

The Identification of Carotenoids and Testing of Carotenoid Antioxidants from Sand Lobster (*Panulirus homarus*) Egg Extract

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Abstract

Sand lobsters (*Panulirus homarus*) are organisms that play an important role in supporting a society's economy. Lobsters have a business appeal due to their high demand, enjoyable taste, as well as significant nutritional content. It is interesting that sand lobster eggs have a yellow orange color as an indicator of the presence of carotenoid content. Related with advancements in research and the utilization of carotenoids, this research identifies carotenoids and tests carotenoid antioxidants from sand lobster (*Panulirus homarus*) egg extract. In identifying carotenoids in sand lobster egg extract, HPLC (high performance liquid chromatography) (shimadzu LC-10AD, Japan) is used. In testing carotenoid antioxidants from sand lobster eggs, a DPPH method is utilized. The identification results with HPLC reveal that the sample contains carotenoids. The kinds of carotenoids identified are dinoxanthin, diadinoxanthin, zeaxanthin, lutein, astaxanthin, and violaxanthin. Among the six kinds of carotenoids identified, lutein is the type of carotenoid that has the highest concentration. These carotenoid compounds are detected in wavelengths of 400-500 nm. The test results of antioxidant power reveal that to hamper free radicals (IC_{50}), a sample concentration of $6675.25 \mu\text{g}\cdot\text{ml}^{-1}$ is needed. The ability to hinder free radicals from a sample extract is mostly conducted by lutein, zeaxanthin, and astaxanthin. If seen from the analytical results, it can be surmised that sand lobster eggs contain carotenoids that can be used as a carotenoid source for humans.

Keywords : Sand Lobster, HPLC, Carotenoids, DPPH, Antioxidant

Introduction

The tropical characteristic of the archipelago water makes it possible for various kinds of lobsters to live, including sand lobsters (*Panulirus homarus*). The distribution of this lobster species is supported by the presence of several other kinds of lobsters like tropical rock lobsters (*Panulirus ornatus*), pronghorn spiny lobsters (*Panulirus penicillatus*), scalloped spiny lobsters (*Panulirus homarus*), and painted spiny lobsters (*Panulirus versicolor*), which are societal cultivation objects (Subani et al., 1993). It can be said that Indonesian waters are an appropriate climate for lobsters to grow and develop.

Sand lobsters are from the crustacean family and have the potential to stimulate economic and industrial advancements. The increasing demand and high nutritional content make sand lobsters a business and source of income for society. Statistical data from Ditjenkan (2011) reveals that

lobsters are considered as an Indonesian export commodity. Sand lobsters (*Panulirus homarus*) are considered as a species which has an export value of up to US \$13 million with prices of US \$6-7.kg⁻¹ (Anonymous 2011a; 2011b). Thus, lobsters do not just drive the society's economy, but they are also a source of state foreign exchange.

One of the important compounds in a lobster's body is carotenoids. These are a group of organic pigments that are fat soluble. Based on the arrangement of components, carotenoids can be divided into two pigment groups, which are xanthophyll (has C, H, O atoms) and carotene (has C, H atoms). For instance, the xanthophyll group has astaxanthin (C₄₀H₅₂O₄). An example of carotene is β -carotene (C₄₀H₅₆) (Britton, 1995). In general, carotenoids cannot be synthesized by animals, including lobsters (Urzua et al., 2011). Carotenoids are available in lobsters through food intake. In a lobster's body, carotenoids play a vital role like in camouflage, status, and mating attraction (Tlustý et

al., 2005). One of the carotenoid pigments that are contained within and play a role in lobster metabolism is astaxanthin. Besides astaxanthin (3,3-dihydroxy-beta, beta-carotene-4,4-dione), there is also crustacyanin (complex protein), which plays a role in determining a lobster's color (Chayen et al., 2000). All of these pigment cells work to determine the color of a lobster's body. These cells are placed in tissues below the epidermis. Based on the description above, it is clear that environmental factors and a lobster's body's metabolism sustainability are two significant components in carotenoid accumulation.

