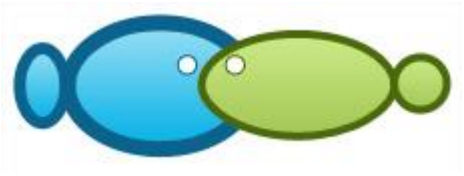

Bioactive peptides from hydrolysates of Indonesian catfish protein isolate: characterization and properties of its antioxidant capacity

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Abstract. Fish protein isolate is an essential food ingredient with a high protein content (at least 90% db). This study aimed to evaluate the properties and the antioxidant capacities of protein hydrolysates from catfish protein isolates (CPI) produced by papain and bromelain enzymes by mixing the CPI with a 0.2 M phosphate buffer in a ratio of 1:100, conditioned with enzyme/substrate concentrations of 1/100 (pH 6 and 37°C for the papain enzyme, pH 7 and 5°C for the bromelain enzyme). The protein hydrolysis was conducted for 0, 30, 60, and 90 min and analyzed for the degree of hydrolysis, peptide chain length and antioxidant capacities through DPPH radical scavenging and ferric reducing power methods. This study showed that the hydrolysis time difference significantly affected the hydrolysis and antioxidant capacity by the papain and bromelain enzymes, except for the bromelain reducing power. The hydrolysis of the CPI for 30 min by papain enzyme resulted in protein hydrolysates with the hydrolysis degree, DPPH radical scavenging and ferric reducing power of 37.77%±2.99, and 59.578%±0.05 (15.83±1.65 mgL^{-1} AEAC), and 0.80±0.02, respectively. While the hydrolysates of CPI by the bromelain enzyme resulted in the characteristics of the hydrolysis degree, DPPH radical scavenging and ferric reduction power of 48.82%±0.90, and 49.52%±6.01 (13.07±0.01 mgL^{-1} AEAC) and 0.44±0.06, respectively. This research can be used as necessary information to develop catfish protein hydrolysates, especially for future functional foods.

Key Words: antioxidants, catfish, hydrolysate, protein isolates, peptide.

Introduction. Catfish is one of the freshwater fish species widely cultivated in Indonesia. The demand for catfish with small to medium size (8-10 pcs kg^{-1}) is much higher than for those of larger size. Oversize catfish is relatively cheap and its preparation process is technically easy. In terms of nutritional composition, catfish contains considerable amount of protein, about 76.6% db, suggesting that the fish is a potential source for protein isolate (Pyz-Łukasik & Paszkiewicz 2018). Fish protein isolate is an essential food ingredient with high protein content (at least 90% db).

Despite having low biological activities in the original structure of protein, additional processes such as enzymatic hydrolysis provide a considerable result in producing bioactive compounds, enabling to convert the protein into functional ingredients. Previous in vitro studies reported biological activities of compounds derived from fish, such as antibacterial activity, inhibitor of angiotensin I-converting enzyme (ACE) and antioxidant (Yusuf et al 2020; Najafian & Babji 2015; Lassoued et al 2015).

Fish protein is developed to produce antioxidant bioactive peptides. Some of the research results related to the fish protein hydrolysate as an antioxidant have been reported in salmon (Neves et al 2016), sardines (Lassoued et al 2015), skipjack tuna (Nalinanon et al 2011) and catfish (Baehaki et al 2015). Antioxidants have an essential role in health as agents preventing the body from overproducing reactive oxygen species (ROS). Antioxidants can also inhibit extensive oxidation reactions by binding to free radicals and highly reactive molecules so that they can inhibit cell damage. The

exploration of natural antioxidant sources is increasing, including the hydrolysates or peptides from catfish.

