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Original Article

Antioxidative peptides from fish sauce by-product: Isolation and characterization

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ABSTRACT

Fish sauce by-product (FSB) refers to solid waste from fish sauce industry. It is composed of nutritionally important biomolecules; however, FSB is currently undervalued. FSB contains natural protein hydrolysate produced from digestion of fish proteins using various proteases from their digestive system and halophiles in the fermentation tank. This study focused on the potential use of FSB from the lowest grade fish sauce production as a source of bioactive peptides. The results showed that the FSB extract contained about 10% (weight per volume) protein and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile was similar to that of fish sauce. The antioxidant activity of the FSB extract was determined using 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. The two most effective fractions had half maximal inhibition values of 0.57 ± 0.05 mg/mL and 1.25 ± 0.16 mg/mL. Upon digestion with Proteinase K, the activity decreased, suggesting that active molecules were derived from proteins or peptides. The low molecular weight FSB fraction contained potent antioxidative molecules, which were identified as PQLLLLL and LLLLLL. The study provided useful information for future development of value-added products from the solid waste produced during fish sauce manufacturing, which is one of the important marine industries in Southeast Asia.

Introduction

By-products from the marine industry are a rich nutritional source as several reports have shown the production of bioactive molecules from those waste includes peptides, proteins, polysaccharides and fatty acid (Jayathilakan et al., 2012). Fish sauce is a major food ingredient in Thailand (Wichaphon et al., 2013), processed annually from about 64,000 t of small fish (Saisithi, 1994). The process involves fermentation during which a high concentration of proteins in the fish are cleaved by proteases from halophilic bacteria and digestive endogenous enzymes (Lopetcharat et al., 2001; Klomklao et al., 2006). After fermentation, the upper liquid layer is filtered as a top-quality product with that remaining used for production of lower quality sauce (Lopetcharat et al., 2001). At the end of the production, fish sauce by-product (FSB) is normally discarded. These final residues could be used as fertilizer, pet food or filler for production of wheat flour extrudates (Han et al., 2002; Pratumwan et al., 2006). However, study of other means of utilization to increasing the residue value is still limited. Pratumwan et al. (2006) indicated that formulated FSB contained 15.37% (weight per weight; w/w) protein content; therefore, this work aimed to explore the presence of bioactive peptides from fish sauce processing waste.

Fermented fish is considered to be a natural protein hydrolysate source and contains a complex mixture of peptides with potential for biological activities (Gildberg, 2004; Thongthai and Gildberg, 2005). Bioactive peptides are short fragments of proteins with 2–20 amino acid residues that possess specific functions, for example, anti-inflammatory, antioxidant, antihypertensive, antimicrobial, and anticancer (Shahidi and Zhong, 2008; Ryan et al., 2011). The specific activity depends on the sequence, types of amino acid and length of peptides and a single peptide can exhibit more than one bioactivity (Meisel and FitzGerald, 2003; Piyadhammaviboon

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et al., 2012). Yin et al. (2005) reported that peptides from fermented minced scombroid mackerel had antioxidative capacity. Isolated peptides from fermented fish sauce showed angiotensin I converting-enzyme inhibition activity (Okamoto et al., 1995; Ichimura et al., 2003; Kim et al., 2016). In addition, bioactive peptides from anchovy sauce possessed immunostimulatory response (Thongthai and Gildberg, 2005). Although there have been several publications on bioactive peptides from liquid fish sauce, the activity of peptides extracted from its waste has not been reported.

The objectives of this work were to identify and characterize the peptides from FSB extract and to evaluate their antioxidant activity. Since the raw material contains a high protein content, it is plausible that a variety of peptide sequences could be produced from several secreted bacterial proteases and fish endoproteases. The current work confirmed that the antioxidant activity was derived from the peptides, as observed by a decrease in the activity after treatment with a broad-specificity protease, Proteinase K. This report presents, for the first time, the bioactivity of FSB peptides from the lowest quality fish sauce waste as a promising source for value-added products or functional food development.