Carotenoids also have an important function in supporting human health. According to Gross (1991), carotenoids are useful as antioxidants, cardiovascular deterrents, osteoporosis, diabetes, as well as natural colorants. The antioxidant ability of carotenoids is utilized to develop the cosmetics and pharmacy industries (Anunciato et al., 2012). Thus, a carotenoid application is a significant part in human civilizations.

Based on the background above, the goal of this research is to determine the carotenoid composition of sand lobster (*Panulirus homarus*) eggs as well as test carotenoid antioxidants of sand lobster eggs.

Material and Methods

This research is conducted to identify the carotenoid content of sand lobster eggs and test the carotenoid antioxidant power of sand lobster eggs. The sample used is sand lobster (*Panulirus homarus*) eggs which are retrieved from a southern beach (Pok Tunggal) in Yogyakarta. The chemical ingredients used consist of methanol, ethyl ether, acetone, KOH, C₆H₈O₆, NaCl, and DPPH.

Extraction

Before the extraction process, a sample is kept for 1x24 hours in the refrigerator. A 20g sample (wet) that has been prepared is crushed with a mortar and pestle until it is refined. Next, the refined sample is extracted using a dissolvent with 50 ml of methanol. The extraction process is repeated once and accompanied with a stirrer to separate the pigments. The extracted pigments are gathered. Then C₆H₈O₆ 1g, NaCl 1g, and 10 ml KOH 60% are added and stirred again for 2 minutes. The pigments are then filtered using Whatman paper no. 1. The filtrate that is obtained is put into separating funnel, added with 30 ml of ethyl ether to separate it. The ethyl ether part is separated and then taken and filtered. For the next stage, the filtrate is steamed

with nitrogen gas (N₂) until it is dry and ready to be analyzed (Khanafari et al., 2007).

Carotenoid Identification Using HPLC

The pigment content from sand lobster egg extract is analyzed with High Performance Liquid Chromatography. The dry sample from the available extraction is dissolved with a methanol 100% dissolvent. A 20 µl sample is injected into HPLC (shimadzu LC-10AD Japan) with a column C18 µ reverse phase (a diameter of 3.9 mm id x 30 cm). A motion phase consists of methanol: acetone (90:10 V.V⁻¹) with a flowing speed of 1 ml.min⁻¹. A running sample is carried out for 40 minutes in a long wave interval of 300-600 nm (Parisenti et al., 2011).

Antioxidant (DPPH) Activity Test

The antioxidant activity test begins by making a series of concentrations of 5000, 1000, 1500, 2000, 2500, and 3000 µg.ml⁻¹. After making the concentration series, next a sample concentration series is devised. A sand lobster egg extract concentration of 8780 µl.ml⁻¹, which is dissolved in methanol, has a concentration series made each consisting of 114, 228, 342, 456, 570, and 684 µl. The sample which has had its concentration arranged is given 3 ml (1:3) of diphenil -2-picryllhydrazil (DPPH) 0.1 mM. The next mixture is homogenized with a vortex for 1 minute as well as incubated for 30 minutes. After this, its absorbance is measured in wavelengths of 517 nm after measuring blank absorbance with methanol. The measurement uses a U-1240 Shimadzu mini UV spectrophotometer (Banerjee et al., 2005). The percentage of obstruction can be calculated with the formula: % of obstruction =

$$\frac{\text{Abs.Blank} - \text{Abs.Sample}}{\text{Abs.Blank}} \times 100\%$$

Data Analysis

The data obtained is analyzed using Matlab R2013a and OriginPro v9.0. The analysis results are compared with a reference (Jeffrey et al., 1997).

Results and Discussion

An identification of carotenoid pigments from a sand lobster egg sample using HPLC reveals that the pigments identified are from the xanthophyll group of carotenoids. The carotenoid content in the sample is strengthened with the presence of an absorption region in the 400-500 nm interval as a carotenoid absorption area (Britton, 1995). The

presence of this carotenoid compound is supported by a yellow extract color. The kinds of carotenoids that are identified in the sample can be seen in the HPLC chromatogram profile figure below.

