European Food Research and Technology
https://doi.org/10.1007/s00217-021-03770-6

ORIGINAL PAPER

Characterization of β-secretase inhibitory extracts from sea cucumber (*Stichopus japonicus*) hydrolysis with their cellular level mechanism in SH-SY5Y cells

Eui-Hyoun Ma[1] · A. U. Rathnayake[2,3] · Jung Kwon Lee[2,4] · Sang-Min Lee[2] · Hee-Guk Byun[2]

Received: 22 January 2021 / Revised: 28 April 2021 / Accepted: 1 May 2021

Abstract
Alzheimer's disease (AD) is the main type of dementia found in the elderly population. The prevention of β-amyloid (Aβ) production by inhibiting β-secretase, which is required for Aβ generation, is the major focus of research in AD therapeutics. In this study, enzymatic extracts obtained from sea cucumber (*Stichopus japonicus*) hydrolysis were investigated for β-secretase inhibitory activities. Sea cucumber hydrolysates were prepared using various enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin). Trypsin hydrolysate (IC_{50}, 93.59 ± 0.68 μg/ml) with the highest β-secretase inhibitory activity was separated into crude polysaccharide (TCP) and protein (TP) by ethanol precipitation. TCP (IC_{50}, 16.13 ± 1.15 μg/ml) exhibited higher β-secretase inhibitory activity than trypsin hydrolysate and TP (IC_{50}, 563.50 ± 25.18 μg/ml). Total sugar content in TCP was 11.58 ± 0.32 g/100 g. Major monosaccharides present in *S. japonicus* were arabinose, fucose, galactose, mannose and ramnose at 1.76 mg/g, 11.98 mg/g, 1.33 mg/g and 0.1 mg/g, respectively. Sulfate and uronic acid contents were 5.69 ± 0.15 g/100 g and 0.49 ± 0.01 g/100 g. Cytotoxicity of TCP different concentrations were tested in SH-SY5Y cells using MTT assay. Treated concentrations at 1, 5, 10, 30, 50, and 100 μg/ml concentrations were not toxic at cellular level. Further, quantitative protein expression reduction of BACE, sAPPβ, β-amyloid, p-JNK, and p-p38 in SH-SY5Y cells were confirmed by Western blot analysis. Therefore, results of this study suggest the potential application of *S. japonicus* in food and biomedicine industries.

Keywords Alzheimer's disease · Amyloid beta · β-secretase · Sea cucumber · Polysaccharide · SH-SY5Y cells

Introduction

The average life expectancy has increased as the level of medical technology has expanded along with the socio-economic development. As a result, proportion of the aging population is increasing. Based on the Organization for Economic Co-operation and Development (OECD) countries, the population older than 65 years old will account for more than 25% of the total population by 2050. Therefore, it is evaluated that social measures against aging are necessary.

Alzheimer's disease was first described in 1906 by Alois Alzheimer, a German psychiatrist [1]. More than 50 million people worldwide suffer from Alzheimer's dementia, and the number is estimated to increase over 150 million in 2050, a threefold increase [2]. According to the 2015 World Alzheimer's Report, the economic cost of dementia in 2050 is reported to be $7.55 trillion [3].

Dementia exists in many different forms: degenerative brain disease, cerebrovascular disease, infectious disease, and deficiency disease. Degenerative brain diseases include Alzheimer's disease, Lewy body dementia, anterior temporal dementia, Huntington's disease, and Parkinson's disease. Cerebral ductal disease-related dementia includes vascular dementia, metabolic disease, hypoxia, and hypoglycemia. Infectious diseases include human mad cow disease, acquired immune deficiency syndrome, and

✉ Hee-Guk Byun
hgbyun@gwnu.ac.kr

1 Wellness Bioindustry, Graduate School, Gangneung-Wonju National University, Gangneung 25457, Republic of Korea

2 Department of Marine Biotechnology, Gangneung-Wonju National University, Gangneung 25457, Republic of Korea

3 Department of Science and Technology, Faculty of Applied Sciences, Uva Wellassa University, Badulla, Sri Lanka

4 Department of Cell Biology & Anatomy, Arnie Charbonneau Cancer Institute, University of Calgary, Calgary, AB, Canada

Published online: 10 June 2021

tuberculosis. Deficiency diseases include Wernicke (Korsakov syndrome). Alzheimer's dementia, a neurodegenerative disease associated with aging, accounts for 60% to 80% of elderly dementia patients. Symptoms consist of memory loss, judgment disorder, cognitive decline, behavioral disorder, emotional instability, speech disorder, delusions, and hallucinations [4, 5].

Among the various causes of Alzheimer's disease, β-amyloid (Aβ) is the most widely known cause. Inhibiting the production and action of Aβ has been proposed as one of the most promising strategies for the treatment of Alzheimer's [6]. Aβ is produced when the amyloid precursor protein (APP) is degraded by β-secretase and γ-secretase. Aβ is produced in small amounts in the general population to protect nerve cells, and they are rapidly decomposed afterwards. However, in patients with Alzheimer's disease, there is an abnormality in the regulation of Aβ expression, resulting in abnormal overproduction of Aβ. Overproduced Aβ accumulates around the brain cells to form plaques. Plaque induces nerve fiber entanglement (NFT), which damages and destroys synapses, the signaling system between nerve brain cells. Aβ forms reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and hydroxy radical that induce oxidative stress and activate the apoptosis pathway, causing damage to nerve cells. These actions result in dementia in Alzheimer's (Fig. 1) [7–9].

Researches to prevent and inhibit the progression of Alzheimer's disease is actively underway. In fact, many pharmaceutical companies are making great efforts to develop new drugs that can change the course of Alzheimer's disease [5]. In addition to the recent research on terrestrial organisms, the proportion of physiological activity studies using marine organisms is increasing. Advanced marine biological material engineering researches that create high added value using marine biological resources are actively conducted. There are many studies that screen various physiological activities by separating and purifying the hydrolysate obtained from hydrolyzing the protein of marine organisms through various enzyme treatments [9–11].

There are 1500 known species of sea cucumbers worldwide. They are member of the echinoderms that include starfish and sea urchins, and are used raw as sashimi or processed into dried products and edibles. In addition, sea cucumbers are believed to help with recovering energy in the summer [12]. It has been reported that sea cucumber extracts or dried products possess various physiological activities, such as antioxidants, antiviral, anticancer, anticoagulant, anti-osteoporosis, and alleviate gastric inflammation [13–16]. Further dietary glucocerebrosides from sea cucumber has identified with the potential ameliorative effects in Alzheimer's disease in vivo [17]. The sea cucumber *S. japonicus* used in this study is mainly inhabiting the coastal sea around Korean, China, Japan, and Russia. This

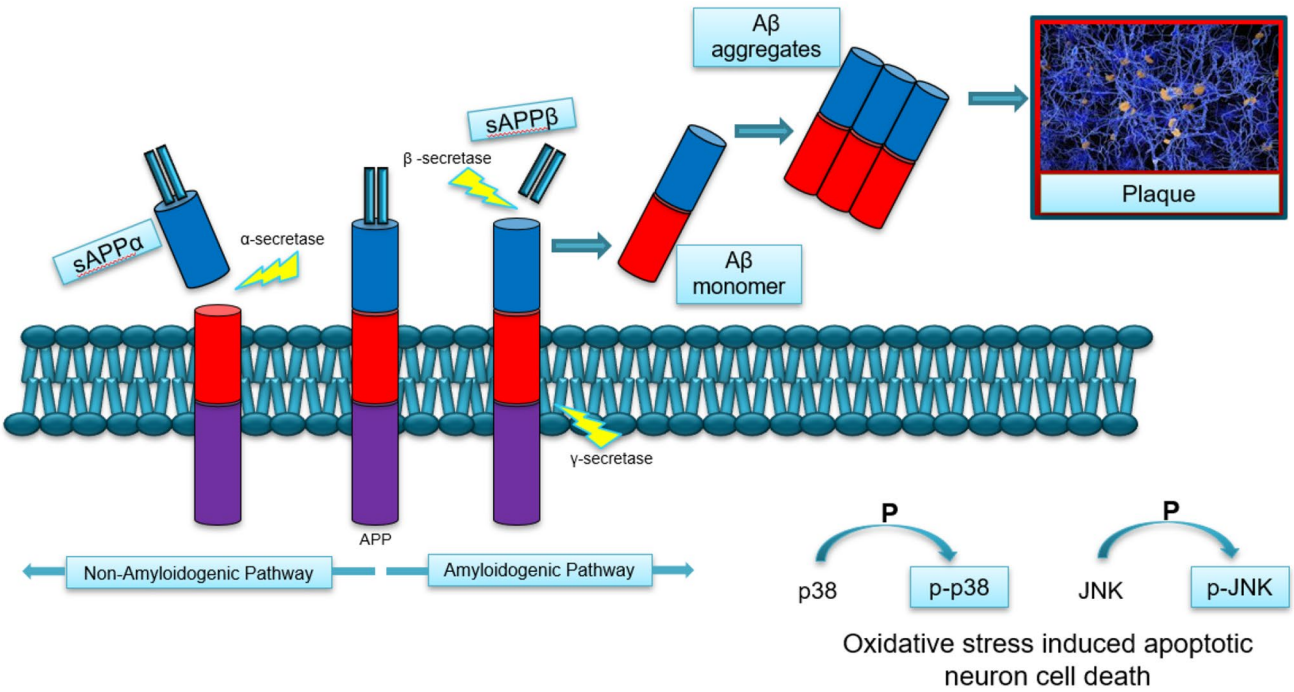

Fig. 1 Schematic diagram of the amyloid and non-amyloid production pathways of APP. In the amyloid generation pathway, APP is cleaved sequentially by β-Secretase and γ-Secretase. Overexpressed and aggregated Aβ form plaques. Plaques damage and destroy nerve cells to reduce function and induce nerve cell death due to oxidative stress

species possesses a range of biologically and pharmacologically important activities such as anti-oxidation, anti-cancer, anti-osteoclastogenesis and anti-coagulation activities. But studies on neuroprotective activities are still lacking [18].

