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Secondary structure changes induced by pulsed electric field affect antioxidant activity of pentapeptides from pine nut (*Pinus koraiensis*) protein

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ABSTRACT

We used a pulsed electric field (PEF) to treat four pentapeptides with similar amino acid sequences (KCHKP, KCHQP, QCHKP, and QCHQP). We then evaluated antioxidant activity of the pentapeptides according to ferric reducing antioxidant power (FRAP) and hydroxyl radical scavenging capacity. Structures of the peptides were determined by high performance liquid chromatography (HPLC), mid-infrared (MIR), circular dichroism (CD) spectroscopy, and nuclear magnetic resonance (NMR). The results indicated that antioxidant activities of KCHQP and QCHKP were increased by PEF, whereas those of KCHKP and QCHQP were reduced. The basic structures and functional groups of peptides were unaffected. PEF treatment reduced the α-helix contents of KCHQP and QCHKP, but increased those of KCHKP and QCHQP. Moreover, the chemical shifts at 14.46 ppm, 8.22 ppm, 7.87 ppm, 7.24 ppm, and 6.13 ppm attributable to hydrogen atoms of QCHKP shifted to the right, but the active hydrogens of QCHQP were not affected by PEF.

1. Introduction

Natural antioxidant peptides are able to inhibit lipid peroxidation and scavenge free radicals. They maintain the free radical balance of the human body and contribute to anti-aging. Natural antioxidant peptides are safe and efficient. Therefore, they have been investigated extensively by researchers, and there have been many studies on antioxidant peptide separation and identification in particular (Wang et al., 2014; Zhang, Mu, & Sun, 2014). The structures of antioxidant peptides have a significant influence on the mechanisms by which they act. Two peptides with the sequences Phe-Tyr-Tyr and Asp-Trp have been identified from lanternfish (*Benthosema pterotum*) hydrolysates (Chai, Chan, Li, Shiau, & Wu, 2013). A peptide with antioxidant activity has been purified from Chinese leek seeds, and the sequence was determined to be Gly-Ser-Gln (Hong, Chen, Hu, Yang, & Wang, 2014). Ghribi et al. (2015) identified two peptides (Asp-His-Gly and Val-Gly-Asp-Ile) from an enzymatic hydrolysate of chickpea (*Cicer arietinum* L.) protein concentrate.

Pulsed electric field (PEF) treatment is an alternative to traditional food processing techniques, and has been applied to pump-able and semifluid foods (Wu, Zhao, Yang, & Yan, 2015). This technology can ensure good product quality and energy utilization because it requires less heat. In the past, most researches in the field of PEF application have focused on sterilization and endogenous enzyme inactivation to ensure product quality and prolong the shelf life of foods. However, with a growing understanding of PEF, the technology has also been used to improve the antioxidant activity of peptides. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition of egg-white polypeptides increased by 28.44% when they were treated by PEF with an electric field intensity of 10 kV/cm and a pulse frequency of 2000 Hz (Wang et al., 2013). The DPPH inhibition of glutathione increased from 81.83% to 97.40% following treatment by PEF (Wang et al., 2014). However, the reason PEF treatment improves antioxidant activity remains unclear.

There are many ways to study protein structures including high performance liquid chromatography (HPLC), circular dichroism (CD) spectroscopy, X-ray stress analysis, infrared spectroscopy, Raman spectrometry, mass spectrum and nuclear magnetic resonance (NMR). Currently, the main analytical methods used to measure changes in protein structure are CD spectroscopy, infrared spectroscopy, etc. The tertiary structure of milk alkaline phosphatase is changed by PEF at 22 kV/cm; the treatment leads to the unfolding of the protein molecules and the exposure of the hydrophobic group (Castro, Swanson, Barbosa-Cánovas, & Dunker, 2001). Neumann and Katchalsky (1972) found that the α-helices proteins can be transformed into random coils by PEF at

