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PRODUCTION OF ACE-INHIBITORY AND ANTIOXIDANT HYDROLYSATES FROM THE FILLET OF HYBRID GROUPER

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Abstract: Hydrolysates from fish muscle have been reported to exhibit various bioactivities including ACE-inhibitory and antioxidant activities. In this study, the effect of hydrolysis conditions on the ACE-inhibitory and antioxidant activities (hydroxyl radical scavenging activity and reducing power) of hydrolysates from the fillet of hybrid grouper (TGGG) was investigated. The fillet was hydrolysed with four different enzymes (Alcalase, proteinase K, trypsin and pepsin) for 48 hours. Part of the hydrolysates were collected at 1, 2, 4, 6, 24, and 48 hours and analysed for several bioactivities. Results obtained showed that Alcalase and proteinase K hydrolysates demonstrated the highest ACE-inhibitory and hydroxyl radical scavenging activity ($p < 0.05$). Pepsin hydrolysate on the other hand exhibited the highest reducing power ($p < 0.05$). Trypsin was the only enzyme where all its bioactivities were affected by the degree of hydrolysis (DH) ($p < 0.05$). Extensive hydrolysis resulted in a higher hydroxyl radical scavenging of pepsin hydrolysate. The results of this study demonstrated that the proper choice of enzymes and optimal hydrolysis duration could potentially enhance the ACE-inhibitory and antioxidant potency of hydrolysates from the TGGG fillet. Further studies to isolate and identify the potent peptides from the fillet hydrolysate are recommended.

Keywords: Antihypertensive, Alcalase, Pepsin, Proteinase K, Trypsin.

Introduction

Grouper is among the most popular fish in aquaculture and a highly traded seafood commodity in the Asia-Pacific region (Shapawi *et al*., 2019). Crossbreeding between male giant grouper (*Epinephelus lanceolatus*) and female tiger grouper (*Epinephelus fuscoguttatus*) resulted in the first global hybrid grouper named TGGG, which inherited positive traits from their parents' species such as fast growing rate and high tolerance to low water salinity (De *et al*., 2014; Firdaus *et al*., 2016). These positive traits have made TGGG a popular fish in the aquaculture and food industries. Our previous study reported that the fillet of TGGG contained high amounts of protein (78.63%), amino acids (63.33 g/100g) and its essential amino acids were comparable to FAO/WHO requirements (Chan *et al*., 2019), which makes it a good substrate for protein hydrolysate production. Utilising fish fillet for the production of hydrolysate can further enhance the value of TGGG by diversifying its usage from the food sector into the nutraceutical sector. To the best of our knowledge, studies on the potential of hydrolysate from TGGG for nutraceutical applications has not been reported to date.

Hydrolysate with multifunctional activities is of interest in the management of different diseases (Neves *et al*., 2017). They may be considered to have added benefits compared to monofunctional hydrolysate, which provide only one activity (Lammi *et al*., 2019). Hypertension has been considered as the most common health problem worldwide, accounting for 9.4 million deaths in 2010 (Lim *et al*., 2012; World Health Organization, 2013). It has been reported that one of the approaches to treat hypertension effectively is via synthetic ACE-inhibitors (captopril, linopril, and enalpril), but the usage of

these drugs has raised much public concern due to their side effects (Wijesekara & Kim, 2010). Therefore, much effort has been put into the search for natural ingredients for the treatment/ prevention of hypertension. Hydrolysates derived from fish are considered as a natural and safe source of ACE-inhibitor (Osajima *et al*., 2009; Olav *et al*., 2014; Neves *et al*., 2017). Their ACE-inhibitory activity was not only seen in *vitro* but *in vivo* as well, proving their possible health benefits as functional ingredients/products for human consumption (Lee *et al*., 2014; Lin *et al*., 2017; Neves *et al*., 2017). A clinical study by Enari *et al*. (2008) found that the systolic blood pressure of mild hypertensive subjects was significantly reduced after four weeks of diet supplemented with salmon hydrolysate, and the antihypertensive effect could last for another two weeks after the treatment was stopped. Besides having ACE-inhibitory activity, fish hydrolysates also demonstrated various antioxidant abilities, such as scavenging of free radicals, power to reduce ferric ion, and chelating of ferrous ion (Dong *et al*., 2008; Phanturat *et al*., 2010; Najafian & Babji, 2014; Zhao *et al*., 2018). Khaled *et al*. (2012) reported that the endogenous antioxidant enzyme activities in the liver of hypercholesterolemic Wister rats were greatly improved after feeding with a diet containing sardinelle hydrolysate for seven weeks. This revealed that the consumption of natural antioxidant supplements can ameliorate oxidative stress.

Enzymatic hydrolysis has been a preferred method to produce hydrolysate because this processes usually require lower hydrolysis temperatures (37-70°C) than the acid/alkaline hydrolysis (> 90°C) that would yield products with reduced nutritional qualities and poor functionalities (Aluko, 2018; Kristinsson & Rasco, 2000). Compared to other hydrolysis methods (chemical hydrolysis and autolysis), this enzyme-specific reaction also allows controllable peptide bonds cleavage due to the enzyme specificity and subsequently could deliver a more reproducible end product (Guerard, 2007). In past studies, a variety of enzymes at different hydrolysis times were investigated in the production of hydrolysates with ACE-inhibitory and antioxidant activities. For example, the study by Ketnawa *et al*. (2016) reported that hydrolysates obtained from the gelatine of giant catfish at 120 min with trypsin exhibited 1.6 times stronger reducing power than the hydrolysate obtained at 10 min. In another study by Ghassem *et al*. (2014), hydrolysate of snakehead fish from Alcalase was found to be the most potent ACE inhibitor than other studied hydrolysates (proteinase K, Neutrase, papain and Flavourzyme). These studies revealed that the bioactivities of the hydrolysates were influenced by several factors such as the enzymes used and the hydrolysis duration.