Therefore, in this study, extracts that can inhibit the action of β-secretase enzyme were isolated and identified using *S. japonicus* collected from the east coast of Sokcho/Goseong, Gangneung, Republic of Korea. Their mechanism of action in reducing Aβ and inhibiting the oxidative apoptosis pathway in SH-SY5Y cells obtained from human bone marrow was further investigated.

Materials and methods

Materials

Enzymes alcalase and neutrase were purchased from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). Other enzymes (α-chymotrypsin, papain, pepsin, and trypsin) were purchased from Sigma Chemical Co. (St. Louis, Missouri). β-secretase and MCA-EVKMDAEFK-(DNP)-NH_2 were purchased from Sigma Chemical Co. (St. Louis, Missouri). Human neuroblastoma SH-SY5Y cell line was obtained from ATCC (The Global Bioresource Center, USA). Cell culture medias and supplementary materials, such as Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (DMEM-F12), penicillin–streptomycin (P/S), fetal bovine serum (FBS), Dulbecco's Phosphate buffer saline (DPBS), and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were purchased from Koram Biotech Co. and Hansol Biotech Co., Ltd. (Gangneung, Korea).

Sample preparation

The raw sea cucumbers of *S. japonicus* collected from the east coast of Sokcho/Goseong, Gangneung, Republic of Korea were soaked in clean distilled water for one hour to induce full expansion (Fig. 2). After cleaning, mouth, anus, and intestines were removed with only the body wall left behind. The body wall was cut into small pieces and freeze-dried. The lyophilized sea cucumber was powdered using grinder and stored at – 80 ℃.

Proximate analysis of *S. japonicus*

The proximate composition of the dried and crushed sea cucumber wall was determined according to the AOAC standard method [19]. The crude protein content of the pulverized body wall of sea cucumber was determined by an automated Kjeldahl system (Buchi, Flawil, Switzerland). The 6.25 conversion factor was used to convert the percentage of Kjeldahl nitrogen to protein content. Ash content was measured with the combustion in a muffle furnace (Thermolyne TM) at 600 ℃ for 4 h (h). Crude lipid content was measured using a Soxhlet extractor (VELP Scientifica, Milano, Italy). The moisture content of the sea cucumber dried powder was estimated by oven drying at 105 ℃ for 6 h. Carbohydrate content was calculated as 100%-(ash, protein, moisture, lipid).

Amino acid analysis of *S. japonicus*

Powdered sample of sea cucumber was acid-hydrolyzed in 6 N HCl at 110℃ for 24 h to analyze amino acid composition. After acid hydrolysis, 5 mL of distilled water was added and HCl was removed using a vacuum concentrator at 40 °C. Amino acids were analyzed using an Agilent 1100 HPLC system (USA) after pre-derivatization with o-Phthaldialdehyde (OPA) and β-mercaptoethanol. Separations were performed with a C18 column (4.6 × 250 mm, 5 µm). Amino acid concentrations of the samples were calculated from the calibration curves based on standard solutions of amino acid (Sigma-Aldrich Co., USA).

Fig. 2 Biological features of *Stichopus japonicus*. Large (20–30 cm length) and contains 20 oral tentacles. Three rows of numerous dense tube feet on the abdominal surface. Six indistinct rows of tall and small papillae along the dorsal and lateral surfaces. Three types of colors exists: red, green, and black

Enzymatic hydrolysis of *S. japonicus*

To produce a β-secretase inhibitory enzyme extract from *S. japonicus*, six enzymes (alcalase, α-chymotrypsin, neutrase, papain, pepsin, trypsin) were enzymatically hydrolyzed under optimal conditions for pH, temperature, and buffer (Table 1) [19]. Hydrolysis was performed at a 2% enzyme/substrate ratio and incubated at an optimal temperature for 6 h while shaking at 130 rpm. At the end of the hydrolysis, solution was heated in a 90 ℃ water bath for 10 min to inactivate enzymes. Then it was filtered through filter paper, lyophilized, and stored in powder form at − 80 °C. The yield of six hydrolysates after freeze drying was calculated as follows:

$$\text{Yield}(\%) = W/W_0 \times 100$$

"W" represents the dry weight of the sample before hydrolysis, and "W_0" is the dry weight of the sample after hydrolysis.

Measurement of β-secretase inhibitory activity

The β-secretase inhibitory activity was measured according to Johnston's method [20], using commercially available fluorescent substrate (MCA-EVKMDAEFK-(DNP)-NH_2). The substrate corresponded to the wild-type APP sequence, and contains a highly fluorescent 7-methoxycoumarin group that is effectively dissipated by resonance energy transfer to the 2,4-dinitrophenyl group. Thus, the substrate can be used to measure the activity of peptidase, which can increase fluorescence by cleaving the amide bond between the fluorescent group and the quencher group. MCA fluorescence is detected when β-secretase cleavage occurs. The activity of β-secretase was measured using a 96-well black plate and a Spectrofluorometer (Molecular Devices). First, 10 µL of β-secretase human and sample, 70 µL of assay 50 mM sodium acetate buffer (pH 4.5) were injected into a 96-black well plate and incubated at 37 ℃. For 5 min. 10 µL of substrate was added to bring the final volume to 100 µL. The fluorescence intensity was measured for 2 h at 5 min intervals at excitation of 340 nm and emission of 430 nm. The β-secretase inhibition rate for the sample was calculated using the following equation:

$$\text{Inhibition}(\%) = \left(1 - \frac{S_I}{C_I}\right) \times 100$$

"C_0" is the initial fluorescence value (time = 0) of the control (enzyme, buffer, substrate), and "C" is the fluorescence value of the control after 2 h of incubation, "S_0" is the initial fluorescence value (time = 0) of the bioactive sample, and "S" is the fluorescence value of the bioactive sample after 2 h of incubation. "C_I" value is the inhibition rate of the control and was determined by calculating $(C-C_0)/C_0 \times 100$. "S_I" is the inhibition rate of the sample and was determined by calculating $(S-S_0)/S_0 \times 100$.

Table 1 Optimum conditions of enzymatic hydrolysis for various enzymes. The hydrolysis of the sea cucumber body wall was performed at a 2% enzyme/substrate ratio

Enzyme	Buffer	pH	Temperature(˚C)
Alcalase	50 mM sodium phosphate	7.0	50
α-chymotrypsin	50 mM sodium phosphate	7.0	37
Neutrase	50 mM sodium phosphate	7.0	50
Papain	50 mM sodium phosphate	7.0	37
Pepsin	20 mM HCl	2.0	37
Trypsin	50 mM sodium phosphate	7.0	37

Separation and purification of hydrolysate

For trypsin hydrolysate, enzymes were deactivated at the end of the hydrolysis by keeping it in boiling water bath for 15 min. Before filtering the hydrolysate, it was kept under 4 ℃ refrigerated condition around 30 min to lower temperature. Afterwards, three volumes of ethanol were added and refrigerated overnight for precipitation. Centrifugation was performed at 10,000 rpm for 15 min to separate supernatant. Supernatant containing ethanol and proteins/peptides was transferred to a concentration flask, and ethanol was removed using a rotary evaporator. Finally, they were freeze-dried. Three consecutive precipitations were done to obtain high purity yield of crude polysaccharide. The protein derived from trypsin hydrolysate was named as TP, and the crude polysaccharide derived from trypsin hydrolysate was named as TCP (Fig. 3).

Physicochemical analysis

Physicochemical properties of TCP were analyzed by measuring the contents of uronic acid, sulfate, glucose, and protein.

