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Chemistry, gelation, and enzymatic modification of seaweed food hydrocolloids

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Abstract

Background: Carrageenan, agar, and alginate are seaweed-derived carbohydrate hydrocolloids which are used as thickening and gelling agents in foods, pharma and biotechnology applications due to their unique gelation properties. These hydrocolloids are extracted commercially from a set of high-yielding, cultivated seaweeds (marine macroalgae). The seaweed type and biological and environmental factors during seaweed growth determine the chemical and rheological hydrocolloid properties. New insight into biosynthesis and microbial degradation of these seaweed-derived hydrocolloids includes discovery of unique enzymes that catalyze specific structural changes in the carbohydrate polymers which affect the hydrocolloid properties.

Scope and Approach: In this review we describe the intricate chemical structures, gelation mechanisms and biosynthesis routes of seaweed hydrocolloids. We provide an overview of novel enzymes and enzyme reactions that catalyze changes in these hydrocolloids, and discuss how this new enzyme knowledge may enable further technological advancements in processing and applications of seaweed hydrocolloids.

Key Findings and Conclusions: The red seaweeds from the genera *Kappaphycus* and *Eucheuma* are key commercial sources of carrageenan, species from the genera *Gracilaria* and *Gelidium* are the main sources of agar, and brown seaweeds from the genera *Laminaria* and *Macrocystis* are currently the main sources of alginate. Recent progress has advanced our understanding of enzymatic reactions involved in the biosynthesis and microbial degradation of these hydrocolloids and especially uncovered unique epimerase, desulfatase, and alginate lyase processes. This new knowledge provides a basis for rethinking the sources and extraction protocols of these hydrocolloids because such enzymes can catalyze distinct molecular changes to improve the physical properties of the hydrocolloids.

28 Keywords: Carrageenans; agar; alginate; epimerase; desulfatase

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29 Introduction

30 Marine macroalgae, notably edible red (phylum Rhodophyta) and brown seaweeds (phylum Ochrophyta,
 31 class Phaeophyceae) are important sources of unique carbohydrate-based hydrocolloids, i.e. carrageenans,
 32 agar and alginate, respectively. Due to their gelation properties these hydrocolloids are used in many value-
 33 added applications in foods, pharma, and biotechnology. These hydrocolloids are considered as food
 34 additives, and are generally recognized as safe by the US Food and Drug Administration and the European
 35 Food Safety Authority, and they have the following E numbers in the European Union: refined carrageenan
 36 has E number E 407 (includes furcelleran), semi-refined carrageenan has E 407a, agar has E 406, and
 37 alginates have several E numbers as follows: E 400 (alginic acid), E 401 (sodium alginate), E 403 (ammonium
 38 alginate), E 404 (calcium alginate) and E 405 (propane-1,2-diol alginate). The safety of carrageenans in
 39 foods has been debated, but based on their safe use history, acute and chronic toxicology tests,
 40 carrageenans are recognized as safe both by the US Food and Drug Administration and the European Food
 41 Safety Authority (David et al., 2018). Carrageenans and agar are commercially extracted from certain red
 42 seaweeds of which the most important sources are carrageenophyte species from the genera *Kappaphycus*
 43 and *Eucheuma* (notably from *Eucheuma cottonii*) and agarophyte species from the genera *Gracilaria* and
 44 *Gelidium* (Porse & Rudolph, 2017). Alginate is commercially extracted from brown seaweed species, most
 45 commonly from the genera *Laminaria*, *Lessonia*, and *Macrocystis* amongst others, but there seems to be
 46 large unused quantities of other alginate-containing seaweeds available for cultivation, e.g. *Saccharina*
 47 species (Porse & Rudolph, 2017). Carrageenans, agar and alginate each constitute a major part of the cell
 48 wall in the respective seaweeds they come from along with cellulose, proteins, ions, and other
 49 polysaccharides (e.g. fucoidans in brown seaweeds), and are associated with osmotic and ionic regulation,
 50 and known to contribute to cell adhesion, physical integrity and immunity of the macroalgae (Deniaud-
 51 Bouët et al., 2014). Green seaweeds (phylum Chlorophyta) accumulate unique sulfated carbohydrates,
 52 namely ulvan and rhamnan sulfate, along with polysaccharides consisting of xylose, arabinose, galactose,
 53 and/or glucuronic acid. These polysaccharides have been reported to have a range of biological activities,
 54 but their viscosity-enhancing properties are low, and to our knowledge green seaweed carbohydrates are
 55 not used commercially as hydrocolloids.

56 Recent market research predicts that the Global market for hydrocolloids will grow to 11.8 billion US\$ by
 57 2025 with an annual compound growth rate (CAGR) of 5% (Market Research 2020); in addition to seaweed
 58 derived hydrocolloids, the food hydrocolloids market includes gelatin, pectin, guar gum, and
 59 microcrystalline cellulose. Based on 2015 data and a CAGR of 5%, we estimate that current total sales value
 60 of carrageenans, agar, and alginate amount to ~1.5 billion US\$ with carrageenan sales accounting for more
 61 than 50% of this value (Rhein-Knudsen, Ale, & Meyer, 2015). Certain bacteria belonging to the genera

62 *Azotobacter* and *Pseudomonas* can also produce alginate (which they use to produce a capsular gel and
63 biofilm), but the bacterial alginate is acetylated and is not employed commercially.

64 Chemical structures and commercial extraction procedures

65 Seaweed hydrocolloid polysaccharides are built of unique carbohydrate monomer structures and consist of
66 either sulfated, dehydrated galactopyranosyl moieties (carrageenans and agar) or uronic acids (alginate).
67 The ability to form gels or viscous solutions depends on the composition and configuration of the
68 polysaccharides. The basic configurations of the different seaweed hydrocolloids are well-defined, but
69 particular structural details such as the extent of 3,6-anhydrogalactopyranosyls in carrageenans and agar
70 and the balance between the uronic acid types in alginate vary with seaweed species, life-stage, season,
71 growth site, and water depth.

73 Carrageenans

74 Carrageenans are linear polysaccharides composed of different types of repeating β -1,4 linked disaccharide
75 D-galactopyranosyl units bound together by α -1,3 linkages. In nature, many types of carrageenans exist
76 which can be distinguished by the amount and position of sulfate ester modifications as well as the content
77 of 3,6-anhydrogalactopyranosyls, unique to red seaweeds. The carrageenans of commercial interest are
78 known as κ -, ι -, and λ -carrageenan (Fig. 1) (Usov & Zelinsky, 2013).

79 The 3,6-anhydro-D-galactopyranosyl moieties are present in κ - and ι -carrageenan, while λ -carrageenan is
80 composed solely of sulfated galactopyranosyl monomers (Fig. 1). Sulfate modifications are present on all
81 the carrageenans where κ -carrageenan has one sulfate ester per dimer, while ι - and λ -carrageenan have
82 two and three sulfate substitutions per dimer, respectively (Fig. 1). The chemical structures described
83 above represent simplified structures. In nature, carrageenans are inherently heterogeneous and contain
84 stretches of their biosynthetic precursors; μ -carrageenan for κ -carrageenan and ν -carrageenan for ι -
85 carrageenan. These precursors contain D-galactopyranosyl 4-sulfate and D-galactopyranosyl 6-sulfate or D-
86 galactopyranosyl 2-6-sulfate but no 3,6-anhydro-D-galactopyranosyls (Usov & Zelinsky, 2013). Adding
87 further to the complexity of the carrageenans, κ/ι hybrid carrageenans exist along with other carrageenans
88 within the same seaweed. Carrageenans may thus contain different diads in the same molecule and
89 different distributions of them depending on the life cycle stage of the seaweed, e.g. the red macroalgae
90 *Chondrus crispus*, commonly known as Irish moss, typically has κ - and ι -carrageenan in the gametophytes
91 (haploid multicellular life-stage) and λ -carrageenan in the sporophytes (diploid multicellular life-stage)
92 (Colln et al., 2013).

Fig. 1 Chemical structures of the three commercially used carrageenans, κ -, ι -, and λ -carrageenan, and agarose and agaropectin, the constituents of agar.

The chemical structures together with the physical gelation properties form the basis for the currently used chemical extraction procedures which involve pH changes and use of cations to promote gelation. However, the extraction technique, i.e. water extraction or combined modification and extraction, may affect the chemical properties of the hydrocolloids. For production of high-value food and pharma carrageenans, the extraction protocol usually involves an alkali-treatment step (Table 1) by which the D-galactopyranosyl 6-sulfates are converted into 3,6-anhydro-D-galactopyranosyls (typically by KOH for κ -carrageenan and NaOH for ι -carrageenan) during the extraction (Istinii, Ohno, & Kusunose, 1994).

