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Antioxidant Activity of Crude Peptides Extracted from Dry-cured Jinhua Ham

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Abstract This study was aimed to investigate the protective effects of Jinhua ham peptide extract against oxidative damage to PC12 cells induced by H_2O_2. Crude peptide C (CPC) presented strong scavenging activities against DPPH radicals *in vitro*. Exposing PC12 cells to H_2O_2 reduced the cell viability, the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), while increased the cell apoptosis. Incubating cells with different concentrations of CPC (200 and 400 μg/mL) enhanced the cell viability and antioxidant enzyme activities. Intracellular reactive oxygen species (ROS) formation, caspase-3 activity and flow cytometric analysis revealed that CPC attenuated the cell apoptosis induced by H_2O_2. These cytoprotective effects of CPC on PC12 cells indicate that Jinhua ham extract might possess the ability to eliminate over-produced ROS in human.

Keywords: *Jinhua ham, antioxidant activity, cytoprotective effects, hydrogen peroxide-induced, PC12 cells*

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1. Introduction

ROS is the main free radicals in organisms mainly including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). [1] Oxidative stress occurs when an imbalance between ROS and antioxidants due to either the accumulation of ROS or the depletion of antioxidants. ROS and oxidative stress have a strong relationship with chronic disease such as coronary heart disease, cancer, and Alzheimer's disease. [2] Therefore, antioxidants have been extensively applied in the food system to increase the stability of lipid and proteins. [3] Currently, synthetic antioxidants are widely utilized including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ). [4] Despite the strong antioxidant activity, various potential side and toxic effects of synthetic antioxidants on the human enzyme system have been reported. [5,6] Therefore, pursuing for natural antioxidants to be applied in the food system has been a hot topic in recent years.

Jinhua ham is a dry-cured product that has increased in popularity due to its unique flavor and typical quality characteristics. [7] During the long ripening time, intense proteolysis was found in ham muscle in which around 10% of muscle proteins including soluble and insoluble proteins could be degraded. [8,9,10] Intense protein degradation results in the accumulation of peptides with different sizes and free amino acids at the end of dry-curing. [11]

Various studies have been conducted to look into antioxidant properties of natural peptides from food protein sources. For instance, antioxidant activity has been found in bluefin leatherjacket, chickpea, egg and fish by-products. [12,13,14,15] Escudero et al. [16] extracted water soluble antioxidant peptides from Spanish dry-cured ham. The peptide that showed high levels of radical-scavenging activity was identified as SAGNPN and the peptide GLAGA showed the higher reducing power. Zhu et al. [17] reported that water soluble extract obtained from dry-cured Jinhua ham showed high levels of antioxidant activity in which the peptide sequenced as Gly–Lys–Phe–Asn–Val exhibited the highest antioxidative activity.

Although many antioxidant peptides have been extracted from dry-cured Jinhua ham, no research has been conducted to investigate the protective effects of these crude peptides against oxidative stress in cells. Therefore, the current study focused on the separation and the purification of antioxidant peptides from Jinhua ham using size-exclusion chromatography (SEC) and the characterization of the peptide fraction by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and amino acid auto-analyzer. Furthermore, the protective effects of peptides against oxidative stress in PC12 cells were further investigated.

2. Materials and Methods

2.1. Materials

Jinhua hams were purchased from Jinzi Ham Company (Jinhua, China). Hydrogen peroxide (H_2O_2), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,5-dihydroxybenzoic acid

(DHB) and glutathione (GSH) were purchased from Sigma chemical company (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

2.2. Processing of Dry-cured Jinhua Ham

According to the traditional techniques, Jinhua hams were processed by natural cooling, salting, soaking and washing, sun-drying, loft-aging, and post-aging. It took 8 months for the whole process in Jinhua of Zhejiang province. Hams were selected as samples randomly at the end of post-aging. Biceps femoris was taken from the Jinhua ham, packed and stored at -20°C before analysis.

2.3. Sample Extraction and Deproteinization

The extraction was performed according to the method of Escudero et al. [16] with slight modifications. Fifty grams of samples, previously removed from their extramuscular fat and connective tissues, were minced. Minced samples were homogenized with 200 mL of 0.1 M phosphate buffer saline (0.2 mol/L, pH 7.2) in a polytron homogenizer (IKA T25 digital ultra-turrax, IKA, Germany; 3 strokes, 15 s each at 20,000 rpm with cooling in ice). The homogenate was then centrifuged at 12,000 g for 20 min at 4°C. After filtering through glass wool, the supernatant was added to 3 volumes of ethanol to remove the protein fraction and the samples were maintained for 12 h at 4°C. The sample was centrifuged again (12,000 g for 20 min at 4°C) and the supernatant was adjusted to pH 7.0 with 1 M NaOH. Finally, the sample extract was stored at -20 °C until use after dried in a vacuum freeze-dryer.

2.4. Size-Exclusion Chromatography

A 5 mL aliquot of the extracted peptide was subjected to SEC using a Sephadex column (5×60 cm) packed with Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden) in order to fractionate the peptides according to their molecular mass. The separation was performed at room temperature using deionized water as eluent at a flow rate of 4 mL/min. Fractions were monitored by an ultraviolet detector (Amersham Biosciences) at 280 nm and 8 mL solutions were collected in every tube by an automatic fraction collector. Collected fractions were dried in a vacuum freeze-dryer.

2.5. Measurement of DPPH Radical Scavenging Activity

The DPPH radical-scavenging activity of the peptide fraction was determined as described by You et al. [18] with slight modifications. Aliquots of samples mixed 1:1 (v/v) with 0.2 mmol/L DPPH (0.02% in ethanol) as sample group, aliquots of samples mixed 1:1 (v/v) with 95% ethanol as control group and aliquots of 95% ethanol mixed 1:1 (v/v) with 0.2 mmol/L DPPH (0.02% in ethanol) as blank group. The solutions were shaken and kept for 30 min protected from sun light at room temperature. The scavenging activity was determined by measuring the absorbance at 517 nm with the multifunctional microplate reader (Spectral Max M2e, MD, USA). The scavenging ability was calculated according to the following equation:

$$\text{DPPH radical}-\text{scavenging activity \%} = 1-\left[(\text{As}-\text{Ac})/(\text{Ab}-\text{Ac})\right]\times 100\%$$

Where As, Ac and Ab represent the absorbencies of the sample, the control and the blank groups, respectively. GSH was used as a control.

