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Chemical and cellular antioxidant activity of two novel peptides designed based on glutathione structure

Longjian Gu [a], Mouming Zhao [a], Wenzhi Li [a,b], Lijun You [a], Jufang Wang [c], Haiyan Wang [d], Jiaoyan Ren [a,*]

[a] College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, Guangdong, China
[b] Infinitus (China) Company Ltd., Guangzhou 510665, China
[c] School of Bioscience & Bioengineering, South China University of Technology, Guangzhou 510640, Guangdong, China
[d] National Institutes of Food and Drug Control 100050, Beijing, China

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ABSTRACT

Two novel peptides, ECH (Glu-Cys-His) and YECG (Tyr-Glu-Cys-Gly), were designed based on glutathione (Glu-Cys-Gly, GSH) and their antioxidant activities were studied. Various chemical methods based on single-electron-transfer (SET) and hydrogen-atom-transfer (HAT) were applied to evaluate the antioxidant activities of the peptides. For SET-based assay, tripeptide ECH displayed the highest DPPH radical scavenging activity (80.16%) and the strongest reducing power (A_{700} = 0.378). Besides, ECH also exhibited the best inhibition activity toward linoleic acid peroxidation with inhibition rate 98.25% at 7th day, which is a HAT-based assay. However, for another two HAT-based assays, it was tetrapeptide YECG that showed extraordinary oxygen radical absorption capacity (ORAC value = 2.42 μM Trolox/μM) and ABTS free radical scavenging ability (8.88 mM Trolox/mM). *In vitro* cultured PC12 cell model also suggested that YECG gave the best protection for PC12 cells to resist H_2O_2-treated necrosis. It was found that the discrepancy of antioxidant capacity between ECH and YECG was caused by the presence of antioxidant amino acids (His/Tyr) and their position in peptide chain. With His located at C-terminal position, ECH demonstrated good electrons donating capacity, while with Tyr at N-terminal position, YECG exhibited strong oxygen radical absorbance capacity.

1. Introduction

There are six major reactive oxygen species (ROS) causing oxidative damage in human body, which are superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), peroxylradicals (ROO·), hydroxyl radical (HO·), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^{-}\cdot$) (Lee et al., 2004). Ordinarily, the living cells have a biological defense system to counteract the assault of these ROS, except that there are too many radicals as well as our body get into mess. This brings the need for antioxidant agents which can prevent oxidative stress and its deleterious effects. Since Marcuse first reported the antioxidant effect of peptides (Marcuse, 1960), hundreds of antioxidant peptides generated from the digestion of dietary proteins have been identified. For example, peptides derived from soybean, wheat gluten, casein, mackerel muscle, squid skin, grass carp muscle, etc., have shown potent antioxidative activities in different oxidative systems (Nam et al., 2008; Ren et al., 2008). Peptides as antioxidant agents not only are safe and healthy, but also can be absorbed quickly and easily.

Although many food-originated antioxidant peptides have been identified, their structure-bioactivity relationships have not fully been understood. In general, there are three factors that mainly affect the antioxidant activities of peptides. Firstly, the molecular weight of peptides can impact the antioxidant activity. Most peptides purified from enzymatic hydrolysates are in the size of 2–20 amino acids and peptides less than 6000 Da are most possibly to show antioxidant activity (Sarmadi and Ismail, 2010). Secondly, the amino acid composition has much contribution to the activity of the antioxidant peptides. For example, Trp, Tyr, and Phe, which contain aromatic residues, can donate protons to electron deficient radical (Rajapakse et al., 2005b) and the imidazole-containing amino acid His has good lipid peroxyl radical trapping capability (Chen et al., 1998). Thirdly, the structurally unique linkage type of peptide chain has been demonstrated to influence antioxidant capacity. For example, tripeptides containing Trp/(Tyr) residues at the C-terminus, or Val/(Leu) at N-terminus, show strong radical scavenging activity (Saito et al., 2003; Elias et al., 2008).

The chemical antioxidant capacity assays can be roughly divided into two categories, single-electron-transfer (SET) reaction

* Corresponding author at: College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, Guangdong, China. Tel./fax: +86 20 22236089.
E-mail address: jyren@scut.edu.cn (J. Ren).

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based assay and hydrogen-atom-transfer (HAT) reaction based assay. SET-based assays include the total phenols assay by Folin Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), total antioxidant potential assay using a Cu (II) complex as an oxidant, and DPPH radical scavenging assay (Huang et al., 2005). HAT-based assays include the inhibition of low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), and crocin bleaching assays. Besides, *in vitro* cultured cell model systems are also valuable methods for antioxidant evaluation of peptides, compared to expensive and time-consuming animal studies. It has been reported that *in vitro* antioxidant model based on rat adrenal pheochromocytoma (PC12) cells is an important method to assess potential health benefits of antioxidant peptides (Ma et al., 2010).

