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## Enzymatically-derived oligo-carrageenans interact with $\alpha$ -Gal antibodies and Galectin-3

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1 **Enzymatically-derived oligo-carrageenans interact with  $\alpha$ -Gal antibodies and**  
2 **Galectin-3**

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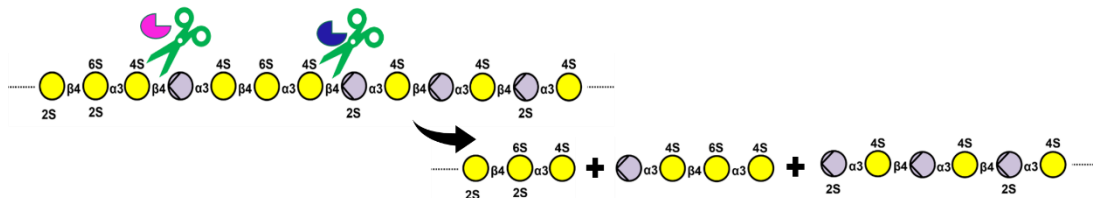
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17 **Abstract**

18 Carrageenans are linear sulfated galactans synthesized in the Gigartinales,  
19 *Rhodophyceae* species with a varied range of biological properties that are of value to the  
20 pharmaceutical and cosmetic sectors. It is unknown how the fine structure of carrageenans  
21 dictates their capacity to affect molecular and cellular responses important to wound healing,  
22 or the ability to mitigate oxidative, hemostatic and inflammatory processes. Here we use  
23 specific endo-carrageenases from the marine bacterium *Zobellia galactanivorans*, to produce  
24 enzymatically defined neo-series oligosaccharides from carrageenans. Further enzymatic  
25 modification of the oligosaccharides was done by treating with the 3,6-anhydro-D-  
26 galactosidases from the same bacterium which hydrolyse non-reducing end 3,6-anhydro-D-  
27 galactose moieties from neo-carrageenan oligosaccharides. Using the enzymatically produced  
28 oligosaccharides, we demonstrate binding to natural human serum antibodies and a  
29 monoclonal anti- $\alpha$ Gal Ab (m86). The significant interactions with the Gal $\alpha$ (1,3)Gal reactive  
30 antibodies produced by humans makes them potential potent inducers of complement-  
31 dependent reactions and attractive for therapeutic applications. We also demonstrate  
32 modulation of the galectin selectivity for the Gal-3 Carbohydrate Recognition Domain (CRD)  
33 relative to Gal-1 which has implications to targeting specific biological pathways regulated by  
34 the galectins.

**1. Endolytic cleavage of carrageenans by specific enzymes**

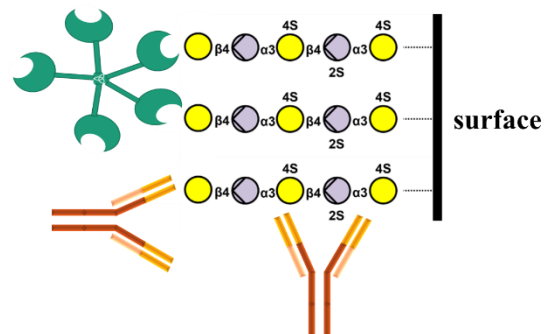


**2. Exolytic carrageenan specific enzymes modify non-reducing ends**



**3. Galectin-3 binds non-reducing ends of carrageenan oligosaccharides**

**Galactose specific antibodies bind along the length and non-reducing ends of oligo-carrageenans**



35

36           **Key words.** carrageenans, carrageenases, 3,6-anhydro-D-galactosidase, glycan-binding  
37 antibody, alpha-Gal antigen, galectin, thrombin, anti-tumour

## 38           **Introduction**

39           The discovery that cellular communication occurs via the ‘sugar code’ which, unlike  
40 nucleic acid and protein code, largely takes place on the cell surface, has led to significant  
41 interest in these conjugate molecules (Gabius, 2018). The major players in sugar  
42 communication are surface bound glycans responsible for the generation of biochemical  
43 messages and carbohydrate-binding proteins transferring those signals into bioactivity. This  
44 communication is informed by protein recognition of sugar epitope structures and topology  
45 (Romero & Gabius, 2019). Among glycan ligands, galactans stand out for their medical  
46 potential, mostly because humans produce staggering amounts of galactan-binding  
47 (glyco)proteins as anti-galactan antibodies and galectins (Galili, 2013; Laaf, Bojarova, Elling,  
48 & Kren, 2019).

