Carrageenan biotechnology

Gerhard A. De Ruiter and Brian Rudolph

In this review, the various aspects of the structure of different carrageenans, water-soluble, sulphated galactans that are isolated from red seaweeds, are described in relation to their functionality as food ingredients. Also, an overview is presented on enzymes that are able to modify carrageenan; such enzymes can be used as potential powerful natural biocatalysts to modify carrageenan structure and alter its functionality. The introduction of biotechnology is considered as a means of obtaining (tailor making) new, specific seaweed polymers to form a new generation of high-quality functional ingredients for foods, cosmetics and pharmaceuticals.

During the past decade, it has been increasingly recognized that marine microorganisms such as bacteria, microalgae and seaweeds represent a largely unexplored source of valuable materials[1]. The various unique carbohydrate residues that are found in marine organisms are especially interesting and emphasize the importance of obtaining further knowledge in this area. So far, the three commercially exploited carbohydrate polymers from marine organisms are: (1) alginates, the mannuronic-acid- and guluronic-acid-containing polymers from brown seaweeds; (2) agar, the D-galactose- and anhydro-L-galactose-containing polymers that are isolated from red seaweeds[2]; and (3) carrageenan. As shown in Fig. 1, carrageenan, together with pectin, is the main natural gelling polysaccharide that is extracted from plants or seaweeds (excluding starch) and used as a high-value food ingredient; estimated worldwide sales amounted to US$263 million in 1997.

Carrageenan is a generic name for a family of natural, water-soluble, sulphated galactans that are isolated from red seaweeds, and exploited on a commercial scale; they are traditionally used as high-quality ingredients in food and cosmetics[3,4]. The three main commercial carrageenans are ι-, κ- and λ-carrageenan, each of these being both a general, commercial name and a name that specifies the major substitution pattern that is present in the 'ideal' galactan backbone (see figure in Box 1). Recently, Knutsen *et al.*[5] proposed an alternative nomenclature for red seaweed polysaccharides that is based on logical abbreviations and the worldwide accepted IUPAC (International Union of Pure and Applied Chemistry) nomenclature[6] (see Box 1). Although this new nomenclature is much better than the use of the word carrageenan with different Greek prefixes, it will be difficult to get it accepted in commercial names for seaweed polymers that are used as food ingredients, because the older system has been in use for so long. In this review, we have used the alternative letter codes for oligomeric structures.

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Important aspects of the science of carrageenans have been summarized in review articles on the biology of seaweeds[3], the rheology and applications of carrageenan[7], and the biomedical applications of marine polysaccharides[8]. Knowledge of the detailed structure of carrageenan is limited. In addition to D-galactose and 3,6-anhydro-D-galactose as the main sugar residues and sulphate as the main substituent, other carbohydrate residues (e.g. xylose, glucose and uronic acids) and substituents (e.g. methyl ethers) are present. This knowledge is important because the structure of carrageenans influences their functional properties, which in turn influence their commercial value.

This review presents an overview of the structures of different carrageenans and the enzymes that are able to modify carrageenan structure. Within the past decade, several enzymes have been shown to modify carrageenans, and processes are becoming available to isolate specific fractions from seaweeds. Enzymes are powerful natural biocatalysts that are being increasingly used to manufacture food ingredients with a specific functionality and consequently higher value. The introduction of biotechnology represents a means to obtain (tailor make) new, specific seaweed polymers that will form a new generation of high-quality functional ingredients for foods, cosmetics and pharmaceuticals.

