Neuroprotective effect of whey protein hydrolysate containing LDIQK on HT22 cells in hydrogen peroxide-induced oxidative stress

Yeok B. Chang,12 Eun-Jin Jung,3 Kyungae Jo,1 Hyung J. Suh,12* and Hyeon-Son Choi4*
1Department of Integrated Biomedical and Life Science, Graduate School, Korea University, Seoul 02841, Republic of Korea
2Transdisciplinary Major in Learning Health Systems, Graduate School, Korea University, Seoul 02841, Republic of Korea
3Department of Food and Biotechnology, Korea University, Sejong 30019, Republic of Korea
4Department of Food Nutrition, Sangmyung University, Seoul 03016, Republic of Korea

ABSTRACT

This study aimed to investigate the neuroprotective effects of whey protein hydrolysate (WPH) containing the pentapeptide leucine-aspartate-isoleucine-glutamine-lysine (LDIQK). Whey protein hydrolysate (50, 100, and 200 µg/mL) demonstrated the ability to restore the viability of HT22 cells subjected to 300 µM hydrogen peroxide (H2O2)-induced oxidative stress. Furthermore, at a concentration of 200 µg/mL, it significantly reduced the increase in reactive oxygen species production and calcium ion (Ca2+) influx induced by H2O2 by 46.1% and 46.2%, respectively. Similarly, the hydrolysate significantly decreased the levels of p-tau, a hallmark of tauopathy, and BCL2 associated X (BAX), a pro-apoptosis factor, while increasing the protein levels of choline acetyltransferase (ChAT), an enzyme involved in acetylcholine synthesis, brain-derived neurotrophic factor (BDNF), a nerve growth factor, and B-cell lymphoma 2 (BCL2), an anti-apoptotic factor. Furthermore, it increased nuclear factor erythroid 2-related factor 2 (Nrf2)-hemoxygenase-1 (HO-1) signaling, which is associated with the antioxidant response, while reducing the activation of mitogen-activated protein kinase (MAPK) signaling pathway components, namely phosphor-extracellular signal-regulated kinases (p-ERK), phosphor-c-Jun N-terminal kinases (p-JNK), and p-p38. Column chromatography and tandem mass spectrometry analysis identified LDIQK as a compound with neuroprotective effects in whey protein hydrolysate; it inhibited Ca2+ influx and regulated the BAX/BCL2 ratio. Collectively, whey protein hydrolysate containing LDIQK demonstrated neuroprotective effects against H2O2-induced neuronal cell damage, suggesting that whey protein hydrolysate or its active peptide, LDIQK, may serve as a potential edible agent for improving cognitive dysfunction.

Keywords: whey protein hydrolysate, LDIQK, neuroprotective effect, HT22 cell, oxidative stress

INTRODUCTION

Advances in medicine and life sciences have contributed to an increase in life expectancy and, consequently, an aging population. Degenerative diseases, such as cognitive dysfunction, cause memory impairment and have become significant social, economic, and health issues (Swerdlow, 2007). Alzheimer’s disease (AD) is the most prevalent type of cognitive dysfunction and emerges as a significant social problem. Cognitive dysfunction is characterized by progressive cognitive decline and language impairment, which interfere with daily life (Sun et al., 2018). Despite extensive research, the cause of cognitive dysfunction in AD remains obscure, and no unified consensus hypothesis exists (Mullane and Williams, 2018). The pathogenesis of cognitive dysfunction is multifactorial and includes neuronal apoptosis in the brain owing to oxidative stress (Citron, 2002), the accumulation of senile plaques composed of amyloid-β protein (Monczor, 2005), the formation of neurofibrillary tangles composed of hyperphosphorylated tau protein (Hutton et al., 1998), and a decrease in the neurotransmitter acetylcholine (ACh) due to acetylcholinesterase (Ballinger et al., 2016). Currently, cognitive dysfunction is treated with therapeutic agents, such as antioxidants (e.g., vitamin E and selegiline) and acetylcholinesterase inhibitors (e.g., Tacrine and Aricept) (Piau et al., 2011; Sano et al., 1997). These treatments can delay or prevent the onset of dementia but cannot cure it. Moreover, these treatments have side effects, such as temporary hepatotoxicity, gastrointestinal disorders, and cardiac bradycardia, underscoring the need for the development of new and safer therapies (Delagarza, 2003).
Several dietary supplements and foods with minimal side effects, such as omega-3 polyunsaturated fatty acids, vitamin D, and flavonoids, improve cognitive function (Hwang et al., 2012). Additionally, peptides derived from sources such as nuts, rice, and milk have antioxidative and cognitive enhancement effects (Murray and FitzGerald, 2007). Among the aforementioned sources, milk protein is considered the most important source of active peptides, and this research focuses on the production and characteristics of milk protein-derived active peptides. In addition, α-lactalbomin, a constituent of milk protein, has been shown to enhance serotonin and cognitive functions (Markus et al., 2002).