Glutathione (GSH, Glu-Cys-Gly) is a well-known multifunctional antioxidant that exists in animals and plants to defend against oxidative stress (Anjum et al., 2011). It has been demonstrated that extracellular supplementation of GSH in culture medium significantly provided useful radical scavengers to abolished all forms of oxidant-mediated cell death (Teramoto et al., 1999; Ueda et al., 1996). In this study, two peptides ECH (Glu-Cys-His) and YECG (Tyr-Glu-Cys-Gly) were designed based on GSH. Antioxidant amino acids, His and Tyr, were included in the new designed peptides ECH or YECG, in order to create new peptides with much stronger antioxidant activity than GSH. Various assay methods including SET reaction based assay (DPPH radical scavenging ability and reducing power) and HAT reaction based assay (inhibition linoleic autoxidation, ORAC and TEAC) were employed to evaluate the antioxidant activity of the three peptides (GSH, ECH and YECG). Additionally, we measured the protection of PC12 cells from hydrogen peroxide induced cytotoxicity by antioxidant these peptides. The relationship between the antioxidant activities and structure properties of the antioxidant peptides were discussed as well.

2. Materials and methods

2.1. Materials

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (Trolox), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), fluorescein disodium, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH), orthophenanthroline, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), linoleic acid and dimethylsulphoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of peptides

GSH (Glu-Cys-Gly), tripeptide ECH (Glu-Cys-His) and tetrapeptide YECG (Tyr-Glu-Cys-Gly) were prepared using L-isomers of each amino acid by solid-phase synthesis with the fluorenylmethoxycarbonyl (Fmoc)-strategy. The synthesis was conducted on an automated simultaneous multiple peptide synthesizer (Symphony, Protein Technologies.Inc) by ChinaPeptides Co. Ltd., (Shanghai, China).

2.3. Determination of the pK_a of thiol groups

The pK_a of thiol groups for three peptides was measured according to Säde Viirlaid (Viirlaid et al., 2009) with some modification. Three milliliter of 0.25 mM peptide solution in 10 mm phosphate buffered saline (pH 7.5) was titrated with 5 μL volumes of 1 M NaOH and plot of the absorbance vs. pH was obtained. Absorbance increased due to the increase in thiolate form of compounds and the pK_a values were calculated from these titration curves. The absorbance of thiol and thiolate concentrations was measured at 240 nm with a spectrophotometer (UV2550, SHIMADZU, Kyoto, Japan).

2.4. Antioxidant activity assays based on single-electron-transfer (SET)

2.4.1. DPPH radical scavenging activities

The DPPH scavenging activity of the peptides was measured by colorimetric method (Shimada et al., 1992). An aliquot of 2 mL of sample solution at different concentrations (0–1.6 mg/mL) was mixed with 2 mL of DPPH solution (0.2 mM in 95% ethanol). The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (UV2550, SHIMADZU, Kyoto, Japan). The ethanol was used as a control. The radical scavenging capacity of the tested samples was measured using the following equation. All determinations were performed in triplicate.

Radical scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100\%$

2.4.2. Reducing power

The reducing power was measured according to the method of Oyaizu (Oyaizu, 1986) with some modification. Two millilitre of sample (at a final concentration of 0.4 mM) was mixed with 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 2% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Afterwards, 2 mL of 10% trichloroacetic acid (TCA) was added to the mixture and was then centrifuged at 3000 rpm for 10 min. The supernatant (2 mL) was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm by a spectrophotometer (UV2550, SHIMADZU, Kyoto, Japan) after the solution stood for 10 min. All determinations were performed in triplicate.

2.5. Antioxidant activity assays based on hydrogen-atom-transfer (HAT)

2.5.1. Inhibition of linoleic acid autoxidation

The lipid peroxidation inhibition capacity of the peptides was measured in a linoleic acid model system (Osawa and Namiki, 1985). Briefly, sample (1.3 mg) was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) and added to a solution containing 0.13 mL of linoleic acid and 10 mL of 99.5% ethanol. Then the total volume was adjusted to 25 mL with distilled water. The mixture was incubated in a conical flask with a screw cap at 60 ± 1 °C dark room. The degree of oxidation was evaluated by measuring ferric thiocyanate values (Mitsuda et al., 1996). Simply speaking, the reaction solution (100 μL) incubated in the linoleic acid model system described above was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 2 M ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm following color development with $FeCl_2$ and thiocyanate at different intervals during the incubation period at 60 ± 1 °C. The inhibition activity was calculated as follows: Inhibition activity (%) = $[(A_{control} - As_{ample})/A_{control}] \times 100\%$ (Dávalos et al., 2004).

2.5.2. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was conducted using fluorescein as fluorescent probe, according to the methods of Ou (Ou et al., 2001) and Davalos (Dávalos et al., 2004) with slight modification. The reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4). Twenty microliters antioxidant [Trolox (1–8 μM) or sample (6 μM)] were mixed with 120 μL fluorescein (70 nM final concentration), and incubated at 37 °C for 2 min. Then 60 μL AAPH (12 mM final concentration) was applied to the mixture. The plate was automatically shaken before the first reading and the fluorescence was recorded every 2 min. The whole assay lasted for 108 min. A Fluoroskan Ascentmicroplate reader (Thermo Electron Corporation, Vantaa, Finland) with 485-P excitation and 520-P emission filters, controlled by Fluoroskan Ascent Software Version 2.6 (Thermo Scientific), was used for fluorescence measurement. Black 96-well microplates (96F untreated, Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4).

