Fishery Technology 62 (2025) : 462 - 473

Optimization of Enzymatic Hydrolysis Conditions for Protein Hydrolysate Production from Indian Squid (*Uroteuthis duvauceli*)

Remya Kumari K. R.[1], Ginson Joseph[2*], Joshy C. G.[3], Ajeeshkumar K. K.[4] and Suseela M.[3]

[1] *National Institute of Fisheries Post Harvest Technology and Training, Vishakhapatnam-530 001, India*

[2] *School of Industrial Fisheries, Cochin University of Science and Technology Lakeside Campus, Cochin-682 016, India*

[3] *Central Institute of Fisheries Technology, Cochin 682 029, India*

[4] *Kelappaji College of Agricultural Engineering and Food Technology, Kerala Agricultural University, Malappuram-679 573, India*

Abstract

This study optimized the enzymatic hydrolysis for producing antioxidant-potent protein hydrolysate from Indian squid (*Uroteuthis duvauceli*) using pepsin, papain, and trypsin, applying response surface methodology. A Box–Behnken design comprising fifteen experimental runs was employed, and the resulting data were used to develop a second-order response surface model. The model adequacy was tested using the coefficient of determination (R^2 = 0.84). Based on the ridge analysis and response surface plots, the ideal hydrolysis conditions for pepsin, papain, and trypsin were 1:100 enzyme-to-substrate ratio, pH: 2, time: 3.15 h, and temperature 37 °C; 1.5:100 enzyme-to-substrate ratio, pH: 6.25, time: 5.15 h, and temperature 60 °C; and 1.4:100 enzyme-to-substrate ratio, pH: 7.25, time: 6 h, and temperature 37 °C, respectively. DPPH activity (0.1 mM) of papain-aided hydrolysate was found to be maximum, 66.34% against trypsin (52.24%) and pepsin (29.49%) aided hydrolysate, which can be attributed to its high content of antioxidant-rich amino acids. The findings support the reliability of the developed model in predicting the antioxidant characteristics of Indian squid protein hydrolysate produced via enzymatic hydrolysis.

Received 2 August 2025; Revised 08 October 2025; Accepted 13 October 2025

Handling Editor: Dr. Remya S.

*Email: ginsonjoseph@cusat.ac.in

Keywords: Indian squid, Proteases, Protein hydrolysate, DPPH activity

Introduction

Marine resources are among the most diverse ecosystems for flora and fauna, with the potential to synthesize bioactive compounds. Isolation and purification of these compounds have been proven useful in various biotechnological and pharmaceutical applications due to their significance in treating lifestyle diseases. There are numerous innovative and standard techniques for extracting bioactive peptides from various biological sources. The limitations of conventional peptide extraction techniques include varying degrees of proteolysis, irregular cleavage profiles, loss of tryptophan and cysteine (Kaur et al., 2022), bitterness due to exposure of hydrophobic groups (Chen, Wei, Hou, Wang, & Ruan, 2025), reduced functionality, decreased nutritional value, and limited use as flavouring agents (Aguilar-Toalá, Quintanar-Guerrero, Liceaga, & Zambrano-Zaragoza, 2022). The use of solvent extraction accompanied by fermentation in synthesizing protein hydrolysates has not received wide approval because of low selectivity, low levels of extraction, residual solvents, and the development of pollutants (Alasalvar, Miyashita, Shahidi, & Wanasundara, 2011). Similar to the chemical process, the traditional method for producing bioactive protein hydrolysates involves using strong acids or alkalis under specific conditions to hydrolyse proteins. According to Hsu (2010), this approach has been utilized to produce fish protein hydrolysates, which are widely used in animal feed and fertilizer applications.

Recently, enzymatic protein hydrolysis has gained significant attention, as the resulting peptides retain enhanced functionalities (Venkatesan, Anil, Kim, & Shim, 2017). Consequently, it has been widely adopted in both nutraceutical and pharmaceutical sectors (Marchbank, Limdi, Mahmood, Elia, & Playford, 2008). Shahidi and Zhong (2008) reported that both natural and commercial proteases have been extensively utilized for generating bioactive peptides. According to Klompong, Benjakul, Kantachote, and Shahidi (2007), the enzymes and cleavage conditions affect the antioxidant capacity of the peptides. Shahidi and Zhong (2008) also reported that the cleavage pattern and resultant peptides will be different for each protease. The quantity of peptides and amino acids that are released from proteins, and the antioxidant potential, depends on proteolytic enzymes and hydrolysis conditions (Wu, Chen, & Shiau, 2003). Among proteases, trypsin, chymotrypsin, papain, pepsin, alcalase, and flavourzyme have been widely used to hydrolyze marine proteins (Pangestuti & Kim, 2017). In proteins, the N-terminal of the acidic and aromatic amino acids is mainly targeted by pepsin (Je, Qian, Lee, Byun, & Kim, 2008), whereas trypsin specifically acts on the carbonyl side of the basic amino acids (Rajapakse, Mendis, Byun, & Kim, 2005). Papain enzyme was used to synthesize protein hydrolysate from fish (Abdulazeez, Ramamoorthy, & Ponnusamy, 2013), and the authors also explained the importance of papain for the preparation of antioxidant marine protein hydrolysate. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical that accepts electrons or hydrogen, and the target compound transforms into diamagnetic molecules at room temperature (Zhong et al., 2011). This may be used to evaluate the antioxidant activity of protein hydrolysates (Wu et al., 2003). Furthermore, it is an inexpensive, accurate, and easy assay that is very popular for evaluating antioxidant potential in protein hydrolysates (Peng, Xiong, & Kong, 2009).