The HT22 mouse hippocampal cell line is used as an in vitro model to study the mechanism of oxidative stress-induced neuronal cell death. Free radicals produced as byproducts of metabolism can exacerbate the damage caused by ischemic brain disease. Hydrogen peroxide (H$_2$O$_2$) is a free radical with superoxide (O$_2$), hydroxy radical (OH$^-$), and other components and is involved in the onset of various diseases, such as cardiovascular diseases and aging (Halliwell, 1989). Oxidative damage caused by free radicals in brain nerve cells is a cause of neurodegenerative diseases, such as AD and stroke (Baynes, 1991). In this study, the neuroprotective effect of neurodegenerative diseases, such as AD and stroke (Baynes, 1991). In this study, the neuroprotective effect of whey protein hydrolysate (WPH), prepared using Alcalase, Prozyme, and Flavorzyme, was investigated in neuronal cells exposed to H$_2$O$_2$-induced oxidative stress. Furthermore, the isolation of a bioactive peptide from WPH and the analysis of a synthesized peptide provided insight into its specific contribution to neuroprotection.

**MATERIALS AND METHODS**

**Preparation of WPH**

Whey protein concentrate (WPC, Seoul, Korea), containing protein of 80% and fat of 8%, was diluted to 20% with distilled water and adjusted to pH 7 using sodium bicarbonate. Diluted WPC was hydrolyzed using 0.2% Alcalase and 0.2% Protamex at 50–55°C for 4 h to obtain the first hydrolysate, followed by 0.2% Flavorzyme at 50–55°C for 15 h to produce the second hydrolysate. The secondary hydrolysate was then heated at 90°C for 10 min, cooled to 25°C, filtered through a 1 μm housing filter, sterilized at 90°C for 30 min, and then spray-dried at an inlet temperature of 190 ± 10°C and an outlet temperature of 95 ± 5°C. The resulting spray-dried powder was passed through an 8000 GAUS magnetic rod to remove metal impurities and then packaged for use as a whey protein hydrolysate (WPH). Packaged WPH was provided by Neocremar Co., Ltd. (Seoul, Korea).

**Amino acid analysis**

After acid hydrolysis of WPH, the amino acid composition was determined using an automatic amino acid analyzer. Briefly, WPH (25 mg) was weighed and placed in a capped tube. Subsequently, 2.5 mL of 6 N HCl was added to the tube, and the mixture was hydrolyzed at 110°C for 24 h. The residual solution was passed through a 3G-4 glass filter to remove undigested materials and then evaporated to dryness at 50°C using a rotary evaporator (N-1110, EYELA, Tokyo, Japan). The resulting residue was dissolved in 25 mL of 0.01 N HCl, and 40 μL of the solution was injected into an automatic amino acid analyzer (Biochrom 30, Cambridge, UK).

**Separation and purification of WPH**

The separation and purification of the active peptide in WPH were performed in a step-by-step manner using anion exchange chromatography, gel filtration chromatography, and reversed-phase chromatography. Initially, whey protein peptides were separated using the Q-Sepharose HiLoad 16/10 ion chromatography column with 25 mM Tris-Cl (pH 8.0) containing 0.6 M NaCl. Among the 7 fractions obtained, the EX-4 fraction was injected into the Superdex peptide 10/30 column and eluted with HPLC-grade distilled water, with peak detection at 280 and 220 nm. The active fraction EX-4/S16 was then subjected to complete solvent evaporation using a Speed Vac. Subsequently, the peptide was separated using the source 5 RPC ST column (4.6 × 150, 4.5 μm) and dissolved in acetonitrile containing 100–300 μL of 0.1% Trifluoroacetic acid (TFA). Peptide separation was confirmed by monitoring peaks at 280 and 220 nm.

**Cell culture and viability assay**

Mouse hippocampus-derived HT22 neuronal cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Welgene, Gyeongsan-si, Korea) containing 10% fetal bovine serum (Welgene, Gyeongsan-si, Korea), 1% penicillin-streptomycin (Welgene, Gyeongsan-si, Korea), and 1% L-glutamate. The cultures were maintained in an incubator under 5% CO$_2$ at 37°C.

To assess the inhibitory effect of WPH on H$_2$O$_2$-induced cell death, cells were seeded in a 96-well plate at a density of 1 × 10$^4$ cells/mL and treated with different concentrations of whey protein hydrolysate (50, 100, 200, 400, and 800 μg/mL) for 2 h. Then, 300 μM H$_2$O$_2$ (St. Louis, MO, USA) was added to induce oxidative stress. After 24 h, cell viability was evaluated using the
WST-1 assay kit (St. Louis, MO, USA) following the manufacturer’s protocol.

