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CARRAGEENAN OLIGOSACCHARIDES: BIOLOGICAL ACTIVITY AND ITS DEVELOPMENT OPPORTUNITIES IN INDONESIA

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Abstract

Oligosaccharides from hydrocolloids especially carrageenan now get a lot of attention because of the abundant raw material availability and various biological activities. It is potentially used in both food and non-food industries. Like oligosaccharides of terrestrial materials, carrageenan oligosaccharides have many biological activities such as antibacterial, antiviral, antioxidant, immunomodulatory, anticancer, and antiinflammatory activity. It can be produced either by physical, chemical or enzymatic methods. This paper reviews articles on carrageenan oligosaccharide, how it is produced, its biological activities and its development opportunities in Indonesia.

Keyword: carrageenan, oligosaccharides, biological activities, development opportunities

Introduction

As an archipelagic country with the longest beach length in the world, the potential production of Indonesia's seaweed is huge. Of the various types of seaweed produced in Indonesia, carrageenan-producing seaweeds *Eucheuma cottonii* and *Eucheuma spinosum* are the highest production species. Seaweed production in Indonesian was 9.9 million ton in 2015 (Anon., 2017[a]). This carrageenan-producing seaweed is very potential to be used as raw material for the production of carrageenan oligosaccharides in the country.

Carrageenan oligosaccharides are sugars of shorter chains with degree of polymerization (dp) $\leq$10, from larger carrageenan polysaccharides (Briones and Sato, 2014). Carrageenans are linear sulfated galactans extracted from many species of red seaweeds and share a common backbone of D-galactose with alternating α(1-3) and β(1-4) linkages (Nyvall, Maud, and Richard, 2009). The carrageenan family shares the same backbone structure, consisting of a repeating disaccharide backbone of alternating 3-linked α-D-galactopyranose (G) and 4-linked R-D-galactopyranose (D), with 3,6-anhydrogalactose residues commonly present (Briones and Sato, 2014).

Oligosaccharides from carrageenan are currently getting large attention due to the abundant availability of raw materials and its many biological activities, therefore the opportunity of application in various fields is very wide. Carrageenan oligosaccharides are stated have a broad range of biological activities such as antibacterial, antiviral, antioxidants, immuno-modulators, antiinflammatory, and antitumor (Chen, Yan, Lin, Wang, and Xu, 2007; Curtois, 2009). In addition, sulfated oligosaccharides are more advantageous than sulfated polysaccharides due to their more homogeneous structure and less toxicity (Chen et al., 2007).

Various methods to produce oligosaccharides have been done chemically, physically and microbiologically. The production of carrageenan oligosaccharides can be carried out with a chemical method by carrageenan depolymerization using acid addition. Another chemical method for production of

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oligosaccharides is depolymerization using free radicals or reductive chemicals (Sun et al., 2015). Carrageenan depolymerization can be carried out by the physical method using temperature or gamma rays, or the biological method using carrageenase enzyme. This paper review studies about carrageenan oligosaccharides, and presents some data about the potency of carrageenan oligosaccharide production in Indonesia.

Production Method of Carrageenan Oligosaccharides

The oligosachharides produced by several methods, mainly are depolymerization of carrageenan polymer by chemical, physical or biological methods as describe in Table 1.

Carrageenan oligosaccharides production with chemical methods can be carried out with mild hydrolysis, hydrolysis with the addition of cation resin, depolymerization by free radicals, partial reductive hydrolysis or synthesis from disaccharides. While physical methods can be carried out by depolymerization using gamma irradiation or microwave equipped with pressure. The production with biological method can be conducted by depolymerization of carrageenan by carrageenase enzymes. These oligosaccharides also can be produced directly from dried seaweed enzimatically using cellulase and carrageenase (Duan, Yu, Liu, Tian, and Mou, 2016).

