Biochemical properties of red tilapia (*Oreochromis niloticus*) protein hydrolysates

Shamloo, M., *Bakar, J., Mat Hashim, D. and Khatib, A.

Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Abstract: The amino-acid composition, 2, 2-Diphenyl-1-picryhydrazyl (DPPH) radical-scavenging activity, and peptide patterns of tilapia protein hydrolysates produced by the enzymatic hydrolysis of Alcalase (AH), Flavourzyme (FH) and Protamex (PH) for 5h using pH-stat method were studied. The ratio of essential amino acids to non-essential amino acids increased after hydrolysis in all samples; however, no significant differences among them were observed. AH had a highest ($P < 0.05$) DPPH radical-scavenging activity, but no significant difference in the DPPH between FH and PH was observed. SDS-PAGE patterns for all the hydrolysates showed significant ($P < 0.05$) reduction in the number and the intensity of the bands with increasing time of hydrolysis. Flavourzyme showed the lowest rate of hydrolytic activity towards the tilapia mince.

Keywords: DPPH, enzymatic hydrolysis, amino-acid composition, Tilapia

Introduction

The positive effects of antioxidants in reducing cardiovascular diseases, diabetes mellitus, cancer and Alzheimer have been reported (Aruoma, 1998), which was related to the removal of radicals including reactive oxygen and nitrogen species. Hence, this leads to the growing interest to identify antioxidative properties in many foods. Protein hydrolysates from maize zein (Kong and Xiong, 2006), egg-yolk (Sakanaka and Tachibana, 2006), yellow stripe trevally (Klompong *et al*., 2007), cod backbone (Šližyte *et al*., 2009) and soybean (Moure *et al*., 2006) were reported to possess antioxidant activities. These activities which are associated with bioactive peptides could be produced through enzymatic hydrolysis which affects the molecular size, hydrophobicity and polar groups of peptides in the hydrolysates (Adler-Nissen, 1986; Kristinsson and Rasco, 2000a). Commercial enzymes were the most commonly used enzymes for the hydrolytic process.

Although reports on the radical scavenging ability in fish hydrolysates could be found in the literature, it could still be considered relatively less abundant and much less from freshwater fish such as tilapia when compared to reports from other food sources such as from soy beans or dairy based sources. A review of the development and biological activities of marine-derived bioactive peptides, which included the antioxidant activities from marine sources, was reported by Kim and Wijesekara (2010). The 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity of peptides and free amino acids from hydrolysed tuna cooking juice and mackerel muscle hydrolysate were reported by Wu *et al*. (2003). The DPPH activities of hoki (*Johnius belengerii*) skin gelatin was later reported by Mendis *et al*. (2005), and the scavenge reactive oxygen species activities (ROS) of alkali-solubilized tilapia (*Oreochromis niloticus*) protein hydrolysates produced by the hydrolytic actions of Cryotin, protease A 'Amano′ 2, protease N 'Amano′, Neutrase and Flavourzyme were reported by Raghavan *et al*. (2008). Alkaline proteases have been reported to have a greater ability to solubilize fish protein compared to neutral and acidic proteases, with the exception of pepsin (Sugiyama *et al*., 1991).

Although tilapia is abundantly found in the wild and cultured in several parts of the world, and often consumed as fresh fish, however, its acceptability in the fresh and minimally processed forms is still low when compared to the marine fish. Its potential as a substrate for the production of bioactive peptides has not been exhaustively documented, except for the report by Raghavan *et al*. (2008). Therefore, the objectives of this study were to evaluate the biochemical and the functional properties of tilapia hydrolysates produced by the Alcalase, Protamex and Flavourzyme.

Materials and Methods

Materials

Alcalase 2.4 L (2,4 AU-A/g), Protamex (1.5 AU-NH/g) and Flavourzyme 500 L (500 LAPU/g), were obtained from Novozyme (Bagsvaerd, Denmark), Sodium dodecyl sulphate (SDS) and 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH), were purchased from Sigma-Aldrich, Inc. (St. Louis, Mo., U.S.A.). All other chemicals used in the experiments were of analytical grade.

