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Research Article

Antioxidative and neuroprotective effects of phosvitin phosphopeptides against H_2O_2-induced oxidative stress in SH-SY5Y cells

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Abstract: The antioxidant and protective effects of phosvitin phosphopeptides (PPPs) in human neuroblastoma were studied. Phosvitin from chicken egg yolk was pretreated using high-temperature mild pressure (HTMP) conditions and then hydrolyzed using trypsin (T), Multifect 14L (M), and trypsin + Multifect 14L (TM) to prepare 4 PPPs treatments (HTMP, HTMP-T, HTMP-M, and HTMP-TM). The antioxidant and protective properties of PPPs were assessed using chemical analyses and human neuroblastoma (SH-SY-5Y) cells. All PPPs exhibited reducing power and radical scavenging, metal-chelating, β-carotene bleaching inhibitory, and lipid peroxidation inhibitory activities, but HTMP-TM had the highest antioxidant activity. HTMP-TM increased cell viability and reduced reactive oxygen species production and apoptosis in stressed (H_2O_2-treated) SH-SY-5Y cells. Additionally, HTMP-TM downregulated B-cell lymphoma-2 (Bcl-2)-associated X protein/Bcl-2 and caspase-3 expression but upregulated catalase expression in oxidatively stressed SH-SY-5Y cells. It was concluded that HTMP-TM exerted protective effects through their antioxidative and anti-apoptotic properties in oxidatively stressed SH-SY-5Y cells.

Keywords: phosvitin phosphopeptides; antioxidant activity; neuroblastoma cell; protective effect; anti-apoptotic properties

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1 Introduction

The incidence of neurodegenerative diseases, such as Alzheimer's and Pakinsons's diseases, memory impairment, anxiety, and depression, is growing as the average lifespan of humans increases[1]. Oxidative stress-induced cell damage is closely related to neurodegenerative disorders[2]. High oxygen and unsaturated fatty acid concentrations increase lipid peroxidation and oxidative stress in the nervous systems[3]. Reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH), are constantly generated in the organs and can cause oxidative stress-induced cell death, especially in the brain, during metabolic processes[4–5]. Excessive ROS can induce deoxyribonucleic acid mutation, protein damage, and membrane phospholipid oxidation, which can result in disrupting cellular functions and lead to various diseases[4]. ROS lowers mitochondrial membrane potential (MMP), which initiates cell-apoptosis signaling pathways and releases cytochrome c and several pro-apoptotic factors, such as B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), which activate downstream effectors, such as caspase-3[6–7]. Our body tries to maintain redox homeostasis as a normal antioxidant defense mechanism. However, the antioxidant enzymes in our body, such as catalase, superoxide dismutase, and glutathione peroxidase, which remove ROS, are not sufficient to protect the cells from oxidative damage[3,8]. Therefore, dietary intake of antioxidant materials is necessary to alleviate oxidative stress. Recently, the demand for natural antioxidants has increased significantly to address oxidative stress in our bodies[4,9–10].

The natural antioxidants derived from food proteins can be easily digested, absorbed, and excreted[11]. The gastrointestinal (GI, pepsin, trypsin, and chymotrypsin) and non-GI (papain, alcalase, neutrase, and thermolysin) proteases in the stomach and small intestine can hydrolyze food proteins and produce bioactive peptides, which can exhibit diverse biological activities depending upon their sizes, amino acid sequences, and compositions[11–14]. Moreover, the peptides produced by multiple proteinases can higher functional activity than those produced by a single enzyme. Studies indicated that hydrolysates with antioxidant activities obtained from *Pleurotus geesteranus*, grass carp skin, and whey have peptides with cytoprotective effects[4,15–16]. Also, the tryptic hydrolysis of phosvitin produced phosphopeptides that protected human intestinal epithelial cells from H_2O_2-mediated oxidative damage[17].

The egg is rich in proteins, lipids, vitamins, and minerals. Phosvitin, the major phosphoprotein in egg yolk, has a molecular weight of 30–45 kDa[17–18] and is composed of 217 amino acids, of which 128 are phosphorylated (123 serines, 4 threonines, and 1 tyrosine). Serine accounts for 57% of the total amino acid residues in phosvitin, and almost all of them are phosphorylated. Phosphopeptides are known to have very strong antioxidant activity. Phosvitin is the best substrate for phosphopeptide production[18]. Phosvitin is extremely difficult to hydrolyze with enzymes because its negatively charged phosphate groups act as

shields, preventing enzymes from accessing its peptide bonds. However, using the high-temperature mild pressure (HTMP) pretreatment, Huang et al.[19] successfully cleaved phosvitin into random oligopeptides, which helped further breakdown of the oligopeptides into small phosphopeptides using enzymes.

