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# Effects of simulated gastrointestinal digestion on chickpea protein, and its hydrolysate physicochemical properties, erythrocyte haemolysis inhibition, and chemical antioxidant activity

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**Abstract**

Chickpea protein and its hydrolysates have good antioxidant activity. Proteins and peptides are degraded and modified in the gastrointestinal tract before being digested and absorbed, a process that changes their physicochemical and biological properties. Changes in structure and antioxidant capacity of the chickpea protein and its hydrolysates after simulated gastrointestinal digestion were studied. The secondary structure, amino acid composition, and chemical antioxidant activity of chickpea protein and its hydrolysates were determined, and their protective effects on AAPH-induced erythrocyte oxidative damage were studied. Results showed that chickpea protein hydrolysate was easier to be digested by the gastrointestinal tract than chickpea protein. After digestion, the chemical antioxidant capacity of chickpea protein and its hydrolysates increased in a dose-dependent manner, and showed a protective effect against erythrocytes AAPH-induced oxidative damage. Chickpea protein and its hydrolysates can be used as natural antioxidants to promote healthy digestion.

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## Introduction

Chickpea (*Cicer arietinum* L.) is an important Leguminosae crop. It is the third most important grain globally after soybean and pea, and mainly used as a food source for humans. Chickpea protein (18.4 – 29.0%) has high solubility and digestibility (89.0%), and is mainly composed of gliadin (56.6%) and glutenin (21.9%) (Boye *et al.*, 2010). The high nutritional value of chickpeas is usually affected by some antinutritional factors such as trypsin inhibitor, haemagglutinin, tannin, phytic acid, and saponin (Alajaji and El-Adawy, 2006). However, there are ways to reduce these factors such as germination. Studies have shown that natural and germinated chickpea protein significantly improves the cooking quality of protein-rich noodles, and gluten-free rice flour enriched with 5 g/100 g of the germinated protein shows better sensory acceptance (Sofi *et al.*, 2020). Milan-Noris *et al.* (2018) studied the anti-inflammatory potential of peptides and isoflavones from gastrointestinal digestive products of cooked or germinated chickpeas. Results showed that germinated chickpea protein concentrate had anti-inflammatory potential in the lower intestine, which may help to prevent intestinal inflammatory diseases. However, the degradation of germinated chickpea protein and its hydrolysates in the digestive system and the possible antioxidant activity need to be further studied.

The production of reactive oxygen species (ROS) is one of the leading causes of oxidation *in vivo* (Ahn *et al*., 2014). The body can maintain the balance of ROS production and consumption through an inherent defence system. Excessive ROS or damage to the body's defence system will lead to oxidative stress. At present, the main method to evaluate antioxidant activity in scavenging free radicals are scavenging peroxy radical (ROO), $ABTS^{+}$, DPPH, and hydroxyl radical. Due to the different scavenging principles of various free radicals, it is impossible to judge their antioxidant activities. Therefore, in addition to *in vitro* chemical analyses, some physiological models are also needed. Mature erythrocytes have a simple structure; no nucleus or organelles, and no complex metabolic pathways. Their main task is to produce coenzyme factors to maintain the osmotic pressure balance, and resist

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oxidative stress (Zhang *et al.*, 2016). Therefore, the erythrocyte model is often used to study the protective effect of antioxidants on oxidative damage (Ma *et al.*, 2014; Baccarin *et al.*, 2015).

Proteins and peptides need to be digested and absorbed through the mouth and stomach into the intestines. The digestion of proteins and peptides through the stomach and intestine will lead to changes in the composition and activity of amino acids. Therefore, it is necessary to study the biological activity changes of the chickpea protein and its hydrolysates after being absorbed by the human body through gastrointestinal (GI) digestion. The *in vitro* gastrointestinal digestion model is a research method used to replace *in vivo* digestion (Orsini Delgado *et al.*, 2016). At present, it has been used to study the stability of antioxidant peptides or proteins in simulated gastrointestinal digestion (Xiao *et al.*, 2020; Zhang *et al.*, 2020). However, as far as we know, comparative studies on the changes of antioxidant capacity of proteins and their hydrolysates during *in vitro* gastrointestinal digestion are rare (Wen *et al.*, 2020). On the other hand, there are only a few reports on the antioxidant activities of proteins and peptides, and the protection of erythrocytes against oxidative damage during simulated *in vitro* digestion (Pepe *et al.*, 2016).

In the present work, the changes of secondary structure and amino acids of the chickpea protein (CP) and its hydrolysates (HCP) during *in vitro* digestion were analysed to determine the relationship between these characteristics and their antioxidant activities. We also used duck erythrocytes to further verify the protective effect of CP and HCP GI digests on AAPH-induced oxidative damage of erythrocytes (haemolysis, haemoglobin oxidation, lipid peroxidation, and GSH and CAT depletion). The aim was to provide more information about the antioxidant behaviour of GI digests of CP and HCP in physiological systems.

## Materials and methods

### *Materials*

Chickpea was purchased from Mulei County, Xinjiang. The 2,20-azobis (2-methylpropiona mide)-dihydrochloride (AAPH) was purchased from Xinjiang Hengchao Biotechnology Co., Ltd. (Xinjiang, China). The L-glutathione (GSH), pepsin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), microscale malondialdehyde (MDA) assay kit, CAT, and GSH assay kits were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). The trypsin was purchased from Beijing Biotopped Technology Co., Ltd. (Beijing, China). Duck blood was bought from Xinjiang farmer's market. All other chemicals and reagents used were of analytical grade, and obtained in China.

### *Preparation of CP and HCP*

The germinated chickpea powder was prepared using germinated chickpeas as raw material, dried at low temperature, crushed by a high-speed pulveriser, sieved through a 60-mesh sieve, and stored at 4°C after degreasing.