**Analysis of intracellular reactive oxygen species (ROS) levels**

To analyze the ROS scavenging activity of WPH, the diacetylchlorofluorescein diacetate (DCF-DA. St. Louis, MO, USA) assay (Brandt and Keston, 1965) was conducted using HT22 cells. The HT22 cells were seeded at a density of $1 \times 10^5$ cells/mL in a 6-well plate and cultured for 24 h at 37°C in a 5% CO$_2$ incubator. Subsequently, WPH (50, 100, and 200 µg/mL) was added for 2 h, followed by exposure to 300 µM H$_2$O$_2$ for 24 h. After removing the media, the cells were incubated with 10 µM DCF-DA solution for 30 min. Fluorescent DCF from cells were dissolved using DMSO. The fluorescence level was evaluated at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a fluorescence microplate reader (SpectraMaxM3; Molecular Devices, Sunnyvale, CA, USA).

**Analysis of intracellular calcium ions (Ca$^{2+}$)**

The concentration of intracellular Ca$^{2+}$ in HT22 cells was measured using Fura-2AM, a Ca$^{2+}$-sensitive fluorophore. The HT22 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells/mL, incubated for 24 h at 37°C in a 5% CO$_2$ incubator, and treated with WPH (50, 100, and 200 µg/mL) or LDIQK (1.25, 2.5, 5, and 10 µg/mL) for 2 h, followed by 300 µM H$_2$O$_2$ for 24 h. To measure intracellular Ca$^{2+}$ concentrations, HT22 cells were incubated with 10 µL of 20 µM Fura-2AM for 1 h in a 5% CO$_2$ incubator at 37°C. Then, the medium was removed, and the cells were lysed with 1.0% Triton X-100 at 37°C for 15 min and washed thrice with phosphate-buffered saline. The lysed solution was transferred to a 96-well plate and the fluorescence intensity (excitation 340 nm, emission 510 nm) was measured using a fluorescence microplate reader.

**Western blotting**

HT22 cells were plated in a 6-well plate at a density of $1 \times 10^5$ cells/mL and cultured for 24 h at 37°C in a 5% CO$_2$ incubator. Subsequently, cells were treated with WPH (50, 100, and 200 µg/mL) for 2 h, followed by 300 µM H$_2$O$_2$ for 24 h. Then, 600 µL of lysis buffer containing 200 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 1% NP40, 1 mM PMSF, 1 mM Na$_3$VO$_4$, and protease inhibitor cocktail was added, and the mixture was homogenized and centrifuged at 10 000 x g for 5 min at 4°C to collect the supernatant.

The protein content in the samples was quantified using the bichinchoninic acid method (Walker, 2009), and 30 µg of protein was loaded onto a 10% SDS-PAGE gel for electrophoresis. The Polyvinylvidene fluoride membrane was blocked with 5% skim milk and bovine serum albumin for 1 h and then incubated with primary antibodies, including α-tubulin (Cell Signaling Technology, Inc., Danvers, MA, USA), BAX (Cell Signaling Technology, Inc.), BCL-2 (Cell Signaling Technology, Inc.), brain-derived neurotrophic factor (BDNF; Cell Signaling Technology, Inc.), phospho-tau (p-tau; Cell Signaling Technology, Inc.), tau (Cell Signaling Technology, Inc.), Nrf2 (Cell Signaling Technology, Inc.), HO-1 (Cell Signaling Technology, Inc.), phospho-extracellular signal-regulated kinase (p-ERK; Cell Signaling Technology, Inc.), phospho-c-Jun N-terminal kinase (JNK; Cell Signaling Technology, Inc.), phospho-p38 (Cell Signaling Technology, Inc.), and choline acetyltransferase (ChAT; Abcam, Cambridge, UK), overnight at 4°C. After 3 washed with Tris-buffered saline containing Tween 20 washes, the membrane was incubated with a secondary antibody (Anti-rabbit IgG, horseradish peroxidase-linked antibody, Cell Signaling Technology, Inc.) for 2 h at room temperature. Protein bands were incubated with SuperSignal Western Blot Enhancer (Thermo Fisher Scientific, Waltham, MA, USA) and then visualized using the FluorChem M Fluorescent Western Imaging System (Protein Simple, San Jose, CA, USA). Antibodies were diluted in 5% skim milk and bovine serum albumin, as per instructions provided by the manufacturer of each antibody.

**Statistical analysis**

All data are expressed as the mean ± standard deviation (SD). Statistical analysis was conducted using SPSS program (SPSS 12.0 for windows, SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using one-way ANOVA followed by Tukey’s multiple range test, and a $P$ value <0.05 was considered statistically significant.