Enzyme selection becomes a pivotal stage in protein hydrolysis as it considerably affects the peptides' amount and bioactivity. Formerly, papain was applied to produce protein hydrolysate from catfish, focusing on amino acid characterization of the resulting catfish hydrolysate (Salamah et al 2012). The research of Nurhayati & Sanapi (2014) focused on the functional properties of catfish protein hydrolysate including solubility, capacity and stability emulsion, and the foam stability. Nevertheless, these studies did not cover the discussion on the antioxidant properties of the protein isolate. In this study, we used papain and bromelain enzymes for the hydrolysis process, which both are from a group of endopeptidase enzymes with the capability to hydrolyze fish protein (Halim et al 2016).

The present study aimed to evaluate the characteristics of catfish protein hydrolysate and its antioxidant capacity. Our experiment evaluated some key indicators such as the hydrolytic activity, peptide chain length and antioxidant activities using DPPH assay and ferric reducing power assay; they are essential in utilizing catfish as a natural source of antioxidants. Scientific evidence is necessary for further studies on the catfish protein hydrolysate, especially for future functional foods.

Material and Method

Materials. Catfish (*Clarias* sp.) of a weight ranging between 457 and 807 g individual^{-1} were collected from a fish farmer in Taman Pagelaran, Bogor, Indonesia, and transported to the laboratory for preparation. Papain (Himedia Laboratories, India) and bromelain (Serva, Germany) were used for hydrolysis. The chemicals for antioxidant activity included DPPH (2,2-diphenyl-l-picrylhydrazyl) (Sigma Aldrich), potassium ferric cyanide (Cica, Japan), FeCl3 (Merck, Germany) and 95% ethanol (PA Merck, Germany).

Processing of catfish protein isolate (CPI). The production of fish protein isolates refers to the method of Yarnpakdee et al (2014), with slight modifications. The white minced catfish meat was added with cold distilled water (2-4°C) at ratio of 1:5. The mixture was crushed in a blender, homogenized, and centrifuged at a speed of 11,000 rpm for 1 min. The addition of 2M of NaOH (under constant stirring) was performed to adjust pH to 11. All stages were performed in cold conditions. The mixture was then centrifuged at 5.000 g for 10 min, at 4-10°C, to separate the supernatant from the residue. 2M of HCl were gradually added and stirred to adjust the pH to 5.5. The solution was subjected to centrifugation at 10.000 g for 20 min at 4-10°C. The filtrate was freeze-dried to yield CPI powder. The powder was vacuum-packed with aluminum foil and stored in freezer at -20°C, prior to hydrolysis.

Processing of catfish protein hydrolysate. The process of fish protein hydrolysate refers to Yarnpakdee et al (2014), by modifying the type of enzymes (papain and bromelain) and the hydrolysis duration (0, 30, 60, 90 min). Briefly, the CPI was added with 0.2M phosphate buffer (pH 6 for papain, pH 7 for bromelain) to reach a final protein concentration of 1% (w/v). The prepared enzymes were added to the CPI (1:100, w/w) at the following conditions: papain at pH 6 and 37°C and bromelain at pH 7 and 50°C. The hydrolysis was performed for 0, 30, 60, 90 min, and finally inactivated at 98°C for 10 min. The resulting liquid protein hydrolysate was centrifuged at 3.800 g in a centrifuge (Hermle Z 383 K, Wehingen, Germany) for 20 min, at 4°C. The supernatant was transferred to 2mL Eppendorf and stored at -20°C for further analysis.

Measurement of the degree of hydrolysis (DH) and peptide chain length (PCL). Measurement of the degree of hydrolysis (DH) followed the method of Hoyle & Merrit (1979) with slight modifications. 2 mL of catfish protein hydrolysate (0.1 mg mL^{-1}) were added with 2 mL of Trichloroacetic acid (TCA) at 20% (w/v) to produce soluble nitrogen in a TCA fraction of 10%. The mixture was homogenized and allowed to stand for 30 min for precipitation, then centrifuged at 7.800 g (Eppendorf 5810R) for 15 min at 4°C. Nitrogen

levels of the samples and supernatants were determined using the Kjeldahl method of the Association of official analytical chemists (AOAC) (2002). The degree of analysis was determined as follows:

$$(\%)\ DH = [(10\ \%TCA - soluble\ nitrogen\ in\ sample/total\ nitrogen\ in\ sample] \times 100$$

Meanwhile, the Peptide Chain Length (PCL) value was determined according to Adler-Nissen & Olsen (1979):

$$PCL = \frac{(100\%)}{DH}$$

Measurement of antioxidant capacity. The antioxidant capacity measurement followed the DPPH and ferric reducing power assays.

