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Guest Articles

Bioactive sugars from red macroalgae: structure, preparation, and applications

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Introduction

Algae is one of the earliest known photosynthetic eukaryotes, commonly found in freshwater and marine environments.[1] It is estimated that approximately 72,000 different algae species exist, although only a small fraction has been identified thus far.[2] Algae can be classified into two main categories: unicellular microalgae and multicellular macroalgae also referred to as seaweeds. The majority of macroalgae are found in the intertidal zone, and they exhibit a higher growth rate than terrestrial plants.[3] Macroalgae are classified into three major groups: red macroalgae (Rhodophyceae), brown macroalgae (Pheophyceae), and green macroalgae (Chlorophyceae). The cell walls of each macroalgae group are composed of different polysaccharides. The cell walls of red macroalgae are composed of agar, carrageenan, and porphyran. The major polysaccharides in brown macroalgae are fucoidan and alginate, whereas ulvan is the major polysaccharide of green macroalgae. Agar, carrageenan, porphyran, ulvan, and fucoidan are sulfated polysaccharides that are exclusively found in marine plants and algae.[4]

Cell wall composition of red macroalgae

Sulfated polysaccharides found in red macroalgae, agar, carrageenan, and porphyran, have distinct structural features. Agar is composed of two major components, agarose and agaropectin. Agarose is a linear heteropolysaccharide composed of D-galactose and 3,6-anhydro-L-galactose (L-AHG) linked with alternative β-1,4 and α-1,3 glycosidic linkages. Repeated units of agarose are known as neoagarobiose [O-3,6-anhydro-α-L-galactopyranosyl (1,3)-D-galactose], a heterodimer of D-galactose and L-AHG linked with a β-1,4 glycosidic linkage. The neoagarobiose units are linked to each other with α-1,3 glycosidic linkages, thereby forming the agarose polymer.[5] Agaropectin is also a linear polysaccharide with a structure similar to agarose. Agaropectin is distinguished from agarose by the presence of chemically modified L-AHG components and a slightly branched structure. The L-AHG units in agaropectin contain sulfoxy or methoxy and pyruvate moieties in their hydroxyl groups, which impart a net negative charge to agaropectin.[6] Agarose is directly responsible for the gelling properties of agar, whereas agaropectin does not contribute to this process.[7]

Porphyran is a linear sulfated polysaccharide comprising D-galactose, L-AHG, 6-O-methyl-D-galactose, and L-galactose-6-sulfate (L-G6S).[8] In contrast to agarose, porphyran contains sulfated and methylated galactose residues in its backbone. The repeating unit of porphyran is composed of α-1,3-linked D-galactose and L-G6S residues. One porphyran moiety is linked to another porphyran or agarose moiety by a β-1,4 linkage. In addition, the galactan backbone is masked by methyl ether groups at the C6 position of the D-galactose residues, regardless of whether D-galactose is linked to L-AHG or L-G6S units.[9]

Carrageenan is a high molecular weight sulfated polygalactan found in several species of red seaweeds of the class Rhodophyceae.[10] Key difference between agarose and carrageenan is the presence of L-AHG in

agarose, in contrast to the 3,6-anhydro-D-galactose (D-AHG) present in carrageenan. The most common forms of carrageenan are designated as kappa (κ), iota (ι), and lambda (λ) carrageenan. κ-Carrageenan is composed of a repeating unit of α-1,3-linked D-galactose-4-sulfate (D-G4S) and D-AHG. This repeating unit is linked to each other by β-1,4 linkage.[11] ι-Carrageenan is similar but with the D-AHG being sulfated at the C2.[12] λ-Carrageenan has repeating units of α-1,3 linked D-galactose-2-sulfate and D-galactose-2,6-disulfate. These repeating units are linked to each other by β-1,4 glycosidic linkages.[13] Further, λ-carrageenan is composed only with differently sulfated D-galactose residues. The L or D isomers of anhydrogalactose are not present in λ-carrageenan.[13]