Based on the HPLC chromatogram profile in Figure 1 above, it shows that not all chromatogram peaks are identified, so that it can be assumed that the unidentifiable peaks are those which have experienced degradation. The peak levels depict the concentrations of the compounds. After comparison tests are done with references, there are 6 peaks identified as carotenoids. The 6 peaks consist of dinoxanthin, diadinoxanthin, zeaxanthin, lutein, astaxanthin, and violaxanthin. The HPLC chromatogram profile reveals that lutein has the highest peak. This clarifies the high concentration of the lutein compound. The high lutein concentration is not separate from the dissolvent role in maintaining the lutein integrity. The level of methanol polarity that approaches the lutein polarity keeps lutein stable. Using this kind of a dissolvent in an HPLC analysis is one of the components that influence absorption and concentration through an elution power and resolution process. The elution power and resolution is determined by the polarity of the whole dissolvent. In an HPLC analysis, a mixture of methanol dissolvent is used: acetone 90:10 as a motion phase. Lutein is a carotenoid that is included in the xanthophyll group. This compound has 9 until 13 conjugated bonds. By having these conjugated bond chains, it makes lutein able to be degraded and also function as an antioxidant in a biological system (Young and George, 1993). The chemical structure of lutein that has a dominant hydroxyl cluster is a special characteristic of the lutein compound. The presence of a hydroxyl cluster in lutein makes a lutein compound more polar compared with α - β -carotene.

A profile of the analysis results regarding the carotenoid pigment composition of sand lobster eggs is provided in Table 1 below. The sample peaks of the HPLC chromatogram are identified based on comparisons with the reference.

In referring to the reference of Jeffrey *et al.* (1997), with an absorbance of 416, 438, and 467 nm, then the first peak of the sample with an absorbance of 420, 442, and 472 nm is identified as a dinoxanthin type of carotenoid. For the second spectrum based on the reference of Jeffrey *et al.* (1997), with an absorbance of 445.5 and 474 nm, then the second peak of the sample with an absorbance of 421, 449, and 474 nm is identified as a diadinoxanthin type of carotenoid. Next, for the third peak based on the reference of Jeffrey *et al.* (1997), with an absorbance of 422, 450, and 481 nm, then the third peak of the sample with an absorbance of 415, 449, and 474 is identified as a zeaxanthin type of carotenoid.

For the fourth spectrum, if the standard in the reference of Jeffrey *et al.* (1997) has an absorbance of 418, 444, and 474 nm, then the fourth peak of the sample with an absorbance of 417, 443, and 472 is identified as a lutein type of carotenoid. Next, for the fifth peak based on the reference of Jeffrey *et al.* (1997), with an absorbance of 478 nm, then the fifth spectrum of the sample with an absorbance of 474 is identified as an astaxanthin type of carotenoid. Then for the sixth peak departing from the reference of Jeffrey *et al.* (1997), with an absorbance of 415, 440, and 469 nm, the sixth spectrum of the sample with an absorbance of 414, 439, and 464 nm is identified as a violaxanthin type of carotenoid.

