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ORIGINAL PAPER

Fractionation and identification of novel antioxidant peptides from fermented fish (pekasam)

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Abstract

The goals of this study were to determine antioxidant activities of water soluble fractions of a Loma fermented fish extract and identify the novel antioxidant peptides by HPLC connected to the tandem mass spectrometry (LC/MS/MS). The antioxidant activities of Loma fermented fish (LFF) were measured by evaluating 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities. Antioxidant peptides from LFF were separated using ultrafiltration, size exclusion chromatography and HPLC. Purified fraction (FIII 3), which exhibited the strongest ABTS radical scavenging activity, with the IC_{50} value of 0.636 mg/ml, had higher antioxidant activity than the LFF sample without fractionation (with IC_{50} of 2.24 mg/ml). Two novel peptides were identified as Ala-Ile-Pro-Pro-His-Pro-Tyr-Pro (AIPPHPYP) and Ile-Ala-Glu-Val-Phe-Leu-Ile-Tre-Asp-Pro-Lys (IAEVFLITDPK). The IAEVFLITDPK peptide demonstrated higher antioxidant activity than the AIPPHPYP. The presence of hydrophobic amino acids (Ile, Ala and Pro), and basic amino acids, (Lys) in the peptide sequences is believed to contribute to the high antioxidant activity of the LFF. Therefore, these peptides may have the potential as a natural functional ingredient in food and pharmaceutical industries.

Keywords Fermented fish · Antioxidant · Peptide purification · Peptide sequencing · LC–MS/MS

Introduction

Fish and seafood are great sources of functional food ingredients, such as antioxidants and bioactive peptides [1]. Peptides with antioxidant activity might serve as functional ingredients to enhance the safety and shelf life of food. These peptides can increase human health and inhibit disease when they are used as functional food ingredients, or nutraceuticals and pharmaceuticals [2, 3]. Bioactive peptides usually include 2–20 amino acid residues. The peptides obtained from fish have nutrient utilization. In addition, bioactive peptides based on their amino acid composition and sequences may be implicated in various biological properties [4] including inhibition of angiotensin-I-converting enzyme (ACE) [5], antioxidant [6, 7], antimicrobial [8] and anticoagulant [9] activities. Oxidation is an important source of adverse quality changes that can influence the texture, flavor and color in food products, causes food spoilage and reduces nutritive value.

Bioactive peptides can increase during food fermentation by proteolytic microorganisms [10, 11]. Therefore, the fermentation of food materials enhances the antioxidant activity of food products. Several industrial dairy starter cultures are employed for microbial fermentation of food proteins.

In addition to acid production by lactic acid bacterium (LAB), starter strains exert proteolytic and lipolytic activities during fermentation. The fermentation with extremely proteolytic strains of LAB is the single most effectual way to increase the quantity of biopeptides in fermented fish products [12]. Bioactive peptides are inactive within the sequences of the parent proteins. They are released by enzymatic hydrolysis and then they may exert various physiological functions. The choice of strains is highly important to release effective bioactive peptides. The highly proteolytic activity of the strain will destroy the product and the right specificity of the strain give high numbers of active peptides. Pekasam is a fermented fish product from Malaysia produced with lactic acid bacteria activity. It is prepared

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from freshwater fish such as Loma, lampam, tengalan, tilapia, and etc. Fermentation also leads to an improvement in amino acid balance and increases protein content [18]. Hassan [13] reported that the moisture, protein, fat, ash, and salt contents of a commercial sample of pekasam fall within the range of 57.0–73.0, 15.0–25.0, 3.0–8.0, 6.0–14.0, and 10.0–16.0%, respectively.

Pekasam as traditional fermented food is widely consumed in Malaysia. Therefore, the effects of fermentation using *Lactobacillus plantarum* on the hydrolysis of fish protein, which can potentially enhance its antioxidant activity, were reported in the present study. Numerous studies have reported that peptides from various food sources possess antioxidant activities, but antioxidant properties of fermented fish have not yet been studied. *L. plantarum* IFRPD P15 effectively promotes high acidity productivity for pekasam production. Hence, we chose *L. plantarum* IFRPD P15 as potential starter culture of Pekasam. The aim of the current study was to purify antioxidative peptides obtained from Loma (*Thynnichthys thynnoides*) fermented fish using ultrafiltration, size exclusion chromatography and RF-HPLC. The sequences of purified fractions with the highest antioxidant activity were identified using HPLC–ESI Q-TOF–MS/MS.

Materials and methods

Fermentation of fish

Fresh water fish sample namely Loma purchased from Kiah Pekasam Enterprise was used in this study. The *L. plantarum* IFRPD P15 strain was a gift from Universiti Putra Malaysia and was stored at 4 °C on slants of MRS agar before inoculation. *L. plantarum* IFRPD P15, as inocula, was prepared by transferring a loopful of an overnight culture from deMan Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) into 10 ml MRS broth and incubated at 27 °C for 24 h. One hundred microliter of the 24 h old culture was transferred into 10 ml MRS broth and incubated at 27 °C for 16 h. Cells were harvested by centrifugation (5000×*g* for 10 min at 4 °C) and washed twice with 0.85% (w/v) NaCl. Finally, the cell pellets, with an initial number of viable cells of 1.5×10^9 cfu/g, re-suspended at a 1:1 ratio in 15 ml of 10% (w/v) skim milk served as the isolate inocula. Thereafter, fishes were placed in plastic tanks and coarse salt was added (ratio fish: salt, 1.5:1), samples were weighted down using screw to prevent them from floating due to the accumulation of liquid as a result of autolysis. The samples were kept for two weeks at room temperature. After 2 weeks the fish samples were washed and mixed with roasted rice, tamarind, brown sugar and starter culture. The mixture was fermented for 15 days in an airtight container to obtain a pH 4.9–5.6 at 27 °C and *pekasam* was obtained. Three batches of sample were collected at different times to conduct a repeated experiment.

