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Potravinarstvo Slovak Journal of Food Sciences
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Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 14, 2020, p. 379-384
https://doi.org/10.5219/1264
Received: 23 December 2019. Accepted: 26 February 2020.
Available online: 28 June 2020 at www.potravinarstvo.com

ISSN 1337-0960 (online)

ANTIOXIDANT ACTIVITIES OF SNAKEHEAD (*CHANNA STRIATA*) FISH SKIN: PEPTIDES HYDROLYSIS USING PROTEASE TP2 ISOLATE FROM SWAMP PLANT SILAGE

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ABSTRACT

The purpose of this research was to study the antioxidants activities of peptides from skin fish of snakehead (*Channa striata*), using hydrolysis of protease TP2 isolate from swamp plant silage. This research 5 treatments hydrolysis time (0, 30, 60, 90, 120 min, respectively), with two replicates, which included several stages of preparation and pre-treatment of the snakehead fish skin production of protease enzymes which were isolated from swamp water, preparation of protein hydrolysates, measurement of hydrolysis degrees, analysis of peptides content and analysis of the antioxidant activity. Results showed that the treatment had given a significant effect on the 5% level of the degree of hydrolysis production (13.98% – 27.08%), with peptides content of 2.73% – 3.78% and antioxidant activity (10.75% – 20.7%). The results of the degree of hydrolysis indicate that the longer the hydrolysis time, the percent degree of hydrolysis will increase. Peptide content and antioxidant activity were increased with increasing hydrolysis time.

Keywords: hydrolysis time; protein hydrolysates; skin; snakehead (*Channa striata*); antioxidant

INTRODUCTION

Snakehead fish in South Sumatra is generally used as basic ingredients of the typical Palembang food industry, namely pempek, kerupuk, and kemplang. The processing process produces waste, one of which is skin waste. The waste is still underutilized due to lack of technological equipment, low commercial value, and a lack of application to the waste **(Blanco et al., 2007)**.

Unused waste contains very important nutritional compounds such as protein content (collagen and keratin) and mineral composition **(Moller et al., 2008)**. This waste has the potential to be used as a protein hydrolysate containing bioactive peptides. Several studies have shown that fish protein hydrolysates have functional properties as antihypertensive, anticancer, antimicrobial, and antioxidant. Bioactive peptides can be obtained by several methods of hydrolysis, namely hydrolysis with digestive enzymes and hydrolysis by proteolytic enzymes produced by microorganisms or plants **(Korhonen, 2009)**.

The bioactive activity of peptides is very diverse and is determined by the sequence of amino acids that make up it. Some bioactive peptides can be precursors of proteins or peptides that will be active when hydrolysed from natural proteins through enzymatic hydrolysis in the digestion, fermentation, and processing processes **(Korhonen, 2009)**. Bioactive peptides have several mechanisms of antioxidants, among others: as radical scavenging (free radical deterrent), a mineral chelating, metal-reducing agents and protectors **(Elias et al., 2008)**. The antioxidant activity of bioactive peptides is strongly influenced by the natural nature and composition of the relevant peptide fragments **(Phelan et al., 2009)**. This is very much determined by the specificity of the protease enzyme used **(Korhonen and Pihlanto, 2006)**. The potential for peptides as antioxidants is not only limited to the prevention of risk factors for degenerative diseases, but also for cosmetic composition and food preservation **(Samaranayaka and Li-Chan, 2011)**.

Proteases are hydrolytic enzymes that can break down peptide bonds between amino acids. Protease enzymes hydrolyse peptide bonds specifically from their original proteins, then produce peptides with sequences and diverse functional properties **(Gonzalez-Rabade et al., 2011)**. One source of proteases are some microorganisms that have been known to produce proteases for commercial applications are *Bacillus, Lactobacillus, Pyrococcus, Termonospora, Rhizopus, Mucor, Endothia* and *Aspergillus* **(Rao et al., 1998)**. In this study using TP2 isolates from swamp plant silage which had a high protease enzyme activity **(Baehaki et al., 2018)**. Protease enzymes are used to hydrolyse proteins in snakehead fish skin and then the hydrolysates produced are tested for antioxidant activity. Antioxidants are known to inhibit the work of free radicals so that the search for antioxidants

from snakehead fish skin is an effort to optimize the use of natural materials in Indonesian waters.

Scientific hypothesis

Protease from swamp plant silage isolates can be used for hydrolysis of snakehead fish skin (*Channa striata*) to produces peptide with antioxidant activity. Degree hydrolysis, peptide content and antioxidant activity increase with increasing hydrolysis time.

