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Development and enhancement of antioxidant peptides from spontaneous *plaa-som* fermentation co-stimulated with Chiangrai Phulae pineapple enzymatic reaction

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Abstract

The present work aimed to search for a released peptide from proteolytic action on a silver barb fish muscular protein that confers health benefit through antioxidation activity. Changes in the physicochemical, microbiological, and protein characteristics of *plaa-som* samples during eight days of both spontaneous traditional fermentation (Batch 1; B_1) and spontaneous fermentation with the addition of pineapple (Batch 2; B_2) were determined. Results showed a correlation between an increase in the total acidity and bacterial counts with the length of fermentation duration, where the pH gradually decreased at the end of fermentation. Protein hydrolysis during fermentation was indicated by an increase in the amount of TCA-soluble peptide contents that peaked on day 5 (D_5) in both batches (B_1D_5 and B_2D_5), which displayed their highest DPPH radical-scavenging inhibition of *plaa-som* protein hydrolysates (PSPHs). Twelve peptide fractions of the best PSPH were separated by ultrafiltration using molecular weight cut off (MWCO) at 3 and 10 kDa, and they were also purified by size exclusion chromatography. Results demonstrated that stronger peptides B_2D_5 - 3 kDa - F_1 and B_1D_5 - 10 kDa - F_1 were arranged in 12 peptides, which exhibited the highest reducing power, more than their radical-scavenging inhibition ($p < 0.05$). Therefore, it was concluded that both peptides obtained from PSPH have released antioxidative peptides that could be beneficial towards consumer's health, particularly the spontaneous *plaa-som* fermented with the addition of pineapple.

Introduction

Fish meat is high in proteins and fats, but low in carbohydrates. The protein content of both freshwater and seawater fish is mainly sarcoplasmic and myofibrillar proteins, ranging from 22.0 to 25.2% for fresh fish fillets of tuna and silver barb (*Barbonymus gonionotus*; *plaa-ta-pian*), respectively (Burgaard and Jørgensen, 2011; Chadong *et al*., 2015). Silver barb is an indigenous fish to Southeast Asia. In Thailand, it is a popular freshwater fish that has been consumed by the locals since ancient times. Silver barb is a common barb species, and one of the ten most cultured fish species in Thailand. The Thai Department of Fisheries has encouraged its use as a commercial fish for consumption and trade. It can also be processed into *plaa-som*, a traditional fermented fish product (FAO, 2012).

Plaa-som is a well-known homemade Thai fermented fish product, obtained from the maturation of whole fish after cleaning and soaking in 15 - 20% of saline solution mixed with sea salt, steamed sticky rice, and mashed garlic according to the Thai Industrial Standard Institute (TISI). The most popular fish used in *plaa-som* production are *plaa-ta-pian*, *plaa-sa-waai* (*Pangasius sutchi*; iridescent shark catfish), and *plaa-te-po* (*P. larnaudii*; spot shark catfish) (TISI, 2003). *Plaa-som* plays important part in the dietary trend of the locals. During the fermentation of *plaa-som*, proteolytic lactic acid bacteria (PLAB) will naturally hydrolyse the fish protein (Kopermsub and Yunchalard, 2008; Saithong *et al*., 2010).