Abbreviations: PEF, pulsed electric field; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; MIR, mid-infrared; CD, circular dichroism; EPR, electron paramagnetic resonance

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20 kV/cm. Yeom, Zhang, and Dunne (1999) used CD spectroscopy to show that PEF treatment induces the α-helix content of papain. Zhong, Wu, Wang, and Zhang (2007) found that peroxidase activity decreased with increasing electric field intensity during treatment with PEF, and CD spectroscopy indicated that the α-helix content of the peroxidase decreased by 35.1%. These studies have revealed structural changes to protein molecules following PEF treatment. Furthermore, several researchers have reported the effects of PEF treatment on peptide structure. Liang, Li, Lin, and Wang (2017) found that PEF-treated soybean antioxidant peptides lack the characteristic absorbance of N—H and C═C. The secondary structures (a-helix, β-turn, and random coil) of the SHCMN peptide is affected by PEF (Lin, Liang, Li, Xing, & Yuan, 2016). However, this research does not reveal the mechanism by which the structural changes affect the activity of antioxidant peptides following PEF treatment. Therefore, in the present study we used HPLC, MIR, CD, and NMR to investigate the structures of PEF-treated peptides from pine nut (*Pinus koraiensis* Sieb. et Zucc) proteins. Our objective was to determine the mechanism underlying the changes in antioxidant activity following treatment of the peptides with PEF. This study may improve our understanding of antioxidant peptides and provide a scientific basis for developing highly efficient antioxidant products. It may also increase the scope of PEF applications.

2. Materials and methods

2.1. Materials and reagents

Four pine nut peptides Lys-Cys-His-Lys-Pro (KCHKP), Lys-Cys-His-Gln-Pro (KCHQP), Gln-Cys-His-Lys-Pro (QCHKP), and Gln-Cys-His-Gln-Pro (QCHQP) were chemosynthetic by Shanghai Yaoqiang Biological Technology Co. (Shanghai, China). The molecular weight of KCHKP, KCHQP, QCHKP and QCHQP is 611.72 Da, and the purity is 98.25%, 98.31%, 98.60% and 98.09%, respectively (Lin et al., 2017). Potassium bromide powder (KBr), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), acetonitrile, trifluoroacetic acid, dimethyl sulfoxide-D6 (DMSO-D6), and 99.8% of D_2O were purchased from Sigma Chemicals Co. (USA). All other chemical reagents were purchased from Peking Chemical Plant (Beijing, China) and of analytical grade purity.

2.2. PEF treatment of peptides

The PEF system designed by Yongguang Yin was used to treat the antioxidant peptides (Jin et al., 2011). This system comprises a high-voltage repetitive pulse generator, a coaxial liquid material treatment chamber, a fiber-optic temperature sensor and a data acquisition system (Lin et al., 2011). The frequency of the instrument can be varied between 1000 and 3000 Hz, and a digital oscillograph is used to display the bipolar pulse waveform. The PEF system can be used to continuously process fluid samples. The peptides were dissolved in deionized water to a concentration of 8 mg/mL before PEF processing. The PEF pump and circuit were washed with deionized water, then ethanol, and finally with deionized water again; this was repeated three times before use. The peptide solution was pumped into the PEF system at a flow rate of 3.2 mL/min. The high-voltage pulse was then turned on. The PEF treatment conditions were varied (1800 Hz, 5 kV/cm; 1800 Hz, 10 kV/cm; 1800 Hz, 15 kV/cm; 1800 Hz, 20 kV/cm; 2400 Hz, 5 kV/cm; 2400 Hz, 10 kV/cm; 2400 Hz, 15 kV/cm; 2400 Hz, 20 kV/cm) and the corresponding peptide solutions (10 mL) were collected.