Uronic acid content was measured by the method of Filisetti-Cozzi [21], and the absorbance for each concentration was measured at 535 nm using D-glucoronic acid as a standard. At first, 4 M sulfamic-potassium sulfamate (pH 1.6) and sulfuric acid was added into D-gluconic acid and acidified at 100 °C for 20 min, then it was cooled down quickly using an ice water bath for 1 minute. Afterwards, uronic acid content was calculated by referring to the absorbance value of the standard.

Sulfate content was measured by the method of Dodgson [22], and the potassium sulfate was used as the standard. At first, 0.5 M HCl was added into the sample to be

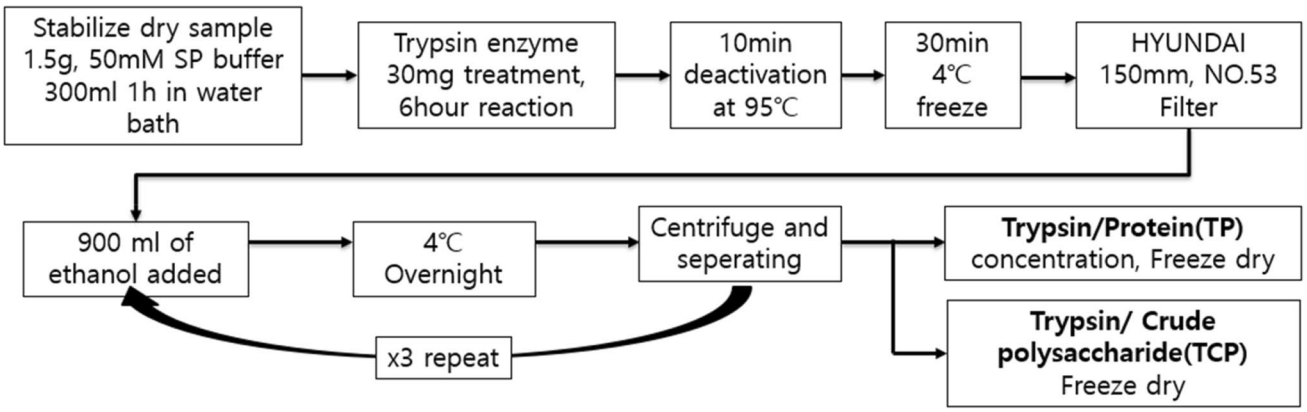

Fig. 3 Schematic diagram of TP and TCP fraction obtained from enzymatic hydrolysate of *S. japonicus*

measured for the standard and the content in each glass tube. Then, it was acid-decomposed at 105 °C for 5 h. After dilution, 3% (w/v) TCA and $BaCl_2$-gelatin solution were added and let to react at room temperature for 20 min, and the absorbance was measured at 360 nm. The sulfate content was measured by referring to the absorbance value of the standard.

For the total carbohydrate content, the phenol–sulfuric acid method was referred in Dubois [23]. Glucose was used as a standard. 5% phenol solution and 95% sulfuric acid were added into each sample and standard, and reacted at room temperature for 20 min. The absorbance was measured at 490 nm and the content of glucose was calculated using the absorbance value of the standard.

For the protein content, the Lawry method [24] was referenced, and the standard used was albumin monomer, bovine (BSA). The absorbance was measured at 750 nm using a spectrophotometer, and the protein content of the sample to be measured was calculated using the absorbance value of the standard.

Monosaccharide composition analysis

For pretreatment, 72% of H_2SO_4 was added to the sample and stirred at 230 rpm for 2 h at 30 °C. Then, the sample was diluted with distilled water and autoclave (121 °C for 1 h). When the internal temperature was below 60 °C, the sample was taken out and filtered with a 0.2 μm syringe filter. Monosaccharide analysis was performed using HPAEC (High Performance Anion-Exchange Chromatography, ICS-5000, Dionex co., USA) equipped with an amperometric detector. 25 μL of the sample was injected into the column of the CarboPac PA-1 (250 × 4 mm, Dionex co., USA), and 18 mM NaOH solution was used as the mobile phase. The flow rate was set to 1.0 mL/min, and the column temperature was set at 25 °C.

Cell culture

The human neuroblastoma SH-SY5Y cell line was purchased through the ATCC. The media used for cell culture was Dulbecco's modified Eagle medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin–streptomycin (Gibco, USA). The cells were incubated at 37 °C and 5% CO_2, and media was changed every 2 days. The subculture was carried out at 80–90% confluence. For immunocytochemical MTT analysis, cell plate wells were set at a density of 1×10^4cells per well. For Western blot analysis, cells were plated in special well plates treated with tissue culture at a density of 1×10^6 cells/well.

MTT assay

The MTT assay for evaluating the toxicity of enzyme extracts were followed as described by Lowry et.al with slight modifications [25]. For viability analysis, cell seeding was performed for 24 h in a 37 °C incubator using DMEM F-12 Ham supplemented with 1% penicillin. Each well was treated with various concentrations of TCP (1, 5, 10, 30, 50, and 100 μg/mL) and incubated for 24 h. Afterwards, they were washed with DPBS and incubated for 3 h by adding MTT solution (5 mg/mL). Then, the MTT solution was carefully removed and 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystal. Readings were taken at 540 nm using a spectrophotometer and the cell viability was calculated with the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

"Abs_{sample}" is the absorbance value of the well with the treated cells. "$Abs_{control}$" is the absorbance of the well without processing cells.

Western blot analysis

Western blot was performed to confirm the amount of protein expression. Cells were seeded on a six-well plate and allowed time for SH-SY5Y cells to adhere for 48 h. Afterwards, the wells excluding the blank were treated with 1 µg/mL of lipid polysaccharide (LPS) for 1 h and washed with DPBS. Samples were diluted in DMEM at various concentrations (10, 20, 30, 50 µg/mL) and treated for 24 h. Cells were washed with DPBS and incubated with trypsin for 5 min at 37 °C under 5% CO_2 to detach the adherent cells. Then they were harvested and the proteins were extracted through a homogenizer directly in a cold lysis buffer (Sigma, USA) containing a protease inhibitor cocktail (Sigma, USA). The protein content of the supernatant was measured using a BCA Protein Assay Kit (Thermo Scientific, IL, USA). In a PCR tube, 20 µg of protein and 5 × loading buffer were mixed and heated at 95 ℃ for 5 min before loading onto the gel. Proteins were isolated on 10% SDS polyacrylamide gels according to the molecular weight of the detected protein. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, USA). The PVDF membranes were then blocked with 5% skim milk in Tris-buffered saline plus 0.05% Tween-20 (TBST) for 1 h at room temperature. Then the membranes were permitted to overnight incubation with primary antibodies (β-Amyloid (B-4): sc-28365, BACE (61-3E7): sc-33711, p-JNK (G-7): sc-6254, p-p38 (E-1): sc-166182, β-Actin (C4): sc-47778 (Santa Cruz, California, USA), and rabbit anti-sAPPβ 813,401 (BioLegend, California, USA).) at 4 °C followed by horseradish peroxidase (HRP) secondary antibody (m-IgGκ BP-HRP: sc-516102, Santa Cruz, California, USA) for 2 h at room temperature. Finally, protein bands were detected with an ECL detection reagent according to the manufacturer's instruction (Thermo scientific, Illinois, USA). Blots were visualized on Chemidoc using the Image Lab program (Bio-Rad Laboratories, California, USA). The bands were normalized to β-actin levels and the intensity of the bands were measured using an Image Lab TM 6.0.1 analysis software.

Statistical analysis

Data are presented as mean ± S.D. in at least three independent experiments. Statistical analysis of data was performed using SPSS 12.0 (SPSS Inc., USA). Results were evaluated with the Statistical Analysis System's Analysis of Variance (ANOVA) procedure.

Results and discussion

Proximate composition of *S. japonicus*

The proximate composition of *S. japonicus* is 45.16% ash, 39.36% protein, 8.10% carbohydrates, 6.73% moisture, and 0.52% lipid (as shown in Table 2). In another study of sea cucumbers, *Parastichopus parvimensis* contained 23.9% ash, 60.7% protein, 2.4% carbohydrate, 2.0% moisture, and 2.4% lipid; *Apostichopus californicus* contained 16.1% ash, 69.6% protein, 2.7% carbohydrate, 5.5% moisture, and 2.81% lipid; *Stichopus herrmanni* contained 37.9% ash, 47.0% protein, 10.2% moisture, and 0.8% lipid; *Thelenota ananas* contained 25.1% ash, 55.2% protein, 15.1% moisture, and 1.9% lipid; and *Holothuria fuscogilva* contained 26.4% ash, 57.8% protein, 11.6% moisture, and 0.3% lipid [26]. In comparison, *S. japonicus* is relatively low in protein, but high in ash and carbohydrates. The lyophilized *Isostichopus badionotusd* was measured as ash 60.3 ± 6.1%, and protein 36.7 ± 7.5%, 5.3% moisture, and lipid 0.6 ± 0.3% [27]. The proximate composition was very similar between *I. badionotusd* and *S. japonicus*, so the difference in content components is judged as specific.

