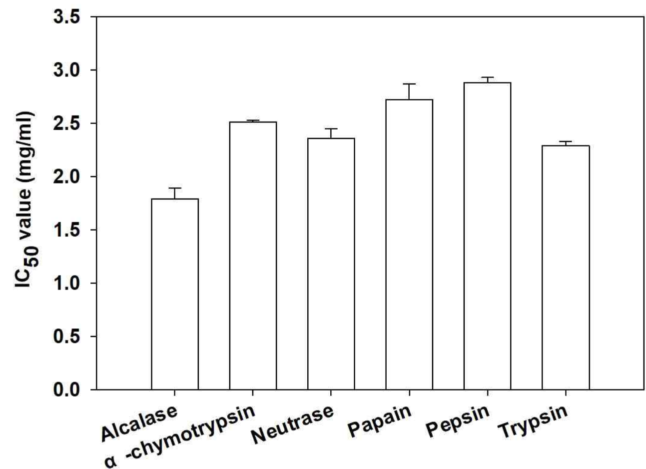


Figure 1. IC_{50} value of β-secretase inhibitory activity of blackfin flounder muscle hydrolysate.

β-secretase inhibitory peptide from blackfin flounder muscle hydrolysate. First step, Alcalase hydrolysate was separated into six fractions (A-F) on a Sephadex G-25 chromatography column (Fig. 2I). Among separated fractions, E had the highest β-secretase inhibitory activity at 0.24 mg/mL (Fig. 2I). Further separation of fraction E was conducted using RP-HPLC on a ODS column, where for the four fractions (F1-F4) different β-secretase inhibitory activities were obtained (Fig. 2II). Among separated fractions, fraction F1 revealed the highest β -secretase inhibitory activity with IC_{50} value of 91.40 μ g/mL (Fig. 2II). Fraction F1-1, with strongest β-secretase inhibitory activity was purified further by using RP-HPLC on the C18 analytical column a linear gradient of acetonitrile (0-15%) for 50 min at a flow rate of 1.0 ml/min (Fig. 2III). The IC_{50} value of the purified peptide was 74.60 μg/mL, 23.99-fold compared to the Alcalase hydrolysate (1.79 mg/mL) using the three-step purification procedure (Table 3). Single peptide fraction that ex-

Table 3. Purification of β-secretase inhibitory peptide from blackfin flounder muscle hydrolysate obtained by Alcalase treatment.

Purification step	IC_{50} value (μg/mL)	Purification fold*
Alcalase hydrolysate	1790.10 ± 0.02	1.00
Sephadex gel filtration (E)	240.02 ± 0.11	7.46
RP-HPLC (F-1)	91.40 ± 0.09	19.58
Purified peptide (F1-1)	74.60 ± 0.03	23.99

