# Optimized Preparation of Duck Blood Cell Short Peptides and Their Properties (Postprint)

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## Abstract

This study aimed to screen commercial proteases with high hydrolysis efficiency and good decolorization effect, establish an optimized process for hemoglobin short peptides, compare changes in nutritional characteristics before and after enzymatic hydrolysis, investigate their functional properties and in vitro antioxidant capacity, and develop functional hemoglobin short peptide products, thereby providing theoretical basis and technical reference for the high-value transformation, utilization, and in-depth exploitation of poultry blood resources. The effects of enzyme type, enzyme concentration, temperature, pH, and hydrolysis time on the degree of hydrolysis (DH), decolorization degree, and hydrolysate yield were compared, and orthogonal experimental design was employed to optimize the best process for hemoglobin short peptides, followed by evaluation of their nutritional value, functional properties, and antioxidant performance. Acid protease was identified as the optimal hydrolytic enzyme, with the optimal process parameters for hydrolyzing duck hemoglobin to prepare short peptides being: enzyme dosage of 6,000 U/g, temperature of 50 °C, pH of 3.5, and hydrolysis time of 7.0 h. Under these conditions, the degree of hydrolysis was (25.10±0.65)%, and the hydrolysate yield was (60.09±1.77)%. The molecular weight distribution of the hydrolysates was analyzed by high-performance liquid chromatography. The results indicated that enzymatic hydrolysis had a significant degrading effect on hemoglobin, with the hydrolysate primarily consisting of short peptides below 3 kDa, among which those below 1 kDa accounted for the majority (62.82%). The hemoglobin short peptide powder exhibited a milky white color, complete amino acid composition, and rich essential amino acid content (53.31%). The solubility of duck hemoglobin after enzymatic hydrolysis was substantially improved (>60%), and it also possessed good emulsifying stability. The hemoglobin short peptides demonstrated strong free radical scavenging capacity, which increased with hemoglobin concentration, showing enhanced DPPH• radical and superoxide anion scavenging abilities, along with gradually increasing reducing power. These results demon-

strate that acid protease can effectively hydrolyze duck hemoglobin to obtain milky white hemoglobin short peptides rich in amino acids, with good solubility and antioxidant activity, which can be applied as functional ingredients in food and feed.

## Full Text

# Optimized Preparation and Characterization of Duck Blood Corpuscle Short Peptides

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**Abstract:** This study aimed to screen commercial proteases with high hydrolytic efficiency and effective decolorization, establish an optimized process for preparing blood corpuscle short peptides, compare changes in nutritional characteristics before and after enzymatic hydrolysis, and evaluate the functional properties and in vitro antioxidant capacity to develop functional blood corpuscle short peptide products and provide theoretical basis and technical reference for the high-value conversion and utilization of poultry blood resources. The effects of enzyme type, enzyme concentration, temperature, pH, and hydrolysis time on the degree of hydrolysis (DH), decolorization, and hydrolysate yield were investigated, and orthogonal experimental design was employed to optimize the preparation conditions. The nutritional value, functional properties, and antioxidant capacity of the resulting blood corpuscle short peptides were subsequently evaluated. Acidic protease was identified as the optimal enzyme, with the following optimal parameters for hydrolyzing duck blood corpuscle protein: enzyme dosage of 6,000 U/g, temperature of 50 °C, pH of 3.5, and hydrolysis time of 7.0 h. Under these conditions, the degree of hydrolysis was (25.10 ± 0.65)% and the hydrolysate yield was (60.09 ± 1.77)%. High-performance liquid chromatography analysis of the molecular weight distribution revealed that enzymatic hydrolysis caused significant degradation of blood corpuscle proteins, with the hydrolysate consisting primarily of short peptides below 3 ku, most of which (62.82%) were below 1 ku. The blood corpuscle short peptide powder exhibited a milky white color, complete amino acid profile, and abundant essential amino acids (53.31%). The solubility of duck blood corpuscle protein was substantially improved after enzymatic hydrolysis (>60%), and the product demonstrated good emulsifying stability. The blood corpuscle short peptides showed strong free radical scavenging capacity, with DPPH• and superoxide anion scavenging abilities, as well as reducing power, increasing with protein concentration. These results demonstrate that acidic protease can effectively hydrolyze duck blood corpuscle protein to produce milky white short peptides rich in amino acids, with good solubility and antioxidant activity, making them

suitable as functional ingredients in food and feed applications.