Extraction and deproteinization

Fifty grams of the fermented fish were minced and then homogenized in a stomacher with 200 ml of 0.01 N HCl for 8 min. The homogenate was centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was filtrated through glass wool and deproteinized by adding 3 volumes of ethanol (to remove water-insoluble fractions). The sample was held for 20 min at 4 °C and then, centrifuged again at 12,000×*g* for 20 min at 4 °C. The supernatant was dried in a rotary evaporator and dissolved in 25 ml of 0.01 N HCl, filtered through a 0.45 µm nylon membrane to obtain Loma fermented fish (LFF) extract. Then, LFF extract was dried in a rotary evaporator and stored at 4 °C.

Chemical compositions

Chemical compositions (moisture, crude protein, crude ash and lipid contents) of samples from Loma fish and Lima fermented fish extract powder were determined in triplicate for each sample according to the methods 950.46, 992.15, 900.02A, 960.39, respectively of the Association of Official Analytical Chemists [14]. Nitrogen is converted to crude protein content by a factor of 6.25.

Amino acid composition

Amino-acid compositions were characterized using the method of Alaiz, Navarro, Girón and Vioque [15], with slight changes. The total amount of all amino-acid residues was measured following hydrolysis with 6-N hydrochloric acid at 110 ± 1 °C for 24 h and the total content of cysteine and cystine were determined by oxidizing the protein with performic acid [16]. Alkaline hydrolysis was also performed to quantify the level of Tryptophan [17]. The amino acids were separated using Waters AccQ.Tag amino acid analyzer (Waters Corporation, Ireland).

Antioxidant activities of LFF extract

DPPH radical scavenging activity assay

Free radical scavenging activity was evaluated with some modifications according to the method of Wu et al. [18]. A 95% ethanol was added to 1.5 ml of 0.15 mM DPPH and mixed with 1.5 ml volume of sample. After vigorous mixing, the mixture was kept at room temperature for 30 min in the dark. Afterward, the absorbance at 517 nm was measured.

The percentage of inhibition activity was calculated from the following equation:

$$\text{DPPH radical-scavenging activity}\,(\%) = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)/\text{Abs}_{\text{control}}\right] \times 100$$

Calculation of 50% inhibitory concentration (IC_{50}): The concentration (mg/ml) of the fractions required to scavenge 50% of the DPPH free radicals was determined using Microsoft Excel 2010. On linear graph section of percentage of inhibition according the concentration of the protein a trend line equation: $y = ax + b$ was determined. IC_{50} was calculated by transforming the equation above and the expression x at which y-value is 50% was accepted as unknown.

Reducing power assay

Antioxidant activity of LFF extract was estimated using the method of reducing power assay described by Oyaizu [19] with slight modifications. A 1 ml of sample solution (at a concentration of 1 mg/ml) was dissolved in 0.2 M phosphate buffer with pH 6.6 (2 ml) and 1% potassium ferricyanide solution (1 ml). The mixture was incubated for 20 min at 50 °C. Aliquot of 10% TCA (1 ml) was added to the reaction mixture, and the mixture was then centrifuged at 1500×*g* for 10 min at 4 °C. The upper layer of the solution (1 ml) was then mixed with distilled water (1 ml) and 0.1% ferric-chloride solution (200 µl). After allowing this mixture to react for 10 min, the absorbance was measured at 700 nm.

ABTS radical-scavenging activity assay

Scavenging activity of the sample against ABTS radical was evaluated spectrophotometrically using ABTS radical cation decolorization assay described by Re et al. [20], with slight modifications. The absorbance values were taken at 734 nm. The percentage inhibition was calculated by using the equation:

$$\text{ABTS radical-scavenging activity}\,(\%) = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)/\text{Abs}_{\text{control}}\right] \times 100$$

