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# Correlation of antioxidant activity and protein alteration of silver BARB protein hydrolysates

Srisan Phupaboon[1,2], Weera Piyatheerawong[2] and Sirinda Yunchalard[2,*]

[1]Graduate School, Khon Kaen University, Khon Kaen, Thailand
[2]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand
[*]Corresponding author: sirinda@kku.ac.th

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## Abstract

The antioxidant effects of silver BARB (*Barbonymus gonionotus*) protein hydrolysates (SBPHs) were investigated under various trypsin and protease treatments. Biochemical characterization and antioxidant activities of 5% (w/v) SBPHs were examined from both fresh fish (FF) and saline-soaked fish (SF) proteins. The highest levels of trichloroacetic acid (TCA)-soluble peptide contents, 57.7 and 58.6 mg/g BARB hydrolysate (BH), were obtained from FF-trypsin and SF-trypsin, respectively, while the highest levels of free amino nitrogen (FAN) contents, 1.9 and 1.8 µmoL tyrosine/g BH, were respectively found after incubation for 150 min. Additionally, these treatments degraded large proteins into small molecular weights sizes of less than 10.5 kDa after 60 to 150 min of incubation. The highest percentage of 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging inhibition of FF-trypsin, 38%, was obtained after 120 min of incubation. Similarly, the highest ferric reducing antioxidant power (FRAP) capacities for both SF-trypsin and FF-trypsin were, respectively, 186.3 and 185.4 µmoL TE/g BH after 120 min of incubation. Furthermore, SF-trypsin exhibited the highest 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging inhibition, 91.0%, after 90 min of incubation. These results suggest that the by-products of SBPHs obtained from trypsin, especially natural antioxidant compounds, may be useful for consumer health if used in functional foods.

**Keywords:** Fish muscle protein, Protein hydrolysate extract, Bioactive compound, Functional food, Physicochemical characteristics

## 1. Introduction

At present, more consumers have turned to fish protein rather than meat since fish is readily available in the market and is produced in large quantities from aquaculture with lower costs than livestock. More importantly, fish have short breeding periods and therefore higher productivity. It is well established that fish is the richest source of protein because it contains a higher protein content than most animal sources of meat with verified satiating effects [1]. Furthermore, fish proteins are digestible and have outstanding essential amino acid profiles that are quite close to the human nutritional requirements set by the World Health Organization (WHO). However, there are many freshwater fish species found in Thailand, such as *Oreochromis niloticus* (plaa-nile), *Catla catla* (plaa-ka-ho), *Labeo rohita* (plaa-yi-sok), *Cirrhinus mrigala* (plaa-nuan-chan), *Cyprinus carpio* (plaa-nai), *Pangasianodon hypophthalmus* (plaa-sa-way), and *Barbonymus gonionotus* (plaa-ta-pien), that do not deliver their full nutritional potential upon direct human consumption. Proteolytic pre-treatments are required to obtain their full nutritional benefits [2].

Enzymatic hydrolysis with proteolytic enzymes is a process in which proteolytic enzymes facilitate the cleavage of peptide bonds in protein molecules in the presence of water [3,4]. The process has been commonly employed as a basic method to catalyze the digestion of fish protein into fish protein hydrolysates, a mixture of free amino acids and oligopeptides or polypeptides [4]. Production of fish protein hydrolysates (FPH) has been further investigated. Studies have shown that the characteristics of these hydrolysates are mostly dependent on the degree of protein hydrolysis (DH) resulting from reaction conditions and the specificity between substrate

and enzyme [5,6]. This process creates a number of peptides in which their nutritional and physicochemical properties as well as their biological activities are enhanced beyond that of their original native proteins [7].