**Keywords:** blood corpuscle short peptide; acidic protease; functional property; antioxidant activity

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Blood, a byproduct of poultry meat processing, accounts for 6%-8% of body weight and is rich in nutritional value, with high protein content (17%-22%) and low fat content (0.15%-0.20%), earning it the reputation of "liquid meat" [1-2]. As a major livestock and poultry farming country, China produced 11.9 billion poultry birds in 2013, yielding up to 1.19 million tons of blood. However, aside from partial processing into low-value products such as blood tofu and blood meal, and limited use in biochemical pharmaceuticals for producing heme, superoxide dismutase, and peptone, product development remains monotonous with low degree of deep processing, wasting valuable protein resources. Blood corpuscle protein is the predominant protein in blood, comprising approximately two-thirds of total protein content. Its development and utilization have been hindered by its bright red color, strong metallic taste, poor storability, and low digestive utilization rate.

Enzymatic protein hydrolysis has emerged as an important direction for efficient utilization of blood corpuscle protein, capable of breaking down poorly digestible macromolecular proteins into peptides and L-amino acids. The resulting blood corpuscle short peptides exhibit various physiological activities, including angiotensin-converting enzyme (ACE) inhibition [3-4], opioid activity [5-6], bacterial growth stimulation [7], analgesic effects [8], antimicrobial properties [9-10], and antioxidant activity [11-12], significantly enhancing their nutritional value and functional properties. Therefore, optimizing the enzymatic hydrolysis process, developing highly functional short peptide products, and investigating their nutritional characteristics, functional properties, and antioxidant capacity hold important theoretical and practical significance for the high-value utilization and promotion of poultry blood resources. Due to its mild reaction conditions, controllable hydrolysis process, and high nutritional value of products, enzymatic methods have been widely adopted by researchers to hydrolyze various proteins for high-value products. Deng et al. [13] used neutral protease and papain for compound hydrolysis of porcine hemoglobin, achieving a DH of 19.1% after 10 h. Guo et al. [14] demonstrated that combined hydrolysis of porcine hemoglobin with pancreatin and flavourzyme achieved a total nitrogen recovery of 97.69%, though the molecular weight distribution of the hydrolysate was scattered across >15 ku and <1 ku ranges, indicating room for improvement in short peptide content. Sun et al. [15] hydrolyzed porcine hemoglobin with different proteases, finding alcalase most effective followed by pepsin, suggesting that acidic or alkaline conditions enhance protein hydrolysis and yield antioxidant peptide fragments. Yu et al. [1] studied compound hydrolysis of porcine hemoglobin with neutral protease and papain, achieving a

DH of 24.5% and total nitrogen recovery of 74.64%. In et al. [16] reported that approximately 98% of heme iron existed in protein precipitates at low pH (3-5) and high DH (>20%), indicating that extensive hydrolysis under acidic conditions facilitates heme iron removal and improves hydrolysate color. However, most current research focuses on potential physiological activities of blood corpuscle protein, while problems such as poor color, palatability, and digestibility caused by heme iron remain inadequately addressed. How to obtain more light-colored, odorless short peptides with high DH has become a critical technical challenge in production processes. Moreover, research on enzymatic hydrolysis of duck blood corpuscle protein for short peptide preparation and investigation of its functional properties and antioxidant capacity remains limited. This study utilized duck blood corpuscle protein as raw material to examine the effects of enzyme type, enzyme dosage, pH, temperature, and time on DH and hydrolysate yield. Orthogonal experimental design was employed to optimize the hydrolysis conditions for duck blood corpuscle protein, aiming to obtain blood corpuscle short peptides with good color, no off-flavors, easy absorption, and balanced amino acid composition. The functional properties and antioxidant activity of the product were also evaluated to provide theoretical basis and technical support for industrial production of blood corpuscle short peptides.

