



HAL
open science

α -Carrageenan: An alternative route for the heterogenous phase degradation of hybrid ι -/ κ -carrageenan

Laurent Poulet, Sophie Mathieu, Sophie Drouillard, Laurine Buon, Mélanie Loiodice, William Helbert

► To cite this version:

Laurent Poulet, Sophie Mathieu, Sophie Drouillard, Laurine Buon, Mélanie Loiodice, et al.. α -Carrageenan: An alternative route for the heterogenous phase degradation of hybrid ι -/ κ -carrageenan. Algal Research - Biomass, Biofuels and Bioproducts, 2023, 71, pp.103049. 10.1016/j.algal.2023.103049 . hal-04668282

HAL Id: hal-04668282

<https://hal.science/hal-04668282v1>

Submitted on 6 Aug 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

α -Carrageenan: an alternative route for the heterogenous phase degradation of
hybrid ι -/ κ -carrageenan

Laurent Poulet^{1#}, Sophie Mathieu^{1#}, Sophie Drouillard¹, Laurine Buon¹, Mélanie Touvrey¹,
William Helbert^{1*}

¹ CERMAV, CNRS and Grenoble Alpes Université, BP53, 38000 Grenoble Cedex 9, France

The authors have equally contributed to the work

*corresponding author: william.helbert@cermav.cnrs.fr

Abstract:

Carrageenans are sulfated galactans densely packed in the cell wall of numerous red algae (Rhodophyta). The most abundant in algae and the most exploited industrially for their gelling properties are the κ - (kappa) and the ι - (iota)-carrageenans which the carrabiose repetition units are substituted by one or two sulfate ester groups. These polyanionic polysaccharides represent an important marine biomass and are carbon source for a number of marine bacteria. One polysaccharide utilization loci, a cluster of genes co-localized in the genome of the marine strain *Paraglaciecola atlantica* T6c, encompasses several glycoside hydrolases and sulfatases dedicated to the degradation of carrageenan. In addition to one ι -carrageenase and three carrageenan sulfatases, already characterized, we discovered the first endo- α -carrageenase and an exo- α -carrageenase sulfatase which allowed describing an alternate way to the degradation of ι -carrageenan without ι -carrageenase. α -Carrageenan didn't make gels in presence of salt suggesting that desulfation of ι -carrageenan probably helps to solubilize the gelling carrageenan. The degradation of the κ -carrageenan fraction of hybrid κ -/ ι -carrageenan was also obtained without κ -carrageenase.

Keywords: carrageenan catabolism, iota-carrageenan, alpha-carrageenase, sulfatases, hybrid-carrageenan, *Paraglaciecola atlantica* T6c

1. Introduction

Carrageenans are sulfated galactans densely packed, embedding the cellulose microfibrils network in the cell wall of red algae (Rhodophyta) [1]. They are the most abundant component of the matrix and can represent up to 50% of the algal dry weight. Carrageenans are made up of linear chain disaccharide repetition units, named carrabioses, as elementary building blocks. Carrabiose units are composed of D-galactose (G) linked alternatively by $\alpha(1\rightarrow3)$ and $\beta(1\rightarrow4)$ bonds and classified according to the number and position of sulfated esters (S) and by the occurrence of 3,6 anhydro ring in the α -linked residues (DA-unit). Among algae, the most abundant carrabiose units are kappa- (κ , DA-G4S) and iota- (ι , DA2S-G4S) carrabioses found in the so-called κ - and ι -carrageenans, respectively (Figure 1).

Except very rare example such as the alkali-treated κ -carrageenan of *Hypnea musciformis* (Wulfen) J.V. Lamouroux 1813, which contains 95% of κ -carrabiose units [2, 3], carrageenans are hybrid structures made of several repetition units. In fact, the terms κ - and ι -carrageenans define polysaccharides composed mainly of κ - and ι -carrabioses, respectively, and which show characteristic gelling properties. Hybridity of carrageenan chains was demonstrated by analyses of the enzymatic degradation products [4, 5, 6] and allowed better understanding of the diversity of their rheological properties.

Because of the complexity of the carrageenan structure, enzymatic degradation of the polysaccharide required a portfolio of enzymes including glycoside hydrolases dedicated to the specific cleavage of the glycosidic bonds between the galactose residues and sulfatases which catalyzes the removal of sulfate ester groups. The first step of the degradation is catalyzed by κ - or ι -carrageenases [7] which are specific to the β -linked galactose of the κ - and ι -carrabiose unit, respectively. Because carrageenan adopts helical conformation forming a three-dimensional solid network of pseudo-crystalline fibers in the algal cell wall [1], κ - and ι -carrageenases have active site topology as well as processive mode of action adapted to the heterogeneous phase degradation [8, 9].

In several marine bacterial strains, it was shown that the genes coding for carrageenases were located in gene clusters or “polysaccharide utilization loci” (PUL) neighboring other glycoside hydrolases, sulfatases and enzymes involved in galactose and anhydrogalactose metabolism pathway. Investigation of these genes encoding enzymes acting in concert allowed deciphering all the steps leading to the complete degradation of κ - and ι -blocks to D-galactose and D-anhydrogalactose [10, 11, 12]. In all cases, desulfatation of the oligo- κ - and oligo- ι -carrageenans by 4O-sulfatases which convert ι - to α -carrabioses or κ - to β -carrabiose was necessary. Desulfatation of α -carrabiose units was obtained with an additional 2O-sulfatase giving β -carrabiose. β -carrageenase, galactosidases and anhydrogalactosidases allowed then degrading the neutral β -carrabiose units to monosaccharide.

The gene *Patl_0879* coding for the *Paraglaiecola atlantica* T6c previously called [*Pseudoalteromonas*] *atlantica* T6c ι -carrageenase (GH82) [13] is located in a cluster of gene encompassing several sulfatases (S1_7: *Patl_0891*; S1_19: *Patl_0889* and *Patl_0895* according to the SulfAtlas classification: <https://sulfatlas.sb-roscoff.fr/sulfatlas/?execution=e6s1>) known to be active on carrageenan [14, 15] (Figure 2). In addition, several genes were predicted to code for glycoside hydrolases and classified in CAZy (<http://www.cazy.org/>) [16], (GH16_13: *Patl_0880*; GH167; *Patl_0885*, GH127: *Patl_0910*) and genes involved in the D-anhydrogalactose metabolism (*Patl_0896* to *Patl_0899*) were also present in this cluster of genes suggesting their role in the carrageenan metabolism. Therefore, in the same vein of previous studies, we have undertaken the characterization of the carrageenan degradation system of *P. atlantica* T6c.

2. Material and methods

2.1. Gene cloning, protein expression and purification

Genomic DNA of *Pseudoalteromonas atlantica* T6c (ATCC® BAA-1087D5™) used as template for the cloning the gene of interest was provided by American Type Culture Collection (ATCC). Glycoside hydrolases and hypothetical proteins of the carrageenan cluster were cloned in pET32a, pET28a, pET20b (Novagen, USA) or pFO4 [17] for heterologous expression in *E. coli* BL21(DE3). Cells harboring the recombinant expression plasmids were cultured on Luria Bertani (LB) medium supplemented with 50 μ g/mL kanamycin (pET28a plasmid) or 100 μ g/mL ampicillin (pET20b, pET32a and pFO4 plasmids) until the OD_{600nm} reached 0.6 in a shaking incubator at 180 rpm and 37°C. After the addition of 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to induce heterologous expression, the temperature was cooled to 20°C and maintained overnight. Cultures were centrifuged (6000 g for 5 min) and the bacterial pellet was suspended in buffer A (20 mM Tris pH 8, 500 mM NaCl) before lysis with a French press. Insoluble fractions were removed by centrifugation at 40,000 g for 30 min at 4°C.

Sulfatases genes were cloned in pFO4 plasmid and co-transformed with pRSF-FGE plasmid, a gift from Marc Wold (Addgene plasmid # 102615; <http://n2t.net/addgene:102615>; RRID:Addgene_102615) into BL21(DE3). Cells were grown in the presence of kanamycin (pRSF-FGE) and ampicillin (pFO4) in LB medium a shaking incubator at 180 rpm and 37°C. At OD_{600nm} of 0.6, the temperature was decreased to 20 °C and L-arabinose (1% w/v final concentration) was added to induce the expression of FGE, followed 30 min later by addition of IPTG to induce the expression of sulfatase. The cultures were then maintained overnight at 20°C.

