

Double enzyme hydrolysis for producing antioxidant peptide from egg white: Optimization, evaluation, and potential allergenicity

Jin Yuan^{1,2,3} | Ying Zheng^{1,4} | Yong Wu^{1,2} | Hongbing Chen^{1,2} | Ping Tong¹  | Jinyan Gao³

¹State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, China

²Sino-German Joint Research Institute, Nanchang University, Nanchang, China

³Department of Food Science, Nanchang University, Nanchang, China

⁴Athena Institute of Holistic Wellness, Wuyi University, Nanping, China

Correspondence

Ping Tong, State Key Laboratory of Food Science and Technology, Nanchang University, Nanjing Dong Lu 235, Nanchang 330047, China.
Email: tongping@ncu.edu.cn

Funding information

Key Research Program of Jiangxi Province, Grant/Award Number: 20161BBG70062; National Natural Science Foundation of China, Grant/Award Number: 31660436; National Key Research and Development Program of China, Grant/Award Number: 2018YFD0400304

Abstract

Egg white is a good source of high-quality proteins in food products and an excellent source of antioxidant active peptides through hydrolysis. The hydrolysis conditions for the preparation of potent antioxidant peptides from egg white with chymotrypsin and pepsin were optimized by the response surface methodology. The antioxidant activity and potential allergenicity of the prepared peptides were evaluated by the oxidative damage model and IgE-binding capability, respectively. After ultrafiltration, the peptide produced using the optimized parameters (preheating time of 3.16 hr, hydrolysis time of 3 hr, a sample/solution ratio of 10%, multiple enzymes ratio (E1/E2) of 1.7:1, and E/S of 0.4%) showed antioxidant activity of was 30.86 $\mu\text{mol AAE/g DW}$ and with low potential for allergenicity. The optimized method is efficient and economical and may be applied to the industrial production of antioxidant peptides to obtain nutraceutical and pharmaceutical agents with low sensitivity. However, further in vivo studies must be conducted.

Practical applications

Egg is consumed as an excellent source of protein. Antioxidant peptides released from egg white is considered to be used in food preservation and human health. Few researches on the optimization of egg peptides were aimed to obtain practical techniques used in the food industry. In this paper, egg white hydrolysis peptide with high antioxidant property and low potential allergenicity was prepared after the optimization of double enzyme hydrolysis. The products could be a natural health care products derived from a dietary source and considered using in additive during food production and health food. And the methods used are economical and energy-saving and could be developed to utilize in the food industry.

KEYWORDS

antioxidant peptide, egg white, hydrolysis, optimization

1 | INTRODUCTION

Reactive oxygen species (ROS) are produced during metabolism and include superoxide, hydrogen peroxide, and hydroxyl radicals, which are unstable and react quickly with other groups or biological macromolecules in an organism. ROS could result in DNA mutation, protein damage, and membrane phospholipid oxidation, which produces oxidative damages to cellular structures and tissues (Huang, Du, & Brekken, 2017). Several chronic diseases, such as aging, diabetes, arthritis, atherosclerosis, Alzheimer's disease, cancer, and other neurodegenerative disorders, could be induced by the accumulation of free radicals in the body (Gu et al., 2015). Some synthesized antioxidants, such as butylated hydroxytoluene and butylated hydroxyanisole, play an important role in preventing oxidative damages in an organism and delaying autooxidation in food commodities (Grażyna, Hanna, Adam, & Magdalena, 2017). Moreover, natural antioxidants derived from a dietary source have attracted increasing attention considering the potential adverse effects and toxicity of artificial synthetic antioxidants (Chi, Hu, Wang, Li, & Ding, 2015). Accordingly, scholars have focused on exploiting antioxidants that are harmless to humans.

Egg white is a complete source of proteins and bioactive peptides. Antioxidant peptides are released by hydrolysis of egg proteins with one or several proteases (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004). Peptides have higher antioxidant activity than proteins possibly due to the destruction of the tertiary structure of the former during enzymatic hydrolysis (Elias, Kellerby, & Decker, 2008). Moreover, many peptides, such as LDEPDPL, NIQTDDFRT, AEERYP, and DEDTQAMP, have been isolated and proven to have strong antioxidant activities (Nimalaratne, Bandara, & Wu, 2015). However, researches on egg bioactive peptides has been only focused on the isolation of individual peptide with the highest antioxidant activity (Abeyrathne, Huang, & Ahn, 2018; Ghassem, Arihara, Mohammadi, Sani, & Babji, 2017) and the related methods are inapplicable to the food industry. Few studies have attempted to optimize the hydrolysis conditions for producing antioxidant peptides at a large scale. In some works, ultrasound and high-intensity pulsed electric field were used in pretreatment to prepare egg white peptide by hydrolysis (Castro, Soares, & Sato, 2015; Liang, Zhang, & Lin, 2017; Stefanović et al., 2014). Although these methods could be used to prepare peptides with high antioxidant activity, which consume a large amount of energy and have complicated operation. In this regard, an economical and energy-saving method for producing antioxidant peptides from egg whites must be developed to utilize antioxidant products from natural sources in the food industry.

Eggs are one of the most common allergens. Egg allergy affects 0.5% to 2.5% of young children (Sicherer & Sampson, 2018). Egg-allergic patients may also be allergic to products containing egg proteins and even protein fragments. Therefore, the potential allergenicity of prepared antioxidant peptides must be evaluated considering the possible consumption of patients allergic to eggs.

In consequence, this work aimed to prepare antioxidant peptide from egg white and evaluate their antioxidant properties and

potential allergenicity. The response surface methodology (RSM) was applied to optimize hydrolysis conditions according to ferric reducing antioxidant potential (FRAP) assay results. FRAP assay was used to evaluate antioxidant activity because of its advantages in the determination of single-electron transfer from all kinds of ROS scavengers (Benzie & Strain, 1996). A Caco-2 cell model was employed in our in vitro experiments due to its functional and structural similarity to human intestinal epithelial cells (Yamamoto, Kushima, Kisaki, Fujiyama, & Okabe, 2003).

2 | MATERIALS AND METHODS

2.1 | Materials and chemical reagents

Trypsin, α -chymotrypsin, pepsin, neutral protease, and Alcalase 2.4L were purchased from Novozymes Co. (Copenhagen, Denmark). Potassium persulfate, fluorescein sodium salt (FL), and 2,2-azobis (2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Aladdin (Shanghai, China). Fresh eggs were purchased from a local market in Nanchang.

2.2 | Selection of multiple enzymes

Liquid egg white (LEW) was diluted to 1:4 by adding distilled water and heated in a water bath at 95°C for 10 min to denature egg white proteins. The pH was adjusted for five enzymes separately with other conditions (Table S1). All the hydrolysates were subjected to FRAP assay after enzyme inactivation. The precipitate was removed by centrifugation at 8,000g for 20 min.

Three of five enzymes with highly antioxidative hydrolysates were selected for double enzyme optimization. The double enzymatic hydrolysis is carried out as follows: The preheated egg white is hydrolyzed by the enzyme 1 for half of the total hydrolysis time, then heated to inactivate enzyme 1, and further hydrolyzed by the enzyme 2.

2.3 | Optimization of enzymatic hydrolysis by RSM

Single-factor optimization of the enzymes selected was conducted using five parameters: Sample/solution ratio (egg white to water), E/S ratio, E1/E2 ratio, preheating time, and hydrolysis time. In the RSM design, three conditions with considerable effects on the antioxidant were selected to fit in a central composite rotatable design (CCRD) and the enzymatic hydrolysis process was optimized by RSM.

Three independent variables and three levels were used to form a Box-Behnken design including 20 experimental runs (Table S2) according to the method of Dahaghin, Mousavi, and Sajjadi (2017). The three selected conditions were randomized to minimize accidental changes in the observed response. Results were presented by a second-order quadratic equation as follows:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (1)$$

where Y is the response function (FRAP value); β_0 , β_i , and β_{ij} are the coefficients of the model; X_i and X_j are the levels of independent variables.

Experimental data were analyzed statistically using Design Expert 7.0 (Stat-Ease, Inc., Minneapolis, MN, USA). The statistical significance of the terms in the regression equations was determined by ANOVA (Table S3). The above RSM was then expressed in the form of three-dimensional surface plots to illustrate the relationship between the responses and the conditions. The point optimization methodology was employed to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

2.4 | Preparation of low molecule antioxidant peptide

The egg white hydrolysates prepared by RSM were fractionated through ultrafiltration with a molecular weight cut off at 3,000 Da (Millipore, Billerica, USA). The permeated component was detected to be peptide with low molecular weight (PLMW, less than 3,000 Da) and lyophilized for further analysis.