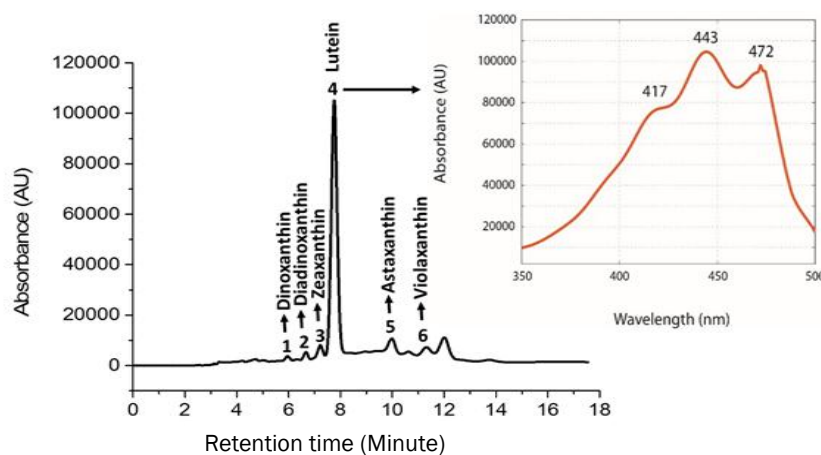


Figure 1. The HPLC chromatogram profile of sand lobster (*Panulirus homarus*) egg extract which has 6 peaks is identified by observing the spectrum in the 446 nm wavelength. Among the 6 peaks, the lutein compound concentration is most dominant compared with the other 5 compounds: dinoxanthin, diadinoxanthin, zeaxanthin, astaxanthin, and violaxanthin.

Table 1. The Composition of Sand Lobster (*Panulirus homarus*) egg extrat pigment 400-500 nm

No	Minute	Absorbance			Solvent	Pigment	Reference
		I	II	III			
1	5,97	420	442	472	Methanol	<i>Dinoxanthin</i>	Loeblich, and Smith(1968)
2	6,66	421	449	474	Methanol	<i>Diadinoxanthin</i>	Johansen et al. (1974)
3	7,23	415	449	474	Methanol	<i>Zeaxanthin</i>	Karrer, and Jucker(1948)
4	7,75	417	443	472	Methanol	<i>Lutein</i>	Karrer, and Jucker(1948)
5	9,97		474		Methanol	<i>Astaxanthin</i>	Repeta, and Gagosian(1982)
6	11,31	414	439	464	Methanol	<i>Violaxanthin</i>	Karrer, and Jucker(1948)

Looking at the data from the sample analysis results and the reference data above, there is a clear shift in absorbance between the sample and reference as a comparison. This shift can be caused by the methanol dissolvent correction factor that can move 1-8 nm (Britton, 1995), and it can also be influenced by the high and low sample concentration value. This absorbance shift, according to Britton (1995), is a chromophore interaction that is bound as carotenoid hydrophobic molecules, so that there is a band shift to another direction which causes absorption value differentiation. In addition, the absorbance shift is also influenced by the presence of an electron π to π^* transition, where one of the bound electrons from a conjugated system is excited from the π orbital to its π^* antibody orbital. In the carotenoid compound, the π electron is significantly delocalized, keeping in mind that its dual bound conjugated system is very long. This causes an excitation process to occur with relatively low energy, so that in general the carotenoid compound becomes more colorful and has the potential to experience a shift in absorbance (Britton, 1995).

It is also explained by Keenan (2005, in Susilowati, 2008) that the occurrence of a shift in wavelengths as well as absorbance between the sample with literature as a comparison is caused by the presence of electron transitions. Electrons which are easily excited by light it appears are usually found in a molecule that has several atoms connected by alternating double and single bonds. This kind of a process causes differences in the sample absorbance with reference.

Antioxidant Test (DPPH)

An antioxidant test of sand lobster (*Panulirus homarus*) eggs is conducted by determining the value % of inhibition (IC_{50}). The inhibition concentration (IC_{50}) is a dissolved concentration that provides DPPH inhibited power of 50%. The IC_{50} value is used to reveal the antioxidant activity of a tested material with a DPPH free radical inhibition method (Molyneux, 2004).

Table 2 describes making a sample concentration series, measuring a DPPH solution, blank, and value of inhibited percentage obtained. The inhibition percent values of each carotenoid extract concentration can be seen in Table 2.

The IC_{50} value is calculated by interpolating the percentage of inhibition (50%) inside the curve of the relationship between the test solution concentration with the percentage of inhibition. From the data between the solution concentration and inhibition percentage, a regression line formula is devised. The Inhibition Concentration (IC_{50}) Curve or inhibition value % is the Y axis and the concentration series is the X axis. The curve in the relationship between the test solution and the inhibition percentage can be seen in Figure 2.

