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Effects of fermentation periods on antioxidant and angiotensin I-converting enzyme inhibitory activities of peptides from fish sauce by-products

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ABSTRACT

Manufacturing of fish sauce results in a large number of underutilized by-products. The fish sauce by-products (FSBs) contain peptides derived from enzymatic hydrolysis of the fish proteins by proteases from their gastrointestinal tract and halophilic microorganisms. Commercial fish sauce can be divided into different grades based on their fermentation period. The 1st, 2nd and 3rd grade FSBs are solid wastes produced after the 1st, 2nd and 3rd grade fish sauce fermentation. This work aims to evaluate the effect of fermentation periods on antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of the FSBs. Based on bioassays against different mechanisms, the 1st grade FSB possessed stronger DPPH and hydroxyl radical scavenging activities and reducing ability than the 2nd and 3rd grades. The top two grades of FSB showed higher % ACE inhibition than the 3rd grade FSB. All FSBs had equally strong H_2O_2 quenching and iron chelating abilities. It was observed that FSB fractions with smaller molecular weight and higher hydrophobicity possessed higher antioxidant and anti-ACE activities. This work demonstrated that the manufacturing fermentation time affected activities of FSB peptides. Understanding of composition and bioactivities of the FSBs may lead to the development of value-added functional products from these manufacturing wastes.

1. Introduction

Fish sauce is an essential food ingredient commonly used worldwide. It is composed of various nitrogen-containing compounds, including amino acids with an extraordinarily high content of lysine. Anchovies are widely used as major raw materials for fish sauce manufacturing (Lopetcharat, Choi, Park, & Daeschel, 2001). During the process of fish sauce fermentation, two or three parts of marine fish are mixed with one part of salt. Fish sauce can be gradually generated by the functions of proteolytic enzymes from fish and secreted enzymes from halophilic bacteria (Saisithi, 1994). In industry, the processing period of the 1st grade fish sauce ranges from 12 to 18 months. After the 1st period of fermentation, the supernatant is filtered to yield the 1st grade fish sauce and the solid residue becomes the 1st grade fish sauce by-product (FSB). The remaining FSB can be further used to produce the 2nd and 3rd grade fish sauces, respectively, using each fermentation period of 1–4 months (Choksawangkarn, Phiphattananukoon, Jaresitthikunchai, & Roytrakul, 2018; Lopetcharat et al., 2001).

FSB is considered as an industrial waste normally traded as low-value fillers for fertilizers or animal feeds (Pratumwan, Jangchud, Wuttijumnong, & Wisarayut, 2006). The FSB contains 10–15% (w/w) of proteins, considered as natural hydrolysate cleaved by proteases in the system (Saisithi, 1994). Specific sequences and lengths of peptides residing in the hydrolysates from marine sources have shown various bioactivities, such as antioxidant, antiinflammatory, antimicrobial, antihypertensive and anticancer activities (Harnedy & FitzGerald, 2012). Fish sauce has been reported to possess antioxidant and angiotensin I-converting enzyme (ACE) inhibitory peptides (Najafian & Babji, 2019; Sasaki et al., 2013), however, there is less information about bioactive peptides found in its by-products. Recently, antioxidant properties of peptides from the lowest grade FSB has been reported, though other biological activities still remain to be studied

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Table 1
Proximate composition of FSB extract from the 1st, 2nd and 3rd grades.

Composition	% (w/w) of each composition		
	1st FSB	2nd FSB	3rd FSB
Ashes	59.59 ± 0.36^{c}	68.96 ± 0.99^{a}	62.33 ± 0.66^{b}
Moisture	27.67 ± 0.69^{a}	19.45 ± 0.54^{c}	25.16 ± 0.81^{b}
Protein	7.81 ± 0.13^{b}	5.44 ± 0.33^{c}	8.52 ± 0.25^{a}
Carbohydrate	5.67 ± 0.95$^{a, b}$	5.99 ± 1.14^{a}	3.75 ± 0.20^{b}
Lipid	0.17 ± 0.00^{b}	0.16 ± 0.01^{b}	0.24 ± 0.01^{a}
Fiber	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.22 ± 0.00^{a}

The data were presented as the mean ± SD from triplicate results. Values in the same row with different letters are significantly different at $P < 0.05$.

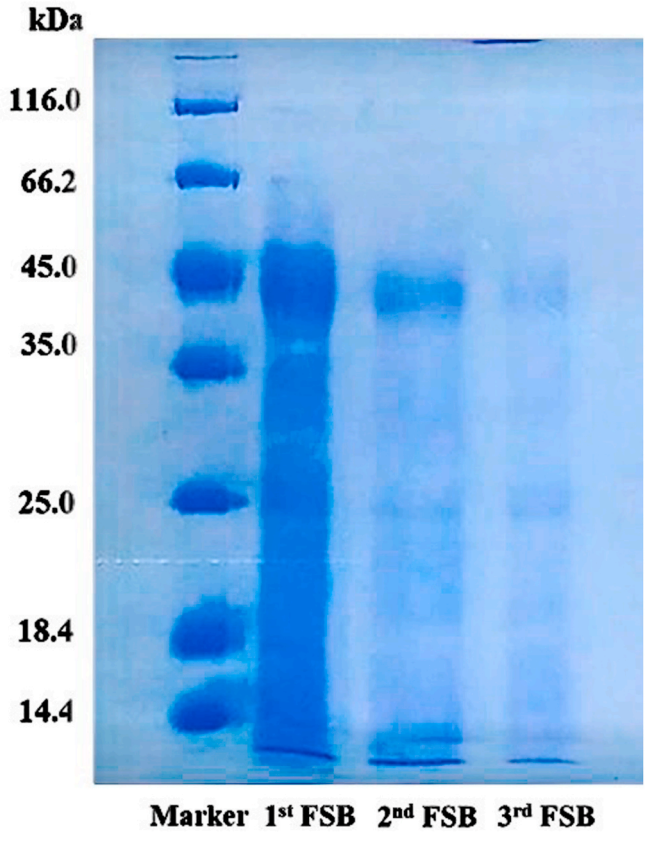

Fig. 1. SDS-PAGE pattern of FSB extracts from the 1st, 2nd and 3rd grades. Lane 1, Marker; Lane 2, crude from 1st FSB; Lane 3, crude from 2nd FSB and Lane 4, crude from 3rd FSB.