MATERIAL AND METHODOLOGY

Materials

The materials used in this study were snakehead fish (*Channa striata*) size ±500 g (weight), TP2 isolate, trichloroacetic acid (Merck, Germany), NaOH (Merck, Germany), DPPH and nutrient Agar (Merck, Germany). The tools used include pH meter, OHAUS analytical balance, incubator, micropipette (Single Channel Capp 10-100 Ul, USA), autoclave (Hirayama, Japan), hotplate (Cimarec, United Kingdom) and spectrophotometer.

Methods

Preparation of Snakehead fish Skin

The preparation of snakehead fish skin is done according to the method of **Liu et al. (2015)**. Preparation is done by separating the skin from other parts such as scales and the rest of the meat. The skin is cut to the size of approximately 1 x 1 cm^2 using scissors. The first stage is the pre-treatment process with NaOH solution which aims to eliminate non-collagen proteins and other impurities such as fat, minerals, pigments, and odours. The fish skin of *Channa striata* is soaked in NaOH solution with a concentration of 0.05 M for 6 h and every 2 h the NaOH solution is replaced with the ratio between the skin and NaOH solution is 1:10 (w/v). The fish skin of *Channa striata* immersed in selected NaOH is washed to near neutral pH.

Refresher Culture

The way the culture of refresher works is as follows: The culture used in this study was in the form of culture stored in the refrigerator. Therefore, the culture must be refreshed first. Approximately 1 ose was scratched after being transferred into a test tube containing 10 mL of sterile NB media, then incubated at 37 ℃ for 24 h. Furthermore, 0.1 mL of the test tube containing the culture was taken and put in another test tube containing 10 mL of sterile LB media to be incubated at 37 ℃ for 24 h. Incubated cultures are ready for use.

Protease Production from TP2 Isolate

The production of protease enzymes uses the method of **Baehaki, Rinto and Budiman (2011)** modified, carried out as follows: TP2 isolates were inoculated on 10 mL of Luria Bertani Broth (LB) media with 1% tripton composition, 1% NaCl, and 0.5% yeast extract. LB media taken 10% of the amount of media then added to the new Luria Bertani Broth (LB) media as a medium for producing proteases. The media is then incubated in the shaker incubator for 45 h, at 37 ℃ at a speed of 120 rpm. The extraction of the protease enzyme was carried out by centrifuging the medium of bacterial growth at a speed of 3000 rpm for 15 min at 4 ℃. The supernatant is an enzyme extract that will be used to hydrolyse protein.

Hydrolysates Production

The preparation of protein hydrolysates was carried out according to the method of **Bhaskar et al. (2008)**. The raw material in the form of fish skin pre-treatment has been mixed with a pH 7 buffer until homogeneous by comparison (1:10). The protease enzyme is added with a concentration of 20% (v/v). The mixture is then hydrolysed at 55 ℃ for 0, 30, 60, 90, and 120 min using a water bath shaker, during the hydrolysis process the sample is stirred regularly. The results of hydrolysis are included in the water bath to inactivate the enzyme at a temperature of 85 ℃ for 20 min. Samples were centrifuged for 20 min at 10 ℃ with a speed of 6000 rpm to separate the dissolved fraction (supernatant) and the non-soluble fraction (pellet). The protein hydrolysate of snakehead fish skin (*Channa striata*) produced was frozen, before being used analysed.

Degree of Hydrolysis.

The degree of hydrolysis is calculated based on the percentage ratio of trichloroacetic acid (TCA) according to the method of **Hoyle and Merritt (1994)**. 20 mL of protein supernatant/hydrolysate added 20% TCA (w/v) as much as 20 mL. Then was centrifuged at a speed of 8,000 rpm at 4 ℃ for 10 min. The resulting supernatant was analysed for protein content. The degree of hydrolysis can be calculated using the following formula:

$$\% \text{ DH} = \frac{\text{100 x dissolved nitrogen in TCA 10\%}}{\text{Total nitrogen in the sample}}$$

Analysis of Peptide Content

Analysis of extract peptide levels was carried out using the formol titration method **(Wikandari and Yuanita, 2016)**, as follows: A total of 5 mL extracts of the sample are put in 100 mL Erlenmeyer. Then extract the sample added 10 mL aqua bides and ±0.5 mL PP indicator. Then the sample was titrated with 0.1 N NaOH until it is pink. Samples added 1 mL of 40% formaldehyde solution and titrated with NaOH.

$$\%N = \frac{a}{B \times 10} \times \text{NaOH} \times \text{Ar N} \times \text{FP}$$

