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Protective effect of a 3 kDa peptide obtained from beef myofibrillar protein using alkaline-AK on neuronal cells

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ABSTRACT

The protective effect of two 3 kDa peptide fractions (AK3KF1 and AK3KF2), obtained from beef myofibrillar protein using an inexpensive enzyme (alkaline-AK) on human neuronal cells (SH-SY5Y) against H_2O_2-induced apoptosis was investigated. These peptides were isolated and further separated by fast protein liquid chromatography (FPLC), and their protective effect against H_2O_2-mediated cell death was measured by determining cell viability, nitric oxide (NO) production, mitochondrial membrane potential (MMP), apoptosis, morphological changes in cell nuclei, and *in vitro* antioxidant assays. The results indicated that treatment with peptide fractions increased cell viability and MMP, and decreased NO production, fragmentation of cell nuclei, and apoptosis in H_2O_2-treated SH-SY5Y cells. This is the first study to report neuroprotective effects of a peptide obtained from beef myofibrillar protein. The peptide sequence was identified as Thr-Gln-Lys-Lys-Val-Ile-Phe-Cys (TQKKVIFC). Thus, these findings suggest that TQKKVIFC can prevent neuronal cell death and could be useful in preventing neurodegenerative diseases.

1. Introduction

With the increase in the aging population, the prevalence of a number of aging neurodegenerative diseases, such as Alzheimer's disease (AD), and Parkinson's disease (PD) is on the rise (Uttara et al., 2009). The main symptoms of these neurodegenerative diseases include neurocognitive decline and memory impairment, which arise due to the cholinergic system and mitochondrial dysfunction, and oxidative stress (Haider et al., 2014; Szeto, 2006). Reactive oxygen species (ROS), which include hydroxyl radicals, hydrogen peroxide (H_2O_2), and superoxide anions ($O_2^{-}\bullet$) are known to contribute to human diseases (Kim et al., 2015). It is reported that NO production leads to apoptosis in neuronal cells which results in neurodegenerative disorders, such as AD, and PD (Hu and Zhu, 2014; Panthi et al., 2018). Additionally, oxidative damage in cells is promoted by nitric oxide (NO) and a three step chain reaction, involving the continuous conversion between hydroxyl radicals, H_2O_2, superoxide radicals ($^{\cdot}O_2^{-}$), and oxygen (O_2) (Šimić et al., 2000; Turrens, 2003). Previous studies have demonstrated that oxidative stress plays a key role in the pathogenesis of neurodegenerative diseases (Liu et al., 2017; Reynolds et al., 2007). For this reason, several potential therapeutic targets aiming to prevent or treat neurodegenerative disorders have been studied in several *in vitro* and *in vivo* studies (Bhullar and Rupasinghe, 2013; Kornberg et al., 2010; Yoshida et al., 1994). The mechanisms of action of neuroprotective agents include inhibition of cell degeneration, inflammation, and production of NO, scavenging of free radicals, and blocking of the pathways leading to apoptosis (de los Rios et al., 2018; Levi and Brimble, 2004). However, these neuroprotective agents have occasionally been reported to induce side effects, such as increased bleeding, mental disorders, and inflammatory reactions due to intravenous injections (De Keyser et al., 1999; Jain, 2011).

Various natural peptides derived from many food protein hydrolysates exhibit several beneficial activities, such as antioxidant, neuroprotective or memory impairment, anti-hypertensive, anti-inflammatory, and antimicrobial activities (Katayama and Nakamura, 2019; Lee and Hur, 2017; Min et al., 2017; Taha et al., 2017). Among several other beneficial health effects, bioactive peptides are also known to exhibit protective effects against neuronal cell death in neurotoxicity-induced mouse models, thereby suggesting that these peptides can be developed as effective neuroprotective agents (Feng et al., 2018; Lu et al., 2010). Although use for peptide as neuroprotective agent is limited in terms of penetration of pharmacological peptide through blood brain barrier (BBB), several studies have demonstrated a close association between the prevention of neurodegenerative diseases and antioxidant activity (Gelain et al., 2012; Jin et al., 2013).

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Abbreviations

FPLC	fast protein liquid chromatography
MMP	mitochondrial membrane potential
NO	nitric oxide
AD	Alzheimer's disease
PD	Parkinson's disease
ROS	reactive oxygen species
H_2O_2	hydrogen peroxide
MEM	minimal essential media
FBS	fetal bovine serum
$NaNO_2$	sodium nitrite
JC-1	tetraethylbenzimidazolylcarbocyanine iodide
Aβ	β-amyloid
NMDA	N-methyl-D-aspartate

However, the protective effect of peptides with antioxidant activity, derived from beef myofibrillar protein using inexpensive enzymes, such as alkaline-AK, on neuronal cells have not been reported extensively. Moreover, the mechanism by which these peptides exert neuroprotective effects against oxidative stress-induced neuronal cells is not known. Therefore, the aims of this study were to 1) determine the antioxidant potential of peptides with a molecular weight of less than 3 kDa, obtained using an inexpensive enzyme, such as alkaline-AK, by performing *in vitro* radical scavenging assays, 2) to evaluate their neuroprotective effects against H_2O_2-induced apoptosis in human neuronal SH-SY5Y cells, and 3) and to identify the peptide sequences.

2. Materials and methods

2.1. Materials

All chemicals and reagents were of analytical grade. Annexin V-FITC/PI cell apoptosis detection kit was purchased from TransGen Biotech (Beijing, China). Propidium iodide (PI), trypan blue, XTT sodium salt (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342 solution was procured from BD Biosciences, USA. JC-1 mitochondrial membrane potential assay kit was purchased from Abcam (Cambridge, UK). Minimal essential media (MEM) was purchased from Welgene Inc. (Daegu, Korea). Fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies (Carlsbad, CA, USA). All other reagents used in this study were of the highest grade available commercially.

