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ORIGINAL ARTICLE

Optimization of hydrolysis conditions, isolation, and identification of neuroprotective peptides derived from seahorse *Hippocampus trimaculatus*

Ratih Pangestuti · BoMi Ryu · SWA. Himaya · Se-Kwon Kim

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Abstract *Hippocampus trimaculatus* is one of the most heavily traded seahorse species for traditional medicine purposes in many countries. In the present study, we showed neuroprotective effects of peptide derived from *H. trimaculatus* against amyloid-β_{42} ($A\beta_{42}$) toxicity which are central to the pathogenesis of Alzheimer's diseases (AD). Firstly, *H. trimaculatus* was separately hydrolyzed by four different enzymes and tested for their protective effect on $A\beta_{42}$-induced neurotoxicity in differentiated PC12 cells. Pronase E hydrolysate exerted highest protection with cell viability value of 88.33 ± 3.33 %. Furthermore, we used response surface methodology to optimize pronase E hydrolysis conditions and found that temperature at 36.69 °C with the hydrolysis time 20.01 h, enzyme to substrate (*E*/*S*) ratio of 2.02 % and pH 7.34 were the most optimum conditions. Following several purification steps, *H. trimaculatus*-derived neuroprotective peptides (HTP-1) sequence was identified as Gly-Thr-Glu-Asp-Glu-Leu-Asp-Lys (906.4 Da). HTP-1 protected PC12 cells from $A\beta_{42}$-induced neuronal death with the cell viability value of 85.52 ± 2.22 % and up-regulated pro-survival gene (Bcl-2) expressions. These results suggest that HTP-1 has the potential to be used in treatment of neurodegenerative diseases, particularly AD. Identification, characterization, and synthesis of bioactive components derived from *H. trimaculatus* have the potential to replace or at least complement the use of seahorse as traditional medicine, which further may become an approach to minimize seahorse exploitation in traditional medicine.

Keywords *H. trimaculatus* · $A\beta_{42}$ · PC12 · RSM · Pronase E · HTP-1

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Introduction

Seahorses are teleosts (bony fish) belong to the *Syngnathidae* of Chordata phylum (Lourie et al. 1999; Zhang et al. 2003). They are important in ecological, economical, medicinal, and cultural terms (Vincent et al. 2011). Among seahorses, *Hippocampus trimaculatus* (Fig. 1) are highly valued and most heavily traded species for traditional medicine purposes in many countries such as China, Japan, Korea, Indonesia, Philippines, Vietnam, Brazil, and Latin America. Seahorses used in traditional medicine are generally ground to powder which may be applied directly or mixed with warm water; hence, the most active components tend to be either fat (lipid) soluble or water soluble to one degree or another. Previous studies on the seahorses bioactive components mainly focused on analysis of trace elements, amino acids, and organic solvent-soluble components (Zhang et al. 2003). In contrast, health benefit effects of seahorse water-soluble components such as polysaccharides, proteins, and bioactive peptides remain unidentified.

Bioactive peptides are specific protein fragments that have a positive impact on a body function or condition and ultimately may influence human health, which expected to be provided by a safe, reliable, and consistent oral delivery

Fig. 1 Representative image of *Hippocampus trimaculatus*. One special feature of this species is the "three spots" on their dorsolateral trunk surface

system (Ryu et al. 2010). Bioactive peptides derived from marine organisms may produce by three methods such as solvent extraction, enzymatic hydrolysis, and microbial fermentation (Kim and Wijesekara 2010). In food and pharmaceutical industries, enzymatic hydrolysis method is more preferred, because of lack of residual organic solvents or toxic chemicals and microbial contaminations in the products (Wijesinghe and Jeon 2011). Enzymatic hydrolysis can be obtained by in vitro hydrolysis of protein sources using appropriate proteolytic enzymes derived from microbes, plants, and animals (Aneiros and Garateix 2004). The physicochemical conditions of the reaction media, such as, temperature, hydrolysis time, enzyme to substrate (*E*/*S*) ratio, and pH of the protein solution, must then be adjusted to optimize the activity of the enzyme used. Hence, for the efficient recovery and to obtain peptides with desired biological activity and functional property, a suitable method of enzyme optimization is needed.

Alzheimer's disease (AD) is the most common, age related, progressive neurodegenerative disorder which is characterized by senile plaques, neurofibrillary tangles, and the loss of neurons and synapses (LaFerla et al. 2007). Accumulation of β-amyloid (Aβ) within the senile plaques is thought to be primary cause of the cognitive dysfunction that occurs in AD. Abnormal accumulation of Aβ resulting in the formation of toxic oligomers is the result of an imbalance between the levels of Aβ production, aggregation, and clearance. Elevated levels of Aβ are present in the brains of individuals with either sporadic or familial form of AD. Recent studies have demonstrated that in AD brains and in cultures of neurons exposed to Aβ, the dying cells display the characteristics of apoptosis (Christensen et al. 2010). Therefore, Aβ is thought to be responsible in part for the neuronal loss in AD and identification of neuroprotective peptides that prevent Aβ-induced neuronal apoptosis is an important area in AD research (Pangestuti and Kim 2011).

PC12 cells are cloned of rat pheochromocytoma cells line that retains a number of chromaffin cell characteristics, such as the presence of nicotinic cholinergic receptors. PC12 cells are a useful model for the study of neuronal development, they can be induced to differentiate toward cholinergic neurons after exposure to nerve growth factor (NGF) (Margioris et al. 1992). For this reason, $A\beta_{42}$-induced PC12 cell death constitutes an excellent model for the screening and subsequent evaluation of the potent candidate for neurodegenerative therapies, particularly AD patients. Presently, identification of seahorse bioactive peptides and their neuroprotective mechanisms are not fully elucidated yet. In the present study, response surface methodology (RSM) was applied to optimize the hydrolysis conditions with the purpose of obtaining the most active peptides derived from *H. trimaculatus* which protect PC12 cells from $A\beta_{42}$-induced neuronal apoptosis. Furthermore, other objectives in this study were to isolate and identify neuroprotective peptides derived from *H. trimaculatus* enzymatic hydrolysates.

Materials and methods

Seahorses samples

Hippocampus. trimaculatus were collected along the Karimun Jawa Island coast of Indonesia during the period of May 2011. *H. trimaculatus* were identified by Prof. Ocky Karna Radjasa, Ph. D (Marine biologist, Diponegoro University, Indonesia). The samples were washed with tap water to remove salt, epiphytes, and sand on the surface of the samples and dried in the shade for 2 weeks. Voucher specimens were deposited in the author's laboratory. Dried *H. trimaculatus* were freeze dried for 3 days and ground until become a powder.

Reagents

Cell culture medium [Dulbecco's Modified Eagle's Medium (DMEM)], penicillin/streptomycin, fetal bovine serum (FBS) and the other materials required for culturing cells

were purchased from Gibco BRL, Life Technologies (Grand Island, US). Horse serum, nerve growth factor (NGF), bovine serum albumin (BSA), proteolytic enzyme (trypsin, α-chymotrypsin, papain and pronase E), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), poly-D-lysine, $A\beta_{42}$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemical and reagents used in this study were of analytical grade.