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Proteolysis is a part of the proteolytic system of PLAB which assists the growth of lactic acid bacteria (LAB) (Griffiths and Tellez, 2013; Toe *et al.*, 2019). The fish protein must be hydrolysed to release amino acids or smaller peptides during fermentation. The by-products produced by the action of enzymes further serve as precursors for the formation of bioactive peptides such as antioxidative peptides (Chalamaiah *et al.*, 2015; Phupaboon *et al.*, 2020; Prabha *et al.*, 2020), antimicrobial peptides (Masso-Silva and Diamond, 2014; Bashir *et al.*, 2020), ACE-inhibitory peptides (Abachi *et al.*, 2019), and antihypertensive peptides (Abachi *et al.*, 2019; Yathisha *et al.*, 2019). These peptides were found to be at relatively higher levels in fermented fish products as compared to those in meat and milk products (Saithong *et al*., 2010). Presently, there are relatively few studies on fish protein hydrolysates (FPH) produced with proteolytic enzymes in food formulation. FPH has been shown to have excellent functional properties including high solubility, good emulsifying property, and good water-binding property (Chalamaiah *et al.*, 2012; 2015). Various studies have established that FPH from freshwater or saltwater fishes have potential antioxidant peptides, for example, silver barb (Phupaboon *et al.*, 2020), silver carp (Ashraf *et al.*, 2011), grass carp (Ren *et al*., 2008), mackerel (Wu *et al*., 2003), and yellow stripe trevally (Klompong *et al*., 2012). Recent evidence suggests that potential antioxidant peptides could be hydrolysed by different enzymes from animal (*e.g.* pepsin and trypsin), plant (*e.g.* papain, ficin, and bromelain), and microbial (*e.g.* alcalase, flavourzyme, neutrase, and proteinase) sources (Kasankala *et al*., 2012; Klompong *et al*., 2012).

In the present work, pineapple (*Ananas comosus*) fruit was used due to its physicochemical property and bromelain enzyme activity which is a cysteine-endopeptidase enzyme with broad specificity (Ren *et al*., 2008). Besides, some properties of pineapples have also been used to preserve fermented fish, and increase the production of antioxidant peptides and functional activities (Ren *et al*., 2008; Elavarasan *et al*., 2014). In the present work, the detection and characterisation of antioxidative peptides released during *plaa-som* fermentation were conducted with the aim to find which process could be best to generate antioxidative peptides from fish muscular protein by considering the different actions of spontaneous microorganisms and plant enzyme, as a Chiangrai Phulae pineapple additive was added during *plaa-som* fermentation. It is hoped that consumers who consume fish-based products such as *plaa-som* on a regular basis could benefit from its health benefits.

Materials and methods

Chemicals

All chemicals used in the present work were of analytical grade, and purchased from Sigma-Aldrich Co. LLC (St. Louis, Missouri, USA): methanol, trichloroacetic acid, Folin-Ciocalteu's phenol reagent, bovine serum albumin, tyrosine, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, and 6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Preparation of plaa-som sample

Fish samples weighing around 400 - 500 g were purchased from a local market at Ubonrat Dam in Khon Kaen, Thailand. The fish samples were kept fresh on ice, and quickly transported to the laboratory within 30 min, where they were immediately prepared. The fish samples were gutted, scaled, slit along both sides of the trunk, and thoroughly washed with clean tap water for several times. Subsequently, the prepared fish fillets were soaked in 20% of brine solution for approximately 2 h. The fermentation processes were divided into two batches. Spontaneous fermentation (B_1) was prepared by mixing 20 kg of fish fillets with 5% (w/w) freshly crushed garlic, 10% of steamed sticky rice, and 10% of salts. Batch 2 (B_2) consisted of 10 kg of fish fillets from B_1 mixed with 1% of minced Chiangrai Phulae pineapple. All ingredients were thoroughly mixed, packed into a plastic bag, and pressed to remove air before sealing with rubber bands. Finally, the bags were placed in a covered plastic box, and left to ferment at 35°C. Fermentation lasted for 8, and sub-samples (*plaa-som*; fermented fish fillet product) collected each day were stored at -20°C until further analyses.

Physicochemical and microbiological analyses

The pH and total acidity were determined following the method of AOAC (2000). *Plaa-som* samples (5.0 g) were collected every day, homogenised with 45 mL of CO_2-free distilled water, and filtered through rough filter paper. The filtrate was directly determined using a pH meter. Titratable

acidity (TA) in the form of lactic acid equivalents was determined using titration. The filtrate was titrated against 0.1 N NaOH with 1% of phenolphthalein solution as an indicator.

