JURNAL PERIKANAN DAN KELAUTAN
Volume 28 No. 2, June 2023: 248-254

e-issn : 2721-8902
p-issn : 0853-7607

Antioxidant Activity of Bioactive Peptides on Protein Hydrolyze of Bombay Duck (*Harpodon nehereus*) from Papain Hydrolysis

Aktifitas Antioksidan Peptida Bioaktif Hidrolisat Protein Ikan Lomek (Harpodon nehereus) dari Hidrolisis Papain

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Received
27 April 2023

Accepted
30 May 2023

Abstract

This study aimed to examine the antioxidant activity of bioactive peptides on protein hydrolyzes of Bombay duck (*Harpodon nehereus*)from hydrolysis using papain enzyme. This research used an experimental method, with a wholly randomized non-factorial design consisting of enzyme concentration factors (1,5, 2, and 2,5%). Parameters tested include chemical composition tests of Bombay duck's meat, flour, and protein hydrolyze, identification of short peptide chains, and antioxidant activity. The results showed that Bombay duck protein hydrolyzes with papain enzyme concentration. There was a significant difference, where the enzyme concentration of 2,5% has the highest degree of hydrolysis of 69,18%. The chemical composition of meat was water 73.15% (ww), protein 81.97% (dw), ash 5.57% (dw), and fat 4,49% (dw). The chemical composition of flour Bombay duck was water 8.32% (ww), protein 79.88% (dw), ash 4,89% (dw), and fat 3,41% (dw). The best treatment of Bombay duck protein hydrolyze at an enzyme concentration of 2.5% resulted in a chemical composition of water 6.73% (ww), protein 80,56 % (dw), ash 5,53 % (dw) and fat 3,11 % (dw). The results of the identification of peptide compounds were L-Pro-L-Ile, L-Leu-L-pro, L-Arg-D-Pro, Leu-Ala-Arg-Leu, and L-Thr-L-pro-L-lys. Furthermore, DPPH free radicals (IC50) scavenging power was 5.19%.

Keywords: Protein hydrolyzate, Bombay duck, Concentration, Papain enzyme.

Abstrak

Penelitian ini bertujuan untuk mengkaji aktifitas antioksidan peptida bioaktif pada hidrolisat protein ikan lomek (*Harpodon nehereus*) dari hidrolisis yang menggunakan enzim papain. Penelitian ini menggunakan metode eksperimen, dengan Rancangan Acak Lengkap terdiri dari faktor konsentrasi enzim (1,5, 2, dan 2,5%). Parameter yang diuji meliputi uji komposisi kimia daging, tepung dan hidrolisat protein ikan lomek, identifikasi rantai peptida pendek dan aktifitas antioksidan. Hasil penelitian menunjukkan hidrolisat protein ikan lomek dengan konsentrasi enzim papain terdapat perbedaan yang signifikan, dimana konsentrasi enzim 2,5% memiliki derajat hidrolisis yang tertinggi sebesar 69,18%. Adapun komposisi kimia pada daging lomek adalah air 73,15% (bb), protein 81,97% (bk), abu 5,57% (bk) dan lemak 4,49% (bk). Komposisi kimia tepung ikan lomek adalah air 8,32% (bb), protein 79,88% (bk), abu 4,89% (bk) dan lemak 3,41% (bk). Perlakuan terbaik hidrolisat protein ikan lomek pada konsentrasi enzim 2,5% menghasilkan komposisi kimia berupa air 6,73 % (bb), protein 80,56 % (bk), abu 5,53 % (bk) dan lemak 3,11 % (bk). Hasil identifikasi senyawa peptida adalah L-Pro-L-Ile, L-Leu-L-pro, L-Arg-D-Pro, Leu-Ala-Arg-Leu dan L-Thr-L-pro-L-lys.

Lebih lanjut daya peredaman radikal bebas DPPH (IC_{50}) sebesar 5,19%.

Kata Kunci : Hidrolisat protein, Ikan lomek, Konsentrasi, Enzim papain

1. Introduction

Bombay duck (*Harpodon nehereus*) is one of the endemic fish on the northeast coast of Riau. Bombay duck is widely processed into various types of dishes as well as raw materials in the manufacture of fishery products. However, information and knowledge about the benefits and properties of this fish are not widely known. This fish is a good source of protein and macro-nutrients containing 66.8% (wt) protein (Gita, 2020), so this fish can be utilized as raw material in functional food. One form of a functional food product is fish protein hydrolysate.