Analysis of the antioxidant capacity of 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity. DPPH assay followed method of Najafian & Babji (2015) with a slight modification of sample types. The concentration of DPPH (Sigma-Aldrich, USA) solution used was 0.15 mM. A total of 0.5 mL fish protein hydrolysate of 0.1 mg mL^{-1} protein concentration and 0.15 mM of DPPH were added to 1.5 mL ethanol at 95% and transferred into a test tube. The mixture was vortexed, then stored in a dark condition at room temperature, for 30 min, before the absorbance detection at 517 nm, using a UV-Vis spectrophotometer (Thermo Scientific 150, USA). The blanks as control were prepared in the same way, with a phosphate buffer used to replace the sample. The ascorbic acid concentration was used as the standard at 0, 2.5, 5, 10, 20 ppm. The radical scavenging capacity (%) was calculated as follows:

$$(\%)Inhibition=[(A-B)/A]x100$$

Where:
A-control absorbance;
B-sample absorbance.

Analysis of ferric reducing power. Catfish protein hydrolysate assays of iron reduction were carried out according to the protocol of Oyaizu (1998). A sample (1 mL) with a protein concentration of 0.1 mg mL^{-1} was added to 1 mL at a concentration of 0.2M of phosphate buffer (pH 6.6) and 1 mL of potassium ferric cyanide ($K_3(FeCN)_6$) at 1%. The mixture was incubated at 50°C for 20 min, then added with 1 mL of TCA at 10%. An aliquot (1 mL) of 10% trichloroacetic acid was added to the mixture, followed by centrifugation at 1.761 g for 10 min. The upper layer of solution (1 mL) was mixed with 1 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance was read at 700 nm by a UV-Vis spectrophotometer (Thermo Scientific 150, USA). The higher the absorbance, the stronger the ferric reduction.

Data analysis. The treatment used for determining the antioxidant activity of each enzyme (papain and bromelain) referred to the difference in the hydrolysis time (0, 30, 60, 90 min). The test results were statistically evaluated using the analysis of variance (ANOVA) with the IBM SPSS version 22 software (Chicago, IL, USA). The significance of the differences between means was determined using the Duncan test, at a 5% level.

Results and Discussions. The experiment tested two protease enzymes, i.e. papain and bromelain. Before the hydrolysis, each enzyme was tested for its activity to estimate the actual proteolytic activity, which was of 2.25 IU for papain and 1.20 IU for bromelain The use of enzymes during the hydrolysis process was equal, to obtain the same enzyme activity. The determination of the hydrolysis time is based on the antioxidant capacity of the crude hydrolysate produced. The hydrolysis process character is explained based on the degree of hydrolysis and the length of the peptide chain (PCL) produced.

Hydrolysis degree. The hydrolysis degree is the number of peptide bonds resulting from hydrolysis, compared to the original protein. The factors affecting the hydrolysis level include the enzyme/substrate ratio, pH, temperature and hydrolysis time Villamil et al (2017). Besides, the type of enzymes and their activity are also an essential factor. The maximum enzyme activity is influenced by the optimum temperature and pH.

The measurement of the hydrolysis degree indicates the hydrolysis reaction's effectivity while also showing the amount of peptide bond cleavages. The hydrolysis degree (%) is the most significant indicator to evaluate the protein breakdown rate. In this regard, the TCA method is the most commonly used method in determining the estimated value of the degree of hydrolysis (%) of a protein. The calculation is based on a ratio of dissolved nitrogen in the hydrolysate (in 10% TCA) compared to the nitrogen in the sample. The value of the degree of hydrolysis by papain and bromelain enzymes is shown in Figure 1.