2.6. Characterization of Peptide Fraction

The molecular mass distribution of the peak with the highest antioxidant activity from SEC fractionation was carried out using MALDI-TOF mass spectrometry (Voyager DE-STR, Applied Biosystems, Foster City, CA, USA) as described by Caprioli et al. [31] with slight modifications. Desorption/ionization was obtained by using a 337 nm nitrogen laser with a 3 ns pulse width. Available accelerating potential was in the range of +20/-20 kV. Laser power was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratios, and mass calibration was achieved using DHB as an external standard (about 0.05% mass accuracy can be achieved). In the post source decay experiment, the precursor ions (sodium adducts) were isolated using a timed ion selector. The spectra shown generally represented the sums of 50 laser shots, unless otherwise stated. Examination and processing of mass spectral data was performed by Data Explorer 4.0 software supplied by Applied Biosystems.

The amino acid composition of the peptide fraction was analyzed with an amino acid auto-analyzer (L-8900, Hitachi, Japan) as described by Xu et al. [32] with slight modifications. The sample was hydrolyzed using 6.0 M HCl in a sealed-vacuum ampoule for 24 h at 110 °C and then dryness in a bath at 45°C under a stream of nitrogen to remove the excess acid. Residue was dissolved in 10 mL of 0.02 M HCl and filtered with 0.22 μm film. About 1 mL of liquid sample was injected into an auto-sampler bottle and placed in amino acid auto-analyzer. Amino acids were determined using a cation separation column (4.6× 60mm) with flow rates of 0.45mL/min (buffer) and 0.25mL/min (reagent) at wavelengths of 440 and 570nm. The amount of each sample was calculated with reference to the standard sample by the EZChrom Elite software (Hitachi, Japan), and the content for each amino acid was expressed as gram of amino acids per 100 gram of samples (g/100 g).

2.7. Cell Culture and Cell Viability Assay

The PC12 cells (culture collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in growth medium (Dulbecco's Modified Eagle's Medium) (DMEM; Gibco, Burlington, ON, Canada) with 20 % fetal bovine serum (FBS; Hyclone, UT, USA) and 1 % penicillin-streptomycin (Gibco) in an incubator (95% air and 5% CO_2) at 37°C. During the logarithmic growth phase, the cells were collected for further experiments.

The viability of the cells was measured using a MTT Cell Proliferation and Cytotoxicity Assay Kit (Jiancheng Biochemical, Nanjing, China). The PC12 cells were plated at the density of 4×10^4 cells/well in 96-well cell culture plates (Costar Corning, Rochester, NY, USA) and incubated for 24 h before the three different experimental treatments were administered. The first group of cells was

treated with different concentrations of crude peptide (50, 100, 200, 400, 800 and 1600 μg/mL) to detect whether the crude peptide was cytotoxic toward PC12 cells. The second group was treated with different H_2O_2 concentrations (50, 100, 200, 400 and 800 μM) to select the optimum H_2O_2 concentration. The third group was pretreated with different crude peptide concentrations (50, 100, 200 and 400 μg/mL) and then subjected to 400 μM H_2O_2 to investigate the protective effects of crude peptide on PC12 cells. After exposure, the media was removed and changed to 100 μL MTT/well for 4 h incubation. The viable cell number was proportional to the production of formazan which was solubilized in isopropanol and absorbed strongly at 563 nm.

2.8. Activity of Cellular Antioxidant Enzymes

The activities of the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were determined using the commercial assay kits (Jiancheng Biochemical, Nanjing, China) according to the manufacturer instructions. SOD activity was determined based on the theory that superoxide ions produced from the oxidation of xanthine could react with tetrazolium to form a formazan dye that absorbs strongly at 550 nm. SOD could lower the rate of formazan dye formation by reacting with superoxide ions. The quantity of SOD that required for 50% inhibition of the formazan formation was defined as one unit of SOD. CAT activity was assayed by detecting peroxidase yielding end products which oxidized N-(4-antipyryl)-3-chloro-5-sulphonate-*p*-benzoquinone-monoimine into a chromophore that absorbed strongly at 405 nm. The decomposition of 1 μM H_2O_2 per second was defined as one unit of CAT. GSH-Px activity was determined by the catalyzing GSH into oxidized glutathione (GSSG) in the presence of *t*-Bu-OOH. Then GSSG was reduced by NADPH in the presence of glutathione reductase, and the oxidation of NADPH to $NADP^+$ that absorbed strongly at 340 nm. The oxidation of 1 nmol/L GSH was defined as one unit.

2.9. Cellular ROS determination by 2′,7′-dichlorofluorescein Diacetate (DCFH-DA)

DCFH-DA is an oxidation sensitive dye and was used to determine the intracellular formation of ROS. [19] The PC12 cells, which were cultured in six-well plates at a density of 4×10^5 cells/mL, were collected after treating with different concentrations of sample (200 and 400 μg/mL) and 400 μM H_2O_2. Old medium was removed and cells were washed with PBS twice. Then the medium was replaced with fresh incubated medium containing 1 mL of 10 μM DCFH-DA for 20 min at 37 °C and was shaken every 5 min. Due to the oxidation of DCFH in the presence of ROS, the formation of fluorescent dichlorofluorescein (DCF) was analyzed by monitoring the fluorescence intensity (excitation at 488 nm and emission at 525 nm).

2.10. Annexin V-FITC and PI Staining Assay

H_2O_2-induced apoptotic death of PC12 cells was quantified by flow cytometry with Annexin V-FITC and PI staining. Briefly, the PC12 cells were cultured in six-well plates at a density of 4×10^5 cells/mL. After the treatment with different concentrations of CPC (200 and 400 μg/mL) and 400 μM H_2O_2, the cells were collected and washed with PBS twice and then subjected to Annexin V and PI staining by using MACS's Annexin V-FITC Kit (Milthenyi Biotec, Teterow, German). The fluorescence of the cells was determined via flow cytometry (FACSCalibur, Becton Dickson, NJ, USA).

