J Food Sci Technol (May 2022) 59(5):1769–1780
https://doi.org/10.1007/s13197-021-05188-0

ORIGINAL ARTICLE

# Production of egg white hydrolysate by digestion with pineapple bromelain: optimization, evaluation and antioxidant activity study

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Revised: 19 June 2021 / Accepted: 22 June 2021 / Published online: 27 June 2021


**Abstract** Chicken egg white is known to be an excellent source of good quality proteins to make hydrolysate with potential bioactive properties. Enzymatic digestion is a well-known method to produce protein hydrolysates; however, the type of enzyme determines the bioactive potential of the protein hydrolysates due to difference in their catalytic specificity. In this study, process optimization, production and evaluation of whole egg white protein hydrolysate (WEWPH) using pineapple bromelain through the Box-Behnken design were carried out. The design experiment ($r^2$ = 0.9557) displayed a significant ($p < 0.01$) effect of pH of egg white (9.0), hydrolysis time (24 h), and enzyme/substrate ratio (3.2 unit/g substrate) on hydrolysis and to form bioactive WEWPH. Antioxidant activity of the WEWPH was confirmed by DPPH radical scavenging assay. Gel filtration chromatography, SDS-PAGE and FTIR spectroscopy analysis of WEWPH revealed the digestion of egg white and the integrity of WEWPH in terms of secondary structure. The WEWPH exhibited strong scavenging activities of DPPH ($EC_{50}$ = 238.3 µg/ml), ABTS ABTS ($EC_{50}$ = 54.9 µg/ml), peroxyl ($EC_{50}$ = 391.6 µg/ml) and superoxide radicals. The WEWPH also displayed reducing power and singlet oxygen quenching activity. These results reveal that the bioactive WEWPH could be a promising ingredient in health food and nutraceuticals.

**Keywords** Chicken egg · Pineapple crown · Bromelain · Egg white hydrolysate · Antioxidant activity

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## Introduction

Foods contain various components and bioactive molecules vital for human nutrition and health (Galanakis 2021). Enzymatic hydrolysis has been received much interest in recent years in the production of protein hydrolysate from food source and is regularly used to enhance the functional and biological properties of food proteins (Liu et al. 2018). Consequently, there are more demands for bioactive protein hydrolysate derived from natural sources by health food and nutraceutical industries in recent years (Chang et al. 2018). Subsequent the disastrous effect of the COVID-19 pandemic, immunity is among people's utmost concern in the period and therefore, consumers are keen to change their diets to healthier alternatives (Galanakis 2020). The production and characterization of protein hydrolysates from various food sources with different bioactivities, particularly antioxidant activity have been reported (Miguel and Aleixandre 2006; Ma et al. 2020; Ashaolu 2020; Pan et al. 2020). However, the study concerning bioactive protein hydrolysates with antioxidant activity from animal products is limited. Among various animal-derived food sources, poultry egg particularly from chicken is considered a nutrient-rich food with well-balanced essential amino acid composition (Liu et al. 2018). Further, the chicken egg is one of the most popular foods that are consumed all over the world, despite religion and

ethnic group (Chang et al. 2018). The whole egg consists of about 13% proteins, which are essentially distributed between egg white/albumin (63%) and yolk (27.5%). The egg white is composed of several functionally active proteins and considered a vital source of bioactive protein hydrolysate and peptides with potential health food applications (Chang et al. 2018; Liu et al. 2018; Chen et al. 2020; Zhang et al. 2020). In addition the COVID-19 pandemic created chances and challenges for the commercialization of novel health foods and nutraceuticals containing targeted bioactive molecules (Galanakis et al. 2020, 2021).

Enzymatic hydrolysis and production of bioactive hydrolysate/peptides from egg white proteins with beneficial health effects have been reported (Miguel and Aleixandre 2006; Venkatachalam and Nagarajan 2019; Zhang et al. 2020). Nevertheless, most studies have used isolated protein fractions of the egg white as the substrate for enzymatic hydrolysis and production of biofunctional peptides (Online Resource 1). The use of whole egg white protein as the substrate for enzymatic hydrolysis using enzymes such as alcalase, flavourzyme, pepsin, bromelain, trypsin, α-chymotrypsin, papain, etc. and production of bioactive hydrolysate has reported (Miguel and Aleixandre 2006; Arsha 2019).

Response surface methodology (RSM) is an assembly of robust and helpful statistical procedures to elucidate/calculate interactions among the variables and optimize complex multi-variable processes compared to the conventional one-variable-at-a-time technique (Khodashenas and Jouki 2020). RSM has widely used in different stages of process optimization in biological processes including in the preparation of protein hydrolysates (Lanfang et al. 2017; Auwal et al. 2017). The Box-Behnken design of RSM is considered very competent and straightforward to organize and elucidate the experimental model compared to other methodologies (Lanfang et al. 2017; Chen et al. 2020).

Bromelain is a complex mixture of proteolytic enzymes extracted from the pineapple plant (*Ananas comosus* L. [Merr.]). Currently, the stem of the pineapple plant is the principal source of commercially available bromelain. Like the stem and fruit of the pineapple plant, the pineapple crown is also a rich source of bromelain. Currently, most of the pineapple crowns produced by pineapple farming are discarding as waste, except in very small quantity, which is used for propagation in some country (Liu et al. 2017). The potential proteolytic activity of the bromelain has been well accepted in the food industry, as evidenced by its long history in meat tenderization. Furthermore, the use of bromelain in developing bioactive peptides from fish proteins has been reported (Salampessy et al. 2010; Elavarasan et al. 2014). The pineapple is an important fruit of India and ranks fifth in the world productions of pineapples in 2019 (APADA 2020), and most of the pineapple fruits produced are consumed domestically. Thus, pineapple farming in India generates a huge quantity of pineapple crowns, which are discarding as waste with no commercial value. Despite several reports on the applications of bromelain in various filed (Salampessy et al. 2010; Arshad et al. 2014; Elavarasan et al. 2014), little work has been carried out to exploit the bromelain, particularly from pineapple crown for the production of egg white hydrolysate (Arsha 2019). Concerning all the aspects presented above and to rise the uses of egg white protein in health food and also to increase the extra value of pineapple crown, the objectives of this investigation were formulated. In this study, whole egg white protein (WEWP) from hen egg was hydrolyzed concerning the production of bioactive whole egg white protein hydrolysate (WEWPH) by using the bromelain from the pineapple crown and optimized the hydrolysis conditions with the help of Box-Behnken design of RSM. Further, the prepared WEWPH was analyzed for its properties towards its applications in health food.