Figure 1 showed that the hydrolysis time exerted a significantly different effect ($p<0.05$) on the concentration of dissolved nitrogen in percent by papain and bromelain enzymes. The hydrolysis at 90 min showed the highest hydrolysis degree, namely 69.65%±2.28 for papain and 53.90%±5.19 for bromelain, while the lowest one was attributed to the hydrolysis at 30 min, namely 37.77%±2.99 and 48.82%±0.90, respectively. Nurhayati & Sanapi reported an optimum hydrolysis degree of 47.24% and Borges-Contreras et al (2019) reported a hydrolysis degree of 67% of the tilapia protein. Several other researchers reported lower hydrolysis values including sea slug protein hydrolysate by 27% (Ulagesan et al 2018), tilapia protein hydrolysate by 23.40% (Foh et al 2010). This study was not much different from the results of the study Tan et al (2019).

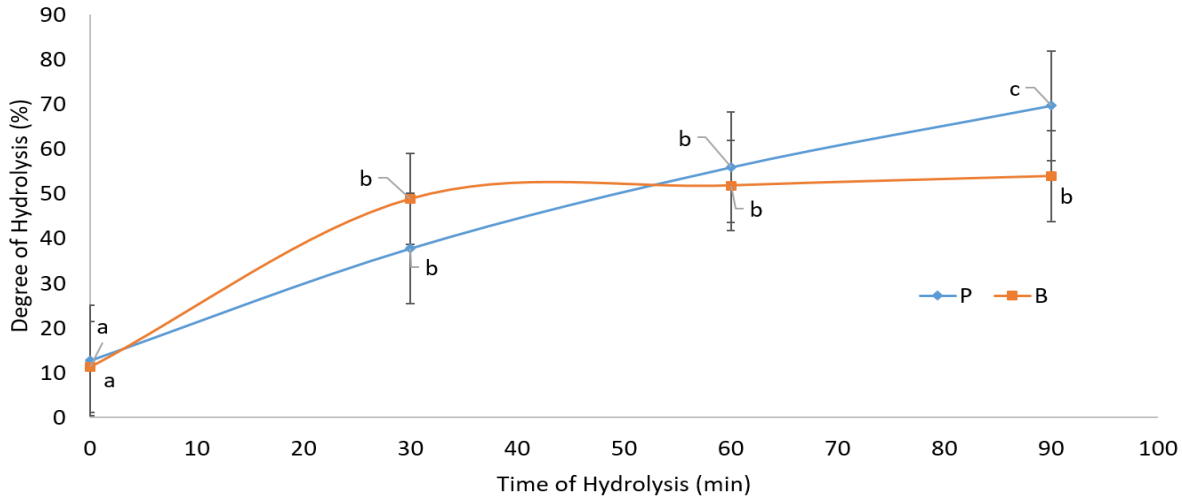

Figure 1. Hydrolysis degree of catfish hydrolysate by papain (P) and bromelain (B). Different letters in each hydrolysis time shows a significant difference ($p<0.05$); n=2.

The hydrolysis time can dictate the hydrolysis degree: by papain, it increases with the time of hydrolysis; by bromelain, it reaches a peak at 30 min of hydrolysis. Furthermore, the hydrolysis rate increases until it reaches a constant phase. The results of the current work were similar to previous reports on protein by-products of catfish (Tan et al 2019) and squid (Hamzeh et al 2016).