(Choksawangkarn et al., 2018).

In this work, the effect of different fermentation periods on the anti-ACE and antioxidant properties of the FSBs were investigated. ACE is one of the main causes of hypertension, which is a major chronic disease that leads to cardiovascular morbidity and mortality. Synthetic ACE inhibitors have been used as therapeutics. However, many side effects were found, including headache, allergic reaction, and taste disturbance (Lee & Hur, 2017). Therefore, ACE inhibitors derived from natural products have been explored as an alternative supplement to decrease blood pressure.

Free radicals are highly reactive molecular species that can attack biomolecules. Excess free radicals cause a number of diseases such as hypertension, heart disease and aging (Carocho & Ferreira, 2013). Antioxidant activities of protein hydrolysate from marine by-products are currently of interest for reducing the risk of these diseases. Active peptides from various fish species including anchovy have been reported to possess antioxidant activities (Song, Wei, Zhang, Yang, & Wang, 2011).

The aims of this study are to evaluate the ACE inhibitory and antioxidant activities of the FSB extracts from the 1st, 2nd and 3rd grades of fish sauce production and to study the effect of fermentation time of the FSB extracts on these two bioactivities. In the future, undervalued fish sauce industrial waste could be developed as functional food to reduce the incidence of diseases.

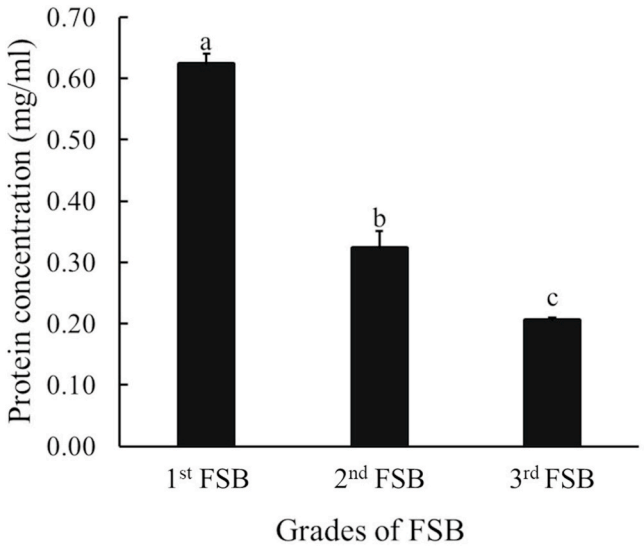

Fig. 3. Protein concentration from the 1st, 2nd, and 3rd grades of FSB. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

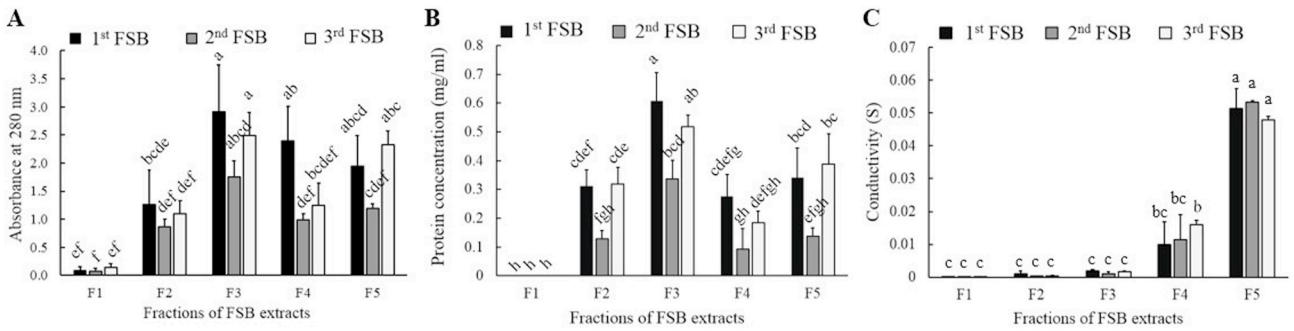

Fig. 2. Absorbance at 280 nm (A), protein concentration (B) and conductivity (C) of fraction 1 to 5 from the 1st, 2nd, and 3rd grades of FSB. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