All reaction mixtures were prepared in duplicate and at least three dependent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

$$AUC = 1 + \sum_{i=1}^{i=54} Fi/F0$$

where F_0 is the initial fluorescence reading at 0 min and F_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = AUC_{antioxidant} - AUC_{blank}$$

The regression equation between net AUC and antioxidant concentration was calculated. The slope of the equation was used to calculate the ORAC value by using Trolox curve obtained for each assay. Final ORAC values were expressed as μmol Trolox equivalent/μmol antioxidant. Assays were carried out in triplicate.

2.5.3. Trolox equivalent antioxidant capacity (TEAC)

The assay was conducted according to the method described by Re (Re et al., 1999). ABTS radical cation ($ABTS^{\cdot+}$) was produced by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration in 10 mL of water) and keeping the mixture in dark at room temperature for 12–16 h before use.

Table 1
Effect of synthetic peptides on single-electron-transfer (SET) reaction and hydrogen-atom-transfer (HAT) reaction based antioxidant activities.

Peptides	Single-electron-transfer (SET) reaction based assay*		Hydrogen-atom-transfer (HAT) reaction based assay*		
	DPPH radical scavenging capacity (%)	Reducing power (absorbance at 700 nm)	Inhibition of linoleic acid autoxidation at 7th-day (%)	ORAC value (μM Trolox/μM)	$ABTS^{\cdot+}$ radical scavenging capacity (mM Trolox/ mM)
GSH (Glu-Cys-Gly)	78.74 ± 0.06[a]	0.252 ± 0.07[a]	23.38 ± 0.00[a]	0.76 ± 0.02[a]	2.39 ± 0.03[a]
ECH (Glu-Cys-His)	80.16 ± 0.10[b]	0.378 ± 0.09[b]	98.25 ± 0.01[b]	0.69 ± 0.01[a]	2.43 ± 0.02[a]
YECG(Tyr-Glu-Cys-Gly)	59.14 ± 0.36[c]	0.290 ± 0.09[c]	65.57 ± 0.02[c]	2.42 ± 0.08[b]	8.88 ± 0.10[b]

In each column, values with same superscripts indicate no significant difference.
* Values are expressed as means ± S.D.

The solution was diluted in 0.2 M PBS (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm after equilibration at 30 °C. Forty microliters of sample or Trolox was added to 1 mL of diluted $ABTS^{\cdot+}$ solution and incubated at 30 °C for 6 min. Scavenging of the radical was evaluated by monitoring the decrease of absorbance at 734 nm using spectrophotometer (UV2550, SHIMADZU, Kyoto, Japan). All determinations were carried out in triplicate, and their average was used as a datum point. The relative activity was calculated using the Trolox calibration curve and converted to the Trolox equivalent antioxidant capacity (TEAC) value. The millimole concentration of antioxidants giving the same percentage change of absorbance of the $ABTS^{\cdot+}$ as that of 1 mM Trolox was regarded as TEAC.

2.6. Cell culture study

2.6.1. Cell culture

Rat pheochromocytoma cell line (PC12 cells) was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM containing 10% (v/v) fetal bovine serum and 100 U/mL each of penicillin and streptomycin in a fully humidified atmosphere in a 5% CO_2, 37 °C incubator.

2.6.2. Cytotoxic assessment of H_2O_2

To determine the radical-induced cytotoxicity, cells were seeded in a transparent 96-well plate of 5×10^3 cells/well and pre-incubated with DMEM for 36 h, then cells were treated with various concentrations of H_2O_2 and incubated for 2 h. Cell viability was determined using MTT method that assesses the ability of the cell's succinate dehydrogenase to convert MTT into visible formazan crystals (Mosmann, 1983). Cells in each well were rinsed with sodium phosphate buffer (pH 7.2) and the supernatant was discarded by the multichannel pipette (Eppendorf, Germany), then 90 μL DMEM and 10 μL MTT (0.5 mg/mL final concentration) were added and incubated (37 °C) in the dark for 4 h. Finally, the medium with MTT was replaced with dimethyl sulphoxide (150 μL) to solubilise the formazan crystals, and the absorption value was determined at 490 nm by using a Multiskan spectrum reader (Thermo Fisher Scientific Inc., Finland).

2.6.3. Cytotoxic assessment of the synthesized peptides

Cells were seeded in a 96-well plate of 5×10^3 cells/well and incubated for 24 h. Then, the cells were treated with the peptides dissolved in sterile distilled water at different concentrations. The cells were then incubated for an additional 12 h at 37 °C. Cell viability was determined using MTT method as described above.

2.6.4. The protection of synthesized peptides on H_2O_2-induced cytotoxicity

Cells were seeded in a 96-well plate of 5×10^3 cells/well and incubated for 24 h. Then, the peptides dissolved in sterile distilled water at different concentrations were added. The cells were then incubated for an additional 12 h and then 700 μM H_2O_2 (final concentration) was added and incubated for another 2 h at 37 °C. Cell survival, expressed as the percentage of viable cells among all counted cells, was evaluated by morphological inspection under phase-contrast microscope (EVOS f1, AMG, America) and determined using MTT method.

