SUPPLEMENTARY MATERIAL

Antioxidant activity of arrowhead protein hydrolysates produced by a novel multi-frequency S-type ultrasound-assisted enzymolysis

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Abstract: Effects of multi-frequency S-type ultrasound (MFSU) assisted arrowhead protein (AP) hydrolysis on the antioxidant activity of its hydrolysates were studied. The results showed the DPPH• and ABTS•+ scavenging activity of hydrolysates obtained with dual frequency ultrasound (20/40 kHz) was 63.61% and 65.11%, respectively, and was higher than that noted for hydrolysates acquired with assistance of other mode (single and triple frequency ultrasound). Compared with hydrolysates without ultrasonic treatment, products of AP hydrolysis assisted by dual frequency ultrasound (20/40 kHz) could significantly alleviate oxidative stress induced by H2O2.
in RAW 264.7 cells, mainly embodied in improving the survival rate and increasing the activity of antioxidant enzymes (CAT and SOD). Taken together, these results showed that MFSU-assisted enzymatic treatment can significantly improve the antioxidant activity of AP hydrolysates. Thus, the development of the novel MFSU could lay a foundation for assisting the protein enzymolysis in food and pharmaceutical industries.

**Keywords:** Arrowhead protein; Multi-frequency S-type ultrasound; Antioxidant activity; Ultrasound-assisted enzymolysis.

**Experimental**

**Materials**

Arrowhead was obtained from Hezhi Fang Biology Technology Co., (Yangzhou, China) and the main components were starch (63.54%), protein (11.93%), Fat (1.1%), ash (2.43%), water (5.4%), crude fiber (4.64%) and some trace elements. The spherical tuber of arrowhead was peeled, diced, dried, ground, pulverized, and passed through a 100 mesh sieve. The sieved arrowhead powder was kept in -20℃ refrigerators until further investigation. Alcalase 2.4 L (2×10^5 U/g) was obtained from Novozymes Co. Ltd. (Tianjin, China). All the other chemicals and solvents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China) and were analytical grade.

**Arrowhead protein extraction**

The method of AP extraction was following the previous method (Zhu et al. 2006) with minor modification. The arrowhead powder was dispersed into distilled water (1:20), and the pH of the mixture was adjusted to 9.5 by using 1 M NaOH. Stirring continuously for 4 hours, then the mixture solution was centrifuged at 4,500g for thirty minutes. The supernatant was adjusted the pH to 4.0 with 1.0 mol/L HCl to precipitate the protein. The precipitates were dispersed in distilled water, and its pH
was adjusted to 7 by using 1 M HCl and lyophilized for further analysis. The protein content of precipitate was 91.6% according to the Kjeldahl method.

**MFSU-assisted and traditional enzymolysis**

The MFSU device diagram is shown in Figure S1A, which was produced by Jiangsu University and mainly composed with two parts (ultrasonic generator, and circulation pump). In addition, Figure S1B-D showed the diagrams with mechanism of action of the MFSU device. Ultrasonic waves can produce cavitation bubbles in the process of spread, and the cavitation bubble will be changed as the change of ultrasonic frequency.

The experimental conditions were carried out according to the following methods:

An aliquot (1L) of AP solution (3 g/L) was prepared and hydrolyzed with 10 mL alcalase (activity 23,400 U/mL) at pH 9.0 and 50 °C. The MFSU-assisted enzymatic hydrolysis of protein solution was carried out under the three frequency mode (Single frequency, double frequency, and triple frequency) as follow: enzymolysis time of 120 min, ultrasonic power of 100 W. After completion, the enzyme in solution need be inactivated in a water bath (100°C) for 10 min and cooled it down to room temperature. The hydrolys solution was adjusted pH to neutral by using 1 M HCl and lyophilized for further analysis.

**DPPH• scavenging capacity assay**

DPPH• scavenging activity of AP hydrolysate were determined by the previous method (Brand-Williams et al. 1995). The samples (0.09375-3 mg/mL) were dissolved in 500 μL of distilled water and mixed with 500 μL of 99.5% ethanol and 125 μL of DPPH• solution (200 μmol/L). The mixtures reacted in the dark at room temperature for 30 min. The absorbance was determined at 517 nm. DPPH• scavenging activity was calculated as follow:

\[
\text{DPPH• scavenging capacity (%) = } \left( \frac{\text{Absorance of Control} - \text{Absorance of sample}}{\text{Absorance of control}} \right) \times 100
\]
The control was used the same manner, except that the samples were replaced by distilled water. All the experiments were carried out in triplicate.

