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Qualitative Protein Hydrolyzed from *Nerita undata* in *supralittoral rocks* and *mezolittoral zone* of Hasa Cape using TLC

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Abstract

Gastropods are the sources of protein for coastal communities in the Maluku islands. We conducted analysis quantitative and qualitative of protein in *Nerita undata* meat from Hasa Cape in Saparua Island. The percentage of protein was analyzed by the Kjeldahl method. Qualitative analysis began with breaking peptide bonds in protein to amino acid components by sulphuric acid and barium hydroxide hydrolyzed. We identified amino acid compounds by using thin-layer chromatography (TLC) in butanol-acetic acid-water (8:1:1 v/v) as eluent. The result showed that *N. undata* meat contains 11.15% of protein and twelve amino acid compounds. There are seven essential amino acids in *N. undata* meat from supralittoral rocks and mezolittoral zone of Hasa cape is a source of quality protein. Thus, this species can be considered as a source of high-quality protein.

Keywords: Amino acid, hydrolysis, Kjeldahl method, Nerita undata, protein.

INTRODUCTION

Food can provide a source of oil and protein. Oil sources can be obtained from plants such as Tengkawang (*Shorea Sumatrana*) (Riski, Maulana, Permana, Lestari, & Tarigan, 2020), and from animals it can be obtained from seafood. Seafood provides oil and also protein. Sources of oil can come fish ex. silver fish (*Mene maculata*) (Tahya, Tahya, & Kainama, 2019). High-quality protein can come from seafood non fish. High-quality protein that contains essential amino acids and is required for the growth and maintenance of the human body (Fagbuaro, Oso, Edward, & Ogunleye, 2006; Domingo, 2016; (Wells et al., 2017)). Essential amino acids, also known as indispensable amino acids, are components that humans and other vertebrates cannot synthesize from metabolic intermediates. These amino acids must be supplied from an exogenous diet because the human body lacks required the metabolic pathways to synthesize.

Proteins contain 20 different amino acids including essential and non-essential of nutrition importance. Both types are indispensable for the nutrition of cells and normal cell and organ function. Shellfish also provide high-quality protein with all the dietary essential amino acids for the maintenance and growth of the human body. Accordingly, shellfish should be considered a low-fat, high-protein food that can be included in a low-fat diet (King, Childs, Dorsett, Ostrander, & Monsen, 1990). The *Nerita undata* is present a great amount in Hasa Cape of Saparua Island. Based on empirical evidence of local communities, the *N.undata* is consumed as food. The meat of species was processed for an alternative protein source.

N.undata consumption only occurs during extreme weather so availability of fish is limited. Therefore, it is crucial to know the essential amino acid compounds contained in *N. undata* meat. The study aim is to determine qualitative analysis of protein in *N.undata* from Hasa Cape in Saparua Island.

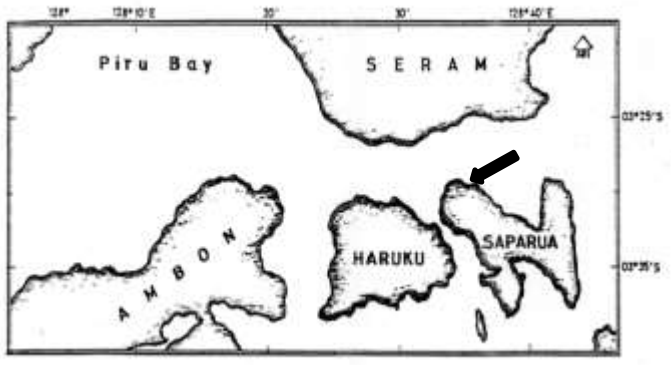

Figure 1. Data location of *N. undata*

The amino acids were identified with the conventional method by using thin-layer chromatography (TLC). Bhawani et al., (2012)

reported the ability of TLC to detect the type of amino acid in a sample as an indicator of protein quality (Bhawani et al., 2012). The result of this study is the first report of amino acid and protein composition from gastropods of these supralitoral and mesolitoral rocks in Saparua coastal. Nevertheless, these are preliminary data for the classification and prediction of the protein biosynthesis pathway in *N.undata*.