49           A common galactose-based sequence found in the majority of mammals is the  $\alpha$ Gal  
50 epitope,  $\alpha$ -Galp-(1 $\rightarrow$ 3)- $\beta$ -Galp-(1 $\rightarrow$ 4)-GlcPNAc-R. It is an antigen that has been eliminated  
51 from human cells during primate evolution. Synchronically with the elimination, the evolution  
52 of molecules capable of reacting with the excluded glycan occurred, *i.e.* natural anti- $\alpha$ Gal  
53 antibodies (Macher & Galili, 2008). Maintenance of high levels of natural anti- $\alpha$ Gal  
54 antibodies is believed to be due to continual environmental exposure, for instance, to  
55 members of the adult human gut microbiome producing an  $\alpha$ Gal epitope (e.g. *Escherichia*  
56 *coli*, *Haemophilus influenza* and *Lactobacillus* species) (Montassier et al., 2019). It is  
57 estimated that the amount of anti- $\alpha$ Gal antibodies reaches up to 1-3% of all circulating Ig  
58 produced by about 1% of all B cells, ensuring a strong innate immunity reaction. Upon  
59 precipitation of surfaces marked with the epitope, the constant Fc parts of Ig triggers the  
60 natural immunity into action to battle virus and tumours or recruit and activate tissue  
61 macrophages for skin regeneration (Galili, 2013), thus making the antibodies interesting  
62 targets for improved wound healing.

63           Galectins are an ancient family of endogenous soluble  $\beta$ -galactoside-binding proteins  
64 abundantly produced in humans (13 out of the 19 known are human) (Laaf et al., 2019). Once  
65 outside the cells, galectins bind cell surface glycoproteins through their carbohydrate  
66 recognition domains (CRDs). CRDs can operate in a traditional or nontraditional mode but  
67 bind  $\beta$ -galactoside containing epitopes of di- to tetra-saccharides abundant in N- and O-  
68 glycans found on glycoproteins and glycolipids of vertebrate tissues. The traditional mode is

69 mediated through direct connection with a receptor glycan to restrict mobility of  
70 transmembrane receptors such as nutrient transporters, cytokines and adhesion receptors. The  
71 nontraditional mode assembles multiple glycosylated partners and forms a dynamic and gel-  
72 like layer of membrane organization — often termed a lattice — that regulates diffusion,  
73 complex formation and domain interactions in the plasma membrane (Nabi, Shankar, &  
74 Dennis, 2015). Both modes translate glycan-encoded information into biological cues with  
75 modulating effects on intracellular glycoprotein trafficking, cell-matrix adhesion, cell  
76 signaling, cancer growth, angiogenesis and effector functions of immune cells (Rabinovich &  
77 Toscano, 2009). The galactins have complex biological functions and sometimes opposing  
78 effects. Tuning their impacts is tissue, cell type and context-dependent, but yet also attractive  
79 as pharmacological targets. Inhibition of extracellular Gal-1 by glycomimetic substances  
80 holds potential for pharmacotherapy of several disorders such as skin cancer and hypertrophic  
81 scar formation (Astorgues-Xerri et al., 2014; Blanchard, Bum-Erdene, Bohari, & Yu, 2016;  
82 Kirkpatrick et al., 2021). Moreover, Gal-3 is considered a preserver of the tumour  
83 microenvironment, thus playing an important role in cancer growth (Filipova et al., 2020).

84        Epitopes for galectins and anti- $\alpha$ Gal antibodies contain  $\beta$ -galactose in their motifs and  
85 the  $\alpha$ Gal antigen has proven to be a ligand for galectins as well (Gal-1 and Gal-3)  
86 (Hirabayashi et al., 2002). Seaweed matrix polysaccharides are potential epitope mimics as  
87 they have regiospecific sulfations (Ficko-Blean, Hervé, & Michel, 2015) but differ  
88 structurally from animal sulfated mucosal glycans and glycosaminoglycans. Biologically  
89 active substances derived from seaweeds are often sulfated carbohydrates covering multiple  
90 biological properties like anticoagulant, antitumor, immunomodulatory and many others  
91 (Deniaud-Bouet, Hardouin, Potin, Kloareg, & Herve, 2017; Huang, Tan, & Nie, 2022).  
92 Among these algal sulfated polysaccharides, carrageenans are a natural source of galactose-  
93 containing glycans with an appealing array of biological properties (Bhattacharyya et al.,  
94 2010; Carlucci et al., 1997; Cheong, Qiu, Du, Liu, & Khan, 2018; Cosenza, Navarro, Pujol,  
95 Damonte, & Stortz, 2015). Notably, they exhibit strong antiviral properties when they are  
96 administered topically to the mucosa of the nasal cavity and the upper respiratory tract (Graf  
97 et al., 2018; Pereira & Critchley, 2020). These linear polymers are composed of alternating 3-  
98 linked  $\beta$ -D-galactopyranosyl units (G-unit) and 4-linked  $\alpha$ -D-galactopyranosyl units (D-unit).  
99 In gelling carrageenans the latter is mainly 4-linked 3,6-anhydro- $\alpha$ -D-galactopyranosyl (DA-  
100 unit) [The designation of G-, D-, DA- is made in accordance to the nomenclature proposed by  
101 Knutsen and co-authors (Knutsen, Myslabodski, Larsen, & Usov, 1994)]. The repeating