Based on the data of the DPPH analysis above, to reach the IC_{50} value, a sample concentration value of $6675.25 \mu\text{g.ml}^{-1}$ is needed. In other words, the sample concentration value is $6675.25 \mu\text{g.ml}^{-1}$ as a value that is able to inhibit free radicals. This value is obtained by using a linear equation above. It can be concluded that the concentration value which progressively increases, has a correlation or is linear with the inhibition value that is connected in the linear line. The inhibition of free radicals from sand lobster egg extract is determined by various antioxidant compounds that are contained in sand lobster egg extract.

Referring to the lutein HPLC data as pigments which have a high concentration, it is ascertained to have a big contribution in inhibiting free radicals. Lutein and astaxanthin are two kinds of carotenoids that have good free radical inhibition power. The capacity to inhibit free radicals was emphasized in research results by Mozaffarieh et al. (2003) and Madhavi and Kagan (2002), who claimed that consuming 6–20 mg of lutein can prevent the occurrence of oxidative reactions in the eyes. Darlington and Stone (2000), mentioned that astaxanthin has a poliene chain that is able to absorb free radicals that cause cellular structure macromolecular disruptions. Besides the role of lutein and astaxanthin, other kinds of carotenoids

Table 2. Data of inhibition percentage from each sample concentration

[$\mu\text{g.ml}^{-1}$]	A517nm	Afk	A(count)	% Inhibition
0	0,719	0	0,719	0
500	0,721	0,006	0,715	0,556328
1000	0,698	0,012	0,686	4,589708
1500	0,675	0,019	0,656	8,76217
2000	0,657	0,028	0,629	12,51739
2500	0,635	0,036	0,599	16,68985
3000	0,617	0,046	0,571	20,58414

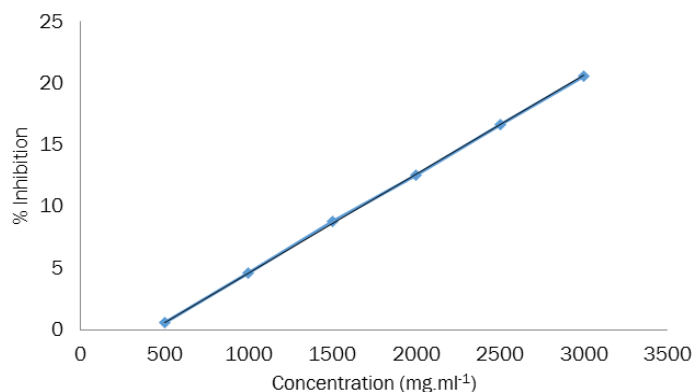


Figure 2. The curve in the relationship between a carotenoid test solution and inhibition percentage. The inhibition power (y) as a function as concentration (x) is obtained from the formula $y = ax + b$. The value of the coefficient a is 8×10^{-3} , and the value of coefficient b is -3.4 .

like zeaxanthin also have the capacity to be good antioxidants. Thus, it is appropriate that the sample used for an antioxidant test has the capacity to inhibit free radicals.

The ability to exhibit good antioxidant power from carotenoids clearly opens development opportunities in the cosmetics, pharmacy, and food industries (Anunciato *et al.*, 2012). In the future, it is expected that developing carotenoid applications can respond to societal needs. It is also hoped that society realizes the importance of consuming lobsters.

Conclusion

Based on the research data and identification results, it can be concluded that carotenoids are contained in sand lobster (*Panulirus homarus*) eggs. The dominant carotenoids are the xanthophyll group carotenoids. The carotenoid antioxidant test from sand lobster eggs reveals there is an ability to counter free radicals. Looking at the capacity of the sand lobster (*Panulirus homarus*) egg sample, in the future it is hoped that society utilizes sand lobsters (*Panulirus homarus*) as well as possible as a carotenoid source to fulfill nutritional intake.

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