Some studies proclaim that proteases and their incubation parameters, including enzyme-to-substrate ratio, pH, and hydrolysis time, profoundly influence the antioxidant potential of the synthesized peptides; however, the optimum hydrolysis level could not be established (Peña-Ramos & Xiong, 2003; Wu et al., 2003). Response surface methodology (RSM), which is an economical approach, can model large process parameters by generating copious data from a few experimental trials through proper selection of model equations (Chen & Chen, 2025). Due to a lack of literature on enzymatic hydrolysis to produce antioxidant-potent squid protein hydrolysate (Mendis, Rajapakse, Byun, & Kim, 2005; Chen et al., 2010), in particular, from Indian squid (*Uroteuthis duvauceli*), this research aimed to produce antioxidant-potent Indian squid protein hydrolysate using protease. Further, RSM was applied for the optimization of cleavage conditions for pepsin, papain, and trypsin, and also determined the potential of each protease for the synthesis of better antioxidant squid protein hydrolysate. The findings from this study could make a significant contribution by promoting the effective utilization of underexploited cephalopod resources and producing bioactive peptides with promising applications in functional foods and nutraceutical formulations.

Materials and Methods

The Indian squid (*Uroteuthis duvauceli*) samples, sourced from the Fish Market at Kochi, Kerala, had an average length of 570 ± 14 mm and weight of 445 ± 15 g. Dissected squid mantle meat was used for the analysis. Muscle protein extraction and the yield percentage calculation followed a modified method based on Sathe and Salunkhe (1981). The analysis of amino acid composition was performed in accordance with the procedure established by Ishida, Fujita, and Asai (1981). For conducting the response surface methodology experiments on hydrolysis, the protein extract was homogenized with deionized water, adjusted to the desired pH, and incubated with the respective proteases at specified temperatures. The hydrolysates were then centrifuged, and the supernatants were freeze-dried for subsequent analyses. The DPPH radical scavenging activity of squid protein hydrolysate (SPH) was assessed using a modified method based on Kitts and Weiler (2003).

$$\text{Yield (\%) wet basis} = \frac{\text{Weight of protein powder (g)}}{\text{Weight of fish minced used (g)}} \times 100$$

A Box-Behnken response surface design with three process variables at three levels, including three centre points, was utilized for the experimental design (Myers & Montgomery, 2002). The antioxidant activity (AOA) of each hydrolysate was assessed through 15 experimental runs, employing a second-order response surface regression model to optimize squid protein hydrolysis conditions. The

Table 1. The Box-Behnken design for optimizing the hydrolysis conditions of pepsin hydrolysate with DPPH activity

Run number	Coded levels of variable			Experimental values			Response value
	X_1	X_2	X_3	E/S ratio (%)	pH	Time (h)	AOA (%)
1	0	-1	+1	1.25	1.5	6.00	58.01
2	-1	+1	0	0.50	2.5	3.25	45.67
3	-1	-1	0	0.50	1.5	3.25	63.28
4	-1	0	+1	0.50	2.0	6.00	32.58
5	0	0	0	1.25	2.0	3.25	28.97
6	0	0	0	1.25	2.0	3.25	30.62
7	+1	-1	0	2.00	1.5	3.25	41.01
8	-1	0	-1	0.50	2.0	0.30	25.51
9	0	-1	-1	1.25	1.5	0.30	43.79
10	0	+1	+1	1.25	2.5	6.00	47.25
11	+1	0	-1	2.00	2.0	0.30	30.47
12	+1	+1	0	2.00	2.5	3.25	32.05
13	0	+1	-1	1.25	2.5	0.30	23.4
14	0	0	0	1.25	2.0	3.25	28.89
15	+1	0	+1	2.00	2.0	6.00	36.87

Table 2. The Box-Behnken design for optimizing the hydrolysis conditions of papain hydrolysate with DPPH activity

Run number	Coded levels of variable			Experimental values			Response value
	X_1	X_2	X_3	E/S ratio (%)	pH	Time (h)	AOA (%)
1	+1	0	-1	2.00	6.0	0.30	28.51
2	-1	-1	0	0.50	5.0	3.25	66.34
3	0	-1	+1	1.25	5.0	6.00	81.57
4	0	+1	+1	1.25	7.0	6.00	82.63
5	0	0	0	1.25	6.0	3.25	61.39
6	0	0	0	1.25	6.0	3.25	65.54
7	+1	0	+1	2.00	6.0	6.00	77.97
8	+1	+1	0	2.00	7.0	3.25	74.85
9	0	0	0	1.25	6.0	3.25	72.08
10	0	+1	-1	1.25	7.0	0.30	38.42
11	-1	+1	0	0.50	7.0	3.25	79.41
12	-1	0	-1	0.50	6.0	0.30	83.96
13	0	-1	-1	1.25	5.0	0.30	48.32
14	-1	0	+1	0.50	6.0	6.00	79.24
15	+1	-1	0	2.00	5.0	3.25	63.76

Table 3. The Box-Behnken design for optimizing the hydrolysis conditions of trypsin hydrolysate with DPPH activity

Run number	Coded levels of variable			Experimental values			Response value
	X_1	X_2	X_3	E/S ratio (%)	pH	Time (h)	AOA (%)
1	0	-1	+1	1.25	6.0	6.00	75.64
2	0	0	0	1.25	7.5	3.25	56.14
3	0	0	0	1.25	7.5	3.25	58.90
4	0	-1	-1	1.25	6.0	0.50	63.35
5	0	+1	+1	1.25	9.0	6.00	76.91
6	0	+1	-1	1.25	9.0	0.30	51.91
7	+1	0	+1	2.00	7.5	6.00	75.00
8	+1	-1	0	2.00	6.0	3.25	69.28
9	-1	0	+1	0.50	7.5	6.00	77.97
10	0	0	0	1.25	7.5	3.25	47.67
11	-1	+1	0	0.50	9.0	3.25	56.78
12	-1	-1	0	0.50	6.0	3.25	56.57
13	+1	0	-1	2.00	7.5	0.30	40.25
14	-1	0	-1	0.50	7.5	0.30	61.23
15	+1	+1	0	2.00	9.0	3.25	56.99