PC12 cell culture and differentiation assay

PC12 cell line was obtained from American Type Culture Collection (Manassas, USA). The cells were grown in T-75 tissue culture cell flasks SPL Life Science (Seoul, Republic of Korea) at 5 % CO_2 and 37 °C humidified atmosphere using DMEM supplemented with 10 % horse serum and 5 % FBS, 2-mM glutamine and 100-μg/ml penicillin–streptomycin. For differentiation experiments, cells were placed on poly-D-lysine-coated dish. Cells were differentiated into neuronal cells by growing the cells in serum-free medium with NGF (100 ng/ml) and BSA (1 %) for 14 days.

Preparation of $A\beta_{42}$ oligomers

Oligomeric $A\beta_{42}$ was prepared as previously described (Resende et al. 2008). Synthetic $A\beta_{42}$ was dissolved in HFIP to 1 mmol/l. The HFIP was then removed in hood for 24 h, then evaporated using a Speed Vac and the dried HFIP film was stored at −20 °C. The peptide film was resuspended to make a 5 mmol/l solution in anhydrous DMSO. $A\beta_{42}$ oligomers were prepared by diluting the solution in medium without phenol red to a concentration of 100 μM and incubated overnight at 4 °C.

Enzyme selections

In order to get the optimum enzyme to hydrolyze *H. trimaculatus*, four protease enzyme (trypsin, α-chymotrypsin, papain, and pronase E) were used. In brief, *H. trimaculatus* powder (250 mg) was homogenized and enzymatically hydrolyzed with various selected enzyme. At enzyme to substrate ratio of 1/100 (w/w), and then mixed. The mixture was incubated for 6 h at each selected temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. Lyophilized hydrolysates were then checked for their effect on $A\beta_{42}$-induced neurotoxicity in PC12 cells.

Cell viability assay

In brief, cells were seeded into 96-well plates at a density of 2×10^4 cells/well and incubated with serum-free medium in the presence of different sample concentration. After incubation for 24 h, 100 μl of MTT (0.5 mg/ml final concentration) was added and incubation was continued for another 4 h. MTT is used as an indicator of cell viability through its mitochondrial reduction to formazan (Rajapakse et al. 2008). Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in DMSO and the absorbance was measured at 540 nm using enzyme-linked immunosorbent assay (ELISA) microplate reader (Tecan Autria GmbH). Relative cell viability was calculated compared with the absorbance of the untreated control group.

Response surface methodology

In order to optimize the enzyme hydrolysis experiments, *H. trimaculatus* were incubated in 100-ml conical flasks with working volume 25 mL. Experiments sought to establish the relationship with between $A\beta_{42}$-induced neurotoxicity in PC12 cells with four independent variables, including temperature (X_1, °C), time (X_2, min), *E/S* (X_3, %), and pH (X_4). The three levels of each variable were coded as −1, 0, +1 which corresponds to the lowest, middle and the higher values, respectively (Table 1). A central composite design with four variables at three levels and a total of 27 sets was used for the study. Design experiments and dependent variables are presented in Table 2.

Regression analysis and analysis of variance (ANOVA) were performed using design of experiment-8 (DOE-8) software. A second-order polynomial used to fit the response to independent variable is shown in the following equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j \quad (1)$$

where Y is the responsible variable, β_0 is the constant coefficient (intercept); β_i is the linear coefficient (main effect); β_{ii} is the quadratic coefficient; β_{ij} is the two factors interaction coefficient.

Table 1 Independent factors and their coded and actual levels used in RSM studies for optimizing hydrolysis conditions using pronase E

Factor		Levels		
		−1	0	+1
Temperature (°C)	X_1	35	45	55
Time (h)	X_2	6	12	24
E/S (%; w/v)	X_3	1	2	3
pH	X_4	6	7	8

Table 2 Actual levels of independence variables along with the observed values for the response variable, PC12 cell viability (*Y*)

Run	Type	Factor (X_1) Temperature (°C)	Factor (X_2) Time (h)	Factor (X_3) *E/S* (%)	Factor (X_4) pH	Responses (*Y*) Cell viability (%)
1	Factorial	−1	−1	−1	−1	88.40
2	Factorial	1	−1	−1	−1	78.12
3	Factorial	−1	1	−1	−1	79.24
4	Factorial	1	1	−1	−1	80.15
5	Factorial	−1	−1	1	−1	81.23
6	Factorial	1	−1	1	−1	91.09
7	Factorial	−1	1	1	−1	85.33
8	Factorial	1	1	1	−1	84.27
9	Factorial	−1	−1	−1	1	85.82
10	Factorial	1	−1	−1	1	80.17
11	Factorial	−1	1	−1	1	90.28
12	Factorial	1	1	−1	1	80.32
13	Factorial	−1	−1	1	1	79.98
14	Factorial	1	−1	1	1	87.86
15	Factorial	−1	1	1	1	84.12
16	Factorial	1	1	1	1	85.46
17	Axial	−1	0	0	0	91.98
18	Axial	1	0	0	0	88.02
19	Axial	0	−1	0	0	91.05
20	Axial	0	1	0	0	90.41
21	Axial	0	0	−1	0	60.40
22	Axial	0	0	1	0	89.88
23	Axial	0	0	0	−1	95.80
24	Axial	0	0	0	1	95.97
25	Center	0	0	0	0	94.34
26	Center	0	0	0	0	94.34
27	Center	0	0	0	0	94.34

Preparation of enzymatic hydrolysates

Protein hydrolysate from *H. trimaculatus* was prepared by employing the optimized conditions obtained from RSM and then heated in a boiling water bath at 100 °C for 10 min to inactivate the enzyme. Lyophilized hydrolysates were stored at −80 °C and freeze dried until used.

Purification of neuroprotective peptide from *H. trimaculatus*

Ion exchange chromatography

Active peptide was purified from proteolytic *H. trimaculatus* hyrolysates using fast protein liquid chromatography (FPLC) (AKTA, Amersham Bioscience Co., Uppsala, Sweden) on a Hiprep 16/10 DEAE FF ion exchange column. The lyophilized *H. trimaculatus* (40 mg/ml) was loaded on the column equilibrated with 20-mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0–2 M) in the same buffer at a flow rate 4 ml/min. The UV absorbance was monitored at 280 nm, and collected by the auto collector at volume of 5 ml. Then, each fraction was pooled according to the peaks and concentrated using a rotary evaporator. Each pooled fraction was analyzed for neuroprotective effect against $A\beta_{42}$-induced neurotoxicity in PC12 cells and the strongest neuroprotective active fraction was lyophilized and used for further purification.

High performance liquid chromatography

The fraction exhibiting the strongest radical scavenging activity was further purified using reversed-phase high performance liquid chromatography (RP-HPLC) (Dionex Ltd., Sunnyvale, CA, USA) on a Nucleosil C_{18} (10 × 250 mm, Mackerey Nagel-Nucleosil, Duren, Germany) column with a linear gradient of acetonitrile

(0–20 % in 45 min) at a flow rate of 1.0 ml/min. Elution peaks were detected at 215 nm, and the active peak was concentrated using a rotary evaporator. Potent peaks were collected, evaluated radical scavenging activity, and then lyophilized. The potent fractions were applied to Promesphere C_{18} (20 × 250 mm, Kyoto, Japan) column with a linear gradient of acetonitrile at flow rate 1 ml/min for 40 min. Each peak was detected and confirmed of their activity. Then, the pure peptide was subjected to amino acid sequence analysis.