### 1.1 Experimental Materials

Fresh duck blood corpuscle protein was provided by Beijing Hongshun Yangyuan Biotechnology Co., Ltd., stored at -20 °C in the dark, and thawed overnight at 4 °C before use. Acidic protease (5×10 U/g) was purchased from Beijing Donghua Qiangsheng Biotechnology Co., Ltd.; neutral protease (4×10 U/g), alkaline protease (4×10 U/g), papain (8×10 U/g), and flavourzyme (1×10 U/g) were obtained from Nanning Pangbo Bioengineering Co., Ltd.; o-phthaldialdehyde (OPA) was from Sinopharm Chemical Reagent Co., Ltd.; sodium dodecyl sulfate (SDS), dithiothreitol (DTT), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•), and pyrogallol were from Sigma-Aldrich (USA); all other reagents were chemically pure.

### 1.2 Main Instruments

SD-BASIC spray dryer (LabPlant, UK); GL-20G-C high-speed refrigerated centrifuge (Shanghai Anting Scientific Instrument Factory); HYG-A thermostatic oscillator (Taicang Experimental Equipment Factory, Jiangsu); TU-1810 UV-Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd.).

#### 1.3.1 Protease Screening

Five proteases (acidic protease, neutral protease, alkaline protease, papain, and flavourzyme) were screened based on protein degree of hydrolysis. Blood corpuscle protein was prepared as a 16% solution at 45 °C. Initial pH was 3.0 for acidic protease, 8.0 for alkaline protease, and 7.0 for other proteases. Hydrolysis

time was 5 h for all treatments, and the degree of hydrolysis was measured. All treatments were performed in triplicate.

### 1.3.2 Process Flow

Controlling the degree of hydrolysis is a critical step in the enzymatic hydrolysis of duck blood corpuscle protein. The basic process is shown in Figure 1 [Figure 1: see original paper], with key points as follows:

1) **Hemolysis and membrane disruption**: Blood corpuscle protein was dissolved in 0.1 mol/L HCl (blood corpuscle protein:HCl = 3:2, m/v) and deionized water to prepare a 16% protein solution. After mixing uniformly, the solution was incubated at 40 °C in a 200 r/min shaker for 30 min to disrupt blood cells and denature proteins.

2) **Enzymatic hydrolysis**: The hemolyzed solution was cooled to room temperature, and pH was adjusted to the predetermined value with 85% lactic acid or 4 mol/L NaOH. Appropriate protease was added, and hydrolysis was carried out in a thermostatic shaker at 200 r/min.

3) **Enzyme inactivation**: After reaction completion, the mixture was heated in a 90 °C water bath for 15 min to inactivate the enzyme.

4) **Centrifugation**: The mixture was centrifuged at 6,000 r/min for 10 min at 4 °C to remove insoluble materials, and the supernatant was collected as duck blood corpuscle protein hydrolysate.

5) **Spray drying**: The supernatant was spray-dried at an inlet temperature of 110–130 °C and outlet temperature of 60–80 °C to produce blood corpuscle short peptide powder.

### 1.3.3 Degree of Hydrolysis Determination

Degree of hydrolysis refers to the percentage of peptide bonds cleaved during protein hydrolysis. The OPA method [17] was used: 0.4 mL of appropriately diluted hydrolysate was mixed with 3 mL OPA reagent, reacted for exactly 2 min, and absorbance was measured at 340 nm.

### 1.3.4 Hydrolysate Yield Determination

Hydrolysate yield (HY) was determined using the method of Low et al. [18]. The supernatant obtained after centrifugation was weighed, and its weight percentage represented the hydrolysate yield.

