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‘Wader’ (*Rasbora jacobsoni*) Protein Hydrolysates: Production, Biochemical, and Functional Properties

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Abstract

Considerable the amounts of Wader (*Rasbora jacobsoni*) are abundant in Indonesia for all year and also have a low economic value. By using Biduri (*Calotropis gigantea*) protease, production of wader protein hydrolysate (WPH) as food ingredients and industrial product may be possible. Hydrolysis condition was proposed depend on the effects of the time (t) (0, 1.5, and 3 hours), and enzyme concentration (E) (1, 2, and 3% (v/w)) on the degree of soluble protein. Significant differences were observed on chemical and biochemical characteristics of WPH. In addition, functional properties of WPH are described, including solubility, rancidity, maillard, water-holding capacity, emulsification, and foam-forming capacity. The composition of amino acids was observed by high performance liquid chromatography (HPLC). The highest proteolytic activity was at 3 hours and 3% (v/w) of enzyme concentration. The WPH contained of high protein solubility (65.90 mg/ml) and glutamate acids (12.72% (w/w)). The properties indicated that WPH was suitable for using as flavor enhancer with wide range of applications in the food industry.

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1. Introduction

Rasbora jacobsoni or commonly known in Indonesia as Wader is one of the abundant freshwater fishes and cheaper compared to other freshwater fishes. Though wader is on a high demand in Indonesia, yet its commercial value is still low. This is due to the utilization of cultures wader is still limited to be local consumption as side dishes. An alternative to enhance the commercial value of wader is by generating wader protein hydrolysates (WPH). WPH can be a source of raw material for flavor enhancer, ketchup, and other food ingredient.

Protein hydrolysates are produce from protein sources by heating with acid or preferably, addition of proteolytic enzymes (Bucci and Unlu, 2000). Hydrolysis decreases the peptide size, so making hydrolysates is the most available amino acid source for protein biosynthesis (Gildberg and Stenberg, 2001). Enzyme hydrolysis is strongly preferred over strictly chemical methods for producing hydrolysates in nutritional application (Lahl and Braun, 1994). Hydrolysis of protein by proteolytic enzymes (often referred as protease) is a widely used technique to modify the physicochemical properties (e.g., emulsification, solubility, etc.) and sensory properties of food proteins (Panyam and Kilara, 1996). Enzymatic hydrolysis of proteins produces a decrease in peptide size, which can modify functional characteristics of the proteins and improve their quality (Petersen, 1981).

Several proteases can be used potentially for making hydrolysates. Some of these are site-specific enzymes. Because of their specificity, the types of polypeptide fragments released in the hydrolysate differ between proteases. A number of different enzymes have been used for hydrolysing of fish. There are plant-derived enzyme such as papain (Shahidi et al., 1995) and biduri protease (Witono et al., 2014) and animal-originated enzymes for example trypsin (Simpson et al., 1998). Enzymes of microbial sources have been also applied such *Bacillus subtilis* that produced alkaline proteinase (Glazer, 1967). Protease from Biduri (*Calotropis gigantea*) be characterized as exopeptidase and has been proven to be one of the enzyme used in the preparation of seafish protein hydrolysates (Witono et al., 2014). Biduri is one kind shrub with 0.5 – 3 meters height that grows in place with dry periods such as Indonesia. The objectives of this study were to produce wader protein hydrolysates (WPH) using biduri protease in order to characterize its biochemical properties and functional properties as affected by hydrolysis time and enzyme concentration.

2. Materials and Methods

2.1. Materials

Wader (*Rasbora jacobsoni*) was purchased from Tanjung Market, a local market of Jember district, Indonesia. Biduri (*Calotropis gigantea*) obtained from the coast of Papuma beach, Jember, Indonesia as source of exopeptidase enzyme. Amino acid standards were purchased from Pierce (Rockford, IL, USA). Soluble casein, standard tyrosin and other chemical of analytical grade were purchased from Sigma (Sigma – Aldrich).

2.2. Preparation of enzyme extract

The protease produced from latex of Biduri plant. Latex diluted with buffer phosphate pH 7.0 (1:1 (v/v)). The solution was centrifuged at 1000 rpm for 10 min at the room temperature. Remove the sludge, and supernatant was crude enzyme of biduri. Stored the enzyme at 4°C until be used. The activity of biduri protease was 1200 U/g.