Purification of antioxidant peptides from the *pekasam*

The LFF extract was filtered through 10 kDa ultrafiltration membrane followed by a 3 kDa membrane and fractionated into three fractions as < 10, 3–10, < 3 kDa UF hydrolysates. Subsequently, the sample of 250 mg containing peptides below 3 kDa was dissolved in 10 mM sodium phosphate buffer, pH 7.2 (2 ml), separated on a Hiprep 26/60 sephacryl S-100 HR gel filtration column, (26 × 600 mm) (GE Healthcare, UK), eluted with the same buffer, pH 7.2 at a flow rate of 1 ml/min and detected at 280 nm, respectively. Standards for determining molecular weights with standard curve were conalbumin (75,000 Da, 70 min), Carbonic anhydrase (29,000 Da, 105 min), Ribonulease A (13,700 Da, 140 min), Aprotinin (6500 Da, 210 min) and Hippuryl-histydilleucine (429.5 Da, 270 min). The fraction showing the highest antioxidant activity was further purified onto an XBridge BEH 130 Prep C_{18} (10 × 250 mm) column (Waters, USA). The Waters HPLC system consists of a pump (*Waters 600 controller pump*), collector (*Waters 2767 Sample Manager*) and detector (*Waters 2998 Photodiode Array Detector*). The antioxidant peptides were eluted with linear gradient of 100% eluent 0.1% TFA in deionize water (A) for 5 min and with the following increasing eluent 0.1% (v/v) TFA in 100% (v/v) acetonitrile solution (B): 0–5 min, 0% eluent B; 5–10 min, 0–45% eluent B; 10–30 min, 45–65% eluent B; 30–35 min, 65–100% eluent B; 35–40 min, 100% eluent B; 40–45 min, 100–0% eluent B at a flow rate of 4.50 mg/min. The peaks were monitored at 214 nm and collected separately and then concentrated using centrifugal concentrator at 130 rpm for 3 h. The concentrated sample was used for DPPH and ABTS radical scavenging activity and the reducing power assays.

Identification of peptides by LC/MS/MS

Samples were identified using nano-ESI with an Agilent 6520 Accurate-Mass Q-TOF LC/MS. The antioxidant peptides were dissolved in 0.1% formic acid in water and 1 µl of test sample was loaded onto C_{18} analytical column, (75 × 150 mm) (Agilent part no: G4240-62010). A 0.1% formic acid (FA) in water and 90% acetonitrile (ACN) in water containing 0.1% formic acid were eluent A and B, respectively. Spectra were recorded over the mass/charge (m/z) range of 110–3000. Since enzyme type was unknown, the search was conducted without the information of enzyme, taxonomy and modification. Data was processed with Agilent Spectrum Mill MS Proteomics Workbench software packages. Database search was SwissProt.MAR.2013.fasta.

Peptide synthesis

Based on the above analysis, selected peptides were synthesized by First BASE Laboratories Sdn Bhd, Malaysia for further tests. The synthesized peptides were then purified by RP-HPLC on a Monitor C18, Column Engineering

(150 × 4.6 mm) column reequilibration (flow rate, 1 ml/min; monitoring 214 nm).

Statistical analyses

All analyses were conducted in at least three independent trials from three different batches of fish for fermentation. A one-way analysis of variance (ANOVA) was used, and separation of means was compared using Duncan's multiple range tests. Data were analyzed using SPSS (SPSS 18.0 for Windows, SPSS Inc.). The significant level was considered ($p < 0.05$).

Results and discussion

Chemical compositions

The chemical compositions of the LF and the dried LFF extract are shown in Table 1. The proximate composition shows that content of moisture (77.34 ± 1.15%) and fat (3.10 ± 0.98%) of LF is high whereas, content of the ash (1.80 ± 0.16%) and protein (16.15 ± 1.25%) is low. Furthermore, the ash, moisture, lipid and protein contents of the LFF are 0.80 ± 0.08, 3.13 ± 0.33, 0.29 ± 0.08 and 91.37 ± 0.89%, respectively. Lima fermented fish extract was dried after deproteinization process to measure protein, fat, ash and moisture of water soluble fractions of LFF extract. Therefore, Low moisture content of LFF extract was due to use of dried LFF extract. In general, protein content of the LFF significantly increased after extraction compared to Loma fish that the differences in protein content were due to increasing water soluble protein content and the produced peptides during fermentation and extraction processes.

Evaluation of the antioxidant activity of LFF extract

DPPH is a comparatively stable free radical that can accept an electron or hydrogen from natural bioactive peptides to become more stable and a less reactive molecule [21].

Table 1 Chemical compositions of fresh Loma fish (LF) and dried Loma fermented fish (LFF) extract

Chemical compositions	LF	LFF
Moisture	77.34 ± 1.15[a]	3.13 ± 0.33[b]
Protein	16.15 ± 1.25[b]	91.37 ± 0.87[a]
Ash	1.80 ± 0.16[a]	0.80 ± 0.08[a]
Fat	3.1 ± 0.98[a]	0.29 ± 0.08[b]

Values shown are the mean ± standard deviation of three replicates

[a, b]Mean of % in the same row without a common superscript letter differ significantly ($p < 0.05$)

Table 2 illustrates that LFF extract is able to decrease the stable radical DPPH (purple) to yellow. When the concentration of LFF extract reached 2 mg/ml, the scavenging of DPPH radical was 68.81 ± 0.76% and the IC_{50} value for the LFF extract was 1.36 mg/ml. The result suggested that the LFF extract possessed hydrogen donating capacity and acted as antioxidant. However, the fermented fish from Loma had a lower radical-scavenging activity than BHT (86.20 ± 0.83%) at the concentration of 2 mg/ml. Antioxidant activity of LF was very low (1.40%) and IC_{50} values were not calculated. The results revealed that antioxidant peptides were inactive within the sequences of their parent protein, but released by enzymatic hydrolysis. The antioxidant activity is affected by changes in the composition, concentration and sizes of free amino acids and small peptides [18].

Table 2 represents ABTS radical scavenging activity assay for LFF extract. The ABTS radicals are reduced to a certain degree on a given timescale by adding antioxidants to the radical cation solution. This depends on the duration of the reaction and concentration of the antioxidant Liu et al. [22]. The observed IC50 values for the ABTS radical-scavenging activity were approximately 2.24 mg/ml. The results indicated that the LFF extract donated hydrogen, reacted with the free radicals to produce stable products and terminated the radical chain reaction. ABTS radical scavenging activity of LF was very low (1.28%) and IC_{50} values were not calculated.