### 1.3.5 Peptide Molecular Weight Distribution Detection

High-performance liquid chromatography was used to determine the molecular weight distribution of blood corpuscle protein and peptides. Prepared peptide samples and standards (molecular weights of 6,500, 13,700, and 43,000 u) were

injected (100  L) with a detection time of 50 min. The percentage of each molecular weight fraction was calculated based on peak values and areas.

### 1.3.6 Color Measurement

Color was measured using a Minolta CR-410 colorimeter (Japan) following the method of Bhaskar et al. [19]. Lightness (L*) represents black-white (brightness), with higher values indicating whiter/brighter appearance; redness (a)* represents green-red, with higher values indicating redder color; yellowness (b*) represents blue-yellow, with higher values indicating more yellow color. Whiteness index (WI) was calculated as:

WI = 100 -[(100 -L)$^2$ + $a^2$ + b*$^2$]/2

### 1.3.7 Amino Acid Composition and Hydrophobicity Analysis

Amino acid content was determined using an AminoPac PA10 amino acid analysis column with integrated amperometric ion chromatography.

### 1.3.8 Bitterness Evaluation of Blood Corpuscle Short Peptides

Bitterness evaluation comprised two parts: (1) Amino acid hydrophobicity analysis based on the amino acid composition, where the hydrophobicity value Q of the protein was calculated as the sum of individual amino acid hydrophobicity values ΔQ according to Ney' s [20] Q rule; (2) Sensory evaluation, where 1 g of blood corpuscle short peptide was dissolved in 10 mL sterile water and evaluated by six trained panelists for bitterness or off-flavors.

### 1.3.9 Functional Properties Determination

#### 1.3.9.1 Solubility Measurement

Following the method of Liu et al. [21], 0.30 g of powder was dissolved in 30 mL deionized water. pH was adjusted (3–8) with 6 mol/L HCl or 6 mol/L NaOH, stirred for 30 min, and centrifuged at 3,000 r/min for 15 min. Nitrogen content in the supernatant was measured by the Bradford method, and solubility was expressed as the percentage of total nitrogen content.

#### 1.3.9.2 Emulsifying Activity and Stability Measurement

The turbidity method [18] was used. At different pH values (3–8), 15 mL of 0.2% (m/v) sample was mixed with 5 mL vegetable oil, homogenized at 20,000 r/min for 1 min at room temperature. Immediately, 50  L of the bottom emulsion was mixed with 5 mL of 0.1% SDS, and absorbance was measured at 500 nm (A ). After standing for 15 min, absorbance was measured again (A  ). Emulsifying activity index (EAI) and emulsifying stability index (ESI) were calculated as:

EAI (m$^2$/g) = (2 × 2.303 × A ) / (0.25 × c × 100)
ESI (min) = A  × 15 / (A  -A  )

where c is the protein concentration of the sample.

#### 1.3.9.3 Foaming Capacity and Stability Measurement [21]

0.5% (m/v) powder was dissolved in 30 mL of 0.02 mol/L citrate-phosphate buffer (pH 3–8), homogenized at 20,000 r/min for 1 min, and allowed to stand for 10 min. Volumes before and after homogenization were recorded. Foaming capacity and foam stability were calculated as:

Foaming capacity (%) = [(V  -V ) / V ] × 100
Foam stability (%) = [(V  -V ) / V ] × 100

where V  is the initial volume, V  is the volume immediately after homogenization, and V  is the volume after standing for 10 min.

### 1.3.10 Antioxidant Activity Assays

#### 1.3.10.1 DPPH • Scavenging Activity [22]

1.5 mL of peptide solution at various concentrations was mixed with 1.5 mL DPPH • solution (1.0 mmol/L), reacted in the dark at room temperature for 30 min, and absorbance was measured at 517 nm.

#### 1.3.10.2 Superoxide Anion Scavenging Activity [23]

0.1 mL of peptide solution at various concentrations was added to 2.8 mL Tris-HCl-EDTA buffer (pH 8.2), incubated at 25 °C for 10 min, then 0.1 mL of 3 mmol/L pyrogallol solution was added and mixed rapidly. Absorbance was measured at 325 nm every 30 s for 5 min. A regression equation of absorbance versus time was established to determine the slope. Scavenging rate was calculated as:

Scavenging rate (%) = [(V_control -V_sample) / V_control] × 100

where V_control is the autoxidation rate of pyrogallol in the control group ( A/min), and V_sample is the oxidation rate in the sample group ( A/min).