2.2. Preparation of peptide fractions using inexpensive enzymes

Hydrolysates obtained from beef myofibrillar protein were processed according to the method described previously (Lee and Hur, 2017). Briefly, Alkaline-AK, a protease Alkaline-AK (180–200 KU/g solid) obtained by fermenting soybean meals with *Bacillus methylotrophicus*, was used as an enzyme to obtain hydrolysate. The myofibrillar protein was hydrolyzed by using 0.2% Alkaline-AK (w/w) (pH 11) at 60 °C for 8 h. The hydrolysate was then heated at 90 °C for 15 min to inactivate the endogenous enzymes. Hydrolysates were subjected to ultrafiltration using Amicon® Ultra (Millipore, Billerica, MA, USA) and membranes with a molecular mass cut-off of < 3 kDa were used to exclude all the novel peptides having molecular weight of more than 3 kDa. Novel peptides thus obtained by using AK3K were lyophilized at −80 °C for 3 days and stored at −20 °C. These novel peptides were purified by using FPLC on a GPC column (HiPrep 26/60 Sephacryl S-100 HR). The mobile phase was a 0.15 M NaCl buffer containing 0.05 M Tris-HCl buffer (pH 6.8) with a flow rate of 2.0 ml/min. Absorbance of eluted fractions was detected at 280 nm, and the collected fractions were lyophilized at −80 °C for 3 days. Fraction F1 of AK3K (AK3KF1), and F2 of AK3K (AK3KF2) were analyzed for their antioxidant potential and also their neuroprotective effect against H_2O_2-treated human neuronal cells.

2.3. Antioxidant activities of peptide fractions

2.3.1. ABTS radical scavenging activity

The ABTS radical scavenging activity of AK3KF1 and AK3KF2 was determined using a previously described method (Arnao et al., 2001). The ABTS radicals were produced by reacting 7 mM of ABTS with 2.45 mM of potassium persulfate, and stored at room temperature in dark for 12 h. The $ABTS^{\bullet+}$ solution was diluted with PBS (pH 7.4) to obtain absorbance of 0.70 ± 0.02 at 734 nm. Five microliter of 1 mg/ml solution of AK3KF1 and AK3KF2 was added to 445 µl of $ABTS^{\bullet+}$ solution and mixed thoroughly for 10 min. The $ABTS^{\bullet+}$ scavenging activity was determined spectrophotometrically at 734 nm. The extraction solution instead of the peptide fractions was used as control. The ABTS radical scavenging activity was calculated as:

$$ABTS^{\bullet+} \text{ scavenging activity } (\%) = [1 - (A_{sample} / A_{control})) \times 100$$

Where A_{sample} and $A_{control}$ were the absorbance of the sample and control, respectively.

2.3.2. DPPH radical scavenging activity

The DPPH radical scavenging activity of AK3KF1 and AK3KF2 was measured using a previously described colorimetric method (Blois, 1958). Fifty microliter of 1 mg/ml solution of AK3KF1 and AK3KF2 was mixed with 50 µl of DPPH reagent in methanol. The reaction mixture was incubated in dark for 25 min, and DPPH scavenging activity was measured at 517 nm on Sunrise™ microplate reader (Tecan, Männedorf, Switzerland). Distilled water was used as a control. The DPPH radical scavenging activity was determined as follows:

$$DPPH^{\bullet} \text{ radical scavenging activity } (\%) = [1 - (A_{sample} / A_{control})) \times 100$$

Where A_{sample} and $A_{control}$ were the absorbance of the sample and control, respectively.

2.3.3. Iron chelating

The iron chelating ability of AK3KF1 and AK3KF2 was assessed by using a previously reported method (Decker and Welch, 1990) with slight modifications. Briefly, for the chelation test, 250 µl of 1 mg/ml solution of AK3KF1 and AK3KF2 was mixed with 50 µl of 600 µM $FeCl_2$ and 450 µl of methanol, and vortexed for 1 min. This mixture was allowed to react for 10 min. Subsequently, 50 µl of 5 mM Ferrozine solution was added to the sample and allowed to react for 10 min. The resulting sample was assessed at 562 nm on Sunrise™ microplate reader (Tecan, Männedorf, Switzerland). 0.25 ml of distilled water, 50 µl $FeCl_2$, and 50 µl of 5 mM ferrozine solution was used as control. The iron chelating activity was calculated as follows:

$$\text{Chelating activity } (\%) = [1 - (A_{sample} / A_{control})) \times 100$$

Where A_{sample} and $A_{control}$ were the absorbance of the sample and control, respectively.

2.3.4. Nitrite scavenging activity

Nitrite scavenging activity of AK3KF1 and AK3KF2 was determined by using a previously described method (Kato et al., 1987). Briefly, 0.5 ml of 1 mg/ml solution of AK3KF1 and AK3KF2 were mixed with

0.25 ml of 2 mM sodium nitrite ($NaNO_2$), and 100 mM HCl buffer (adjusted to pH 2.0) and the final volume was made up to 2.5 ml. After incubation at 37 °C for 1 h, 50 μl of this solution was mixed with 0.25 ml of distilled water and 50 μl of Griess reagent and incubated for 20 min. The absorbance was read at 540 nm and nitrite scavenging activity was calculated as follows:

$$\text{Nitrite scavenging activity (\%)} = [1 - (A_{sample} / A_{control})) \times 100$$

Where A_{sample} and $A_{control}$ were the absorbance of the sample and control, respectively.

2.3.5. Reducing power

The reducing power of AK3KF1 and AK3KF2 was determined using a previously reported method (Oyaizu, 1988). Briefly, 125 μl of 1 mg/ml solution of AK3KF1 and AK3KF2 was mixed with 0.2 M PBS (pH 6.6) and potassium ferricyanide (10 mg/ml) (1:1) and incubated at 50 °C for 20 min in dark. After incubation, 125 μl of 10% trichloroacetic acid (TCA) was added to the mixture, and centrifuged at 1000 × *g* for 10 min. Subsequently, 0.1 ml of supernatant was mixed with 0.1 ml of distilled water and 0.1% ferric chloride, and the absorbance was read at 700 nm.

2.4. Neuroprotective effect of peptide fractions on human neuronal SH-SY5Y cells

2.4.1. Cell viability

The effect of peptide fractions on cell viability was assessed using the XTT assay. In our preliminary study, the higher concentration than these concentrations (0.25-0.5 mg/ml) showed cell cytotoxicity on SH-SY5Y cell (data not shown). Thus, we used the concentrations (0.25-0.5 mg/mL) that did not influence on SH-SY5Y cell. Briefly, human neuronal SH-SY5Y cells were seeded in 96-well plates at a density of 0.5×10^6 cells/well in MEM culture medium containing 10% FBS, 1% penicillin/streptomycin, and 25 mM HEPES, and incubated at 37 °C in a humidified incubator with 5% CO_2. After 24 h of incubation, cells were treated with 0.25 and 0.5 mg/ml AK3KF1 and AK3KF2, respectively, for 2 h, and then incubation with 0.2 mM H_2O_2 for 60 min. After incubation, 0.25 ml of XTT reagent was added to each well of the 96-well plate, and the samples were incubated for 2 h at 37 °C. The absorbance was read at 450 nm using Sunrise™ microplate reader (Tecan, Männedorf, Switzerland).