Although phosvitin phosphopeptides (PPPs) are reported to have excellent potential as calcium delivery agents, antioxidants, anticancer agents, anti-caries agents, and immune modulators, studies on their preparation and functional activities are quite limited, mainly because the hydrolysis of phosvitin was difficult until recently. Recently, Cui et al.[20] isolated PPPs from phosvitin hydrolysates and identified a novel bifunctional peptide that reduces oxidative stress and promotes calcium uptake into cells. Some PPPs isolated from phosvitin hydrolysates showed anticoagulant effects[21], while others demonstrated excellent calcium-binding capacity and promoted osteogenic differentiation in MC3T3-E1 cells[22]. Lee et al.[23] reported that PPPs significantly inhibited elastase activity and inhibited nitric oxide production in the lipopolysaccharide stimulated RAW264.7 macrophages. Lee et al.[24] studied the immunomodulatory effect of PPPs in forced swimming-induced immune defunct BALB/c mice and found that PPPs enhanced the natural killer cell cytotoxicity and exerted potent immunomodulatory effects.

This study determined the antioxidant activities of PPPs on the H_2O_2-induced cell damage in human neuroblastoma SH-SY5Y cells.

2 Materials and methods

2.1 Materials

Phosvitin was prepared from chicken egg yolk using the method that we have developed[25]. Multifect 14L (food grade, mainly thermolysin, 160 U/g) was purchased from Genencor (Rochester, NY, USA). Trypsin type I from bovine pancreas (EC3.4.21.4, ~15 500 U/mg prot), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), linoleic acid, ethanol, and H_2O_2 were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.2 Sample preparation

A 5% (*m*/*m*) lyophilized phosvitin solution was pretreated under HTMP conditions (121 °C and 151.99 kPa for 30 min)[19]. The HTMP-pretreated phosvitin was hydrolyzed using trypsin (pH 8.0, 40 °C), Multifect 14L (pH 8.0, 70 °C), and trypsin + Multifect 14L for 6 h, for each enzyme, in a water bath. The enzyme to substrate ratio was 1:50 (*m*/*m*). In the two-step hydrolysis, the second enzyme hydrolysis was performed after inactivating the first enzyme in a boiling water bath for 10 min. The hydrolysates were abbreviated as HTMP (HTMP pretreatment alone), HTMP-M (HTMP pretreatment then Multifect 14L hydrolysis), HTMP-T (HTMP pretreatment then trypsin hydrolysis), and HTMP-TM (HTMP pretreatment then trypsin and Multifect 14L hydrolysis in sequence). Trypsin and Multifect 14L were selected for the enzymatic hydrolysis of phosvitin because our previous study with various enzymes and enzyme combinations indicated they produced the highest hydrolysis[19].

2.3 Radical scavenging activity determination

2.3.1 DPPH radical scavenging activity

Aqueous PPPs solutions (0.5 mL, 1 and 2 mg/mL) were mixed with the same volume (0.5 mL) of DPPH solution (24 mg in 100 mL methanol), diluted to an $OD_{517\ nm}$ of 1.20 ± 0.05, and incubated at 37 °C in the dark. After 60 min, the absorbance was measured at 517 nm. Ascorbic acid (AA; 1 mg/mL) was used as a positive control[10]. DPPH radical scavenging activity was calculated by equation (1):

$$\text{Radical scavenging activity}\,(\%) = \left(1 - \frac{OD_{Sample}}{OD_{Control}}\right) \times 100 \quad (1)$$

2.3.2 ABTS cation radical scavenging activity

The ABTS stock solution containing 7 mmol/L ABTS and 2.5 mmol/L potassium persulfate was prepared and incubated in the dark for 18 h. The ABTS stock solution was diluted to an $OD_{734\ nm}$ of 0.70 ± 0.02 using 0.2 mol/L sodium phosphate buffer (pH 7.4) before use. Then, 0.1 mL of PPPs solution (1 and 2 mg/mL) was mixed with 0.9 mL of diluted ABTS solution and incubated at 37 °C for 10 min in the dark, and the absorbance was measured at 734 nm. AA (1 mg/mL) was used as a positive control[26]. ABTS cation radical scavenging activity was calculated by equation (1).

2.4 Metal-chelating activity determination

2.4.1 Cu^{2+}-chelating activity

The Cu^{2+}-chelating activity of PPPs was measured according to the method of Gallegos-Tintoré et al.[27] with modifications. Thirty microliters of PPPs solution (1 and 2 mg/mL) were added to a 96-well microplate well containing 250 μL of $CuSO_4$ (10 μg/mL $CuSO_4$ dissolved in 50 mmol/L sodium acetate buffer, pH 6.0) and 6.25 μL of 4 mmol/L pyrocatechol violet. The absorbance was measured at 632 nm after 10 min, with ethylenediaminetetraacetic acid (EDTA, 1 mg/mL) as a positive control[21]. Cu^{2+}-chelating activity was calculated by equation (2):

$$\text{Chelating activity}\,(\%) = \left(1 - \frac{OD_{Sample}}{OD_{Control}}\right) \times 100 \quad (2)$$

2.4.2 Fe^{2+}-chelating activity

Two hundred microliters of PPPs solution (1 and 2 mg/mL) were added to a 96-well microplate well containing 50 μL of 100 mmol/L sodium acetate buffer (pH 4.9) and 25 μL of 600 μmol/L $FeCl_2$ solution. After 15 min, 12.5 μL of 40 mmol/L ferrozine solution was added to the mixture, and the absorbance at 562 nm was measured. EDTA (1 mg/mL) was used as a positive control[21]. Fe^{2+}-chelating activity was calculated by equation (2).