Materials and methods

Fish sauce and fish sauce by-product material

Fish sauce and fish sauce by-product (FSB) were provided by Pichai Fish-Sauce Co., Ltd. (Chonburi, Thailand). First-grade fish sauce represented the supernatant of a mixture of two-parts whole fish (anchovy) and one-part sodium chloride, by weight, after fermentation for 12 mth. The remainder was allowed to ferment for an additional 3 mth and the supernatant was considered as second-grade fish sauce. The supernatant after a final 3 mth of fermentation was considered third-grade fish sauce, which was used in the present study. The FSB sample in this study was the solid waste remaining after the third fermentation period and was kept at 4 °C.

Extraction of fish sauce by-product

In preparation for use in this study, 10 g of solid FSB was dissolved in 10 mL of deionized H_2O (Millipore; Billerica, MA, USA) and stirred at room temperature for 30 min. The extract was filtered through grade 1 Whatman filter paper. The flow-through was then desalted using PD-10 desalting columns containing Sephadex™ G-25 medium (GE Healthcare; Piscataway, NJ, USA), following the manufacturer's procedure with a slight modification. The column was equilibrated with a 10-column volume of Type II H_2O, and 2.5 mL of FSB extract was added. Six fractions (each 3 mL) were collected for further analysis. Dissolved proteins/peptides from each fraction were determined using Lowry's protein assay (Lowry et al., 1951).

Determination of protein profile using sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The protein profiles of FSB extract and fish sauce were investigated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on a standard procedure. FSB extract and the lowest-grade fish sauce (10 μg each) were loaded onto the 13.5% (weight per volume; w/v) polyacrylamide gel and run at 100 V for 60 min. Gels were stained using 0.1% (w/v) Coomassie blue in 40% methanol:10% acetic acid:50% H_2O solution to visualize protein bands for 30 min and destained using 10% methanol:10% acetic acid:80% H_2O solution.

Proximate analysis of the fish sauce by-product

Chemical composition of protein, lipid, crude fiber, moisture and ash of the FSB were determined according to standard protocols. The protein content was determined using the Kjeldahl procedure (AOAC International, 2000). Lipid content was estimated using the Soxhlet lipid extraction method (AOAC International, 1997). The percentages of crude fiber, moisture and ash were determined according to the official method of analysis (AOAC International, 1997). The carbohydrate content was estimated by subtraction of protein, lipid, crude fiber, moisture and ash. All experiments were performed in triplicate.

Determination of total protein content in fish sauce by-product extract

The protein concentration was determined by Lowry's method with slight modification, using ovalbumin as a standard protein (Lowry et al., 1951). A portion of 200 μL of standard solutions (0.2 μg/μL, 0.5 μg/μL, 0.75 μg/μL, 1 μg/μL, 1.25 μg/μL) or sample solutions were added to 1 mL of Lowry's reagent and incubated for 15 min at room temperature. After that, 100 μL Folin-Ciocalteu reagent was added to each solution and the mixtures were incubated for 30 min at room temperature. After incubation, absorbance at 750 nm recorded using a spectrophotometer (Thermo Fisher Scientific; Madison, WI, USA) was used to construct a standard curve to calculate the protein concentrations. Three replicate experiments were carried out.

Determination of total phenolic content in fish sauce by-product extract

The total phenolic content was estimated using the Folin-Ciocalteu method (Blainski et al., 2013) with slight modifications. Twenty microliters of each fraction of FSB extract was incubated with 900 μΛ of 10% (volume per volume; v/v) Folin-Ciocalteu reagent at room temperature for 5 min. A solution of 7.5% (w/v) $NaHCO_3$ (600 μΛ) was added to the mixture and incubated at 50 °C for 10 min. The total volume was adjusted to 3 mL and absorbance was measured at 765 nm. Gallic acid concentrations of 0.1 μg/mL, 0.2 μg/mL, 0.3 μg/mL, 0.4 μg/mL and 0.5 μg/mL were used as standards and the result were reported as the concentration equivalent to gallic acid. Experiments were performed in triplicate.

Determination of α-amino group and free tyrosine in fish sauce by-product extract

The presence of the α-amino group was evaluated using the Ninhydrin method (Yemm et al., 1955) with modifications. Each FSB extract fraction (500 μL) was mixed with 500 μL of 0.1 M sodium citrate buffer, pH 5.0, and 500 μL of Ninhydrin reagent. The mixtures were incubated at 100 °C for 15 min. Glycine solutions at the concentration of 0.0125 μg/mL, 0.025 μg/mL, 0.05 μg/mL and 0.1 mg/mL were used as the positive control.