## Materials and methods

### Materials

Pineapple crowned fruit of Kew variety fully matured with 3/4 yellow colour were procured from a local fruit market (Mysuru, India). Fruit uniform in size (∼ 1.0 kg average weight) and devoid of disease symptom or mechanical injury was selected. The crown was removed from the fruit just prior to its use. Chicken eggs (non-fertilized table eggs) from a local supermarket (Mysuru, India) were procured. ABTS, 1,3-diphenyl-isobenzofuran (DPIBF), DPPH, pyrogallol red (PGR), Trolox, α-tocopherol, Sephacryl S-400 HR (Sigma Aldrich Co., St. Louis, MO, USA), standard molecular weight (Mw) marker, bovine serum albumin (BSA), (Himedia Lab., Mumbai, India), tertiary butyl hydroquinone (TBHQ), Hammerstein casein, and tyrosine (Sisco Research Lab., Mumbai, India) were procured. All other chemicals and reagents used were analytical grade with 99% or greater purity.

### Preparation of bromelain from pineapple crown

Pineapple crowns (∼ 125 g average weight) were washed first with potable water, further by demineralized water and air-dried for 3 h. Then, the washed pineapple crown was chopped into small pieces (∼ 1.5 × 1.5 cm size), and the bromelain was extracted by homogenizing the pineapple crown pieces (5 kg) in 5 L of chilled (4 °C) sodium-phosphate buffer (0.1 M, pH 7.0) using a wet grinder for

4–5 min at room temperature (32 ± 2 °C). The resulting blend was filtered with two-fold gauze and then centrifuged at 12,000 × *g* for 20 min at 4 °C. Then, the clear supernatant was collected as the bromelain from the pineapple crown and stored at − 20 °C.

### Analysis of the bromelain from the pineapple crown

The proteolytic activity of the bromelain was measured by using the method reported by Murachi (1970) using Hammerstein casein as the substrate and tyrosine as the standard (100 µg/ml) and expressed the activity as casein digestion unit (CDU). One unit of CDU was defined as the quantity of enzyme needed to release a product equal to 1 µg of tyrosine/ min/ ml sample in the specified conditions of the assay. The bromelain concentration determined is 32.02 ± 1.4 CDU/ml. The protein content of the bromelain extract was estimated by the method of Lowry et al (1951) using BSA as the standard (50–500 µg).

### Enzymatic hydrolysis and preparation of whole egg white protein hydrolysate (WEWPH)

The eggs collected from the supermarket were washed with potable water and air-dried. Then, the washed eggs were wiped with absolute ethyl alcohol and egg whites were manually separated from the whole egg using a sterile egg yolk separator under aseptic condition using a laminar air flow cabinet. The pooled whole egg white was homogenized gently. Afterwords, 100 ml of the whole egg white protein (WEWP) in a sterile 250 ml Erlenmeyer conical flask (autoclaved with cotton plug) was heated at 65 ± 2 °C using a water bath for 10 min to deactivate any native enzyme(s) present in the WEWP. After cooling to room temperature, it was mixed with bromelain from the pineapple crown at different enzyme/substrate (E/S) ratio (CDU/g WEWP) and incubated at 37 ± 2 °C in a reciprocal shaking water bath under gentle shaking. Duplicate aliquots were withdrawn at a defined time of hydrolysis up to 26 h and stopped the hydrolysis reaction by heating at 80 ± 2 °C using a water bath for 8 min. The obtained WEWPH was immediately cooled in ice-chilled water and stored at − 20 °C. The resultant WEWPH was freeze-dried using a laboratory scale lyophilizer (Scanvac Cool Safe, Vassingerød, Denmark) and stored at − 20 °C.

### Optimization of enzyme treatment conditions by design experiment

The Box-Behnken design of RSM was used in the design experiment. The model was applied to the independent factors/variables [pH of WEWP (X1), hydrolysis time (X2; h), E/S ratio (X3)] using a three-factor-three level (-1, 0 and + 1) with 1 block and 15 runs (Table 1). The average of the antioxidant peptides formation measured as the %DPPH radical (%DPPH·) scavenging activity from three separate runs was taken as the response/dependent factor. For all these experiments, sterile conical flask (100 ml) with 25 ml of WEWP was used. To analyze the experiment data and to derive the polynomial model equation, the statistical software package STATISTICA version 7.1 (Statistica 2005) was used. The fitted polynomial model was analyzed based upon the model capability ($R^2$), regression factor significance (*p*-value), and analysis for lack of fit. The significance range was set at *p*-value < 0.05. The data were evaluated by means of the quadratic polynomial regression model as given in Eq. 1:

$$Y = \beta 0 + \left( \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \right) \quad (1)$$

where Y is the dependent/response variable, β0 is the constant, βi, βii and βij are the linear, quadratic and interactive coefficients generated by the model and Xi and Xj are the independent variables, respectively.

### Analysis of WEWPH

#### *Degree of hydrolysis (DH)*

DH, considered as the percentage of peptide bonds cleaved, was estimated by the O-phthaldialdehyde method (Nielsen et al. 2001) and expressed in terms of per cent of the degree of hydrolysis (%DH). Total peptides (amino groups) content in the WEWP was estimated by using serine as the standard. The %DH was calculated using the equation: DH (%) = Amino groups in WEWPH/total amino groups in WEWP × 100. DH analysis was carried to determine the rate of hydrolysis of egg white by digestion with bromelain.

#### *Gel filtration chromatography*

Gel exclusion chromatography of the water-soluble peptides mixture from the WEWPH was carried out on Sephacryl S-400 HR column (260 × 15 mm) and eluted at a flow rate of 0.33 ml/min with phosphate buffer (pH 7.0, 50 mM). The amount of protein/peptides was monitored using UV detection at 280 nm by using tyrosine as the standard and the antioxidant activity using %DPPH· scavenging assay for each fraction. Gel filtration chromatography was performed to separate and elutes the antioxidant active fractions from the WEWPH.

