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by Ratnawati Ratnawati

Ultrasound-Assisted Ultra-Mild-Acid Hydrolisis of κ-Carrageenan

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INTRODUCTION

κ-carrageenan, extracted from red seaweed *Kappaphycus alvarezii*, is widely used as stabilizer, thickener, gelling agent, and emulsifier in food, pharmaceutical, cosmetic, and textile industries (Campo *et al*., 2009). The utilization of κ-carrageenan can be expanded, especially in biomedical application, when the molecular weight is lower. Many groups of researchers have applied the low molecular weight fractions (LMWF) of κ-carrageenan in biomedical applications as tumor growth inhibitor (Yao *et al*., 2014), antivirus (Chiu *et al*., 2012), anticoagulant (Silva *et al*., 2010; Gomez-Ordonez *et al*., 2014), and antioxidant (de Souza *et al*., 2012; Gomez-Ordonez *et al*., 2014).

The LMWF of κ-carrageenan used in the biomedical application has an average molecular weight less than 20 kDa (de Souza *et al*., 2012). With such small molecular weight, the LMWF of κ-carrageenan can penetrate into human cells more effectively compared to that of the original high molecular weight κ-carrageenan (Wijesekara *et al*., 2011). The negative charge of the LMWF of κ-carrageenan may interact with the positive charge of the outer wall of virus or cell, hindering the virus to penetrate into the cell (Bondu *et al*., 2010).

The commercial κ-carrageenan is an average molecular weight of 100-1000 kDa (Campo *et al.*, 2009). Thermal depolymerization (Lai *et al.*, 2000), acid-catalyzed hydrolysis (Karlsson and Singh, 1999; Yuan and Song, 2005), enzymatic hydrolysis (Haijin *et al.*, 2003), irradiation (De Souza *et al.*, 2012; Abad *et al.*, 2010), and sonication (Taghizadeh *et al.*, 2015; Ratnawati *et al.*, 2016) are widely used as depolymerization techniques. Among all techniques, acid-hydrolysis is the most common technique used to depolymerize polysaccharides as it is the cheapest. However, a large amount of acid is usually involved, and it will raise pollution issue. Most of the acid hydrolysis processes are conducted at pH lower than 2 (Singh and Jacobson, 1994; Hjerde *et al.*, 1996; Myslabodski *et al.*, 1996; Karlsson and Singh, 1999). Karlsson and Singh (1999) categorized pH 2 as a mild condition.

Compared to other techniques, ultrasonication is simple, cheap, energy-efficient, and suitable for food processing (Akyuz *et al.*, 2008). Ultrasonication can be used as a technique for depolymerization. When ultrasound wave is applied to a dilute polymer solution, it generates pressure wave causing a cavitation which consists of the nucleation, growth, and collapse of microbubbles. A segment of a polymer molecule in the vicinity of a collapsing bubble moves with much higher velocity than other segments located farther. The velocity gradient causes the polymer chain to stretch and eventually a cleavage at the midpoint of the molecular chain could happen (Akyüz *et al.*, 2008; Caruso *et al.*, 2009). The collapse of the microbubbles can also cause instantaneous extreme temperature and pressure with a lifetime in the order of 10^{-10} sec. The pressure and the temperature in the bubble could reach as high as 1,000 atm and 5,000 K, respectively, while the temperature in the vicinity of the bubbles can reach 1,000 K (Suslick and Flannigan, 2008). The hot spot created during the bubble collapse may cause water to undergo homolytic cleavage giving hydrogen and hydroxyl radicals (Didenko *et al.*, 1999).

To the best of our knowledge, there are no studies concerning the effect of pH and temperature on ultrasound-assisted hydrolysis of κ-carrageenan up to the moment. So, this work was intended to study the effect of temperature, pH, and time on the number-average molecular weight of depolymerized κ-carrageenan. The ultrasound-assisted ultra-mild acid-hydrolysis of κ-carrageenan was conducted at pH higher than 2.

MATERIALS AND METHODS
Materials

The material used in this work was semi-refined κ-carrageenan obtained from CV. Karagenan Indonesia, Semarang, Indonesia, which was refined prior to the ultrasonication. Other materials used were hydrochloric acid solution 37% (E. Merck, Cat. No.100314), distilled water, and isopropyl alcohol (E. Merck, Cat. No.818766).