Several studies reported slightly different results, regarding the degree of hydrolysis. A low hydrolytic activity by papain, i.e. 22.2%, was recorded in the mackerel skin (Abdulazeez & Ramamoorthy 2013). In the protein hydrolysis of meriga fish eggs, the optimum degree of hydrolysis by papain was reported at 90°C, reaching up to 17.10% (Chalamaiah et al 2010). Hsu et al (2011) stated that the protease enzymes XXIII and papain produced hydrolysis degrees of 30.2 and 20.4%, respectively, in the hydrolysis of the red tuna meat. The hydrolysis time at 0 min is also studied in this work,

enabling to evaluate the enzymatic activity despite absence of commercial proteolytic enzymes. The hydrolytic activity by papain enzyme is more effective in the hydrolysis of the CPI. Noticeably, proteolytic enzymes also naturally occur in the fish meat and most of them are still present in the isolated product. Another study explained a positive correlation between the hydrolysis time and the degree of hydrolysis (Baehaki et al 2015). The fish protein enzymatic hydrolysate contains a mixture of free amino acids, dipeptides, tripeptides and oligopeptides, which raise the number of polar compounds and solubility. The enzymatic treatment of the hydrolysate can substantially improve its functionality and bioactivity compared to the untreated protein.

Peptide Chain Length (PCL). The higher the hydrolysis degree, the lower the height of the peptide chain. The PCL for a hydrolysis time of 30 min is 2.68±0.18 for the papain and 2.06±0.01 for the bromelain. Figure 2 shows PCL of hydrolyzed catfish protein by papain and bromelain. Regarding the PCL, both enzymes show a similar pattern at each hydrolysis time point.

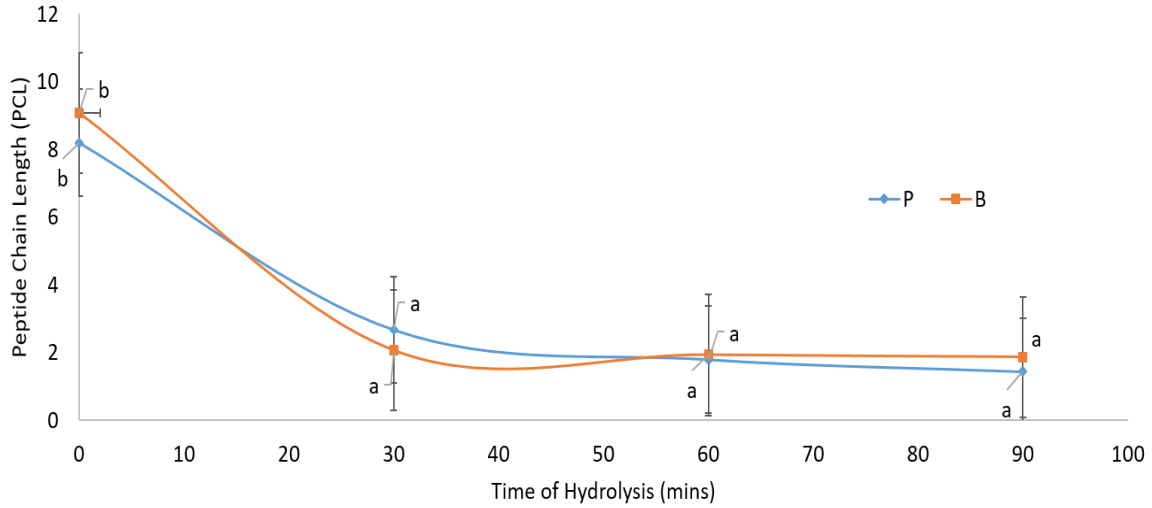

Figure 2. Catfish hydrolysate peptide chain length by papain (P) and bromelain (B). Different letters in each hydrolysis time show significant difference at $p<0.05$; n=2.

A longer hydrolysis time resulted in a shorter PCL value. Other studies reported that the peptide chain lengths of engraved catfish (*Nemapteryx caelata*) for 2 hours hydrolysate were 2.52 and 1.86 (Binsi et al 2015). In this study, the PCL for a hydrolysis of 90 min is 1.44 for the papain and 1.87 for the bromelain. In the hydrolysis of fish protein, the CPI peptide bonds will mostly be hydrolyzed rapidly, in the initial phase of hydrolysis. The enzymatic hydrolysis will decrease with the hydrolysis time until it reaches the stationary phase (with no real hydrolysis activity). The peptide size length affects the hydrolyzed protein characteristics, functional properties, as well as absorption rate, including the nutritional value of a protein. Peptides in the form of di- and tripeptides are more rapidly absorbed in the jejunum than free amino acids (Hinsberger & Sandhu 2004).