Biologically active hydrolysates can be promoted as food additives providing a value-added type of product, a so-called functional food [8,10]. Particularly, bioactive peptides refer to short peptide sequences of approximately 2 to 30 amino acids that are enzymatically released from polypeptides or protein from any source [2,7-9]. These so-called bioactive peptides are specific peptide fragments that exhibit a constructive function in providing a beneficial influence upon health after consumption [8,9]. For example, some bioactive peptides can produce physiological effects as antihypertensive, immunomodulating, anti-thrombotic, antioxidative, anticancer and antimicrobial actions [7,10,11].

The silver BARB species was selected for use in the current study. This species is found in all freshwater resources throughout the Kingdom of Thailand. Accordingly, its consumption represents the concept of having plenty and prosperity, reflected in the well-known Thai phrase “Nai Narm Mee Plaa” “Nai Naa Mee Khao” (There are fish in the water and rice in the fields). Similarly, another well-known Thai phrase “Gin Khao Gin Plaa” (Eat rice and eat fish) also indicates the importance of fish consumption. The Department of Fisheries of Thailand has promoted the consumption of freshwater fish, including developing marketing plans to promote freshwater fish farming as one of the top ten industries considering cultivation of other fish species as well. Freshwater fish can be processed into Thai fermented foods such as *plaa-raa* and *plaa-som*. The current research work aimed to investigate the mechanism of BARB protein hydrolysis catalyzed by commercial proteolytic enzymes, trypsin and protease. Both enzymes provide antioxidant activities in terms of radical scavenging and reducing power. These activities were analyzed using the triple methods, DPPH radical-scavenging activity, FRAP capacity, and ABTS radical-scavenging activity.

## 2. Materials and methods

### *2.1 Chemicals*

The commercial enzymes used included trypsin (from porcine pancreas, 96.5 U/mg solid, CAS No.: 9002-07-7) and protease (from *A. saitoi*, ≥ 0.6 U/mg solid, CAS No.: 9025-49-4) obtained from Sigma Chemical Co. (St. Louis, MO). Methanol and acetic acid were purchased from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium persulfate, and ferric chloride hexahydrate were obtained from Sigma Chemical Co. (St. Louis, MO).

### *2.2 Fish samples*

Silver BARB (Barbonymus gonionotus) fish were purchased from local markets in Khon Kean, Thailand. The length and weight of all fish were between 40 and 50 cm and 400 to 500 g, respectively. The fish were brought to the laboratory under an iced condition at a fish:ice ratio of 1:1 (w/w) to retain the quality and integrity of the fish protein.

### *2.3 Preparation of silver BARB protein powder* (SBPP)

Preparations of SBPP were divided according different states to form two distinct groups. The first was fresh fish (FF) used in an upstream process of fermented *plaa-som* production, while the second consisted of fish soaked in a 20% (w/v) of brine for 2 h (SF). Next, all the fish were washed with water and dressed. The fish meat was separated and blended using a food blender (SG model, Toyo Seikan Kaisha Limited, Tokyo, Japan). The minced fish was transformed into fish protein powder using a freeze-drying technique. SBPP was used as a substrate for fish protein hydrolysis using commercial proteolytic enzymes.

### *2.4 Preparation of SBPH*

Five grams of each SBPP were added to a 0.1 M phosphate buffer (pH 7.0 ± 0.2) resulting in 100 mL mixtures that were each continuously stirred for 5-10 min. The enzymatic reaction was started by adding commercial proteolytic enzymes, trypsin (from porcine pancreas, 96.5 U/mg solid) or protease (from *A. saitoi*, ≥ 0.6 U/mg solid), at an enzyme to protein substrate ratio of 1% (w/w). Hydrolysis was carried out at 37 °C, and a sample was taken every 30 min for 3 h. The reaction was terminated by heating the mixture in a boiling water bath (Julabo TW20, Germany) for 15 min. The slurry was centrifuged at 10,000 g and 4 °C for 15 min to remove insoluble protein. The supernatants (SBPHs) were collected and lyophilized using a speed-vacuum technique and stored at 4 °C until use to evaluate protein alteration and measurement of antioxidant activity.