METHODOLOGY

Materials and Instruments

N. undata was collected from Hasa Cape, Saparua Island. Chemicals using in this study were pure analysis from Merck: petrolium ether, sulphuric acid (H_2SO_4), Barium hydroxide, $Ba(OH)_2$, Amino acids standard, buthanol (C_4H_9OH), acetic acid glacial (CH_3COOH), HCl, Nynhidrin, Ethanol, Mercury oxide (HgO), Sodium sulphate (Na_2SO_4), Sodium thiosulphate ($Na_2S_2O_3$), Boric acid (H_3BO_3), zink, methyl red-methylene blue indicator, TLC silica gel 60 F_{254} Preparative sheets 20x20 cm^2. The equipment: soxhlet set, volumetric flask, three-neck round flask, buret, Kjeldahl apparatus, erlenmeyer, CAMAG Flat bottom TLC chamber and destilation set from pyrex.

Methods

Isolation of Water and Fat in *N. undata* Meat (NuM)

NuM was cut into small pieces, then 1 g dried in the oven at 110^{o}C for 3 hours. The dried NuM obtained was extracted with a soxhlet apparatus until lipid was dissolved in petroleum ether for 72 hours.

Acidic Hydrolysis of Protein NuM

N.undata meat fat free refluxed with concentrated sulphuric acid at 110 ^{o}C for 24 hours. The solution neutralized by adding barium hydroxide slowly and filtered to the mark of 50 mL in a measuring flask.

Basic Hydrolysis of protein NuM

N.undata meat fat free refluxed with 22.8 g barium hydroxide in 10 mL aquadest at 110 ^{o}C for 24 hours. The solution neutralized by adding sulphuric acid slowly and filtered to the mark of 50 mL in a measuring flask.

Analysis compounds of amino acids in NuM

TLC Chamber containing the mobile phase as eluent of butanol-acetic acid-water (80 mL:10 mL:10 mL v/v) saturated for an hour. On the other side TLC was activated by heating in the oven for 2 minutes at 85 ^{o}C. Spot 1 µL of standard amino acid solution and 2 µL sample NuM hydrolyzed on TLC, respectively. The TLC was put into the chamber and has been left completely eluted at plate. TLC was drying in room temperature and then sprayed with 0.1% of ninhydrin in ethanol. TLC was heating in the oven for 10 minutes at 80 ^{o}C. The spots of sample NuM then was comparing to 17 standard amino acids.

Analysis of protein content of NuM

NuM protein content can be known by measuring the amount of Nitrogen total. Nitrogen was determined by the Kjeldahl method as described by Babu et al., 2010. NuM 1 g of that has been mashed into a 500 mL Kjeldahl flask and added 10 mL of sulphuric acid. 5 g of a mixture of Na_2SO_4-Hg (20:1) was added as a catalyst (Babu, Venkatesan, & Rajagopal, 2011). The destruction process uses a Kjeldahl flask and is carried out in a fume hood until it stops smoking. The mixture is continued heating at high temperature until the liquid is colorless and added an hour before cooling. After cooling, add 140 mL of aqua dest and 35 mL of NaOH-$Na_2S_2O_3$ and granules of zinc. Distillation was carried out and 100 mL of distillate was accommodated in an Erlenmeyer containing 25 mL of a colorless solution of boric acid and a few drops of methyl red-methylene blue indicator. Furthermore, the obtained solution was titrated with 0.1 N HCl. The blank solution was prepared by replacing NuM with aquadest where the destruction, distillation, and titration. This procedure is done in duplicate.

Data Analysis

The water, fat and protein contents of samples was determined using Equation 1, 2, 3 dan 4 respectively.

$$\%\ \text{Water} = \frac{W0-W1}{W0} \text{x } 100 \quad (1)$$

$$\%\ \text{Fat} = \frac{\text{W fat}}{\text{W sample}}\ x\ 100 \quad (2)$$

$$\%\ \text{N} = \frac{\text{V HCl blank} - \text{V HCl sample x N HCl x } 14{,}08}{\text{W sample x } 1000} x\ 100 \quad (3)$$

$$\%\ \text{Protein} = \%\ \text{N}\ x\ \text{Conversion factor } (6.25) \quad (4)$$

RESULTS AND DISCUSSION

Description of *N. undata* Line

Gastropod samples was collected from Tanjung Hasa (Figure 1). Then the samples was identified the taxonomically at the Invertebrate Laboratory of LIPI Ambon.