Determination of amino acid sequence

The accurate molecular mass and amino acid sequence of the purified peptide was determined using the Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled with an electrospray ionization (Wijesinghe and Jeon) source. The purified peptides were separately infused into the electrospray source after being dissolved in methanol/water (1:1, v/v), and its molecular mass was determined by the doubly charged $(M+2H)^{2+}$ state in the mass spectrum. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectrometry analysis.

Proximate composition

The proximate compositions (moisture, fat, protein, and ash) of the raw material were measured according to the procedure of the Association of Official Analytical Chemist (AOAC 1997). The moisture content was determined by drying the samples, at 105 °C to a constant weight. Crude fat was determined by the Soxhlet method; crude protein content was calculated using the Kjeldahl method; crude carbohydrates was determined by phenol–sulfuric acid method; and crude ash content was calculated using the muffle-furnace technique.

Amino acid analysis of *H. trimaculatus*

The freeze-dried *H. trimaculatus* (50 mg) was hydrolyzed for 24 h in 6 N HCl containing 0.1 % thioglyocolic acids at 110 °C in vacuum. Amino acids which derivatized with phenylisothiocyanate were identified and quantified using automatic amino acid analyzer (Biochrom 20, Pharamacia Biotech, UK).

Semiquantitative reverse transcription polymerase chain reaction

Total RNA was extracted from PC12 cells treated with $A\beta_{42}$ in the presence or absence of hydrolysates or bioactive peptides using TRIzol® reagent, as reported in the manufacturer's manual. Equal amount of RNA (2 μg) was used for each cDNA synthesis reaction. 1 μl oligo dT primer (10 μM) was added and the final volume were adjusted to 13 μl with DEPC water. The solution was then incubated for 10 min at 70 °C and was then snap-cooled on ice. Once the samples had the condensation spun down, a master mix containing 1x reverse transcriptase (RT) buffer, 1 mM dNTPs, 140 U of murine Moloney leukemia virus (MMLV) reverse transcriptase, and 40 U of RNase inhibitor were added and mixed using a pipette tips. The samples were then incubated for a further 1 h at 42 °C. PCR was carried out in an automatic Whatman thermocycler (Biometra, Kent, UK). PCR products were then electrophoresed on 1 % agarose gels, visualized by ethidium bromide staining and quantified using AlphaEAse® gel image-analysis software (Alpha Innotech, San Lendro, CA, USA). Single stranded cDNA was amplified by PCR with specific primers for Bcl-2 forward and reverse primer: 5′-TGGGATGCCTTTGTGGAACT-3′ and 5′-CAGCCAGG AGAAATCAAACAGA-3′; β-actin forward and reverse primer: 5′-GTTGGGATGAACCAGAAGGA-3′ and 5′-CTTACAATTTCCCGCTCTGC-3′. The following PCR conditions were applied for all amplifications: 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extended at 72 °C for 30 s. The resulting cDNA was separated by electrophoresis on 1 % agarose gel for 15 min at 100 V, followed visualization under UV light after ethidium bromide staining. Band intensities were quantified with Multi gauge Software (Fujifilm Life Science, Tokyo, Japan) and band of specific genes were normalized using β-actin as references.

Statistical analysis

The data were presented as mean ± SD ($n = 3$). Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The statistical software package, SPSS v.16 (SPSS Inc., Chicago, IL, USA), was used for the analysis.

Table 3 Proximate compositions of *H. trimaculatus*

Compositions	Contents (%)
Crude protein	75.75 ± 0.13
Crude lipid	4.02 ± 0.03
Crude carbohydrates	2.04 ± 0.5
Ash	18.27 ± 0.11
Total	100

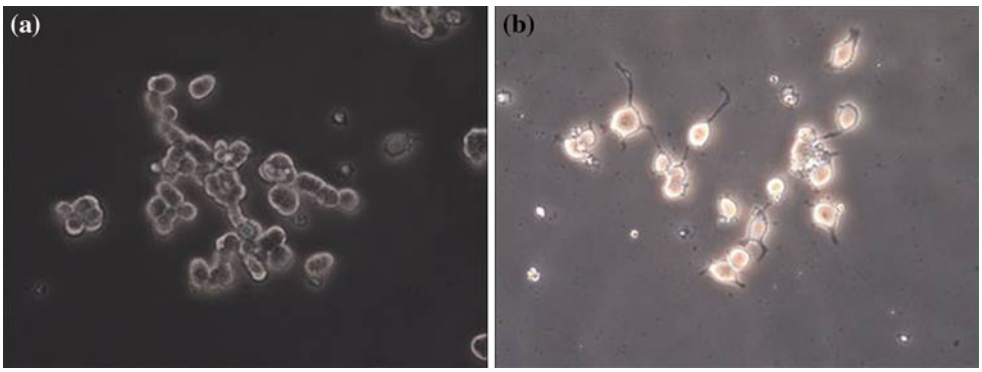

Fig. 2 NGF-induced differentiation of PC12 cells. Cells were plated on poly-D-lysine-coated plates at a low density of (2×10^4 cells/ml) and then left untreated (**a**), or treated with 10 ng/ml of NGF (**b**), data correspond to three independent experiments

Results

Proximate analysis of *H. trimaculatus*

The proximate compositions of *H. trimaculatus* were expressed in percentage of dry weight and presented in Table 3. Crude protein contents of *H. trimaculatus* are notably high (75.75 ± 0.13 %) compared to lipid, carbohydrates, and ash contents. The analysis revealed that protein is the highest proximate content in *H. trimaculatus* suggesting that *H. trimaculatus* are an excellent source of protein which can be hydrolyzed to obtain neuroprotective peptides.

Preparation of *H. trimaculatus* enzymatic hydrolysates

PC12 cells have been extensively used as a model for studying neurites formation and differentiation. Upon NGF treatments, PC12 cells stop dividing, form neurites, and undergo differentiation. NGF treatment of PC12 cells caused striking neurite formation. As shown in Fig. 2, after treatment with 10 ng/ml NGF for 14 days, PC12 cells successfully differentiate into neurons and the neurites formation were quiet long and extensive. These differentiated PC12 cells were used in all experiments in this study.

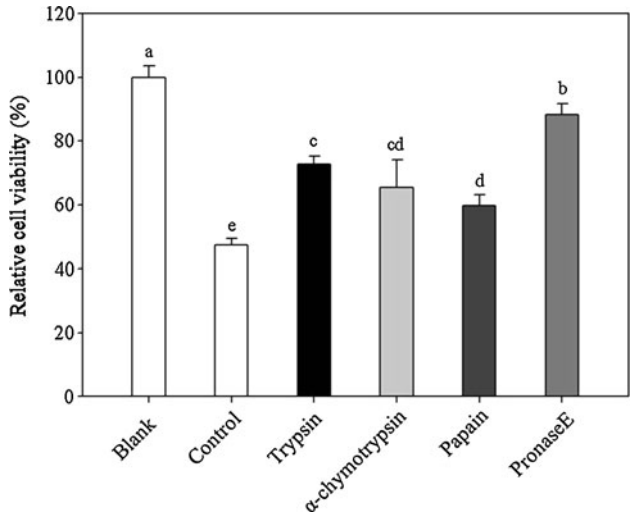

Fig. 3 Neuroprotective effects of *H. trimaculatus* enzymatic hydrolysates (100 μg/ml) against $A\beta_{42}$-induced neurotoxicity in PC12 cells. Cell viability was detected by MTT assay and quantified as percentage compared to blank. Values correspond to mean ± SD from three independent experiments. *a–e letters* in each sample are different significantly by Duncan's multiple range test ($p < 0.05$)