Catfish fish protein hydrolysates antioxidant capacity-DPPH radical scavenging capacity. The capacity of the DPPH radical scavenger for catfish protein hydrolysate is based on the resulting inhibition value. The higher the inhibition value, the stronger the antioxidant capacity. The average capacity of inhibition of the catfish protein peptides is shown in Figure 3.

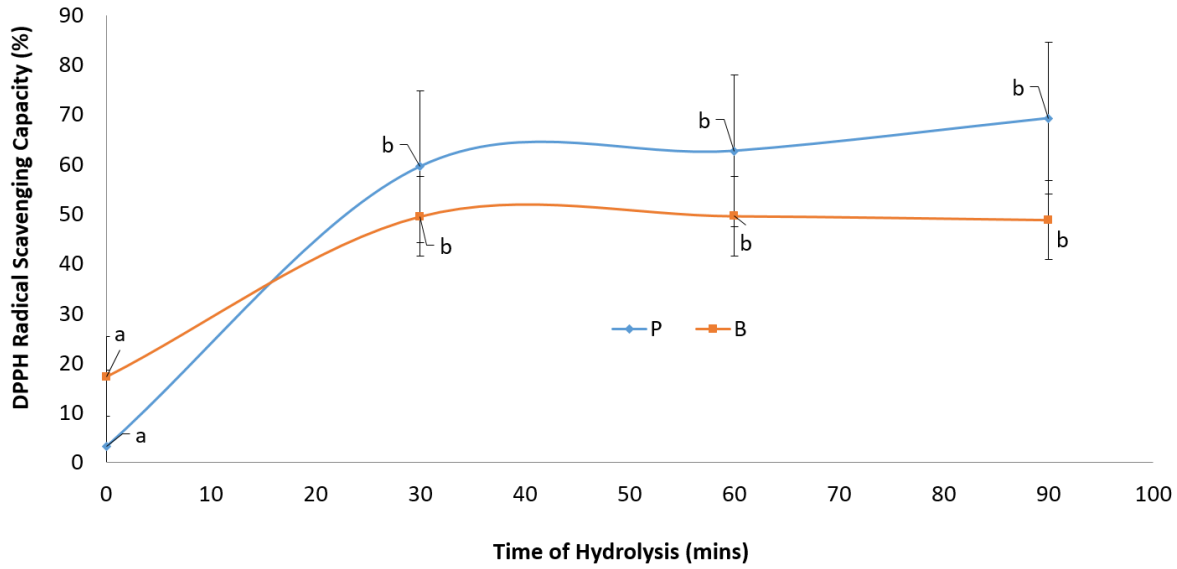

Figure 3. DPPH radical scavenging capacity of catfish peptides by papain (P), bromelain (B). Different letters in each hydrolysis time show significant difference at $p<0.05$; n=2.