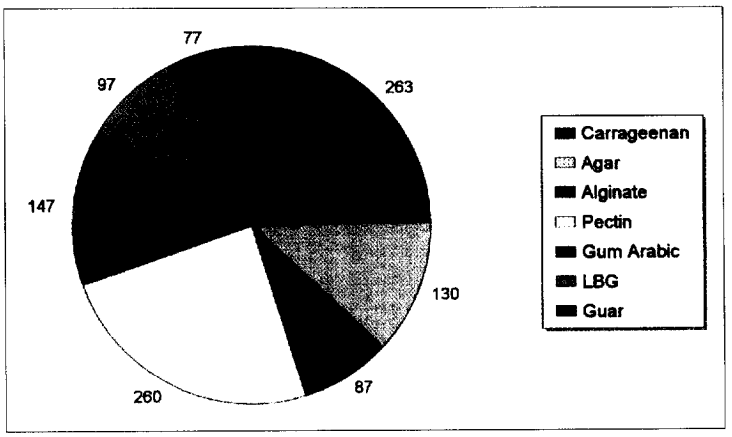

Fig. 1
Estimates for the 1997 world market (in million US$) of natural gelling polysaccharides that are extracted from plants and seaweeds (excluding starch) and used as high-value ingredients in the food industry; LBG, locust bean gum. (D. Seisun, unpublished; data presented at the 'Food Hydrocolloids 97' conference in Nice, France.)

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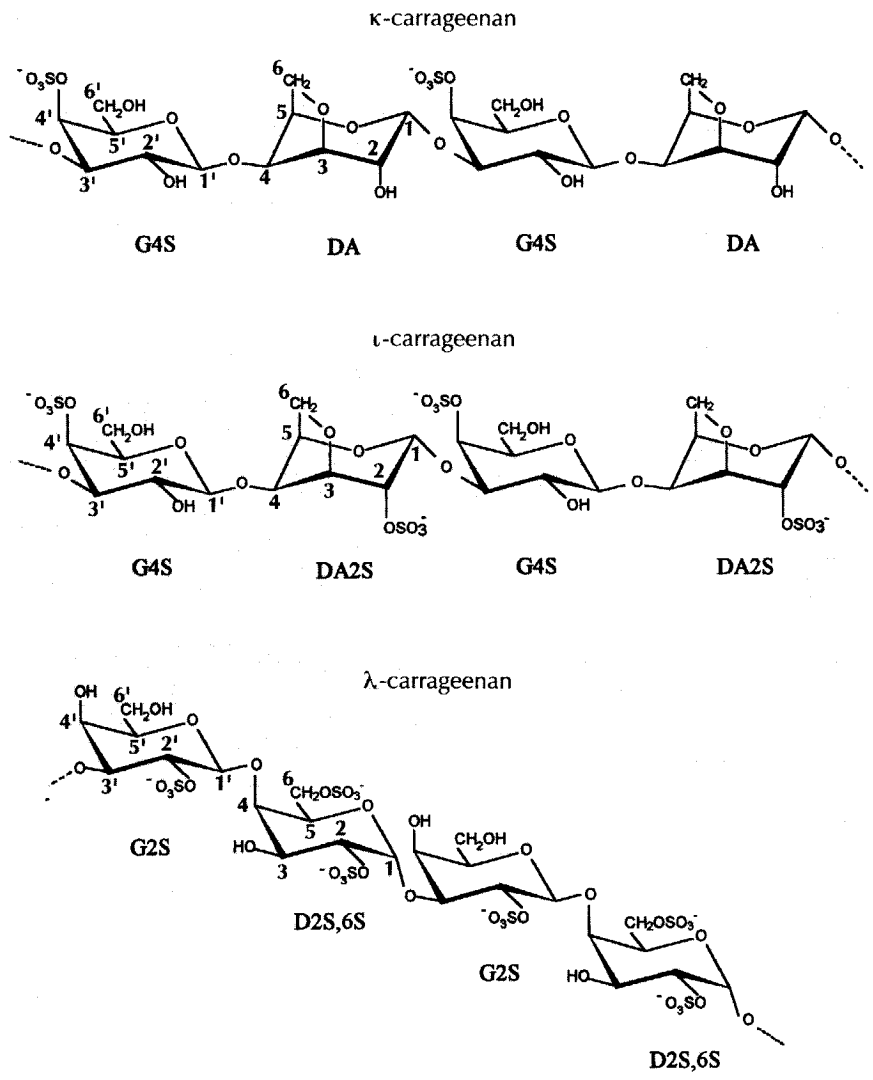
Box 1. Nomenclature of carrageenan