102 disaccharide units of G, D and/or DA interchanging along the carrageenan polymer resemble  
103 the sequence of the  $\alpha$ Gal disaccharide [ $\alpha$ -Galp-(1 $\rightarrow$ 3)- $\beta$ -Galp-(1 $\rightarrow$ 4)]. A recent investigation  
104 determined that carrageenan polymers bind to total human serum antibodies with a preference  
105 for IgM (Sokolova et al., 2021). This finding led to the conclusion that carrageenans present a  
106 highly multifaceted ligand platform for human serum antibody binding as well as galectin  
107 inhibitors; however, the molecular basis of the interaction of carrageenans with anti- $\alpha$ Gal-  
108 antibodies has never been investigated.

109 Understanding the fine structural characteristics of carrageenans influencing *in vitro*  
110 biological properties could allow the creation of carrageenan-based structures with controlled  
111 and reproducible activities with pharmaceutical potential. This can be approached by using  
112 carrageenan-specific enzymes with their regio-, stereo-, and enantio-selectivity, mild reaction  
113 conditions, and possibility of sequential or one-pot reactions. The model marine bacterium  
114 *Zobellia galactanivorans* (Barbeyron et al., 2016) is capable of catabolizing carrageenans by  
115 means of a multifaceted carrageenan-induced regulon containing a non-canonical  
116 polysaccharide utilization locus (PUL) (Ficko-Blean et al., 2017). *Z. galactanivorans*  
117 produces endolytic enzymes capable of releasing oligo-carrageenans. The family GH16  $\kappa$ -  
118 carrageenases (Matard-Mann et al., 2017; Michel, Chantalat, Duee, et al., 2001) and family  
119 GH82  $\iota$ -carrageenases (Barbeyron, Michel, Potin, Henrissat, & Kloareg, 2000; Michel,  
120 Chantalat, Fanchon, et al., 2001) are glycoside hydrolases that specifically cleave  $\beta$ -1,4  
121 linkages within carrageenan chains, producing varying lengths of oligosaccharides with DA  
122 moiety at the non-reducing end G moiety at the reducing end. The sulfation motifs on the  
123 released oligosaccharides will vary according to the used carrageenases. To this end, both the  
124 carrageenan oligosaccharides and the  $\alpha$ Gal epitope, have non-reducing end galactose-based  
125 structures, though the non-reducing end terminal 3,6-anhydro- $\alpha$ -D-galactose structure has  
126 undergone a ring flip due to the steric restraints of the 3,6-anhydro-bridge.

127 The exolytic 3,6-anhydro-D-galactosidase enzymes (ZgGH127-1, ZgGH127-2,  
128 ZgGH127-3 and ZgGH129), were recently discovered in the first described carrageenan PUL  
129 (Ficko-Blean et al., 2017). They are specific for the removal of the  $\alpha$ -1,3-DA monosaccharide  
130 residue from the non-reducing end, this results in a non-reducing end  $\beta$ -1,4-G residue after  
131 enzymatic hydrolysis. One 3,6-anhydro-D-galactosidase, ZgGH129, has undergone a  
132 thorough kinetic characterization (Wallace, Guee, et al., 2020) and its fine specificity has also  
133 been examined, revealing a preference for unsulfated neo- $\beta$ -carrabiose motifs on the non-  
134 reducing end (Wallace, Ficko-Blean, & Stubbs, 2020; Wallace, Guee, et al., 2020). While the

135 catalytic activity of the other 3,6-anhydro-D-galactosidases from the GH127 family has been  
136 confirmed, qualitative analyses indicate they may not all share the same fine specificities  
137 (Ficko-Blean et al., 2017).

