Chicken Egg White—Advancing from Food to Skin Health Therapy: Optimization of Hydrolysis Condition and Identification of Tyrosinase Inhibitor Peptides

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Abstract: Active fragments (bioactive peptides) from the chicken egg white proteins were expected to exert tyrosinase inhibitory activities in which skin hyperpigmentation could be prevented. Egg white was hydrolyzed by trypsin, chymotrypsin and the combination of both enzymes. The enzyme treatments achieved >50% degree of hydrolysis (DH) at substrate-to-enzyme (S/E) ratio of 10–30 (w/w) and hydrolysis time of 2–5 h. A crossed D-optimal experimental design was then used to determine the optimal enzyme composition, S/E ratio and hydrolysis time in order to yield hydrolysates with strong monophenolase and diphenolase inhibitory activities. The optimized conditions 55% trypsin, 45% chymotrypsin, S/E 10:1 w/w and 2 h achieved 45.9% monophenolase activity inhibition whereas 100% trypsin, S/E 22.13:1 w/w and 3.18 h achieved 48.1% diphenolase activity inhibition. LC/MS and MS/MS analyses identified the peptide sequences and the subsequent screening had identified 7 peptides (ILELPFASGDLLML, GYSLGNWVCAAK, YFGYTGALRCLV, HIATNAVLFFGR, FMMFESQNKDLLFK, SGALHCLK and YFGYTGALR) as the potential inhibitor peptides. These peptides were able to bind to H85, H94, H259, H263, and H296 (hotspots for active residues) as well as F92, M280 and F292 (stabilizing residues) of tyrosinase based on structure-activity relationship analysis. These findings demonstrated the potential of egg white-derived bioactive peptides as skin health therapy.

Keywords: bioactive peptide; crossed D-optimal design; diphenolase inhibitory activity; egg white; monophenolase inhibitory activity; pigmentation; tyrosinase

1. Introduction

The excess melanin production and deposition in the melanocytes and keratinocytes cause hyperpigmentation, leading to uneven skin tones. Tyrosinase (E.C. 1.14.18.1) plays a key role in melanin synthesis (melanogenesis) by catalyzing the rate-limiting hydroxylation of \( \text{L-tirosine} \) to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to dopaquinone, via the monophenolase and diphenolase reactions, respectively [1]. The inhibition of tyrosinase using a nature-derived agent is hence of huge cosmeceutical demand, as some potent skin-lightening ingredients including hydroquinone and heavy metals have been ascribed with harmful side effects and banned for use in certain countries [2,3]. Since the discovery of a potential protein or peptide with tyrosinase inhibitory activity from lemon skin extract in 2006 [4], reports on peptide-based skin lightening agents has increased by leaps and bounds. The anti-pigmentation mechanisms of peptides include tyrosinase inhibition [5], copper chelation [6] and melanogenesis pathway regulation [7]. Peptide was also incorporated as an active ingredient in commercial skin lightening products such as

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β-White™ (contains oligopeptide-68) and Melanostatine™ 5 (contains nonapeptide-1) marketed by Lucas Meyer Cosmetics. The emergence of bioactive peptides as a new class of therapeutic agent is nonetheless endowed by their relatively small size, low toxicity, fast clearance and high specificity in inhibiting protein-ligand interactions [8].

Anti-pigmentation peptides have been discovered from various natural protein sources including rice bran [6,9] and marine microalgae [10]. Yet, there has been no relevant study on the anti-pigmentation effect of chicken egg white-derived peptides, although this readily available protein has been traditionally used as facial masks to boost skin health. It should be also noted that the active fragments are usually encrypted in the parent protein and required to be released in order to exhibit higher rate of the bioactivity. Enzymatic hydrolysis was therefore employed in this study to release the bioactive peptides from the egg white. In addition, the hydrolysis conditions such as the enzyme used, enzyme composition, substrate-to-enzyme (S/E) ratio and hydrolysis time in order to produce egg white hydrolysates that exhibit the highest monophenolase and diphenolase inhibitory activities should be optimized using D-optimal experimental design. The crossed D-optimal approach conjugates the mixture component (enzyme composition) and process factors (S/E ratio and hydrolysis time) in a single experimental design and generates a relatively smaller number of experimental runs, which has made it more viable in terms of cost and time, especially when each variable comes with multiple levels [11]. In fact, crossed D-optimal design had been successfully implemented to optimize different hydrolysis conditions for other purposes [12,13]. Therefore, this technique was used in this study.

Enzymes used in this study were trypsin and chymotrypsin due to their specific cleavage preferences, i.e., trypsin selectively cleaves at the C-terminal of arginine and lysine [14] whereas chymotrypsin predominantly cleaves at the C-terminal of the aromatic phenylalanine, leucine, methionine, tryptophan and tyrosine [15]. The peptides released may thus fulfil the characteristics of strong tyrosinase inhibitors such as the presence of an aromatic C-terminal residue tyrosine, tryptophan or phenylalanine [9,16] or one or more arginine in combination with phenylalanine, valine, alanine and leucine in the peptide sequence as delineated by Schurink, van Berkel, Wichers and Boeriu [17]. Therefore, the objectives of present study were to determine the optimum hydrolysis conditions of egg white using trypsin and/or chymotrypsin that yield the protein hydrolysates with tyrosinase inhibitory activities followed by peptide sequence identification as well as to investigate the structure-activity relationship between the identified inhibitor peptides and tyrosinase.

2. Materials and Methods

2.1. Chemicals

Egg white powder was purchased from a local market (Penang, Malaysia). Trypsin (EC 3.4.21.4, 10,000 U/mg) and α-chymotrypsin (EC 3.4.21.1, 40 U/mg) from bovine pancreas, tyrosinase (EC 1.14.18.1, 7164 U/mg) from Agaricus bisporus were purchased from Sigma-Aldrich. Other chemicals and reagents used were of analytical grade and purchased from Sigma-Aldrich unless otherwise stated.

2.2. Enzymatic Hydrolysis of Egg White Proteins

The enzymatic hydrolysis of egg white was conducted according to the protocol of Miguel, Recio, Gomez-Ruiz, Ramos and Lopez-Fandino [18] with modifications. Briefly, egg white powder was dissolved in 0.05 M sodium phosphate buffer (pH 7.8 or 8.0, depending on the digestive enzyme used) to 5 mg/mL and boiled at 95 °C for 30 min. Subsequently, designated enzyme treatments at various substrate-to-enzyme (S/E) ratios were added to the substrate solution and incubated for different durations at 37 °C with constant shaking of 300 rpm. The details of the experiment will be elaborated in Sections 2.2.1 and 2.2.2. The resultant hydrolysate was then boiled at 95 °C for 30 min to terminate the reaction and centrifuged at 4500× g for 15 min. The supernatant was collected and stored at −20 °C until further analysis. Heat-inactivated enzymes were used as the control treatment.
2.2.1. Single-Factor Experiment

The effects of enzyme composition, S/E ratio and hydrolysis time on the degree of hydrolysis (DH) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein band profiling of egg white hydrolysate were studied as preliminary work to investigate the hydrolysis condition required to produce sufficient peptides from egg white. Below are the details of the experiment:

The effect of enzyme composition was investigated based on the hydrolysis of egg white using designated enzyme treatments: 100% trypsin (T), 100% chymotrypsin (C), and 50% trypsin + 50% chymotrypsin (T+C). Phosphate buffer at pH 7.8 was used for T whereas pH 8.0 was used for C and T+C treatments. All these compositions were used for the investigations of the effects of S/E ratio and hydrolysis time. During the investigation of the effect of S/E ratio, the ratio of 10:1, 20:1, 30:1, 40:1 and 50:1 (w/w) were studied whereas the hydrolysis time was fixed at 3 h. On the other hand, 0.5, 1, 2, 3, 4 and 5 h were studied during the investigation of the effect of hydrolysis period and the S/E ratio fixed at 30:1 (w/w).

The DH of the hydrolysate was determined by measuring the soluble protein content in 10% trichloroacetic acid (TCA) (Fisher Chemicals™, Leicestershire, UK) according to the protocol of Baharuddin, Halim and Sarbon [19] with modifications. Briefly, the hydrolysate was dissolved in an equal volume of 20% TCA and incubated at room temperature for 30 min. The sample was then centrifuged at 3000 × g for 10 min. The pellet was suspended in 0.5 mL 0.1 M NaOH and subjected to Bradford assay to determine the amount of soluble protein in the hydrolysate. Briefly, 5 µL of the hydrolysate was added with 250 µL of Bradford reagent and incubated at room temperature for 10 min. The absorbance was then measured at 595 nm using a spectrophotometer (Spectramax M5, Molecular Devices, San Jose, CA, USA). Each measurement was autozeroed against a blank containing Bradford reagent and 0.1 M NaOH. The DH was calculated using the following equation:

\[
\text{Degree of hydrolysis (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

where \(A_{\text{control}}\) denotes the absorbance of the system containing Bradford reagent, NaOH and undigested egg white powder; \(A_{\text{sample}}\) denotes the absorbance of the system containing Bradford reagent, NaOH and egg white hydrolysate.

To evaluate the protein profile after enzymatic hydrolysis, SDS-PAGE analysis was conducted using 4% stacking gel and 12% resolving gel. Briefly, 10 µL of sample was added with 10 µL 2× Laemmli buffer (Bio-Rad Laboratories Inc., Hercules, CA, USA) and 1 µL 2-mercaptoethanol followed by incubation at 95 °C for 15 min. Then, 10 µL of the mixture was loaded into the well and the set was run at 80 V for 15 min followed by 120 V for 1 h. Opti-Protein XL Marker G266 (abm Inc., Richmond, BC, Canada) with a molecular weight range of 10 to 245 kDa was used as the standard protein marker. The gel was stained overnight using staining solution (50% ddH\(_2\)O, 40% methanol, 10% acetic acid, 0.1% Coomassie Blue) and destained using destaining solution (50% ddH\(_2\)O, 40% methanol, 10% acetic acid) until blue protein bands were visible against a clear background. The image of the gel was captured using Fujifilm LAS-3000 Imager (Fujifilm, Tokyo, Japan). The molecular weights of the protein bands were analyzed using Multi Gauge software version 3.0 (Fujifilm, Tokyo, Japan).