Figure 3 showed that protein hydrolysis increases the DPPH radicals scavenging capacity. A hydrolysis time of 30 min significantly increased the antioxidant capacity, but a hydrolysis from 30 to 90 min did not show a significant difference. The hydrolysis time essentially influenced DPPH radical scavenging capacity. Hydrolysis by papain increased with the hydrolysis time. With bromelain, the highest capacity was obtained for a hydrolysis time of 30 min as for a hydrolysis time of 30 to 90 min (no significant difference). The hydrolysis time and activity of certain enzymes produce peptides with the highest DPPH radical scavenging activity. The peptides produced have amino acid residues that can donate hydrogen, captured by DPPH radicals. The yield of DPPH radical scavenging capacity of catfish peptides hydrolysis by papain is higher than that of catfish peptides hydrolysate using the bromelain. This difference indicates that the stem bromelain enzyme ability to break peptide bonds during the hydrolysis process is lower than that of the papain enzyme. The difference in the hydrolysate character produced is related to the ability of the papain enzyme to work during the hydrolysis process. The enzyme papain has a sulfhydryl group (-SH) as an active site, which is found in the amino acids cysteine 25 and histidine 159 and is a major part of the catalytic process. This amino acid has the capacity as an antioxidant. Papain also cleaves proteins at the carboxyl side of the amino acids valine, lysine, and arginine by the active site of papain (Suhartono 1989). While the bromelain enzyme has an active site only found in the amino acid cysteine. Bromelain enzymes hydrolyze proteins on the carbonyl side of the amino acids lysine, alanine, tyrosine, and glycine (Sawano et al 2008). These conditions can affect the bioactive activity of the hydrolysate during the hydrolysis process.

Catfish peptides hydrolyzed with papain and bromelain enzymes had the highest DPPH radical scavenging capacity of 69.23%±8.08 (18.49±2.22 mg L^{-1} AEAC) and 59.58%±0.05 (15.83±1.65 mg L^{-1} AEAC) with a hydrolysis time of 90 min and 30 min, respectively. In contrast, the lowest capacity was 49.52%±2.43 (13.07±0.01 mg L^{-1} AEAC) and 48.81%±6.01 (12.71±0.67 mg L^{-1} AEAC), respectively, at a hydrolysis time of 30 min and 90 min. The difference in the DPPH radical scavenging capacity relates to the difference in the optimum pH required for each enzymatic activity, ultimately affecting the produced peptide. In this study, papain worked at pH of 7, while bromelain was best at pH of 6. The value of antioxidant capacity is higher in acidic conditions and lower in neutral and alkaline conditions (Giuliana et al 2015).

Catfish protein hydrolysate with the papain enzyme showed a highly increased antioxidant activity (Najafian & Babji 2015). It was also reported that papain is a protease which shows a specific substrate preference for the antioxidant activity on myofibril protein hydrolysate. In addition to the pH, the factors affecting the value of

antioxidant inhibition by the DPPH test included the type of samples and the enzyme specificity (Borawska et al 2015). The effect of antioxidants on the DPPH radicals is caused by their ability to donate hydrogen. Antioxidant compounds donate hydrogen when dealing with DPPH radicals, then these radicals are captured, which is indicated by the color changes from purple to yellow and by the absorbance decreases.

Reducing power. The ferric ions reducing power is used to measure the capacity of antioxidants, by determining the compound's ability to reduce ferric (III) iron to ferrous (II) iron. The reducing power of peptides showed their ability as electron donors. The higher the absorbance value, the stronger the antioxidant capacity. The results of reducing capacity of the catfish peptide are presented in Figure 4.