The preparation of germinated chickpea protein was according to Feng *et al.* (2019) with some modification. The germinated chickpea powder was mixed with water in a ratio of 1:10 (w/v), pH was adjusted to 9 with 0.5 M NaOH solution, mixed by stirring for 1 h, centrifuged at 4,000 *g* for 10 min, and the resulting precipitation was repeatedly extracted twice at a ratio of 1:5 (w/v). The three supernatants were mixed, adjusted to pH 4.3 with 0.5 M HCl, and centrifuged at 8,000 *g* for 10 min. The precipitate was washed with distilled water, the pH value of the protein slurry was adjusted to 7.0, freeze-dried for 48 h, and stored at -20°C.

The preparation of HCP was according to Yang *et al.* (2020) with some modifications. The germinated chickpea protein was mixed with water at the ratio of 1:50 (g/v), and the pH value of the solution was adjusted to 10, 2% (w/w) alkaline protease was added, and the enzymatic reaction was allowed to be processed for 1 h. During the enzymatic hydrolysis, 0.1 M NaOH was added to the system to maintain a constant pH value. After enzymolysis, the enzyme was inactivated by boiling for 10 min, and centrifuged at 7,000 rpm for 10 min. The supernatant (HCP) was collected, lyophilised, and stored at -20°C until further analysis.

### *In vitro GI digestion of CP and HCP*

The *in vitro* GI digestion was carried out according to Sun *et al.* (2018) with some modifications. First, CP and HCP were dissolved in distilled water at the concentration of 30 mg/mL, the pH was adjusted to 2.0 with 1 M HCl, and then 4% (w/w) pepsin was added. After incubation at 37°C for 2 h, the pH was adjusted to 5.3 with a 0.9 M $NaHCO_3$ solution, then adjusted to pH 7.5 with a 0.5 M NaOH solution. Trypsin 4% (w/w) was added, stirred

evenly, and incubated at 37°C for 2 h. Then, the pH value of the system was adjusted to 7.0 with 1 M HCl, and the enzyme was inactivated by boiling for 10 min. After centrifugation at 8,000 rpm for 10 min, the supernatant was freeze-dried, and stored at -20°C further analysis.

*Fourier transform infrared spectroscopy (FTIR) analysis*

All samples (2 - 3 mg) were mixed with 150 mg of KBr, ground, and the mixture was pressed into a transparent sheet by a tablet press. The FTIR spectra of CP, HCP, GCP, and GHCP were recorded at 25°C using a 2 $cm^{-1}$ resolution VERTEX 70V FTIR spectrometer (Bruker, Germany). Spectra were analysed with PeakFit (v4.12, Systat Software, Inc., San Jose, CA, USA) and Omnic (ver. 6.1a, Thermo Fisher Scientific, Waltham, MA, USA).

*Amino acid analysis*

The L8900 amino acid automatic analyser was used for amino acid analysis. Each sample was hydrolysed at 110°C with 6 M HCl in a sealed vacuum glass tube for 24 h. The digested sample was put into a 25 mL volumetric flask, and the volume was diluted with deionised water. Then, 5 mL of the digestive solution was dried at 60°C, and diluted with sodium acetate buffer solution (pH 6.4). The results of amino acid composition analysis were expressed in mg/g sample.

*Determination of chemical antioxidant activity*

*DPPH radical scavenging activity assay*

The DPPH radical scavenging activity of samples was evaluated according to Liu *et al.* (2018). The test mixture consisted of 2 mL of different concentrations of sample or GSH and 2 mL DPPH (0.1 mM in absolute ethanol). The control group consisted of 2 mL DPPH (0.1 mM in absolute ethanol) and 2 mL absolute ethanol, while the blank group consisted of 2 mL sample or GSH and 2 mL absolute ethanol. After mixing, the mixture was reacted at room temperature in the dark for 30 min, centrifuged (8,000 rpm, 5 min), and absorbance was measured at 517 nm. DPPH radical scavenging rate was calculated using Eq. 1:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100 \quad \text{(Eq. 1)}$$

where, $A_0$, $A_1$, and $A_2$ = absorbance of the blank group, absorbance of the experimental group, and absorbance of the control group, respectively.

*Hydroxyl radical scavenging activity assay*

The determination of hydroxyl radical scavenging activity was according to Sheng *et al.* (2019) with slight modifications. In the experimental group, 1 mL of 6.0 mM $Fe_SO_4$, 1 mL of 6.0 mM $H_2O_2$, 1 mL of different concentrations of samples or GSH, and 1 mL of 6.0 mM salicylic acid were added into the test tube. After 25 min at 37°C, samples were centrifugated (8,000 rpm, 5 min), and the absorbance was measured at 510 nm. In the blank group, distilled water was used. In the control group, distilled water was added instead of $H_2O_2$. The hydroxyl radical scavenging rate was calculated using Eq. 2:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100 \quad \text{(Eq. 2)}$$

where, $A_0$, $A_1$, and $A_2$ = absorbance of the blank group, absorbance of the experimental group, and absorbance of the control group, respectively.

*Reducing power assay*

The reducing power measurement was performed according to Zhang *et al.* (2018) with slight modifications. In short, different samples or GSH concentrations, 0.2 M pH 6.6 phosphate buffer, and 1% potassium ferricyanide ($K_3Fe(CN)_6$) solution were added to a water bath (50°C) for 20 min. One volume of 10% trichloroacetic acid (TCA) solution was added to all samples, let stand for 10 min, and centrifugated (3,000 rpm, 10 min). The centrifuged supernatant (2 mL) was removed and mixed with one volume of distilled water and 0.4 mL of 0.1% $FeCl_3$ solution, then incubated in a water bath (50°C) for 10 min. The system solution changes from yellow to blue, and absorbance was at 700 nm. Distilled water was used to replace the sample as a blank group. The higher the absorbance value of the sample, the stronger the reduction power.

*Preparation of the erythrocyte sample*

The reference was slightly modified (Zheng *et al.*, 2016b). Fresh duck blood (10 mL) with anticoagulant was centrifuged at 1,200 *g* at 4°C for 10 min, and the serum was removed. The remaining

erythrocytes were washed three times with PBS at pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$) until the supernatant was clear. Erythrocytes were diluted in nine times the volumes of PBS to prepare a 10% erythrocyte suspension.