Our earlier study found that the hydrolysates from the head and bones of TGGG exhibited ACE-inhibitory and antioxidant activities (Chan *et al*., 2020), but the activities were lower than the by-products of catfish, tilapia and skipjack tuna (skin, skeleton and dark muscle) reported in the literature (Mahmoodani *et al*., 2014; Chi *et al*., 2015; Borges-Contreras *et al*., 2019). Two studies by the same group of researchers (Lassoued et al., 2015a; 2015b) found that hydrolysate from the muscle of thornback ray exhibited higher ACE-inhibitory (ACE: 89% vs 78%) and antioxidant activities (reducing power: 1.39 absorbance unit vs 0.67 absorbance unit at 700 nm) than its skin. This showed that the capacity of the bioactivities is influenced by primary peptide sequences of the substrate which is dependent on the source of protein (Harnedy & FitzGerald, 2011). This, therefore, leads to the attempt to compare the dual functionalities of the hydrolysate from the fillet of TGGG to its by-product. Based on our observations on the head and bones of TGGG, the potency of the hydrolysates was affected by the type of enzyme and duration of hydrolysis. Comparing proteinase K, trypsin, and pepsin, Alcalase was found to generate hydrolysate with the highest bioactivity at shorter reaction time (Chan *et al*., 2020). It is known that the bioactivities of hydrolysates are highly dependent on the sequences and molecular mass of the peptides formed as governed by the hydrolysis conditions (Sila & Bougatef, 2016). Therefore, in this

study, similar hydrolysis conditions used in our previous study were employed for the fillet (enzyme: Alcalase, proteinase K, trypsin, pepsin; hydrolysis duration: 1 – 48 hours) and to determine if this different substrate could offer stronger bioactivities than the by-product of TGGG.

Materials and Methods

Sample Collection

The TGGG, of body weight of 800-850g were purchased from the hatchery of Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah. The fish were packed in ice and immediately transported to the laboratory. Upon arrival at the laboratory, the fish was descaled, eviscerated and filleted. The fish fillets were collected and washed thoroughly using distilled water before frozen using liquid nitrogen. The frozen fish fillets were then lyophilised using a freeze dryer (FreeZone 6 L, Labconco Corporation, MO, USA) before being grounded using a blender and stored at -20°C for further analysis.

Preparation of Protein Hydrolysate

The freeze-dried fish fillets were mixed with different buffer solutions (1:50, w/v) and separately hydrolysed using four enzymes, i.e. Alcalase (EC 3.4.21.14), proteinase K (EC 3.4.21.64), trypsin (EC 3.4.21.4), and pepsin (EC 3.4.23.1) (Sigma-Aldrich, MO, USA) under their respective optimum pH and temperature (Table 1). The enzyme was added into the reaction mixture at an enzyme/substrate ratio of 1% (w/w) to initiate the hydrolysis and the hydrolysis was carried out in a temperature-controlled shaking incubator for 48 hours. At the designated time, i.e. 1, 2, 4, 6, 24 and 48 hours of hydrolysis, hydrolysate was collected and the enzyme was inactivated by heating at 95°C for 15 min in a water bath (Fisherbrand Isotemp GPD 20, Fisher Scientific UK Limited, Loughborough, UK). After cooling down, the hydrolysate was centrifuged at 10,000 x g at 4°C for 20 min (Sorvall Biofuge Primo R, Thermo Fisher Scientific Incorporation, MA, USA). The resultant supernatant was lyophilised and then kept at -20°C for degree of hydrolysis (DH), ACE-inhibitory, hydroxyl radical scavenging and reducing power determinations.

Determination of Degree of Hydrolysis

The DH was determined using the o-phthaldiadehyde (OPA) method as described by Nielsen *et al*. (2001) and Mirzaei *et al*. (2015). The OPA reagent comprised 0.1 M di-sodium tetraborate decahydrate, 1% of sodium dodecyl sulphate (SDS), 40 mg OPA (in 1 mL of ethanol), and 5.7 mM dithiothreitol (DTT). Then the mixture was adjusted to a final volume of 200 mL with distilled water. This OPA reagent must be freshly prepared for use. Hydrolysate and serine standard (0.4 mL) were mixed with 3 mL OPA reagent and the mixture was shaken for 5 sec. The absorbance of the mixture was measured at 340 nm using UV-Vis spectrophotometer (Lambda 35, Perkin Elmer, MA, USA) after 2 min of incubation at room temperature. The DH was calculated using the following equation: $DH = [h_t - h_0 / h_{tot}] \times 100\%$, in which h_t is the amount of amino group released at time, t, h_0 is the amount of amino group in the supernatant at time 0 hour, and h_{tot} is the total amount of amino group in the sample. The value h_{tot} was obtained from acid hydrolysis of fillet using 6 N HCl at 110°C for 24 hours.