Cultures (200 mL) were centrifuged at 6,000 g for 15 min and the bacterial pellet was suspended in 10 ml of buffer A (20 mM Tris pH 8, 500 mM NaCl, 20 mM Imidazole). The cells were lysed using a cell disrupter (Constant system Ltd). Insoluble fractions were removed by centrifugation at 20,000 g during 30 min at 4°C and the supernatant was loaded on a 1 mL HisTrap™ HP column

(GE Healthcare) connected to a NGC chromatography system (BioRad). The proteins were eluted with a imidazole gradient from 20 to 300 mM gradient of imidazole. Purification were followed by a size exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column mounted on the same chromatography system and eluting in buffer B (10 mM Tris-HCl pH 8, 50 mM NaCl).

2.2. Enzymology

2.2.1. Degradation kinetics

Enzymatic assays were carried out by incubating 1.4 mL of carrageenan or oligo-carrageenan (0.2% w/v in 50 mM Tris-HCl pH 8, 1 mM CaCl₂) with a few microliters, ranging from 15 to 50 μ L, of the purified enzymes at 25°C. The protein concentration of the purified enzyme ranged from 5 to 70 μ M. The production of reducing ends was measured using the ferricyanide method. Aliquots (100 μ L) were transferred to a 900 μ L ferricyanide solution (0.3 g K₃[Fe(CN)₆], 24 g Na₂CO₃, 1 mL 5 M NaOH, qsp 1 L) which stopped the enzymatic reaction. The solution was heated to 100°C for 10 min and, after cooling to room temperature, the absorbance of sample was measured at 420 nm with a CARY50 Bio UV/Vis spectrophotometer (Varian).

2.2.2. Analytical chromatography

After filtration (0.2 μ m), samples were eluted on 0.1 M NaCl pre-equilibrated Superdex S200 10/300 and Superdex peptide 10/300 (GE Healthcare) columns mounted in series and connected to a high-performance liquid chromatography (HPLC) Ultimate 3000 system (Thermo Fischer). The injection volume was 20 μ L and the elution was performed at 0.4 mL/min in the same eluent. Oligosaccharides were detected by differential refractometry (Iota 2 differential refractive index detector, Precision Instruments) and UV spectrometry at 235 nm (Thermo Fischer).

2.2.3. Semi-preparative chromatography

End-products were purified on a semi-preparative size-exclusion chromatography system which consisted of a Knauer pump (pump model 100), a HW40 Toyopearl column (120 x 16 mm; Tosoh Corporation), a refractive index detector (Iota 2, Precision Instruments) and a fraction collector (Foxy R1) mounted in series. Elution was conducted at a flow rate of 0.4 mL/min at room temperature using 100 mM (NH₄)₂CO₃ as the eluent. The fractions containing pure oligosaccharides (mono-, di- or tetrasaccharides: DP1, DP2, DP4, respectively) were collected and freeze-dried.

2.3. ¹H NMR

Samples were exchanged twice with D₂O and were transferred to a 5 mm NMR tube. ¹H NMR spectra were recorded at 323 K using an Avance III 400 MHz spectrometer (Bruker). Chemical shifts are expressed in ppm in reference to water. The HOD signal was not suppressed.

3. Results

The cluster of genes (*Patl_0879* to *Patl_0911*; Genebank: CP000388.1) investigated encompassed various predicted glycoside hydrolases, sulfatases and enzymes involved in D-anhydro-galactose metabolism (*Patl_0896* to *Patl_0899*). In addition, two transcriptional regulators (*Patl_0894*, *Patl_0901*), one transporter (*Patl_0900*) and one TonB-dependent receptor (*Patl_0887*) were also predicted. All the genes coding for glycoside hydrolases, sulfatases and hypothetical proteins were cloned and heterologous expressed in *Escherichia coli* (Table 1).

As previously shown [13], the ι -carrageenase (*Patl_0879*, GH82) is an endo-acting enzyme giving neo- ι -tetracarrabiose (DP4) and neo- ι -carrabiose (DP2) as smallest end-products. ι -Carrageenan and oligo- ι -carrageenans were then converted in α -carrageenan and oligo- α -carrageenans using the endo-4O- ι -carrageenan sulfatase (*Patl_0889*, S1_19) following the same procedure of [14]. These ι - and α -carrageenan substrates were characterized by ^1H NMR to validate their chemical structure. In Figure 3 are presented the ^1H NMR of the ι - and α -carrageenan which signals were ascribed according to [14]. The function of the overexpressed proteins of the carrageenan cluster were screened, at first, using these well characterized substrates.

3.1. Novel endo- α -carrageenases (*Patl_0880*, GH16_13)

Functional screening of the predicted GH16 encoded by the gene *Patl_0880* revealed it was specifically active on α -carrageenan. Degradation kinetics monitored by size exclusion chromatography showed that the high molecular weight of α -carrageenan substrate (15-20 min) was degraded into oligosaccharides as the degradation proceeded in agreement with an endo-mode of action of the enzyme (Figure 4A). The end-products were purified by gel permeation chromatography and characterized by ^1H NMR (Supplementary Figure 1). The ^1H NMR spectra (Figure 4B) were straightforwardly attributed to neo- α -carrabiose and neo- α -carratetraose and annotated according to the previous work on oligo- α -carrageenans [13].

Patl_0880 was classified in the GH16 CAZy family which encompasses several galactanases such as agarases and carrageenases. The enzyme was recently grouped in the sub-family GH16_13 [18] containing already characterized κ - and β -carrageenases from the marine bacteria *Paraglaciicola hydrolytica* S66^T [11]. Therefore, we have also tested the activity of the enzyme on κ -carrageenan and furcellaran – a hybrid κ -/ β -carrageenan. No activity even weak was observed by the method of the reducing sugar or by chromatography confirming the high specificity of the enzyme for α -carrageenan. The recent observation by Hettle and co-workers [19] that the degradation of ι -carrageenan was possible when combining a sulfatase and a GH16_13 of *Pseudoalteromonas fuliginea* PS47 homologous to *Patl_0889* and *Patl_0880*, respectively, suggests that the α -carrageenase activity is probably present in several families of the order *Alteromonadales*.

3.2. Novel exo-2O- α -neocarrabiose sulfatase (*Patl_0888*, S1_81)

Most of the characterized carrageenan sulfatases were specific of the position 4 of the β -D-galactose of κ - or ι -carrageenan. Only two sulfatases were found to be active on the sulfate located at the position 2 of the D-anhydrogalactose residue of oligo- α -carrageenans. The first discovered, the sulfatase *zobellia_3151*, belongs to the S1_17 sub-family [20] which was active on oligo- α -carrageenans [10]. The second sulfatase was grouped in the S1_81 sub-family [19]. *P. atlantica* T6c *Patl_0888* sulfatase which belongs to the S1_81 sub-family, was active on *p*-nitrophenyl-sulfate and on oligo- α -carrageenans as demonstrated by chromatography and ^1H NMR experiments (Figure 5).

Incubation of the mixture of oligo- α -carrageenans with the *Patl_0888* sulfatase leads to a loss of molecular weight of the oligosaccharides which eluted slower in gel permeation chromatography (Figure 5A). Isolated oligosaccharides analyzed by ^1H NMR highlighted the desulfation of the position 2 of the anhydro-galactose at the non-reducing end. The chemical shift of the anomeric proton of the DA2S residue located at the non-reducing end (DA2Snr: 5.21 ppm) was shifted to 5.07 ppm accordingly to the desulfation (Figure 4B). The chemical shifts observed were similar to those reported to β -carrabiose residues [21].

3.3. Two paths for the degradation of ι -carrageenan

The combination of the endo-4O- ι -carrageenan sulfatase (*Patl_0889*) and the endo- α -carrageenase (*Patl_0880*) give oligo- α -carrageenans which can be subjected to further desulfation by the exo-2O-neo- α -carrageenan sulfatase (*Patl_0888*). Therefore, in parallel to the more common ι -carrageenan degradation pathway involving GH82 ι -carrageenase (*Patl_0879*) followed by the desulfation of oligo- ι -carrageenans, an alternate way to produce oligo-carrageenans from ι -carrageenan was evidenced. Further investigations of the *P. atlantica* T6c cluster revealed that the genes encoded all the enzymes required to completely digest the oligo- α - and ι -carrageenans (Figure 6).