*2.5 Biochemical analysis of SBPH*

SBPHs (2.5 g) were homogenized with 50 mL of deionized water. The total protein content of these homogenates was determined as described by Lowry et al. [12] and expressed as mg/g BARB hydrolysate (BH) by comparing the results to a standard curve of BSA at 660 nm. The free amino nitrogen (FAN) content of supernatants was determined according to the method of Wylie and Johnson [13]. A standard curve of glycine was derived and expressed as mg/g BH. Trichloroacetic acid-soluble peptide (TCA-soluble peptides) contents were estimated by adding TCA at a concentration of 10% (w/v) and supernatants in ratio 1:1, storing the mixture at 4 °C for 24 h, and subsequently centrifuging it at 10,000 g and 4 °C for 10 min. The TCA-soluble peptides were quantified using the Lowry method, measuring absorbance at 660 nm. The quantity of the peptides was expressed as µM tyrosine/g BH by comparing measurements to a standard curve using tyrosine. All analyses were carried out in triplicate.

*2.6. Gel electrophoretic analysis of protein alteration*

The supernatants of SBPH at various times were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) employing a 4% stacking gel and a 15% separating gel, according to the method of Laemmli [14]. The hydrolysate supernatants, containing 4 mg/mL protein, were mixed with a sample buffer and placed in a boiling water bath for 4 min. Then, approximate 15 µL of samples were loaded onto the gel and subjected to electrophoresis at a constant current, 15 mA per gel, using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the SDS-polyacrylamide gel was stained with 0.1% (w/v) Coomassie® brilliant blue R250 and de-stained in a 10:40% mixture (v/v) of acetic acid and methanol in water at a moderate shaker speed (Guangzhou Cancare Medical, China) overnight. The band area was quantified by scanning gels with a gel documentation system (Bio-Rad, USA).

*2.7 Determination of antioxidant activity*

*2.7.1 DPPH radical-scavenging activity*

DPPH radical-scavenging activity was determined via a DPPH assay, as described by Binsan et al. [15] with slight modification. A 1.5 mL sample of FPH was added to 1.5 mL of 0.15 mm 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. The mixture was vigorously mixed and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The activity was expressed as percent radical-scavenging inhibition.

*2.7.2 FRAP capacity*

FRAP was measured according to the method of Benzie and Strain [16]. A 0.15 mL aliquot of a FPH sample was added to 2.85 mL of a FRAP solution and kept for 30 min in the dark. The product absorbance at 593 nm was recorded. A standard curve was prepared using Trolox in concentrations ranging from 50 to 1000 µM. The activity was calculated as µmoL TE/g BH.

*2.7.3 ABTS radical-scavenging activity*

ABTS radical-scavenging activity was determined using an ABTS assay according to the method of Arnao et al. [17] with slight modification. A 0.15 mL aliquot of a FPH sample was added to 2.85 mL of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then recorded at 734 nm using a spectrophotometer. The ABTS radical-scavenging activity was expressed as percent radical-scavenging inhibition.

## 3. Results and discussion

*3.1 Biochemical results*

Figure 1 presents the total soluble protein (TSP) content of SBPH from hydrolysis with trypsin and protease. It can be seen that the TSP content in both FF and SF decreased with incubation time. The initial TSP contents were found to be in the range of 13.0 to 13.7 mg/g BH after 30 min. The proteolysis of all SBPP samples in this

study occurred under hydrolysis at 37 °C and the protein content was gradually decreased to nearly 10 mg/g BH after 150 min of incubation. Generally, there were two components of fish muscle protein, myofibrillar and sarcoplasmic proteins [18,19]. Fish muscle can be dissolved in water to produce a crude protein containing these components. Additionally, the reduction of protein content in the fish samples was due to enzymatic reactions resulting from microbial activity in the fermentation process and also the acidic conditions during fermentation [20,21].

