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Study on antioxidant activity of chicken plasma protein hydrolysates

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ABSTRACT

This study optimised the hydrolysis process of chicken plasma protein and explored the *in vivo* antioxidant activity of its hydrolysates. The results showed that alkaline protease provided the highest degree of hydrolysis (19.30%), the best antioxidant effect *in vitro*. The optimal hydrolysis process of alkaline protease was: temperature 50 °C, time 8 h, [E]/[S] 7000 U g^{-1}, pH 7.5. Antioxidant studies *in vivo* showed that the low, medium, and high dose groups significantly reduced the serum MDA and protein carbonyl content ($P < 0.05$) and significantly increased the serum SOD and GSH contents ($P < 0.05$). The results of HE staining of the liver showed that the liver cells in the model group were severely damaged, but the chicken plasma protein hydrolysates could alleviate this pathological damage. Chicken plasma protein hydrolysis products had certain antioxidant activity.

KEYWORDS

chicken blood, enzymatic hydrolysis, plasma protein hydrolysates, antioxidant activity

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1. INTRODUCTION

The method of enzymatic hydrolysis could convert high molecular weight low-value by-product proteins into hydrolysates with certain biological activity (Ali et al., 2016). The polypeptides in hydrolysates had a variety of biological activities, including the treatment of cancer (Xing et al., 2016; Yaghoubzadeh et al., 2019), malaria (Adewole and Adebayo, 2016), high blood pressure (Indiano-Romacho et al., 2016), diabetes (Huang et al., 2015), and other diseases, as well as antibacterial (Nath et al., 2020) and antioxidant activities (Xue et al., 2015). Antioxidant peptides could scavenge free radicals in the body and reduced the damage of free radicals to cells. It protected the body from oxidative damage associated with cardiovascular disease and cancer (Abeyrathne et al., 2018). Several studies had shown that peptides derived from natural proteins in food had been proven to be more easily absorbed and safer for the body than chemically synthesised peptides (Rizzello et al., 2016; Gluvic and Ulrih, 2019). Therefore, in recent years, there had been more and more studies on the antioxidant activity of antioxidant peptides and protein hydrolysates, and peptides derived from beans (Ngoh and Gan, 2016), meat (Kim et al., 2018), and milk (El-Fattah et al., 2016) had been widely studied. Sheep, goat, and rabbit plasma hydrolysates also showed antioxidant activity, and rabbit plasma hydrolysates had the strongest antioxidant activity (Kumar et al., 2022). Nath et al. (2020) used papain and cysteine protease hydrolysis of soy milk to prepare a polypeptide with antioxidant activity. Yang et al. (2020a) extracted hydrolysates from duck plasma (DPH) to identify seven novel peptides, of which EVGK exhibited the highest Fe^{2+} chelating capacity (16.35%) and RCLQ showed the highest reducing power.

Blood is rich in proteins, which are non-allergenic compared to legume and milk proteins. Plasma accounts for 65–70% of whole blood and contains more than 3,000 proteins, with serum albumin, globulin, and fibrinogen being the most abundant (Zou et al., 2018). Plasma albumin has the smallest molecular weight among the 3 types of proteins but is the most abundant in plasma (Chang et al., 2019). Animal blood meal is commonly used as livestock feed, nutritional supplements (Lynch et al., 2017), or for the extraction of heme, hematoporphyrin derivatives, superoxide dismutase, etc. Chicken blood is an edible meat by-product that accounts for 3–5% of chicken body weight (Zheng et al., 2018). It is usually used for animal feed (Seo et al., 2015), but a small amount is also directly disposed of, resulting in problems such as waste of resources and environmental pollution. However, as a protein-rich material, it has the potential to be processed into a high value-added product, and effective use could not only reduce environmental pollution but also increased the income of the slaughtering industry. Studies has shown that animal blood hydrolysis products have certain biological activities (Ofori and Hsieh, 2014; Seo et al., 2015; Chen et al., 2019). Therefore, in this study, chicken plasma was used as raw material, the best hydrolysis protease was first screened, and the hydrolysis conditions were optimised through single factor and orthogonality to separate and prepare hydrolysates with high antioxidant activity. Finally, a mouse D-galactose oxidative damage model was established to detect the effects of hydrolysates on the content of lipid oxidation products (MDA), protein carbonyl content, reducing glutathione content (GSH), an antioxidant enzyme activity (SOD) in mouse plasma, total antioxidant capacity, and other indicators. At the same time, histopathological sections were observed to study the *in vivo* antioxidant activity of plasma protein hydrolysates. The aims of the work was to provide the experimental basis and technical reference for the efficient use of poultry plasma and to provide data support for developing poultry plasma proteolysis products into antioxidant functional food additives.

