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Antioxidant Activities of Squid Protein Hydrolysates Prepared with Papain Using Response Surface Methodology

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Abstract Squid protein hydrolysates (SPH) were prepared from the Indian squid *Loligo duvauceli* using papain. Response surface methodology (RSM) was used for optimization of hydrolysis conditions, including temperature, time, and the enzyme-substrate ratio using DPPH radical scavenging activity as a response. The amino acid composition of SPH was compared with raw squid muscle. *In vitro* antioxidant activities were evaluated based on reducing power, metal chelation, ABTS, hydroxyl radical, and superoxide anion radical scavenging assays. SPH exhibited good ABTS radical scavenging activities of 96.50±0.90%, superoxide anion radical scavenging activities of 96.4±0.89%, reducing powers of 0.71±0.02, moderate hydroxyl radical scavenging activities of 64.03±2.11%, and metal chelating activities of 52.04±1.02%. *In vivo* antioxidant activities determined using a sardine minced model system showed 42% reduction in formation of secondary oxidative products as thiobarbituric acid reactive substances (TBARS), almost equivalent to reduction by ascorbic acid of 41.42% at 400 ppm.

Keywords: antioxidant activity, squid protein hydrolysate, response surface methodology, meat model system, papain

Introduction

In recent years, there has been an increased demand for health beneficial products derived from fish based protein substances. Fish protein hydrolysates (FPH) are products generally prepared using proteolytic enzymes and have been well documented in the last decade for health promoting antioxidant, antihypertensive, anti-proliferative, anticoagulant, antimicrobial, anticancer, and antidiabetic activities (1,2). These properties are believed to be due to the presence of peptides in FPHs with a size ranging from 3-20 amino acid residues (2). These peptides are referred to as bioactive peptides and associated bioactive properties differ based on molecular weight, structure, amino acid composition, and sequence (2). Among bioactive properties of FPHs, antioxidant activities have received attention over the years as lipid oxidation is a major biochemical problem occurring in food and living systems. In food systems, oxidation of lipids leads to rancidity, which is often characterized by undesirable flavors, odors, and production of toxic substances that affect product quality and nutritional value (3).

In living systems, reactive oxygen species (ROS) generated during metabolic processes attack membrane lipid, protein, and DNA macromolecules that may lead to cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases (3). Therefore, many synthetic commercial antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and propyl gallate (PG), have been used to retard the oxidation processes. However, synthetic antioxidants must be used under strict regulation due to potential health hazards (2). Hence, research has focused on searching for natural antioxidants that have no potential harmful effects.

Antioxidant properties of peptides present in FPHs are governed by hydrolysis conditions, viz., temperature, time, the enzyme/substrate ratio (E/S ratio), pH, enzyme type, and nature of substrate (4). Hence, hydrolysis conditions need to be optimized for production of FPH with antioxidant properties. Response surface methodology (RSM) is a statistical tool that has been used for optimization of hydrolysis conditions for production of FPH (4).

Squid is an important fishery in India with an annual production of 83,223 tons in 2014 (5). Protein hydrolysates prepared from squid are expected to provide unique nutraceutical activities. Although there has been some work on antioxidant properties of hydrolysates of different squid species (4,6), the Indian squid *Loligo duvauceli*

remains unstudied. Therefore, antioxidant properties of hydrolysates prepared from Indian squid were investigated. Preliminary studies on proximate composition of Indian squid exhibits 16% protein and 0.38% fat, which is desirable for preparation of SPH as a high fat content can physically interfere with proper hydrolysis (Proximate data is not given).

Materials and Methods

Materials The Indian squid *Loligo duvauceli* was used for hydrolysate preparation. The oil sardine *Sardinella longiceps* was used as a meat model study system. Squids and sardines were procured from Thoothukudi Fishing Harbor, Tamilnadu, India in March of 2015 and transported to the laboratory on ice where they were washed twice with chilled water at 4±1°C and heads, tentacles, skin, and gut contents were removed. Squid mantle tissues were then rinsed in chilled potable water at 4±1°C and used for preparation of squid protein hydrolysates.