Box-Behnken designs employed to optimize the hydrolysis conditions of pepsin, papain, and trypsin hydrolysates based on DPPH antioxidant activity are presented in Tables 1, 2 & 3, respectively. The enzyme-to-substrate (E/S) ratio (X1), pH of the solution (X2), and process time (X3) were optimized. Equation 1 represents the model used to separate total variability into linear, quadratic, and interaction components of the process parameters. Ridge analysis was conducted to predict responses at various distances from the centre of the design space, with optimization based on ridge scores, response surface plots, and desirability functions. A validation study under optimized conditions analyzed AOA using one-way ANOVA, with significant results at the 5% level ($p < 0.05$), followed by Duncan's test for comparing the mean AOA values of different proteases. All statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., 2012).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum b_{ij} X_i X_{j,i\neq j=1,2,3} \text{.....(1)}$$

Results and Discussion

The protein content of squid muscle was found to be 17.5%. The yield of protein concentrate extracted from Indian squid muscle was 10.2% and exhibited a protein content of 90.38%. The distribution and composition of amino acids in the squid protein concentrate were determined and presented in Fig. 1. The figure shows several antioxidant-potent amino acids, including aspartic acid, glutamate, lysine, leucine, valine, phenylalanine, histidine, methionine, alanine, tyrosine, and cystine, which can scavenge free radicals (Nazeer, Deeptha, Jaiganesh, Sampathkumar, & Naqash, 2011) and were present at 14.08%, 13.94%, 6.98%, 6.79%, 6.5%, 6.05%, 5.95%, 1.43%, 1.34%, and 0.37%, respectively. Among the amino acids with antioxidant potential, higher concentrations (14.08%) of aspartic acid and relatively lower concentrations (0.26%) of proline were identified. The availability of these potent antioxidant amino acids would contribute to the strong antioxidant characteristics of the isolated squid protein hydrolysate.

The linear, quadratic, and interaction effects of hydrolysis conditions on antioxidant activity in the pepsin hydrolysate of squid muscle protein explained the total variability in antioxidant activity. An R^2 value of 0.83 was significant for the second-order response surface regression. Results from the linear effects of the E/S ratio, pH, and hydrolysis

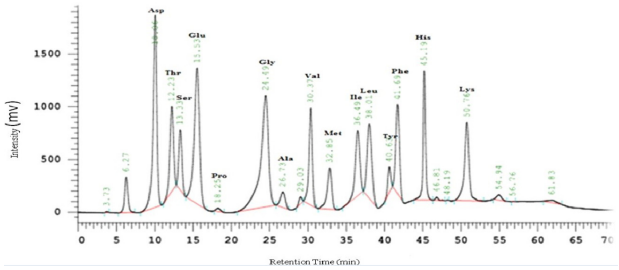

Fig. 1. Quantitative Distribution of Amino Acids in Indian Squid Protein Concentrate

time showed decreasing antioxidant activity in the pepsin hydrolysate, while quadratic effects revealed increasing antioxidant activity, except for time. Similarly, the increasing trend of the interaction effects of the independent variables was not observed for the E/S ratio and hydrolysis time (Equation 2).

AOA of pepsin = $309.26-25.18X_1-249.07X_2^{**}-0.82X_3+3.79X_1^2+55.53X_2^{2*}-0.03X_3^2+5.77X_1X_2-0.08X_1X_3+1.69X_2X_3$, $R^2=0.83$.....(2)

Response surface plots of the predicted antioxidant activity in pepsin hydrolysate as a function of changes in hydrolysis conditions at pH 2, an E/S ratio of 1.25, and a time of 3.15 hours are illustrated in Fig. 2(A), 2(B), and 2(C), respectively. Significant dose- and pH-dependent changes in pepsin hydrolysate antioxidant activity were observed at a fixed time of 3.15 hours. This decrease in antioxidant activity with increasing E/S ratio and pH up to 2.2 may be due to over-hydrolysis at higher enzyme concentrations and reduced pepsin activity at suboptimal pH, resulting in fewer bioactive peptides. The change in antioxidant activity was negligible initially at a fixed pH of 2, but increased with time at E/S ratios starting at 1.25%. This

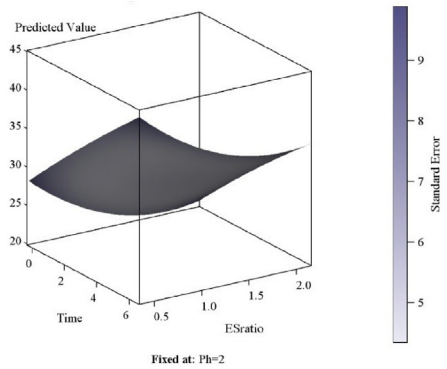

Fig. 2(A)

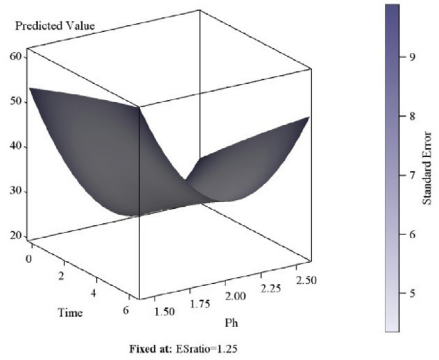

Fig. 2(B)

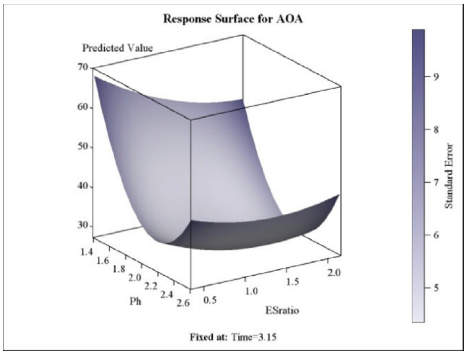

Fig. 2(C)

Fig. 2(A), 2(B), and 2(C). Response surface plot showing the effect of hydrolysis conditions at a fixed pH of 2, a fixed E/S ratio of 1.25%, and a fixed time of 3.15 hours, respectively, on the AOA of pepsin hydrolysate.

increase may be attributed to the gradual release of medium-sized peptides rich in antioxidant amino acids over time. Similarly, at a fixed E/S concentration of 1.25%, antioxidant activity also increased slightly over time, which reflects the progressive generation of antioxidant peptides without over-degradation. In contrast, antioxidant activity decreased significantly at a pH level of 2 and increased with higher pH levels. This trend is likely due to the narrow pH optimum of pepsin, where slight increase in pH enhance peptide solubility and exposure of antioxidant residues, improving peptide activity.