Fish protein hydrolysates are produced through a hydrolysis degradation process either chemically or enzymatically as a catalyst. The use of enzymes in protein hydrolysis is more beneficial than the use of acids and bases. This is because enzymes are more effective on the target protein that is broken down and safe for the utilization of the product (Li et al., 2008). Papain enzyme is one of the protease enzymes that catalyze the reaction of polypeptide chain breakdown in proteins by hydrolyzing peptide bonds into simpler compounds, such as long-chain peptides, short-chain peptides, and amino acids (Nurhayati et al., 2014).

The simple compounds produced in protein hydrolysates are bioactive compounds that have the potential as antioxidants with their ability to reduce free radicals (Samaranayaka & Li-Chan, 2011). The antioxidant activity of protein hydrolyses depends on the composition and amino acid sequence of the peptides (Widadi, 2011).

Based on the description above, it is necessary to conduct research on the preparation of Bombay duck protein hydrolysate using papain enzymes, so that further identification of short-chain peptide chains that function as antioxidants can be determined.

2. Material and Method

2.1. Methods

The research method used was experimental with a completely randomized design (CRD) consisting of 3 levels, namely A_1 (1.5% papain enzyme), A_2 (2% papain enzyme), and A_3 (2.5% papain enzyme).

2.2. Research Materials

The main raw material used was Bombay duck in the form of meat with a weight of 200-300 g/head from Dumai, Riau. Chemicals used for the preparation of protein hydrolyzes are papain enzyme with enzyme activity of 105,000 U/g (*Xian Lyphar Biotech*) and distilled water solution. Chemicals for the hydrolyses degree test were HCl, NaOH, H_2SO_4, Cu complex, chloroform, NaOH, PP indicator, H_2BO_3, mixed indicator (methylene red-blue), TCA (*trichloroacetic acid*) 20%. Antioxidant activity testing using DDPH *(2,2-diphenyl-1-picrylhydrazyl)* and Fenton reagent.

Furthermore, the equipment used in the analysis of short peptide chains is (LC-MS Qtof) to obtain chromatography and identified with Masslynx Version 4.1 software and ChemSpider website while antioxidants are spectrophotometers with the brand 2000-1 Smart Spectro.

2.3. Data Analysis

The parameters tested included analysis of the chemical composition of Bombay duck meat and flour, optimum conditions for fish protein hydrolysed based on the degree of hydrolysis parameter and characteristics of Bombay duck protein hydrolysed based on chemical composition, short peptide chain chemistry, and antioxidant activity test.

3. Result and Discussion

3.1. Characteristics of Bombay Duck (H.nehereus)

Bombay ducks obtained from Dumai waters have the characteristics of having a long torpedo-like body, small eyes, eye distance close to the tip of the nose, wide mouth with pointed teeth, scales on the rib line extending beyond the curve of the caudal fin sheet, the beginning of the anal fin behind the back, the front upper jaw bone extends and removes the upper jaw bone from the edge of the mouth, the bones of the gill lid are perfect, sometimes not bubbling swimming (Rahardjo et al., 2011).

Bombay duck mainly consists of meat, head, bones, gills, and viscera. Proportion is used to obtain the percentage of Bombay duck body parts. Proportion is an important parameter in the utilization of Bombay duck body as raw material for a product. The proportion of Bombay duck body parts is presented in Table 1.

Table 1. Body part proportions of Bombay duck

Composition	Meat	Flour
Water (bb)	73,15±0,13	8,32±0,31
Protein (wt)	81,97±0,51	79,88±1,33
Ash (wt)	5,57±0,34	4,89±0,72
Fat (wt)	4,49±0,43	3,41±0,46

Description:1) bb= wet weight; 2) bk= dry weight

3.2. Chemical Composition of Bombay Duck Meat and Fishmeal

This research is related to protein hydrolysis, therefore the total protein content in meat and flour needs to be analyzed first as raw materials in the preparation of hydrolysates. In addition, other chemical compositions need to be tested. Such as proximate (water content, ash, fat) further presented in Table 2.