2.5 Reducing power determination

Two hundred microliters of PPPs (1 mg/mL), 200 μL of phosphate-buffered saline (PBS), and 200 μL of 1% (*m*/*m*) potassium ferricyanide solution were mixed and reacted for 20 min in a 50 °C-water bath. Subsequently, 200 μL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3 000 × *g* for 10 min. The upper layer (200 μL) was mixed with 200 μL of 0.1% (*m*/*m*) ferric chloride solution and reacted for 10 min. The absorbance of the reaction mixture was read at 700 nm. The reducing power was calculated based on the AA standard curve[10].

2.6 Lipid oxidation inhibitory activity determination

2.6.1 Preparation of reaction mixture

The reaction mixture was prepared by mixing PPPs solution (1.948 mL, 1 mg/mL), linoleic acid (1% (*V*/*V*), 52 μL), anhydrous

ethanol (4 mL), and sodium phosphate buffer (4 mL, 50 mmol/L, pH 7.0) in a test tube according to Sarbon et al.[28]. The mixture was incubated at 37 °C in the dark for up to 9 days and used for the ferric thiocyanate (FTC) assay and the thiobarbituric acid reactive substances (TBARS) assay. Butylated hydroxytoluene (BHT; 1 mg/mL) was used as a positive control.

2.6.2 FTC assay

An aliquot (25 μL) of the reaction mixture, 1.175 mL of ethanol (70%), 25 μL of ammonium thiocyanate (30%), and 25 μL of ferrous chloride (20 mmol/L) solutions were mixed in a test tube. The absorbance at 500 nm was measured at 24 h intervals until the control reached its maximum absorbance. The FTC inhibitory activity of PPPs was calculated by equation (3):

$$\text{Inhibition activity}\,(\%) = \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100 \quad (3)$$

2.6.3 TBARS assay

The reaction mixture (50 μL), distilled water (0.8 mL), 20% acetic acid (pH 3.5, 1.5 mL), sodium dodecyl sulfate (8.1%, 0.2 mL), and 0.8% 2-thiobarbituric acid (1.5 mL) solutions were transferred to a test tube, heated at 90 °C for 60 min, and cooled to room temperature. The solution was centrifuged at 4 000 × *g* for 10 min, and the absorbance was read at 532 nm. The TBARS inhibitory activity of PPPs was calculated by equation (3).

2.6.4 β-Carotene bleaching inhibitory activity determination

β-Carotene (1.5 mg) was dissolved in a solution containing 33 μL of linoleic acid, 150 μL of Tween-80, and 10 mL of chloroform. The chloroform was evaporated, and a β-carotene solution with $OD_{470\,nm}$ of 2.0 ± 0.1 was prepared using distilled water. One hundred microliters of PPPs (1 mg/mL) and 1 mL of β-carotene solution were mixed, incubated at 50 °C for 150 min in a water bath, and the absorbance was read at 470 nm at a 30 min interval[26]. The β-carotene bleaching inhibitory activity of PPPs was calculated by equation (3).

2.7 Cell culture

The human neuroblastoma SH-SY5Y cell line (ATCC CRL-2266) was obtained from the American Type Culture Collection and cultured with Dulbecco's Modified Eagle medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% streptomycin/penicillin solution (Hyclone) at 37 °C in a 5% CO_2 incubator.

2.8 Cell viability determination

The SH-SY5Y cells (1 × 10^5 cells/well) were seeded in a 96-well plate, incubated at 37 °C for 24 h, and then treated with PPPs (0.25, 0.5, 1, and 2 mg/mL). After 24 h, thiazolyl blue tetrazolium bromide (MTT) solution (20 μL, 2 mg/mL) was added to each well. The plate was incubated for 4 h. After removing the medium, the formazan crystals in the well were dissolved using 100 μL of dimethyl sulfoxide (DMSO), and the absorbance was read at 570 nm. A similar analysis was performed to assess the cytotoxicity of H_2O_2 (100–400 μmol/L) on SH-SY5Y cells.

The cytoprotective effect of PPPs on H_2O_2-treated SH-SY5Y cells was determined as described below: the PPPs from HTMP-TM (0.25, 0.5, and 1 mg/mL) were added to SH-SY5Y cells 4 h before H_2O_2 (300 μmol/L) treatment, and the cells were incubated for 20 h. Finally, 20 μL of MTT solution (2 mg/mL) was added to each well, and the plate was incubated for 4 h. After removing the medium, the formazan crystals were dissolved with DMSO, and the absorbance was read at 570 nm using a microplate reader. Cell viability was calculated by equation (4):

$$\text{Cell viability}\,(\%) = \frac{OD_{Sample}}{OD_{Control}} \times 100 \quad (4)$$

Where control refers to a sample without PPPs.