The alkali induced modification mechanism converting D-galactopyranosyl 2,6-sulfate to 3,6-anhydro-D-galactopyranosyl 2-sulfate, i.e. ν -carrageenan to ι -carrageenan (outlined in Fig. 2) was originally proposed by Ciancia et al. (1993). The reaction starts with ionization of the C₃ OH on the sulfated α -galactopyranosyl residue, creating a charge repulsion between the ionized oxygen and the C₃ sulfate (Fig. 2). To situate these charged components as far away from each other as possible, the chair conformation changes from 4C_1 to

1C_4 , arranging the charges in an axial position that enables an intramolecular S_N2 reaction with the formation of the 3,6-anhydro ring and the concurrent displacement of the C_6 sulfate group (Fig. 2). It is proposed that the analogous conversion of D-galactopyranosyl 6-sulfate to 3,6-anhydro-D-galactopyranosyl, i.e. from the μ -carrageenan precursor to κ -carrageenan (to be discussed later), occurs via an additional ionization of the C_2 OH, which thus creates a similar repulsion effect. The conversion from 4C_1 to 1C_4 and notably the loss of a sulfate group and the 3,6-anhydro ring formation (Fig. 2) changes the hydrophobicity of the polysaccharides enabling helix induced gel formation (Ciancia, Nosedá, Matulewicz, & Cerezo, 1993).

Fig. 2 Proposed mechanism for alkali modification of D-galactopyranosyl 2,6-sulfate to 3,6-anhydro-D-galactopyranosyl 2-sulfate, i.e. ν -carrageenan to ι -carrageenan.

Following modification, the carrageenans are extracted from the seaweeds in boiling water to minimize viscosity, separated from the seaweed residuals by filtration, and recovered by alcohol precipitation, outlined in Table 1. Native carrageenan, i.e. non-modified carrageenan, can be extracted directly in boiling water followed by filtration and alcohol precipitation. The exact reaction conditions vary between reported studies but generally carrageenans and agar are modified at 80-90 °C for 1-3 hours, for carrageenans typically with 6 % KOH (for agar usually with 5-6 % NaOH), followed by washing and pH adjustment to prevent depolymerization, before they are extracted in boiling water for 1-3 hours (Table 1).

Table 1 Overview of processing steps and conditions for carrageenan, agar, and alginate extraction summarized from Istini et al., 1994 and Calumpong, Maypa, & Magbanua, 1999.

Process	Carrageenan extraction	Agar extraction	Alginate extraction
Raw material	Drying, milling	Drying, milling	Drying, milling
Alkali-modification	KOH/NaOH, 80-90 °C	NaOH, 80-90 °C	
Phenolic compounds removal			Formaldehyde, 20 °C
Conversion to alginic acid			HCl, room temp.
Extraction	H ₂ O, pH 7.5-8.5, 99 °C	H ₂ O, pH 6-6.5, 99 °C	NaCO ₃ , 99 °C
Filtration and precipitation	Isopropanol	Isopropanol	Isopropanol
Refined hydrocolloid	Drying/freeze-drying	Drying/freeze-drying	Drying/freeze-drying

Differences in the extraction procedures affect the sulfate and 3,6-anhydrogalactosyls content, which may change from 20 to 16 % and 22 to 40 %, respectively, for carrageenan extracted from *Hypnea musciformis* (Rhein-Knudsen, Ale, Ajallouei, Yu, & Meyer, 2017). Analogously, the 3,6-anhydrogalactosyl content could be increased from 28 to 33 % using 6 % KOH for alkali-modification during extraction (Moses, Anandhakumar, & Shanmugam, 2015).

The traditional extraction techniques require chemical treatments, high temperatures, and long reaction times and may have effects on the hydrocolloid products, resulting in reduced functional properties, due to degradation of the polysaccharides during extraction and yield losses. Alternative extraction methods, such as enzyme-assisted extraction, microwave-assisted extraction, and ultrasound-assisted extraction have been proposed and have been extensively reviewed recently by Khalil et al. (2018).

Agar

Like carrageenan, agar is composed of a linear backbone chain of galactopyranosyl moieties, but whereas the 3,6-anhydrogalactopyranosyl is in the D-configuration in carrageenans, it is in the L-configuration in agar (Fig. 1). Araki (1958) was the first to solve the primary structure of agar and determined that it consists of two fractions: agarose, having a neutral unmodified backbone built up by D-galactosyl and 3,6-anhydro-L-galactosyls, and a fraction which he termed agaropectin, which consists of D-galactosyls and L-galactosyls (Fig. 1). Modifications on agaropectin include sulfate, pyruvate and to a lesser extent various sugar molecules. Sulfation of C6 in the L-galactosyl residues is found in most agarophytes, but is particularly abundant in agar extracted from *Porphyra* species, hence the extracted agar from these species is referred to as porphyran. However, porphyran is considered the precursor of agar. Methylations on the O6 of D-galactopyranosyls of porphyran have been detected. Whereas the percentage of sulfate is normally high in carrageenans, agar normally has less than 12 % sulfate substitutions (Usov & Zelinsky, 2013).

Like for carrageenans, the chemical composition of agar varies depending on extraction technique, i.e. water extraction or combined alkali modification and extraction (Table 1). The proposed mechanism for alkali modification of agar is not readily available in the literature, but is presumed to follow the same principles and essentially be similar to that proposed for carrageenan modification (Ciancia et al., 1993): ionization that results in electrostatic repulsion to destabilize the 4C_1 chair conformation allowing anhydro-ring formation by nucleophilic displacement of the sulfate at position 6. Reductive acid hydrolysis of agar extracted with water at 90 °C for 45 min from *Gracilaria birdiae* showed that this agar contained around 65 % galactose, 25 % 3,6-anhydrogalactose and 8 % sulfate (Souza et al., 2012), but in another study, water extraction at 90 °C for 4 h gave agar derived from *Gracilaria gracilis* that contained 28 % galactose, 35 %

anhydrogalactose and only 4 % sulfate (Rodríguez, Matulewicz, Nosedá, Ducatti, & Leonardi, 2009). Water extraction (100 °C, 3 h) of agar from *Hydropuntia cornea* gave a 3,6-anhydrogalactose and sulfate content of 35 % and 6 %, respectively, while agar extracted from *Hydropuntia dentata* contained 18 % anhydrogalactose and 8 % sulfate (Pereira-Pacheco, Robledo, Rodríguez-Carvajal, & Freile-Pelegrín, 2007; Rhein-Knudsen, Ale, Ajalloueiian, Yu, et al., 2017). Applying alkali-modification of the porphyran blocks during extraction changed the composition of the *Hydropuntia dentata* agar to 35 % anhydrogalactose and 5 % sulfate (Rhein-Knudsen, Ale, Ajalloueiian, Yu, et al., 2017).

Alginate

Alginate from seaweeds represents a family of negatively charged unbranched polysaccharides built via 1,4-glycosidic linking of the two uronic acids, β -D-mannuronic (M) and α -L-guluronic acid (G), which are C5 epimers. The mannuronic acid residues form β -1,4 linkages to give blocks of mannuronic acids in a linear conformation, whereas the α -1,4 linkages formed by the guluronic acids provide a folded structure due to the introduction of a steric hindrance around the carboxyl groups (Fig. 3).

The uronic acids are arranged in segments of varying proportions of GG, MM, and MG-blocks (Fig. 3) that vary depending on the type, age, and life-stage of the macroalgae as a result of the way the alginate is synthesized in the algae (discussed below). The M/G ratios of alginates from different species of *Sargassum* have thus been reported to vary from 0.2-1.2 and alginate from e.g. *Sargassum fluitans* from Cuba and Florida have been reported to have widely different M/G ratios of 0.52 and 1.18, respectively, (Torres et al., 2007). *Padina* sp. from Ghana has been shown to contain alginate having high M/G ratios of 1.5 (Rhein-Knudsen, Ale, Ajalloueiian, & Meyer, 2017), but alginate from *Padina pavonica* collected in Egypt has been reported to contain higher amounts of guluronic than mannuronic acid (Pereira, Gheda, & Ribeiro-Claro, 2013).

Fig. 3 Stylized chemical structure of alginate composed of α -L-guluronic acid and β -D-mannuronic acid residues shown in stretches of GG-blocks, MM-blocks, and MG-blocks.

Extraction protocols for alginate differ significantly from those of carrageenan and agar, Table 1, as the alginate is bound in the cell wall as insoluble alginate salts, i.e. calcium or magnesium alginate. Current extraction techniques therefore rely on conversion of these insoluble alginates to soluble ones. Normally, the counter ions are removed using acid to form insoluble alginic acid, and the alginates are subsequently extracted as sodium alginates. In addition, formaldehyde addition is used to remove phenolics (Table 1). The sodium alginate is separated from the seaweed residuals by filtration or centrifugation and then recovered by precipitation in alcohol (Calumpong, Maypa, & Magbanua, 1999).

Seaweed hydrocolloid biosynthesis

Although the biosynthetic routes and detailed conversions involved in seaweed hydrocolloids synthesis are yet to be understood in detail, recent sequencing, gene annotation, and investigations of the enzymes involved in cell-wall polysaccharide synthesis in the red seaweed *Chondrus crispus* and the brown seaweed *Ectocarpus siliculosus* have cast important new light on the synthesis of hydrocolloid polysaccharides in red and brown macroalgae (Collén et al., 2013; Michel, Tonon, Scornet, Cock, & Kloareg, 2010).