2. MATERIALS AND METHODS

2.1. Materials and reagents

For the study, 3-month-old Highland brown chicken (Lanzhou Ruiyuan Agricultural Technology Co., Ltd.) and 8-week-old Kunming mice (Lanzhou University Laboratory Animal Center) were obtained. For the experiments the following kits were used: lipid oxidation product malondialdehyde (MDA) kit, total superoxide dismutase (SOD) determination kit, reduced glutathione (GSH) determination kit, cyanide methemoglobin determination kit, and total antioxidant capacity determination kit (Nanjing Jianjian Institute of Biological Engineering). Pepsin (Pep) was derived from porcine gastric mucosa with enzyme activity of 200,000 U g^{-1}. Alkaline protease (Alk) was a proteolytic enzyme produced through fermentation by *Bacillus licheniformis* with enzyme activity of 200,000 U g^{-1}). Trypsin (Try) was derived from porcine pancreas with enzyme activity of 200,000 U g^{-1}). Papain (Pap) was extracted from the unripe fruit of the papaya plant with enzyme activity of 100,000 U g^{-1}. Neutral proteases (Neu) was derived from *Bacillus subtilis* with enzyme activity of 200,000 U g^{-1} (Novozymes Biotechnology Limited). Other reagents used were chemicals of analytical grade.

2.2. Preparation of chicken blood and plasma protein

After weighing, the chickens were bled from the neck and slaughtered, the whole blood was centrifuged and the plasma was separated. Ammonium sulphate was added at a ratio of 3.5%, the pH value was adjusted to 6, and the supernatant was obtained by centrifugation for 10 min at 3,500 r.p.m. and 4 °C. The supernatant was put into a dialysis bag and dialysed for 24 h. The dialysed solution was centrifuged at 3,500 r.p.m. and 4 °C for 10 min. The supernatant was freeze-dried, and the dried supernatant was used as plasma protein powder (Gao et al., 2020).

2.3. Preparation of chicken plasma protein hydrolysates

A certain amount of plasma protein powder was weighed, formulated into a 5% (w/v, protein concentration) aqueous solution, and heated to the desired temperature in a water bath. The pH was then adjusted and protease was added for hydrolysis. The hydrolysis process took place in a constant temperature water bath, and 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl was continuously added to maintain the pH within the specified range (the difference between the top and bottom was not more than 0.1). After the set time, the reaction system was kept at 100 °C for 10 min to inactivate the enzyme, then a certain amount of 10% TCA solution was added, the hydrolysates were refrigerated and centrifuged at 4,000 r.p.m. for 10 min, and the supernatant was taken for detection (Jang and Lee, 2005).

2.4. Screening of protease

Five hydrolases, Pep, Alk, Try, Pap, and Neu, were selected. Under the same conditions of substrate concentration and hydrolysis time, the degree of hydrolysis of chicken plasma protein and the *in vitro* antioxidant activity of hydrolysed products were determined at the optimum temperature and pH of the enzyme. The best protease was screened on the basis of degree of hydrolysis. Among them, the *in vitro* antioxidant activity measurement included superoxide radical scavenging rate, reducing capacity, DPPH radical scavenging capacity, hydroxyl radical

scavenging activity, and Iron chelating activity. The screening reaction table of enzymes is shown in Table 1. The pH value and reaction temperature of each enzyme were based on the conditions recommended in its manual.