2.3. Enzymatic hydrolysis

For enzymatic hydrolysis, wader fillet was mixed with NaOH 0.1 N (1:4 (w/v)). The mixture was adjusted to the enzyme pH of 7 and temperature of 55°C. The sample was added enzyme with difference concentration (1%, 2%, and 3% (v/w) and incubated for 0, 1.5 and 3 hours. All solution was heated at 90°C for 10 min to inactivate the protease. The solution was dried at the temperature 60°C for 24 hours and then grinded until the size reach 60 mesh. The dried wader protein hydrolysates (WPH) stored at room temperature for further experiments.

2.4. Biochemical properties

Crude protein content was determined using Kjeldahl method, AOAC 981.10 (AOAC, 2005). One gram of sample, one Kjeldahl catalyst tablet and 10mL H₂SO₄ were put into Kjeldahl tube and digested for 2 hour at 420°C. The product was then made basic with 30% (w/v) NaOH before distillation into 0.1M HCl and titration against 0.25 M NaOH. The factor used to convert nitrogen into crude protein was 6.25. Moisture content was determined with a modified version of the AOAC 925.04 (AOAC, 2005). 10g of samples were oven-dried at 105°C for 24 hour and the water content of the sample was gravimetrically determined. Fat content was determined by using Soxhlet extractor (Behrotest, Behr Labor Technik Gro bH, Dusseldorf, Germany). The dried sample was inserted into a Soxhlet tube and petroleum ether was recycled through the sample for 2 hour. Remaining ether was evaporated and the sample was dried at 105°C overnight. Fat content was then calculated gravimetrically. The ash content was analyzed using a modified version of AOAC 938.08 (AOAC, 2005). The water and fat free sample was combusted in the furnace at 550°C for 12 hr and the ash content was determined gravimetrically.

2.5. Functional properties

Solubility and nitrogen solubility index were calculated to determine the solubility of protein hydrolysates, following the procedure of Morr (1985). Maillard value was determined according to the method proposed by Subagio et al. (2002), and the rancidity by Hofmann et al. (1999) method. Foaming capacity was determined by dissolved one gram of WPH in 100 ml of distilled water. Then suspensions were whipped at a low speed in blender for 1 min at room temperature (22°C) and poured into 100 ml cylinder. To determine foam stability (FS), foam volume was recorded 30 min after whipping and calculated (Kabirullah and Wills, 1983).

2.6. Amino acid profile

Amino acid analysis of plant samples was based on methodology previously reported (Vázquez-Ortiz et al., 2007). Powdered samples (3 mg) were hydrolyzed with HCl 6 M at 150°C during 6 hours. After hydrolysis, the acid was removed by rotary evaporation (RE500 Yamato Scientific America Inc.). Sample was resuspended on 2 mL of sodium citrate buffer pH 2.2. Sample derivatization was achieved adding o-phthalaldehyde (OPA) 7.5 mM to the sample on citrate buffer (OPA reagent contains β-mercaptoethanol and Brij 35). The HPLC method precision and accuracy was evaluated using external and internal standard. The amino acid reference standard consisted on fifteen amino acids (0.05 μmoles mL⁻¹ each amino acid) and was utilized to determine the retention times for each amino acid. As well, internal standard aminobutyric (0.05 μmoles mL⁻¹) was added to amino acid reference standard and each plant sample to normalize and quantify the amino acid content.

A gradient mobile phase of sodium acetate 0.1 M pH 7.2 and methanol (9:1) elute sample for amino acid separation through C18 column reversed-phase octadecyl dimethylsilane particles (100 × 4.6 mm × 1/4" Microsorb 100-3 C18). Fluorescence detection was realized using an excitation-emission wavelength of 360 and 455 nm respectively. Star Chromatography work station (Varian version 5.51) software was used to achieve amino acid peak integration.

2.7. Statistical Analysis

Data were analyzed using the analysis of variance procedure. Mean difference was determined using the least significant difference (LSD) multiple range test (SPSS 16.0 Version). Significance of difference was established at $p < 0.05$.