Patl_0885 is grouped in the CAZy GH167 family which encompasses members cleaving the neo- β -carrabiose unit located at the non-reducing end of hybrid neo- κ -/ β -carratetraose. We demonstrated that *Patl_0885* cleaved the β -carrabiose unit at the non-reducing end of hybrid neo- β -/ α -carratetraose by the chromatography experiment presented Figure 7A. The degradation products were characterized by ^1H NMR confirming the hydrolysis of the glycosidic linkage between the β - and α -carrabiose units (Figure 7B). We have also tested the degradation of neo- κ -/ β -carratetraose by the *P. atlantica* T6c GH167 and observed the production of β - and κ -carrabiose units confirming the observations of Hettle and co-workers [19].

Therefore, our results suggested that *patl_0885* can accommodate in the catalytic subsite +1 either D-anhydro-galactose of the κ -carrabiose or 2O-sulfate-D-anhydro-galactose of the α -carrabiose. We didn't observe the degradation of β -carrabiose located inside hybrid κ -/ β -carrageenan (e.g.

furcellaran). The endo- character of GH167 enzymes was not evidenced and is in agreement with the pocket topology of the active site revealed by crystallography investigations [19]. Therefore, these enzymes should be more adequately named exo-neo- β -carrabiose hydrolase instead of β -carrageenase as previously proposed.

Patl_0910 is grouped in GH127 family and presented 57.6% identity with *Zobellia galactinovorans* Dsij^T D-anhydrogalactosidase (*zobellia_3150*) [10]. The enzyme was active on the neo-oligo- β -/ α -carrageenans and removed the D-anhydrogalactose located at the non-reducing end of the neo-oligo- β -/ α -carrageenans giving a trisaccharide. The enzyme was also active on hybrid β -/ κ -tetrasaccharide suggesting again that the different sulfation moieties were accommodated in the enzyme active site.

3.4. Degradation of hybrid oligo- κ -/ ι -carrageenans

Inspection of *P. atlantica* T6c genome didn't allow evidencing any potential κ -carrageenases suggesting that the bacterium is probably unable to degraded κ -carrageenan. However, the occurrence of 4O- κ -carrageenan sulfatase (*Patl_0891*, S1_7; *Patl_0895*, S1_19) and glycoside hydrolases active on β -carrabiose moieties suggested that the marine strain was able to degrade, at least, oligo- κ -carrageenans. In this context, we tested the hypothesis that the set of enzymes found in the carrageenan cluster was sufficient to catabolize hybrid oligo- ι -/ κ -carrageenans.

The hybrid ι -/ κ -carrageenan extracted from the red algae *Chondrus crispus* Stackhouse 1797, is composed of about 40/60 ι -/ κ -carrabiose units [5]. Incubation of this carrageenan with the ι -carrageenase leads to the production of standard oligo- ι -carrageenans and hybrid oligo- ι -/ κ -carrageenans. Hybrid oligosaccharides were purified by chromatography and their structures were determined by NMR (Figure 8). The three oligosaccharides: ι - κ , ι - κ - κ and ι - κ - ι , have systematically a ι -carrabiose moiety at the non-reducing end. The occurrence of both κ - or ι -carrabiose units at the reducing end suggested that the active site of the ι -carrageenase can accommodate both DA2S of ι -carrabiose and DA of κ -carrabiose at subiste -2.

Degradation scheme of a hybrid ι -/ κ -carrageenan is presented in Figure 9. The ι -carrabiose moieties located at the non-reducing end, were cleaved following a similar scheme demonstrated for standard oligo- ι -carrageenans, which includes the complete desulfation of the non-reducing moiety giving a β -carrabiose unit then eliminated by the exo- β -carrabiose hydrolase (*Patl_0885*, GH167). After removing the ι -carrabiose unit, the κ -carrabiose moiety then located at the non-reducing end, become accessible to desulfation the two 4O- κ -carrabiose sulfatases (*Patl_0891*, S1_7; *Patl_0895*, S1_19) giving β -carrabiose which was then subjected to degradation by the exo- β -neocarrabiose hydrolase (GH167, *Patl_0885*).

4. Discussion

Cell wall matrix of carrageenophyte algae is made of a skeleton of cellulose microfibrils embedded with densely packed carrageenan [1]. The enzymatic degradation of the solid and recalcitrant cell wall is therefore catalyzed by enzymes adapted to heterogeneous phase degradation. In the case of the ι -carrageenase of *Alteromonas fortis* 1^T, it was demonstrated that the degradation of crystalline ι -carrageenan proceeded according to a processive mode of action. This mode of action was explained by the conformational flexibility of three protein domains - A, B, and C - allowing to shape the active site in tunnel-like topology in presence of substrate [9]. The three domains observed in *A. fortis* 1^T ι -carrageenase were not present in all ι -carrageenases and, therefore, the tunnel shape topology requires for processivity can't be obtained with enzymes lacking notably the domain A [9, 22]. *P. atlantica* T6c ι -carrageenase lacks the domain A and presented the characteristics of an endo-enzyme without or low processive character.

The discovery of an endo- α -carrageenase (*patl_0880*) active on α -carrageenan obtained by the conversion of ι -carrageenan in α -carrageenan by the endo-4O- ι -carrageenan sulfatase (*Patl_0889*, S1_19) showed that the degradation of ι -carrageenan without ι -carrageenase is possible. The oligo- α -carrageenans produced were then subjected to desulfation by a new exo-2O- α -carrageenan sulfatase (*Patl_0888*, S1_81) which completed the route of degradation of α -carrageenan. Combination of these three enzymes demonstrated that ι -carrageenan can be degraded without ι -carrageenase and that the first step of carrageenan degradation can be a desulfation step instead of hydrolysis. These results demonstrated that, in the case of *P. atlantica* T6c, ι -carrageenan was degraded *via* two different co-occurring pathways.

The ι -carrageenase (*patl_0979*, GH82), the α -carrageenases (*patl_0880*, GH16_13) and the endo-4O- ι -carrageenan sulfatase (*Patl_0889*, S1_19) harbored a signal peptide suggesting they are secreted (Table1) in agreement with the degradation of the polysaccharides located outside the bacterium cell. The produced oligo- α -carrageenans are then degraded by enzymes predicted to be anchor at the surface of the outer membrane such the exo-neo- β -carrabiose hydrolase (GH167, *Patl_0885*) which present a lipoprotein signal peptide (SPII) or in the cytoplasm (e.g. no signal peptide). These predictions suggest that the degradation of the ι -carrageenan proceed from the exterior of the bacterium cell where is present the carrageenan substrate (e.g. algal cell wall) toward the interior of the cell where the galactose residues are catabolized.

Hybrid ι -/ α -carrageenan was first reported in the red algae *Catenella nipae* Zanardini 1872 [23, 24]. The α -carrageenan didn't show gelling properties in 0.1 M NaCl in contrast with κ -carrageenan which also carries one sulfate ester by carrabiose units [25]. We also didn't observe the

formation of strong gels with α - or ι - α -carrageenans as those obtained with κ -carrageenan in presence of KCl, CaCl₂ and other ions. This suggests that α -carrageenan has probably very weak gelling properties and that the conversion of ι - to α -carrageenan may help in solubilizing ι -carrageenan densely packed in algal cell wall. Therefore, we hypothesized that the combination of ι -carrageenase and α -carrageenase pathways could be an efficient way to disintegrate the matrix cell wall.

