



## The potential of hydrolyzed, concentrated, and isolated protein from *Acetes erythraeus* as natural antioxidant

<sup>1</sup>Suparmi, <sup>2</sup>Irwan Effendi, <sup>2</sup>Nursyirwani, <sup>1</sup>Dewita, <sup>1</sup>Santhy W. Sidauruk, <sup>3</sup>Windarti

<sup>1</sup> Department of Fishery Products Technology, Faculty of Fisheries and Marine Science, Universitas Riau, Pekanbaru, Riau, Indonesia; <sup>2</sup> Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Riau, Pekanbaru, Riau, Indonesia; <sup>3</sup> Department of Water Resources Management, Faculty of Fisheries and Marine Science, Universitas Riau, Pekanbaru, Riau, Indonesia. Corresponding author: Suparmi, teten\_58@yahoo.co.id

**Abstract.** Rebon shrimp (*Acetes erythraeus*) is a small shrimp that is available in Riau Province, Indonesia. It can potentially be used as a source of hydrolyzed, concentrated and isolated proteins. These may produce bioactive compounds that are able to act as antioxidants. This study has been conducted to identify the antioxidant activities of the hydrolyzed, concentrated and isolated proteins from Rebon shrimp. The hydrolyzed protein was obtained using papain enzyme 15%, the concentrated protein was produced using isopropyl alcohol (1:3) and the isolated protein was produced by the pH 11 and pH 5 combination method. The antioxidant activity of all products was identified using the DPPH method. Results showed that the hydrolyzed protein contains terpenoids, flavonoids, alkaloids and phenolic compounds. The flavonoid content was 2.2356 mg g<sup>-1</sup> and the phenolic content was 4.2352 mg g<sup>-1</sup>. The molecule weights ranged from 5.62 to 71.19 kDa. The highest antioxidant activity was found in the hydrolyzed protein (IC<sub>50</sub> value was 0.20 mg mL<sup>-1</sup>).

**Key Words:** alkaloid, bioactive compounds, flavonoid, Rebon shrimp, terpenoid.

**Introduction.** Rebon (*Acetes erythraeus*) is present in Riau Province, Indonesia. The shrimp is available, producing a high amount of catch, 3215.4 tons ND 8462.2 tons in 2015 and 2016, respectively (Riau District Marine and Fisheries Service 2017). Its economic value is relatively low, as the price of fresh shrimp is less than 0.35 USD for 1 kg. Because of this, the shrimp is sometimes categorized as "marginal shrimp".

Even though *A. erythraeus* is categorized as a non-important commercial shrimp, its nutritional quality and quantity, especially the crude protein content, are not different from those of other shrimps. Its nutritional value is relatively high, with a crude protein content of 16.2%, 1.3% crude fat, 79% water, 2.6% calcium, 1% phosphorus and 2.2% Fe (Suparmi et al 2017). *A. erythraeus* is a small sized shrimp with a relatively soft shell. When processing, the shell waste is reduced, almost all body parts being processed.

So far, the use of *A. erythraeus* is not optimal, especially in Riau. It is rarely sold fresh or used as a main ingredient in Indonesian dishes. Instead, it is traditionally processed into dried shrimp, shrimp paste or shrimp sauce, commonly used as a spice.

As *A. erythraeus* is rich in proteins, its economic value can increase. By using non-conventional or modern processing methods, the shrimp can be used as a source for hydrolyzed proteins or concentrated proteins through enzymatic hydrolysis processes, or acidic, alkaline and fermentation processes (Jemil et al 2014). These products are commonly used as food additives. Moreover, results of several studies indicate that peptides present in the hydrolyzed protein act as bioactive compounds and could act as antioxidants (Luo et al 2012; Abdulazeez et al 2014; Tanuja et al 2014; Baehaki et al 2015).

Wijaya (1996) stated that natural antioxidants can be obtained from ocean resources. As Indonesia is rich in ocean resources, it is possible to discover new sources of antioxidants from the ocean, including Rebon shrimp. Luo et al (2012) discovered that hydrolyzed protein with antioxidant activities can be used as a replacement of synthetic antioxidants such as butylated hydroxytoluen (BHT) and butylated hydroxyanisole (BHA). These synthetic antioxidants may unfortunately trigger DNA damage and are toxic, so new resources can be explored.