* Relative value of reciprocal of β-secretase inhibitory activity by IC_{50}

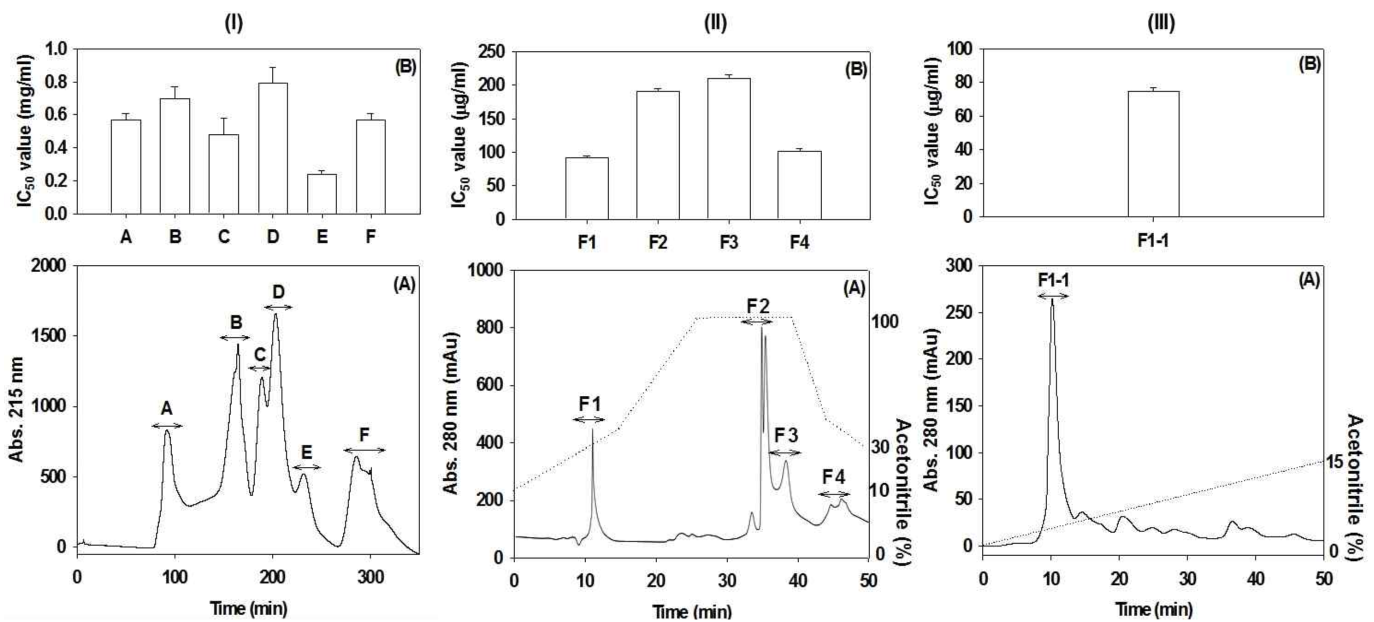


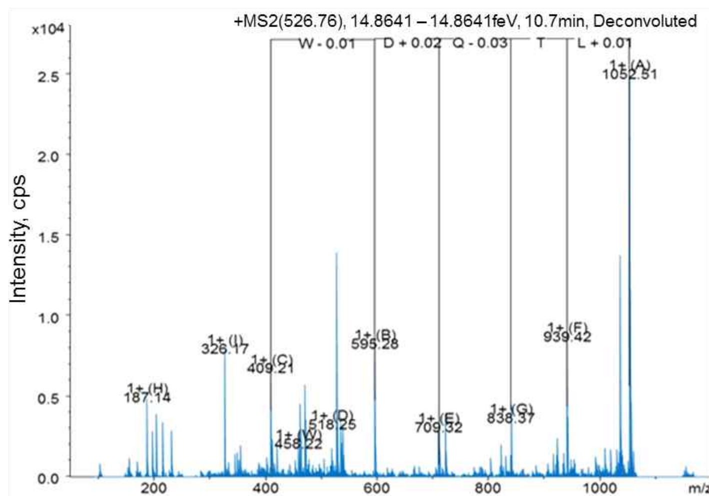
Figure 2. The steps for purification of β-secretase inhibitory peptide from blackfin flounder muscle hydrolysate. (I) Sephadex G-25 Gel filtration chromatogram of hydrolysates. Gel filtration chromatogram of hydrolysates prepared with blackfin flounder muscle (A). Separation was performed with 1.5 mL/min and collected at a fraction volume of 7.5 mL. The fractions isolated by Sephadex G-25 Gel column were separated (A-F) and β-secretase activity determined as upper panel (B). (II,III) Reverse phase-HPLC chromatograms of the potent β-secretase inhibitory fractions from the previous steps. The lower panels (A) of each pair show the chromatography results of the separated fractions while the top panels (B) of each pair represent the β-secretase inhibitory activity of the separated fractions in terms of their IC_{50} values expressed in mg/mL (I) or μg/mL (II, III).

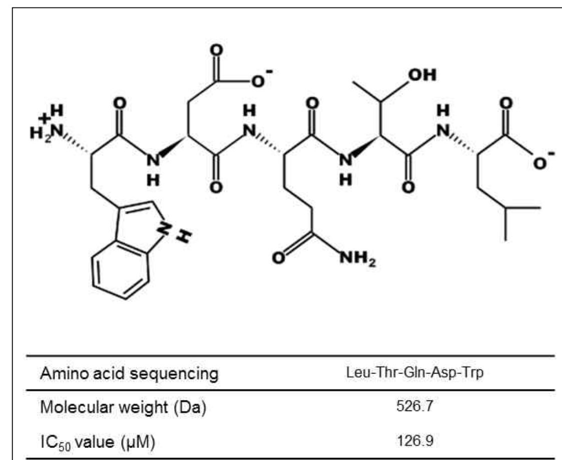
hibited β-secretase inhibitory activity, was finally purified on an analytical HPLC column and their amino acid sequences were determined by N–terminal sequencing analysis.