The reducing-power assay evaluates the capacity of an antioxidant compound to donate an electron or hydrogen atom. As shown in Table 2, LFF extract has the capacity to donate electrons. At a concentration of 1 mg/ml, the reducing power of LFF was 0.351. However, the samples showed lower reducing power than BHT (1.65 ± 0.01%) at the concentration of 1 mg/ml.

The results showed that antioxidant activities of LF have increased using fermentation. Therefore, the protein hydrolysates from LFF extract acted as hydrogen donors, terminating the radical chain reaction by reacting with the free

Table 2 Antioxidant activities of Loma fish (LF) and Loma fermented fish (LFF) extract

Sample	IC_{50} (mg/ml)		
	DPPH radical scavenging activity	ABTS radical scavenging activity	Reducing power[a]
LF	–[b]	–[b]	0.06 ± 0.009
LFF	1.36 ± 0.66	2.24 ± 0.58	0.351 ± 0.008

Values are reported as the mean ± SD, experiments were performed in triplicate

[a]Reducing power was measured at a concentration of 1 mg/ml

[b]DPPH and ABTS radical scavenging activities of LF were too low (1.40 and 1.28%, respectively), IC_{50} value was not calculated

radicals to produce stable products. In the fermentation of fish, proteins are broken down into polypeptides by both the muscle's endogenous and microbial enzymes and this degradation of proteins is primarily controlled by pH [23]. Protein proteolysis, resulting in peptides and free amino acids, clearly has an impact on fermented fish and contributes to the development of volatile and non-volatile flavours. It has been reported [24] that *Lactobacillus casei* and *L. plantarum* can break down and hydrolyze sarcoplasmic and myofibrillar proteins.

Ultrafiltration

Three fractions were found after ultrafiltration: LFF-I (MW < 10 kDa), LFF-II (3 kDa < MW < 10 kDa), and LFF-III (MW < 3 kDa). The DPPH and ABTS radical-scavenging activities and reducing power of LFF and the three fractions prepared are shown in Fig. 1a–c. It is clear that the LFF-I, II, and III fractions had stronger DPPH radical scavenging activity than LFF extract. The ultrafiltration fraction LFF-III particularly exhibited significantly better DPPH radical scavenging (53.52%) compared to the other two fractions (45 and 47.8%), respectively ($p < 0.05$).

The results of the ABTS radical scavenging activity assay are presented in Fig. 1b. At a concentration of 1 mg/ml, the ABTS radical-scavenging activity of the LFF-III (61.23%) was significantly stronger than the LFF-II (55.2%) and LFF-I (47%). This finding demonstrated that, the fractions with MW < 3 kDa had significant antioxidant activities than those with MW > 3 kDa, as reported by Liu et al. and Ji et al. [22, 25]. During centrifugation and ultrafiltration, the peptides obtained from *pekasam* containing water-soluble low molecular weight peptides can react with ABTS water-soluble radicals but not with the DPPH lipid-soluble radicals [26].

Reducing power ability of LFF extract and three fractions obtained by ultrafiltration are represented in Fig. 1c. The results showed that all the ultrafiltration fractions (LFF-I, II and III) exhibited significantly higher antioxidant activity than LFF ($p < 0.05$). Zou et al. [27] reported that the access to long chain of amino acids is far and the procedure becomes more complicated with longer peptides. These results were similar to those of Ranathunga et al. [28] which reported the peptides with lower molecular weights have higher antioxidative properties and can easily react with lipid radicals to reduce the radical-mediated lipid peroxidation Furthermore, Wu et al. [18] reported that peptides with a molecular weight of approximately 1400 Da showed a higher in vitro antioxidant activity than those of the 900 and 200 Da peptides.

Size exclusion chromatography (SEC)

According to the results of antioxidant activities of fermented fish from Loma fraction, LFF-III was chosen for further purification. Figure 2 and Table 3 present the antioxidant activity (IC50) and size exclusion chromatography elution profile of the LFF-III fraction. Through the separation of gel filtration chromatography, the fraction of LFF-III was separated into other fractions (FI–FIV).

After pooling each fraction, the antioxidant activity of lyophilized fraction was examined. FIII fraction possessed the highest DPPH and ABTS radical scavenging activities with IC_{50} of 1.38 and 1.12 mg/ml, respectively