2.5. Optimisation of chicken plasma protein hydrolysis process

Four factors, temperature (A), time (B), pH (C), and enzyme-to-substrate ratio ([E]/[S]) (D), were selected to carry out a 5-level single factor test to study the effect of the above factors on chicken plasma. The influence of the degree of proteolysis was used to obtain the optimal process conditions for the enzyme. Based on a single-factor experiment, four factors including enzymolysis temperature, enzymolysis time, [E]/[S], and pH were selected, and an L9(34) orthogonal table was used to conduct the orthogonal experiment with 4 factors and 3 levels to optimise the enzyme technological conditions for solubilising chicken plasma protein. The single factor experimental design table is shown in Table 2.

2.6. Determination of the degree of protein hydrolysis (DH)

The degree of protein hydrolysis (DH) was determined by the ninhydrin colorimetric method (Pearce et al., 2010) and calculated from Eq. (1).

$$DH\% = \frac{A_2 - A_0}{A_1 - A_0} \times 100 \tag{1}$$

where A_0: the number of free amino acids contained in the chicken plasma protein solution before hydrolysis, mmol; A_1: the number of free amino acids contained in chicken plasma protein after complete hydrolysis, mmol; A_2: the number of free amino acids contained in the chicken plasma protein hydrolysates, mmol.

Table 1. The screening reaction table of enzymes

Protease	Temperature (°C)	pH	Substrate concentration (%)	Time (h)	[E]/[S] (U g^{-1})
Pepsin	37	2	5	6	6,000
Alkaline protease	45	7.5	5	6	6,000
Trypsin	37	5.5	5	6	6,000
Papain	40	7.0	5	6	6,000
Neutral protease	45	7.0	5	6	6,000

Table 2. The single factor experimental design table

	Factors			
Levels	A Time (h)	B Temperature (°C)	C pH	D [E]/[S] (U g^{-1})
1	2	35	6	3,000
2	4	40	6.5	4,000
3	6	45	7	5,000
4	8	50	7.5	6,000
5	10	55	8	7,000

2.7. Animal model establishment

All procedures regarding animal treatment were approved by the Experiment Animal Care and Use Committee of Gansu Province, China. Sixty 8-week-old Kunming mice (35 ± 5 g) were randomly divided into 6 groups, namely the low-dose protein hydrolysates group (10 times the recommended amount for humans), the medium-dose group (20 times), and the high-dose group (30 times), positive control group (vitamin C), blank control group, and model control group (physiological saline). Except for the blank control group, the other groups were injected with D-galactose into the abdominal cavity of the neck and back. The injection volume was 0.1 mL/10 g, once a day for 6 consecutive weeks. The positive control and low-dose groups were given the same amount of gastric administration. While giving the test samples, the model control group and each dose group continued to inject D-galactose (0.1 mL/10 g) intraperitoneally for 42 days. The mice were weighed and recorded at the same time every day.

2.8. Determination of antioxidant indices

The mice were sacrificed, and plasma and liver were collected and weighed. The mouse plasma lipid oxidation products (MDA), protein carbonyls, reduced glutathione (GSH), and antioxidant enzyme activity (SOD) were determined as antioxidant ability and other indicators by the kit method.

2.9. Observation of the pathological section of the liver

Haematoxylin and eosin staining was used to stain pathological sections of the liver (Asokan et al., 2019). The specific steps were as follows: sample collection → fixation → dehydration → transparency → waxing → embedded → baked slices → dewaxing → dying → dehydration → transparent → sealing slices → baked slices → observation under the microscope.

2.10. Statistical analyses

IBM SPSS Statistics 21 software was used for statistical analyses, ANOVA was used to compare means, and Duncan post-mortem tests were used to analyse differences at a significance level of 0.05. Origin 2018 software (Originlab Corporation) was used to draw diagrams. All experiments or analyses were carried out in triplicate, and the data were reported as the mean ± standard error of the mean.