Although carrageenan oligosaccharides can be produced chemically, physically and biologically, each method has advantages and disadvantages. The production of carrageenan oligosaccharides using chemical and physical methods has an advantage because of its lower cost compared to biological method. While the disadvantage of this method is producing diverse oligosaccharides so that their biological activity is often less specific to a particular target. To produce a uniform degree of polymerization, an advance procedure such as chromatography is required. Although the production carrageenan oligosaccharides using biological method or with the aid of enzymes are more expensive, the enzymes work more specifically to produce a more uniform product of oligosaccharides. Therefore, the biological activity of the carrageenan oligosaccharides produced biologically is often higher than that produced chemically or physically. In addition, the enzymatical method results in a non-reducing end in the product, which is thought to have better biological activity than the reducing end (Figure 1). This is in line with those found in alginates oligosaccharide (AOS) which show that AOS produced enzymatically has better immunomodulatory activity than that produced chemically (Iwamoto et al, 2005).

Biological Activity of Carrageenan Olligosaccharides

Oligosaccharides are reported to have a wide range of biological activities such as antibacterial, antiviral, antioxidants, immunomodulators, antiinflamatory and antitumor (Chen et al*.,* 2007; Curtois, 2009). Compared to carrageenan polysaccharides, the oligosaccharides has advantages that they are structurally more homogeneous and exhibit less toxicity due to have a lower anticoagulant activity and easier to be excrete. Moreover, many activities can be resulted from depolymerization process, such as anticoagulant and antiviral activity (Yamada et al., 1997). Chen et al. (2007) suggested that to increase the bioactivity of carrageenan can be achieved by reducing its molecular weight.

Antioxidant Activity

Several studies on antioxidant activity of oligosaccharides and their derivatives have been conducted previously. Yuan et al. (2005) conducted research on the antioxidant activity of carrageenan oligosaccharide and its derivatives against three radical systems namely hydroxy radicals, superoxide radicals, and DPPH radicals scavenging activity. The results show that carrageenan oligosaccharide derivatives exhibited higher antioxidant activity than its polysaccharides source. All carrageenan oligosaccharides derivatives showed significant improvement of antioxidant activities, some of it are the phosphorylated and low-Degree sulfation acetylated derivatives, the oversulfated and acetylated derivatives, and the phosphorylated derivatives. Another research (Yuan et al., 2006[b]) showed that carrageenan oligosaccharides modified by chemical method can increase their antioxidant activity in vitro. Carrageenan oligosaccharides and their derivatives exhibited positive activity as antioxidants both in vitro or cell system.

Sun et al*.* (2009), evaluated the antioxidant activity of carrageenan degradation product produced oxidatively. Antioxidant activity was observed from scavenging activity against superoxide radicals. The results showed that the smaller the molecular weight of carrageenan, the higher is the resulting antioxidant activity.

Sun et al*.* (2015) also observed the relationship between the method of carrageenan oligosaccharide