Table 1: Conditions for the hydrolysis of TGGG fillet

Enzymes	Buffer	pH	Temperature (°C)
Alcalase	0.1 M Na_2HPO_4-NaH_2PO_4	7.5	50
Proteinase K	0.1 M Na_2HPO_4-NaH_2PO_4	7	37
Trypsin	0.1 M Na_2HPO_4-NaH_2PO_4	8	37
Pepsin	0.1 M KCl-HCl	2	37

Determination of ACE-inhibitory Activity

The ACE-inhibitory activity of hydrolysates was measured using the method of Cushman & Cheung (1971) and Forghani *et al*. (2012) with some modifications. Fifty microliters of hydrolysate (1 mg/mL) were first pre-incubated with 150 µL of hippuryl-histidyl-leucine (HHL) (8.3 mM) at 37°C for 5 min in a water bath. Subsequently, 50 µL of ACE (25 mU/mL) was added to the mixture, which was incubated at 37°C for 60 min in a water bath. After 60 min, the reaction was terminated by adding 250 µL of HCl (1 N) to the mixture. The released hippuric acid was extracted by addition of 1.5 mL of ethyl acetate followed by centrifugation at 2000 x g at 4°C for 10 min. The extracted layer (1 mL) was transferred to a test tube and was evaporated to dryness at 40°C using a vacuum oven (VD 23, Binder GmbH, Tuttlingen, Germany). Then, 1 mL of distilled water was added to the test tubes and mixed for 1 min. The absorbance of the mixture was measured at 228 nm using UV-Vis spectrophotometer. The ACE-inhibitory activity was calculated using the following equation: ACE-inhibitory activity (%) = [(A – B) / (A – C)] × 100%, where A is the absorbance of the test sample without hydrolysate, B is the absorbance of the test sample with hydrolysate, and C is the absorbance of reaction blank (HCl was added prior to the addition of ACE).

Determination of Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of hydrolysates was measured using the method described by Jumeri & Kim (2011). Reaction mixture containing 0.1 mL $FeS0_4$ (10 mM), 0.1 mL EDTA (10 mM), 0.5 mL 2-deoxyribose (10 mM), and 0.9 mL sodium phosphate buffer (0.1 M, pH 7.4), were mixed with 0.2 mL of sample aliquot (2 mg/mL). The reaction was initiated by addition of 0.2 mL H_2O_2 (10 mM) and this mixture was incubated at 37°C for 90 min in a water bath. After 90 min, 1 mL of cold trichloroacetic acid (2.8%, w/v) and 1 mL of thiobarbituric acid (1%, w/v) were added to the mixture to stop the reaction. This mixture was then heated in a boiling water bath for 15 min. The absorbance of the resulting pink colour was measured at 532 nm against a reagent blank using UV-Vis spectrophotometer. The hydroxyl radical scavenging activity was calculated using the following equation: hydroxyl radical scavenging activity (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100\%$, whereby $A_{control}$ is the absorbance of reaction mixture without hydrolysate, A_{sample} is the absorbance of reaction mixture with hydrolysate.

Determination of Reducing Power

The ability of hydrolysates to reduce ferric ion (Fe^{3+}) was evaluated by the method of Najafian & Babji (2014). An aliquot of sample (0.2 mL) with concentration of 0.25 mg/mL was added to 0.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 mL of potassium ferricyanide solution (1%, w/v). The reaction mixture was then incubated at 50°C for 20 min in a water bath. After that, 0.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture, and the reaction mixture was centrifuged at 3500 x g at 4°C for 10 min. Then, 0.5 mL of supernatant was mixed with 0.5 mL distilled water and 0.1 mL ferric chloride (0.1%, w/v). The reaction mixture was incubated in the dark at room temperature for 2 min. The absorbance of the solution was then measured at 700 nm against blank using a UV-Vis spectrophotometer. The results were expressed as absorbance unit at 700 nm, in which the higher the absorbance unit, the higher the reducing power.

Statistical Analysis

All the measurements were carried out in triplicates. The data was presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey test were performed using SPSS version 25. A Pearson correlation test was used to study the relationship between the DH and bioactivities of hydrolysate for each enzyme. The differences between means were considered significant at $p < 0.05$.

Results and Discussion

Degree of Hydrolysis

As shown in Figure 1, at the end of 48 hours hydrolysis, Alcalase produced the hydrolysate with the highest DH (92.67%) ($p < 0.05$) from the fillet of TGGG, followed by proteinase K (57.92%), trypsin (12.74%), and pepsin (8.90%). Comparing to our earlier work on the head and bones of TGGG, the effect of types of enzymes on the DH shares a similar trend in terms of the hydrolysis efficiency; however, the DH values of the hydrolysates obtained from the fillet by each enzyme are higher than the by-products (Chan *et al*., 2020). The higher protein and total amino acid content in the fish fillet resulted in more peptide bonds during hydrolysis as compared to the head and bones (protein: 61.93%; total amino acids: 33.69 g/100g) (Chan *et al*., 2019). Similar findings were reported by Roslan *et al*. (2018) whereby the hydrolysate derived from the fillet of tilapia (DH: 31.25%) by Alcalase was also found higher than its by-product (DH: 25.59%). It is worth noting that the fillet was almost completely hydrolysed by Alcalase after 48 hours of hydrolysis, suggesting that the broad spectrum of Alcalase (cleaved at C-terminal with large uncharged amino acids) was favourable toward the protein of fillet and thus able to cleave the protein effectively. In comparison to Alcalase and proteinase K, trypsin and pepsin were more specific and have limited cleavage sites (Adler-Nissen, 1993; Vercruysse *et al*., 2005), therefore limited the DH. Previous studies comparing Alcalase with other enzymes also found that Alcalase exhibited higher DH values (Chi *et al*., 2015; Borges-Contreras *et al*., 2019). However, trypsin with a comparatively narrower spectrum than Alcalase was reported to yield 1.8 times higher DH thanchum salmon (Lee *et al*., 2014). The availability of susceptible bonds in chum salmon for cleaving by trypsin could be another factor which resulted in higher DH value. Even though considered as a broad spectrum enzyme, proteinase K only achieved 62.50% of the DH of Alcalase at the end of the hydrolysis (Figure 1). This may be attributed to the low availability of aliphatic and aromatic amino acids at the C-terminal (cleavage sites of proteinase K) of the protein derived from the fillet of TGGG (Adler-Nissen, 1993; Vercruysse *et al*., 2005). Similar observation was reported in the skin of grass carp, whereby proteinase K displayed seven times lower DH than Alcalase (Yi *et al*., 2017). The disparities in findings suggested that it is important to select the appropriate enzyme to ensure hydrolysis for a specific type of protein.