*Erythrocyte haemolysis assay*

Briefly, 200 μL of 10% erythrocyte suspension was pre-incubated with 200 μL of sample or PBS at 37°C for 30 min. Then, 400 μL of 100 mM AAPH was added, and the mixture was incubated at 37°C for 6 h. AAPH untreated erythrocytes incubated in PBS were used as the control group. Each reaction mixture was cultured at 37°C for 1, 2, 3, 4, 5, and 6 h. After incubation, 200 μL of the reaction mixture was diluted with 1 mL of PBS, and centrifuged at 1,500 *g* for 10 min. The absorbance of the supernatant ($A_{PBS}$) at 540 nm was measured. The same volume of the reaction mixture was treated with 1 mL of distilled water to obtain complete haemolysis. The absorbance of the supernatant ($A_{water}$) was determined under the same conditions. The haemolysis rate was calculated using Eq. 3:

$$\text{Hemolysis}\ (\%) = A_{PBS}/A_{waters} \times 100 \qquad \text{(Eq. 3)}$$

*Measurement of haemoglobin oxidation*

Determination of haemoglobin oxidation rate was performed according to Zheng *et al.*, 2016b. Briefly, 200 μL of cultured erythrocyte suspension was dissolved in 1 mL of distilled water, centrifuged at 2,500 *g* for 5 min, and 900 μL of the supernatant was taken to determine the absorbance at 630 nm ($A_{630}$) and 700 nm ($A_{700}$). Then, 45 μL of a 5% $K_3Fe(CN)_6$ solution was added to the erythrocyte suspension, the absorbance at 630 and 700 nm was measured and recorded as $A_{metHb630}$ and $A_{metHb700}$. The percentage of metHb in erythrocytes was calculated using Eq. 4:

$$\text{metHb}(\%) = [(A_{630} - A_{700})/(A_{metHb630} - A_{metHb700})] \times 100 \qquad \text{(Eq. 4)}$$

*Measurement of MDA content, GSH, and CAT levels*

Briefly, 200 μL of cultured erythrocyte suspension were dissolved with 1 mL of precooled ultrapure water, and centrifuged at 3,500 rpm for 5 min. Next, the supernatant was removed, and 10 μL of the precipitate was diluted in 990 μL of precooled ultrapure water to make the total volume of 1 mL. The suspension was then stored at -80°C until further analysis (Zhan *et al.*, 2021).

The protein content was determined by Coomassie brilliant blue method. The MDA kit determined the MDA content. The GSH content and CAT activity were determined by GSH and CAT kits, respectively. Erythrocyte MDA value was expressed as nmol/mg protein, GSH content was expressed as μg/mg protein, and CAT activity was expressed as U/mg protein.

*Statistical analysis*

All experiments were carried out in triplicate, and results were reported as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) using SPSS version 20.0 (SPSS Inc., Chicago, IL). Significant differences were determined using Duncan's Multiple Range Test ($p < 0.05$).

## Results and discussion

*FTIR analysis*

FTIR can be used to analyse the changes of amide I region (1600 - 1700 $cm^{-1}$) to study the relative changes of protein secondary structure (Zhang *et al.*, 2021). The FTIR spectra of CP and HCP during digestion were deconvoluted, as shown in Figure 1; the secondary structures of CP and HCP are shown in Table 1.

Table 1 shows that the secondary structure of CP and HCP changed significantly after gastrointestinal digestion ($p < 0.05$). The relative content of β-turn structures of CP and HCP increased significantly ($p < 0.05$) from 30.42 ± 0.60 and 37.41 ± 0.55% to 34.57 ± 0.59 and 43.79 ± 0.70%, respectively, during *in vitro* digestion, while the relative content of random coils did not change significantly during hydrolysis. When compared with CP, the β-sheets structure of HCP decreased significantly from 22.84 ± 0.65 to 9.41 ± 0.50%, while the α-helix structures increased significantly from 21.52 ± 0.55 to 26.57 ± 0.50%, thus indicating that trypsin hydrolysis extended the structure of HCP. This is similar to result from Xu *et al.* (2016) using circular dichroism (CD) spectrum to study the secondary structure changes of rice protein during hydrolysis. β-sheet is a relatively stable structure, while α -helix, β-turn, and random coil are relatively flexible (Yie *et al.*, 2006). These results indicated that HCP opened the β-sheet structure in the hydrolysis

process, thus making the structure loose, facilitating the binding between the enzyme and HCP, and providing the possibility to hydrolyse more active peptides.

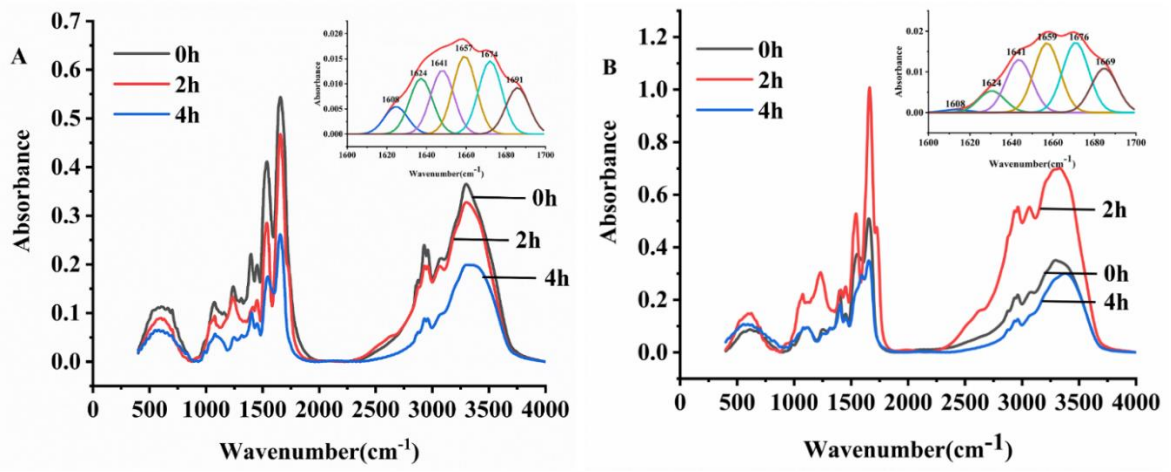


**Figure 1.** The amide region I in Fourier transform infrared (FTIR) spectra of **(A)** CP and **(B)** HCP at different digestion times. α-helix (1649 - 1661 $cm^{-1}$, yellow); β-sheet (1610 - 1637 $cm^{-1}$, blue and green); β-turn (1665 - 1700 $cm^{-1}$, cyan and brown); random coils (1637 - 1648 $cm^{-1}$, purple).