*A. erythraeus* may be a potential natural antioxidant source. A modern method to produce hydrolyzed, concentrated and isolated proteins from Rebon shrimp has not been applied until now, to our knowledge. By applying the modern method, the proteins can be isolated and it used in food diversification. The aim of the study was to understand the potential of *A. erythraeus* as a source of hydrolyzed, concentrated and isolated protein and their potential as antioxidants.

## Material and Method

**Materials.** *A. erythraeus* shrimp obtained from the Bagan siapi-api Regency, Riau Province, was used in this study. The study was conducted in the Fish Processing Technology Laboratory, Fisheries and Marine Science Faculty, Riau University, Indonesia, in March 2018. Materials used include: papain enzyme, NaOH, NaHCO<sub>3</sub>, isopropyl, magnesium, methanol, Dragendroff reagen, Bouchardat and Mayer reactive agents, sodium dodesyl sulphate (SDS), amonium persulphate (APS), tetramethylenediamine (TEMED). Laboratory equipment used is: incubator (IS900 Yamato), autoclave (SM52 Yamato), stainless steel blender, centrifuge (Beckman 7B-6 type), oven (Memmert-Germany), pH meter (HM-205), magnetic stirrer, spectrophotometer UV-vis and freeze dryer.

**Methods.** This study used an experimental method to obtain the proteins, namely hydrolyzed protein (HP), concentrated protein (CP), and isolated protein (IP), and to identify the antioxidant roles of the proteins. The proteins from the shrimp were obtained through hydrolysis as follows:

**Hydrolyzed protein production.** The production of hydrolyzed protein was conducted following Nurhayati et al (2007). First, 300 g of shrimp was rinsed thoroughly using running water and then it was minced using a meat grinder. 300 mL of distilled water (1:1 w/v) was added, and the mix was homogenized and heated for 15 minutes, at 60°C. Then the pH was adjusted to reach the optimum point (7.0) by the addition of NaOH 0.5 N. Papain enzyme (15% of sample weight) was added. The mixture was incubated for 4 hours, at 60°C, in order to optimize the enzyme reaction. After the hydrolysis process, the sample was heated using a water bath for 15 minutes, at 85°C. The sample was centrifuged for 15 minutes at 15000 rpm for separating the liquid and solid materials. The hydrolyzed protein was the supernatant produced in this process.

**Concentrated protein production.** The concentrated protein production was based on Dewita & Syahrul (2014). To obtain the concentrated protein, 300 g of fresh shrimp was rinsed using running water, and minced. 1.5% of sample weight NaHCO<sub>3</sub> was added and the pH was adjusted to 7.4-7.8. NaCl (2% of shrimp weight) was added. The next step was the sample extraction using isoprophyl alcohol (the ratio of minced shrimp to isoprophyl alcohol was 1:3) for 10 hours, at 5°C. After the extracting process, the mixture was pressed to separate the NaHCO<sub>3</sub>, and then the pressed sample was dried for 24 hours in the oven, at 55°C. The dried sample was powdered by using a blender and was sieved using a sieve with an eye diameter of 250 microns to obtain concentrated protein.

**Isolated protein production.** The isolated protein production was based on Karnila (2012). This product was made by washing 500 g of fresh shrimp thoroughly in running water. The shrimp was dried for 24 hours at 60°C, in an oven. The dried sample was minced into powder using a blender, and was sieved using a sieve with an eye diameter

of 250 microns. Then 100 g of Rebon powder was suspended in AquaDest - the ratio of powder to AquaDest was 1:1.5 (w/v) - and the pH was adjusted by adding NaOH 1 N progressively to reach pH 11. The mixture was heated and stirred at 55°C for 30 minutes, then it was centrifuged for 15 minutes at 15000 rpm. The pH of the supernatant produced was adjusted by progressively adding HCl 1 N to reach pH 5. The supernatant was centrifuged at 15000 rpm for 15 minutes. The supernatant was removed and the particles obtained (the shrimp isolated protein) was dried using a freeze dryer.