Kingdom : Animalia
Phylum : Mollusca
Class : Gastropods
Ordo : Archaegastropods
Family : Neritideae
Genus : Nerita
Species : *Nerita undata*
Local name : Bia langsat (Saparua)

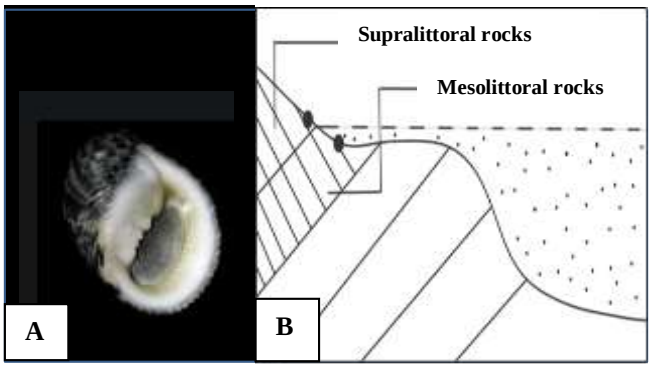

Figure 2. (A) Shell; (B) Living zone of *N. undata*

N. undata have spire of the shell is short, while the bottom is swollen. On the outside of the lip, there is a thickening with teeth as in the columella (Figure 2A) (Nouet, Cotte, Cuif, Dauphin, & Salomé, 2012). This species size of 2-3 cm. Tend to move freely in search of food at low tide and moderate temperatures are mostly found at night. They generally live in the supralittoral zone and cling to dead coral (Figure 2B).

Composition of Water and Fat in NuM

The initial analysis in this study was to separate water and fat from the sample before being hydrolyzed of protein in NuM. The gravimetric method is used to determine the percentage of water lost during the drying process. The NuM was heated at constant temperature causes a decrease in the sample mass 5.28±0.15g to 0.94±0.15g (Table 1). This study showed that petroleum ether as a non-polar solvent has released fat on *N.undata* meat on soxhlet extraction for 72 hours. Dry weight (W1) of NuM 0.94±0.32 g contains fat weight (Fw) 0.007±0.29 g equivalent to 76% of fat (0.76%) (Table 1, Figure 3). The results of the analysis showed that NuM contains water (82.0%) higher than fat (Figure 3). The large water content must be removed from the sample so that no emulsion occurred during the fat extraction process. However, the low contained fat molecules must be released from the sample. This step is taken to prevent the formation of tailings or spots on the widening of the TLC.

Table 1. Percentage of Water, fat, and N in NuM

Sample	Percentage of Water		
	Wo(g)	W1(g)	Water (%)
	5.28±0.15	0.94±0.15	82.0±0.15
	Percentage of Fat		
	W1 (g)	Wf	Fat (%)
NuM	0.94±0.32	0.007±0.29	0.76±0.30
	Percentage of Nitrogen		
	W1 (g)	Titrant (mL)	Nitrogen (%)
	1.05±0.21	13.86±0.12	1.85±1.35

Wo: wet weight, W1: dry weight, Wf: weight of fat

Protein of NuM

The Kjeldahl method is used to analyze crude protein in foot ingredients indirectly. The percentage of nitrogen is equivalent to 6.25 mg of protein (conversion factor). The principle of protein analysis using the Kjeldahl procedure that has been carried out in this study through three major steps is digestion, distillation, and titration. The aim of the digestion procedure is to break all nitrogen bonds in the NuM and convert all of the organically bonded nitrogen into ammonium ions. Organic carbon and hydrogen from NuM formed carbon dioxide and water. Digestion meat *N. undata* is hydrolyzed with 98% sulfuric acid and heated in the mixture. The step aims to break down other substances found in the NuM (eq.5). In this process, the organic material from NuM carbonizes which can be visualized by the transformation of the NuM into black foam. During the digestion, the foam decomposes and finally, a clear liquid indicated the completion of the chemical reaction. Zink is added in order to increase the speed and efficiency of the digestion procedure. After digestion is completed the NuM is allowed to cool to room temperature, then dilute with water and transferred to the distillation unit.

