Changes in Antioxidant Activity of Peptides Identified from Brown Rice Hydrolysates under Different Conditions and Their Protective Effects against AAPH-Induced Oxidative Stress in Human Erythrocytes

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ABSTRACT: Four antioxidant peptides (Ile-Tyr, Leu-Tyr, Val-Tyr, and Tyr-Leu-Ala), identified from brown rice protein hydrolysates, showed strong ROO· and ABTS+ scavenging activities. Changes in the antioxidant activity of peptides and GSH (control) under different processing conditions, namely, NaCl concentration, temperature, pH, and gastrointestinal proteases, were evaluated by the oxygen radical absorbance capacity assay and the Trolox equivalent antioxidant capacity assay. Results indicated that with the increase in NaCl concentration, temperature, and pH (beyond neutral), the antioxidant activity of the peptides decreased, while the decrease was lower than that of GSH. The antioxidant activity of the four antioxidant peptides changed slightly after in vitro digestion, indicating a relatively high digestion resistance. The protective effect on the oxidative damage model of 2,2-azobis(2-methylpropionamide)-dihydrochloride-induced human red blood cells was also studied. Leu-Tyr and Tyr-Leu-Ala could alleviate but not totally inhibit oxidative damage in red blood cells, and their protective effects were dependent on concentration.

1. INTRODUCTION

Food-derived antioxidant peptides could act as antioxidative agents to control various oxidative processes in the human body as well as in food.1,2 The antioxidant potential of the rice and by-product-derived protein hydrolysates and peptides has been widely demonstrated in various oxidative systems, such as free radical scavenging activity, reducing power assay, and metal ion-chelating capacity.3 Selenium-enriched rice protein hydrolysates showed the potential antioxidant activity evaluated by the cellular antioxidant activity test.4,5 The peptide Tyr-Ser-Lys derived from rice bran protein exhibited strong DPPH scavenging activities and reducing power assay.6 Bioactive peptides should remain intact before reaching the target organ to exert biological effects.7 Peptide structures can be changed when they are influenced by physical or chemical factors, such as heat, high pressure, extreme pH, salt ions, metal ions, and protease, leading to the changes in activity.8 Peptides may undergo deamidation, hydrolysis, oxidation, cyclization, and other reactions during processing and storage, resulting in the loss of antioxidant activities.9 In digestion, antioxidant peptides easily come into contact with chemical substances, such as Fe and Cu food derivatives, which affect peptide activities. Moreover, peptides may be further hydrolyzed by gastrointestinal enzymes thereby altering their activities.10 Ao and Li11 reported that a casein hydrolysate fraction with high basic amino acid content exhibited stronger degradation during digestion, releasing about 34% of free

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amino acids. By contrast, the fraction containing high acidic amino acids showed better digestive stability. Therefore, the antioxidant activity of peptides under different conditions should be determined for food applications.

Cell culture models provide a cost-effective and relatively fast approach that addresses the issues of uptake, distribution, and metabolism of antioxidant compounds. In recent years, different types of cell models have been established, the red blood cell model of which is widely used.12−14 When it is used to evaluate the protective effects of antioxidants on oxidative damage in cells, the cells must be initially subjected to oxidative damage. 2,2-Azobis(2-methylpropionamide)-dihydrochloride (AAPH) is an azo-based free radical initiator that decomposes at 37 °C to produce ROO·, leading to oxidative damage in cells.15 AAPH is good for relatively constant decomposition to generate free radicals. Therefore, it is commonly used to establish oxidative damage models of red blood cells. Sun et al.16 reported that the purified fraction P3 from leaf proteins hydrolysates showed the highest hemolysis inhibition ability, which could be ascribed to containing the Tyr residues at the C-terminal region. Similarly, findings revealed that dipeptides Trp-Gly, Tyr-Gly, Met-Gly, and Cys-Gly exhibited protective effects against AAPH-induced oxidation of hemoglobin in a dose-dependent manner.12

Our previous studies showed that Se-enriched brown rice protein hydrolysates can be used as natural foodborne antioxidants.17 Besides the reported Se-containing antioxidant peptide SeMet-Pro-Ser, the peptides Ile-Tyr, Leu-Tyr, Val-Tyr, and Tyr-Leu-Ala have also been identified from the Se-enriched brown rice protein hydrolysates and were the most potent antioxidant peptides. The objective of this study was to evaluate the antioxidant activity of the four peptides against NaCl, temperature, pH, and gastrointestinal digestion by measuring the oxygen radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) values and explore Leu-Tyr and Tyr-Leu-Ala protective effects against AAPH-induced oxidative damage in human erythrocytes.