2.3. Ferric reducing antioxidant power (FRAP) test

We used the ferric reducing antioxidant power (FRAP) test to evaluate antioxidant activity, based on the procedure reported by Müller, Fröhlich, and Böhm (2011) with some modifications for the use of 96-well microplates. The FRAP reagent comprised acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl, and 20 mmol iron chloride solution in the ratio 10:1:1 (v/v). 400 μL of FRAP reagent were added to 100 μL of sample. The absorbance of the tested samples were measured at 593 nm 4 min later. The $FeSO_4{\cdot}7H_2O$ with various concentrations was used to prepare standard curve. The FRAP values of sample were expressed as mmol Fe^{2+}/mg.

2.4. Determination of hydroxyl radical scavenging ability by electron paramagnetic resonance (EPR)

The antioxidant activity of the peptides (at a concentration of 5 mg/mL) was evaluated by measuring their ability to scavenge hydroxyl radicals using the method described by Morel et al. (1995) with some modifications. The hydroxyl radicals in the samples (KCHKP, KCHQP, QCHKP, and QCHQP) were investigated before and after PEF treatment using an X-band EMXnano EPR spectrometer (Bruker BioSpin) at 25 °C. The EPR measurement conditions were: sweep width 200 G; modulation frequency 100 kHz; modulation amplitude 1.0 G; receiver gain 1×10^6; time constant 1.28 ms; sweep time 30.0 s; and an accumulation of three scans.

2.5. Investigation of the peptides using HPLC

The peptides were investigated using an LC-20AD HPLC system (Shimadzu Corp., Japan). A kromasil 100-5 C18 column (250 × 4.6 mm, 5 μm; AkzoNobel Corp, Sweden) was used to identify the peptides using the method described by Wang and Li (2017), with some modifications. The wavelength was set at 220 nm. Mobile phase A comprised 0.1% trifluoroacetic acid in 100% acetonitrile; mobile phase B comprised 0.1% trifluoroacetic acid in 100% water. The peptide elution gradients were as follows. KCHKP: mobile phase A was adjusted from 5% to 30% over 25 min; KCHQP: mobile phase A was adjusted from 4% to 29% over 25 min; QCHKP: mobile phase A was adjusted from 8% to 33% over 25 min; QCHQP: mobile phase A was adjusted from 5% to 30% over 25 min. The peptide solution (10 μL) was injected at a concentration of 1 mg/mL.

2.6. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

The NMR spectroscopy was carried out according to the method described by Delaglio et al. (1995). The ^{1}H NMR and ^{13}C NMR spectra were obtained using an AVANCE III 500 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) with a 5 mm broad band fluorine observation probe (BBFO) and z-gradients. First, a solution of peptide QCHKP was treated by PEF at a pulse frequency of 2400 Hz and an electric field intensity 20 kV/cm. The treated solution was then dried in a vacuum freeze drier. The freeze-dried QCHKP (25 mg) was dissolved in 600 μL of D_2O, and its ^{1}H NMR and ^{13}C NMR spectra were obtained. Next, PEF-treated and untreated QCHKP and QCHQP peptides (10 mg each) were dissolved in 600 μL of DMSO-D6, and their ^{1}H NMR spectra were obtained. A drop of D_2O was then added to the untreated QCHKP and QCHQP solutions to obtain the ^{1}H NMR spectra.

2.7. Functional groups analysis by MIR

The MIR spectroscopy method described by Lin et al. (2013) was used for the analysis of functional groups in the peptides. The PEF-treated samples were dried in a vacuum freeze drier purchased from the Hangzhou Creative Vacuum Freeze Drying Equipment Plant (Hangzhou, China). The MIR spectra were obtained using a IRPrestige-21 Fourier transform infrared spectrometer (Shimadzu, Japan) at a resolution of 4 cm^{-1} over the range 4000–400 cm^{-1}. A KBr (dried at 130 °C for 8 h) spectrum was used as a background, and the testing samples were prepared by mixing 2 mg of the dried peptides with 200 mg of KBr. The samples were then placed in the sample compartment with automatic accessory recognition at a scanning speed of