2.9 Intracellular ROS accumulation determination

SH-SY5Y cells (8 × 10^5 cells/mL) were seeded in the wells of a 24-well plate, incubated for 24 h, pretreated with HTMP-TM (1 mg/mL) for 4 h, and incubated with or without H_2O_2 (300 μmol/L) for 2 h. Then, the cells were treated with 10 μmol/L 2′,7′-dichlorofluorescein diacetate at 37 °C for 30 min, and their images were observed using a fluorescence microscope (Nikon, Tokyo, Japan).

2.10 Detection of apoptosis by Hoechst 33342 staining

The SH-SY5Y cells were pretreated with HTMP-TM (1 mg/mL) for 4 h and cultured with or without H_2O_2 (300 μmol/L) for another 2 h. The cells were stained with Hoechst 33342 solution (5 μg/mL) for 30 min, and the apoptosis was observed under a fluorescence microscope.

2.11 Quantification of relative mRNA expression using quantitative real-time polymerase chain reaction (qRT-PCR)

The SH-SY5Y cells, pretreated with HTMP-TM (1 mg/mL) for 4 h, were cultured with or without H_2O_2 (300 μmol/L) for an additional 20 h, and the total RNA was isolated using the RNeasy® Mini kit (QIAGEN, Hilden, Germany). The RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA according to the manufacturer's instructions. The SYBR Green PCR Master mix with cDNA and primers was combined, and the qRT-PCR was run with the following programmed thermal cycle conditions: 95 °C for 10 min for polymerase activation, 40 cycles of 95 °C for 20 s, and 60 °C for 20 s. The circulation threshold (Ct) value was normalized to *GAPDH* housekeeping gene. The relative gene expression level was determined using the $2^{-\Delta\Delta Ct}$ method.

2.12 Detection of protein expression using Western blot

The SH-SY5Y cells were lysed using the radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA). Cell proteins were extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%). The separated proteins were transferred (for 1 h at 1 A at 4 °C) to a polyvinylidene fluoride membrane, incubated with the primary (Bax and Bcl-2) and the secondary (horseradish peroxidase-conjugated) antibody. The diluted (1:5 000) primary and secondary antibodies were incubated in blocking buffer for 1 h and 45 min, respectively, at room temperature. After incubation, the membrane was washed 3 times (10 min each) with 0.05% Tween-20 in PBS. The immunoreactive protein bands were exposed to X-ray film. GAPDH was used as the loading control.

2.13 Statistical analysis

The SPSS software version 18 (SPSS Inc.) was used for statistical analysis. The one-way analysis of variance was used for multiple groups. The results were expressed as the mean ± standard deviation (SD) of three replications. The Duncan's multiple range test was used to evaluate the significance level ($P < 0.05$).

3 Results and discussion

3.1 Radical scavenging and metal-chelating activities of PPPs

The radical scavenging activities of PPPs indicated that the HTMP (41.77%) had significantly lower DPPH radical scavenging activity than HTMP-M (45.48%), HTMP-T (46.80%), and HTMP-TM (45.42%) ($P < 0.05$) at 2 mg/mL (Figure 1A). The PPPs at 1 and 2 mg/mL, HTMP (1 mg/mL: 36.53% and 2 mg/mL: 57.67%) showed the lowest ABTS cation radical scavenging activities, and HTMP-TM (1 mg/mL: 61.20% and 2 mg/mL: 77.77%) showed the highest activities ($P < 0.05$, Figure 1B).

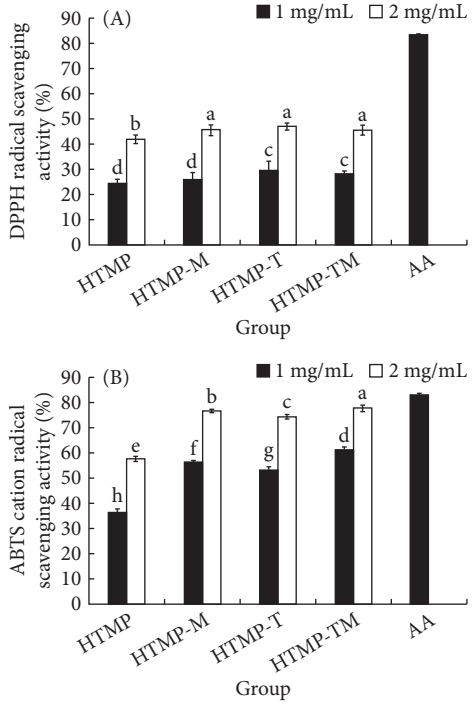

Figure 1 (A) DPPH radical and (B) ABTS cation radical scavenging activities of PPPs. AA (1 mg/mL) was used as a positive control. The results are expressed as the mean ± SD of independent experiments. Different lowercase letters on each bar indicate a statistically significant difference ($P < 0.05$).

The metal-chelating activities of PPPs (Figure 2) indicated that the HTMP-TM (1 mg/mL: 43.25% and 2 mg/mL: 51.13%) showed the highest Cu^{2+}-chelating activities and HTMP (1 mg/mL: 27.51% and 2 mg/mL: 36.68%) showed the lowest activities ($P < 0.05$). The HTMP-M (1 mg/mL: 38.42% and 2 mg/mL: 46.57%) showed similar Cu^{2+}-chelating activities to HTMP-T (1 mg/mL: 38.51% and 2 mg/mL: 46.48%) (Figure 2A). At 1 and 2 mg/mL, HTMP (1 mg/mL: 52.39%; 2 mg/mL: 55.11%) showed the lowest Fe^{2+}-chelating activity among the four treatments. At a 2 mg/mL content, HTMP-M, HTMP-T, and HTMP-TM exhibited chelating activities of 58.39%, 60.13%, and 61.26%, respectively (Figure 2B).