$$\text{Protein (-N) NuM} + H_2SO_4 \xrightarrow{Zn} (NH_4)_2SO_4 + CO_2 + H_2O \quad (5)$$

$$(NH_4)_2SO_4 + 2NaOH \rightleftharpoons 2NH_3 + (NH_4)_2SO_4 + 2H_2O \quad (6)$$

$$NH_3 + HCl \longrightarrow NH_4Cl \quad (7)$$

Furthermore, the distillation and titration step following general principles of the Kjeldahl method. Both of the distillation and titration in this study are described in reaction (Equation 6 and Equation 7).

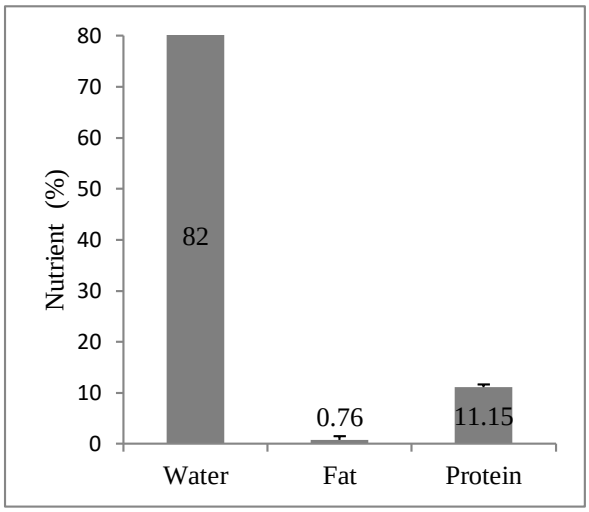

Figure 3. Water, fat and Protein of *N. undata*

The volume of hydrochloric acid used for ammonia titration in this study was 13.86 ±0.12 mL (Table 2). Based on eq.3 then the calculation of percent nitrogen in NuM is 1.85±1.35 % (Table 2). The percentage of protein in NuM of 11.15±1.35% (Fig.3) is highly determined by the amount of nitrogen (eq. 4). The shows that species NuM contains nutrients that can be used to meet animals other than fish, squid, shrimp, eggs, and others. So that, protein supply for the human body can be obtained from NuM. The results show that the percentage protein of NuM is almost the same as Lambis lambis from Waisarisa (15.52%) and Suli (16.97%) (Leiwakabessy & Lewerissa, 2017)

Amino acids in NuM

Peptide bonds linking amino acid monomers can be broken by the hydrolysis of protein. Hydrolysis can be performed by either chemical or enzymatic means, while chemical hydrolysis can be performed under either acidic or basic conditions. This process is refluxed *N.undata* meat in 6 M sulphuric acid without oxygen at 110 ^{o}C in 22 hours to breaking peptide bonds from protein. However, this process caused the destruction of the tryptophan (**Trp**) amino acid. The further, hydrolyzed using $Ba(OH)_2$ to digest the damaged **Trp** due to acid hydrolysis.

In this study using TLC for detection of amino acids components in both hydrolyzed acid and is based of protein in NuM an analysis was carried out. The use of TLC based on several advantages such as fast, inexpensive, and versatile separation technique with many practical considerations that contribute to its effectiveness (Bhawani et al., 2012).

The use of eluent caused the separation of amino acid components in NuM and compared Rf with the standard. The Rf at chromatograms TLC of amino acid profiles of N. undata and standard are presented in Fig.4. The sulphuric acid hydrolysis of NuM resulted in eleven spots with Rf suitable for **Cys**, **Lys**, **His**, **Asn**, **Ser**, **Thr, Ala**, **Val**, **Tir**, **Leu,** and **Phe**. Ten spots as barium hydroxide hydrolized with Rf suitable for **Cys**, **Lys**, **His**, **Asp**, **Ala**, **Val**, **Tir**, **Leu**, **Phe** and **Trp**, respectively.