Characterization of the blackfin flounder muscle β-secretase inhibitory peptide

The purified F1-1 fraction was analyzed by ESI.MS for molecular mass determination and ESI.MS/MS for peptide characterization. Amino acid sequence of purified bioactive purified peptide from this fraction was identified to be Leu–Thr–Gln–Asp–Trp and the IC_{50} value of this purified β-secretase inhibitory peptide was 126.93 μM. Molecular mass of purified β-secretase inhibitor was estimated at 526.7 Da by ESI.MS. According to studies, peptidic inhibitors were targeted as β-secretase inhibitors. Lee *et al.* [29] identified the amino acid sequence of a purified β-secretase inhibitory peptide (IC_{50} value = 2.59 μM) from *Saccharomyces cerevisiae* as Gly–Pro–Leu–Gly–Pro–Ile–Gly–Ser by N–terminal sequence analysis. Lee *et al.* [30] found that the amino acid sequence of a purified β-secretase inhibitory peptide from skate skin hydrolysate (IC_{50} value = 24.26 μM) was Gln–Gly–Thr–Arg–Pro–Leu–Arg–Gly–Pro–Glu–Phe–Leu with N–terminal sequence analysis, but reported the need to reduce the size of this peptide to overcome metabolic instability. Lee *et al.* [30] found that a synthesized peptide (Pro–Glu–Phe–Leu) revealed the highest β-secretase inhibitory activity (IC_{50} value = 14.66 μM), The IC_{50} value of Pro–Glu–Phe–Leu had high inhibitory activity compared to original peptide (Gln–Gly–Thr–Arg–Pro–Leu–Arg–Gly–Pro–Glu–Phe–Leu). Li-Chan *et al.* [31] found that a synthesized peptide (Leu–Phe–His) revealed the highest β-secretase inhibitory activity (IC_{50} value = 34.11 μM). The IC_{50} value of the synthetic peptides improved over the original peptide isolated from shrimp waste hydrolysate (Asp–Val–Leu–Phe–His, IC_{50} value = 92.70 μM). Therefore, the amino acid composition, sequence, and length may be critical characteristics for determining

the β-secretase inhibitory activity of peptides. The C‒ and N‒terminal amino acid residues of inhibitory peptides may interact with subsites and active site of β-secretase. Turner *et al.* [32] found that each subsite can accommodate multiple residues. The S1 subsite is the most stringent, preferring residues in the order of Leu > Phe > Met > Tyr. The preferences of other of subsites are as follows: S2 (Asp > Asn > Met), S3 (Ile > Val > Leu), and S4 (Glu > Gln > Asp). The S1 site can accommodate a Leu or Phe side chain, while S2 can accommodate polar and hydrophobic residues. Similarly, S3 prefers branched hydrophobic side chains and S4 accommodates polar acidic side chains [33]. These isolated peptides from blackfin flounder muscle could be useful in the study of the mechanisms of Alzheimer’s disease.





Amino acid sequencing	Leu-Thr-Gln-Asp-Trp
Molecular weight (Da)	526.7
IC_{50} value (μM)	126.9

Figure 3. Identification of molecular weight and amino acid sequence of the purified peptide from blackfin flounder muscle hydrolysate. MS/MS experiments were performed on a Q-TOF tandem mass spectrometer equipped with a nano-ESI source.

Conclusion

In this study, blackfin flounder muscle protein was hydrolysed using enzymatic hydrolysis with various enzymes and β-secretase inhibitory activity of peptide was determined and peptides were purified by using chromatography. Finally, β-secretase inhibitory peptide with five-amino acids from Alcalase hydrolysate of blackfin flounder muscle protein was purified. Results revealed that the purified peptide had significant inhibition of β-secretase activity with IC_{50} value of 126.93 μM. Results of this study suggest that the β-secretase inhibitory peptide from blackfin flounder muscle protein has potential health benefits for use in Alzheimer’s diseases.

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