Research Article

Optimization of Enzyme-assisted Extraction of Bioactive Peptides from Whiteleg Shrimp (*Litopenaeus vannamei*) Head Waste Using Box-Behnken Design

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Abstract
Whiteleg shrimp (Litopeneaus vannamei) processing lines discard huge quantities of head wastes, which are the protein-rich source. Enzymatic hydrolysis has been studied for decades to salvage discarded whiteleg shrimp head waste to produce high-added value products applicable to animal industry. Alcalase 2.5 L, which is widely applied on marine materials, was used to hydrolyze whiteleg shrimp head waste protein and then purified by cold absolute ethanol. Hydrolysis conditions including enzyme/substrate (E/S) ratio, incubation temperature and duration were preliminarily screened (E/S ratio 0–2.5%, temperature 50–70 °C, duration 30–90 min) before conducting optimization using Box-Behnken design. After optimization, collected protein hydrolysate reached an experimental yield of 61.64% on dry basis under the optimal conditions as treated by Alcalase 2.5 L at 0.9% E/S ratio and incubated at 62 °C for 65 min. Protein hydrolysate performed bioactivity including DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity (3.88 ± 0.10 mg Trolox equivalents g dry matter^{-1}), reducing power (19.20 ± 0.52 mg Trolox equivalents g dry matter^{-1}) and α-amylase inhibitory activity (10.74 ± 0.65% inhibition at 50 mg mL^{-1}).

Keywords: Alcalase, α-amylase inhibition, Antioxidant, Protein hydrolysate, Whiteleg shrimp head waste

1 Introduction

Whiteleg shrimp (Litopenaeus vannamei) is one of the most prominent and important species in Vietnam shrimp farming. Shrimp processing industry inevitably comes along with the discarding of high amounts of shrimp wastes every year. Globally, the production of shrimp reached 5.03 million tonnes in 2020 [1]. Vietnam ranked third in shrimp production accounting for 13% of global shrimp production meaning that total shrimp amount was approximately 684 thousand tonnes [2]. The shrimp wastes consisting of exoskeleton, cephalothorax and tail account for 45–60% of the whole shrimp weight. Corresponding to the huge quantity of shrimp in Vietnam, there has been approximately 325 thousand tonnes per year and about 1000 tonnes of shrimp waste discharged from shrimp processing line every day which could cause environment-related problems [3]. Valorization of potential biopolymers from food wastes and converting them into value-added products is a promising way to adapt BCG concept (bio, circular and green economy) towards sustainable development in the industry [4]

For decades, various studies have shown that shrimp wastes are a considerable source of protein and available bioactive compounds full of health benefits. Shrimp waste protein hydrolysate was shown to contain significant bioactive peptides inhibiting the proliferation of human cancer cells, particularly, small-sized peptides with molecular weight of lower than 10 kDa exhibited alone the inhibition of 55% liver cancer cells and 60% colon cancer cells [5]. Bioactive peptides also performed the ability to inhibit the α-amylase, which is the enzyme playing an initial role in starch digestion

converting starch into dextrins, by attaching to the active site of α-amylase preventing from starch binding, leading to inactivation of α-amylase [6]. *In vitro* antidiabetic property by α-amylase inhibitory activity measurement was conducted on shrimp shell waste reaching 43.4% of inhibition rate at 76.9 mg mL^{-1} [7]. Besides antitumor and antidiabetic properties, bioactive peptides extracted from shrimp waste proteins were also proven to act as antioxidant, anti-inflammatory and antimicrobial factors as well as nutritional, sensory and functional factors [8]. Many studies showed that peptides extracted from shrimp by-products waste were the potential antioxidant source [9], [10]. Bioactive peptides perform antimicrobial due to the presence of a proline rich N-terminal region [5]. Antioxidative peptides were likely to contain low molecular weight and consisted of aromatic as well as hydrophobic amino acids [11], [12]. Fractions of peptides having molecular weight of lower than 3 kDa gave higher hydroxyl radical scavenging activity as well as higher ACE (angiotensin-converting enzyme) inhibitory activity [13]. Low molecular weight peptides with hydrophobic amino acids (Leu and Val) in N-terminal regions are potentially bioactive. Furthermore, sulfur-containing amino acids (Cys and Met), phenylic-ring-containing amino acids (Phe, Trp, Tyr) and His residue contribute greatly to the bioactivity of peptides by highly free radicals scavenging ability [12]. Carotenoid derivatives from protein-astaxanthin complexes hydrolysis and histidine-dipeptide (carnosine and anserine) also contribute to antioxidative properties of shrimp processing discards [14]. Free radical quenching ability of protein hydrolysate was reported not to be linked with degree of hydrolysis.