Table 1. Methods of carrageenan oligosaccharides production

	Methods	Procedures	References
	Chemically		
1	Mild hydrolysis	A solution of 1-2% carrageenan in 0.1 M HCl was heated at 60°C for 3 hours or at 37°C for 24 h. Depolymerization reaction was terminated by netralization with 0.1 M NaOH. The solution was then filtered, desalted and freeze dried or precipitated with 5 volumes of ethanol and followed by centrifugation to obtain dry oligosaccharides.	Briones and Sato, 2013; Kalitnik et al., 2015
2	Hydrolisis with the addition of cation resin	1% carrageenan solution in aquadest containing 20% cation exchange resin, heated and maintained at 90°C for 6 hours. The solution then neutralized, desalted and freeze dried.	Chen et al. , 2007
3	Depolymerization by free radicals	Solution of 4% carrageenan solution in 3% H_2O_2 was incubated for 12 hours at 40°C. The liquid was cooled and the oligosaccharides was precipitated with ethanol 5 times the volume of the solution and freeze dried+G16	Sun et al ., 2010
4	Partial reductive hydrolysis	100 mL of solution with 1% carrageenan was heated at 60°C. The borane-4-methylmorpholine complex solution was added, followed by addition of 10 ml of 2 mol/L trifluoroaceticacid solution. The solution was incubated 12 hours at 65°C. After the reaction, the acid and water were evaporated, the oligosaccharides dissolved in a small amount of water, dialyzed, salt removed and freeze dried	Sun et al., 2015
	Physically		
1	Gamma irradiation	The carrageenan was dissolved in aquadest to form 4% solution, then heated at 80°C. Furthermore, gamma ray irradiation was given at a dose of 10kGy/hr	Relleve et al., 2005
2	Microwave with pressure	The carrageenan solution at the concentration of 0.5% was microwaved for 4 min at 10 atm. The resulting irradiated solution is then freeze dried to obtain a dry oligosaccharide	Zhou et al, 2006
3	Biologically: Enzymatic depolymerization	The 0.2% carrageenan solution in 0.05 M mol/L tris HCl buffer pH 7 incubated with the enzyme carrageenase 10 ug /ml for 12 hours at 40°C or 1 L subtrate (2% carrageenan) + 10 ml 200 units concentrating enzyme for 6 h at 26°C. Enzyme inactivation is carried out by heating at 100°C. The product is dialyzed, removed by salt and dried	Sun et al., 2015; Duan, Yu, Liu, Tian, & Mou, 2016; Hu, Jiang, Aubree, Boulenguer., & Critchley, 2006

production and its antioxidant activity. The antioxidant assay included superoxide radical scavenging activity, hydroxyl radical scavenging activity, reducing power and DPPH radical scavenging activity. The results show that antioxidant activity of carrageenan oligosaccharides has a positive correlation with the