3. Results and Discussion

3.1. Protein solubility

Solubility is one of the most valuable functional properties of food proteins in general. Highly soluble protein is required to obtain optimum functionalities. Greater protein solubility generally correlates to good gelling, foaming, water-holding capacity, viscosity, gelation, foaming and emulsifying properties. These properties are determined by the physicochemical characteristics such as amino acid composition molecular size, structural conformation of the protein, and inter- and intra-molecular interactions as well as environmental factors such as pH, temperature, and ionic strength (Kinsella, 1979; Lampart-Szczapa, 2001).

Protein solubility level of WPH using biduri protease at various concentration and hydrolysis time ranged between 44.78 mg/ml to 65.90 mg/ml (Fig 1). The solubility protein values obtained from all treatments increased with increasing the concentration enzymes and the hydrolysis time (Fig 2). Interaction effect was significant at all interaction ($p > 0.05$). The protease breaks down fish proteins into amino acids soluble. The changes occurring during the hydrolysis process include protein and dry matter solubility, liberation of free α -amino groups, changes in the molecular weights of the peptides hydrolysates and also in the composition of amino acids (Soufi-Kechaou et al., 2009). Increase of protease concentration was associated with a quadratic increase in soluble nitrogen at fish protein hydrolysates (Rebeca et al., 1991). In this study, the highest enzyme concentration and hydrolysis time is the best treatment with the highest levels of soluble protein (65.90 mg/ml).

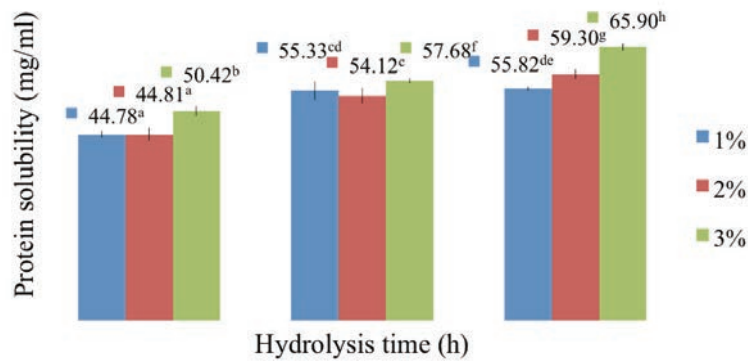


Fig 1. Protein solubility of wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis

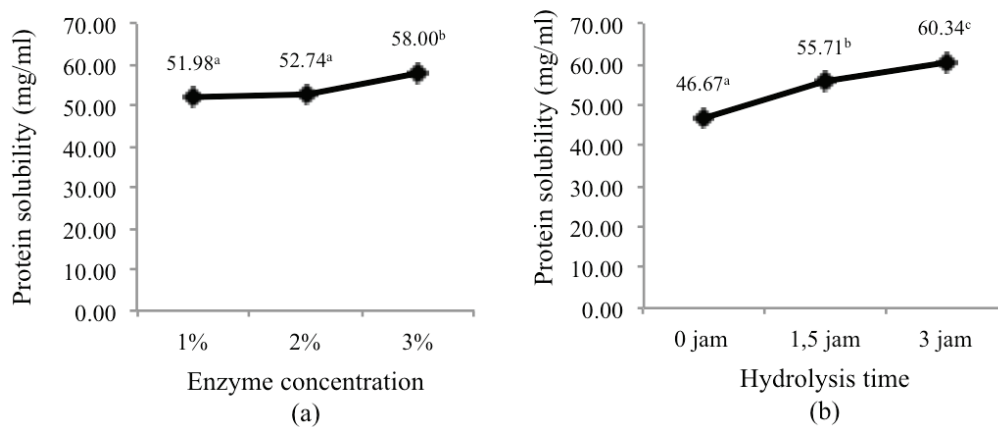


Fig 2. (a) The effect of increasing enzyme concentration and (b) hydrolysis time on the values of protein solubility in WPH (jam = hour).

Long time of hydrolysis also affects the levels of WPH soluble protein. This result was similar to those reported by Vijaya et al. (2002) and Haslaniza (2010) which indicated an increase in DH when incubation time was increased so that the protein content of the higher dissolved.