**Antioxidant activity test.** The antioxidant activities of the hydrolyzed protein, concentrated protein, and isolated protein products obtained were tested using the DPPH method, based on Li et al (2007). To test the activities, 10 mg of concentrated or isolated proteins, or 10 mL of hydrolyzed protein were diluted with methanol PA, in series concentrations (100, 200, 400, 800 ppm). The solution from each concentration (160 µL) was mixed with 40 µL DPPH (0.3 mg mL<sup>-1</sup>). The mixture was incubated in a dark room, at 37°C for 30 minutes. The absorbance was measured at 517 nm wavelength, and ascorbic acid was used as a control. Sample concentration and the inhibition percentage were plotted in a linear regression curve and the 50% inhibition concentration (IC<sub>50</sub>) was calculated in mg mL<sup>-1</sup>. The absorbance of the blanko liquid was measured as a basis to calculate the inhibition percentage. The results of absorbance were calculated using the following formula:

$$\% \text{ inhibition} = \frac{(\text{blanko absorbance} - \text{sample absorbance})}{\text{blanko absorbance}} \times 100$$

The data was then plotted for linear regression, with the sample concentration on the X axis and the inhibition values on the Y axis to find out the IC<sub>50</sub> (50% inhibition concentration value).

**Natural compound identification.** Several tests were conducted to identify the presence of natural compounds from the obtained products, including steroids/terpenoids, alkaloids, and flavonoids. The tests conducted are:

1. Steroid/terpenoid identification test: 1 mL of sample solution (hydrolyzed protein, concentrated protein, and isolated protein have been macerated with methanol) was dried up at room temperature for 24 hours. The dried sample was mixed with Lieberman-Burchard reagent. The presence of steroids and terpenoids was indicated by the changing of the solution color to blue.

2. Alkaloid identification test: 3 mL of sample solution were added to 1 mL HCl 2 N and 6 mL of AquaDest. The solution was heated for 2 minutes, cooled at room temperature and filtered. The filtrate was checked for the presence of alkaloid compounds by using Dragendroff, Bouchardat and Mayer reagents. Formation of precipitates indicates the presence of alkaloid compounds.

3. Flavonoid identification test: 2 mL of the extract was added to magnesium powder and 2 mL of HCl 2 N. The presence of flavonoids was indicated by the changing of the color of the solution to orange or red.

**The profile of protein molecular weight.** The weight of hydrolyzed, concentrated, and isolated protein molecule from the Rebon shrimp was identified using a Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method based on Laemmli (1970).

## Results and Discussion

**Natural compound in the Rebon shrimp products.** Results of the natural compound identification tests showed that the hydrolyzed, concentrated and isolated protein from the Rebon shrimp contain alkaloid and other compounds (Table 1).

The content of terpenoid compounds in the hydrolyzed, concentrated and isolated proteins is generally the same, adequate (+++). The terpenoid and saponin content was also mainly the same (+). The hydrolyzed proteins contain more flavonoids (++)

alkaloids (++++) and phenolics (++++). Sjahid (2008) stated that the flavonoid compound acts as a good reductant compound able to deter many oxidation reactions, in enzymatic or in the non-enzymatic processes. Hanani et al (2005) mentioned that the alkaloid compounds may act as antioxidants. The amount of flavonoids and phenolic compounds of the products are presented in Table 2.

Table 1

The content of natural chemical compounds of hydrolyzed, concentrated, and isolated protein from Rebon shrimp (*Acetes erythraeus*)

Compound	Hydrolyzed protein	Concentrated protein	Isolated protein
Terpenoid/Steroid	+++	+++	+++
Flavonoid	++	+	+
Alkaloid	+++	++	++
Phenolic	++++	+++	++
Saponin	+	+	+

Note: + - present; ++ - low; +++ - adequate; ++++ - abundant.

The content of flavonoids is more than half of the phenolic compounds in the tested products (Table 2). The content of flavonoid and phenolic compounds in the hydrolyzed proteins was higher than those of other products. This fact suggested that the hydrolyzed protein of the Rebon shrimp has the potential to be used as a basic material for antioxidants. According to Karadeniz et al (2005), phenolic compounds have various biological effects and roles, such as antioxidant activities through reduction mechanisms, free radical scavengers, chelating metals, hampering the formation of singlet oxygen and electron donors. As the phenolics, flavonoid compounds have the potential to capture free radicals. Pratama et al (2013) mentioned that the antioxidant activity of a food resource is affected by its chemical compounds such as amino acids and phenolic compounds. Moreover, Sayuti & Yenrina (2015) stated that antioxidant components such as phenolics and flavonoids are natural antioxidant substances present in food sources.