2.7. Statistical analysis

All of the assays were carried out in triplicate. Data were analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was done to determine the significance of the main effects. Significant differences ($P < 0.05$) between means were identified using least significant difference (LSD) procedures.

3. Result

3.1. The pK_a of thiol groups for peptides

The pK_a values reflect the reactivity of thiol groups in GSH, ECH and YECG. The results showed that the pK_a of GSH was 9.1 ± 0.2, which is in agreement with other reports (Viirlaid et al., 2009). The pKa for ECH and YECG was 9.0 ± 0.2 and 9.4 ± 0.2, respectively. The results indicated that the thiol groups have similar acidity in GSH and ECH. With the presence of Tyr at N-terminal of YECG, there was an increase in its pK_a value, indicating that Tyr had influence on chemical properties of Cys residue and contributed to the antioxidant activities of YECG based on hydrogen-atom-transfer.

3.2. Single-electron-transfer (SET) reaction based antioxidant assay

DPPH radical scavenging capacity assay was used for the primary characterization of the scavenging potential of peptides, which is SET reaction based assay. According to the results shown in Table 1, ECH (Glu-Cys-His) demonstrated the strongest DPPH radical scavenging activity (80.16%) as followed by GSH (78.74%) and YECG (Tyr-Glu-Cys-Gly) (59.14%).

The reducing power assay is also SET reaction based assay. Table 1 gives the reducing power of the three synthetic peptides determined at 700 nm. At the concentration of 0.4 mM, ECH exhibited the highest reducing power (A_{700} = 0.378), which is better than those of the GSH (A_{700} = 0.252) and YECG (A_{700} = 0.290).

3.3. Hydrogen-atom-transfer (HAT) reaction based antioxidant assay

The capacity of the peptides to inhibit lipid peroxidation in a linoleic acid oxidation system was investigated using ferric thiocyanate (FTC) method, which is HAT reaction based assay. As shown in Fig. 1, all the three peptides, GSH, ECH (Glu-Cys-His) and YECG (Tyr-Glu-Cys-Gly), exerted significant ($P < 0.05$) lipid peroxidation inhibitory activity in the linoleic acid system. Without the protec-

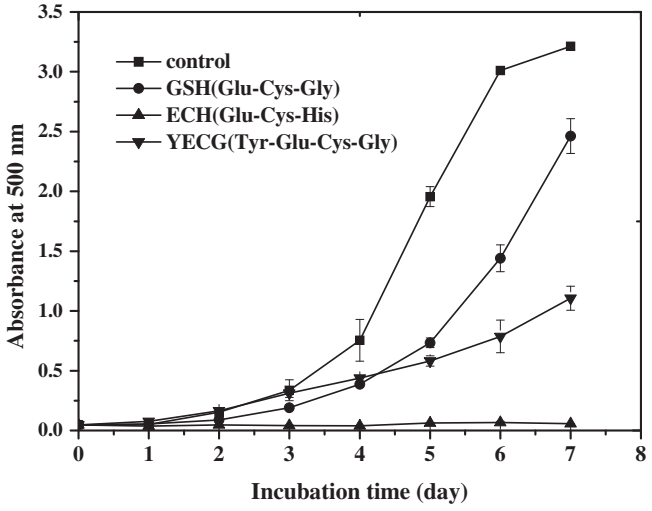

Fig. 1. Inhibition of linoleic acid autoxidation by the three synthetic peptides. The degree of linoleic acid oxidation was measured by ferric thiocyanate (FTC) method at every 24 h interval. Vertical bars indicate mean values ± SD.

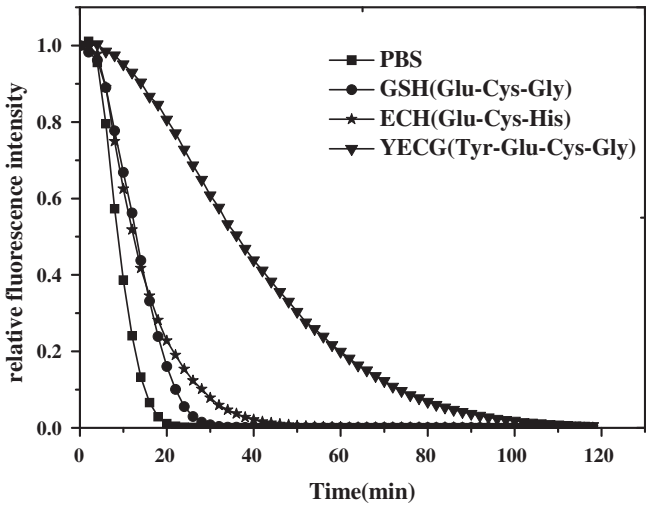

Fig. 2. Radical scavenging activity of synthetic peptides toward peroxyl radicals (ORAC assay).

tion of peptides, the auto-oxidation of linoleic acid increased rapidly, and reached very high value on the last day (see control in Fig. 1). At the 7th day, the inhibition rates were 23.38%, 98.25%, 65.57% for GSH, ECH and YECG, respectively (Table 1). ECH was found to exhibit extraordinary inhibitory activity during the seven days of oxidative reaction.