*Corresponding author.
Email: *jamilah@putra.upm.edu.my*
Tel: 603-89468368; Fax: 603-89423552

Production of protein hydrolysates

Fresh red Tilapia (*Oreochromis niloticus*) bought from the local wet market in Selangor, Malaysia were transported immediately to the laboratory on ice. Upon arrival, the samples were washed and filleted. The fillets were packed in polyethylene bags and freeze-stored at -20°C until used. The fillets (60 g) were thawed and 240 mL of distilled water was added before the homogenization. They were then homogenized in the Waring blender (Model 32BL79, USA) at 11,000 rpm for 1 min. The homogenate (in the ratio of 1:4 substrate to distilled water) was pre-incubated at 60, 55 or 50°C for 20 min prior to the enzymatic hydrolysis to reach the recommended optimum temperature for the respective enzymes. Alcalase, Flavourzyme and Protamex were then added to the homogenates. The hydrolytic reaction was started by the addition 2% (w/w) of the Alcalase or Flavourzyme or Protamex and the hydrolysis was carried out at 60°C, pH 8.0 for Alcalase; at 55°C, pH 7 for Flavourzyme and at 50°C, pH 7.5 for Protamex using the pH-stat method (Adler-Nissen, 1986). Hydrolysis was carried out for 0.25, 0.5, 1, 2, 3, 4, and 5 h. The pH of the mixture was kept constant throughout the hydrolysis. The reaction mixtures were heated in a water bath at 90°C for 15 min with occasional agitation for the termination of the reaction.

All samples were cooled immediately in ice and the pHs of the samples were subsequently adjusted to 7.0 using 1 M HCl. Hydrolysates were then centrifuged at 7000 rpm for 15 min in a refrigerated high speed centrifuge (KUBOTA, 7800). Supernatants were collected and freeze-dried using a freeze-dryer (LABCONCO, 7753032, U.S.A.). The protein hydrolysate powders obtained at the different periods of hydrolysis were packed in polyethylene bags and kept under vacuum at 4°C in dessicators until use.

Degree of hydrolysis (DH)

The DH of hydrolyzed protein was determined using the pH-stat method (Adler-Nissen, 1986).

DH was then calculated as follows:

$$DH\ (\%) = B \times N_b \times 1/\alpha \times 1/M_p \times 1/h_{tot} \times 100$$

Where B was the amount of alkali consumed (ml), N_b was the normality of alkali, M_p was the mass of the substrate (protein (gr), %N ×6.25), $1/\alpha$ was the calibration factors for pH-stat, and h_{tot} was the content of peptide bonds. For tilapia protein, h_{tot} = 8.6 mmolg^{-1} of protein (Adler-Nissen, 1986).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970). All aliquots of AH, FH and PH from different times of hydrolysis were dissolved in deionised distilled water and the dilutions were adjusted to obtain a protein concentration of 50 mgmL^{-1}. Sample solutions were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol) and heated at 90°C for 3 min before loading. Aliquots of 10 μl were loaded into individual wells and a constant current was passed through the gel for 1 h to obtain separation of the peptides. Protein markers (3.4 to 212 kDa) were used for molecular weight determination.

Determination of DPPH radical scavenging ability

The method described by Kitts and Weiler (2003) was used to assess the DPPH radical scavenging capacity. A 0.1 mM DPPH solution in ethanol was mixed with various amounts (10, 20, 30, 40, 50 μgmL^{-1}) of AH, FH, and PH and allowed to stand at ambient temperature for 30 min. The absorbance of the mixture was then read at 517 nm using spectrophotometer (SHIMADZU, UV-1650PC). The scavenging percentage was calculated according to the following equation:

$$\%DPPH° = (A_{control} - A_{test})^{*}100 / A_{control}$$

Where the $A_{control}$ is the absorbance of the control (DPPH° solution without the test sample), and A_{test} was the absorbance of the test sample.

Then the radical scavenging activity was calculated as the concentration needed to induce 50% of the original scavenging activity (EC_{50}). The term half maximal effective concentration (EC_{50}) refers to the concentration of a compound where 50% of its maximal effect is observed.

Determination of amino acid composition

The samples were hydrolysed, then derivatised prior to the injection for analysis. The hydrolysis was carried out by mixing 0.2 g sample with 5 ml 6 N HCl at 110°C for 24 h. Upon completion, 4 ml of the internal standard (AABA) was added to the residues and then made up to 100 ml by deionised water. The determination of the resulting amino acids was carried out by Waters-Pico Tag Amino Acid Analyzer High Performance Liquid Chromatography (Waters 2690/5, Waters Co., Milford, USA) system.

Derivatisation

Derivatisation was carried out acording to the vendor's recommendation using AccQ-Fluor

Reagent kit (Waters Co., Milford, USA). 10 µl of standard solution or hydrolysed samples were mixed with 70 µl of borate buffer and 20 µl AccQ reagent. The mixture was incubated at room temperature for 1 min and tranferred to an auto-sampling vial. After 10 min of incubation at 55°C, 5 µl of the standard and samples were automatically injected into the HPLC column for peak separation and identification.