Suitable enzymes for marine protein hydrolysis can be selected according to various criteria, such as physicochemical properties of the resulting hydrolysates, flavor extraction, or reduction of bitterness (Chabeaud et al. 2009). In this study, *H. trimaculatus* was separately hydrolyzed using trypsin, α-chymotrypsin, papain, and pronase E enzymes. The hydrolysates were tested for their protective effect on $A\beta_{42}$-induced PC12 cell death. Exposure to $A\beta_{42}$ at a concentration of 20 μM for 24 h reduced PC12 cell viability to 47.45 ± 2.05 % (Fig. 3). Interestingly, *H. trimaculatus* enzymatic hydrolysates treatment (100 μg/ml) showed protection against neuronal death. Notably, pronase E hydrolysate exerted strongest protection with cell viability value of 88.33 ± 3.33 % ($p < 0.05$). Based on this result, pronase E hydrolysates were selected as potential enzyme to isolate and characterize neuroprotective peptides derived from *H. trimaculatus*.

H. trimaculatus hydrolysis conditions by RSM

The most important parameters affecting the enzyme efficiency are temperature, time, *E*/*S* ratio and pH. RSM were used to optimize the hydrolysis conditions for the preparation of neuroprotective peptides from *H. trimaculatus*. According to the optimal working conditions of pronase E, the studied pH and temperature ranged from 6 to 8 and 35 to 55 °C, respectively. Meanwhile, enzyme to substrate ratio ranged from 1 to 3 % and the hydrolysis carried out between 6 and 24 h and PC12 cell viability was selected as dependent variables (Table 2).

Table 4 Analysis of variance table of neuroprotective value as affected by temperature, time, *E*/*S* ratio, and pH during optimization experiments using pronase E

Responses	Sources	*df*	SS	MS	*F* value	*p* value
Cell viability	Regression					
	Linear	4	331.96	82.99	1.48	0.2379
	Quadratic	4	1,180.95	295.24	65.43	0.0001[a]
	Cross-product	6	152.37	25.40	0.39	0.8786
	Total model	14	1,665.29	403.63	67.3	
	Residual					
	Lack of fit	10	66.35	6.63	24.88	0.0012
	Pure error	5	1.33	0.27		
	Total error	5	65.02	6.36		
	Total	19	1,730.31	410.02		

Coefficient of determination (R^2) = 0.9609; adjusted R^2 = 0.9245

df degree of freedom, *SS* sum of square, *MS* mean of square

[a] Significant at 95 % confidence degree ($p < 0.05$)

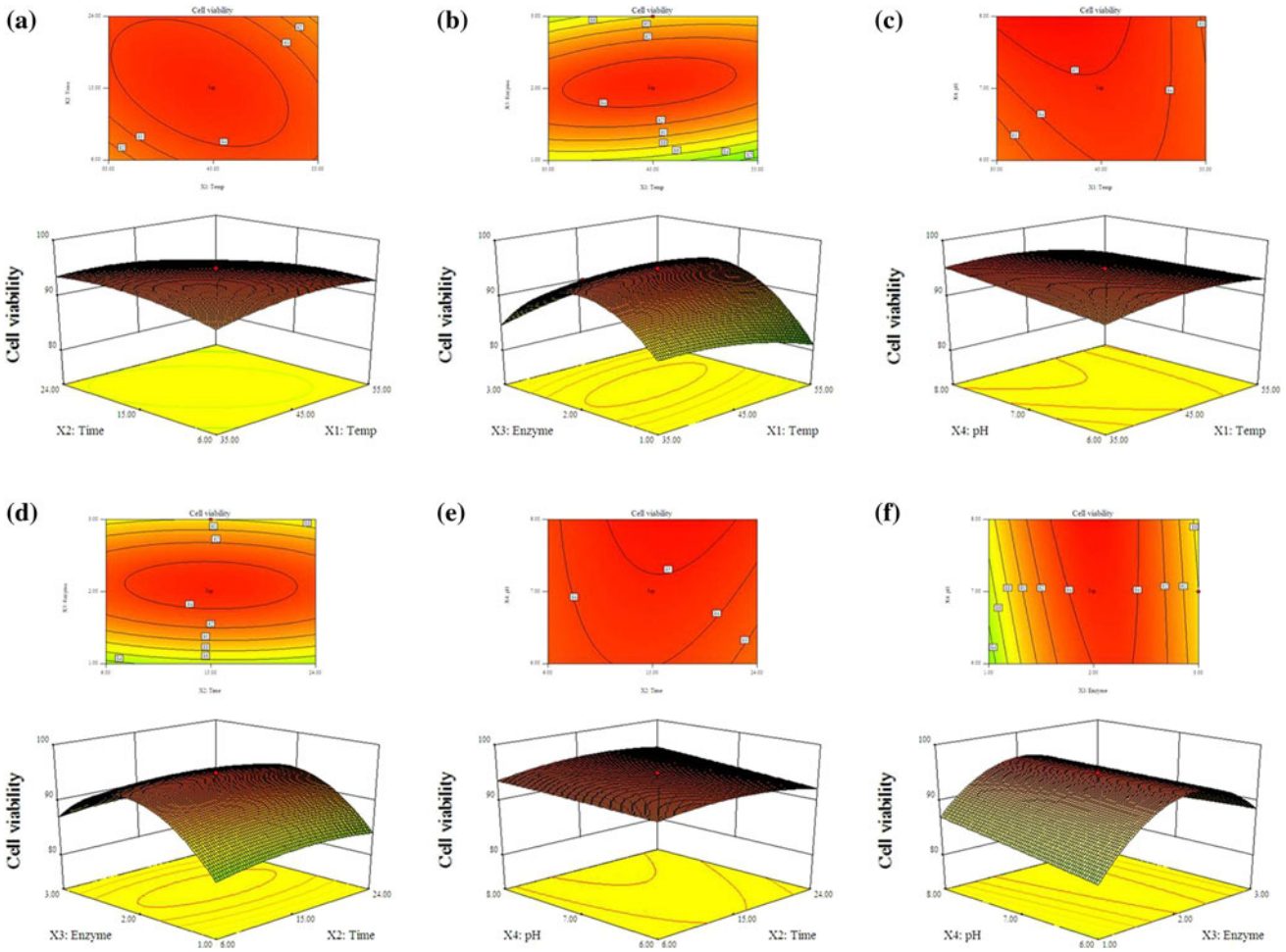

Fig. 4 Response surface contour plot and response surface plot for the effects of variables on neuroprotective activity against $A\beta_{42}$-induced neurotoxicity in PC12 cells: temperature and time (**a**), temperature and *E*/*S* ratio (**b**), temperature and pH (**c**), time and *E*/*S* ratio (**d**), time and pH (**e**), *E*/*S* ratio and pH (**f**)