**Table 1** Box-Behnken design matrix with observed and predicted values of dependent variable (a) Analysis of variance (ANOVA) table (b) for the antioxidant (measured as % DPPH radical scavenging activity) production by bromelain digestion of whole egg white protein (WEWP) as function of pH of the WEWP substrate (X1), hydrolysis time (X2) and enzyme/substrate (E/S, CDU/g WEWP) ratio (X3)

a

| Run | Independent variables | | | | | | Dependent variable | |
|---|---|---|---|---|---|---|---|---|
| | pH (X1) | | Time (h, X2) | | E/S ratio (%, X3) | | % DPPH radical scavenging activity | |
| | Coded | Actual | Coded | Actual | Coded | Actual | Observed | Predicted |
| 1 | − 1 | 5 | − 1 | 1 | 0 | 80.0 | 41.53 ± 0.96 | 41.05 |
| 2 | 1 | 9 | − 1 | 1 | 0 | 80.0 | 54.59 ± 1.2 | 52.10 |
| 3 | − 1 | 5 | 1 | 47 | 0 | 80.0 | 28.42 ± 0.64 | 30.91 |
| 4 | 1 | 9 | 1 | 47 | 0 | 80.0 | 55.16 ± 0.86 | 55.64 |
| 5 | − 1 | 5 | 0 | 24 | − 1 | 3.2 | 49.31 ± 0.62 | 46.21 |
| 6 | 1 | 9 | 0 | 24 | − 1 | 3.2 | 73.48 ± 1.6 | 72.39 |
| 7 | − 1 | 5 | 0 | 24 | 1 | 156.8 | 42.14 ± 0.74 | 43.23 |
| 8 | 1 | 9 | 0 | 24 | 1 | 156.8 | 49.72 ± 0.36 | 52.82 |
| 9 | 0 | 7 | − 1 | 1 | − 1 | 3.2 | 51.42 ± 1.4 | 55.01 |
| 10 | 0 | 7 | 1 | 47 | − 1 | 3.2 | 50.02 ± 0.85 | 50.63 |
| 11 | 0 | 7 | − 1 | 1 | 1 | 156.8 | 43.26 ± 0.24 | 42.65 |
| 12 | 0 | 7 | 1 | 47 | 1 | 156.8 | 44.02 ± 0.62 | 40.44 |
| 13 | 0 | 7 | 0 | 24 | 0 | 80.0 | 58.14 ± 0.91 | 57.61 |
| 14 | 0 | 7 | 0 | 24 | 0 | 80.0 | 56.10 ± 0.73 | 57.61 |
| 15 | 0 | 7 | 0 | 24 | 0 | 80.0 | 58.60 ± 0.65 | 57.61 |

b

| Variables | Sum of squares | Degree of freedom | Mean square | F-ratio | *P*-value | Significant |
|---|---|---|---|---|---|---|
| X1 (Linier, L) | 639.984 | 1 | 639.984 | 362.993 | 0.00274 | * |
| (Quadratic, Q) | 35.570 | 1 | 35.569 | 20.175 | 0.04616 | * |
| X2 (L) | 21.705 | 1 | 21.704 | 12.311 | 0.07250 | |
| (Q) | 339.221 | 1 | 339.221 | 192.403 | 0.00515 | * |
| X3 (L) | 254.272 | 1 | 254.272 | 144.221 | 0.00686 | * |
| (Q) | 2.659 | 1 | 2.6595 | 1.5084 | 0.34429 | |
| X1 L by X2 L | 46.759 | 1 | 46.7585 | 26.5210 | 0.03569 | * |
| X1 L by X3 L | 68.773 | 1 | 68.7725 | 39.0071 | 0.02469 | * |
| X2 L by X3 L | 1.168 | 1 | 1.1681 | 0.6625 | 0.50116 | |
| Lack of fit | 61.033 | 3 | 20.3443 | 11.5391 | 0.08079 | |
| Pure error | 3.526 | 2 | 1.7631 | | | |
| Total SS | 1457.253 | 14 | | | | |

* $p$-value $< 0.05$

*Electrophoresis analysis*

The SDS-PAGE was performed with the Tricine-SDS-PAGE method according to Schägger and Von Jagow (1987). The wide range of Mw standard from 11 to 245 kDa was used as the marker. SDS-PAGE analysis was performed to determine the Mw of the proteins/peptides present in the WEWPH.

*SEM analysis*

The freeze-dried samples (WEWPH and non-hydrolysed WEWP) were mounted on the double-sided conducting tape fixed on the SEM sample holder and sputtered with gold (~ 100 Å). Then, the surface morphology and ultrastructure of the sample was examined by using the SEM (LEO Electron Microscopy Ltd., Cambridge, UK) at a 20 kV. SEM analysis was carried to observe any alter in the ultrastructure of WEWPH particles (Alipoorfard et al. 2020).

*Attenuated total reflectance (ATR)-FTIR spectra analysis*

ATR-FTIR spectra examination of freeze-dried WEWPH and non-hydrolysed WEWP were done by the ATR method using the FTIR spectrophotometer (IFS 25 TENSOR II,

Bruker, Germany). The spectra in the range of 4000–500 $cm^{-1}$ were observed at room temperature in 16 scans at a resolution of 4 $cm^{-1}$ against a background spectrum of a clean empty cell. FTIR spectra analysis was performed to determine the integrity of WEWPH in terms of secondary structure.

### Antioxidant potential analysis

To understand the antioxidant potentials of the WEWPH performed the DPPH radical (DPPH·), ABTS radical ($ABTS^{\cdot+}$), Peroxyl radical ($HO_2\cdot$), superoxide anion ($O_2^{\cdot-}$) scavenging assays to evaluate the radical scavenging capability. The radical scavenging potential of the WEWPH was expressed in $EC_{50}$ value, except for $O_2^{\cdot-}$. The calculated $EC_{50}$ value stand for the amount of sample/antioxidant required to scavenge 50% (half maximum) radicals in the reaction mixture (Jouki et al. 2020). For the metal chelating potential, ferric reducing antioxidant power (FRAP) assay was carried out. Besides, the singlet oxygen quenching ability of the WEWPH was also assayed.

*DPPH radical scavenging assay* The DPPH· scavenging activity of the WEWPH was determined by the procedure described previously (Heo et al. 2005). TBHQ was used as the positive control.

*ABTS radical scavenging (Trolox Equivalent Antioxidant Capacity, TEAC) assay* The reaction was performed using the ABTS assay described by Re et al. (1999), which involves the formation of ABTS radical cation ($ABTS^{\cdot+}$) by oxidation of the ABTS solution by $K_2S_2O_8$. α-Tocopherol was used as the positive control.

*Peroxyl radical scavenging assay* The $HO_2\cdot$ scavenging potential of the WEWPH was measured by the inhibition of PGR oxidation by the $HO_2\cdot$ formed from AAPH as detailed by Lopez-Alarcon and Lissi (2005), and TBHQ was used as the positive control.

*Superoxide anion scavenging assay* The $O_2^{\cdot-}$ scavenging potential of the WEWPH was estimated by calculating the inhibition of pyrogallol auto-oxidation by the procedure reported (Heo et al. 2005). TBHQ was used as the reference compound.

