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RESEARCH ARTICLE

Effect of Crystallization and Maceration Time on Antioxidant Activity of Ethanolic Extract from Brown Algae *Sargassum*sp

***Bambang Budi Sasmito, Sri Kumalaningsih, Susinggih W and Hardoko**

1.Agricultural Science, Post-graduateProgram - Faculty of Agricultural, Brawijaya University; Faculty of Fisheries and Marine Sciences, Brawijaya University Malang - East Java, Indonesia

2, 3. Faculty of Agricultural Technology, Brawijaya University, Malang - East Java, Indonesia

4. Faculty of Fisheries and Marine Sciences, Brawijaya University Malang - East Java, Indonesia

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****Corresponding Author***

Bambang Budi Sasmito

Brown algae as a source of bioactive has been known for a long time; antioxidants is one of the compounds that have an important role to maintain good health.The research was conducted to determine the effect of crystallization by freezing method and time of maceration on the antioxidant activity and the yield of the ethanolic extract from brown algae. Mashed fresh brown algae has been frozen at -18° C for 6 hours and partially treated without freezing. Furthermore macerated using ethanol at a ratio of 1:3 (brown algae: ethanol, w/v) for 6, 12, 18 and 24 hours. The antioxidant activity and total polyphenols content (TPC) of ethanolic extracts (EE) were determined by using IC_{50} (inhibition against DPPH) and Folin-Ciocalteu reagents, respectively. The results showed that the crystallization by freezing (-18° C for 6 hours) resulted in a high of antioxidant activity, TPC and yield of the ethanolic extract.Crystallization by the freezing combined with maceration time of 18 and 24 hours, in addition to the highest ethanolic extract were obtained, total polyphenol content and alsothe highest in antioxidant activity
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Introduction

Brown algae has abundance and a very wide distribution in almost all Indonesian waters (Handayani et al., 2004). Sargassumis a genus that is widely available in the east coastal waters of Maduraisland. Exploration of natural resources to obtain bioactive source of antioxidants in particular has become an important issue, because their role as inhibitors and free radical scavengers.At least over the past 10 years, researchers havebecome increasingly interested in natural antioxidant (Bambang et al., 2013, Burtin, 2003, Gan et al., 2010, Shanab, 2007). The chief reason for this interest is the natural antioxidant properties, their great abundance in foods, and their probable role in the prevention of various diseases associated with oxidative stress (Junzeng et al., 2007, Lamia et al., 2011).Many studies have reported that brown algae used as a potential source of natural antioxidants (Matsukawa et al., 1997, Maria et al., 2009; Nakamura et al. 1996; Zahra et al., 2007).Natural antioxidants are increasingly in demand by the public, because in addition to having a high ability to scavenge free radicals and there also safer to use.

Bioactive compounds are classified as polyphenols from brown algae has been known as a powerful natural antioxidant.Many research have been done on the particular brown algae *Sargassum*sp still exploratory, so it has not obtained an effectively extraction techniques. Maceration in bioactive extraction is regarded as one of the simplest method, and it was many used by workers in lab activity for explorative studies. For bioactive extraction like antioxidantusing the method is required a relatively long time.Several studies have been conducted for the extraction of antioxidants takes maceration for 3 days (Boonchumet al., 2011; Suryaningrum et al., 2006). If the time required

to extract is too long at room temperature conditions, can lead to the possibility of antioxidants extracted a lot of oxidative change occurs and decreasing of the quality.

Efforts to shorten maceration in order to stay awake antioxidant qualities, i.e. by wounding the cell walls of brown algae with crystallization by slow freezing.In generally, freezing injury occurs at temperatures below the freezing point of water (Galindo et al., 2007). Crystallization at lower temperature -6° C has been shown to injure cell (Sukumaran and Weiser, 1972). In generally,physical damage of cells influenced by temperature or duration of freezing.Besides the influence of the material properties, freezing injury also triggered by a combination of freezing temperatures and freezing period (Andersson et al., 2003; Susan and Carlos, 2005).In the preliminary study, brown algae frozen at a temperature of -18° C for 6 hours has shown a lot of freezing injury.With the physical damage of the algal cells are expected to assist the extraction of bioactive compounds, especially antioxidants from the algae cells.Crystallization by freezing was used in the extraction of natural antioxidants has several advantages, such as reducing the risk of thermal oxidation.

This research is aimed to know influence of freezing and maceration time on the yield, total polyphenol content and antioxidant activity of ethanolic extracts from brown algae.In these studies have been conducted using a sample freezing temperature of -18° C for 6 hours based on the results of the preliminary.Further maceration carried out for less than 3 times the 24 hours to determine whether freezing combined with the duration of maceration able to accelerate the extraction of antioxidants from brown algae.