**Table 1.** Secondary structure of CP and HCP as determined by Fourier transform infrared (FTIR) spectra at indifferent digestion times.

| Digestive process | | α-helix (%) | β-sheet (%) | β-turn (%) | Random coils (%) |
|---|---|---|---|---|---|
| CP | 0 h | 23.31 ± 0.65$^{bc}$ | 24.29 ± 0.66$^{a}$ | 30.42 ± 0.60$^{d}$ | 21.90 ± 0.80$^{a}$ |
| | 2 h | 24.12 ± 0.85$^{b}$ | 22.25 ± 0.66$^{b}$ | 31.30 ± 0.60$^{d}$ | 22.36 ± 0.65$^{a}$ |
| | 4 h | 22.58 ± 0.50$^{cd}$ | 24.26 ± 0.66$^{a}$ | 34.57 ± 0.59$^{c}$ | 18.53 ± 0.50$^{c}$ |
| HCP | 0 h | 21.52 ± 0.55$^{d}$ | 22.84 ± 0.65$^{b}$ | 37.41 ± 0.55$^{b}$ | 18.20 ± 0.70$^{c}$ |
| | 2 h | 26.01 ± 0.90$^{a}$ | 16.32 ± 0.60$^{c}$ | 37.56 ± 0.46$^{b}$ | 20.09 ± 0.90$^{b}$ |
| | 4 h | 26.57 ± 0.50$^{a}$ | 9.41 ± 0.50$^{d}$ | 43.79 ± 0.70$^{a}$ | 20.56 ± 0.35$^{b}$ |

Data are mean ± standard deviation (SD) of three replicates ($n$ = 3). Means followed by different lowercase superscripts are significantly different between groups ($p < 0.05$).

*Amino acid analysis*

Amino acids are the building blocks of proteins, and closely related to life activities. They are also the main component of foods; therefore, amino acid balance plays a vital role in body health.

As shown in Table 2, undigested CP and HCP have similar amino acid compositions. Among them, Glu was the most abundant, followed by Asp. The total amino acid contents (TAA) of HCP and CP were higher than that of the GI digested products ($p < 0.05$), consistent with the results of mulberry leaf protein and its hydrolysate (Sun *et al.*, 2018). After intestinal digestion, the amino acid content of CP was significantly lower than that after gastric digestion, thus indicating that chickpea protein was mainly digested in the stomach, while for its hydrolysate was the opposite. After 2 h of gastric digestion, the TAA content of HCP decreased significantly from 77.27 ± 3.56 to 62.92 ± 15.25 mg/g (decreased by 18.6%, $p < 0.05$). In contrast, there was no significant difference in TAA content between CP and its gastric digestive products (decreased by 8.5%, $p > 0.05$). The results showed that HCP released more free amino acids than CP after gastric digestion, and CP was more tolerant to gastric digestion than HCP.

In addition, the contents of hydrophobic amino acids (HAA) and aromatic amino acids (AAA) in HCP and CP decreased gradually during GI digestion, especially during gastric digestion. Similar results were obtained during the digestion of casein peptides (Ao and Li, 2013). When compared with CP, the content of Met in HAA of HCP was higher. Studies have found that hydrophobic amino acids (HAA) such as Pro, Val, Ala, Ile, Leu, Phe, and Met can act

as antioxidants by increasing the solubility of peptides in lipids, thus promoting better interaction with free radicals that cause oxidative damage, thereby improving the antioxidant capacity of small peptides (Alashi *et al.*, 2014; Bustamante *et al.*, 2021). For example, endogenous protease was used to hydrolyse squid viscera, and the relative content of hydrophobic amino acids in the hydrolysate reached 38.52%, which was helpful to form hydrophobic structure, and had significant antioxidant activity (Renard *et al.*, 2000).