The total viable count (TVC) and lactic acid bacteria (LAB) count were performed by the modified drop plate technique described by Speack (1976). Briefly, 5 g of *plaa-som* samples were aseptically homogenised using a stomacher for 5 min. Serial dilutions of the homogenate were prepared with sterile saline-peptone water, and about 10 µL of supernatant were dropped onto plate count agar (PCA) plates and modified-MRS with 1.0% of calcium carbonate (MRS-$CaCO_3$) agar plates by using the drop plate technique. Each plate was inoculated at 35°C for 24 - 48 h in triplicates for both TVC and LAB counts. After the incubation period, PCA and MRS-$CaCO_3$ plates with 3 - 30 colonies were counted, and the results were calculated in log CFU/g of the sample at different sampling days of *plaa-som* fermentation.

Preparation of plaa-som protein hydrolysates (PSPH)

The minced *plaa-som* from each sampling day was mixed with DI water at a ratio of 1:2, and homogenised at high speed for extraction. The homogenate was filtered and centrifuged at 10,000 *g* for 10 min at 4°C to separate the soluble and insoluble proteins. The solution (approximately 30 mL) of each hydrolysate was freeze-dried following the procedure of Eyela speed freeze-dryer (Shanghai, China), placed in sealed bags, and stored at -20°C until further analyses.

Total protein and TCA-soluble peptide analyses

Analyses were performed in 96-well microtiter plates in the maximum volume of 250 µL. Each PSPH sample of 0.3 g was dissolved in 1.5 mL of DI water, and mixed using a mixer at a constant speed. The mixture was subsequently filtered and centrifuged at 10,000 *g* for 10 min at 4°C. The total protein content of the supernatant was determined according to Lowry *et al*. (1951), and bovine serum albumin was used as a standard. The soluble peptide content was quantified using a modified method. The supernatant was precipitated with chilled 10% trichloroacetic acid (TCA). Centrifugation was performed at 10,000 *g* for 10 min. Then, 20 µL of supernatant was added with 100 µL of 0.4 M Na_2CO_3 and 50 µL of Folin-Ciocalteu's phenol reagent to the mixture of each well. The absorption of the solution was measured by a spectrophotometer at 595 nm. The amount of peptide was expressed as the µmoL tyrosine content.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The SDS-PAGE analysis was performed using a modified method of Laemmli (1970) using a 5% stacking gel and 15% resolving of polyacrylamide gel. PSPH was mixed with loading buffer in the final concentration of 1×, and boiled at 100°C for 5 min. Aliquots of 15 - 20 µL of samples were loaded into each well on stacking gels, and electrophoresis was performed at 100 V. Finally, SDS-polyacrylamide gel was stained with 0.1% Coomassie® brilliant blue R250, and de-stained in a mixture of 10% acetic acid and 40% methanol in water overnight.

Antioxidative activities

DPPH radical-scavenging activity

The DPPH radical-scavenging activity was slightly modified using 96 microtiter plate assays following the method described by Binsan *et al*. (2008). An aliquot of 50 µL of PSPH samples or purified peptides were added to each well (200 µL) of 0.15 mM freshly DPPH in methanol. Following incubation in the dark for 30 min, the absorbance of the supernatant was measured at 490 nm using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories Ltd., California, USA). The control was prepared by mixing a DPPH solution with DI water instead of the sample. The activity was expressed as (%) DPPH radical-scavenging inhibition.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was slightly modified using a microtiter plate assay following the method described by Benzie and Strain (1996). An aliquot of 50 µL from each sample was mixed with 200 µL of FRAP solution, and incubated for 30 min in the dark. The absorbance of the solution was measured by a microplate reader at 595 nm. The standard curve was prepared using Trolox ranging from 50 to 1000 µM. The activity was expressed as µmoL TE/g sample.

Purification of antioxidant peptides

The peptide fractions of the PSPH were separated using column chromatography as described by Wu *et al*. (2003). The freeze-dried hydrolysate (0.3 g of each sample) was dissolved in 1.5 mL of filtered DI water, and subjected to ultrafiltration using

membrane with a molecular weight cut off (MWCO) at 3 and 10 kDa. The fractions were subjected to semi-preparative HPLC using reverse-phase C_{18} (Φ 7.8 × 300 mm) column (Waters Corporation, USA). The chromatographic conditions were as follows: the flow rate was 0.8 mL/min with a linear gradient consisted of (A) acetonitrile containing 0.1% of trifluoroacetic acid (TFA), and (B) filtered DI water containing 0.1% TFA. The UV detector measured the absorbance between 215 and 280 nm. All fractions were collected and lyophilised at 4°C. The lyophilised fractions were resuspended in filtered DI water, and antioxidative activity was determined through DPPH radical scavenging inhibition and FRAP capacity.