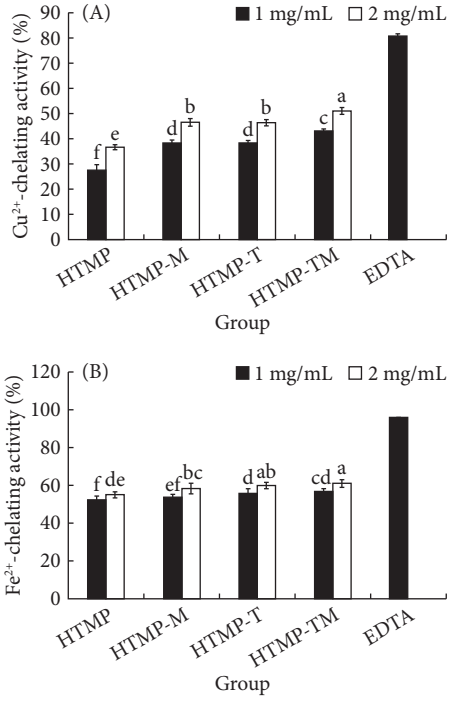

Figure 2 (A) Cu^{2+}-chelating and (B) Fe^{2+}-chelating activities of PPPs. EDTA (1 mg/mL) was used as a positive control. The results are expressed as the mean ± SD of independent experiments. Different lowercase letters on each bar indicate a statistically significant difference between values ($P < 0.05$).

Antioxidant materials quench DPPH radical and ABTS cation radical by transferring electrons or hydrogen atoms[8,29]. The heavy and transition metals can generate free radicals, which may cause oxidative stress. Thus, metals like copper, iron, and lead should be eliminated[8]. Among the metal ions, Fe^{2+} plays the most important role in producing ·OH through the Fenton reaction[29]. This study showed that all the PPPs produced (HTMP, HTMP-M, HTMP-T, and HTMP-TM) showed high metal-chelating abilities. As shown in Table 1, the reducing powers of HTMP, HTMP-M, HTMP-T, and HTMP-TM were 7.13, 9.48, 10.00, and 9.61 mg AAE/g, respectively, and the HTMP had the lowest reducing power ($P < 0.05$).

3.2 Lipid peroxidation inhibitory and β-carotene bleaching inhibitory activities of PPPs

The antioxidant activities of PPPs in the linoleic emulsion system suggested that oxidation was significantly inhibited by the PPPs,

Table 1 Reducing powers (mg AAE/g) of PPPs.

Sample	HTMP	HTMP-M	HTMP-T	HTMP-TM
Reducing powers	7.13 ± 1.25^{b}	9.48 ± 0.80^{a}	10.00 ± 0.90^{a}	9.61 ± 0.32^{a}

Note: The reducing powers were expressed as milligrams of ascorbic acid equivalent (AAE) per gram of PPPs (mg AAE/g). The results are expressed as the mean ± SD of independent experiments ($n = 3$). Different lowercase letters within a row indicate significant differences ($P < 0.05$).

regardless of the production method of PPPs. After 8 days of storage, PPPs' lipid peroxidation inhibitory activities were 34.28%–41.63% as measured by the FTC assay (Figure 3A). Lipid oxidation was also markedly inhibited by the PPPs, and the inhibitory activities of PPPs were 30.59%–33.88% after 8 days of storage (TBARS assay, Figure 3B). The TBARS in the control group decreased rapidly compared with those in the PPPs and BHT groups (Figure 3B). All four PPPs significantly inhibited β-carotene bleaching, but the HTMP treatment showed the lowest inhibitory activity ($P < 0.05$) (Figure 3C). The HTMP-TM showed high antioxidant activities and was selected for further studies on the Human Neuroblastoma (SH-SY5Y) cells.

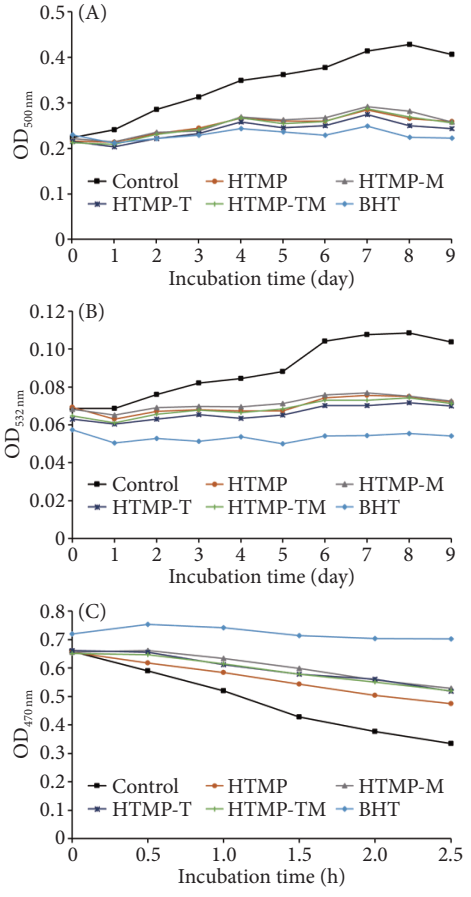

Figure 3 Lipid peroxidation measured by (A) FTC, (B) TBARS, and (C) β-carotene bleaching inhibitory activities of PPPs. BHT (1 mg/mL) was used as a positive control.