**Table 2.** Changes in the amino acid composition of CP and HCP during GI digestion.

| Amino acid | Amino acid content (mg/g sample) | | | | | |
|---|---|---|---|---|---|---|
| | CP | | | HCP | | |
| | 0 h | 2 h | 4 h | 0 h | 2 h | 4 h |
| Asp | 10.03 ± 0.03$^{a}$ | 9.51 ± 0.06$^{a}$ | 7.47 ± 0.10$^{b}$ | 9.25 ± 0.46$^{a}$ | 7.54 ± 1.83$^{b}$ | 6.77 ± 0.08$^{b}$ |
| Thr | 2.64 ± 0.01$^{a}$ | 2.23 ± 0.02$^{b}$ | 1.86 ± 0.02$^{c}$ | 2.46 ± 0.10$^{a}$ | 2.02 ± 0.47$^{b}$ | 1.83 ± 0.28$^{c}$ |
| Ser | 4.59 ± 0.03$^{a}$ | 4.32 ± 0.02$^{a}$ | 3.46 ± 0.06$^{b}$ | 4.22 ± 0.21$^{a}$ | 3.47 ± 0.79$^{b}$ | 3.11 ± 0.05$^{b}$ |
| Glu | 14.67 ± 0.07$^{a}$ | 14.09 ± 0.05$^{a}$ | 11.37 ± 0.16$^{b}$ | 13.68 ± 0.67$^{a}$ | 11.13 ± 2.74$^{b}$ | 9.98 ± 0.13$^{c}$ |
| Gly | 3.15 ± 0.01$^{a}$ | 2.78 ± 0.01$^{a}$ | 2.27 ± 0.03$^{b}$ | 2.85 ± 0.14$^{a}$ | 2.31 ± 0.54$^{b}$ | 2.06 ± 0.03$^{b}$ |
| Ala | 3.50 ± 0.02$^{a}$ | 3.14 ± 0.01$^{a}$ | 2.49 ± 0.04$^{b}$ | 3.21 ± 0.15$^{a}$ | 2.63 ± 0.61$^{b}$ | 2.36 ± 0.04$^{b}$ |
| Cys | 1.83 ± 0.03$^{a}$ | 1.75 ± 0.10$^{a}$ | 1.76 ± 0.04$^{a}$ | 1.91 ± 0.02$^{a}$ | 1.11 ± 0.21$^{b}$ | 1.13 ± 0.02$^{b}$ |
| Val | 3.44 ± 0.03$^{a}$ | 2.96 ± 0.02$^{b}$ | 2.47 ± 0.03$^{b}$ | 3.10 ± 0.11$^{ab}$ | 2.89 ± 0.66$^{bc}$ | 2.74 ± 0.03$^{bc}$ |
| Met | 0.56 ± 0.07$^{a}$ | 0.48 ± 0.06$^{b}$ | 0.41 ± 0.01$^{c}$ | 0.75 ± 0.07$^{a}$ | 0.58 ± 0.15$^{b}$ | 0.46 ± 0.02$^{c}$ |
| Ile | 3.73 ± 0.01$^{a}$ | 3.09 ± 0.02$^{b}$ | 2.56 ± 0.03$^{c}$ | 3.26 ± 0.14$^{a}$ | 2.67 ± 0.68$^{b}$ | 2.42 ± 0.03$^{b}$ |
| Leu | 6.93 ± 0.02$^{a}$ | 6.11 ± 0.03$^{a}$ | 4.93 ± 0.07$^{b}$ | 6.08 ± 0.29$^{a}$ | 4.93 ± 1.18$^{b}$ | 4.45 ± 0.06$^{b}$ |
| Tyr | 2.46 ± 0.02$^{a}$ | 2.08 ± 0.04$^{b}$ | 1.70 ± 0.04$^{c}$ | 2.29 ± 0.10$^{a}$ | 1.87 ± 0.47$^{b}$ | 1.70 ± 0.02$^{b}$ |
| Phe | 5.68 ± 0.03$^{a}$ | 5.16 ± 0.03$^{a}$ | 4.19 ± 0.05$^{b}$ | 5.06 ± 0.18$^{a}$ | 4.17 ± 1.00$^{b}$ | 3.79 ± 0.05$^{c}$ |
| Lys | 5.55 ± 0.02$^{a}$ | 5.01 ± 0.03$^{a}$ | 3.75 ± 0.07$^{b}$ | 5.04 ± 0.23$^{a}$ | 4.09 ± 1.03$^{b}$ | 3.62 ± 0.06$^{c}$ |
| His | 2.42 ± 0.13$^{a}$ | 2.20 ± 0.02$^{a}$ | 1.82 ± 0.14$^{b}$ | 2.18 ± 0.15$^{a}$ | 1.83 ± 0.50$^{b}$ | 1.76 ± 0.14$^{b}$ |
| Arg | 8.17 ± 0.01$^{a}$ | 7.71 ± 0.03$^{a}$ | 5.94 ± 0.08$^{b}$ | 7.07 ± 0.38$^{a}$ | 5.72 ± 1.48$^{b}$ | 5.17 ± 0.11$^{b}$ |
| Pro | 3.89 ± 0.02$^{a}$ | 3.49 ± 0.07$^{a}$ | 2.74 ± 0.05$^{b}$ | 3.49 ± 0.19$^{a}$ | 2.80 ± 0.71$^{b}$ | 2.48 ± 0.10$^{b}$ |
| TAA | 83.21 ± 0.31$^{a}$ | 76.12 ± 0.40$^{a}$ | 62.20 ± 0.93$^{b}$ | 75.90 ± 3.54$^{a}$ | 61.73 ± 15.03$^{b}$ | 55.82 ± 0.83$^{c}$ |
| HAA | 32.00 ± 0.05$^{a}$ | 28.28 ± 0.28$^{b}$ | 23.25 ± 0.35$^{c}$ | 29.14 ± 1.22$^{a}$ | 23.63 ± 5.66$^{b}$ | 21.52 ± 0.30$^{b}$ |
| AAA | 8.13 ± 0.06$^{a}$ | 7.24 ± 0.63$^{a}$ | 5.89 ± 0.08$^{b}$ | 7.35 ± 0.28$^{a}$ | 6.04 ± 1.46$^{b}$ | 5.49 ± 0.07$^{b}$ |

Data are mean ± standard deviation (SD) of three replicates ($n$ = 3). Means in a row followed by different lowercase superscripts are significantly different ($p$ < 0.05). TAA = total amino acids; HAA = hydrophobic amino acids (Ala, Val, Ile, Leu, Tyr, Phe, Pro, Met, and Cys); AAA = aromatic amino acids (Phe and Tyr); CP = chickpea protein; and HCP = hydrolysates of CP.

*Chemical antioxidant activity of GCP, GDCP, GHCP, and GDHCP*

The free radical scavenging and reducing abilities of GI digestion products of CP and GCP were dose-dependent (Figure 2). GCP and GHCP were the gastric digestion of CP and HCP, while GDCP and GDHCP were the gastrointestinal digestion of CP and HCP, respectively.

As shown in Figure 2A, with the increase in sample concentration, the DPPH scavenging activity increased continuously during simulated digestion. The chickpea protein and its hydrolysates were more fully digested during gastric digestion than during intestinal digestion. This was closely related to the role of pepsin and gastric digestive fluid. Different types of enzymes lead to functional changes of the enzymatic hydrolysates. The DPPH radical scavenging abilities of GCP and GHCP were significantly higher than that of GDCP and GDHCP. At the concentration of 2.0 mg/mL, GCP and GHCP scavenging capacity reached 68.24 ± 0.10 and 60.64 ± 0.42%, respectively. The result is similar to the gastrointestinal digestion products of rice bran protein (Cho, 2020), but lower than the DPPH radical

scavenging capacity of control GSH (98.29 ± 0.002%). Since GCP and GHCP had high HAA and AAA contents, these amino acids could relatively quickly scavenge DPPH free radicals.