Material and Methods

Brown algae

Brown algae (*Sargassumfilipendula*) was collected from Sumenep East Java using a coolbox during transport to the lab.Brawijaya University – Malang, East Java Indonesia. Sample of brown algaewashed and drained in the shade below the room temperature. The sample mixed by usea blender and stored in an airtight container until time of used.

Chemycals and reagents

Ethanol (as solvent), Folin-Ciocalteu, 1.1-diphenyl-2-picryhydrazyl (DPPH), galic acid reagents were obtained from Sigma Chemical Co, USA. All chemicals were of analytical grade.

Preparation of sample extracts

Extraction was carried out by the maceration method using ethanol as solvent with a ratio 1:3 (w/v) of sample and ethanol. A part of the sample crystallized by using the slow freezing temperature of -18° C for 6 hours, while the other part is stored at room temperature (25° C).A part of the sample crystallized by using the slow freezing temperature of -18° C for 6 hours, while the other part is stored at room temperature (25° C). Furthermore, each sample was divided in 4 parts for maceration (6, 12, 18 and 24 hours) using ethanol as a solvent.100 g of sample is introduced into a beaker glass that was filled 300 ml of ethanol and then covered tightly with aluminum foil. During the maceration is agitated using a shaker at approximately 75 per minute.The extracts obtained were separated by using rotaryvacuumevaporator under controlled temperatures ± 60° C. Ethanolic extract (EE) measured the percentage yield, total polyphenol content (TPC) and antioxidant activity (IC_{50}).Brown algae tissue before and after crystallized (with slow freezing) has been observed microscopically.

Determination of Total Polyphenol Content (TPC).

The total polyphenol content of ethanolic extracts (ethanolic) were determined by using Folin-Ciocalteu reagent (Chandler &Dodds, 1983). The ethanolic extracts (250 μl) each of sample was mixed with 1250 μldeionized water, 250 μl ethanol and 125 μlof the Folin-Ciocalteu reagent. The mixture was incubated at room temperature for 5 min and then 250 μl of 5% sodium carbonate solution was added. The mixture was allowed to stand for 1 hour in the dark at room temperature. The absorbance of the discolored productwas measured with a spectrophotometer at 725 nm. The Total phenolic content (TPC) of the ethanolic extracts were determined from regression equation of standard curve $y = 0.0085\,x - 0.018$ and expressed as mg of galicacid equivalents (GAE) per g of sample.

Antioxidant activity (DPPH radical scavenging activity assay)

The DPPH radical scavenging activitywas assessed according to the method described by Shen et al.,(2010). An aliquot (0.1 ml) of each sample with different concentration (25, 50, 100 and 200 ppm) was added to 3.0 ml of ethanolic DPPH solution. The mixture of each samplewas shaken, left to stand for 30 minutesat room temperature in

a dark room. The scavenging effect on the DPPH radical was read using spectrophotometer (SpectroquantPharo 300) at 517 nm. The capability to scavenging the DPPH radical was calculated by the following equation:

Percentage of the DPPH scavenging = $\left[\frac{Ao-As}{Ao}\right] x\ 100$

Where, A_C =absorbance of control and A_S = absorbance of sample solution. The DPPH solution without sample solution was used as control. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated by using the plotted graph of RSA against the concentration of samples.

Statistical analyses

The factorial completely randomized design was used in this experiment. The treatments were used: (1) pre-treatment of the material is divided into two, namely the condition without crystallization (WC) and the crystallization (C), and (2) time of maceration (MT): 6, 12, 18 and 24 hours. Data analysis were used analysis of variance (Anova, using Design Expert 7.0 software) and significant differences between the means of parameters were determined by using Least Significant Different (LSD) test.

Result and Discussion

Yield of the ethanolic extract

The results of the analysis of variance has shown that the yield is significantly influenced by the treatment of crystallization, maceration time and a combination of both (P <0:01). The mean of yields that have been obtained by a combination of crystallization and maceration time are presented in Table 1.