The nomenclature of carrageenan with the Greek prefixes (see figure) is used universally, including in regulation and legislation, but it is becoming increasingly confusing from a scientific point of view because it is not logical, is inflexible and does not make it possible to describe polymers unambiguously. To solve this problem, Knutsen *et al.*[5] proposed an alternative nomenclature for carrageenan and agar that is logical, systematic and flexible, and is also based on the worldwide accepted IUPAC (International Union of Pure and Applied Chemistry) nomenclature[6] (see table). Based on this nomenclature, 'ideal' [i.e. a structure based on a perfect alternating sequence of α(1→4)-linked 3,6-anhydro-D-galactopyranose residues and β(1→3)-linked D-galactopyranose residues] κ-carrageenan is called carrageenose 4′-sulphate (letter code of dimeric structure: G4S-DA); 'ideal' ι-carrageenan is called carrageenose 2,4′-disulphate (letter code of dimeric structure: G4S-DA2S); and 'ideal' λ-carrageenan is called carrageenan 2,6,2′-trisulphate (letter code of dimeric structure: G2S-D2S,6S).

Alternative letter code nomenclature of the main galactopyranose monomers and oligomers present in different carrageenans, as developed by Knutsen *et al.*[5]

Letter code	Occurrence in carrageenans	Full IUPAC name (recommended in 1996)[a]
G	β	3-Linked β-D-galactopyranose
D	Not found	4-Linked α-D-galactopyranose
DA	κ, β	4-Linked 3,6-anhydro-α-D-galactopyranose
S	κ, λ, ι	Sulphate ester ($O\text{-}SO_3^-$)
G2S	λ	3-Linked β-D-galactopyranose 2-sulphate
G4S	κ, ι	3-Linked β-D-galactopyranose 4-sulphate
DA2S	ι	4-Linked 3,6-anhydro-α-D-galactopyranose 2-sulphate
D2S,6S	λ	4-Linked α-D-galactopyranose 2,6-disulphate

[a]Taken from Ref. 6



Schematic representation of the different 'ideal' structures of κ-, ι- and λ-carrageenans. The letter codes refer to the table (left). The small numbers on the first pair of residues indicate the carbon-atom numbering system.

Seaweed raw materials

All of the seaweeds that produce carrageenan as their main cell-wall material belong to the red algae, or *Rhodophyta*. There is almost no information available on the biosynthesis of carrageenans and the genetics of the seaweed plant cell wall. Such details are essential to understand the structure of carrageenans better. Different seaweed species produce different carrageenans, and this, together with the availability of the seaweeds, determines their commercial worth. The commercial cultivation of the tropical seaweed *Kappaphycus alvarezii*, known in the trade as *Eucheuma cottonii* (or Cottonii), in the mid-1960s provided a breakthrough, which boosted carrageenan production. This seaweed still accounts for the largest consumption worldwide[9]. Cottonii yields a relatively homogeneous κ-carrageenan after alkali treatment. Another important species is *Eucheuma denticulatum* (trade name Spinosum) as shown in Fig. 2, which yields ι-carrageenan upon alkali extraction. Other carrageenans are obtained from different species from the *Gigartina* and *Chondrus* genera. These seaweeds have a life cycle with alternating gametophytic (male and female; 1n: one pair of chromosomes each) and sporophytic (2n: two pairs of chromosomes) phases; peculiarly, the sporophytic plants produce λ-carrageenan, whereas the gametophytic plants produce a hybrid of κ- and ι-carrageenan. What is commercially known as λ-carrageenan (not restricted to the 'ideal' structure G2S-D2S,6S; see Box 1) is mainly obtained from *Gigartina pistillata* from Morocco and also from cultivated sporophytic *Chondrus crispus* from Canada. Most of the seaweeds from temperate regions are harvested from natural populations in areas where they exist in relatively pure populations and in large quantities. *C. crispus* is mainly harvested in the Eastern provinces of Canada and in France, and *Gigartina radula* in Chile. *G. radula* produces a carrageenan that is similar to that produced from *Chondrus*[10]. The total world production of seaweeds for carrageenan extraction is currently ~120 million kg (dry weight, 'as sold'), yielding ~23 million kg of carrageenans with an estimated value of ~US$263 million in 1997 (Fig. 1).