Methods of Depolymerization

Five hundred milligrams of refined κ-carrageenan was dissolved in 100 ml of distilled water at ± 70°C with stirring for ± 15 min. The solution was then cooled to specified temperatures (30, 40, 50, dan 60°C) and the pH was adjusted to 3 and 6 by adding dilute HCl solution. The solution was transferred to a 40 mm diameter and 120 mm height glass vessel. The vessel was placed in an ultrasound device, Krisbow type KLS 303365, with a frequency of 42 kHz. The glass was placed right above the ultrasonic transducer. The ultrasonication was run at various times (0, 8, 16, 24, and 32 mins), after which a sample was analyzed for its viscosity using ubbelohde-type capillary viscometer (Canon-Fenske). The viscosity measurement for each sample was conducted in triplicate.

Molecular Weight Determination

The number-average molecular weight of κ-carrageenan was determined by Mark-Houwink equation relating intrinsic viscosity to the molecular weight (Lai *et al.*, 2000) as follows:

$$[\eta] = k_{MH} \cdot M^a \qquad (1)$$

where $[\eta]$ is intrinsic viscosity (ml g^{-1}), k_{MH} and a are constants, and M is the number-average molecular weight (g mol^{-1}). The values of k_{MH} and a for the κ-carrageenan solution in water at 25°C are 0.00778 and 0.90, respectively (Vreeman *et al.*, 1980; Rochas *et al.*, 1990). The intrinsic viscosity is obtained using Huggins equation:

$$\eta_{sp}/C = [\eta] + k_H [\eta]^2 C \qquad (2)$$

where η_{sp}, C, and k_H are specific viscosity (dimensionless), concentration of the solution (g ml^{-1}), and Huggins constant (dimensionless) of which value is 0.3 (Vreeman *et al.*, 1980).

RESULTS AND DISCUSSION
Effect of Temperature on Molecular Weight

The initial number-average molecular weight of κ-carrageenan is 629 kDa. After sonication at initial pH of 6 and 3, the molecular weight decreases with time, as depicted in Figures 1 and 2, respectively.

It is shown in Figure 1 that at initial pH of 6, after 32 min of ultrasonication at 30, 40, 50, and 60°C, the number-average molecular weight of κ-carrageenan decreases to 201, 182, 112, and 107 kDa, respectively. In term of molecular weight reduction, the molecular weight reduces by 68.0, 71.1, 82.2, and 83.1%, respectively. Similar results are also observed for pH of 3, as depicted in Figure 2. The molecular weight drops to 115, 98, 52, and 14 kDa or 81.7, 84.5, 91.7, and 97.7% reduction of molecular weight when the ultrasonication is carried out for 32 min at 30, 40, 50, and 60°C, respectively. The results show that the molecular weight of the treated κ-carrageenan is lower

as the sonication temperature is higher. It means that temperature has a positive effect on the depolymerization.
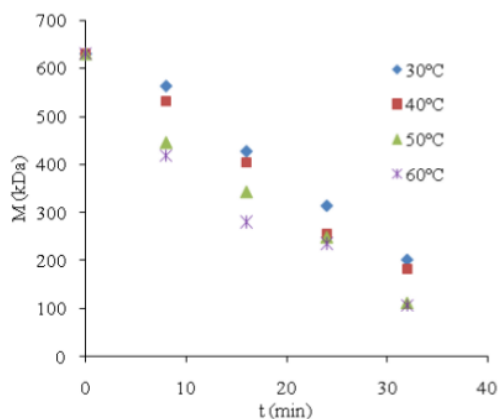

Figure 1. Effect of temperature on the number-average molecular weight of κ-carrageenan upon ultrasound-assisted hydrolysis at initial pH 6
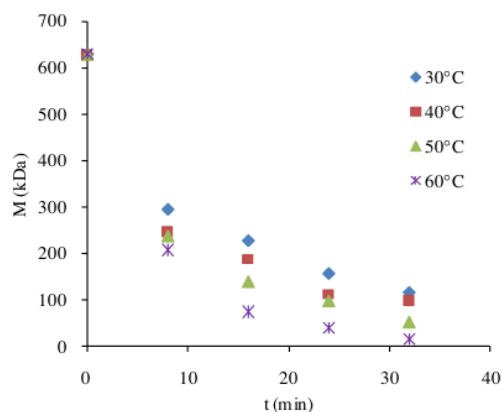