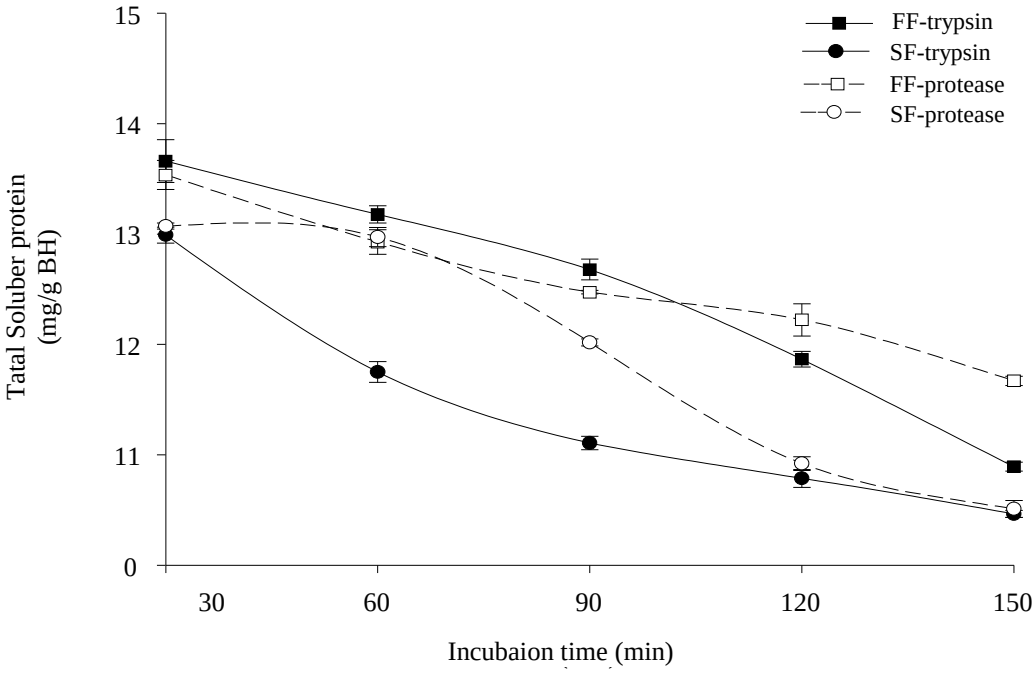


**Figure 1** TSP content of SBPH after hydrolysis with trypsin and protease after various incubation times.

Furthermore, there was a correlation between TCA-soluble peptides and the FAN contents of soluble SBPH. As depicted in Figure 2, both TCA-soluble peptide and FAN contents in all SPPH samples increased over the incubation period. The TCA-soluble peptide content was established as the fraction with the highest peptide content in the treatment of FF-trypsin and FS-trypsin, 57.7 and 58.6 µmoL tyrosine/g BH after incubation at 37 ºC for 150 min. The FAN content was found to be highest (1.9 mg/g BH) in the FF-trypsin treatment after 150 min of incubation. This study clearly shows that the fish proteins are degraded by proteolytic enzymes. Alternatively, the resulting by-products obtained from enzymatic actions throughout the incubation period include free amino acids and soluble peptides. Similar results have been reported by researchers [18,22] who found that fermented pork (nham) and fermented fish (plaa-som) products demonstrated an increase in the amount of non-protein nitrogen (NPN) compounds due to degradation of meat or fish muscles. Moreover, previous research studies reported similar findings in other fermented fish species such as *Yu-lu* [23] and *Suan-yu* [20], in which there were increased amounts of TCA-soluble peptides as well. Increasing the peptide and free amino acid contents leads to development of the characteristic flavors and aromas of fermented fish products [24].