Chromatographic run

Separations of amino acid peaks were achieved on a AccQ Tag C18 RP column 3.9 x 150 mm (Waters Co., Milford, USA) using a gradient run. The eluent system consisted of two components: AccQ Tag concentrate as Eluent A and 60% acetonitrile as Eluent B. The gradient condition was programmed as follows: 100% A in 0.5 min, 98% A in 14.5 min, 90% A in 4 min, 87% A in 13 min, 65% A in 2 min, 0% A in 3 min, followed by 100% A for 13 min at the flow rate of 1 mlmin^{-1}. Detection was achieved using a fluorescence detector (FD) (Waters 2475, Waters Co., Milford, USA). Blank test, standards and samples were evaluated at Eλ=250 nm and Em=395 nm. The amino acids were identified using Waters Auto analyzer (Waters 2690/5, Waters Co., Milford, USA) system.

Statistical analysis

All data collected were analyzed using Analysis of Variance (ANOVA) and Tukey's Multiple Test to determine the significant difference among the means using statistical analyses package (Minitab Version 14, 2008).

Results and Discussion

*Enzymatic hydrolysis of tilapia muscle*

The hydrolysis of red tilapia protein by Protamex, Alcalase and Flavourzyme progressed rapidly during the first 30 min for all the samples and a gradual increase thereafter (Figure 1), which indicated that the maximum cleavage of the peptides had occurred within the first 30 min of the hydrolysis. The percentages of DH after 300 min of hydrolysis were 23.3%, 20.1% and 11.3%, for Protamex, Alcalase and Flavourzyme, respectively. Mackie (1974) reported that after an initial rapid phase of hydrolysis, the rate of hydrolysis tends to decrease then entering a stationary phase. The shape of the hydrolysis curve obtained in this study was similar to those previously reported for Herring (Sathivel *et al.*, 2003) and for Meriga egg protein hydrolysates (Chalamaiah *et al.*, 2010). Among the three enzymes, Protamex gave the highest DH values throughout the hydrolysis period

and significant differences ($P < 0.05$) in DH were obtained between FH with PH and AH. No significant difference for DH was observed between PH and AH. The higher DH indicated higher proteolytic activity. Alkaline proteases such as Alcalase and Protamex have been reported to exhibit higher activities than neutral or acid proteases, such as papain or pepsin (Sugiyama *et al.*, 1991).
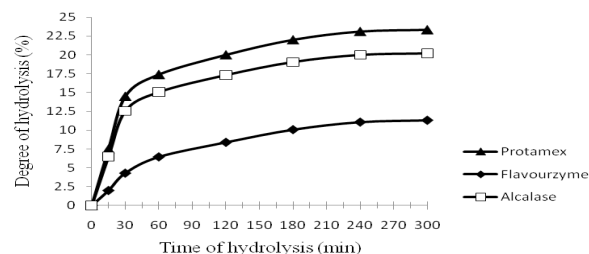
Figure 1. Effect of time on degree of hydrolysis (DH) of tilapia hydrolysates by Protamex, Alcalase and Flavourzyme

The correlation between %DH and time (min) of hydrolysis for Protamex (pH=7.5, T=50°C, E/S=2%), Alcalase (pH=8, T=60°C, E/S=2%), and Flavourzyme (pH=7, T=55°C, E/S=2%) are as shown in Figure 2. The result showed that there was no significant difference ($P < 0.05$) between the rate of hydrolytic activity for Alcalase and Protamex on the tilapia homogenates. The rate of hydrolysis by Flavourzyme was significantly ($P < 0.05$) less than that of Alcalase and Protamex.
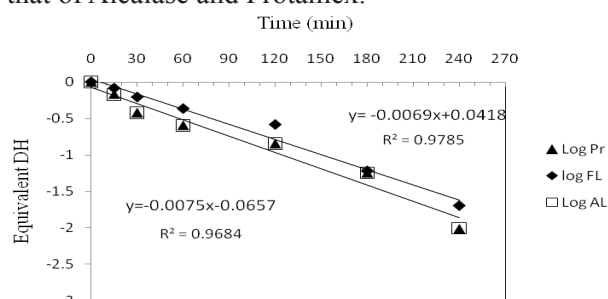
Figure 2. Linearity between time and equivalent degree of hydrolysis for Alcalase, Protamex and Flavourzyme