138 Since carrageenans have modified Gal $\alpha$ (1,3)Gal motifs and internal  $\beta$ -galactose in their  
139 motifs, they are of strong interest to be able to intervene in the sugar code. Thus, the aim of the  
140 study is to explore the possibilities of carrageenan-based galactans to interact with the human  
141 galactoside binding proteins Galectins 1 and 3 (Gal-1 and Gal-3) and human serum glycan  
142 binding antibodies. Four gelling carrageenans have been chosen, commercial  $\kappa$ - and  $\iota$ -types  
143 and natural hybrid carrageenans extracted under mild conditions from *Chondrus crispus* and  
144 *Furcellaria lumbricalis*. The enzymatic treatments to change the carrageenan structures  
145 enabled us to postulate which galactan components, either terminal or along the glycan chain  
146 itself, are responsible for binding to the galactoside-specific proteins. Using the carrageenan-  
147 specific marine bacterial enzymes, we demonstrate modulation of binding to natural human  
148 antibodies, including a monoclonal anti- $\alpha$ Gal Ab (m86) since natural human anti- $\alpha$ Gal  
149 antibodies include multiple subsets with slightly differing specificities (Bernth Jensen et al.,  
150 2021). We also demonstrate modulation of the selectivity for Gal-3 CRD over Gal-1, using  
151 the galectin inhibitor thiodigalactoside, which has implications to targeting specific biological  
152 pathways regulated by the galectins.

153

## 154 **Materials and methods**

155  $\kappa$ -Carrageenan was a kind gift from Sanofi Bio-Industries.

156 *The reagents purchased from Sigma, St. Louis, USA.*  $\iota$ -Carrageenan from *Euchema*  
157 *denticulatum* (cat no. C1138, lot no. SLCC4631), Ampicillin sodium salt (cat no. A9518), R-  
158 2A Agar (cat no. 17209, lot no. BCCB8737), sodium deoxycholate (cat no. D6750, lot no.  
159 MKBS0373V), sucrose (cat no. S0389, lot no. SLCF6079), lysozyme (cat no. L6876),  
160 Ammonium persulfate, for electrophoresis (cat no. A3678, lot no. 113K0656), silver nitrate  
161 (cat no. 31630, lot no. STBJ5526), Human Serum (cat no. P2918, lot no. SLCC8367), Anti-  
162 Human IgM ( $\mu$ -chain specific)-Peroxidase antibody produced in goat (cat no. A6907, lot no.  
163 SLCH6187), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (cat no. A 3219, lot no.  
164 MKCM9914), Streptavidin-HRP Solution (ORO3L, lot no. 3792178), Thiodigalactoside (cat.  
165 no. SML2310, lot no. 0000093539), Thrombin Inhibitor Screening Kit (Fluorometric) (cat.  
166 no. MAK243), Factor Xa Inhibitor Screening Kit (Fluorometric) (cat. no. MAK239)

167 *Reagents from other companies.* Celite 545 (Diatomeaceous silica for filtration) was  
168 from PROLABO (cat no. 22552.290, lot no. 93103, Paris, France), tryptone (cat no. BP9727,  
169 lot no. 137-676, Fisher BioReagents, USA), yeast extract (cat no. 1349-9539, lot no. A1985,  
170 Fisher Scientific, USA ), DNase I (cat no. 18405J, Interchim, France), imidazole (cat no.  
171 G3H4N2, lot no. 211276, ThermoScientific, Germany), N,N,N',N'-  
172 tetramethylethylenediamine (TEMED) (cat no. 87689, Fluka, USA), acrylamide/bis-  
173 acrylamide (37.5:1) solution 30% (cat no. 1610158, Bio-Rad Inc., France), Alcian Blue (cat  
174 no. J60122, lot no. U19F032, AlfaAesar, Germany), Carbo-bind plate (Costar, Corning Inc.,  
175 NY, USA), 96-well tissue culture treated sterilized polystyrene Imagelock microplate (cat no.  
176 4379, Sartorius, Essen BioScience, Ann Arbor, MI, USA). Human VEGF DuoSet ELISA (cat  
177 no. DY293B, R&D Systems, USA). Gal $\alpha$ 1-3Gal $\beta$ 1-4Glc carbohydrate sequence (cat no.  
178 OG44766, Biosynth Carbosynth)

179 *Reagents from Abcam.* Biotinylation Kit / Biotin Conjugation Kit (Fast, Type B) -  
180 Lightning-Link® (ab201796) composed of Quencher reagent, ab273995, lot no. GR3401609-  
181 1, Modifier reagent, ab273994, lot no. GR3404190-4; Biotin (Typ B) Conjugation Mix,  
182 ab274078, lot no. GR3395269-1. Human Galectin 1 Matched Antibody Pair Kit (ab218170)  
183 or Human Galectin 1 Detector antibody (Abcam, SNT-12499, lot no. 2101008040). Human  
184 Galectin 3 Detector antibody (Abcam, cat no. ab 270341, lot no. GR3405992-1). Human  
185 Galectin 3 Capture antibody (ab270340, lot no. GR3404762-1). human monoclonal antibodies  
186 to Alpha Gal [m86] (ab281899, lot no. GR3422108-1). TMB ELISA Substrate (High  
187 Sensitivity) (cat. no. ab171523, lot no. 2101010452). Recombinant human Galectin 1 protein  
188 (ab50237, lot no. GR322443-22 and GR322443-23). Recombinant human Galectin 3 protein  
189 (active) (ab89487, lot no. GR339273-2).