Chemicals 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), phenazine-methosulfate (PMS), and sodium salicylate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Ferrozine, nitroblue-tetrezolium chloride (NBT), β-nicotinamide adenine dinucleotide (NADH), ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ferrous sulfate ($FeSO_4$), ethylene diamine tetra acetic acid (EDTA), papain, butylated hydroxyl anisole (BHA), thiobarbituric acid (TBA), ascorbic acid (ASA), and Tris were purchased from Merck (Darmstadt, Germany).

Optimization of hydrolysis conditions using RSM Optimization of squid protein hydrolysis conditions using the RSM function of Design-Expert 7.0.0 was performed following the method described by Fang *et al*. (4). SPH was prepared with papain. Temperature (A), time (B), and the E/S ratio (C) were independent variables and the DPPH radical scavenging activity was the response. A Box-Behnken Design (BBD) was applied with 3 variables at 3 levels to achieve 15 runs (Table 1).

Squid mantle tissues were homogenized using an Ultra-Turrax T25 high speed homogenizer (Janke & Kunkel, Staufen, Germany) and mixed with distilled water at a ratio of 1:2. The enzyme, papain, was added at a pre-determined E/S ratio and the reaction mixture was incubated in a water bath shaker (T03/WBS; Technico, Chennai, India) under varying temperatures for different durations of hydrolysis (Table 1). The reaction was stopped by heating the mixture in a water bath set at 90±2°C for 20 min. SPH was then filtered through Whatman No.4 filter paper and stored in airtight containers at −20°C in deep freezer (Bluestar, Chennai, India). The DPPH free radical scavenging activity of SPH was determined following the method described by Yen and Wu (7). Based on optimum conditions obtained using the RSM model, SPH was produced and subjected to antioxidant activity assays.

Amino acid composition of SPH The amino acid composition of SPH prepared under optimum conditions was determined and compared with raw squid muscle following the method of Ishida *et al*. (8). Ten mg of SPH and raw squid sample was transferred to an ampule (Borosil Glass Works Ltd., Ahmedabad, India) sealed under a stream of nitrogen gas, and hydrolyzed using 6 N HCl. The hydrolyzed samples were filtered through 0.2 μm filter and derivatized using an AccQ. Tag Ultra derivatization kit (Waters Corp., Milford, MA, USA). Amino acid derivatives were then separated using a Waters ACQUITY-UPLC 1.7 μm, 2.1x100 mm column fitted with an AccQ. Tag Ultra C18 derivatization kit for stepwise elution. Amino acids were quantified based on absorbance values at 260 nm using a tunable UV detector

Table 1. A Box–Behnken design for optimization of hydrolysis conditions

Coded values of variable				Experimental values			Response
Run	A	B	C	Temperature (°C)	Time (min)	E/S ratio (%)	DPPH (%)
1	-1	-1	0	30	20	1.5	60.37
2	1	-1	0	60	20	1.5	61.69
3	-1	1	0	30	60	1.5	62.14
4	1	1	0	60	60	1.5	62.36
5	-1	0	-1	30	40	0.5	63.56
6	1	0	-1	60	40	0.5	64.72
7	-1	0	1	30	40	2.5	67.59
8	1	0	1	60	40	2.5	69.64
9	0	-1	-1	45	20	0.5	59.94
10	0	1	-1	45	60	0.5	57.58
11	0	-1	1	45	20	2.5	61.63
12	0	1	1	45	60	2.5	64.31
13	0	0	0	45	40	1.5	71.21
14	0	0	0	45	40	1.5	71.48
15	0	0	0	45	40	1.5	71.58

and analyzed using Empower 2 Software (Waters Corp.). Amino acid standards were also run simultaneously for calibration. Amino acids present in SPH were represented as mg/g.

Antioxidant properties of SPH

***Reducing power assay*:** The ability of SPH to reduce ferric iron was determined following the method described by Oyaiza (9). A 1 mL aliquot of SPH containing 5-25 mg of protein/mL was mixed with 2.5 mL of 0.2 M phosphate buffer at pH 6.6 and 2.5 mL of 1% (w/v) potassium ferriccyanide. The reaction mixture was incubated at 50°C for 30 min in a water bath (T03/SWB; Technico, Chennai, India) and the reaction was stopped via addition of 2.5 mL of 10% (w/v) trichloroacetic acid (TCA). Then, a 2.5 mL solution was pipetted out and mixed with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) $FeCl_3$ solution, followed by incubation for 10 min at room temperature and the absorbance was measured at 700 nm using a UV-Vis spectrophotometer (V-530; Jasco, Easton, MD, USA). Distilled water and BHA at 200 ppm were used as a control and a positive control, respectively. Higher absorbance values of the reaction mixture indicated higher reducing powers.