SDS-PAGE of tilapia protein hydrolysates

Peptides pattern distribution showed that in all the hydrolysates, the observed peptide bands had molecular weight (MW) of less than 166 kDa, which were different from the fish muscle which had two major bands with molecular weights of 212 and 166 kDa (Figure 3). At 30 min of hydrolysis, heavy chains (~212 kDa) were completely broken down into smaller MW peptides. As the proteolytic breakdown progressed, peptides with MW <14.4 kDa were more obvious. This could be related to the degree of hydrolysis which increased with the time of hydrolysis.
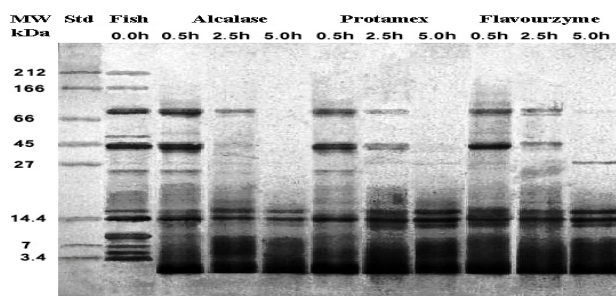
Figure 3. SDS-PAGE patterns of protein hydrolysates from using Alcalase, Protamex and Flavourzyme during three different time of hydrolysis. (MW) Molecular weight markers

The intensity of molecular masses equal or larger than 45 kDa decreased in all the hydrolysates significantly at 2.5 h compared to 0.5 h of hydrolysis. All hydrolysates had several intense bands between 14.4 kDa and 27 kDa in the 2.5 h of hydrolysis. Disappearance of peptides with apparent MW of 27 kDa was observed after 5.0 h of hydrolysis with the exception of the Flavourzyme hydrolyzed samples. Similar trends had been reported by other researchers for hydrolysates prepared from salmon (Kristinsson and Rasco, 2000b) and catfish (Theodore *et al.*, 2008).

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical-scavenging activity

The scavenging activity of protein hydrolysates compared to vitamin C for DPPH radical is shown in Table 1. DPPH activity of vitamin C did not shown significant change throughout the hydrolysis period. All treatments produced hydrolysates had significantly ($P < 0.05$) higher DPPH activities compared to tilapia muscle. Hydrolysates prepared using Alcalase showed significantly (P < 0.05) higher DPPH activities as compared to Flavourzyme and Protamex hydrolysates. The Scavenging capacity of AH was the highest with EC_{50} value of 4.45 (mg/ml) at 4.0 h of hydrolysis followed by FH with the EC_{50} value of 32.21 (mg/ml). Statistically, there was no significant difference in the scavenging activities between Flavourzyme and Protamex hydrolysates.

The result thus showed that the red tilapia hydrolysates contained substances which were electron donors and could react with free radicals to convert them to more stable product. Therefore, the produced hydrolysates could be a potential source of antioxidant in the diet. Ngo *et al*. (2010) suggested that peptide derived from Nile tilapia (*O. niloticus*) scale gelatin showed properties against oxidative stress and could be used as a functional food ingredient.

Table 1. Scavenging capacity of DPPH radical of fish protein hydrolysates

Time of Hydrolysis	EC_{50} (mg/ml)				
	No treatment	Alcalase	Flavourzyme	Protamex	Vit C
0.5 h	351.31 ± 4.49[aA]	46.23 ± 2.01[aB]	189.74 ± 2.65[aC]	199.49 ± 3. 2[aC]	3.63 ± 0.02[aD]
1.0 h	338.53 ± 3.23[bA]	25.29 ± 1.1[bB]	112.31 ± 1.82[bC]	109.22 ± 2.73[bC]	3.55 ± 0.21[aD]
2.0 h	334.53 ± 4.2[bA]	18.72 ± 2.5[bB]	74.91 ± 3.2[cC]	84.23 ± 3.8[cC]	3.8 ± 0.1[aD]
3.0 h	331.57 ± 3.7[bA]	8.980 ± 1.2[cB]	39.46 ± 1.42[dC]	43.15 ± 2.5[dC]	3.89 ± 0.13[aD]
4.0 h	318.53 ± 2.21[cA]	4.45 ± 0.2[dB]	32.21 ± 0.93[dC]	35.63 ± 1.62[dC]	3.91 ± 0.18[aB]
5.0 h	327.21 ± 3.56[dA]	5.95 ± 0.75[dB]	34.52 ± 1.83[dC]	37.82 ± 2.15[dC]	3.85 ± 0.09[aD]

Values were means ± SD from triplicate determinations.
Means within each row sharing same upper case were not significantly different ($P < 0.05$).
Means within each column sharing same lower case were not significantly different ($P < 0.05$).