Enzyme-assisted hydrolysis exhibits more advantages than chemical methods using alkali and acid. Alkaline hydrolysis can cause racemization turning L-amino acids into D-amino acids which are non-absorbable to human body [15], [16] whereas protein products hydrolyzed by acid under harsh conditions often exhibit poor nutritional functionalities and off-flavor characteristics due to the huge loss of essential amino acids [17]. Alcalase, flavourzyme, protamex and neutrase are commonly used proteases for fishery protein hydrolysis. Amongst these proteases, Alcalase was the most efficient enzyme giving the highest protein recovery of 60% whereas other enzymes treatment gave results of 50–55% protein recovered [9]. Alcalase was proven to give both higher protein and astaxanthin recovery compared with the usage of pancreatin in the research of De Holanda and Netto [18]. Alcalase (also known as Subtilisin) is sorted as a serine protease, mostly performing extensive nucleophilic attacks on the middle positions of the protein chain. Alcalase can be highly active and recognize a wide range of amino acids including Glu, Met, Leu, Tyr, Lys and Gln at the P1 position where on the C-terminal of the active site [8]. Alcalase also preferentially cleavages the peptide bonds containing hydrophobic amino acids. Due to the high amino acids recognition ability, especially hydrophobic amino acids, Alcalase is used to obtain smaller peptides with general hydrophobic terminals, which were reported to have higher antioxidative properties. *Bacillus subtilis* was originally involved in the production of Alcalase but nowadays, the enzyme is commercially obtained by the submerged fermentation using *Bacillus licheniformis* [8]. Alcalase is stable and active at alkaline pH and moderately hot temperature which does not exceed 70 °C. Temperature, pH, durations and enzyme/substrate (E/S) ratio are concerned parameters during enzyme-assisted hydrolysis [9].

Alcalase, a commercial enzyme having the ability to cleavage proteins to release desirable peptides with the properties of short chain and high antioxidant activity, is used to break down protein in whiteleg shrimp head waste to collect smaller peptides performing bioactivity. Alcalase working conditions including E/S ratio, incubation temperature and duration were concerned factors that needed optimization to obtain protein hydrolysate with the highest yield. Subsequently, bioactivity such as DPPH free radical scavenging activity, reducing power and α-amylase inhibitory activity were also tested on protein hydrolysate after optimization. Moreover, previous publications on collecting animal proteins by Alcalase-assisted approach were mostly terminated in the aqueous phase, which was scarcely separated by precipitation. This research aimed at protein after being separated by using ethanol. Overall, the achievement is to enrich the database of shrimp head post-harvesting studies and to contribute to the field of food waste treatments creating the biological values applicable to the food and animal industries.

2 Materials and Methods

2.1 *Materials and chemicals*

Whiteleg shrimp head waste (WSHW) powder provided by Vinafood Company, Vietnam. Alcalase enzyme 2.5 L from *Bacillus licheniformis* (enzyme activity of 2.5 AU-A/g, working pH range 6.5–10, working temperature range 60–75 °C) provided by Novozymes A/S, Bagsværd, Denmark. All chemicals and reagents used were of analytical grade.

2.3 *Sample preparation*

Each 10 g of dried WSHW powder was weighed and put in an erlenmeyer flask, then mixed with distilled water at a ratio of 1:4 (w/v) before treating the enzyme. The extraction method was conducted according to Muoi and Vy [19] as follows with some slight modifications.

2.3 *Alcalase-assisted hydrolysis under varying conditions*

2.3.1 Effects of E/S ratios

E/S ratio, which was reported to highly significantly impact protein yield followed by temperature and duration, was investigated in ranges of 0 to 2.5% [10], [20]. Those flasks were sealed and shaken in the shaking water bath (Maxstudy 18, Daihan, South Korea) at a set temperature of 60 °C for 1 h. After completing hydrolysis, samples were thermally deactivated at 90 °C for 10 min using a waterbath (WCB-22, Daihan, South Korea) to prevent further hydrolysis and centrifuged (Universal 320R, Hettich, Germany) at 25 °C, 1164 × g for 10 min. Absolute ethanol (Chemsol, Vietnam) was used to separate oligopeptides out of aqueous hydrolysate with a hydrolysate/ethanol ratio of 1:5 (v/v) at 4 °C for 1 h. The mixture after being centrifuged at 4 °C, 6082 × g in 15 min was decanted and left solid which was referred to as the protein hydrolysate.