**RESULTS**

**Amino acid composition of WPH**

The total amino acid content of WPH was 80.11 ± 2.41 g/100 g (Table 1). Aspartic acid, Threonine, Glutamic acid, Leucine, and Lysine exhibited relatively high levels, each exceeding 5.0 g/100g. Among the analyzed amino acids, the content of glutamic acid was the highest at 14.49 ± 0.45 g/100 g, while that of cysteine was the lowest at 0.69 ± 0.02 g/100 g in WPH.
Effect of WPH on HT22 cell viability and ROS production

Whey protein hydrolysate significantly increased the survival rate of HT22 cells compared with the normal group (NOR) ($P < 0.05$). In addition, WPH at concentrations ranging from 50 to 800 mg/mL increased cell viability by 25% to 39%, indicating no cytotoxicity by WPH at all concentrations (Figure 1A). Hydrogen peroxide (300 mM) induced HT22 cell death, reducing cell viability by 37.4% compared with the normal group (NOR). Treatment with WPH mitigated H$_2$O$_2$-mediated cytotoxicity by increasing cell viability in a dose-dependent manner up to 200 mg/mL (Supplementary Figure 1 and Figure 1B). In particular, 50, 100, and 200 mg/mL WPH significantly increased cell viability by 46.5%, 61.9%, and 68.2% ($P < 0.001$), respectively, compared with the control group (CON) (Figure 1B). The cell viability at 100, 200, 400, 800 mg/mL of WPH remained comparable to the levels observed in the normal group (Figure 1B). Hence, subsequent experiments were carried out using WPH at concentrations of 50, 100 and 200 mg/mL, revealing a dose-dependent relationship (Figure 1B). In addition, H$_2$O$_2$ (CON) increased ROS generation in cells by 84.8% compared with the normal group (NOR). On the contrary, WPH significantly attenuated H$_2$O$_2$-induced ROS production. In particular, 50, 100, and 200 mg/mL WPH reduced intracellular ROS levels by 25.8%, 30.6%, and 46.1%, respectively (Figure 2). These results indicate that WPH enhances the survival of cells in H$_2$O$_2$-induced oxidative stress, confirming the protective effect of WPH against ROS-mediated oxidative stress.

Effect of WPH on intracellular Ca$^{2+}$ concentration in HT22 cells

Hydrogen peroxide significantly increased intracellular Ca$^{2+}$ concentration (2.3-fold) in HT22 cells compared with the normal group (NOR) (Figure 3), indicating that H$_2$O$_2$ triggered a pro-apoptotic state in neuronal cells by disrupting calcium homeostasis. However, WPH at concentrations of 50, 100, and 200 mg/mL reduced H$_2$O$_2$-mediated ROS production in Ca$^{2+}$ influx (34.3%, 33.2%, and 46.2%, respectively; $P < 0.001$, Figure 3). Collectively, these findings suggest that WPH inhibits ROS-induced Ca$^{2+}$ influx.

Effects of WPH on apoptosis-related factors in HT22 cells

Hydrogen peroxide significantly increased the BAX/BCL2 ratio, indicating that H$_2$O$_2$-induced neuronal cell apoptosis. However, 50, 100, and 200 mg/mL WPH...
significantly reduced the BAX/BCL2 ratio (111.8%, 10.4%, and 39.6%, respectively; Figure 4A and B), demonstrating that WPH suppresses neuronal cell apoptosis by regulating apoptosis-related factors in oxidative stress conditions.

**Effects of WPH on neurologic factors in HT22 cells**

Whey protein hydrolysate regulated factors related to neuronal functions (p-Tau, ChAT, and BDNF). H$_2$O$_2$ significantly downregulated ChAT and BDNF, suggesting impaired brain plasticity and neurotransmitter production. However, WPH reversed H$_2$O$_2$-induced downregulation of ChAT and BDNF. In particular, 50, 100, 200 mg/mL WPH increased protein levels of ChAT (29.8%, 53.7%, and 2.4-fold, respectively; $P<0.05$, Figure 4A and C) and BDNF (18.82%, 94.3%, and 3.0-fold, respectively, Figure 4A and D), indicating that WPH has the potential to improve neuronal functions impaired by ROS. Hydrogen peroxide significantly increased (2.6-fold) the p-tau/tau protein ratio, a neurodegenerative marker, in HT22 cells compared with the normal group (NOR). WPH (50, 100, and 200 mg/mL) dose-dependently decreased the p-tau/tau ratio (28.4%, 40.8%, and 52.2%, respectively; $P<0.001$, Figure 4A and E). This finding suggests that WPH suppresses neuronal degeneration by reducing p-tau levels.
**Effects of WPH on antioxidant response factors in HT22 cells**

