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Effects of Enzymatic Hydrolysis on the Antioxidative and Antihypertensive Activities from Red Tilapia Fish Protein

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Abstract

In this research, activities of antioxidative and antihypertensive peptides, derived from Red Tilapia meat protein (*Oreochromis niloticus*) by alcalase and thermolysin enzymes were evaluated. The hydrolysis process was performed from 0 - 4 hours at 37°C, pH 7.4. Two hours of hydrolysis with thermolysin and alcalase resulted in degree of hydrolysis of 76.29% and 63.49%, respectively. Hydrolysates obtained after 1 hour and 2 hours hydrolysis were chosen for further study on the bioactive activities. Results showed that thermolysin enzyme yielded higher antioxidant activities than alcalase enzyme based on ABTS and reducing power assays, before and after ultrafiltration for concentrated cut-off interval of hydrolysates. For antihypertensive assay, thermolysin enzyme yielded higher inhibition of ACE enzyme activities after 1 hour hydrolysis while alcalase enzyme yielded higher inhibition activities after 2 hours hydrolysis. Chosen cut-off interval of hydrolysates showed that, thermolysin hydrolysates have a strong inhibition effects towards ACE enzyme than alcalase hydrolysates. Based on SDS-PAGE test, the hydrolysates obtained by alcalase appeared as a smear and have concentrated the most with molecular weight within range ≤14 kDa. Hydrolysates obtained by thermolysin appeared as a band and have concentrated the most with molecular weight of 14 kDa and 3 kDa. Hydrolysates from Red Tilapia may contribute as a health promoting ingredient, to improve shelf-life of functional food and as an ingredient which have the antihypertensive effects towards mild hypertension patients, in functional foods.

Keywords: Alcalase; Thermolysin; Antioxidative; Antihypertensive; Protein hydrolysate

Introduction

Peptides containing antioxidative and antihypertensive activities have been focused by researchers, mainly due to their involvement in food quality and health of consumers. Antioxidative peptide could prevent the occurence of oxidation process and further improve shelf-life of the foods involved. Antihypertensive peptide would act as an inhibitor to ACE enzyme, the main protein component in hypertension reactivity. This bioactive peptide can be used as a functional ingredient in food component and to be delivered to consumers [1].

In previous study, this bioactive peptide was determined in various source of protein like plant, milk, egg, meat, chicken and fish [2-5]. Considering fish having a higher biological value than other protein sources, the specific components of fish protein like sarcoplasmic, myofibrillar, collagen, skin, bone and visceral protein had been extracted to study the bioactive activity from the peptides obtained [4,6-9]. The studies resulted in varied range of value on bioactivities depending on the source of sample used.

Extraction process to purify specific components of fish protein consuming time. The yield of extraction obtained usually is very low. Fortunately, the activity of bioactive peptide from different component of fish protein can be estimated and compared based on the studies conducted. Fish-derived bioactive peptides based on their structural properties, amino acid composition and sequences, may be involved in various biological functions. The use of fish protein as a whole as in fish fillet might carry a higher value of bioactivities. Red Tilapia used in this study was chosen due to the growing demand for live fish and tilapia fillet in domestic market. Thus, Red Tilapia is more readily available than other freshwater fish.

Bioactive peptides can be produced by solvent extraction, enzymatic hydrolysis or microbial fermentation of food proteins. However, the enzymatic hydrolysis method is much preferred because other methods can leave residual organic solvents or toxic chemicals in the products [10]. Most study showed that alcalase and thermolysin enzymes used in preparation of protein hydrolysates have higher activity of antioxidative and antihypertensive, respectively, instead of other enzymes. In this study, the alcalase and thermolysin were compared to determine the antioxidative and antihypertensive activities obtained from hydrolysates of Red Tilapia whole protein.

Materials and Methods

Chemicals and reagents

Thermolysin (Bacillus thermoproteolyticus rokko, EC3.4.24.27), Alcalase® (Bacillus licheniformis, ≥2.4 AU/g), trichloroacetic acid (TCA), o-phthaldialdehyde (OPA), 1,1-diphenyl-2-picryl-hidrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and other chemicals of analytical grade were purchased from Sigma (Sigma–Aldrich Chemical Co., St Louis, MO, USA). Ultrafiltration membranes of 10 kDa (Vivaflow 200) and 3 kDa (Vivaflow 50) molecular weight cut-off (MWCO) were purchased from Sartorius (Vivaflow, Sartorius, Germany).