Analysis of variance (ANOVA) analysis was performed to analyze the response function of the experimental data. The coefficient and *p* value of linear polynomials (X_1, X_2, X_3), quadratic polynomial ($X_1 X_1$, $X_2 X_2$, $X_3 X_3$), cross polynomial ($X_1 X_2$, $X_1 X_3$, $X_2 X_3$) and constant are presented in (Table 4). The ANOVA result showed that *E*/*S* ratio (X_3) had relatively more significant effect compared to other factors ($p < 0.05$). In addition, in terms of second-order polynomial, X_3^2 was the most significant effect. The statistical analysis revealed that the model was significant ($p < 0.05$) with a satisfactory value

of determination coefficient ($R^2 = 0.9609$), indicating that 96.02 % of the variability in the response could be explained in the following equation:

$$Y = 35.44X_3 + 0.22X_1 \cdot X_3 + 8.24X_3^2 - 12.99 \quad (2)$$

The interaction of the four parameters and their optimal levels affecting PC12 cell viability were further analyzed by RSM. A response surface quadratic model was drawn and the statistical analysis for the linear, the quadratic and the interaction of four variables was demonstrated. The contour plots and three dimension graphs were presented in Fig. 4. The p value for model was <0.0001 suggesting that the model was significant and could be used to monitor the optimization. Among the four variables, *E*/*S* ratio (X_3) exerted highest significant effect on protective effect against $A\beta_{42}$-induced neurotoxicity in PC12 cells within 99 % confidence interval ($p = 0.0074 < 0.01$). As shown in Fig. 4, the cell viability increased until *E*/*S* ratio (X_3) reached an optimum point and then decreased following the increase ratio. In terms of temperature (X_1), both low (35 °C) to high (55 °C) showed almost similar neuroprotective activity. Meanwhile, time (X_2) and pH (X_4) both did not show any significant effects.

Optimization and validation of hydrolysis conditions at pilot scale

In order to obtain the maximum neuroprotective activity of the hydrolysates, the model was performed using RSM auto-analysis software by setting PC12 cell viability value (Y) as the dependent variable. According to the RSM auto-analysis, PC12 cell viability of 94.61 ± 2.12 % was obtained by treatment with 100 μg/ml of *H. trimaculatus* hydrolysates which were hydrolyzed by the following conditions: $X_1 = 36.69$ °C; $X_2 = 20.01$ h; $X_3 = 2.02$ %, and $X_4 = 7.34$. To confirm the model validity, three independent assays were performed under optimal conditions given above. As shown in Fig. 5, the experimental PC12 cell viability value was 92.46 ± 4.59 % by the treatment with 100 μg/ml *H. trimaculatus* hydrolysates. The experimental cell viability value was in agreement with the value predicted by the model with 95 % confidence interval. The above result confirmed that the model was suitable for the estimation of experimental value.

In addition, *H. trimaculatus* hydrolysates also increase the expression of pro-survival (Bcl-2) gene expression as analyzed by RT-PCR. Thus, expression of Bcl-2 may account for the increased cell viability observed on PC12 cell by *H. trimaculatus* hydrolysates treatment. Hence, *H. trimaculatus* hydrolysates could be used to purify neuroprotective peptide for further experiments.

Preparation and identification of neuroprotective peptide from *H. trimaculatus* hydrolysates

The lyophilized *H. trimaculatus* hydrolysates were dissolved in 20-mM sodium acetate buffer (pH 4) and loaded into a Hiprep 16/10 DEAE FF anion exchange column

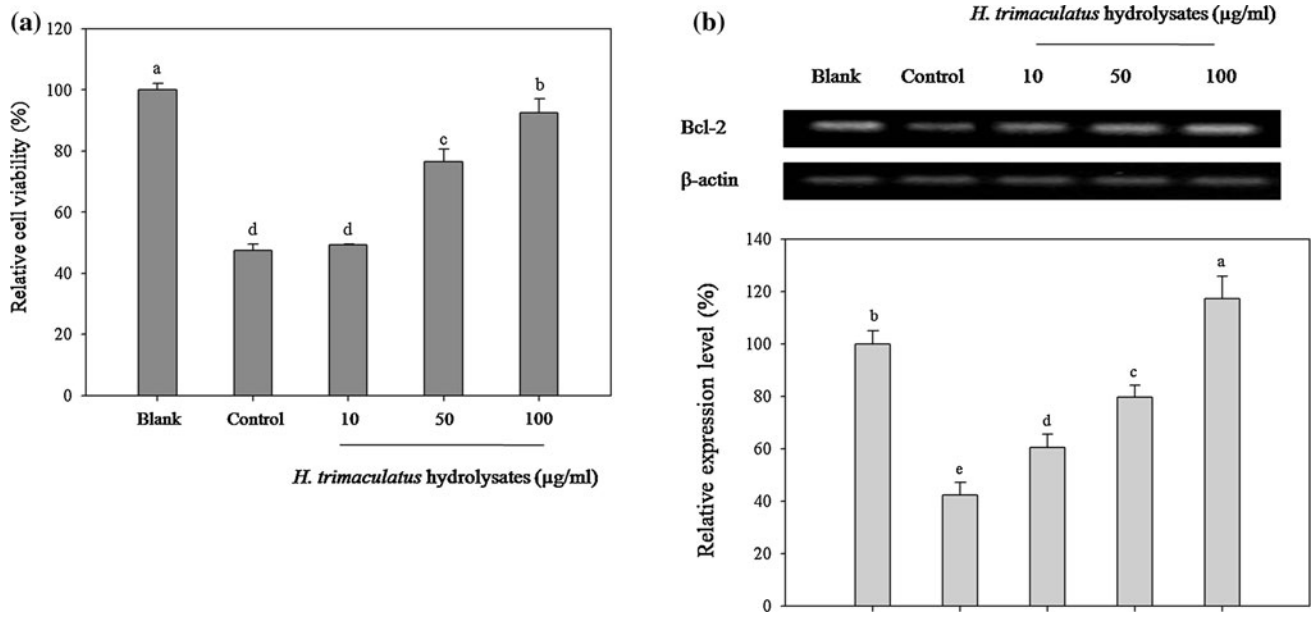

Fig. 5 Neuroprotective effect of *H. trimaculatus* enzymatic hydrolysates on $A\beta_{42}$-induced PC12 cell death. Cell viability was detected by MTT assay and quantified as percentage compared to non-treated group (**a**), In parallel experiments, the expressions of Bcl-2 mRNA were measured by RT-PCR (**b**), β-actin was used as an internal control for PCR analysis. Values correspond to mean ± SD from three independent experiments. *a–e* letter in each sample are different significantly by Duncan's multiple range test ($p < 0.05$)

using a linear gradient of NaCl (0–2 M). Elution peak was monitored at 280 nm and each fraction was collected every 4 ml. As shown in Fig. 6, the *H. trimaculatus* hydrolysates spectrum was separated into five fractions. Each fraction was pooled, lyophilized, and its activity was measured by MTT and PCR assay. Among the five fractions tested, fraction V (Fr. V) possess the highest protection against $A\beta_{42}$-induced cell death with the cell viability value of 85.52 ± 2.22 %, respectively (Fig. 6b). Furthermore, Fr. V also showed relatively strong Bcl-2 mRNA expressions in PC12 cells. Therefore, Fr. V was selected as active fraction for further purifications by HPLC.