Because of their industrial interest and their abundancy, the most documented carrageenans are hybrid κ - ι -carrageenans. Despite few alkali extracted carrageenans composed of mainly one type of carrabiose residue such as the κ -carrageenan of *Kappaphycus alvarezzii* (Doty) Doty ex P.C. Silva, 1996 (κ - ι -carrabioses: 94/6 mol/mol) and the ι -carrageenan of *Eucheuma denticulatum* (Burm.f.) Collins & Hervey, 1917 (κ - ι - μ -carrabioses: 88/6/4 mol/mol/mol), carrageenans are usually hybrid structure. Hybridity of carrageenan was evidenced by analyses of their enzymatic degradation products [6]. The enzymatic degradation of hybrid carrageenan by *Pseudoalteromonas carrageenovora*^{9(T)} κ -carrageenase lead to three main fractions: standard oligo- κ -carrageenans, hybrid oligo- κ - ι -carrageenans and a resistant fraction made of ι -carrabiose rich fragments. The proportion of these fractions is strongly dependent of the composition (i.e. amount of κ -carrabiose moiety) and the distribution of the carrabiose units (e.g. blockwise, random). The hybrid oligo- κ - ι -carrageenans were systematically terminated at the reducing and non-reducing ends by a κ -carrabiose unit, in agreement with the modality of the substrate recognition by the enzyme [6].

Conversely, the degradation of hybrid κ - ι -carrageenans by ι -carrageenase produced standard oligo- ι -carrageenans, hybrid oligo- κ - ι -carrageenans and a resistant fraction made of κ -carrabioses rich fragments. We observed that the hybrid oligo- κ - ι -carrageenans obtained after the incubation with *P. atlantica* T6c ι -carrageenase were terminated by a ι -carrabiose unit at both reducing and non-reducing ends. This differed from the hybrid oligosaccharides prepared with *Alteromonas fortis* 1^T ι -carrageenase which were terminated by ι -carratetraose at the non-reducing end and ι -carrabiose at the reducing end [6].

The recent studies on the enzymatic degradation of hybrid carrageenan by complex cluster of genes or “polysaccharide utilization loci” (PUL) explained the degradation of κ - and ι -carrageenan fractions by independent route starting with the action of κ - and ι -carrageenases. In this context, at first glance, the carrageenan cluster of *P. atlantica* T6c seemed specialized in the degradation of ι -carrageenan because of the absence of κ - or λ -carrageenases, for example. In addition, it was reported by Préchoux and co-workers [14, 15] that the production of κ - and ι -carrageenan sulfatases were induced when the bacterium was grown in presence of ι -carrageenan while κ - and λ -carrageenan had weak or no effect on the enzymes production, therefore, reinforcing the apparent specialization of the *P. atlantica* T6c toward the ι -carrageenan degradation. Then, the lacking enzymes could be provided

by other bacteria co-localized at the surface of algae and acting in partnership to digest the cell wall. When taking into account the hybridity of the carrageenan and the structure of the hybrid degradation products of the ι -carrageenase, we found that *P. altanlica* T6c had all the enzymatic tools to digest the hybrid oligo- ι - κ -carrageenans. Therefore, the number of enzymes required to catabolize carrageenan is probably less important than what the complexity of carrageenan suggest. We have demonstrated that the degradation of ι -carrageenan without ι -carrageenase by the α -carrageenase route was possible. Ultimately, our results suggest that the degradation of hybrid κ -/ ι -carrageenans could be possible without κ - or ι -carrageenase.

Acknowledgments

This work was supported by the French National Research Agency (Grant ANR-17-CE20-0032-01, PULmarin). W.H. have received support from the Glyco@Alps Cross-Disciplinary Program (Grant ANR-15-IDEX- 02), Labex ARCANE, and Grenoble Graduate School in Chemistry, Biology, and Health (Grant ANR-17-EURE-0003).

References

1. J.S.Craigie, The cell wall. In: Cole KM, Sheath RG (eds) *Biology of the red algae*. Cambridge University Press, Cambridge, UK,1990 pp 221–257
2. H.J Bixler., K. Johndro , R. Falshaw, Kappa-2 carrageenan: structure and performance of commercial extracts. II. Performance in two simulated dairy applications. *Food hydrocolloid* 15 (2001) 619–630.
3. F. van de Velde, A.S. Antipova, H.S. Rollema, T.V. Burova, N.V. Grinberg, L. Pereira, P.M. Gilsenan, R.H. Tromp, B. Rudolph, V.Y. Grinberg, The structure of κ / ι -hybrid carrageenans II. Coil-helix transition as a function of chain composition. *Carbohydr. Res.* 340 (2005) 1113–1129
4. C. Greer, W. Yaphe, Enzymatic analysis of carrageenans: structure of carrageenan from *Eucheuma nudum*. *Hydrobiologia* 116/117 (1984) 563–567.
5. M. Guibet, P. Boulenguer, J. Mazoyer, N. Kervarec, A. Antonopoulos, M. Lafosse, W. Helbert, Composition and distribution of carrabiose moieties in hybrid κ -/ ι -carrageenans using carrageenases. *Biomacromolecules*, 9 (2008) 408-15
6. D. Jouanneau., P. Boulenguer, J. Mazoyer, W. Helbert, Hybridity of carrageenans water- and alkali-extracted from *Chondracanthus chamissoi*, *Mazzaella laminarioides*, *Sarcothalia crispata* and *S. radula*. *J. Appl. Phycol.* 23 (2011) 105-114

7. G. Michel, P. Nyvall-Collen, T. Barbeyron, M. Czjzek, W. Helbert W., Bioconversion of red seaweed galactans : a focus on bacterial agarases and carrageenases. *Appl. Microbiol. Biotechnol.* 71 (2006) 23-33
8. M. Lemoine, P. Nyvall Collén, W. Helbert, Physical state of κ -carrageenan modulates the mode of action of κ -carrageenase from *Pseudoalteromonas carrageenovora*. *Biochem. J.* 419 (2009) 545-553
9. G. Michel, W. Helbert, R. Kahn, O. Dideberg, B. Kloareg, The structural bases of the processive degradation of ι -carrageenan, a main cell wall polysaccharide of red algae. *J. Mol. Biol.* 334 (2003) 421–433
10. E. Ficko-Blean, A. Préchoux, F. Thomas, T. Rochat, R. Larocque, Y. Zhu, M. Stam, S. Génicot, M. Jam, A. Calteau, B. Viart, D. Ropartz, D. Pérez-Pascual, G. Correc, M. Matard-Mann, K.A. Stubbs, H. Rogniaux, A. Jeudy, T. Barbeyron, C. Médigue, M. Czjzek, D. Vallenet, M.J. McBride, E. Duchaud, G. Michel, Carrageenan catabolism is encoded by a complex regulon in marine heterotrophic bacteria. *Nature communications* 8 (2017) 1685, doi:10.1038/s41467-017-939 01832-6.
11. M. Schultz-Johansen M., P.K. Bech, R.C. Hennessy, M.A. Glaring, T. Barbeyron, M. Czjzek, P. Stougaard, A novel enzyme portfolio for red algal polysaccharide degradation in the marine bacterium *Paraglaciecola hydrolytica* S66^T encoded in a sizeable polysaccharide utilization locus. *Front. Microbiol.* 9 (2018) 839 doi: 10.3389/fmicb.2018.00839
12. L. Christiansen, D. Pathiraja, P.K. Bech, M. Schultz-Johansen., R. Hennessy, D. Teze, I.G. Choi, P. Stougaard, A Multifunctional Polysaccharide Utilization Gene Cluster in *Colwellia echini* Encodes Enzymes for the Complete Degradation of kappa-Carrageenan, iota-Carrageenan, and Hybrid beta/kappa-Carrageenan. *Msphere* 5, e00792-19, DOI: 10.1128/mSphere.00792-19
13. A. Préchoux A., W. Helbert, Preparation and detailed NMR analyses of a series of oligo- α -carrageenans. *Carbohydr Polym.* 101 (2014) 864-870
14. A. Préchoux, S. Genicot, H. Rogniaux, W. Helbert Controlling carrageenan structure using a formylglycine-dependent sulfatase, an endo-4S-iota-carrageenan sulfatase. *Marine Biotech.* 15 (2013) 265-274
15. A. Préchoux, S. Genicot, H. Rogniaux, W. Helbert, Enzyme-assisted preparation of furcellaran-like κ -/ β -carrageenan. *Marine Biotech* 18 (2016) 133-143
16. E. Drula E., M.-L. Garron, S. Dogan, V. Lombard, B. Henrissat, N. Terrapon, The carbohydrate-active enzyme database: functions and literature *Nucleic Acids Res* 50 (2022) D571–D577.
17. A. Groisillier, C. Hervé, A. Jeudy, E. Rebuffet, P.F.Pluchon, Y. Chevolut, D. Flament, C. Geslin, I.M. Morgado, D. Power, M. Branno, H. Moreau, G. Michel, C. Boyen, M. Czjzek., MARINE-