The oxygen radical absorption capacity (ORAC values) of the peptides was evaluated as well, which is also HAT reaction based assay. Fig. 2 depicted the effect of peptides on the time-dependent decay of fluorescein induced by AAPH. The tetrapeptide YECG was found to have the highest inhibition of fluorescein decay and thus showed the highest ORAC value 2.42 μM Trolox/μM peptide (see Table 1). However, the two tripeptides, GSH and ECH, were not that effective to inhibit the fluorescein decay and therefore, exhibited low ORAC values as 0.76 and 0.69 μM Trolox/μM peptide, respectively (Table 1). Compared with GSH (Glu-Cys-Gly), the presence of Try at N-terminal of the tetrapeptide YECG (Tyr-Glu-Cys-Gly) might contribute to the enhancement of its scavenging activity against oxygen radicals.

The ABTS radical cation decolorization assay (TEAC assay), which is HAT reaction based assay too, has been widely applied to evaluate the total antioxidative activity (Kong and Xiong, 2006). As shown in Table 1, the $ABTS^{\cdot+}$ free radical scavenging ability of YECG (8.88 mM Trolox/mM) was much stronger than those of the GSH (2.39 mM Trolox/mM) and ECH (2.43 mM Trolox/mM).

3.4. The ability of synthesized peptides to resist H_2O_2-inducing PC12 cell oxidation

The protection capacity of the three synthetic peptides on radical-mediated cellular injuries was assessed. The cell culture experiment was performed using rat adrenal pheochromocytoma (PC12) by exposing them to H_2O_2-inducing oxidative damage. Cytotoxicity was determined by the MTT assay. In Fig. 3A, the minimum dose for H_2O_2 to significantly decrease the cell viability of PC12 cells was 400 μM, and it exhibited a concentration-dependent decrease on cell viability. The viability of PC12 cells decreased to 40% as caused by the oxidation of 700 μM H_2O_2, and there was an approximately 97.5% decrease in the cell viability after the dosage of 1.0 mM H_2O_2 treatment. Fig. 3B showed that the three synthetic peptides, GSH, ECH and YECG, had no negative effect on the cell viability of PC12 cells at the concentration from 0.001 mM to 0.01 mM, which proved that synthetic peptides themselves has no cytotoxicity on