2.11. Measurement of Caspase-3 Activity

Activity of caspase-3 was determined by using fluorometric assay kits (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Briefly, the PC12 cells were cultured in six-well plates at a density of 4×10^5 cells/mL. After the treatment with different concentrations of CPC (200 and 400 μg/mL) and 400 μM H_2O_2, the cells were removed and added with 25 μl Caspase-Glo® 3/7/kit. After incubation at 37 °C for 1 h, the activity was determined using multifunctional microplate reader (Spectral Max M2e, MD, USA). Data were expressed as percentage of the control and the control group was designed as 100%.

2.12. Statistical Analysis

All experiments were carried out with triplicate sample analysis. Three independent experimental trials (replications) were conducted. Statistical analyses were carried out using IBM SPSS 20.0 (IBM, NY, USA). The statistical significance of the date was determined by one-way ANOVA followed by Dunnett's test with a $P < 0.05$ taken as value of significance.

3. Results and Discussion

3.1. Separation of Crude Peptide and Their Free Radical Scavenging Activity

During the long ripening process (8-10 months), muscle protein and fat are hydrolyzed to some extent by endogenous enzymes. Many small peptides, free amino acids, free fatty acids and volatiles are produced which eventually contribute to the unique flavor of Jinhua ham. [20] The peptide content extracted from dry-cured Jinhua ham in this experiment was 12.94%. Many peptide fractions extracted from China and Spanish dry-cured ham have been reported to possess antihypertensive effect and antioxidant activity [10,17,21].

Size-exclusion chromatography was employed to fractionate the water soluble peptide extract from Jinhua ham in the present study (Figure 1A). Crude peptide was separated into five fractions crude peptide A (CPA), crude peptide B (CPB), CPC, crude peptide D (CPD) and crude peptide E (CPE) on the basis of their molecular weights. The DPPH and superoxide anion radical scavenging assays were carried out to determine the antioxidant activity of the five fractions (Figure 1B). Crude peptide exhibited high DPPH scavenging activity in a dose-dependent manner, in which CPC presented the highest DPPH scavenging activity (72.45% at 4 mg/mL) followed by CPB (54.55%), CPA (34.32%), CPD (30.23%), and CPE (22.29%). Due to the higher DPPH radical scavenging activities compared to other crude peptide fractions, CPC was used for further analysis.