2. RESULTS AND DISCUSSION

2.1. Influence of NaCl on Antioxidant Peptide.

Different NaCl concentrations were adopted to treat five antioxidant peptides, and the changes in their activities are shown in Figure 1. The antioxidant activity of Ile-Tyr decreased as the NaCl concentrations increase. When the NaCl concentration increased to 8%, the antioxidant activity of Ile-Tyr was the lowest (Figure 1A). When the NaCl concentration reached 2%, the antioxidant activity of Leu-Tyr did not significantly differ from that of the control group (0% NaCl). When the NaCl concentration increased to 4%,

Figure 1. Influence of NaCl on antioxidant peptides (A) Ile-Tyr, (B) Leu-Tyr, (C) Val-Tyr, (D) Tyr-Leu-Ala, and (E) GSH. (Times): TEAC, (solid square): ORAC. Each bar represents mean ± standard errors (n = 3). Mean values denoted by different letters are significantly different (P < 0.05).
the antioxidant activity of Leu-Tyr significantly decreased. When the concentration further increased, the antioxidant activity of Leu-Tyr remained the same (Figure 1B). The antioxidant activity of Val-Tyr could be significantly lost at high NaCl concentrations, and its ORAC value was reduced by 0.21 μmol TE/μmol compared with that of the control group. Conversely, when the NaCl concentration was lower than 6%, the antioxidant activity of Val-Tyr was not influenced by NaCl concentration changes (Figure 1C). The antioxidant activity of Tyr-Leu-Ala decreased as the NaCl concentrations increased. Different NaCl concentrations affected the ABTS⁺ and ROO· scavenging ability to varying degrees. When the NaCl concentration reached 2%, the ROO· scavenging ability of Tyr-Leu-Ala decreased, whereas ABTS⁺ scavenging ability remained unchanged. When the NaCl concentration increased to 6%, its TEAC value was also reduced (Figure 1D). The antioxidant activity of GSH decreased as the NaCl concentrations increased (Figure 1E).

The interaction of a salt ion with a peptide or other nonpolar genes, especially a π-electron on an aromatic side chain, is one of the mechanisms of protein denaturation. It may be one of the causes of the decrease in the activity of the above-mentioned antioxidant peptides. Liu et al. showed that the antioxidant activity of the enzymatic hydrolysate of male silkworm moth decreases as the NaCl concentration increases. The presence of salt decreases the solubility of peptides and proteins, thereby decreasing antioxidant activities. Zhu et al. found that the Fe²⁺ chelation capability loss of antioxidant peptides was up to 25% when the NaCl concentration was 8% probably because high NaCl concentrations act on some peptides with the ability to chelate transition-metal ions. Therefore, the structure was changed by the chelate ionic ability of the peptide, resulting in the loss of its activity.

In summary, the antioxidant activities of the five peptides decreased with the increased concentration of NaCl. The sensitivity of the different peptides differs for the same salt content; meanwhile, its antioxidant activity is influenced by the salt content and type in different aspects and degrees. However, the five peptides can still maintain high antioxidant activities.