Ramp gradient of acetonitrile (0–30 %) was chosen for Fr. V purification. HPLC separated Fr. V into five fractions (Fr. V-1 and Fr. V-5) (Fig. 7a). Compared to the other fractions, Fr. V-2 showed highest protective effect against $A\beta_{42}$-induced neurotoxicity in PC12 cells with the value of 79.43 ± 3.59 %. Hence, Fr. V-2 was further purified by HPLC under 0–20 % gradient of acetonitrile (Fig. 8). In the next step, the target peptides (Fr. V-2) were pooled and further purified with isocratic gradient of acetonitrile (15 %) containing 0.1 % TFA (Fig. 8b). Following several purification steps, *H. trimaculatus*–derived neuroprotective peptides (HTP-1) sequence was identified as Gly-Thr-Glu-Asp-Glu-Leu-Asp-Lys (906.4 Da, +25.39 kcal mol^{-1} hydrophobicity).

Discussion

Seahorses are believed to have many medicinal and health benefits for human, including anti-aging, anti-fatigue, appetite enhancement, healing of pregnant woman, strengthening the kidneys, and neuroprotective effects (Li et al. 2008). Despite seahorse has long been used as traditional medicine, there were few reports about seahorse in terms of isolation, characterization, and health benefit effect of its water-soluble ingredients, such as bioactive

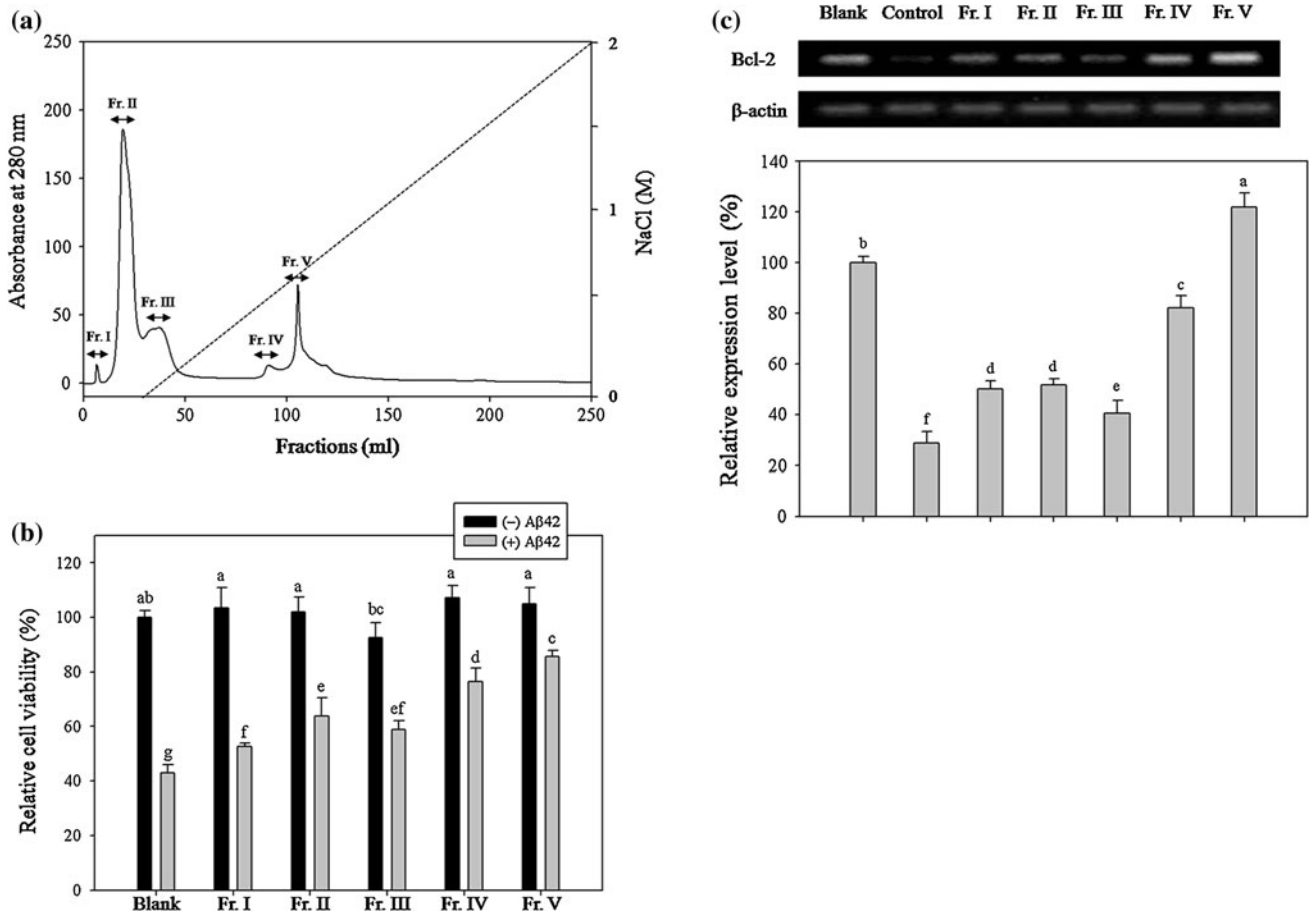

Fig. 6 Separation of neuroprotective peptide of *H. trimaculatus* enzymatic hydrolysates by Hiprep 16/10 DEAE FF anion exchange chromatography. Elution was carried out with a linear gradient of NaCl (0–2 M) at a flow rate of 1 ml/min (**a**). Neuroprotective effect of *H. trimaculatus* fractions on $A\beta_{42}$-induced PC12 cell death. Cell viability was detected by MTT assay. Viability of cells was quantified as a percentage compared to non-treated group (**b**). In parallel experiments, the expressions of Bcl-2 mRNA were measured by RT-PCR (**c**). β-Actin was used as an internal control for PCR analysis. Values correspond to mean ± SD from three independent experiments. *a–g letter* in each sample are different significantly by Duncan's multiple range test ($p < 0.05$)