- EXPRESS: taking advantage of high throughput cloning and expression strategies for the post-genomic analysis of marine organisms. *Microb Cell Fact* 9 (2010) 45.
18. A.H. Viborg, N. Terrapon, V. Lombard, G. Michel, M. Czjzek, B. Henrissat, H. Brumer, A subfamily roadmap of the evolutionarily diverse glycoside hydrolase family 16 (GH16). *J Biol Chem.* 294 (2019) 15973-15986. doi:10.1074/jbc.RA119.010619
 19. A.G. Hettle, J.K. Hobbs, B. Pluvinage, C. Vickers, K.T. Abe, O. Salama-Alber, B.E. McGuire, J.H. Hehemann, J.P.M. Hui, F. Berrue, A. Banskota, J. Zhang, E.M. Bottos, J. Van Hamme, A.B. Boraston, Insights into the κ/ι -carrageenan metabolism pathway of some marine *Pseudoalteromonas* species. *Commun. Biol.* 2 (2019) 474. doi: 10.1038/s42003-019-0721-y
 20. T. Barbeyron, L. Brillet-Guéguen, W. Carré, C. Carrière, C. Caron, M. Czjzek, M. Hoebeke, G. Michel, Matching the diversity of sulfated biomolecules: Creation of a classification database for sulfatases reflecting their substrate specificity. *PLoS ONE* (2016) 11(10): e0164846.
 21. G. Correc, A. Barabanova, R. Tuvikene, K. Truus, I. Yermak, W. Helbert, Comparison of the structures of hybrid κ - β -carrageenans extracted from *Furcellaria lumbricalis* and *Tichocarpus crinitus*. *Carbohydr. Polym.* 88 (2012) 31–36
 22. E. Rebuffet, T. Barbeyron, A. Jeudy, M. Jam, M. Czjzek, G. Michel, Identification of catalytic residues and mechanistic analysis of family GH82 iota-carrageenases. *Biochemistry* 7 (2010) 7590-7599.
 23. R. Falshaw, R.H. Furneaux, H. Wong, M. Liao, A. Bacic, S., Chandrkrachang, Structural analysis of carrageenans from Burmese and Thai samples of *Catenella nipae* Zanardini. *Carbohydr. Res.* 285 (1996) 81–98
 24. E. Zablackis., G.A. Santos, The carrageenan of *Catenella-nipae* Zanardini, a marine red alga. *Bot. Mar.* 29 (1986) 319–322
 25. A. Bourgoïn, E. Zablackis, J.B. Polic, Characterization of α -carrageenan solution behavior by field-flow fractionation and multiangle light scattering. *Food Hydrocolloids* 22 (2008) 1607–1611

Table 1: List of the genes located in the ι -carrageenase polysaccharide utilization loci investigated in this study. Signal peptide prediction were indicated. SPI: "Standard" secretory signal peptides. SPII: Lipoprotein signal peptides.

Locus	Genebank	CAZy/ SulfAtlas	SignalP 5.0 prediction	Yield	Fonction	
<i>Patl_0879</i>	ABG39405.1	GH82	SPI	++	endo- ι -carrageenan hydrolase	Préchoux <i>et al.</i> , 2014
<i>Patl_0880</i>	ABG39406.1	GH16	SPI	++	endo- α -carrageenan hydrolase	This study
<i>Patl_0881</i>	ABG39407.1	S1_16	SPII	+	unknown	
<i>Patl_0884</i>	ABG39410.1		SPII	-	unknown	
<i>Patl_0885</i>	ABG39411.1	GH167	SPII	++	Exo-neo- β -carrabiose hydrolase	This study
<i>Patl_0886</i>	ABG39412.1		SPII	-	unknown	
<i>Patl_0888</i>	ABG39414.1	S1_81.	SPI	++	Exo-2O- ι -carrabiose sulfatase	This study
<i>Patl_0889</i>	ABG39415.1	S1_19	SPI	++	Endo-4O- ι -carrageenan sulfatase	Préchoux <i>et al.</i> , 2013
<i>Patl_0890</i>	ABG39416.1		SPII	++	unknown	
<i>Patl_0891</i>	ABG39417.1	S1_7	SPII	++	Exo-4O- κ -carrabiose sulfatase	Préchoux <i>et al.</i> , 2016
<i>Patl_0893</i>	ABG39419.1		SPII	++	unknown	
<i>Patl_0895</i>	ABG39421.1	S1_19	SPI	++	Exo-4O- κ -carrabiose sulfatase	Préchoux <i>et al.</i> , 2016
<i>Patl_0910</i>	ABG39436.1	GH127	No SP	++	α -D-anhydro-galactosidase	This study
<i>Patl_0911</i>	ABG39437.1			++	unknown	

++ good and very good expression of soluble protein, + low expression, - inclusion body

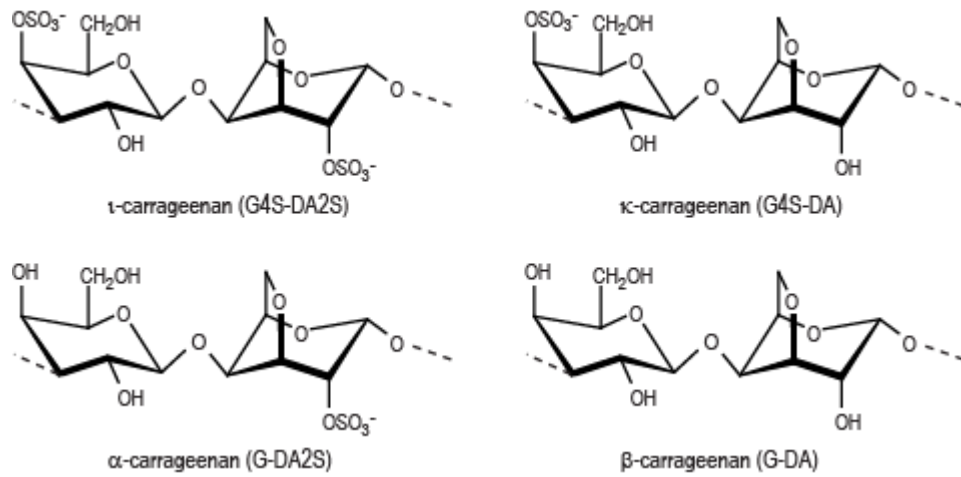


Figure 1: Chemical structure of the main carrabiose repetition units encountered in ι - κ - α - and β -carrageenans.

Paraglaciecola atlantica T6c (locus *Patl_0879* to *Patl_0911*)

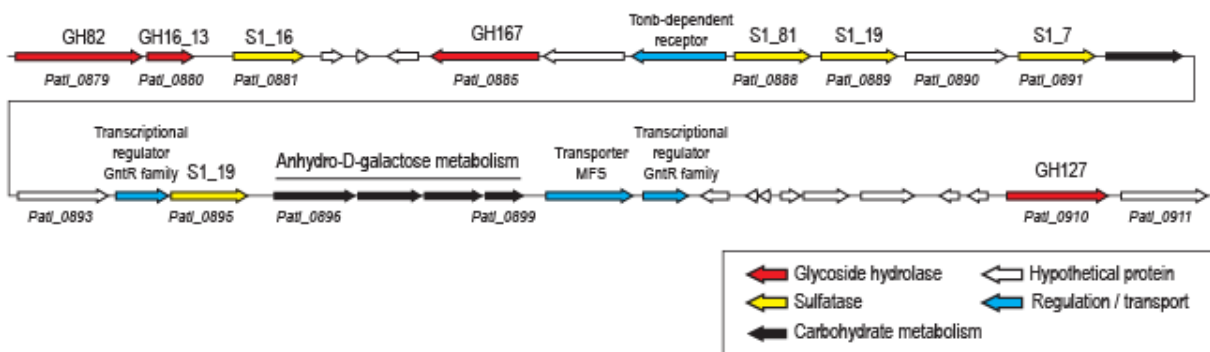


Figure 2: Polysaccharide utilizing loci dedicated to the degradation of carrageenan found in the genome of *Paraglaciecola atlantica* T6c.