As shown in Figure 2B, with the increase in sample concentration, the scavenging ability of hydroxyl radical increased significantly ($p < 0.05$). Contrary to DPPH, GDCP and GDHCP had significantly higher hydroxyl radical scavenging capacity than GCP and GHCP. At the concentration of 2 mg/mL, the scavenging rates of GDCP and GDHCP were 49.08 ± 0.14 and 83.46 ± 0.14%, respectively, much higher than that of GSH (28.20 ± 0.11%). Intestinal digestion has a significant effect on the scavenging ability of hydroxyl radicals. This might have been due to the different types of enzymes, different kinds of proteases have different cleavage sites for protein, so the functional properties of products and peptides after enzymatic hydrolysis were also different. The enzymatic hydrolysis of trypsin, and the increase of small molecular peptides further enhanced the free radical scavenging ability of digestive products, thus suggesting that chickpea protein and its hydrolysates could become more effective hydrogen or electron donors after digestion *in vitro* (You *et al.*, 2010).

In general, there is a positive correlation between reducing capacity and antioxidant capacity. The reducing power determination can test and evaluate the potential of antioxidants to provide electrons or hydrogen atoms. As shown in Figure 2C, at the same concentration, the reducing ability of the four samples was lower than that of GSH (2.317 ± 0.08), which was similar to DPPH radical scavenging ability. When the concentration of GHCP was 2 mg/mL, the reducing power of GHCP was the strongest with 0.914 ± 0.11, followed by GDCP with 0.684 ± 0.1. The reducing power of GCP and GDHCP were 0.21 ± 0.02 and 0.201 ± 0.10, respectively, and were higher than the same concentration of rice bran protein GI digested products (Cho, 2020). Udenigwe and Aluko (2011) found that hydrophobic and acidic amino acids (Asp, Glu) were significantly positively correlated with reducing ability. Table 2 shows that the contents of HAA and acidic amino acids in GHCP and GDCP were significantly higher than those in GDHCP.

Altogether, these results showed that different antioxidant methods had different antioxidant outcomes. For example, GHCP could effectively scavenge DPPH free radicals, but its hydroxyl radical scavenging ability was weak; contrary, GDCP could effectively scavenge hydroxyl radicals, but its DPPH free radical scavenging ability was weak. Therefore, a variety of tests should be carried out to study the antioxidant activity of samples. In the present work, AAPH-induced erythrocyte oxidative damage cell model was used to evaluate the antioxidant activity of these substances.

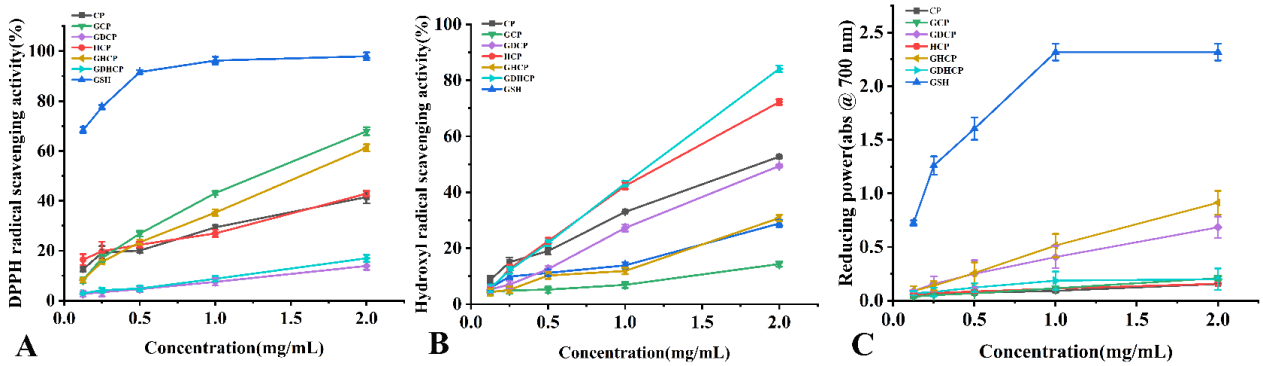


**Figure 2.** The **(A)** DPPH, **(B)** hydroxyl radical scavenging activities, and **(C)** reducing power of GCP, GDCP, GHCP, GDHCP, and GSH at various concentrations. GCP and GHCP = gastric digestion of CP and HCP; GDCP and GDHCP = gastrointestinal digestion of CP and HCP, respectively.

*Protective effects of GCP, GDCP, GHCP, and GDHCP against AAPH-induced haemolysis*

AAPH can decompose at 37°C, and produce peroxy radicals, which can attack the erythrocyte membrane, and induce lipid and protein peroxidation, thus leading to haemolysis. Therefore, the antioxidant activity of the samples could be evaluated by detecting the haemolysis inhibition (Wang *et al.*, 2017; Duranti *et al.*, 2018). To better evaluate the antioxidant effect of gastrointestinal digestive products of CP and HCP, the protective effect of four samples on AAPH-induced haemolysis was studied.

As shown in Figure 3A, the protective effect of the samples at different time points on AAPH-induced haemolysis was evaluated. In the absence of AAPH, there was no haemolysis within 6 h, and erythrocytes remained stable (8.03 ± 0.22%). When AAPH was added, haemolysis was time-dependent (13.50 ± 0.37% after 1 h and 65.75 ± 0.40% after 6 h). In the early stage, the protective effect of each sample was GDHCP > GDCP > GHCP > GCP > GSH. For example, after incubation for 3 h, the haemolysis rates of pre-treated erythrocytes with GCP, GDCP, GHCP, and GDHCP were 46.47 ± 0.31, 21.40 ± 0.84, 36.64 ± 0.84, and 14.70 ± 0.38%, respectively, significantly lower than joining only the AAPH group (51.30 ± 1.04%) ($p < 0.05$). This is consistent with the results of the hydroxyl radical scavenging ability. Results showed that GDHCP and GDCP had protective effects on AAPH-induced haemolysis. After incubation for more than 5 h, there was no significant difference in the protective effect of samples on haemolysis ($p > 0.05$). These results showed that these samples could only delay the haemolysis process but could not inhibit haemolysis at the test concentration (Zhang *et al.*, 2014).