Protein hydrolysate preparation, degree of hydrolysis and quantification of peptide.

Red Tilapia (*O. niloticus*) was obtained at Kajang Wet Market,

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Selangor. For hydrolysis process, the fish fillet was mixed with distilled water (2:100 w/v) and was adjusted to optimal pH and temperature for thermolysin and alcalase (pH 7.4; 37°C). Ratio of enzyme to sample was 1:100 (v/w). The hydrolysis was performed for 0 to 4 hours in a shaking waterbath incubator. Enzyme reaction was inactivated with temperature at 90°C for 10 minutes. The samples were then centrifuged at 3000 × *g* for 20 minutes and the soluble hydrolysates were freeze-dried.

Degree of hydrolysis (DH) of protein hydrolysates obtained was analyzed according to percent of trichloroacetic acid (TCA) method as described by Hoyle and Merritt [11]. The supernatant was analyzed for soluble nitrogen using Kjeldahl method (Kjeltec 2100, Foss, Denmark). The percent DH was expressed as follows:

%DH=(10% TCA - soluble N in sample/total N in sample) × 100

Peptide content were measured by method of Church et al. [12] with some modifications using o-phthaldialdehyde (OPA) spectrophotometric assay. 50 µl of hydrolysate containing 5-100 µg protein was mixed with 2 ml of OPA reagent and incubated for 2 min at ambient temperature. The absorbance at 340 nm was measured with a spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). Leucine was used as a standard.

Measurement of antioxidant and ACE inhibition activities upon the fish peptide.

DPPH (1,1-diphenyl-2-picryl-hidrazyl) radical-scavenging activity of both enzymatic hydrolysates were determined by DPPH assay, as described by Binsan et al. [13]. To the sample (1.5 ml), 1.5 ml of 0.15 mM DPPH in 95% ethanol were added. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a UV-1800 spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The activity was expressed as µmol Trolox equivalents (TE)/g protein of hydrolysate.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical-scavenging activity of both enzymatic hydrolysates were determined by ABTS assay, as described by Binsan et al. [13]. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hours at room temperature in the dark. Sample (150 µl) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 hours in the dark. The absorbance was then measured at 734 nm using a spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The activity was expressed as µmol Trolox equivalents (TE)/g protein of hydrolysate.

Ability of the hydrolysates to reduce $Ferro^{3+}$ to $Ferro^{2+}$ was measured spectrophotometrically by the method of Oyaizu [14] with some modifications. A volume of 2 mL of sample (5 mg/mL) was mixed with 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After that, 2 mL 10% TCA was added to the reaction. Then 2 mL from each incubated mixture was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride in test tubes. After a 10 min reaction, the absorbance of the resulting solution was measured at 700 nm with spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). Increase in absorbance of the reaction indicates an increased reducing power.

Activity of ACE inhibition was measured by the method of Belovic et al. [15]. 50 µL of ACE solution (100 mU/mL) was incubated with 50 µL of borate buffer (or tested sample, 1 mg/ml) at 37°C for 10 min. After the addition of 150 µL of substrate (8.3 mM Hip His Leu in borate buffer), the reaction mix was incubated for 80 min at 37°C. The reaction was terminated by the addition of 250 µL of 1 M HCl. The resulting hippuric acid was extracted with 3 × 500 µL of ethyl acetate and centrifugated at 800 g for 15 min. 750 µL of the upper layer was transferred into test tube and evaporated under air flow at 37°C. The hippuric acid was dissolved in 1 mL of distilled water, and the absorbance was measured at 228 nm using spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan).