Excessive lipid peroxidation of cell membranes can cause cell injury[3]. Antioxidants inhibit hydroperoxides (the primary lipid oxidation products) in the fatty acid emulsion system. When hydroperoxides react with Fe^{2+}, they are degraded into secondary products, such as carbonyl compounds, and Fe^{3+} is produced. The condensation of 2-thiobarbituric acid with aldehydes such as malonaldehyde, one of the important secondary lipid oxidation products, is used to assess lipid oxidation[28]. The β-carotene bleaching assay involves decolorization of β-carotene by lipid peroxyl radicals. However, the decolorization of β-carotene is slowed in the presence of antioxidants[8]. Milk casein phosphopeptides scavenge peroxyl radicals and chelate transition metals[30]. Phosphorylation is involved in antioxidant activity due to the metal-binding properties and the phosphate group's hydrogen-atom transfer properties[31]. Also, the amounts of negatively charged, hydrophobic, basic, and acidic amino acids in the hydrolysate were related to antioxidant properties. The small peptides in the casein hydrolysate contained more basic and acidic amino acids, which played a key role in chelating metal ions[2,4].

Small peptides (< 3 kDa) are more bioavailable and readily digestible than intact proteins. Therefore, enzymatic proteolysis produces bioactive peptides from parent proteins and improves intestinal absorption. Bioactive peptides possess a variety of functionalities depending on their sequence, size, hydrophobicity, and composition[13,32]. Heat pretreatment and enzymatic hydrolysis are common methods for producing bioactive peptides from proteins[33]. Heating modifies protein structure, exposing hydrolysis sites previously inaccessible to enzymes[12]. The hydrolysis of proteins improves their functional activities, and hydrolysates produced by multiple enzymes showed higher functional activities than those produced by a single enzyme[33]. Due to its structural characteristics, phosvitin is extremely difficult to hydrolyze using enzymes. However, Huang et al.[19] developed novel HTMP pre-treatment-enzyme combinations to produce phosphopeptides from phosvitin. The HTMP pretreatment improved the subsequent enzymatic hydrolysis of phosvitin. In a previous study, the HTMP-pretreated phosvitin hydrolyzed with trypsin + Multifect 14L showed the highest hydrolysis, followed by trypsin and Multifect 14L alone[19]. In contrast, phosvitin with HTMP pretreatment alone showed the lowest hydrolysis level[10]. The antioxidant activity of bioactive peptides varies significantly depending upon the enzyme used for hydrolysis, amino acid sequences, and peptide sizes[34]. Therefore, the difference in antioxidant activities of PPPs could be related to the degree of breakdown of phosvitin into smaller peptides.

3.3 Effects of HTMP-TM and H_2O_2 on the viability of SH-SY5Y cells

The oxidative stress-induced cell death in the human neuroblastoma SH-SY5Y cell line is commonly used to assess products' neuroprotective properties *in vitro*, and this cell line is not restricted to specific neuronal disease research[35–36]. Undifferentiated SH-SY5Y cells are more appropriate for investigating neuroprotective effects, as differentiation may increase antioxidant enzyme activity. However, undifferentiated SH-SY5Y cells are highly susceptible to neuroprotective agents and simulate physiological neuron cell conditions[37–39]. Also, H_2O_2 was used to induce general oxidative stress, as this study focused on the cytoprotective effects of PPPs on oxidative stress-induced cell damage, not on any specific neuronal diseases[40].

HTMP-TM at up to 1 mg/mL had no cytotoxic effect on SH-SY5Y cells, as evaluated by the MTT assay (Figure 4A). The cell viability of the SH-SY5Y cells treated with 100–400 μmol/L H_2O_2 was almost half that of the control group at 300 μmol/L of H_2O_2 treatment (Figure 4B). Thus, H_2O_2 concentration at 300 μmol/L (IC_{50}) was chosen for the subsequent study.