In terms of hydrolysis rate, proteinase K showed the fastest hydrolysis rate during the

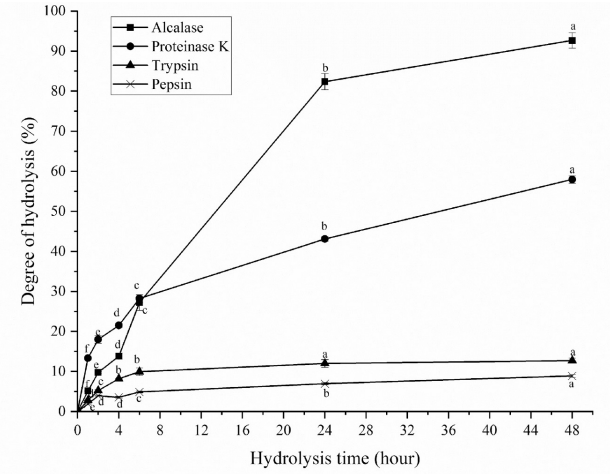

Figure 1: The degree of hydrolysis of fillet hydrolysed with four different enzymes, i.e. Alcalase, proteinase K, trypsin, and pepsin. Means with different letters (a – f) within the same line pattern indicate significant difference at $p < 0.05$

first 6 hours of treatment, followed by a linear increment with slower rate until the end of hydrolysis (Figure 1). Alcalase, on the other hand, exhibited a slower hydrolysis rate than proteinase K at the beginning of hydrolysis. However, starting at the sixth hour, the hydrolysis rate of Alcalase increased tremendously until the end of the process, surpassing the DH of proteinase K. The hydrolysis rate of trypsin and pepsin was comparatively lower. The hydrolysis rate of trypsin increased steadily for the first 6 hours of treatment and slowed down reaching to a plateau at the 48-hour mark. Pepsin displayed a similar hydrolysis pattern as trypsin, but at a rate 1.5 times slower than trypsin. The high hydrolysis rate exhibited by the enzymes at the beginning of reaction indicates the availability of peptides bonds, while the reduction of reaction rate at the later stage is attributed to the decrease in susceptible peptides bonds (Ketnawa *et al*., 2016).

ACE-inhibitory Activity

No ACE-inhibitory activity was detected in the unhydrolysed fillet (data not shown), indicating that a hydrolysis process was needed to generate hydrolysate with improved bioactivity. Depending on the type of enzyme used, the duration of hydrolysis was found to influence the ACE-inhibitory activity of the hydrolysate. At the end of the hydrolysis, Alcalase hydrolysate displayed the highest ACE-inhibitory activity (85.52%) ($p < 0.05$), followed by proteinase K, trypsin and pepsin (Figure 2A). High ACE-inhibitory activity was also reported in Alcalase hydrolysate of other food sources such as snakehead fish (Ghassem *et al*., 2014) and salmon (Ahn *et al*., 2012). This observation was dissimilar to the previously reported study on the head and bones of TGGG (Chan *et al*., 2020), whereby proteinase K (55.92%) and pepsin (52.38%) hydrolysates showed the highest ACE-inhibitory activity instead of Alcalase (39.71%). Other than the difference in enzyme efficiency, the fillet hydrolysates also exhibited higher ACE-inhibitory activity in comparison to the head and bone hydrolysates, suggesting that the ACE-inhibiting peptides derived from the fillet of TGGG were more potent. This finding however disagrees with the results reported by Roslan *et al*. (2018) in which both hydrolysates obtained from the muscle and by-product of tilapia were found to have similar ACE-inhibitory activity. Since fish fillet is mainly made up of myofibrillar and sarcoplasmic protein, whereas fish head and bones consist of collagen and gelatine (Harnedy & FitzGerald, 2011; Petricorena, 2015), therefore the combination of peptides in the hydrolysates obtained could be different and hence the ACE-inhibitory activity.

As portrayed in Figure 2A, the ACE-inhibitory activity of Alcalase hydrolysate increased by 37.6% at the completion of 2 hours of hydrolysis. Hydrolysis beyond 2 hours up to 6 hours showed slight decline in the ACE-inhibitory activity, but the reduction was statistically insignificant ($p > 0.05$). The ACE-inhibitory activity then increased to the highest activity of 81.46% ($p < 0.05$) after 24 hours of hydrolysis and this inhibitory activity was maintained until the end of hydrolysis. This observation suggests that extended hydrolysis resulted in the peptide sequences with stronger inhibitory activity. Other than the sequences of the peptides, the chain length of the peptides may also contribute to this stronger ACE-inhibitory activity. Prolonged hydrolysis led to excessive cleavage on the fillet which may result in the increased number of peptides with relatively shorter chain length (with potent peptide sequences). Previous studies have reported that hydrolysates with higher amounts of short-chain peptides demonstrated greater ACE-inhibitory activity (Byun & Kim, 2001; Neves *et al*., 2017). The effect of hydrolysis duration on the ACE-inhibitory activity of proteinase K hydrolysates was seen only during the first 4 hours of hydrolysis. The ACE-inhibitory activity increased continuously up till the fourth hour, where the highest inhibitory activity was recorded (84.88%) ($p < 0.05$) and no significant difference was reported thereafter. Pepsin hydrolysate showed gradual increase of ACE-inhibitory activity for the first 24 hours of hydrolysis. Extended hydrolysis