#### 1.3.10.3 Reducing Power [11]

1.0 mL sample was mixed with 1.0 mL sodium phosphate solution (pH 6.6) and 1.0 mL of 1% potassium ferricyanide, incubated at 50 °C for 20 min. After adding 1.0 mL of 10% trichloroacetic acid (TCA) solution, the mixture was centrifuged at 5,000 r/min for 10 min. The supernatant (2.0 mL) was mixed with 2.0 mL deionized water and 0.4 mL of 0.1% ferric chloride, allowed to stand at room temperature for 10 min, and absorbance was measured at 700 nm.

### 1.4 Statistical Analysis

Experimental data were expressed as mean ± standard deviation. JMP 10.0 was used for experimental design and data analysis, and Origin Pro 9.0 was used for graph preparation.

## 2.1 Enzyme Screening

Five proteases were screened based on degree of hydrolysis, with results shown in Figure 2 [Figure 2: see original paper]. Overall, flavourzyme showed the lowest initial DH but the fastest hydrolysis rate. Acidic protease exhibited the highest initial and overall DH, followed by neutral protease. Considering the high cost of neutral protease and the red-brown color of its hydrolysate requiring additional decolorization methods, acidic protease was selected as the tool enzyme for subsequent experiments.

## 2.2 Orthogonal Experiment for Enzymatic Hydrolysis

Based on preliminary single-factor experiments, an orthogonal experiment was designed using variance analysis to determine optimal hydrolysis conditions for duck blood corpuscle protein. Four factors were selected: enzyme dosage, pH, temperature, and hydrolysis time (Table 1 ). K and k values represent the total and average values of DH and hydrolysate yield for each factor level, while R values represent the range. Larger K or k values indicate preferred factor levels, and larger R values indicate greater influence of that factor.

For DH, the order of factor influence was: hydrolysis time (D) > temperature (A) > enzyme dosage (C) > pH (B), with hydrolysis time being the most influential and pH the least. Visual analysis indicated the optimal combination for acidic protease hydrolysis was: enzyme dosage 6,000 U/g, temperature 50 °C, pH 3.5, and hydrolysis time 7 h. For hydrolysate yield, pH was the most influential factor, with the order: pH (B) > temperature (A) > enzyme dosage (C) > time (D). The optimal combination for yield was identical to that for DH.

Combining these results, the optimal conditions were: temperature 50 °C, pH 3.5, enzyme dosage 6,000 U/g, and hydrolysis time 7 h. These conditions corresponded to treatment combination #16 in Table 1, yielding a DH of (25.10 ± 0.65)% and hydrolysate yield of (60.09 ± 1.77)%.

**Table 1** Results of orthogonal experiment for enzymatic hydrolysis of blood corpuscle protein

[Table content with Temperature, E/S, pH, Time, DH, and Hydrolysate yield data]

Note: Data are means ± SD of three replicates; K, k represent total and average values of DH and hydrolysate yield for each factor level; R represents the range; Q indicates the optimal level of each factor.

## 2.3 Molecular Weight Distribution of Blood Corpuscle Protein and Short Peptides

High-performance liquid chromatography (HPLC) analysis of molecular weight distribution showed that blood corpuscle protein had short elution times with relatively single peaks, predominantly large molecular weight peptides (>10 ku)

accounting for about 82.34%. After acidic protease hydrolysis, the molecular weight distribution shifted from high (20–80 ku) to low (<10 ku) ranges (Table 2 ). Large molecular weight proteins were hydrolyzed into smaller peptides, with small peptide content increasing significantly. Peptides below 10 ku accounted for 97.65%, an 80% increase compared to unhydrolyzed protein. Peptides below 3 ku represented approximately 81.89%, about 6.1 times that of unhydrolyzed blood corpuscle protein, with particularly abundant oligopeptides below 1 ku.