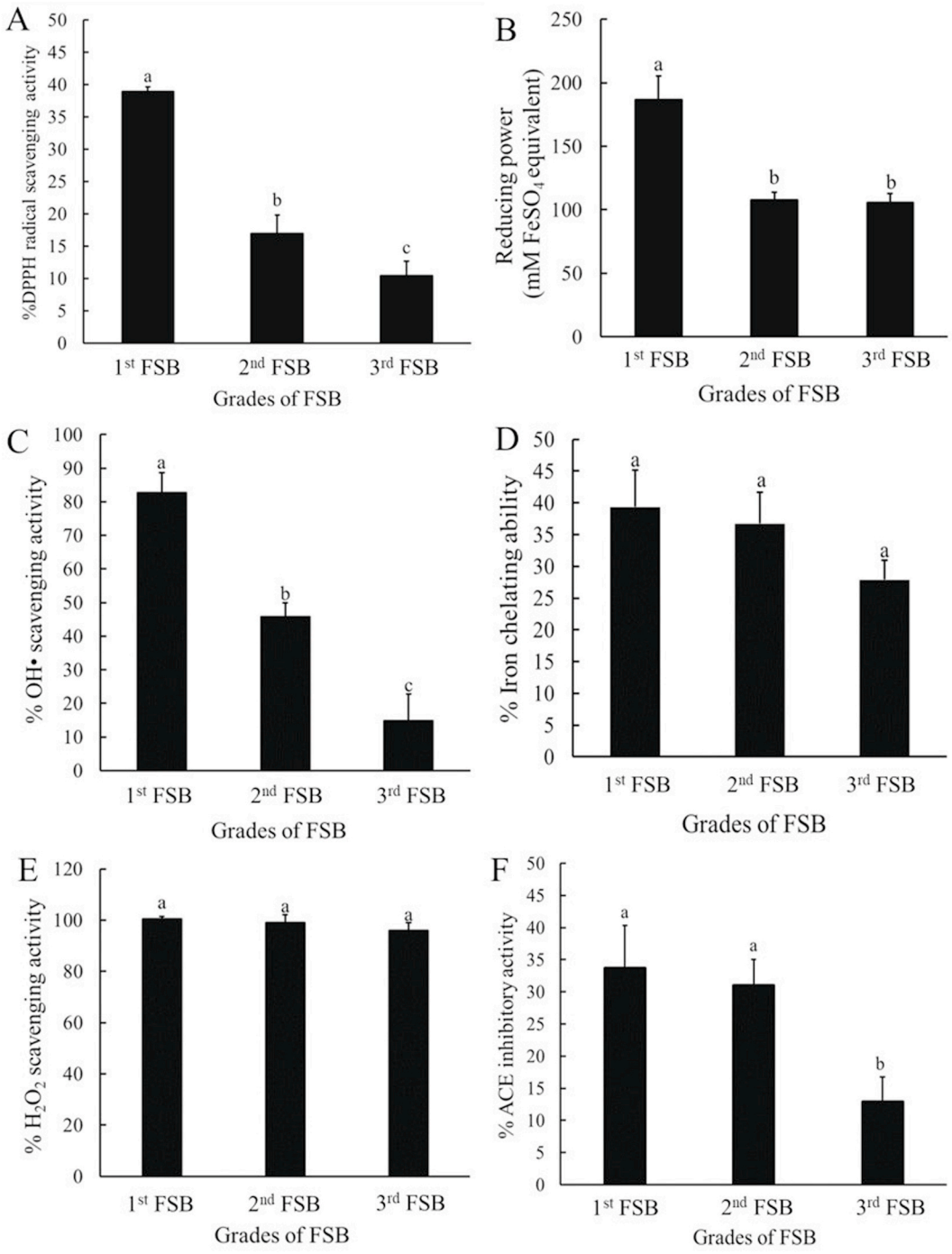

Fig. 4. Biological activities from the 1st, 2nd and 3rd grades of FSB including (A) DPPH radical scavenging activity, (B) ferric reducing antioxidant power, (C) hydroxyl radical scavenging activity, (D) iron chelating activity, (E) hydrogen peroxide quenching activity and (F) ACE inhibitory activity. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

Table 2
IC_{50} or EC_{50} values of antioxidant and ACE inhibitory properties from the 1st, 2nd and 3rd grades of FSB.

FSB	IC_{50} (mg/ml) from DPPH radical scavenging assay	IC_{50} (mg/ml) from hydroxyl radical scavenging assay	EC_{50} (mg/ml) from iron chelation assay	IC_{50} (mg/ml) from H_2O_2 scavenging assay	IC_{50} (mg/ml) from ACE inhibitory assay
1st FSB	0.32 ± 0.02^{b}	0.78 ± 0.07^{a}	0.12 ± 0.02^{c}	0.07 ± 0.01^{a}	0.72 ± 0.27^{b}
2nd FSB	1.05 ± 0.08^{a}	0.36 ± 0.01^{b}	1.00 ± 0.12^{b}	0.06 ± 0.01^{a}	0.62 ± 0.07^{b}
3rd FSB	0.96 ± 0.11^{a}	0.77 ± 0.06^{a}	$>1.5^{a}$	0.03 ± 0.01^{b}	$>1.5^{a}$

The data were presented as the mean ± SD from triplicate results. Values in the same column with different letters are significantly different at $P < 0.05$.

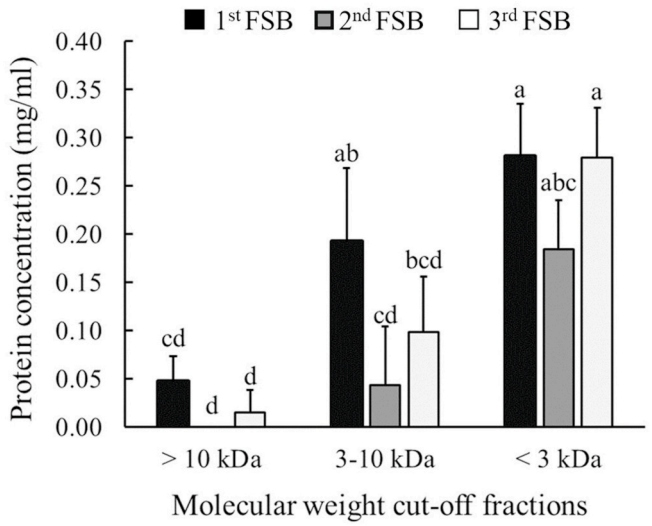

Fig. 5. Protein concentration of different molecular weight cut-off fractions from the 1st, 2nd and 3rd grades of FSB. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

2. Materials and methods

2.1. Materials

ACE, N-hippuryl-his-leu (HHL), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-Triazine (TPTZ), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), $FeCl_3$, ferrozine, $FeSO_4$, and ascorbic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). H_2O_2, acetonitrile and methanol were purchased from Merck Millipore (Darmstadt, Germany). Sephadex G-15 was purchased from GE Healthcare (Uppsala, Sweden). Distilled water was obtained from a Milli-Q® water purification system (Darmstadt, Germany). Acrylamide: bis-Acrylamide (29:1) solution, SDS, the gel loading buffer, glycine, tetramethylethylenediamine and ammonium persulfate were purchased from Bio-Rad (Hercules, CA, USA).

2.2. Raw materials and composition analysis

Fish sauce by-products from Anchovy (*Encrasicholina* sp. and *Stolephorus* sp.) were obtained from Pichai Fish Sauce Co.,Ltd. (Chonburi, Thailand). Whole fish were mixed with NaCl at the ratio of 2:1 (w/w) and incubated in a fermentation tank for 12 months to produce the 1st grade fish sauce and the 1st grade FSB. The 1st grade FSB was further used for the 2nd period of fermentation of 3 months. The supernatant was filtered to yield the 2nd grade fish sauce and its by-product. Then, the 3rd grade fish sauce and by-product were produced from the 2nd grade FSB after 3 months of incubation. The fiber, ashes, moisture, protein and fat contents of the samples were determined for each fermentation period (AOAC, 1997; AOAC, 2000). The remaining percentage indicated the carbohydrate content.

2.3. Crude FSB extraction

The extraction of crude FSB was performed according to Choksawangkarn et al. (2018). FSB (10 g) from the 1st, 2nd and 3rd grades were dissolved in 10 mL distilled H_2O and homogenized for 30 min. The homogenized samples were filtered by Whatman® filter paper No1 and immediately subjected to the SDS-PAGE or the fractionation procedures.