for another 24 hours caused the decline of the ACE-inhibitory activity, to a value similar as after 4 hours of hydrolysis ($p > 0.05$). Unlike the hydrolysates produced by the other three enzymes, hydrolysate generated by trypsin was the only hydrolysate which recorded a continuous decline of ACE-inhibitory activity during the first 4 hours of hydrolysis, i.e. from 46.06% to 30.67% ($p > 0.05$). The ACE-inhibitory activity later rose gradually until the end of the treatment, where the highest inhibitory activity was reported (61.12%, $p < 0.05$). This observation may be attributed to the changing peptide profiles (such as peptides size and sequences) (Ketnawa *et al*., 2017; Neves *et al*., 2017) in the hydrolysate because of continuous hydrolysis. The comparatively lower ACE-inhibitory activity reported in hydrolysate before 6 hours of hydrolysis indicates that the combination of peptides obtained during that period exhibited a lower capacity.

All the fillet hydrolysates exhibited positive correlations between the DH and ACE-inhibitory activity (Figure 2B) as observed in Alcalase ($r = 0.8047$) ($p < 0.01$), pepsin ($r = 0.6655$) ($p < 0.01$), trypsin ($r = 0.5169$) ($p < 0.05$), and proteinase K hydrolysates ($r = 0.3612$) ($p > 0.05$). These findings are unlike the observations reported for hydrolysates originating from the head and bones, in which only proteinase K and pepsin hydrolysates exhibited positive correlations, whereas Alcalase and trypsin hydrolysates displayed negative correlations (Chan *et al*., 2020). The high correlation coefficient exhibited by Alcalase hydrolysate of fillet indicated that its ACE-inhibitory activity was very much dependent on the extent of hydrolysis. The DH is known to affect the peptide chain length,

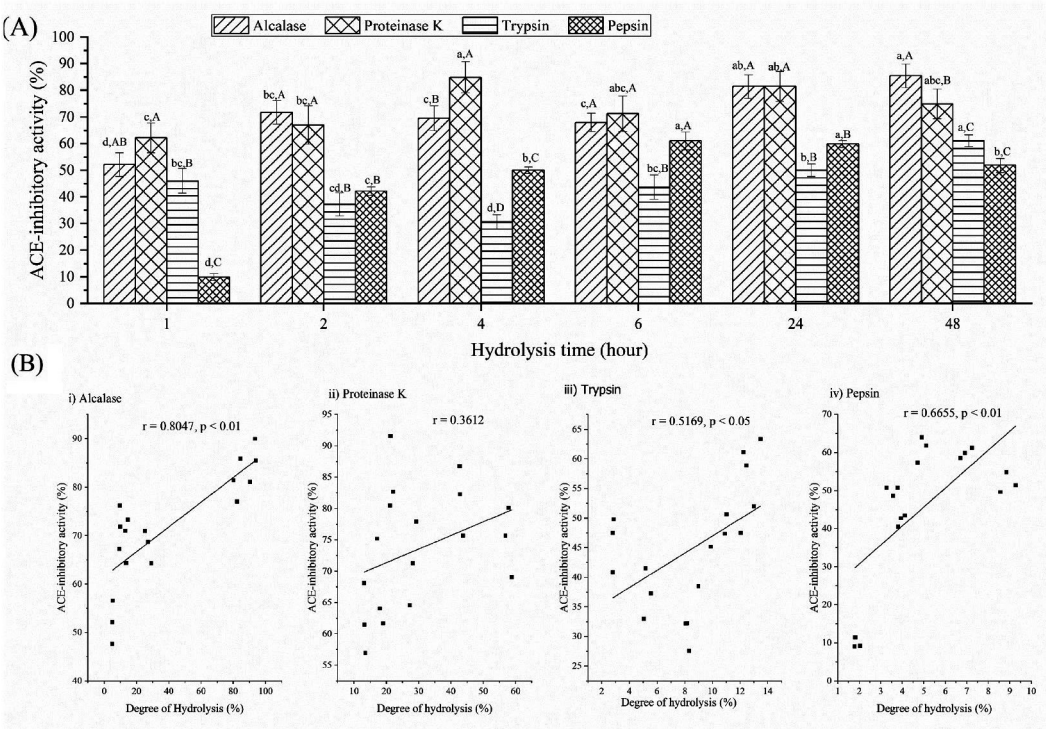

Figure 2: (A) The ACE-inhibitory activity of Alcalase, proteinase K, trypsin and pepsin hydrolysates during 48 hours of hydrolysis. Means with different lowercase letters (a – d) indicate significant difference between hydrolysis time for the same enzyme ($p < 0.05$). Means with different uppercase letters (A – D) indicate significant difference between enzymes within the same hydrolysis time ($p < 0.05$). (B) The correlation plots between DH and ACE-inhibitory activity for four different hydrolysates, i.e. i) Alcalase, ii) Proteinase K, iii) Trypsin and iv) Pepsin

as well as the exposure of the terminal amino groups of the products obtained (Ketnawa *et al*., 2016). A study by Neves *et al*. (2017) on salmon gelatine showed the increment of DH from 15 to 25% in Alcalase hydrolysate led to the increased number of short-chain peptides (molecular weight less than 1 kDa) as well as ACE-inhibitory capacity. Besides DH, the type of enzyme used for hydrolysate production could be another important factor that affected the ACE-inhibitory activity as the enzyme specificity could affect the peptide sequences of the resultant hydrolysate. This can be seen in the ACE-inhibitory activity exhibited by Alcalase and trypsin hydrolysates, though having similar DH (~ 9.80%) at 2- and 6-hour mark respectively (Figure 1), Alcalase hydrolysate exhibited 1.6 times higher inhibitory activity than trypsin (Figure 2A).