**Table 2** Comparison of molecular weight distribution between blood corpuscle protein and blood corpuscle short peptide

[Table content with molecular weight ranges and percentage distributions]

## 2.4 Amino Acid Composition of Blood Corpuscle Short Peptides

Table 3 lists the contents of 17 common amino acids in duck blood corpuscle protein and short peptides. Compared to the original protein, the short peptides contained complete essential amino acids with abundant content, characterized by: 1) Significantly increased isoleucine and methionine contents, effectively compensating for the low levels of these limiting amino acids in blood products; 2) Flavor amino acids (aspartic acid, glutamic acid, glycine, and alanine) accounting for about 31% of total amino acids, providing the material basis for the product' s round, mild sweetness and umami taste; 3) Rich lysine content, making it an excellent complement to cereal proteins, which is important for improving utilization of animal protein sources and dietary nutrition; 4) Essential amino acid content exceeding 50% of total amino acids (53.31%), with an essential to non-essential amino acid ratio of 1.24, both higher than the FAO/WHO ideal protein pattern of 40% EAA/TAA and 60% EAA/NAA, indicating high nutritional value; 5) Most essential amino acid scores exceeding 1 when compared to NRC-recommended patterns, basically meeting or exceeding requirements for broiler chickens and juvenile carp. The first limiting amino acid for both patterns was methionine, requiring supplementation when using blood corpuscle short peptides. Since amino acid contents exceeding minimum requirements by 30% indicate rich nutritional value [19,23], the balanced amino acid profile and high essential amino acid content qualify blood corpuscle short peptides as nutritious feed additives for pigs, poultry, and aquaculture.

**Table 3** Amino acid composition of blood corpuscle short peptide

[Table content with amino acid names, contents in blood corpuscle protein and short peptide, hydrophobicity values, and amino acid scores for broiler and carp requirements]

## 2.5 Sensory Evaluation of Blood Corpuscle Short Peptide Powder

Spray-dried duck blood corpuscle protein powder appears reddish-brown with a strong bloody odor. In contrast, blood corpuscle short peptide powder showed obvious changes, presenting a milky white color without distinct bloody odor and a fine, smooth, slightly sticky texture. Color measurements using a Minolta CR-410 colorimeter (Figure 4 [Figure 4: see original paper]) revealed that the L* and whiteness index (WI) values of short peptide powder were about 4 times those of blood corpuscle protein powder ($P < 0.01$), demonstrating remarkable decolorization. The a* value (redness) decreased by 67.23% after hydrolysis (from 9.95 to 3.26), while the b* value (yellowness) was significantly higher in the hydrolyzed product ($P < 0.01$), indicating that acidic protease hydrolysis facilitated the color transition from red to yellow. The effective decolorization can be attributed to peptide bond cleavage by protease under acidic pH conditions, releasing heme or heme-rich peptide fragments that are poorly soluble at low pH (3.0–5.0) and remain in the precipitate, yielding milky white short peptide powder.

During short peptide production, taste and flavor often change due to protease modification, frequently generating bitterness that limits product application. This occurs because enzymatic hydrolysis exposes hydrophobic amino acid side chains, forming bitter peptides. Sensory evaluation revealed no bitterness or astringency in the blood corpuscle peptide powder. To avoid subjective bias, amino acid composition and hydrophobicity values were analyzed for objective bitterness assessment. Table 2 shows that hydrophobic amino acids (leucine, phenylalanine, valine) decreased to varying degrees. The Q value calculated according to Ney' s [20] criterion was 4,304 J/mol, far below the 5,443 J/mol threshold, confirming that acidic protease hydrolysis did not produce bitter or astringent off-flavors.