190 ÄKTA Avant Chromatographic System equipped with Unicorn™ software, SP  
191 Histrap™ FF column (5ml), Sephadex 200 gel packing were purchased from GE Healthcare  
192 Co., Ltd. (Chicago, IL, USA).

### 193 **Polysaccharide extraction**

194 *C. crispus* gametophytes and *F. lumbricalis* were harvested during low tide along the  
195 shore of Roscoff (Brittany, France) on December 15<sup>th</sup>, 2020 and September 3<sup>rd</sup>, 2015,  
196 respectively. After the harvest the algae were washed with water to remove impurities dried at  
197 40°C for 24 h, ground in powder by waring blender and weighed. The polysaccharides from  
198 both algae were isolated by aqueous extraction in milliQ water at 80°C for 4 h (w:v = 30 g : 2  
199 L). To remove the algal biomass residue, Celite 545 (20 g) was added to the resultant solution



200 followed by filtration by a paper filter or nylon membrane. The carrageenan polymer was  
201 recovered by precipitation with 4 volumes of ethanol, washed with ethanol and then dried  
202 overnight in a 45 °C oven. In order to remove excess salt and non-polymeric materials, the  
203 carrageenans were dialyzed (12-14 kDa MWCO Spectra/Por ®) against distilled water for 3  
204 days at room temperature followed by precipitation with ethanol (v : v = 1 : 4) and drying at  
205 45°C overnight. The carrageenan yield was calculated from the percentage of dried algae.

### 206 **Nuclear magnetic resonance**

207 One dimensional <sup>1</sup>H NMR spectra were recorded at 353K on a BRUKER AVANCE III  
208 HD 500 spectrometer equipped with an indirect 5 mm probehead BBFO 1H/ [BB]. NMR  
209 analyses were performed on samples dissolved in 700 uL of D<sub>2</sub>O 100%. The spectra were  
210 performed according BRUKER's pulse programs with standard pulse sequences of 2 s of  
211 delay, a 30° pulse and 64 scans. Chemical shifts were expressed in ppm relative to TMS as  
212 external reference.

### 213 **Media**

214 LB-media: Luria-Bertani medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g, 1 L H<sub>2</sub>O,  
215 autoclaved for 20 min at 115°C), LB/Amp-media: LB-media supplemented with ampicillin  
216 sodium salt (0.01%, w : v = 0.1 g : 1 L), LB/ Amp/agar-media: LB-media supplemented with  
217 agar (1.5 %) autoclaved for 15 min at 121°C after cooling down to 45-50°C ampicillin sodium  
218 salt was added (0.01 % w/v). ZYP 5052 media: After autoclavation of ZY solution (10 g  
219 tryptone, 5 g yeast extract, 925 mL water) the media was supplemented with the following  
220 solutions 1 mL 1M MgSO<sub>4</sub>, 1 mL 1000×trace metal mixture (1×conc = 50 μM Fe, 20 μM Ca,  
221 10 μM Mn, 10 μM Zn, 2 μM Co, 2 μM Cu, 2 μM Ni, 2 μM Mo, 2 μM Se, 2 μM H<sub>3</sub>BO<sub>3</sub>), 20  
222 mL 50×5052 (1×5052 = 0.5 % glycerol, 0.05% glucose, 0.2% α-lactose), 50 mL 20×NPS  
223 (1×NPS = 100 mM PO<sub>4</sub>, 25 mM SO<sub>4</sub>, 50 mM NH<sub>4</sub>, 100 mM Na, 50 mM K) + 1 mL  
224 ampicillin stock (100 mg/mL).