Amino acid composition of *S. japonicus*

The amino acid composition of *S. japonicus* are shown in Table 3. Glycine and glutamic acid were the highest at 15.72% and 14.92%, respectively, and aspartic acid was at 9.77%. After that, 8.38%, 7.06%, and 6.91% were in the order of proline, alanine, and arabinonic acid. According to other sea cucumber research results, eight dried sea cucumbers (*Actinopyga mauritiana, Actinopyga caerulea, Bohadschia argus, Holothuria fuscogilva, Holothuria fuscopunctata, S. herrmanni, Thelenota ananas*) had glutamic acid and glycine as the highest content in the amino acid composition [26]. *Oncorhynchus keta* was measured in the order of glutamic acid 14.56%, glycine 9.15%, and aspartic acid 8.56%. For *Oncorhynchus gorbuscha*, glutamic acid was measured to be 17.78 ± 0.02%, aspartic acid was 9.89 ± 0.03%, and glycine was 7.11%. [27]. For *I. badionotusd*, the contents

Table 2 Proximate composition of dried *S. japonicus* body wall

Compositions	Contents (% of dry weight)
Ash	45.16 ± 1.17
Protein	39.36 ± 2.89
Carbohydrate	8.10 ± 3.73
Moisture	6.73 ± 0.52
Lipid	0.52 ± 0.29

Table 3 Amino acid compositions of *S. japonicus* body wall

Amino acids	Contents (%)	Amino acids	Contents (%)
Asp	9.77	Ile	2.18
Thr	4.59	Leu	3.91
Ser	5.16	Tyr	1.91
Glu	14.92	Phe	2.49
Gly	15.72	Lys	2.98
Ala	6.91	His	0.98
Cys	1.13	Arg	7.06
Val	3.10	HyP	4.95
Met	2.27	Pro	8.38

Table 4 Separation and purification yield of TP and TCP from enzymatic extract of *S. japonicus*

Sample	Yield (%)
TP	49.04 ± 11.80
TCP	40.52 ± 7.52

consisted of glycine 215.0 g/kg, glutamic acid 158.2 g/kg, and aspartic acid 100.0 g/k [28]. Therefore, glutamic acid, glycine, and aspartic acid can be regarded as the major non-essentials that are contained in the amino acid composition analysis of sea cucumbers.

Enzymatic hydrolysis of *S. japonicus*

Freeze-dried *S. japonicus* powder was hydrolyzed to obtain an enzyme extract that inhibits the action of β-secretase enzyme. Six enzymes (alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin) were used. Hydrolysis was performed under optimal conditions of each enzyme. Yields obtained in hydrolysis were 69.72%, 70.08%, 77.26%, 75.18%, 59.14%, and 84.54% for alcalase, α-chymotrypsin, neutrase, papain, pepsin, and Trypsin, respectively (Table 4). Trypsin hydrolysate showed significantly the highest yield, and pepsin hydrolysate had the lowest yield. In addition, neutrase and papain were relatively high, followed by alcalase and α-chymotrypsin. Enzymatic hydrolysis has been useful to obtain physiological active peptides based on proteins. Previous study on enzymatic hydrolysis of *Arctoscopus japonicus* using alcalase enzyme exhibited the yield of 77.87 ± 0.27% [28]. Use of skate skin (*Raja Kenojei*) gave the yield of 79.02% for neutrase enzyme hydrolysis [29]. Comparatively the yield for trypsin hydrolysate of *S. japonicus* (84.54 ± 3.30%) obtained in this study was in higher than previous studies. The high yield in hydrolysis research has high economic value and can be easily used industrially. This is an alternative approach to evaluating economic value by converting edible parts or wastes of living organisms into hydrolysates [31]. Hydrolysis, which produces enzyme extracts as hydrolysates is essential and important for studying bioactive peptides [32] (Fig. 4).

Measurement β-Secretase inhibitory activity of hydrolysis

The IC_{50} values for trypsin hydrolysate and α-chymotrypsin hydrolysate were 93.59 µg/ml and 94.46 µg/ml, respectively. Statistical analysis revealed much lower IC_{50} values in α-chymotrypsin and trypsin hydrolysates (Fig. 5). Therefore, further studies were conducted using trypsin hydrolysate, which exhibited a higher yield than α-chymotrypsin. Some previous studies have been reported for β-secretase inhibitory activity against enzymatic hydrolysates. The IC_{50} value for β-secretase inhibition with the use of trypsin hydrolysate from *Holothuria spinifera* was 81.94 ± 0.04 µg/mL [33]. Whereas it was 0.56 mg/mL for nutrase hydrolysate obtained from skate skin hydrolysis. [31]. Further, IC_{50} value for β-secretase inhibition from alcalase hydrolysate from *Arctoscopus japonicus* was 0.52 mg/mL [30]. Another study of sea hare (*Aplysia kurodai*) hydrolysate exhibited IC_{50} values of 0.34 mg/mL, 0.44 mg/mL, and 0.51 mg/mL, respectively, for trypsin, α-chymotrypsin, and neutrase hydrolysates [34]. Protamex hydrolysate of shrimp (*Pandalopsis dispar*) waste was exhibited IC_{50} value of 0.54 mg/mL comparatively [35]. Therefore, compared with previous studies, it can be confirmed that the enzyme hydrolysate derived from *S. japonicus* has a high level of β-secretase inhibitory activity.

Separation and purification of hydrolysate

Among the IC_{50} values of β-secretase inhibitory activity of *S. japonicus* enzyme hydrolysate, trypsin hydrolysate (93.59 ± 0.68 µg / ml) was the lowest. Therefore, the trypsin hydrolysate was separated and purified through ethanol precipitation. The trypsin hydrolysate was separated into TP and TCP, and tested to measure the β-secretase inhibitory activity. The IC_{50} value of β-secretase inhibitory activity of TP was 563.50 ± 25.18 µg/ml, and the TCP IC_{50}

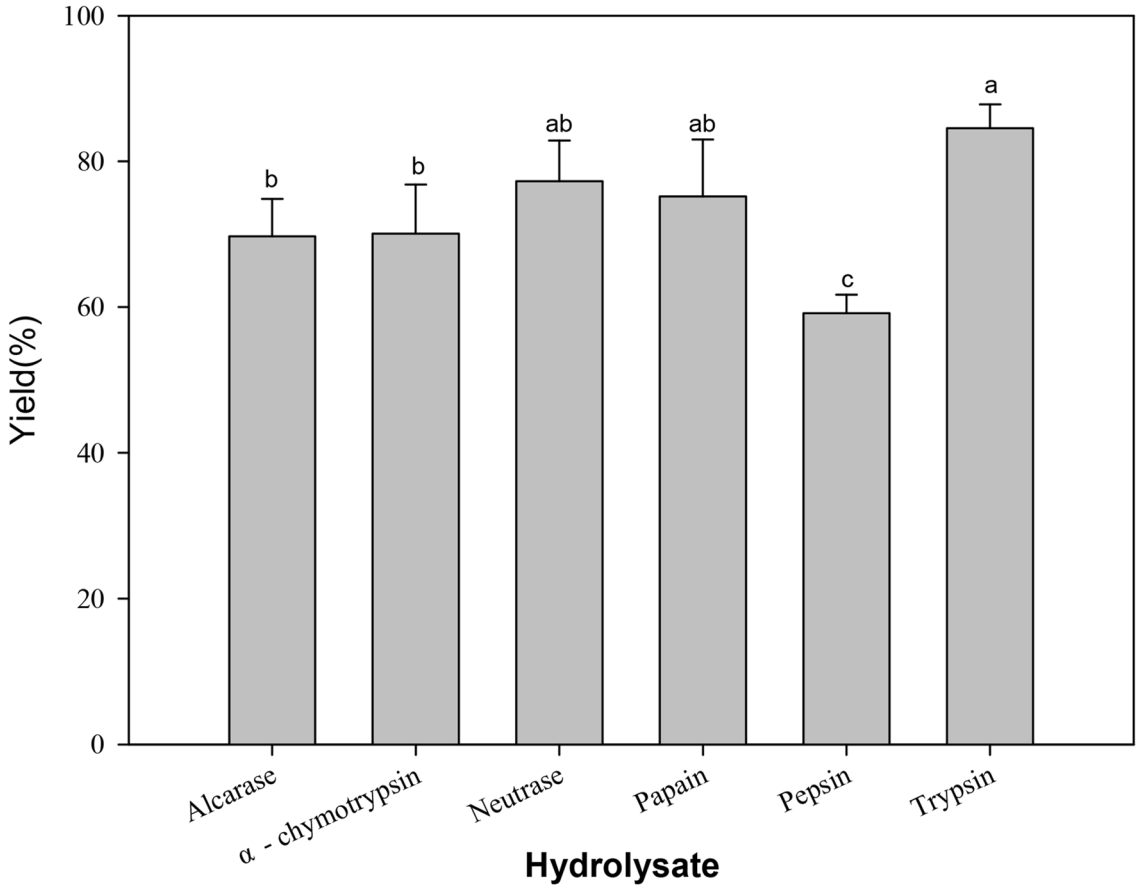

Fig. 4 Yields of *S. japonicus* enzymatic hydrolysates. Values are expressed as the mean ± SD of three independent decisions. ($p < 0.05$)