2.3.2 Effects of incubation temperature

Alcalase was used at the best E/S ratio from the above experiment. Temperature was studied at 50, 55, 60, 65 and 70 °C. Thermal deactivation and precipitation were designed as described in the study of the E/S ratio.

2.3.3 Effects of incubation duration

Alcalase was added to the sample at selected previous optimum conditions. The reaction time varied every 15 min from 30 to 90 min. Parameters of deactivation and precipitation were correspondingly set as above.

2.4 *Acid hydrolysis*

Acid hydrolysis as a traditional method was conducted to compare with investigated approach of Alcalase-assisted hydrolysis in terms of protein yield and bioactivity. Hydrochloric acid was used for hydrolysis with a concentration of 3 M at 90 °C for 90 min then terminated by NaOH to reach final pH of 5 [21]. The supernatant was collected after centrifuging at 1164 × g at 25 °C for 10 min. Cold absolute ethanol was used to separate oligopeptides out of aqueous protein hydrolysate with a ratio of 1:5 (sample/solvent, v/v) at 4 °C for 1 hour. Protein hydrolysate was collected after centrifuging at 4 °C, 6082 × g for 15 min.

2.5 *Box behnken design*

To optimize hydrolysis conditions using Response Surface Methodology, Box-Behnken design (BBD) was used in this regard. Due to higher order response surfaces and fewer runs required, there were several studies on shrimp waste using Box-Behnken design to predict enzyme working conditions [7], [9] The level giving the most effective response and two aside significantly different levels of each factor including E/S ratios, incubation temperatures and durations, were chosen from preliminary screening experiments and coded as –1, 0 and +1 corresponding to low, middle and high value, respectively. Protein yield on a dry basis (db) was taken as a response. Optimization experiments followed Box-Behnken design with 15 runs in total including 12 factorial runs and 3 centerpoint runs. Experiments were triplicated and single effects as well as interactive impacts of factors were assessed on protein yield in db as a response. The model of regression analysis was constructed in the form of quadratic polynomial Equation (1):

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

Where Y was response variable (protein yield in *db*), X was independent variable (E/S ratio, incubation temperature and duration) and β represented a set of regression coefficients.

2.6 *Analytical analysis*

2.6.1 Determination of proximate composition

Proximate composition of WSHW powder including moisture, ash, crude fat and crude protein was determined according to AOAC method (2000) with slight modification. Moisture content was measured by the forced draft oven (UNE700, Memmert, Germany) at a temperature of 105 °C until the weight of sample was constant. Soxhlet apparatus (R 106S, Behr, Germany) was used to determine the total crude fat content in WSHW. Crude protein content was determined by Kjeldahl method (S 2, Behr, Germany) with chosen conversion factor for shrimp head of 5.6 [22]. WSHW powder was burned at 550 °C using the muffle furnace (FHX-14, Daihan, South Korea) to determine the ash content. All proximate composition tests were carried out in triplicate and reported on a dry weight basis.

2.6.2 Determination of protein yield

The soluble protein content of collected protein hydrolysate was determined by colorimetric Hartree-Lowry assay [23] with some modifications. In detail, 100 mL solution A was prepared with 2 g of Na_2CO_3 in 0.1 M NaOH, 100 mL solution B was prepared with 0.5 g of $CuSO_4.5H_2O$ in 1% sodium citrate. Solution C was prepared by mixing solution A and B at the ratio 49:1 (v/v) only used for the day of experimenting. Bovine serum albumin 0.1% (Biobasic, Canada) was used to build the calibration curve at the concentration range of 0 – 250 µg/mL. Precipitated protein was dissolved in 25 mL nanopure water and diluted 100-folded for quantification. Two mL of solution C was added to 0.4 mL samples, shaken well and kept for 10 min to create the coprous-protein complex. Then, 0.2 mL of 1 N Folin-Ciocalteu reagent (Merck, Germany) was added consecutively, shaken well and kept for 10 min. Finally, 2 mL distilled water was added, shaken well and kept for 5 min before measuring at an absorbance wavelength of 750 nm by UV-Vis spectrophotometer (V730, J∧s.co, Japan). Protein yield was calculated as the Equation (2):

$$Protein\ yield\ (\%db) = \frac{Protein\ content\ of\ crude\ hydrolysate}{Weight\ of\ dry\ matter\ in\ crude\ hydrolysate} \times 100\% \quad (2)$$

2.6.3 Determination of DPPH free radical scavenging activity

The antioxidant activity was measured by the capacity of scavenging free radical from DPPH of the obtained peptides. The method of measuring antioxidant activity was referenced from Zhao *et al.* [24] with minor modifications. One and a half mL of 50-fold diluted sample was placed in a test tube and 2 mL of 0.1 mM methanolic DPPH solution (Alfar Aesar, UK) was added. The mixture was shaken well and placed in the dark at room temperature for 30 min before the spectrophotometric measurement with an absorption wavelength of 517 nm. Trolox (Sigma-Aldrich, USA) was used as standards at the range of 0–50 µM. Sample was replaced by pure methanol as the blank.