Statistical analysis

All results were expressed as mean ± standard deviation, and analysed by the SPSS version 16.0 statistical program. The information was analysed by utilising one-way (ANOVA). Results with $p < 0.05$ indicated significant difference.

Results and discussion

Physicochemical characteristics during plaa-som fermentation

Figure 1A shows the pH and total acidity (TA). The pH levels of both B_1 and B_2 were initially found at 6.32 ± 0.01 and 6.19 ± 0.01, respectively. During fermentation, the pH levels of both batches gradually decreased. At the end of fermentation (day 8), the pH levels were 4.44 ± 0.01 (B_1) and 4.33 ± 0.00 (B_2). This is consistent with the characteristics of *plaa-som* as prescribed by the Thai community product standards (TISI, 2003). Generally, pH is regarded as an indicating factor to ensure the safety of fermented products (Riebroy *et al*., 2004; Kopermsub and Yunchalard, 2008; Chadong *et al*., 2015).

The TA, calculated as lactic acid, indicated a continuous increase in both batches during *plaa-som* fermentation. The initial TA of B_1 increased from 0.17% to 0.90%, while the initial TA of B_2 increased from 0.19 to 1.42% (Figure 1A). The high level of TA was mainly associated with the major organic acid content such as lactic acid, which was produced via fermentation, and continued to accumulate throughout fermentation. There are several factors affecting the acidity of *plaa-som* such as growth, fermentation action of the fermenting microorganisms, type, and amount of ingredients available in the recipe (Kopermsub and Yunchalard, 2008; Chadong *et al*., 2015). Previous studies have reported that the TA of *plaa-som* made from Thai red tilapia fish ranged from 1.6 to 2.3% after 120 h of fermentation, which was at a higher level than the level found in the *plaa-som* prepared in the present work. Likewise, the TA of *som-fuk* has been reported to be in the range of 1.4 to 2.5% after 72 h of fermentation (Riebroy *et al*., 2004; Saithong *et al*., 2010).