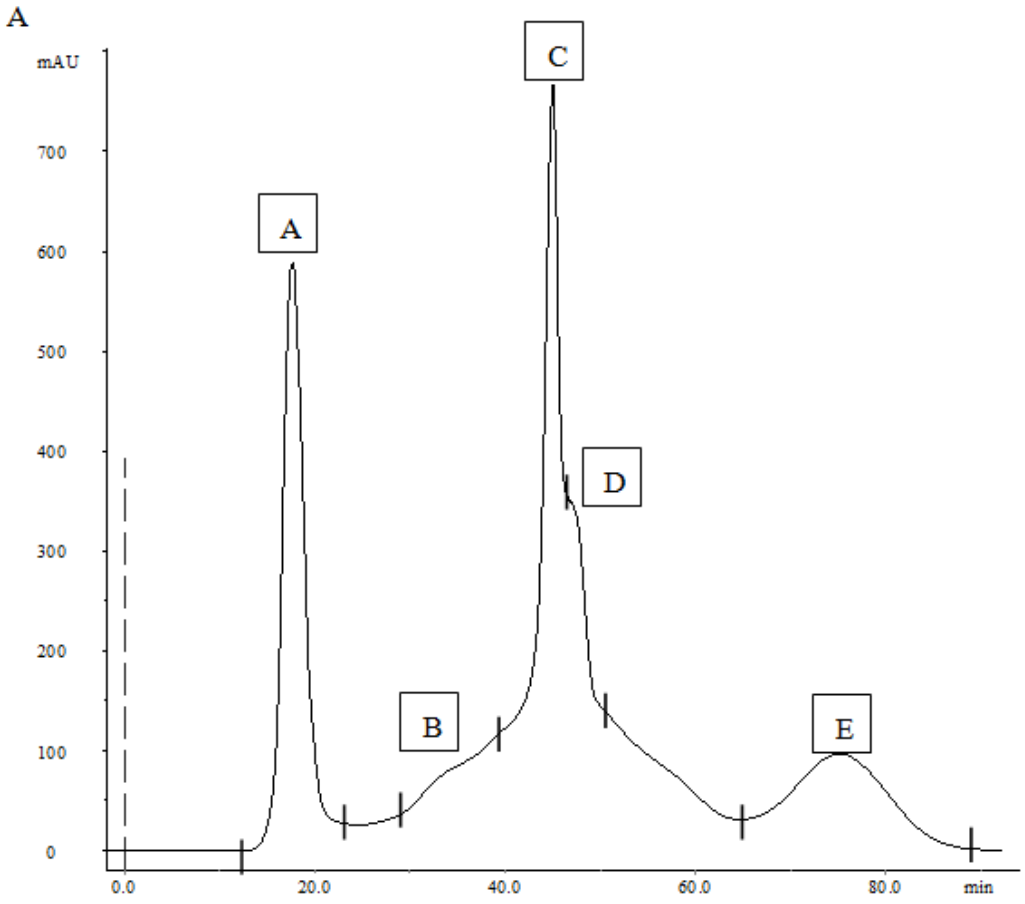

Figure 1. (A) Elution profile of crude peptide on the Sephadex G-25 column

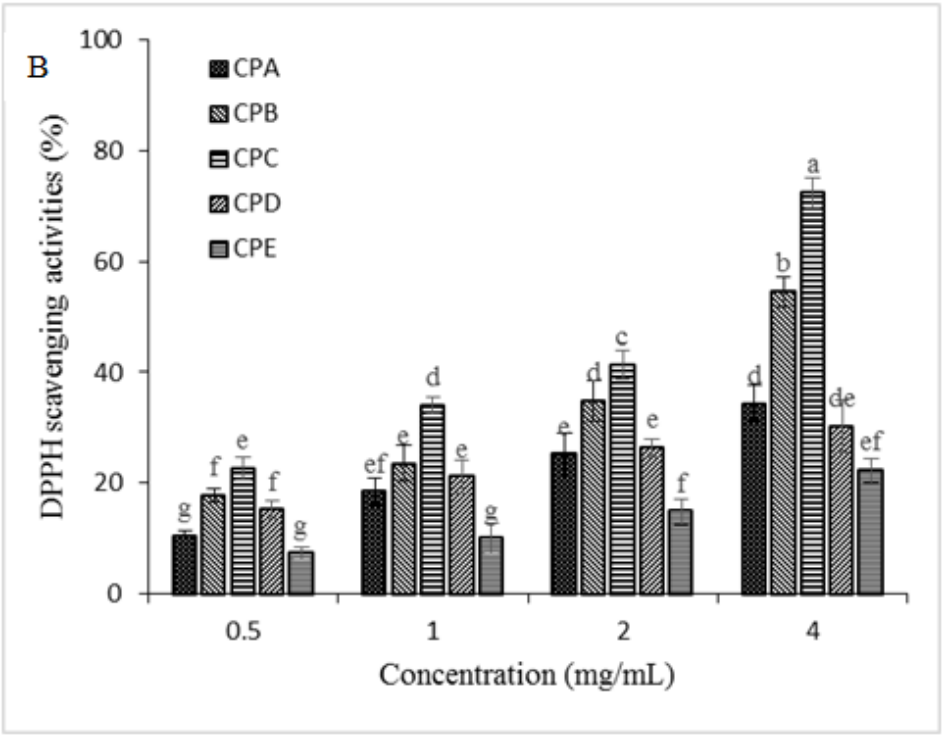

Figure 1. (B) DPPH radical scavenging activity. All assays were carried out at the concentration of 0.5, 1, 2 and 4 mg/mL. The error bars refer to the standard deviations obtained from the triplicate sample analysis. Means with different letters (a-g) differ significantly (P <0.05)

3.2. Characterization of Peptide Fraction

Amino acid composition is the foundation of the physiological functions of the peptides which are associated with their R groups. Amino acids with nucleophilic sulphur-containing side chains, aromatic side chains, or imidazole-containing side chains would be the most reactive ones. [22] As shown in Table 1, the CPC

possessed high amount of His, Phe, Glu, Ala and Pro which accounted for 59.67% of the total amino acids. Hydrophobic amino acids including Ala, Phe and Pro play a crucial role in the radical scavenging capacity and amino acids with aromatic residues, including His and Phe can donate protons to electron deficient radicals. [23] This property improves the radical-scavenging properties of the amino acid residues [24].

The molecular mass distribution of CPC was carried out using MALDI-TOF mass spectrometry. As shown in Figure 2, CPC possessed major peptides in the range of 400-1000 Da. It was reported that the majority of the antioxidative peptides derived from food sources have molecular weights ranging from 500 to 1800 Da. [25] The amino acid composition and the molecular mass distribution results agree with the date from free radical scavenging assay indicating that CPC may possess strong antioxidant activity.

Table 1. Amino acid composition of CPC

Amino acid	(g/100g)
	Sample
Aspartic acid	3.21 ± 0.42
Threonine	2.28 ± 0.11
Serine	1.81 ± 0.18
Glutamic acid	8.95 ± 0.53
Proline	7.30 ± 0.62
Glycine	3.85 ± 0.14
Alanine	4.47 ± 0.43
Cystine	8.90 ± 0.80
Valine	2.57 ± 0.14
Methionine	2.08 ± 0.24
Isoleucine	2.05 ± 0.34
Leucine	3.76 ± 0.35
Tyrosine	3.02 ± 0.32
Phenylalanine	11.5 ±1.47
Histidine	10.6 ± 1.76
Lysine	1.81 ± 0.14
Arginine	1.66 ± 0.25
Total	79.8 ± 5.12

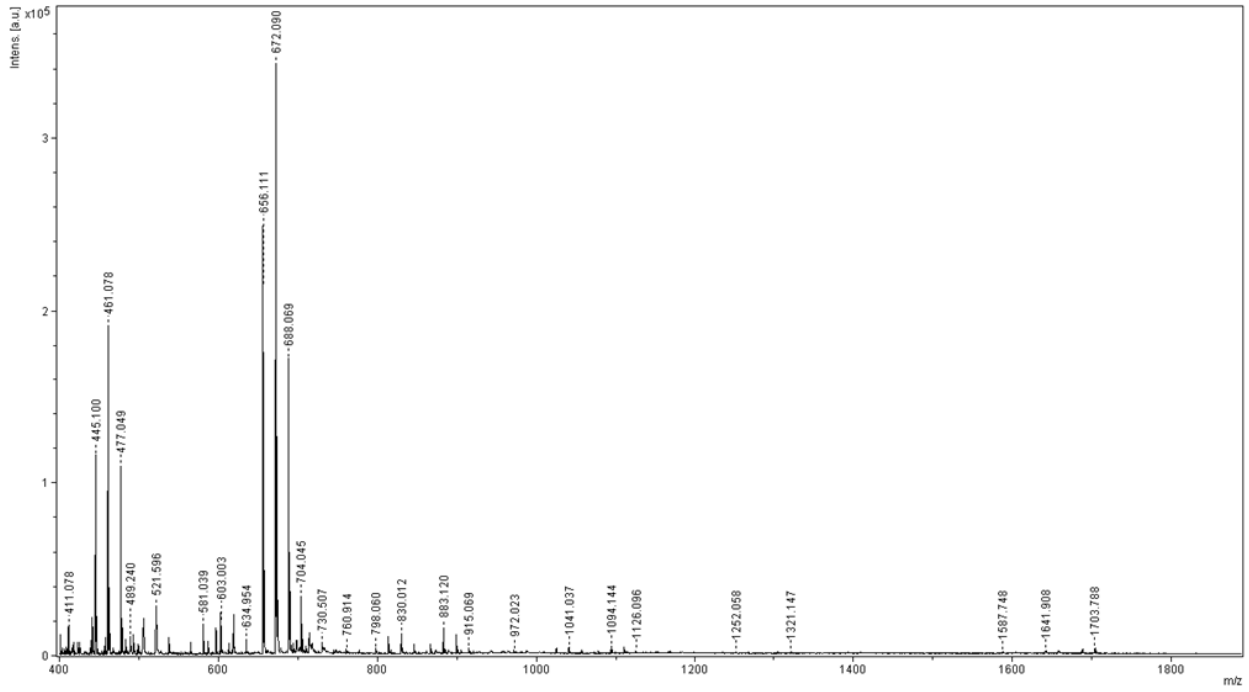

Figure 2. MALDI-TOF mass spectrometry spectrum of CPC

3.3. Cell Viability

Reactive oxygen species such as superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) may perturb natural antioxidant defense systems of cells leading to the damage to most biological macromolecules. [26] Hydrogen peroxide is thought to be the major precursor of highly reactive free radicals, which has been reported to induce apoptosis in cells of the central nervous system. [2] Hence, H_2O_2 was used to induce oxidative stress in the model with PC12 cells in the present study.