2.6.4 Determination of reducing power

The potential of protein hydrolysate to reduce Fe^{3+} was measured following method described by Irshad and others [25] with some modifications. The mixture including 0.5 mL of the 50-fold diluted sample, 0.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 mL of $K_3[Fe(CN)_6]$ 1% (Acros Organics, Belgium) was incubated in a waterbath at 50 °C for 20 min. The reaction was terminated by adding 0.5 mL of trichloroacetic acid 10% (Fisher Scientific, Belgium). Two mL of distilled water and 0.1 mL of FeCl3 0.1% were consecutively added and shaken well. After 10 min, the mixture was measured using a UV spectrophotometer (V730, J∧s.co, Japan) at 700 nm. Trolox was used to construct the standard curve at various concentrations at 0, 10, 20, 30, 40 and 50 µM in this test. Reducing power was represented as mg TE g dry matter^{-1}.

2.6.5 Determination of α-amylase inhibitory activity

The measurement of α-amylase inhibitory activity was conducted as described by Yuan *et al.*, [7] with some modifications. The mixture of 0.1 mL of sample and 0.1 mL of α-amylase 0.5 mg mL^{-1} (Sigma-Aldrich, USA) was incubated at 25 °C for 20 min. Subsequently, 0.1 mL of 2% starch solution (VWR Prolabo, France) was added to the mixture and re-incubated at 25 °C for 5 min. To stop the reaction, 0.1 mL of DNS (Biobasic, Canada), prepared by dissolving 1 g 3,5-dinitrosalicylic acid in 50 mL distilled water then adding 30 g of sodium potassium tartrate tetrahydrate and 20 mL NaOH 2N, was used and boiled at 90 °C for 10 min. Three mL of distilled water was added before the spectrophotometer measuring at 540 nm. The rate of α-amylase inhibition was calculated as the Equation (3):

$$\%\,Inhibition\ rate = \left(1 - \frac{A_s}{A_0}\right) \times 100\% \quad (3)$$

where A_s and A_0 were absorbances of protein sample and blank (distilled water), respectively.

2.7 *Statistical analysis*

Significant differences among levels of factors were analyzed by SPSS Statistics Software (version 25, IBM, USA) using LSD test at a 95% level of confidence to detect the effects of Alcalase-assisted hydrolysis conditions on yield of protein hydrolysate obtained from whiteleg shrimp head waste. ANOVA results of the quadratic model and 3D surface plot visualization from optimization were obtained by Design-Expert Software (version 13, Stat-Ease, USA)

3 Results and Discussions

3.1 *Proximate composition*

Commercial dried whiteleg shrimp head waste powder provided by Vinafood Company had a bisque color and smelled slightly fishy. There was no strange odor indicating the deterioration detected in used powder. The particle size of used powder was in the range of 120–125 microns in diameter. Table 1 showed the results of proximate composition of WSHW powder. Relatively low moisture content (5.54 ± 0.07% db) was advantageous preventing the invasion of microorganisms for easier storage and latter usage [26]. Prominent compositions in the material were crude protein and ash content concurring well with the study of Ibrahim and others [27] that two major components of dried shrimp head meat were also protein and mineral (47.75% and 15.73% in dry weight basis, respectively). Crude fat content in dy basis was in agreement with finding that whiteleg shrimp head waste contained 8.3% of crude fat [28]. In some other papers, crude fat content of whiteleg shrimp was reported to be lower such as 4.7% db [29]; 7.6% db [30]. The differences in proximate composition of materials of governed by many other external factors such as species, age, feeding, cultivation practice as well as processing methods [31]

Table 1: Proximate composition of WSHW powder

Proximate Composition	Content in db (%)
Moisture	5.54 ± 0.07
Crude protein	48.94 ± 0.27
Crude fat	8.77 ± 0.42
Ash	20.01 ± 0.54