2.4.2. Nitric oxide (NO) production

Production of NO was assessed by measuring the levels of accumulated nitrite using Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamide in 5% phosphoric acid). Briefly, human neuronal SH-SY5Y cells were seeded in 96-well plates at a density of 0.1×10^6 cells/well in MEM culture medium containing 10% FBS, 1% penicillin/streptomycin, and 25 mM HEPES, and then incubated at 37 °C in a humidified incubator with 5% CO_2. After 24 h of incubation, cells were treated with 0.25, and 0.5 mg/ml concentration of AK3KF1 and AK3KF2 for 2 h, followed by incubation with 0.2 mM H_2O_2 for 60 min. After incubation, 0.1 ml of culture medium was collected in a fresh 96-well plate and equal volume of Griess reagent was added. Samples were incubated for 10 min at room temperature, and the absorbance was read at 540 nm using a microplate reader.

2.4.3. Hoechst 33342 staining

The morphology of apoptotic nuclei was assessed by using Hoechst 33342 staining. Cells were seeded in 24-well plates at a density of 0.5×10^6 cells/well. After incubation, cells were treated with 0.25 and 0.5 mg/ml concentration of AK3KF1 and AK3KF2 for 2 h, followed by incubation with 200 μM H_2O_2 for 1 h. After treatment, 0.1 ml of 0.5 μg/ml Hoechst 33342 solution was added to each well for 15 min. Changes in the morphology of apoptotic nuclei were observed with a fluorescent microscope in a visual field (× 400 magnification).

2.4.4. Mitochondrial membrane potential (MMP, $\Delta\psi_m$)

The effect of peptide fractions on the mitochondrial function was assessed by performing the JC-1 assay. JC-1 (tetraethylbenzimidazolylcarbocyanine iodide), which is known as a dual membrane potential sensitive probe, was used to detect MMP (Cao et al., 2010). Briefly, human neuronal SH-SY5Y cells were placed in 96-well plates at a density of 0.1×10^6 cells/well in MEM culture medium containing 10% FBS, 1% penicillin/streptomycin, and 25 mM HEPES, and then incubated at 37 °C in a humidified incubator with 5% CO_2. After 24 h of incubation, cells were treated with 0.25 and 0.5 mg/ml AK3KF1 and AK3KF2, respectively, for 2 h, followed by incubation with 0.2 mM H_2O_2 for 60 min. Cells were then washed with 1× dilution buffer, and incubated with 0.02 mM of JC-1 solution prepared in 1× dilution buffer, for 10 min at 37 °C. Stained cells were then washed with 1× dilution buffer twice. The green fluorescence of JC-1was measured at an excitation and emission wavelength of 475 and 530 nm, respectively, and the red fluorescence was measured at an excitation and emission wavelength of 530 and 590 nm, respectively, using SpectraMax® M2 microplate reader. The ratio of red (JC-1 aggregates)/green (JC-1 monomers) fluorescent intensities were calculated by JC-1 staining assay.

2.4.5. Detection of apoptosis using Annexin V-PI staining

Human neuronal SH-SY5Y cells were plated in 6-well plates at a density of 2×10^6 cells/ml and treated with 0.25 and 0.5 mg/ml AK3KF1 and AK3KF2, respectively, for 2 h followed by incubation with 0.2 mM H_2O_2 for 60 min. The cells were harvested, washed twice with cold PBS, and then centrifuged at 500 × *g* for 5 min at 4 °C. Cells were suspended in 0.1 ml of 1× Annexin V-binding buffer, 2 μl of Annexin V-FITC, and 2 μl of PI solution, and mixed gently. Cells were incubated at 20–25 °C for 15 min in dark, and 0.4 ml of 1× Annexin V-binding buffer was added. The stained cells were then measured by flow cytometry within 1 h of staining.

2.5. Identification of neuroprotective peptide

In order to get the selected peptide mass fingerprint of the AK3KF1obtained by HMPH, Nano LC-MS/MS analysis was performed with Easy n-LC (Thermo Fisher San Jose, CA, USA) and a LTQ Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA, USA) equipped with a nano-electrospray source. Samples were separated on a C_{18} nanobore column (150 mm × 0.1 mm, 3 μm pore size; Agilent). The mobile phase A for LC separation was 0.1% formic acid, 3% acetonitrile in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 0% B to 32% B in 23 min, 32% B to 60% B in 3 min, 95% B in 3 min, and 100% A in 6 min. The flow rate was maintained at 1.5 μl/min. Mass spectra were acquired using data-dependent acquisition with a full mass scan (350–1200 *m/z*) followed by 10 MS/MS scans. For MS1 full scans, the orbitrap resolution was 15,000 and the automatic gain control (AGC) was 2×10^5. For MS/MS in the LTQ, the AGC was 1×10^4.

2.6. Statistical analyses

All statistical analyses were performed using one-way analysis of variance using SPSS 20.0 (IBM, Armonk, NY, USA). Tukey's multiple comparisons test was used to determine significance of differences between means of different experimental groups, and a p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Antioxidant activity of peptide fractions obtained from beef myofibrillar protein

The peptides with a molecular mass of less than 3 kDa obtained from beef myofibrillar protein using alkaline-AK enzyme (AK3K) were isolated using FPLC on a GPC column (HiPrep 26/60 Sephacryl S-100 HR). Elution profile of two peptide fractions (AK3KF1 and AK3KF2) is shown in Fig. 1A.

Antioxidant potential of the AK3K peptide fractions (AK3KF1 and AK3KF2) was determined based on ABTS and DPPH radical scavenging activities, iron chelating activity, nitrite scavenging activity, and the reducing power assay (Fig. 1B–F). The ABTS radical scavenging activity of both the AK3K peptide fractions (AK3KF1 and AK3KF2) was more than 70%, however, AK3KF1 exhibited a significantly higher ABTS radical scavenging activity than did the AK3KF2 fraction. Furthermore, AK3KF1 had a significantly higher iron chelating activity, nitrite scavenging activity, and reducing power than did the AK3KF1 fraction. On the other hand, AK3KF2 exhibited a significantly higher DPPH radical scavenging activity compared to the AK3KF1 fraction.