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of all the hydrolysates was also found higher than the unhydrolysed fillet (data not shown). At the end of hydrolysis, all the hydrolysates exhibited similar hydroxyl radical scavenging activity (41.49 – 48.34%) ($p > 0.05$) except for pepsin, where its radical scavenging activity was 50% lower (22.59%, $p < 0.05$) (Figure 3A). Pepsin hydrolysates obtained from the head and bones of TGGG also exhibited the lowest hydroxyl radical scavenging activity as compared to the hydrolysates obtained from the other three enzymes (Chan *et al*., 2020).

The hydroxyl radical scavenging activity of hydrolysates catalysed by Alcalase and trypsin exhibited less variation as compared to proteinase K and pepsin throughout the

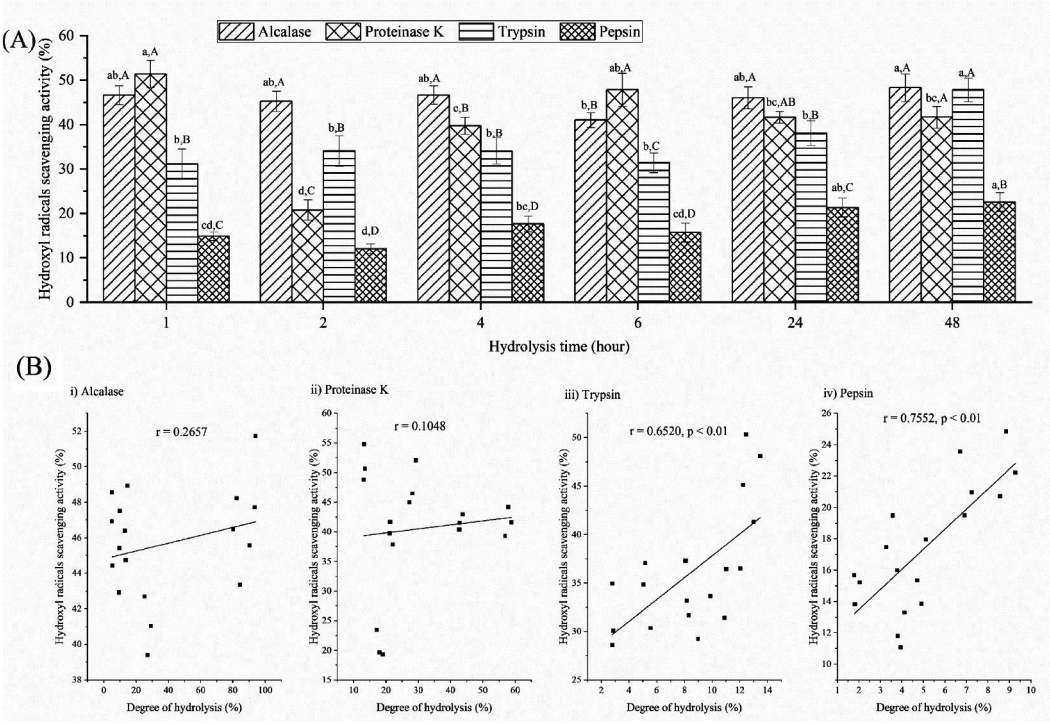

Figure 3: (A) The hydroxyl radical scavenging activity of Alcalase, proteinase K, trypsin, and pepsin hydrolysates during 48 hours of hydrolysis. Means with different lowercase letters (a – d) indicate significant difference between hydrolysis time for the same enzyme ($p < 0.05$). Means with different uppercase letters (A – D) indicate significant difference between enzymes within the same hydrolysis time ($p < 0.05$). (B) The correlation plots between DH and hydroxyl radical scavenging activity for four different hydrolysates, i.e. i) Alcalase, ii) Proteinase K, iii) Trypsin and iv) Pepsin

hydrolysis. The hydroxyl radical scavenging activity of Alcalase did not differ much until 48 hours of hydrolysis, whereby 15% higher activity was obtained (Figure 3A). Likewise, for trypsin hydrolysate, the significant improvement of hydroxyl radical scavenging activity was also only seen after 48 hours of hydrolysis, in which the scavenging activity increased from 38.07% to 47.85% ($p < 0.05$). The minimal changes of hydroxyl radical scavenging activity in these two hydrolysates throughout the digestion suggests that instead of hydrolysis duration, other factors such as the peptide sequences may play a more important role in contributing to the scavenging activity of these hydrolysates (Sarmadi & Ismail, 2010). This is likely due to specificity of Alcalase and trypsin which could continuously release mixture of peptides that contained amino acids with potent radical scavenging activity such as arginine, histidine tyrosine, and phenylalanine at the carboxyl end of the peptide chains (Sila & Bougatef, 2016). The accumulation of these potent peptides over time may contribute to the significant increment of hydroxyl radical scavenging capacity in Trypsin hydrolysate at the end of hydrolysis. Proteinase K hydrolysate recorded the highest hydroxyl radical scavenging activity at the first hour of hydrolysis (51.40%, $p < 0.05$), nonetheless the bioactivity dropped more than 50% when the hydrolysis time was extended to 2 hours. Continuous hydrolysis from 4 to 6 hours gradually raised the radical scavenging activity; hydrolysis beyond this point did not further enhance the radical scavenging activity. The first 6 hours of hydrolysis did not cause much change to the hydroxyl radical scavenging activity of pepsin hydrolysate. After 24 hours of hydrolysis, the radical scavenging activity was elevated from 15.72 to 21.34%, achieving the highest activity ($p < 0.05$) and prolonged hydrolysis until 48 hours did not affect the radical scavenging activity.