The oligosaccharides generated by the breakdown of α-1,3 linkages and β-1,4 linkages in agar and carrageenan polymers exhibit distinct structural and chemical characteristics. The hydrolysis of α-1,3 linkages in agar results in the generation of short oligosaccharide chains with L-AHG at the reducing end, which are known as agarooligosaccharides (AOS). In contrast, hydrolysis of α-1,3 linkages in carrageenan generates oligosaccharides with D-AHG at the reducing end. The hydrolysis of β-1,4 linkages in both agar and carrageenan results in the generation of oligosaccharides that contain D-galactose at the reducing end. Agar-derived oligosaccharides with D-galactose at the reducing end are designated as neoagarooligosaccharides (NAOS).[14]

Pretreatment of red algal polysaccharides

Prior to the extraction and breakdown of algal polysaccharides, pretreatment is conducted to remove low-molecular-weight compounds, pigments, and lipids by washing powdered seaweeds with ethanol or chloroform. The extraction of polysaccharides from red seaweeds is conducted via microwave-assisted extraction, ultrasonic-assisted extraction, and enzymatic-assisted extraction.[15] Out of these three methods, microwave assisted extraction and ultrasonic assisted extraction are regarded as high efficiency extraction methods to obtain high yield of polysaccharides from seaweeds.

Preparation of bioactive sugars from agar

The breakdown of agar polymer to oligosaccharides and subsequently to monosaccharides is regarded as a two-step process. The liquefaction of agar, which is the depolymerization of agar to oligosaccharides, is a process that is conducted mainly by chemical or enzymatic methods. The subsequent breakdown of agar oligosaccharides into monosaccharides is referred to as saccharification, which is primarily achieved using enzymatic methods.[16]

Acid hydrolysis of agar is carried out under mild conditions, typically using weak acids or ionic liquids. To hydrolyze agar under mild conditions, low concentrations of strong acids, short reaction times, or low temperatures are commonly employed.[16] Mild acid hydrolysis randomly cleaves the α-1,3 linkages, thus yielding a broad range of AOS with degree of polymerization (DP) ranging from 4 to 22. However, the L-AHG moiety at the reducing end of AOS is spontaneously hydrolyzed under acidic conditions, yielding odd-numbered AOS.[17] Furthermore, the L-AHG released during acid hydrolysis is readily converted to the toxic byproduct 5-hydroxymethylfuraldehyde (HMF).[7,17] Hydrolysis of agar with mild acids, such as acetic acid, also preferentially cleaves the α-1,3 linkages, yielding even-numbered AOS. In contrast to strong acids, weak acid hydrolysis minimizes L-AHG loss and the generation of toxic byproducts.[18] Bisulfate ionic liquids derived from the reaction of sulfuric acid and choline chloride were employed to hydrolyze agar. Ionic liquids are known to yield the monosaccharides D-galactose and L-AHG.[19] However, ionic liquids are not a cost-effective option due to their high cost and limited recycling.

In contrast to chemical methods, enzymatic hydrolysis employs specific enzymes to break the agar polymer at specific points, thereby generating oligosaccharides with specific sizes. Two types of agar-hydrolyzing enzymes have been identified: β-agarase (EC 3.2.1.81) and α-agarase (EC 3.2.1.158).[20] β-Agarase cleaves the β-1,4 glycosidic linkages of the agar polymer, yielding even-numbered NAOS. In contrast, α-agarase cleaves the α-1,3 glycosidic linkages, yielding even-numbered AOS. β-Agarases that have been functionally characterized belong to glycoside hydrolase (GH) families 16, 50, 86, and 118. Each GH family of agarases