Paper chromatography was used to evaluate the free tyrosine. Solutions of 0.1% tyrosine, 0.1% aspartic acid, 0.1% valine, 0.1% phenylalanine, 0.1% leucine, 0.1% arginine and 0.1% cysteine were used as standards to determine the R_f values. The standards and FSB extract fractions were spotted on the filter paper and placed in the chamber containing mobile phase solution (65% 1-butanol, 10% acetic acid and 25% H_2O). The separation of each amino acid in the stationary phase was visualized using 0.2% Ninhydrin staining. The presence of free tyrosine in the FSB samples was examined based on its R_f value.

Determination of antioxidant activity of fish sauce by-product extract

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay (Molyneux, 2004). FSB solution (50 μL) was mixed with 100 μL of 0.2 mM DPPH in methanol. The mixture was incubated at room temperature in the dark for 30 min prior to monitoring absorbance at 517 nm. Ascorbic acid (20 μg/mL) was used as the positive control. DPPH scavenging activity was calculated using Equation (1):

$$\% \text{ DPPH inhibition} = \{[(A - B) - (C - D)]/(A - B)\} \times 100 \quad (1)$$

where A, B, C and D are the absorbance of DPPH without FSB extract, methanol, DPPH with FSB extract, and FSB extract without DPPH, respectively. The half maximal inhibitory concentration (IC_{50}) using the FSB protein concentration was in the range 0.1–1.6 μg/μL based on at least five data points.

Preparation of proteinase K digested fish sauce by-product extract

Fractions from gel filtration chromatography that showed high antioxidant activity were subjected to enzymatic treatment using 50 μg/mL Proteinase K in a buffer solution containing 10 mM $CaCl_2$, 100 mM Tris-HCl and pH 7.5. The digestion was performed at 37 °C for 16 hr. The DPPH radical scavenging activity of the same fractions was evaluated both before and after the enzymatic treatment.

Fractionation of fish sauce by-product extract by ultrafiltration technique

The FSB extract was separated based on molecular weight using 10 kDa and 3 kDa molecular weight cut-off (MWCO) filters as per the manufacturer's suggestion (Millipore; Billerica, MA, USA). FSB extract (400 μl) was applied to a pre-equilibrated 10 kDa MWCO filter and centrifuged at 12,500 rpm for 30 min. Filtrate was then added to a pre-equilibrated 3 kDa MWCO filter and centrifuged at 12,500 rpm for 30 min. The protein concentration and DPPH radical scavenging properties were determined for FSB subfractions with MW > 10 kDa, 10 kDa > MW > 3 kDa, and MW < 3 kDa.

Fractionation of fish sauce by-product extract using high performance liquid chromatography

Ultrafiltrates were fractionated using a Waters 600 HPLC system equipped with a Waters 2478 dual λ UV-visible detector (Waters Corp.; Milford, MA, USA). Fraction 2 filtrate was injected into a Zorbax 300SB-C18 column with dimension 4.6 mm × 250 mm (Agilent Technologies; Palo Alto, CA, USA), and eluted using an isocratic solvent system of 2.5% acetonitrile, 97.4% H_2O and 0.1% formic acid with a flow rate of 1 mL/min for 30 min. Absorbance at 215 nm and 280 nm was measured during sample elution and each fraction was collected for further experiments.

Peptide identification using liquid chromatography-mass spectrometry

Fractionated peptides were subjected to liquid chromatography-mass spectrometry (LC-MS/MS) analysis for the sequence identification using an HCTultra PTM Discovery System (Bruker Daltonics Ltd; Hamburg, Germany) interfaced with an UltiMate 3000 LC System (Dionex Ltd.; Altrincham, UK). The collected subfractions were injected into a nanocolumn (PepSwift monolithic column, 100 μm × 50 mm). Peptides were eluted with a linear gradient from 10% to 70% solvent B (80% acetonitrile, 19.9% H_2O and 0.1% formic acid) for 13 min; while 0.1% formic acid was used as solvent A. Each experiment was performed for 20 min; the column was pre-equilibrated at 10% solvent B prior to the gradient and washed at 90% solvent B after the gradient. Mass spectrometry was performed in a data-dependent mode with a mass range of 300–1500 m/z, using three average scans and the maximum of five precursor ion selection. The mass range of the precursor ion scan was set to 50–3000 m/z.