Figure 2. Effect of temperature on the number-average molecular weight of κ-carrageenan upon ultrasound-assisted hydrolysis at initial pH 3

The results are different from those obtained by several groups of researchers who reported that temperature has a negative effect on ultrasound-assisted depolymerization in organic solvents (McCoy and Madras, 1997; Mahalik and Madras, 2005; Daraboina and Madras, 2009). Ultrasonic degradation of organic polymers can occur through a homolytic cleavage mechanism as a result of the collapse of microbubbles. The bond cleavage leads to the formation of two radicals. These radicals will immediately either react with other radicals or attack other polymers to yield polymer radicals. The reaction among radicals can take place through a coupling mechanism to form a new molecule or disproportionation mechanism to form two new

molecules. The homolytic bond breaking occurs due to shear forces as a result of cavitation. As the temperature increases, more solvent vapor enters the bubbles and reduces the jet effect of the collapsing bubbles. As a result, the depolymerization reduces as the temperature increases or temperature has a negative effect (Caruso *et al*., 2009).

Many other groups of researchers reported similar results to this work in which temperature as a positive effect on depolymerization (Kasaai *et al*., 2008; Ma *et al*., 2011; Savitri *et al*., 2014; Prajapat *et al*., 2016). It can be explained as follows. The mechanism of polymer chain scission may occur through the second mechanism, i.e. bond cleavage by hydrogen and hydroxyl radicals. Both radicals are formed by thermal cleavage of water due to hot spot during the collapse of microbubbles, according to the following reactions:

$$H_2O \rightarrow H\bullet + OH\bullet \qquad (3)$$
$$H\bullet + H\bullet \rightarrow H_2 \qquad (4)$$
$$OH\bullet + OH\bullet \rightarrow H_2O_2 \qquad (5)$$
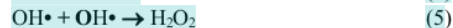
Both $OH\bullet$ and H_2O_2 are strong oxidizing agents. Even in a very low concentration, they can oxidize organic polymers, such as κ-carrageenan, resulting in random scission of the polymer (Gogate and Prajapat, 2015). Like other chemical reactions, this oxidation reaction is faster as the temperature becomes higher (Li *et al*., 2010).

Another mechanism that most possibly occurs is chain scission by H^+ ion, as the reaction was conducted in an acidic environment. Myslabodski *et al*. (1996) conducted acid-hydrolysis of κ-carrageenan at various temperatures and pHs. They reported that to reduce the average molecular weight of κ-carrageenan by 25%, the hydrolysis at pH 6 and at temperatures of 50, 70, and 90°C required 12 days, 6 h, and 1.4 h, respectively. Hydrolysis at different pH gave similar results, in which the reaction was faster at higher temperatures. It means that temperature has a positive effect on depolymerization.

According to Yoon (2010), the hydrolysis mechanism of galactan from seaweed extracts proceeds like that of cellulose. The glycosidic oxygen in the β-1,4-glycosidic bond rapidly interacts with the proton (H^+) of the acid catalyst to form a conjugated acid. Then, the C-O bond undergoes a slow cleavage to form two fragments, for which one fragment has OH as the end group while the other fragment ends with a cyclic carbocation. Finally, a rapid addition of a water molecule to the carbocation occurs, resulting in a stable final fragment by releasing the proton. These reaction proceeds faster as the temperature becomes higher (Xiang *et al*., 2003).