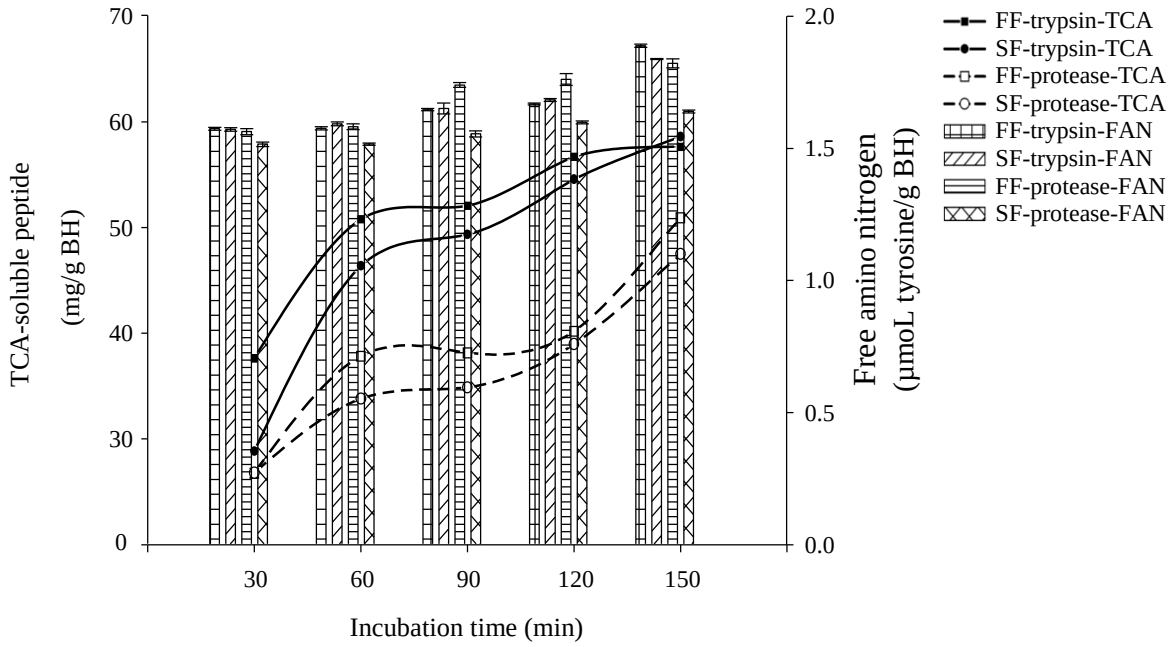


**Figure 2** TCA-soluble peptides and FAN contents of SBPH after hydrolysis with trypsin and protease after various incubation times.

*3.2 Electrophoretic pattern of proteins*

The patterns of protein were obviously different in FF and FS. There are several components of protein demonstrated in Figure 3 (A) and (B). Five major types of proteins can be found in both FF and FS protein powder, consisting of a myosin light chain 3 (MLC 3), myosin light chain 2 (MLC 2), tropomyosin, actin, and myosin heavy chain (MHC) with masses of approximately 16, 19, 35, 42, and 210 kDa, respectively. In this study, all of the proteins were completely hydrolyzed with protease and trypsin after 150 min of incubation. This result suggests that MHC is potentially one of the most susceptible proteins for decomposition into free amino acids or short peptides. A similar result was observed during the *plaa-som* fermentation of silver BARB fish after 120 h of fermentation, and a simultaneous increase in small peptides (<1.3 kDa and 18-21 kDa) was detected in their fermented products [22,25].