Ultrafiltration and measurement of molecular weight distribution

Hydrolysate solution was filtered by 0.2 µm membrane and separated into large and small molecular weight fractions by ultrafiltration at 4°C using 10 kDa molecular weight cut-off (MWCO) membrane (Vivaflow 200, Sartorius, Germany) followed by 3 kDa MWCO (Vivaflow 50) membrane to enrich specific hydrolysate fractions. Both membranes were activated by spinning 100 ml of deionized water prior to use. This permeate was defined as small peptides with molecular weight less than 3000 Da. The eluent was then lyophilised and kept for use in further experiment.

The molecular weight distribution of red tilapia protein hydrolysates at different DH with concentration of sample 10 mg/ml, were analysed by Trycine– SDS–PAGE according to Schägger and Von Jagow [16], using a 5% stacking gel and a 16% resolving gel.

Statistical analysis

All data collected was analyzed using analysis of variance (ANOVA) and Duncan's multiple range tests. Significant differences in means between samples were determined at 5% confidence level ($P<0.05$).

Results and Discussion

Degree of hydrolysis and peptide content

Specific activities of antioxidative and antihypertensive peptides are closely related to degree of hydrolysis of the parent protein. The yield of enzymatic hydrolysis in this study showed that DH sample is higher with thermolysin enzyme than alcalase enzyme, as shown in Table 1. DH for both hydrolysates, with thermolysin and alcalase, after two hours had achieved 76.29% and 63.49%, respectively. Quantification of peptide content was done to observe the increasing chain of peptides in hydrolysates produced within the range of hydrolysis time and enzymes used. The content of peptides in the varied time was directly proportional to the increased of protein DH, as shown in Table 1.

Hydrolysis time (hour)	Thermolysin		Alcalase	
	DH (%)	Peptide content (mg/ml)	DH (%)	Peptide content (mg/ml)
0.5	27.41	2.62 ± 0.17^{d}	26.85	2.03 ± 0.11^{d}
1	47.3	4.78 ± 0.15^{c}	34.19	3.35 ± 0.07^{c}
2	76.29	7.92 ± 0.03^{b}	63.49	6.53 ± 0.23^{b}
3	88.01	9.20 ± 0.25^{a}	80.66	8.40 ± 0.13^{a}
4	92.58	9.70 ± 0.28^{a}	85.3	8.90 ± 0.27^{a}

^{abcd}Different lowercase letters indicate significant differences ($P<0.05$) between samples (n=2).

Table 1: Effect of hydrolysis time and different enzymes (Thermolysin and Alcalase) on degree of hydrolysis and peptide content of *O. niloticus* protein hydrolysate (n=2).

Decrease in the rate of hydrolysis as in the third hours and further can be explained with the inhibition of enzyme used by the hydrolysate itself which formed at the high degree of protein hydrolysis. The hydrolysis products tend to act as a potent competitive substrates towards unfinished and partially hydrolysed fish protein [17]. Rate and pattern of protein hydrolysis are dependants on cutting sites of enzyme and the ability of the enzyme to be attached to peptide bonds involved. Based on Kilic-Apar and Ozbek [18], alcalase has a high ability in hydrolysis and solubilize protein compared to others. Following the specifications of enzyme cutting site, alcalase has a random and broad range than thermolysin which has much specific site.

Thermolysin showed a higher capacity of protein hydrolysate production than alcalase enzyme. DH with thermolysin demonstrate that this enzyme have a much better binding site and more efficient than alcalase. Yet the difference in cutting site between enzymes can affect the function of protein hydrolysate obtained. Bioactivity of peptide is dependent on the sequence of amino acids in hydrolysate produced at certain time which carry the activity of interest. Hydrolysates from both enzymes at one and two hours was chosen based on the DH obtained for further analysis in determination of antioxidative and antihypertensive activity.

Antioxidant activities from fish peptides

DPPH radical scavenging method has generally been used to demonstrate the ability of hydrogen donor by protein hydrolysates to scavenge the free radical. As shown in Table 2, antioxidative activities are higher in hydrolysates at one hour for both enzymes at 1 mg/ml thermolysin and alcalase at 38.98% and 38.89%, respectively. Hydrolysates with size cut-off 10 kDa and below showed high antioxidative activity with alcalase than thermolysin. On the contrary, hydrolysates with size cut-off 3 kDa and below showed otherwise. Based on the value of antioxidative activities, results showed that most hydrolysates with high activities was within size cut-off 10 kDa, which contain more bound amino acids and potentially involved in scavenging activity.