2.2.2. Optimization of Hydrolysis Conditions for Monophenolase and Diphenolase Inhibitory Activities

Crossed D-optimal design was used to optimize the hydrolysis parameters to yield egg white hydrolysates with the highest tyrosinase inhibitory activities (i.e., the monophenolase inhibitory activity (Y) and diphenolase inhibitory activity (Z)). The enzyme composition (trypsin, \(X_1\); chymotrypsin, \(X_2\)) represents the mixture component whereas S/E ratio (\(X_3\)) and hydrolysis time (\(X_4\)) represents the process factors. Based on the results of single-factor experiment (Section 2.2.1), the levels of the variables were chosen and coded, as shown in Table 1. These variables generated 28 experimental runs. Data analysis and calculation of predicted response were conducted using Design-Expert software
Foods 2020, 9, 1312 (version 6.0, Minneapolis, MN, USA). Five confirmation experiments were performed to verify the optimized condition.

Table 1. Parameters and levels for crossed D-optimal design of the monophenolase and diphenolase inhibitory activities.

| Variable                     | Coded Variable | Coded Variable Level |
|------------------------------|----------------|----------------------|
| Trypsin composition (%)      | X₁             | −1 −0.5 0 0.5 1      |
| Chymotrypsin composition (%) | X₂             | −1 −0.5 0 0.5 1      |
| S/E ratio (w/w)              | X₃             | −1 −0.5 0 0.5 1      |
| Hydrolysis time (h)          | X₄             | −1 −0.5 0 0.5 1      |

2.3. Determination of Tyrosinase Inhibitory Activities

2.3.1. Monophenolase Inhibitory Activity

The monophenolase inhibitory activity was performed according to Takahashi, Takara, Toyozato and Wada [20] with slight modifications. Briefly, 10 µL of sample and 180 µL of 50 mM potassium phosphate buffer (pH 6.8) containing 0.5 mM L-tyrosine were added to a 96-well plate and incubated at 30 °C for 10 min. The reaction was started by the addition of 1 µL tyrosinase (6250 U/mL) and immediately monitored at 470 nm at every 20 s for 15 min with a constant temperature of 30 °C throughout the reaction. Each measurement was autozeroed against a blank containing L-tyrosine. The monophenolase inhibitory activity is calculated as follows:

\[
\text{Monophenolase inhibitory activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 (2)
\]

where \(A_{\text{control}}\) denotes the absorbance of the system containing tyrosinase and L-tyrosine; \(A_{\text{sample}}\) denotes the absorbance of the system containing tyrosinase, L-tyrosine and sample.

2.3.2. Diphenolase Inhibitory Activity

The diphenolase inhibitory activity was performed according to Takahashi, Takara, Toyozato and Wada [20] with slight modifications. Briefly, 10 µL of sample and 180 µL of 50 mM potassium phosphate buffer (pH 6.8) containing 0.5 mM L-DOPA were added to a 96-well plate and incubated at 30 °C for 10 min. The reaction was started by the addition of 1 µL tyrosinase (6250 U/mL) and immediately monitored at 470 nm at every 10 s for 1 min with a constant temperature 30 °C throughout the reaction. Each measurement was autozeroed against a blank containing L-DOPA. The diphenolase inhibitory activity is calculated as follows:

\[
\text{Diphenolase inhibitory activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 (3)
\]

where \(A_{\text{control}}\) denotes the absorbance of the system containing tyrosinase and L-DOPA; \(A_{\text{sample}}\) denotes the absorbance of the system containing tyrosinase, L-DOPA and sample.

2.4. Identification of Bioactive Peptides

The samples produced using the optimized parameters (Section 2.2.2) were subjected to LC/MS and MS/MS analyses using Easy-nLC II system (Thermo Scientific, San Jose, CA, USA) coupled with LTQ Orbitrap Velos. The chromatographic separation and mass spectrometry (MS) parameters were set up according to Siow and Gan [21]. Data acquisition was conducted using Xcalibur version 2.1. Peptide sequencing and identification based on the spectra acquired were performed using PEAKS Studio version 7.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) [22]. The error mass tolerance
allowed for precursor and fragmented ions were 0.1 and 0.8 Da, respectively. Enzyme was not specified in the peaks search against SwissProt2019 database and the false discovery rate (FDR) was estimated with decoy-fusion method. PeptideRanker web server (http://bioware.ucd.ie/, accessed on 6th July 2020) was used to screen for potential biologically active peptides where peptides with PeptideRanker score >0.5 were considered potentially active [23] and hence selected for further analysis. Protein-peptide docking was then performed using PepSite 2 web server (http://pepsite2.russelllab.org/, accessed on 7th July 2020) to predict the potential peptide binding sites on the protein molecule [24]. The server requires inputs for a protein structure in pdb format and a peptide sequence for prediction. The three-dimensional crystal structure of mushroom tyrosinase (PDB ID: 2Y9X) was obtained from the RCSB Protein Data Bank (PDB) at https://www.rcsb.org (accessed on 10th July). For peptide sequence input, peptides with >10 residues were split into equal portions since the maximum size of peptide accepted by PepSite 2 server was 10 residues. The predicted protein–peptide binding spots were ranked according to statistical significance where a \( p \)-value < 0.25 implies significant binding interaction.

2.5. Statistical Analysis

The study was performed in replicates. Statistical analysis was conducted using SPSS version 20.0 (SPSS Institute, Chicago, IL, USA). The results were analyzed using one-way ANOVA. \( p \)-value less than 0.05 implies a significant difference between sample means. T-test was conducted to analyze the significant \( (p < 0.05) \) difference between the experimental and predicted results for model validation.

3. Results and Discussion

3.1. Single-Factor Experiment

The SDS-PAGE profile of the protein bands after enzymatic hydrolysis using different enzyme compositions, S/E ratios and hydrolysis times was shown in Figure 1. Generally, protein bands with MW ranging from 11–48 kDa and >245 kDa were observed in control treatments (L1). According to Abdou, Kim and Sato [25], the possible egg white proteins within or near the molecular weight range include ovomucin (230–8300 kDa), ovomacroglobulin (760–900 kDa), ovotransferrin (77.7 kDa), avidin (60 kDa), ovoinhibitor (49 kDa), ovoglobulin G3 (45 kDa), ovalbumin (44.5 kDa), ovoflavoprotein (32–36 kDa), ovoglobulin G2 (36 kDa), ovomucoid (28 kDa), ovoglobulin G2 (24.4 kDa), lysozyme (14.4 kDa) and cystatin (12.7 kDa). Ovalbumin-related protein X and Y, on the other hand, share similar molecular weight of 50 kDa [26,27]. Notably, the >245 kDa bands were absent after enzymatic hydrolysis, suggesting the successful cleavage of large molecular weight proteins ovomucin and ovomacroglobulin into smaller protein fragments. For T treatment, the protein bands observed after hydrolysis were <17 kDa (Figure 1a). An 11–17 kDa band was observed at \( t = 0.5 \) and 1 h (Figure 1a, Lane 2 and 3) which is likely due to the presence of either lysozyme, cystatin or a subunit (15.6 kDa) of the tetrameric avidin. Protein bands of >25 kDa were also absent as the S/E ratio decreased from 50 to 10 (w/w) (Figure 1, Lane 8–12) where S/E 10 (w/w) treatment (Figure 1a, Lane 8) showed no observable bands at MW > 11 kDa. A <11 kDa band was found in all hydrolysates regardless of the hydrolysis time and S/E ratio, yet there were no reports on egg white proteins within this molecular weight range. This protein band may be contributed by a new, uncharacterized protein or the hydrolysis product from the aforementioned egg white proteins. Similar observations were recorded in C treatment (Figure 1b) yet 17–48 kDa protein bands were observed at S/E 40 and 50 (w/w) (Figure 1b, Lane 11 and 12), suggesting the incomplete hydrolysis of ovalbumin, ovoflavoprotein, ovomucoid or ovogloboprotein when low amount of enzyme was used. Abeyrathne, Lee, Jo, Nam and Ahn [28] had reported the inability of 1% chymotrypsin to hydrolyze 20 mg/mL ovalbumin even up to 24 h. Apart from incomplete hydrolysis by chymotrypsin, the presence of ovoflavoprotein and ovomucoid could be attributed by their high thermal stability as boiling at 100 °C for 30 min could not denature the protein structures [25,29]. In contrast, \( T + C \) treatment showed complete digestion of large MW proteins (25–48 kDa) even when the shortest hydrolysis time at \( t = 0.5 \) h (Figure 1c, Lane 2).
and the least amount of enzyme at S/E 50 (w/w) (Figure 1c, Lane 12) were used. This implies enzyme combination using trypsin and chymotrypsin was more effective in releasing smaller peptides from large protein molecules compared to individual enzyme treatments.