3.2. Biochemical properties

Proximate compositions of WPH at various hydrolysis time and enzyme concentration are shown in Table 1. The protein content of raw material was 12.56%. After hydrolysis, the protein content increase significantly with increasing enzyme concentration and hydrolysis time. The highest protein content (65.84 % db) was at 3% enzyme concentration and 3 hours hydrolysis. This is because during the hydrolysis process, which split proteins into short peptides that cause more changes in the protein group that is increasing NH_3^+ and COO^- thus also increase the solubility of the protein, resulting in increased protein content (Nielsen, 1997). Nitrogen recovery, as an index of nitrogen solubilization and hydrolysis yield, increased with increasing hydrolysis time ($P < 0.05$). Liaset et al. (2002) reported that nitrogen recovery increased with increasing time. The DH of yellow stripe trevally was found to increase with longer the incubation time and higher enzyme concentration (Klompong et al., 2007).

Table 1 Proximate composition of Wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis time.

Treatments	Crude Protein (db % w/w)	Fat (db % w/w)	Moisture (wb % w/w)	Ash (db % w/w)
1% enzyme; 0 h	60.35 ± 3.48	20.97 ± 0.82	8.54 ± 0.40	4.89 ± 0.54
2% enzyme; 0 h	60.57 ± 3.82	23.27 ± 0.91	8.51 ± 0.31	4.86 ± 0.20
3% enzyme; 0 h	61.26 ± 4.43	22.98 ± 0.78	9.24 ± 0.31	6.24 ± 0.47
1 % enzyme; 1.5 h	61.46 ± 5.43	17.48 ± 0.61	9.05 ± 0.39	4.86 ± 0.19
2 % enzyme; 1.5 h	64.38 ± 9.52	22.56 ± 0.04	9.43 ± 0.27	5.70 ± 0.14
3 % enzyme; 1.5 h	65.22 ± 4.22	21.34 ± 0.92	9.86 ± 0.23	6.48 ± 0.14
1 % enzyme; 3 h	64.65 ± 4.29	22.02 ± 1.03	7.57 ± 0.25	4.65 ± 0.15
2 % enzyme; 3 h	65.32 ± 4.81	13.71 ± 0.54	7.75 ± 0.34	5.85 ± 0.43
3 % enzyme; 3 h	65.84 ± 4.64	14.26 ± 0.43	8.15 ± 0.27	4.58 ± 0.06

The lipid content of WPH ranged from 13.71% to 23.37%. Decreasing lipid content in the hydrolysates might increase stability of the material toward lipid oxidation and might also enhance products stability (Diniz and Martin, 1997; Kristinsson and Rasco, 2000). The ash content of WPH ranged from 4.58% to 6.48%. The addition of alkali (NaOH) in the manufacture of WPH may increase the ash content of the hydrolyzsates. Gesualdo and Li-Chan (1999) (stated that the mixing of acid and alkali compounds in a solution of the protein hydrolysates will lead to the formation of a salt compound, so as to increase the ash content on protein hydrolysates (Liceaga-Gesualdo and Li-Chan, 1999).

3.3. Emulsifying capacity and stability

Emulsifying capacity is defined as the ability of a material to form an emulsion. WPH emulsifying capacity range between 1.44 m^2/g to 4.85 m^2/g (Fig 3).

Emulsifying capacity of WPH increased with increasing enzyme concentrations but decreased with the longer time of hydrolysis (Fig 4). Overall the emulsifying capacity of interaction various enzyme concentration and hydrolysis time of WPH tends to decrease. This is in accordance to Gbogouri et al (2004) which stated that the hydrolysates has a high degree of hydrolysis (DH) will have low emulsifying capacity and stability because of the small size of the peptide (Gbogouri et al., 2004).