Liquid chromatography-mass spectrometry data analysis

The MS/MS spectra files were converted to *.mgf files and submitted to the De Novo sequence identification using PEAKS (Bioinformatics Solution Inc.; Waterloo, ON, Canada). Precursor ion mass tolerance was set to 1.0 Da and the product ion mass tolerance was 0.6 Da. Oxidation of methionine was allowed as a variable modification. Reported peptides were filtered using an average local confidence of 90% and a local confidence of 70% for each residue.

Statistical analysis

Data were analyzed using the GraphPad Prism software version 5.0 (GraphPad; San Diego, CA, USA). A paired *t*-test was used for comparison of two sets of data. For the dataset with more than two samples, one-way analysis of variance followed by Tukey's multiple comparison test was used. For both analyses, differences between means at the 5% confidence level (p value < 0.05) were considered statistically significant.

Results and discussion

Proximate compositions of the fish sauce by-product

The solid FSB from the lowest-grade (third-grade) fish sauce was composed of protein, lipid, fiber, moisture, crude ash and carbohydrate as shown in Table 1. The crude protein content was approximately 10% (w/w) using the Kjeldahl method. Crude ash represented the greatest proximate component of the FSB, which could have been derived from the fish backbone and salt used during the manufacturing process.

Comparison of protein content and profile of fish sauce and fish sauce by-product extract

The total protein concentration determined using Lowry's protein assay indicated that the protein concentration of the fish sauce (21.8 μg/μL) was about three times higher than in 10% (w/v) FSB extract (7.2 μg/μL) as shown in Fig. 1A. The profile of proteins dissolved in the fish sauce and 10% (w/v) FSB extract are shown in Fig. 1B. SDS-PAGE indicated that the types of proteins dissolved in the fish sauce were similar to those that remained in the FSB. Most of the proteins had a molecular weight lower than 45 kDa; however, small peptides were below the level of detection. Larger proteins

Table 1
Proximate analysis (mean ± SD; $n = 3$) of fish sauce by-product composition.

Component	Composition (% weight per weight)
Protein	10.22 ± 0.02
Lipid	1.26 ± 0.03
Fiber	0.07 ± 0.01
Moisture	25.17 ± 0.94
Ash	63.19 ± 0.50
Carbohydrate	0.09 ± 1.25

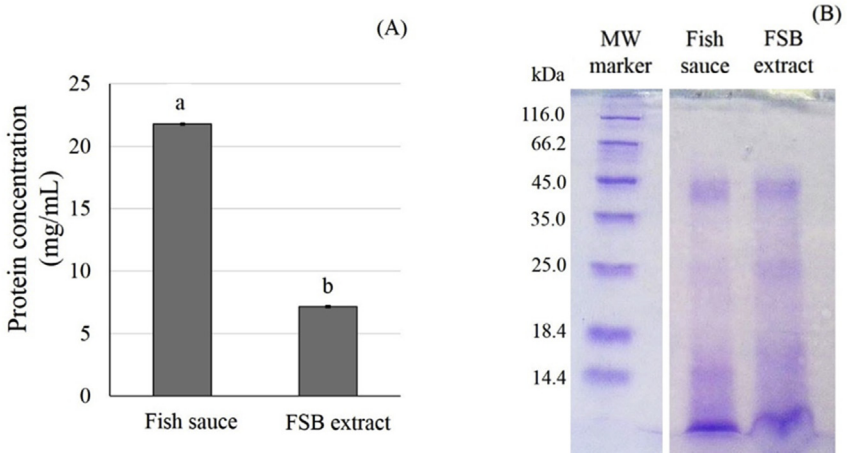

Fig. 1. (A) Protein concentration of the lowest-grade fish sauce and its fish sauce by-product (FSB) extract, where bars show mean +SD ($n = 3$) and different lowercase letters above bars indicate means with significant ($p < 0.05$) difference; (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing protein patterns from the fish sauce and its FSB extract.