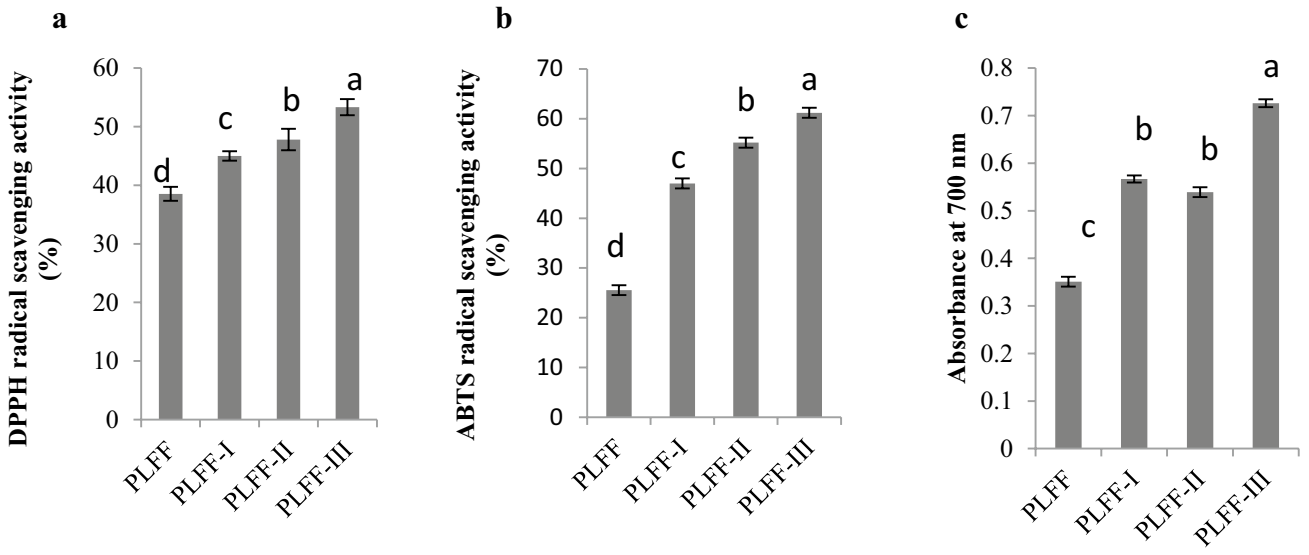

Fig. 1 Antioxidant activities of LFF extract and three fractions. **a** DPPH radical-scavenging activity; **b** ABTS radical-scavenging activity; **c** reducing power ability. The values were represented as means of three independent experiments ± S.D

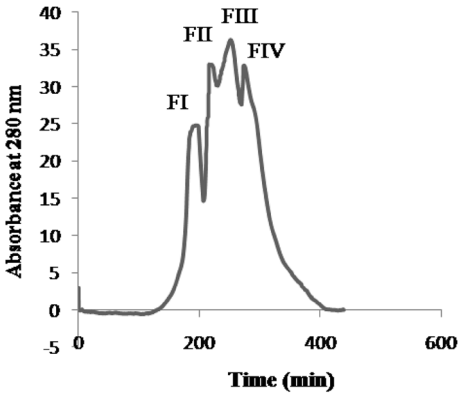

Fig. 2 Size exclusion chromatography elution profile of the LFF-III fraction

Table 3 Antioxidant activity (IC_{50}) of fractions obtained from LFF-III using gel filtration chromatography

Fractions	IC_{50} (mg/ml)		
	DPPH radical scavenging activity	ABTS radical scavenging activity	Reducing power
FI	33.82 ± 1.37^{a}	21.94 ± 1.68^{a}	0.06 ± 0.004^{c}
FII	27.11 ± 1.01^{b}	19.69 ± 1.22^{b}	0.08 ± 0.008^{c}
FIII	1.38 ± 1.14^{d}	1.12 ± 0.30^{d}	0.581 ± 0.006^{a}
FIV	11.41 ± 0.98^{c}	6.09 ± 0.81^{c}	0.197 ± 0.006^{b}

Values are reported as the mean ± SD, experiments were performed in triplicate

In each column different letters mean significant differences ($p < 0.05$)

and reducing power with an absorbance of 0.581 at a concentration of 1 mg/ml, followed by the fraction of FI, FII, and FIV. With reference to the molecular weight standard curve, it was found that FIII has the molecular weight of fractions ranging 500–1000 kDa, while the molecular weight of fraction of FIV < 500 kDa. This suggests that antioxidant activity of fraction from LFF extract with molecular weight of < 500 kDa is very low. FIII fraction possessed the highest radical scavenging activity. Wu et al. [18] reported that peptides with molecular weight of 1400 Da have more potent antioxidant activity in vitro than the peptides with molecular weight of between 200 and 900 Da.

Reversed-phased high performance liquid chromatography (RP-HPLC)

Figure 3 and Table 4 illustrated the elution profile of RP-HPLC for the FIII fraction of LFF extract and the antioxidant activity (IC_{50}). Three fractions (FIII 1–FIII 3) were separated from FIII fraction using RP-HPLC.

Among them, the fraction FIII 3 in elution time of 12.5 min demonstrated the highest DPPH and ABTS radical scavenging activities, with IC_{50} of 1.04 and 0.636 mg/ml, respectively and reducing power with an absorbance of 0.595 at a concentration of 1 mg/ml ($p < 0.05$). Antioxidant peptides from LFF extract were separated by three successive separation methods and their antioxidant activity was 2.46 times more than that of LFF extract.