Fig. 2(D), 2(E), and 2(F) represent the response contour graphs depicting the impact of hydrolysis conditions at a constant time of 3.15 hours, a pH of 2, and an E/S ratio of 1.25%, respectively, on the antioxidant activity of pepsin hydrolysate derived from squid protein. The limitations in antioxidant activity of pepsin hydrolysate were influenced by variations in the E/S ratio and pH, with the activity maximized at a pH higher than 2.2 (Fig. 2D). Similarly, an increasing interaction between the E/S ratio and time was observed to regulate the evolution of antioxidant activity (Fig. 2E). The combined effect of hydrolysis time and pH also significantly affected the antioxidant properties of pepsin hydrolysate (Fig. 2F). The optimal conditions for pepsin hydrolysis of squid protein were established at an enzyme-to-substrate (E/S) ratio of 1:100, pH 2, and a hydrolysis time of 3.15 hours in a hydrated system. Under these conditions, the hydrolysate exhibited an antioxidant activity of 29.49%. These parameters were optimized using ridge analysis to identify the combination of variables that maximized the response.

The variability in antioxidant activity of papain hydrolysate was modeled using a second-order response surface regression, which accounted for the linear, quadratic, and interaction effects of

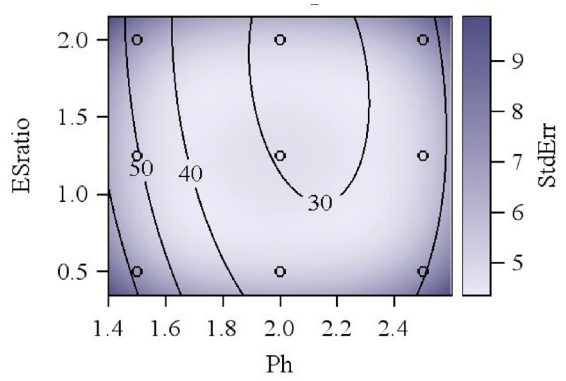

Fig. 2(D)

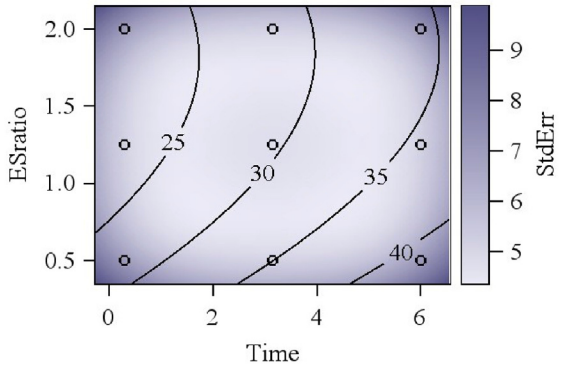

Fig. 2(E)

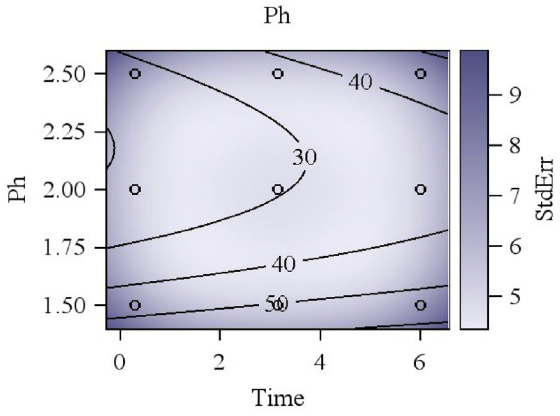

Fig. 2(F)

Fig. 2(D), 2(E), and 2(F). Response contour plots showing the effect of hydrolysis conditions at a fixed time of 3.15 hours, a fixed pH of 2, and a fixed E/S ratio of 1.25%, respectively, on the AOA of pepsin hydrolysate.

hydrolysis conditions on squid muscle protein, yielding a significant coefficient of determination (R^2 = 0.84). The AOA of papain hydrolysate varied linearly with the independent variables and non-linearly with the hydrolysis conditions, except for time. However, the interaction effects generally showed a decreasing trend in antioxidant activity, except when considering the interaction between time and E/S ratio (Equation 3).

$$\text{AOA of papain} = 99.34 - 47.62X_1 - 0.70X_2 - 5.51X_3 + 8.39X_1^2 + 0.035X_2^2 - 0.45X_3^2 - 0.66X_1X_2 + 6.34X_1X_3 - 0.96X_2X_3,\ R^2 = 0.84 \quad \text{.....(3)}$$

The changes in hydrolysis conditions for predicting the antioxidant activity (AOA) values of papain hydrolysate are presented in the response surface plots (Fig. 3A, 3B, and 3C). At a fixed hydrolysis time of 3.15 hours, a slight increase in AOA was observed with rising pH. This may be due to enhanced enzyme activity and improved solubility of protein substrates at slightly higher pH, leading to greater release of antioxidant peptides. Similarly, AOA increased as the E/S ratios ranged from 1% to 1.5%. This increase likely reflects the generation of an optimal concentration of bioactive peptides, whereas too low or excessively high E/S ratios may limit peptide formation or cause over-hydrolysis. At a constant pH of 6, a downward parabolic trend in AOA was noted as a function of time. This could be attributed to the initial accumulation of antioxidant peptides followed by partial degradation or formation of smaller peptides with reduced activity over prolonged hydrolysis. E/S ratios up to 1% showed neither activity nor AOA, but above this concentration, AOA increased, which indicates that a minimum enzyme concentration is required to release sufficient antioxidant peptides from the substrate. A similar initial upward trend in AOA