The effect of WPH on the protein levels of Nrf2 and HO-1, both of which are antioxidant response factors, was examined under oxidative stress conditions. Treatment with H2O2 significantly reduced the protein levels of Nrf2 and HO-1 by 80.5% and 55.8%, respectively (Figure 4A, and F), demonstrating that H2O2 suppressed the antioxidant response. However, WPH (50, 100, and 200 mg/mL) increased the protein levels of Nrf2 (2.0-, 2.2-, and 4.4-fold, respectively) and HO-1 (2.3-, 2.7-, and 4.1-fold, respectively, Figure 4A and G) compared with the control group (CON). In addition, nuclear Nrf2 protein increased by over 2.1-fold with the WPH treatment (Supplementary Figure 3). These results indicate that WPH enhances the antioxidant response under oxidative stress conditions.

**Effects of WPH on the mitogen-activated protein kinase (MAPK) signaling pathway in HT22 cells**

The effects of WPH on the MAPK signaling pathway under oxidative stress conditions were evaluated. Hydrogen peroxide increased the protein expression of p-ERK, p-JNK, and p-p38, which are key components of the MAPK signaling pathway, compared with the normal group (NOR) (3.53-fold, 4.14-fold, and 3.01-fold, respectively; \( P < 0.01 \), Figure 4G-I). Whey protein hydrolysate treatment decreased the protein levels of these MAPKs in a dose-dependent manner. Contrarily, 50, 100, and 200 mg/mL WPH decreased p-ERK expression by 19.1%, 31.3%, and 53.5%, p-JNK expression by 58.7%, 64.5% and 74.7%, and p-p38 expression by 41.2%, 46.3%, and 58.3%, respectively, compared with the H2O2-treated control group (\( P < 0.01 \), Figure 4G-I). These results indicate that WPH effectively suppresses oxidative stress-induced activation of MAPKs.

**Isolation and purification of WPH**

Neuroprotective compounds derived from WPH were isolated and identified using chromatography and tandem mass spectrometry, respectively. The Q-Sepharose HiLoad 16/10 ion chromatography analysis column and 25 mM Tris-Cl (pH 8.0) solvent were used for WPH analysis. The results revealed 7 distinct fractions (EX-1–EX-7; Figure 5A). Among these fractions, the EX-4 fraction showed the most significant reduction in Ca\(^{2+}\) concentration in H2O2-treated HT22 cells (36.2%) compared with the control group (CON) (Figure 5B). Further fractionation of EX-4 using the Superdex peptide 10/30 column generated fractions S14 to S21 (Figure 5C). Among these fractions, S16 demonstrated the most pronounced decrease in Ca\(^{2+}\) concentration (40.0%) (Figure 5D). The amino acid sequence of peptides derived from the S16 fraction was obtained using tandem mass spectrometry and found the LDIQK peptide with 99% confidence (Figure 5E and F). This peptide corresponded to the sequence f26–30 of β-lactoglobulin. Furthermore, HPLC analysis of LDIQK in WPH at a retention time of 10.4 min (Supplementary Figure 4) showed that the LDIQK content was 14.2 ± 0.7 mg/g (Supplementary Table 1).

**Effect of LDIQK on H2O2-induced apoptosis in HT22 cells**

To confirm whether WPH-derived LDIQK was an active substance, its effect on apoptosis markers and Ca\(^{2+}\) influx in H2O2-treated HT22 cells was evaluated. LDIQK (20 and 50 mg/mL) significantly decreased the BAX/Bcl-2 ratio (30.4% and 60.9%, respectively) compared with the control group (CON) (\( P < 0.001 \), Figure 6A and B). Similarly, LDIQK significantly decreased H2O2-induced Ca\(^{2+}\) influx. In particular, 10 mg/mL LDIQK treatment showed a 45.9%-reduction in Ca\(^{2+}\) influx compared with the control group (CON) (\( P < 0.05 \), Figure 6A and C). These results suggest that the LDIQK peptide is an active substance in WPH with neuroprotective effects.
Figure 4. Effect of whey protein hydrolysate on the apoptosis response in hydrogen peroxide-treated HT22 cells. NOR: normal, CON: control, WPH: whey protein hydrolysate. HT22 cells of all groups except the NOR group were treated with 300 µg/mL hydrogen peroxide. Data are presented as the mean ± SD *P < 0.05, ***P < 0.001 vs. NOR, and *P < 0.05, **P < 0.01, ###P < 0.001 vs. CON by Tukey’s multiple test.
DISCUSSION