Note: Data are presented as mean ± standard deviation

3.2 *Effects of extraction conditions on protein yield*

3.2.1 Effects of E/S ratio

Figure 1 showed the trend of protein yield in db of protein hydrolysate extracted from whiteleg shrimp head waste at several E/S ratios from 0 (blank sample) to 2.5%. The E/S ratio impacted significantly on yield of protein hydrolysate extracted from WSHW (p-value < 0.05). Generally, protein content fluctuated in the range of 48.19 ± 1.87% to 59.22 ± 1.39% db. As shown in Figure 1, all samples treated with Alcalase were higher than the blank one without Alcalase. Amongst them, the treatment of 1% E/S ratio resulted in the highest value (59.22 ± 1.39%). For the yield and protein recovery of protein hydrolysate extracted from the same species of shrimp, 1% of Alcalase was also proven to be more efficient compared to 0.5% [32]. Saidi *et al.*, [33] used Alcalase at a ratio of 1% for tuna dark muscle hydrolysis to get the highest protein recovery. The excess of enzyme dose (more than 1%) had an adverse

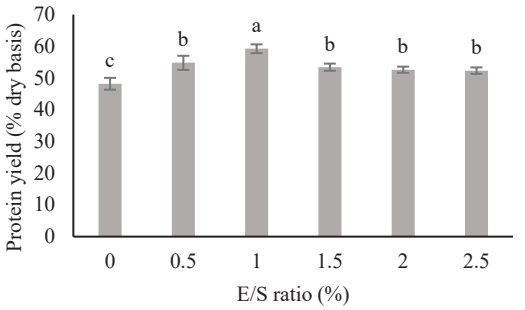

Figure 1: Effects of E/S ratio on protein yield. Different superscripts indicate significant differences among levels (p-value < 0.05).

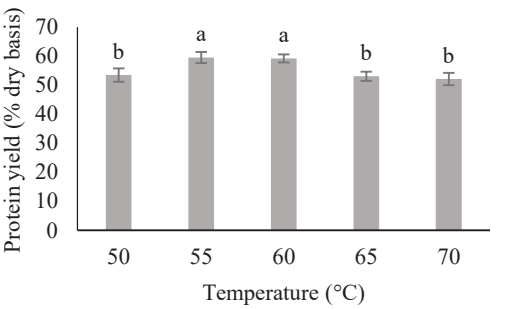

Figure 2: Effects of incubation temperature on protein yield. Different superscripts indicate significant differences among levels (p-value < 0.05).

effect on hydrolysis causing a decrease in protein yield. This was due to the fact that the availability of substrate (peptide bonds) was limited during hydrolysis [34]. Moreover, products, which are smaller sized peptides, acted as inhibitory factors to block the active site of Alcalase, leading to enzyme deactivation [34]. The downward trend beyond the optimal E/S ratio might be explained by protein quantification method. Lowry-Hartree method is partly based on the principle of Biuret reaction of peptide bonds [23]. A higher dose of Alcalase aggressively produced smaller sized peptides molecules and amino acids with lesser peptide bonds, thus, reducing the absorbance recorded.

3.2.2 Effects of incubation temperature

Protein yield pattern changes at different temperatures ranging from 50 to 70 °C were outlined in Figure 2. A temperature of 55 °C was optimum for hydrolysis reaching the highest protein yield (59.53 ± 1.92%), which was not significantly different from that incubated at 60 °C (59.22 ± 1.39%). Indeed, temperatures at 55–60 °C were proven to be the best thermal working condition of Alcalase for several types of materials, particularly marine by-products. This confirmed the findings that 55 °C was suggested in hydrolysis of shrimp cephalothorax to recover 66.8% of protein [35]. Alcalase was also used at 55 °C resulting in the most efficient protein yield and recovery of other fish by-products such as Caspian white fish viscera, tail, backbones and fines [36]; silver cyprinid [37] and salmon skin [20]. At 60 °C, Alcalase worked the most efficiently on hydrolysis of shortfin scad yielding 90.48% protein [38]. As samples were treated beyond the optimum temperature of Alcalase, the yields had the tendency to decline from 58.86 ± 2.13% to 52.12 ± 2.13%. Under elevated temperatures, enzymes, which are proteins by nature, were destabilized by changing the shape of the active site and then blocking substrates from binding; thus total enzyme activity was reduced [39].