Where:
a = Titration Volume Formol; b = Sample Weight; fp = Dilution Factor

Analysis of Antioxidant Activities with DPPH Method

Testing of antioxidant activity using the DPPH method which refers to **(Shimada et al., 1992)** is as follows: The samples tested for determining the highest antioxidant activity were protein hydrolysate filtrate which was diluted 20 times with ethanol solvent p.a. The sample solution and the comparative antioxidant solution that was made each were taken as much as 1.5 mL and reacted with 1.5 mL of 0.1 mM DPPH solution in a test tube. The mixture is then vortexed and incubated at 37 ℃ for 30 minutes and the absorbance is measured at a wavelength of 571 nm to determine its inhibitory percent. The results of absorbance

measurements and to determine antioxidant activity are expressed in the formula:

$$\% \text{ Inhibition} = \frac{\text{blank absorbance - sample absorbance}}{\text{blank absorbance}} \times 100$$

Statistical analysis

All experiments were carried out in triplicate and the results are reported as means with standard deviations. The experimental data were subjected to analysis of variance (Duncan's test), at the confidence level of 0.05 using the SPSS software (IBM, USA).

RESULTS AND DISCUSSION

Degree of Hydrolysis

The degree of hydrolysis is a parameter that shows the ability of proteases to break down proteins by comparing amino nitrogen with total nitrogen, the degree of hydrolysis can be used as an indicator of the success of the hydrolysis process **(Hasnaliza et al., 2010)**. Hydrolysis conditions are generally influenced by substrate concentration, enzyme concentration, temperature, pH, and time **(Muchtadi et al., 1992)**. Different hydrolysis times will produce different types of free amino acids and peptides which can be seen from the percentage of hydrolysis. The value of the degree of hydrolysis changes during the hydrolysis process. The percentage value of the hydrolysis degree of snakehead fish skin (*Channa striata*) can be seen in Figure 1.

The results of the degree of hydrolysis indicate that the longer the hydrolysis time, the percent degree of hydrolysis will increase. The smallest hydrolysis degree found in the treatment the 0 min hydrolysis time was 13.98% and the highest value of hydrolysis degree in the treatment 90 min hydrolysis time was 27.08%. The degree of hydrolysis of snakehead fish skin increased faster in the first 30 min, however, after hydrolysis time of 30 to 90 min there was an increase but not significant (p <0.05). This might be due to the hydrolysis time that was used not long. In the study of **Gomez-Guillen et al. (2010)**, the hydrolysis level of gelatine hydrolysate of tuna skin and squid skin using alkalase has a maximum hydrolysis degree value of 47.52% incubated for 150 min and 43.46% after incubation for 110 min.

In this study, there was a decrease in the value of hydrolysis degrees in the treatment of 120 min hydrolysis time with a percentage of hydrolysis degree of 24.97%. This is in line with the research of **Khirzin et al. (2015)**, on the hydrolysis of collagen protein peptides from gamma sea cucumber using the pepsin enzyme, decreasing the degree of hydrolysis starting from the treatment when hydrolysis 180 min at 54.54%. Decreasing the degree of hydrolysis was caused by several conditions including a decrease in the concentration of available peptide bonds to be hydrolysed, decreased enzyme activity, and the inhibition of the substrate hydrolysis process by the products produced **(Guerard et al., 2001)**. Literature studies show that there was a relationship between the degree of hydrolysis and its bioactivity, generally its antioxidant activity **(Klompong et al., 2007**) and ACE inhibitors **(Chen et al., 2012)**.

Peptide Content

Peptides are composed of two or more amino acids that form a bond. If the number of amino acids below 50 molecules are called a peptide if more than 50 molecules are called proteins. Bioactive peptides have extensive biological functions and are beneficial for health, which can function as antimicrobial, antihypertensive, antioxidant, anticytotoxic, and mineral transporting activities **(Korhonen and Pihlanto, 2006)**. Fish skin contains collagen which has three polypeptides (α-chains) in the form of triple helix and can be a source of protein needs animal for the body **(Gelse et al., 2003)**. Peptide level analysis was carried out by the formol titration method **(Wikandari and Yuanita, 2016)**.

The purpose of this hydrolysis was to produce peptides with lower molecular weights to produce peptides with higher antioxidant activity. The average value of the peptide content contained in snakehead fish hydrolysate was shown in Figure 2. The results of the research on the determination of peptide content, the average value of protein hydrolysate peptide content of snakehead fish skin produced ranged from 2.73 to 3.78%. The hydrolysis time treatment of 0 min was not significantly different from the hydrolysis time treatment of 30 and 60 min but it was significantly different from the hydrolysis time treatment of 90 and 120 min. This shows that hydrolysis time that was not long (30 and 60 min) produces the same peptide content as without hydrolysis (0 min), but the use of 90 and 120 min of hydrolysis time results in increased peptide content.