### 225 **Cloning, enzyme production and purification**

226 All genes coding for endolytic and exolytic carrageenan-related enzymes were cloned  
227 from genomic DNA extracted from the marine bacterium *Zobellia galactanivorans*  
228 (Barbeyron et al., 2016) into the ampicillin resistant plasmid pFO4 as previously described  
229 (Groisillier et al., 2010). The pFO4 plasmid incorporates a 6His tag on the N-terminus of the  
230 proteins for immobilized metal affinity chromatography (IMAC). The plasmids were  
231 transformed into the *E. coli* BL21 (DE3) strain for protein expression. The list of recombinant

232 enzymes is the following :  $\kappa$ -Carrageenase (Label: ZgGH16, locus ID: Zgal\_236, MW: 33.5  
233 kDa) (Matard-Mann et al., 2017),  $\iota$ -carrageenase (ZgGH82, Zgal\_4265, MW: 51.0 kDa  
234 (Barbeyron et al., 2000; Groisillier et al., 2010) and four 3,6-anhydro-D-galactosidases (3  
235 GH127 enzymes: ZGAL\_3147, MW: 77.0 kDa; ZGAL\_3148, MW: 75.0 kDa; ZGAL\_3150,  
236 MW: 78.8 kDa; and one GH129 enzyme: ZGAL\_3152, MW: 77.8 kDa) (Ficko-Blean et al.,  
237 2017; Wallace, Ficko-Blean, et al., 2020; Wallace, Guee, et al., 2020).

238 Prior to experimental use, the *E. coli* BL21(DE3) strains containing the target plasmids  
239 were maintained as frozen glycerol stocks at  $-80^{\circ}\text{C}$ . The culture cells were streaked out on  
240 LB/Ampicillin agar plates and incubated overnight at  $37^{\circ}\text{C}$ . The next day single cell colonies  
241 from the plates were used to initiate the starter cultures in 5 mL LB/Ampicillin media,  
242 incubated overnight at  $37^{\circ}\text{C}$  and shaking at 175 rpm. The starter culture (1mL) was used to  
243 inoculate 1 L of ZYP 5052 media (Studier, 2005) and which was cultivated 180 rpm and  
244  $20^{\circ}\text{C}$ . After 3 days, the cells were harvested by centrifugation (3000 g, 30 min,  $4^{\circ}\text{C}$ ).

245 A chemical lysis procedure was used to lyse the cells. First, the harvested cells were  
246 dissolved in 10 mL of resuspension buffer (25 % sucrose, 50mM Tris pH 8.0). After 10 min  
247 on ice with lysozyme (150  $\mu\text{L}$ , 100 mg/mL), 20 mL of lysis buffer containing 100 mM NaCl,  
248 1% Na deoxycholate, 1% Triton X-100, 20 mM Tris pH 8.0 was added and incubated 5 min  
249 on ice. The final step of the chemical lysis consisted in the addition of  $\text{MgCl}_2$  (to 5mM) and  
250 DNase I (150  $\mu\text{L}$ , 500 000 Units/mL in  $\text{CaCl}_2$  10 mM,  $\text{MgCl}_2$  10 mM, Tris 10 mM pH8) at  
251 room temperature for 5 minutes. The lysed cells were centrifuged at 20 000 g for 45 min at  
252  $4^{\circ}\text{C}$  and the supernatants containing the soluble protein were passed through 0.2  $\mu\text{m}$  filter.

253 Enzyme purification was performed by means of an ÄKTA Avant system with a 5 mL  
254 HisTrap FF column immobilized with  $\text{Ni}^{2+}$ . After loading the column with the filtered  
255 supernatant, the column was washed with 20 mM Tris pH 8.0 and 100 mM NaCl until there  
256 was no longer a change in the UV. The proteins were eluted in a linear imidazole gradient up  
257 to 0.5M imidazole, 25 mM Tris pH 8, 50mM NaCl. A second purification step was performed  
258 on a Sephadex-200 gel filtration column by isocratic elution with 50 mM HEPES pH 7.5 and  
259 100 mM NaCl. Purity of the protein samples was analyzed by SDS-PAGE.

## 260 **Digestion of polymers by endolytic enzymes**

261 Endolytic carrageenases ( $\kappa$ - and  $\iota$ -carrageenases) were incubated with the carrageenan  
262 polymer samples to release oligosaccharides. The carrageenans were treated three different  
263 ways: 1) digested with  $\iota$ -carrageenase, 2) digested with  $\kappa$ -carrageenase or 3) digested with  $\kappa$ -

264 and ι-carrageenase enzymes sequentially. All samples were kept in identical conditions (100  
265 rpm, 30°C, 0.5% carrageenan solutions (3 g/600 mL) in 50 mM NH<sub>4</sub>HCO<sub>3</sub> at pH 7). The  
266 sequential reactions were started by treatment with ι-carrageenase (1.2 μg/mL) over 5 days,  
267 heat denaturing for 25 mins at 70°C, followed by treatment with κ-carrageenase (1.2 μg/mL)  
268 over 4 days. The single enzymatic reactions were done by adding either ι-carrageenase (1.2  
269 μg/mL) or κ-carrageenase (1.2 μg/mL). All the reactions were stopped by heating for 25 min  
270 at 70°C in a water bath.