Effect of pH on Molecular Weight

For the sake of comparison, the results of the hydrolysis at 30°C and pH 6 are depicted in Figure 3 along with those obtained at 30°C and pH 3. It is obvious in Figure 3 that at the same reaction time, the

molecular weight of κ-carrageenan decreases at lower pH. At 30°C, ultrasonication of the κ-carrageenan solution with initial pH of 6 for 32 min gives a final molecular weight of 201 kDa or 68.0% reduction in molecular weight. Meanwhile, the same process with initial pH of 3 for 32 min results in a final molecular weight of 115 kDa or 81.8% reduction in molecular weight. The same effect was also observed for other temperatures, although it is not shown in this report.
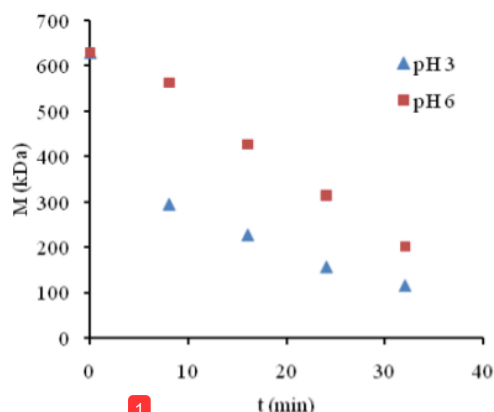

Figure 3. Effect of pH on the number-average molecular weight of κ-carrageenan upon ultrasound-assisted hydrolysis at 30°C

Myslabodski *et al.* (1996) who hydrolyzed κ-carrageenan at different temperature with various pHs, reported similar results to this work. It was reported that to reduce 25% of molecular weight, the hydrolysis with pH 6, 5, 4, and 3 required 12 days, 2 days, 8 h, and 1.4 h respectively. It means that the hydrolysis reaction proceeds faster at lower pH.

The effect of pH on the number-average molecular weight can also be viewed in terms of rate of reaction as well as the amount of hydrolysis product. Lenihal *et al.* (2010) hydrolyzed lignocellulosic biomass from potato peels using phosphoric acid as the catalyst with various concentrations, i.e. 2.5, 5.0, 7.5, and 10% w/w which are equivalent to pH of 1.40, 1.23, 1.14, and 1.10 respectively. They reported that the reaction rate increased as the acid concentration rose or the pH decreased. Kumar *et al.* (2015) who hydrolyzed sugarcane bagasse using sulfuric acid as the catalyst reported that the hydrolysates (xylose, glucose, and furfural) increase as the percentage of sulfuric acid increases.

As it was previously mentioned that in an acidic hydrolysis, the proton (H^{+}) can rapidly attacks oxygenin the β-1,4-glycosidic bond (Xiang *et al.*, 2003). As the pH becomes lower, the concentration of the proton becomes higher and thus more β-1,4-glycosidic bond being attacked. The overall observed result is that reaction becomes faster as the pH decreases.

Effect of Ultrasound on Molecular Weight

To determine whether the ultrasound irradiation affects the depolymerization of κ-carrageenan, an acid hydrolysis at 60°C and pH 3 without ultrasound irradiation was conducted for 32 min. The final number-average molecular weight obtained was 48 kDa or it was reduced by 92.0%. Meanwhile, the ultrasound-irradiated process with the same pH, temperature, and time gave gave a final number-average molecular weight of 14 kDa or 97.7% of reduction, as it is presented in Figure 2. It indicates that ultrasound irradiation contributes to the depolymerization process.

It was previously explained that the ultrasound treatment may contribute to midpoint scission of the κ-carrageenan molecules as a result of a solvatodynamic effect of the collapse of microbubbles (Akyüz*et al.*, 2008; Caruso *et al.*, 2009). When ultrasound irradiation is applied to water, the cavitational effect would also lead to the homolytic cleavage of water molecules leading to the formation of hydroxyl and hydrogen radicals which further react to form hydrogen peroxide according to equations (3)-(5). Hydroxyl radical and hydrogen peroxide are powerful oxidant. They can readily attack the κ-carrageenan molecules, causing fragmentation to smaller oligomers (Gogate and Prajapat, 2015). The overall effect is that the ultrasound increases the degree of depolymerization.

CONCLUSION

An ultrasound-assisted acid hydrolysis of κ-carrageenan has been conducted. The results showed that ultrasound treatment, temperature, and pH have positive effects on the depolymerization. The highest percentage of molecular weight reduction was achieved through depolymerization at 60°C with pH of 3 for 32 min, *i.e.* 97.7% or equivalent to a number-average molecular weight of 14 kDa.

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