The PC12 cells were firstly exposed to different concentrations of H_2O_2 (50, 100, 200, 400, 600 and 800 μM) to determine the optimal H_2O_2 concentration to induce oxidative damage in cells. The H_2O_2-induced cytotoxciity was concentration dependent (Figure 3A), and the cell viability reduced significantly with the increase of H_2O_2 concentration ($P < 0.05$). Lower concentration of H_2O_2 (0-200 μM) decreased the cell viability, while the effect was relatively low. However, higher conecntrations of H_2O_2 (400-800 μM) further significantly reduced the cell viability ($P < 0.05$), and all cells were killed at the concentration of 800 μM. In the presence of 400 μM H_2O_2, the cell viability was moderately decreased and thus 400 μM H_2O_2 was used as the concentration to damage the PC12 cells for following experiments.

The PC12 cells were secondly treated with various concentrations of CPC (0-1600 μg/mL) to determine whether CPC has cytotoxicity to the cells. As shown in Figure 3B, 0-400 μg/mL CPC showed no significant cytotocity in the PC12 cells ($P > 0.05$). Nevertheless, CPC was considered to be toxic when the concentration was higher 800 μg/mL ($P < 0.05$). Therefore, the concentration of 50, 100, 200, and 400 μg/mL were chosen for the subsequent assays.

The PC12 cells were lastly pretreated with different crude peptide concentrations (50, 100, 200 and 400 μg/mL) and then subjected to 400 μM H_2O_2 to investigate the protective effects of crude peptide on PC12 cells. As shown in Figure 3C, the cell viability decreased to 48.19%

after exposure to 400 μM H_2O_2. However, when pretreated with CPC significantly improved the cell viability in a dose-dependent manner in which the cell viability was repaired from 48.19% to 52.24%, 60.98%, 67,44% and 80.23% at the concentrations of 50, 100, 200 and 400 μg/mL, respectively.

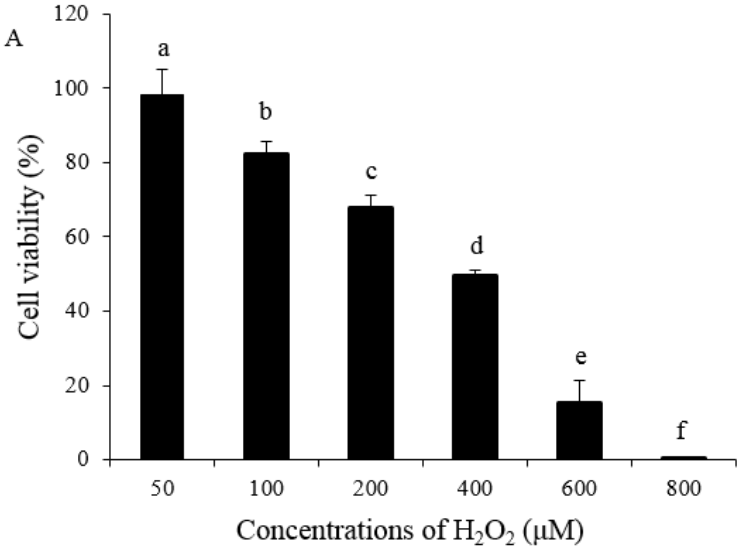

Figure 3. (A) The effects of the different H_2O_2 concentrations PC12 cell viability

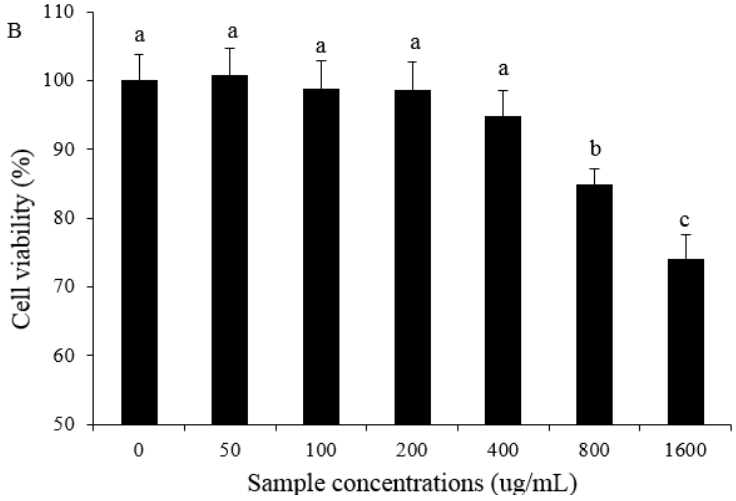

Figure 3. (B) The effects of the different CPC concentrations PC12 cell viability

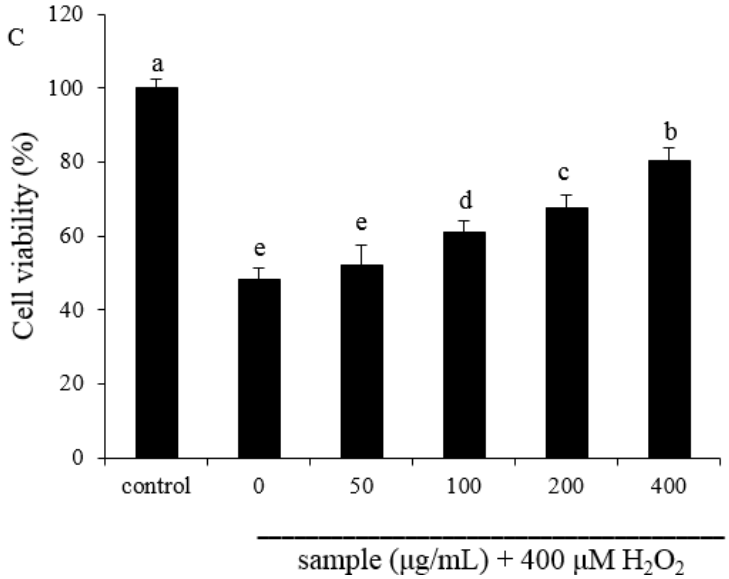

Figure 3. (C) the protective effects of CPC on PC12 cells oxidative stress by H_2O_2. The error bars refer to the standard deviations obtained from the triplicate sample analysis. Means with different letters (a-e) differ significantly ($P < 0.05$)