Table 1. The yield of the ethanolic extracts (EE) from brown algae (Sargassumsp)

Treatments	Yield (%, dry matter)	
WC-MT_6	0.6343 ± 0.27^a	Data are expressed as mean ± SD, standard deviation. Means within each column with different superscript letters (a-e) differ highly significant (p < 0.01).
WC-MT_{12}	0.7797 ± 0.11^a	
WC-MT_{18}	1.3580 ± 0.09^b	
WC-MT_{24}	2.1950 ± 0.54^c	
C-MT_6	2.1527 ± 0.18^c	
C-MT_{12}	4.1810 ± 0.10^d	
C-MT_{18}	4.9320 ± 0.21^e	
C-MT_{24}	5.1293 ± 0.43^e	

Table 1, showed that the ethanolic extracts obtained by a combination of freezing treatment with maceration time for 24 hours (C-MT_{24}) produced the highest yield of ethanolic extract. However, there was no significantly difference with the product resulting from the combination of freezing treatment and maceration time for 18 hours (C-MT_{18}). Ethanolic extracts obtained from CMT_{24} and CMT_{18}were 5.1293 ± 0.43 and 4.9320 ± 0.21 percent, respectively.These results are much higher than the resulting ethanolic extracts of other treatments, without freezing. Lowest results obtained from treatment without freezing combined with maceration for 6 and 12 hours. The yield of ethanolic extracts have obtained by both of the combination treatments (WC-MT_6 and WC-MT_{12}) were 0.6343 ± 0.27 and 0.7797 ± 0.11 percent, respectively.Overall, the ethanolic extract was resulted by the combination treatment between freezing and maceration times more than the yield obtained from other treatments. These was due to crystallization using the method of freezing has caused injury to the cells, so that can assist the extraction with maceration method. Even cell injury could be done during and post thawing of materials (Palta et al., 1977), so it will have a shorter time required for maceration.

If the ice is formed in the space between the cells at a certain temperature, it causes the water withdrawn from the cell. As a result, an increase in solute concentration and a decrease in cell water potential until a new equilibrium is reached (Valentini et al., 1990). Furthermore, when thawing occurs shrinkage of cells, and this causes the osmotic pressure inside the cell becomes lower. Under such conditions, the diffusion of the solvent into the cell will become easier, so that more is produced during extraction. In addition to physical injury due to freezing could occur in the cell wall, so that when maceration solvent diffusion into cells very easily. This estimate is supported by a micrograph of the brown algae tissue (Figure 1), where many injuries occur after freezing the cells.

Figure 1. Thallus tissue of brown algae (A) before and (B) after crystallization (temperature -18° C for 6 hours).

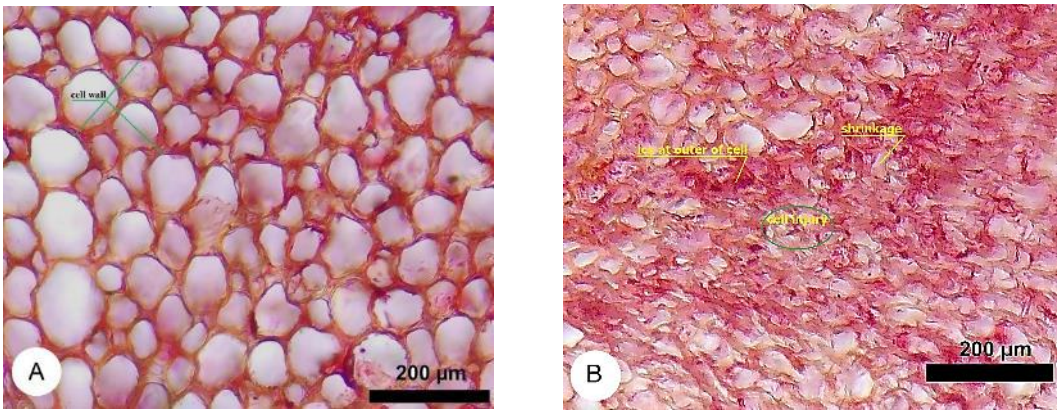

Figure 1 (A) was showed that the cell wall of the thallus tissue had still intact before crystallized. Freezing injury has occurred after the algae were frozen at -18° C for 6 hours.It is known that slow freezing has caused damage to cells and lead to occur "drip" when thawed.The cell wall damage caused by swelling of the fluid volume of intra-and inter-cellular. Fig. 1 (B) above has shown that more than 60 percent of the cells were damaged, so the cell structure becomes irregular.Damage to these cells and the amount varies greatly depending on many factors such as material type, temperature and duration or period of freezing (Andersson et al., 2003; Susan and Carlos, 2005). Paltaet al. (1977) has been reported that the onion cell injury can reach 75 per cent. In addition, also occured at the freezing temperature is lower than - 4° C there is a decrease in the osmotic pressure of the fluid inside and outside cells. The decrease in the osmotic pressure reaches about 40 per cent at a temperature of -11° C. In similar condition would easily of solvent diffused into the sap of cell. Cytoplasm or intracellular fluid will come out with its contents that occur during thawing. In this study was proved that the yield of ethanolic extract obtained from freezing treatment more than the results of other treatments.