2.2. Influence of Temperature on Antioxidant Peptide. The effects of temperature on the antioxidant activities of the five peptides are shown in Figure 2. When the temperature was lower than 60 °C, the change in temperature had no significant effects on the antioxidant activity of Ile-Tyr. At 80 °C, the Ile-Tyr antioxidant activity slightly increased. When the temperature continuously increased (>80 °C), no significant differences were observed between the antioxidant activities of Ile-Tyr and those of the unheated group (25 °C) (Figure 2A). The effect of temperature on the antioxidant...
activity of Leu-Tyr was consistent with that of Ile-Tyr (Figure 2B). The antioxidant activity of Val-Tyr initially increased and then decreased. At 100 °C, the ORAC value of Val-Tyr decreased by 0.19 μmol TE/μmol compared with that of the unheated group (Figure 2C). The antioxidant activity of Tyr-Leu-Ala slightly increased because of heating, and the TEAC value of Tyr-Leu-Ala was slightly higher than that of the unheated group after it was heated at 100 °C (Figure 2D). As the temperature increased, the ABTS·+ and ROO· scavenging abilities of GSH decreased. When the temperature increased from 25 to 100 °C, the ORAC value of GSH decreased from 0.55 ± 0.05 to 0.16 ± 0.06 μmol TE/μmol and the TEAC value decreased from 2.26 ± 0.05 to 0.63 ± 0.04 μmol TE/μmol (Figure 2E), indicating that GSH showed adverse heat antioxidant activity. The radical scavenging of GSH can act mainly through a single electron transfer mechanism, while GSH could undergo side chain modifications leading to cleavage of thiol (S–H) bonds and formation of the disulfide bond under higher temperature treatment.7

For short-chain and low-molecular-weight peptides, their activities are altered by high temperatures because of changes in the secondary structure of peptides, considering that they do not have tertiary and quaternary structures.19 Jang et al.20 found that the activity of the antioxidant peptides initially increased and then decreased as temperature increased by heating Ala-Thr-Ser-His-His. When the temperature reached 100 °C, the DPPH· scavenging ability was still as high as 66%. Therefore, although high temperatures can destroy the GSH antioxidant activity, other activities of antioxidant peptides slightly changed after heating, suggesting that the four peptides are stable in heat processing. Most food processing technologies involve heat treatment; thus, these antioxidant peptides are promising for use in the food industry.

2.3. Influence of pH on Antioxidant Peptide. Changes in pH affect electrostatic interactions between charged amino acids. For example, H+ and OH− interact with charged regions of proteins, destroying the hydrogen bond between amino acids, leading to protein or peptide denaturation and changes in its biological activity.21 The changes in the activity of the five peptides after treatment at different pH levels are shown in Figure 3. At neutral pH, the antioxidant activity of these peptides was relatively high, but their antioxidant activity decreased significantly under peracid or over-based conditions. For example, the TEAC value of Leu-Tyr was 1.48 ± 0.01 μmol TE/μmol at pH 6, while it was 1.34 ± 0.00 μmol TE/μmol at pH 12 (Figure 3B). The effect of pH on the ROO· scavenging ability of Val-Tyr, whose ORAC value varied from 2.06 to 2.49 μmol TE/μmol, was significant (Figure 3C). When the pH was increased to 8, the ABTS·+ scavenging activity of GSH sharply declined and at pH 12, the activity was reduced by 40% compared with that under the neutral pH condition. (Figure 3E).

Figure 3. Influence of pH on antioxidant peptides (A) Ile-Tyr, (B) Leu-Tyr, (C) Val-Tyr, (D) Tyr-Leu-Ala, and (E) GSH. (Times): TEAC, (solid square): ORAC. Each bar represents mean ± standard errors (n = 3). Mean values denoted by different letters are significantly different (P < 0.05).
The antioxidant activity of the five peptides was most stable under or close to neutral conditions. By contrast, their antioxidant activity can be reduced in super acid or alkaline environments. Zhu et al.\textsuperscript{19} found that an alkaline environment significantly affects the DPPH· scavenging rate of the Jinhua ham antioxidant peptide. Liu et al.\textsuperscript{9} also proposed that the loss of the antioxidant activity of the peptides may be due to the occurrence of racemization, deamination, and variation of the degradation pathway. Therefore, each peptide has its optimum pH range. Beyond this range, peptide structure changes, and its antioxidant activity is affected.

\textbf{2.4. Influence of In Vitro Gastrointestinal Digestion on Antioxidant Peptide.} Some foodborne bioactive peptides lose their activity upon digestion. The digestive stability of antioxidant peptides is usually assessed on the basis of changes in their activity after they undergo digestion in vitro. As shown in Figure 4, the antioxidant activities of the five peptides slightly changed when pepsin, trypsin, and chymotrypsin act alone or successively on the peptides. These results indicated that they have good antioxidant activity in the gastrointestinal digestive system.