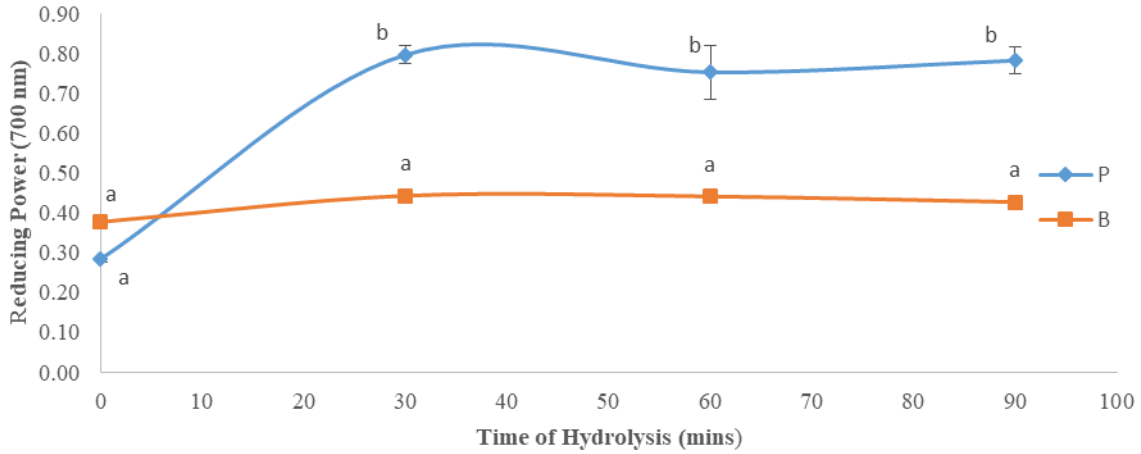

Figure 4. Reducing power capacity of catfish peptides by papain (P), bromelain (B). Different letters in each hydrolysis time show significant difference at ($p<0.05$); n=2.

Figure 4 showed that the catfish peptides hydrolyzed by papain exhibited a high reducing power, while the hydrolysis with bromelain showed a lower capacity as reducing power. The hydrolysis time of 30 min resulted in the optimum ability of the enzyme in reducing ferric iron, with an absorbance of 0.80±0.02 and 0.44±0.06, respectively.

A longer hydrolysis time showed a decrease in the reducing power. A decreased or increased ferric reducing capacity of a fish protein hydrolysate closely related to the exposure to the amino acid-dense side of the electron chain group, such as charged groups or poles during the hydrolysis process (Pezeshk et al 2018). The capacity to reduce peptide catfish by the papain enzyme hydrolysis was higher than by the bromelain enzyme peptide hydrolysis. Catfish hydrolysate treated with papain showed its ability as an electron donor. Previous work reported on the reducing ability of catfish collagen hydrolysate using papain (Baehaki et al 2015). Furthermore, a study by Sarmadi & Ismail (2010) indicated that several aromatic amino acids (Phe, Tyr, His and Trp) could convert radicals into stable molecules by donating electrons.

Several researchers have reported on the reduction power of catfish waste by papain enzyme treatment. The reduction power of the catfish sarcoplasmic hydrolysate was 0.29 (Najafian & Babji 2014) and the catfish myofibril hydrolysate reducing power was 0.32 (Najafian & Babji 2015). Some factors that influence a peptide reducing power include peptide hydrophilic properties determined by the sulfuric acid and the hydrophilic residues content in the N- or C-terminal and peptide sequence.

Based on our observations in this study, the catfish protein isolate hydrolysate has a natural antioxidant capacity. Besides, the hydrolysis process using the papain enzyme is practical. Future research needs to be carried out by catfish protein hydrolysates fractionation and sequencing to determine the antioxidant amino acid sequence.

Conclusions. The hydrolysis of the CPI for 30 min using papain provides a satisfying result in the catfish protein hydrolysate manufacturing. The hydrolysis time significantly affected the hydrolysis of the catfish protein hydrolysates produced with papain. Meanwhile, the peptide chain length and antioxidant capacities (DPPH method and ferric reducing power) of the papain and bromelain-treated hydrolysates were not significantly affected by different hydrolysis time (30, 60 and 90 min). The hydrolysis of the CPI for 30 min by papain enzyme resulted in catfish protein hydrolysate with an antioxidant capacity of 59.578±0.05% (15.83±1.65 mg L^{-1} AEAC) scavenging DPPH radicals and ferric reducing power of 0.80±0.02. The hydrolyzate of CPI by the bromelain enzyme resulted in an antioxidant capacity of 49.52±6.01% (13.07±0.01 mg L^{-1} AEAC) scavenging DPPH radicals and ferric reducing power of 0.44±0.06. The findings of this study indicate that catfish protein hydrolysate with papain enzyme has better potential as an antioxidant. A future research on catfish peptide fractionation and sequencing is envisaged in order to determine sequence responsible for the antioxidant activity.

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Conflict of interest. The authors declare no conflict of interest.

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