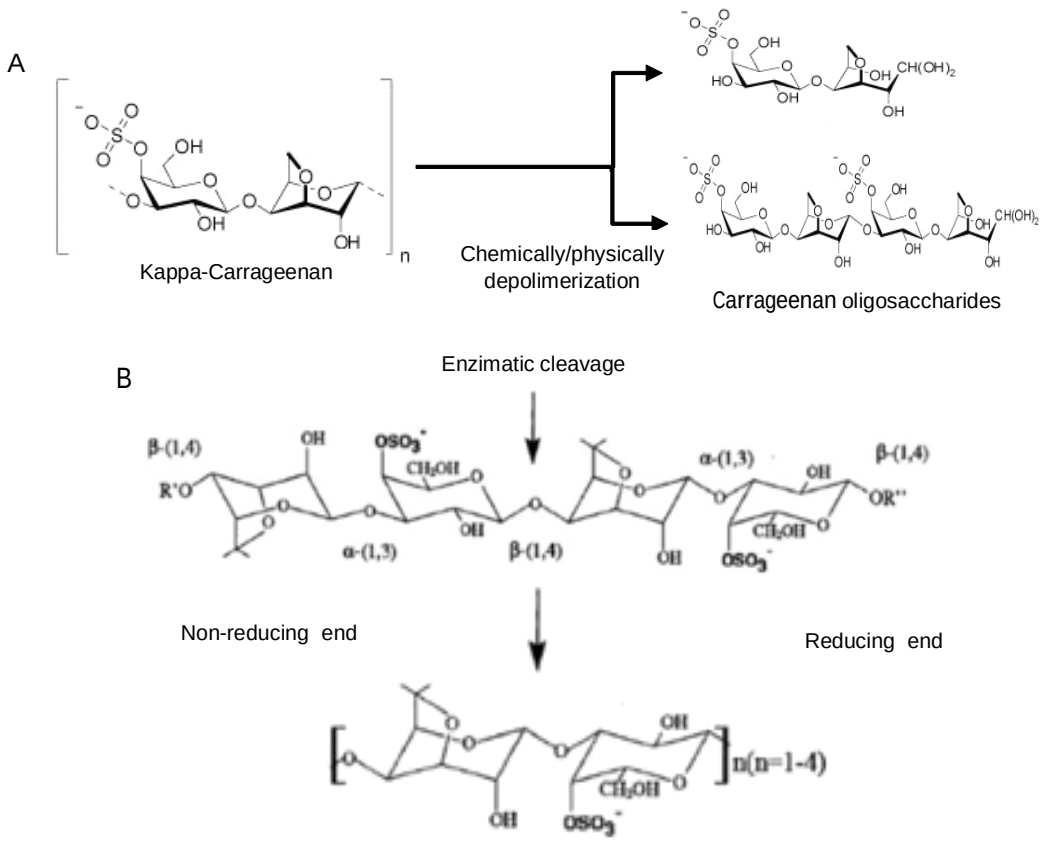

Figure 1. Depolimerization of kappa carrageenan by chemical/physical depolimarization (A) and enzymatical cleavage (B) to produce carrageenan oligosaccharides (Source: Ducatti et al., 2011; Yu et al., 2002; Zhu & Ning, 2015).

content of reducing sugar, the degree of polymerization, the content of sulfated groups, and the structure of reducing end.

Kumar, Ganesan, and Rao (2008) showed that antioxidant activity not only generated by carrageenan oligosaccharides but also from *Kappaphycus alvarezii* extract. Therefore the authors argue that the antioxidant activity in carrageenan oligosaccharides may actually also be contributed by the content of the extracted compounds such as phenolic compounds in the production process of carrageenan oligosaccharide from seaweed. The antioxidant activity of carrageenan oligosaccharides is probably a combination of oligosaccharide activity and several other activities from compounds present in products such as phenolic compounds.

Antitumor Activity

Some researchers have reported the activity of carrageenan oligosaccharides as antitumor (Haijin, Xiaolu, and Huashi, 2003; Yuan and Song, 2005; Hu et al, 2006; Yuan et al, 2006[a]; Yuan et al, 2011; Chen et al, 2009). The anti-tumor activity of k-carrageenan oligosaccharides with different molecular weight was also studied by using Sarcoma 180 tumor in mouse (Haijin et al., 2003). An oral application of carrageenan oligosaccharides with a molecular weight of 1726, shows reduction on tumor growth. The anti-tumor activity of the non-sulfonated and light-sulfonated were much more than that of high-sulfonated carrageenan preparation. This oligosaccharide was effective in stimulating the antioxidation capacity and eliminating the free radicals hazard. The research shows unique effects on immunological regulation, especially the phagocytosis index and phagocytosis ratio of macrophage, which boost the anti-tumor activity.

Carrageenan oligosaccharides from *Kappaphycus striatum* was also reported to have antitumor activity against three human neoplastic cell lines (KB, BGC, and Hela). Oligosaccharide fractions were preparated by gel permeation chromatography and one of the fraction showed relatively higher antitumor activity against the three cancer cells compared to its polysaccharides (Yuan & Song, 2005).

Hu et al. (2006) evaluated antitumor activity of carrageenan oligosaccharides produced from gelling carrageenan and thickening carrageenan, which were modified enzymatically with k-carrageenase. The carrageenan oligosaccharides produced from the thickening carrageenan showed much higher catalase activity and tumor inhibition to the sarcoma 180 cell, compared with the control group. The antitumor activity of this carrageenan oligosaccharides may be inisiated by organ-mediated defense reactions since there are significantly increase in the mass of immune organs.

Yuan et al (2006[a]) has evaluated the antitumor activity of carrageenan oligosaccharides with several parameters such as natural killer activity, interleukin-2, tumor necrosis factor alpha (TNF-α) and transplantable sarcoma S180 growth. It mentioned that carrageenan oligosaccharides inhibited tumor growth (sarcoma S180) accompanied by increasing natural killer activity, increased phagocytosis macrofage, serumal TNF-α and IL-2 levels in mice tested. Yuan et al. (2011) continued to study the antitumor activity of carrageenan oligosaccharide derivatives. The results exhibit that the treatment with different carrageenan oligosaccharide derivatives give different results on the growth rate of sarcoma S180 inhibition. The sulfated oligosaccharide at doses 200μgg per day exhibited the maximum tumor weight inhibition as 54.12% and stimulated nature killer cells activity up to 76.1%. The activity of carrageenan oligosaccharide derivatives was both significantly higher than the original oligosaccharides. It suggested that modification of carrageenan oligosaccharides by chemical method (especially sulfation) can rise their antitumor effect and boost their antitumor immunity.