Fig. 2
Photograph of the red seaweed Spinosum (*Eucheuma denticulatum*), which is harvested in the Philippines and used for ι-carrageenan production. (Photograph provided by B. Rudolph.)

Structure of carrageenans

Sulphate esters

Carrageenans are linear, partially sulphated galactans that are mainly composed of repeating dimers of an α(1→4)-linked D-galactopyranose (D) or 3,6-anhydro-D-galactopyranose residue (DA) and a β(1→3)-linked D-galactopyranose residue (G), as shown in the figure in Box 1. The sulphate groups are covalently coupled via ester linkages to the carbon atoms C-2, C-4 or C-6 of individual galactose residues. The amount of $-O-SO_3^-$ in sulphated polysaccharides can be considerable and varies in the range of 0–41% (w/w), resulting in highly negatively charged polymers. The three main repeating dimer structures of carrageenans are G4S-DA (main dimeric structure of κ-carrageenan), G4S-DA2S (ι-carrageenan) and G2S-D2S,6S (λ-carrageenan), as schematically shown in the figure in Box 1. The κ-, ι- and λ-carrageenan dimers have one, two and three sulphate ester groups, respectively, resulting in respective calculated sulphate contents of 20%, 33% and 41% (w/w). Typically, commercial κ-carrageenan contains 22% (w/w) of sulphate, ι-carrageenan 32% (w/w) and λ-carrageenan 38% (w/w), although large variations can occur owing to differences between seaweed species or batches. These small but real differences between the sulphate levels of each of the three main carrageenan types and the levels expected on the basis of their 'ideal' structures clearly indicate that carrageenan samples are not 'ideal' structures; rather, each is a complex galactose-based polysaccharide that has different quantities of sulphate esters at different positions and with different distributions.

Because the sulphate groups are such an important structural substituent of the galactose backbone, reproducible and rapid analysis of the sulphate ester content is important for studying: (1) seaweed raw materials, (2) the rheological properties of the polymers, (3) their applications in foods and cosmetics, and (4) their possible biomedical applications. The officially used method for determining the sulphate content in carrageenans is based on the selective hydrolysis of the sulphate ester by acid and subsequent selective precipitation of the sulphate ions as barium sulphate, which is then measured by weighing or by turbidimetry as described by the FAO/WHO Joint Expert Committee on Food Additives (JECFA), and most recently updated in 1992 (Ref. 11). This method has disadvantages: it is laborious and requires gram quantities of sample. Within the past two decades, some alternatives to this method have been described, including elemental analysis, infrared (IR) spectroscopy[12], methylation analysis[13] and NMR[14,15].

A promising development in this area is the use of high-performance liquid chromatography (HPLC) technology to measure the free sulphate that is liberated from the carrageenan polymers after acid treatment; this is an alternative to the selective precipitation of the liberated sulphate as barium sulphate. Typically, this analysis is

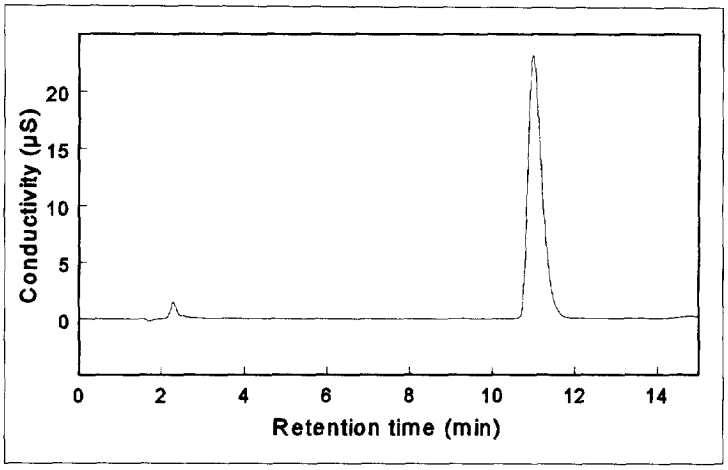


Fig. 3

Chromatogram of the analysis of the free sulphate liberated from an ι-carrageenan by using the anion-exchange chromatography high-performance liquid chromatography system with conductivity detection using the ASRS membrane. The peak represents ~128 ppm (mg/l) of free sulphate. (Figure kindly provided by Mrs Corien Jol and Mrs Bea Penninkhof; Analytical Science Division, Hercules European Research Center, The Netherlands.)