2.5 | Antioxidant assays

2.5.1 | FRAP assay

FRAP assay was conducted according to the method discussed earlier (Li et al., 2012). Briefly, 20 μ l of the standard or sample was mixed with 300 μ l of ferric-TPTZ reagent [300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ at a ratio of 10:1:1 (v/v/v)] and placed in the wells. The plate was incubated at 37°C for the specific duration of the reaction. Absorbance was recorded at 593 nm for 30 min. Six concentrations, namely, 25, 50, 100, 200, 400, and 800 μ M, were used to prepare the standard curve of L-ascorbic acid. Antioxidant activity is expressed as micro-mole ascorbic acid equivalent (AAE) per gram dry weight of the egg white protein (μ mol AAE/g DW). Strong absorption indicates a high reducing power of the phytochemical and high antioxidant activity.

2.5.2 | Superoxide anion radical-scavenging activity assay

The superoxide anion radical-scavenging activity of prepared egg white peptide (EWP) was measured by quantifying the inhibition of the autoxidation of pyrogallol according to the available method (Ak & Gulcin, 2008). In brief, 0.1 ml of aliquot of 0.5 g/L EWP and 2.8 ml

of 50 mM Tris-HCl buffer (pH 8.2) were mixed and preheated at 25°C for 20 min in a water bath. The reaction was initiated by incubating with 0.05 ml of 3 mM pyrogallol (dissolved in 10 mM HCl) for 4 min. The reaction was then terminated by adding 1 ml of 8 mM HCl. Absorbance was recorded at 325 nm.

2.6 | Amino acid ratio analysis

The amino acid content of peptides and egg white was measured by an Amino Acid Analyzer (Sykam GmbH, Eresing, Germany) following the Sykam manual for amino acid analyzer S433D. According to the guide book, the peptides were hydrolyzed by 6 M HCl at 110°C for 24 hr. The hydrolysates were then evaporated to dryness and dissolved in 3 ml of sodium citrate buffer (pH 2.2) for amino acid analysis.

2.7 | CCK-8 proliferation assay

Caco-2 cells were precultured in 96-well plates at a density of 5×10^4 cells/ml for 4 hr and incubated with or without PLMW. Cell proliferation was assessed using the Cell Counting Kit-8 kit (CCK-8 kit, Dojindo Molecular Technologies, Inc., Shanghai, China). All operations followed the manufacturer's instructions.

2.8 | Oxidative damage model

2.8.1 | Induction of oxidative stress

The induction of oxidative stress was determined using the method described by Shi, Kovacs-Nolan, Jiang, Tsao, and Mine (2014) with a slight modification. Cells cultured for 20 generations were used at a concentration of 2×10^5 cells/ml and cultivated in 96-well culture plates (NEST Biotechnology, Wuxi, China). The cells were grown for 5–7 days and the medium replaced every 2 days. The cells were then treated with various concentrations of antioxidant peptides of egg whites in DMEM/F-12 (a mixture of Dulbecco's modified Eagle's medium mixture and Ham's F 12 nutrient medium; Thermo Fisher Scientific, Waltham, USA) with 5% fetal bovine serum for 2 hr. The cells were washed using Hank's Balanced Salt Solution (Thermo Fisher Scientific, Waltham, USA), followed by the addition of 1 mM H_2O_2 with prepared peptide for 6 hr. After 8 hr, the culture supernatants were collected to measure interleukin-8 (IL-8) production.

2.8.2 | Interleukin-8 immunoassay

The IL-8 concentrations of collected cell supernatants were analyzed by a Porcine IL-8 ELISA kit according to the manufacturer's instructions (RAB0321, Sigma, Saint Louis, USA).

2.9 | Evaluation of potential allergenicity

2.9.1 | IgG binding capacity assessment

An IgG binding capacity test was performed by competitive inhibition ELISA according to the methods of Ma et al. (Ma, Chen, Gao, Hu, & Li, 2013; Ma, Gao, & Chen, 2013) to analyze the potential antigenicity of the prepared peptide. Untreated egg white (2 µg/ml) in 50 mM sodium carbonate buffer (pH 9.6) was precoated overnight at 4°C. Prepared peptides were added to compete with egg whites. A heated but unhydrolyzed egg white was used as control.

2.9.2 | IgE binding capacity assessment

Similar to the IgG binding capacity test, the IgE binding capacity test was conducted by competitive inhibition ELISA to assess the potential allergenicity of the prepared peptides (Ma, Gao, et al., 2013). A serum pool was prepared from 11 egg-allergic patients upon obtaining informed consent. Sera were collected and mixed in equal volumes (Table S3).

2.10 | Statistical analysis

Data were analyzed using repeated measures two-way ANOVA to calculate *p* values among groups (SPSS for Windows, version 20.0, Microsoft Corp., Chicago, IL, USA). Design-Expert software 8.0 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to build an RSM model. Means were separated using Duncan's multiple range test at *p* < .05.

3 | RESULTS

3.1 | Selection of multiple enzymes

As shown in Figure 1A, LEW was hydrolyzed by trypsin, chymotrypsin, pepsin, Alcalase 2.4L, and neutral proteinase. Antioxidant activity was assessed using the FRAP value. Three enzymes, namely, Alcalase 2.4L, chymotrypsin, and pepsin, which could produce highly antioxidative peptide, were selected for double enzyme analysis (Figure 1B) under the conditions and are shown in Table S1. EWP hydrolyzed by chymotrypsin and pepsin had higher FRAP values than the other EWP. Accordingly, these two enzymes were selected for subsequent optimizations.

3.2 | Effect of hydrolysis conditions on the antioxidant capacity of EWP

The effects of different hydrolysis times on FRAP value are shown in Figure 2A. The sample/solution ratio at 10%, *E*/*S* ratio at 0.4%, *E*₁/*E*₂ (chymotrypsin: pepsin) ratio at 1:1, and preheating time at 20 min (Table S2) were employed during optimizations. FRAP value was increased following the hydrolysis time from 1 hr to 3 hr but gradually decreased after 3 hr with hydrolysis for a long time resulting in the disruption of the antioxidant activity. The hydrolysis time of 3 hr was selected as the most appropriate.

The maximum FRAP value of the hydrolysates with sample/solution ratio from 5% to 30% of LEW was shown at 10% of LEW (Figure 2B). The other hydrolysis parameters were the same as before. Therefore, the sample/solution ratio of 10% was selected as the center point in the response surface experiment.

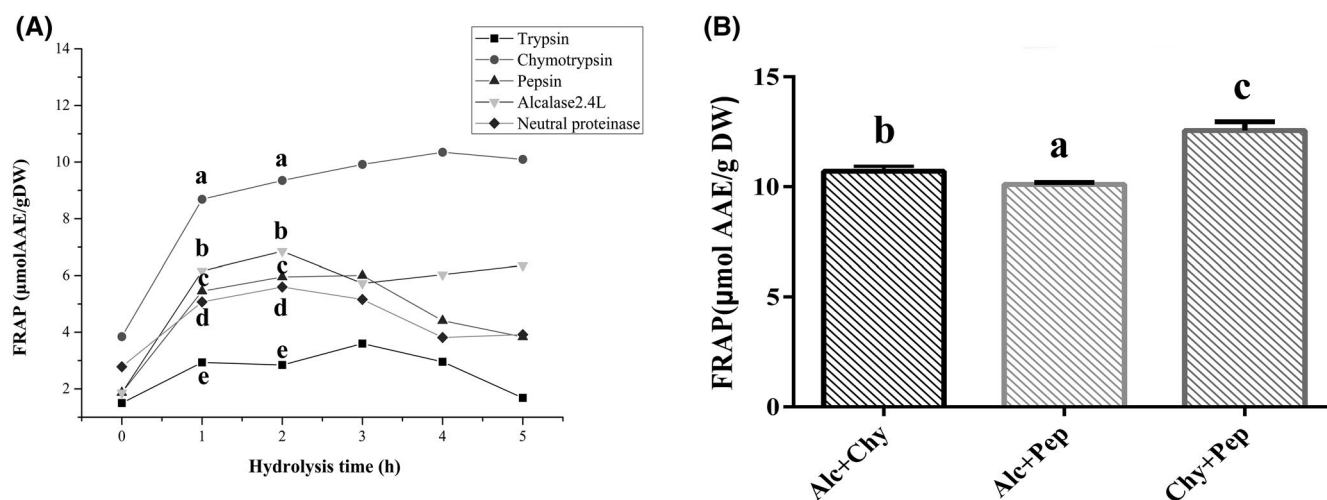


FIGURE 1 Selection of single and combined enzymes. (A) FRAP value of hydrolysates generated by the single enzyme at different hydrolysis times; (B) FRAP value of peptides generated by combined enzymes. The same letter denotes that the variance of the two samples was not significant (*p* > .05); different letters denote that the variance of the two samples was significant (*p* < .05)