### 271 **Carbohydrate polyacrylamide gel electrophoresis**

272 Carbohydrate polyacrylamide gel electrophoresis (C-PAGE) (Zablackis & Perez, 1990)  
273 was used to qualitatively visualize the polydispersity of charged oligo-carrageenans after  
274 digestion with endolytic enzymes (ι- and/or κ-carrageenases). The concentration of  
275 acrylamide in the stacking and running gels was 6% and 27%, respectively. The upper and  
276 lower buffer chambers contained 50 mM Tris, pH 8.7 with 1 mM EDTA. The samples (20  
277 μL, 0.5 %) were mixed with 60 μL of sucrose-phenol red solution (10 % sucrose, 0.1 %  
278 phenol red), after that 2-3 μL of the mixture were loaded, and the samples were  
279 electrophoresed at ambient temperature and 200 V until 75% of the migration front of the gel  
280 length (visualized with the phenol red). The gels were placed into a big petri dish. The  
281 carbohydrates were fixed and stained in the gel with 0.5% alcian blue in milliQ H<sub>2</sub>O (10  
282 minutes, with agitation) and destained in milliQ H<sub>2</sub>O with agitation until the gel showed clear  
283 again. The silver staining of the glycans was performed by silver nitrate (0.4%, 10 min,  
284 without agitation). After washing 3 times with H<sub>2</sub>O under agitation, a solution of sodium  
285 carbonate/formaldehyde (7 g sodium carbonate and 80 μL formaldehyde in 100 mL milliQ  
286 H<sub>2</sub>O) was added, manually agitated and the reaction was stopped with 1 mL acetic acid added  
287 directly to the petri dish.

### 288 **Reducing sugar assay**

289 A reducing sugar assay was performed to measure the level of hydrolysis of the samples  
290 by the enzymes following the method developed by Lever (Lever, 1972). It is based on the  
291 reaction of reducing ends of sugars with p-hydroxybenzoic acid hydrazide (PAHBAH) which  
292 is stored at 5% in 0.5M HCl and prior to the assay diluted 10-fold in 0.5 M NaOH. The test  
293 was done in triplicate by heating the mixture of the sample (20 μL) and the PAHBAH reagent  
294 (180 μL) at 100°C for 5 min. The absorbance of the reaction mixtures was detected at 410 nm  
295 by a Spark 10 M microplate reader (Tecan, France).

## 296 **Modification of carrageenans by 3,6-anhydro-D-galactosidases**

297 The oligosaccharides from *C. crispus* and *F. lumbricalis* (0.7 % solutions in H<sub>2</sub>O)  
298 produced after endolytic digestion by  $\kappa$ - and  $\iota$ -carrageenases were treated with 3,6-anhydro-D-  
299 galactosidases. The enzymes were added to their respective solutions two times at 24 h  
300 intervals and incubated a total of 72 h at 25 °C. After the first enzyme addition the  
301 concentration of GH127-2, GH127-3, GH129 was 0.05 mg/mL and of GH127-1 was 0.005  
302 mg/mL. After the second enzyme addition the final enzyme concentration was 0.1 mg/mL for  
303 GH127-2, GH127-3, GH129 and 0.01 mg/mL for GH127-1. Controls contained only enzyme  
304 buffer solution in a volume equal to the volume of enzymes added. Carrageenan polymers  
305 were also treated with a mixture of the 3,6-anhydro-D-galactosidases with final concentrations  
306 of 0.025 mg/mL for GH127-2, GH127-3, GH129 and 0.0025 mg/mL for GH127-1. Controls  
307 contained only enzyme buffer solution in a volume equal to the volume of enzymes added.

## 308 **Exolytic 3,6-anhydro-D-galactosidase activity on artificial substrate**

309 The activity of the 3,6-anhydro-D-galactosidases was tested on an artificial substrate 4-  
310 nitrophenyl 3,6-anhydro- $\alpha$ -D-galactopyranoside (3,6-ADG-pNP) (Wallace, Guee, et al.,  
311 2020). The enzymes (ZgGH127-1, ZgGH127-2, ZgGH127-3 and ZgGH129, 5  $\mu$ g/mL, final)  
312 were incubated with 2 mM 3,6-ADG-pNP in water in an endpoint digestion over 2 days at  
313 ambient temperature, the development of the color reaction with the substrate was registered  
314 at 405 nm. The blank was with water instead of the 3,6-anhydro-D-galactosidases and control  
315 was with boiled mixture of enzymes.