**Figure 1.** SDS-PAGE protein band profiling of albumin hydrolysate by (a) 100% trypsin, T; and (b) 100% chymotrypsin, C and (c) 50% trypsin + 50% chymotrypsin, T+C treatments at various substrate-to-enzyme (S/E) ratios and hydrolysis times. L1, control; L2, t = 0.5 h; L3, t = 1 h; L4, t = 2 h; L5, t = 3 h; L6, t = 4 h; L7, t = 5 h; L8, S/E = 10 (w/w); L9, S/E = 20 (w/w); L10, S/E = 30 (w/w); L11, S/E = 40 (w/w); L12, S/E = 50 (w/w).
DH is an indication of peptide bond cleavage in a protein hydrolysate and is vital in modulating the composition and properties of the peptides produced. The effects of different enzyme compositions, S/E ratio and hydrolysis time on the DH of egg white were summarized in Figure 2. Overall, it was observed that a lower S/E ratio and longer hydrolysis time contributed to higher DH in all T, C and T+C enzyme treatments. The highest DH was recorded in the T+C treatments at S/E ratio 10 (88.3 ± 0.4%; Figure 2a) and t = 5 h (86.1 ± 1.7%; Figure 2b). This could be due to the synergistic effect between the trypsin and chymotrypsin as the enzymes have different preferential cleavage sites. For example, the simultaneous treatment with trypsin and chymotrypsin had significantly reduced the time required for total hydrolysis of cheese whey proteins [30]. Chymotrypsin may cleave the bulky side chains of egg white proteins, exposing more cleavage sites for trypsin actions. Moreover, the resistance of albumin to trypsin digestion was overcome by boiling at 95 °C for 30 min before enzymatic hydrolysis. Heat treatment may have partially denatured or altered the tertiary structure of albumin, making the protein more accessible to both trypsin and chymotrypsin cleavage. A high DH corresponds to more peptide production and is often related to high bioactivity. For instance, Noh and Suh [31] who hydrolyzed egg white liquid using Alcalase, Neutrase, Protamex, Flavourzyme, Collupulin and Ficin had reported a positive correlation between DH and antioxidant activity. Alcalase hydrolysate produced at S/E 50 w/w and 24 h recorded the highest DH (43.2%) and free radical scavenging effects (82.5%) compared to other enzyme treatments. Moreover, high radical scavenging effect (ORAC value 1193.12 and DPPH value 19.05 Trolox EQ µmol g⁻¹) were recorded when the DH of egg white were higher than 50% [32]. Similar conclusion was drawn by Chen, Chi, Zhao and Xu [33] where the antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of egg white protein hydrolysate increased as the DH increased. Thus, together with the SDS-PAGE analysis result, the S/E ratio of 10–30 (w/w) and hydrolysis time of 2–5 h were selected for optimization study as these ranges recorded egg white hydrolysates with small peptides (MW < 17 kDa) and DH >50%.
This is because the summation of X\(^2\) suggested by the software.

The experimental and predicted responses of the 28 generated runs, presented as the mean of triplicate experiments, are shown in Table 2. There was a close agreement between the experimental and predicted responses. X\(_1\) was designated as the slack variable and removed from the model. Hence, a significant (p < 0.05) \(X_2\) reflects the significance of \(X_1\). Square root transformation of data was performed to normalize the data as suggested by the software.

### Table 2. Crossed D-optimal experimental design with the actual and predicted responses for (a) monophenolase and (b) diphenolase inhibitory activities.

| Run | Enzyme Composition (%) | S/E Ratio, \(X_1\) | Hydrolysis Time, \(X_4\) (h) | (a) Monophenolase Inhibitory Activity (%) | (b) Diphenolase Inhibitory Activity (%) |
|-----|------------------------|-------------------|-----------------|---------------------------------------|---------------------------------------|
|     | Trypsin, \(X_1\) | Chymotrypsin, \(X_2\) | Experimental (\(y_1\)) | Predicted \(^*\) (\(y_3\)) | Experimental (\(y_2\)) | Predicted \(^*\) (\(y_4\)) |
| 1   | 100                    | 0 10              | 5                | 32.5 ± 2.8 | 35.6 | 30.7 ± 1.7 | 33.6 |
| 2   | 50                     | 100 20            | 3.5               | 20.8 ± 1.8 | 20.2 | 44.3 ± 4.5 | 44.1 |
| 3   | 0                      | 100 10            | 2                 | 15.2 ± 1.4 | 12.7 | 31.4 ± 3.5 | 32.1 |
| 4   | 0                      | 100 10            | 2                 | 31.6 ± 2.4 | 30.5 | 34.2 ± 4.0 | 33.8 |
| 5   | 100                    | 0 30              | 2                 | 24.7 ± 1.3 | 24.3 | 35.7 ± 2.1 | 36.1 |
| 6   | 0                      | 100 30            | 5                 | 39.1 ± 3.4 | 38.5 | 40.6 ± 1.8 | 40.9 |
| 7   | 0                      | 100 30            | 5                 | 18.9 ± 1.8 | 19.6 | 30.2 ± 2.3 | 31.1 |
| 8   | 100                    | 0 20              | 2                 | 39.5 ± 4.7 | 30.7 | 45.5 ± 3.6 | 48.1 |
| 9   | 0                      | 100 30            | 2                 | 20.0 ± 3.8 | 19.6 | 32.0 ± 0.5 | 31.1 |
| 10  | 50                     | 100 10            | 5                 | 37.2 ± 2.5 | 37.5 | 32.8 ± 0.9 | 31.7 |
| 11  | 0                      | 100 10            | 2                 | 30.2 ± 5.0 | 30.5 | 33.7 ± 1.7 | 33.8 |
| 12  | 50                     | 100 30            | 5                 | 26.1 ± 3.7 | 25.3 | 28.8 ± 2.7 | 30.0 |
| 13  | 100                    | 0 30              | 3.5               | 26.3 ± 3.5 | 27.3 | 32.1 ± 1.7 | 37.3 |
| 14  | 100                    | 0 30              | 2                 | 30.3 ± 4.5 | 32.8 | 35.8 ± 3.9 | 35.6 |
| 15  | 50                     | 100 20            | 2                 | 15.8 ± 3.8 | 16.9 | 44.7 ± 3.9 | 42.5 |
| 16  | 50                     | 100 30            | 3.5               | 19.2 ± 2.8 | 21.0 | 43.4 ± 3.8 | 44.3 |
| 17  | 75                     | 25 25             | 2.75              | 26.1 ± 3.5 | 23.5 | 49.6 ± 3.2 | 47.0 |

Figure 2. Effects of enzyme treatments (100% trypsin, T; 100% chymotrypsin, C and 50% trypsin + 50% chymotrypsin, T+C) on the degree of hydrolysis of egg white proteins at various (a) substrate-to-enzyme (S/E) ratio where the hydrolysis time is fixed at t = 3 h and (b) hydrolysis time where the S/E ratio is fixed at 30 (w/w). Results were reported as means with error bars representing the standard deviations of triplicate experiments.

### 3.2. Optimization of Tyrosinase Inhibitory Activities

The experimental and predicted responses of the 28 generated runs, presented as the mean of triplicate experiments, are shown in Table 2. There was a close agreement between the experimental and predicted responses. Square root transformation of data was performed to normalize the data as suggested by the software.
Table 2. Cont.

| Run | Trypsin, X1 (%) | Chymotrypsin, X2 (%) | S/E Ratio, X3 | Hydrolysis Time, X4 (h) | (a) Monophenolase Inhibitory Activity (%) | Predicted * (y1) | (b) Diphenolase Inhibitory Activity (%) | Predicted # (z1) |
|-----|----------------|----------------------|---------------|--------------------------|------------------------------------------|-----------------|----------------------------------------|-----------------|
| 18  | 0              | 100                  | 20            | 5                        | 21.8 ± 3.6                               | 23.9            | 36.2 ± 3.4                              | 35.1            |
| 19  | 25             | 75                   | 15            | 2.75                     | 19.0 ± 2.1                               | 20.9            | 31.8 ± 2.5                              | 35.0            |
| 20  | 0              | 100                  | 30            | 5                        | 38.4 ± 0.6                               | 38.5            | 41.6 ± 2.2                              | 40.9            |
| 21  | 0              | 100                  | 10            | 5                        | 43.4 ± 4.0                               | 41.5            | 46.6 ± 2.0                              | 48.7            |
| 22  | 0              | 100                  | 10            | 3.5                      | 37.4 ± 4.5                               | 39.7            | 41.7 ± 2.7                              | 39.3            |
| 23  | 75             | 25                   | 20            | 5                        | 48.0 ± 4.1                               | 48.1            | 35.9 ± 2.8                              | 34.5            |
| 24  | 100            | 0                    | 30            | 5                        | 44.7 ± 0.2                               | 44.4            | 25.0 ± 2.3                              | 25.2            |
| 25  | 100            | 0                    | 10            | 5                        | 38.7 ± 0.7                               | 35.6            | 35.7 ± 4.2                              | 33.6            |
| 26  | 100            | 0                    | 10            | 2                        | 35.2 ± 1.3                               | 32.8            | 36.9 ± 1.4                              | 35.6            |
| 27  | 50             | 50                   | 10            | 2                        | 47.2 ± 2.2                               | 45.9            | 37.7 ± 2.5                              | 38.6            |
| 28  | 75             | 25                   | 15            | 3.5                      | 36.1 ± 1.4                               | 34.6            | 39.1 ± 1.7                              | 39.7            |

Note: Data is presented as the mean ± standard deviation of triplicate experiments; * predicted using Equation (4); # predicted using Equation (5).

Based on Table 2a, the experimental monophenolase inhibitory activity ranged from 15.2–48.0% under various test conditions, with the highest inhibition (47.989%) recorded at $X_1 = 75\%$, $X_2 = 25\%$, $X_3 = 25 \text{w/w}$ and $X_4 = 5 \text{h}$. On the other hand, the experimental diphenolase inhibitory activity were ranging from 25.0–49.6% under various test conditions, with the highest inhibition (49.6%) recorded at $X_1 = 75\%$, $X_2 = 25\%$, $X_3 = 25 \text{w/w}$ and $X_4 = 2.75 \text{h}$ (Table 2b). Analysis of variance (ANOVA) was then conducted to evaluate the significance of the coefficient models at a 95% confidence interval (Table 3). The crossed reduced quadratic × cubic model was selected as it recorded a $p$-value of $<0.0001$ for both monophenolase and diphenolase inhibitory activities. The insignificant lack of fit $p$-value of 0.3348 (Table 3a) and 0.2906 (Table 3b) were observed for monophenolase and diphenolase inhibitory activities, respectively, indicating a well-fitted model that is adequate to describe the observed data. In addition, the values of coefficient of determination, $R^2$ and adjusted $R^2$ observed were 0.967 and 0.920, respectively (for monophenolase inhibitory activity), and 0.934 and 0.863, respectively (for diphenolase inhibitory activity), suggesting an excellent fit to the selected model. The coefficient of variation (CV) measures the dispersion of data around the mean. Both models had a low CV of 4.611 and 2.996%, respectively, implying a high precision and low degree of variation of the experiment performed.