Cellular oxidative stress occurs when an imbalance between ROS and cellular antioxidant defenses due to either the accumulation of ROS or the depletion of antioxidants. The accumulation of ROS could result in lipid peroxidation, protein oxidation and DNA damage. [27] Consequently, pursuing for antioxidants, which can scavenge free radicals and attenuate ROS-induced oxidative stress, has been a hot topic in recent years. The present findings indicate that the improved PC12 cell viability by pretreating with CPC can be attributed to their ability to against ROS.

3.4. Effect of CPC on Intracellular Antioxidant Enzymes

The antioxidant enzyme system in cells mainly consisted of CAT, SOD and GSH-Px. Hence, the activities of CAT, SOD and GSH-Px were determined after the pretreatment of CPC with or without the addition of 400 μM H_2O_2 in PC12 cells. As shown in Figure 4, the CAT, SOD and GSH-Px activities were decreased by 58%, 71% and 47% compared to the control after exposure to 400 μM H_2O_2, respectively. These results indicate that the untreated cells could not withstand the oxidative injury caused by H_2O_2 as previously reported. [27] Nevertheless, pretreating cells with CPC before the oxidative damage alleviated the decrease in antioxidant enzyme activities. Compared to only H_2O_2-treated cells, cells pretreated with 200 μg/mL CPC showed a relatively steady increase in CAT activity, while 400 μg/mL CPC significantly increased the CAT activity ($P < 0.05$). The similar effect was found in SOD and GSH-Px activities. The SOD activity increased by 67% and 143%, and the GSH-Px activity increased by 18% and 38% in cells pretreated with 200 μg/mL and 400 μg/mL of CPC, respectively ($P < 0.05$).

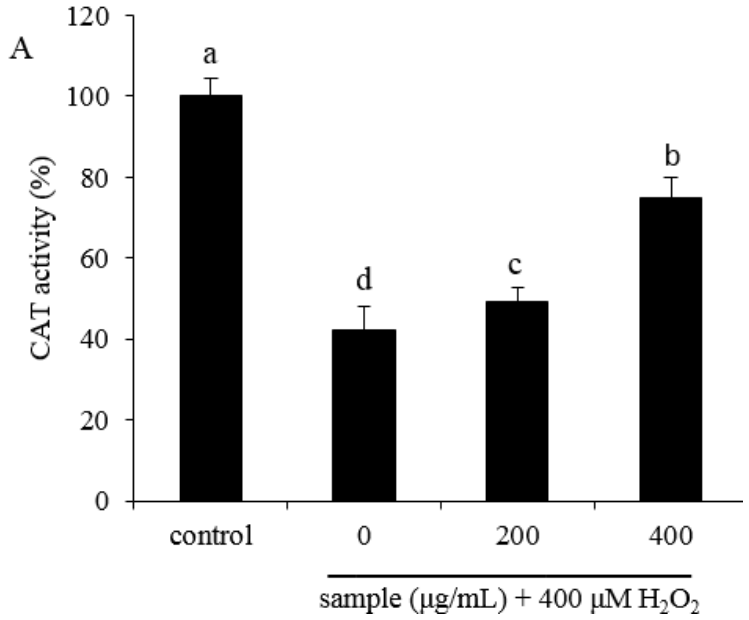

Figure 4. (A) The effects of the CPC on the CAT activity in PC12 cells subjected to H_2O_2-induced oxidative damage

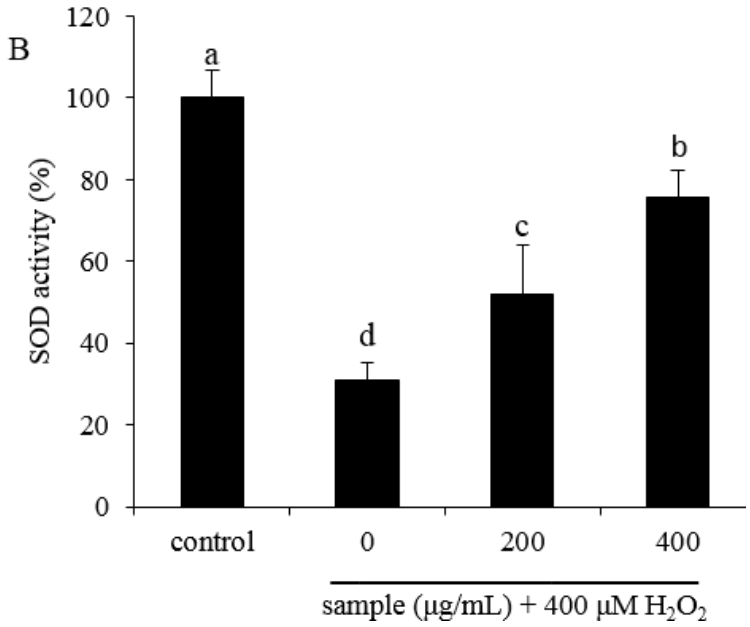

Figure 4. (B) The effects of the CPC on the SOD activity in PC12 cells subjected to H_2O_2-induced oxidative damage