Carrageenan biosynthesis

It has been proposed that three classes of enzymes are involved in carrageenan biosynthesis: 1) galactosyl transferases (α and β), catalyzing the polymerization of the galactose backbone; 2) sulfotransferases, adding sulfate substitutions to the newly synthesized galactose chains; 3) Gal-6-sulfurylases, sometimes referred to as sulfohydrolases, catalyzing the removal of sulfate at position 6 in the α -galactopyranosyl monomer and subsequent formation of the 3,6-anhydro bridge (Fig. 4). The first two reactions, i.e. galactose polymerization and sulfate substitution, have been shown to occur in the Golgi apparatus, though none of the related enzymes have been characterized. Only a few steps of carrageenan biosynthesis, namely those involving the key galactose-sulfurylase reactions, have at this point in time been demonstrated experimentally with the isolation and characterization of the carrageenan specific enzymes (Genicot-Joncour et al., 2009; Lipinska, Collén, Krueger-Hadfield, Mora, & Ficko-Blean, 2020). Sulfurylase activity, which is unique to red seaweeds, was first demonstrated by Wong & Craigie (1978), who characterized an enzyme from the red seaweed *C. crispus* responsible for the conversion of μ -carrageenan into κ -carrageenan (Wong & Craigie, 1978) (the first experimental data supporting the sulfurylase activity was already obtained by Rees et al. (1961), though with specific activity on porphyran, the precursor for agar (Rees, 1961)).

The presence of an enzyme catalyzing the conversion of ν -carrageenan to ι -carrageenan was then demonstrated in the red seaweed *Calliblepharis jubata* by Zinoun et al. (1997) (Zinoun, Diouris, Potin,

Floch, & Deslandes, 1997). Similarly, carrageenan sulfurylase activities have been demonstrated in *Betaphycus gelatinus* (Wang et al., 2014), *Eucheuma striatum* (Qin, Ma, Lou, & Wang, 2015), and *Gigartina stellata* (Lawson & Rees, 1970). Genicot-Joncour et al. (2009) purified two enzymes, sulfurylase I and sulfurylase II, from *C. crispus* that catalyze conversion of ν -carrageenan to ι -carrageenan (Genicot-Joncour et al., 2009). The enzymatic mechanism behind the conversion of galactopyranosyl 6-sulfate to 3,6-anhydrogalactopyranosyl monomers is still unknown. However, this conversion is proposed to occur through a nucleophilic substitution in which the C₆ sulfate is removed and replaced by the hydroxyl group at position 3 (C₃ OH) on the sulfated α -galactopyranosyl monomer, in a reaction similar to that which occurs during chemical modification of carrageenans using an alkali (Ciancia et al., 1993; Genicot-Joncour et al., 2009). The reaction by sulfurylases, i.e. the conversion of the sulfated galactopyranosyl monomers in the precursors, alters the hydrophobicity of the sugars and inverts the chair conformation from 4C_1 to 1C_4 , which consequently gives the carrageenan the ability to form gels by helix formation (Fig. 4) (Genicot-Joncour et al., 2009).

Fig. 4 Conversion of precursor μ -carrageenan into κ -carrageenan, a reaction catalyzed by sulfurylase enzymes.

Agar biosynthesis

The biosynthesis of agar in macroalgae is not understood in detail because the enzymes responsible for galactose polymerization and potential substitutions are yet to be characterized. Nevertheless, it is known that the proposed biosynthesis pathway begins with glycosylation of the alternating D- and L-galactose monomers in the Golgi apparatus, followed by potential sulfation by sulfotransferases, after which the polymers are transferred to the cell wall matrix for further modification of the precursor components by sulfurylases (Lee et al., 2017).

Sulfurylase activity, namely the modification of precursors by removal of sulfate and subsequent formation of an anhydro-bridge (illustrated for κ -carrageenan in Fig. 4), was demonstrated early by Rees et al. (1961)

on porphyrin, the biosynthetic precursor for agar, using an enzyme extract from the red seaweed *Porphyra umbilicalis* (Rees, 1961). Similar sulfurylase activity was more recently reported in *Gracilaria dura* (Shukla, Kumar, Prasad, Reddy, & Jha, 2011). The conversion of galactopyranosyl 6-sulfate to 3,6-anhydrogalactopyranosyl was verified *in vivo* in another porphyrin-containing red seaweed, *Gracilaria chilensis* (Hemmingson, Furneaux, & Wong, 1996).

Alginate biosynthesis

Alginate is known to be synthesized in brown seaweeds via initial polymerization of D-mannuronic acid residues in the Golgi apparatus (Michel et al., 2010). Guluronic acid monomers are then generated *in situ* in the mannuronate polymers by mannuronan C5-epimerase (EC 5.1.3.37) catalyzed C-5 epimerization of D-mannuronic acid into L-guluronic acid (Nyvall et al., 2003). Algal C5-epimerase genes encoding AlgG-type epimerases (discussed further below), have recently been identified in several brown seaweeds, i.e. *L. digitata*, *Saccharina japonica* and *E. siliculosus*, and recent work by Fischl et al. (2016) and Inoue et al. (2016) has included successful heterologous expression of mannuronan C5-epimerases from *E. siliculosus* and *S. japonica* in *E. coli* and insect cells, respectively (Fischl et al., 2016; Inoue et al., 2016). Yet, detailed knowledge of alginate biosynthesis in brown macroalgae is still somewhat limited. In contrast, the alginate synthesis pathway, including the multiprotein complex involved and the function of mannuronan C5-epimerases, is well characterized in alginate-producing bacteria, i.e. *Azotobacter vinelandii* and *Pseudomonas* spp., and has recently been eminently reviewed by Moradali, Ghods, & Rehm (2018).

Gel-formation

Carrageenans

The exact mechanism of carrageenan gelation, i.e. the intermolecular interactions and ionic effects, is still a matter of debate. However, it is generally accepted that the gelation process involves a coil-helix transition and double helix formation followed by aggregation which is promoted by specific ions (Fig. 5). This gelation model is called the “domain model” and was originally proposed by Morris et al. (1980) (Morris, Rees, & Robinson, 1980).

Carrageenans in solutions at high temperatures adopt random coil structures due to electrostatic repulsions between adjacent negatively charged chains. Upon cooling, the 3,6-anhydro-D-galactopyranosyl containing polymers change conformation to helix structures. Due to 1C_4 chair conformation, the 3,6-anhydro-galactopyranosyl units present in κ - and ι -carrageenan allow the carrageenan chains to form α -helices. Occurrence of stretches without 3,6-anhydrogalactopyranosyl units, i.e. the precursors μ - and ν -carrageenan having sulfated galactopyranosyl units in the 4C_1 conformation, causes “kinks” in the chains, as

273 it prevents helix formation and in consequence inhibits gelation (Van de Velde, Knutsen, Usov, Rollema, &
 274 Cerezo, 2002).

275

276 **Fig. 5** The gelation mechanism for κ -carrageenan in the presence of potassium ions.

277 Further cooling and presence of cations leads to aggregation of the helical carrageenan chains and
 278 consequently to the formation of a stable three dimensional network formed by the intermolecular
 279 interactions between adjacent carrageenan chains. The mechanisms which govern the conformational
 280 transitions are still highly debated and controversial. It is generally accepted that κ -carrageenans change
 281 conformation to a double-helical structure prior to gelation (Yuguchi, Thu Thuy, Urakawa, & Kajiware,
 282 2002). Helix formation, and the adherent gelation, probably occur because of intra- and intermolecular
 283 interaction associations, i.e. hydrogen bonds and van der Waals forces of attraction, between hydroxyl
 284 groups and potentially also the hemiacetal oxygen, as observed for other gelling polysaccharides, e.g.
 285 starch (Tako, Tamaki, Teruya, & Takeda, 2014). In an attempt to resolve the conformational transition for ι -
 286 carrageenan, Schefer et al. (2014) performed atomic force microscopy (AFM) imaging of ι -carrageenan.

Their results strongly support the conclusion that ι-carrageenan in salt solution consists of an intramolecular single-stranded helix and not a double-stranded one as previously claimed. However, possible intermolecular dimeric double helix formation upon increasing polymer concentration cannot be excluded, and a double helix has been proposed by earlier studies supported by X-ray differentiation and differential scanning calorimetry (Schefer, Adamcik, & Mezzenga, 2014).

The binding of ions plays an important role in the aggregation process because binding of ions decreases the effective charge density of the hydrocolloids and hence eases aggregation by decreasing electrostatic repulsion. Different molecular interactions between the carrageenan chains and the ions have been proposed. For example, Tako et al. (2014) described that large univalent cations such as K^+ participate in both intramolecular and intermolecular associations that occur via a combination of cation bridges between the cation and the sulfate on the galactopyranosyl 4-sulfate moieties and electrostatic forces between the cation and the ether-bound oxygen on the adjacent 3,6-anhydrogalactopyranosyl moieties (Tako et al., 2014), whereas others have speculated that the ions interact with two sulfate groups on opposing chains through ionic bonds (Tecante & Núñez, 2012). Nevertheless, due to the charge of the carrageenan chains, it is not surprising that the electrostatic repulsion is shielded by the ions and it can thus be assumed that the charged ions function to stabilize the junction zones between the helices without hindering aggregation.