Table 3. ANOVA for crossed reduced quadratic × cubic model: estimated regression model of the relationship between the mixture component ($X_1$, $X_2$), process variables ($X_3$, $X_4$) and the response variables (a) monophenolase inhibitory activity (Y) and (b) diphenolase inhibitory activity (Z).

| Source      | Sum of Squares | DF | Mean Square | F Value | Prob > F |
|-------------|----------------|----|-------------|---------|----------|
| Model       | 20.668         | 16 | 1.292       | 20.447  | <0.0001  |
| $X_1$       | 1.678          | 1  | 1.678       | 26.563  | 0.0003   |
| $X_2$       | 0.009          | 1  | 0.009       | 0.139   | 0.7167   |
| $X_3$       | 4.155          | 1  | 4.155       | 65.766  | <0.0001  |
| $X_4$       | 0.075          | 1  | 0.075       | 1.189   | 0.2988   |
| $X_1^2$     | 1.469          | 1  | 1.469       | 23.260  | 0.0005   |
| $X_2^2$     | 0.258          | 1  | 0.258       | 4.079   | 0.0685   |
| $X_3^2$     | 1.728          | 1  | 1.728       | 27.346  | 0.0003   |
| $X_4^2$     | 0.973          | 1  | 0.973       | 15.396  | 0.0024   |
| $X_1X_2$    | 0.873          | 1  | 0.873       | 13.812  | 0.0034   |
| $X_1^2X_3$  | 1.489          | 1  | 1.489       | 23.572  | 0.0005   |
| $X_1^2X_4$  | 1.222          | 1  | 1.222       | 19.342  | 0.0011   |
| $X_2X_3$    | 5.107          | 1  | 5.107       | 80.846  | <0.0001  |
| $X_2X_4$    | 0.217          | 1  | 0.217       | 3.442   | 0.0905   |
| $X_3^2X_4$  | 2.024          | 1  | 2.024       | 32.038  | 0.0001   |
Table 3. Cont.

| Source         | Sum of Squares | DF | Mean Square | F Value | Prob > F |
|----------------|----------------|----|-------------|---------|----------|
| $X_2X_4^2$     | 0.005          | 1  | 0.005       | 0.071   | 0.7943   |
| $X_2X_3X_4$   | 0.073          | 1  | 0.073       | 1.148   | 0.3069   |
| Residual       | 0.695          | 11 | 0.063       |         |          |
| Lack of Fit    | 0.447          | 6  | 0.075       | 1.507   | 0.3348   |
| Pure Error     | 0.247          | 5  | 0.049       |         |          |
| Cor Total      | 21.362         | 27 |             |         |          |
| $R^2$          | 0.967          |    |             |         |          |
| Adjusted $R^2$| 0.920          |    |             |         |          |
| C.V.           | 4.611          |    |             |         |          |

(b) Diphenolase inhibitory activity ($Z$)

| Source         | Sum of Squares | DF | Mean Square | F Value | Prob > F |
|----------------|----------------|----|-------------|---------|----------|
| Model          | 6.078          | 14 | 0.434       | 13.106  | <0.0001  |
| $X_2$          | 0.185          | 1  | 0.185       | 5.573   | 0.0345   |
| $X_3$          | 0.071          | 1  | 0.071       | 2.143   | 0.1669   |
| $X_4$          | 0.519          | 1  | 0.519       | 15.658  | 0.0016   |
| $X_2^2$        | 0.010          | 1  | 0.010       | 0.316   | 0.5833   |
| $X_3^2$        | 1.283          | 1  | 1.283       | 38.730  | <0.0001  |
| $X_4^2$        | 0.214          | 1  | 0.214       | 6.454   | 0.0246   |
| $X_2X_3$       | 0.235          | 1  | 0.235       | 7.104   | 0.0194   |
| $X_2X_4$       | 0.385          | 1  | 0.385       | 11.632  | 0.0046   |
| $X_3X_4$       | 0.258          | 1  | 0.258       | 7.799   | 0.0152   |
| $X_2^2X_3$     | 0.263          | 1  | 0.263       | 7.939   | 0.0145   |
| $X_2^2X_4$     | 1.060          | 1  | 1.060       | 31.990  | <0.0001  |
| $X_2X_3^2$     | 1.771          | 1  | 1.771       | 53.473  | <0.0001  |
| $X_3X_4^2$     | 0.314          | 1  | 0.314       | 9.481   | 0.0088   |
| $X_2X_3X_4$    | 0.040          | 1  | 0.040       | 1.198   | 0.2936   |
| Residual       | 0.431          | 13 | 0.033       |         |          |
| Lack of Fit    | 0.315          | 8  | 0.039       | 1.696   | 0.2906   |
| Pure Error     | 0.116          | 5  | 0.023       |         |          |
| Cor Total      | 6.508          | 27 |             |         |          |
| $R^2$          | 0.934          |    |             |         |          |
| Adjusted $R^2$| 0.863          |    |             |         |          |
| C.V.           | 2.996          |    |             |         |          |

The significant ($p < 0.05$) effect on the monophenolase inhibitory activity was contributed by the linear terms $X_4$ and $X_2$, quadratic term $X_3^2$ and various interaction terms $X_2X_3$, $X_2X_4$, $X_3X_4$, $X_2^2X_3$, $X_2^2X_4$, $X_2X_3^2$ and $X_3^2X_4$, whereas, the significant ($p < 0.05$) effect on the diphenolase inhibitory activity was contributed by the linear terms $X_4$ and $X_2$, quadratic terms $X_3^2$ and $X_4^2$ alongside various interaction terms $X_2X_3$, $X_2X_4$, $X_3X_4$, $X_2^2X_3$, $X_2^2X_4$, $X_2X_3^2$ and $X_3X_4^2$. This suggested the hydrolysis time, enzyme compositions, as well as their interactions with S/E ratio, played a prominent role in the inhibition of these activities. To better fit the model, backward elimination step was carried out to eliminate the non-significant terms. The final response equations for monophenolase and diphenolase inhibitory activities in coded variables are given in Equations (4) and (5), respectively.

\[
\sqrt{\text{Monophenolase inhibitory activity}} = 6.269 - 2.711x_2 + 1.395x_4 - 1.084x_2^2 + 0.612x_4^2 - 3.157x_2x_3 - 2.128x_2x_4 + 0.289x_3x_4 + 2.835x_2^2x_3 + 2.334x_2^2x_4 + 3.45x_2X_3^2 - 0.926x_2x_4^2 - 0.938x_3^2x_4
\]

\[
\sqrt{\text{Diphenolase inhibitory activity}} = 6.943 - 1.258x_2 - 0.269x_4 - 0.952x_3^2 - 0.269x_4^2 + 1.301x_2x_3 - 1.472x_2x_4 - 0.139x_3x_4 - 1.267x_2^2x_3 + 2.244x_2^2x_4 + 1.726x_2x_3^2
\]
3.3. Verification of Predictive Models

Various combination of hydrolysis parameters was suggested to verify the suitability of the predictive models. Taking into consideration the cost, efficiency and feasibility of the experiment, the optimal condition (desirability value of 0.949, which indicating that the suggested condition is close to the desired process condition-minimum amount of enzymes, minimum hydrolysis time and maximum inhibitory activities) to achieve monophenolase inhibitory activity of 45.9% corresponded to 55% trypsin, 45% chymotrypsin, S/E ratio 10:1 (w/w) and hydrolysis time 2 h was examined. The experimental value of 45.3% was found close with no significant (p > 0.05) difference with the predicted value (45.9%). The DH determined was 84.8 ± 1.8%. For diphenolase inhibitory activity, the optimal condition suggested was 100% trypsin, 0% chymotrypsin, S/E ratio 22.13:1 (w/w) and hydrolysis time 3.18 h. The experimental value of 48.1% was found close with no significant (p > 0.05) difference with the predicted value (48.1%). The degree of hydrolysis determined was 64.0 ± 2.1%. Therefore, this model was valid for the optimization of monophenolase and diphenolase inhibitory activities from egg white. It was also observed that in monophenolase inhibitory activity optimization, even though 48.0% was achieved by sample run 23 (Table 2), 5 h of hydrolysis time was required, whereas 2 h of hydrolysis time could achieve 45.3% (the difference by only 2.7%) in the optimized condition. In the optimization of diphenolase inhibitory activity, the optimized sample achieved 48.1% activity by using only trypsin (lower in cost) compared to the sample run 17 (49.6%) in which the difference was only 1.5%. Therefore, it was suggested that the optimization process had managed to achieve the goal of study.

3.4. Identification of Bioactive Peptides

There were 139 and 189 peptides identified for monophenolase and diphenolase inhibitory activities, respectively, using PEAKS Studio (Appendix A Table A1). For monophenolase inhibitory activity, 76, 36, 4, 7, 11, 4 and 1 peptides were identified from ovalbumin, ovotransferrin, ovomucoid, ovalbumin-related protein X, ovalbumin-related protein Y, Ovomucin, and cystatin, respectively, where they were found to achieve 77%, 45%, 42%, 38%, 32%, 16% and 12% coverages of the corresponding protein sequences (Appendix A Table A1a). For diphenolase activity, 81, 54, 9, 12, 4, 8, 20 and 2 peptides identified were found to match 81%, 63%, 54%, 38%, 29%, 28%, 26% and 2% sequence coverages of ovalbumin, ovotransferrin, lysozyme, ovomucoid, ovalbumin-related protein X, ovalbumin-related protein Y, Ovomucin and ovostatin, respectively (Appendix A Table A1b). Subsequently, PeptideRanker web server was used to screen for potential biologically active peptides since the likeliness of being bioactive is usually governed by specific structural characteristics of peptide [23]. The use of PeptideRanker for initial screening and prediction had been proven successful to identify bioactive peptides with wide array of bioactivities [34–36]. Therefore, there were 7 and 21 peptides (PeptideRanker score >0.5) shortlisted for monophenolase and diphenolase inhibitory activities, respectively. The shortlisted peptides were further subjected to structure-activity relationship analysis with mushroom tyrosinase using the PepSite2 web server.