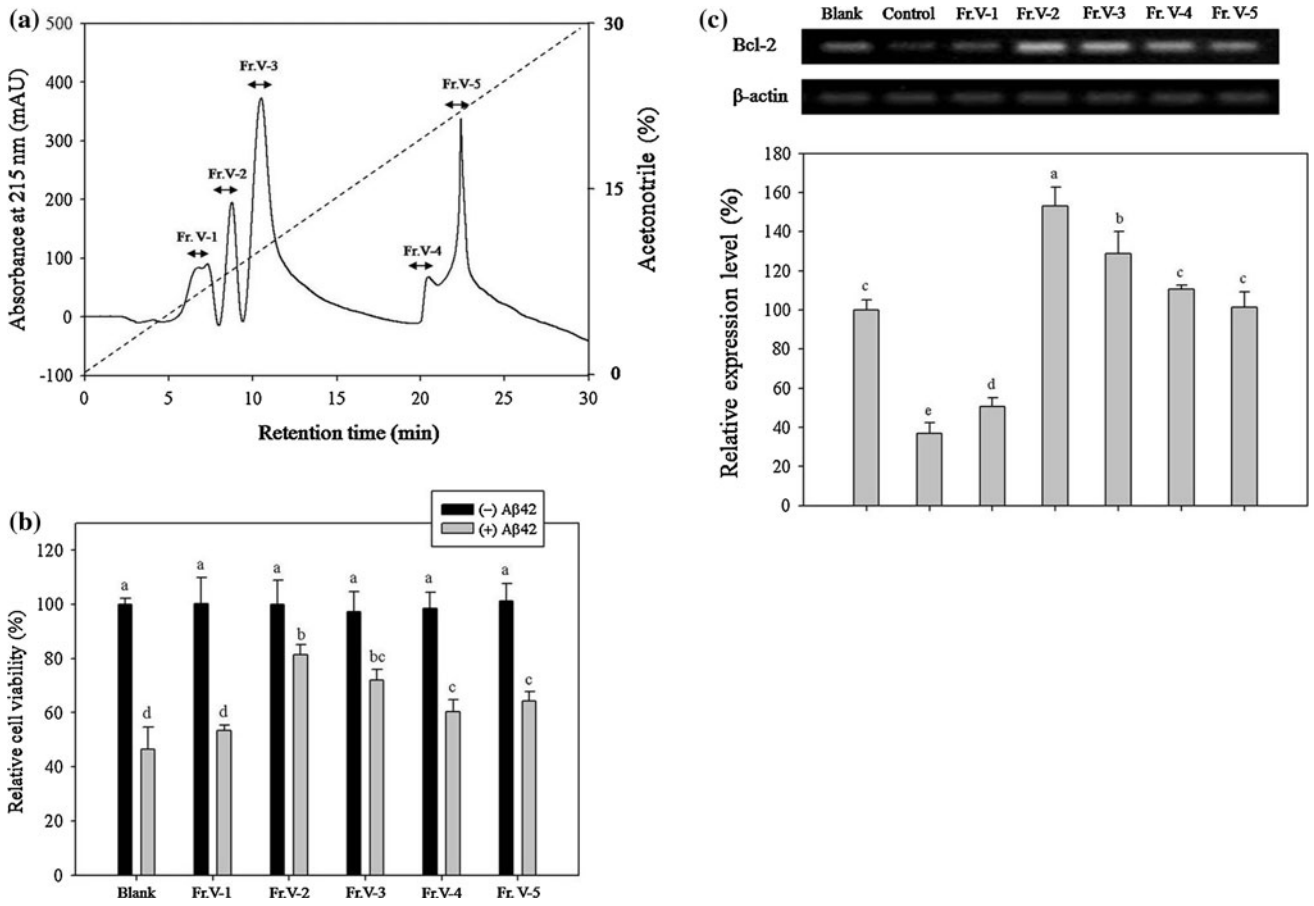

Fig. 7 Separation of neuroprotective peptide of *H. trimaculatus* by RP-HPLC. RP-HPLC pattern of fraction V-1 to V-5 of *H. trimaculatus* enzymatic hydrolysates with 0–20 % acetonitrile as the mobile phase at 1 ml/min flow rate, using UV detector at 215 nm (**a**). Cell viability was detected by MTT assay and quantified as a percentage compared to non-treated group (**b**). In parallel experiments, the expressions of Bcl-2 mRNA were measured by RT-PCR (**c**). β-Actin was used as an internal control for PCR analysis. Values correspond to mean ± SD from three independent experiments. *a–g* letter in each sample are different significantly by Duncan's multiple range test ($p < 0.05$)

peptides. In the present study, using protease, neuroprotective peptides were isolated, optimized, and characterized. To the best of our knowledge, this is the first report about optimization, isolation, and identification of neuroprotective peptides derived from *H. trimaculatus*.

The proximate compositions of *H. trimaculatus* are characterized by high crude protein contents (75.75 % of dry weight). Lin et al. (2008) reported that crude protein levels of six seahorse species (*H. kuda*, *H. trimaculatus*, *H. kellogi*, *H. spinosissims*, *H. histrix*, *H. comes*) were high, ranging from 68.10 to 78.30 %. The nutritional composition of fish is strongly affected by the composition of their food compositions (Lourie et al. 1999). In marine environment, the diets of adult seahorses consist mostly of copepods, small shrimp, and amphipods (Payne and Rippingale 2000). In addition to food consumptions, other factor such as species, genetics, size, reproductive status, as well as the environmental characteristics can also influence the proximate compositions of seahorses (Lin et al. 2009).

In the present study, enzymatic method was used to hydrolyze bioactive peptides from *H. trimaculatus*. Enzyme utilization to produce fish protein hydrolysates has at least one common characteristic: they should be food grade and if they are microbial origin, the producing organisms should be non-pathogenic. The choice of substrate, protease employed and degree to which the protein gets hydrolyzed generally affects the physiochemical properties of the resulting hydrolysates (Mullally et al. 1995). Considering those reasons, *H. trimaculatus* were hydrolyzed with four different enzymes (trypsin, α-chymotrypsin, papain, and pronase E). The highest neuroprotective activity was obtained from pronase E hydrolysates. Pronase E—a protease from *Streptomyces griseus*; Actinase E—displays a wide range of substrate specificity. Pronase E is an enzyme mixture of at least three proteolytic activities (carboxypeptidases, aminopeptidases,s and endopeptidases) including an extracellular serine protease (Ryu et al. 2010). Pronase E has been proven to be one of