Chen et al. (2007) also discovered that λ-carrageenan oligosaccharides (λ-CO) have an preventing activity of angiogenesis, or the formation of new blood vessels that are the initial phases of the development of cancer. Significant inhibition of vessel progression was detected at 200 µg/pellet. λ-CO treated samples have a less of capillary plexus and connective tissue than control samples. The viability of cells of λ-CO treated samples also significantly decreased even at a low concentration. Among three types of cancer cells tested, HUVEC is the most sensitive to λ-CO. Additionally, endothelial cell penetration and relocation also inhibited by the treatment with λ-CO at low concentration (150-300 µg/mL). These findings exhibit that λ-CO is a potential angiogenesis inhibitor that has sinergic effects on inhibiting invasion, migration, and proliferation of cancer cell.

Chen et al (2009) studied the cytotoxic activity effects of λ-oligosaccharides carrageenan (λ-CO) on human umbilical vein endothelial cells (HUVECs), and the mechanism of inhibition of cell proliferation by the oligosaccharides. Its showed that λ-CO obtained reactive oxygen species (ROS) production and accompanied by the multiplication of early apoptotic cells, nuclear morphology changes and cell cycle arrest at the S and G2 / M phases. λ-CO induced a depolarization of mitochondrial transmembrane potential. λ-CO also induced active-regulation of p53 and Bax, depressed-regulation of Bcl-2 and inisiation of caspase-9 and -3. These results suggest that exposure to a high concentration of λ-CO stimulates the mitochondrial-mediated apoptotic pathway and cell cycle prevent by generation of ROS.

From the above study it is seen that many reports suggest that carrageenan oligosaccharides have potential anti-tumor activity. These activities not only looked directly at the effects of tumor suppression, but it also showed that carrageenan oligosaccharides improved some factors that could inhibit tumor development such as immune system improvement seen from increased production of tumor necrosis factor, natural killer activity, increased antioxidant activity and inhibition of angiogenesis activity. Nevertheless, further research on antitumor activity in vivo still needs to be conducted.

Antibacterial Antivirus Activity

Wang et al. (2012) evaluated the antibacterial activity of k-carrageenan oligosaccharides produced enzymatically against *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Penicillium citrinum* and *Mucor*.sp The antibacterial activity assay were conducted by measuring the inhibitory zone diameter. The results show that ê-carrageenan oligosaccharides have antibacterial activity against the five bacteria tested. The antibacterial activities of κ-carrageenan against *Saccharomyces cerevisiae* was higher than against the other bacteria.

Wang et al. (2011) also reported the antiviral activity of carrageenan oligosaccharides agains influenza A1H1virus and its activity mechanism. He exhibited that carrageenan oligosaccharide with a molecullar weight 2 kDa (CO-1) efectively reduce influenza A1H1virus replication. Furthemore, this oligosaccharides constrained influenza A1H1virus replication superior than that of 3 kDa and 5 kDa carrageenan oligosaccharide (CO-2 and CO-3). CO-1 inactivated virus particles after pretreatment, without bind to the surface of MDCK cells. In addition, CO-1 also constrained protein expression. Unlike the carrageenan polysaccharide, CO-1 could penetrate into MDCK cells, and did not interfere with IAV

adsorbtion. The ability of carrageenan oligosaccharides to inhibit IAV intracellular replication could be a new anti-influenza alternative therapy.

From those studies it is seen that carrageenan oligosaccharides have anti-bacterial and anti-virus activity. From several reports, it is also seen that the low molecular weight of carrageenan oligosaccharides is positively correlated with the activity power. Therefore, to utilize carragenan oligosaccharides as antibacterial or antiviral agents, further studies of how effective polymerization degrees and how efficient depolymerization processes need to be continued.