The p-values of mushroom tyrosinase-peptide binding interactions predicted by PepSite 2 web server were summarized in Table 4. A smaller p-value signifies higher potential of peptide binding to the enzyme. The smallest p-value was recorded by ADHPF (0.002658), AFKDEDTKAMPF (0.02053) and ILELPFASGDLLML (0.03464) whereas the largest p-value was recorded by DGSGGCIKP (0.1274) for monophenolase inhibitory activity (Table 4a). For diphenolase activity, SDFHLGFPPGK (0.009412), FDGRSK (0.01312) and FNCSSAGPGAIGSEC (0.01614) were among the peptides with the smallest p-values whereas YFGYTGALR had recorded the largest p-value of 0.2364 (Table 4b). Overall, all peptides showed significant (p < 0.25) binding interactions with mushroom tyrosinase. Notably, phenylalanine, leucine and alanine were frequently observed in the peptide sequences. Phenylalanine may act as the pseudo-substrate of tyrosinase since it is structurally identical to tyrosine, the natural substrate of tyrosinase. Besides, the hydrophobic side chains of leucine and alanine may interact directly with the hydrophobic binding pocket of tyrosinase to cause enzyme inhibition. According to
Strothkamp, Jolley and Mason [37], mushroom tyrosinase is a tetramer comprising of two H subunits and two L subunits. The L subunit is the product of Orf239342 gene and possesses a lectin-like structure, hence annotated as mushroom tyrosinase associated lectin-like protein. It comprises residues 9–28 and 35–150 of ORF239342 protein and is arranged into 12 antiparallel β-strands that is located away from the tyrosinase catalytic site, suggesting an insignificant role in enzyme activity [38]. The L subunit was postulated to provide innate immunity against bacterial infection [39] and act as a cofactor in melanin production [40]. In contrast, the H subunit originates from ppo3 gene and covers residues 2–392 of PPO3. This subunit is made up of 13 α-helices, 8 short β-strands and loops that structured the catalytically essential tyrosinase core domain [38,41]. The H subunit houses a binuclear copper active site where copper A is coordinately bonded with H61, H85 and H94 whereas copper B to H 259, H263 and H296. The 6 histidine residues form a hydrophobic binding pocket at the bottom of the H subunit and H263 is postulated to regulate proper orientation of incoming substrate [38]. The structural rigidity of the binding pocket is maintained by several interactions between the 6 histidine and their neighboring residues. For instance, the side chain rotational freedom of H85 is restricted through the formation of a thioether bond with the side chain of C83. This thioether bond stabilizes H85 and is also suggested to optimize redox potential as well as to facilitate rapid electron transfer for the redox reactions occurring in the binuclear copper site [38,42]. Furthermore, the presence of F90 confers structural constraints to H94, H259 and H296 while F292 limits the side chain flexibility of H61, H263 and H296 [38]. The interaction between M280 and the aromatic ring of histidines also stabilizes the protein structure [43] and this residue may aid in copper incorporation into the binding pocket [44]. Notably, the ILELPFASGDLLML for monophenolase inhibitory activity (Table 4a) and GYSLGNWVCAAK, YFGYTGALRCLV, HIATNAVLFFGR, FMMFESQNKDLLFK, SGALHCLK and YFGYTGALR for diphenolase inhibitory activity (Table 4b) were found to interact with H61, H85, H94, H259, H263, and H296 (hotspot residues) and F92, F292 and M280 (stabilizing residues) of mushroom tyrosinase. The peptide interactions with the hotspot residues may weaken or hinder enzyme binding with its putative substrate whereas peptide interactions with the stabilizing residues may disrupt the integrity of active site, which reduces the catalytic potency of the enzyme. Thus, ILELPFASGDLLML, GYSLGNWVCAAK, YFGYTGALRCLV, HIATNAVLFFGR, FMMFESQNKDLLFK, SGALHCLK and YFGYTGALR represent potential tyrosinase inhibitory peptides.
Table 4. List of potential biologically active egg white-derived peptides shortlisted for (a) monophenolase and (b) diphenolase inhibitory activities using PeptideRanker web server and their potential binding sites on mushroom tyrosinase predicted using PepSite 2 web server.

| No. | Peptide Sequence | Egg Protein Fragment | Peptide Length | Potential Binding Sites of Mushroom Tyrosinase (PDB ID: 2Y9X) | PepSite 2 p-Value |
|-----|------------------|----------------------|----------------|-------------------------------------------------------------|------------------|
|     |                  |                      |                | (a) Monophenolase inhibitory activity                        |                  |
| 1   | ADHPF            | Ovalbumin            | 5              | Y140, K389, H390                                          | 0.002658         |
| 2   | AFKDEDTKAMPF     | Ovalbumin            | 12             | N22, F135, D137, Y140, R301, P366, D367, W386, H390, Y391, Y36, L40, F54, G58, H61, H85, F90, H94, W101, Q133, H259, H263, M280, H285, A286, A287, F288, D289, F290, F292, W293, H296 | 0.02053          |
| 3   | ILELPSGDDLML     | Ovalbumin-related protein X | 14          | H263, M280, H285, A286, A287, F288, D289, F290, F292, W293, H296 | 0.03464          |
| 4   | DKLPFGED         | Ovalbumin            | 8              | Y140, P370, Y382, W386, K389, H390                         | 0.05277          |
| 5   | FKLPFGED         | Ovalbumin            | 9              | Y140, P370, Y382, W386, K389, H390                         | 0.0722           |
| 6   | DMLPDGEQSGTSVN  | Ovalbumin            | 20             | Y140, T233, R301, M309, D367, Y382, N384, W386, H388, K389, H390 | 0.08107          |
| 7   | DGSSGCIPK        | Ovomucin             | 9              | Y140, R313, D367, Y382, W386, K389, H390                   | 0.1274           |
|     |                  |                      |                | (b) Diphenolase inhibitory activity                          |                  |
| 1   | SDFHLCPPGBK      | Ovotransferrin       | 11             | H61, H85, H94, 197, Y140, H259, H263, M280, V283, A287, F292, W293, H296, H61, H85, F90, Y97, Y140, R301, P366, D367, W386, H390, Y391 | 0.009412         |
| 2   | FDGRSR           | Ovotransferrin       | 6              | D137, R301, P366, D367, W386, H390, Y391                   | 0.01312          |
| 3   | FNCSAGFAGMCGSEC  | Ovotransferrin       | 15             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.01614          |
| 4   | MYOQGFLR         | Ovomucin             | 8              | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.01614          |
| 5   | GYSLGNWVCAAK     | Lysozyme             | 12             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.01832          |
| 6   | DLEKDFSAIMLK     | Ovotransferrin       | 12             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.02538          |
| 7   | CQCOQSKGHPPEK    | Ovotransferrin       | 14             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.02578          |
| 8   | ADHPFLF          | Ovalbumin            | 7              | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.03051          |
| 9   | SGAFHCLK         | Ovotransferrin       | 8              | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.03994          |
| 10  | YFGYTGALRCLVL    | Ovotransferrin       | 12             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.04488          |
| 11  | HATNAVLFGR       | Ovalbumin            | 12             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.04707          |
| 12  | FKDEDTQAMPFR     | Ovalbumin            | 12             | H61, H85, H94, 197, Y140, R301, P366, D367, W386, H390, Y391 | 0.05594          |
Table 4. Cont.

| No. | Peptide Sequence | Egg Protein Fragment | Peptide Length | Potential Binding Sites of Mushroom Tyrosinase (PDB ID: 2Y9X) | PepSite 2 \( p \)-Value |
|-----|------------------|----------------------|----------------|-------------------------------------------------------------|--------------------------|
| 13  | FMMFESQNKDLFK    | Ovotransferrin       | 14             | H61, N81, Y92, C83, T84, H65, F90, W93, H94, Y97, D137, Y140, H259, H263, M280, A286, A287, F292, W293, H296 | 0.06287 |
| 14  | FDKLPFGCD        | Ovalbumin            | 9              | Y140, P370, Y382, W386, K389, H390                          | 0.07225 |
| 15  | SMLVLPDEVGQLESINFEK | Ovalbumin          | 24             | D137, Y140, R301, D367, P370, Y382, W386, K389, H390, Y391 | 0.08195 |
| 16  | SGGYSAFHCLK      | Ovotransferrin       | 11             | Y140, R301, P366, D367, Y382, W386, H390                    | 0.0849 |
| 17  | SGGQFSLSTVKEC    | Ovomucin             | 14             | D137, Y140, R301, D367, W386, H390, Y391                    | 0.08667 |
| 18  | SGGALHCLK        | Ovotransferrin       | 8              | H61, N81, Y92, C83, T84, H65, W93, H94, R95, Y97, E98, H259, H263, A286, A287, F292, W293, H296 | 0.1247 |
| 19  | SSCICS           | Ovomucin             | 6              | N22, F135, D137, R301, P366, D367, W386, H390              | 0.1938 |
| 20  | SSGELDCVCT       | Ovomucin             | 9              | D137, Y140, R301, D367, W386, H390, Y391                    | 0.2086 |
| 21  | YFGYTGALR        | Ovotransferrin       | 9              | H61, H85, H94, Y97, H259, H263, M280, V283, A286, A287, F292, W293, H295, H296, V299 | 0.2364 |

Note: Underlined residues are actively involved in binding interaction with mushroom tyrosinase; Residues in bold indicate mushroom tyrosinase hotspots; Residues in italics indicate stabilizing residues.
On the other hand, majority of the peptides were also found to bind to Y140, W386 and H390 (Table 4) which were not within the mushroom tyrosinase substrate binding pocket. Hassani Hagnbeen and Fazli [45] reported two mixed-type inhibitors of tyrosinase, phthalic acid and cinnamic acid, each bound to different binding sites of the enzyme. For instance, phthalic acid formed hydrogen bonds with W136, W141 and G149 and van der Waals interactions with D137, W138, G139, Y140, F147 and F224 whereas cinnamic acid form hydrogen bonds with Q307 and D312 and van der Waals interactions with T308, Y311, V313, Y314, E356 and W358. Jung et al. [46] also reported a mixed-type tyrosinase inhibitor, (E)-2-(2,4-dihydroxybenzylidene)-2,3-dihydro-1H-inden-1-one (BID3) which formed a hydrogen bond with Y140 and interacted hydrophobically with L24, F147 and I148. These findings suggest potential peptide interaction with non-specific binding site of the enzyme since the allosteric site of mushroom tyrosinase has yet to be identified.