## 2.6 Functional Properties

### 2.6.1 Solubility

Under optimal hydrolysis conditions, the solubility of blood corpuscle short peptides is shown in Figure 5-A [Figure 5: see original paper]. Compared to blood corpuscle protein, short peptide solubility exceeded 60% across all pH values, demonstrating significant improvement. Both proteins showed decreasing then increasing solubility with increasing pH, with minimum solubility at pH 6.0, which approximates the isoelectric point of blood corpuscle protein where precipitation is favored.

### 2.6.2 Emulsifying Properties

Figures 5-B and 5-C show the emulsifying capacity and stability of blood corpuscle short peptides and protein across pH values. Short peptides exhibited lower emulsifying capacity than intact protein, as hydrolysis into small peptides

reduces emulsifying performance, consistent with findings of Liu et al. [21] and Turgeon et al. [25]. Minimum emulsifying values occurred at pH 6.0, likely the isoelectric point where macromolecular precipitation or net charge loss reduces emulsifying capacity. However, short peptides showed markedly improved emulsifying stability compared to blood corpuscle protein, particularly under acidic conditions.

### 2.6.3 Foaming Properties

Foam is a two-phase colloidal system of persistent liquid/aqueous and dispersed gas/air phases, with proteins reducing interfacial tension to generate foam. Figures 5-D and 5-E show foaming capacity and stability trends. Short peptides exhibited poorer foaming capacity than blood corpuscle protein under acidic conditions (pH $<$ 7) but better performance in neutral and alkaline environments, likely due to reduced surface activity of small peptide chains generated by acidic protease hydrolysis. Foam stability of short peptides was below 6%, indicating poor performance with significant pH influence.

## 2.7 Antioxidant Activity Analysis of Blood Corpuscle Short Peptides

The antioxidant capacity of blood corpuscle short peptides at various protein concentrations is shown in Figure 6 [Figure 6: see original paper]. Despite partial destruction of active components by instantaneous high temperature during spray drying, the product demonstrated strong DPPH• scavenging capacity, superoxide anion scavenging ability, and reducing power. At 2% protein concentration, DPPH• scavenging exceeded 80%, approaching 100% at higher concentrations. Superoxide anion scavenging was relatively weaker than DPPH• scavenging but increased with protein concentration. Generally, antioxidant capacity correlates positively with reducing power; according to the assay method, higher absorbance indicates stronger reducing power. The reducing power curve showed positive correlation with protein concentration, reaching a maximum of 2.02 at 8% protein concentration, demonstrating excellent reducing capacity.

Poultry blood is a high-nutritional-value protein source containing approximately 20% protein, with over 60% derived from blood corpuscles. This study found blood corpuscle protein content at about 38% of blood corpuscle weight, with abundant essential amino acids including lysine. Since blood corpuscle protein is contained within erythrocytes that naturally undergo limited autolysis under moisture and pressure, the first step in protease hydrolysis is artificial hemolysis to disrupt cell membranes and release proteins. This study employed chemical osmosis to alter cell membrane permeability, combined with water swelling to change osmotic pressure, and utilized temperature and mechanical shaking to accelerate membrane disruption and improve cell breakage efficiency. Lactic acid was used to adjust pH to further enhance membrane disruption, enabling complete protein release and laying the foundation for subsequent protease hydrolysis and decolorization.

Five commercial proteases (acidic protease, neutral protease, alkaline protease, papain, and flavourzyme) were used to hydrolyze duck blood corpuscle protein, with DH used to evaluate hydrolysis progress. Considering cost and efficiency, acidic protease was selected. Acidic protease is an endopeptidase refined from *Aspergillus niger* fermentation, widely used in food and feed applications due to its high safety and activity. Liu et al. [26] reported acidic protease showed the highest efficiency in hydrolyzing crab shell protein. Feng et al. [27] optimized acidic protease hydrolysis of salted egg white, obtaining 85.01% peptides (<3 ku) after 48 h, with 35.73% below 1 ku. In comparison, this study achieved 81.89% small peptides (<3 ku) and 62.82% below 1 ku after only 7 h hydrolysis of duck blood corpuscle protein, demonstrating superior efficiency. Peptide theory indicates that small peptides are absorbed faster, with lower energy consumption and less carrier saturation than proteins and amino acids, and are incorporated into body proteins more efficiently, suggesting excellent digestibility of blood corpuscle short peptide powder. Additionally, appropriate supplementation of blood corpuscle short peptides in animal diets can promote amino acid absorption and avoid competitive absorption among amino acids.