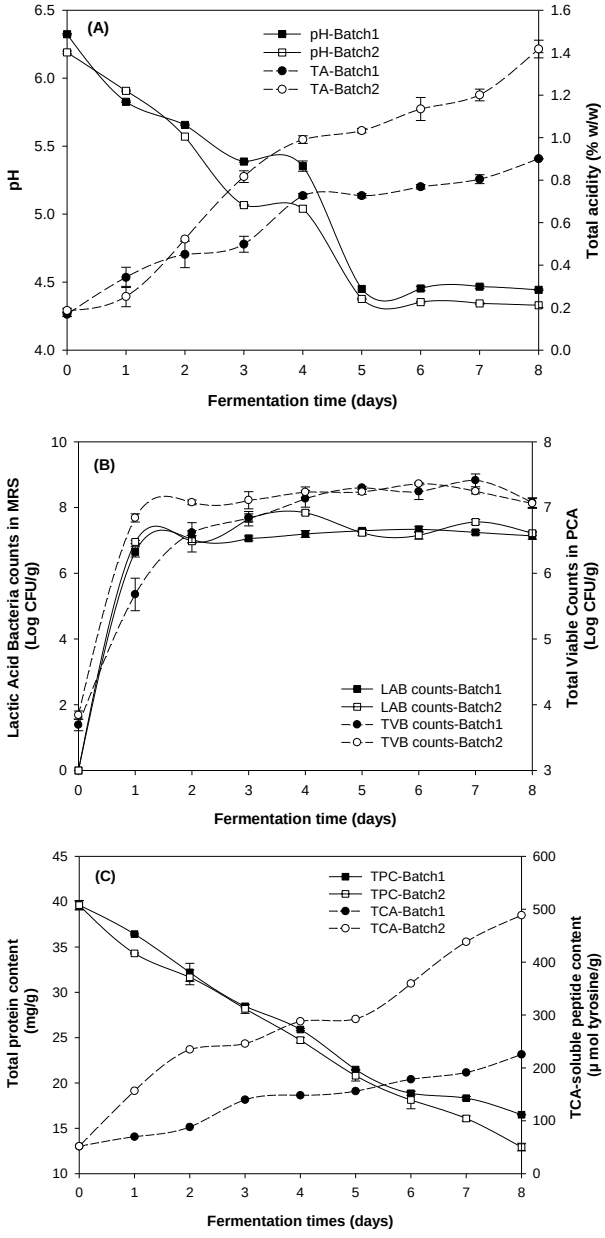

Figure 1. Changes in physiochemical and microbiological characteristics of PSPHs during *plaa-som* fermentation: **(A)** pH and total acidity; **(B)** lactic acid bacterial count and total viable bacterial count; and **(C)** total protein and TCA-soluble peptide contents. Bars represent ± SD obtained from three replicates ($n = 3$).

Microbiological changes during plaa-som fermentation

Based on the microbiological changes observed during *plaa-som* fermentation, two stages were apparent namely start stage and maturation stage, comparative to the reports of Kopermsub and Yunchalard (2008). The LAB and TVC counts during the fermentation are shown in Figure 1B. For B_1, both LAB and TVC counts were found to have slightly increased from log 1 to log 6.8 CFU/g after one day of fermentation, and then increased to log 7.2 CFU/g at the end of fermentation. For B_2, both LAB and TVC counts increased from log 3.8 CFU/g at the start of fermentation to log 7.1 CFU/g at the end of fermentation. Both fermentation batches showed a dramatic increase in LAB counts, where both batches reached a maximum level of log 7.3 CFU/g after five days of fermentation. For TVC counts, both batches reached the maximum levels of log 7.8 and 7.4 CFU/g at 4 and 6 days of fermentation, respectively. These results exceeded those reported by Kopermsub and Yunchalard (2008) and Saithong *et al*. (2010); LAB (log 6.3 CFU/g) and TVC (log 6.8 CFU/g). It can be concluded that the addition of pineapple in *plaa-som* fermentation in the present work did not affect the increase in the number of LAB during the process; the LAB were still dominant and controlled both the physicochemical and microbiological changes that appeared during the fermentation (Kopermsub and Yunchalard 2008; Xu *et al.*, 2010, 2021).

Alternation of silver BARB during plaa-som fermentation

Figure 1C displays fish protein hydrolysis and peptide formation throughout fermentation at 35°C. An increase was observed in the amount of TCA-soluble peptide content that reached 225.5 ± 0.02 and 488.6 ± 0.03 µmoL tyrosine/g by the end of fermentation, respectively. Concurrently, the initial protein contents of both batches (39.6 ± 0.51 mg/g) steadily decreased to 16.5 ± 0.03 and 12.9 ± 0.42 mg/g by the end of fermentation.

The protein changes during *plaa-som* fermentation as assessed by SDS-PAGE are shown in Figure 2.