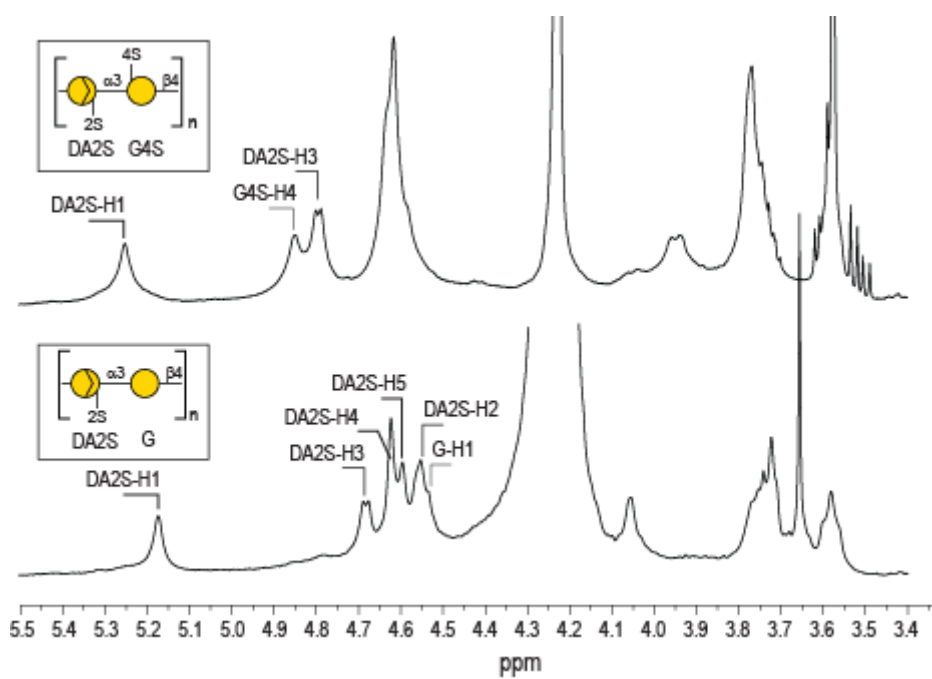


Figure 3: ¹H NMR of ι-carrageenan (top) and α-carrageenan (bottom) obtained after incubation of ι-carrageenan with the endo-4O-ι-carrageenan sulfatase (*Patl_0889*, S1_19). Attribution of the anomeric protons were indicated on the spectra.

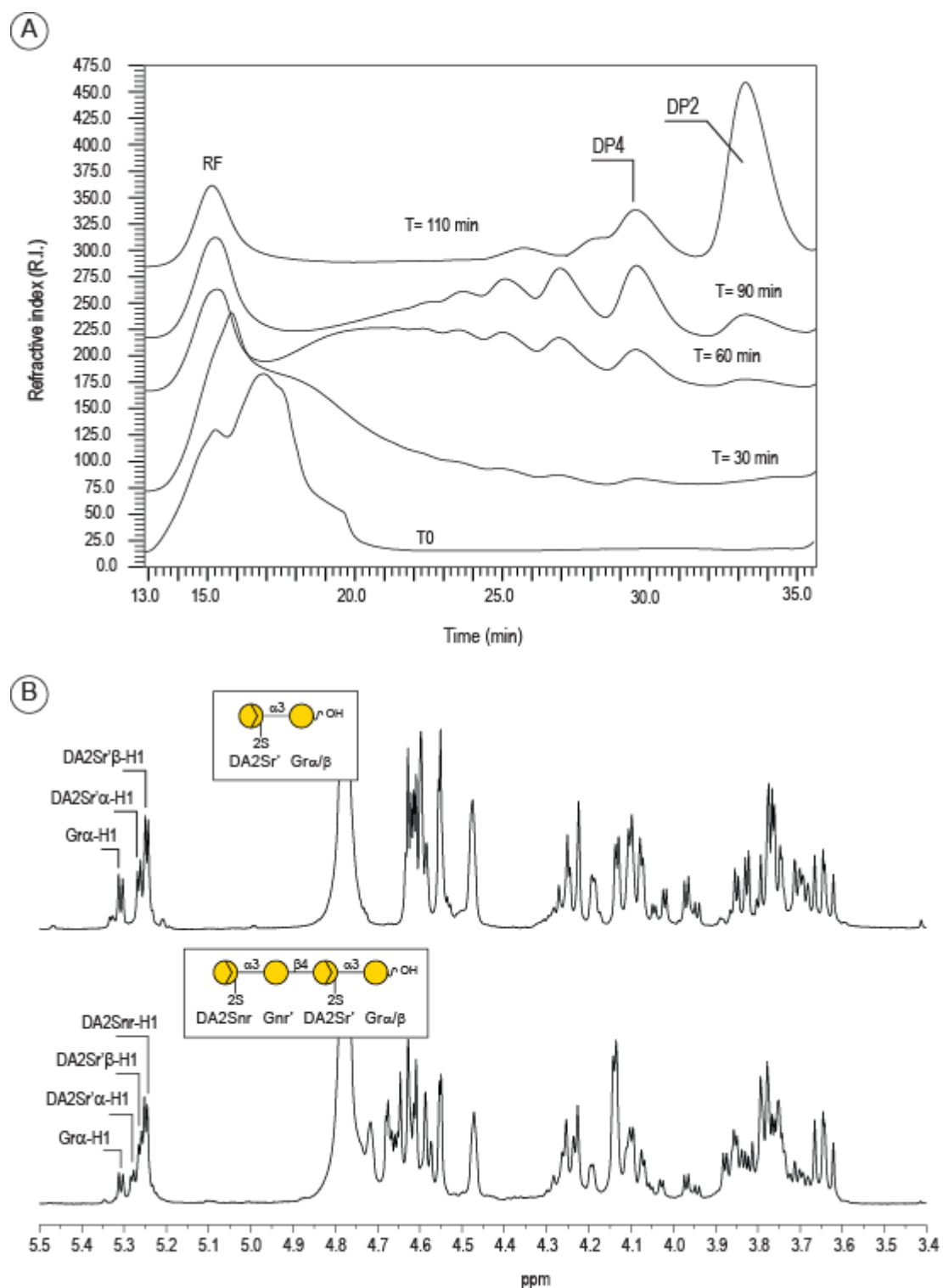


Figure 4: Degradation of α -carrageenan by the endo- α -carrageenase (*Patl_0880*, GH16_13). **A**) Degradation kinetic monitored by size exclusion chromatography. **B**) The end-products, neo- α -carrabiose (DP2) and neo- α -carratetraose (DP4), were analysed by ^1H NMR. The anomeric protons were annotated on the spectra.