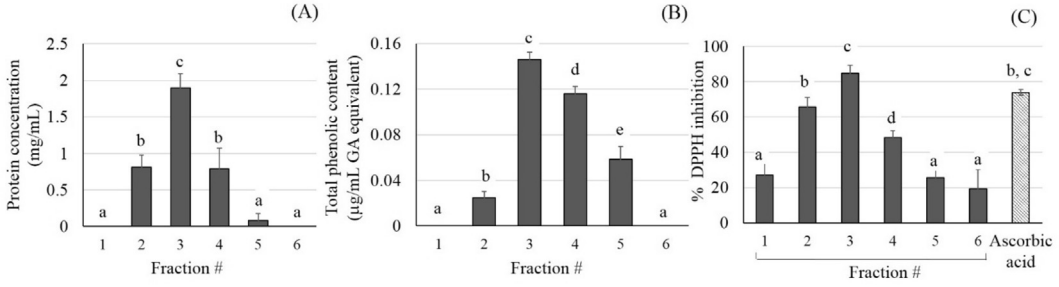

Fig. 2. (A) Protein content; (B) total phenolic content present as gallic acid (GA) concentration equivalent; (C) percentage 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition of the gel filtration fractions of extracted fish sauce by-product and 20 μg/mL ascorbic acid, where each bar represents mean + SD ($n = 3$) and different lowercase letters represent significant ($p < 0.05$) differences among means.

were not present in the system since the third-grade fish sauce and FSB extract in this experiment had been fermented for a long period (18 mth). The starting proteins could have been degraded by indigenous and microbial enzymes, including endogenous and exogenous proteases (Lopetcharat et al., 2001). This finding was in agreement with previously published reports which showed a significant increase in the degree of hydrolysis of the Pacific whiting fish sauce after a fermentation period of 9 mth (Tungkawachara et al., 2003) and a rise in the amount of trichloroacetic acid-soluble peptides from traditional Chinese fish sauce after a fermentation period of 6 mth (Jiang et al., 2007). Similar observations of an increasing degree of hydrolysis and a smaller peptide content were also reported in other fermentation products such as the salted shrimp paste (Faithong and Benjakul, 2014; Pongsetkul et al., 2017). Based on the current qualitative and quantitative analysis, the abundant proteins/peptides had not completely dissolved in the third-grade fish sauce, and still remained to be extracted in its processing waste. With agitation in water during extraction, more proteins/peptides could be released from the solid waste.

Proteins and phenolic content after gel-filtration

After desalting and fractionation of the FSB extract using a gel filtration column, the protein concentration was determined using a Lowry's protein assay. The highest protein/peptide concentration was in fraction 3, followed by fraction 2 and fraction 4 which all contained similar amounts (Fig. 2A). This was in accord with the Ninhydrin test, which showed the highest concentration of α–amino containing compounds in fraction 3 (Table 2). The total phenolic concentration determined using the Folin-Ciocalteu method was also highest in fraction 3 (0.14 ± 0.006 μg/μL gallic acid equivalent) compared using a one-way analysis of variance and Tukey's multiple comparison test; however, it was significantly lower in fraction 2 (0.019 ± 0.005 μg/μL gallic acid equivalent) (Fig. 2B). It was possible that the phenolic content resulted from tyrosine residues in the proteins/peptides or free tyrosine. The results from paper chromatography stained using Ninhydrin reagent confirmed that there was free tyrosine present in fractions 3, 4 and 5 (Table 2). The presence of tyrosine, either in solution or residing in the peptides, could lead to a strong antioxidant property since it contained a phenolic side chain (Liu et al., 2016; Matsui et al., 2018).

Table 2
Chemical analyses for α–amino group (Ninhydrin test) and free tyrosine using paper chromatography, followed by Ninhydrin staining.

Fraction number	Ninhydrin test[a]	Free tyrosine tested using paper chromatography[a]
1	–	–
2	++	–
3	+++	+
4	+	+
5	–	+
6	–	–

[a] (+) indicate the darkness of blue color from the test.

Table 3
Half maximal inhibitory concentration (IC_{50}) values of fraction 2 and fraction 3, where values indicate mean ± SD ($n = 3$).