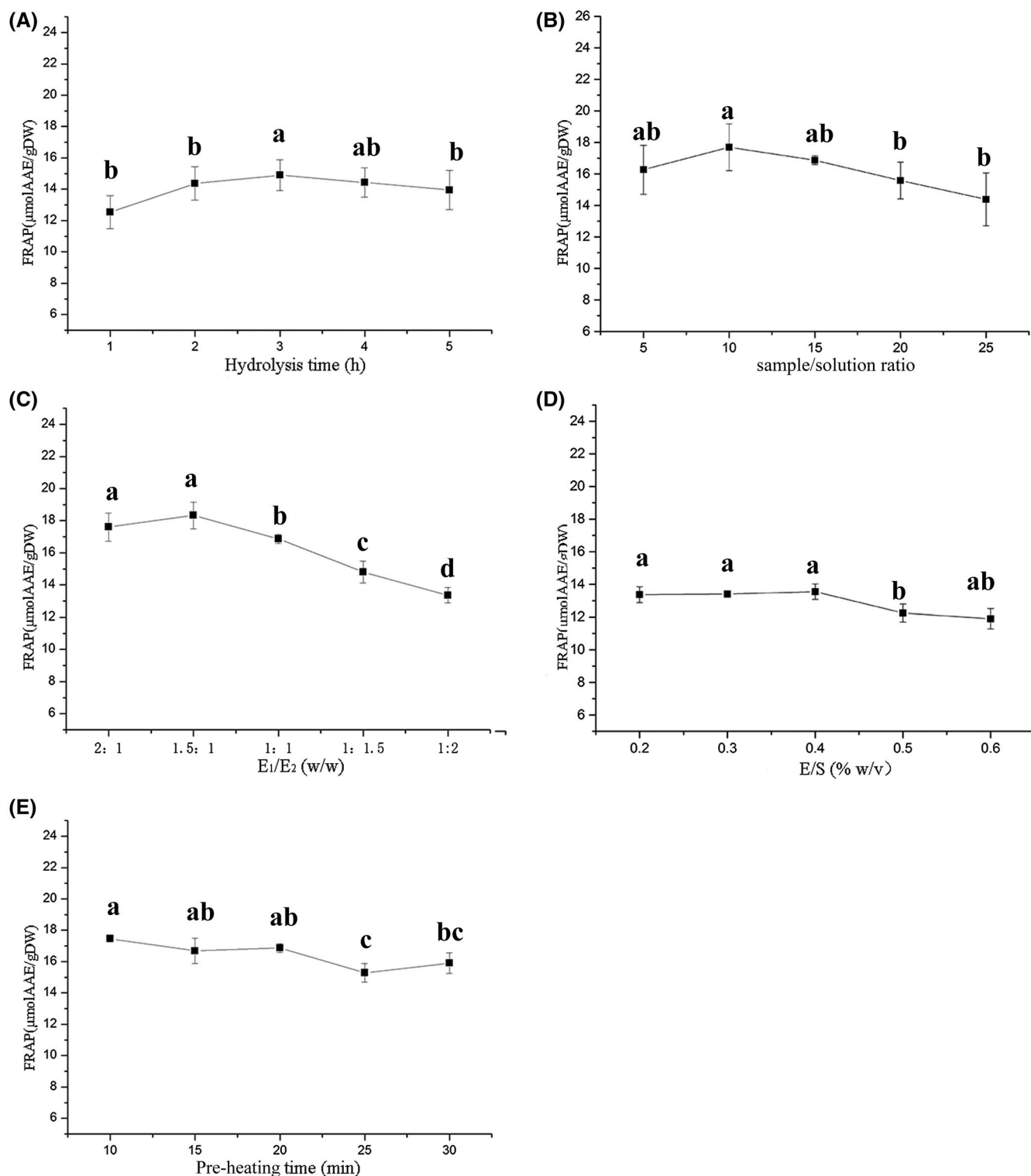


FIGURE 2 Effect of different conditions on the FRAP value of EWP. (A) hydrolysis time; (B) sample/solution ratio (egg white to water); (C) E_1/E_2 (ratio of chymotrypsin to pepsin); (D) E/S (ratio of enzyme to substrate); (E) preheating time. The same letter denotes that the variance of the two samples was not significant ($p > .05$); different letters denote that the variance of the two samples was significant ($p < .05$)

The effects of different ratios of double enzymes (chymotrypsin: pepsin) on the FRAP value of EWP are shown in Figure 2C with the same hydrolysis parameters. The FRAP value of the hydrolysates significantly increased when the E_1/E_2 (chymotrypsin: pepsin) ratio varied from 2:1 to 1.5:1 and then decreased from 1.5:1 to 1:2. Hence,

chymotrypsin and pepsin at the ratio of 1.5:1 were considered as the optimal double enzyme ratio.

Various E/S ratios are investigated in Figure 2D. The other hydrolysis parameters were the same as before. No significant difference was found among different groups ($p < .05$), indicating that E/S ratio

had almost no effect on antioxidant activity and thus was not selected as a center point. The E/S ratio at 0.4% was selected as the prepared condition.

The effects of different preheating times from 10 to 30 min on antioxidant activity are indicated in Figure 2E. Other hydrolysis parameters remained the same. The FRAP value increased when the preheating time was 10 min; however, no significant difference was observed when compared with 15 and 20 min ($p > .05$). Therefore, preheating time was not selected as the optimum condition for the further response surface design.

3.3 | Model fitting and optimization for the FRAP value of EWP

The three-dimensional response surface plots were analyzed by Design-Expert version 8.0.6 using multiple regression analysis. Results of the central composite and design response are shown in Table 1. The correlation between response variables and test variables related to the second-order polynomial equation is described as follows:

$$Y = 13.54 + 0.058X_1 + 0.039X_2 + 0.11X_3 + 0.11X_1X_2 + 0.15X_2X_3 + 0.19X_1X_3 - 0.41X_1^2 - 0.64X_2^2 - 0.37X_3^2$$

TABLE 1 Central composite experimental design and response for FRAP value of LEWH

No.	Coded factor level			FRAP ($\mu\text{mol AAE/gDW}$)
	X1	X2	X3	
1	2.5	7	1.7	11.9853
2	3	10	1.45	13.0948
3	3	10	1.45	13.6982
4	3	10	1.87	12.4975
5	3.5	7	1.7	12.0403
6	2.16	10	1.45	11.8624
7	3.5	13	1.2	11.0654
8	2.5	13	1.2	11.5221
9	2.5	7	1.2	12.2602
10	3	4.95	1.45	11.7139
11	3	10	1.45	13.6214
12	2.5	13	1.7	12.3398
13	3	10	1.03	11.2938
14	3	10	1.45	13.8356
15	3.5	7	1.2	11.0067
16	3.84	10	1.45	12.8511
17	3.5	13	1.7	12.478
18	3	15.05	1.45	11.6631
19	3	10	1.45	13.0449
20	3	10	1.45	13.6148

where Y represents the FRAP value of EWP; and X_1 , X_2 , and X_3 represent hydrolysis time, sample/solution ratio, and E/S ratio, respectively.

According to the results of Design-Expert software, the identified optimal parameters for the hydrolysis condition generated from the model (Figure 3) were as follows: The hydrolysis time was 3.16 hr; the sample/solution ratio of LEW was 10.52%; and the E_1/E_2 ratio was 1.7:1. The FRAP value predicted by the model under optimal conditions was $13.33 \mu\text{mol AAE/g DW}$. To verify the accuracy of the prediction, a verification test was performed, obtaining $13.46 \pm 0.456 \mu\text{mol AAE/g DW}$, which indicated that the experimental values were consistent with the predicted ones. The accuracy of the response surface model was tested by the ANOVA assay (Table S3). The variance of the quadratic regression model showed that the determination coefficient (R^2) was .9076, indicating that 90.76% of the total variance can be explained by the model, suggesting consistency between predicted values and experimental data. In addition, the p value of the model was lower than 0.0004, indicating that the response surface model was fitting and accurate.

3.4 | Antioxidant activity of different molecular weight components of peptides

The antioxidant activity of different fractions of the prepared peptides using the optimal hydrolysis parameters was measured by the FRAP assay and superoxide anion radical-scavenging activity assay as a reference (Figure 4). Compared with heated egg white (HEW), EWP, and components greater than 3,000 Da (HLMW), PLMW showed the highest antioxidant activity at $30.86 \mu\text{mol AAE/g DW}$.