Table 2. Proximate meat and flour of Bombay duck

Fish parts	Percentage (%)
Meat	45,40 ± 0,11
Head	30,83 ± 0,1
Bones	15,26 ± 0,12
Gills	5,18 ± 0,06
Viscera	3,30 ± 0,07

Table 2 shows that the Bombay duck has a relatively high water content of 73.15 ± 0.13% (bb). The protein content is relatively high at 81.97 ± 0.51%. Ash content ranged from 5.57 ± 0.34% while fat content was relatively low at 4.89 ± 0.43%. Differences in environmental conditions, habitat, sex, and age will result in differences in proximate content in fish meat. The high protein content indicates that Bombay duck has potential as a raw material for fish protein hydrolyzes.

The high proximate content of Bombay duck meat, especially water content, determines the freshness and durability of the food. Fish meat that has high water content is also at risk of spoilage if not handled properly (Winarno, 2008). Table 2 shows that the moisture content of Bombay duck flour showed a decrease to 8.32 ± 0.31% (bb). The drying process in flour-making effectively reduces the moisture content in Bombay duck meat. Most of the water will evaporate when in contact with heat during the drying process so the water content contained in food ingredients will also decrease (Widadi, 2011).

The protein content of Bombay duck flour is relatively high at 79.88 ± 1.33%. There was a protein loss of 2.09%. Protein loss in the heating process is thought to occur by the solubility of protein during the heating process. The Ash content in Bombay duck flour was 4.15 ± 0.72%. Not much different from the ash content of Bombay duck meat, it is suspected that the heating process does not affect the mineral content contained in the heated fish meat. So flour is dominated by mineral content (Zahroh, 2015). The fat content of Bombay duck is quite low, as well as the fat content of the resulting Bombay duck flour. The heating process does not affect the fat content found in fishmeal. The difference in fat content in meat and flour is caused by the percentage of water content left behind (Nurhayati et al., 2012).

3.3. Characteristics of Bombay Duck Protein Hydrolysate

Determination of optimum conditions (papain enzyme concentration) in the process of hydrolysis of Bombay duck protein by calculating the degree of hydrolysis. The average value of the degree of hydrolysis of Bombay duck protein hydrolyses with different concentrations of papain enzyme is presented in Figure 1.

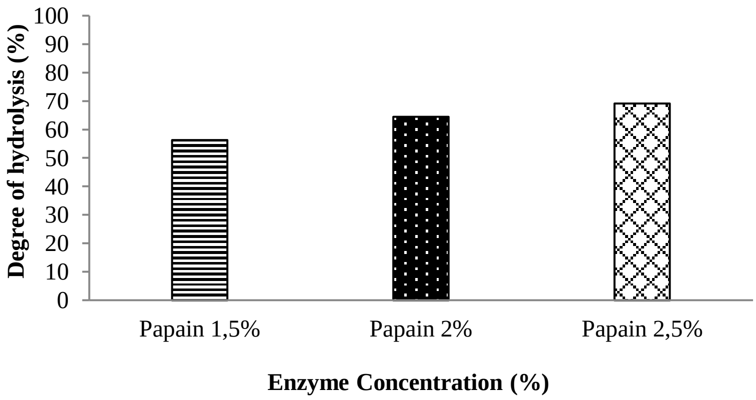


Figure 1. Effect of enzyme concentration on the hydrolysis of Bombay duck

Figure 1 shows the difference in the degree of hydrolysis value for each concentration of papain enzyme used. The greater the concentration of papain enzyme added, the greater the degree of hydrolysis of protein hydrolyses. The highest degree of hydrolysis was obtained from a 2.5% papain enzyme concentration of 69.18%. While the lowest degree of hydrolysis was obtained from a 1.5% papain enzyme concentration of 56.34%.

One Way Anova test showed that different concentrations of papain enzyme significantly affected the degree of hydrolysis produced ($p<0.05$). Duncan's further test results showed that enzyme concentrations of 1.5% and 2, and 2.5% were significantly different. The pattern of increasing the degree of hydrolysis at enzyme concentrations of 1.5% and 2% increased rapidly, but there was a slowdown in the enzyme concentration of 2.5%. This is inseparable from the peptide bonds available to be hydrolyzed. Charoenphun et al. (2013) stated that the increase in the degree of hydrolysis was due to the increase in peptides and amino acids dissolved in TCA as a result of the breaking of peptide bonds during the protein hydrolysis process. Meanwhile, the slowing down of protein hydrolysis is caused by a decrease in the concentration of peptide bonds that can be hydrolyzed by the enzyme.