performed on an HPLC system using an AS12A anion-exchange column (Dionex Corp., Sunnyvale, CA, USA) with sodium carbonate–sodium bicarbonate solution (pH of ~10.5) as the eluent, followed by conductivity detection. Conductivity detection is achieved using a recently described new, membrane-based electrolytic suppressor device[16] (ASRS; Dionex Corp.), which reduces the background conductivity dramatically, and allows the accurate detection of free sulphate (see Fig. 3).

3,6-Anhydro bridges in galactose

κ- and ι-Carrageenans contain 3,6-anhydro bridges in the α(1→4)-linked D-galactopyranose residue, whereas λ-carrageenan does not have an anhydro bridge. The non-gelling μ- and ν-carrageenans (carrageenan-6,4′-disulphate and carrageenan-2,6,4′-trisulphate, respectively) are the natural precursors that are present in seaweeds that contain κ- and ι-carrageenans, respectively, and have a sulphate ester group at C-6 of the α(1→4)-linked D-galactopyranose residue of the dimeric unit. The 3,6-anhydro bridges are formed by the elimination of the sulphate from the C-6 sulphate ester of the precursors and the concomitant formation of the 3,6-anhydro bridge. This formation is based on a two-step mechanism[17]. First, the α-linked galactose 6-sulphate (D6S) converts from the $^4C_1$-conformation (equatorial 6-O-SO_3^--Gal) into the $^1C_4$-conformation by the increase in temperature; this puts both the 6-O-SO_3^--Gal and the C3-OH into an axial position. The strong alkali present simultaneously ionizes the free hydroxyl groups (at C-2 and C-3 in κ-carrageenan; at C-3 in ι-carrageenan) of the α(1→4)-linked D-galactopyranose, producing O^- anions. The second step is the nucleophilic displacement reaction of C6-sulphate by the action of the O3 anion, resulting in the formation of the 3,6-anhydro bridge in the same galactose residue (DA) and the release of the sulphate group at C-6 (Ref. 17). In κ-carrageenan, only the O3 anion is in a favourable stereochemical axial position, and is therefore the only anion that reacts.

Helix formation

The sulphate ester groups and the 3,6-anhydrogalactose rings are essential for the physicochemical properties of the respective carrageenans, their helix formation and consequently their rheological properties and applications[18,19]. Typically, κ-carrageenan forms firm and brittle gels that can undergo syneresis (exudation of water), whereas ι-carrageenan forms elastic and soft gels that usually do not undergo syneresis; the non-gelling λ-carrageenans are used as thickening agents.

The gelation of a carrageenan solution is induced by cooling a hot solution that contains gel-inducing cations such as K^+ and Ca^{2+}. The Na^+-form of κ- and ι-carrageenans does not yield a gel, but the addition of a gelling salt instantly induces gelation. A detailed mechanism of gelation is not yet available, but there is a consensus that gel formation involves a conformational transition from random coil to helix form[19]. Carrageenans have a significant reactivity with milk proteins, making them valuable hydrocolloids for use in the dairy industry. Unlike starches or cellulosics, carrageenans are not susceptible to degradation by enzymes such as cellulases and other food enzymes; thus, carrageenans are used in toothpaste in tropical regions.

X-ray crystallographic data of sulphated monosaccharides have been used to evaluate the conformational energies of the repeating units of the κ-, ι- and λ-carrageenan polymers, taking into account the rotation around the sulphate groups. Based on computer modelling, it has been predicted that all three carrageenans are highly flexible. The differences in the physical properties of carrageenans cannot arise solely from electrostatic sulphate-induced differences in the random coil configuration of these polymers, but must also result from the conformational parameters of isolated chains and the effects of interchain interactions[18].