3. RESULTS AND DISCUSSION

3.1. Screening of protease

Papain, pepsin, trypsin, neutral protease, and alkaline protease were used to hydrolyse chicken plasma protein. The results of degree of hydrolysis and antioxidant activities of each hydrolysed liquid are shown in Fig. 1. Alkaline protease had the highest degree of hydrolysis of chicken plasma protein (19.30%), and papain had the lowest degree of hydrolysis (11.75%). The five protease hydrolysates all had specific antioxidant activities, but alkaline protease had the best antioxidant effect *in vitro*. Its superoxide radical scavenging rate was 76.63%, reducing power was 0.82%, DPPH free radical scavenging ability was 75.70%, and hydroxyl radical scavenging

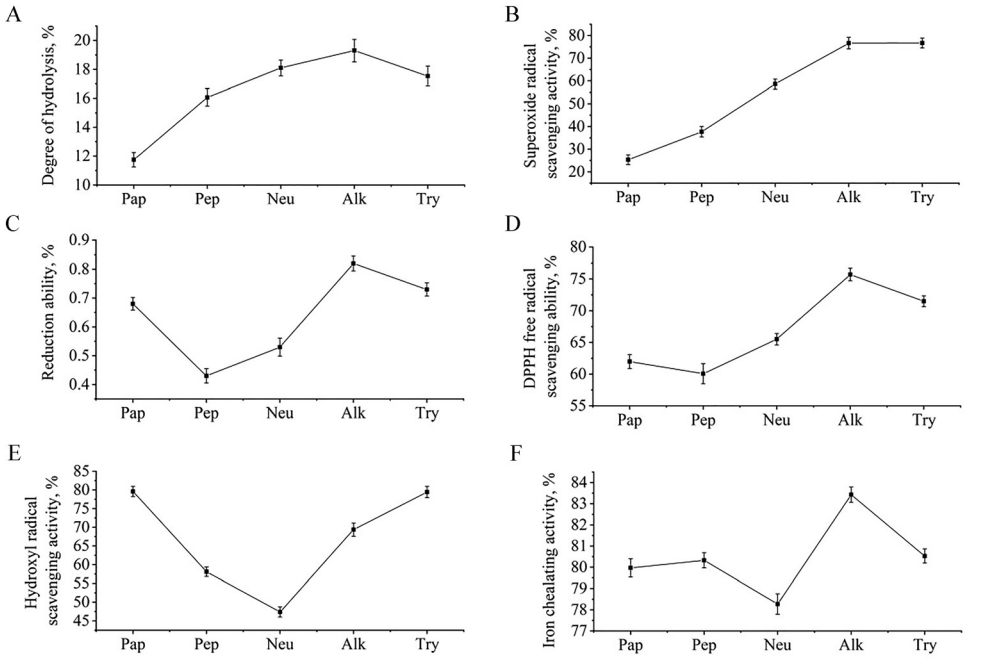

Fig. 1. Hydrolysis degree (A), superoxide anion scavenging ability (B), reduction ability (C), DPPH free radical scavenging ability (D), hydroxyl radical scavenging activity (E), iron chelating activity (F) of 5 types of protease hydrolysed liquids

Pap: papain; Pep: pepsin; Try: trypsin; Neu: neutral protease; Alk: alkaline protease.

capacity was 76.63%. The free radical scavenging activity was 69.37%, and the ability to chelate metal Fe^{2+} ions was 88.43%. Therefore, alkaline protease was selected to hydrolyse chicken plasma protein.

3.2. Optimisation of chicken plasma protein hydrolysis process

The results of protease hydrolysis showed that the hydrolysis effect of alkaline protease was the best and the hydrolysates had good antioxidant activities *in vitro*. Therefore, alkaline protease was selected for single factor and orthogonal experiments. The single factor experiment results of alkaline protease hydrolysing plasma protein are shown in Fig. 2. As the pH increased, the degree of hydrolysis initially showed an upward trend, and the degree of hydrolysis reached the maximum when the pH was 7.5 and then gradually decreased. With the increase of the reaction temperature, the degree of hydrolysis first increased and then decreased. The degree of hydrolysis was the maximum at the temperature of 45 °C. As the concentration ratio of enzyme to substrate ([E]/[S]) increased, the degree of hydrolysis first increased and then decreased, and the degree of hydrolysis was maximum at [E]/[S] of 6,000 U g^{-1}. With the prolongation of the reaction time, hydrolysis gradually increased. After 6 h, the degree of hydrolysis gradually became smooth and no longer increased. Therefore, 6 h was chosen as the system reaction time.