***ABTS radical scavenging activity*:** The ABTS radical scavenging activity of SPH was determined following the method described by Binsan *et al*. (10). An ABTS stock solution was prepared via mixing equal volumes of 7.4 mM ABTS and 2.6 mM potassium persulfate and allowing a reaction for 12 h at room temperature in the dark. Then, 1 mL of the ABTS stock solution was mixed with 50 mL of methanol to obtain an absorbance of 1.1±0.02 units at 734 nm measured using a UV-Vis spectrophotometer (V-530; Jasco). A 150 μL aliquot of each SPH containing 5-25 mg of protein/mL was mixed with 2.85 mL of the ABTS stock solution and the mixture was allowed to react at room temperature for 2 h in the dark. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer (V-530; Jasco). Distilled water and BHA at 200 ppm were used as a control and a positive control, respectively. The ABTS scavenging activity was calculated as:

$$\text{ABTS scavenging activity (\%)} = 1-\left(\frac{Abs_{sample}}{Abs_{control}}\right)\times 100$$

***Metal chelating activity*:** The metal chelating ability of SPH for chelation of ferrous iron was assessed using the method of Decker and Welch (11). A 1 mL aliquot of SPH was mixed with 3.7 mL of distilled water then, 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added. The reaction mixture was held for 10 min at room temperature before reading the absorbance at 562 nm using a UV-Vis spectrophotometer (V-530; Jasco). Distilled water and EDTA at 250 ppm were used as a control and a positive control, respectively. The metal chelating activity was calculated as:

$$\text{Metal chelating activity (\%)} = 1-\left(\frac{Abs_{sample}}{Abs_{control}}\right)\times 100$$

***Hydroxyl radical scavenging activity*:** The hydroxyl radical scavenging activity of SPH was assayed following the method described by Smirnoff and Cumbes (12). A 1 mL aliquot of SPH was mixed with 1.0 mL of 1.5 mM $FeSO_4$, 0.7 mL of 6 mM hydrogen peroxide, and 0.3 mL of 20 mM sodium salicylate. The reaction mixture was then incubated for 1 h at 37°C in a water bath. After incubation, the absorbance of the hydroxylated salicylate complex was measured at 562 nm using a UV-Vis spectrophotometer (V-530; Jasco). Distilled water and ASA at 400 ppm were used as a control and a positive control, respectively. The scavenging activity of the hydroxyl radical was calculated as:

$$\text{Hydroxyl radical scavenging activity (\%)} = \left[1-\left(\frac{Abs_{sample}-Abs_{sample\ control}}{Abs_{control}}\right)\times 100\right]$$

where, $Abs_{sample\ control}$ is the absorbance value without sodium salicylate.

***Superoxide anion radical scavenging activity*:** The superoxide anion radical scavenging activity of SPH was determined following the method described by Liu *et al*. (13). A 1 mL aliquot of SPH was mixed with 1 mL of a 50 μM NBT solution and 1 mL of a 78 μM NADH solution. A reaction was initiated via addition of 1 mL of a 10 μM PMS solution. The reaction mixture was incubated in a water bath at 25°C for 5 min. The absorbance was measured at 560 nm using a double beam UV-Vis spectrophotometer (V-530; Jasco). Tris-HCl at 16 mM and pH 8.0 and ASA at 400 ppm were used as a control and a positive control, respectively. The superoxide anion radical scavenging activity was calculated as:

$$\text{Superoxide anion radical scavenging activity (\%)} = 1-\left(\frac{Abs_{sample}}{Abs_{control}}\right)\times 100$$

Antioxidant activity in a sardine meat model system Sardine meat was minced using an Ultra-Turrax T25 high speed homogenizer (Janke & Kunkel). Minced fish was divided into 2 portions of 750 g each, then 25 mL of SPH was added to obtain a final concentration of 50 mg/kg of minced sardine meat. Meat without SPH served as a control and minced sardine meat with ASA at 400 ppm was used as a positive control. Both control and treated minced meat were separately placed in polythene bags, placed in styrofoam boxes containing ice, and stored in a chiller at 5°C. For determination of the extent of lipid oxidation, changes in thiobarbituric acid reactive substance (TBARS) values for 10 days were analyzed following the method described by Raghavan and Hultin (14).