exhibits a distinctive hydrolytic pattern. GH16 β-agarase is an endo-type agarase that specifically recognizes and cleaves β-1,4 linkages within the agar polymer. GH16 β-agarase yields NAOS longer than neoagarododecaose (DP>12). These NAOS are further hydrolyzed to neoagarohexose (DP 6) and neoagarotetraose (DP 4) by the same enzyme.[21] GH16 β-agarase from *Zobellia galactanivorans* DsiJ, *Saccharophagus degradans* 2-40, and *Microbulbifer thermotolerans JAMB-A94 are some of the well characterized GH16* β-agarases.[21–23] GH50 β-agarases exhibit both endo- and exo-lytic agarolytic activity. These enzymes use both agar and short NAOS as the substrate and produce neoagarobiose (DP 2) as the major product.[24] Some of the most well-characterized GH50 enzymes are those from *S. degradans* 2-40, *Phocaeicola plebeius* DSM 17135, and *Agarivorans gilvus*.[24–26] GH86 β-agarases produce a wide range of NAOS including neoagarobiose, neoagarotetraose, and neoagarohexaose by hydrolyzing agar. The GH86 β-agarases of *S. degradans* 2-40, *Bacteroides uniformis* NP1, and *M. thermotolerans* JAMB-A94 have been extensively studied and well characterized.[27–29] GH118 β-agarases produce neoagarooctaose (DP 8) and neoagarodecaose (DP 10) as major products. The GH118 β-agarase of *Vibrio* sp. PO 303 and *Pseudoalteromonas* sp. CY 24 are among the well characterized GH 118 β-agarases.[30,31]

α-Agarase catalyzes the hydrolysis of α-1,3 glycosidic linkages present in agar. α-Agarase belongs to the GH 96 family of hydrolases and yields AOS, including agarotetraose (DP 4) and agarohexaose (DP 6). Some of the well-characterized GH 96 α-agarases are those of *Colwellia echini* A3, *Thalassotalea agarivorans* JAMB-A33, and *Thalassomonas* sp. LD5.[32–34] Short AOS and NAOS produced by chemical and enzymatic methods are completely saccharified into D-galactose and L-AHG by enzymatic methods.[18] Short NAOS are converted to neoagarobiose by GH50 β-agarase. Neoagarobiose has a α-1,3 glycosidic linkage which is hydrolyzed by GH117 family of enzymes.[35] Short AOS are converted to NAOS by GH2 family hydrolases, which remove the D-galactose at the non-reducing end.[14] Subsequently, these NAOS are subjected to hydrolysis by GH50 and GH117 family agarases.

Biological activities of agar

NAOS produced by the enzymatic hydrolysis of agar showed prebiotic effects. Specially, NAOS with DP 8, 10, and 12 showed higher prebiotic effect than NAOS with DP 4 and DP 6. The longer NAOS has been observed to stimulate the growth of probiotic gut bacteria, including *Bifidobacterium adolescentis* ATCC 15703, *B. bifidum* ATCC 29521, *B. longum* SMU 27001, *Lactobacillus acidophilus* AMU 28001, and L. *delbrueckii*.[36] AOS derived from the acid hydrolysis of agar has also shown prebiotic effect on B. adolescentis 1.2190 and *B. infantis* 1.2202. Agar-derived AOS has demonstrated antioxidant, anti-inflammatory, carcinostatic, and hepatoprotective activities.[37] Studies have demonstrated that AOS suppresses the induction of TNF-α and induces heme oxygenase I, thereby inhibiting inflammatory responses and oxidative damage.[38] *In vitro* studies have demonstrated that L-AHG exhibits anti-inflammatory activity by inhibiting nitrite production in RAW 264.7 cells.[39] L-AHG and neoagarobiose can be employed in the field of cosmeceuticals as a skin-whitening and moisturizing agent. *In vitro* studies have demonstrated that L-AHG and neoagarobiose suppress melanin production.[39,40] Furthermore, L-AHG has been shown to have stronger skin whitening activity than arbutin, a commonly used skin whitening agent. Both L-AHG and neoagarobiose demonstrated moisturizing activity.[39,40] L-AHG was found to induce the expression of hyaluronan synthase 2, a key enzyme in hyaluronic acid synthesis. Moreover, neoagarotetraose has demonstrated comparable skin whitening activity to arbutin.[41] Finally, L-AHG has demonstrated anticariogenic activity by inhibiting the growth of *Streptococcus mutans*. *Streptococcus mutans* is one of the primary microorganisms responsible for dental caries. *S. mutans* is unable to ferment L-AHG, and L-AHG inhibits the cell growth and lactic acid production. The anticariogenic effect of L-AHG is significantly greater than that of xylitol, which is one of the most effective anticariogenic sugar substitutes.[42]