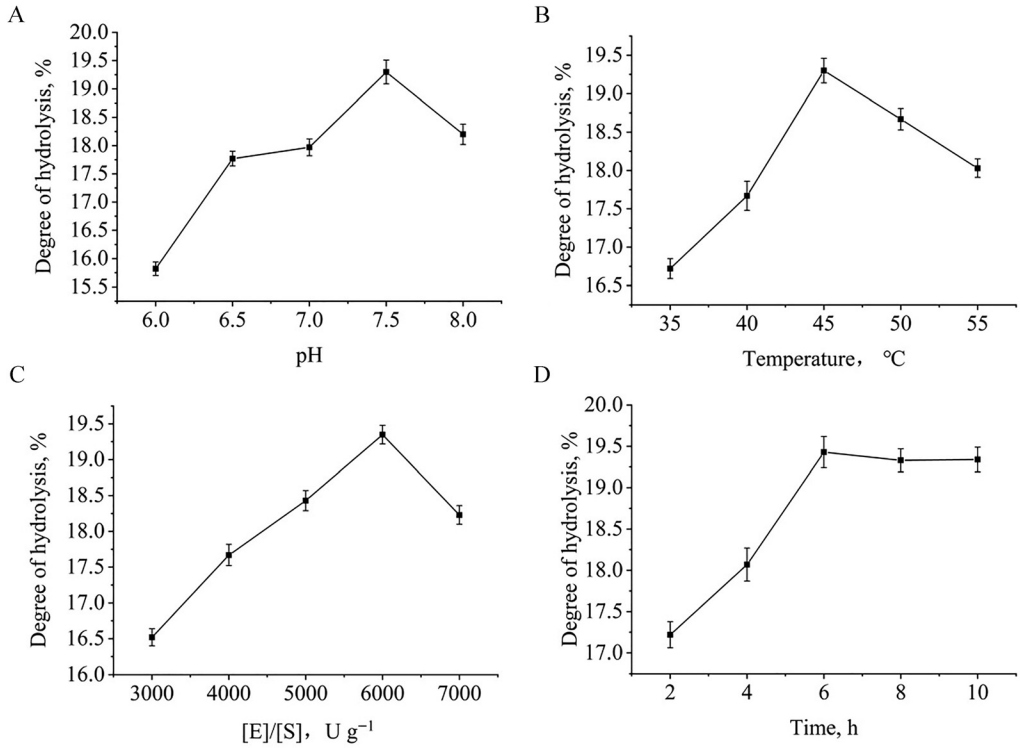

Fig. 2. Effects of pH (A), temperature (B), [E]/[S] (C), and time (D) on the hydrolysis of chicken plasma protein by alkaline protease
Significant impact ($P < 0.05$).

A 4-factor 3-level orthogonal experiment was carried out, based on a single-factor experiment. The results are shown in Tables 3 and 4. The order of the range of the 4 factors was: RD > RC > RA > RB, indicating that [E]/[S] had the most significant influence on the degree of hydrolysis, followed by pH, enzymatic hydrolysis time, and enzymatic hydrolysis temperature had almost negligible effect. According to the results of the range analysis, the optimal process condition combination of the enzymatic extraction method was A3B3C2D3, indicating that the enzymatic hydrolysis temperature was 50 °C, the enzymatic hydrolysis time was 8 h, [E]/[S] was 7,000 U g^{-1}, and the pH was 7.5. The analysis of variance showed that only [E]/[S] had a significant effect on the degree of hydrolysis ($P < 0.05$). This process had good repeatability and could be used to hydrolyse chicken plasma protein.