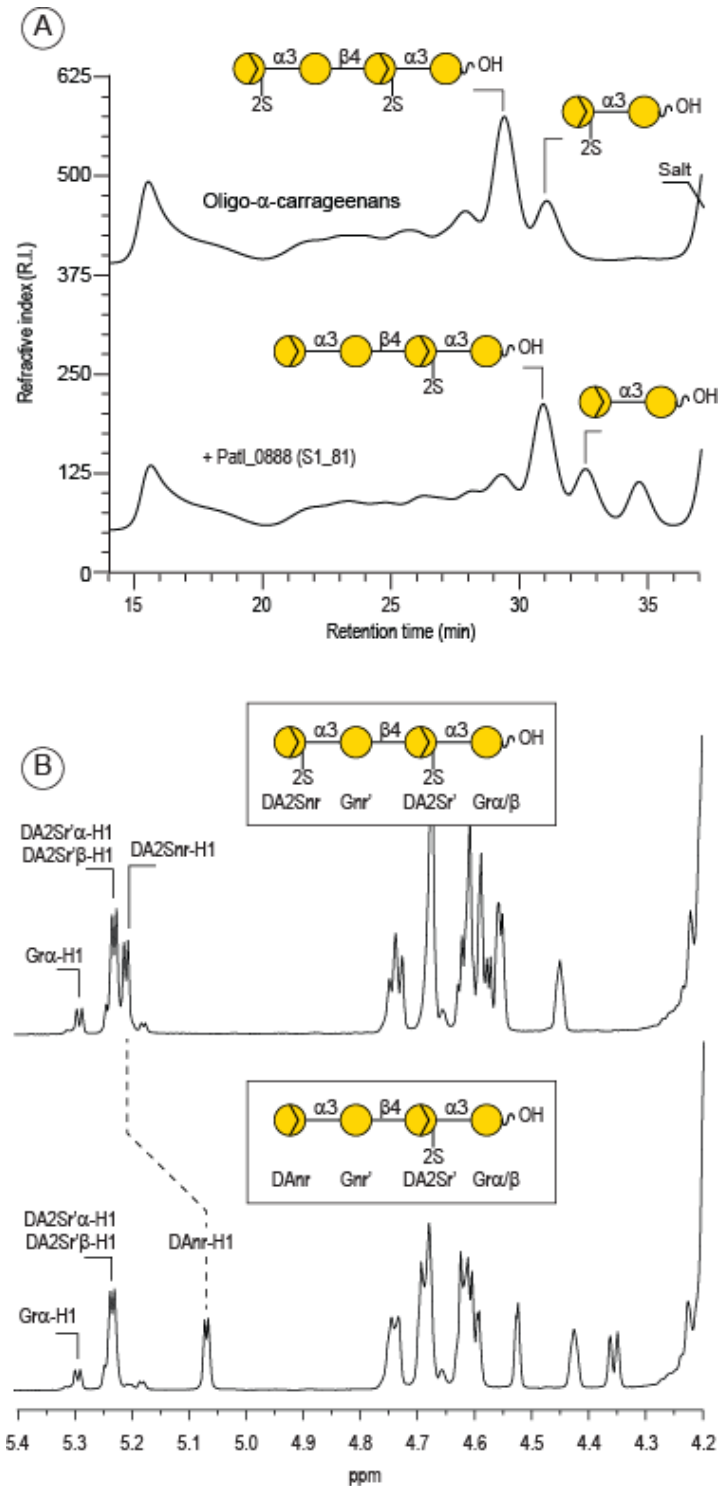


Figure 5: Desulfation of oligo- α -carrageenans by the exo-2O- α -carrageenan sulfatase (*Patl_0888*, S1_81). **A)** Comparison of the size exclusion chromatograms of oligo- α -carrageenans before and after incubation with the sulfatase. **B)** Comparison of the ^1H NMR spectra of the neo- α -carratetraose (top) and the desulfated tetrasaccharide (bottom). The shift of the anomeric proton of the anhydrogalactose located at the non-reducing end (DA2Snr-H1 to DAnr-H1) demonstrates the specific desulfation at the non-reducing end by the exo-acting sulfatase.

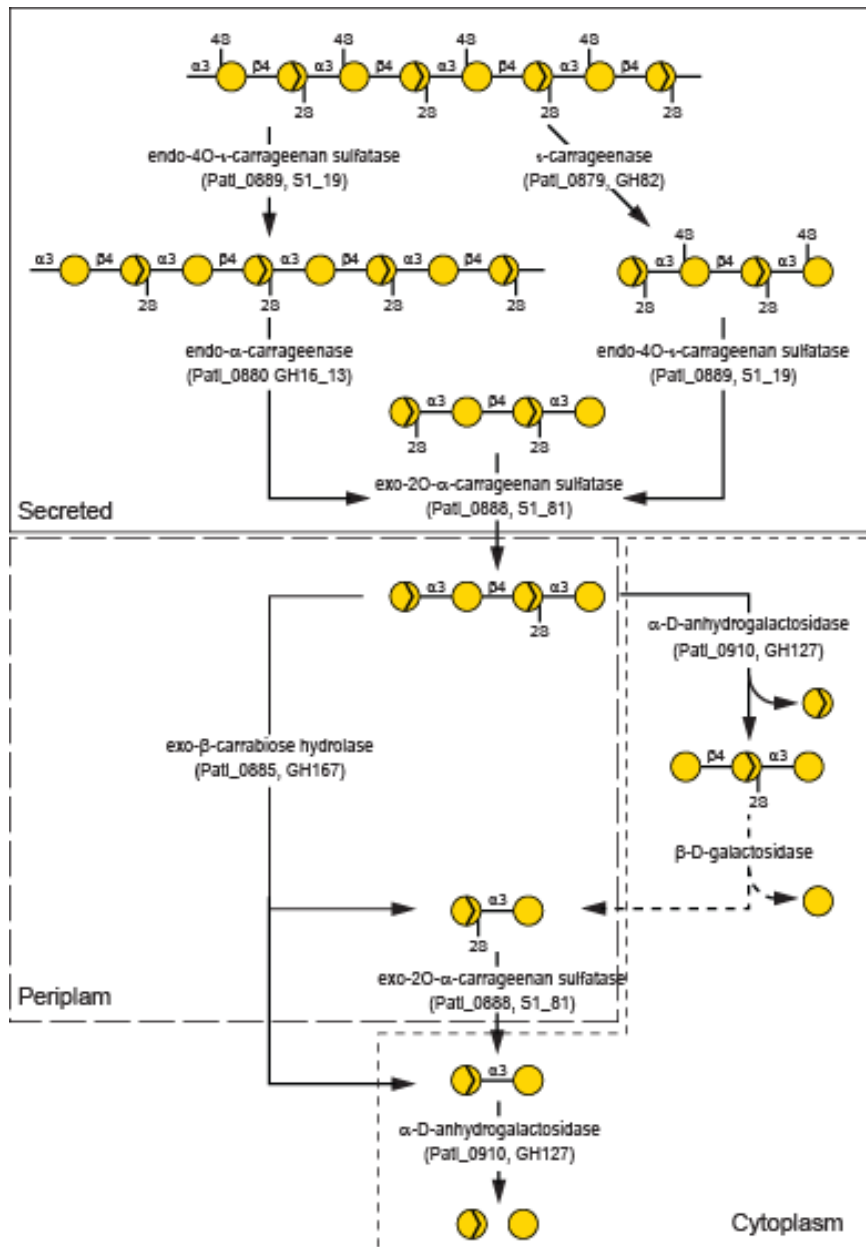


Figure 6: Degradation pathways of ι -carrageenan. Two possible routes are involving; one side, the endo-4O- ι -carrageenan sulfatase (*Patl_0889*, S1_19) and the endo- α -carrageenase (*Patl_0880*, GH16_13) and another side, the endo- ι -carrageenase (*Patl_0879*, GH82) and the endo-4O- ι -carrageenan sulfatase (*Patl_0889*, S1_19).