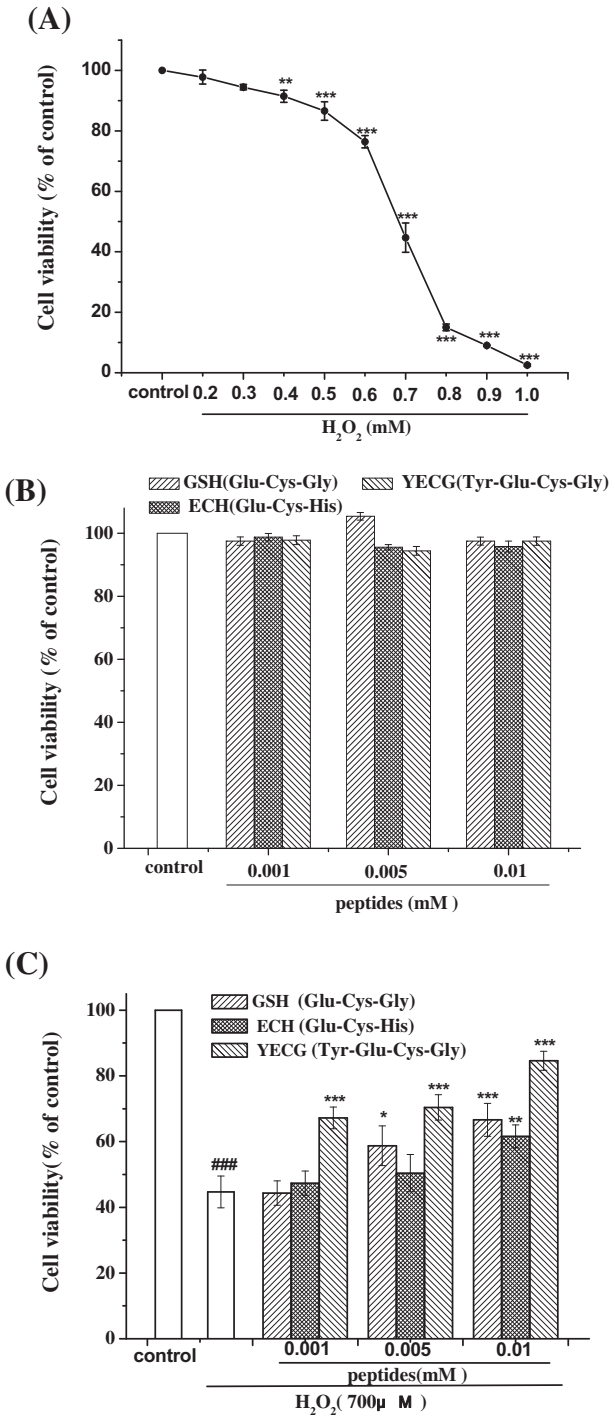

Fig. 3. Cell viability of PC12 cells. (A) The cytotoxicity of H_2O_2 on viability in normal cells. Cells were treated with H_2O_2 at the indicated concentrations (from 0.2 to 1 mM) and after 2 h, cell viability was assessed by MTT assay. $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the control group. (B) The cytotoxicity of the synthetic peptides on viability in normal cells. Cells were treated with the synthetic peptides at the indicated concentrations (0.001, 0.005 and 0.01 mM) and after 12 h, cell viability was assessed by MTT assay. $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the control group. (C) Protective effect of the synthetic peptides on H_2O_2-induced oxidative damage in normal cells. The viability of cells after H_2O_2 treatment was assessed by MTT assay. Vertical bars indicate mean values ± SD. $^{\#\#\#}p < 0.001$ compared with the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the H_2O_2 treated group.

PC12 cell at the concentration less than 0.01 mM. Hydrogen peroxide (H_2O_2) could induce significantly decrease of cell viability as compared with the control group (Fig. 3C), while the synthetic

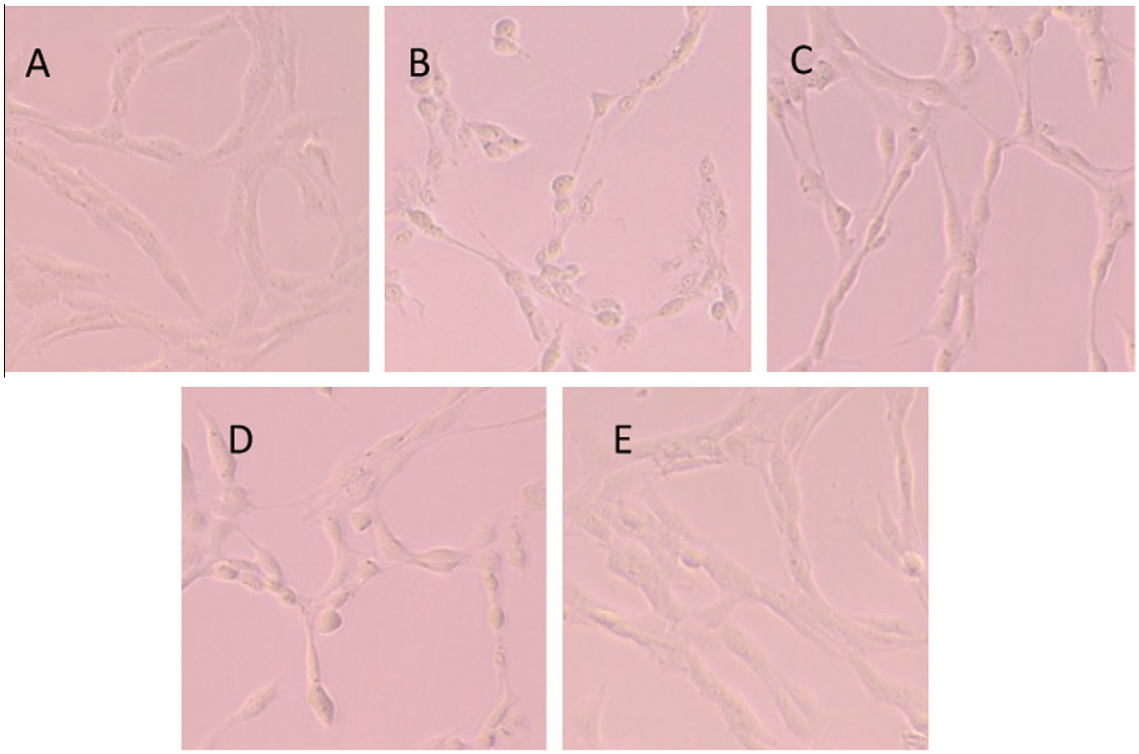

Fig. 4. Morphological characteristics of PC12 cells after H_2O_2 treatment in the absence or presence of peptides. (A) No treatment; (B) PC12 cells were exposed to 700 μM H_2O_2 for 2 h; (C) PC12 cells were treated with 0.01 mM GSH (Glu-Cys-Gly) for 12 h before exposed to H_2O_2 treatment (700 μM, 2 h); (D) PC12 cells were treated with 0.01 mM ECH (Glu-Cys-His) for 12 h before exposed to H_2O_2 treatment (700 μM, 2 h); (E) PC12 cells were treated with 0.01 mM YECG (Tyr-Glu-Cys-Gly) for 12 h before exposed to H_2O_2 treatment (700 μM, 2 h).

peptides (0.01 mM) exhibited good protection on H_2O_2-induced PC12 cell oxidation. All of the three peptides had a dose-dependent protective effect in cell survival enhancement. Among them, the tetrapeptide YECG demonstrated the best ability to resist H_2O_2-induced cell injuries and it was effective even at very low concentration (0.001 mM), while for GSH and ECH, they was only effective at relative high concentration (0.01 mM) and showed no positive effect at low concentrations (0.001 or 0.005 mM).