3.3. Antioxidant activity of chicken plasma protein hydrolysates *in vivo*

3.3.1. The effect of plasma protein hydrolysates on mouse body weight and liver. The effects of chicken plasma protein hydrolysates on mouse body weight and liver weight are shown in Table 5. The mice in the low-dose group and model control group lost weight, while the middle-dose group, high-dose group, positive control group, blank control group, and model control

Table 3. Orthogonal experiment results

Experiment number	Factor				Degree of hydrolysis (%)
	A Time (h)	B Temperature (°C)	C pH	D [E]/[S] (U g^{-1})	
1	4	40	7	5,000	14.7
2	4	45	7.5	6,000	19.5
3	4	50	8	7,000	17.8
4	6	40	7.5	7,000	19.7
5	6	45	8	5,000	14.0
6	6	50	7	6,000	18.4
7	8	40	8	6,000	18.0
8	8	45	7	7,000	19.2
9	8	50	7.5	5,000	17.9
k1	17.33	17.46	17.43	15.53	
k2	17.36	17.57	19.03	18.63	
k3	18.37	18.03	16.60	18.90	
R	1.03	0.56	2.433	3.367	

Table 4. Analysis of variance

Factor	Q	f	F ratio	F	Sig
Time	2.069	2	3.769	19.00	
Temperature	0.539	2	1.000	19.00	
pH	8.167	2	16.714	19.00	
[E]/[S]	19.016	2	38.281	19.00	*
Error	0.55	2			

*: Significant at ($P < 0.05$).

Table 5. Mice body weight and liver weight

Group	Starting weight (g)	End weight (g)	Liver weight (g)
L	35.51 ± 4.28	34.50 ± 4.11	1.59 ± 0.07^{c}
M	35.84 ± 4.57	36.25 ± 5.28	1.70 ± 0.08^{b}
H	36.84 ± 4.82	39.28 ± 5.07	0.87 ± 0.18^{a}
MC	34.81 ± 5.56	3,394 ± 6.21	1.54 ± 0.14^{c}
VC	35.97 ± 3.91	36.17 ± 5.24	1.67 ± 0.09^{b}
B	33.89 ± 2.51	33.93 ± 3.02	1.50 ± 0.10^{c}

L: low-dose group; M: medium-dose group; H; high-dose group; MC: model group; VC: positive control vitamin C group; B: blank control group; different lowercase letters between different groups indicate significant differences ($P < 0.05$)

group gained weight. The liver weight of mice in the middle-dose, high-dose, and positive control groups was significantly higher than that of the other groups ($P < 0.05$).

3.3.2. The effect of chicken plasma protein hydrolysates on oxidative factors in mouse serum. The effects of chicken plasma protein hydrolysates on mouse serum MDA, protein carbonyl, SOD, and GSH are shown in Fig. 3. Compared with the blank control group, the serum MDA and protein carbonyl content of the model group increased significantly ($P < 0.05$) and the content of SOD and GSH decreased significantly ($P < 0.05$). Compared with the model group, the low, medium, and high dose groups significantly reduced the serum MDA and protein carbonyl contents ($P < 0.05$) and significantly increased the serum SOD and GSH contents ($P < 0.05$). The plasma levels of MDA and GSH in the middle-dose group were not significantly different from those in the positive control group and the blank control group ($P > 0.05$). It showed that the antioxidant activity of the medium-dose chicken plasma protein hydrolysates was consistent with the VC effect, and the chicken plasma protein hydrolysates could alleviate the aging of mice.