3.2. Protective effect of peptide fractions on cell viability and NO production in H_2O_2-treated neuronal cells

Both AK3K peptide fractions (AK3KF1 and AK3KF2) exhibited protective effects against H_2O_2 treated human neuronal SH-SY5Y cells. Effect of AK3K peptide fractions on cell viability and NO production in H_2O_2 induced neuronal cells was determined by using XTT assay and Griess reagent, respectively (Fig. 2A and B). Treatment with 0.25 and 0.5 mg/ml AK3KF1 and AK3KF2, respectively, significantly increased ($p < 0.05$) cell viability compared to the H_2O_2 treated SH-SY5Y cells. Additionally, treatment with 0.25 and 0.5 mg/ml concentration of AK3K peptide fractions (AK3KF1 and AK3KF2) inhibited NO production in SH-SY5Y cells compared to the H_2O_2 treated cells ($p < 0.001$).

3.3. Effect of peptide fractions on the morphology of cell nuclei in H_2O_2 treated neuronal cells

The protective effect of 0.25 and 0.5 mg/ml AK3KF1 and AK3KF2, respectively, on H_2O_2 treated neuronal cells was confirmed by morphological assessment of cell nuclei using Hoechst 33342 staining (Fig. 3). Treatment with both the AK3K peptide fractions exhibited protective effects on neuronal cells by reducing cell shrinkage and nuclei condensation compared to the H_2O_2 treated neuronal cells. Furthermore, nuclear condensation was found to be lower in cells

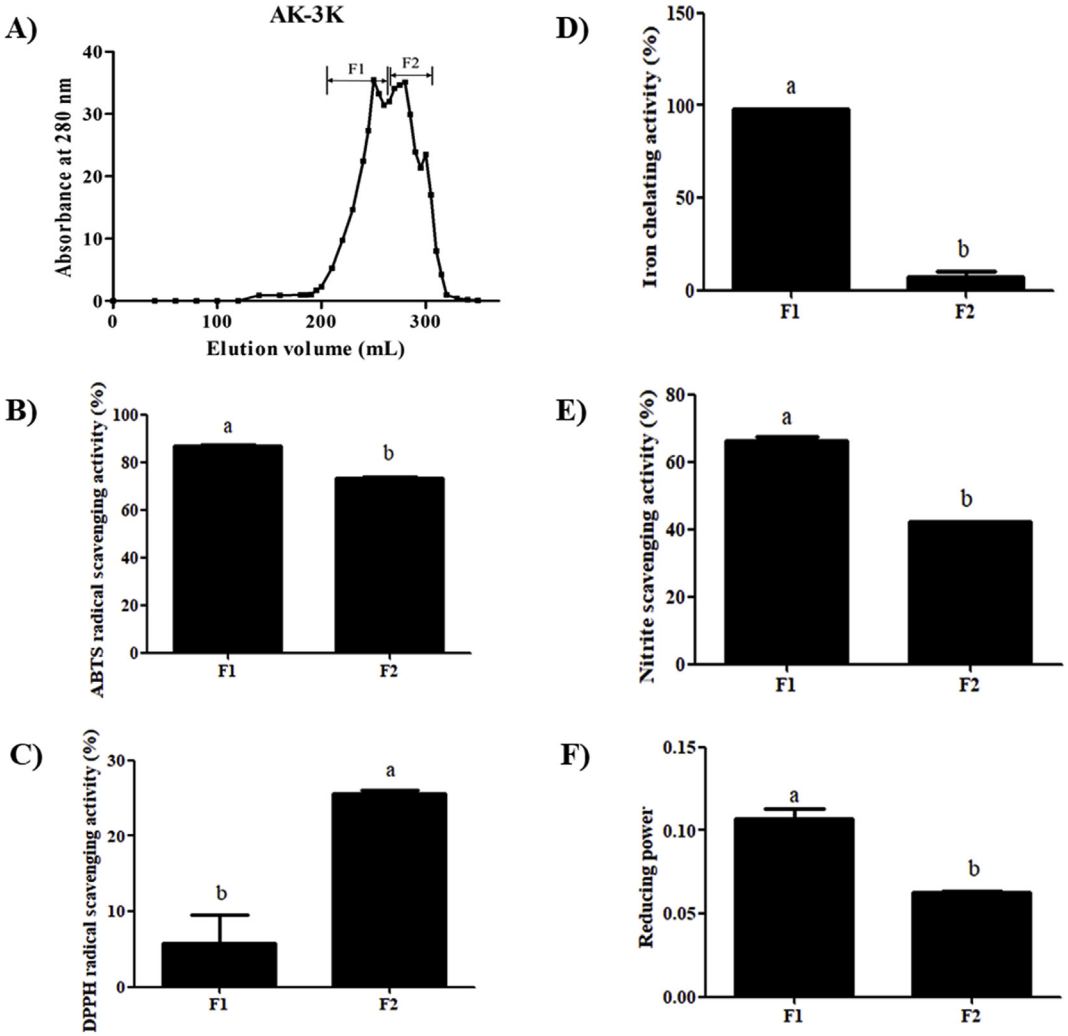

Fig. 1. (A) Elution profile of peptide fractions with molecular weight of less than 3 kDa obtained from beef myofibrillar protein using alkaline-AK by FPLC on GPC. (B) ABTS radical scavenging activity, (C) DPPH radical scavenging activity, (D) iron chelating activity, (E) nitrite scavenging activity, and (F) reducing power of AK3KF1 and AK3KF2. [a-b] Dissimilar alphabets in superscript indicate significant difference at $p < 0.05$.