Among the four hydrolysates, only trypsin ($r = 0.6520$) and pepsin ($r = 0.7552$) ($p < 0.01$) hydrolysates recorded significant positive correlations between the DH and hydroxyl radical scavenging activity (Figure 3B). On the other hand, proteinase K hydrolysate from the head and bone of TGGG also exhibited positive correlation similar to trypsin hydrolysate, while pepsin hydrolysate showed negative correlation (Chan *et al*., 2020). The significant correlations exhibited by trypsin and pepsin hydrolysates indicated that their hydroxyl radical scavenging activity was influenced by the DH. The DH dependence of hydroxyl radical scavenging activity was similar to that reported for several fishes, such as blacktip shark skin (Kittiphattanabawon *et al*., 2012) and silver carp (Dong *et al*., 2008). However, since not all hydrolysates exhibited significant correlation, there may be factors other than DH affecting the radical scavenging activity (You *et al*., 2009; Sarmadi & Ismail, 2010). This observation was in accordance with a study reported by Lima *et al*. (2019), whereby the hydroxyl radical scavenging activity of Alcalase hydrolysate of stripped weakfish was not significantly affected even when the DH was increased from 5 to 15%. In another study on loach, the hydroxyl radical scavenging activity of papain hydrolysate was affected by the amount of antioxidant amino acids (tyrosine, methionine, histidine, lysine, and tryptophan) instead of the DH (You *et al*., 2009).

Reducing Power

Similar to other investigated bioactivities, higher reducing power was also reported in the hydrolysates rather than the unhydrolysed counterpart (data not shown). Unlike hydroxyl radical scavenging activity, pepsin-generated hydrolysate exhibited twice higher reducing power ($p < 0.05$) than other hydrolysates at the end of hydrolysis (Figure 4A). The high reducing power of pepsin hydrolysate indicated that this hydrolysate contained peptides that exhibited strong electron and hydrogen donating ability (Alemán *et al*., 2011). Proteinase K on the other hand, exhibited the highest reducing power when used to hydrolyse the head and bones of TGGG (Chan *et al*., 2020). The difference between the protein structure of fillet and by-products of TGGG as mentioned earlier could be the reason for this discrepancy.

The highest reducing power ($p < 0.05$) of pepsin hydrolysate was recorded at the second hour of hydrolysis, but it was significantly reduced by 30% (from 0.1669 to 0.1170 absorbance unit) ($p < 0.05$) after another two hours of hydrolysis. Further hydrolysis to 6 hours raised the reducing power to 0.1410 absorbance unit but with no significant changes until the end of hydrolysis ($p > 0.05$). Overall, Alcalase hydrolysate demonstrated the weakest reducing power throughout the treatment, except after 48 hours of hydrolysis; its reducing power was similar to proteinase K and trypsin hydrolysates ($p > 0.05$). For proteinase K hydrolysate, the reducing power decreased gradually for the first 4 hours before rising to a higher level (0.0584 absorbance unit) ($p < 0.05$) after 6 hours of hydrolysis. Prolonged hydrolysis to 24 hours decreased the reducing power but was then brought up to 0.0608 absorbance unit ($p < 0.05$) at the end of hydrolysis. Two-fold increase in the reducing power of trypsin hydrolysate (from 0.0443 to 0.0855 absorbance unit) ($p < 0.05$) was observed after 4 hours of hydrolysis, but the activity was dropped back to its initial capacity (0.0295 absorbance unit) after 6 hours of hydrolysis. Prolonged hydrolysis of up to 24 hours led to the highest reducing power (0.1193 absorbance unit) ($p < 0.05$) but the reducing power was again reduced to 0.0673 absorbance unit after 48 hours of hydrolysis.

The reducing power of the fillet hydrolysates was positively correlated to the DH, similar to that reported for the head and bone hydrolysates (Chan *et al*., 2020). Moderate significant correlations were reported in the hydrolysates from Alcalase ($r = 0.5253$) and trypsin ($r = 0.4918$) ($p < 0.05$), whereas the