4. Conclusions

In this study, egg white has been proven to be more than just a food component. The optimization of enzymatic hydrolysis conditions, LC/MS MS/MS peptide identification and sequencing followed by structure-activity relationship analyses had corroborated the potential of this food protein as a source for the production of anti-tyrosinase peptides to prevent skin hyperpigmentation. Nonetheless, the monophenolase and diphenolase inhibitory peptides identified will next be chemically synthesized and validated for their in vitro anti-tyrosinase efficacies before proceeding to in vivo assays to examine their effects on the melanogenesis pathway regulatory proteins.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. List of egg white-derived peptides for (a) monophenolase and (b) diphenolase inhibitory activities with their corresponding PeptideRanker scores.

| No. | Egg Protein Sequence Coverage | Peptide | Peptide Sequence Number | Score |
|-----|------------------------------|---------|--------------------------|-------|
| (a) Monophenolase inhibitory activity |
| 1   | ADHPF *                      | 361–365 | 0.8592 |
| 2   | FDKLPGFGD *                  | 60–68   | 0.6817 |
| 3   | FDKLPFGDFSIEAQGSTVN *        | 60–79   | 0.5429 |
| 4   | AFKDEDTKAMPF *               | 188–199 | 0.5332 |
| 5   | DKLPGFGD *                   | 61–68   | 0.5174 |
| 6   | MSALAM                       | 36–41   | 0.4941 |
| 7   | RGGLPEINF                    | 127–135 | 0.4842 |
| 8   | YILPEYL                      | 112–119 | 0.4732 |
| 9   | AFKDEDTQAMPF                 | 188–199 | 0.4636 |
| 10  | VLLPDEVSGLEKLESIINF          | 244–262 | 0.4494 |
| 11  | QVLPDEVSGLEQULESIINF         | 243–262 | 0.4335 |
| 12  | KEDTQAMPF                    | 190–199 | 0.4113 |
| 13  | FDKLPFGDSIEAQ               | 60–73   | 0.4067 |
| 14  | YPLPELYLVCKELY              | 112–126 | 0.3960 |
| 15  | VLPHEVSQLEQLESIINF          | 244–262 | 0.3712 |
| 16  | LQPLDEVSGLEQLESIINF         | 243–262 | 0.3593 |
| 17  | LPDEVSGLEQLESIINF           | 246–262 | 0.3544 |
| 18  | MAMGITDVF                   | 299–307 | 0.3250 |
| 19  | SSSANLSGISSAESLK            | 308–323 | 0.3207 |
Table A1. Cont.

| No. | Egg Protein Sequence Coverage | Peptide | Peptide Sequence Number | Score |
|-----|-------------------------------|---------|-------------------------|-------|
| (a) Monophenolase inhibitory activity |
| 20  | LVLLPDEVSGLEQLESINFE         | 243–262 | 0.3189                  |
| 21  | VHHANENIFY                   | 21–30   | 0.3132                  |
| 22  | VTEQESKPVQMMYXQLF            | 210–218 | 0.3126                  |
| 23  | VHANENIF                     | 21–29   | 0.2953                  |
| 24  | GGLPINFQTAADQAR              | 128–143 | 0.2810                  |
| 25  | VLLPDEVSGLEQLESINFE          | 244–263 | 0.2738                  |
| 26  | VASMAEKMK                    | 220–229 | 0.2728                  |
| 27  | AAHAEINEAGR                  | 330–340 | 0.2679                  |
| 28  | AEERYPILPEYL                 | 108–119 | 0.2648                  |
| 29  | YPILEPYLQ                    | 112–120 | 0.2638                  |
| 30  | ISQAVHAAHAEINEAGR            | 324–340 | 0.2599                  |
| 31  | SANLSGIIASESLK               | 310–323 | 0.2537                  |
| 32  | SVVESQTNQIR                  | 148–159 | 0.2523                  |
| 33  | AKFDEDTQAMP                  | 189–198 | 0.2456                  |
| 34  | SGIassaelsk                  | 314–323 | 0.2242                  |
| 35  | MAMGITDVFFSSANLSGIS          | 299–317 | 0.2239                  |
| 36  | INSVESQTNQIIIR              | 146–159 | 0.2193                  |
| 37  | LYAERYIPILPEYL               | 106–119 | 0.2015                  |
| 38  | SGIassaesl                   | 314–322 | 0.1982                  |
| 39  | VLLPDEVSGLEQLESINFE          | 244–264 | 0.1885                  |
| 40  | VLLPDEVSGLEQIS              | 244–256 | 0.1835                  |
| 41  | VLLPDEVSGLEQIS              | 313–323 | 0.1793                  |
| 42  | HAEINEAGR                    | 332–340 | 0.1789                  |
| 43  | SAEAGVEDAASVEEF              | 345–359 | 0.1784                  |
| 44  | VLLPDEVSGLEQESIN           | 244–261 | 0.1723                  |
| 45  | EVVSGSAEAVGDAASVSEEF         | 341–360 | 0.1703                  |
| 46  | NVLQPSSVHSQTAM              | 160–173 | 0.1699                  |
| 47  | AMGITDVFFS                  | 300–309 | 0.1674                  |
| 48  | SSNVMEEM                    | 270–276 | 0.1638                  |
| 49  | EVVSGSAEAVGDAASVSEEF         | 341–359 | 0.1629                  |
| 50  | SGIassaesl                   | 314–323 | 0.1544                  |
| 51  | ELINSWVESQTNQIR             | 144–159 | 0.1482                  |
| 52  | SGIassaesl                   | 314–323 | 0.1469                  |
| 53  | VTEQESKPVQMM                 | 201–212 | 0.1453                  |
| 54  | AEINEAGR                     | 333–340 | 0.1437                  |
| 55  | VLPQSSVDSQTAM               | 161–173 | 0.1431                  |
| 56  | VESQTNQIIIR                 | 150–159 | 0.1401                  |
| 57  | NLQPSSVDSQTAM               | 160–173 | 0.1362                  |
| 58  | VLLPDEVSGLEQLESR            | 244–259 | 0.1282                  |
| 59  | VLLPDEVSGLEKESIINF          | 244–264 | 0.1189                  |
| 60  | VASMAEK                     | 220–227 | 0.1086                  |
| 61  | ISQAVH                      | 324–329 | 0.1070                  |
| 62  | TSSNVMEER                   | 269–277 | 0.1050                  |
| 63  | VLLPHEVSGLEQLES             | 244–258 | 0.0986                  |
| 64  | QITKPNDVY                   | 90–98   | 0.0968                  |
| 65  | DILNQITKPNDVY               | 86–98   | 0.0951                  |
| 66  | VLLPDEVSGLEQLES             | 244–258 | 0.0916                  |
| 67  | NQITKPNDVY                  | 89–98   | 0.0879                  |
| 68  | LTEWTSSNVMEER               | 265–277 | 0.0807                  |
| 69  | VTEQESKPVQMK                | 201–212 | 0.0805                  |
| 70  | RVTEQESKPVQMM               | 200–211 | 0.0787                  |
| 71  | NQITEPNDVY                  | 89–98   | 0.0742                  |
| 72  | VTEQESKPVQ                 | 201–211 | 0.0712                  |
| 73  | VTEQESKPV                   | 201–209 | 0.0395                  |
| 74  | NVLQPSSVDSQTAMVLVNAIVFK     | 160–182 | 0.0365                  |
| 75  | NVLQPSSVDSQTAMVLVNAIVF      | 160–181 | 0.0218                  |
| 76  | KTSSNVWNINLKL               | 458–468 | 0.4883                  |
| 77  | GEADAVALHGGLVY              | 406–419 | 0.4290                  |
| 78  | NLQMDDFELL                  | 579–588 | 0.4262                  |
| 79  | NLKMDDFELL                  | 579–588 | 0.3988                  |
| No. | Egg Protein Sequence Coverage | Peptide | Peptide Sequence Number | Score  |
|-----|-------------------------------|---------|-------------------------|--------|
| 81  |                               | KDQLTPSPR | 352–360                | 0.3329 |
| 82  |                               | VPSLMHSQLY | 329–338                | 0.3155 |
| 83  |                               | GAIEWEGIESGSVEKAVAK | 155–173            | 0.2986 |
| 84  |                               | KGeadavaldgglvy | 405–419            | 0.2793 |
| 85  |                               | TAGVCGLVPVMAER | 420–433              | 0.2662 |
| 86  |                               | VPSLMDSQLY | 329–338                | 0.2612 |
| 87  |                               | AQSDFGVTDK | 289–298                | 0.2525 |
| 88  |                               | GAIEWEGIESGSVEQAVAK | 155–173            | 0.2308 |
| 89  |                               | RVPSLMDSQLY | 328–338              | 0.2249 |
| 90  |                               | VPVMAER | 427–433                | 0.1784 |
| 91  |                               | VAAHAVVAR | 266–274                | 0.1704 |
| 92  |                               | GAIEWEGIESGSVEQAVAE | 155–173            | 0.1683 |
| 93  |                               | AIEWEGIESGSVEQAVAK | 156–173            | 0.1624 |
| 94  |                               | LKPIAAEYY | 93–101                | 0.1209 |
| 95  |                               | HAVVPRPEK | 611–619                | 0.1184 |
| 96  |                               | TVNDQLGK | 124–131                | 0.1055 |
| 97  |                               | AVVVPRPEK | 612–619                | 0.0953 |
| 98  |                               | HTTVNENAPDQDKEYELL | 229–246            | 0.0946 |
| 99  |                               | EIEGIEGSVEQAVAK | 160–173              | 0.0919 |
| 100 |                               | TDERPASY | 443–450                | 0.0891 |
| 101 |                               | TKHSTVEENGTGK | 559–571            | 0.0865 |
| 102 |                               | TVNDLQGK | 124–131                | 0.0847 |
| 103 |                               | DLTQQER | 44–50                  | 0.0846 |
| 104 |                               | TVQHSTVEENGTGK | 559–571            | 0.0835 |
| 105 |                               | EGIESGSVEQAVAK | 160–173              | 0.0768 |
| 106 |                               | TVISSL | 682–688                | 0.0710 |
| 107 |                               | HHTVNENAPDQDKEYELL | 229–246            | 0.0630 |
| 108 |                               | EIEGIESGSVEQAVAE | 160–173              | 0.0600 |
| 109 |                               | VVVPRPEK | 613–619                | 0.0580 |
| 110 |                               | TVEENGTGK | 563–571                | 0.0491 |
| 111 |                               | ISDAVHGF | 324–332                | 0.4692 |
| 112 |                               | MISDAVHGF | 323–332              | 0.4224 |
| 113 |                               | VAAHAVVARDNDNQVEDIW | 266–283            | 0.2846 |
| 114 |                               | HSLELEEFR | 354–362              | 0.2286 |
| 115 | Ovalbumin-related protein Y   | SLEIADKLY | 99–107                | 0.1898 |
| (32%)|                               | VLLPDEVSLER | 244–255              | 0.1871 |
| 116 |                               | VLLPDEVSLERIEKTN | 244–261            | 0.1605 |
| 117 |                               | MEVNEEGTATGSTGAIGNIK | 333–353             | 0.1211 |
| 118 |                               | TGGVEEVNFK | 127–136              | 0.1200 |
| 119 |                               | NVATLPAEK | 219–227                | 0.1152 |
| 120 |                               | VAAHAVVARDNDNQVEDIW | 74–87              | 0.8643 |
| 121 | Ovalbumin-related protein X   | ILELPASDGDLML | 74–87                | 0.8223 |
| (38%)|                               | TGISSAEHLK | 158–167              | 0.1152 |
| 122 |                               | NVATLPAEK | 63–71                  | 0.1084 |
| 123 |                               | VLLPDEVSDLER | 88–99             | 0.1070 |
| 124 |                               | VLLPDEVSLERIEK | 166–173            | 0.1070 |
| 125 |                               | AGSTGVIEDIK | 187–197            | 0.1025 |
| 126 |                               | VTKQESKPVQV | 45–55                | 0.0833 |
| 127 |                               | FPNAVTDKEGK | 32–41                | 0.3269 |
| 128 |                               | DLRPICGTIDGVTY | 49–61              | 0.2154 |
| 129 |                               | VEQGASVDR | 137–146                | 0.0903 |
| 130 |                               | VEQGASVDR | 137–146                | 0.0734 |
| 131 |                               | DGSGGCIPK | 814–822                | 0.6103 |
| 132 |                               | VTDSF | 1591–1595             | 0.2004 |
| 133 |                               | SNSLVLTPQA | 1494–1503           | 0.1384 |
| 134 |                               | IOQEATDPGAEK | 941–952            | 0.1215 |
| 135 |                               | LLGAPPVDENEDEQLQR | 30–46              | 0.2450 |
Table A1. Cont.