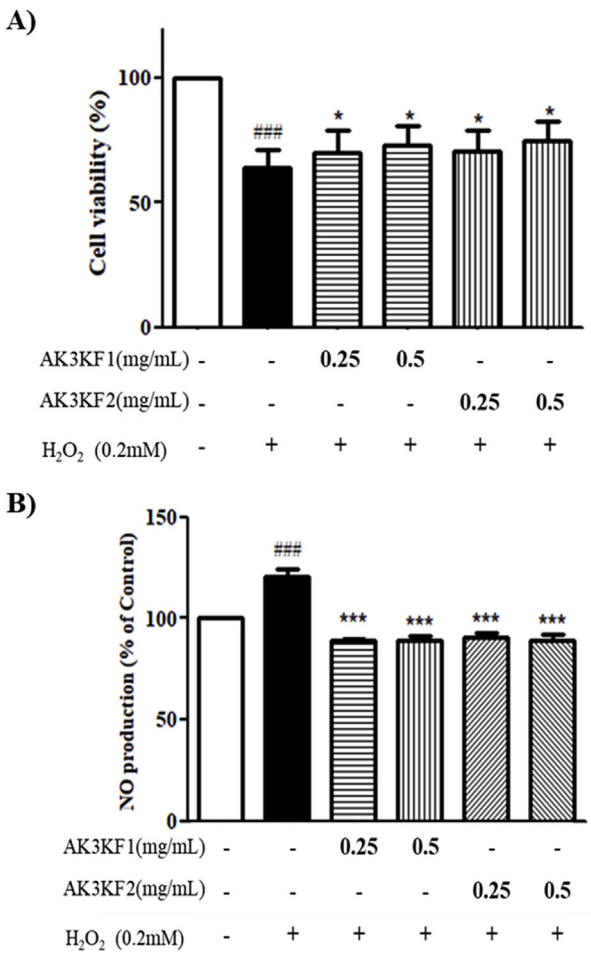

Fig. 2. Effects of < 3 kDa peptide fractions (0.25 and 0.5 mg/ml) obtained from beef myofibrillar protein using alkaline-AK on inhibition of (A) cell viability and (B) NO production in H_2O_2 damaged human neuronal SH-SY5Y cells. Data is presented as mean ± standard deviation ($n = 3$). $^{###}p < 0.001$ compared to the control cells. $^{*}p < 0.05$ compared to the H_2O_2-treated cells.

treated with AK3KF1 than in the cells treated with AK3KF2.

3.4. Effects of peptide fractions on MMP (Δψm) in H_2O_2 treated neuronal cells

Assessment of MMP (JC-1 aggregates/monomers) serves as an indicator of apoptosis or mitochondrial dysfunction-associated cell death (Wang et al., 2010). The energized MMP, which indicates normal mitochondrial function, increases the concentration of the JC-1 aggregates, responsible for the red fluorescence in the mitochondrial matrix, while apoptotic cells leak the monomeric form of JC-1, responsible for the green fluorescence in the cytosol (Reers et al., 1991). To determine the neuroprotective effect of AK3K peptide fractions (AK3KF1 and AK3KF2) on SH-SY5Y cells against H_2O_2-induced apoptosis, MMP was measured by JC-1 staining assay (Fig. 4). We observed that MMP (JC-1 aggregates/monomer) decreased in H_2O_2 treated cells, and treatment with 0.5 mg/ml AK3KF1 significantly increased MMP compared to that of the H_2O_2 treated cells ($p < 0.05$). These results indicated that AK3KF1 reduced H_2O_2 induced neuronal cell damage and restored MMP. Based on these results, we further determined the effect of AK3K peptide fractions against neuronal cell death using the Annexin V/PI staining assay.

3.5. Anti-apoptotic effect of peptide fractions on H_2O_2 induced neuronal cells

The apoptotic rate of H_2O_2 induced neuronal cells was assessed by Annexin V/PI staining and analyzed by flow cytometry. As shown in Fig. 5A, the lower left quadrant represents the viable cells and the upper and lower right quadrants represent the damaged cells related with necrosis or apoptosis. Treatment with 0.2 mM H_2O_2 increased the percentage of apoptotic cells to 86.80 ± 10.33% (Fig. 5B), which decreased to 18.82 ± 19.77% and 43.32 ± 16.94% in the presence of AK3KF1 and AK3KF2 peptide fractions, respectively. Thus, the AK3KF1 fraction showed a significantly stronger neuroprotective effect compared to the AK3KF2 fraction in H_2O_2treated neuronal cells ($p < 0.05$). These results indicate that AK3KF1 protected neuronal cells against H_2O_2 induced oxidative stress.

3.6. Identification of the sequence of the neuroprotective peptide

Since our results confirmed that the AK3KF1 had significantly high antioxidant potential and exhibited a stronger neuroprotective effect compared to the AK3KF2 fraction in H_2O_2 induced neuronal cells, the amino acid sequence of AK3KF1 was analyzed using the LTQ Orbitrap XL mass spectrometer. The sequence of the peptide was identified as Thr-Gln-Lys-Lys-Val-Ile-Phe-Cys with a molecular weight of 965.54 Da (Fig. 6).

4. Discussions

This study was conducted to determine the neuroprotective effects of peptide fractions obtained from beef myofibrillar protein and to identify the amino acid sequence of the resulting peptides. In this study, AK3K peptide fractions exhibited antioxidant activities as assessed by their ability to scavenge ABTS and DPPH radicals, inhibition of NO production, iron chelation and reducing power. Among the two AK3K peptide fractions, AK3KF1 exhibited stronger antioxidant activity compared to the AK3KF2.

The excessive production of free radicals and excessive amounts of transition metals in the brain contribute to neuronal cell death (Lee et al., 2007), therefore, scavenging free radicals and chelation of transition metals can inhibit neurodegenerative diseases (Chan et al., 2016). Previous studies reported that amino acids such as Gln, Lys, and Pro, and hydrophobic amino acids derived from natural food sources possess free radical scavenging activity, reducing power, and inhibit lipid peroxidation (He et al., 2012; Najafian and Babji, 2015; Zou et al., 2016). Negatively-charged acidic amino acids and aromatic amino acids with phenol structures, Phe, Trp, and Tyr, inhibit production of free radicals by donating protons to the electron deficient free radicals, thereby neutralizing them (Rajapakse et al., 2005; Zou et al., 2016). Additionally, amino acids such as Cys, Ser, His, Asp, and Gln, can participate by binding with metal ions (Walters et al., 2018). Peptides chelate metals through the coordination of ions with the carbonyl group of the peptide bonds, and with the free electrons on the nitrogen atoms (Walters et al., 2018). Therefore, we hypothesized that the possible antioxidant mechanisms of the peptide isolated in this study could be related to its amino acid sequence and the results of our study were in agreement with those of the previous studies. Several studies (Ghassem et al., 2017; Sheih et al., 2009) also reported that the peptide structure, amino acid sequence, and hydrophobicity of bioactive peptides contribute to their antioxidant activity. Hydrophobic peptides can easily be absorbed through the lipid layer membranes and thereby exhibit high radical scavenging activity (Lee and Hur, 2019).