3.5 | Amino acids ratio analysis of peptides

Amino acid analysis was performed, as shown in Figure 5. Several amino acids, such as Phe, His, Tyr, Met, and Lys, have generally been regarded as an important factor in the antioxidant activities of peptides. As shown in Figure 5, the content of the five amino acids in PLMW was 25.34%, whereas, in EWP, it was 24.72%, partly explaining why the antioxidant activity of PLMW was stronger than in EWP.

3.6 | Proliferation of Caco-2 cells with PLMW

To determine the effects of PLMW on cell proliferation in Caco-2 cells, dose-dependent incubation experiments were performed (Figure 6). Caco-2 cells were stimulated by 100–1600 $\mu\text{g/ml}$ of PLMW, and cell proliferation was assessed by the CCK-8 assay. No significant difference among groups indicated that PLMW had no proliferation toxicity to Caco-2 cells.

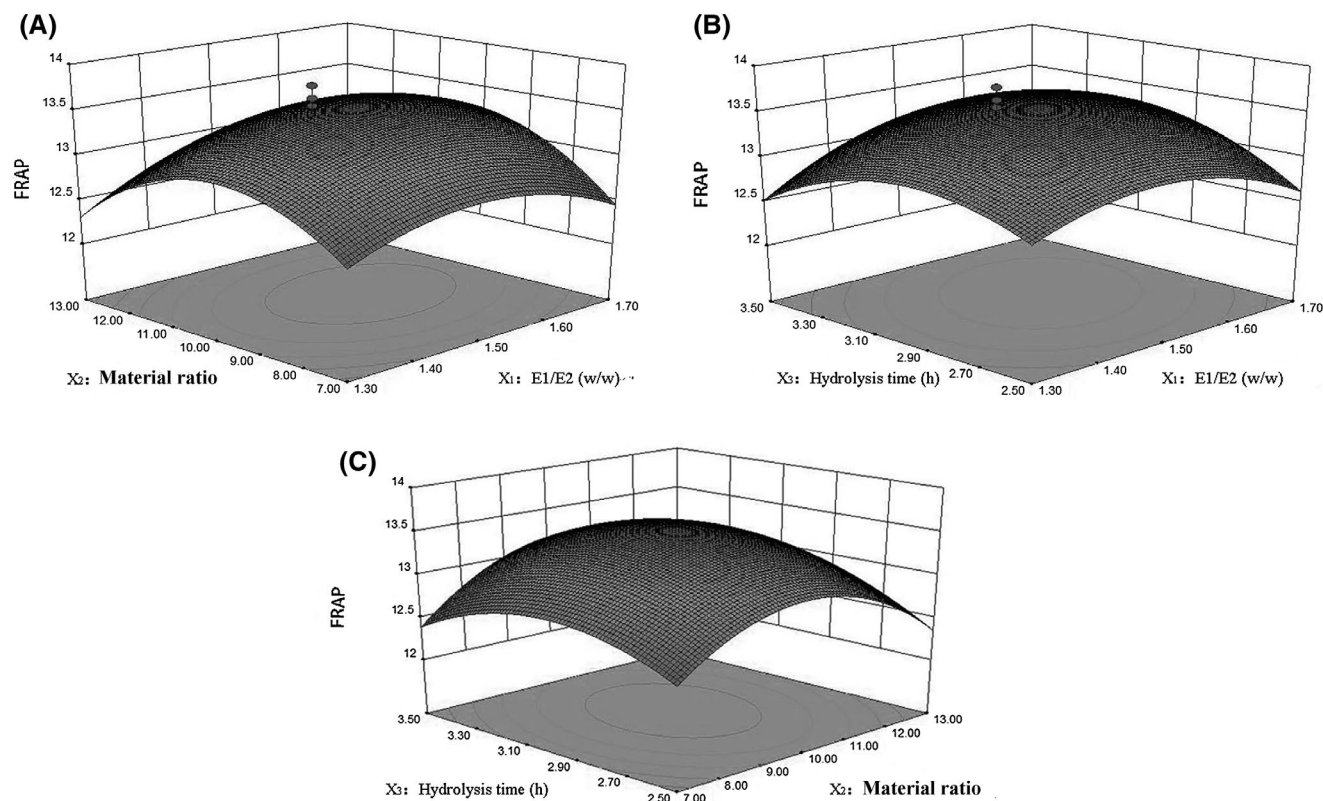


FIGURE 3 Response surface plot for the effect of the interaction of enzyme to substrate (E/S) ratio, multiple enzymes ratio (E1/E2), and hydrolysis time of egg white hydrolysates on the FRAP value. The same letter denotes that the variance of the two samples was not significant ($p > .05$); different letters denote that the variance of the two samples was significant ($p < .05$)

3.7 | Effect of antioxidant peptides on H₂O₂-induced IL-8 expression in Caco-2 cells

A Caco-2 cell oxidation stimulation model was used for further evaluation of the antioxidant capacity of the peptides. In Caco-2 cells, IL-8 can be drastically increased when stimulated by H₂O₂, making it a key indicator of oxidative stress (Shi et al., 2014). A series of concentrations of PLMW were added into the Caco-2 cells model. Considerable inhibition of IL-8 production was detected in Caco-2 cells that were treated with different concentrations of antioxidant peptides, ranging from 100 to 1,600 µg/ml. Moreover, the inhibition was correlated with the concentration of the peptide, leading to the gradual inhibition of oxidative damage (Figure 6). PLMW was proven effective in protecting cells from oxidative damage caused by H₂O₂.

3.8 | Potential allergenicity of antioxidant peptide

To assess the potential allergenicity of antioxidant peptide, the IgG and IgE binding abilities were tested by competitive inhibition ELISA (Figure 7). The IgG binding abilities of PLMW and EWP were considerably lower than that in heated but unhydrolyzed egg white (Figure 7A). However, no significant difference was observed in the IgE binding abilities of EWP and egg white. The IgE binding ability of PLMW was significantly lower than that of HEW (Figure 7B). These

results suggested that the potential allergenicity of PLMW was remarkably reduced after hydrolysis and ultrafiltration.

4 | DISCUSSION

Egg white is not only a source of high-quality proteins but also a good natural material for producing antioxidant peptides. The released peptides could exhibit specific biological activities after hydrolysis, fermentation, or enzymolysis (You & Wu, 2011). Enzymatic hydrolysis is one of the most widely used methods to produce bioactive peptides due to its mild reaction conditions and uncomplicated operations.

The different proteases leads to different peptides segments. Alcalase 2.4L, chymotrypsin, pepsin, trypsin, and neutral protease were used individually or in combinations to prepare antioxidant peptides from hen's eggs in previous works (Abeyrathne, Lee, Jo, Suh, & Ahn, 2016; Carrillo et al., 2016; Memarpoor-Yazdi, Asoodeh, & Chamani, 2012; Wang et al., 2012; Zhang, Zhang, Chen, & Zheng, 2017). Accordingly, the antioxidant activity of different kinds of enzymolysis was tested in our enzyme selection work. The hydrolysate of chymotrypsin, pepsin, and Alcalase 2.4L individually had higher antioxidant activity in the FRAP assay (Figure 1A). Moreover, the combination of chymotrypsin and pepsin showed higher antioxidant activity than the combination of chymotrypsin and Alcalase 2.4L