The results of the degree of hydrolysis in this study indicate that the enzyme concentration affects the decomposition of proteins into peptides and amino acids, thus it can be seen that the most efficient papain enzyme concentration to produce the degree of hydrolysis of Bombay duck protein is using an enzyme concentration of 2.5%.

3.4. Proximate Analysis of Bombay Duck Protein Hydrolysate

The results of the proximate analysis of the resulting Bombay duck protein hydrolysate are presented in Table 3.

Table 3. Proximate protein hydrolyses of Bombay duck

Composition	Percentage (%)
Water (bb)	6,73 ± 0,18
Ash (wt)	5,53 ± 0,31
Protein (wt)	80,56 ± 0,68
Fat (wt)	3,11 ± 0,57

Moisture content shows the stability in product storage, the higher the moisture content, the shorter or less durable the product storage. One of the efforts to reduce moisture content is drying. *Freeze drying* is effective because it can achieve very low moisture content with the risk of protein damage because the drying process occurs at very low temperatures (Widadi, 2011).

Bombay duck protein hydrolyses has a protein content of 80.56%. The analytical method used is the Kjeldahl method which uses the amount of nitrogen as a conversion in the calculation of total protein content (crude protein). This method determines the total organic nitrogen in the sample and not just the protein, so it cannot determine the quality of the protein contained in the sample (Henni et al., 2015).

The addition of alkaline compounds, such as NaOH, and or acidic compounds, such as HCl, in the protein hydrolysis process aims to achieve the optimum pH value of the enzyme and keep the pH constant during the hydrolysis process so that the breaking of peptide bonds by enzymes can continue. Gesualdo & Li-Chan (1999) stated that mixing acidic and alkaline compounds in the protein hydrolyses solution will cause the formation of salt compounds, thus increasing the ash content of the protein hydrolyses.

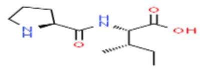
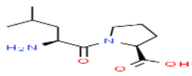
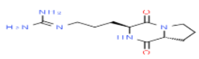
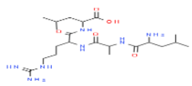
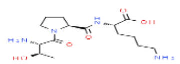
The fat content of Bombay duck protein hydrolyses was 3.11%. Some of the fat contained in the protein hydrolyses is thought to be separated from the undissolved protein during centrifugation. Protein hydrolyzes that have a low-fat content are generally more stable against reactions to fat oxidation compared to fish protein hydrolyzes that have high-fat content (Nilsang et al., 2005). The rapid changes in fish tissue structure caused the fat content to decrease. Types and amino acid content of Bombay duck protein hydrolysate.

3.5. Short Chain Peptides

Enzymatic hydrolysis of protein will produce hydrolyzes containing short-chain peptides and free amino acids, through the breaking of peptide chain bonds. The higher the enzyme concentration is given to Bombay duck protein hydrolysate, the higher the degree of hydrolysis value. The results of LC-MS QTOF analysis obtained 25 chromatogram peaks for the hydrolyses obtained using 2.5% papain enzyme. Identification using reference standards based on mass spectrum fragment patterns, obtained 5 peptide compounds from protein hydrolyses using 2.5% papain enzyme concentration (Table 4).

Peptide compounds found in the use of 2.5% papain enzyme with a degree of hydrolysis of 61.97% are L-Pro-L-Ile, L-Leu-L-pro, L-Arg-D-Pro, Leu-Ala-Arg-Leu, and L-Thr-L-pro-L-lys. The use of 2.5% enzyme concentration gave diversity to the amino acid skeleton of the resulting peptides. Nakamura et al. (1995) explained that bioactive peptides with Pro-Ile or Pro-Arg amino acid composition have physiological functions as angiotensin-converting enzyme inhibitors (ACEI). Angiotensin-converting enzyme inhibitor (ACEI) drugs are known to be quite effective and have been widely used as antihypertensives and antioxidants (Desi et al., 2020). Bioactive peptides that have the amino acid composition Val-Lys-Glu-Ala-Met-Ala-Pro-Lys have physiological functions as antioxidants (Hernandez-Ledesma et al., 2004). Research conducted by Bougatef et al. (2010) has identified seven antioxidant peptides (Leu-Ala-Arg Leu, Gly-Gly-Glu, Leu-His-Tyr, Gly-Ala-His, Gly-Ala-Trp-Ala, Pro-His-Tyr-Leu, and Gly-Ala-Leu-Ala-His) in protein hydrolysates obtained from sardine processing waste.