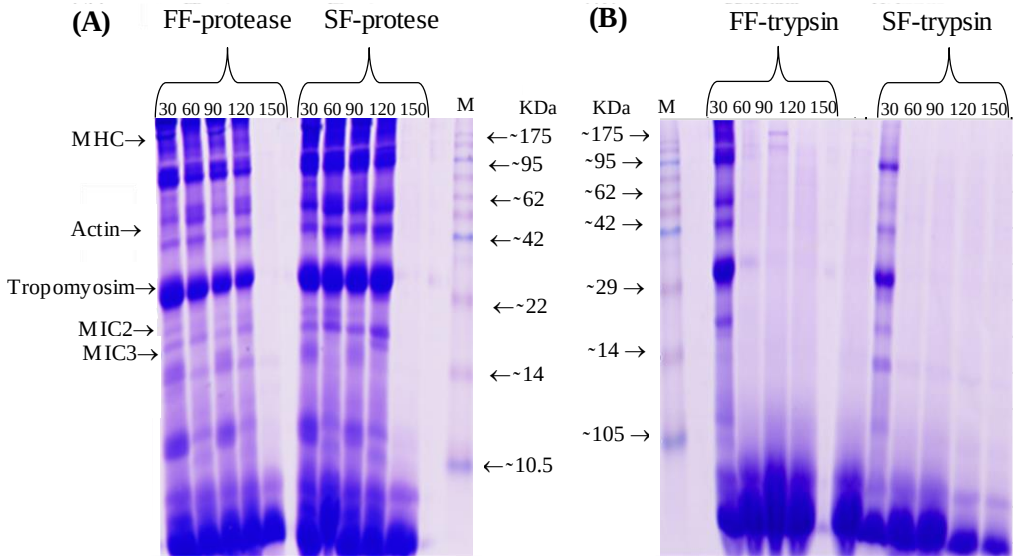


**Figure 3** SDS-PAGE patterns of both protein samples during hydrolysis catalyzed by protease (A) and trypsin (B) after various incubation times, within MHC = myosin heavy chain, MLC 2 = myosin light chain 2, MLC 3 = myosin light chain 3. M = chromatein prestained protein ladder.

*3.3 Antioxidant properties of SBPHs*

*3.3.1 DPPH radical scavenging activity*

The ability of SBPH to inhibit the oxidation of DPPH radical scavenging activity is shown in Figure 4. The treatment of FF-trypsin exhibited a significantly higher radical scavenging activity, 38%, with 120 min of incubation that decreased to 34% after 150 min of incubation. The results of these tests suggest that the peptides present in hydrolysates could donate protons or electrons that interact with free radicals and reduce them to stable forms [26]. Furthermore, previous work [27] examined the hydrolysis of freshwater fish protein hydrolysates of *Catla catla*, *Lebeo rohita*, and *Cirrhinus mrigala* using flavorzyme. They found that the highest radical-scavenging activity was in the range from 50-82%, observed after 1 h incubation. This finding is in agreement with Slizyte et al. [9], who reported that salmon backbone hydrolysates showed a 50% radical-scavenging activity after 20 min of hydrolysis in a mixture of bromelain, papain, and trypsin, which is a more efficient catalyst for degrading proteins.

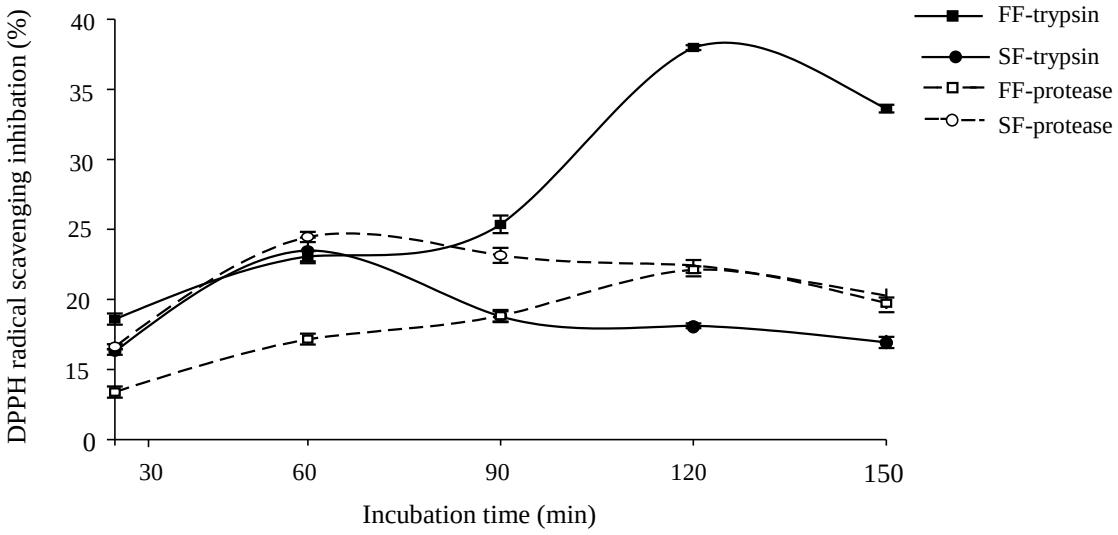


**Figure 4** DPPH radical-scavenging inhibition of SBPH after hydrolysis using protease and trypsin after various reaction times.