Study by Bougatef et al. [19] reported that hydrolysates obtained with low molecular weight of protease showed the highest radical scavenging activity, 76.6% with concentration 3 mg/ml, compared to other enzymes. Foh et al. [20] showed the hydrolysates with alcalase have a higher scavenging activities than flavourzyme and nutrease, 86.67%, with concentration 5 mg/ml. The involvement of enzyme that can produce low molecular weight of peptides seems to give a potentially high antioxidative activity. In this study, thermolysin showed a high antioxidative activity compared to alcalase enzyme.

Relative ability of antioxidative peptides have been measured by ABTS radical scavenging activity [21]. Hydrolysate with thermolysin gave a higher scavenging activity than alcalase for one and two hours of hydrolysates, at 1 mg/ml, as shown in Table 2. Studies at low molecular weight cut-off (10 kDa and 3 kDa) also showed that hydrolysates with thermolysin gave a higher scavenging activity than alcalase enzyme.

According to Tang et al. [5], the capacity of ABTS radical scavenging which soluble in water is not dependent on molecular weight of the hydrolysates. Furthermore, the ability to scavenge DPPH radical which soluble in ethanol and O_2 is dependent on molecular weight of the hydrolysates. Previous studies have showed a mixture of varied molecular weight of protein fractions had given a contribution in bioactivity of peptides. The studies had proved that protein fractions containing low molecular weight generally have the antioxidative peptides which potentially gave a high antioxidative activities [22-24]. In this study, hydrolysates after one hour hydrolysis already have high scavenging activity compared to the parent protein and hydrolysates with molecular weight cut-off 3 kDa and below.

Reducing power capacity of a compound can be a significant indicator on a potential antioxidative activity within the compound involved [25]. Hydrolysates with reducing ability may reduce ferricyanide complex (Fe^{3+}) to a ferrous form (Fe^{2+}) [19]. In this study, hydrolysates with thermolysin showed higher reducing power capacity than alcalase enzyme with concentration 2 mg/ml, as shown in Figure 1. Hydrolysates with thermolysin at both molecular weight cut-off (10 kDa and 3 kDa) also was higher than alcalase enzyme. At molecular weight cut-off 3 kDa, hydrolysates with thermolysin gave a significant higher than alcalase on reducing power capacities.

A compound with reducing power ability belongs to an electron donor group and can reduce the intermediate oxidative species in lipid oxidation process. This compound can also act as a primary and secondary antioxidant. Results obtained in this study again demonstrated that hydrolysates after one hour of hydrolysis without ultrafiltration had reducing capacity nearly similar with hydrolysates of molecular weight cut-off 10 kDa and below, as in the DPPH and ABTS radical scavenging activities.

Wang et al. [26] reported that protein hydrolysates obtained also gave a high antioxidative activities after one hour hydrolysis whereas after two hydrolysis, the activities had drop and remain low in further hydrolysis. According to Zhu et al. [27], hydrolysates with low molecular weight that can give a potentially high reducing activities showed the ability of the hydrolysates to bind ferum and pro-oxidative ferum and further reduce their oxidation power in oxidation process.

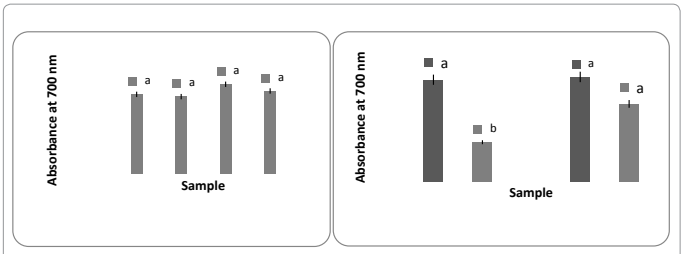
ACE inhibition activity from fish peptides