2.4. Investigation of protein profile using gel electrophoresis

The protein profiles of FSB extracts were determined by SDS-PAGE (Laemmli, 1970). Briefly, 10 μL of FSB extracts and the Pierce™ standard protein marker (Thermo Fisher Scientific, Rockford, IL, USA) were loaded onto the discontinuous polyacrylamide gel, comprising 5% stacking gel and 12% separating gel. The gel was run at a constant voltage of 100 V for 60 min, using the Bio-Rad Mini-PROTEAN® electrophoresis system (Hercules, CA, USA). Protein bands were visualized by Coomassie blue staining.

2.5. Fractionation of FSB extracts

FSB extracts were fractionated by Sephadex G-15 gel filtration chromatography. The column dimension was 1.5 × 100 cm and the medium were packed to a height of 50 cm. Crude FSB extract (4 mL) was loaded onto the column and eluted with H_2O at a flow rate of 0.5 mL/min. The first 20 mL of eluted solution was discarded. Five 10 mL fractions of FSB solution were collected for each sample. Protein concentration was evaluated by measuring the absorbance at 280 nm and protein assay (Lowry, Rosebrough, Farr, & Randall, 1951). The salt concentration was determined using conductivity measurements. The gel filtration fractions from each FSB grade with high protein content and low salt content (F2–F4) were combined for bioactivity analyses.

2.6. In vitro biological activities

2.6.1. DPPH radical scavenging assay

DPPH radical scavenging activity was determined according to the method from Molyneux (2004) with slight modifications. A 50 μL aliquot of FSB solution or ascorbic acid as a positive control, were mixed with 100 μL of 0.2 mmol/L DPPH in methanol. The mixtures were incubated in the dark for 30 min and the absorbance was measured at 517 nm. The %inhibition was calculated by Eq. (1) and the half maximal inhibitory concentration (IC_{50}) values were determined.

$$\%\ \text{inhibition or \%chelation} = ((A_{control} - A_{sample})/ A_{control}) * 100 \qquad \text{Eq. (1)}$$

where $A_{control}$ is the absorbance of the control that contained all reagents except the test samples. A_{sample} is the absorbance of the FSB extract with reagents added.

2.6.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out using the method from Benzie and Strain (1999) with modifications. FRAP solution was prepared by mixing 7 mL of 0.28 mol/L acetate buffer (pH 3.6), 700 μL of 5 mg/mL $FeCl_3$ and 700 μL of 10 mmol/L TPTZ in 40 mmol/L HCl. FSB solution (20 μL) was mixed with 180 μL of FRAP solution and incubated for 30 min in the dark. The absorbance was measured at 596 nm $FeSO_4$ at different concentrations (15, 30, 60, 120, 240 and 480 μM) were used as standards and the result was expressed as mmol/L $FeSO_4$ equivalents.

2.6.3. Hydroxyl radical (OH·) scavenging assay

Hydroxyl radical scavenging activity was performed according to Wang, Zhang, Zhang, and Li (2008) with modifications. The reaction mixture consisted of 66 μL of FSB extract, 67 μL of 1.5 mmol/L $FeSO_4$, 47 μL of 6 mmol/L H_2O_2 and 20 μL of 20 mmol/L sodium salicylate. The mixture was stored in the dark for 1 h at 37 °C and the absorbance at 562 nm was monitored. Ascorbic acid was used as a positive control. The activity was calculated using Eq. (1) and the IC_{50} values were determined.

2.6.4. Iron chelation assay

Iron chelating activity was determined according to Carter (1971) using 10 mmol/L EDTA as a positive control. The samples were mixed with 60 μL of 0.8 mmol/L $FeCl_2$ and 25 μL 40 mmol/L ferrozine. The