The effects of different cations on carrageenan gelation have been extensively studied. Monovalent cations that induce κ-carrageenan gelation include K^+ , Rb^+ , Cs^+ and high amounts of Na^+ and Li^+ . Divalent cations such as Ca^{2+} , Ba^{2+} and Mg^{2+} likewise have the ability to induce gel formation. Divalent cations have been shown to increase the gel strength of κ-carrageenan better than monovalent cations, with the exception of potassium that probably binds specifically to the κ-carrageenans because K^+ are more effective at shielding the electrostatic repulsion (Thrimawithana, Young, Dunstan, & Alany, 2010). Likewise, cation-specific effects are observed for ι-carrageenan aggregation where the divalent ion Ca^{2+} shows a very strong helix-stabilizing effect. Calcium analogously cross-links the two ι-carrageenan helices, presumably by salt bridges. However, the charged sulfate esters on the other side of the monomer encourage an extended conformation via a repulsion effect of the negative sulfate groups and thus inhibit gelation while promoting viscosity in the solution (Wu & Imai, 2012). Ion-specificity seems to be related to the specific type of carrageenan and the possible interaction between these two components. It is suggested that the radius of the cation seems to be most important for potential interaction with κ-carrageenan, which forms the strongest gels with potassium, whereas the valence of the cations seems to have a higher effect for cation-dependent ι-carrageenan gelation (Rinaudo, Karimian, & Milas, 1979). Improvements in analytical

techniques over the years have resulted in many discoveries related to hydrocolloid gelation, but much remains to be determined in order to obtain a greater understanding of the molecular events of this process. Improved insight into the molecular details would ideally lead to a greater understanding of polymer conformation transition, and such knowledge could potentially be used for designing carrageenans with specific properties, for example by controlling polymer-cation ratio.

Agar

The physico-chemical properties of agar are outstanding amongst the seaweed hydrocolloids. The chemical structure of agar (i.e. specifically of agarose, which is purified agar devoid of agaropectin), basically consists of D-galactopyranosyl residues and 3,6-anhydro-L-galactopyranosyl monomers bound together by alternating α -1,3 and β -1,4 linkages (Fig. 1). This structural configuration allows the polysaccharide to form helices comparable with carrageenans (Usov & Zelinsky, 2013). It is alluring to assume that agar gelation likewise occurs through the aggregation of these helices, though without the help from ions. Tako and Nakamura (1988) have proposed a mechanism for α -helix formation of agar during gel formation in which the helix is stabilized by hydrogen bonds between the ether-bond and the C₂ OH in the anhydro-L-galactopyranosyl residues opposite one another. As for the carrageenans, the presence of its biological precursor porphyran, inhibits gel formation due to the lack of anhydrogalactopyranosyl residues. The aggregation of the agar helices (agarose chains) is more favorable than for carrageenans and relies entirely on intermolecular and intramolecular hydrogen bonding in a structural network which has been compared to a tetrahedral ice-like structure (Tako et al., 2014), giving agar the highest gel strength of the red seaweed hydrocolloids in pure water (Tako & Nakamura, 1988).

Alginate

Alginate gelation is normally described by the “egg-box” model originally proposed by Grant et al. in 1973. In the presence of divalent ions, mostly Ca²⁺, the ions can bind to the carboxyl groups in alginate and act as cross-linkers that stabilize the alginate chains by formation of a gel network (Fig. 6). The gel network is described as the “egg-box” because gelation predominantly involves GG-blocks into which the ions fit like eggs in a box (Grant, Morris, Rees, Smith, & Thom, 1973). Alginate gels can also be prepared with other ions and the affinity of alginate towards divalent ions decreases in the following order: Pb > Cu > Cd > Ba > Sr > Ca > Co, Ni, Zn > Mn (depending on charge and ionic radius); however, calcium is normally used for inducing alginate gelation (Mørch, Donati, Strand, & Skjåk-Bræk, 2006).

348
349 **Fig. 6** The gelation mechanism for alginate in the presence of calcium ions.

350 The calcium ions preferentially bind to the GG-blocks, as the structure of the GG-blocks allows a high
351 degree of coordination of the divalent ions. Binding of ions thus results in tightly held junctions between
352 aligned GG-blocks. The MG-blocks also participate in gel formation although these blocks form weaker
353 junctions and thus weaker gels. The relative amount of guluronate residues, more precisely the amount of
354 polyguluronic acid blocks (GG-blocks), and hence the M/G ratio thus has a profound impact on the gelation
355 properties of alginate: alginates with low M/G ratio generally produce firm and brittle gels, whereas
356 alginates with high M/G ratios form more elastic gels (Mørch et al., 2006).

357 Gel properties

358 Minor changes at the molecular level have a huge impact on the gelling properties of the seaweed
359 hydrocolloids, i.e. carrageenans with the same amount of precursor units may show different rheological

properties as a result of precursor distribution. In addition, molecular weight, lengths of chains, and ionic contents and strengths all have significant effects on the gelation abilities of these carbohydrates.

Carrageenans

Mtolera and Buriyo (2004) tested the seasonal variations of alkali modified carrageenans extracted from Tanzanian *Hypnea musciformis* and showed that gel strengths at 1 % w/v in 0.1 M KCl estimated at 25 °C ranged from 77-198 g/cm². Similar differences were observed in gelling and melting temperatures which ranged from 50-59 °C and 67-70 °C, respectively (Mtolera & Buriyo, 2004). These authors did not correlate the gelation properties with the composition of the carrageenans extracted in different seasons, but this has been done with carrageenans extracted from *Hypnea musciformis* in Ghana. Here, gelation properties were estimated using 1.5 % carrageenan with 1 % KCl, and gelling and melting temperatures were found to be ~36 °C and 60 °C, respectively. Gelling and melting temperatures were shown to increase with increasing amounts of 3,6-anhydrogalactose (Rhein-Knudsen, Ale, Ajallouei, Yu, et al., 2017). Similar results have been obtained for κ-carrageenan extracted from the commercially used *Kappaphycus alvarezii* and it was also shown that gel strength increases with increasing KCl concentration (Manuhara, Praseptianga, & Riyanto, 2016). Rheological analyses of κ- and ι-carrageenan gels with different ions have demonstrated that increasing counter ion concentration above a certain threshold has a negative effect on gel formation; this phenomenon is interpreted as indicative of a maximum packing or cross-linking density of the carrageenans (Thrimawithana et al., 2010).

Agar

Gelling and melting temperatures have been determined for agar extracted from *Gracilaria vermiculophylla* both by using water and by combined extraction and modification: Native agar exhibits gelling and melting temperatures at 22 °C and 60 °C, whereas modified agar gelled and melted at 37 °C and 98 °C, respectively (Arvizu-Higuera, Rodríguez-Montesinos, Murillo-Álvarez, Muñoz-Ochoa, & Hernández-Carmona, 2008). Similar differences were observed for gel strengths estimated at 22 °C, namely 72 g/cm² for the native agar and 1064 g/cm² for the alkali-modified agar owing to the differences in 3,6-anhydrogalactose contents (Arvizu-Higuera, Rodríguez-Montesinos, Murillo-Álvarez, Muñoz-Ochoa, & Hernández-Carmona, 2008). Along with the differences in 3,6-anhydrogalactose contents, the differences in macroscopic properties are due to differences in amounts of methyl substitutions and molecular weights. As for all the seaweed hydrocolloids, the higher the molecular weight, the greater the probability of forming a stable gel network through molecular interactions. A comparison of agar samples having varying amounts of methyl substitutions has shown that amount and position of methylations affect the gelation mechanism and

allows it to occur at different temperatures (Praiboon, Chirapart, Akakabe, Bhumibhamon, & Kajiware, 2006).