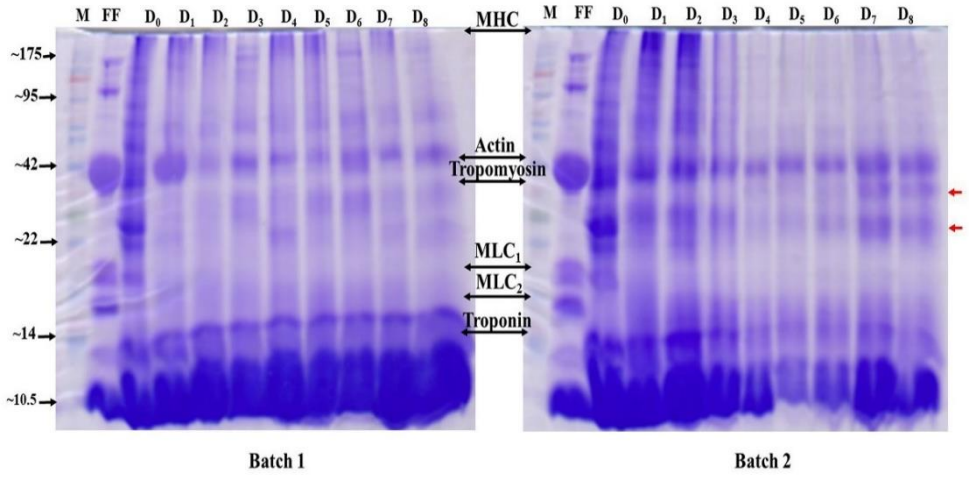

Figure 2. Protein profiles of PSPHs during *plaa-som* fermentation. M = protein ladder; FF = fresh fish protein; MHC = myosin heavy chain; MLC_1 and MLC_2 = myosin light chain 1 and 2; and D_{1-8} = days of fermentation. Red arrow is a protein that has changed.

The results showed high molecular weight protein of approximately 210 kDa, as myosin heavy chain (MHC) was gradually hydrolysed after the fermentation started. This is consistent with the study of MHC degradation during *plaa-som* fermentation and the changes of frozen Pacific whiting fish protein (Chadong *et al.*, 2015; Phupaboon *et al.*, 2020). Also, when compared to other proteins of fresh fish (FF) such as actin, tropomyosin, myosin light chain 1 (MLC_1), and myosin light chain 2 (MLC_2), the concentration of protein bands and sizes decreased gradually after one day of fermentation. These results exhibited short proteins or peptides smaller than 42 to 22 kDa, which could be seen in B_2 after fermentation for seven and eight days (Figure 2), respectively. The decrease in protein content depends on proteolytic action from microorganisms and endoproteolytic enzymes during fermentation (Nie *et al*., 2014; Xu *et al*., 2010; Zeng *et al*., 2013). Previous studies suggested that not only muscle endoproteolytic

enzymes, such as calpain and cathepsin, but also microorganisms producing proteolytic enzymes, in particular, proteolytic lactic acid bacteria (PLAB), contribute to fish protein degradation during processing. Besides, the present work also demonstrated that the fish muscle protein was degraded by the action of bromelain in B_2, higher than that by PLAB's activity alone (B_1). Other studies also investigated protein hydrolysis by detecting soluble peptide contents in various types of fermented fish, for example, *som-fuk* (Riebroy *et al.*, 2004; Anal, 2019), grass carp sausage (Nie *et al*., 2014), and fermented silver carp fish (Kasankala *et al*., 2012) which were released from muscular proteins, and had the potential as antioxidative peptides.

Antioxidant activity of lyophilised PSPH fraction

The DPPH radical-scavenging inhibitions of PSPHs obtained during *plaa-som* fermentation are shown in Figure 3. During fermentation, both B_1 and B_2 yielded highest radical-scavenging inhibition at 56.6 and 65.3% after five days of fermentation, respectively, before slightly decreasing towards the end of fermentation. These were significantly higher than the DPPH radical-scavenging inhibition of the control, which was in the range of 16.3 to 22.0%. Consequently, B_1D_5 and B_2D_5 were selected and subjected to ultrafiltration using a membrane with a different size of MWCO at 3 and 10 kDa. Four potential MWCO filtrates (B_1D_5 - 10 kDa, B_2D_5 - 10 kDa, B_1D_5 - 3 kDa, and B_2D_5 - 3 kDa) were subjected to peptide purification and separated by size exclusion chromatography on reversed-phase C_{18} columns using HPLC techniques. Results revealed 12 fractions consisting of B_1D_5 - 3 kDa ($F_1 \rightarrow F_3$), B_1D_5 - 10k Da ($F_1 \rightarrow F_4$), B_2D_5 - 3 kDa ($F_1 \rightarrow F_2$), and B_2D_5 - 10 kDa ($F_1 \rightarrow F_3$), which are presented in Table 1.