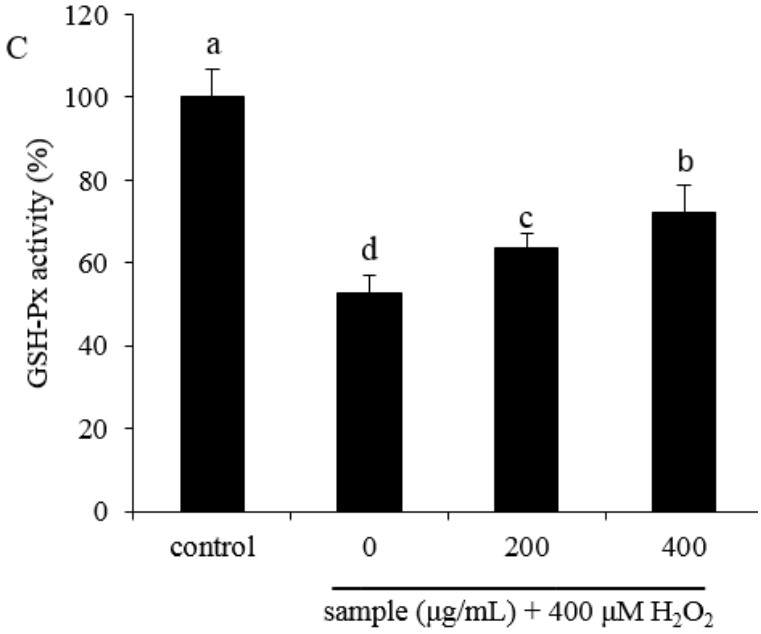

Figure 4. (C) The effects of the CPC on the GSH-Px activity in PC12 cells subjected to H_2O_2-induced oxidative damage. The error bars refer to the standard deviations obtained from the triplicate sample analysis. Means with different letters (a-d) differ significantly ($P < 0.05$)

Many other extracts are capable of modulating the endogenous antioxidant enzymes in different cells including eggshell membrane extracts, [22] whey protein hydrolysates [28] and black currant extract. [27] The mechanism of the cytoprotective effect of CPC might be relevant with the direct free radical scavenging activity, which mitigates redundant accumulation of ROS in cells. Additionally, the enhanced CAT, SOD and GSH-Px activities were in accordance with the raising in cell viability. In conclusion, our results indicate that CPC could restrain H_2O_2-induced cell damage by raising the activity of endogenous antioxidant enzymes.

3.5. Effects of Crude Peptides on the Inhibition of Intracellular ROS Formation

To further understand whether the observed cytoprotective effect of CPC is attributed to the decrease of oxidative stress, the effect of CPC on intracellular ROS generation was measured. The changes of intracellular ROS in CPC-treated and untreated PC12 cells were detected with the stain of DCFH-DA by Microplate Spectrophotometer (Figure 5). The 400 μM H_2O_2 significantly enhanced ROS generation, compared with control, indicating that a significant number of cells were damaged as a result of oxidative stress (P <0.05). Nevertheless, CPC-treated cells had remarkably lower fluorescence intensity. Compared to only H_2O_2-treated cells, intracellular ROS generation was decreased by 25% and 36% in cells pretreated with 200 and 400 μg/mL of CPC, respectively (P <0.05). These results were similar to the intracellular antioxidant enzymes results presented in Figure 4 indicating that CPC may have a protective role in PC12 cells against the oxidative damage from ROS.

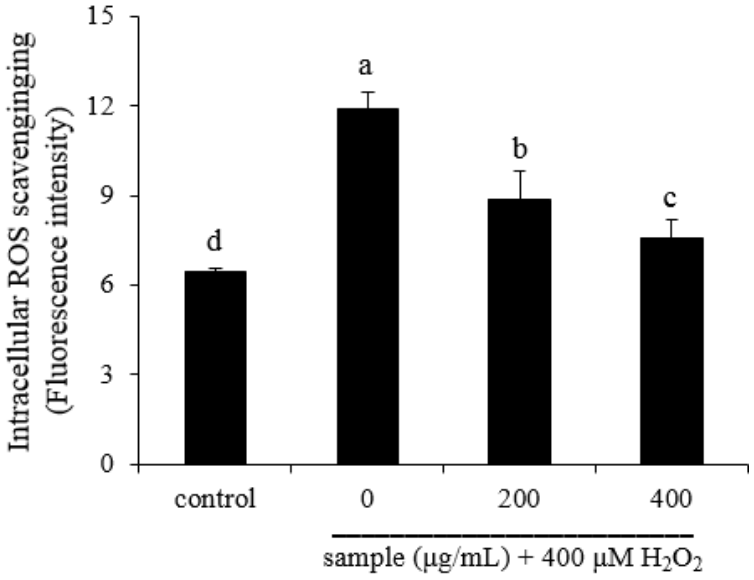

Figure 5. Intracellular ROS scavenging activity of CPC. Increase in fluoresecence intensity of the DCFH-DA-labelled PC12 cells treated with CPC (200 and 400 μg/mL) after oxidation induction by H_2O_2 (400 μM). The error bars refer to the standard deviations obtained from the triplicate sample analysis. Means with different letters (a-d) differ significantly ($P < 0.05$)

3.6. CPC Attenuated H_2O_2-induced Apoptosis in PC12 Cells

Caspase-3 is the key executor during apoptotic programing. [29] Therefore, the effect of CPC on caspase-3 activity in H_2O_2-induced PC12 cells was determined. As shown in Figure 6, after treatment with 400 μM H_2O_2 for 24 h, the activity of caspase-3 increased by 208% compared to the control (P < 0.05). However, when PC12 cells were incubated with H_2O_2 in the presence of CPC at the concentrations of 200 and 400 μg/mL, caspase-3 activity decreases by 69% and 64%, respectively (P < 0.05) suggesting that H_2O_2 induced caspase-3 activation could be significantly inhibited by CPC.

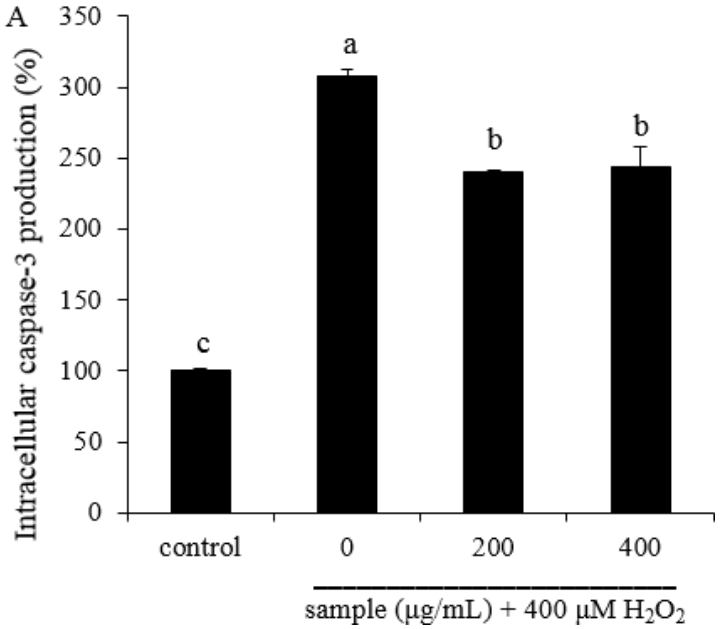

Figure 6. (A) The effect of the CPC on the intracellular caspase-3 activity in PC12 cells subjected to H_2O_2-induced apoptosis. Means with different letters (a-c) differ significantly (P < 0.05)