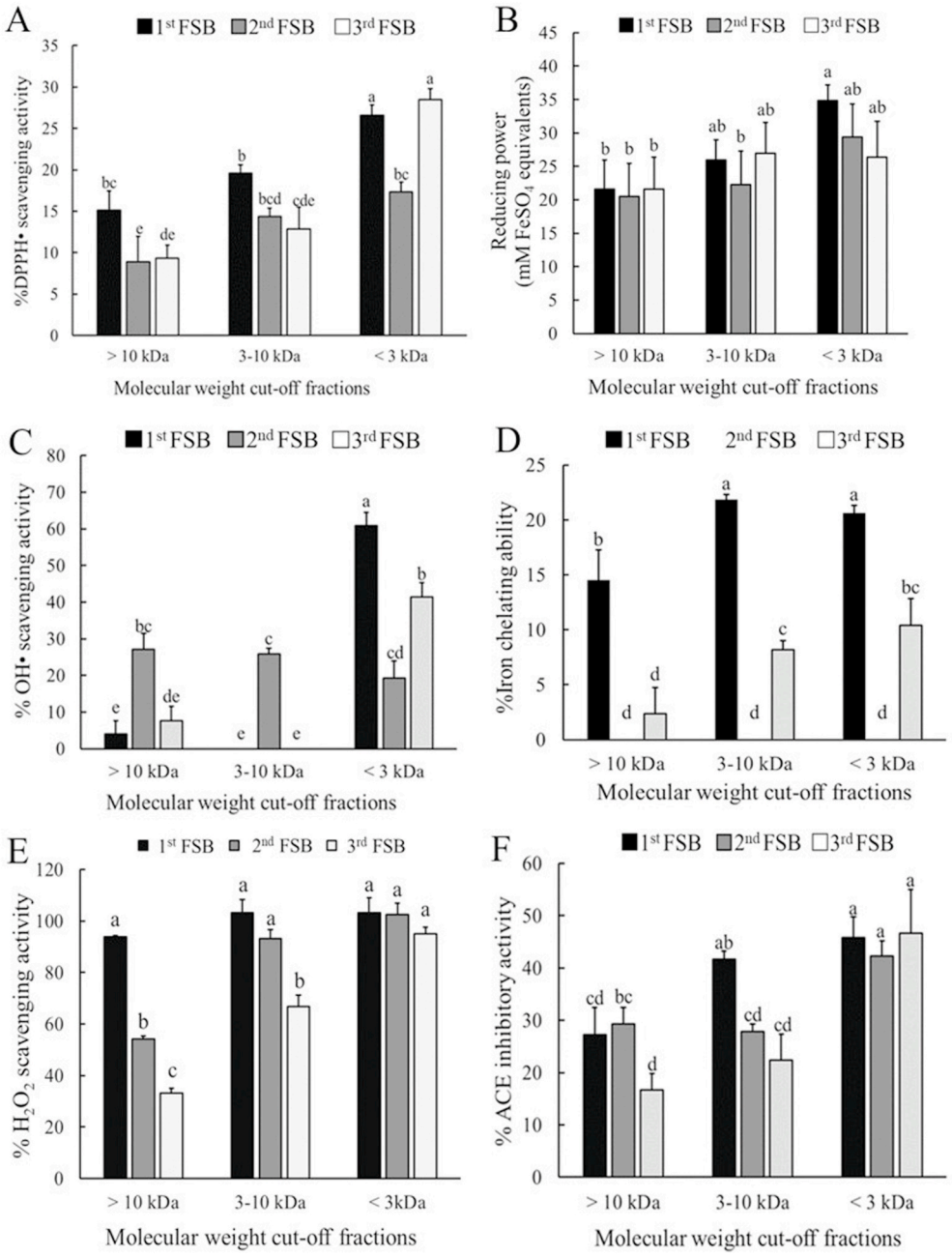

Fig. 6. Biological activities of different molecular wright cut-off fractions from the 1st, 2nd and 3rd grades of FSB including (A) DPPH radical scavenging activity, (B) ferric reducing antioxidant power, (C) hydroxyl radical scavenging activity, (D) iron chelating activity, (E) hydrogen peroxide quenching activity and (F) ACE inhibitory activity. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

mixture was incubated at room temperature for 30 min prior to absorbance measurement at 562 nm. The % chelation was calculated using Eq. (1) and the half maximal effective concentration (EC_{50}) values were evaluated.

2.6.5. H_2O_2 scavenging assay

H_2O_2 scavenging activity was determined according to Karnjanapratum and Benjakul (2015) with modifications. FSB solution (500 μL) was mixed with 41.5 μL of 100 mmol/L H_2O_2 in 0.1 mol/L phosphate buffer, pH 7.4. The reaction was incubated for 40 min at room temperature and the absorbance was measured at 230 nm. Trolox solution was used as the standard. The % H_2O_2 scavenging activity was calculated using Eq. (1) and the IC_{50} values were determined.

2.6.6. ACE inhibitory activity

Anti-ACE activity was determined as described by Cushman and Cheung (1971) with slight modifications. FSB solution (25 μL) was dissolved with 50 μL of 50 mmol/L sodium borate, pH 8.3. The mixture was preincubated with 50 μL of 25 mU/mL ACE (EC.3.4.15.1) at 37 °C for 5 min, followed by addition of 150 μL of 8.3 mmol/L HHL. The reaction was performed at 37 °C for 1 h and stopped by adding 250 μL 1 M HCl. Hippuric acid was extracted into 1.5 mL ethyl acetate and separated by centrifugation at 800×*g* for 15 min. The amount of hippuric acid was determined by measuring the absorbance at 228 nm. The ACE inhibitory activity was calculated using Eq. (1) and the IC_{50} values were determined.

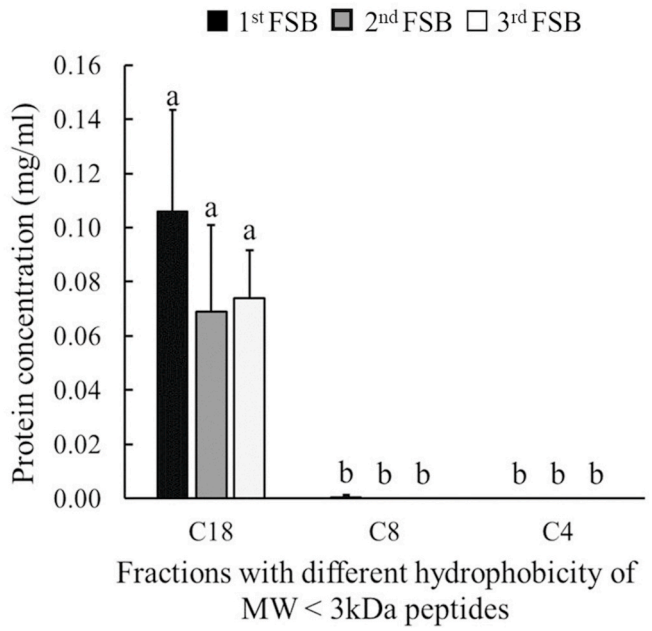

Fig. 7. Protein concentration of fractions with different hydrophobicity of MW < 3 kDa peptide from the 1st, 2nd and 3rd grades of FSB. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

2.7. Peptide fractionation by ultrafiltration

The FSB extracts were further fractionated using ultrafiltration membranes (Millipore Corporation, Bedford, MA, USA). FSB solution (400 μL) from the combined fractions was centrifuged at 14000×*g* for 20 min. Ten kDa molecular weight cut-off (MWCO) was used to separate FSB solution into > 10 kDa retentate and <10 kDa filtrate. The <10 kDa filtrate was further separated using 3 kDa MWCO to obtain 3–10 kDa retentate and <3 kDa filtrate. The three fractions (>10 kDa, 3–10 kDa and <3 kDa) were investigated for bioactivities.

2.8. Reversed phase spin columns chromatography

Spin column chromatography was used to separate FSB fractions based on their hydrophobicity since the column could be customized with different matrices (C18, C8 and C4). This method is suitable for peptide separation because it helps minimize the sample loss and is compatible with the downstream mass spectrometry analysis (Rusnak, Zhou, & Hathaway, 2000). The separation was carried out in the Macro spin column™, following the manufacturer's protocol (Harvard apparatus, Holliston, MA, USA). The spin column with C18 matrix (10 μm particle size, 300 Å pore size) was used first to fractionate the FSB extracts. The flow-through fraction from the C18 column was separated using a C8 spin column (5 μm particle size, 300 Å pore size), followed by a C4 spin column (4.5 μm particle size, 300 Å pore size). Bioactivities of the eluted fraction from each matrix (C18, C8 and C4) were determined and the fraction with high activities were selected for a sequence determination.