*Reducing power assay* Reducing power of the WEWPH was estimated by the procedure reported (Heo et al., 2005). The positive control used was TBHQ.

*Singlet oxygen quenching assay* The singlet oxygen ($^1O_2$) was formed by a peroxidase, $H_2O_2$, and halide system and $^1O_2$ quenching potential was determined by the oxidation of DPIBF at 420 nm by the procedure reported (Piatt et al. 1977). Then quenching potential of the WEWPH was compared with the α-tocopherol using as the positive control.

### Statistical analysis

All the experiments and calculations were carried out in triplicates, except for SEM and are reported average value ± standard deviation. Analysis of Variance (ANOVA) was performed with the $p$-value $< 0.05$ using the Statistica version 7.1 statistical software package (Statistica 2005).

## Results and discussion

### Preparation of WEWPH by using bromelain from the pineapple crown

The proteolytic activity and protein content of the bromelain from the pineapple crown was found to be 32.02 ± 1.4 CDU/ml and 2.9 ± 0.06 µg/µl, respectively. As shown in Fig. 1a, both E/S ratio and enzyme treatment time had a significant ($p < 0.05$) effect on the %DPPH· scavenging activity of the obtained protein hydrolysate. The antioxidant activity of WEWPH was increased ($p < 0.05$) with the increase of enzyme concentration. It was observed that the formation of antioxidant activity (measured as the %DPPH· scavenging activity) in the hydrolysate at the E/S ratio of ∼ 32.0 CDU/g WEWP was comparatively ($p < 0.05$) high as compared to the E/S ratio of ∼ 80.0 CDU/g WEWP. In contrast to %DPPH· scavenging activity, the %DH of the hydrolysate was increased with hydrolysis time at the high E/S ratio, but at low E/S level, the %DH changed steadily with hydrolysis time. These results indicated that the suitable hydrolysis environments to make the hydrolysate with the greater antioxidant potential were differing from the suitable hydrolysis conditions to attain the maximum %DH of the hydrolysate. Further, it was observed that the end %DH does not control the antioxidant potential of the protein hydrolysate. These results might be because of the presence of hydrophobic peptides at higher %DH was higher than these peptides at lower %DH. Comparable findings have been reported (Lanfang et al. 2017).

In order to validate the reproducibility, experiments were carried out to produce WEWPH using the bromelain from different batches of pineapple crown. It was observed that the bromelain from different batches (n = 3) of pineapple crown provided reproducible ($p < 0.05$) results in the production of WEWPH and the %DPPH· scavenging activity.

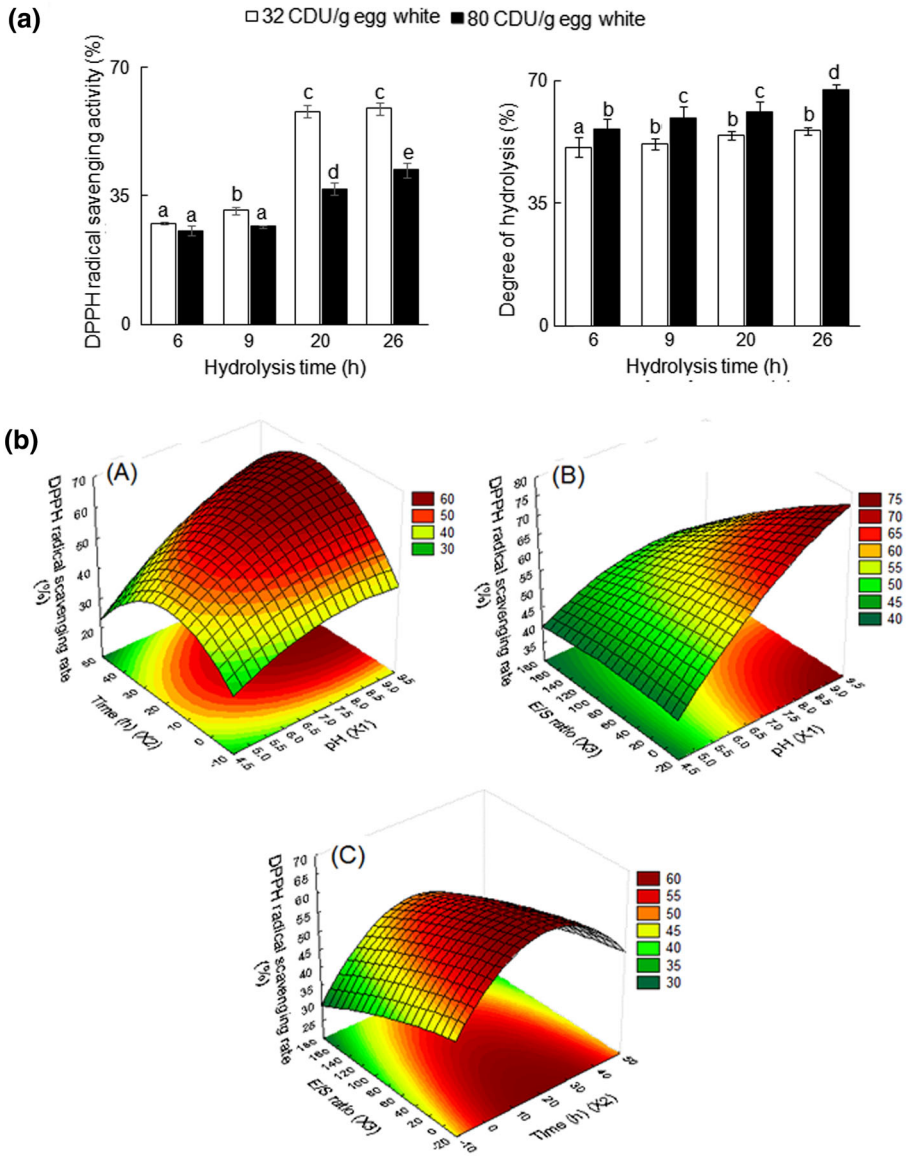


**Fig. 1** Time scale of bromelain digestion of whole egg white protein and production of antioxidant whole egg white protein hydrolysate (WEWPH) (**a**) and response surface plots of the model (**b**) used in Box-Behnken experimental design for the antioxidant WEWPH production. Different lowercase letters in the graph indicates values are significantly different from each other and the significance accepted at $p < 0.05$