3.2.3 Effects of incubation duration

The influence of incubation duration on protein yield was depicted in Figure 3 with yield values varying from 48.96 ± 1.80% to 59.53 ± 1.92%. Incubation duration significantly impacted the protein content of precipitated crude extract from shrimp head waste (p-value < 0.05). The protein content reached the highest with a duration of 60 min (59.53 ± 1.92% db) whereas 30 min was insufficient for the course of hydrolysis when the substrate source was abundant. Plant-based materials including rice bran and defatted soybean were hydrolyzed with Alcalase for 60 min giving the highest degree of hydrolysis [40]. Dey and Dora [9] had preliminary screening experiments on shrimp waste using Alcalase and incubation time of 60 min was the best condition for further protein yield optimization. Protein hydrolysis by Alcalase lasting for hours was also handled in many studies to get high yield, such as fresh shrimp head waste in 3 h [41]; freeze-dried shrimp head meat powder in 4 h [19]. The positive impact of Alcalase was nullified as the hydrolysis duration was prolonged. As illustrated in Figure 3, protein yield increased initially for the first 60 min then remained for the next 15 min (p-value > 0.05) before dropping. This could be elucidated by both

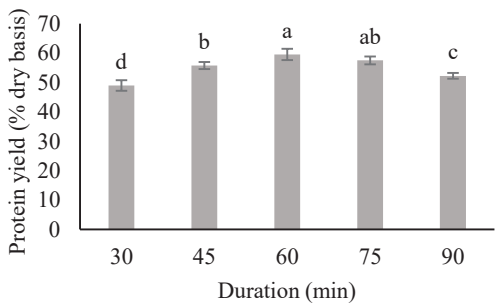

Figure 3: Effects of incubation duration on protein yield. Different superscripts indicate significant differences among levels (p-value < 0.05).

enzyme saturation and formed product inhibition acting as a substrate competitor over a long period of time [9].

3.3 *Optimization using Box-Behnken design*

After screening experiments, hydrolysis conditions were chosen for subsequent optimization by using Box-Behnken design. Those included E/S ratios in range of 0.5–1.5%, incubation temperatures from 50–70 °C and hydrolysis durations of 45, 60, 75 min, which were coded and designed as shown in Table 2 and 3 with protein yield (db) as average response result of triplicates.

3.3.1 Regression analysis

As shown in Table 4 and 5, the regression model was successfully evaluated and reliable to optimize hydrolysis conditions for the highest protein yield when the model was highly significant (p-value < 0.05) and the lack of fit was insignificant (p-value > 0.05) indicating the adequacy of pure error. The adjusted coefficient of determination was relatively good (R_{adj}^2 = 0.9592) showing that only 4.08% of variability could not be elucidated by the model. There was a reasonable agreement between adjusted R^2 and predicted R^2 when those values were not different from each other by an interval of 0.2. Desirable Adeq Precision which was much higher than 4 implied an adequate signal for the model. The empirical mathematical relationship among variables was constructed as below with most of the coefficients presented (p-value < 0.05) except for the interactive relationship of temperature and duration (X_2X_3) as the Equation (4):

Table 2: Symbols and coded levels of chosen variables for BBD

Variables	Symbols	Coded Levels		
		–1	0	+1
E/S ratio (%)	X_1	0.5	1	1.5
Temperature (°C)	X_2	50	60	70
Duration (min)	X_3	45	60	75

Table 3: BBD matrix with observed response (protein yield in db)

Standard Order	Run Order	Independent Variables			Response Y (% db)
		X_1 (%)	X_2 (°C)	X_3 (min)	
11	1	1	50	75	52.08
9	2	1	50	45	48.57
2	3	1.5	50	60	51.94
5	4	0.5	60	45	54.48
7	5	0.5	60	75	61.23
13	6	1	60	60	62.69
10	7	1	70	45	54.40
3	8	0.5	70	60	59.27
14	9	1	60	60	62.34
12	10	1	70	75	57.76
8	11	1.5	60	75	52.38
4	12	1.5	70	60	53.84
1	13	0.5	50	60	49.89
15	14	1	60	60	61.59
6	15	1.5	60	45	55.48

Table 4: Analysis of Variance for response surface quadratic model

Source	Sum of Squares	df	Mean Square	p-value
Model	300.01	9	33.33	0.0005
X_1	15.76	1	15.76	0.0084
X_2	64.92	1	64.92	0.0004
X_3	13.83	1	13.83	0.0109
X_1X_2	13.97	1	13.97	0.0107
X_1X_3	24.30	1	24.30	0.0034
X_2X_3	0.0066	1	0.0066	0.9345
X_1^2	30.89	1	30.89	0.0020
X_2^2	115.03	1	115.03	< 0.0001
X_3^2	43.30	1	43.30	0.0009
Residual	4.44	5	0.8875	
Lack of Fit	3.81	3	1.27	0.2057
Pure Error	0.6315	2	0.3158	
Cor Total	304.45	14		

Table 5: Fit statistics

Std. Dev.	0.9421	R^2	0.9854
Mean	55.86	Adjusted R^2	0.9592
C.V. %	1.69	Predicted R^2	0.7953
		Adeq Precision	17.1739

$$Y\ (\%db) = 62.21 - 1.40X_1 + 2.85X_2 + 1.31X_3 - 1.87X_1X_2 - 2.46X_1X_3 - 0.0407X_2X_3 - 2.89X_1^2 - 5.58X_2^2 - 3.42X_3^2 \quad (4)$$

Where Y was the protein yield response (% db); X_1, X_2 and X_3 were linear factors including E/S ratio, temperature and duration, respectively; X_1X_2 and X_1X_3 indicated the interaction between factors; X_12, X_2^2 and X_1^2 were quadratic terms of factors; and associated coefficients with each term.