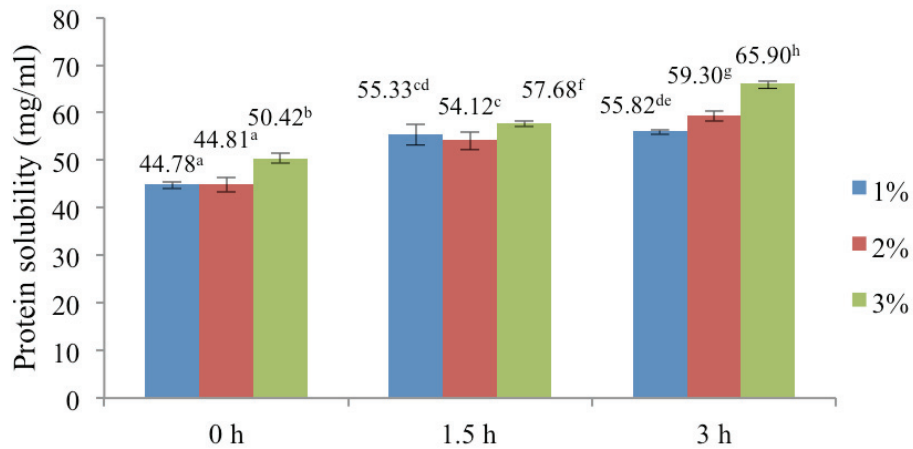


Fig 3. Emulsifying capacity of Wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis

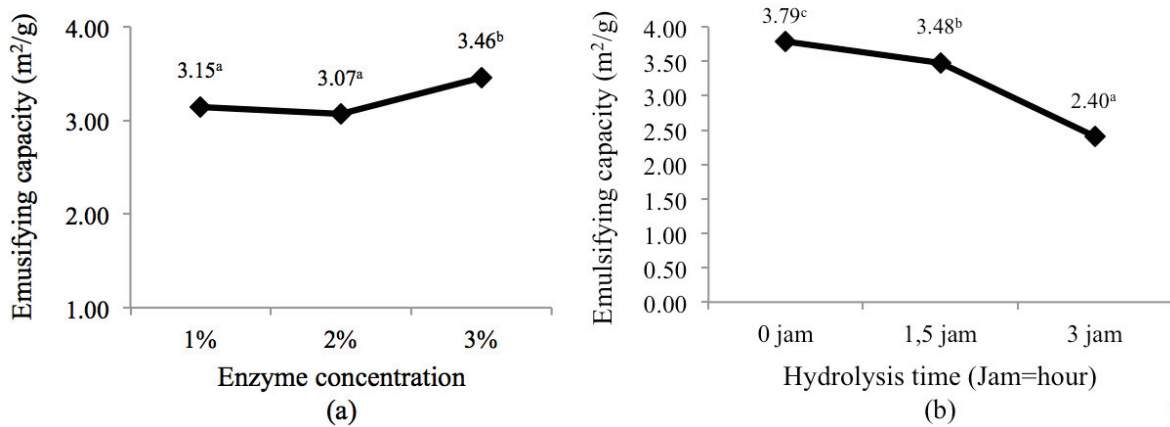


Fig 4. (a) The effect of increasing enzyme concentration and (b) hydrolysis time on the values of emulsifying capacity in WPH

Emulsifying stability is the ability of a material to maintain the formed of emulsion. Emulsifying stability of WPH ranged between 58.81 up to 211.40 hours (Fig 5). Gbogouri et al. (2004) stated that it would be better emulsifying stability at a low degree of hydrolysis (Gbogouri et al., 2004). This is because the lengths of peptides that are formed are absorbed in a layer of oil and trigger the formation of small oil droplets, consequently higher emulsifying stability.

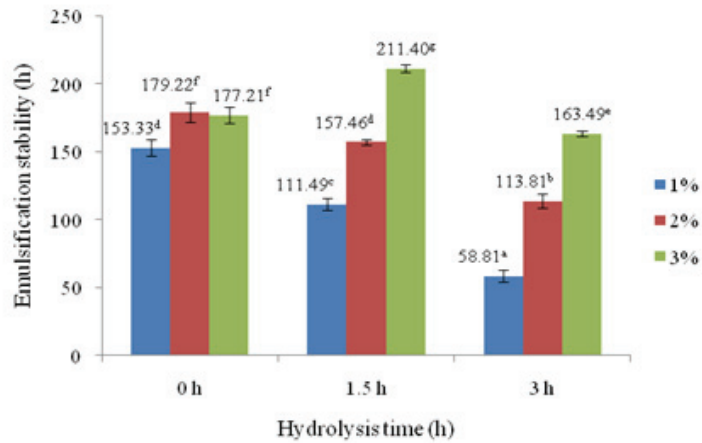


Fig 5. Emulsifying stability of Wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis

3.4. Foaming capacity and stability

Foams were gaseous droplets encapsulated by a liquid film containing soluble surfactant protein resulting in reduced interfacial tension between gas and water. The foaming capacity of WPH ranges from 258.02 to 259.22 %, but the foaming stability relatively low ranges from 15.03 – 24.02 s (Fig 6, 7). A good foaming capacity might attribute to an increase in the surface activity, which is due to partial proteolysis that produced greater number of polypeptide chain and therefore allowed more air to be incorporated (Kuehler and Stine, 1974). But increasing of enzyme concentration and hydrolysis time has a lower foaming capacity (Fig 6). This may be due to the small size of peptides that produce with extensive hydrolysis would lower its surface activity and thus hinder the formation of a stable firm around the gas bubbles, and also by the apparition of hydrophilic peptides during extensive hydrolysis (Kuehler and Stine, 1974).

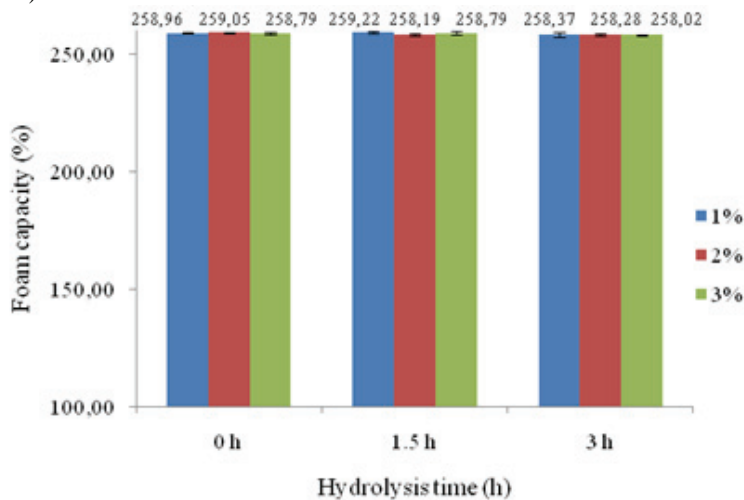


Fig 6. Foam capacity of Wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis

Foam stability is the ability of a protein in maintaining the foam in a certain time. Foam stability of WPH ranged from 15.03% to 24.02% (Fig 7). Foam stability depends on the film’s nature and reflects the extent of protein-protein interaction within the matrix (Mutlangi et al., 1996). Foam stability can be enhanced by flexible protein

domains that increased the viscosity of the aqueous phase, protein concentration and film thickness (Phillips et al., 1994). Shahidi *et al.* (1995) reported capelin protein hydrolysate possessed good foaming properties of 90% at lower DH but the foaming stability is very poor (0% after 60 min) (Shahidi et al., 1995). The longer the hydrolysis time increase the foam stability. This is because the longer the hydrolysis time will broke more protein into short peptides and amino acids so leads to reduced molecular weight of the protein. Reduced molecular weight of the protein can improve the stability of the foam (Liceaga-Gesualdo and Li-Chan, 1999).

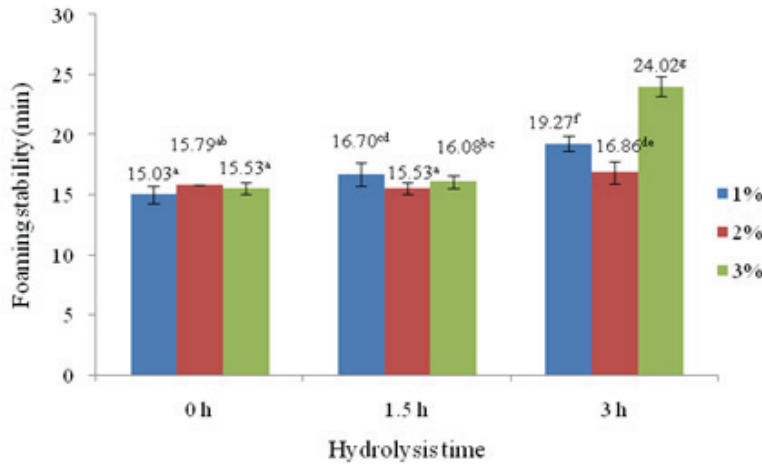


Fig 7. Foam stability of Wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis

3.5. Maillard and Rancidity

Maillard and rancidity of WPH at various hydrolysis time and enzyme concentration are shown in Table 2. Maillard reaction (non-enzymatic browning) involves the reaction of aldehydes and amines with through various reactions. Maillard reactions to form compounds taste of food and dark colour. Maillard value ranges from 0.86 to 1.64. the higher concentration of enzyme and hydrolysis time have the higher of maillard value. Hydrolysis process would produce an amine group which is a substrate Maillard reaction, which amine groups of proteins binds to the aldehyde group or ketone of reducing sugars thus formed polymer nitrogenous brown or called melanoidin (Witono et al., 2014) which resulted in increasing the value of maillard on WPH.

Table 2 Maillard and rancidity of Wader protein hydrolysates (WPH) at various enzyme concentration and hydrolysis

Treatments	Maillard	Rancidity (mmol/kg)
1% enzyme; 0 h	0.91 ± 0.07	0.09 ± 0.00
2% enzyme; 0 h	0.86 ± 0.04	0.07 ± 0.00
3% enzyme; 0 h	1.09 ± 0.05	0.05 ± 0.00
1 % enzyme; 1.5 h	1.31 ± 0.05	0.11 ± 0.00
2 % enzyme; 1.5 h	1.59 ± 0.08	0.10 ± 0.01
3 % enzyme; 1.5 h	1.68 ± 0.08	0.06 ± 0.00
1 % enzyme; 3 h	1.13 ± 0.02	0.15 ± 0.01
2 % enzyme; 3 h	1.16 ± 0.04	0.13 ± 0.01
3 % enzyme; 3 h	1.64 ± 0.09	0.21 ± 0.01

The rancidity level of WPH indicated by the TBA for the formation of red color condensation between two molecules of TBA with 1 molecule malonaldehida. Increasing of enzyme concentration decreases the rancidity of WPH, but increasing of hydrolysis time increases the rancidity of WPH (Fig 8). Protease extracted directly from plants biduri contain of polyphenols and flavonoids which are antioxidants, so the more the Biduri protease is used, the content of polyphenols and flavonoids increasingly and decrease the rancidity levels of WPH. Rancidity can occur due to hydrolysis reaction with water. Hydrolysis processes will produce free fatty acids that will combine water molecules with fat molecule, so if one fatty acids has be freed resulting in the formation of rancidity in the hydrolysates (Murano, 2004).

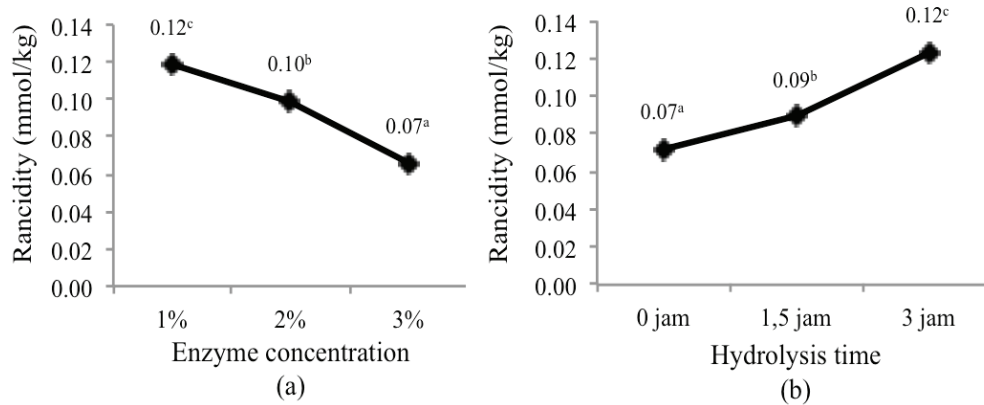


Fig 8. (a) The effect of increasing enzyme concentration and (b) hydrolysis time on the values of rancidity in WPH (jam=hour)