2.9. Identification of amino acid sequence using LC-MS/MS

The sample was analyzed using an Ultimate 3000 Nano LC system (Thermo Scientific, UK) coupled to a Q-TOF impact II™ mass spectrometer (Bruker Daltonics, Germany). One microlitre of fractionated peptides (50 ng) was separated on an Acclaim PepMap RSLC C18 analytical column (75 μm × 15 cm, 2 μm, 100 Å, Thermo Scientific, UK) confined in a thermostat oven at 60 °C. A linear gradient elution method at a flow rate of 0.3 μL/min was used, where solvent A was 0.1% formic acid in H_2O and solvent B was 0.1% formic acid in 80% acetonitrile. The conditions used were as follows; equilibration from 0 to 4.0 min using 5% B, separation from 4.0 to 45.0 min using 5–60% B, washing period from 45.0 to 50.0 min using 60%–95% B and re-equilibration from 50.0 to 60.0 min using 5% B. Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Nitrogen was supplied as a drying gas at the flow rate of 50 L/h. Mass spectra were acquired in the positive mode at a frequency of 2 Hz over the m/z range of 150–2200 (Compass 1.9 software, Bruker Daltonics). All spectra were acquired for 40 min using a data-dependent method by selecting the most abundant precursor ions for fragmentation. Collision-induced dissociation was performed at a voltage of 10 eV using nitrogen as the collision gas.

The raw LC-MS spectra were submitted to *de novo* peptide sequencing by using PEAKS studio X (Bioinformatics Solutions Inc., Canada). The precursor mass tolerance was fixed to 0.1 Da and fragment mass tolerance was fixed to 0.01 Da. Variable modifications (oxidation (M), deamination (NQ) and protein acetyl N-term) were allowed. The peptide identification threshold was set to an average local confidence (ALC) score of 95. Potential bioactivity of these peptides was predicted using the PeptideRanker software (http://bioware.ucd.ie/~compass/biowareweb/). This tool provides the probability of the peptides being bioactive based on the training set from available bioactive peptide databases, which include but is not limited to the antioxidant and anti-ACE peptides (Mooney, Haslam, Pollastri, & Shields, 2012).

2.10. Statistical analysis

All results were obtained from at least three replicate experiments and analyzed with Minitab ver. 18 software. One-way analysis of variance (ANOVA) and Tukey's tests were conducted to determine the differences between the bioactivities of FSB extracts from the three grades from different experimental groups ($p < 0.05$).

3. Results and discussions

3.1. Proximate composition of FSB extract

The chemical composition of solid FSB from the 1st, 2nd and 3rd grades are shown in Table 1. The major component was shown to be crude ashes, which mainly resulted from salt and the fish backbone. In agreement with a previous report, the percent compositions decreased in this order, inorganic ashes, moisture, protein, carbohydrate, lipid and fiber, respectively (Choksawangkarn et al., 2018). The results demonstrated that all three grades of FSB contained a range of protein contents between 5.44 ± 0.33% and 8.52 ± 0.25% and could be used as a source of peptides. It is noticeable that all proximate compositions varied among the different grades. This observation could result from the metabolic changes in the fermentation tank due to the dynamic variation of the bacterial community across fermentation periods. This hypothesis could be supported by the published report that the microbiome of traditional Chinese fish sauce was altered during different fermentation stages, resulting in the transformation of the fish sauce compositions (Du, Zhang, Gu, Song, & Gao, 2019).

3.2. Protein profiles of FSB extracts from the 1st, 2nd and 3rd grades

MW of proteins in the 1st, 2nd and 3rd grades of crude FSB were investigated by SDS-PAGE (Fig. 1). All three grades contained major protein bands with approximate MW of 45 and 25 kDa. The results revealed that all grades of FSB extracts had a similar pattern of proteins but with different amounts. The band intensity of proteins from the 1st grade was higher than the 2nd and the 3rd grades, indicating a decrease in protein quantity with lowering FSB grades. In contrast, using the Kjeldahl method to estimate total proteins suggested a different result (Table 1), with the 3rd grade FSB containing the highest proximate