Most peptides of more than three amino acids are extracellularly hydrolyzed by enzymes in the brush border membrane of the intestinal epithelium. Dipeptides, tripeptides, and peptides containing proline residues can partially resist the enzymatic attack.\textsuperscript{10} Chen and Li\textsuperscript{22} studied the effect of molecular weight distribution on the gastrointestinal digestion stability of casein antioxidant peptides. The components with a molecular weight of less than 1000 Da exhibited a strong antioxidant survival ability by changing the ABTS\textsuperscript{+} and hydroxyl radical scavenging abilities and ORAC value before and after digestion. The ABTS\textsuperscript{+} scavenging ability of Val-Ser-Ala-Phe-Leu-Ala isolated from a Chinese chestnut hydrolysate was significantly improved after it undergoes gastrointestinal digestion, whereas the activities of the four other peptides slightly changed.\textsuperscript{23} Wong et al.\textsuperscript{24} proposed that the Pro-containing peptides are not damaged by gastrointestinal digestive enzymes. SNAAC was degraded to form the fragment SNAA after gastric digestion and gastrointestinal digestion; the formation of the peptide dimer through the disulfide bond increased the solubility, resistance against proteases, and antioxidant activity of the peptide.\textsuperscript{8} However, a comprehensive and systematic understanding of the relationship between amino acid composition or sequence of peptides and their resistance to gastrointestinal digestive enzymes has yet to be achieved.

In summary, a single peptide of a small molecule has a strong resistance to digestion. The activities of mixed peptides are more susceptible to gastrointestinal digestive enzymes because of their complex composition. The five antioxidant peptides in this study still maintain good activity after gastrointestinal digestion. Therefore, these peptides show potential for the development of functional foods or drugs.

\textbf{2.5. Influence of Antioxidant Peptide on the Hemolysis Rate of AAPH-Induced Human Red Blood Cells.} Protective effects of Leu-Tyr and Tyr-Leu-Ala on
AAPH-induced hemolysis in human red blood cells were evaluated (Figure 5). When human red blood cells were only incubated in PBS, they were very stable with limited hemolysis observed for up to 5 h. The cells were oxidatively damaged by AAPH. When the injury time was 1 h, the hemolysis rate was as high as 22.83%, which was significantly higher than that of the normal control group. As oxidative damage was prolonged, the hemolysis rate of red blood cells in the AAPH group continuously increased.

Human red blood cells were preincubated with Leu-Tyr for 30 min and then exposed to AAPH for 1, 3, or 5 h for oxidative damage. In this way, the hemolysis of red blood cells can be effectively alleviated (Figure 5A). When the concentration of Leu-Tyr was added at 0.5 mmol/L and the oxidative damage duration lasted 1 h, the hemolysis rate was 10.56%, which was significantly lower than that of the AAPH group (22.83%), although it was significantly higher than that of the normal control group. The hemolysis rate of red blood cells increased significantly when they were cultivated for 3 and 5 h at the same concentration, but their hemolysis rate was still lower than that of the AAPH group. When the concentration of Leu-Tyr was 1.0 mmol/L and the oxidative damage lasted for 1 h, the hemolysis rate of red blood cells was 4.60%, which was not significantly different from that of the normal control group. As the time of injury was extended, the hemolysis rate of red blood cells increased significantly. When the injury time lasted for 5 h, the hemolysis rate was 15.88%, which was significantly higher than that of the normal control group (3.48%), but the degree of oxidative damage was significantly lower than that of the red blood cells with an added concentration of 0.5 mmol/L, indicating that the addition of Leu-Tyr can effectively alleviate the hemolysis of red blood cells and the added amount affects the hemolysis rate of red blood cells. The higher the added amount was, the more obvious the inhibition of hemolysis would be.