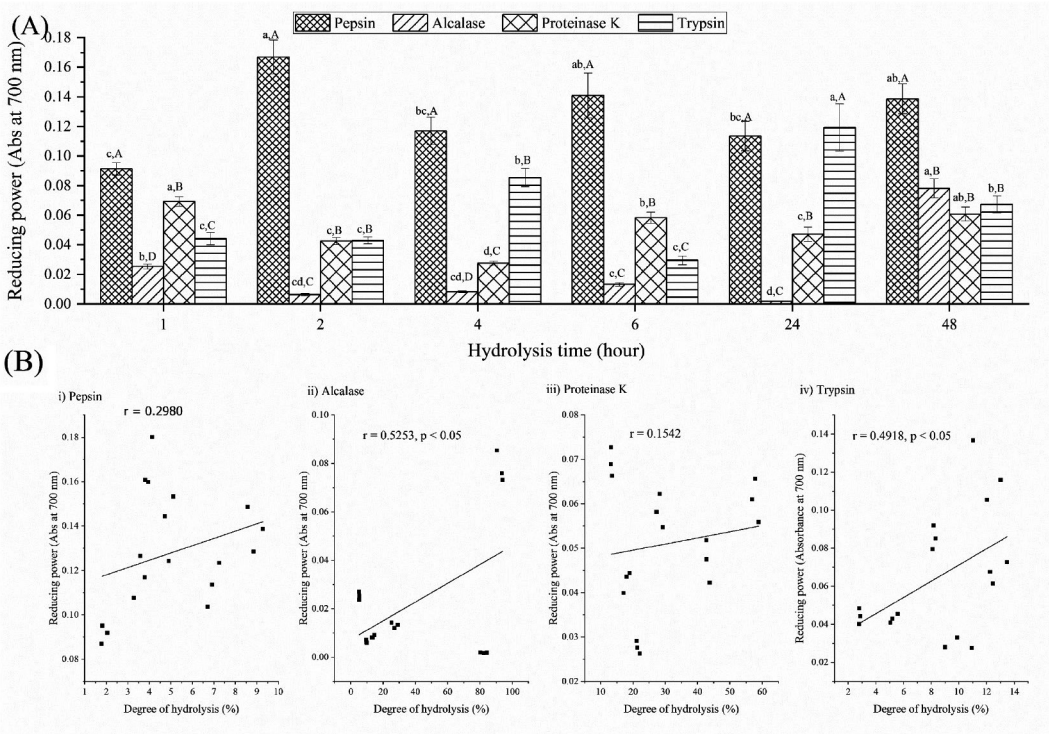

Figure 4: (A) The reducing power of pepsin, Alcalase, proteinase K and trypsin hydrolysates during 48 hours of hydrolysis. Means with different lowercase letters (a – d) indicate significant difference between hydrolysis time for the same enzyme ($p < 0.05$). Means with different uppercase letters (A – D) indicate significant difference between enzymes within the same hydrolysis time ($p < 0.05$). (B) The correlation plots between DH and reducing power for four different hydrolysates, i.e. i) Pepsin, ii) Alcalase, iii) Proteinase K and iv) Trypsin

other two hydrolysates exhibited insignificant correlations (Figure 4B). The moderate correlation coefficient suggests the reducing power of Alcalase and trypsin hydrolysates was partly influenced by the extent of hydrolysis. As mentioned earlier, there may be several other factors besides DH that are associated with the antioxidant potency of hydrolysates (Ketnawa *et al*., 2016). However, there are a number of studies that disagreed with the current finding, whereby a strong relationship was reported between the reducing power of hydrolysate with DH. For example, Phanturat *et al*. (2010) reported that the reducing power of Alcalase-treated gelatine hydrolysate of bigeye snapper increased in accordance with the increment of DH from 5 to 25%. In another study, an inverse correlation between reducing power and DH was found in the Alcalase hydrolysate of yellow stripe trevally and stripped weakfish.

Overall, hydrolysates from TGGG fillet reported better dual-functionalities than the by-product. The ACE inhibition of hydrolysate from the fillet was 84.88% whereas the by-product was 59.12%. Meanwhile, for antioxidant activities, the fillet hydrolysate has 44% higher reducing power (0.1669 absorbance unit vs 0.1160 absorbance unit) and 21% higher hydroxyl radical scavenging activity (51.40% vs 42.47%) than the by-product hydrolysate. However, in order to achieve the maximum activities for ACE-inhibitory and reducing power, longer hydrolysis time (more than 2 hours) was required as opposed to the head and bone hydrolysate (1 hour). On the other hand, shorter hydrolysis time was needed by the fillet to reach the highest hydroxyl radical scavenging activity. Even though by-product is a comparatively cheaper source of protein, but the higher bioactivities shown by the fillet hydrolysates suggest its potential to be further developed into nutraceutical products.

Conclusion

This study reveals that the hydrolysates obtained from the fillet of TGGG may exhibit varying degrees of ACE-inhibitory and antioxidant activities, depending upon the type of enzyme and duration used in the hydrolysis treatment. Alcalase could hydrolyse the fish fillet most effectively, followed by proteinase K, trypsin, and pepsin. Both Alcalase and proteinase K have potential to be used to produce dual-functional hydrolysate with high ACE-inhibitory and hydroxyl radical scavenging activities. The strongest reducing power was reported in pepsin hydrolysate, while trypsin could produce hydrolysate with moderate potency as compared to other enzymes. The DH in various hydrolysates was also seen to exert different effects on the ACE-inhibitory, hydroxyl radical scavenging and reducing power. All the three bioactivities of trypsin hydrolysate were correlated to its DH values. In contrast, these bioactivities were independent of its DH value for proteinase K hydrolysate. Extended hydrolysis improved the ACE-inhibitory activity as well as the reducing power of Alcalase hydrolysate. The reducing power of pepsin hydrolysate was the only bioactivity not affected by the extent of hydrolysis. These variations demonstrated that the proper choice of enzymes and optimal hydrolysis duration could potentially enhance the ACE-inhibitory and antioxidant potency of hydrolysate derived from the fillet of TGGG. This study demonstrates that the fillet of TGGG can be used to produce hydrolysate with better ACE-inhibitory and antioxidant activities than its by-product. Therefore, further studies that focus on the isolation and identification of the peptides responsible for these bioactivities are recommended.

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