Fig. 3 RP-HPLC elution profile of the FIII fraction

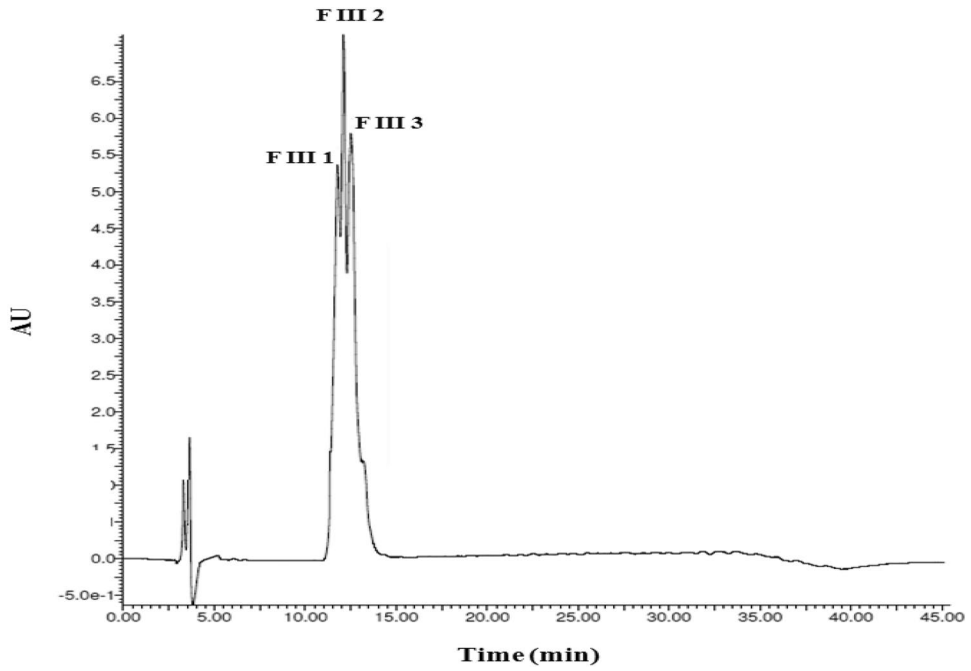

Table 4 Antioxidant activity (IC_{50}) of fractions obtained from the FIII using RP-HPLC

Fractions	IC_{50} (mg/ml)		
	DPPH radical scavenging activity	ABTS radical scavenging activity	Reducing power
FIII 1	27.13 ± 1.22^{a}	17.65 ± 1.12^{a}	0.08 ± 0.009^{c}
FIII 2	15.31 ± 1.61^{b}	8.72 ± 1.63^{a}	0.126 ± 0.006^{b}
FIII 3	1.04 ± 0.90^{c}	0.636 ± 0.02^{a}	0.595 ± 0.008^{a}

Values are reported as the mean ± SD, experiments were performed in triplicate

In each column different letters mean significant differences ($p < 0.05$)

Table 5 Amino acid (AA) compositions of the Loma fish and Loma fermented fish fractions (%)

AA	LF[1]	LFF-III[2]	FIII 3
Asp	9.49 ± 0.11^{c}	9.59 ± 0.11^{b}	9.67 ± 0.19^{a}
Ser	3.45 ± 0.13^{a}	2.87 ± 0.15^{b}	2.47 ± 0.14^{c}
Glu	12.57 ± 0.17^{c}	12.64 ± 0.14^{b}	12.73 ± 0.10^{a}
Gly	6.73 ± 0.14^{a}	5.81 ± 0.14^{b}	5.02 ± 0.16^{c}
His*	2.24 ± 0.21^{c}	2.71 ± 0.18^{b}	2.91 ± 0.14^{a}
Arg	4.60 ± 0.16^{a}	3.40 ± 0.16^{b}	2.60 ± 0.15^{c}
Thr*	4.79 ± 0.15^{a}	4.25 ± 0.17^{c}	4.42 ± 0.19^{b}
Ala	9.86 ± 0.08^{c}	10.25 ± 0.18^{b}	10.68 ± 0.11^{a}
Pro	9.33 ± 0.12^{c}	9.48 ± 0.15^{b}	9.62 ± 0.16^{a}
Tyr	1.35 ± 0.19^{c}	1.42 ± 0.08^{b}	1.46 ± 0.17^{a}
Val*	5.05 ± 0.08^{c}	5.39 ± 0.12^{b}	5.57 ± 0.15^{a}
Met*	2.13 ± 0.17^{c}	2.44 ± 0.15^{b}	2.69 ± 0.15^{a}
Lys*	10.20 ± 0.18^{c}	10.53 ± 0.13^{b}	10.82 ± 0.11^{a}
Ile*	4.06 ± 0.13^{c}	4.16 ± 0.13^{b}	4.56 ± 0.17^{a}
Leu*	7.78 ± 0.15^{c}	8.23 ± 0.16^{b}	8.46 ± 0.11^{a}
Phe*	5.05 ± 0.08^{b}	5.48 ± 0.09^{a}	5.43 ± 0.16^{a}
Cys	0.91 ± 0.09^{c}	1.22 ± 0.07^{b}	1.33 ± 0.15^{a}
Trp*	0.42 ± 0.16^{c}	0.510 ± 0.11^{b}	0.58 ± 0.18^{a}
TEAA[3]	42.63^{c}	43.70^{b}	45.44^{a}
HAA[4]	44.61^{c}	46.85^{b}	48.47^{a}

The values were represented as means of three independent experiments ± S.D

In each column different letters mean significant differences ($p < 0.05$)

*Essential amino acid

[1]LF: Loma fish

[2]LFF-III and FIII 3: Fractions with the highest antioxidant activities obtained from ultrafiltration (LFF-III) and RP-HPLC (FIII 3)

[3]Total essential amino acids

[4]Hydrophobic amino acids (Ala, Val, Met, Ile, Leu, Phe, Pro and Tyr)