Table 4. Short-chain peptide compounds in Bombay duck protein hydrolyses

No.	Compound	Molecular formula	Structure of the compound	Retention Time
1	L-Pro-L-Ile	$C H N O_{112023}$		1.701
2	L-Leu-L-pro	$C H N O_{112023}$		2,569
3	Cyclo(L-Arg-D-pro)	$C H N O_{111952}$		3,235
4	Leu-Ala-Arg-Leu	$C H N O_{214175}$		5,231
5	L-Thr-L-pro-L-lys	$C H N O_{152945}$		5,891

The main factor influencing antioxidant activity is that peptides have amino acid sequences that contain many hydrophobic, aromatic amino acids and those with sulfur groups. Amino acids that are hydrophobic (Gly, ala, val, leu, and pro). Aromatic amino acids (Phe, Tyr, and Trp), amino acids have sulfur groups (Cys and met) (Zou et al., 2016). This may affect the antioxidant activity of the peptides. The hydrolysis process affects the antioxidant activity; the hydrolysis process can either induce or eliminate the antioxidant activity of a peptide (Costa et al., 2012). In this study, the hydrolysis process elicited antioxidant activity from Bombay duck protein hydrolysate.

3.6. Antioxidant Activity

The free radical scavenging test (DDPH) by antioxidants used in general is the 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) method because DPPH is a stable free radical that gives maximum absorbance at 520 mm. Antioxidant activity in this test is shown from its ability to reduce DPPH free radicals, thus forming colorless DPPH-free non-radicals. The ability of Bombay duck protein hydrolyses using papain enzyme concentrations of 1.5%, 2%, and 2.5% was 83.11%. 86.80%, and 87.57% (Figure 2).

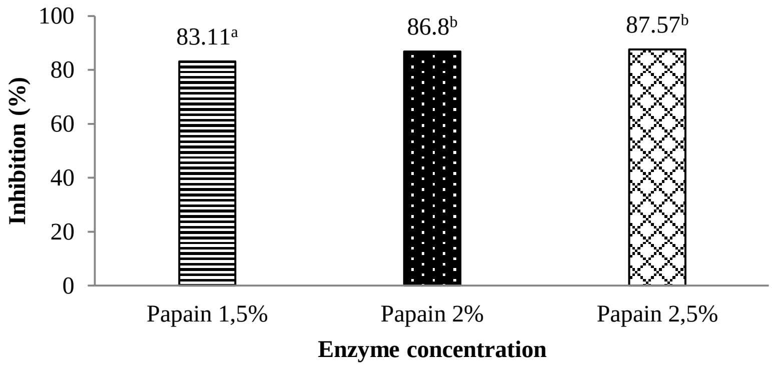


Figure 2. Percentage of antioxidant activity of Bombay duck protein hydrolysate against DPPH free radical scavenging

One Way Anova test showed that there was an effect of papain enzyme concentration on the preparation of protein hydrolyses on its ability to reduce DPPH free radicals ($p<0.05$). Duncan's further test results showed that there was a difference in the use of 1.5% papain enzyme concentration with 2% and 2.5% papain enzyme concentration in the preparation of protein hydrolyses on its ability to reduce DPPH free radicals. In contrast, the use of papain enzyme concentrations of 2 and 2.5% did not show any difference. The use of 2.5% papain enzyme gave a relatively greater DPPH free radical scavenging value compared to the others but was not significantly different from the use of 2% papain enzyme. This shows that the peptides produced from protein breakdown are more functional.

In the antioxidant silencing test of protein hydrolyzes against DPPH free radicals at various concentrations mentioned above, the IC_{50} value (effective concentration of fish protein hydrolyses required to reduce 50% of total DPPH) can be determined. The IC_{50} values of fish protein hydrolyses using 1.5%, 2%, and 2.5% papain enzyme with a degree of hydrolysis of 56.34%, 64.51%, and 69.18%, respectively, to reduce DPPH free radicals as presented in Figure 3 were 11.15% v/v, 8.75% v/v, and 5.19% v/v.