value was 16.13 ± 1.15 μg / ml. The TCP IC_{50} value was significantly lower than that of trypsin hydrolysate and TP, and the purification fold value for trypsin hydrolysate was 5.80 (Fig. 6). The TCP was diluted by various concentrations (5, 20, 30 μg/ml) and various fluorescence from 0 to 120 min were measured. The relative intensity value at zero minutes5 was 3500–3700 for the control and samples. As time passed, the control gradually increased to 8200, and the increase in fluorescence intensity was inhibited to 7300 at 5 μg/ml, 5100 at 20 μg/ml, and 4000 at 30 μg/ml depending on the concentration of the sample (Fig. 7). Studies on β-secretase inhibitory activity using samples obtained through ethanol precipitation using sea cucumber are insufficient compared with other studies. When treated with 100 μg/mL, the fruiting polysaccharides derived from *Psoriasis mushrooms* isolated using the ethanol precipitation method inhibited β-secretase by 48% compared to the control [36]. Natural substances or extracts capable of inhibiting the action of β-secretase play the most important role in producing Aβ; hence, having the potential to be developed as pharmaceutical materials for Alzheimer's.

Physicochemical analysis

The total sugar content, protein ($y = 0.0011x + 0.1339$, $R^2 = 0.9947$), uronic acid ($y = 0.0067x + 0.0038$, $R^2 = 0.9984$), and sulfate content ($y = 0.3537x - 0.0008$, $R^2 = 0.9988$) of TCP samples are shown in Table 5. The total sugar content was 11.58 ± 0.32 g/100 g, protein content was 5.83 ± 0.89 g/100 g, uronic acid content was 0.49 ± 00.1 g/100 g, and the sulfate content was 5.69 ± 0.15 g/100 g. The uronic acid and sulfate content are essential analysis to determine whether the sugar has biological and pharmacological effects. The uronic acid and sulfate content of polysaccharides obtained through enzymatic digestion are thought to exhibit physiological activities, such as immune and anticomplementary activities, anticancer, and antithrombotic effects [37, 38]. In another study, the physicochemical analysis of the water-soluble protein sulfide fucan (PSF) complex isolated from the body wall of *S. japonicus* showed that the sulfate and uronic acid content in the crude state was 16.0 ± 0.6 g/100 g and 0.8 ± 0.3 g/100 g, respectively. Afterwards, the fractions were obtained from

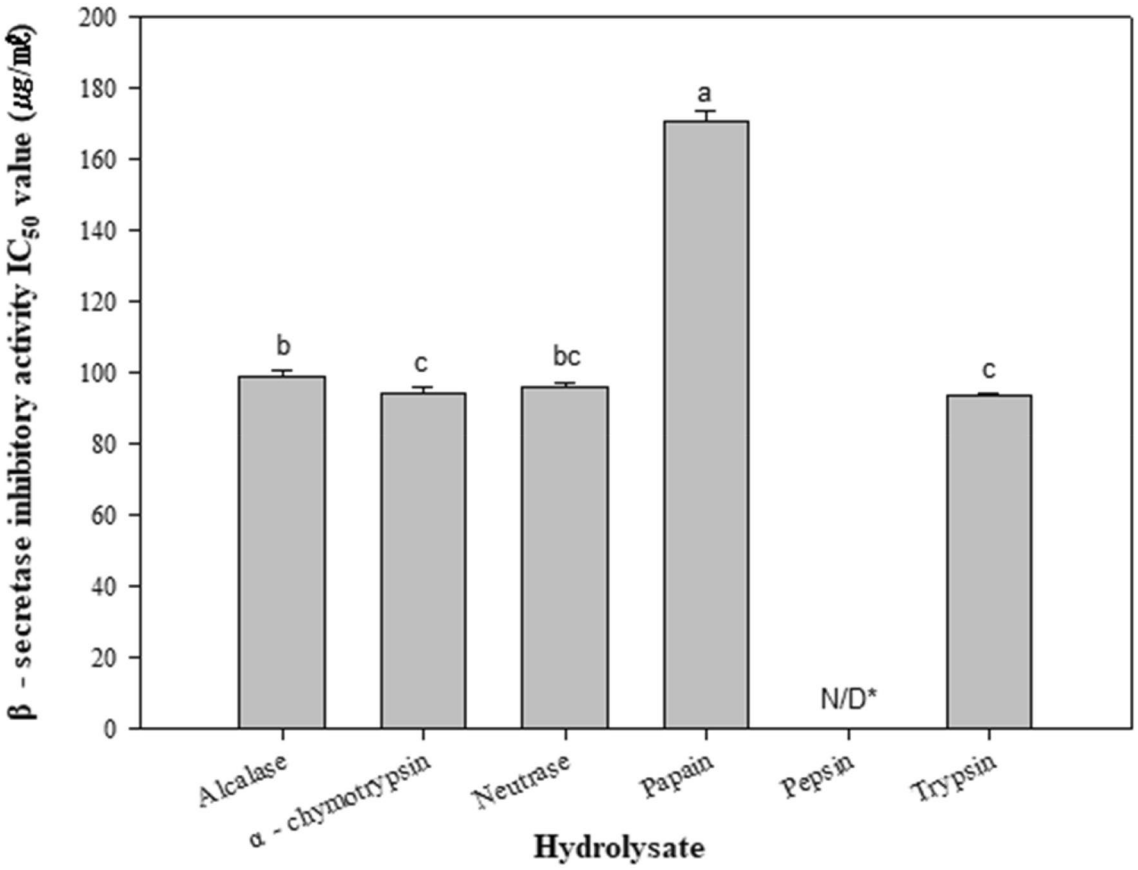

Fig. 5 IC_{50} (μg/mL) values for β-secretase inhibition by six different hydrolysates. Values are expressed as the mean ± SD of three independent decisions. ($p < 0.05$). (*N/D**: non-detected)

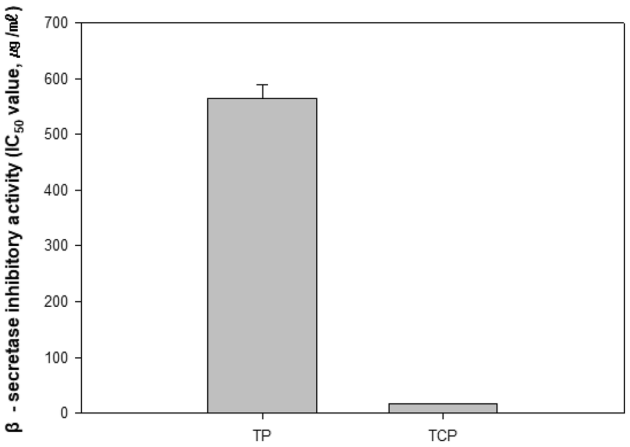

Fig. 6 IC_{50} (μg/mL) values for β-secretase inhibitory activity of TP and TCP

the separations and purifications using a DEAE Sepharose fast flow column (17–0709-01, GE Healthcare Bio-Science AB, Uppsala, Sweden). The sulfate and uronic acid content of the fractions increased to 35.5 ± 0.4 g / 100 g and 3.7 ± 0.1 g / 100 g. Antioxidant and immune effects have increased. [39]. In another study, when analyzing crude polysaccharides derived from *Styela plicata*, the total sugar content was 28.60%, uronic acid content was 9.88%, sulfate content was 18.03%, and the protein content was 26.47% [40]. Compared with the sea cucumber *S. japonicus*, each of the contents were relatively low, except for the high content of sulfate.