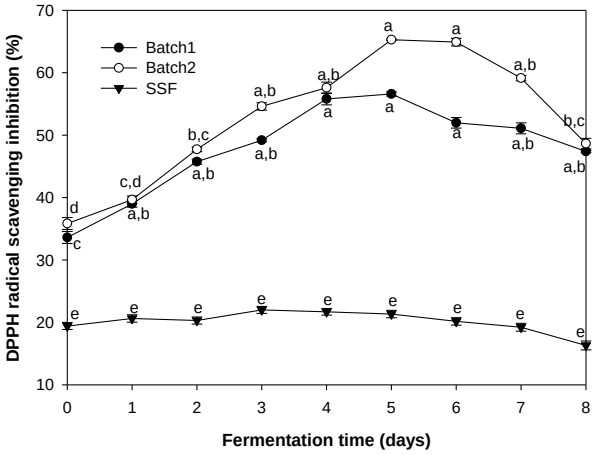

Figure 3. DPPH radical-scavenging activities of PSPHs during *plaa-som* fermentation. Bars represent ± SD obtained from three replicates (n = 3). Lowercase letters indicate significant difference ($p < 0.05$).

Table 1. Fractions and protein contents of PSPHs with different retention times following separation by MWCO and purification by HPLC.

MWCO filtrate	Fraction	Retention time (min)	Protein content* (mg/mL)
B_1D_5 - 3 kDa	F_1	18.0 - 19.0	4.02 ± 0.12
	F_2	21.0 - 22.5	0.79 ± 0.12
	F_3	30.5 - 35.0	0.79 ± 0.98
B_1D_5 - 10 kDa	F_1	18.0 - 20.0	4.85 ± 0.24
	F_2	21.0 - 25.0	0.53 ± 0.08
	F_3	27.0 - 29.0	3.98 ± 0.88
	F_4	31.0 - 37.0	3.39 ± 0.99
B_2D_5 - 3 kDa	F_1	19.0 - 21.5	4.93 ± 0.14
	F_2	28.0 - 34.5	0.55 ± 0.12
B_2D_5 - 10 kDa	F_1	18.0 - 19.5	3.93 ± 0.15
	F_2	20.0 - 25.0	3.98 ± 0.99
	F_3	30.5 - 34.5	0.76 ± 0.16

*mean ± SD from three replicates (n = 3). MWCO = molecular weight cut off, HPLC = high performance liquid chromatography.

Separation and purification of antioxidant peptides

The above results were established from the four potential MWCO filtrates that were purified into 12 fractions by size exclusion chromatography on a C_{18} column. The measured protein content was in the range of 0.53 to 4.93 mg/mL at different retention times of 18.0 to 30.5 min, and was used as peptide preparation data for further analysis of antioxidant activity (Table 1). The fractions of B_1D_5 - 10 kDa - F_1 and B_2D_5 - 3 kDa - F_1 had the highest protein content of 4.85 and 4.93 mg/mL, respectively. Consequently, the protein concentration at 3 mg/mL of each fraction (F) was found to exhibit radical-scavenging inhibition, and FRAP capacity was evaluated according to the seven fractions (B_1D_5 - 10 kDa - F_1, B_1D_5 - 10 kDa - F_3, B_1D_5 - 10 kDa - F_4, B_2D_5 - 10 kDa - F_1, B_2D_5 - 10 kDa - F_2, B_1D_5 - 3 kDa - F_1, and B_2D_5 - 10 kDa - F_1) that were investigated for the antioxidant activities. It can be seen from the data in Figure 4 that 3 kDa fraction presented significantly higher result than 10 kDa fraction in terms of FRAP capacity and DPPH radical-scavenging inhibition.