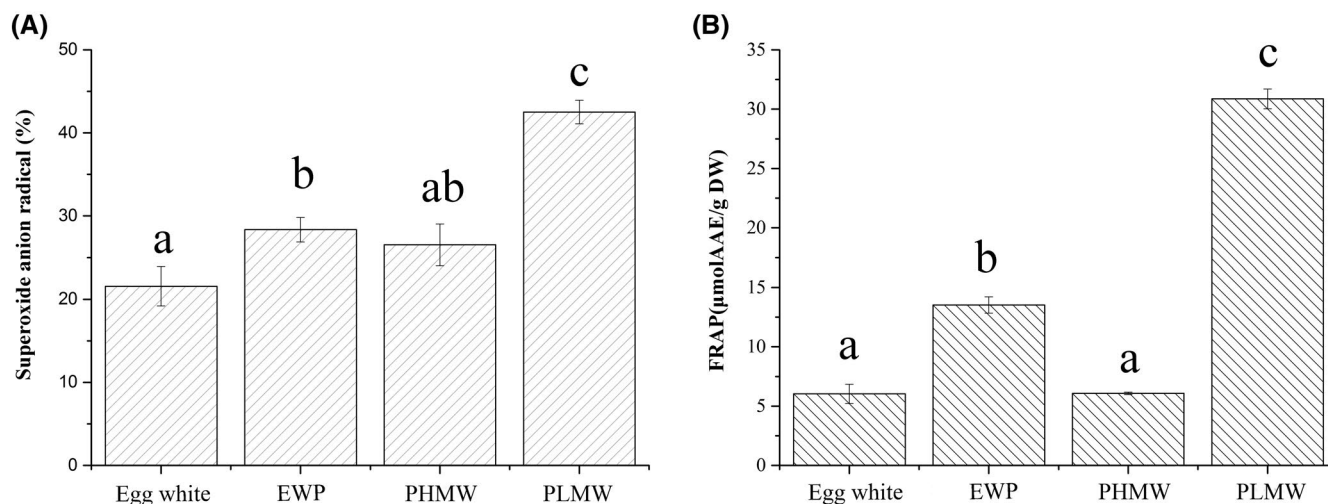


FIGURE 4 Antioxidant activity of different fractions of egg white peptides and egg white by (A) superoxide union radical assay and (B) FRAP assay. EWP: Egg white peptide; PHMW: Peptide more than 3 kDa; PLMW: Peptide less than 3 kDa. The same letter denotes that the variance of the two samples was not significant ($p > .05$); different letters denote that the variance of the two samples was significant ($p < .05$)

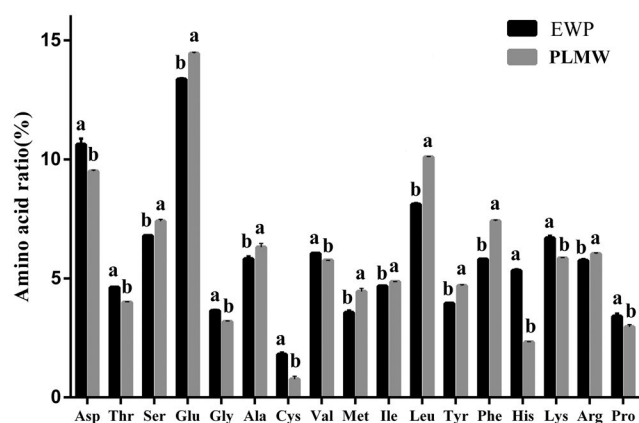


FIGURE 5 Amino acid composition in egg white peptide (EWP) and low-molecule-weight peptide (PLMW, less than 3 kDa). Mean $\pm$ SD with different letter superscripts within the same amino acid indicates that the values differ significantly ($p < .05$). The same letter denotes that the variance of the two samples was not significant ($p > .05$)

(Figure 1B). It was the reason that chymotrypsin tends to hydrolyze aromatic amino acid residues, such as Tyr, Trp, Ile, and hydrophobic residues, such as Met (Narai-Kanayama, Kawamura, Hosono, & Aso, 2009), and the enzyme-cutting site of pepsin is mainly aromatic or Ile residue (Shi, Wang, Zhang, Liu, & Fei, 2017). These aromatic residues can quench the free radicals by electron transfer (Lukacs et al., 2006); thus, can reduce oxygen-free radicals. Furthermore, the antioxidant activity of peptides could be increased due to these active amino acids present at the C-terminus and N-terminus (Wu, Sun, Ding, Zhu, & Lin, 2019). Therefore, the combination of pepsin and chymotrypsin could produce hydrolysate with more aromatic amino acid residues with higher antioxidant activity.

The high antioxidant activity of a hydrolysate could be produced under appropriate hydrolysis conditions according to various

enzymes. Hydrolysis conditions were then optimized through RSM. Three factors, including enzymolysis time, ratio of pepsin and chymotrypsin, and sample/solution ratio, were selected as CCRD, because they had a great influence on the antioxidant activity of the hydrolysates. RSM has been widely used in condition optimization for producing antioxidant peptide from grass carp sarcoplasmic protein (Ren et al., 2008), corn gluten meal (Zhuang, Tang, Dong, Sun, & Liu, 2013), and egg white powder (Lin et al., 2013). In a similar work, chymotrypsin was shown to produce higher antioxidant peptides using the DPPH method and the enzymatic hydrolysis conditions were optimized using RSM (Zhang, Cao, et al., 2019). In our study, three axes of response surface were replaced by hydrolysis time, the ratio of enzyme 1 and enzyme 2, and the ratio of raw water and egg white, according to results of the pre-experiment. To verify the accuracy of the model, the FRAP value of peptide prepared under the optimized conditions by RSM was determined and showed that a higher activity existed compared with that in our pre-experiment. Moreover, the ANOVA for the response surface quadratic model performed in Table S3 confirmed the validity of the model. These findings imply a more remarkable effect on the optimization of preparation.

The biological activities of peptides are related to many factors. First, the molecular weight is the main factor. Low molecular weight peptides (less than 6 kDa) with 2–20 amino acids were more bioactive than their parent large polypeptide or protein (Davalos et al., 2004). Low molecular weight peptides separated through ultrafiltration were proven to have the highest antioxidant activity (Figure 4). The high antioxidant activity of peptides with low molecular weight was likely to be related to the destruction of its two-stage structure (Elias et al., 2008). Similar to our results, many of the purified peptides with high antioxidant activity are less than 3,000 kDa (Duan et al., 2014; Ngho & Gan, 2016; Nimalaratne et al., 2015; Zhang, Wang, et al., 2019; Zhang, Cao, Cao, Sun, Sun, & Xu, 2019).

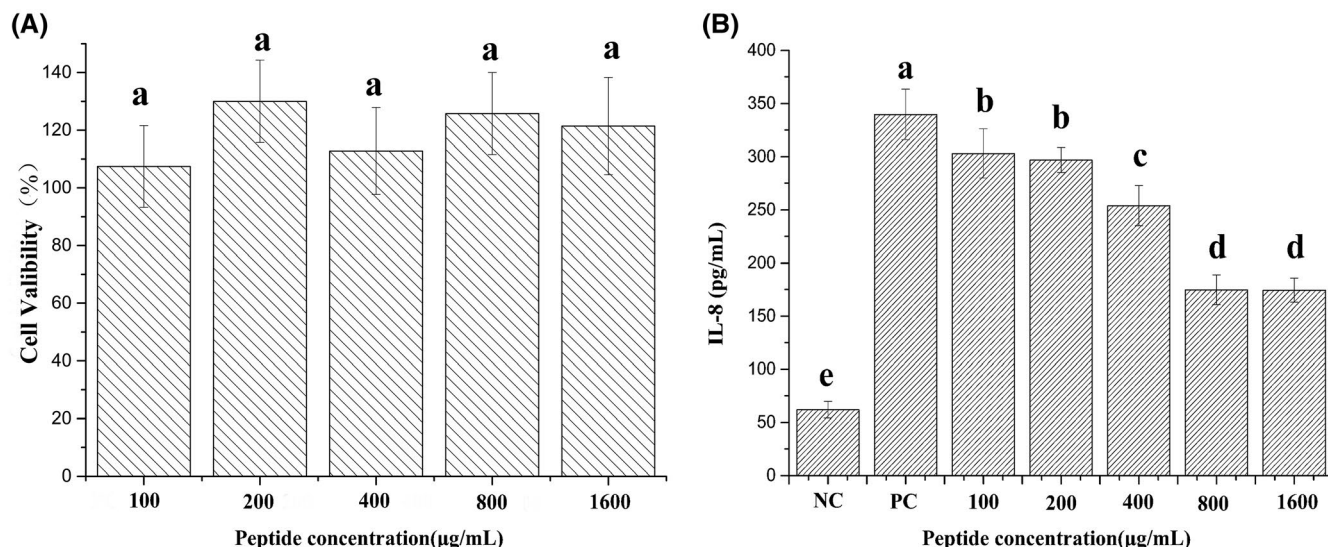


FIGURE 6 Protection of peptide (PLMW: Peptide less than 3 kDa) against oxidative damages in cells. (A) Proliferation of Caco-2 cells with PLMW assessed by the CCK-8 assay; (B) effect of PLMW on IL-8 expression in Caco-2 cells induced by H_2O_2 NC: Negative control, PC: Positive control (H_2O_2 without peptide). The same letter denotes that the variance of the two samples was not significant ($p > .05$); different letters denote that the variance of the two samples was significant ($p < .05$)