Immunomodulator Activity

Immunomodulation and antitumor activity of k-carrageenan oligosaccharides also been reported (Yuan et al., 2006[a]). Macrophage phagocytosis, quantitative haemolyisis of sheep red blood cell, spleen lymphocyte proliferation, the activity of natural killer (NK) cell, production of interleukin-2, and tumor necrosis factor-α (TNF-α) were investigated. The research showed that carrageenan oligosaccharides significantly increased antibody secreted by spleen cells, proliferation of spleen lymphocyte, TNF-α level, serumal IL-2, macrophage phagocytosis and NK cells activity.

The immunomodulatory activity of k-carrageenan oligosaccharides (KOS) also has been reported by Zao et al., (2014). He obtained that k-carrageenan oligosaccharides could defend the activation of microglial cells by lipopolisaccharides (LPS). He proposed that the inhibition function was correlated to the content of sulfate group of KOS, that compete with LPS. He also described that the cell viability, arginase and TNF-α released by LPS-activated microglia cell can be inhibited by KOS and desulfated derivatives of KOS (DSK). He also found that the effect of DSK was lower than that of KOS. The immunomodulatory activation mechanism is including the binding of KOS on the cell surface firstly, and then they enter into the cell to the nucleus, spread over the entire cell finally.

Immune response mechanism of mouse monocytes/macrophages treated with κ-carrageenan polysaccharide also has been reported by Shu et al., (2017). He reported that κ-carrageenan polysaccharide stimulates RAW 264.7 cells as well as boosts cell proliferation accompanied by the improvement of tumor necrosis factor-α (TNF-α) secretion and cells' phagocytic capability. More over, the use of TLR4-specific inhibitors can significantly fasilitate the increased TNF-α secretion induced by ê-carrageenan polysaccharide. The RAW 264.7 cells treated with ê-carrageenan polysaccharide indicate the expression of upregulated TLR4 and translocation of main subunit of NF-êB (p65). These results promote the immunomodulatory function of ê-carrageenan polysaccharide in RAW 264.7 cells.

The results of these studies show that carrageenan oligosaccharides (COS) have immunomodulatory activity demonstrated by their ability to increase the activity of some immune cells such as macrophage phagocytosis, lymphocyte proliferation, natural killer cells activity, and their ability to activate several types of interleukins such as interleukin-2, tumor necrosis factor-α (TNF-α), including formation of antibody screeted by spleen cells. From some of these activities, it can be concluded that carrageenan oligosaccharides potential to be utilized as immunomodulator substance to regulate the human immune system. Nevertheless, most immunnomodulatory studies are still conducted in-vitro, so further research to see its invivo activity still need to be continued.

Antiinflammatory Activity

Kalitnik et al., (2015) evaluated the effect of κ/β low molecular weight carrageenan oligosaccharides (1.7 kDa) and its source carrageenan polysaccharide (400 kDa) on the production of antiinflammatory cytokine IL-10 by in vivo and ex vivo assay. This research showed that both samples of carrageenan stimulate the induction of IL-10 in human and in mice blood cells, and this stimulation were depended on their molecular weights. Carrageenan polysaccharide (administered orally) stimulated less IL-10 in vivo production than low molecular weight carrageenan. Nevertheless, the ex vivo assay exhibited that cytokine-induced activity of carrageenan polysaccharide did not depend on its concentration.

Yermak et al. (2012) also reported that κ/βcarrageenan can induces the synthesis of anti-inflammatory cytokine IL-10 in human blood cells. Even at low concentrations (10ng/mL), the activity of activity of κ/βcarrageenan was greater than that of LPS. IL-10 is a great potential substance for use in the treatment of inflammatory and immune illnesses, so the synthesis of this substance in human blood cell is regarded as the ability of carrageenan to work as an anti inflammatory compound.