In this study, we assessed the neuroprotective effects of whey protein hydrolysate (WPH) on HT22 cells in H₂O₂-induced oxidative stress. In the context of neurodegeneration, oxidative stress, which is characterized by excessive ROS production, plays a pivotal role in inducing neuronal cell death. Oxidative stress activates apoptotic signaling pathways, which subsequently trigger programmed cell death. This process is the central mechanism of neuronal cell death (Ryter et al., 2007; Wang and Qin, 2010). Hydrogen peroxide serves as a signal transmitter to regulate cell functions; however, as it is easily oxidized, it can be converted into hydroxyl radicals, which induce oxidative stress, ROS generation, and an increase in intracellular Ca²⁺ levels (Sies, 2017). Therefore, H₂O₂ is extensively used in vitro and in cellular systems to analyze various pharmacological activities, such as drug efficacy or cellular responses, under controlled oxidative stress conditions (Sies, 2017). Additionally, its application provides valuable insights into the effect of oxidative stress or antioxidants on different cellular processes (Sies, 2017). In the case of oxidative stress-induced neuronal damage, free radicals affect...
essential cellular components by peroxidizing lipids, oxidizing proteins, and inducing DNA strand breaks, all of which ultimately cause cell death (Farooqui and Farooqui, 2009). Notably, in this study, WPH inhibited neuronal cell death by suppressing ROS production, which can induce cellular damage. Oxidative stress, strongly associated with Ca²⁺ influx, induces the release of calcium from intracellular components, such as endoplasmic reticulum and mitochondria, thereby increasing the levels of intracellular calcium (Feno et al., 2019). Elevated intracellular calcium levels cause the activation of calcium-dependent enzymes that generate ROS as byproduct (Görlach et al., 2015). In addition, both oxidative stress and abnormal Ca²⁺ influx can induce mitochondrial dysfunction, which further increases oxidative stress, disrupts calcium homeostasis, and creates a feedback loop (Sies, 2017). Calcium ions play an important role as a trigger for the initiation of apoptosis, and elevated cytoplasmic calcium levels promote the permeabilization of mitochondria with the release of pro-apoptotic factors (Sies, 2017).

Bax is a pro-apoptotic factor located in the mitochondrial outer membrane. When activated, it forms pores or channels, through which pro-apoptotic factors, such as cytochrome c, are released from the intermembrane space of mitochondria into the cytoplasm. The release of cytochrome C triggers the activation of caspases, which are proteases that break down cellular proteins, leading to cellular dismantling (Elena-Real et al., 2018). On the contrary, anti-apoptotic proteins, such as Bcl-2, play a crucial role in maintaining mitochondrial permeability and the stability of mitochondrial membrane barrier, preventing the release of pro-apoptotic factors, including cytochrome (Bock and Tait, 2020). Therefore, induction and inhibition of apoptosis are dependent on the balanced expression of Bax and Bcl-2 family proteins. In previous studies, WPH, which was produced through whey protein hydrolysis using trypsin, inhibited apoptosis by downregulating Bax and upregulating Bcl-2 in PC12 cells (Jin et al., 2013). Similarly, this study demonstrated that WPH significantly reduced Bax protein level and increased Bcl-2 protein level in oxidative stress-induced HT22 cells (Figures 4A and 6A). This WPH-mediated regulation of the BAX/BCL2 ratio suppressed neuronal cell death. Additionally, the present study identified LDIQK as a WPH-derived active peptide with apoptosis inhibitory effect.

Nuclear factor erythroid 2-related factor 2 and HO-1 are important signaling molecules that respond to oxidative stress. In particular, Nrf2 is a transcription factor that regulates cellular defense mechanisms against oxidative stress or harmful substances (Balogun et al., 2003). Nrf2 activation upregulates antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, which enable cells to counteract oxidative stress or detoxify toxic compounds (Ngo and Duennwald, 2022). HO-1, one of the downstream targets of Nrf2, catalyzes the breakdown of heme, a hemoglobin molecule, into biliverdin, carbon monoxide, and iron (Gao et al., 2022). These byproducts have antioxidant, anti-inflammatory, and cytoprotective effects (Kundu et al., 2014). Activated Nrf2 promotes the gene expression of various antioxidant enzymes and detoxifying proteins, including HO-1, by translocating to the nucleus and binding to the antioxidant response element of target genes. As a result, Nrf2 activation increases the expression of HO-1. Here, WPH increased the expression of both HO-1 and Nrf2 proteins, indicating that WPH enhances the antioxidant response against oxidative stress (Figure 4E and F). WPH-mediated activation of Nrf2/HO-1 contributes to the reduction of ROS generation (Figure 2). Many studies have reported that food-derived components activate the Nrf2/HO-1 signaling pathway. Brassinin, a brassica-derived compound, activated Nrf2 and its