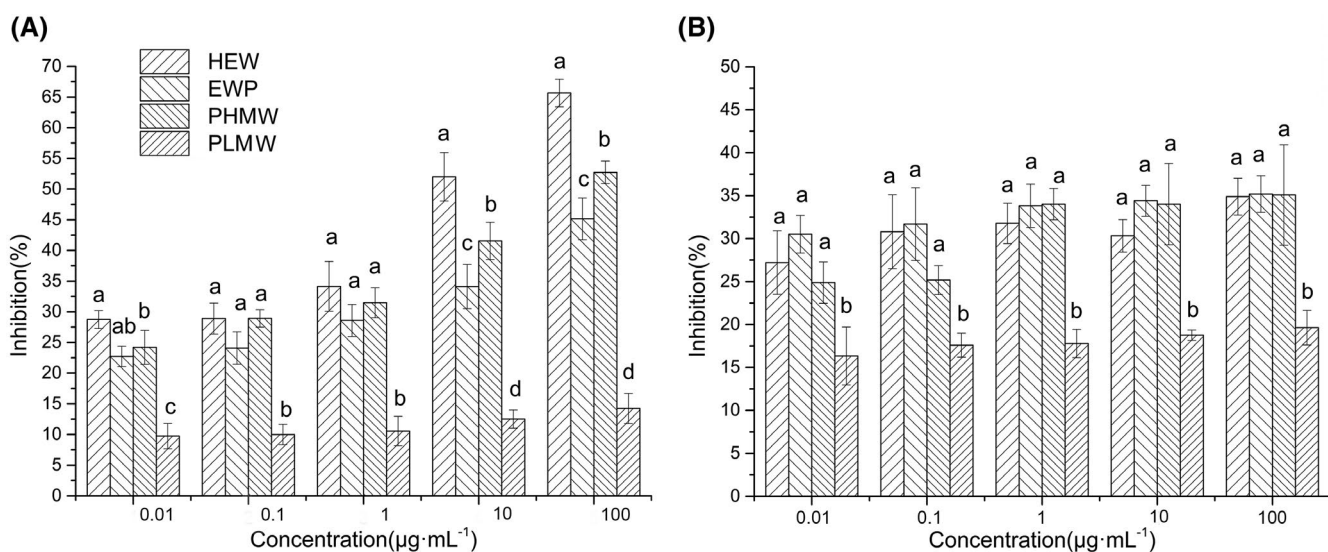


FIGURE 7 IgG (A) and IgE (B) binding abilities of peptides tested by inhibition ELISA. HEW: Heated egg white; EWP: Egg white peptide; PHMW: Peptide more than 3 kDa; PLMW: Peptide less than 3 kDa. The same letter denotes that the variance of the two samples was not significant ($p > .05$); different letters denote that the variance of the two samples was significant ($p < .05$)

The amino acid composition, structure, and hydrophobicity of peptides also has influence on the bioactivity of proteins (Elias et al., 2008; Nwachukwu & Aluko, 2019). Therefore, the amino acid analysis was tested to further explain why the low molecular weight peptide had high antioxidant activity in this study. Aromatic amino acids, such as Tyr and Phe with indole and imidazole groups, were proven to contribute to antioxidant activity by donating protons to electron-deficient radicals and scavenge them efficiently. The amount of Tyr, Trp, Phe, and Ile was higher in PLMW mainly due to the cleaving to the amino acids of pepsin and chymotrypsin. Furthermore, the antioxidant activity could be increased due to

these amino acids present at the C-terminus and N-terminus (Wu et al., 2019). Therefore, the combination of these two enzymes can cleave the egg white into smaller peptide segments with more aromatic amino acid residues.

Several methods, such as oxygen-free radical scavenging, ABTS (2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulphonate]) radical scavenging, DPPH (1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-[2,4,6-trinitrophenyl]hydrazyl) radical scavenging, and hydroxyl radical scavenging, were used to evaluate the antioxidant activity of the samples. However, all the above methods were aimed to evaluate the elimination of a particular kind of ROS. The

FRAP method focused on the determination of the single-electron transfer from all kinds of ROS scavenging in this work. This method was also regarded as simple, fast, and efficient for evaluating antioxidant activity, leading to its global use (Benzie et al., 1996; Canabady-Rochelle et al., 2015; Zhang, Tong et al., 2019). Moreover, the reagents and equipment required in the FRAP assay could easily be accessed, and the result had high reproducibility. Reasonably, the FRAP method was used as the main index to evaluate the antioxidant activity in our hydrolysis optimization. Superoxide anion is produced by O_2 and could be then converted to other ROS (Nimse & Pal, 2015); thus, the superoxide anion radical assay is another method that is widely used to prepare antioxidant peptides from proteins (Nauseef, 2014). A similar tendency between superoxide anion radical assay and FRAP assay was observed (Figure 4). The antioxidant ability of PLMW was almost five times as high as egg white (Figure 4). The antioxidant ability of PHMW was significantly lower than EWP, whereas it was similar to that of egg white. Peptides with low molecular weight seemed to be the main sources of antioxidant egg peptides. Nevertheless, according to the previous optimization experiment with the corresponding surface, the antioxidant activity of egg peptide would decrease after 3.16 hr, indicating that the antioxidant structure in PLMW would be destroyed under long-time hydrolysis.

The antioxidant of peptides detected by FRAP, based on the chemical evaluation of the oxidizing radical or free electrons, can only suggest that the peptide had the ability to reduce the oxidation effect. To evaluate the antioxidant capacity of the prepared peptide in cells, the Caco-2 cell oxidation stimulation model was performed. Before the Caco-2 cell oxidation stimulation experiments, the effect of PLMW on the cell proliferation of Caco-2 cells was determined by the CCK-8 assay. Results indicated that PLMW (100–1600 $\mu\text{g/ml}$) had no proliferation toxicity to Caco-2 cells. Therefore, the concentration from 100 $\mu\text{g/ml}$ to 1,600 $\mu\text{g/ml}$ of PLMW was selected for subsequent oxidative stimulation experiments.

Compare to cells used in H_2O_2 -induced oxidative damage model like HEK-293 cells from human embryo kidney (Zhang, Wang et al., 2019) and HepG2 cells from human liver hepatocellular (He, Bu, Xie, & Liang, 2019), the Caco-2 cell line was originally from human colon adenocarcinoma cells, and had similar morphology, structure, tight junction, and permeability to the small intestinal epithelial cells (Wan, Liu, Yu, Sun, & Li, 2015). The Caco-2 cell line had been widely used in the oxidative stimulation model to evaluate the antioxidant activity of peptides and proteins (Shi et al., 2014; Tonolo et al., 2018). In addition, Caco-2 cells had been reported to produce IL-8, which contribute to inflammation when stimulated by oxidative stress, similar to gastrointestinal epithelial cells (Yamamoto et al., 2003). The positive association between the secretion of IL-8 and oxidative stress suffered by Caco-2 cells was used to evaluate the antioxidant activity of the prepared peptide.

In our oxidative stimulation model, PLMW was tested for the antioxidant activity to protect Caco-2 cells from oxidative damage caused by H_2O_2 . High concentration peptide with lower IL-8

secretion suggested that the peptide could protect the cells from oxidative damage caused by H_2O_2 (Figure 6). These results were consistent with the FRAP value results. Therefore, PLMW could effectively inhibit the free radical oxidation and protect Caco-2 cells from oxidant stress.

Considering that the peptide we prepared would be consumed in the future, the potential effects of peptides on egg-allergic people should be considered. To evaluate the potential allergenicity of prepared antioxidant peptide, IgG and IgE binding capacities were assessed. In high concentrations, the IgG binding ability of EWP was considerably lower than that of HEW, indicating that IgG-specific epitopes of egg white proteins could be distrusted after hydrolysis (Liao et al., 2018). The IgG binding ability of PLMW was considerably lower than that of EWP and PHMW, which indicated that the peptide with less IgG-specific epitopes may be selected after ultrafiltration. The IgE binding capacity of EWP and PHMW was similar to that of HEW, whereas the IgE binding capacity of PLMW was considerably lower than that of HEW, indicating that the potential sensitization of PLMW was remarkably reduced after ultrafiltration. These results showed that after hydrolysis and ultrafiltration, PLMW could be a hypoallergenic egg product that could be consumed by egg patients.

5 | CONCLUSIONS

In this study, egg white antioxidant peptides were prepared after the optimization of double enzyme hydrolysis, followed by ultrafiltration with a molecular weight cut off at 3 kDa. The optimized parameters for producing peptide with high antioxidant activity were as follows: Preheating time at 3.16 hr, hydrolysis time at 3 hr, a sample/solution ratio of 10%, E1/E2 at 1.7:1, and E/S at 0.4%. The antioxidant activity of the product was evaluated by FRAP assay, superoxide anion radical-scavenging activity assay, and Caco-2 oxidative damage model. The potential allergenicity of prepared peptide was evaluated using IgG and IgE binding capacity. Results suggested that PLMW could have high antioxidant property and low sensitivity. Nevertheless, further studies are necessary to assess the antioxidant activity of the antioxidative peptide in vivo to develop prepared egg white antioxidant peptide as nutraceutical and pharmaceutical agents.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (No. 2018YFD0400304), the National Natural Science Foundation of China (No. 31660436), and the Key Research Program of Jiangxi Province, China (No. 20161BBG70062).