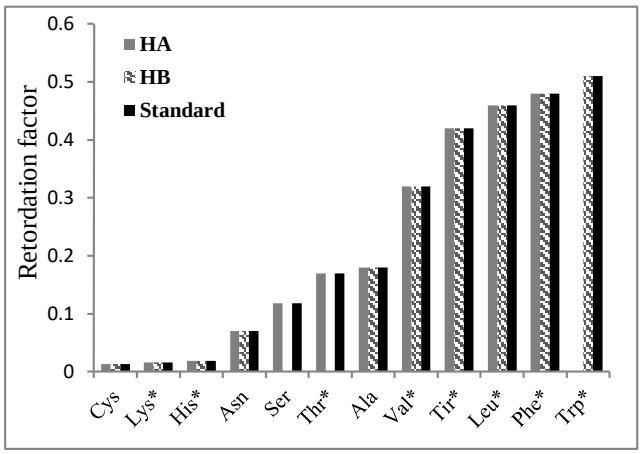

Figure 4. Rf value of amino acids in NuM
HA:(sulphuric acid hydrolized); HB:(barium hidroxide hydrolyzed)*: Essential amino acids.

The amino acid of **Ser** and **Thr** are destroyed while **Trp** appears on barium hydroxide hydrolyzed. Both of these hydrolysis subtances obtained twelve amino acid components were **Cys**, **Lys**, **His**, **Asp**, **Ser**, **Thr**, **Ala**, **Val**, **Tir**, **Leu**, **Phe,** and **Trp**, respectively. The seven components of essential amino acids type in NuM were **Lys**, **His**, **Thr**, **Val**, **Leu**, **Phe**, and **Trp** (Figure 4). Methionine (**Met**) is an amino acid component that plays as a growth stimulant. This study showed that **Met** was not found in NuM. The deficiency of **Met** component can be a change of **Cys** in this species.

In principle, a protein source that can provide essential amino acids for human needs has high quality. The quality of amino acids and their availability determines the nutritional quality of the proteins (Gressler et al., 2010). Animals cannot synthesize all these amino acids of primary metabolisms by themselves, and it will earn through their diet (Joshi, Joung, Fei, & Jander, 2010). These results studies indicate that NuM has high-quality protein which is showed that profile described there are seven essential amino acids (>50%). The species is classified as a high-quality protein source.

In general, the gastropods species were reported to provide an inexpensive source of proteins and amino acids (Babu et al., 2011). The results of this study can be compared with the amounts of essential amino acids

previously reported in gastropods from different locations. NuM contains eight components essential amino acids so that higher protein quality than *C. ramosus* (six components). The data in Table 1 showed that eight essential amino acids contain in *L.lambis* and *Haliotis* sp, respectively from Seram Island (Leiwakabessy & Lewerissa, 2017) the same amount as *N. undata*. The *P. tumindus* reported by Hafiludin et al., 2020 which contains eight essential amino acids components. However Jayaprabha et al., 2016 reported on *T. Brunneus* containing glutamic acid which was not used as a standard for the sample of *N. Undata* (Jayaprabha, 2016). In the genus Nerita, Feng et al., 2021 reported the types of essential amino acids in *N. chameleon* meat are the same as those contained in NuM (Feng, Miao, Li, Guo, & Lü, 2021). Another study reported by Humayun et al., 2020 regarding N. undata scattered in coastal Pakistan contains higher protein (35.67±3.36%) and fat (18.01±0.35%) than that obtained in this study. However, the number and types of essential amino acids were the same as in NuM from Hasa Cape except for Isoleucine (Ile).