Alginate

The gelation properties of alginate are normally accessed by a methodology that relies on gel formation using CaCO_3 and D-glucono- δ -lactone to ensure a gradual calcium release (Draget, Østgaard, & Smidsrød, 1991). Using oscillatory rheology, Fernández Farrés and Norton (2014) made a comparison of the gelling profiles of two different alginates of different molecular size but with equivalent fractions of guluronic acids. The study showed that high molecular weight alginate fluid gels exhibited faster formation kinetics, enhanced viscosities, greater G' and more solid-like behavior than those produced with low molecular weight alginates (Fernández Farrés & Norton, 2014). Funami et al. (2009) made similar experiments on alginate samples of comparable molecular weights and different mannuronic and guluronic acid ratios, and observed that at low calcium concentrations mannuronic rich alginate produced higher G' values than guluronic acid rich alginates, whereas this result was inverted at high calcium concentrations. Hence, alginate gelation is not only dependent on calcium dose but also on the composition and sequence pattern of the alginate. In their study, the mannuronic rich alginate with an M/G ratio of 0.79 showed a G' value of 134.5 Pa at a CaCO_3 dose of 3.75 mM, whereas the guluronic rich alginate with an M/G ratio of 0.41 had a G' of 30.6 Pa - increasing the calcium dose to 15 mM gave G' values of 782.1 Pa and 1997.6 Pa, respectively (Funami et al., 2009). The data were obtained using 0.5 w/v % alginate and 15 mM D-glucono- δ -lactone at 20 °C for 6 h with an applied strain of 1Hz (Funami et al., 2009). In another study, using 1.5 w/v % alginate, 15 mM D-glucono- δ -lactone and 22.5 mM CaCO_3 , alginates extracted from different *Sargassum* samples and having M/G ratios of 0.47 and 0.70 showed G' values of 1.1 Pa and 35.4 Pa, respectively. In the same study, alginate with an M/G ratio of 1.85 from *Padina antillarum* gave a G' value of 376.3 Pa at the same reaction conditions. Due to the higher M/G ratio, it was assumed that this *P. antillarum* alginate would produce gels of lower strength than those with M/G ratios of 0.47 and 0.70 from *Sargassum* (Rhein-Knudsen, Ale, Ajallouei, & Meyer, 2017). Many *Sargassum* alginates have M/G ratios around or below one and high contents of GG-blocks but several studies report poor gelling properties of the *Sargassum* alginates (Mchugh, 2003).

Interactions with other components in food matrices

Interactions with proteins

In the food industry, carrageenans are used extensively in dairy products to adjust viscosity and in addition to the gelation mechanisms outlined above, the carrageenans also work by interaction with casein micelles.

The negatively charged carrageenans are believed to interact directly with κ -casein located at the surface of casein micelles. Both κ - and ι -carrageenans interact in this way with casein micelles, but the complexes differ in texture and microstructure (Arltoft, Ipsen, Madsen, & de Vries, 2007). Alginate is also widely used to reduce fat in different types of restructured meat products because of its emulsion-stabilizing and thickening properties. In model systems with bovine serum albumin, the intermolecular interactions between alginates and proteins have been shown to result in formation of an alginate:bovine serum albumin complex. The complex formation is a result of electrostatic forces between the positively charged stretches in the protein and the negatively charged carboxylic groups of the alginate (Zhao, Li, Carvajal, & Harris, 2009). In complex meat systems, alginate also interacts with proteins. Recently, addition of alginate to frankfurters to partially substitute 25-50% of pork fat and thus decrease overall fat content, was shown to retain cooking yield, hardness, and sensory perception of juiciness (Kang, Wang, Li, Li, & Ma, 2020). Yet, the more detailed assessment of the molecular interactions between the alginate and meat proteins in the frankfurter matrix showed that alginate induced secondary and tertiary structural changes in the meat proteins; the changes included increases in β -sheet, β -turn and random coil structures and were interpreted as being due to hydrophobic interactions and hydrogen-bond formation between the alginate and certain amino acids in the proteins such as tyrosine and tryptophane residues (Kang et al., 2020).

Interactions with aroma compounds and effect on flavour release

Although the main effect and intent of hydrocolloid addition to foods is to modify viscosity and gelation, it is known that the addition may also affect – usually decrease – the perceived flavor (Paravisini and Guichard, 2017). In addition to sensory assessment, direct effects of hydrocolloids additives on aroma compounds and flavour release is objectively measured as a mass transfer or phase partitioning coefficient; this coefficient is expressed as the ratio between the flavor compound concentration in the air phase above the product versus the corresponding flavour concentration in the product at equilibrium (Bylaite, Ilgunaite, Meyer, & Adler-Nissen, 2004). In aqueous systems, addition of ι -carrageenan appears to have a very limited effect on flavour release (Chana, Tromelin, Andriot, & Guichard, 2006). In contrast, a study involving systematic series of volatile, aromatic compounds in λ -carrageenan model solutions has shown that λ -carrageenan slightly suppresses release rates and partitioning coefficients of the most volatile aroma compounds – however, neither viscosity or carrageenan concentration (from 0.1-0.5%) affect the aroma compound partitioning or release rates (Bylaite et al., 2004). The latter result indicates that even if λ -carrageenan addition profoundly increased the macroscopic viscosity, the water mobility and air-water partitioning of volatile aroma compounds essentially remain unchanged.

453 New enzymes that modify seaweed hydrocolloids

454 Enzymatic and microbial carrageenan-modification

455 Carrageenases

456 Carrageenases are glycoside hydrolases that cleave the β -1,4 linkages in the carrageenan backbone to
 457 produce carrageenan-oligos. These enzymes are named according to their substrate specificity, i.e. κ -
 458 carrageenases (EC 3.2.1.83), ι -carrageenases (EC 3.2.1.157), and λ -carrageenases (EC 3.2.1.162). Based on
 459 their amino acid sequences they belong to distinct glycoside hydrolase (GH) families, specifically GH16 (κ -
 460 carrageenases), GH82 (mainly ι -carrageenases), GH150 (λ -carrageenases), and exo-acting carrageenases
 461 classified into GH2, GH127, GH129 (Hettle et al., 2019), and in the recently created family GH167. The
 462 GH167 family has in fact been generated based on the recent discovery of κ/ι -carrageenan specific enzymes
 463 encoded by a carrageenan-specific polysaccharide utilization locus in four *Pseudoalteromonas* species
 464 isolated from red seaweeds (Hettle et al., 2019; Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat,
 465 2014).

466 When it comes to the seaweeds, no carrageenases have been predicted from genome sequence analysis of
 467 the carrageenan-containing red seaweed *C. crispus* (Collén et al., 2013). However, the key enzymatic
 468 conversion steps in the pathway for carrageenan biosynthesis have recently been outlined for *C. crispus*
 469 (red macroalga) (Lipinska et al., 2020). The enzyme battery involved in the carrageenan biosynthesis
 470 includes several galactose-sulfurylases, carbohydrate-sulfotransferases, glycosyltransferases, and *C. crispus*
 471 moreover harbors three genes that appear to encode GH16 hydrolases. However, the GH16 enzymes from
 472 *C. crispus* categorize in the same clade as bacterial agarases, porphyranases, and some other enzymes from
 473 the red algae *Porphyra umbilicalis*; nevertheless, the GH16 hydrolases encoded by *C. crispus* are suggested
 474 involved in remodeling of carrageenan since agar and porphyrin are not present in *C. crispus* (Lipinska et al.,
 475 2020).

476 According to the CAZY.org-database currently known carrageenases thus mainly come from marine
 477 bacteria such as *Flavobacteriaceae*, *Bacteroidites*, and γ -*Proteobacteria*. Explorative screening studies
 478 indicate that enzymatic carrageenan hydrolytic activity is widespread in marine bacteria, notably in
 479 *Bacteroidites* and *Proteobacteria*, isolated from red algae (Kalitnik, Nedashkovskaya, Stenkova, Yermak, &
 480 Kukhlevskiy, 2018).

481 Recently, the genome of the marine bacterium *Colwellia echini* A3^T (isolated from the intestine of a sea
 482 urchin collected in Øresund, Denmark) was shown to harbor two gene clusters (polysaccharide utilization
 483 loci), for degradation of agar and carrageenan, respectively (Christiansen et al., 2020). Notably, the

carrageenolytic gene cluster was reported to encode all the enzymes required for complete degradation of κ -carrageenan and ι -carrageenan as well as furcelleran (Christiansen et al., 2020). Furcelleran is a mixed type of high gelling κ -carrageenan, also used to form gels in pharma and food applications (E number E407). Despite these recent important findings, the GH82 ι -carrageenases from *Alteromonas fortis*, *Cellulophaga* sp., *Microbulbifer thermotolerans*, and *Zobellia galactanivorans* DsijT remain the most studied, especially with regard to their potential for generating carrageenan derived oligomers. Various strains of the marine bacterium *Wenyngzhuangia*, including *W. fucanolytica* and *W. aestuarii* (OF219) are also listed in the CAZY.org-database as encoding endo-acting GH16 κ -carrageenases. For example, a κ -carrageenase from *W. aestuarii* (OF219) has recently been cloned and reported suitable for production of κ -carrageenan oligomers (Shen, Chang, Chen, & Dong, 2018). The production of oligo-carrageenans by enzymes offers advantages over production by acid hydrolysis because of the specificity provided by the enzymes. As mentioned above, there has been controversy over the safety of carrageenans in the past and according to David et al. (2018) mainly three issues still remain partly unsettled: i) there are only limited data on daily intake of carrageenans and a recommended (acceptable) daily dietary intake has not been specified; ii) the translation of data from animal studies to humans has been problematic because carrageenan levels applied in animal models have usually exceeded those used in food products; iii) Recent human studies are based on interventions on only few persons producing only marginal (or no) changes between groups or between onset and final values, making the conclusions uncertain: For example, one human study with 12 ulcerative colitis patients showed that three of the patients who received carrageenan in capsules relapsed, whereas none of the patients who received placebo-containing capsules relapsed (Battacharyya et al., 2017); in another recent study involving 13 prediabetic persons, 8 of the participants followed a carrageenan free diet for 12 weeks, and the remaining 5 participants followed a comparable carrageenan containing diet (carrageenan intake of ca. 250 mg/day). The results indicated that a carrageenan-free diet gave improved glucose tolerance, but did not identify changes in the participants on the carrageenan-containing diet (Feferman, Bharracharyya, Oates, Haggerty, Wang, Varady, & Tobacman, 2020). Although more research on the digestive fate of carrageenans and oligo-carrageenans is indeed warranted, carrageenans are currently fully approved and widely used as food additives. In 2015 the World Health Organization expert committee on Food Additives (JECFA) furthermore reinstated that use of up to 1 g/L of carrageenan even in infant formula is safe and not of concern (David et al., 2018).