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

ORCID

Ping Tong  <https://orcid.org/0000-0002-0298-6411>

REFERENCES

- Abeyrathne, E. D. N. S., Huang, X., & Ahn, D. U. (2018). Antioxidant, angiotensin-converting enzyme inhibitory activity and other functional properties of egg white proteins and their derived peptides—A review. *Poultry Science*, 97(4), 1462–1468. <https://doi.org/10.3382/ps/pex399>
- Abeyrathne, E. D., Lee, H. Y., Jo, C., Suh, J. W., & Ahn, D. U. (2016). Enzymatic hydrolysis of ovomucin and the functional and structural characteristics of peptides in the hydrolysates. *Food Chemistry*, 192, 107–113. <https://doi.org/10.1016/j.foodchem.2015.06.055>
- Ak, T., & Gulcin, I. (2008). Antioxidant and radical scavenging properties of curcumin. *Chemico-Biological Interactions*, 174(1), 27–37. <https://doi.org/10.1016/j.cbi.2008.05.003>
- Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. *Analytical Biochemistry*, 239(1), 70–76. <https://doi.org/10.1006/abio.1996.0292>
- Canabady-Rochelle, L. L., Harscoat-Schiavo, C., Kessler, V., Aymes, A., Fournier, F., & Girardet, J. M. (2015). Determination of reducing power and metal chelating ability of antioxidant peptides: Revisited methods. *Food Chemistry*, 183, 129–135. <https://doi.org/10.1016/j.foodchem.2015.02.147>
- Carrillo, W., Gomez-Ruiz, J. A., Miralles, B., Ramos, M., Barrio, D., & Recio, I. (2016). Identification of antioxidant peptides of hen egg-white lysozyme and evaluation of inhibition of lipid peroxidation and cytotoxicity in the Zebrafish model. *European Food Research and Technology*, 242(10), 1777–1785. <https://doi.org/10.1007/s00217-016-2677-1>
- Chi, C.-F., Hu, F.-Y., Wang, B., Li, T., & Ding, G.-F. (2015). Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle. *Journal of Functional Foods*, 15, 301–313. <https://doi.org/10.1016/j.jff.2015.03.045>
- Dahaghin, Z., Mousavi, H. Z., & Sajjadi, S. M. (2017). A novel magnetic ion imprinted polymer as a selective magnetic solid phase for separation of trace lead(II) ions from agricultural products, and optimization using a Box-Behnken design. *Food Chemistry*, 237, 275–281. <https://doi.org/10.1016/j.foodchem.2017.05.118>
- Davalos, A., Miguel, M., Bartolome, B., & Lopez-Fandino, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection*, 67(9), 1939–1944. <https://doi.org/10.4315/0362-028x-67.9.1939>
- de Castro, R. J. S., & Sato, H. H. (2015). A response surface approach on optimization of hydrolysis parameters for the production of egg white protein hydrolysates with antioxidant activities. *Biocatalysis and Agricultural Biotechnology*, 4(1), 55–62. <https://doi.org/10.1016/j.bcab.2014.07.001>
- Duan, X., Ocen, D., Wu, F., Li, M., Yang, N. A., Xu, J., ... Xu, X. (2014). Purification and characterization of a natural antioxidant peptide from fertilized eggs. *Food Research International*, 56, 18–24. <https://doi.org/10.1016/j.foodres.2013.12.016>
- Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430–441. <https://doi.org/10.1080/10408390701425615>
- Ghassem, M., Arihara, K., Mohammadi, S., Sani, N. A., & Babji, A. S. (2017). Identification of two novel antioxidant peptides from edible bird's nest (*Aerodramus fuciphagus*) protein hydrolysates. *Food & Function*, 8(5), 2046–2052. <https://doi.org/10.1039/c6fo01615d>
- Grazyna, C., Hanna, C., Adam, A., & Magdalena, B. M. (2017). Natural antioxidants in milk and dairy products. *International Journal of Dairy Technology*, 70(2), 165–178. <https://doi.org/10.1111/1471-0307.12359>
- Gu, M. U., Chen, H.-P., Zhao, M.-M., Wang, X. I., Yang, B., Ren, J.-Y., & Su, G.-W. (2015). Identification of antioxidant peptides released from defatted walnut (*Juglans Sigillata* Dode) meal proteins with pancreatin. *LWT—Food Science and Technology*, 60(1), 213–220. <https://doi.org/10.1016/j.lwt.2014.07.052>
- He, Y., Bu, L., Xie, H., & Liang, G. (2019). Antioxidant activities and protective effects of duck embryo peptides against H₂O₂-induced oxidative damage in HepG2 cells. *Poultry Science*, 98(12), 7118–7128. <https://doi.org/10.3382/ps/pez430>
- Huang, H., Du, W., & Brekken, R. A. (2017). Extracellular matrix induction of intracellular reactive oxygen species. *Antioxidants & Redox Signaling*, 27(12), 774–784. <https://doi.org/10.1089/ars.2017.7305>
- Li, H., Deng, Z., Zhu, H., Hu, C., Liu, R., Young, J. C., & Tsao, R. (2012). Highly pigmented vegetables: Anthocyanin compositions and their role in antioxidant activities. *Food Research International*, 46(1), 250–259. <https://doi.org/10.1016/j.foodres.2011.12.014>
- Liang, R., Zhang, Z., & Lin, S. (2017). Effects of pulsed electric field on intracellular antioxidant activity and antioxidant enzyme regulating capacities of pine nut (*Pinus koraiensis*) peptide QDHCH in HepG2 cells. *Food Chemistry*, 237, 793–802. <https://doi.org/10.1016/j.foodchem.2017.05.144>
- Liao, Z.-W., Ye, Y.-H., Wang, H., Chen, Y., Sha, X.-M., Zhang, L. U., ... Tu, Z.-C. (2018). The mechanism of decreased IgG/IgE-binding of ovalbumin by preheating treatment combined with glycation identified by liquid chromatography and high-resolution mass spectrometry. *Journal of Agricultural and Food Chemistry*, 66(41), 10693–10702. <https://doi.org/10.1021/acs.jafc.8b04165>
- Lin, S., Jin, Y., Liu, M., Yang, Y. I., Zhang, M., Guo, Y., ... Yin, Y. (2013). Research on the preparation of antioxidant peptides derived from egg white with assisting of high-intensity pulsed electric field. *Food Chemistry*, 139(1–4), 300–306. <https://doi.org/10.1016/j.foodchem.2013.01.048>
- Lukacs, A., Eker, A. P. M., Byrdin, M., Villette, S., Pan, J., Brettel, K., & Vos, M. H. (2006). Role of the middle residue in the triple tryptophan electron transfer chain of DNA photolyase: Ultrafast spectroscopy of a Trp → Phe mutant. *Journal of Physical Chemistry B*, 110(32), 15654–15658. <https://doi.org/10.1021/jp063686b>
- Ma, X. J., Chen, H. B., Gao, J. Y., Hu, C. Q., & Li, X. (2013). Conformation affects the potential allergenicity of ovalbumin after heating and glycation. *Food Additives and Contaminants Part A—Chemistry Analysis Control Exposure & Risk Assessment*, 30(10), 1684–1692. <https://doi.org/10.1080/19440049.2013.822105>
- Ma, X.-J., Gao, J.-Y., & Chen, H.-B. (2013). Combined effect of glycation and sodium carbonate-bicarbonate buffer concentration on IgG binding, IgE binding and conformation of ovalbumin. *Journal of the Science of Food and Agriculture*, 93(13), 3209–3215. <https://doi.org/10.1002/jsfa.6157>
- Memarpour-Yazdi, M., Asoodeh, A., & Chamani, J. K. (2012). A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. *Journal of Functional Foods*, 4(1), 278–286. <https://doi.org/10.1016/j.jff.2011.12.004>
- Narai-Kanayama, A., Kawamura, Y., Hosono, M., & Aso, K. (2009). Availability of tyrosine amide for alpha-chymotrypsin-catalyzed synthesis of oligo-tyrosine peptides. *Enzyme and Microbial Technology*, 45(6–7), 457–462. <https://doi.org/10.1016/j.enzmictec.2009.08.002>
- Nauseef, W. M. (2014). Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases. *Biochimica Et Biophysica Acta-General Subjects*, 1840(2), 757–767. <https://doi.org/10.1016/j.bbagen.2013.04.040>
- Ngoh, Y.-Y., & Gan, C.-Y. (2016). Enzyme-assisted extraction and identification of antioxidative and alpha-amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris* cv. Pinto). *Food Chemistry*, 190, 331–337. <https://doi.org/10.1016/j.foodchem.2015.05.120>
- Nimalaratne, C., Bandara, N., & Wu, J. (2015). Purification and characterization of antioxidant peptides from enzymatically hydrolyzed chicken egg white. *Food Chemistry*, 188, 467–472. <https://doi.org/10.1016/j.foodchem.2015.05.014>