Statistical analysis All analyses were carried out in triplicate and results are expressed as mean±standard deviation (SD). A one-way analysis of variance (ANOVA) was used for antioxidant assay data. Significant differences were defined as $p<0.05$ for comparison of antioxidant assay triplicate mean values determined using the

Duncan's multiple range test function of SPSS, version 20 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Optimization of hydrolysis conditions using RSM Statistical analysis of the quadratic model and interactions among the 3 variables for response values are presented in Table 2. The *p* value of the model was <0.0001, indicating that the model was significant and could be used for optimization of hydrolysis conditions. Among the 3 independent variables, the *p* value of E/S ratio, C (p<0.0001) was lower than temperature, A (p<0.0137), and time, B (p<0.0830), indicating that the E/S ratio had a higher influence than temperature and time on antioxidant activity.

A lack of fit test was used to determine whether the model was adequate for description of observed data. If the *p* value of the lack of fit is <0.05, the model is adequate (15). In this study, the coefficient of determination (R^2=0.9965) indicated that only 0.35% of total variation was not explained by the model. The value of the adjusted coefficient of determination was used to determine how much the model was significant. The adjusted coefficient of determination (Adj R^2=0.9902) also showed a high degree of significance for the model. Moreover, predicted R^2=0.9477 was in reasonable agreement with Adj R^2=0.9902. The range of the predicted response relative to associated error was measured based on adequate precision. The model was suitable for preparation of SPH using papain. The best explanatory model equation for antioxidant activity based on the response surface quadratic model was:

$$DPPH=71.42+0.59A+0.34B+2.17C-0.27AB+0.22AC+1.26BC-2.14A^2-7.35B^2+2.91C^2$$

where A=temperature, B=time, C=the E/S ratio.

Two dimensional contour plots and 3D response surface plots were used for graphical representation of the regression equation (Fig. 1). Mutual interactions between factors were expressed as counter plots. If a counter plot is circular, interactions between corresponding factors are negligible. An elliptical contour plot shows significant interactions between corresponding variables (16). Patterns of interactions between temperature and time and between the E/S ratio and time are shown in Fig. 1A and 1C, respectively. An elliptical counter plot is evident, which indicates significant interactions between temperature and time and between the E/S ratio and time. A circular counter plot indicates that the interaction between the E/S ratio and temperature was not significant and can be ignored (Fig. 1B). Under optimum conditions, 3D surface plots exhibit convex shapes (17). All response surface plots shown in Fig. 1D, 1E, and 1F are convex, indicating that well-defined optimum conditions were present in the model.

Optimum conditions for a high DPPH activity obtained from the equation were temperature=46°C, time=45 min, and the E/S ratio= 1.71, for which a DPPH antioxidant activity of 71.49% was expected. The validity of the model was confirmed based on 3 assays under optimum conditions. The average antioxidant activity value was 71.46% with a 99% confidence level, which was close to the expected antioxidant activity value. Optimum conditions were used for preparation of SPH for further *in vitro* and *in vivo* anti-oxidative analysis.

Amino acid composition of SPH Amino acid composition is a critical factor that determines antioxidant activity. An amino acid profile provides information on individual amino acids in hydrolysates that are responsible for antioxidant activities. Amino acid compositions of raw squid and SPH are given in Table 3.

Table 2. Analysis of variance for the hydrolysate response of antioxidant activity

Source	Sum of squares	DF	Mean square	*F* value	*p* value
Model	291.45	9	32.38	158.93	<0.0001
A	2.82	1	2.82	13.84	0.0137
B	0.95	1	0.95	4.67	0.0830
C	37.71	1	37.71	185.09	<0.0001
AB	0.30	1	0.30	1.48	0.2774
AC	0.20	1	0.20	0.97	0.3695
BC	6.35	1	6.35	31.17	0.0025
A^2	16.84	1	16.84	82.63	0.0003
B^2	215.97	1	215.97	1059.91	<0.0001
C^2	31.28	1	31.28	153.49	<0.0001
Residual	1.02	5	0.20		
Lack of fit	0.95	3	0.32	8.60	0.1059
Pure error	0.073	2	0.037		
Cor total	292.47	14			
R-Squared	0.9965				
Adj *R*-Squared	0.9902				
Pred *R*-Squared	0.9477				
Adeq Precision	37.021				