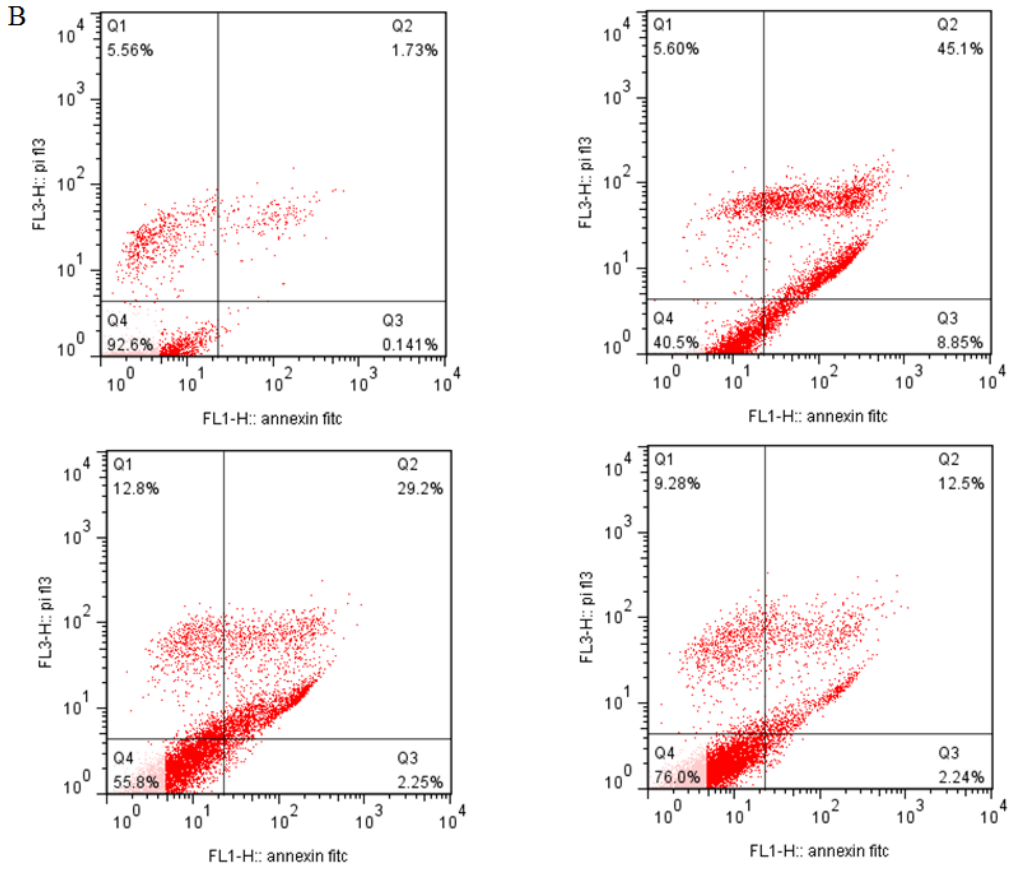

Figure 6. (B) Apoptosis rate in PC12 cells was assessed by flow cytometry using the annexin V-VITC/PI apoptosis assay after different treatments. The Q2 quadrant indicants the percentage of early apoptotic cells (Annexin V positive cells) and the Q3 quadrant indicates the percentage of late apoptotic cells (Annexin V and PI double positive cells). The error bars refer to the standard deviations obtained from the triplicate sample analysis. Means with different letters (a-c) differ significantly (P < 0.05)

In addition to the intracellular caspase-3 production, the protective effect of CPC against H_2O_2-induced apotosis was detected through flow cytometrc analysis Annexin V-FITC and PI staining. As shown in Figure 6, the percentage of apoptotic cells was only 1.87% in the control cells, while the H_2O_2 treated cells showed 8.85% in early apoptosis and 45.1% in late apoptosis. Nevertheless, when pretreating cells with CPC before the oxidative damage attenuated cell apotosis and reduced the percentage of apototic cells to 31.5% and 14.7% in a dose-dependent manner, respectively ($P < 0.05$).

An upsurge in ROS production can cause shifts in the redox state of cells, which is associated with the apoptotic pathway. [30] The increased generation of ROS and caspase-3 activity upon the treatment of the cells with H_2O_2 are indicative of apoptosis. In addition, the flow cytometrci analysis results agreed with the date from the cell viability assay indicating that the ability of CPC to attenuate H_2O_2-induced PC12 cell death was mediated by its antiapopotic activity.

4. Conclusion

Our results suggested that the Jinhua ham peptide extract exhibited strong DPPH radicals scavenging activities. These peptide extract could effectively protect PC12 cells from H_2O_2-induced oxidative damage by modulating the endogenous enzyme related to the antioxidant activity of CPC. Therefore, CPC may possess the ability to attenuate the over-production of ROS. Moreover, this study first reported the cytoprotective effect of crude peptides extracted from dry-cured Jinhua ham. Further researches are required particularly to investigate whether these peptides could regulate the expressions of some genes and proteins involved in the antioxidant mechanism.

Abbreviations USED

H2O2, Hydrogen peroxide; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; GSH, glutathione; ROS, reactive oxygen species; O2-, superoxide anion; •OH, hydroxyl radical; SEC, size-exclusion chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; TBHQ, tert-butylhydroquinone; SEC, size-exclusion chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; DHB, 2,5-dihydroxybenzoic acid; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GSSG, glutathione; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCF, dichlorofluorescein; CPA, crude peptide A; CPB, crude peptide B; CPC, crude peptide C; CPD, crude peptide D; CPE, crude peptide E;

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