Amino acid composition

Table 5 shows the amino acid composition of Loma fish, the fraction of LFF-III from ultrafiltration and the FIII 3 from size exclusion chromatography. The major amino acids of the LF, LFF-III and FIII 3 are Asp (D), Glu (E), Ala (A), Pro (P), Lys (K) and Leu (L). The content of essential amino acids in the FIII 3 fraction increased to 45.44% compared with LFF-III (43.70%) and LF (42.63%) ($p < 0.05$). Acidic amino acids (glutamic and aspartic) with electron-donating ability have the DPPH scavenging and ferric-reducing effects of protein hydrolysates [29]. The hydrophobic amino acid content (Val, Ala, Met, Leu, Ile, Phe, Tyr and Pro) of the LF, LFF-III and FIII 3 is high: 44.61, 46.85 and 48.47%, respectively. The FIII 3 significantly presented the highest hydrophobic amino acid ($p < 0.05$).

Aluko [29] reported that hydrophobic amino acids by increasing the accessibility of the antioxidant peptides to hydrophobic PUFAs could reduce the oxidative damage. According to the results of the present study, hydrophobic amino acids of the FIII 3 including Ala, Leu, Pro, showed the highest content. Sarmadi and Ismail [30] described that Phe, His, Tyr and Trp (aromatic amino acids) can convert radicals into the stable molecules by donating electron. They maintain their own stability by improving the radical-trapping properties of the amino acids residues [31].

Identification of antioxidant peptides by LC–MS–TOF and peptide synthesis

In order to characterize the amino acid sequence, fraction FIII 3 with the highest antioxidant activity was analyzed using HPLC–MS/MS. According to database search and calculation, two novel peptides were characterized to be AIPPHPYP and IAEVFLITDPK, which have not yet been reported (Fig. 4).

Mass error, score and forward–reverse score were 4.6 ppm, 13.01 and −0.06 for the AIPPHPYP and 2.4 ppm, 10.52 and 0.3 for the IAEVFLITDPK, respectively. These peptides contain 7 and 11 amino acid residues. This finding is in agreement with previous studies which reported that peptides are short and usually consist of 2–20 amino acids.

The identified peptides were synthesized, and the antioxidant activity of these peptides was determined (Table 6). All synthesized peptides had antioxidant activity, however IAEVFLITDPK possessed the highest DPPH and ABTS radical scavenging activity with IC_{50} values of 0.897 and 0.594 mg/ml, respectively and reducing power with an absorbance of 0.688.

In both antioxidant peptides from FIII 3, Proline (P), Alanine (A), Leucine (L) and Isoleucine (I) amino acids were present. In addition, IAEVFLITDPK peptide has Glutamic acid (E), Valine (V), Phenylalanine (F) and