In the formula (1), protein yield was directly proportional to incubation temperature and duration with positive coefficients whereas E/S ratio had an inverse proportion to response due to a negative coefficient. Among three variables, incubation temperature was suggested to influence protein yield more substantially when absolute values of coefficients were ranked ($|\beta_2| > |\beta_1| > |\beta_3|$). The interactive relationship between incubation temperature and duration was insignificant and ignored (p-value > 0.05). The negativity of quadratic coefficients implied that 3D parabolic surfaces concaved downward and exposed the extreme areas which were reasonable to figure out the maximum response.

3.3.2 Optimization

The reciprocal interactions between every two factors with the rest factor fixed at zero level were visualized in Figure 4. As visualized in Figure 4 and negative interactive coefficients shown in formula (1), the reciprocal influence of every two factors was observed to be adversely proportional to response meaning that

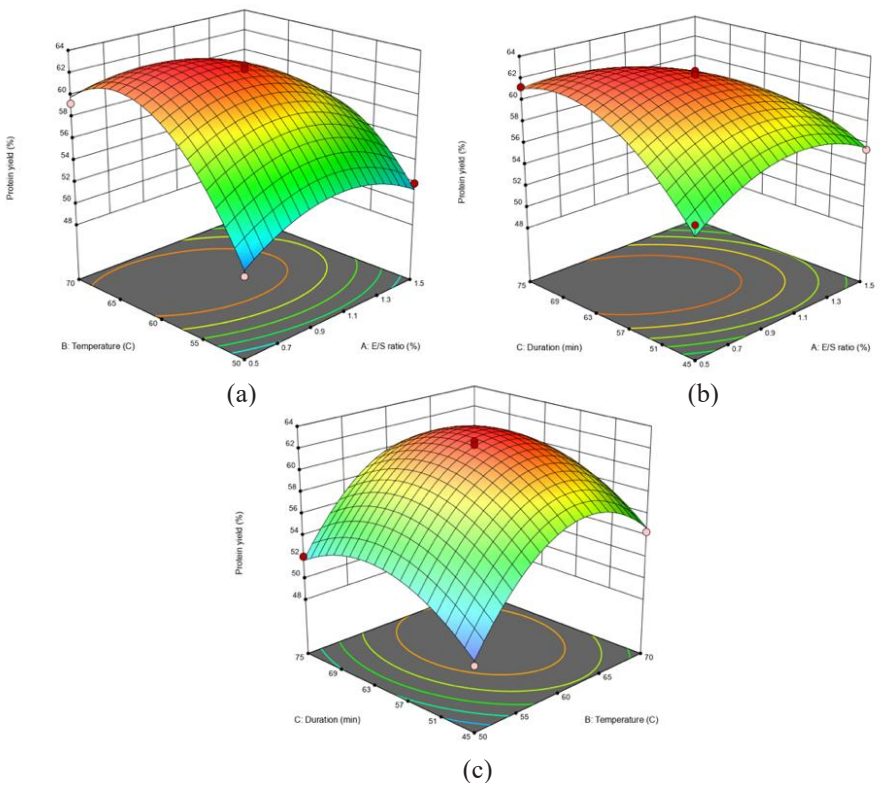

Figure 4: 3D surface plots of response as a function of (a) E/S ratio and temperature, (b) E/S ratio and duration, (c) temperature and duration.

protein yield decreased when both two factors got higher and higher simultaneously beyond the maximum points. Those could be explained by the fact that extreme conditions such as a high E/S ratio, highly thermal heating for a prolonged duration during treating enzyme can destabilize enzyme activity leading to the decrease in protein yield [9], [33], [38].

Experiments under chosen solution to optimization model were conducted with slight modifications to validate the compatibility of the model, with enzyme dose of 0.9% incubated at 62 °C for 65 min. Results of protein yield under optimized experimental conditions were shown in Table 6 which was 61.64%. As expected, the deviation of experimental protein yield was only 2.11% compared to predicted value indicating that the model was appropriate.