Monosaccharide composition

The monosaccharide composition of TCP derived from *S. japonicus* was conducted. Firstly, the TCP was decomposed into a monosaccharide state through acid hydrolysis, and then through the HPAEC (High Performance Anion-Exchange Chromatography, ICS-5000, Dionex co., USA) and amperometric detector. An analysis was performed with CarboPac PA-1 (250 × 4 mm, Dionex co., USA). The results showed a composition of arabinose 1.76 mg/g, fucose 11.98 mg/g, galactose 1.33 mg/g, mannose 1.33 mg/g, and ramnose 0.1 mg/g. Xylose and fructose were not detected (Table 6). Constituent sugar analysis of TCP from *S.*

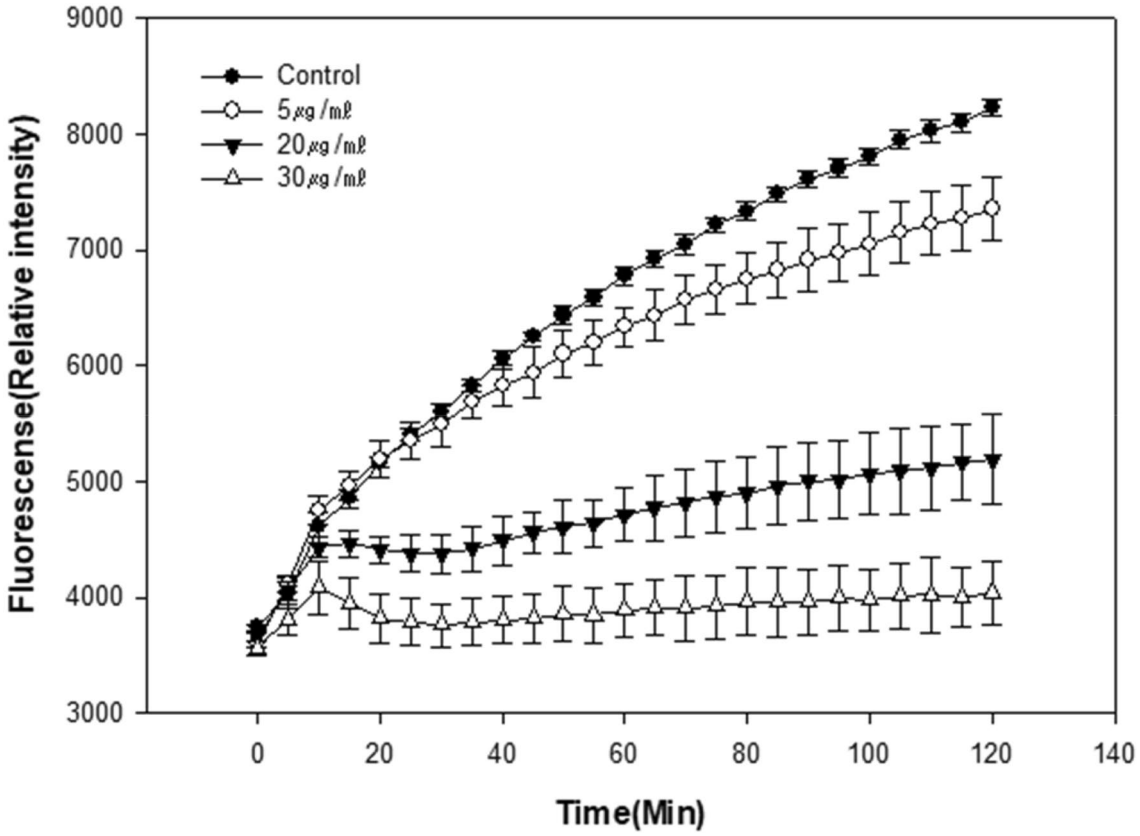

Fig. 7 β-secretase inhibitory activity of TCP obtained from enzymatic extracts of *S. japonicus*

Table 5 Sugar, protein, uronic acid, and sulfate contents of TCP obtained from enzyme extract of *S. japonicus*

Sample	Sugar contents (Glucose)	Protein (BSA[a])	Uronic acid (GA[b])	Sulfate contents (PS[c])
Contents (g/100 g)	11.58 ± 0.32	5.83 ± 0.89	0.49 ± 0.01	5.69 ± 0.15

[a]*BSA* bovine serum albumin

[b]*GA* galacturonic acid

[c]*PS* potassium sulfate

japonicus was performed on six types, but a relatively low content was detected, therefore, further studies on the analysis of monosaccharides are needed. The monosaccharide composition of sea cucumber, *Holothuria leucospilota*, was rhamnose 39.08 mg/g, fucose 35.72 mg/g, glucuronic acid 10.72 mg/g, galactose 8.43 mg/g, glucose 4.23 mg/g, and xylose 1.83 mg/g [41]. The monosaccharide composition of marine organism *Styela plicata* was lactose 44.08 mg/g, glucose 37.63 mg/g, mannose 4.06 mg/g, glucosamine 10.74 mg/g, and galactosamine 3.49 mg/g [40].

MTT assay

MTT analysis was performed to evaluate the cytotoxicity of the TCP to SH-SY5Y cells. Cell seeding 96-well plates were treated with TCP of various concentrations (1, 5, 10, 30, 50, 100μg/ml) for 24 h. Cell viability of more than 85% was observed from all concentrations from 1 to 100μg/ml (Fig. 8). Therefore, it is safe to use up to a concentration of 100μg/ml for research. Compared with other studies, the cell viability of SH-SY5Y cells of the sulfate polysaccharide isolated from *S. japonicus* was measured to be more than 80%

Table 6 Monosaccharide compositions of TCP obtained from enzymatic extract of *S. japonicus*

	Arabinose	Fucose	Galactose	Mannose	Rhamnose	Xylose	Fructose
Contents (mg/g)	1.76 ± 0.13	11.98 ± 0.55	1.33 ± 0.02	1.33 ± 0.02	0.1 ± 0.01	N/D[a]	N/D[a]

N/D[a]: non-detected

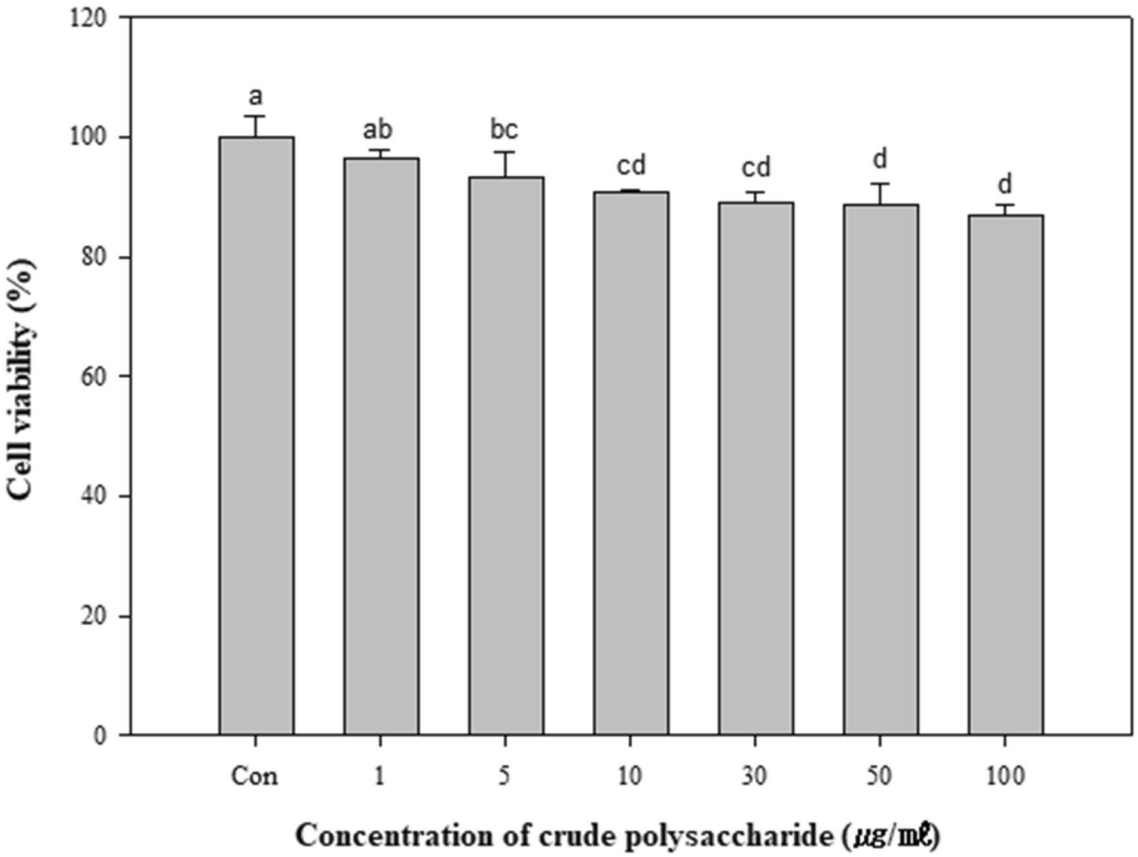

Fig. 8 Effect of TCP obtained from enzymatic extract of *S. japonicus* on the viability of SH-SY5Y cells. Treatment concentrations of 1, 5, 10, 30, 50, 100 µg/ml of TCP. Values are expressed as the mean ± SD of three independent decisions ($p < 0.05$)

at the concentration treated with 75µM to 500µM. Sulfate polysaccharides isolated from *S. japonicus* have also been studied to inhibit the decrease in cell viability due to oxidative stressors caused by 6-hydroxydopamine [42].