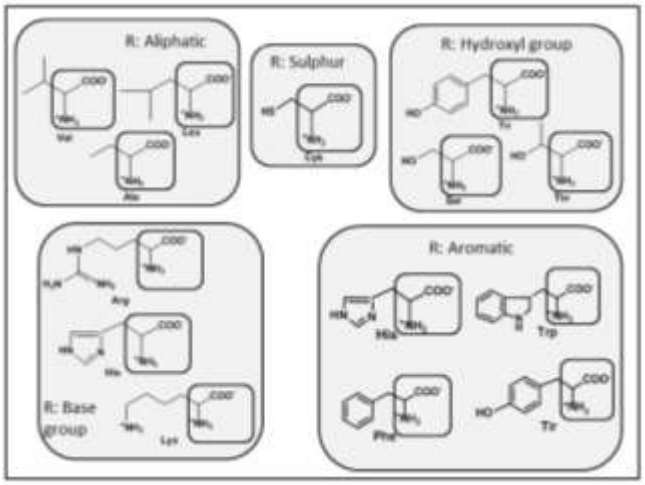

Figure 4**.** Groups amino acids in NuM

Figure 4 showed that **Val** and **Ala** belong to the type of compounds with R being aliphatic. The three types of amino acids in NuM with R as the hydroxyl group are **Tir**, **Ser,** and **Thr**. Type of amino acids wich R as the base are three compounds **Arg**, **His** and **Lys**, respectively. The **His**, **Trp**, **Tir** and **Phe** are amino acids containing aromatic in the R group. The only amino acid that contains sulfur is **Cys**

Biosynthesis pathway of amino acid in NuM

Twenty amino acids are required for protein synthesis. Animals have the genetic material required to synthesize the enzymes found in the biosynthesis pathways for amino acids. There is likely an evolutionary advantage behind removing the long pathways required to synthesize essential amino acids. Genetic material is required to synthesize these amino acids and relying on the environment to provide these building blocks. Based on the previously reported model (Nelson & Michel, 2008) it can be predicted that the biosynthesis pathway of amino acids in NuM can be predicted as shown in Figure 5.

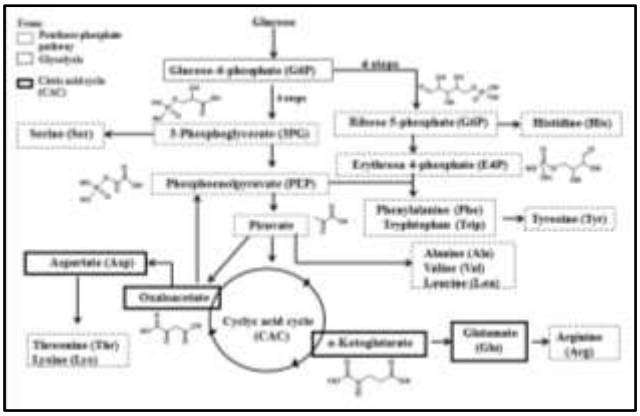

Figure 5**.** Biosynthesis pathway amino acids proposal in NuM based on profile chromatogram of TLC

Amino Acid mostly contains Nitrogens in the form of NH_4^+ and carbon atoms. NH_4^+ group comes from glutamate or glutamine and carbon atoms come from glucose as a monosaccharide. The source of amino groups in Amino Acid is derived from glutamine and glutamate in the process of transamination. The source of carboxyl and hydroxyl groups is derived from monosaccharides (glucose). Individual Amino Acid is a kind of amino acid that is derived from the other amino acid but, it can't convert to be another (Hou & Wu, 2018; Hou, Yin, & Wu, 2015) .

Biosynthesis pathway in *N. undata* predicted showed that Figure 5. The amino acids *N. undata* are derived from Phosphoenolpyruvate and Erythrose 4-Phosphate were **Tir**, **Trp**, and **Phe**. The His is the only amino acid that is derived from ribose 5-phosphate. **Ser** and **Cys** are both of amino acids are derived from 3-Phosphoglycerate. Amino acids biosynthesis by oxaloacetate is **Thr**, **Lys,** and **Arg** from α-ketoglutarate of precursor, respectively. The **Ala**, **Val**, and **Leu** are amino acids groups of biosynthesis by pyruvate precursor.

CONCLUSION

NuM consumed by the people on Saparua Island is a source high-quality protein from supralittoral and mesolittoral zone which is seven essential amino acids. The five types of amino acids in NuM based on R are

metabolic precursors in the biosynthesis of amino acids.

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