To understand the molecular basis of carrageenan degradation, Barbeyron et al. (1994) characterized a κ -carrageenase, the CgkA from the marine bacterium *Pseudoalteromonas carrageenovora* from which several hydrolases involved in the degradation of carrageenan have been purified. The results obtained represent the first sequence determination of a carrageenase. Their deduced protein sequence presented significant

sequence similarities with various β -1,3-1,4-glucanases and CgkA was therefore designated as a new member of the GH16 family (Barbeyron, Henrissat, & Kloareg, 1994). The κ -carrageenase was shown to be endo-hydrolase operating with a molecular mechanism retaining the anomeric configuration when it produces oligo-carrageenans with a degree of polymerization (DP) of 2 and 4 (Potin et al., 1995). A recent report on cloning and characterization of a κ -carrageenase from another *P. carrageenovora* strain (*P. carrageenovora* ASY5) confirms that the main DP2 and DP4 hydrolysis products of this κ -carrageenase action consist of repeating units of β -D-galactopyranose-4-O-sulfate and 3,6-anhydrogalactopyranose moieties (Xiao, Zeng, Li, Zhu, Xiao, & Ni, 2018).

The first crystal structure of a carrageenase was deduced by Michel et al. (2001) who showed that the CgkA from *P. carrageenovora* folds into a curved β -sandwich formed by two closely packed and anti-parallel β -sheets creating a tunnel-shaped active site, which further suggests a processive mode of action. The κ -carrageenan chain is composed of alternating neutral and negatively charged sugars. To accommodate the dual nature of its substrate, CgkA in its active site features both conserved aromatic and basic residues which are predicted to interact with the 3,6-anhydrogalactosyls and galactosyl-4-sulfate moieties, respectively (Michel, Chantalat, Duee, et al., 2001). The same structural features have been observed for the ZgCgkA κ -carrageenase from *Z. galactanivorans*, which was shown to have additional elements such as a CBM and a distinct tunnel topology that resulted in an opposite direction of processivity (Matard-Mann et al., 2017).

Barbeyron et al. (2000) also characterized the first ι -carrageenases from *Z. galactanovorans* and *Alteromonas fortis* and showed that ι -carrageenases, like κ -carrageenases, have an endolytic mode of action but unlike κ -carrageenases, these ι -carrageenases were shown to be inverting hydrolases. The two ι -carrageenases both release oligo-carrageenans DP2 and DP4 as their end-product (Barbeyron, Michel, Potin, Henrissat, & Kloareg, 2000). The three-dimensional structures of the ι -carrageenase (CgiA_Af) from *A. fortis* was later resolved, and it was revealed that the enzyme folds into a right-handed parallel β -helix flanked by two domains, A and B, where A upon binding to substrate shifts toward the β -helix groove to form a tunnel consistent also with the processive behavior of ι -carrageenases. ι -Carrageenan, which consists of only negatively charged sugars, is recognized by CgiA essentially through ionic interactions between its sulfate groups and several conserved arginine residues of the protein (Michel, Chantalat, Fanchon, et al., 2001).

λ -carrageenase activity has also been identified from the marine bacterium *P. carrageeronovora*. Guibet et al. (2007) cloned and characterized the first λ -carrageenase producing oligo-carrageenans DP2 to DP8. Sequence analysis revealed that this λ -carrageenase, referred to as CglA, is the first member of a new

family of GHs which is unrelated to families GH16 and GH82 that contain the κ - and ι -carrageenases, respectively. CglA was demonstrated to proceed according to an endolytic mode of action and a mechanism of inversion of the anomeric configuration. Although the catalytic domain structure of λ -carrageenase remains to be resolved it is likely that this enzyme has a modular architecture with at least two domains connected by a linker, including a β -propeller domain at the N-terminus (Guibet et al., 2007). Apart from representing important scientific insight, this knowledge has important implications for understanding how microbial enzymes may be employed to modify carrageenans for tailor-made physical functionalities.

Sulfatases

The carrageenans are heavily sulfated and, as explained above, these negatively charged ions have effects on the gelation properties of the individual polysaccharides. Re-arrangement or removal of sulfates offers another alternative for modulating the gelation properties of the hydrocolloids. It also offers the potential for production of neutral mono- and oligosaccharides which can be used for energy production for example.

Sulfatases are the enzymes responsible for the removal of sulfates. Compared to the relatively high number of described carrageenases, only a few carrageenan sulfatases have been characterized and, as with the carrageenases, these are all of marine bacterial origin. Sulfates are grouped into three classes based on sequence homology, structure, and mechanism where Type I sulfatases, also known as formylglycine-dependent sulfatases, represent the majority of known sulfatases. These sulfatases require a post-translational conversion of cysteine or serine into a formylglycine amino acid residue that is essential for catalysis, and this conversion is performed by a formylglycine generating enzyme. Other sulfatases include Type II, Fe(II) α -ketoglutarate-dependent alkylsulfatases and Type III, the Zn^{2+} or Mn^{2+} -dependent metallo- β -lactamases (Barbeyron et al., 2016).

All characterized carrageenan sulfatases with one exception have been identified as being formylglycine-dependent sulfatases. The formylglycine-dependent sulfatases adopt a similar fold comprising two (α/β) domains, a large N-terminal domain containing the catalytic pocket, and a smaller C-terminal domain. The active site encompasses the catalytic nucleophile formylglycine and nine additional conserved residues, four acidic/polar residues coordinating a calcium ion which binds and activates the sulfate group of the substrate, and five basic amino acids stabilizing the formylglycine and/or binding the sulfate group (Barbeyron et al., 2016). Two mechanisms for sulfate removal by formyl-glycine dependent sulfatases have been proposed, which differ in the hydration state of the formylglycine. However, both propositions agree that the last step of the reaction involves covalent binding of the formylglycine modified amino acid and

581 sulfate followed by regeneration of formylglycine by hydrolysis, which results in an overall reaction in which
 582 the retention of the carbon is retained (Williams, Denehy, & Krenske, 2014).

583 The first sulfatase targeting seaweed hydrocolloids was carrageenan sulfatase which was discovered by
 584 Weighl and Yaphe (1966) from a protein extract from the marine bacterium *P. carrageenovora* (Weigl &
 585 Yaphe, 1966). This carrageenan sulfatase, called 4-O- κ -carrabiose sulfatase, was shown to have an exo-type
 586 mode of action that removes the sulfate ester at the non-reducing end of oligo- κ -carrageenans (McLean &
 587 Williamson, 1979). Recently, Préchoux et al. (2013) recombinantly expressed a 4S-iota carrageenan
 588 sulfatase purified from *Pseudoalteromonas atlantica* T6c which converts ι -carrabiose into α -carrabiose
 589 repeating units by removing the sulfate ester at position 4 of the D-galactosyl residues through an endo
 590 mode of action. Sequence alignments confirmed that this sulfatase belongs to the Type I formylglycine-
 591 dependent sulfatases, characterized by a cysteine included in a signature sequence of 12 amino acids.
 592 Cloning experiments resulted in enzymes having lower activity than the native protein, which suggests that
 593 only a small fraction of the cysteine or only a low fraction of the overexpressed protein had been
 594 matured in the overexpressed protein. Co-expression of a formylglycine-generating enzyme may be used
 595 to address these issues. κ -carrageenan, which is also composed of galactose-4-sulfate, was not desulfated
 596 by this sulfatase, suggesting that the residue adjacent to galactose- 4-sulfate is important for desulfation
 597 (Préchoux, Genicot, Rogniaux, & Helbert, 2013). Similarly, a formylglycine-dependent endo-4-O- κ -
 598 carrageenan sulfatase identified from *P. atlantica* T6c yielded β -carrabiose repetition moieties (Préchoux,
 599 Genicot, Rogniaux, & Helbert, 2016).