*3.3.2 FRAP capacity*

In this assay, the capability of hydrolysates to reduce $Fe^{3+}$ to $Fe^{2+}$ was determined. This assay is usually used to assess the ability of an antioxidant molecule to donate an electron or hydrogen [26], affecting FS and FF proteins treated with trypsin (Figure 5). The results in this research showed a slight increase in the reducing power from 171.2 to 186.3 and 148.3 to 185.4 µmoL Trolox equivalents (TE)/g BH at the end of incubation times for FS and FF, respectively. Treatment of both FS-trypsin and FF-trypsin exhibited the highest reducing power, 197.0 and 196.3 µmoL TE/g PH, after 120 min incubation, respectively. Numerous studies have found that there is a correlation between antioxidant scavenging activities and reducing power of certain bioactive molecules [2,8,10]. Similarly, previous studies [27,28] also reported that the highest hydrolysis degree for fish protein hydrolysates of *Catla catla* and *Cyprinus carpio* were achieved after 120 min. At this stage, enzymatic activities were increased by the bioactive compounds, resulting in greater reducing power.

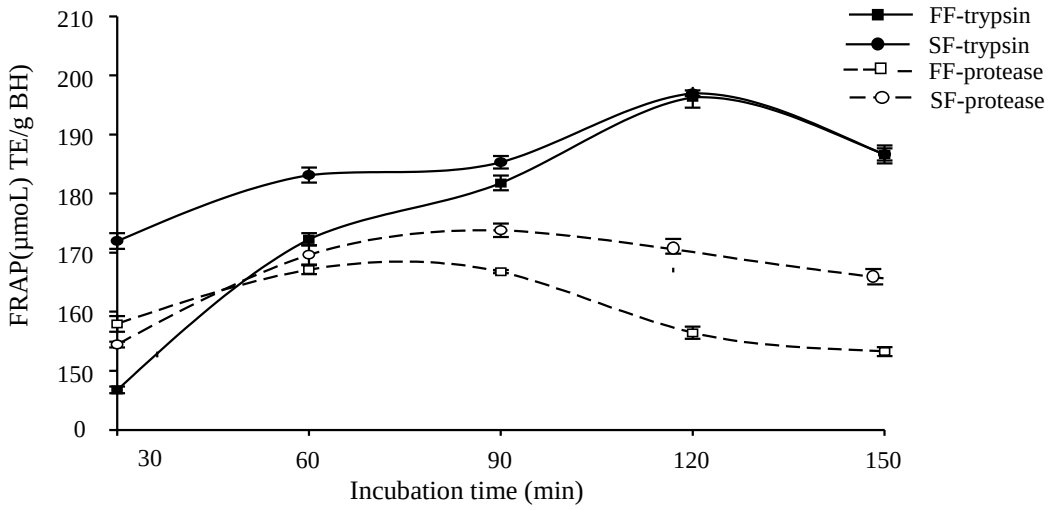


**Figure 5** FRAP of SBPH after hydrolysis using protease and trypsin after various reaction times.

*3.3.3 ABTS radical scavenging activity*

The reactions of the ABTS method can provide estimates of both hydrophilic and lipophilic antioxidants in the sample [26]. An extensive increase in ABTS radical scavenging inhibition that gradually declined in all experiment hydrolysates (both FF and FS treated with trypsin and protease) is shown in Figure 6. Consequently, the effect of ABTS radical-scavenging inhibition was observed in SBPHs obtained from FF and FS treated with trypsin and/or protease after 30 min and once more after 150 min of incubation. Specifically, the ABTS radical scavenging inhibition increased to 91% in the treatment of FS-trypsin, which was comparable to those of FF-trypsin, FF-protease, and FS-protease treatments, which ranged from 86.4-88.6% after 90 min of incubation. A number of studies have found that protein hydrolysates from the muscle of ornate threadfin bream (*Nemipterus hexodon*) and loach (*Misgurnus anguillicaudatus*) proteins treated with pepsin, papain, and protamex also showed strong ABTS and DPPH radical-scavenging activities [29,30].