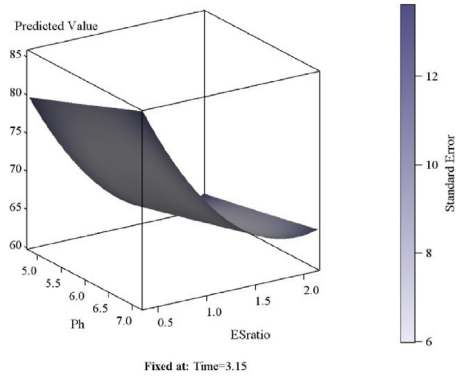

Fig. 3(A)

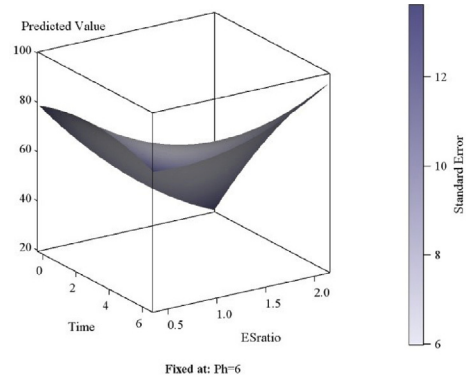

Fig. 3(B)

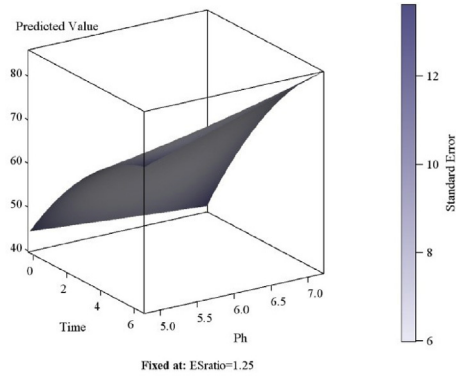

Fig. 3(C)

Fig. 3 (A), 3(B), and 3(C). Response surface plots illustrating the effect of hydrolysis conditions at a fixed time of 3.15 hours, a fixed pH of 6, and a fixed E/S ratio of 1.25%, respectively, on the AOA of papain hydrolysate.

over time and an upward parabolic trend in AOA with variations in pH were reported by Nazeer et al. (2011) at an E/S ratio of 1.25%. This supports the observation that both enzyme concentration and pH critically influence the generation of bioactive peptides contributing to antioxidant activity.

Response contour graphs depicting the antioxidant activity of papain hydrolysate from squid protein as a function of the enzyme/substrate (E/S) ratio and pH at a fixed time of 3.15 hours (Fig. 3D), the E/S ratio and time fixed at pH 6 (Fig. 3E), and pH and time fixed at an E/S ratio of 1.25% (Fig. 3F) are also presented. The combined effect of the E/S ratio and pH demonstrated a decreasing trend in antioxidant activity, while the interaction between pH and time exhibited an increasing trend at a fixed E/S concentration of 1.25%. The AOA of papain hydrolysates decreased with increasing E/S ratios but increased over time. Ridge analysis determined that the optimal conditions for producing papain hydrolysate with enhanced antioxidant properties (66.34%) were an E/S ratio of 1.5:100, a pH of 6.25, and a hydrolysis time of 5.15 hours. Similar findings were also reported by Nazeer et al. (2011).

Hydrolysis conditions of squid muscle protein were modelled using a second-order response surface regression, explaining 85% of the variability in antioxidant activity (AOA). This model utilized linear, quadratic, and interaction effects to analyze the data. Generally, the hydrolysis conditions exhibited a linear effect on AOA, with the exception of the enzyme-to-substrate (E/S) ratio. However, significant quadratic effects of the E/S ratio, pH, and time were observed, leading to an increase in AOA. Similar to the independent variables, the interaction effects among them also positively influenced AOA, except in the cases of pH and E/S ratio (Equation 4).

AOA of trypsin = $183.85 + 7.18X_1 - 30.75X_2 - 10.68X_3 + 2.07X_1^2 + 2X_2^2 + 1.01X_3^2 - 2.78X_1X_2 + 2.11X_1X_3 + 0.74X_2X_3$, $R^2=0.85$.....(4)

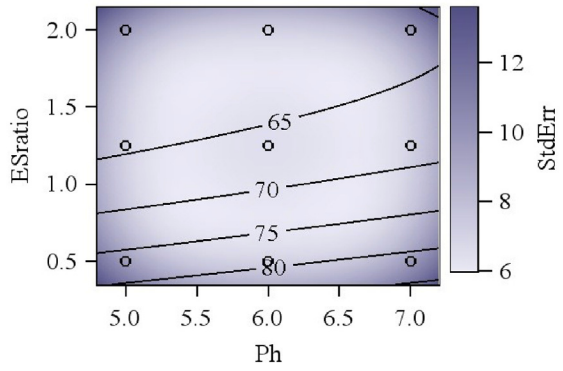

Fig. 3(D)

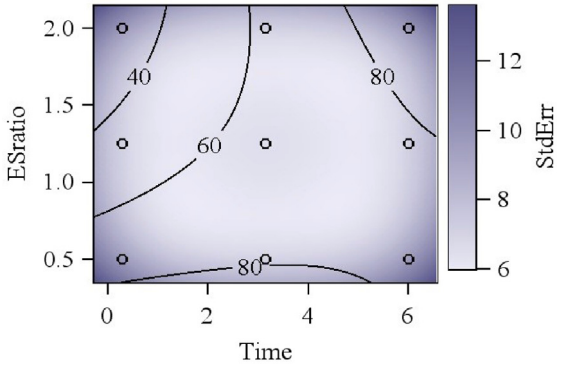

Fig. 3(E)