In this study, treatment with AK3K peptide fractions (AK3KF1 and AK3KF2) increased cell viability and suppressed NO production compared to H_2O_2- treated neuronal SH-SY5Y cells. The β-amyloid (Aβ) peptide, which is known as an AD inducer, has been reported to increase the production of NO which in turn results in potential oxidative

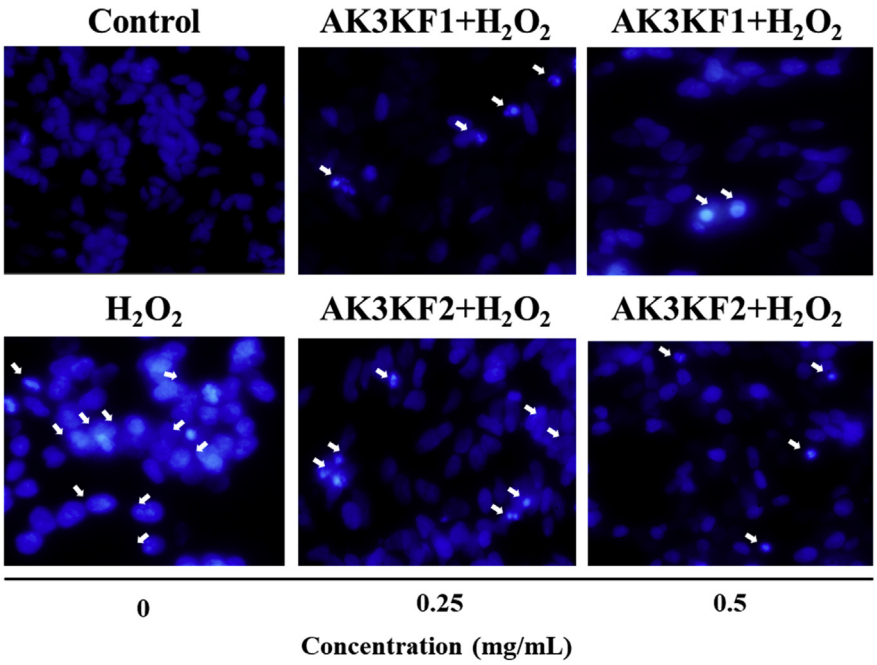

Fig. 3. Effect of 0.25 and 0.5 mg/ml concentration of 3 kDa peptide fractions obtained from beef myofibrillar protein using alkaline-AK on fragmentation of nuclei by Hoechst staining in H_2O_2-treated human neuronal SH-SY5Y cells. Data are given as mean ± standard deviation ($n = 3$).

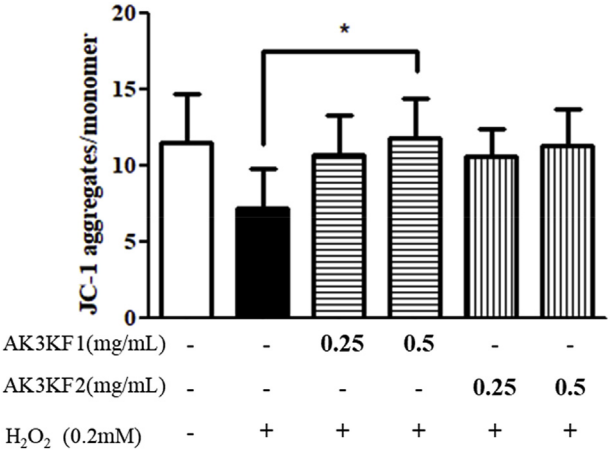

Fig. 4. Effect of 0.25 and 0.5 mg/ml concentration of 3 kDa peptide fractions obtained from beef myofibrillar protein using alkaline-AK on mitochondrial membrane potential by JC-1 staining in H_2O_2 damaged human neuronal SH-SY5Y cells. Data is presented as mean ± standard deviation ($n = 3$). $^{*}p < 0.05$ compared to the H_2O_2-treated cells.

injury to the brain and neuronal SH-SY5Y cells (Akama et al., 1998; Ill-Raga et al., 2010). Ill-Raga et al. (2010) found that the NO donor-treated SH-SY5Y cells were protected by antioxidant molecules, via indirect mechanisms, such as scavenging of free radicals that activate the antioxidant defense mechanism of the neurons against Aβ neurotoxicity. The hydrophobic antioxidants with low molecular weight, such as vitamin E and Coenzyme Q exhibited stronger protection against oxidative damage than the antioxidants with high molecular weight, such as superoxide dismutase and catalase, because hydrophobic and low molecular weight molecules can penetrate cell membranes more easily (Hseu et al., 2002; Szeto, 2006). Several studies investigating the role of inhibition of NO in the brain have reported that NO inhibitors play a major role as effective therapeutic agents for the treatment of neurodegenerative diseases (Mukherjee et al., 2014). Therefore, suppression of NO production could be another possible mechanism for the neuroprotective effect of the peptide isolated in this study. Silverman et al. (1997) also reported that $_{D}$-Phe-$_{D}$-Arg-NO_2-OMe inhibited neuronal NO production due to the presence of basic amino acid residues in the side chain of peptides (Silverman et al., 1997). Previous studies found that the peptides obtained from Lunasin and milk protein were able to protect Caco-2 cells against H_2O_2 induced oxidative stress (García-Nebot et al., 2014; Tonolo et al., 2018). In addition, the peptides with aromatic and basic amino acids can prevent cell death by resonance reaction of their phenol groups (Zhao et al., 2004). Therefore, the neuroprotective effect of AK3K peptide fractions (AK3KF1 and AK3KF2) could be related to their ability to suppress NO production, or scavenge free radicals and penetrate through the cell membrane.

This study demonstrated that the AK3KF1 exhibited neuroprotective effect by decreasing MMP, and inhibiting fragmentation of cell nuclei and apoptosis as assessed by Annexin V/PI staining (Figs. 4 and 5). It is known that mitochondrial function, fragmentation of cell nuclei, and apoptosis are closely related to neuroprotective effects (Morelli et al., 2014) and oxidative stress-induced loss of MMP ($\Delta\psi_m$) results in cellular injury and DNA fragmentation by activating apoptotic pathways (Kruman and Mattson, 1999; Wadia et al., 1998).