The cytoprotective effects of HTMP-TM in H_2O_2-treated SH-SY5Y cells indicated that H_2O_2 at 300 μmol/L significantly reduced cell survival (Figure 4C): only 57.46% of the cells were viable. However, with HTMP-TM (0.25, 0.5, and 1 mg/mL) pretreatment for 4 h prior to H_2O_2 treatment, the cell viability increased significantly ($P < 0.05$). Co-treatment of the cells with the HTMP-TM (1 mg/mL) restored cell survival to 76.44%. The

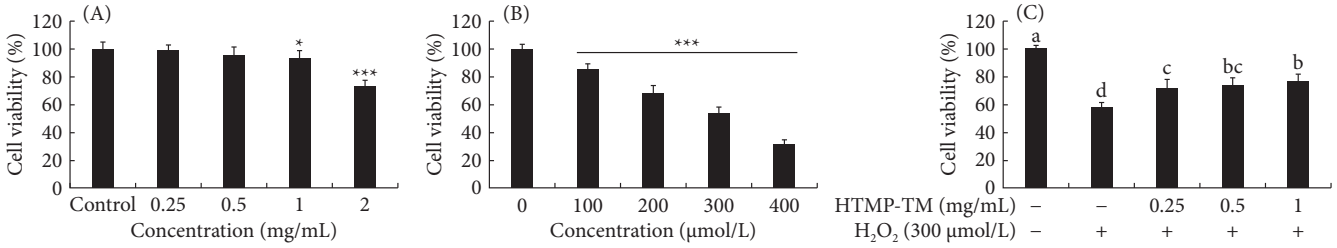

Figure 4 Effects of PPPs produced by the (A) HTMP and HTMP-TM, (B) H_2O_2, and (C) HTMP-TM + H_2O_2 on the viability of SH-SY5Y cells. H_2O_2 (300 μmol/L) was used to induce oxidative stress. The results are expressed as the mean ± SD of independent experiments. $^*P < 0.05$, $^{***}P < 0.001$ *vs.* control. Different lowercase letters on each bar indicate a statistically significant difference between values ($P < 0.05$).

H_2O_2-treated SH-SY5Y cells were shrunken, and their neurites disappeared, an effect attenuated by 1 mg/mL HTMP-TM pretreatment (Figure 4C).

3.4 Effects of HTMP-TM on the intracellular ROS production and cell apoptosis in H_2O_2-treated SH-SY5Y cells

The intracellular ROS production was examined to determine the effects of HTMP-TM on oxidative stress induced by H_2O_2. As shown in cell fluorescence images (Figure 5A), the 300 μmol/L H_2O_2 treatment of SH-SY5Y cells exhibited brighter green fluorescence than the control group because of higher intracellular ROS production. The HTMP-TM treatment of SH-SY5Y cells, followed by 300 μmol/L H_2O_2, attenuated the ROS production compared with H_2O_2 treatment alone. The nuclei of apoptotic cells were observed using the fluorescent dye Hoechst 33342. With H_2O_2, cell apoptosis is very clear. However, cell apoptosis significantly decreased when HTMP-TM was added to the H_2O_2-treated sample (Figure 5B).

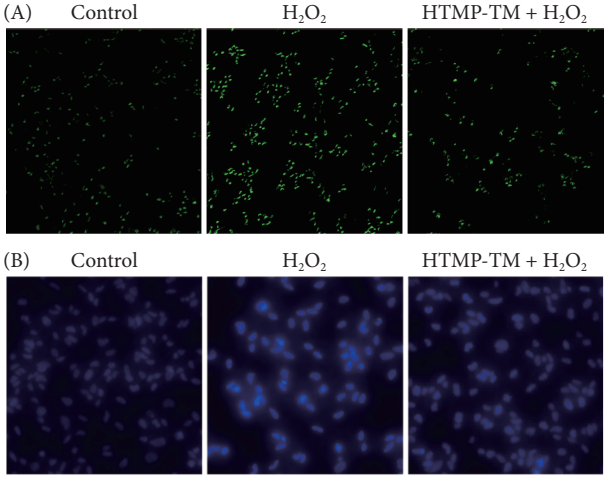

Figure 5 Effects of PPPs produced by HTMP and HTMP-TM on the (A) intracellular ROS production and (B) cell apoptosis in H_2O_2-treated SH-SY5Y cells.

3.5 Effects of HTMP-TM on the Bax, Bcl-2, caspase-3, and catalase expressions in H_2O_2-treated SH-SY5Y cells

To study the mechanisms underlying HTMP-TM's neuroprotective effects, qRT-PCR was used. The *catalase* mRNA expression of H_2O_2 group was significantly lower (0.59-fold) than in the control (Figure 6A). Co-treatment with HTMP-TM increased *catalase* mRNA levels (0.76-fold the control) compared with the H_2O_2-only group. Moreover, *caspase-3* mRNA expression was increased in the SH-SY5Y cells treated with H_2O_2 alone (2.19-fold of the control). With the HTMP-TM treatment, however, the *caspase-3* mRNA level decreased to 1.49-fold of the control in H_2O_2-treated SH-SY5Y cells. Similarly, the *Bax*/*Bcl-2* ratio of mRNA expression drastically increased by 2.23-fold of the control under the H_2O_2 treatment (Figure 6B). The *Bax*/*Bcl-2* ratio of mRNA expression was decreased to 1.33-fold of the control by the HTMP-TM, indicating that HTMP-TM treatment regulated Bax and Bcl-2 protein expressions (Figure 6C).