Table 6: Predicted and experimental response under optimal conditions

	Predicted	Experimental
E/S ratio (%)	0.91	0.90
Temperature (°C)	61.83	62.00
Duration (min)	61.52	65.00
Protein yield (%db)	62.97	61.64

3.4 *Bioactivity of protein hydrolysate under optimal conditions*

Bioactivity of protein hydrolysate extracted under optimized operating conditions was demonstrated in comparison with the control sample undergoing acid hydrolysis (Table 7). Alcalase-assisted hydrolysis gave more bioactive peptides than the traditional acidic hydrolysis method. The α-amylase inhibitory activity of protein hydrolysate extracted by acid was reported not to exist when absorbances of samples and blank were relatively the same. DPPH radical quenching activity of WSHW hydrolysate using Alcalase was relatively higher compared to that of porcine liver protein hydrolysate (2.17 mg TE/g dry matter) [42]. Alcalase-assisted hydrolysis of other materials released antioxidative peptides such as mozzarella whey protein hydrolysates with an antioxidant capacity of 1.18 μmol Trolox/mg protein [43]. The hydrolysis of date seed by Alcalase generated the hydrolysate with a reducing power of 0.51 μmol TE/mg protein [44]. Antioxidant activity of peptides was associated with the molecular weight, composition, structure, sequence and nature of constituent amino acid in peptide chains [14], [45]. To be more specific, antioxidative peptides had low molecular weight and consisted of aromatic as well as hydrophobic amino acids [12], [45]. Regarding anti-α-amylase property, previous research pointed out that shrimp shell waste protein hydrolysate at the concentration of 76.9 mg/mL inhibited 43.4% α-amylase activity [7]. This property, however, performed better in plant-derived material hydrolysis, in particular, defatted watermelon seed hydrolysate displayed high α-amylase inhibitory activity that all inhibition rates were all over 50% at the concentration range from 0.5 to 3 mg/mL [45] Alcalase assisted the hydrolysis by cutting the protein chain and releasing peptide molecules possessing cationic (Lys) and branched-chain residues (Trp, Tyr and Phe), which had the tendency to bind to α-amylase and inhibit enzyme activity causing the less sugar content produced [45]. Additionally, as explained by Awosika and others, narrow-sized peptides released by Alcalase-assisted hydrolysis had the ability to attach to the substrate-binding site of α-amylase blocking the starch interaction [6]. As documented by Aaslyng and others [46], harsh conditions including high temperature and low pH during acid hydrolysis degraded sulfur-containing amino acids in protein and completely destroyed tryptophan, which was reported to own antioxidative property [12], [46]; therefore, protein extracted by acid hydrolysis was not a potentially antioxidative source.

Table 7: Bioactivity of protein hydrolysate under optimal Alcalase-assisted hydrolysis conditions compared with acid hydrolysis

	Alcalase-assisted Hydrolysis	Acid Hydrolysis
DPPH radical scavenging activity (mg TE g dry matter^{-1})	3.88 ± 0.10^{a}	1.67 ± 0.16^{b}
Reducing power (mg TE g dry matter^{-1})	19.20 ± 0.52^{a}	6.92 ± 0.24^{b}
α-amylase inhibitory activity (% inhibition)	10.74 ± 0.65	N/A

Note: N/A: not available
Data are presented as mean ± standard deviation
Data in the same row with different superscript letters are significantly different

4 Conclusions

In this research, enzyme-assisted hydrolysis, particularly, Alcalase 2.5 L was used as an efficient and beneficial

method to get highly antioxidative peptides. Factors including E/S ratio, incubation temperature and incubation duration made significant impacts on yield of collected protein hydrolysate. By using Box-Behnken design associated with Response Surface Methodology, the optimized extraction conditions of 0.9% of Alcalase incubated at 62 °C for 65 min followed by precipitating with cold absolute ethanol giving product with 61.64% protein in db. In addition, bioactivity of collected optimized product was investigated in this study. Protein hydrolysate performed DPPH radical scavenging property (3.88 ± 0.10 mg TE g dry matter^{-1}); high reducing power (19.20 ± 0.52 mg TE g dry matter^{-1}) and α-amylase inhibition of 10.74 ± 0.65%. In the field of enzyme investigation, more factors should be studied to get much more positive and optimal results such as sample/solvent ratio and pH. Protein hydrolysate can be firstly applicable for animal feeding purpose while safety test for consumption and sensory tests are further recommended in case of using such product for human in the future.

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Author Contributions

D.C.: investigation, conducting experiments, data analysis, writing an original draft; V.L.: conducting experiments; H.V.H.: supervising, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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