Table 2

The total content of flavonoid compounds and phenolics in Rebon shrimp (*Acetes erythraeus*) products

Product	Compound ( $\text{mg g}^{-1}$ dry basis)	
	Flavonoid compounds	Phenolic compounds
Hydrolyzed protein	2.2356	4.2352
Concentrated protein	1.7814	3.3810
Isolated protein	1.6300	2.4495

**Molecular weight profile of hydrolyzed, concentrated, and isolated protein.** The results of the SDS-PAGE in identifying the molecule weight of the hydrolyzed, concentrated, and isolated proteins from the Rebon shrimp are various. The weight of hydrolyzed protein ranged from 5.62-71.19 kDa, while that of the concentrated and isolated proteins were 5.38-71.39 kDa and 5.38-71.11 kDa, respectively. The peptide fractures are related to types, concentrations and specifications of enzymes toward the used substrate. The protein molecular weight profile of Rebon shrimp products is presented in Figure 1.

Results obtained indicate that the protein fractures of all the products was <100. The low protein fraction in the hydrolyzed proteins may be caused by the use of the papain enzyme during the hydrolyzing process, as the enzyme may be able to break up the protein fraction. Molecular weight of the hydrolysate may affect the antioxidant properties. Khirzin et al (2015) stated that the antioxidants of sea cucumbers are relatively strong, with an  $\text{IC}_{50}$  value of 2.19, and this may be caused the presence of peptides that have 91.91-95.03 kDa molecular weights and are in coarse form.

According to Mine & Shahidi (2005), protein originating from ocean products, such as bioactive peptides, actively affect human health. The molecule weight of peptides that are active and positively affect human health was <100 kDa, consist of 3-10 amino acids. As the molecule weight is lighter, the protein and amino acid content may increase and the bioactivity of the product would also increase. Belkaaloul et al (2010) stated that in the protein hydrolysis process, by using proteolytic enzymes, the proteins may be broken down into smaller protein fractions and could increase the protein content in the hydrolyzed product.

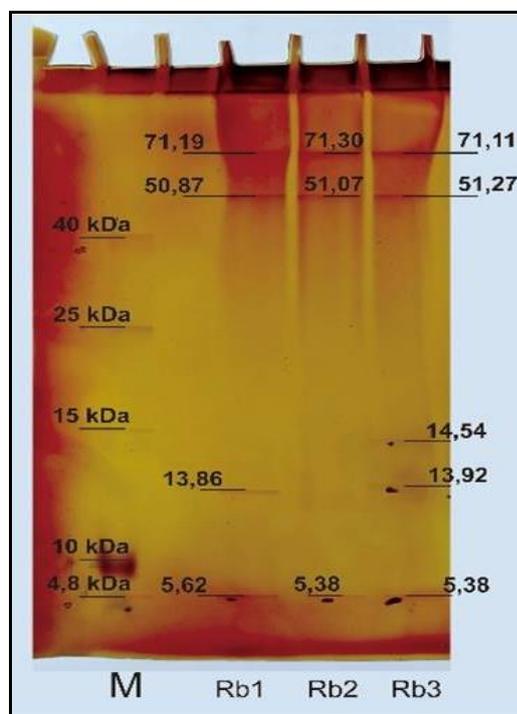


Figure 1. Profile of molecular protein weight of Rebon shrimp (*Acetes erythraeus*) products. Rb1 - hydrolyzed protein; Rb2 - concentrated protein; Rb3 - isolated protein.

**Antioxidant activities of hydrolyzed, concentrated, and isolated proteins from Rebon shrimp.** The results of the antioxidant activity tests on Rebon shrimp products are presented in Table 3.

Table 3  
Antioxidant activity ( $IC_{50}$ ) in the hydrolyzed, concentrated, and isolated protein from the Rebon shrimp (*Acetes erythraeus*)

Product	$IC_{50}$ (mg mL <sup>-1</sup> )			Average
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	
Hydrolyzed protein	0.20	0.18	0.22	0.20±0.02
Concentrated protein	0.45	0.43	0.47	0.45±0.02
Isolated protein	0.58	0.53	0.56	0.35±0.25

Note: R - repetition.