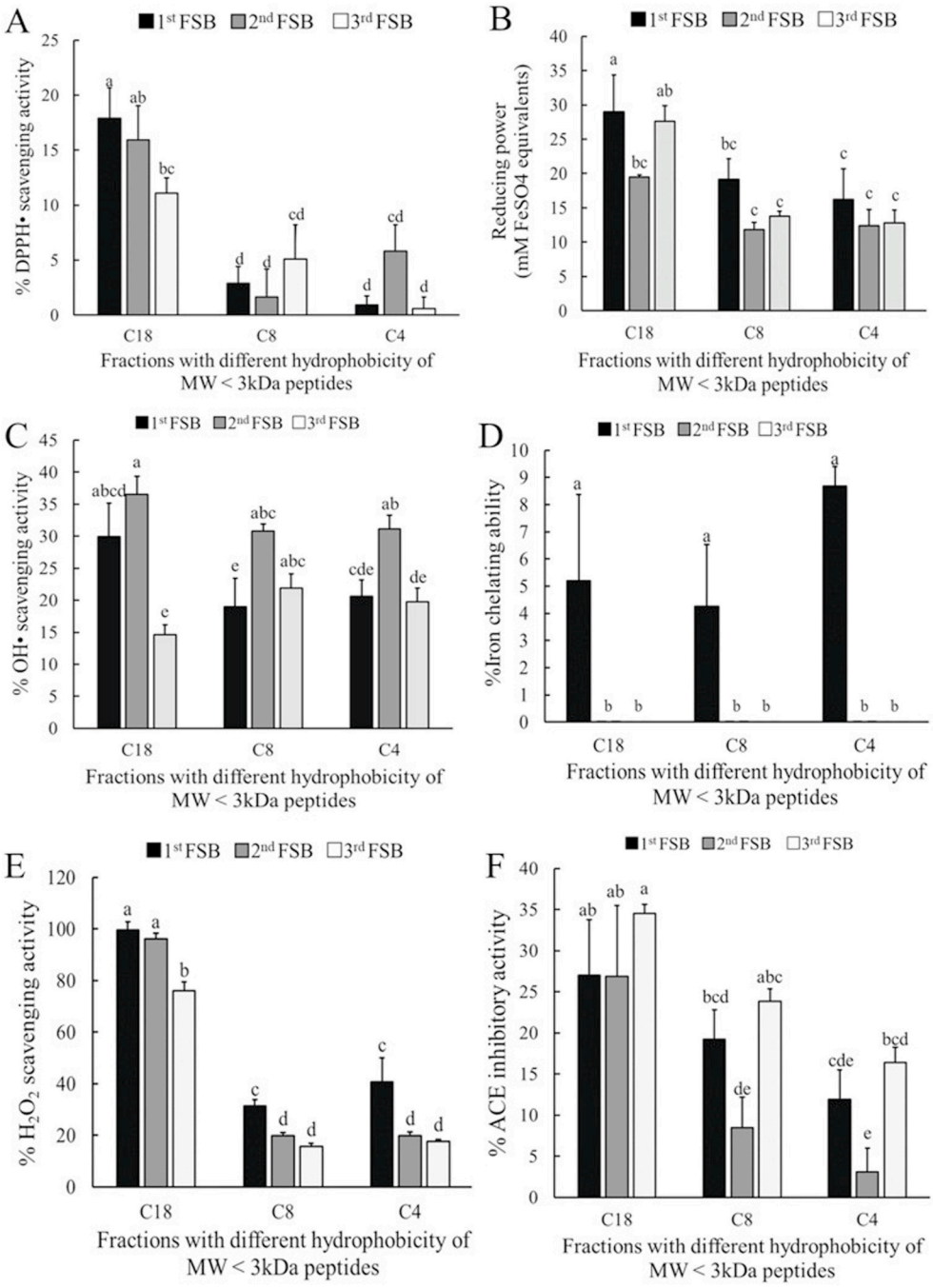

Fig. 8. Biological activities of fractions with different hydrophobicity of MW < 3 kDa peptide from the 1st, 2nd and 3rd grades of FSB, including (A) DPPH radical scavenging activity, (B) ferric reducing antioxidant power, (C) hydroxyl radical scavenging activity, (D) iron chelating activity, (E) hydrogen peroxide quenching activity and (F) ACE inhibitory activity. Data were presented as the mean ± SD from triplicate experiments. Different letters in the charts indicate significant differences ($P < 0.05$).

protein content. It was plausible that large proteins presented in the 1st grade FSB were further cleaved by proteases, producing smaller N-containing compounds that could be detected by the Kjeldahl method, but were not present on the gel. This observation was in agreement with previous reports that longer incubation period between secreted enzyme and protein hydrolysate led to higher degrees of hydrolysis and shorter peptide fragments (Tungkawachara, Park, & Choi, 2003).

3.3. Preparation and desalting of FSB extracts from the 1st, 2nd and 3rd grades

Prior to bioactivity determination, peptides extracted from the 1st, 2nd and 3rd grades of FSB were desalted using gel filtration chromatography. The presence of peptides in five fractions (F1–F5) was monitored by measuring absorbance at 280 nm (Fig. 2A) and Lowry's protein assay (Fig. 2B). Both measurements revealed similar results that F2 to F5 from the three grades of FSB contained higher protein content than F1. Conductivity measurements indicated that F5 possessed the highest salt concentration (Fig. 2C). Therefore, fractions F2 to F4, which contained high concentration of peptides with minimal salt content, were selected and combined for the following analysis.

After desalting, protein concentrations from each grade of FSB was measured. Using the same weight of starting materials, the 1st FSB grade showed the highest protein concentration, followed by the 2nd and 3rd FSB grades, respectively (Fig. 3), which was consistent with the SDS-PAGE results shown in Fig. 1.

Table 3
Peptides identified from the 1st, 2nd and 3rd grades of FSB with Average Local Confidence (ALC) score ≥ 95.

FSB grade:	Peptide sequence	ALC	m/z	charge	Bioactivity score from the PeptideRanker
1st FSB:	MDCGH	96	562.2067	+1	0.64
	MGSH	98	431.1563	+1	0.44
	HCDEE	97	632.1896	+1	0.09
	NEDY	95	540.1268	+1	0.08
	DAEE	95	463.1431	+1	0.04
2nd FSB	FDDR	97	552.2286	+1	0.64
	AGDDPP	95	571.1748	+1	0.60
	DPPDE	97	572.3004	+1	0.36
	EVDCH	96	602.1920	+1	0.13
	EDSH	95	487.1458	+1	0.07
	NPPDE	95	571.1748	+1	0.33
	WGEEY	95	683.2266	+1	0.30
	VEPP	95	441.1541	+1	0.19
3rd FSB	MFCEF	97	676.1763	+1	0.95
	ACCCM	95	530.0809	+1	0.97
	PCGAF	95	494.1967	+1	0.96
	CSMMNQ	95	713.1945	+1	0.53
	KVAMW	95	634.3347	+1	0.58
	VEDVE	97	590.3110	+1	0.02
	DDCAH	97	560.2002	+1	0.40
	LDTGDD	96	635.188	+1	0.10
	GSTTE	96	494.1967	+1	0.05
	AESSS	96	480.174	+1	0.06
	KHCY	95	550.2067	+1	0.46
	MEADPP	95	659.2044	+1	0.41

3.4. Antioxidant and anti-ACE properties of different grades of FSB extracts

Antioxidant activities of FSB from different grades were compared using various methods to evaluate different mechanisms of action. The 1st grade of FSB possessed the highest activities to quench DPPH· and OH· radicals and to reduce the FRAP reagent (Fig. 4A, B, and 4C). Based on the DPPH assay the 1st grade FSB had the strongest ability to donate hydrogen atoms to the free radicals with an IC_{50} value of 0.32 ± 0.02 mg/mL (Table 2). However, it was still less potent than the positive control, which was 16.34 ± 0.72 μg/mL. The strongest reducing ability of FSB was also observed in the 1st grade FSB, which was equivalent to 186.78 ± 18.57 mmol/L $FeSO_4$. Another mechanism that was of interest was the ability to quench OH·, which are known to be highly reactive and constantly produced within cells. The 1st grade FSB was able to effectively quench OH· with 82.79 ± 5.81% inhibition, which was more effective than that of 1 mg/mL ascorbic acid (%inhibition = 75.05 ± 0.49%).

The three activities were suppressed with decreasing concentrations of proteins in the lower grades of FSB (Fig. 3). These results were in agreement with other studies which showed that the activities were directly proportional with the quantity of proteins present (Sripokar, Benjakul, & Klomklao, 2019).