Other research reports on anti-inflammatory activity of carrageenan oligosaccharides are still very limited, so this activity is still not convincing. On the other hand, several other studies have reported contrary results, suggesting that the carrageenan polymer acts

as an inflammatory inducer. Several studies that used carrageenan as inducer of inflammation among others: Amdekar et al., (2015) that use carrageenan as substance to induce acute inflammatory in Winstar rats, Xu et al., (2012) use carrageenan as substance to induce acute inflammatory in Male BALB/c mice, Hafaez et al., (2013) use carrageenan to induce hind-paw oedema of Swiss albino mice, and many other research. Thus, the report on anti-inflammatory activity carrageenan still need to be studied further.

Development Opportunities of Carrageenan Oligosaccharides in Indonesia

Indonesia is the largest producer of red carrageenan seaweed in the world. Production of seaweed in Indonesian increased by 27,88% per year, and the production was 9,9 million ton in 2015 (Anon., 2016; Zakaria et al*.,* 2017). The production of carrageenan oligosaccharide carrageenan can be applied by utilizing carrageenan materials with poor quality such as carrageenan with low polymerization degree, because this product does not require good gel forming ability. If a good carrageenan is intended for export markets, then unqualified carrageenan qualities can be used as raw material for oligosaccharide processing. Assuming the production of carrageenan seaweed is 9.9 million tons, and the yield of carrageenan 15-30%, then at least the national carrageenan production can reach 1.5 – 3.0 million tons per year. Assuming if the quality of carrageenan does not meet the 15% export requirement, the carrageenan available for oligosaccharide processing raw material is equivalent to 223-446 ton. The availability of abundant raw materials will be one of the advantages of domestic oligosaccharide production due to the abundance of raw materials and the low price of seaweed.

In addition, as the country lies in the tropical area, the abundance of carrageenan-produced seaweed species diversity is also quite high. Several seaweed carragenan producer that exist in Indonesia are *Eucheuma cottonii*, *Eucheuma spinosum*, *Eucheuma denticulatum*, *Betaphycus gelatinum*, *Gigartina scottsbergii*, *Gigartina canaliculata*, and *Hypnea musciformis* (Diantariani, 2008; Distantina et al., 2011; Harris, 2017; Kasim *et al*., 2017; Pereira, 2017; Silva, et al., 2017; Sridevi, 2017; Thangavel & Sridevi, 2017). Various types of carrageenan-producing seaweed will produce different product characteristics and sulfate content. Due to the biological activity of oligosaccharides is largely determined by the degree of polymerization, the molecular structure, the content of reducing sugar, and the content and position of the sulfate group (Sun et al., 2015), the potential for producing new carrageenan oligosaccharides with diverse activity will be very high. Therefore, further research opportunities to produce oligosaccharides with certain biological activities will be enormous.

The world's demand for oligosaccharides for food is quite high. For example, the value of galacto oligosaccharide trade (GOS) in the USA is worth 34.2 million tons in 2015, and the world's prebiotics demand for food and beverages in 2011 amounted to 1.8 million tons (Anonymous, 2017[b]; Anonymous 2017[c]). The high world demand for oligosaccharides and the high abundance of raw materials of marine origin to produce oligosaccharides in Indonesia is a great opportunity for industrial development. The cheap price of raw materials will be the competitiveness to capture the world oligosaccharides market share.

Conclusion

Carrageenan oligosaccharides are sugars of shorter chains (dp $\leq$ 10) depolymerized from larger carrageenan polysaccharides. The carrageenan oligosaccharides production by chemical method were carried out with mild hydrolysis, hydrolysis with the addition of cation resin, depolymerization by free radicals, partial reductive hydrolysis or synthesis from disaccharides. The production of oligosaccharides by phisical method were carried out with carrageenan depolymerization using gamma irradiation or microwave with pressure. While oligosaccharide production by biological method may be performed with the help of carrageenase enzymes. Oligosaccharides are reported to have a wide range of biological activities such as antibacterial, antiviral, antioxidants, immunomodulators, and anti tumor. The development of carrageenan oligosaccharide industry in Indonesia is still possible to increase due to the abundance of species, production of carrageenan as the raw materials and the high demand of the world oligosaccharides.

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