Preparation of bioactive sugars from carrageenan

The preparation of carrageenan oligosaccharides can be achieved through chemical, physical, and enzymatic degradation of carrageenan polysaccharides. Chemical hydrolysis is carried out using mild

acid hydrolysis, free radical hydrolysis, and partial reductive hydrolysis. Mild acid hydrolysis using diluted hydrochloric acid and diluted sulfuric acid were employed to obtain carrageenan disaccharides (Carrabiose).[43,44] Low molecular weight κ-carrageenan oligosaccharides were obtained by free radical hydrolysis using hydrogen peroxide.[45] Both mild acid hydrolysis and free radical hydrolysis randomly break α-1,3 linkages and β-1,4 linkages in carrageenan, generating odd- and even-numbered oligosaccharides. Free radical hydrolysis of κ-carrageenan generates oligosaccharides with D-AHG at the reducing end and D-G4S/D-AHG at the nonreducing end. Mild acid hydrolysis generates κ-carrageenan oligosaccharides with D-G4S/D-AHG at the reducing end and D-AHG at the nonreducing end.[46] Trifluoroacetic acid (TFA) and borane-4-methylmorpholine complex are often employed in partial reductive hydrolysis of carrageenan. κ-Carrageenan oligosaccharides ranging from DP 2 to DP 10 were obtained from partial reductive hydrolysis. This method is a more controlled process that specifically breaks α-1,3 linkages and generates even-numbered oligosaccharides with D-AHG at the reducing end.46 Physical methods of carrageenan depolymerization include the use of ultraviolet (UV) radiation, gamma rays, and microwaves.[47–49] Microwave-assisted depolymerization can be employed to preserve the fragile sulfate groups present in carrageenan.49 Physical methods can be readily implemented at a low cost and with minimal environmental impact, although the yields obtained are typically limited.

Enzymatic hydrolysis of carrageenan specifically breaks the β-1,4 linkages in different carrageenan types. Three different types of carrageenan hydrolytic enzymes, namely κ-carrageenase, ι-carrageenase, and λ-carrageenase, are present to hydrolyze κ-, ι-, and λ-carrageenan, respectively.[50] Each of these carrageenase types exhibits high substrate specificity, rendering them incapable of hydrolyzing the other types of carrageenan. The κ-carrageenase (EC 3.2.1.81) belongs to the GH 16 family and is the most extensively characterized type among the carrageenases. κ-Carrageenase is capable of cleaving β-1,4 linkages in κ-carrageenan, resulting in the production of oligosaccharides of DP 2, DP 4, and DP 6 as the predominant products. Notable examples of well-characterized κ-carrageenase enzymes are those of *Z. galactanivorans* DsiJ, *C. echini* A3, and *Paraglaciecola hydrolytica* S66.[51–53] Unlike κ-carrageenase, ι-carrageenase (EC 3.2.1.157), and λ-carrageenase ((EC 3.2.1.162) are not very common in nature. ι-Carrageenase belong to GH 82 family and hydrolyze β-1,4 linkages in ι-carrageenan, whereas λ-carrageenase belongs to GH 150 family and hydrolyze β-1,4 linkages in λ-carrageenan.[54,55] Unlike chemical methods, enzymatic hydrolysis can preserve the sulfate groups in carrageenan oligosaccharides. Sulfatases that remove the sulfate groups from the carrageenan oligosaccharides are also highly specific to their substrate, allowing the preparation of oligosaccharides with a desired sulfation pattern. For instance, carrageenan sulfatases of the S1_7 family remove the sulfate at the 4C position of D-galactose in κ-carrageenan, leaving non-sulfated β-carrageenan oligosaccharides. Carrageenan sulfatases of the S1_19 family remove the sulfate at the 4C position of D-galactose in ι-carrageenan, leaving α-carrageenan oligosaccharides, which are sulfated at the 2C position of the D-AHG moiety.[56,57] Therefore, a combination of specific carrageenase and sulfatase can be used to produce carrageenan oligosaccharides with the desired size and sulfation pattern.