Figure 6. Effect of LDIQK on the apoptosis response in hydrogen peroxide-treated HT22 cells. NOR: normal, CON: control, WPH: whey protein hydrolysate. HT22 cells of all groups except the NOR group were treated with 300 µg/mL hydrogen peroxide. Data are presented as the mean ± SD *P < 0.05, ***P < 0.001 vs. NOR and §P < 0.05, §§P < 0.01, §§§P < 0.001 vs. CON by Tukey’s multiple test.
targets, HO-1 and NQO1, to suppress inflammatory responses (Kang et al., 2019). Moreover, Kim et al. (2019) reported that the feverfew-derived phytochemical parthenolide ameliorated obesity-induced oxidative stress/inflammatory response via the Nrf2-keap1 signaling pathway. Collectively, these studies demonstrate that the antioxidant response is associated with the anti-inflammatory response. Moreover, Nrf2 activation can counteract pro-inflammatory signals by reducing oxidative stress, which is known to trigger the inflammatory response (Gao et al., 2022). In particular, Nrf2 activation reduces the release of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and regulates the expression of NF-κB, a master inflammatory protein that promotes inflammatory cytokine production. In addition, HO-1 and its metabolites, such as carbon monoxide and biliverdin, suppress the expression of NF-κB and its target inflammatory mediators (Gao et al., 2022).

Oxidative stress can activate the MAPK transduction system, which plays a pivotal role in relaying external stimuli into intracellular responses. It comprises 3 distinct families: ERK, p38, and JNK (Choi et al., 2006). These MAPKs present different regulatory actions in apoptosis depending on the cell type and stimuli. JNK and p38 MAPK cascades are known to regulate the apoptotic pathway; they promote cell apoptosis by inducing BAX activation and transmission from the mitochondria, but inhibiting BCL2 function. However, these MAPKs also mediate the anti-apoptotic process. ERK and JNK activation can suppress UVB-induced apoptosis in keratinocytes (Moriyama et al., 2017). In addition, JNK activation ameliorates endoplasmic reticulum stress-induced cell death (Brown et al., 2016). In this study, oxidative stress activated MAPKs, but WPH inhibited MAPK activation by suppressing their phosphorylation. Nrf2 activation has been shown to inhibit the activation of JNK and p38 (Chaiprasongsuk et al., 2017), which in turn contributes to the suppression of inflammatory and apoptotic responses. This is consistent with our results indicating an association between WPH-mediated Nrf2 activation and MAPK inhibition.

In addition to hippocampal cell death, cognitive dysfunction is associated with decreased BDNF expression, hyperphosphorylation of tau protein, and reduced ACh production. The nerve growth factor BDNF plays a vital role in the growth, development, plasticity, and survival of nerve cells (Alonso et al., 1996; Luikart et al., 2008). In the hippocampal region of the brain, aging decreases neuroplasticity and neurogenesis, leading to decreased learning, memory, and cognitive function. This phenomenon further contributes to the development of neurodegenerative diseases, such as AD (Long et al., 2009). In addition to reduced BDNF levels, elevated oxidative stress is one of the factors that causes neurodegenerative diseases. BDNF has a protective role against oxidative stress owing to its antioxidant properties (Wang et al., 2020). Here, WPH significantly increased BDNF protein levels in HT22 cells under oxidative stress conditions, suggesting that WPH has the potential to protect neurons from degeneration.

Tau is a microtubule-associated protein primarily expressed in the central nervous system, particularly in neuronal axons, and functions as a microtubule stabilizer (Drechsel et al., 1992). Abnormal structure of the tau protein owing to hyperphosphorylation leads to neurofibrillary tangle formation, which is a hallmark of AD. The aggregation of hyperphosphorylated tau disrupts BDNF transport and signaling within neurons, affecting neuronal functions (Rosa et al., 2016) and eventually causing cognitive decline and memory impairment. Oxidative stress induces tau protein hyperphosphorylation, resulting in its detachment from microtubules and aggregation into neurofibrillary tangles. In addition, the accumulation of tau aggregates impairs mitochondrial functions, leading to an increase in ROS generation (Haque et al., 2019). The generation of ROS is associated with tau protein aggregation, and therefore contributes to progressive neurodegeneration. We showed that WPH significantly reduced tau phosphorylation (Figure 4D), indicating its protective role against neuronal dysfunctions (Figure 4D).

The neurotransmitter ACh is used by all cholinergic neurons and plays a crucial role in both the peripheral and central nervous systems. Acetylcholinesterase degrades ACh in the synaptic cleft, while the ChAT enzyme synthesizes acetylcholine and is an indicator of cholinergic neuron function (Ferreira-Vieira et al., 2016). In AD, reduced ChAT activity in the cerebral cortex and hippocampus is correlated with the severity and duration of dementia, thus serving as a potential basis for therapeutic intervention. Augmenting ChAT activity in the forebrain cortex and hippocampus halts the progression of mild AD to severe AD (DeKosky et al., 2002). This is because ACh protects neurons from oxidative damage by reducing oxidative stress via the inhibition of ROS production and an increase in the activity of antioxidant enzymes (Sun et al., 2014). Here, WPH increased ChAT levels in HT22 cells in oxidative stress, indicating that WPH can recover cholinergic functions of neurons in oxidative stress (Figure 4B).