Fraction number	IC_{50}
2	0.57 ± 0.05 μg/μL[a]
3	1.25 ± 0.16 μg/μL[b]

[a,b] different letters represent significant ($p < 0.05$) differences among means.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity of fish sauce by-product

The antioxidant activity of each fraction of FSB extract was determined using DPPH radical scavenging assay. The highest DPPH radical scavenging activity was found in fraction 3 (84.8 ± 4.3%), followed by fractions 2, 4, 1, 5 and 6, respectively ($p < 0.05$) as shown in Fig. 2C. The abilities of fraction 2 and fraction 3 to inhibit DPPH radicals were relatively comparable to the positive control (Fig. 2C). Fractions which exhibited >50% DPPH scavenging were chosen for determination of IC_{50} values. The IC_{50} value of fraction 2 was 0.57 ± 0.05 mg/mL or approximately one half that of fraction 3 (1.25 ± 0.16 mg/mL), a clear indication of better efficacy to scavenge DPPH radicals (Table 3). Treatment with Proteinase K was used to assure the antioxidant activity of peptides. The experiment showed that after digestion with Proteinase K, the ability to scavenge DPPH radicals of fraction 2 decreased by 54% and that of fraction 3 decreased by 26%, supporting the claim that the antioxidant activity was raised from peptides in the FSB extract. Fraction 2 was then fractionated using ultrafiltration with 10 kDa and 3 kDa molecular weight cut-off filters. The majority of proteins/peptides had molecular weights lower than 3 kDa (Fig. 3A), and the highest inhibition of the DPPH radicals was in the subfraction with MW < 3 kDa (Fig. 3B). This result was in agreement with a number of previously published reports that similarly small peptides with MW < 3 kDa produced higher antioxidant activity compared to the higher molecular weight fractions (Sun et al., 2011; Ji et al., 2014; Ngoh and Gan, 2016).

The FSB sample with MW < 3 kDa was further fractionated using HPLC with a C18 column equipped with an ultraviolet detector.

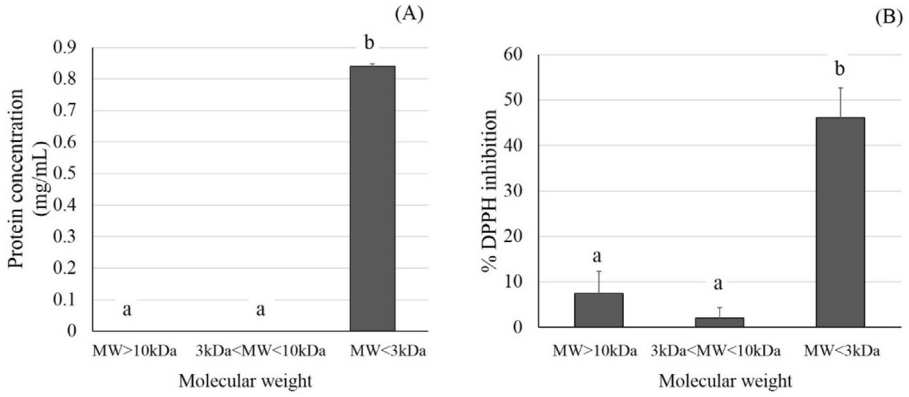

Fig. 3. (A) Molecular weight of proteins in fraction 2; (B) percentage 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition activity of proteins in fraction 2, where each bar represents mean + SD ($n = 3$) and different lowercase letters represent significant ($p < 0.05$) differences among means.