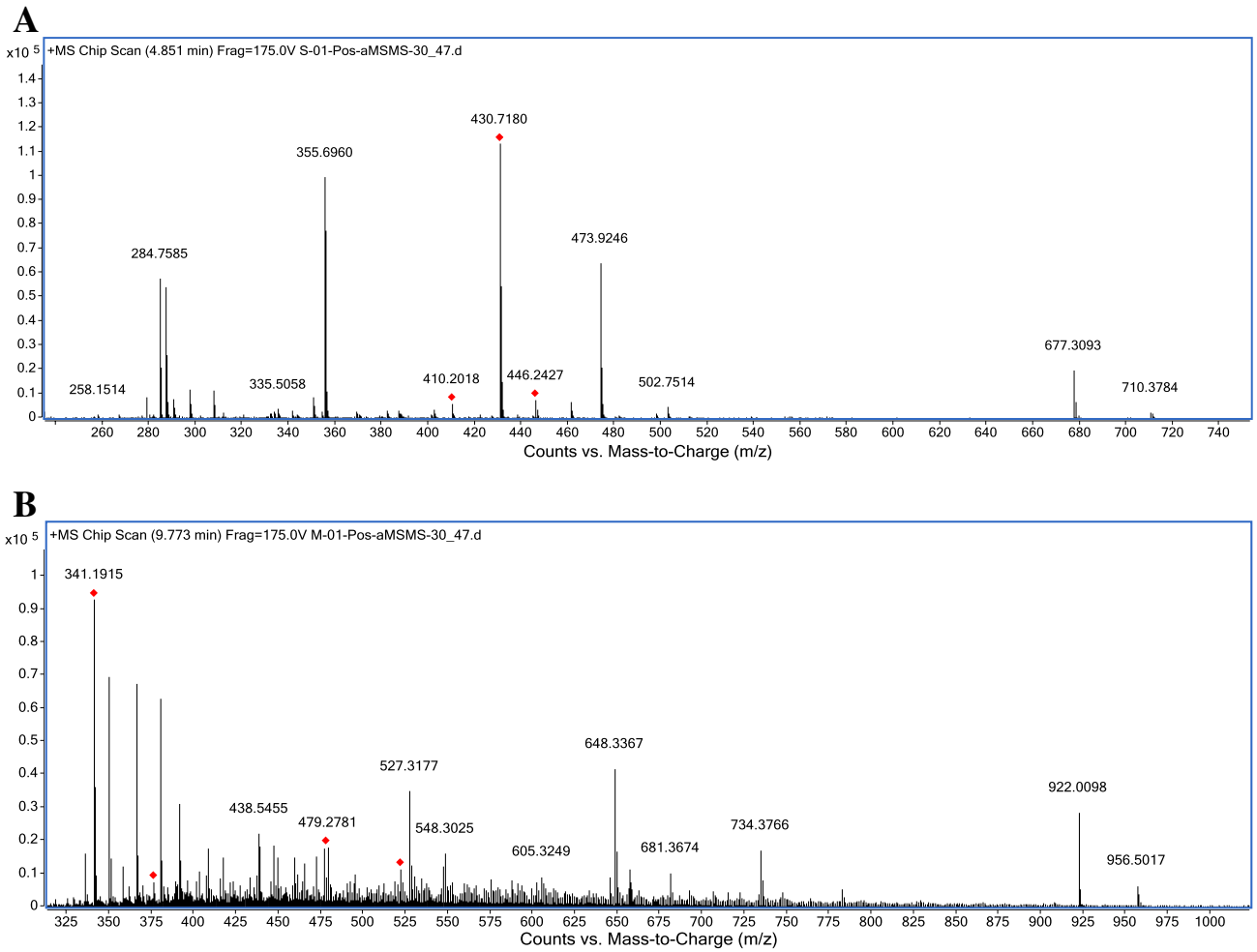

Fig. 4 The mass spectra (MS/MS) of the antioxidant peptide from Loma fermented fish (LFF) extract. **a** AIPPHPYP; **b** IAEVFLITDPK

Table 6 Antioxidant activities of the synthesized peptides from Loma fermented fish (LFF) extract

Synthesized peptides	IC_{50} (mg/ml)		
	DPPH radical scavenging activity	ABTS radical scavenging activity	Reducing power[a]
IAEVFLITDPK	0.897 ± 0.84^{a}	0.594 ± 0.22^{a}	0.688 ± 0.006^{a}
AIPPHPYP	1.38 ± 0.25^{b}	0.873 ± 0.18^{b}	0.456 ± 0.008^{b}

Values are reported as the mean ± SD, experiments were performed in triplicate

In each column different letters mean significant differences ($p < 0.05$)

[a]Reducing power was measured at a concentration of 1 mg/ml

Lysine (K). According to Table 5, the hydrophobic amino acids Phe, Ala, Leu, Val and Pro which are high in LFF fractions have been presented in identified antioxidant peptides. The interactions between amino acids may be major factors for the antioxidant activity. The amphiphilic nature of peptides, besides the hydrophobicity of peptides with high antioxidant activity seems to increase the radical-scavenging activities using increasing peptide solubility while facilitating the interaction and proton exchanges with radical species [27]. A sequence of hydrophobic amino acid residues like Ala or Ile at the N-terminus containing Pro, His, Tyr, Glu, Val, Phe, Leu or Lys is characteristic of antioxidant peptides. In the sequence, Pro tends to interrupt and alter the secondary structure of the peptide molecule, which may augment the availability of the amino acid residues of the peptide sequences to act as antioxidants [27]. Aluko [29] confirmed that the presence of aspartic acid, threonine, valine and isoleucine had high

positive effects in enhancing the DPPH and ABTS radical scavenging effects of food protein hydrolysates [29]. Chi et al. showed that inhabitation of lipid peroxidation and radical scavenging activities are facilitated by the hydrophobic amino acids [32]. Hydrophobic amino acid sequences via donating the protons to free radicals can protect against oxidation. Kleekayai et al. [33] identified Trp-Pro and Ile-Phe in two antioxidative peptides from Thai traditional fermented shrimp pastes. Mendis et al. [34] reported that HGPLGPL as an antioxidant with high ability could be obtained from hydrophobic amino acids through better interaction with fatty acids. In addition, Saiga et al. [35] stated that the existence of amino acids such as Leu, Pro and Gly had higher antioxidant activities. Therefore, the higher antioxidant activity of peptides from the PLFF may be due to the existence of the hydrophobic amino acid Valine (V), Alanine (A) and Proline (P) in the peptide sequences. The presence of hydrophobic amino acids like Ile at the N-terminal and basic amino acid like Lys at C-terminal of IAEVFLITDPK peptide can possess high antioxidant activity. Nikoo et al. [36] suggested that the peptide PAGY derived from Amur sturgeon skin gelatin has inhibition activity against hydroxyl, ABTS and DPPH radicals. However, Aluko [29] reported that the antioxidant activity of the amino acids alone may be weaker than of the same amino acids in peptides. Loma fermented fish (*pekasam*) may serve as a source of novel peptides for natural antioxidants.

Conclusion

The present study demonstrated that the fractionated Loma fermented fish using ultrafiltration, size exclusion chromatography and RP-HPLC have exhibited antioxidant effects. Two novel peptides, AIPPHPYP and IAEVFLITDPK were identified and characterized from fraction FIII 3. The IAEVFLITDPK showed higher antioxidant activity. Generally, identified peptides were rich in acidic (E, D), basic (K) and hydrophobic, Ile (I), Ala (A), Val (V) and Pro (P) amino acids indicating antioxidant properties. This research provided new antioxidative peptide sequences derived from fermented fish products. Consequently, Loma fermented fish (*pekasam*) can be used in the nutraceutical and pharmaceutical industry to extend new functional and medicinal food (in improving public health and preventing ROS-related chronic diseases) with antioxidant effects.

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