Amino acid composition

Amino acid compositions of the fresh tilapia muscle and its hydrolysates are shown in Table 2. In all the hydrolysates, total amino acids increased compared to tilapia muscle, especially for the Alcalase hydrolysate, a significant increased was observed ($P < 0.05$). There were significant differences ($P < 0.05$) between the hydrolysates and the muscle for glutamic acid, theronine, valine, glycine, alanine, and arginine. The hydrolysates were higher in arginine, tyrosine, and methionine but lower in aspartic acid, serine and histidine as compared to fish muscle. Glycine, in particular (50% reduction) was decreased in all the hydrolysates compared to the fish muscle.

The ratio of essential amino acids to non-essential amino acids was increased after hydrolysis. It was 0.5, 0.63, 0.60 and 0.64 for fish muscle, AH, PH and FH, respectively. This finding was close to the report of Klompong *et al*, (2009), which showed that yellow stripe trevally (*selaroides leptolepis*) protein hydrolysates had a higher ratio of essential amino acids to non-essential amino acids compared to the flesh.

Shahidi and others (1995) reported that the composition of protein hydrolysates depended on the type of enzyme used. Changes in size, level and composition of amino acids and small peptides affected the antioxidative activity (Wu *et al.*, 2003). Aromatic amino acids (tyrosine, histidine, tryptophan, and phenylalanine) (Chen *et al.*, 1998; Rajapakse *et al.*, 2005), hydrophobic amino acids (valine, leucine, and alanine) (Rajapakse *et al.*, 2005), and also methionine (Mendis *et al.*, 2005), were reported to play a vital role in antioxidative activities. The

Table 2. Amino Acid composition of fish protein hydrolysates

Amino Acid (g/100 g)[1]	No treatment	Amino Acid in different hydrolysates (g/100 g)		
		Alcalase	Protamex	Flavourzyme
Aspartic acid	6.95±0.20[a]	6.10±0.08[b]	6.03±0.09[b]	5.18±0.03[c]
Serine	3.07±0.08[a]	2.88±0.02[b]	2.67±0.01[c]	2.27±0.01[d]
Glutamic acid	8.18±0.37[a]	13.54±0.15[b]	13.09±0.21[b]	13.81±0.02[b]
Glycine	9.30±0.16[a]	4.41±0.04[b]	4.55±0.06[b]	4.17±0.002[b]
Histidine	4.27±0.04[a]	2.55±0.01[b]	2.02±0.008[c]	1.96±0.015[c]
Arginine	5.33±0.14[a]	6.25±0.02[b]	6.15±0.05[b]	6.52±0.02[b]
Threonine	3.52±0.10[a]	7.38±0.02[b]	7.27±0.02[b]	7.51±0.01[b]
Alanine	4.89±0.11[a]	4.39±0.01[b]	4.36±0.05[b]	4.34±0.003[b]
Proline	2.66±0.03[a]	2.76±0.1[a]	2.72±0.01[a]	2.79±0.003[a]
Cysteine	1.26±0.03[a]	1.36±0.002[b]	1.29±0.007[a]	1.38±0.001[b]
Tyrosine	5.59±0.16[a]	5.69±0.01[a]	5.71±0.03[ab]	6.05±0.004[b]
Valine	1.88±0.06[a]	2.65±0.005[b]	2.49±0.006[c]	2.71±0.000[b]
Methionine	0.90±0.01[a]	1.71±0.00[b]	0.99±0.000[c]	0.97±0.004[c]
Lysine	7.17±0.27[a]	6.40±0.18[b]	6.81±0.15[b]	7.22±0.06[a]
Isoleucine	3.27±0.10[a]	3.26±0.008[a]	2.86±0.02[b]	3.32±0.01[a]
Leucine	6.24±0.22[a]	6.87±0.01[b]	6.51±0.07[ab]	6.94±0.01[b]
Phenylalanine	2.90±0.08[a]	3.01±0.006[a]	2.45±0.01[b]	2.37±0.006[b]
Total	77.37	81.22	77.97	79.51

[1]Values were means ± SD of duplicates.

hydrolysis conditions employed in the processing of protein isolates, the type of protease and the degree of hydrolysis also affected the antioxidant activity (Pena-Ramos *et al*., 2002) as well as the levels and compositions of amino acids sequences and peptides (Wu *et al*., 2003).

Conclusions

Amino acid composition, peptide patterns and radical scavenging activities of AH, PH and FH were different. All the three hydrolysates had higher DPPH activities than unhydrolysed tilapia muscle. The Alcalase hydrolysates exhibited significantly ($P < 0.05$) higher radical scavenging capacity compared to Protamex and Flavourzyme hydrolysates. The highest activity was exhibited by AH. The type of protease employed in the processing of protein hydrtlysis, significantly affected on the molecular weight of peptides produced in SDS-PAGE, scavenging capacity, as well as the total released amino acids after hydrolysis.

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