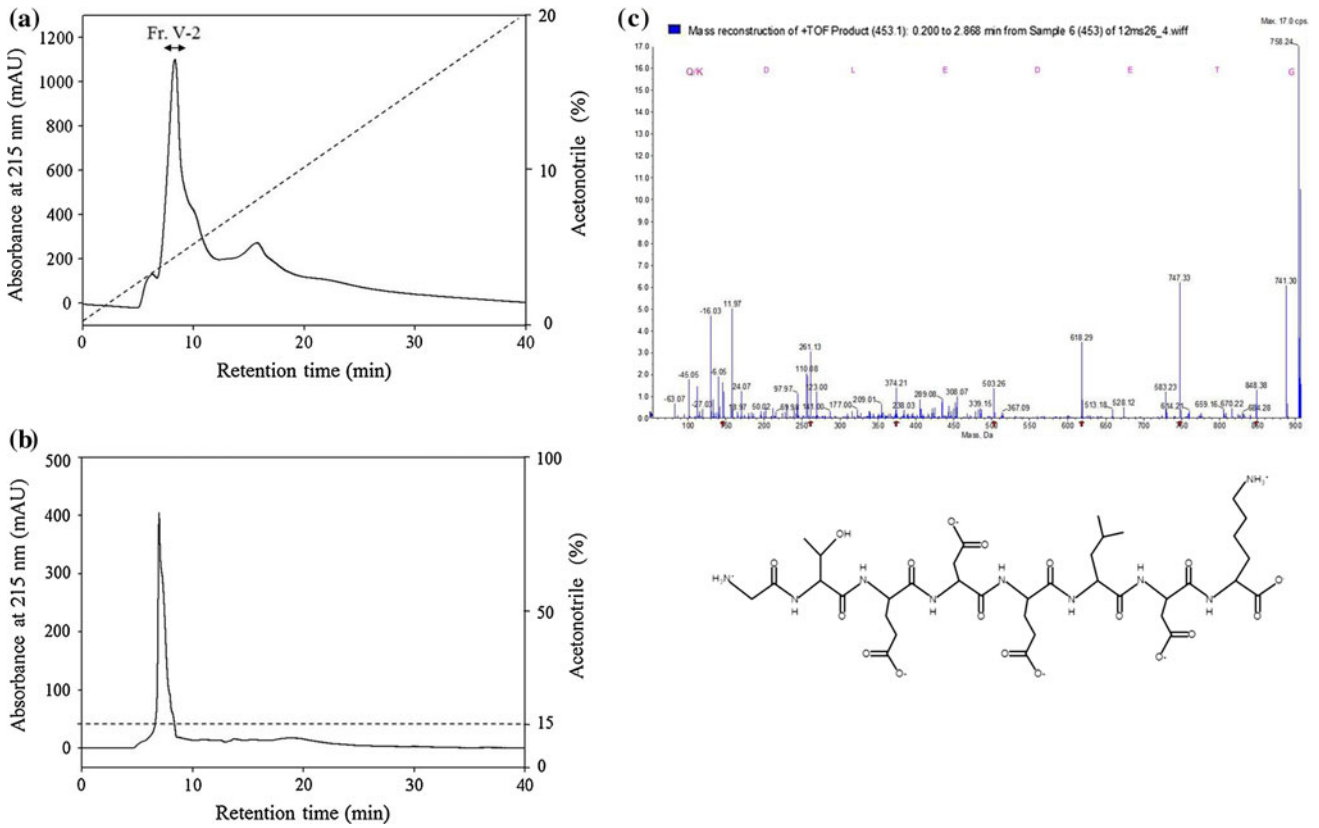

Fig. 8 Chromatogram of RP-HPLC of fraction V-2 of *H. trimaculatus* enzymatic hydrolysates. Elution was performed with the linear gradient of acetonitrile (0–20 %) as the mobile phase at 1 ml/min flow rate, using UV detector at 215 nm (**a**). Further separation of fraction V-2 of *H. trimaculatus* enzymatic hydrolysates. Elution was performed with the isocratic gradient of acetonitrile (15 %) as the mobile phase at 1 ml/min flow rate, using UV detector at 215 nm (**b**). Molecular mass spectra and amino acid sequence of *H. trimaculatus* peptide-1 (HTP-1). MS/MS experiments were performed on a Q-TDF tandem mass spectrometer (Micromass Co., Mancester, UK) equipped with a nano-ESI source. Sequencing of active peptide was done using the PepSeq de novo sequencing algorithm (**c**)

the best enzymes used in the preparation of fish protein hydrolysates by many researchers (Ryu et al. 2010; Kim et al. 2001b; Kim et al. 2001a; Je et al. 2007). From technical and economical point of view, microbial enzymes operating at alkaline pH have been reported to be most efficient in the hydrolysis of fish proteins (Bhaskar et al. 2008). Moreover, fish protein hydrolysates prepared using pronase E had less bitter than the ficin or bromelain hydrolysates (Hevia and Olcott 1977).

Response surface methodology was used to optimize the hydrolysis condition for the preparation of neuroprotective peptides from *H. trimaculatus*. From the RSM result, it may assume that *E*/*S* ratio (X_3) had relatively more significant effect compared to other factors. The results could be due to greater hydrolysis of protein when more protease was added. It may assume that a relatively sufficient amount of *E*/*S* ratio is desirable to promote the neuroprotective effect of the hydrolysates. These results were in agreement with Ren et al. (2008) who found that high enzyme concentration led to an increase in the antioxidant activity by the hydrolysates of grass carp sarcoplasmic protein. It has also been reported that high *E*/*S* ratio led to an increase in the inhibition of the ACE activity of bioactive peptides (Cornelly et al. 2002).

Several studies indicated that the ability of peptide to show neuroprotective activity is closely related to their structure. The structural peptide characteristics required to prevent neuronal death were previously described. First, the neuroprotective property of peptides depends to its composition, structure, and degree hydrophobicity. Increased hydrophobicity of the peptide enhances the antioxidant activity of a peptide, as it allows the peptide to reach hydrophobic targets like cell membranes (Hsu 2010). Furthermore, the peptide should include polarized amino acids at one or both ends to increase its solubility; and the peptide should be between 3 and 15 amino acids long with a molecular weight below 1 kDa (Ashur-Fabian et al. 2003). HTP-1 fulfills the neuroprotective peptides conditions. The structure of HTP-1 is mainly composed of hydrophobic amino acid with relatively high degree of hydrophobicity (+25.39 kcal/mol) and molecular weight below 1 kDa. Peptides with molecular weight 500–1500 Da showed more

potent biological activities compared to peptides above 1500 Da and below 500 Da. With its small molecular size, neuroprotective peptides may have the potential to cross the compromised blood brain barrier in the central nervous system (CNS) (Guan et al. 2004). In addition, N-terminus sequence of HTP-1 consists of three amino acids which is GTE. Interestingly, the N-terminus sequence of HTP-1 is almost similar to insulin-growth factor-1 (IGF-1), a naturally occurring peptide in the CNS. Guan et al. (2004) reported that N-terminal of IGF-1, G-proline (P)-E is found to be neuroprotective. GPE has been demonstrated to stimulate dopamine and acetylcholine release in vitro. Furthermore, GPE can protect neurons when given intracerebroventricularly 2 h after hypoxic–ischemic brain injury in rats (Guan et al. 2004). Neuroprotective activity of GPE at N-terminus included the prevention of neuronal apoptosis, promotion of astrocyte survival and inhibition of microglial proliferation. Thus, structure similarity between HTP-1 and IGF-1 may partially explaine neuroprotective mechanisms of HTP-1 against $A\beta_{42}$-induced neurotoxicity. Furthermore, the cellular mechanisms behind neuroprotective effect of HTP-1 on $A\beta_{42}$-induced neurotoxicity in PC12 cells can be explained by investigating anti-apoptotic gene expressions. In AD, neuronal degeneration may be caused by $A\beta_{42}$-mediated down-regulation of Bcl-2 rendering the neurons vulnerable to age-dependent secondary insults (Mattson and Magnus 2006). Treatment with HTP-1 showed positive effect on Bcl-2 gene expression, suggesting that HTP-1 neuroprotective effect on $A\beta_{42}$-induced neurotoxicity in PC12 cells was mediated through induction of anti-apoptotic gene expression.

Bioactive peptides provide a conceptual breakthrough for future identification of small molecules that mimic the activity of large protein factors. They provide a new horizon for innovative drugs to protect the compromised brain using non-invasive intranasal administration (Gozes 2001). Moreover, bioactive peptides can be synthesized by specialized techniques, known as peptide synthesis. Synthesis of neuroprotective peptides derived from seahorses may provide an alternative to the overexploitation of seahorses as traditional medicines. The result of this study has the potential to replace or at least complement the use of seahorse to treat neurodegenerative diseases which may minimize seahorses (*Hippocampus* sp.) exploitation as traditional medicine.

Conclusion

The findings in this study suggest that HTP-1 exhibits potent neuroprotective activity. HTP-1 may ultimately prove to be useful in treatment of neurodegenerative diseases, particularly in AD. However, further study such as effects other neuronal cells (i.e., glial cells), molecular mechanisms and in vivo tests should be explored. Considering that traditional medicine contributed as the largest consumption of seahorses; identification, characterization, and synthesis of bioactive materials derived from seahorse have the potential to replace or at least complement the use of seahorse to treat neurodegenerative diseases. Thus, may help to may minimize seahorses (*Hippocampus* sp.) exploitation as traditional medicine.

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Conflict of interest The authors declare that there are no conflicts of interest.

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