Biotechnology

Biotechnology is as old as humankind and, as a general term, includes technologies such as fermentation and biocatalysis. Fermentation is traditionally based on the use of growing cells of microorganisms to convert substrates via multi-step reactions into a complex product. Typical examples of fermented products include wine, beer and cheese. Biocatalysis is usually defined as the use of enzymes or enzyme systems to catalyse a highly selective transformation of a single substrate into a defined end product. Consequently, the term polysaccharide biotechnology usually means biocatalytic transformation and can be generally described as: 'the application of enzymes or enzyme systems to convert carbohydrate polymers into value-added new polysaccharides'. The biotechnology of red seaweed polysaccharides, and in particular that of carrageenans, is still in its infancy compared with the biotechnology of alginate and plant carbohydrate polymers such as starch[20] or pectin[21].

Table 1. κ- and ι-Carrageenases that have been described in the literature

Enzyme name	Microorganism	Enzyme properties				Refs
		Mechanism	Cleavage group	Main product	Purity	
κ-Carrageenase	*Pseudomonas carrageenovora* NCMB 302	β-Hydrolase	$(G4S\text{-}DA)_2$	Dimers of different neo-carrabioses	Partially purified	26, 28, 29
κ-Carrageenase	*Pseudomonas carrageenovora* NCMB 302	β-Hydrolase	G4S-DA	ND	Purified	30, a
κ-Carrageenase	*Alteromonas carrageenovora*	β-Hydrolase	G4S-DA	ND	Cloned and sequenced	27, 31
κ-Carrageenase	*Cytophaga* 1k-C783	β-Hydrolase	G4S-DA	ND	Purified	32
κ-Carrageenase	*Cytophaga* strain Dsji	β-Hydrolase	G4S-DA	Tetramer	Purified/cloned and sequenced	33, b
ι-Carrageenase	Unspecified bacterium	β-Hydrolase	G4S-DA2S	Tetramer	Purified	26, 34
ι-Carrageenase	*Cytophaga* strain Dsji	β-Hydrolase	G4S-DA2S	ND	Cloned and sequenced	c

[a] S.H. Knutsen (1992) *Isolation and Analysis of Red Algal Galactans* (PhD thesis), University of Trondheim, Norway
[b] P. Potin (1992) *Recherche, Production, Purification et Caractérisation de Galactane Hydrolases pour la Préparation d'Oligosaccharides des Parois d'Algues Rouges* (PhD thesis), Université de Bretagne Occidentale, Brest, France
[c] T. Barbeyron (1993) *Caractérisation Moléculaire d'Une Sulfatase, d'Une Kappa-carraghénase et d'Une Iota-carraghénase Chez Deux Bactéries Marines: Alteromonas carrageenovora et Cytophaga drobachiensis* (PhD thesis), Université Pierre et Marie Curie, Paris, France
ND, Not determined

Agarases

The first report on the enzymatic modification of sulphated seaweed polysaccharides dates back to 1902, when H.H. Gran[22], from Norway, described the degradation of agar by an enzyme isolated from a seaweed bacterium that he called *Bacillus gelaticus*; consequently, he named the enzyme 'gelase'. Most of this work was carried out in the laboratory of Martinus W. Beijerinck (Delft, The Netherlands). Since then, many agarases, particularly α- and β-agarases, have been isolated, purified and cloned, and are used commercially on a small scale[23]. Especially in Japan, there has always been great interest in agarases and oligosaccharides from agar, which are increasingly being used as functional food ingredients[23]. Two types of oligosaccharides can be formed from agar: the agar-oligosaccharides result from the hydrolysis of the α-linkage, catalysed by dilute acid or α-agarases; the neo-agar-oligosaccharides result from the hydrolysis of the β-linkage, catalysed by β-agarases.