Total polyphenolcontent (TPC)

The amount of total phenolics varied in different treatments andranged from 27.786 ± 0.95 to 36.544 ±0.99 mg GAE/g in the etanolic extract of *Sargassum* sp.The total polyphenol content of the ethanolic extract was significantly different between crystallization treatment, and maceration time ($P < 0.01$). The average of TPC were obtained by a crystallization and maceration time are presented in Table 2.

Table 2. The Total Polyphenol Content and IC_{50} value of the ethanolic extracts from brown algae (Sargassumsp)

Treatments	TPC (mgGAE/g)	IC_{50} (ppm) by DPPH assay	
WC	29.671 ± 3.22^{a}	100.785 ± 21.85^{a}	Data are expressed as mean ± SD, standard deviation. $^{(a–b) \text{ and } (p-r)}$Values within column with same superscripts indicated no significant difference ($P > 0.05$)
C	36.233 ± 6.05^{b}	87.048 ± 19.76^{b}	
MT6	27.786 ± 0.95^{p}	118.982 ± 16.92^{p}	
MT12	31.819 ± 1.87^{pq}	96.427 ± 19.66^{q}	
MT18	35.658 ± 1.64^{qr}	88.802 ± 13.27^{q}	
MT24	36.544 ± 0.99^{r}	71.453 ± 26.28^{r}	

Table 2 shows that the ethanolic extract of crystallization treatment had total polyphenol content more than the results of without crystallization treatment.This possibility is due to cell injury caused by freezing, so the extraction of bioactive antioxidants especially becomes easier.Freezing injury is strongly influenced by the temperature and

duration of freezing as well as other factors such as the type of material. Cell damage increased by the effect of the duration of freezing, as reported by Anderson et al.(2003). Polyphenols as secondary metabolites are part of vacuolization within the cytoplasm. With the injury of cells by freezing would facilitate the release of the cytoplasm and its contents. This conjecture supported by the micrographs (Figure 1) which shows that there is a lot of damage to the cell wall after freezing.

The solubility of a compound is proportional to the time spent to reach the saturation point. Effect of maceration time was very real to the total polyphenol content in the ethanolic extract (P <0:01). Ethanolic extracts obtained from maceration for 24 hours had a higher polyphenol content than the other, but not significantly with the results of maceration for 18 hours.Total polyphenol content of each ethanolic extract was obtained as follows: 36.544 ± 0.99 and 35.658 ± 1.64mgGAE / g sample, respectively.In general there was a tendency to increase in parallel with increasing maceration time.Maceration for 6 hours resulted inethanolic extract with the lowest polyphenol content, i.e.27.786± 0.95mgGAE / g sample.

In this study, the average of total polyphenol content (TPC) is slightly higher than that reported by Heo et al., (2003), where he found the total polyphenol content in brown algae, *Sargassum*sp: 15.60mgGAE/g sample. However, the TPC was slightly lower than that found by Maria et al., (2009), i.e.80.35mgGAE/g sample.

Antioxidant activity (IC_{50} value)

Polyphenolic compounds are natural antioxidants found mostly in many plants and seaweeds.These compounds were one of the most effective antioxidant in brown algae (Burtin, 2003; Maria et al., 2009). Phenolic compounds as important antioxidants because of their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates.The antioxidant activity (IC_{50}) of the ethanolic extract has a close relationship with the total polyphenol content.The pattern of relationships indicates that the high polyphenol content in the extract, the lower the IC_{50} value, or stronger antioxidant activity.Boonchumet al., (2011) found a strong correlation between antioxidant activity and total phenolic content, which are in agreement with studies of Duan et al. (2006) and Jimenez et al. (2001).

However, in this research we found that there was no significantly correlation between the total polyphenol content and the antioxidant activity of the ethanolic extracts obtained from the various treatments (crystallization and time of maceration), r= 0,5968.This possibility was not phenolic compounds in ethanol crude extract having antioxidant activity, such as groups of polysaccharides and pigments. In this study in accordance with the report of Li et al., (2007) where there was no significant relationship between total phenol and antioxidant activity.

The value of IC_{50} varied in different treatments andranged from 71.453± 26.28to 118.982 ± 16.92ppm.The antioxidant activity was strongly influenced by two factors, namely crystallization (C) and maceration time (MT) treatments. The average of IC_{50} value was obtained by a maceration time for 24 hours (MT_{24}) appeared to possess the highest antioxidant activity (p <0.01) in the DPPH test is expressed with lowest of IC_{50} value i.e71.453 ± 26.28 ppm (Table 2).

Conclusion

The conclusion of this study is that the use of freeze crystallization has been shown to accelerate or assist in the extraction of natural antioxidants from brown algae*Sargassum* sp. The combined treatment with macerated for 18 to 24 hours were resulted in the highest ethanolic extract, as well as the total polyphenol content and antioxidant activity.

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