Furthermore, different ABTS and DPPH profiles could be clearly observed. Generally, various bioactive compounds have different behaviors in reaction media that affect their activities. As a result, the different profiles are generated by two major factors, solution pH and the characteristics of the specific absorption spectrum. In the current study, DPPH was prepared in an alcoholic solution and it is sensitive to acidic pH values. Potassium persulfate was used to prepare an ABTS aqueous solution and it can be stabilized over a wide range of pH values [31]. Additionally, ABTS radicals form a blue-green colored solution that can be measured at longer wavelengths (734 nm), whereas the DPPH radical is stable with an absorption band at 515 nm [32].

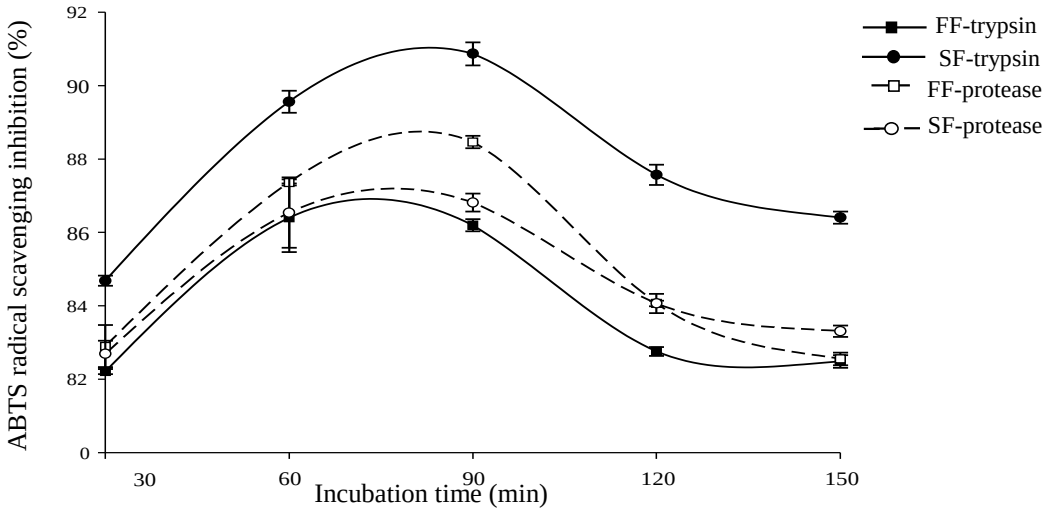


**Figure 6** ABTS radical-scavenging inhibition of SBPH after hydrolysis using protease and trypsin after various reaction times.

## 4. Conclusion

Trypsin was used to treat SBPH and exhibited the strongest radical scavenging activity as a percentage of inhibition and reduction of power capacity. Therefore, their hydrolysates could be used as natural antioxidants to prevent the oxidative reactions that occur during fish fermentation. Additionally, it also enhances the value of fish-based functional foods. However, the sequencing of peptides and their correlation to antioxidant activity should be verified in further research work. Consequently, these verified results will be compared to antioxidant activities of natural enzymes during a spontaneous *plaa-som* fermentation, which is catalyzed by co-enzymes from lactic acid bacteria and natural plant enzymes such as bromelain (from pineapple) or papain (from papaya).

## 5. Acknowledgements

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