Figure 3B shows the protective effects of different concentrations (0.05, 0.25, 0.5, and 1.0 mg/mL) of GCP, GDCP, GHCP, and GDHCP on AAPH-induced haemolysis. It was apparent that the protective effect of the sample on erythrocyte haemolysis induced by AAPH was dose-dependent. The haemolysis rate decreased with the increase in sample concentration. When the sample concentration was 0.05 - 0.5 mg/mL, GDCP had the most robust haemolysis inhibition ability, followed by GDHCP and GHCP. When the concentration increased to 1.0 mg/mL, the haemolysis of erythrocytes pre-treated with GDCP, GHCP, and GDHCP was similar to that of the AAPH untreated group (control group). It was shown that the GI digests of CP and HCP could effectively protect erythrocyte haemolysis at a certain concentration.

*Effects of GCP, GDCP, GHCP, and GDHCP on AAPH-induced haemoglobin oxidation in erythrocytes*

Haemoglobin is the main soluble protein in erythrocytes. Oxidative damage of erythrocytes can lead to haemoglobin oxidation to methaemoglobin, thus reflecting the degree of protein oxidation in cells (Bento *et al.*, 2018). As shown in Figure 3C, the content of metHb in erythrocytes without AAPH treatment was very low, and its content did not exceed 18.02 ± 0.95% during 5 h incubation. After AAPH treatment, metHb in erythrocytes increased in a time-dependent manner. After incubation for 1, 3, and 5 h, the metHb content increased to 48.13 ± 0.90, 62.01 ± 0.99, and 79.99 ± 0.95%, respectively. After adding samples (GCP, GDCP, GHCP, or GDHCP), the level of metHb in erythrocytes significantly decreased ($p < 0.05$), but significantly higher than that in erythrocytes without AAPH treatment. These results suggested that GI digests of CP and HCP had protective effects on haemoglobin oxidation induced by AAPH. GDHCP had the strongest inhibitory effect on metHb, followed by GDCP. GHCP and GCP had the weakest inhibitory effect.

*Effects of GCP, GDCP, GHCP, and GDHCP on AAPH-induced MDA formation in erythrocytes*

When free radicals exist in excess, they will attack the cell membrane, cause cell damage, and cause lipid peroxidation. Lipid peroxidation can produce MDA as the end-product. When MDA accumulates to a certain extent, it will continue to destroy cell membrane structure, thus causing cell metabolic disorder, and leading to cell apoptosis (Yang *et al.*, 2017). Therefore, the effect of antioxidants on lipid peroxidation induced by a free radical chain reaction in erythrocytes was investigated by measuring MDA content in erythrocytes. The effects of GCP, GDCP, GHCP, and GDHCP on MDA content in erythrocytes after AAPH treatment were studied. The results are shown in Figure 3D. The level of MDA in erythrocytes increased significantly in the AAPH group, thus indicating that AAPH treatment significantly induced the formation of MDA in erythrocytes. After incubation with AAPH for 1 h, MDA content increased from 3.21 ± 0.60 to 13.36 ± 1.00 nmol/mg protein, but the increase over time did not change significantly. This was consistent with the result that the haemolysis rate increased significantly after AAPH treatment. After incubation for 3 h, the content of MDA was significantly lower than that in the AAPH treatment group, which was consistent with the result that the haemolysis rate was significantly reduced after sample treatment. These results indicated that GCP, GDCP, GHCP, and GDHCP could effectively eliminate the free radicals produced by AAPH treatment, block the occurrence of lipid peroxidation, and then inhibit the formation of MDA to protect the cell membrane from oxidative damage.