## Enzyme treatment condition optimization using design experiment and model development approach

The Box-Behnken design of RSM was found to be useful in formulating a precise experimental model among significant factors for the production of hydrolysate from WEWP with promising antioxidant activity by using the bromelain from the pineapple crown. The predicted value of the response variable of antioxidant peptides formation (measured as %DPPH· scavenging activity) along with the observed value of the design experiment is presented in Table 1a. Analysis of variance (ANOVA) was used to analyze the response obtained from the experiment (Table 1b). Significant factors were defined by values $p < 0.05$, and the model probability was $p < 0.00006$. This result suggested that the regression model used to clarify the association of antioxidant peptides formation with the variables evaluated was precise. The effectiveness of the fit of the model equation was examined by the determination of coefficient ($r^2$) (0.9557), which depict that the response model can clarify a total variation of 95.57% and also expressed that the design can effectively be applied and accepted. The $p$-value (0.081) and F-value (11.53) observed by the analysis of lack of fit indicated that it had no significant relation to the pure error. These results illustrate that the design was enough to foresee the formation of antioxidant peptides (measured as %DPPH· scavenging potential) at any blend of values of the 3 factors. The regression (2-order polynomial) equation derived is shown in Eq. 2, which explains the normal logarithm of response, as a mean of three factors (independent) and their

functions (linear, quadratic and collective) despite their significance.

$$Y = (-17.52) + (15.71 * X1) + \left(-0.776 * X1^2\right) + (0.253 * X2) + \left(-0.018 * X2^2\right) + (0.52 * X3) + \left(-0.002 * X3^2\right) + (0.74 * X1 * X2) + (-0.086 * X1 * X3) + (0.001 * X2 * X3) \quad (2)$$

where Y stands for the response variable antioxidant peptides formation (measured as %DPPH• scavenging activity), while X1, X2 and X3 are denoting the independent variables such as pH of WEWP substrate, hydrolysis time, and E/S ratio, respectively.

ANOVA (Table 1b) and the Pareto chart (Fig. 2a) for the three factors explained that the quadratic model resulting from the Box-Behnken design could sufficiently be applied to illustrate the factors for antioxidant peptides formation (Y) under a broad choice of the working environment. Of the three independent factors pH of WEWP substrate had a linear ($p < 0.0027$) and quadratic ($p < 0.046$), and hydrolysis time had a linear ($p < 0.0027$), as well as E/S ratio, had a quadratic ($p < 0.0068$) effect on antioxidant peptides formation. The pH of WEWP (X1) had a significant interactive effect with time (X2) ($p < 0.035$) and E/S ratio (X3) ($p < 0.024$) used on the formation of the antioxidant peptides (Table 1b and Fig. 2a).

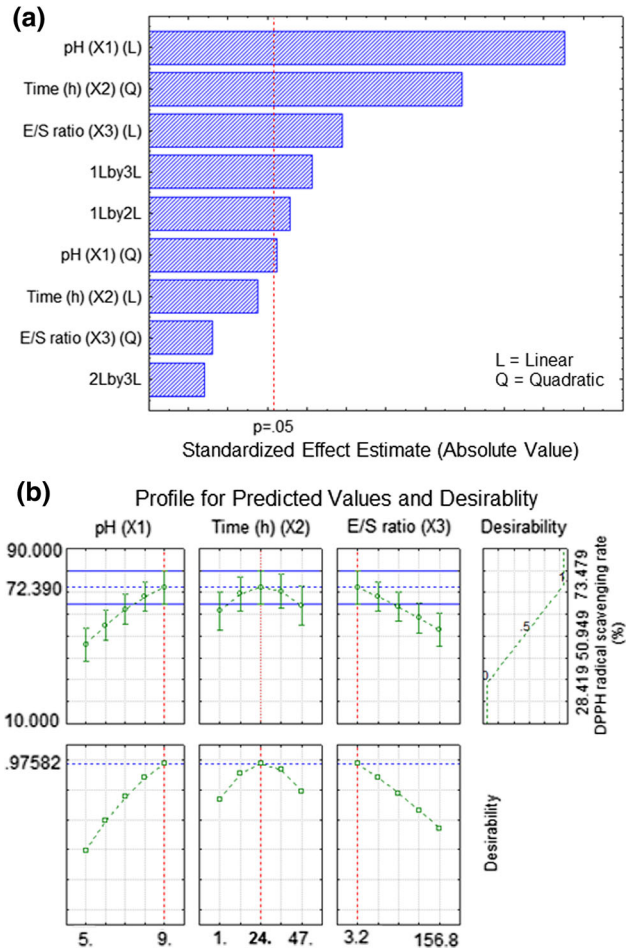


**Fig. 2** Pareto graph of significant variables **(a)** and characteristic of the model used in Box-Behnken experimental design (**b**) for the digestion of whole egg white protein by bromelain and production of antioxidant whole egg white protein hydrolysate

Figure 1b display the response surface and contour plots of antioxidant peptides formation (measured as %DPPH· scavenging activity) which clarifies the association among response and the experimental data, as a means of levels of two variables with the other one variable at its midpoint. Figure 1b (A) exhibit the effect of pH of the WEWP and the hydrolysis time on antioxidant peptides formation. The result depicts that the formation of the antioxidant peptides increased as the pH of the WEWP and the enzyme treatment time increased. However, after 24 h of hydrolysis, a declining tendency was observed. Figure 1b (B) reveals the effect of pH of the WEWP and the E/S ratio on the formation of the antioxidant peptides. The result indicates that the formation of the antioxidant peptides increased as the pH of the WEWP increased and a sharp decreasing trend with the increase of enzyme concentration. Figure 1b (C) shows the effect of enzyme treatment time and the E/S ratio on the formation of antioxidant peptides. The result depicts that the formation of the antioxidant WEWPH increased along with the increase of hydrolysis time up to 24 h followed by a decline, while a sharp decreasing trend was observed with the increase of enzyme concentration. Now, it can be noticed that the formation of the antioxidant peptides (measured as %DPPH· scavenging activity) increased virtually linear with the increasing pH of WEWP and slightly direct with increasing the enzyme treatment time. However, no linear relationship was observed in forming the antioxidant peptides with increasing the E/S ratio.

The optimum levels of independent factors, pH of WEWP substrate (X1), hydrolysis time (X2), E/S ratio (X3), were elucidated from the response surface plots (Fig. 1b) and the desirability profile (Fig. 2b). The experimentally optimized level of independent factors to get the highest quantity of antioxidant peptides was 9.0 pH of WEWP substrate, 24 h hydrolysis time, and 3.2 ± 0.14 CDU/g WEWP substrate. This combination predicted a yield of 72.43%DPPH· scavenging activity. To validate the competence of the model equation, a confirmation test was performed by using the optimized conditions with 9.0 pH of WEWP substrate, 3.2 ± 0.14 CDU/g WEWP substrate and 24 h enzyme treatment time. At this state, an average %DPPH· scavenging potential was 71.92 ± 0.13 (n = 5),

equivalent fit to the value projected (72.43) by the design equation, which established that the developed design was precise for the optimization.