Cell nuclei damage induces chromatin condensation and DNA fragmentation that seen in apoptotic cells (Widlak et al., 2001, 2002). In agreement with this study, they reported that the morphology of nuclear in damaged cells not only was confirmed chromatin condensation but chromatin condensation induced cytoplasmic membrane perforation (Widlak et al., 2002). As above mentioned, these factors such as fragmentation of cell nuclei or DNA are closely related to neuronal cell apoptosis induced oxidative damage such as ROS (Madabhushi et al., 2014; Narciso et al., 2016). Drummond et al. (2017) demonstrated that oxidative stress inhibitor scavenges intracellular ROS and inhibits downstream oxidative damage to both membranes and nuclei fragmentation in SH-SY5Y cells. Recent studies also have indicated that peptides or hydrophobic amino acid protect against cell oxidative damage through their free radical scavenging activity and antioxidant activities (Nurdiani et al., 2017; Zhao et al., 2017). In addition, the results of this study are in agreement with previous studies which have reported the protective effect of peptides/proteins against oxidatively damaged neuronal cell via activation of mitochondria

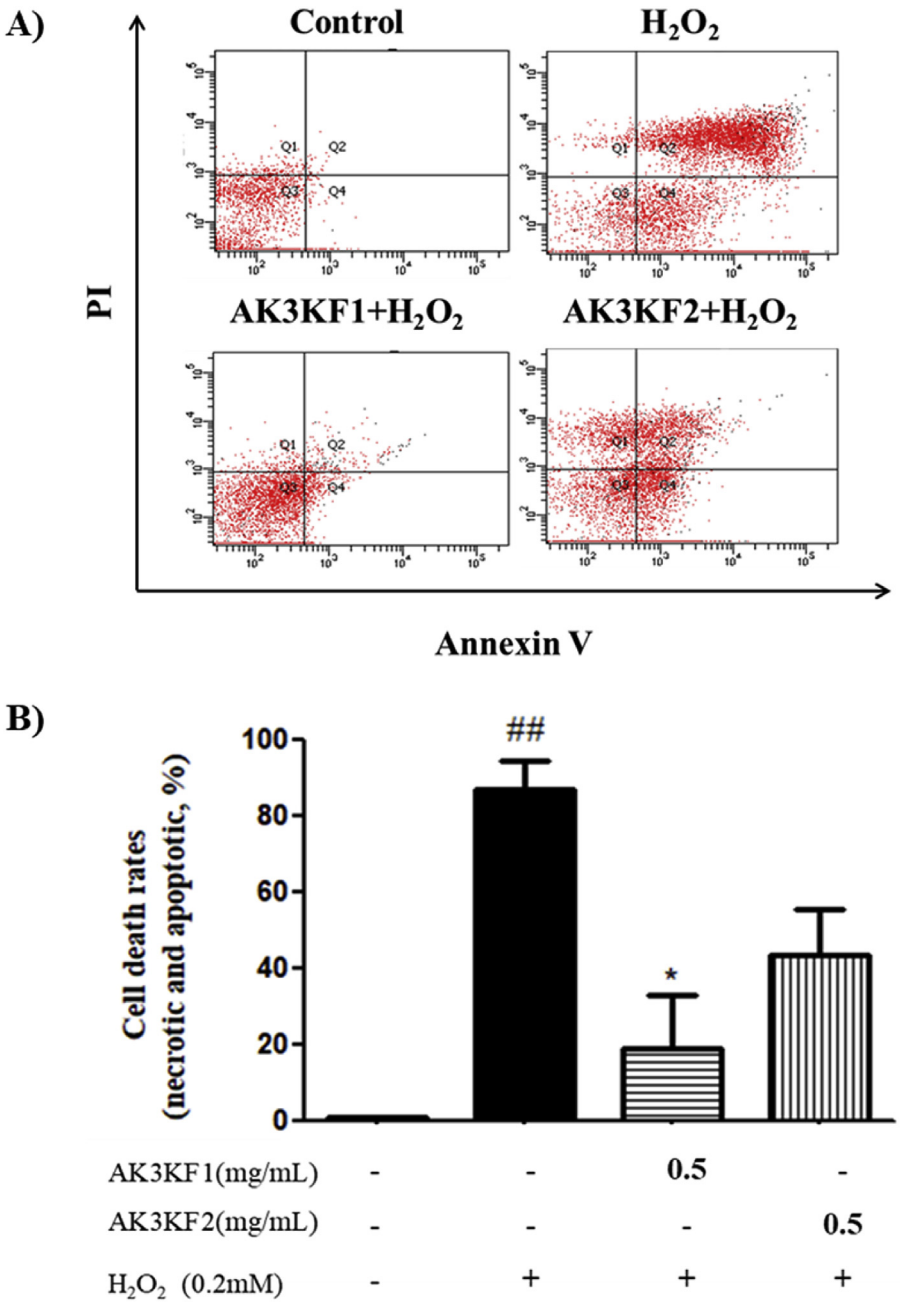

Fig. 5. (A) Flow cytometry analysis, and (B) quantitative analysis of the cell death rate to determine protective effect of 3 kDa peptide fractions on neuronal cells. $^{\#\#}p < 0.001$ compared to the control cells. $^{*}p < 0.05$ compared to the H_2O_2-treated cells.

dependent apoptotic pathways (Jin et al., 2013; Li et al., 2018; Zou et al., 2013). Whey protein hydrolysates protected PC12 cells against oxidative stress by regulating the mitochondrial apoptotic pathways which resulted in inhibition of apoptosis and restoration of MMP (Jin et al., 2013). Zhu et al. (2013) also demonstrated that hydrolysate from wheat germ protected neuronal cells by reducing MMP, nuclear condensation, and apoptosis, and activating antioxidant enzymes. Other studies have also revealed that hydrolysates or peptides reduce cell death by blocking the opening of the mitochondrial permeability transition pores, suppressing the overload of calcium by N-methyl-D-aspartate (NMDA) receptors, and regulating the potential loss of MMP (Gamir-Morralla et al., 2015; Jin et al., 2013; Koya et al., 2000). These studies also suggested that the protective effect of hydrolysates/peptides is strongly related to their antioxidant activity and ability to permeabilize the inner mitochondrial membrane. Therefore, we speculated that the mechanism by which AK3KF1 exerted neuroprotective effects could also be related to its amino acid sequence, antioxidant potential, and also its ability to penetrate the mitochondrial membrane.

Although the main mechanism for the neuroprotective effect of AK3KF1 still remains unclear, we believe that its effect against H_2O_2-induced apoptosis in neuronal cells is due to its ability to restore the MMP, thereby inhibiting the loss of mitochondrial function. Another mechanism could be attributed to its ability to scavenge free radicals induced by H_2O_2, thereby preventing apoptosis. Antioxidant activities including radical scavenging and transition metal chelation by AK3KF1 resulted in reducing H_2O_2-induced oxidative stress in neuronal SH-SY5Y cells.