Western blot analysis

One of the major hypotheses in Alzheimer's disease physiology is the amyloid cascade hypothesis [43]. The sequential action of β-secretase and γ-secretase on the APP causes the formation of Aβ, which causes neuronal cell death [43–45]. Therefore, research aimed at treating Alzheimer's disease by reducing the production of Aβ by inhibiting the action of β-secretase and γ-secretase [44–47]. In addition, Aβ increase the expression level of p-JNK and p-p38 by producing reactive oxygen species, creating oxidation in brain cells. Expression levels of proteins were performed by processing various TCP samples of 10, 20, 30 and 50 µg/mL in a Western blot. It resulted in protein levels that decreased accordingly with the concentration of the TCP fraction, and even a high level of protein expression was decreased (Fig. 9). TCP fraction reduced the expression of β-secretase protein, which in turn reduced the subsequent expression of sAPPβ and Aβ. This also prevented cell damage by reducing the expression levels of p-p38 and p-JNK, which are involved in the apoptosis pathway by oxidative stress. Inhibition of β-secretase activity leads to a decrease in the level of Aβ and can automatically inhibit plaque formation. This can help treat Alzheimer's disease by preventing brain nerve cell damage and the occurrence of reactive oxygen species.

Conclusion

In this study, an enzyme extract was isolated and purified from *S. japonicus* effectively to inhibit the production of substances that cause Alzheimer's disease. Sea cucumber exhibited 39.36% protein and 45.16% ash. The main amino acids present in *S. japonicus* are glycine 15.72% and glutamic acid 14.92%. Among six hydrolysates prepared, trypsin hydrolysate exhibited the highest β-secretase inhibitory activity (IC_{50} value of 93.59 µg/ml) and yield (84.54%). Fraction TCP showed significantly the lowest IC_{50} value for β-secretase inhibitory activity (16.13 µg/ml). As revealed by physicochemical analysis of TCP fraction, total sugar content was 11.58 g/100 g, the protein content was 5.83 g/100 g,

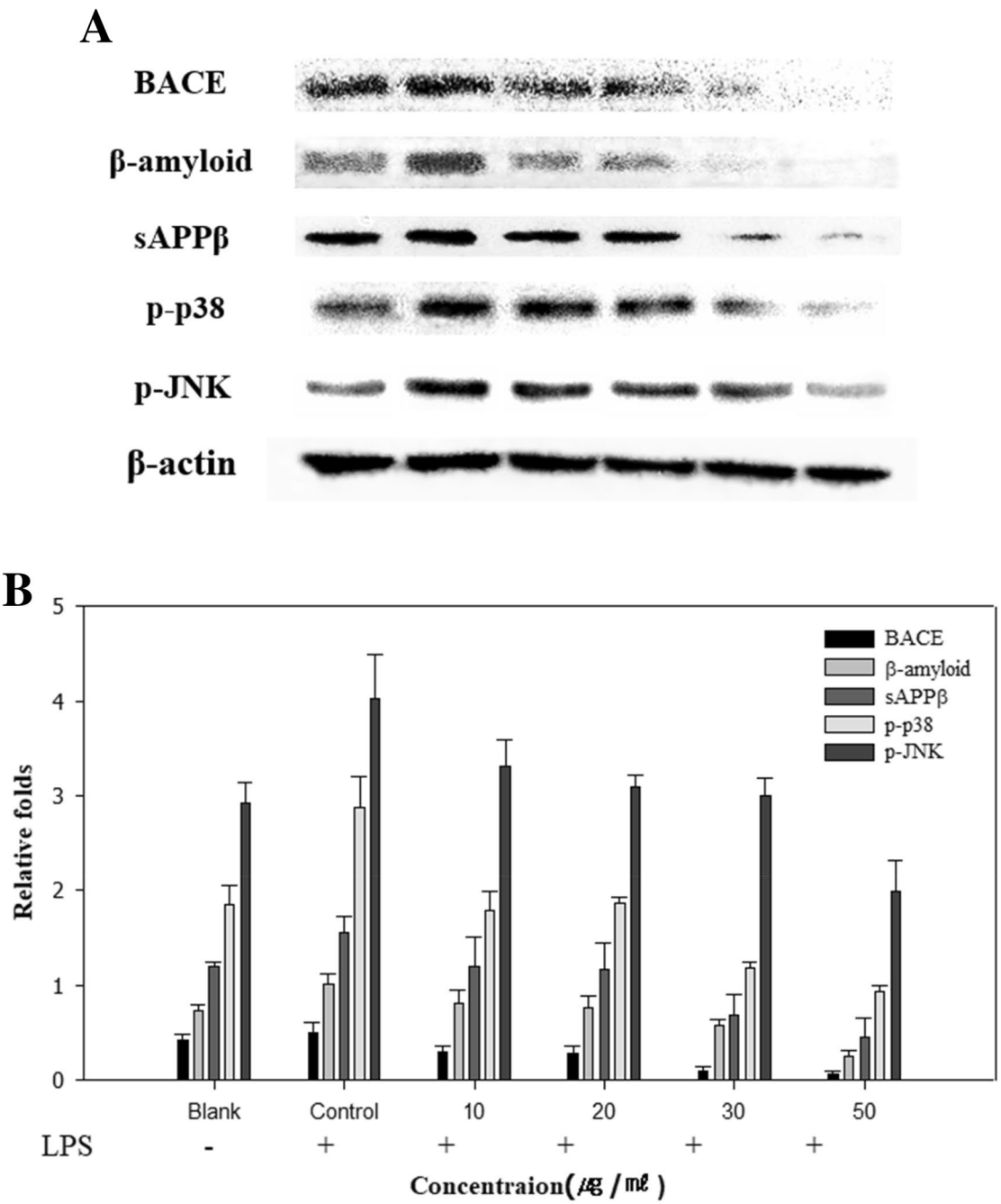

Fig. 9 BACE, amyloid-β, sAPPβ, p-p38, and p-JNK protein levels in SH-SY5Y cells of *S. japonicus* TCP. This is the result of 24 h of TCP processing. Western blot is a representative method of measuring protein levels. **a** The results of quantifying the intensity of the band using Image Lab software **b** After 24 h of processing with TCP. Values are expressed as the mean ± SD of three independent decisions. ($p < 0.05$)

the uronic acid content was 0.49 g/100 g, and the sulfate content was 5.69 g/100 g. Major monosaccharides presented were arabinose 1.76 mg/g, fucose 11.98 mg/g, galactose 1.33 mg/g, mannose 1.33 mg/g, rhamnose 0.10 mg/g, and xylose. Cell were treated with the highest active TCP fractions. There was no cytotoxicity produced by TCP in SH-SY5Y cells at tested concentrations. In SH-SY5Y cells TCP treatment identified to inhibit neuronal degradation by reducing the levels of BACE, Aβ, sAPPβ, p-p38 and p-JNK. In conclusion, sea cucumber (*S. japonicus*) can be used as a promising treatment for Alzheimer's disease by preventing nerve cells damage from Aβ. Therefore, *S. japonicus* is a potential source of food and biomedicine industries.

Acknowledgements This research was funded by the Marine Biotechnology Program of Korea Institute of Marine Science and Technology

Promotion (KIMST) and Ministry of Oceans and Fisheries (MOF) (No. 20140441).

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human or animal subjects.