The morphological characteristics of PC12 cells after H_2O_2 treatment incubated with or without peptides were shown in Fig. 4. As compared with the control (Fig. 4A), PC12 cell demonstrated obvious cell shrinkage and membrane blebbing when exposed to 700 μM H_2O_2 (Fig. 4B), which are associated with the occurrence of apoptotic cell death. With the protection of the synthetic peptides, GSH (Fig. 4C), ECH (Fig. 4D) and YECG (Fig. 4E), most of the cells remained normal morphology. Among them, YECG showed extraordinary protection on cells with most of them exhibited normal morphological characteristics.

4. Discussion

The DPPH radical scavenging activity and reducing power are typical assay methods based on single-electron-transfer (SET) reaction. DPPH is long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation (Huang et al., 2005). Since ethanol was used to dissolve DPPH in the present study, the reaction mechanism of DPPH is based on an election transfer reaction due to the capacity of organic solvents to form strong hydrogen bonds with antioxidants (Boudier et al., 2012). Reducing power assay reflects the ability of antioxidants to donated electron, which is similar with the mechanism of DPPH radical scavenging assay. Many researchers have found that there was a direct correlation between antioxidant activity and reducing power (Duh, 1998). In the present study, ECH (Glu-Cys-His) had the strongest DPPH radical scavenging activity and reducing power (Table 1). According to the electron flow of ECH (see Fig. 5A), with His located at the C-terminal position of the peptide chain, the carboxyl (N- terminal) could enhance the ability of imidazole group in His to provide electrons, and thus contributed to the antioxidant activity of ECH.

The following mentioned three assay methods including the inhibition of linoleic autoxidation, ORAC assay and TEAC assay are all based on HAT reaction, which quantifies hydrogen atom donating capacity. Lipid peroxidation proceeds via radical mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Rajapakse et al., 2005b). The lipid peroxyl radicals (LOO·) could be formed in lipid peroxidation process, and the free radical chain reaction of lipid peroxidation could be terminated by antioxidants which directly scavenge LOO·. The assay about the capacity of peptides to inhibit lipid peroxidation is based on hydrogen-atom-transfer (HAT) reaction (Rajapakse et al., 2005a). As shown in Table 1, ECH (Glu-Cys-**His**) exhibited the highest lipid peroxidation inhibitory activity, which might be due to the presence of His residue in this tripeptide. The activity of His-containing peptides against lipid peroxidation is partly related to its metal ions chelating capability by imidazole ring (Yong and Karel, 1978). Furthermore, the antioxidative activity of His-containing peptides was reported to exceed that of His itself, due to the increased hydrophobicity of the peptides and thus increased the interaction between the peptides and fatty acids.

The ORAC assay measures the inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking activity by hydrogen atom transfer (Ou et al., 2001). The peroxyl radicals (LOO·) would be formed in the lipid peroxidation process, so ORAC assay provides a controllable source of peroxyl radicals with lipids in both food and physiological systems (Sheih et al., 2009).

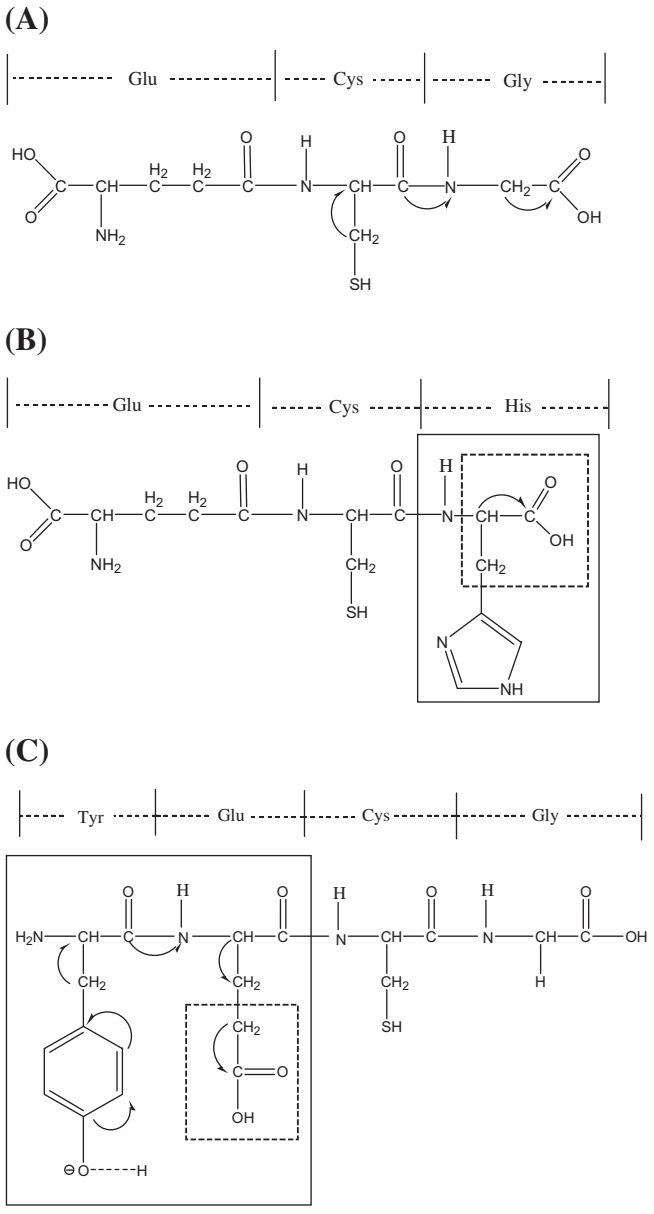

Fig. 5. The flow of electrons in peptides. (A) The flow of electrons of GSH (Glu-Cys-Gly); (B) the flow of electrons of ECH (Glu-Cys-His); (C) the flow of electrons of YECG (Tyr-Glu-Cys-Gly).