600 Genicot et al. (2014) identified another ι -carrageenan sulfatase that also catalyzes the removal of the 4O
 601 sulfate. However, despite the finding that this enzyme has the same specificity as the ι -carrageenan
 602 sulfatase from *P. atlantica*, sequence analysis showed no sequence homology between this enzyme and the
 603 *P. atlantica* sulfatase nor with other known sulfatases, which indicates that this sulfatase could be the first
 604 characterized member of a new sulfatase family (Genicot, Rogniaux, Meslet-Cladière, Barbeyron, & Helbert,
 605 2014). A recent crystal structure of an endo-4S- ι -carrageenan sulfatase from *Pseudoalteromas* sp. PS47
 606 indicated that the sulfate group of the D-galactosyl-2-sulfate is important in recognition, which is also
 607 supported by the specificity provided by the identified sulfatases (Hettle et al., 2018). Recently, the
 608 carrageenan catabolism has been solved by Ficko-Blean et al. (2017) and a formylglycine-dependent ι -
 609 carrageenan sulfatase, the *cgsC* from *Zobellia galactanivorans*, has been discovered. This sulfatase is
 610 specific for the α -carrabiose motifs and removes the 2- linked sulfate group from the 3,6-anhydro-
 611 galactosyls to generate β -carrabiose motifs (Ficko-Blean et al., 2017). Several other putative sulfatases have

been predicted through various genome sequencing programs but the precise functions and substrate specificity remain to be determined by experimental data.

Sulfurylases

The use of sulfurylases, the enzymes involved in the biosynthesis of carrageenans, could be an alternative to alkali treatment in the production of carrageenans. Such enzyme treatments could be used to produce carrageenan products with enhanced gelling properties for high-value uses, such as in pharma as tablet coatings, in drug delivery, and to substitute animal fats in new products and functional foods. In red seaweeds *in vivo* the conversion of sulfated galactose moieties into anhydrogalactose is catalyzed by galactose-6-sulfurylases, as described above. Sulfurylase activity has been demonstrated from various seaweed extracts (Hemmingson et al., 1996; Rees, 1961; Wong & Craigie, 1978) and Genicot-Joncour et al. (2009) have purified two enzymes, sulfurylase I and sulfurylase II, from the red seaweed *Chondrus crispus*, and shown that they each catalyze the conversion of ν -carrageenan to ι -carrageenan. Notably, sulfurylase II was shown to release sulfate during incubation with carrageenans, and this release was correlated with an increase in 3,6-anhydrogalactosyl content and viscosity increase (Genicot-Joncour et al., 2009). Subsequently, using sequence analysis (Colln et al., 2013), the genome of *C. crispus* was shown to contain 11 genes encoding putative galactose-6-sulfurylases, including the sulfurylase I and II characterized by Genicot-Joncour et al. (2009). The functions of these genes have not yet been validated experimentally due to difficulties in obtaining recombinant sulfurylases. Similarly, Wang et al. (2014) have identified a sulfurylase that converts μ -carrageenan into κ -carrageenan from the marine algae *Betaphycus gelatinus*, but to the best of our knowledge this enzyme has not yet been produced recombinantly (Wang et al., 2014).

Enzymatic modification of agar

Agarases

Agarases are classified into α -agarase (E.C. 3.2.1.158) and β -agarase (E.C. 3.2.1.81) according to their cleavage pattern, yet both belong to family GH16. α -agarase catalyzes cleavage of the α -1,3 linkages to produce agaro-oligosaccharides, while β -agarases cleave β -1,4 linkages to produce neoagaro-oligosaccharides. Agarases have been isolated from many different genera of marine bacteria, marine sediments, and other environments.

Two α -agarases, one each from *Alteromonas agarlytica* GGJ1B and *Thalassomonas* sp. JAMB-A33, have been identified and shown to be endo-type agarases with DP4's as their main end-products (Ohta et al., 2005; Potin, Richard, Rochas, & Kloareg, 1993). The α -agarase from *A. agarlytica* (AgaA) has been cloned and sequenced and shown to be unrelated to β -agarases (and also carrageenases) and is classified as a

GH96 family glycoside hydrolases in the CAZy-database. The AgaA33 α -agarase from *Thalassomonas* sp. JAMB-A33 (now renamed to *Thalassotalea agarivorans* JAMBA33) has similarly been sequenced and produced recombinantly and also shown to be part of the GH96 and to hydrolyse both the α -1,3 linkages in agarose and in porphyran (Hatada, Ohta, & Horikoshi, 2006). α -neoagarobiose hydrolases responsible for the production of monomers produced from DP2s have been identified from *S. degradans*, *Z. galactanivorans* and *B. plebius* and belong to family GH117 (Fu & Kim, 2010).

Several β -agarases have been reported that cleave the internal β -1,4 linkages in agar to result in DP2s to DP8s as end products. However, most of the reported endolytic β -agarases belong to the GH16 family and like the κ -carrageenases, including the bacterial β -agarases from *Aquimarina agarilytica* ZC1, *Cellulphaga omnivescoria* W5C, *Flammeovirga* sp., *Z. galactanivorans*, *Pseudomonas* sp. W7 and *Vibrio* sp. PO-303, yield DP4s as their main products, although some neoagarohexaose was also obtained as end product in several cases (product analysis often done by TLC only) (Chen, Lin, Jin, Zeng, & Lin, 2019; Fu & Kim, 2010; Lin, Liu, Lu, Zhao, & Hu, 2017; Ramos, Valdehuesa, Nisola, Le, & Chung, 2018). The same appears to be the case for the β -agarase cloned from environmental DNA obtained from mangrove sediments (Di, Qu, & Zeng, 2018).

The conserved domain of agarases in the GH16 family has been well studied. In the characterized GH16 enzymes, the location of the GH16 module is directly adjacent to the signal peptide and the CBM6 module is in the C-terminal (Dong, Tamaru, & Araki, 2007). The crystal structure of the catalytic domain of a GH16 β -agarase from the marine bacterium *Microbulbifer thermotolerans* JAMB-A94 shows a β -jelly roll composed of two antiparallel β -sheets, with open and extended substrate binding clefts, which proceeds with a molecular mechanism leading to overall retention of the anomeric configuration, similar to that reported for β -agarase from *Z. galactanivorans*, and the GH16 κ -carrageenases (Takagi et al., 2015). The GH16 β -agarase PdAgaC from the marine bacterium *Persicobacter* sp. CCB-QB2 is one of only a handful of endo-acting GH16 β -agarases able to degrade agar completely to produce DP2s. The crystal structure of the PdAgaC reveals that the catalytic domain is made up of a typical β -jelly roll fold with two additional insertions and a well-conserved but wider substrate-binding cleft with some minor changes in comparison to the *Z. galactanivorans* β -agarases (Hafizah, Teh, & Furusawa, 2019). Other GH16 β -agarases that produce DP2s include rAgaA from *Vibrio* sp. PO303 and rAgaB from *Z. galactanivorans* (Fu & Kim, 2010). *Agarivorans albus* is also listed in the CAZY-database as the microbial origin of a GH16 β -agarase. An exo- β -agarase from *Vibrio* sp. JT0107 belonging to the GH50 family was characterized that can degrade not only agarose but also DP4s to DP2s (Sugano, Matsumoto, Kodama, & Noma, 1993). Another β -agarase belonging to the GH50 family is the β -agarase from *Agarivorans* sp. JA-1, showing 98.8 % sequence similarity to the former, and also this β -agarase produces DP2s (D. G. Lee et al., 2006). A crystal structure of a GH50 β -

agarase, the Aga50D from the marine microbe *Saccharophagus degradans*, elaborated by Pluvinae et al. (2013), reveals a $(\beta/\alpha)_8$ -barrel fold that is elaborated with a β -sandwich domain. Enzyme-substrate complexes suggested a catalytic machinery that is most consistent with a retaining catalytic mechanism and a tunnel-shaped active site, suggesting an exo-processive mechanism (Pluvinae, Hehemann, & Boraston, 2013). The first characterization and sequencing of family GH86 β -agarases was the AgaO from *Microbulbifer*-like JAMB-A94, which was characterized to be an endo-type β -agarase degrading agarose and oligo-agarans to DP6s. Likewise, a β -agarase from *Vibrio* sp. PO-303 has been identified and shown to produce DP8's as its main product. Other GH86 β -agarases include the Aga86C and Aga86E from *Saccharophagus degradans* 2-40, but to the extent of our knowledge these agarases have been identified only by genome analysis (Fu & Kim, 2010). Lastly, a β -agarase derived from *Pseudoalteromonas* sp. CY24 has been cloned and characterized. Analyses show that this enzyme degrades agar to produce DP8s and DP10s with an inversion of the anomeric configuration, in contrast to other known agarases that are retaining enzymes. The agarase from *Pseudoalteromonas* sp. CY24 have no known sequence similarity with any known proteins (Ma et al., 2007).

Sulfatases

Compared to carrageenans, agar normally has very few or no sulfate ester modifications such as are normally present on agaropectin or porphyran, the precursors of agar (Usov & Zelinsky, 2013). Enzymatic hydrolysis of the sulfate groups in agaropectin (Fig. 1) could be used to simplify the process of agarose preparation because incorporation of sulfates in agarose causes weakened gel strength as a result of avoidance of cross-links during gel-formation.