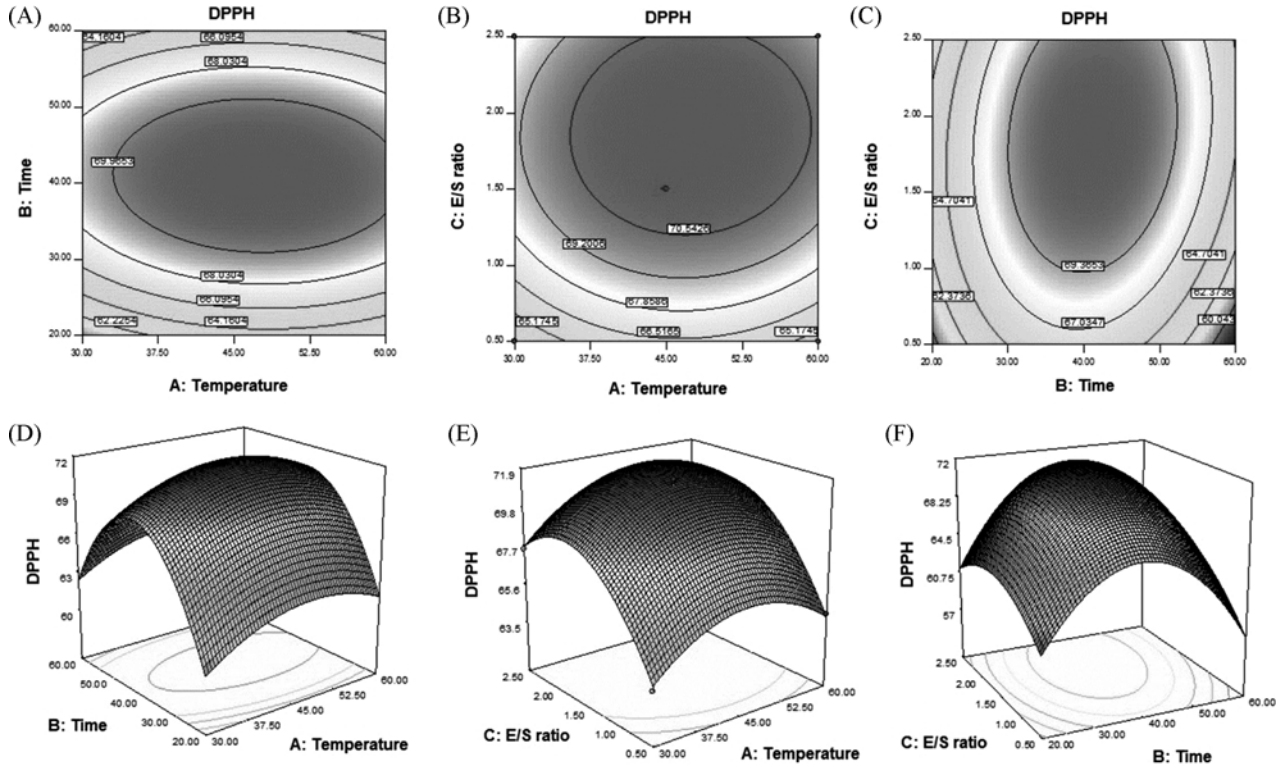

Fig. 1. Contour and 3D response surface plots. Contour plots (A, B, and C) and response surface plots (D, E, and F) for effects of temperature, time, and the E/S ratio on DPPH scavenging activity. (A) and (D) indicate effects of temperature and time, (B) and (E) indicate effects of temperature and the E/S ratio, (C) and (F) indicate effects of time and the E/S ratio.

For SPH, proportions of alanine, lysine, proline, arginine, and glycine were higher than in the raw counterpart, while proportions of histidine, asparagine, and aspartic acid were lower. Papain, being an endopeptidase, cleaves arginine and lysine to yield peptides (18). SPH, therefore, had higher proportions of these amino acids available for antioxidant activities. The total hydrophobic amino acid proportion was 58.37% in SPH. Hydrophobic amino acids, such as alanine, proline, valine, leucine, phenylalanine, and methionine, are reported to play important roles in free radical scavenging mechanisms (6). The total essential amino acid proportion was 43%, indicating that SPH can be used as a supplement for essential amino acids in functional foods.