| No. | Egg Protein Sequence Coverage | Peptide | Peptide Sequence Number | Score  |
|-----|-------------------------------|---------|------------------------|--------|
| (b) Diphenolase inhibitory activity | | | | |
| 1   | ADHPFLF*                      | 361–367 | 0.9699                 |
| 2   | MYQGLFR*                      | 212–219 | 0.8011                 |
| 3   | SMLVLLPDEVSGLEQLESINFEK*       | 241–264 | 0.6983                 |
| 4   | FDKLPGFGD*                    | 60–68   | 0.6817                 |
| 5   | HIATNAVLFGER*                 | 371–382 | 0.5209                 |
| 6   | FKDDETDQAMPFR*                | 189–200 | 0.5001                 |
| 7   | DEDTKAMPFR                    | 191–200 | 0.4978                 |
| 8   | ILELFASGTMS                   | 230–241 | 0.4972                 |
| 9   | DEDTQAMPFR                    | 191–200 | 0.4842                 |
| 10  | MLVLLPDEVSGLEQLESINFEK        | 242–264 | 0.4842                 |
| 11  | YPILPEYLQCVK                  | 112–123 | 0.4769                 |
| 12  | AFKDDETDQAMPFR                | 188–200 | 0.4702                 |
| 13  | SSANLSGISSAESLKR              | 308–323 | 0.3207                 |
| 14  | SQAVHAAHAEINEAGR              | 325–340 | 0.2965                 |
| 15  | VTEQESKPVQMMYQQLFR            | 201–219 | 0.2886                 |
| 16  | GLEPINFQTAADQAR               | 128–143 | 0.2810                 |
| 17  | SQTAMVLVNAIVFK                | 169–182 | 0.2781                 |
| 18  | VASMASEMK                     | 220–229 | 0.2728                 |
| 19  | AAHAINEAGR                    | 330–340 | 0.2679                 |
| 20  | YPILPEYLQ                     | 112–120 | 0.2638                 |
| 21  | QAVHAAHAEINEAGR               | 326–340 | 0.2606                 |
| 22  | ISQAVHAAHAEINEAGR             | 324–340 | 0.2599                 |
| 23  | EAQCGTSNVHSSLR                | 71–85   | 0.2554                 |
| 24  | SANLSGISSAESLKR               | 310–323 | 0.2537                 |
| 25  | SSANLSGISSAESLKR              | 309–323 | 0.2497                 |
| 26  | EVCGSAEAGVDAASVSEEFR          | 341–360 | 0.2424                 |
| 27  | GLEPINFQTAADQAR               | 129–143 | 0.2420                 |
| 28  | VLLPDEVSGLEQLESINFEQ          | 244–264 | 0.2410                 |
| 29  | VLVNAIVFK                     | 174–182 | 0.2362                 |
| 30  | NSQAVHAAHAEINEAGR             | 324–340 | 0.2273                 |
| 31  | ISQAVHAAHAEIN                 | 324–336 | 0.2267                 |
| 32  | DLNQTPTKPDVYVSFLASR           | 86–105  | 0.2229                 |
| 33  | FQTAADQAR                     | 135–143 | 0.2202                 |
| 34  | VLVNAIVFK                     | 174–182 | 0.2049                 |
| 35  | MAMGIDTFVSSSANLSGISSAESLKR    | 299–323 | 0.1963                 |
| 36  | AVHAHAHAINEAGR                | 327–340 | 0.1935                 |
| 37  | SVNVHSSLR                     | 77–85   | 0.1930                 |
| 38  | LCEWTTSSNVMEER                | 265–277 | 0.1886                 |
| 39  | VLLPDEVSGLEQLESINFEK          | 244–264 | 0.1885                 |
| 40  | DLIPINFQTAADQAR               | 129–143 | 0.1854                 |
| 41  | LSGIASSAESL                  | 313–323 | 0.1793                 |
| 42  | HAEINEAGR                     | 332–340 | 0.1789                 |
| 43  | LEPINFQTAADQAR                | 130–143 | 0.1739                 |
| 44  | EVCGSAEAGVDAASVSEEFR          | 341–360 | 0.1703                 |
| 45  | DILNQITKPDVYYSF               | 86–100  | 0.1698                 |
| 46  | EAGVDAASVSEEFR                | 437–360 | 0.1664                 |
| 47  | AEAGVDAASVSEEFR               | 346–360 | 0.1641                 |
| 48  | VHAHAHAINEAGR                 | 328–340 | 0.1633                 |
| 49  | EVCGAEGVDAASVSEEFR            | 341–360 | 0.1628                 |
| 50  | EPINFQTAADQAR                 | 131–143 | 0.1588                 |
| 51  | ISQAVHAAH                     | 324–332 | 0.1572                 |
| 52  | ELINSWVESQTNGIIR             | 144–159 | 0.1482                 |
| 53  | VTEQESKPVQMM                  | 201–212 | 0.1453                 |
| 54  | VTEQESKPVQMMY                | 201–213 | 0.1416                 |
| 55  | RVTQESKPVQMMY                | 200–213 | 0.1413                 |
| 56  | GITHVSSSANLSGISSAESLK         | 302–323 | 0.1401                 |
| 57  | VLVNAIVFE                    | 174–182 | 0.1401                 |
| 58  | AMNGTDVFSSSANLSGISSAESLK     | 300–323 | 0.1395                 |
| 59  | NVLPSSVDQTAM                 | 160–173 | 0.1362                 |
| 60  | EWTSSNVMEER                  | 267–277 | 0.1280                 |
Table A1. Cont.