For the iron chelation and H_2O_2 inhibitory assays, different grades of FSB did not affect their abilities. The presence of both iron and H_2O_2 in the system leads to consequent oxidative damage to the cells due to the Fenton reaction. Thus, either chelating the iron or quenching H_2O_2 could prevent the production of other reactive species. From Fig. 4D and E, the %iron chelation and %H_2O_2 inhibition from all three FSB grades were not statistically different. The ability to chelate iron was in a range of 27.86 ± 3.06% to 39.36 ± 5.85%, which was much lower than that of the positive control; 0.5 mmol/L EDTA provided 99.70 ± 1.41% chelation. All samples revealed potent H_2O_2 scavenging ability within a range of 95.84 ± 3.10% to 100.57 ± 0.86%; while 1 mmol/L Trolox solution exhibited 95.90 ± 7.50% inhibition.

Overall, the 1st grade FSB provided the strongest antioxidant activities, as compared to the two lower grades, using equal amount of the starting FSB. It was plausible that stronger activities were the result of higher concentrations of peptides in the sample (Faithong, Benjakul, Phatcharat, & Binsan, 2010). The 1st grade FSB was produced with shorter period of fermentation as compared to other grades, so the size of peptides could be longer than others. Previously published reports have suggested that extensive digestion could lead to reduction of the antioxidant activities due to the loss of antioxidant structures and the production of free amino acids (Kong & Xiong, 2006).

From the anti-ACE activity determination, the 1st and 2nd grades of FSB exhibited stronger ACE inhibitory activity than the 3rd grade FSB (Fig. 4F and Table 2). Based on the literature, it has been reported that fish sauce contained short ACE inhibitory peptides; LAR, AP, KP and RP (Ichimura, Hu, Aita, & Maruyama, 2003; Sasaki et al., 2013). However, this work reports for the first time that the FSBs could also inhibit ACE activity.

3.5. Effects of MW on bioactivities of FSB extracts

After fractionation, the FSB samples were categorized into three groups; > 10 kDa, 3–10 kDa, and <3 kDa. Based on the protein assay, a majority of peptides had MW lower than 3 kDa (Fig. 5). Comparing the samples with distinct sizes, lower MW peptides from all grades tended to provide greater DPPH radical scavenging, reducing, OH· quenching, iron chelating, H_2O_2 scavenging and ACE inhibitory activities than higher MW peptides (Fig. 6). This observation was in concordance with several previous studies which demonstrated that smaller peptides (<3 kDa) from protein hydrolysate possessed stronger antioxidant and ACE inhibitory activities than the larger peptides (>3 kDa) (Lee & Hur, 2017; Nasir & Sarbon, 2019). Also, small peptides could be absorbed via mucosa of the small intestine (Tkaczewska, Bukowski, & Mak, 2019), so the fractions with MW < 3 kDa are suitable for functional food applications. Comparable to the results from crude FSB, these activities were directly proportional to the protein concentration in each fraction despite the fact that the same amount of FSB from each grade was controlled.

3.6. Effects of hydrophobicity on bioactivities of FSB extracts

The peptides in the low MW fractions were further fractionated based on their hydrophobicity, using reversed phase chromatography. The most hydrophobic fraction was obtained using the C18 matrix and decreasing hydrophobicity was retrieved from the C8 and C4 matrices, respectively. The highest concentration of protein from all FSB grades were found to be the C18 fractions (Fig. 7). The trend was that an increase in hydrophobicity of the peptides led to stronger bioactivities, including DPPH·, OH· and H_2O_2 scavenging activities, reducing power and ACE inhibitory activity (Fig. 8 A, 8B, 8C, 8D and 8F). It was frequently observed that antioxidant properties of the peptides corresponded with the presence of hydrophobic amino acid residues (Zou, He, Li, Tang, & Xia, 2016). These hydrophobic amino acids were also purposed to interact with the active site of ACE to suppress its function, resulting in the inhibitory activity (Ghassem, Babji, Said, Mahmoodani, & Arihara, 2014). However, hydrophobicity did not affect the iron chelating ability of the peptides (Fig. 8 E). One of the reasons could be that both hydrophilic and hydrophobic amino acids play important roles in forming coordination bonds with metal ions, including the imidazole ring of histidine and aromatic rings of phenylalanine, tyrosine and tryptophan.

3.7. Peptide sequence identification

The FSB peptides identified by LC-MS/MS had MWs in the range 431–713 Da and contained 4–6 amino acid residues (Table 3 and Supplementary Fig. S1). Commonly found amino acid residues in these identified peptides were glutamate and aspartate, which are known to be the two most abundant amino acids found in Thai fish sauce (Park et al., 2001). Based on the PeptideRanker bioactivity prediction

(Mooney et al., 2012), 8 out of 25 identified peptides had high potential of being bioactive (score > 0.5), which were MDCGH, FDDR, AGDDPP, MFCEF, ACCCM, PCGAF, CSMMNQ, and KVAMW. A majority of these peptides contained negatively charged residues which were proposed to promote antioxidant activities because of their electron-rich functional groups and aromatic residues which were known to enhance the activity because of their resonance structure (He et al., 2012; Zou et al., 2016). Moreover, the presence of proline and aromatic/branched hydrophobic and positively charged amino acids at the C-terminal position are known to be characteristics of the ACE inhibitors since they are involved in the binding to ACE (Norris & FitzGerald, 2013). The proposed bioactive peptides were found across all three grades of the FSBs particularly in the lowest grade, suggesting the potential application of these underutilized by-products.

4. Conclusions

This study revealed that FSBs could suppress free radicals via hydrogen donation and reduction reactions, chelate iron, quench reactive oxygen species, and reduce the activity of ACE. The FSB fractions with lower molecular weight and higher hydrophobicity showed better antioxidant and anti-ACE properties than the heavier and more hydrophilic fractions. In addition, the 1st grade FSB with the shortest fermentation time had higher bioactivities than the two lower grades of FSB. This research demonstrated that the FSB contained potential antioxidant and ACE inhibitory peptides and could potentially be used as a valuable source for developing functional food products from manufacturing waste.

CRediT authorship contribution statement

Petlada Khositanon: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - original draft, Visualization. **Naphatsawan Panya:** Investigation. **Sittiruk Roytrakul:** Methodology, Data curation, Supervision. **Sucheewin Krobthong:** Investigation. **Salil Chanroj:** Methodology. **Waeowalee Choksawangkarn:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110122.

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