- Nimse, S. B., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Advances*, 5(35), 27986–28006. <https://doi.org/10.1039/c4ra13315c>
- Nwachukwu, I. D., & Aluko, R. E. (2019). Structural and functional properties of food protein-derived antioxidant peptides. *Journal of Food Biochemistry*, 43(1), e12761. <https://doi.org/10.1111/jfbc.12761>
- Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., ... Xue, S. J. (2008). Optimization of antioxidant peptide production from grass carp sarcoplasmic protein using response surface methodology. *LWT—Food Science and Technology*, 41(9), 1624–1632. <https://doi.org/10.1016/j.lwt.2007.11.005>
- Shi, W., Wang, Y., Zhang, H., Liu, Z., & Fei, Z. (2017). Probing deep into the binding mechanisms of folic acid with alpha-amylase, pepsin and trypsin: An experimental and computational study. *Food Chemistry*, 226, 128–134. <https://doi.org/10.1016/j.foodchem.2017.01.054>
- Shi, Y., Kovacs-Nolan, J., Jiang, B. O., Tsao, R., & Mine, Y. (2014). Antioxidant activity of enzymatic hydrolysates from eggshell membrane proteins and its protective capacity in human intestinal epithelial Caco-2 cells. *Journal of Functional Foods*, 10, 35–45. <https://doi.org/10.1016/j.jff.2014.05.004>
- Sicherer, S. H., & Sampson, H. A. (2018). Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *Journal of Allergy and Clinical Immunology*, 141(1), 41–58. <https://doi.org/10.1016/j.jaci.2017.11.003>
- Stefanović, A. B., Jovanović, J. R., Grbavčić, S. Ž., Šekuljica, N. Ž., Manojlović, V. B., Bugarski, B. M., & Knežević-Jugović, Z. D. (2014). Impact of ultrasound on egg white proteins as a pretreatment for functional hydrolysates production. *European Food Research and Technology*, 239(6), 979–993. <https://doi.org/10.1007/s00217-014-2295-8>
- Tonolo, F., Sandre, M., Ferro, S., Folda, A., Scalcon, V., Scutari, G., ... Rigobello, M. P. (2018). Milk-derived bioactive peptides protect against oxidative stress in a Caco-2 cell model. *Food & Function*, 9(2), 1245–1253. <https://doi.org/10.1039/c7fo01646h>
- Wan, H., Liu, D., Yu, X., Sun, H., & Li, Y. (2015). A Caco-2 cell-based quantitative antioxidant activity assay for antioxidants. *Food Chemistry*, 175, 601–608. <https://doi.org/10.1016/j.foodchem.2014.11.128>
- Wang, J., Wang, K. E., Lin, S., Zhao, P., Jones, G., Trang, H., ... Ye, H. (2012). Improvement of antioxidant activity of peptides with molecular weights ranging from 1 to 10 kDa by PEF technology. *International Journal of Biological Macromolecules*, 51(3), 244–249. <https://doi.org/10.1016/j.ijbiomac.2012.05.017>
- Wu, D., Sun, N., Ding, J., Zhu, B., & Lin, S. (2019). Evaluation and structure-activity relationship analysis of antioxidant shrimp peptides. *Food Function*, 10(9), 5605–5615. <https://doi.org/10.1039/c9fo01280j>
- Yamamoto, K., Kushima, R., Kisaki, O., Fujiyama, Y., & Okabe, H. (2003). Combined effect of hydrogen peroxide induced oxidative stress and IL-1 alpha on IL-8 production in CaCo-2 cells (a human colon carcinoma cell line) and normal intestinal epithelial cells. *Inflammation*, 27(3), 123–128. <https://doi.org/10.1023/a:1023813710941>
- You, S.-J., & Wu, J. (2011). Angiotensin-I converting enzyme inhibitory and antioxidant activities of egg protein hydrolysates produced with gastrointestinal and nongastrointestinal enzymes. *Journal of Food Science*, 76(6), C801–C807. <https://doi.org/10.1111/j.1750-3841.2011.02228.x>
- Zhang, B., Wang, H., Wang, Y., Yu, Y., Liu, J., Liu, B., & Zhang, T. (2019). Identification of antioxidant peptides derived from egg-white protein and its protective effects on H₂O₂-induced cell damage. *International Journal of Food Science & Technology*, 54(6), 2219–2227. <https://doi.org/10.1111/ijfs.14133>
- Zhang, N., Zhang, C., Chen, Y., & Zheng, B. (2017). Purification and characterization of antioxidant peptides of *Pseudosciaena crocea* protein hydrolysates. *Molecules*, 22(1), 57. <https://doi.org/10.3390/molecules22010057>
- Zhang, Q., Tong, X., Li, Y., Wang, H., Wang, Z., Qi, B., & Jiang, L. (2019). Purification and characterization of antioxidant peptides from alcalase-hydrolyzed soybean (*Glycine max* L.) hydrolysate and their cytoprotective effects in human intestinal Caco-2 cells. *Journal of Agricultural and Food Chemistry*, 67(20), 5772–5781. <https://doi.org/10.1021/acs.jafc.9b01235>
- Zhang, X., Cao, D., Sun, X., Sun, S., & Xu, N. (2019). Preparation and identification of antioxidant peptides from protein hydrolysate of marine alga *Gracilariopsis lemaneiformis*. *Journal of Applied Phycology*, 31(4), 2585–2596. <https://doi.org/10.1007/s10811-019-1746-9>
- Zhuang, H., Tang, N., Dong, S. T., Sun, B., & Liu, J. B. (2013). Optimisation of antioxidant peptide preparation from corn gluten meal. *Journal of the Science of Food and Agriculture*, 93(13), 3264–3270. <https://doi.org/10.1002/jsfa.6170>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Yuan J, Zheng Y, Wu Y, Chen H, Tong P, Gao J. Double enzyme hydrolysis for producing antioxidant peptide from egg white: Optimization, evaluation, and potential allergenicity. *J Food Biochem*. 2019;00:e13113. <https://doi.org/10.1111/jfbc.13113>

1 **Supporting Information:**

2 **Table S1** The hydrolysis conditions of five different kinds of enzyme

Species	Temperature()	pH	E/S (% w/v)
Alkalize 2.4L	50	9	0.4
Trypsin	37	8	0.4
Chymotrypsin	37	8	0.4
Neutral Protease	50	7	0.4
Pepsin	37	2	0.4

3 **Table S2** Independent variables and their levels used in the response surface design

Independent variables	Symbol	Coded factor level				
		-2	-1	0	+1	+2
Hydrolysis time (h)	X ₁	1	2	3	4	5
sample/solution ratio (%)	X ₂	5	10	15	20	25
E/S ratio (% w/v)	X ₃	0.2	0.3	0.4	0.5	0.6
E ₁ /E ₂ (w/w)	X ₄	2:1	1.5:1	1:1	1:1.5	1:2
Preheating time (min)	X ₅	10	15	20	25	30

4 **Table S3** ANOVA for response surface quadratic model for the FRAP value of LEWH

Term	Sum of Squares	DOF	MS	F-value	P-value
Model	13.76	9	1.53	10.92	0.0004
X ₁	0.22	1	0.22	1.6	0.234
X ₂	0.019	1	0.019	0.14	0.7186
X ₃	0.043	1	0.043	0.31	0.591

X_1X_2	0.27	1	0.27	1.93	0.1946
X_1X_3	0.45	1	0.45	3.23	0.1024
X_2X_3	0.097	1	0.097	0.69	0.4252
X_1^2	4.73	1	4.73	33.78	0.0002
X_2^2	6.01	1	6.01	42.9	< 0.0001
X_3^2	2.42	1	2.42	17.28	0.002
Lack of Fit	0.85	5	0.17	1.55	0.3219
Residual	1.4	10	0.14		
Pure Error	0.55	5	0.11		
Cor Total	15.18	19			
R^2					0.9076
R^2_{Adj}					0.8245

5

6