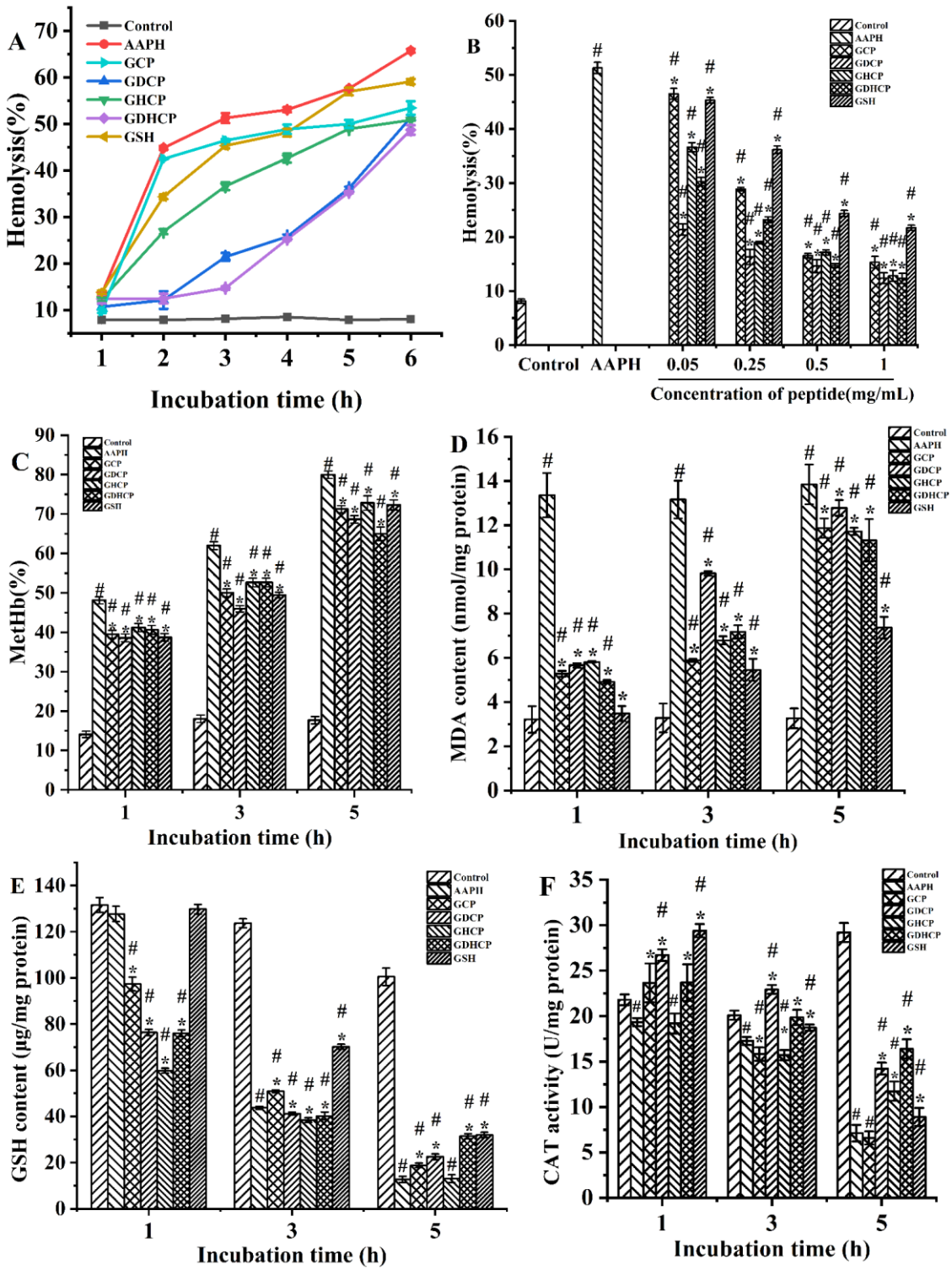


**Figure 3. (A)** Protective effects of GCP, GDCP, GHCP, and GDHCP against AAPH-induced haemolysis in duck erythrocytes. **(B)** Protective effects of GCP, GDCP, GHCP, and GDHCP at different concentrations (0.05, 0.25, 0.5, and 1.0 mg/mL) against AAPH-induced haemolysis in duck erythrocytes. **(C)** Effects of GCP, GDCP, GHCP, and GDHCP on the oxidation of haemoglobin to metHb. **(D)** Formation of MDA, **(E)** levels of GSH, and **(F)** CAT in erythrocytes after AAPH-induced oxidative damage. #significant difference from control group ($p < 0.05$), *significant difference from AAPH group ($p < 0.05$).

When incubated for 5 h, the protective effect on MDA was not obvious. This may be because after incubation for 5 h, the samples had no obvious protective effect against MDA formation.

*Effects of GCP, GDCP, GHCP, and GDHCP on AAPH-induced changes in GSH level of erythrocytes*

GSH is the most critical non-enzymatic antioxidant defence system in cells. GSH provides the first defence barrier during oxidative stress (Zou *et al*., 2001). Therefore, one way to prevent oxidative damage is to reduce the consumption of GSH (Alvarez-Suarez *et al.*, 2012). Figure 3E shows the effects of GCP, GDCP, GHCP, and GDHCP on AAPH-induced changes in erythrocyte GSH levels. In the group without AAPH, the level of GSH in erythrocytes was 131.59 ± 3.16 μg/mg protein. There was no significant change within 5 h ($p > 0.05$). The intracellular GSH level significantly decreased after AAPH treatment ($p < 0.05$), and decreased to 121.74 ± 3.26, 43.77 ± 0.61, and 12.69 ± 1.40 μg/mg protein at 1, 3, and 5 h, respectively. After adding GCP, GDCP, GHCP, and GDHCP, the GSH level in cells was depleted in a time-dependent manner. It was apparent that the sample had a significant protective effect on the intracellular GSH level within 1 h; however, after incubation for 3 and 5 h, the sample had no significant protective effect on the intracellular GSH depletion. These results suggested that GI digests of CP and HCP could delay the depletion of GSH in cells.

*Effects of GCP, GDCP, GHCP, and GDHCP on AAPH-induced changes in CAT level of erythrocytes*

In addition to the non-enzymatic antioxidant system, there are also some antioxidant enzymes in erythrocytes such as SOD, CAT, and GSH-Px, which can resist oxidative stress and protect the body from free radical attack through a synergistic effect. CAT can remove intracellular and extracellular $H_2O_2$ by reducing it to $H_2O$ (Moran, 1997). Unlike GSH, CAT is not very sensitive to AAPH-induced oxidative damage. This is due to the limited production of $H_2O_2$ in the process of oxidative stress induced by AAPH (Zheng *et al.*, 2016a). As shown in Figure 3F, the CAT level in erythrocytes treated with AAPH was lower than that in the control group. After adding different samples, the CAT level in erythrocytes increased significantly ($p < 0.05$). After incubation for 5 h, the protective effect of the sample on CAT level was not noticeable.

## Conclusion

CP and HCP had different effects on gastrointestinal digestion. CP was more easily digested by the stomach, while HCP was the opposite. After gastrointestinal digestion, the antioxidant activity of hydroxyl free radical of GI digestive products of HCP was the strongest, while the reducing ability of gastric digestive products of HCP was also the strongest. On the contrary, the scavenging ability of DPPH free radical of gastric digestive products of CP was the strongest. GI digestive products of both had good chemical antioxidant capacity in a dose-dependent manner. In the AAPH-induced haemolysis test, GI digests of CP and HCP showed dose-dependent protective effects. They could protect erythrocytes from AAPH-induced haemoglobin oxidation, and slow down MDA production and the depletion of GSH in erythrocytes momentarily. CAT activity did not change significantly. GI digests of CP and HCP may be used as direct free radical scavengers to protect erythrocytes from AAPH induced oxidative damage. The present work assisted in understanding the changes in physicochemical properties and antioxidant activities of proteins and peptides during gastrointestinal digestion. Moreover, it provided valuable information on the application of chickpea as a natural antioxidant.

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