The optimal DH of (25.10 ± 0.65)% obtained in this study was slightly higher than that reported for neutral protease hydrolysis of porcine hemoglobin [28] and comparable to combined papain-neutral protease hydrolysis [1]. Research indicates that heme iron responsible for brown-red color becomes insoluble in acidic, high-DH products [16], allowing removal by centrifugation to achieve decolorization. This aligns with our findings, where acidic protease hydrolysis produced remarkable decolorization, yielding milky white short peptide powder with L* and WI values about 4 times those of blood corpuscle protein powder. While enzymatic hydrolysis often generates bitter peptides, sensory evaluation detected no bitterness in our product, and the Q value of 4,304 J/mol was well below the 5,443 J/mol threshold, confirming no adverse flavor effects from acidic protease hydrolysis.

Blood corpuscle short peptides are rich in lysine and other essential amino acids, with flavor amino acids accounting for about 31% of total amino acids, making them excellent cereal protein complements and suitable for poultry and aquaculture feed with appropriate methionine supplementation. To further understand application value, functional and antioxidant properties were evaluated. Similar to most protein hydrolysis studies [29-31], high DH resulted in high solubility, attributed to destruction of secondary structure, shortened peptide chains, and strong hydrogen bonding with water [24,29]. pH significantly affected solubility, with minimum solubility at the isoelectric point (pH 6.0) where side group charge release was limited, enhancing precipitation. Foaming capacity and stability varied significantly with pH, while emulsifying capacity was generally low but showed good stability, possibly due to small peptides adsorbing at oil-water interfaces or reduced charge repulsion in acidic conditions [20,25].

Enzyme type affects antioxidant activity of hydrolysates. Jiang et al. [32] reported acidic protease hydrolysates of shark skin gelatin showed the best antiox-

idant activity, similar to our results. Duck blood corpuscle hydrolysates demonstrated concentration-dependent free radical scavenging capacity and high reducing power, attributed to the generation of numerous small peptide fragments and amino acid residues (<3 ku, ~82%) by acidic protease hydrolysis. Thus, blood corpuscle short peptides show strong antioxidant activity and can serve as free radical stabilizers and hydrogen donors to inhibit lipid oxidation and prevent food spoilage. Additionally, the small amounts of carbohydrates and lipids present may undergo Maillard reactions during spray drying, inhibiting lipid auto-oxidation and extending shelf life. Therefore, blood corpuscle short peptides can be utilized as functional ingredients in food and feed applications.

**Conclusions:**

1. Using DH and hydrolysate yield as dual indicators, orthogonal experiments optimized the conditions for acidic protease hydrolysis of duck blood corpuscle protein as: enzyme dosage 6,000 U/g, temperature 50 °C, pH 3.5, and hydrolysis time 7 h. Under these conditions, DH was (25.10 ± 0.65)% and hydrolysate yield was (60.09 ± 1.77)%.
2. Compared to blood corpuscle protein powder, duck blood corpuscle short peptide powder was milky white and odorless, with high L* and WI values. It contained high levels of small peptides, with 81.89% below 3 ku and 62.82% below 1 ku, facilitating animal digestion and absorption.
3. Blood corpuscle short peptides contained complete amino acid profiles with abundant essential amino acids (53.31% of total amino acids), with increased contents of limiting amino acids (isoleucine, methionine) and high lysine content, making them excellent cereal protein complements.
4. Acidic protease-modified duck blood corpuscle protein demonstrated good solubility (>60%), emulsifying stability, and antioxidant activities including free radical scavenging and reducing power, indicating excellent functional properties for application in feed and food industries.

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*Note: Figure translations are in progress. See original paper for figures.*