Table 3. Amino acid composition of raw squid muscle and squid protein hydrolysates (mg/g)

Amino acids	Squid muscle	Squid protein hydrolysate
Histidine	15.64±0.62	2.24±0.44
Asparagine	12.63±0.50	5.81±0.04
Taurine	57.97±3.27	56.65±0.15
Serine	9.69±1.23	6.43±0.01
Arginine	171.43±6.91	205.20±1.01
Glycine	53.65±1.10	72.37±0.15
Aspartic acid	77.40±0.91	44.57±0.05
Glutamic acid	124.62±4.66	129.42±1.10
Threonine	18.79±1.10	18.39±0.02
Alanine	66.86±2.28	88.14±0.11
Proline	23.69±2.11	31.61±0.51
HyLys1	7.78±0.52	6.37±0.10
Cysteine	0.09±0.01	0.11±0.01
Lysine	67.86±1.99	82.90±0.51
Tyrosine	4.02±0.67	3.22±0.04
Methionine	71.01±2.01	73.70±1.00
Valine	35.87±1.00	34.67±.64
Isoleucine	43.33±2.22	38.78±0.32
Leucine	67.09±1.01	64.45±0.91
Phenylalanine	22.97±0.93	23.37±0.23

Antioxidant properties of SPH

***Reducing power activity*:** The reducing power of SPH is based on reduction of iron (III) to iron (II) via donation of an electron. The SPH concentration influences reduction of the ferric cyanide complex. The reducing power significantly (p<0.05) increased with an increasing SPH protein concentration (Fig. 2A). A similar increasing trend was reported for sardine heads and visceral protein hydrolysates prepared using alcalase, and smooth hound muscle hydrolysates prepared using pepsin (19,20). However, there were no significant (p>0.05) differences in activities beyond 20.0 mg/mL of SPH.

Hydrophobic amino acids enhanced reducing powers in soybean hydrolysates (21), and levels of hydrophobic amino acids were high in SPH. Histidine, methionine, tyrosine, lysine, and tryptophan in loach peptides showed strong reducing powers (22) and these amino acids