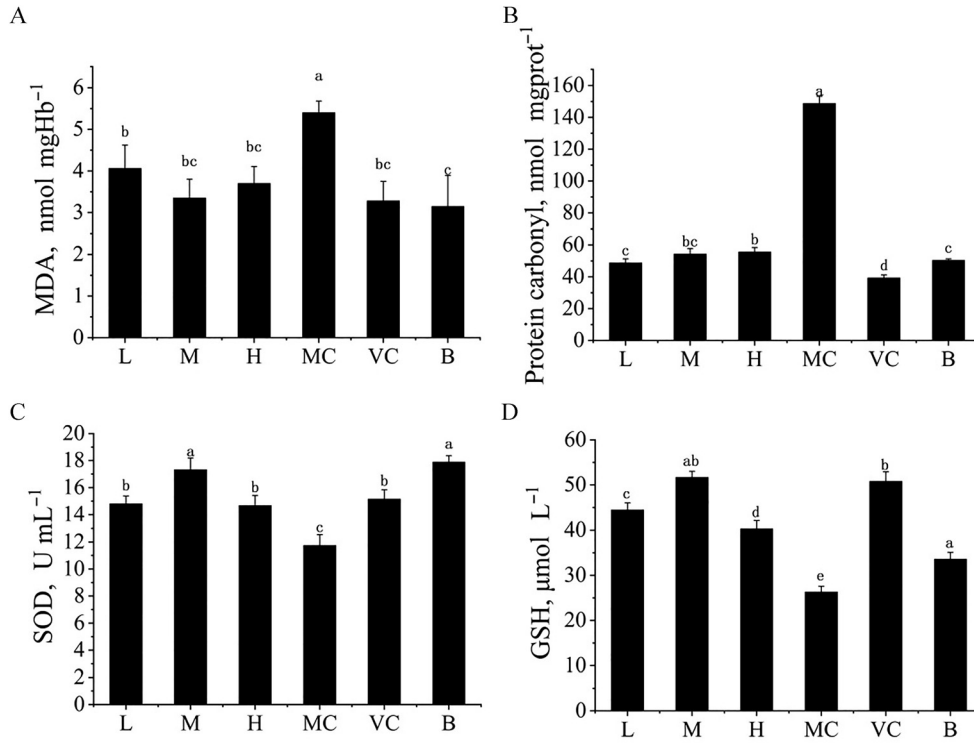

Fig. 3. Effects of chicken plasma protein hydrolysates on MDA (A), protein carbonyl (B), SOD (C), and GSH (D) in mouse serum

MDA: malondialdehyde; GSH: glutathione; SOD: superoxide dismutase. Different lowercase letters indicate significant differences between groups ($P < 0.05$), and the same lowercase letters indicate no significant differences between groups ($P > 0.05$).

3.3.3. Observation of haematoxylin and eosin staining of mouse liver. The pathological section of mice liver is shown in Fig. 4. By observing the HE stained sections of the liver of six groups of mice, it was found that the outline of the liver cells in group B was complete, the nuclei of the liver cells were lightly stained, and the liver lobules were normal, and the hepatocyte cords were transparent. In the VC group, the outline of hepatocytes was complete, the nuclei of hepatocytes were stained, the liver lobules were normal, and there were many new cells. The outline of MC hepatocytes still existed, but the cells were swollen, the cytoplasm was loosened, and red granular material appeared, the cells became watery, and some of the nuclei were dissolved. The outline of liver cells in group L was complete, the nuclei of liver cells were lightly stained, the liver lobules were normal, and the hepatocyte cords were transparent. The contours of liver cells in group M are complete, the nuclei of liver cells were lightly stained, the liver lobules were normal, the hepatocyte cords were transparent, and the space between liver sinuses was ample. The contours of hepatocytes in group H were complete, the nuclei of hepatocytes were stained, the liver lobules were expected, there were many new cells, and the central vein was