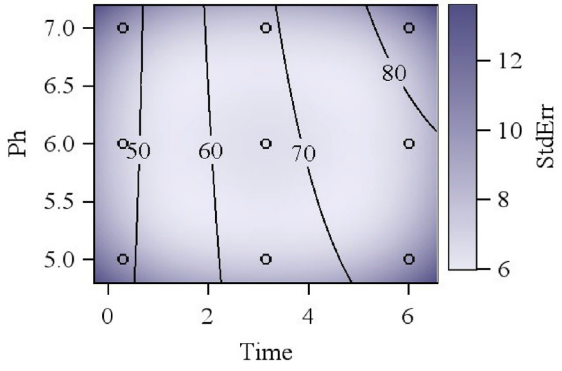

Fig. 3(F)

Fig. 3(D), 3(E), and 3(F). Response contour plots showing the effect of hydrolysis conditions at a fixed time of 3.15 hours, a fixed pH of 6, and a fixed E/S ratio of 1.25%, respectively, on the AOA of papain hydrolysate

Response surface plots, i.e., Fig. 4(A), 4(B), and 4(C), respectively, illustrate the effect of hydrolysis conditions at a fixed time (3.15 h), pH (7.5), and E/S ratio (1.25%) on the predicted values of antioxidant activity (AOA) of trypsin hydrolysate. The antioxidant activity exhibited an upward parabolic pattern in relation to pH. This may be due to the optimal activity of trypsin and improved solubility of protein substrates at moderate pH, which promotes the release of bioactive peptides. AOA decreased with increasing E/S concentration, which could result from over-hydrolysis at higher enzyme concentrations, producing smaller peptides or amino acids with lower radical-scavenging activity. An upward parabolic trend was also observed in the interaction between the E/S ratio and time, with AOA increasing concurrently with higher E/S concentrations. This suggests that an optimal balance between enzyme amount and hydrolysis duration is necessary to maximize peptide formation without over-degradation. Additionally, a similar upward parabolic trend in AOA over time was evident, with higher antioxidant activity corresponding to changes in pH. This likely reflects the gradual accumulation of medium-sized antioxidant peptides as hydrolysis progresses under near-optimal pH conditions.

The response contour plot in Fig. 4(D) depicts the influence of E/S ratio and pH on the antioxidant activity (AOA) of trypsin hydrolysate at a constant hydrolysis time of 3.15 hours. The AOA is found in higher levels in samples with lower E/S concentrations and pH levels. The effect of the E/S ratio and time at this pH (7.5) is shown in Fig. 4(E) as a curve of AOA as a function of trypsin hydrolysate. A higher AOA was obtained using an E/S ratio between 0.5% and 1.3% and a hydrolysis time of 6

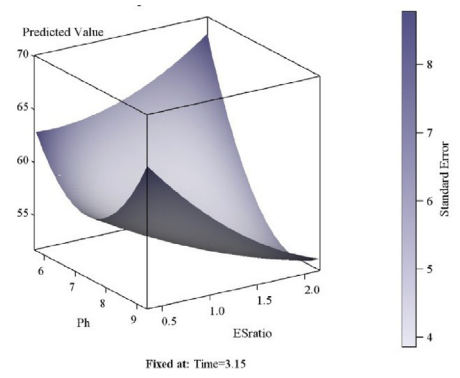

Fig. 4(A)

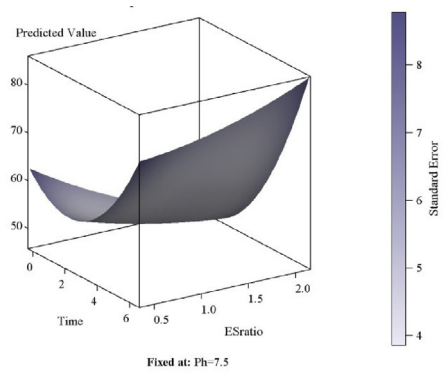

Fig. 4(B)

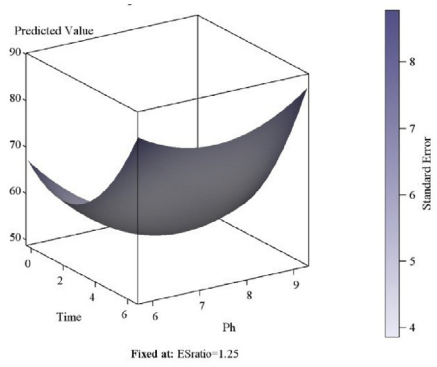

Fig. 4(C)

Fig. 4 (A), 4(B), and 4(C). Response surface plots illustrating the effect of hydrolysis condition at a fixed time of 3.15 hours, a fixed pH of 7.5, and a fixed E/S ratio of 1.25%, respectively, on the AOA of trypsin hydrolysate.

hours. Similarly, in experiments at constant E/S, lower pH levels and longer experimental times led to higher AOA for trypsin hydrolysate (Fig. 4F). Ridge analysis identified the optimal conditions for producing trypsin hydrolysate with superior antioxidant properties (54.24%) at an E/S ratio of 1.4%, pH 7.25, and a hydrolysis time of 6 hours.