3.6. Amino acids composition of WPH

Fig 9 showed the amino acid composition of the best WPH treatments (3% of enzyme concentration and 3 hours of hydrolysis time). The hydrolysate were rich in glutamic acid (12.72 %), aspartic acid (7.66%), lysine (6.17%) where their composition was 42.39%. It was also noted that the hydrophobic amino acids, leucine and isoleucine was 3.18 and 3.06% respectively. The increase in hydrophobic amino acids is important due to the effects that these have on the functional properties of food proteins. Besides that, an increase amount of hydrophobic amino acids would also increase the solubility in lipid and therefore enhance the antioxidative activity (Rajapakse et al., 2005; Zhu et al., 2006).

According to Chen et al. (1996), amino acids such as tyrosine, histidine, lysine, tryptophan and methionine were generally accepted as antioxidants (Chen et al., 1996). Based on the research of whey protein isolate fractions (38), the delay of lipid oxidation was found to be related to the presence of histidine and hydrophobic amino acids. Thus, the result showed that the WPH also may be a good source of antioxidant to be incorporated into other products as supplement due to the presence of hydrophobic amino acids. Several studies has reported that the essential amino acid composition of the fish protein hydrolysate were higher than the recommended value for a human adult. Including those of herring (29), grass carp skin and round scad muscle hydrolysate (Thiansilakul et al., 2007).

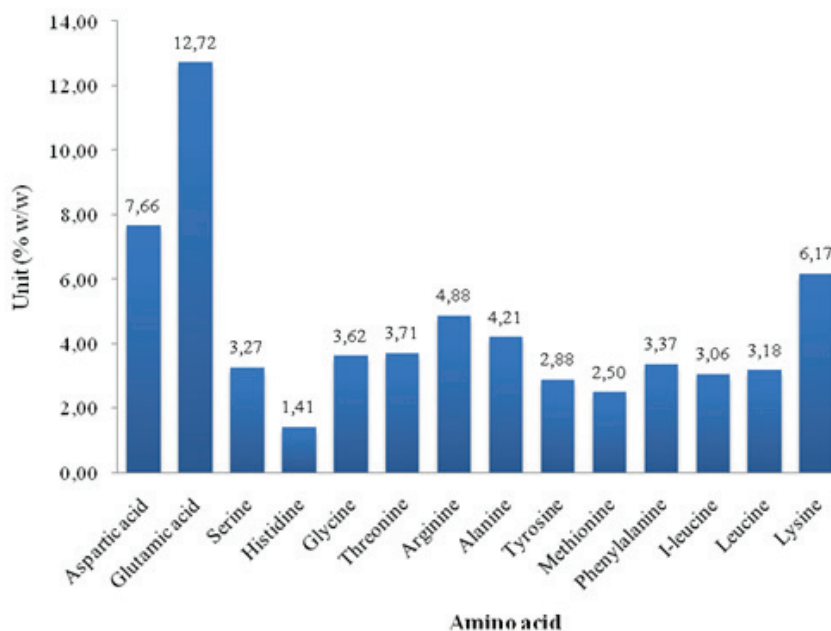


Fig 9. Amino acid composition of WPH (3% of enzyme concentration and 3 hours of hydrolysis time)

4. Conclusion

Protein solubility of WPH was significantly affected by enzyme concentration and hydrolysis time. Proximate composition showed significant difference in ash and fat content, but not in moisture and protein content. Emulsifying capacity increased with increasing of enzyme concentration but decreased with increasing the hydrolysis time. Foaming capacity was proportional to the increasing of enzyme concentration and hydrolysis time but foam stability was opposite. This study showed that the extent of hydrolysis had greatly influenced the amino acid content, maillard and rancidity. The high content of glutamic acid makes it good alternatives to be used as flavor enhancer. High solubility and good foaming properties makes it also good to be used as food ingredients as well as emulsifiers in food industry.

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