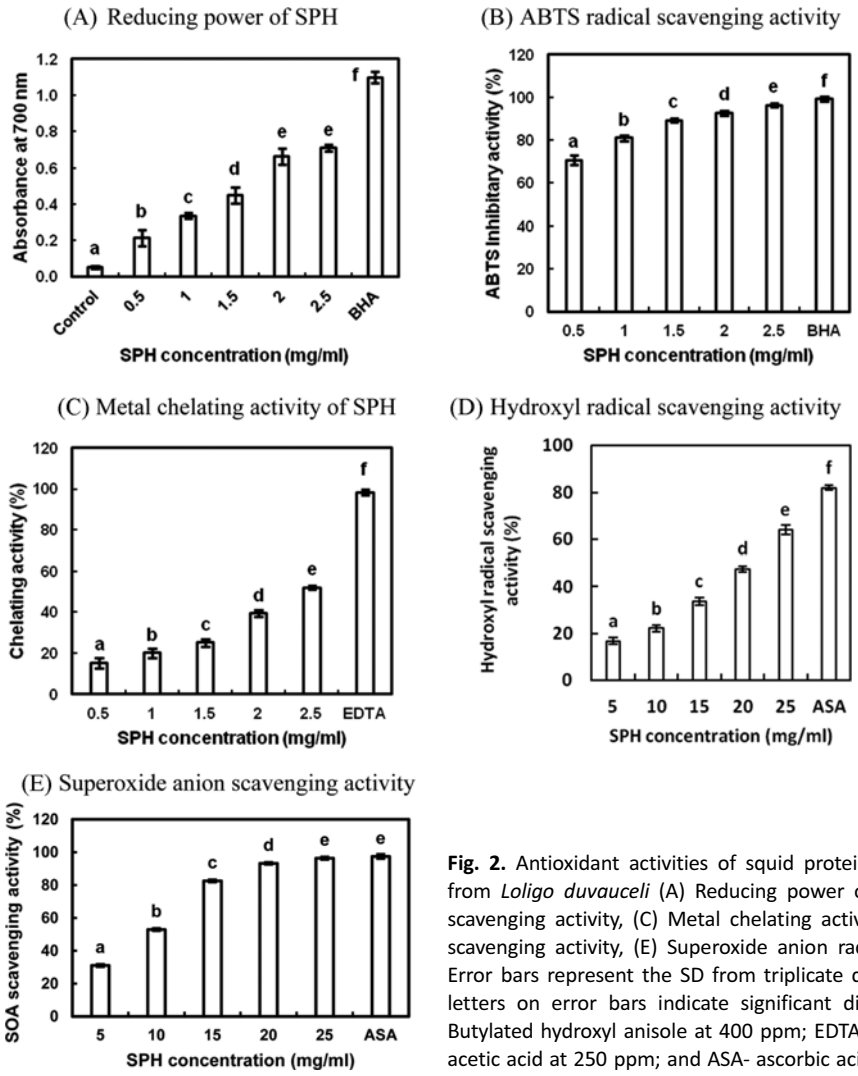

Fig. 2. Antioxidant activities of squid protein hydrolysates prepared from *Loligo duvauceli* (A) Reducing power of SPH, (B) ABTS radical scavenging activity, (C) Metal chelating activity, (D) Hydroxyl radical scavenging activity, (E) Superoxide anion radical scavenging activity. Error bars represent the SD from triplicate determinations. Different letters on error bars indicate significant difference (p<0.05). BHA- Butylated hydroxyl anisole at 400 ppm; EDTA- Ethylene diamine tetra acetic acid at 250 ppm; and ASA- ascorbic acid at 400 ppm.

were also present in SPH. Thus, SPH contained peptides capable of donating electrons to reduce iron (III) to iron (II). The iron reducing power of SPH (0.71±0.02) was slightly lower than the activity shown by 200 ppm BHA (1.10±0.03).

***ABTS radical scavenging activity*:** There was a significant (p<0.05) increase in the ABTS radical scavenging activities of SPH with increasing protein concentrations from 5 to 25 mg/mL (Fig. 2B). The highest activity of 96.50±0.90% was observed at a concentration of 25 mg/mL, which was almost equivalent to the BHA activity of 99.40±0.99% at 200 ppm. Ornate threadfin bream hydrolysates prepared with pepsin extracted from skipjack tuna had higher ABTS activities due to the presence of hydrophilic amino acids (23). SPH also contained nearly 40% hydrophilic amino acids that might have contributed to a strong ABTS activity.

***Metal chelating activity*:** The metal chelating activity of SPH increased significantly (p<0.05) with an increasing concentration of SPH (Fig. 2C). SPH had a metal chelating activity of 52.04±1.02% at 25 mg/mL. Li *et al*. (24) reported that grass carp protein hydrolysates prepared with papain showed a 50% metal chelating activity at a concentration of just 0.81 mg/mL. Transition metal ions that act as electron donors can react quickly with peroxides to form alkoxyl radicals (25). Antioxidant peptides can, thus, retard the oxidation reaction by chelation of transition metal ions. Charged ions present in the acidic amino acids, aspartic acid and glutamic acid, interact with metal ions and retard pro-oxidant activities (26). In this study, SPH contained 17% acidic amino acids, which can become involved in chelation of metal ions. Mendis *et al*. (27) reported that the imidazole ring present in histidine is also involved in metal ion chelation. However, the histidine level in SPH was negligible. The presence of hydrophobic amino acids was also reported to provide a metal chelation activity, which could have accounted for the metal chelating ability of SPH (28).

***Hydroxyl radical scavenging activity*:** The hydroxyl radical is a potent reactive oxygen species capable of initiating lipid peroxidation directly via abstraction of hydrogen from fatty acids. The hydroxyl radical scavenging activity increased significantly (p<0.05) with an increasing protein concentration (Fig. 2D). The highest activity of 64.03±2.11% was observed at an SPH concentration of 25 mg/mL. Li

et al. (24) reported a 50% hydroxyl radical activity at a concentration of 8.12 mg/mL for grass carp protein hydrolysates prepared with papain.