Design experiment and optimization of hydrolysis conditions for the preparation of protein hydrolysate with antioxidant activity from stonefish using bromelain was reported by Auwal et al (2017). Recently, Chen et al. (2020) has reported the optimization of egg white protein hydrolysis by neutral protease using Box-Behnken design and optimum conditions were in substrate concentration of 3 g egg white protein powder in 100 ml, pH of 6.5, [E/S] of 5.32% to obtain 9.61% DH. However, as on date, no work was reported on optimization of hydrolysis conditions and production of bioactive protein hydrolysate from hen egg whole white protein through the enzymatic method by RSM approach.

## Analysis of WEWPH

### *SEM analysis*

The SEM analysis of WEWPH showed distinct differences in the microstructure and surface morphology between the control and the hydrolyzed samples (Fig. 3a). The freeze-dried WEWPH particles had a spongy/rubbery structure formed by the dispersal of air bubbles during quick dehydration. While the control (non-enzyme treated freeze-dried WEWP) had a sheet shape with glassy surfaces and solid hard structure (Fig. 3a).

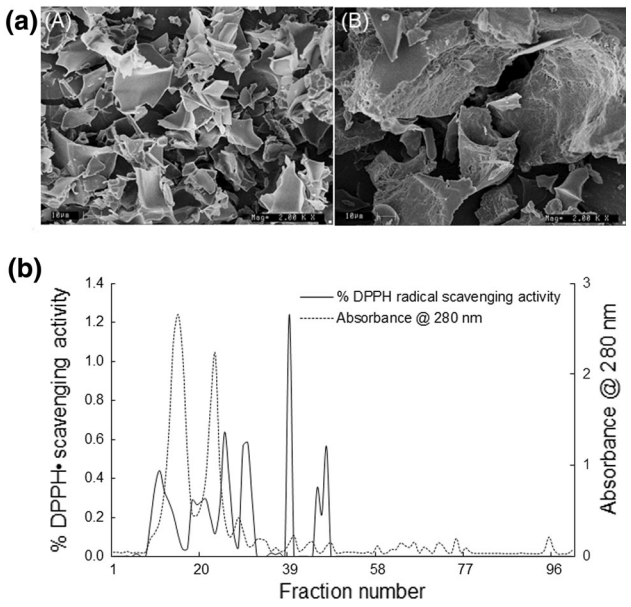


**Fig. 3** SEM (**a**) of freeze dried whole egg white protein (A) and whole egg white protein hydrolysate (WEWPH) (B) and gel filtration chromatogram of WEWPH (**b**)

### *Gel filtration chromatography*

Gel filtration is an efficient tool for separating macromolecules, including peptides based on molecular weight and has been extensively used in mixed constituent separations or salt exclusion. As shown in Fig. 3b, the elution profile revealed four major and two minor symmetrical peaks from gel filtration chromatography, indicating that the water-soluble peptides from the freeze-dried WEWPH were not homogeneous. The WEWPH prepared using bromelain hydrolysis was found to be a complex blend and some diverse peptides with antioxidant capacity (Fig. 3b).

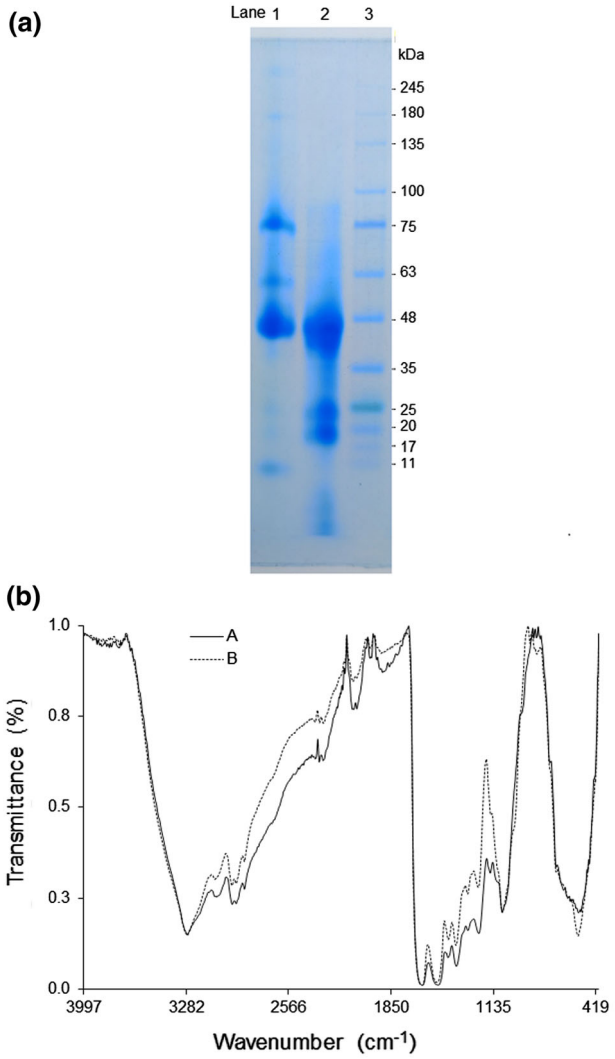


**Fig. 4** SDS-PAGE pattern of non-hydrolyzed whole egg white protein (WEWP) (Lane 1), whole egg white protein hydrolysate (WEWPH) (Lane 2), standard protein Mw marker (Lane 3) (**a**) and ATR-FTIR spectrum of WEWPH (—— A) and WEWP (—— B) (**b**)

*Electrophoresis analysis*

SDS-PAGE profile of the control (non-hydrolyzed WEWP) (Lane 1) and WEWPH (Lane 2) samples are shown in Fig. 4a. The numbers of bands (protein/peptides) of the WEWPH increased as compared to the control in the SDS-PAGE pattern. It depicted the polypeptides with higher Mw successfully cleaved during enzymatic hydrolysis and resulting in lower Mw peptides with high antioxidant activity. Remarkably, more band numbers were observed in the region with lower Mw (< 40 kDa) (Fig. 4a). The result explained that the Mw of control was within the range of 45–260 kDa. However, the average Mw of the hydrolysate were < 40 kDa as compared to the protein/peptide marker (Lane 3). It is documented that to get food derived protein hydrolysate with improved bioactivity, the peptides in the hydrolysate should occur more in low Mw species and quantity of free amino acids as little as possible.