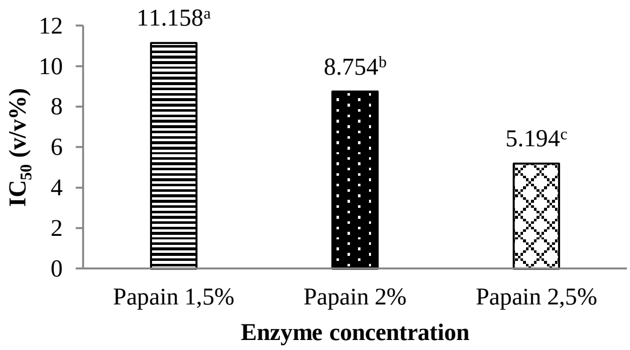


Figure 3. IC_{50} activity of DPPH free radical silencing of Bombay duck protein hydrolysate

Based on this Figure 3, shows that Bombay duck protein hydrolyses has very strong antioxidant activity because the IC value is <50 (Molyneux, 2004). One Way Anova test obtained a probability value smaller than the significance level of 0.05 ($p<0.05$) indicates that the effect of papain enzyme concentration on the preparation of protein hydrolyzes on its ability to reduce 50% of the total DPPH radicals. Duncan's further test results show that there is a difference in the use of each concentration of papain enzyme 1.5%, 2%, and 2.5% on its ability to reduce 50% of the total DPPH radicals.

The DPPH free radical scavenging value increased significantly with the increase in enzyme concentration. When the enzyme concentration increased from 1.5% to 2%, the degree of hydrolysis increased from 56.34% to 64.51%, the DPPH free radical scavenging activity decreased from 11.15% v/v to 8.75% v/v. Likewise, when the enzyme concentration increased to 2.5%, the degree of hydrolysis was 69.18%, and the DPPH free radical scavenging activity decreased to 5.19% v/v.

The increase in DPPH free radical scavenging mentioned above is inseparable from the presence of amino acid compounds and peptides in the protein hydrolysates obtained (Table 4). The oxidative susceptibility of amino acid residues to free radical attack is largely determined by the side chains of their functional groups, such as amino acids and proteins whose side chains are hydrophobic (Gly, ala, val, leu, and pro), sulfur-containing (Met and Cys) and aromatic (Phe, Tyr, and Trp). According to Li et al. (2008); Pownall et al. (2010), hydrophobic and aromatic amino acids are useful as DPPH-free radical antioxidants.

Fish protein hydrolysate obtained with 2.5% papain enzyme concentration contains a variety of peptide compounds (L-Pro-L-Ile, L-Leu-L-pro, L-Arg-D-Pro, Leu-Ala-Arg-Leu, and L-Thr-L-pro-L-lys).
From the above, it can be stated that fish protein hydrolysate using papain enzyme with different concentrations produces amino acids and peptides with increased potential to absorb DPPH radicals and 2.5% papain enzyme concentration has better potential to reduce DPPH radicals than others.

4. Conclusions

This study has shown that Bombay duck flour (*Harpodon nehereus*) contains relatively high protein content of 79.88% (wt), relatively low moisture content of 8.32% (wt), fat content of 3.41% (wt) and ash content of 4.15% (wt), so it has the potential to be used as a source of raw materials to be used as a Fish Protein Hydrolysate product. The optimum condition in the use of papain enzymes can be seen from the highest degree of hydrolysis, namely the use of 2.5% papain enzyme concentration. The resulting degree of hydrolysis was 69.18%. The peptide compounds identified in the hydrolysate produced using 2.5% papain concentration are L-Pro-L-Ile, L-Leu-L-pro, L-Arg-D-Pro, Leu-Ala-Arg-Leu, and L-Thr-L-pro-L-lys. The protein hydrolysate obtained with 2.5% papain enzyme concentration can reduce DPPH free radicals with an IC_{50} value of 5.19% v/v. So overall, it can be said that the hydrolysis of Bombay duck protein using papain enzyme produces amino acids and peptides that contribute to relatively large antioxidant properties.

5. Suggestion

This study has shown that hydrolyses of peptide Bombay duck protein can be developed into functional food as an antioxidant. Before its direct use, it is necessary to study its potential in vivo. So that its use can be formed in microencapsulation.

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