Carrageenan-modifying enzymes

As far as we are aware, the first enzyme that was found to modify carrageenans was described in 1943 by the Japanese researcher Mori[24], followed 12 years later by the Canadians Yaphe and Baxter[25]. Since that time, many carrageenan-modifying enzymes have been described, as listed in Table 1, most of which have been developed recently in the laboratory of Bernard Kloareg (CNRS Station Biologique, Roscoff, France). A considerable number of these enzymes have been purified from marine bacteria such as *Alteromonas carrageenovora* (previously called *Pseudomonas carrageenovora*) and from various algae (e.g. *Gigartina* spp.).

The best-characterized enzyme is the κ-carrageenase isolated from *A. carrageenovora* [Refs 26 and 27, and S.H. Knutsen (1992) *Isolation and Analysis of Red Algal Galactans* (PhD thesis), University of Trondheim, Norway]. This enzyme acts as an endo-enzyme, specifically hydrolysing the β-glycosidic linkage between 3,6-anhydro-D-galactose and D-galactose; this results in the formation of neo-carrabiose oligosaccharides with the letter code DA-G4S and with 3-linked β-D-galactopyranose 4-sulphate as the reducing end (see Fig. 4). The lytic mechanism and specificity of this enzyme were revealed by detailed analysis of the reaction products by NMR and, based on the amino acid sequence, it was classified in family 16 of the glycosyl transferases in the system designed by Bernard Henrissat[27]. To our knowledge, no enzymes have been reported to be able to cleave the glycosidic linkage of the resultant neo-carrabiose dimers, or to cleave the 3,6-anhydro-α-D-galactose linkage, resulting in a reducing end of 3,6-anhydro-galactose residues. Many of the enzymes listed in Table 1 have not been purified to homogeneity and are not very well characterized. Furthermore, none of these carrageenan-modifying enzymes is commercially available.

Future developments

Biotechnology is developing rapidly as a sophisticated tool to design polysaccharides and oligosaccharides that have a specific functionality. It is already used on a commercial scale to manufacture plant carbohydrate polymers such as starch, guar gum or pectin. Limited amounts of marine macroalgal polysaccharides, mainly alginates, are currently modified using enzymes and are commercially available. It is likely that this trend will increase in the future, because biotechnology may be the only feasible means to meet the needs of the market, which continuously requests specific polymers for specific applications at low cost. Furthermore, the increasing attention that is being