*ATR-FTIR spectra analysis*

ATR-FTIR spectrum of the freeze-dried WEWPH (A) and control (non-hydrolyzed WEWP) (B) samples are shown in Fig. 4b. The ATR-FTIR spectra revealed an O–H and C-H stretching vibrations at 3400 ~ 3200 $cm^{-1}$ and 3000 ~ 2800 $cm^{-1}$, respectively. Absorption peaks from 1630 $cm^{-1}$ to ~ 1539 $cm^{-1}$ were attributed to Amide I and Amide II, which are common to the absorption of protein. Amide I band was primarily because of the C=O stretch vibrations. The stretching vibration of C–N in the amide was observed at 1398 $cm^{-1}$. The absorbance crest at 1200 ~ 1000 $cm^{-1}$ revealed the existence of ring vibrations superimposed with stretch vibrations of C–OH and C–O–C. Based on the ATR-FTIR spectra study, there were no obvious changes between control WEWP and WEWPH sample in terms of secondary structure. This possibly caused since the enzymatic hydrolysis reduced the size of egg white protein molecules by just cleaving of the polypeptide chains.

*Antioxidant potential evaluation*

The activities of antioxidants possibly credited to various mechanisms such as free radical scavenging capacity, reducing/metal chelation capacity, prevention of chain initiation, decomposition of peroxides, or a collective result of one or more and these antioxidant potentials of a compound can be evaluated by different methods. Therefore, to determine the antioxidant potentials of the WEWPH, the DPPH·, $ABTS^{\cdot+}$, $HO_2\cdot$, $O_2\cdot^-$ scavenging activity, ferric reducing antioxidant power (FRAP) and singlet oxygen quenching ability of the WEWPH were analyzed.

*DPPH· scavenging activity* The DPPH· is a stable free radical broadly explored for measuring the radical scavenging potential of protein hydrolysates and bioactive peptides as well as other compounds due to the quick and reproducible results. The effect of antioxidants on DPPH is owing to their hydrogen-donating capacity. DPPH· scavenging potential of the WEWPH is denoted in Fig. 5a. The result showed a dose-dependent manner inhibition of the DPPH· and increased linearly with an increase in the concentration of the sample. The $EC_{50}$ value for DPPH· scavenging activity was found to be 238.3 µg/ml. The $EC_{50}$ value of the TBHQ was found to be 2.86 µg/ml.

*$ABTS^{\cdot+}$ scavenging/TEAC assay* The $ABTS^{\cdot+}$scavenging/TEAC assay is one of the simplest and widely applied indirect method for screening natural compounds, food products, hydrolysates and other extracts on their antioxidant capacity (Arts et al. 2004). The $ABTS^{\cdot+}$ scavenging potential of the WEWPH is presented in Fig. 5b. The peptides expressed the $ABTS^{\cdot+}$ scavenging activity in a concentration-dependent manner. The obtained $EC_{50}$ value was 54.9 µg/ml. The $EC_{50}$ value of the Trolox was calculated to be 2.01 µg/ml. Results revealed that the peptides present in the WEWPH could deactivate reactive oxygen species, either by the decrease through electron transfer or by radical scavenging through hydrogen atom transport, resulting in more stable species (Prior et al. 2005).

*Peroxyl radical scavenging activity* The peroxyl radicals ($HO_2\cdot$), are exceptionally active radicals, which can react with other radicals and lipid hydroperoxides. The $HO_2\cdot$ scavenging potential displayed dosage dependency as the activity increased with the increasing quantity of the sample (Fig. 5c). The $EC_{50}$ value calculated was found to be 391.61 µg/ml. TBHQ was used as the standard. Higher $HO_2\cdot$ scavenging shown by this peptides extracts indicates the fact that these peptide extracts have chain breaking (sulfur containing amino acid like cystine) antioxidant properties.

*Superoxide anion scavenging activity* The superoxide anion ($O_2\cdot^-$) is very harmful to cellular components (Collin 2019). In organisms, generally, the $O_2\cdot^-$ is changed to hydrogen peroxide using superoxide dismutase enzyme. With a lack of transition metal ions, hydrogen peroxide is quite constant. But, $O_2\cdot^-$ can be created by the response of superoxide by hydrogen peroxide in the occurrence of metal ions, typically Fe and Cu (Collin 2019). As shown in Fig. 5d, $O_2\cdot^-$ scavenging activity was not displayed any concentration-dependency as the activity decreased with the increasing quantity of the sample. But the $O_2\cdot^-$ scavenging activity increased with time. The superoxide anion scavenging activity of the WEWPH indicates it's useful in declining the probable toxicity of $O_2\cdot^-$.

*Reducing power* The reducing power capacity of a material is often used as an important marker of its possible