In the validation study, the experimental antioxidant activity values of papain, trypsin, and pepsin hydrolysates processed under optimal hydrolysis conditions were 65.8%, 53.6%, and 28.4%, respectively (Fig. 5). These results align closely with the predictions from the second-order response surface regression model (i.e., 66.34%, 54.24% and 29.49%, respectively) demonstrating a reasonable percentage error range. Among the protein hydrolysates, the DPPH scavenging activity was lowest for pepsin, intermediate for trypsin, and highest for papain. This result correlates well with the findings of Fang, Xie, Chen, Yu, and Chen (2012), who also standardized the enzymatic hydrolysis conditions for flying squid using RSM and observed the highest antioxidant properties in papain-digested peptides. The authors reported that papain hydrolysates of flying squid exhibited approximately 70% DPPH scavenging activity, significantly higher than those produced using pepsin and trypsin, highlighting the enzyme's superior cleavage efficiency in releasing hydrophobic and aromatic amino acid residues that enhance radical-scavenging potential. Similarly, Ren et al. (2008) employed RSM to optimize protease hydrolysis conditions for synthesizing antioxidant peptides from grass carp hydrolysates and also found the highest antioxidant activity in papain-digested hydrolysates. The study demonstrated that papain treatment yielded hydrolysates with higher antioxidant activity due to the generation of low-molecular-weight peptides rich in tyrosine, tryptophan, and phenylalanine, which effectively donate

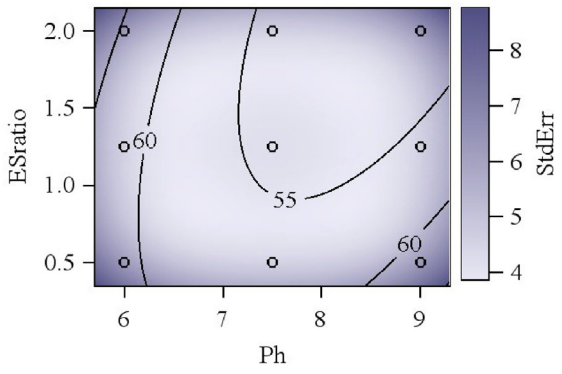

Fig. 4(D)

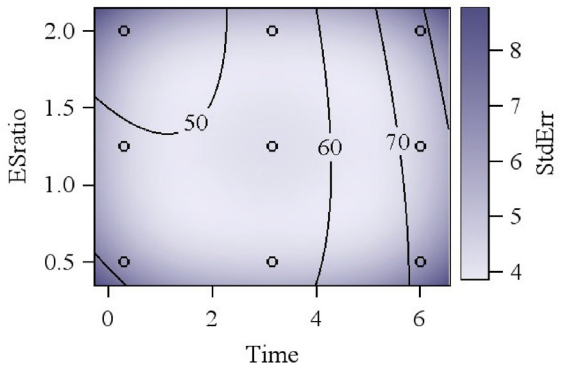

Fig. 4(E)

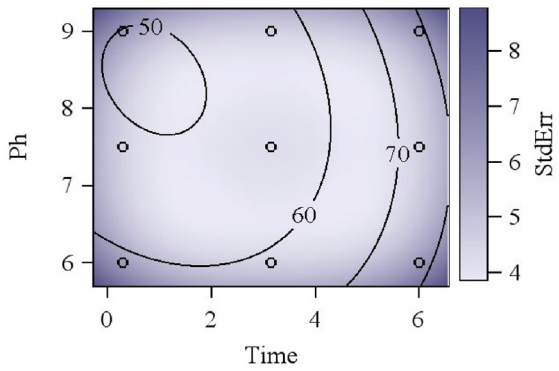

Fig. 4(F)

Fig. 4(D), 4(E), and 4(F). Response contour plots showing the effect of hydrolysis condition at a fixed time of 3.15 hours, a fixed pH of 7.5, and a fixed E/S ratio of 1.25%, respectively, on the AOA of trypsin hydrolysate.

hydrogen atoms to neutralize free radicals. These findings corroborate the results of the present study, suggesting that the broad substrate specificity of papain promotes the generation of potent antioxidant peptides from Indian squid.

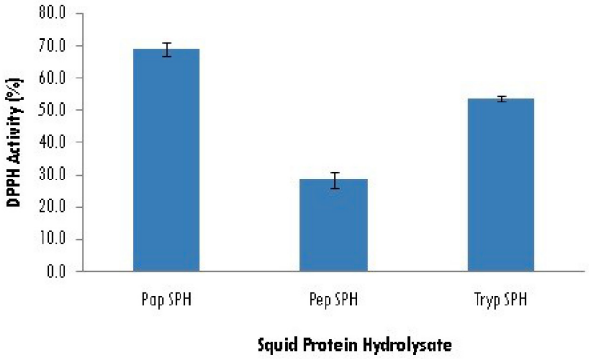

Fig. 5. DPPH scavenging activity of squid protein hydrolysates

The hydrolysis conditions associated with protease treatment were found to play a critical role in determining the antioxidant properties of squid protein hydrolysates. Amino acids from the squid protein concentrate were successfully isolated as potent antioxidants. Total variability in antioxidant activity was described by a second-order response surface model through the linear, quadratic, and interaction terms of hydrolysis parameters. The model was suitable for predicting the response variable from the independent variables, as indicated by a high coefficient of determination (R^2).

Based on antioxidant activity and results from ridge and multiple response analysis, the optimal hydrolysis conditions were identified as follows: For papain, an enzyme/substrate (E/S) ratio of 1.5:100, a pH of 6.25, and a time of 5.15 hours were optimal. For pepsin, optimal conditions were determined to be an E/S ratio of 1:100, pH 2, and a hydrolysis duration of 3.15 hours. In contrast, trypsin exhibited optimal activity at an E/S ratio of 1.4:100 and pH 7 and time 6 hours. Among these, the papain-digested squid protein hydrolysate exhibited the highest antioxidant activity, whereas longer hydrolysis time compared to pepsin may lead to the formation of bitter peptides. These findings strongly correlate the predicted antioxidant activity values by the model and the experimental results, which further supports the demonstrated model's high applicability in optimizing protease hydrolysis conditions in Indian Squid.