Antioxidative peptides containing aromatic amino acid residues (Trp and Tyr) in the sequence have strong radical scavenging activities due to the labile hydrogen atom (Najafian and Babji, 2011). As shown in Fig. 2, YECG (**Tyr**-Glu-Cys-Gly) effectively inhibited of the fluorescein decay and thus showed the highest ORAC value among the three peptides (Table 1). The Tyr at the N-terminal contributed to the antioxidant activity of YECG. Tyr acts as hydrogen donor by containing hydroxyl, which also exists in polyphenol and synthetic antioxidants such as butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT) (Wang et al., 2011; Winata and Lorenz, 1996). According to the electron flow of YECG shown in Fig. 5B, the carboxyl of Glu that located next to Tyr could induce the releasing of hydrogen atom of phenolic hydroxyl in Tyr to reach a more stable form of YECG.

ORAC assay has a mechanistic similarity to the lipid peroxidation assay, but in the present study, the tetrapeptide YECG showed the highest ORAC value, while the tripeptide ECH exhibited the highest lipid peroxidation inhibitory activity (Table 1). There are two reasons for this discrepancy and firstly, the concentration of the peroxyl radicals was much less in lipid peroxidation assay than that in ORAC assay. The ORAC assay are carried out with an artificial oxidant (AAPH) added to initiate the reaction, whereas in linoleic acid model system, the reaction occurs without extra added oxidant (Huang et al., 2005). Secondly, the hydrophobicity of the peptides is different. The partition coefficients of three peptides were −3.2, −3.5 and −2.6 for GSH, ECH and YECG, respectively. The reaction medium was the mixture of linoleic acid, ethanol, phosphate buffer and distilled water. Basically, the mixture is hydrophilicity. Since ECH has the highest hydrophilicity and also contains special perssad, it got the greatest diffusion coefficient in the medium. Therefore, ECH demonstrated the strongest inhibition activity to linoleic acid oxidation.

The TEAC assay is also based on HAT reaction. As shown in Table.1, all the three peptides showed good $ABTS^{\cdot+}$ free radical scavenging ability and compared with the other two, YECG exhibited the highest activity. It has been reported that amino acids demonstrated the $ABTS^{\cdot+}$ free radical scavenging reactivity followed the order Cys > Trp > Tyr > His, and that a labile hydrogen atom (e.g. SH, NH, and OH) was required for the reaction to occur (Liu et al., 2010). The more labile hydrogen atom, the more vulnerable to reaction (Aliaga and Lissi, 2000). So with the crucial amino acid residue Cys in the sequence of peptides, the three peptides demonstrated good $ABTS^{\cdot+}$ free radical scavenging ability. Obviously, the presence of Tyr at the N-terminal of YECG enhanced its $ABTS^{\cdot+}$ free radical scavenging ability compared with other two peptides.

The capability of the antioxidant peptides to scavenge free radicals in a cellular model was also evaluated. Cell culture models used for antioxidant research are particularly important since the mechanism of the action of antioxidants in human health promotion go beyond the antioxidant activity of scavenging free radicals (Liu and Finley, 2005). H_2O_2 is the most valuable exogenous ROS generator, although it does not have the properties of radical for itself. In particular, it is a potential source for $HO^{\cdot}$, which is one of the most dangerous radicals (Kanno et al., 2003). Therefore, H_2O_2 can serve as a typical chemical for investigation of oxidative stress and apoptosis (Kanno et al., 2000). In our study, it was found that extracellular supplementation of the synthetic peptides in culture media significantly abolished the H_2O_2-induced cell death (Fig. 3C). Research revealed that small di- and tri-peptides can easily get into the cell (Vermeirssen et al., 2002), and protect against cell death by enzymatic pathway or scavenging free radicals. YECG demonstrated the best resistance to H_2O_2-induced cells necrosis, while the biological mechanism of peptides on cells needs to be further studied.

In conclusion, this study found that the presence of antioxidant amino acids such as His or Tyr, and their position in peptide chain (C-/N- terminal), could influence the antioxidant capacity of the peptides. With His located at the C-terminal position of the peptide chain, ECH (Glu-Cys-**His**) demonstrated good electrons donating capacity, while with Tyr at N-terminal position, YECG (**Tyr**-Glu-Cys-Gly) exhibited strong oxygen radical absorbance capacity. These results suggest that one single chemical assay could not accurately reflect the antioxidant capacity of the peptides, so different assay methods should be applied for comprehensive evaluation of antioxidant activity.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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