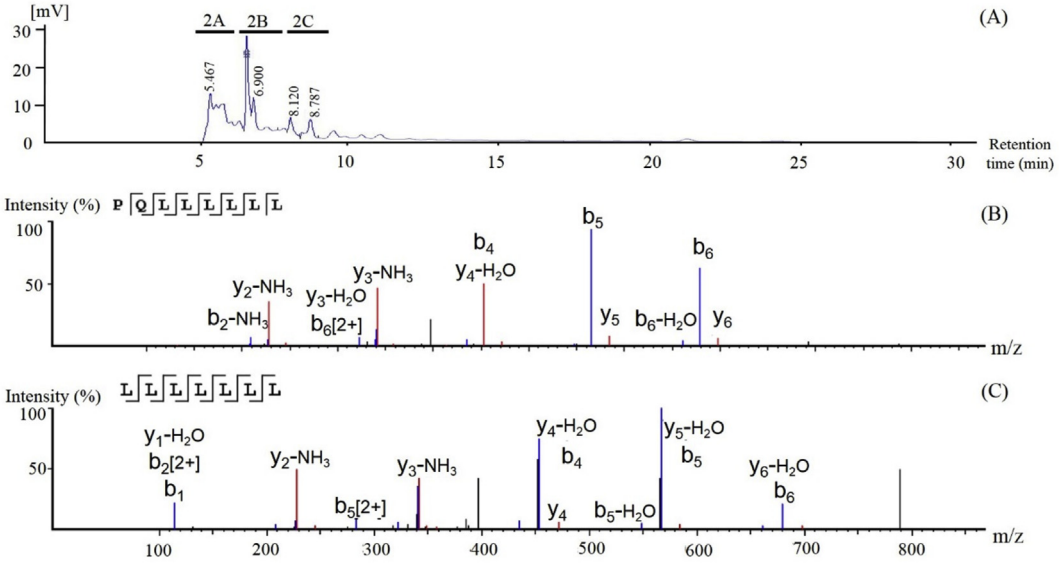

Fig. 4. (A) High performance liquid chromatography chromatogram showing fish sauce by-product subfractions 2A 2B and 2C; (B) liquid chromatography-mass spectra of peptide PQLLLLLL (m/z = 462.26, z = +2) from fraction 2A; (C) liquid chromatography-mass spectra of peptide LLLLLL (m/z = 405.73, z = +2) from fraction 2B; identified b-ions and y-ions are indicated in the spectra.

Table 4
2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of high performance liquid chromatography (HPLC) subfractions and their corresponding peptides identified using liquid chromatography-mass spectrometry.

HPLC subfraction	% DPPH inhibition ($n = 3$)	Peptide sequence	Average local confidence	m/z	Charge state
2A	59.05 ± 2.42	PQLLLLLL	93%	462.26	+2
2B	63.39 ± 4.99	LLLLLLL	99%	405.73	+2
2C	68.43 ± 1.44	–	–	–	–

The HPLC subfractions were collected according to absorbance at 215 nm and 280 nm. The majority of the extract was eluted at the beginning of the run. The first three fractions were collected and designated as subfractions 2A (5.00–6.50 min), 2B (6.50–7.00 min) and 2C (7.00–8.50 min) as shown in Fig. 4A. The DPPH scavenging activity was determined as 59.05 ± 2.42%, 63.39 ± 4.99%, and 68.43 ± 1.44% for subfractions 2A, 2B and 2C, respectively (Table 4). Fractions were identified using LC-ESI-MS/MS and the tandem mass spectra were interpreted using *de novo* sequencing with the PEAKS software package (Zhang et al., 2012). The MS/MS spectra of a peptide from subfractions 2A and 2B are shown in Fig. 4B, C, respectively. The peptides identified with average local confidence (ALC) higher than 90% were PQLLLLLL (ALC = 93%) and LLLLLLL (ALC = 99%). It is notable that both these peptides contain repetitive hydrophobic amino acids, accounting for 87.5–100% of all residues. Similar observations have been stated in a number of studies that hydrophobic amino acids, including leucine and proline, play significant roles in radical inhibition via hydrogen atom or electron transfer, as well as scavenging reactive oxygen species (Zhuang et al., 2013; Najafian and Babji, 2014; Ngoh and Gan, 2016). In addition, the peptide identified in the subfraction 2B contained the N-terminal leucine, which is known to be one of the important characteristics of strong antioxidant peptides (Zou et al., 2016). This was in agreement with Chen et al. (1995), who discussed the presence of leucine at the N-termini of potent radical scavenging peptides from a soybean protein. In addition, the peptide from the subfraction 2A contained proline and leucine residues, which were found to be two of the three most abundant amino acids presented in the 42 selected radical inhibitory peptides from previously published reports (Zou et al., 2016).

In conclusion, the current work has provided evidence that antioxidative peptides could be identified from solid waste from the fish sauce industry. The results revealed that the fish sauce by-product contained a high amount of proteins/peptides and had antioxidant activity. Most proteins/peptides had relatively low molecular weights, in line with the most potent antioxidant activity. Two peptide sequences (PQLLLLLL and LLLLLLL) were identified after fractionation using gel-filtration, the molecular weight cut-off filter, RP-HPLC and identification using LC-MS/MS. To increase the value of the FSB, these extracted peptides could be used as a functional ingredient in fish sauce or other food products.

Conflict of interest

The authors declare that there are no conflicts of interest.

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