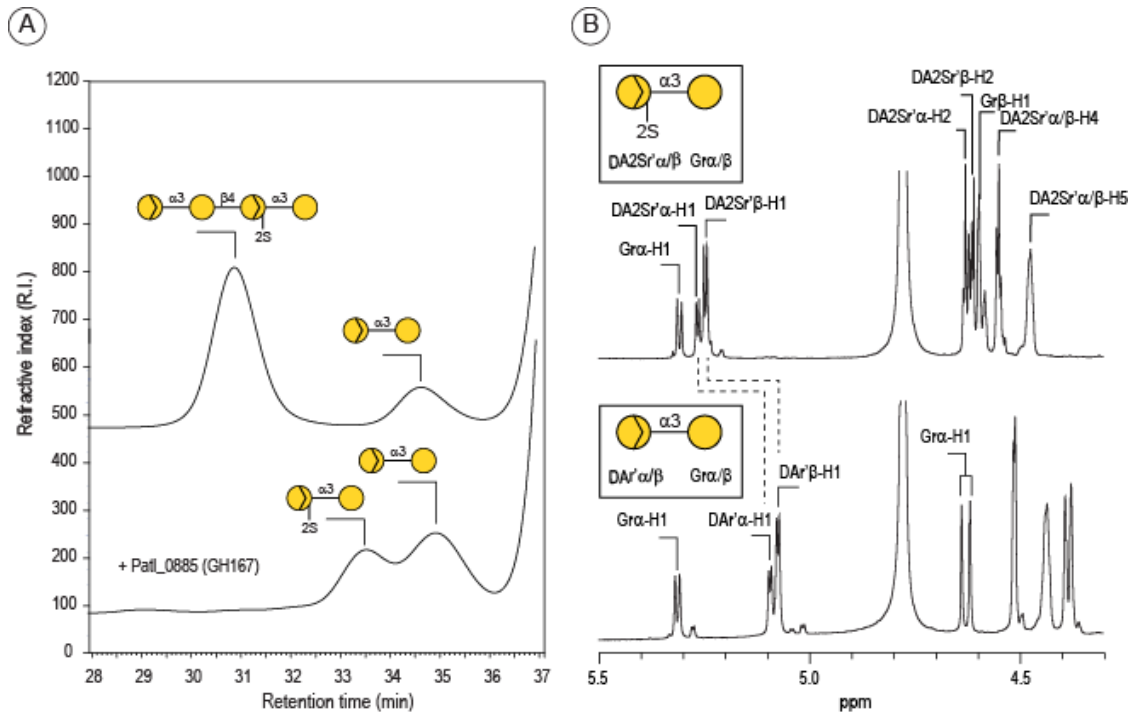


Figure 7: Cleavage of the neo- β -carrabiose unit by the GH167 exo- β -carrabiose hydrolase (*Patl_0885*). **A)** Size exclusion chromatogram of the degradation of desulfated oligo- α -carrageenan. **B)** ^1H NMR spectra of the degradation products.

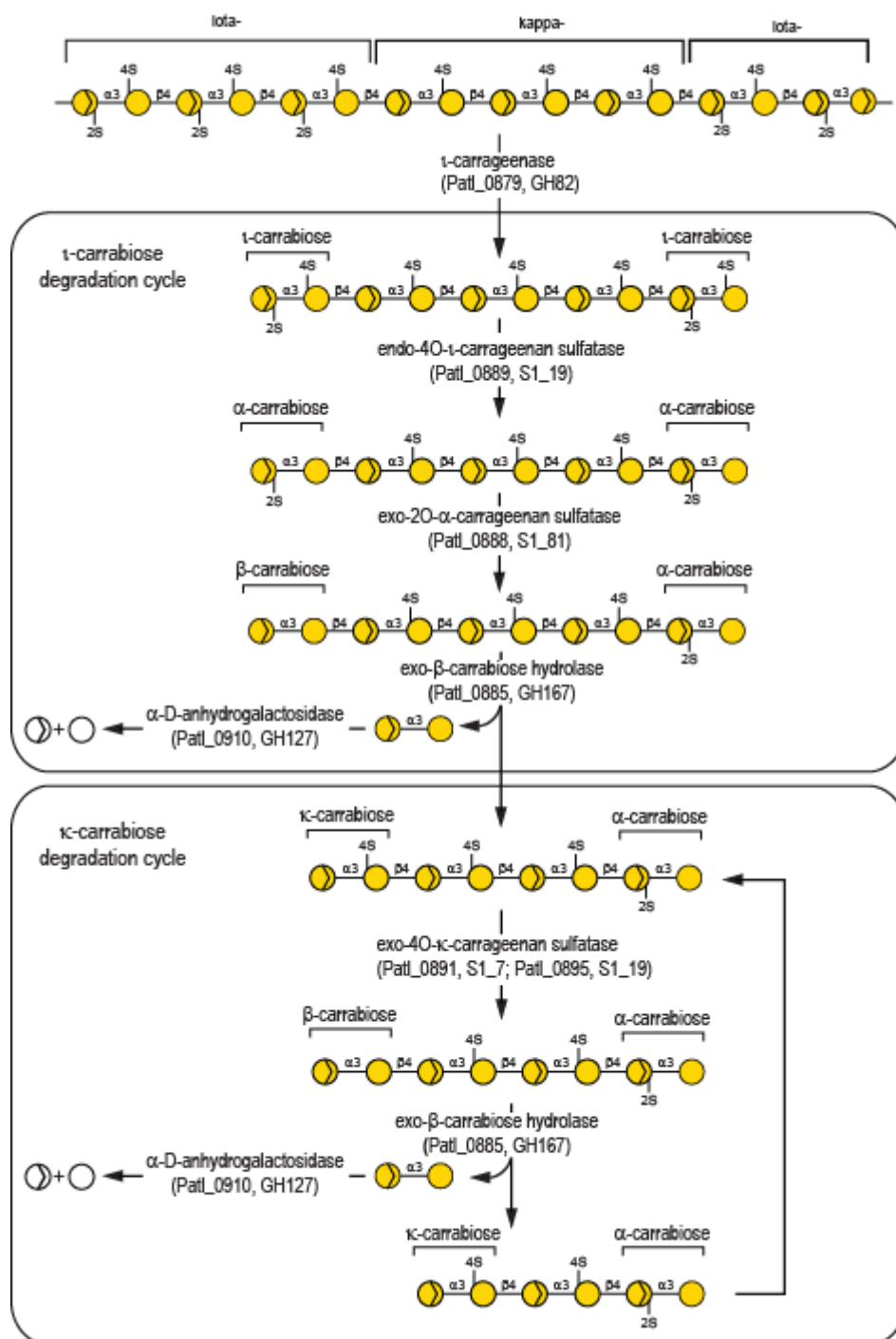
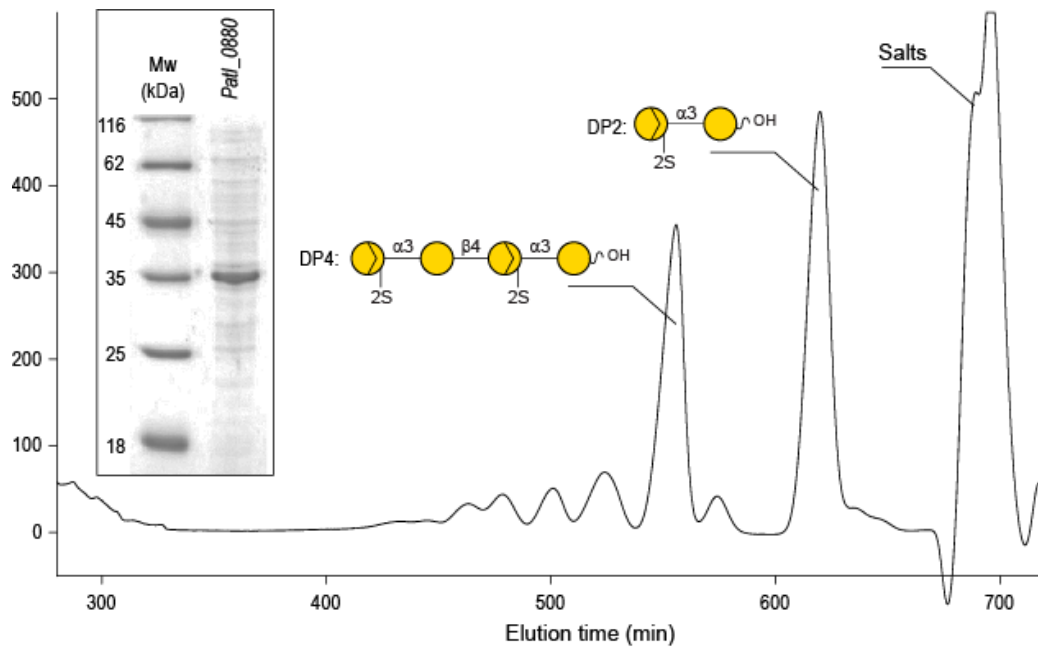


Figure 9: Degradation pathway of the hybrid oligo-ι/κ-carrageenan. Two cycles involving exo-acting enzymes allow degrading ι- and κ-carrabiose moieties located at the non-reducing ends of oligosaccharides.



Supplementary Figure 1: Preparative gel permeation chromatography of oligo- α -carrageenans obtained after extensive degradation of the α -carrageenan by the endo- α -carrageenan hydrolase (*PatI_0880*, GH16_13). The fraction corresponding to the DP2 and DP4 were isolated and characterized by NMR. Inset: Polyacrylamide gel electrophoresis of the purified enzyme. Mw: Molecular weight