References

1. Möller HJ, Graeber MB (1998) The case described by Alois Alzheimer in 1911. Eur Arch Psychiatry Clin Neurosci 248(3):111–122
2. Lalut J, Payan H, Davis A, Lecoutey C, Legay R, Santos S-D J, Claeysen S, Dallemagne P, Rochais C (2020) Rational design of novel benzisoxazole derivatives with acetylcholinesterase inhibitory and serotoninergic 5-HT 4 receptors activities for the treatment of Alzheimer's disease. Sci Rep 10(1):1–11
3. Prince MJ, Wimo A, Guerchet MM, Ali GC, Wu Y-T, Prina M (2015) World Alzheimer Report 2015 - The Global Impact of Dementia: an analysis of prevalence, incidence, cost and trends. Alzheimer's Disease International, London, p 84
4. Tian XY, Zhao Y, Yu SS, Fang WS (2010) BACE1 (Beta-Secretase) inhibitory phenolic acids and a novel sesquiterpenoid from homalomena occulta. Chem Biodivers 7(4):984–992
5. Liu XC, Bai CQ, Liu QZ, Liu ZL (2014) Evaluation of nematicidal activity of the essential oil of *Homalomena occulta* (Lour.) Schott rhizome and its major constituents against *Meloidogyne incognita* (kofoid and white) chitwood. J Entomol Zool Stud 2(4):182–186
6. Han SH (2009) Novel pharmacotherapies for Alzheimer's disease. J Korean Med Assoc 52(11):1059–1068
7. Citron M (2004) β-Secretase inhibition for the treatment of Alzheimer's disease–promise and challenge. Trends Pharmacol Sci 25(2):92–97
8. Son HJ et al (2016) "In vitro screening for anti-dementia activities of seaweed extracts", journal of the korean society of food science and nutrition. Korean Soc Food Sci Nutr 45(7):966–972. https://doi.org/10.3746/jkfn.2016.45.7.966
9. Selkoe DJ (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399(6738):A23–A31
10. Hwang DY, Chae KR, Kang TS, Hwang JH, Lim CH, Kang HK, Goo JS, Lee MR, Lim HJ, Min SH, Cho JY (2002) Alterations in behavior, amyloid p-42, caspase-3, and Cox-2 in mutant PS2 transgenic mouse model of Alzheimer's disease. FASEB J 16(8):805–813
11. Casteels P, Ampe C, Jacobs F, Vaeck M, Tempst PJTEJ (1989) Apidaecins: antibacterial peptides from honeybees. EMBO J 8(8):2387–2391
12. Lee SY, Hur SJ (2017) Antihypertensive peptides from animal products, marine organisms, and plants. Food Chem 228:506–517
13. Ryu HS, Moon JH, Suh JS (1997) Chemical compositions of glycoprotein and chondroitin sulfates from sea cucumber (*Stichopus japonicus*). J Korean Soc Food Sci Nutr 26:72–80
14. Mou J, Li Q, Qi X, Yang J (2018) Structural comparison, antioxidant and anti-inflammatory properties of fucosylated chondroitin sulfate of three edible sea cucumbers. Carbohyd Polym 185:41–47
15. Chen J, Ren Y, Wang G, Xia B, Li Y (2018) Dietary supplementation of biofloc influences growth performance, physiological stress, antioxidant status and immune response of juvenile sea cucumber Apostichopus japonicus (Selenka). Fish Shellfish Immunol 72:143–152
16. Xu C, Zhang R, Wen Z (2018) Bioactive compounds and biological functions of sea cucumbers as potential functional foods. J Funct Foods 49:73–84
17. Huo D, Sun L, Ru X, Zhang L, Lin C, Liu S, Xin X, Yang H (2018) Impact of hypoxia stress on the physiological responses of sea cucumber Apostichopus japonicus: respiration, digestion, immunity and oxidative damage. Peer J 6:e4651
18. Song Y, Cong P, Lu L, Wang Y, Tang Q, Zhang H, Xu J, Xue C (2017) Effects of dietary glucocerebrosides from sea cucumber on the brain sphingolipid profiles of mouse models of Alzheimer's disease. Food Funct 8(3):1271–1281
19. Oh GW, Ko SC, Lee DH, Heo SJ, Jung WK (2017) Biological activities and biomedical potential of sea cucumber (*Stichopus japonicus*): a review. Fish Aquat Sci 20(1):1–17
20. Official AOAC (2005) Methods of analysis of AOAC International AOAC INTERNATIONAL, Maryland, USA
21. Johnston JA, Liu WW, Coulson DTR, Todd S, Murphy S, Brennan S, Foy CJ, Craig D, Irvine GB, Passmore AP (2008) Platelet β-secretase activity is increased in Alzheimer's disease. Neurobiol Aging 29(5):661–668
22. Filisetti-Cozzi TM, Carpita NC (1991) Measurement of uronic acids without interference from neutral sugars. Anal Biochem 197(1):157–162
23. Dodgson KS, Price RG (1962) A note on the determination of the ester sulphate content of sulphated polysaccharides. Biochem J 84(1):106–110
24. Dubois M, Gilles KA, Hamilton JK, Rebers PT, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28(3):350–356
25. Miller RE, Cantor SM (1951) Aconitic acid, a by-product in the manufacture of sugar. Adv Carbohydr Chem 6:231–249
26. Datki Z, Juhász A, Gálfi M, Soós K, Papp R, Zádori D, Penke B (2003) Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells. Brain Res Bull 62(3):223–229
27. Wen J, Hu C, Fan S (2010) Chemical composition and nutritional quality of sea cucumbers. J Sci Food Agric 90(14):2469–2474
28. Yoon HD, Karaulova EP, Shulgina LV, Yakush EV, Mok JS, Lee SS, Xie C, Kim JG (2015) Nutritional value and bioactive properties of enzymatic hydrolysates prepared from the livers of *Oncorhynchus keta* and *Oncorhynchus gorbuscha* (*Pacific salmon*). Fish Aquat Sci 18(1):13–20
29. Pérez-Vega JA, Olivera-Castillo L, Gómez-Ruiz JÁ, Hernández-Ledesma B (2013) Release of multifunctional peptides by gastrointestinal digestion of sea cucumber (*Isostichopus badionotus*). J Funct Foods 5(2):869–877
30. Park SBN, Kim SR, Byun HG (2018) Characterization of a new anti-dementia β-secretase inhibitory peptide from *Arctoscopus japonicus*. J Chitin Chitosan 23(4):220–227
31. Lee JK, Li-Chan EC, Byun HG (2015) Characterization of β-secretase inhibitory peptide purified from skate skin protein hydrolysate. Eur Food Res Technol 240(1):129–136
32. Kristinsson HG, Rasco BA (2000) Fish protein hydrolysates: production, biochemical, and functional properties. Crit Rev Food Sci Nutr 40(1):43–81
33. Ou K, Liu Y, Zhang L, Yang X, Huang Z, Nout MR, Liang J (2010) Effect of neutrase, alcalase, and papain hydrolysis of whey protein concentrates on iron uptake by Caco-2 cells. J Agric Food Chem 58(8):4894–4900
34. Rathnayake AU, Ma EH, Abuine R, Byun HG (2020) Purification of β-secretase inhibitory peptides from sea cucumber (*Holothuria spinifera*) enzymatic hydrolysates. 한국수산과학회 양식분과 학술대회, p 241

35. Lee JK, Kim SR, Byun HG (2018) Purification and characterization of β-secretase inhibitory peptide from sea hare (*Aplysia kurodai*) by enzymatic hydrolysis. Fish Aquat Sci 21(1):1–8
36. Li-Chan EC, Cheung IW, Byun HG (2016) Shrimp (*Pandalopsis dispar*) waste hydrolysate as a source of novel β-secretase inhibitors. Fish Aquat Sci 19(1):1–7
37. Jo HS, Choi DJ, Chung MJ, Park JK, Park YI (2012) Characterization and β-secretase inhibitory activity of water-soluble polysaccharides isolated from *Phellinus linteus* fruiting body. Korean J Mycol 40(4):229–234
38. Kumar V, Nagar S, Tripathi YC (2014) Do assorted approaches aid in estimation of uronic acids? Case studies on *Tinospora sinensis* polysaccharides. Int J Biol Macromol 70:360–363
39. Tao Y, Zhang Y, Zhang L (2009) Chemical modification and antitumor activities of two polysaccharide–protein complexes from pleurotus tuber-regium. Int J Biol Macromol 45(2):109–115
40. Cao RA, Surayot U, You S (2017) Structural characterization of immunostimulating protein-sulfated fucan complex extracted from the body wall of a sea cucumber, *Stichopus japonicus*. Int J Biol Macromol 99:539–548
41. Lee DH, Hong JH (2019) Physicochemical properties and immunomodulatory effects of polysaccharides isolated from *Styela plicata*. Korean J Food Preserv 26(4):416–424
42. Yuan Y, Li C, Zheng Q, Wu J, Zhu K, Shen X, Cao J (2019) Effect of simulated gastrointestinal digestion in vitro on the antioxidant activity, molecular weight and microstructure of polysaccharides from a tropical sea cucumber (*Holothuria leucospilota*). Food Hydrocoll 89:735–741
43. Rathnayake AU, Abuine R, Kim YJ, Byun HG (2019) Anti-Alzheimer's materials isolated from marine bio-resources: a review. Curr Alzheimer Res 16(10):895–906
44. Cui C, Cui N, Wang P, Song S, Liang H, Ji A (2016) Neuroprotective effect of sulfated polysaccharide isolated from sea cucumber Stichopus japonicus on 6-OHDA-induced death in SH-SY5Y through inhibition of MAPK and NF-κB and activation of PI3K/Akt signaling pathways. Biochem Biophys Res Commun 470(2):375–383
45. Abuine R, Rathnayake AU, Byun HG (2021) A peptide fraction of olive flounder (*Paralichthys olivaceus*) skin hydrolysate inhibits amyloid-β generation in SH-SY5Y cells via suppression of BACE1 expression. Int J Pept Res Ther 27(1):627–639
46. Palop JJ, Mucke L (2010) Amyloid-β–induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. Nat Neurosci 13(7):812
47. Giacobini E, Gold G (2013) Alzheimer disease therapy—moving from amyloid-β to tau. Nat Rev Neurol 9(12):677–686