This also accords with our earlier observations, which showed that 3 kDa fraction of grass carp hydrolysate from alcalase digestion exhibited the highest hydroxyl radical-scavenging and lipid peroxidation activities of more than a fraction of 5 and 10 kDa (Kim *et al.*, 2009; Wang *et al.*, 2021). B_2D_5 - 3 kDa - F_1 indicated as the highest radical-scavenging inhibition against DPPH of 37.8%, and the highest reducing power of 70.1 µmoL TE/mg F. B_1D_5 - 10 kDa - F_1 also indicated the highest radical-scavenging inhibition and reducing the power of 32.0% and 61.0 µmoL TE/mg F, respectively. This is consistent with the report of Rajapakse *et al*. (2005), where the hydrophobic peptide approximately ≤ 3 to ≤ 10 kDa was displayed within the fish protein hydrolysates, and had a more prominent effect on the observed antioxidant activities. Nevertheless, to the best of our knowledge, there was no report available regarding the use of silver barb for *plaa-som* fermentation and the investigation of their antioxidant properties. However, many studies have found that antioxidant peptides were hydrolysed by the different enzymes namely trypsin, alcalase, flavourzyme, neutrase, papain, and bromelain in the range of temperature at 50 - 60°C and pH at 6.8 - 8.0 obtained from various muscle protein hydrolysates. For example, silver barb protein (Phupaboon *et al.*, 2020), silver carp protein (Ashraf *et al.*, 2011; Qiu *et al.*, 2014), grass carp protein (Ren *et al*., 2008), mackerel protein (Wu *et al.*, 2003; Bashir *et al.*, 2020), and yellow stripe trevally protein (Klompong *et al*., 2012) have exhibited high radical-scavenging activity and reducing power. The peptide sequences of mackerel protein and grass carp protein hydrolysates were A-L-S-T-W-T-L-Q-L-G-S-T-S-F-S-A-S-P-M and P-S-K-Y-E-P-F-V, respectively, by using mass spectrometry (Ren *et al.*, 2008; Bashir *et al.*, 2020). Additionally, many studies have reported high antioxidant peptides from other species such as hoki, jumbo squid, Alaska pollack, and yellowfin sole with peptide sequences of G-S-T-V-P-G-A-T-H-P-A-C-P-A-P-A (Kim *et al.*, 2007), T-C-T-S-l-S (Sudhakar and Nazeer, 2015), L-P-H-S-G-T (Je *et al*., 2005), and R-P-D-F-D-L-E-P-P (Jun *et al*., 2004), respectively.

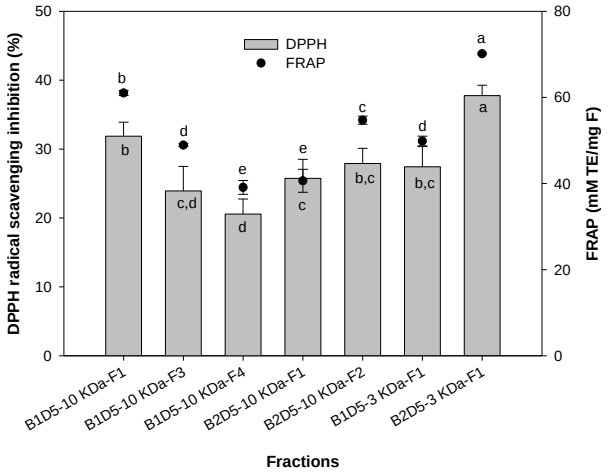

Figure 4. Fractions of DPPH radical scavenging activity and FRAP capacity of different fractions of peptide extract following separation by MWCO and purification by HPLC. Values are mean ± SD obtained from three replicates (n = 3). Lowercase letters in same column indicate significant difference ($p < 0.05$).

The present work demonstrated that PSPH peptides occurred in both *plaa-som* fermentation batches, and they had the potential to be bioactive compounds for human consumption. Based on these data, there is sufficient reason for product development of protein hydrolysates and bioactive peptides as they are both economically important and well established in the food industry and consumers.

Conclusion

The present work demonstrated spontaneous *plaa-som* fermentation added with Chiangrai Phulae pineapple (*plaa-som sap-pa-rod*) as a newly fermented fish product. During fermentation, bromelain co-reacted with the proteolytic enzyme from spontaneous microorganisms, especially PLAB that exhibited strong antioxidative activities. It was concluded that pineapples did not only increase the bioavailability of the product, but it also added value to agricultural products (pineapples). Pineapple can also be used as a supplementary raw material in the development of fermented fish products to provide a variety of flavours, textures, a new technique of food preservation, as well as to promote better quality of life of consumers. However, further research to identify PSPH peptides using mass spectrophotometry is still needed. In addition, sensory and nutritional assessments of these fish hydrolysate products must also be conducted in the future.

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