The protection against AAPH-induced oxidative damage of human red blood cells was also observed when Tyr-Leu-Ala was used to preculture the red blood cells (Figure 5B). With the addition of Tyr-Leu-Ala, the hemolysis was significantly retarded in a time-dependent manner. For instance, after incubation for 3 h, the hemolysis did not occur for red blood cells pretreated with Tyr-Leu-Ala at 1.0 mmol/L. After incubation for more than 5 h, the hemolysis rate was slightly higher than that of the normal control group, indicating that Tyr-Leu-Ala could but not completely inhibit the hemolysis. Zheng et al.12 reported that Tyr and Trp could quench the ABTS·∗ and ROO· by transferring a proton to electron-deficient radicals and could protect erythrocytes against AAPH-induced hemolysis and retard the oxidation of hemoglobin. Hydrophobic amino acids including Phe, Ile, and Val could promote the entry of peptides into cells by interaction with membrane lipid bilayers and terminate radical chain reactions through proton donation.25

2.6. Influence of Antioxidant Peptide on the AAPH-Induced Hemoglobin Oxidation Rate in Human Red Blood Cells. Oxidative damage of hemoglobin results in formation of metHb, which could decrease the ability of erythrocytes to transport oxygen.26 The effects of two antioxidant peptides on the AAPH-induced hemoglobin oxidation rate in human red blood cells are shown in Figure 6. When the human hemoglobin was cultured under normal conditions for 5 h, the hemoglobin oxidation rate was basically unchanged. However, AAPH posed a significant oxidizing effect on hemoglobin. The hemoglobin oxidation rate significantly increased as the culture time was extended. When the culture time was 5 h, the hemoglobin oxidation rate of the AAPH group reached 50%. It was indicated that ROO· produced by the thermal decomposition of AAPH attacked erythrocyte membrane components and caused changes in the structure and function of cell membranes. Hemoglobin dissolved in cells is oxidized to methemoglobin.
3. CONCLUSIONS

In conclusions, the results indicated that with the increase in NaCl concentration, temperature, and pH (beyond neutral), the antioxidant activity of the peptides decreased, while GSH showed more decrease than the peptides. The four peptides presented good resistance to gastrointestinal digestion and are relatively stable. Further study on cell activities of Leu-Tyr and Tyr-Leu-Ala was also performed. The two peptides can effectively inhibit the oxidative damage of AAPH on human red blood cells at millimolar concentrations, and the hemolysis rate can be significantly influenced by antioxidant peptide concentrations.

4. EXPERIMENTAL PROCEDURE

4.1. Materials. Peptides Ile-Tyr, Leu-Tyr, Val-Tyr, and Tyr-Leu-Ala (purity >95%) were prepared by ChinaPeptides (Shanghai, China). GSH, fluorescein disodium salt (FL), AAPH, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), and 2,2-azino-bis-(3-ethylbenzo- thiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich (USA). The rest of the chemicals used were pure reagents.

4.2. ORAC Assay. ORAC value measurement of peptides was performed as per the previously reported procedure.27,28 The results were expressed as μmol TE/μmol of peptide.

4.3. TEAC Assay. The TEAC assay was performed according to Re et al.29 with a slight modification. All samples and reagents were prepared in PBS (50 mmol/L, pH 7.4). Briefly, 50 μL of samples and buffer (blank) was first added to a 96-well plate followed by 150 μL of ABTS+ solution (absorbance, ~0.7 at 734 nm) and then incubated at 37 °C for 30 min. The absorbance was measured at 734 nm. ABTS+ scavenging activity was calculated using the following formula:

radical scavenging activity (%) = \[
\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

A series of concentrations of Trolox replaced samples to obtain a standard curve. The TEAC values of the samples were calculated on the basis of the relationship between the ABTS+ scavenging activity of the sample and the curve equation and were expressed as μmol TE/μmol of peptide.

4.4. Antioxidant Activity of Antioxidant Peptides against NaCl, Temperature, pH, and Gastrointestinal Digestion. The antioxidant activity test referenced the method of Jang et al.20 with minor modifications.

4.4.1. Effect of NaCl. Peptide solutions were exposed to 100 °C for 10 min at different NaCl concentrations (0%, 2%, 4%, 6%, and 8% (w/w)) and then rapidly cooled to room temperature on ice. ORAC and TEAC values of the treated peptides were measured, as described above.