We isolated and identified the peptide LDIQK as an active compound capable of protecting neuronal cells from oxidative stress. Numerous studies have highlighted the diverse biological activities of bioactive peptides derived from various sources. Bioactive peptides can be acquired using different methods, including enzyme
hydrolysis, microbial fermentation, chemical synthesis, and recombinant production (Akbarian et al., 2022). Milk protein is an ideal source of bioactive peptides, and they exhibit various biological effects, such as antibacterial, antioxidant, anti-hypertensive, and immunomodulatory effects. In addition, milk-derived opioid peptides exhibit morphine-like activity in the central nervous system (Akbarian et al., 2022). Additionally, milk peptides show therapeutic effect against SARS-CoV-2 (Pradeep et al., 2021). Among the biological activities of peptides, antioxidant activity against free radicals or ROS is widely recognized. Peptides with antioxidant activity typically consist of 5–16 amino acids and have a molecular size ranging from 500 to 1500 Da (Akbarian et al., 2022). Antioxidant peptides derived from a food source are considered safe and beneficial owing to low molecular weight, cost-effectiveness, and easy absorption. Moreover, these peptides may exhibit neuroprotective activities as they can effectively inhibit oxidative stress. Furthermore, numerous studies have reported peptides derived from natural sources with neuroprotective effects. Considering that certain neurotransmitters and neurological factors are peptides, it is reasonable to assume that food-derived bioactive peptides may exhibit neuroprotective effects. For example, WPH regulated matrix metalloproteinase activity and mitochondrial membrane permeability to protect brain cells from oxidative stress (Jin et al., 2013). Li et al. (2016) showed that peptides derived from soybean, walnut, and peanut following papain-mediated hydrolysis exhibit neuroprotective effects via the inhibition of apoptosis and improvement in impaired memory. These peptides commonly consist of hydrophobic amino acids, with Asp and Glu residues at the N-terminal, and have a low molecular weight (below 1 kDa) (Akbarian et al., 2022). These characteristics of peptides with neuroprotective effects align with those of LDIQK, which was isolated in this study. However, although the inhibitory effect of LDIQK on apoptotic markers and calcium influx have been confirmed (Figure 6), further studies on LDIQK are warranted to confirm its neuroprotective effects. Moreover, this study lacks animal- and clinical-based data. Therefore, experiments involving animal or clinical models to validate the neuroprotective effects of WPH or LDIQK are crucial for gaining a more comprehensive understanding. The present study not only highlighted the inhibitory effects of LDIQK on calcium influx but also demonstrated comparable or lesser effects in the other peptide fractions from WPH. This suggests the potential neuroprotective properties of these alternative fractions. Therefore, delving into the specific mechanisms governing their ability to inhibit calcium influx or apoptosis could yield valuable insights into their neuroprotective potentials. In addition, as a bioactive peptide can exhibit multiple biological activities (Akbarian et al., 2022), LDIQK should be analyzed for other potential biological activities, such as antimicrobial, anti-inflammatory, anti-hypertensive, immunomodulatory, and anti-aging activities.

CONCLUSIONS

Whey protein hydrolysate exhibited a protective effect against neuronal cell damage. Whey protein hydrolysate effectively suppressed oxidative stress-induced ROS production and calcium influx. Furthermore, WPH significantly reduced the BAX/BCL-2 ratio and the expression of MAPKs and p-tau, but increased protein levels of Nrf2, HO-1, BDNF, and ChAT. The neuroprotective effects of WPH may be attributed to the pentapeptide, LDIQK. Collectively, both WPH and its active compound LDIQK hold substantial potential as neuroprotective agents for the management of neurodegenerative diseases.

ACKNOWLEDGMENTS

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (IPET) through (High Value-added Food Technology Development Program), funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (N0.322011-4).

REFERENCES


Rosa, E., S. Mahendram, Y. D. Ke, L. M. Ittner, S. D. Ginsberg, and M. Fahnstock. 2016. Tau downregulates BDNF expression in ani-
Chang et al.: Neuroprotective effect of whey protein hydrolysate


ORCIDS

Yeok B. Chang © https://orcid.org/0000-0002-2445-5750
Hyung J. Suh © https://orcid.org/0000-0001-8869-3929
Hyeon-Son Choi © https://orcid.org/0000-0002-0165-7886