Antihypertensive assay was done based on hydrolysis of 'hippuryl-L-histidyl-L-leucine' (HHL) by angiotensin converting enzyme (ACE). The inhibition activity by hydrolysates from red tilapia fillet was shown in Table 3. Hydrolysates with thermolysin after one hour hydrolysis showed higher inhibition activities than two hours hydrolysis (79.41%), while hydrolysates with alcalase after two hours hydrolysis showed

Samples	Size interval	DPPH activity (%)		ABTS activity (%)	
		Thermolysin	Alcalase	Thermolysin	Alcalase
Native protein	-	12.31 ± 2.03^{d}		14.81 ± 0.69^{d}	
Hydrolysates After hydrolysis.	1	38.98 ± 0.70^{b}	38.89 ± 0.71^{b}	44.78 ± 0.06^{b}	40.41 ± 0.16^{b}
(chosen time interval (hour))	2	34.70 ± 0.52^{c}	31.09 ± 0.66^{c}	37.82 ± 0.09^{c}	34.64 ± 0.13^{c}
After ultrafiltration	10 kDa	42.51 ± 0.67^{a}	44.30 ± 0.01^{a}	54.00 ± 0.01^{a}	44.60 ± 0.04^{a}
(diff. membrane cut-off (MWCO))	3kDa	14.70 ± 0.01^{d}	11.50 ± 0.06^{e}	20.00 ± 0.07^{d}	13.60 ± 0.03^{e}

^{abcde}Different lowercase letters indicate significant differences (P<0.05) between samples.

Table 2: Effect of hydrolysis time and low molecular weight (after one hour hydrolysis of protein) with different enzymes on anti-oxidant activity of *O. niloticus* protein hydrolysate with DPPH and ABTS assay (n=3).



[ab]Different lowercase letters indicate significant differences (P<0.05) between samples.
a is the hydrolysate by alcalase after 1 hour hydrolysis; a is the hydrolysate by alcalase after 2 hours; a is the hydrolysate by thermolysin after 1 hour; a is the hydrolysate after 2 hours; a is the hydrolysate by alcalase with 10 kDa molecular weight (MW) cut-off; b is the hydrolysate by alcalase with 3 kDa MW cut-off; b is the hydrolysate by thermolysin with 10 kDa MW cut-off; b is the hydrolysate with 3 kDa MW cut-off.

Figure 1: Effect of hydrolysis time and different enzymes on antioxidative activity of *O. niloticus* protein hydrolysate with reducing power assay; (b) Antioxidative activity on low molecular weight of *O. niloticus* protein hydrolysate after one hour hydrolysis with reducing power assay (n=3).

Samples	Size interval	Inhibition activity (%)	
		Thermolysin	Alcalase
Hydrolysates after hydrolysis.	1	79.41 ± 0.14[a]	55.15 ± 0.25[b]
(chosen time interval (hour))	2	58.09 ± 0.11[d]	78.68 ± 0.17[a]
After ultrafiltration	10 kDa	60.30 ± 0.23[c]	22.11 ± 0.11[d]
(diff. membrane cut-off (MWCO))	3kDa	66.33 ± 0.15[b]	37.19 ± 0.13[c]

[abcde]Different lowercase letters indicate significant differences (P<0.05) between samples.

Table 3: Effect of hydrolysis time and low molecular weight (after one hour hydrolysis of protein) with different enzymes on antihypertensive activity of *O. niloticus* protein hydrolysate by inhibition of ACE enzyme (n=3).

high inhibition activities than one hour hydrolysis, (78.68%). Studies on low molecular weight cut-off showed the hydrolysates with 3 kDa and below have the inhibition activities better than 10 kDa and below for both hydrolysates (with thermolysin and alcalase).

Based on the results obtained, the use of thermolysin enzyme to produce ACE inhibitory peptides is more efficient than alcalase. Ghassem et al. [6] explained that thermolysin enzyme may produce a shorter sequence of peptide, which contribute better in ACE inhibitory activity and the peptides also usually incorporated in low molecular weight of peptides (≤3 kDa). Another study had been conducted on peptides with various range of molecular weight cut-off, 10, 5, 3 dan 1 kDa, by Campos et al. [28]. The results showed that the most high molecular weight cut-off have the lowest activities while the most low molecular weight cut-off have the highest activities on inhibition of ACE enzyme.