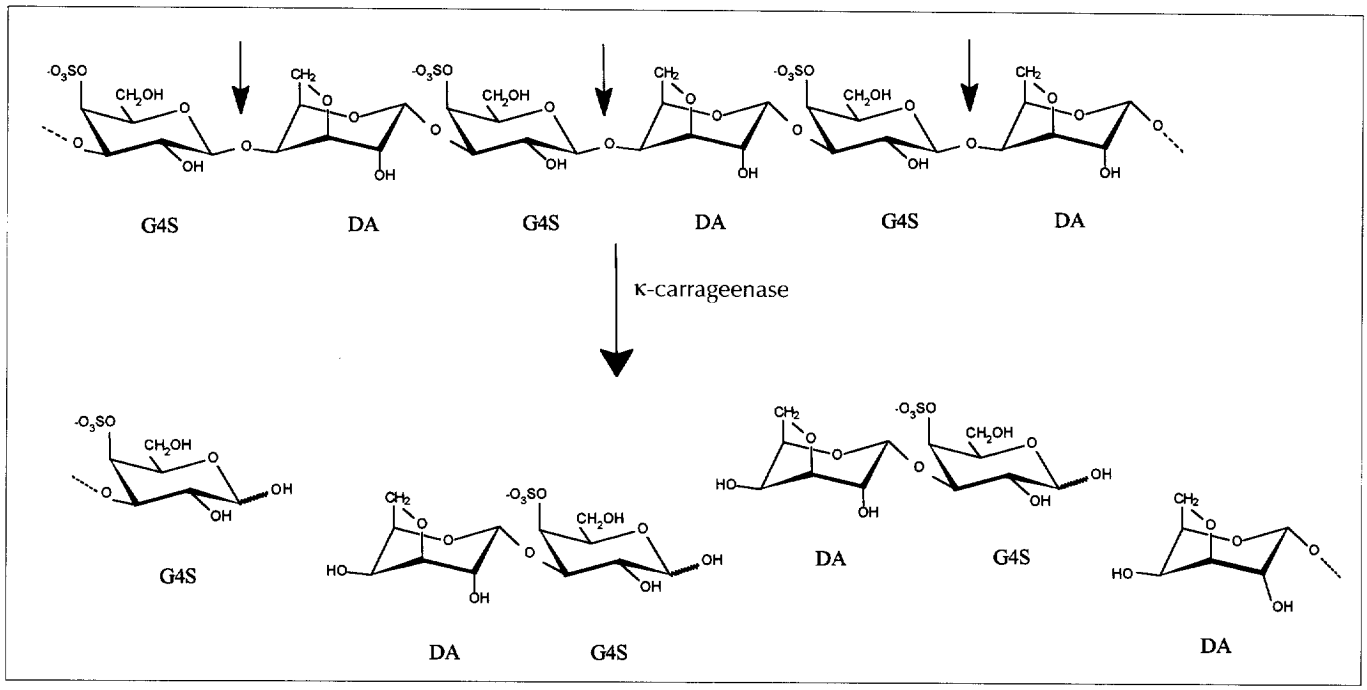


Fig. 4

Example of the specific degradation of κ-carrageenan by a κ-carrageenase that specifically hydrolyses the β-glycosidic linkage between 3,6-anhydro-D-galactose and D-galactose, resulting in the formation of neo-carrabiose oligosaccharides with the letter code DA-G4S and with 3-linked β-D-galactopyranose 4-sulphate as the reducing end groups.

paid to the possible biomedical applications of well-characterized carbohydrates isolated from marine algae, as indicated by the explosion in the number of scientific articles and patents in this area, suggests that in the future these compounds will have pharmaceutical applications. Also, it can be expected that enzymes will be increasingly used as sophisticated tools for the detailed structural analysis of seaweed polymers, which will be essential to understand their physicochemical properties.

In conclusion, the short-term benefits of the use of biotechnology in the manufacture of marine carbohydrates will be: the development of new polymers that have specific functional properties, the better utilization of the seaweed materials that remain after carrageenan extraction, and the improvement of carrageenan extraction processes. In the longer term, it can be expected that biotechnology will allow: the transfer of algal genes that code for useful enzymes to other microorganisms, and the manufacture of specific biologically active compounds. Biotechnology will be an increasingly important tool for producing (tailor making) new, specific marine carbohydrates that will form a new generation of high-quality functional ingredients for use in foods, cosmetics and pharmaceuticals.

Acknowledgements

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Review

Antibody- and lectin-based assays for the rapid analysis of food-grade gums and thickeners

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The main objectives of this brief review are to: (1) provide a literature update on the analytical methods currently used to identify and estimate the concentration of food-grade gums and thickeners; (2) provide a detailed account of some novel techniques based on antibodies (monoclonal and polyclonal) and lectins that have been developed for a range of polysaccharides (e.g. galactomannans, xanthan gum, carrageenans and gum arabic); and (3) shed some light on probable future developments in this field.

Polysaccharide additives are widely used in foods as stabilizing, gelling and thickening agents. Typical polysaccharide additives include guar gum, locust bean gum (LBG), carrageenans, xanthan gum, gum arabic and alginates (see Table 1). The analysis of gums and thickeners for food use is required for several reasons, including: (1) to verify compliance with legislative requirements and maximum permitted levels; (2) to confirm the authenticity of raw materials received from suppliers; and (3) to improve understanding of the basic mechanisms of polysaccharide functionality and nature during product formulation and processing. For example, work has been reported on the analysis of polysaccharide functionality and the mechanisms of gel formation in relation to proteins[1], antioxidants[2] and synergistic reactions with other polysaccharides[3]. Regarding the current legislation, working documents have been issued by the Council of the European Communities (e.g. document III/9040/90 plus addendum, September 1990) specifying the maximum levels of various polysaccharides that are permitted in foods. In addition, the UK Food Advisory Committee's review of emulsifiers and stabilizers recommended the reclassification of food-grade carrageenans from Group A (additives acceptable) into Group B (provisionally acceptable)[4].

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