The  $IC_{50}$  of each product is different. The  $IC_{50}$  value of the hydrolyzed protein was 0.20 mg mL<sup>-1</sup>, lower than those of the concentrated protein (0.45 mg mL<sup>-1</sup>) and isolated protein (0.35 mg mL<sup>-1</sup>). The low  $IC_{50}$  value in the hydrolyzed protein may be caused by the use of papain enzyme during the process, producing peptides with stronger antioxidant properties. Li et al (2007) stated that the difference in antioxidant activity might be affected by structure composition and hydrophobicity of the amino acids that

construct the peptide, and the type of enzyme that hydrolyzed the product (Qian et al 2008).

The IC<sub>50</sub> value of the hydrolyzed protein was relatively low (0.20 mg mL<sup>-1</sup>), indicating that the antioxidant activity of the product is strong. This may be due to the use of enzymes during the hydrolysis process, as the enzymes may have produced more peptides with high antioxidant activity. Fan et al (2012) stated that the deterring act of DPPH free radical in hydrolyzed protein from bones of Nile tilapia (*Oreochromis niloticus*) that was isolated using enzymatic processes is strong (IC<sub>50</sub> - 1.92 mg mL<sup>-1</sup>).

The antioxidant activity IC<sub>50</sub> of the hydrolyzed protein from Rebon shrimp is stronger than that of other products. According to Torino et al (2013), the antioxidant activities of the hydrolyzed protein are strongly affected by the type of peptides that were formed through the hydrolysis process. Wu et al (2003) stated that the molecular weight and amino acid composition of peptides formed during hydrolysis process strongly affect the antioxidant activity of hydrolyzed proteins. The antioxidant activities of hydrolyzed protein from Indian mackerel (*Rastrelliger kanagurta*) have a strong potency, being able to deter the fat oxidation process, with a strength almost equal to that of synthetic antioxidants such as BHT (Abdulazeez et al 2014). Thus, the hydrolyzed protein from fishery products that has bioactive peptides is a potential natural antioxidant and might be developed into commercial food additives. Similar results were obtained by Wang et al (2014), who stated that the IC<sub>50</sub> value of hydrolyzed protein from fish that was isolated through enzymatic processes was 0.283, indicating a strong action in deterring the DPPH free radicals.

**Conclusions.** The hydrolyzed, concentrated, and isolated protein can be obtained from the Rebon shrimp. In the protein products, there are abundant natural compounds present, consisting of terpenoids, flavonoids, alkaloids, phenolics, and saponins. The flavonoid content of hydrolyzed protein is 2.2352 mg g<sup>-1</sup> and the phenolic content is 4.2352 mg g<sup>-1</sup>. The molecule weight profile was <10 kDa. The IC<sub>50</sub> value of the antioxidants from hydrolyzed protein of Rebon shrimp is 0.20 mg mL<sup>-1</sup>, indicating that the hydrolyzed protein is a potential natural antioxidant.

**Acknowledgements.** This research is financially supported by the LPPM University of Riau, Pekanbaru, Indonesia, through a research grant from 2018. We would like to thank the team members, students, and colleagues that helped and supported this research.

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Received: 29 December 2019. Accepted: 08 February 2020. Published online: 16 May 2020.

Authors:

Suparmi, Department of Fishery Products Technology, Faculty of Fisheries and Marine Science, Universitas Riau, 28293 Riau, Indonesia, e-mail: teten\_58@yahoo.co.id

Irwan Effendi, Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Riau, 28293 Riau, Indonesia, e-mail: helpingirwan@gmail.com

Nursyirwani, Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Riau, 28293 Riau, Indonesia, e-mail: nursyirwani\_adnan@yahoo.com

Dewita, Department of Fishery Products Technology, Faculty of Fisheries and Marine Science, Universitas Riau, 28293 Riau, Indonesia, e-mail: bucharidewita@gmail.com

Santhy Wisuda Sidauruk, Department of Fishery Products Technology, Faculty of Fisheries and Marine Science, Universitas Riau, 28293 Riau, Indonesia, e-mail: santhy.sidauruk@lecturer.unri.ac.id

Windarti, Department of Water Resources Management, Faculty of Fisheries and Marine Science, Universitas Riau, 28293 Riau, Indonesia, e-mail: windarti.unri@gmail.com

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How to cite this article:

Suparmi, Effendi I., Nursyirwani, Dewita, Sidauruk S. W., Windarti, 2020 The potential of hydrolyzed, concentrated, and isolated protein from *Acetes erythraeus* as natural antioxidant. *AAFL Bioflux* 13(3):1292-1299.