**ABTS•+ radical scavenging capacity assay**

The ABTS•+ scavenging activities of AP hydrolysates were measured by previous method (Thaipong et al. 2006) with minor modification. The different concentrations of samples (0.09375-3 mg/mL, 100 μL) were mixed with 100 μL of the diluted ABTS•+ solution. The mixtures were reacted in the dark at room temperature. The reduction of ABTS•+ were measured at 734 nm. ABTS•+ scavenging capacity was measured as follow:

ABTS•+ scavenging capacity (%)

\[
\text{ABTS•+ scavenging capacity (%)} = \frac{\text{Absorance of Control} - \text{Absorance of sample}}{\text{Absorance of control}} \times 100
\]

The control was used the same manner, except that the samples were replaced by distilled water. The entire experiments were conducted in triplicate.

**Cell culture**

RAW 264.7 cell line was obtained from American Type Culture Collection (ATCC; Arlington, USA). Cells were generally cultured in DMEM medium. It is worth noting that the medium contains 100 units/mL penicillin and 100 g/mL streptomycin. The condition of cell culture was at atmosphere of 5% CO$_2$ and the temperature of 37 °C.

**Cell viability assay**

AP hydrolysates were used to measure the cytoprotective effect on RAW 264.7 cells by using MTT assay. The RAW 264.7 cells with a density of $2 \times 10^5$ were incubated in 96 well plate. Different concentrations of AP hydrolysates (0.125-1.0 mg/mL) were incubated into the RAW 264.7 cells for 2 hours. After that, remove the medium and wash it with PBS solution for two times. Then, H$_2$O$_2$ (0.5 mM) was added to the cells for incubation (6h). Subsequently, the cells were continued to incubate 4 h with MTT instead of H$_2$O$_2$ in constant temperature incubator. Finally, the MTT solution was
removed and the formazan was dissolved with 150 μL of DMSO. The ELISA reader (TECAN, Switzerland) was used to determine the absorbance at 590 nm. The cell viability rate was calculated as follow:

\[
\text{Cell viability rate (\%)} = \left( \frac{\text{experimental absorbance}}{\text{control absorbance}} \right) \times 100
\]

**Antioxidant enzyme activity**

The antioxidant enzyme activity was measured by the SOD assay kit and the Catalase (CAT) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The experimental method refers to the instruction manual of the kit. Briefly, CAT activity was determined by the production of formaldehyde produced by the reaction of the sample with methanol and H₂O₂. The absorbance was measured at a wavelength of 540 nm by using a microplate reader. In addition, the determination of SOD activity was based on the ability of the sample to inhibit O²⁻ produced by the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the amount that reduces the absorbance at 450 nm by 50%.

**Statistical analysis**

All experiments were performed in triplicate. Data were expressed as means ± SD. The statistical analyses were performed for multiple comparison analysis with Duncan’s tests by using SPSS 18.0 software (SPSS Inc. Chicago, IL, USA). \( p<0.05 \) was considered as statistically significant.

**Reference**

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Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of food composition and analysis. 19:669-675.

Zhu K, Zhou H, Qian H. 2006. Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. Process Biochemistry. 41:1296-1302.
Figure S1. Schematic diagrams of the multi-frequency S-type ultrasound equipment (A); Single frequency ultrasound (B); Double frequency ultrasound (C), note: lowercase a, b represents individual waveforms of different frequencies, and lowercase c represents the intersection waveform of simultaneous operation at different frequencies; Triple frequency ultrasound (D), lowercase a, b, c represents...
individual waveforms of different frequencies, and lowercase d represents the intersection waveform of simultaneous operation at different frequencies.

**Figure S2.** Effect of MFSU-assisted enzymolysis of AP on the DPPH• scavenging activity of AP hydrolysates (A: Single frequency ultrasound, B and C: Double frequency ultrasound, D: Triple frequency ultrasound). Untreated represents arrowhead protein hydrolysates obtained without ultrasonic treatment. Vertical bars indicate mean values ± SD (n=3). Different lowercase letters represent significant (p<0.05) differences between hydrolysates.
Figure S3. Effect of MFSU-assisted enzymolysis of AP on the ABTS•+ scavenging activity of AP hydrolysates. (A: Single frequency ultrasound, B and C: Double frequency ultrasound, D: Triple frequency ultrasound). Untreated represents arrowhead protein hydrolysates obtained without ultrasonic treatment. Vertical bars indicate mean values ± SD (n=3). Different lowercase letters represent significant (p<0.05) differences between hydrolysates.
Figure S4. Effects of MFSU-assisted enzymolysis treated on cellular antioxidative capacity of AP. A: Cells were incubated in medium containing various concentrations of AP and AP hydrolysates (0.125-1 mg/mL) for 24 h and cell viability was measured with an MTT assay. B: Cytoprotective effect of AP after \( \text{H}_2\text{O}_2 \)-induced oxidative damage in RAW 264.7 cells. C and D: Effects of AP on the activities of SOD and CAT after \( \text{H}_2\text{O}_2 \)-induced oxidative damage in RAW 264.7 cells. Untreated represents arrowhead protein. APHPs represent arrowhead protein hydrolysates obtained without ultrasonic treatment. Vertical bars indicate mean values ± SD (n=3). Different lowercase letters represent significant (\( p < 0.05 \)) differences between hydrolysates.