The presence of phenylalanine in peptides allowed high hydroxyl radical scavenging activities due to a reaction of the aromatic ring with hydroxyl radicals to form a stable compound. Tyrosine at the C-terminal was also responsible for hydroxyl radical scavenging activity, based on 3 peptides sequenced from royal jelly protein hydrolysates (29). Suetsuna *et al*. (30) reported that phenolic groups in phenylalanine and tyrosine contributed to hydroxyl radical scavenging activities via electron donation. Substantial amounts of phenylalanine and tyrosine were also present in SPH, indicating a hydroxyl radical scavenging activity.

***Superoxide anion radical scavenging activity*:** The superoxide anion is a weak oxidant that produces hydroxyl radicals and singlet oxygen. Superoxide anion radical scavenging activities of SPH increased significantly (p<0.05) with an increasing protein concentration (Fig. 2E). The highest scavenging activity was 96.40±0.89% at 25 mg/mL, which was close to the 97.42±1.4% activity of 400 ppm ascorbic acid. Different mechanisms have been cited for actions of hydrolysates on superoxide anions. Phenolic rings present in the aromatic amino acids tyrosine and phenylalanine can act as a superoxide anion radical scavenger (31). SPH contained 1.63% tyrosine and 2.93% phenylalanine. Studies of giant squid skin gelatin and casein peptides containing glutamic acid and leucine at the C-terminal position also showed high superoxide anion radical scavenging activities (28,30). Glutamic acid and leucine represented 21% of the total amino acids in SPH. Li *et al*. (32) related the presence of hydrophobic amino acids in chickpea protein hydrolysates to oxygen scavenging activities. More specifically, the basic amino acids histidine, arginine, and lysine have been suggested to have high oxygen scavenging activities in mussel hydrolysates (33). In SPH, the presence of high concentrations of arginine and lysine was unique, compared with raw squid. Although different amino acids have been reported, glutamic acid, leucine, arginine, and lysine were responsible for the superoxide anion radical scavenging activity of SPH.

Antioxidant activity in a sardine meat model system Formation of secondary lipid oxidation products during a period of ice storage in minced sardine meat treated with SPH was monitored based on measurement of TBARS values (Fig. 3), which increased from day 1 to day 8 and, thereafter, levelled off. Formation of TBARS indicated decomposition of primary products into secondary products, especially aldehydes. A decrease in TBARS values beyond the day 8 was probably due to breakdown of aldehydes into volatile compounds. TBARS values increased in minced meat model systems containing SPH at a slower rate than in model systems containing control meat. SPH exhibited a 42.35% reduction in TBARS values, compared with controls, almost equivalent to ASA value of 41.42%. Shahidi *et al*. (34) reported an approximate 44.4% reduction in lipid oxidation for capelin fish protein hydrolysates prepared with papain and added to a pork minced model system at 2% level. Kittiphattanabawon *et al*. (35) reported that blacktip shark skin gelatin hydrolysates prepared with papain reduced lipid oxidation in a comminuted pork meat model system at 500 and 1,000 ppm levels. In this study, SPH retarded lipid oxidation via antioxidant activities and, therefore, can serve as an antioxidant in formulated meat products.

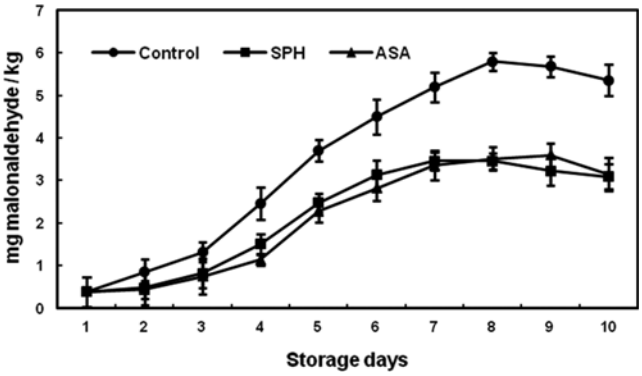

Fig. 3. Effect of squid protein hydrolysates on TBARS formation in an oil sardine minced meat model system. Ascorbic acid (ASA) at 400 ppm was used as a positive control and water was used as a control. Error bars represent standard deviations from triplicate determinations.

Disclosure The authors declare no conflict of interest.

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