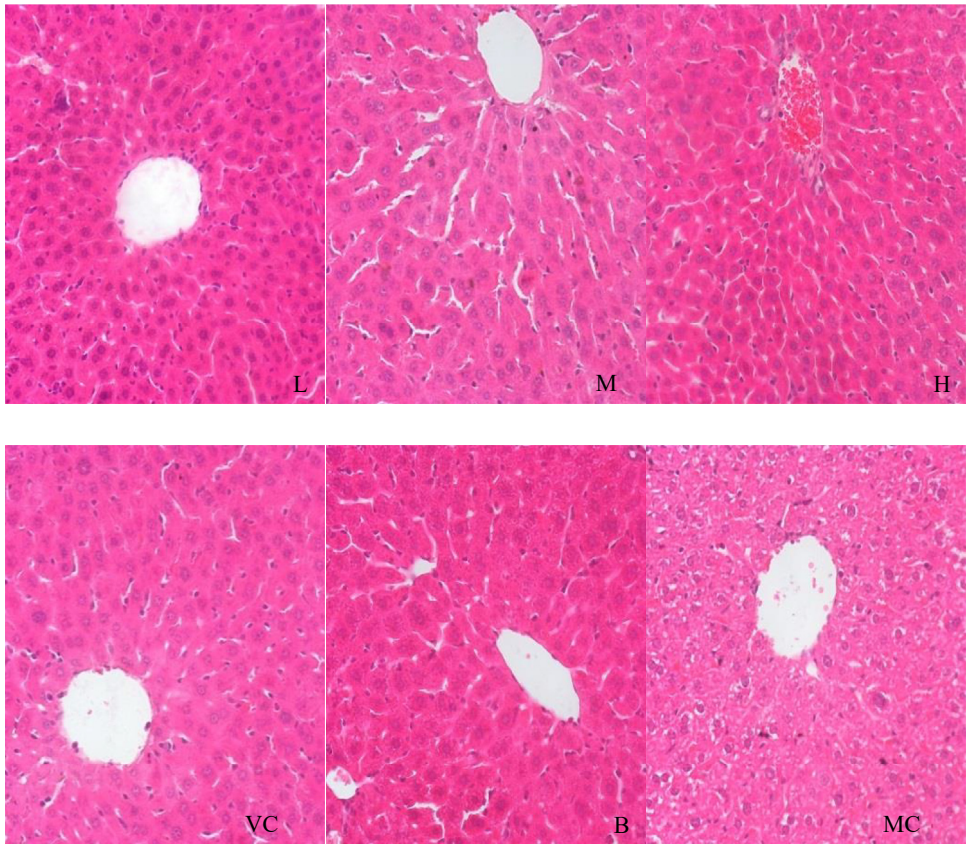

Fig. 4. Observation of pathological section of mouse liver
L: low-dose group; M: medium-dose group; H: high-dose group; VC: positive control group; B: blank control group; MC: model control group; magnification: 40×.

congested. It could be seen that the liver cells in the model group were severely damaged and produced specific pathological changes, but the chicken plasma protein hydrolysates could alleviate this pathological damage.

4. CONCLUSIONS

Hydrolysate fractions or bio-active peptides from natural proteins are considered safer as food additives than those synthesised chemically. Therefore, it was of certain significance to carry out the study of hydrolysis to produce bioactive substances using the by-products of food processing as raw materials. In this study, the protease with the best effect on the hydrolysis of chicken plasma was first screened.

The results showed that the hydrolysis product of chicken plasma protein obtained by five kind of protease had certain antioxidant activities *in vitro*. On the whole, alkaline protease not only had the highest degree of hydrolysis but also had high antioxidant activity *in vitro* of hydrolysate products, so basic proteases were selected for follow-up experiments. Univariate and orthogonal results showed that the optimal process conditions for alkaline protease hydrolysis were temperature of 50 °C, time of 8 h, [E]/[S] of 7,000 U g^{-1}, and pH 7.5. The results of the study are inconsistent with the results of Yang et al. (2020b), who used alkaline protease to hydrolyse duck blood, and the pH environment required for this experiment was low, which may be related to the different plasma protein content and species. *In vivo* antioxidant activity studies had shown that the antioxidant activity of medium-dose chicken plasma proteolysis was consistent with the VC effect, which could alleviate aging in mice. The tissue sectioning results showed that the hepatocytes in the model group were severely damaged causing certain pathological changes, but the chicken plasma protein hydrolysis products could alleviate this pathological damage. In summary, it was shown that chicken plasma hydrolysis products had certain antioxidant activities. However, this study only studied the antioxidant activity of chicken plasma protein hydrolysates and did not isolate and purify their components. The need to identify specific peptides with antioxidant activity requires further exploration.

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