Molecular weight distribution

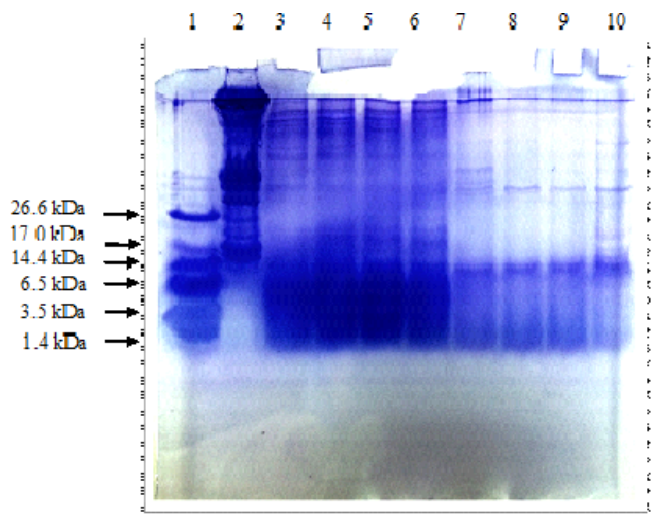
Polyacrylamide gel electrophoresis test with sodium dodecyl sulphate (SDS-PAGE) have been conducted to observe the distribution of molecular weight of the hydrolysates obtained. Results on electrophoresis gel were presented in Figure 2. Hydrolysates with alcalase had appeared as a smear (containing a broad range and varied molecular weight) and concentrated within the range of 16.6 kDa and ≤9.1 kDa. Hydrolysates with thermolysin had appeared as a band (containing a much uniform molecular weight of peptides) and concentrated around 9.1 kDa and 1.4 kDa, respectively.

The results obtained through electrophoresis gel showed that the production of hydrolysates with thermolysin may give much uniform molecular weight of peptides than alcalase. Antioxidative and antihypertensive activities from thermolysin's peptides also was higher than alcalase's peptides. The yield of alcalase's peptides can be referred to the ability of alcalase in hydrolysis process with a broad range of specificity towards the peptide bond especially residues without big charges [29]. Due to this characteristic, the use of alcalase enzyme had resulted in hydrolysates with a complex molecular weight and further appeared as a smear on the gel.

Furthermore, although the intensity of smear by alcalase's hydrolysate was clearer than the band by thermolysin's hydrolysate, which shows high content of peptides, the antioxidative and antihypertensive activities seems to be higher from thermolysin's hydrolysate than alcalase's hydrolysate. These results proved that the bioactivities involved within the hydrolysates are mainly based on the sequence of peptides within the hydrolysates produced at a specific DH.

Conclusion

Protein hydrolysates of Red Tilapia (*O. niloticus*) have demonstrated some potential antioxidative and antihypertensive activities. Based on the results above, high antioxidative and antihypertensive activities and uniform distribution of molecular weight of peptides were obtained with thermolysin at one hour of hydrolysis. Hydrolysates produced by thermolysin enzyme after one hour hydrolysis showed significant difference with alcalase in the antioxidative and antihypertensive assay. Freshwater fish protein hydrolysates could be used as a health enhancing ingredient in functional foods and can be considered to replace synthetic chemicals that are generally used in food industry, nutraceutical and pharmaseutical need.



1: C=standard protein; 2: Protein before hydrolysis; 3: Hydrolysate by alcalase after 1 hour hydrolysis; 4: Hydrolysate by alcalase after 2 hours hydrolysis; 5: Hydrolysate by alcalase after 3 hours hydrolysis; 6: Hydrolysate by alcalase after 4 hours hydrolysis; 7: Hydrolysate by thermolysinafter 1 hour hydrolysis; 8: Hydrolysate by thermolysinafter 2 hours hydrolysis; 9: Hydrolysate by thermolysinafter 3 hours hydrolysis; 10: Hydrolysate by thermolysinafter 4 hours hydrolysis.

Figure 2: Analysis of molecular weight distribution for *O. niloticus* protein and protein hydrolysates.

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