Sulfatases responsible for sulfate removal in agar originating from *Marinomonas* sp. FW-1, *P. carrageenovora*, *Thermotoga maritima* and *Sphingomonas* sp. AS6330 have for example been shown to be able to release sulfates following incubation on agar, and the sulfatases from *Marinomonas*, *P. carrageenovora* and *Sphingomonas* have even been shown to double the gel strength of agar following enzymatic reaction (Byun et al., 2004; Lim et al., 2004; Wang, Duan, & Fu, 2016). The agar sulfatases in the literature are normally referred to as arylsulfatases, which refers to action on 4-methylumbelliferyl sulfate or on the aromatic sulfate ester p-nitrophenyl sulfate that are used as standard substrates for activity detection, though the described enzymes are still Type I sulfurylases. The *P. carrageenovora* arylsulfatase (EC 3.1.6.1) displays homology to type III sulfurylases of the metallo- β -lactamase superfamily, though (Barbeyron et al., 2016). Since selective enzymatic desulfation with arylsulfatase can improve agar gelation, notably the arylsulfatase from *P. carrageenovora* has been investigated recently: The enzyme has been recombinantly expressed in *E. coli* and engineered by single amino acid substitution to exhibit improved

thermostability (using error-prone PCR) (Zhu, Yin, Liu, Li, Chen, Li, Xiao, & Ni, 2019). Another mutant of the enzyme was found to have 33% improved enzyme activity at 55°C, pH 8 (Zhu, Liang, Li, Ni, Li, Li, & Jiang, 2020). Sulfatase activities from several other bacteria have been observed but no information is available on the mechanism and substrate specificity of the agar-sulfatases, but sequencing programs have lately revealed new hydrocolloid sulfatases which may have potential to drive future functional characterization studies.

Enzymatic modifications of alginate

Alginate Lyases

Alginate oligosaccharides, produced by alginate lyase reaction on alginate, have long been known to promote plant growth and exhibit bioactivity as antitumor agents (Wong, Preston, & Schiller, 2000). More recently, interest in using brown macroalgae for bioenergy and biorefining processes has sparked renewed interest in alginate lyase discovery and characterization.

Alginate lyases (EC 4.2.2.-) catalyze depolymerization of alginates via a β -elimination reaction and are divided into G-block specific (EC 4.2.2.11) and M-block specific (EC 4.2.2.3) enzymes depending on the reaction they catalyze, though several of the alginate lyases appear to have activity towards both G- and M-blocks in alginate, albeit acting with higher rate on either poly-mannuronate or poly-guluronate (Manns et al., 2016). Alginate lyases are produced by different bacteria, fungi, and seaweeds, and categorized in many different polysaccharide lyase families, PL5, PL6, PL7, PL14, PL15, PL17, and PL18. Substrate specificities and modes of action of the alginate lyases vary between families and family diversity implies that alginate lyases are structurally diverse. Most bacterial endolytic alginate lyases belong to family PL5 or PL7, while the most exolytic alginate lyases are assigned to family PL15 or PL17. The lyases isolated from marine mollusks and viruses are grouped in the PL14 family (Zhu & Yin, 2015). The polysaccharide lyase families PL5, PL15, and PL17 have been found to have an $(\alpha/\alpha)_n$ toroid fold structure, PL6 has a β -helix fold structure, while PL7, PL14 and PL18 are folded as β -jelly rolls and have been extensively reviewed by Garron and Cygler (2014). Most examples of characterized enzymes come from family PL7, where G-, M-, and MG-specific alginate lyases have been reported from various different organisms (Garron & Cygler, 2014).

Recently, we discovered that the marine fungus *Paradendryphiella salina* encodes three putative PL7 alginate lyases, and that one of these enzymes has particular affinity for M-block degradation (Pilgaard et al., 2019). An endo-lytic PL7 alginate lyase with similar polymannuronate preference has also been identified from *Flavobacterium* sp. albeit this enzyme also had moderate activity towards polyguluronic acid (Inoue et al., 2014; Manns et al., 2016). Another PL7 alginate lyase from a *Flavobacterium* sp. with

preferred activity on polyguluronate over polymannuronate has recently been characterized: the alginate degradation products obtained with this enzyme were DP5s, DP6s, and DP7s (Huang et al., 2013). *Sphingomonas* species A1 encodes several alginate lyases, A1-I, A1-II, A1-III, and A1-IV, with the A1-III being the first PL5 alginate lyase with a resolved structure. This PL5 A1-III alginate lyase acts primarily on polymannuronic acids with DP4s as the minimum substrate and produces DP2s and DP3s and also catalyzes depolymerization of acetylated alginates produced by bacteria. In contrast, the *Sphingomonas* derived PL7 A1-II enzyme has higher propensity for poly-guluronate than for poly-mannuronate (Manns et al., 2016). The AlyGC, a PL6 alginate lyase from the marine bacterium *Glaciecola chathamensis* has been characterized and also shows preference towards polyguluronic-acid blocks in alginate (Xu et al., 2017). The alginate lyase vAL-1 from *Chorella* virus shows two pH-dependent modes of action, endo and exo, to release DP6s and DP2s at pH 7 and 10, respectively (Ogura et al., 2009). The *Agrobacterium tumefaciens* alginate lyase Atu3025, is categorized as a PL15 lyase and acts on alginate polysaccharides and oligosaccharides exolytically as does the PL17 family Alg17C from *Saccharophagus degradans* 2-40 (Ochiai, Yamasaki, Mikami, Hashimoto, & Murata, 2010; Park, Jagtap, & Nair, 2014). Finally, a bifunctional family PL18 alginate lyase from *Pseudomalteromonas elyakovi* has been characterized, which produces DP3s to DP8s on alginate (Ma, Chi, Li, & Wu, 2008).

C5 Epimerases

As early as in 1969, Haug et Larsen described the first bacterial mannuronan C5-epimerase, an AlgE-type epimerase from *A. vinelandii*, which attracted attention due to its ability to increase the guluronic acid content in alginates *in vitro* (Haug & Larsen, 1969). Mannuronan C-5 epimerases can thus be used to control the composition and distribution of mannuronic and guluronic acids along the alginate chain and thus be applied to produce alginates with higher gel strength or with gel strength designed to specific applications. Several AlgE-type C5-epimerases have since been identified and characterized and shown to introduce different guluronic acid distribution patterns and be expressed differentially during the cell life-cycle (Høidal, Svanem, Gimmestad, & Valla, 2000). AlgG-Type epimerase is the other type of epimerase which has been identified in bacteria, and also the ones that are related to the bacterial AlgG-type epimerase. This epimerase is periplasmic and encoded in the alginate biosynthesis gene cluster in bacteria and results in secretion of alginates with some guluronic acid residues (Michel et al., 2010; Nyvall et al., 2003).

Conclusion and Future Trends

Recent insight into the *in vivo* biosynthesis and maturation of seaweed hydrocolloid carbohydrates combined with new discoveries of microbial enzymes that catalyze molecular changes and

depolymerisation of these polysaccharides, have provided a new foundation for targeted enzymatic tailoring of seaweed hydrocolloid functionality. These options may pave the way for new tailor-made functional hydrocolloids in functional foods and in new nutritional applications, and expand the use of hydrocolloids in low-fat and animal/meat-free products. We also anticipate that introduction of post-harvest enzymatic modification of the hydrocolloids can help develop and commercially viable biorefineries using a wider range of seaweeds.

One aspect of the seaweed industry, which has not been covered in this review, is the possible use of seaweed hydrocolloids in non-food applications, e.g. in various polymer composites. A range of such potential applications have been proposed in the literature such as use of κ -carrageenan in nano-composite cathodes in microbial fuel cells, agar in optoelectronic materials, and alginate in various packaging and polymer-composites, reviewed by Jumaidin et al. (2018). Likewise, for the sake of completion it should also be mentioned that only a limited proportion of the seaweed residues resulting from the hydrocolloid extraction are utilized, e.g. as organic fertilizers. However, significant research efforts are currently invested in exploring the properties and the potential of seaweed extraction residues for various non-food uses, e.g. carrageenan waste may be useful as renewable filler in eco-friendly materials (Jumaidin et al., 2017). Indeed, an expanded, advanced utilization of seaweed hydrocolloids in food, biotech, and pharma applications, including use of enzymes for functional optimization, may drive the development of new sustainable processes and products based on seaweeds.

CrediT author statement

NRK and ASM: Conceptualization. NRK: Original draft preparation. NRK: Visualization. ASM: Supervision. NRK and ASM: Writing, Reviewing, Editing.

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Highlights

- Chemical structures of seaweed-derived hydrocolloids carrageenans, agar, alginate
- Illustrated gelation mechanisms of seaweed-derived hydrocolloids
- Update of biosynthesis of cell wall hydrocolloid carbohydrates in seaweeds
- New microbial enzymes that catalyze modifications of carrageenans, agar, alginate