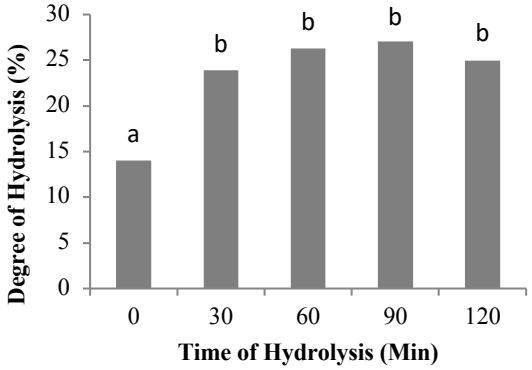

Figure 1 Degree of Hydrolysis of Snakehead Fish (*Channa striata*) Protein.

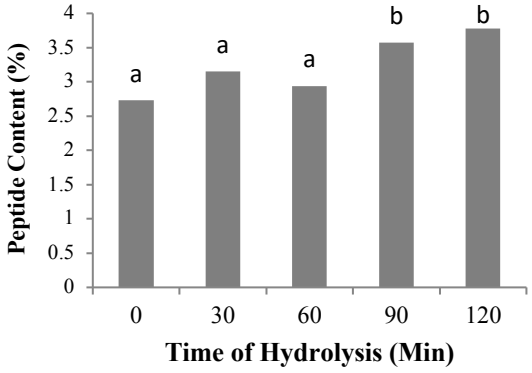

Figure 2 Peptide Content of Hydrolysis Protein from Snakehead Fish (*Channa striata*).

Determination of peptide content was carried out using formol titration, the end point of the titration if the colour changes to pink colour. Peptide bioactivity was influenced by molecular size and amino acid composition **(Gomez-Guillen et al., 2011)**. **Putalan (2018)** states that the hydrolysis time can increase the concentration of peptide content in the selar fish hydrolysate protein. **Nielsen et al. (1997)** also state that peptide content increase as the degree of hydrolysis increases, this was because during the process of hydrolysis the protein was broken down into simpler peptides.

Antioxidant Activity with DPPH Method

In this study, the antioxidant activity of protein hydrolysate of snakehead fish skin was measured using the DPPH method. DPPH which has the molecular formula $C_{18}H_{12}N_5O_6$ and molecular mas was 394.33, DPPH was a stable free radical that can react with other radicals to form more stable compounds **(Molyneux, 2004)**. DPPH can also react with hydrogen atoms to form a stable reduced DPPH (diphenyl picrylhydrazine). A compound can be said to have antioxidant activity if the compound is able to donate its hydrogen atom **(Molyneux, 2004)**. The DPPH method can be used to test solid or liquid samples and is not specific to certain antioxidant components **(Baehaki et al., 2015)**. Percent of DPPH free radical inhibition of snakehead fish skin protein hydrolysate was shown in Figure 3.

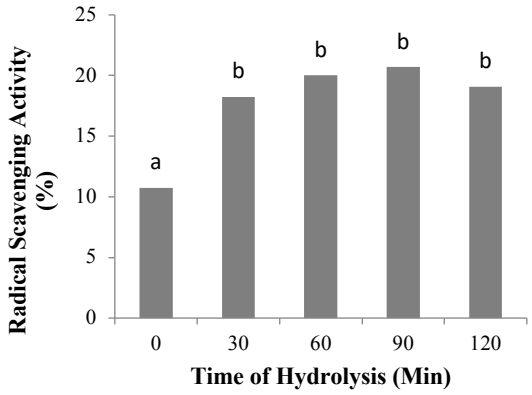

Figure 3 Radical scavenning activity of hydrolyze from Snakehead Fish (*Channa striata*).

The results of the study showed that the highest antioxidant activity produced had a percentage of 20.7% in the treatment of 90 min hydrolysis time. Figure 3 shows the difference between radical scavenging activity (antioxidant activity) without hydrolysis (0 min) and hydrolysis (30 to 120 min). The hydrolysis time used (30 to 120 min) produces radical scavenging activity that was not significantly different (p <0.05), this may be due to the hydrolysis time was used not long and the enzyme was used crude enzyme from TP2 isolate.

Several studies on the antioxidant activity of proteins have been carried out such egg yolk protein **(Park et al. 2001)**, Alaska Pollack skin gelatine hydrolysate **(Kim et al. 2001)**, pork protein **(Carlsen et al. 2003)**, yellowfin fish protein **(Jun et al. 2004)** and collagen from skin fish **(Baehaki et al., 2016)**.

CONCLUSION

Protease from swamp plant silage isolates can be used for hydrolysis of snakehead fish skin (*Channa striata*) to produces peptide with antioxidant activity. The difference in hydrolysis time in the preparation of snakehead fish skin hydrolysates has a significant effect on the degree of hydrolysis, peptide content, and antioxidant activity (p <0.05).

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Acknowledgments:

This work was supported by profesi grant Universitas Sriwijaya and Ministry of Research, Technology and Higher Education, Republic of Indonesia.

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