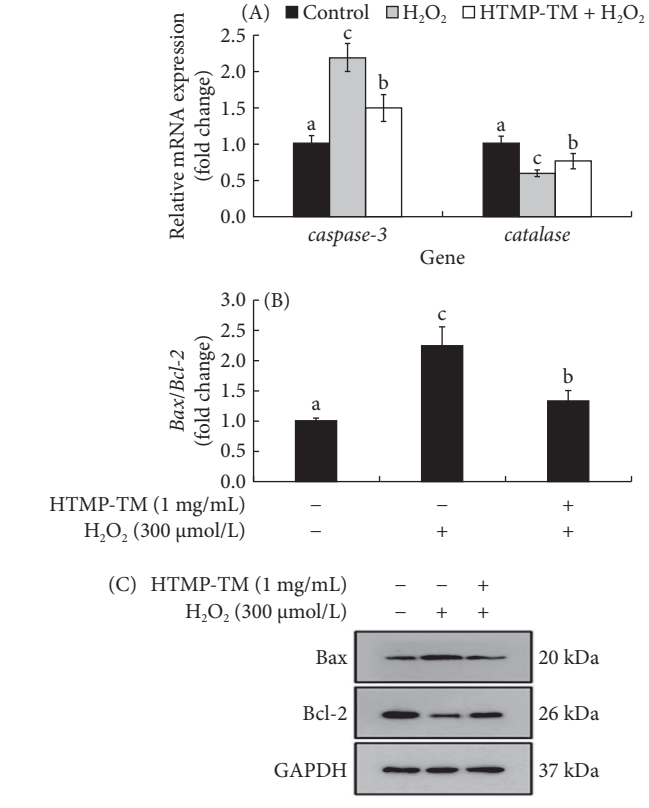

Figure 6 Effects of PPPs produced by HTMP and HTMP-TM on the (A) relative mRNA expression of *caspase-3* and *catalase*, (B) *Bax*/*Bcl-2* mRNA expression ratio, and (C) Bax and Bcl-2 protein expression in H_2O_2-treated SH-SY5Y cells. GAPDH was used as the loading control. The results are expressed as the mean ± SD of independent experiments. Different lowercase letters on each bar indicate a statistically significant difference between values ($P < 0.05$).

The overproduction of intracellular ROS by oxidative stress disrupts normal redox signaling[4]. Catalase reduces ROS production by converting H_2O_2 to water[1]. The Bcl-2 protein family is involved in the apoptotic process. The ratio of two major apoptosis-related proteins, Bcl-2 and Bax[1–2], is critical for evaluating

cell sensitivity to apoptotic stimuli[42]. Under oxidative stress, Bax, a cytosolic pro-apoptotic protein, translocates from the cytosol to mitochondria and forms Bax dimers. This apoptosis signaling pathway triggers ROS production, depolarizes MMP, and releases cytochrome c from mitochondria. In addition, caspase-3, one of the caspase family proteins in the cytosol, is activated by cytochrome c as the key executor of apoptosis[41]. However, Bcl-2 antagonizes pro-apoptotic proteins[5,43]. Bioactive peptides or hydrolysates with neuroprotective effects are reported to block apoptosis-related factors such as Bax protein, caspases, and factors associated with DNA fragmentation[44–45]. Lee et al.[11] reported that the hydrophobic fraction of whey protein hydrolysates reduced caspase-3 expression but increased Bcl-2 expression. Also, a peptide containing hydrophobic amino acids exerted neuroprotective effects via its antioxidant capacity, as evidenced by free radical scavenging activity that reduced ROS production[11]. Liu et al.[46] also reported that PPPs alleviate the oxidative stress on osteoblasts. Therefore, our results indicated that the HTMP-TM had anti-apoptotic properties in oxidatively stressed SH-SY5Y cells.

4 Conclusion

The PPPs exhibited reducing power, DPPH radical and ABTS cation radical scavenging, metal-chelating, β-carotene bleaching inhibitory, and lipid peroxidation inhibitory activities. The PPPs prepared by HTMP pretreatment-enzymatic hydrolysis combinations, especially the two-step hydrolysis, were significantly more effective in exerting antioxidant activity than those prepared by HTMP pretreatment alone. The HTMP-TM exhibited cytoprotective effects against H_2O_2-induced cytotoxicity, inhibiting ROS production and apoptosis in oxidatively stressed SH-SY5Y cells. The HTMP-TM exhibited cytoprotective effects against H_2O_2-induced cytotoxicity, inhibiting ROS production and apoptosis in oxidatively stressed SH-SY5Y cells. Therefore, PPPs are promising functional ingredients against H_2O_2-induced neurotoxicity. However, more *in vivo* studies are needed to demonstrate the value of PPPs as value-added food/nutraceutical ingredients. Also, functional activities, dosage, delivery route, and safety of the PPPs in animals or humans require more work.

Conflict of interest

The authors declared that there is no conflict of interest.

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Author contributions

Ji-Eun Lee: Writing original draft, methodology, formal analysis. Jae Hoon Lee: Methodology, validation, data curation. Dong Uk Ahn: Conceptualization, resources, writing review, editing. Kee-Tae Kim: Investigation, software, validation. Hyun-Dong Paik: Supervision, resources, writing review, editing.

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