Further we determined the amino acid sequence of AK3KF1 using LTQ Orbitrap XL mass spectrometer. The sequence of the

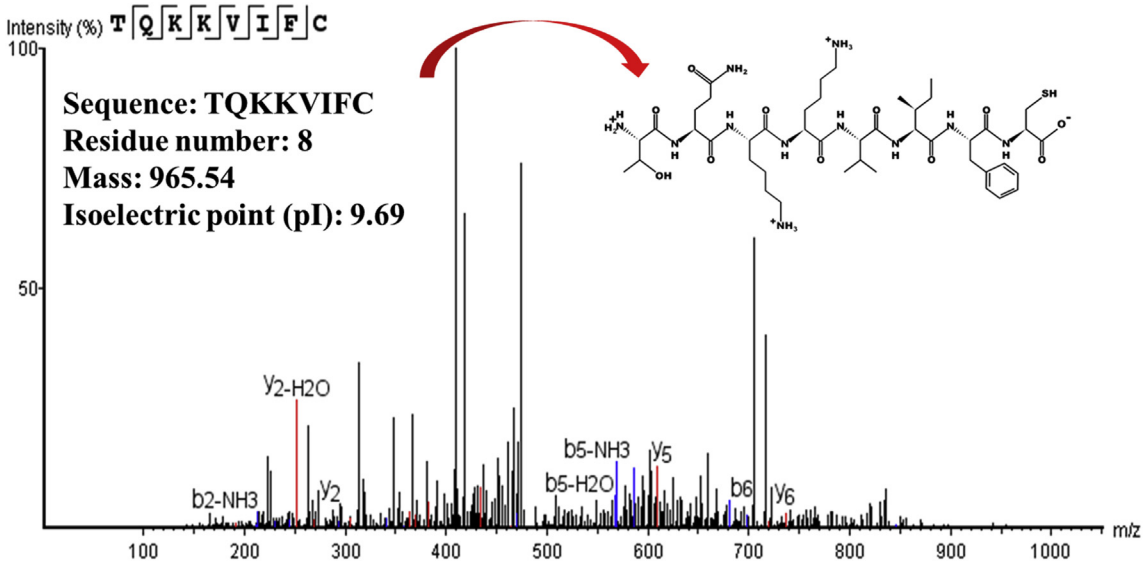

Fig. 6. Identification of molecular mass and amino acid sequence of AK3KF1. MS/MS was performed on a LTQ Orbitrap XL mass spectrometer.

neuroprotective peptide was identified as Thr-Gln-Lys-Lys-Val-Ile-Phe-Cys with a molecular weight of 965.54 Da. This is the first study to report neuroprotective effects of a peptide obtained from beef myofibrillar protein using alkaline-AK. Previous studies have suggested that there is a relationship between neuroprotective effect of peptides and their structural characteristics, such as hydrophobicity or low molecular weight (Brenneman and Gozes, 1996; Festoff et al., 1996; Pangestuti et al., 2013). Furthermore, it has been previously explained that the characteristics of a peptide are associated with inhibition of Aβ formation or amyloid neurotoxicity (Poduslo et al., 1999; Soto, 1999). These studies suggested that the main characteristics of peptides include hydrophobic and polarized amino acids for improving solubility, and a chain of 2–15 amino acid residues which leads to inhibition of Aβ aggregation by blocking β-sheet formation required for Aβ structure. In addition to this, previous studies have also demonstrated that neuroprotective peptides not only comprise of hydrophobic amino acids and have molecular weight below 1 kDa, but they also block the β-sheet of Aβ, and penetrate blood brain barrier (BBB) in the central nervous system (CNS) (Ashur-Fabian et al., 2003; Jehle et al., 2008; Pangestuti et al., 2013). The blood brain barrier (BBB), semipermeable border that separate the circulating blood in brain and extracellular fluid in the central nervous systems (CNS), is formed by endothelial cells (Daneman and Prat, 2015). Therapeutic molecule into CNS is limited for developing neurological agents due to their poor ability to cross the BBB. For this reason, complementary studies such as cell penetrating peptide and drug transport have been reported in *in vitro* and *in vivo* (Stalmans et al., 2015; Weksler et al., 2013; Zou et al., 2013). Cell penetrating peptides (CPP) contain whether positive charge amino acids such as arginine and lysine residue or the amphipathic peptides such as hydrophilic and hydrophobic residues (Stalmans et al., 2013). The penetration capacity of CPP is higher as the cationic residue their size is smaller than other transport systems (Mäe and Langel, 2006; Zou et al., 2013). Therefore, the peptide in this study may be penetrates into BBB as a peptide with CPPs characteristics through an amino acid structure or a small size. However, we suggest that this study need to further studies with cell line model such as cerebral microvessel or microvascular endothelial cells and human trials will be essential in order to validate the effectiveness of this peptide.

The antioxidant activity of peptides can be attributed to the aliphatic residues, such as Leu, Ile, Val and Phe, which can easily donate protons to free radicals (Zhu et al., 2008). Zhao et al. (2017) demonstrated that peptides with Pro-Ala-Tyr-Cys-Ser and Cys-Tyr-Gly-Ser-Tyr sequences protected PC12 cells against glutamate-induced apoptosis via inhibiting ROS production owing to the presence of Tyr and Cys residues which have ability to quench free radicals (Zhao et al., 2017). Therefore, the neuroprotective effect of peptide isolated from beef myofibrillar protein in this study with amino acid sequence as Thr-Gln-Lys-Lys-Val-Ile-Phe-Cys could be associated with composition of amino acids affecting its hydrophobicity, affinity or permeability into cell membrane, free radical scavenging activity and ability to donate protons and chelate metal ions. Thus our results indicate that the peptide (Thr-Gln-Lys-Lys-Val-Ile-Phe-Cys) obtained from beef myofibrillar protein using alkaline-AK exhibits neuroprotective effects against oxidative stress-induced apoptosis in human neuronal cells.

5. Conclusions

This study demonstrated the neuroprotective effect of two < 3 kDa peptide fractions (AK3KF1 and AK3KF2) obtained from beef myofibrillar protein using an inexpensive enzyme (alkaline-AK) on human neuronal SH-SY5Y cells. The two peptide fractions (AK3KF1 and AK3KF2) showed neuroprotective effect by increasing cell viability, inhibiting NO production and fragmentation of cell nuclei, increasing MMP, reducing apoptosis, and ability to scavenge free radicals. Furthermore, AK3KF1 exhibited stronger neuroprotective effect than the AK3KF2. The sequence of AK3KF1 was identified as Thr-Gln-Lys-Lys-Val-Ile-Phe-Cys with a molecular weight of 965.54 Da. The neuroprotective effect of this peptide could possibly be related to amino acid composition, hydrophobicity, and ability of peptides to penetrate through the cell membranes.

Conflicts of interest

The authors declare no conflicts of interest.

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