Acknowledgments

The authors are indebted to the Director, ICAR-CIFT, Kochi, for granting permission to conduct the research at the institute and for supporting the publication of this paper in a reputed journal.

References

Abdulazeez, S. S., Ramamoorthy, B., & Ponnusamy, P. (2013). Proximate analysis and production of protein hydrolysate from King Fish of Arabian Gulf Coast-Saudi Arabia. *International Journal of Pharmacy and Biological Sciences*, *3*(1), 138-144.

Aguilar-Toalá, J. E., Quintanar-Guerrero, D., Liceaga, A. M., & Zambrano-Zaragoza, M. L. (2022). Encapsulation of bioactive peptides: A strategy to improve the bioactivities and stability of bioactive peptides. *RSC Advances, 12*, Article 111060.

Alasalvar, C., Miyashita, K., Shahidi, F., & Wanasundara, U. (2011). *Handbook of seafood quality, safety and health applications*. John Wiley & Sons.

Chen, H. Y., & Chen, C. (2025). A Study of the Response Surface Methodology Model with Regression Analysis in Three Fields of Engineering. *Applied System Innovation, 8*, Article 99.

Chen, J., Wei, C., Hou, J., Wang, J., & Ruan, Q. (2025). Debittering and antioxidant improvement of soy protein hydrolysates using curcumin as hydrophobic core. *Journal of the Science of Food and Agriculture, 105*(4), 2506–2514.

Chen, X. E., Xie, N. N., Fang, X. B., Yu, H., Ya-mei, J., & Zhen-da, L. (2010). Antioxidant activity and molecular weight distribution of in vitro gastrointestinal digestive hydrolysate from Flying squid (*Ommastrephes batramii*) skin-gelatin. *Food Science, 31*, 123-130.

Fang, X., Xie, N., Chen, X., Yu, H., & Chen, J. (2012). Optimization of antioxidant hydrolysate production from flying squid muscle protein using response surface methodology. *Food and Bioproducts Processing, 90*(4), 676-682.

Hsu, K. C. (2010). Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product. *Food Chemistry, 122*(1), 42-48.

Ishida, Y., Fujita, T., & Asai, K. (1981). New detection and separation method for amino acids by high-performance liquid chromatography. *Journal of Chromatography A, 204*, 143-148.

Je, J. Y., Qian, Z. J., Lee, S. H., Byun, H. G., & Kim, S. K. (2008). Purification and antioxidant properties of bigeye tuna (*Thunnus obesus*) dark muscle peptide on free radical-mediated oxidative systems. *Journal of Medicinal Food, 11*(4), 629-637.

Kaur, L., Mao, B., Beniwal, A.S., Abhilasha; Kaur, R., Chian, F.M., & Singh, J. (2022). Alternative proteins vs animal proteins: The influence of structure and processing on their gastro-small intestinal digestion. *Trends in Food Science & Technology, 122*, 275–286.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design*, *9*(16), 1309-1323.

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, *102*(4), 1317-1327.

Marchbank, T., Limdi, J. K., Mahmood, A., Elia, G., & Playford, R. J. (2008). Clinical trial: protective effect of a commercial fish protein hydrolysate against indomethacin (NSAID) induced small intestinal injury. *Alimentary Pharmacology & Therapeutics*, *28*(6), 799-804.

Mendis, E., Rajapakse, N., Byun, H. G., & Kim, S. K. (2005). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their in vitro antioxidant effects. *Life Sciences*, *77*(17), 2166-2178.

Myers, R. H., & Montgomery, D. C. (2002). Response surface methodology: process and product optimization using designed experiments (2nd ed.). Wiley. New York.

Nazeer, R. A., Deeptha, R., Jaiganesh, R., Sampathkumar, N. S., & Naqash, S. Y. (2011). Radical scavenging activity of seela (*Sphyraena barracuda*) and ribbon fish (*Lepturacanthus savala*) backbone protein hydrolysates. *International Journal of Peptide Research and Therapeutics*, *17*(3), 209-216.

Pangestuti, R., & Kim, S. K. (2017). Bioactive peptide of marine origin for the prevention and treatment of non-communicable diseases. *Marine Drugs*, *15*(3), 67.

Peña-Ramos, E. A., & Xiong, Y. L. (2003). Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. *Meat Science*, *64*(3), 259-263.

Peng, X., Xiong, Y. L., & Kong, B. (2009). Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. *Food Chemistry*, *113*(1), 196-201.

Rajapakse, N., Mendis, E., Byun, H. G., & Kim, S. K. (2005). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *The Journal of Nutritional Biochemistry*, *16*(9), 562-569.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., & Xue, S. J. (2008). Optimization of antioxidant peptide production from grass carp sarcoplasmic protein using response surface methodology. *LWT-Food Science and Technology*, *41*(9), 1624-1632.

SAS Institute Inc. (2012). *SAS: Statistical software* (Version 9.3). SAS Institute Inc., USA.

Sathe, S. K., & Salunkhe, D. K. (1981). Functional properties of the great northern bean (*Phaseolus vulgaris L.*) proteins: emulsion, foaming, viscosity, and gelation properties. *Journal of Food Science*, *46*(1), 71-81.

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International*, *91*(4), 914-931.

Venkatesan, J., Anil, S., Kim, S. K., & Shim, M. S. (2017). Marine fish proteins and peptides for cosmeceuticals: A review. *Marine Drugs*, *15*(5), 143.

Wu, H. C., Chen, H. M., & Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, *36*(9-10), 949-957.

Zhong, K., Gao, X. L., Xu, Z. J., Gao, H., Fan, S., Yamaguchi, I., Li, L. H., & Chen, R. J. (2011). Antioxidant activity of a novel Streptomyces strain Eri12 isolated from the rhizosphere of *Rhizoma curcumae* Longae. *Current Research in Bacteriology, 4*(2), 63-72. https://doi.org/10.3923/crb.2011.63.72.