4.4.2. Effect of Temperature. Peptide solutions were placed in a water bath for 2 h at 25, 40, 60, 80, and 100 °C and then rapidly cooled to room temperature on ice.

4.4.3. Effect of pH. The pH values of peptides were changed to 2, 4, 6, 8, 10, and 12 at room temperature for 1 h. The pH was adjusted to 7.0 by adding 1 mol/L HCl or NaOH.

4.4.4. Simulated Gastrointestinal Digestion. The antioxidant activity of peptides against in vitro gastrointestinal proteases was performed, as described below. The pepsin was prepared in KCl–HCl (0.1 mol/L, pH 2.0), and trypsin and chymotrypsin were prepared in K₃PO₄ (0.1 mol/L, pH 8.0). In brief, 0.2 mL of peptide solutions was combined with an equal amount of 0.05% (w/v) pepsin, trypsin, and chymotrypsin and then incubated at 37 °C for 4 h. Next, the resulting samples were adjusted to pH 7.0 by adding 1 mol/L HCl or NaOH and centrifuged (10,000 g for 30 min). ORAC and TEAC values of the treated peptides were measured. A two-stage digestion model was established. The pepsin digest prepared above was centrifuged, dried in a vacuum dryer, and dissolved in 0.2 mL of distilled water. The solution was combined with 0.025% (w/v) trypsin and chymotrypsin and incubated at 37 °C for 4 h. The above process was then repeated.

4.5. Erythrocytes Preparation and Experimental Protocol. Human blood was collected from healthy donors. The blood was centrifuged (3000 g for 10 min) at 4 °C. The cells were washed three times with phosphate buffer (pH 7.4) and centrifuged (3000 g for 10 min). The cells were diluted to 10% with phosphate buffer and were used in the subsequent experiment.

The above erythrocytes were subjected to the following treatments: control group; AAPH-only group (20 mmol/L AAPH); AAPH plus Leu-Tyr (20 mmol/L AAPH and 0.5 or 1 mmol/L Leu-Tyr); and AAPH plus Tyr-Leu-Ala (20 mmol/L AAPH and 0.5 or 1 mmol/L Tyr-Leu-Ala). AAPH, Leu-Tyr, and Tyr-Leu-Ala were dissolved in phosphate buffer.

4.6. Erythrocyte Hemolysis Assay. The erythrocyte hemolysis assay was carried out according to the method described by Zheng et al.30 A 200 μL solution of treated erythrocytes was mixed with 1 mL of phosphate buffer, and then centrifuged (3000 g for 10 min) at 4 °C. The absorbance of the supernatant was measured at 450 nm. Control samples were prepared by adding deionized water. The percent of erythrocyte hemolysis was calculated as follows:

\[
\text{hemolysis rate (％)} = \frac{A_{\text{PBS}} - A_{\text{water}}}{A_{\text{water}}} \times 100
\]

4.7. Measurement of Hemoglobin Oxidation. The measurement of hemoglobin oxidation was performed according to the method of Jarosiewicz et al.30 A 200 μL solution of treated erythrocytes was mixed with 1 mL of deionized water and then centrifuged (3000 g for 10 min) at 4 °C. The absorbance of the supernatant was measured at 630 and 700 nm. In addition, 200 μL of supernatant and a 10 μL solution of K3[Fe(CN)6] (5%, w/w) were added to a 96-well plate and the samples were reasayed for absorbance at the same wavelengths (positive control). The hemoglobin oxidation rate was calculated as follows:
hemoglobin oxidation rate (%) = \frac{A_{630} - A_{700}}{B_{630} - B_{700}} \times 100

(3)

where $A_{630}$ is the absorbance of hemoglobin in the sample tested at 630 nm, $A_{700}$ is the absorbance of hemoglobin in the sample tested at 700 nm, $B_{630}$ is the absorbance of hemoglobin in the positive control tested at 630 nm, and $B_{700}$ is the absorbance of hemoglobin in the positive control tested at 700 nm.

4.8. Statistical Analysis. All tests were conducted in triplicate, and data were represented as mean ± SD. One-way ANOVA was performed by the Statistical Analysis System software 9.0 (SAS, USA) using Duncan’s multiple range test. Significance was established at the level of $P < 0.05$.

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