| No. | Egg Protein Sequence Coverage | Peptide | Peptide Sequence Number | Score |
|-----|--------------------------------|---------|-------------------------|-------|
| 61  | GTSVN VHSSLR                  | 75–85   | 0.1274                  |
| 62  | LQPSVDQSQTAMLVNAIVFK          | 162–182 | 0.1259                  |
| 63  | AMGTDVFSSANLSGISSAESEKL       | 300–323 | 0.1239                  |
| 64  | VTEQESKPKVM                   | 201–211 | 0.1172                  |
| 65  | GITDVFSSANLSGISSAESEKL        | 302–323 | 0.1089                  |
| 66  | VASMAEK                       | 200–227 | 0.1086                  |
| 67  | TSSNVMEER                     | 269–277 | 0.1050                  |
| 68  | DLINQTKPNDVY                  | 86–98   | 0.0951                  |
| 69  | LTEWTSNVMEER                  | 265–277 | 0.0807                  |
| 70  | ELINSWVEQTN                   | 144–155 | 0.0798                  |
| 71  | TEWTSSNVMEER                  | 266–277 | 0.0763                  |
| 72  | IYAEER                        | 106–111 | 0.0759                  |
| 73  | EAVGDAASV5                    | 347–356 | 0.0755                  |
| 74  | VASMAEK                       | 220–227 | 0.0721                  |
| 75  | VTEQESKPVQOM                  | 201–211 | 0.0712                  |
| 76  | TQINK                         | 52–56   | 0.0607                  |
| 77  | NVKVR                         | 55–59   | 0.0591                  |
| 78  | VTEQESKPQVMQMYQIGLFRVASMAEK   | 201–227 | 0.0512                  |
| 79  | NVLKPSVDQSQTAMLVNAIVFK        | 160–182 | 0.0486                  |
| 80  | NVLQPSVDQSQTAMLVNAIVFK        | 160–182 | 0.0365                  |
| 81  | SDHFLGFPGPK *                | 299–309 | 0.8619                  |
| 82  | SCYGAFHCLK *                 | 208–218 | 0.8239                  |
| 83  | SCAHCLK *                     | 211–218 | 0.8038                  |
| 84  | CQLQSGGGIPPEK *              | 518–531 | 0.6874                  |
| 85  | YFQYGTALRCLV *               | 540–551 | 0.6541                  |
| 86  | DLLFKDAMIL *                 | 216–327 | 0.6400                  |
| 87  | SGALHCLK *                    | 211–218 | 0.5971                  |
| 88  | FMMFESQKDLFLK *              | 644–657 | 0.5892                  |
| 89  | YFGYTALR *                   | 540–548 | 0.5801                  |
| 90  | KDSNVWNNLK                   | 458–468 | 0.4863                  |
| 91  | DDNVEWDSLK                   | 275–288 | 0.4641                  |
| 92  | SGIPPEK                       | 524–531 | 0.4622                  |
| 93  | NIPGTLHHR                    | 145–155 | 0.4328                  |
| 94  | DLLFKDAMIL *                 | 316–327 | 0.4322                  |
| 95  | SAIQSMR                       | 345–351 | 0.4293                  |
| 96  | ANVMDYR                       | 595–601 | 0.4255                  |
| 97  | FFASASCPGATIEQK              | 174–188 | 0.4152                  |
| 98  | NIPGTLHHR                    | 145–154 | 0.3756                  |
| 99  | TSCHTGLGR                    | 132–140 | 0.3624                  |
| 100 | GAIEWREGIESGSGVEQAVAKFFSASCPG| 155–183 | 0.3503                  |
| 101 | FMMFESKNK                    | 644–652 | 0.3416                  |
| 102 | KDQLTPSPR                    | 352–360 | 0.3329                  |
| 103 | FMMFESQNK                    | 644–652 | 0.3265                  |
| 104 | GDVAFVK                      | 222–228 | 0.3236                  |
| 105 | EAGLAPYK                     | 85–92   | 0.3173                  |
| 106 | GLIHNK                       | 488–493 | 0.3124                  |
| 107 | VEDIWSFSLSE                  | 278–288 | 0.3066                  |
| 108 | GAIEWREGIESGSGVEKAVAK        | 155–173 | 0.2986                  |
| 109 | DKGDVAEVK                    | 219–228 | 0.2939                  |
| 110 | SDFGVDTK                     | 291–298 | 0.2580                  |
| 111 | AQDSDFGVT DK                 | 289–298 | 0.2525                  |
| 112 | GDVAFK                       | 222–228 | 0.2525                  |
| 113 | TDERPASYF                    | 443–451 | 0.2356                  |
| 114 | GAIEWREGIESGSGVEQAVAK        | 155–173 | 0.2308                  |
| 115 | AAHAVAR                      | 267–274 | 0.2295                  |
| 116 | GAIEWREGIESGSGVEQAVAK        | 155–173 | 0.2076                  |
| 117 | GAIEWREGIESGSGVEQAVAK        | 155–173 | 0.2067                  |
| 118 | AQDSFGVDT E                  | 289–298 | 0.1852                  |
| 119 | FYTVISSLLKTCP NS             | 680–693 | 0.1792                  |
| 120 | VAAHAVVAR                    | 266–274 | 0.1704                  |

(b) Diphenolase inhibitory activity
Table A1. Cont.

| No. | Egg Protein Sequence Coverage | Peptide | Peptide Sequence Number | Score |
|-----|-------------------------------|---------|-------------------------|-------|
| 121 | (-) | GAIEWEGIESGSVEQVAEE | 155–173 | 0.1683 |
| 122 | FGVHGSEK | 634–641 | 0.1601 |
| 123 | FGVNCSEKSK | 634–643 | 0.1364 |
| 124 | RFGVNGSEK | 633–641 | 0.1351 |
| 125 | GDVAFQHQSTVEENTGGK | 554–571 | 0.1323 |
| 126 | KGTEFTVNDLQGK | 119–131 | 0.1190 |
| 127 | GTEFTVNDLQGK | 120–131 | 0.1166 |
| 128 | DVAFIQHSTVEENTGGK | 555–571 | 0.1158 |
| 129 | KCVAS | 531–535 | 0.1136 |
| 130 | TDERPASY | 443–450 | 0.0891 |
| 131 | LKPAAAEYEHTEGTSYY | 93–112 | 0.0753 |
| 132 | YTVSSLK | 681–688 | 0.0742 |
| 133 | CTVDETK | 390–397 | 0.0495 |
| 134 | HTTVNENADQPK | 229–240 | 0.0390 |
| 135 | FPNATDEKK | 32–41 | 0.3269 |
| 136 | VMVLCNR | 107–113 | 0.2887 |
| 137 | GASVDR | 140–146 | 0.2203 |
| 138 | SIEFTGNIK | 71–80 | 0.1899 |
| 139 | AVVESNTLTLHFGK | 194–209 | 0.1597 |
| 140 | FCNAVYES | 191–198 | 0.1383 |
| 141 | VEQGASVDR | 137–146 | 0.0903 |
| 142 | CAHKVEQ | 133–139 | 0.0902 |
| 143 | VEQGASVDER | 137–146 | 0.0734 |
| 144 | CNFCNAVVESNTLTLHFGK | 189–209 | 0.0623 |
| 145 | VEQGASVDK | 137–145 | 0.0585 |
| 146 | VEQGASVDE | 137–145 | 0.0467 |
| 147 | SCCSGLTSTVVC * | 1973–1986 | 0.5560 |
| 148 | FNSSAGPAGIGSEC * | 771–785 | 0.5429 |
| 149 | SSSCICS * | 270–275 | 0.5397 |
| 150 | FDGRSR * | 1000–1005 | 0.5330 |
| 151 | PAQFQLM | 1255–1261 | 0.3931 |
| 152 | KSLSCSLLK | 916–924 | 0.3501 |
| 153 | VTSDCGCK | 2001–2008 | 0.3200 |
| 154 | LEGCYPECS | 1163–1171 | 0.3088 |
| 155 | ECNGSC | 312–317 | 0.2923 |
| 156 | EPSELCK | 1629–1635 | 0.2680 |
| 157 | TCTCNKR | 846–852 | 0.2315 |
| 158 | DTCADPE | 319–325 | 0.2167 |
| 159 | VTDSE | 1591–1595 | 0.2004 |
| 160 | TATGAVEDESSAASFGNSWE | 547–564 | 0.1661 |
| 161 | GTCTSYS | 2044–2050 | 0.1278 |
| 162 | IQETAPGAEK | 941–952 | 0.1215 |
| 163 | EVIVDTLISR | 1722–1731 | 0.1101 |
| 164 | VQVSTVGR | 28–35 | 0.0990 |
| 165 | AVTGTN | 1901–1906 | 0.0685 |
| 166 | GYSLGWNVCAAK * | 40–51 | 0.6563 |
| 167 | HGLEDNYRG | 33–40 | 0.4763 |
| 168 | HGLEDNYR | 33–39 | 0.3753 |
| 169 | GTDVQAWIR | 135–143 | 0.3365 |
| 170 | GILQINSR | 72–79 | 0.2008 |
| 171 | FESNFQTATNR | 52–63 | 0.1820 |
| 172 | SNFTQATNR | 54–63 | 0.1487 |
| 173 | ILQINSR | 73–79 | 0.1212 |
| 174 | NTDGSTDYGILQINSR | 64–79 | 0.0900 |
| 175 | VHNLFK | 80–85 | 0.3580 |
| 176 | HSLEELFR | 354–362 | 0.2286 |
| 177 | VTATLPAEKMUK | 220–229 | 0.1937 |
| 178 | Ovalbumin-related protein Y | KFYTGVEEVFNFK | 124–136 | 0.1917 |
| 179 | VPLLDEVSGLER | 244–255 | 0.1871 |

(b) Diphenolase inhibitory activity
Table A1. Cont.

| No. | Egg Protein Sequence | Peptide | Peptide Sequence Number | Score |
|-----|----------------------|---------|-------------------------|-------|
| 181 | FYTGGVEEVNFK          |         | 125–136                 | 0.1570 |
| 182 | ATGSTGAI             |         | 342–349                 | 0.1262 |
| 183 | TESQMK               |         | 50–55                   | 0.0899 |
| 184 | EKMAPALRLLV          |         | 538–548                 | 0.4863 |
| 185 | LVDKDNISPISK         |         | 379–390                 | 0.1410 |
| 186 | HNPNITIVYPGR         |         | 217–228                 | 0.2810 |
| 187 | ILELPFASGLSMVLVPDEVSNER | 74–99 | 0.1682 |
| 188 | ILELPFASGLSMVLVPDEVSHEL | 74–99 | 0.1607 |
| 189 | ISQAVHGAFMELSEDGIEMAGSTGVEDIK | 168–197 | 0.0118 |

* shortlisted peptides (PeptideRanker score > 0.5) for structure-activity relationship analysis.

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