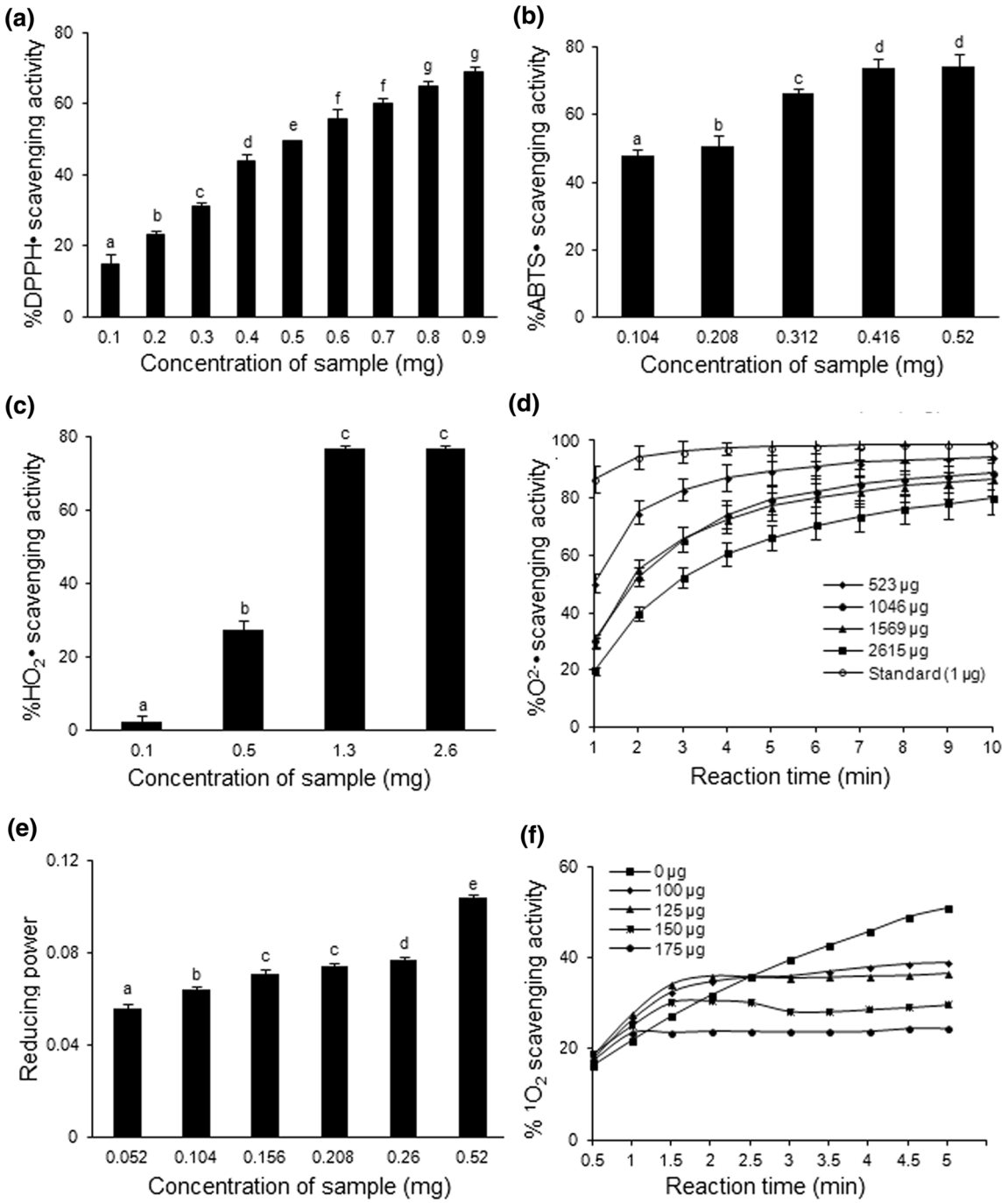


**Fig. 5** Antioxidant activities of the extract of whole egg white protein hydrolysate. DPPH (**a**), ABTS (**b**), peroxyl ($HO_2$) (**c**) and superoxide anion ($O_2^-$) (**d**) radicals scavenging activities (%); reducing power (**e**) and singlet oxygen ($^1O_2$) scavenging activity (**f**). Different lowercase letters in the graph indicates values are significantly different from each other and the significance accepted at $p < 0.05$

antioxidant capacity and the electron donating ability. The reducing power of a compound usually depends on the occurrence of reductones (antioxidants), which exercise the antioxidant ability by cleaving the free radical sequence by providing a hydrogen atom. As shown in Fig. 5e, WEWPH exhibited better reducing power capability similar to the free radical scavenging activity in a dosage-dependent mode which was comparable to that of standards. The antioxidant peptides found in the WEWPH made the decrease of ferric/ferricyanide mixture to the ferrous type and consequently confirmed the reducing power potential.

*Singlet oxygen scavenging* The singlet oxygen ($^1O_2$) is a high power type of oxygen and is identified as one of the reactive oxygen species (ROS). $^1O_2$ is concerned in

numerous diseases, and it induces hyper oxidation and oxygen cytotoxicity as well as decreases antioxidative activity (Kochevar and Redmond 2000). Therefore, the $^1O_2$ quenching potential of natural compounds exhibits their helpfulness as antioxidants. The current study observed that the peptides from WEWPH have excellent quenching activity for $^1O_2$ (Fig. 5f) while was not as competent as the standard.

Antioxidant potential of bioactive egg white hydrolysates and peptides have been reported (Chang et al. 2018; Liu et al. 2018; Venkatachalam and Nagarajan 2019; Zhang et al. 2020; Chen et al. 2020; Ma et al. 2020. The production of antimicrobial peptides by bromelain hydrolysis of leatherjacket (*Meuchenia* sp.) insoluble proteins has reported (Salampessy et al. 2010). In the present study, hydrolysis of WEWP from hen egg by the bromelain derived from the pineapple crown (a by-product/waste from pineapple farming or fruit industry) was explored, on which there is no prior report in the literature. Even though the bromelain, particularly from pineapple stem (stem bromelain), is available commercially, in the study, we have used pineapple crown as the source of bromelain for the development of the bioactive WEWPH from hen egg in order to rise the extra value and use of this underutilized bioresource (the pineapple crown, a by-product/discard of pineapple farming).

## Conclusion

Enzymatic digestion is a well-accepted procedure for improving the additional/nutritional value of food derived proteins. Despite the current interest in the antioxidant activities of food protein hydrolysates to date, there is not much information on the enzyme digestion conditions required to obtain hydrolysate enriched with antioxidant peptides from egg white. In this study, hydrolysis of WEWP from the hen egg by using the bromelain from the pineapple crown under the predicted operating conditions derived from the design experiment produced a promising effect on the antioxidant property of the WEWPH. The optimized conditions of bromelain hydrolysis of WEWP from using the time (24 h), pH of WEWP (9.0) and E/S ratio (3.2 ± 0.14 CDU/g WEWP) was established for the highest response of antioxidant active WEWPH by using the Box-Behnken design of RSM. The obtained WEWPH exhibited strong scavenging activities of DPPH·, $ABTS^{\cdot+}$, $HO_2\cdot$, and $O_2^{\cdot-}$ as well as reducing power and singlet oxygen quench. These results indicate that the WEWPH can be used as a promising functional ingredient in the development of health food and nutraceutical products to benefit human health. In addition, the application of bromelain from the pineapple crown discard/waste to the development of bioactive WEWPH would raise the extra value and uses of this underutilize bioresource and also help to improve farmer's income. Though, further in vivo and scale-up studies must be carried out to exploit the WEWPH.

**Supplementary Information**The online version contains supplementary material available at https://doi.org/10.1007/s13197-021-05188-0.

**Acknowledgements** LCJ thank the University Grants Commission (UGC), New Delhi, India, for the award of a Research Fellowship. Authors thank the Director, CSIR-CFTRI, Mysuru, India for encouragement and facilities.

**Author contributions** LCJ carried out the experiments; analyzed the data and drafted the manuscript; TGK performed statistical analysis of the experimental data and edited the manuscript; SPV conceived and designed the study, provided resources, supervised, edited and reviewed manuscript.

**Funding** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Data availability** Available from the corresponding author on reasonable request.

**Declarations**

**Conflicts of interest** All authors declare that they have no conflict of interest.

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