Biological activities of carrageenan

The biological activities of carrageenan oligosaccharides are contingent upon the degree of polymerization, the number and position of the sulfate groups, and the type of sugar moiety at the reducing/non-reducing ends.[46] Several studies have demonstrated the strong antioxidant activities of carrageenan oligosaccharides and their chemical derivatives. κ-Carrageenan oligosaccharides with lower DP show higher antioxidant activity than longer oligosaccharides.[58] These oligosaccharides demonstrate antioxidant activity by scavenging superoxide anions and hydroxyl radicals.[45] κ-Carrabiose, a sulfated disaccharide resulting from the enzymatic hydrolysis of κ-carrageenan, exhibited high antioxidant activity due to its free radical scavenging ability.[59] Short oligosaccharides obtained by the hydrolysis of κ- and λ-carrageenan demonstrated potent anti-tumor activity by inhibiting tumor growth.[60,61] High sulfation levels of the carrageenan oligosaccharides can significantly enhance the anti-tumor activity.[62] κ-Carrabiose has

demonstrated high cytotoxic activity against tumor cells, inducing apoptosis.[63] A high concentration of λ-carrageenan oligosaccharides exerts cytotoxic effects by triggering the production of intracellular reactive oxygen species and inducing apoptosis.[64] Furthermore, short λ-carrageenan oligosaccharides have demonstrated anti-tumor activity by inhibiting angiogenesis and heparanase activity.[65]

Carrageenan oligosaccharides possess a diverse array of immunomodulatory activities. κ-Carrageenan oligosaccharides have been demonstrated to activate the phagocytosis of macrophages, the proliferation of lymphocytes, and to enhance the activity of natural killer (NK) cells.[66] Likewise, λ-carrageenan oligosaccharides can induce the proliferation of lymphocytes and enhance the activity of NK cells.[67] Furthermore, ι-carrageenan oligosaccharides enhance the phagocytosis of neutrophils, cytotoxicity of NK cells, and stimulate lymphocyte proliferation.[68] Low molecular weight λ-carrageenan and κ/β-oligosaccharides have shown anti-inflammatory properties, which are mediated by the induction of the anti-inflammatory cytokine IL-10.60 D-AHG, a monosaccharide derived from carrageenan, exhibit anti-inflammatory activity in in-vitro assays.[39] Short κ-carrageenan oligosaccharides have been demonstrated to exhibit antiviral and antibacterial activities. Research has shown that κ-carrageenan oligosaccharides can inhibit the replication of influenza virus.[69] The acetyl and sulfate content of κ-carrageenan oligosaccharides may influence the anti-viral activity.[70] κ-Carrageenan oligosaccharides obtained by enzymatic hydrolysis has shown antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.[71] Although κ-carrageenan oligosaccharides have demonstrated promising biological activities, their application is constrained by the potential for inducing apoptosis, inflammatory responses in the colon and rectum, and gastrointestinal irritation observed in animal models.[72–74]

Future directions

The exploration of bioactive sugars from red macroalgae presents a promising frontier in both biotechnology and pharmacology. However, chemical, physical, or enzymatic methods commonly employed for the preparation of oligosaccharides often yield a heterogenous mixture of oligosaccharides. Future research should prioritize the development of efficient extraction and purification methods of oligosaccharides to maximize yield, purity, and activity. The bioactivity of these sugars against various pathogens and in modulating immune responses suggests significant therapeutic potential. Future studies should focus on detailed mechanistic investigations to understand their interactions at the molecular level and their effects in complex biological systems. In vivo studies and clinical trials will be necessary to evaluate their efficacy and safety in medical applications. Interdisciplinary collaborations involving marine biology, chemistry, pharmacology, and biotechnology will be essential to fully harness the potential of bioactive sugars from red macroalgae, paving the way for novel therapeutic agents and industrial applications.

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