## 316 **Purification of the carrageenan oligosaccharides**

317 Carrageenan oligosaccharides were ultrafiltrated via a 10 kDa CutOff membrane on  
318 Amicon® Ultra-15 10K centrifugal filter devices for volumes up to 15 mL (cat no.  
319 UFC901008D, UFC901024D, UFC901096D, Millipore, Sigma, USA) at 2500 g.  
320 Monosaccharides under 100 – 500 Da were removed by dialysis with Spectra/Por dialysis  
321 tubes (MWCO 100 - 500 Da) against ultrapure water.

## 322 **High pressure size exclusion chromatography (HPSEC)**

323 Filtered samples (200  $\mu$ L, 0.22  $\mu$ m) were injected on analytical Superdex 200 and  
324 Superdex peptide columns (Cytiva) connected in series. The elution was conducted in 0.1 M  
325 LiNO<sub>3</sub> using an isocratic Thermo Scientific Ultimate 3000 pump working at a flow rate of 0.5  
326 mL/min. The chromatography experiments were monitored using a Wyatt Optilab Rex  
327 refractive index detector. Chromeleon software (Thermo Scientific) was used for data

328 acquisitions. For comparison, in-house purified standards oligosaccharides were also injected  
329 (Guibet et al., 2008; Jouanneau, Boulenguer, Mazoyer, & Helbert, 2010).

### 330 **Thrombin and Factor Xa inhibitor screening capacity of carrageenans**

331 The ability of carrageenans to inhibit the coagulation factors Thrombin and Factor Xa  
332 was tested by fluorometric assay using kits: Thrombin Inhibitor Screening Kit (Fluorometric,  
333 cat. no. MAK243), Factor Xa Inhibitor Screening Kit (Fluorometric, cat. no. MAK239). The  
334 protocol follows Thrombin and Factor Xa enzymes which cleave a synthetic 7-Amino-4-  
335 methylcoumarin (AMC)-based peptide substrate to release fluorophore AMC. Fluorescence  
336 (ex = 350 nm/em = 450 nm) was measured in kinetic mode for 30–60 minutes at 37°C. In the  
337 presence of irreversible inhibitors (GGACK Dihydrochloride for Factor Xa, 10 mM, and  
338 PPACK Dihydrochloride, 2 mM, for Thrombin) the reaction was abolished. The  
339 concentration for carrageenans for anticoagulant activity was 1 mg/ml in water. Both  
340 Thrombin and Factors Xa screening assays were performed and the results calculated  
341 according to the manufacturer's instructions.

### 342 **Binding human serum antibodies and monoclonal human antibodies to $\alpha$ Gal [m86]**

343 To determine the binding ability of normal human serum (NHS) antibodies and  
344 monoclonal antibodies (mAb) to the alpha-Gal [m86] epitope, an indirect ELISA-based  
345 technique using sugars covalently linked to Carbo-bind® plate wells was applied. Briefly,  
346 Carbo-bind® plates were coated with 50  $\mu$ L of carrageenans (0.02, 0.2, and 2 mg/mL in  
347 phosphate-buffered saline (PBS), pH 7.5), sealed with plate seal tape and incubated overnight  
348 at 37°C. The plates were then heated at 60°C for 25 min, rinsed 2-4 times with washing buffer  
349 (1X PBS, 0.05% Tween 20) and blocked with 1 mg/mL of bovine serum albumin (BSA) for  
350 90 min while shaking then washed. Human serum (2000-fold dilution) in 1X PBS containing  
351 0.1 mg/mL BSA was added to the wells and incubated, shaking, for 2 h at room temperature.  
352 The mAb to  $\alpha$ Gal (0.1  $\mu$ g/mL) in 1X PBS containing 0.1 mg/mL BSA was added to the wells  
353 and incubated, shaking, overnight at 4°C with mAb to  $\alpha$ Gal. After washing, horseradish  
354 peroxidase-conjugated goat anti-human IgM ( $\mu$ -chain specific) was diluted 2000-fold in PBS  
355 with 0.1 mg/mL BSA buffer and 50  $\mu$ L was added per well for 1 h at room temperature, 400  
356 rpm shaking. The bound antibodies were detected by the reaction with the TMB substrate and  
357 the absorbance at 450 nm was measured. The mean absorbance of negative control wells  
358 (coating with buffer alone) was used to define the endpoint titer. Commercial Gal $\alpha$ 1-3Gal $\beta$ 1-  
359 4Glc (Biosynth Carbosynth) was used as positive control.

## 360 **Binding human Gal-1 and Gal-3 inhibition assay with thiodigalactoside**

361 The galectin binding assay was done on CARBO-bind® 96-well microwell plates. All  
362 reagents were added at 50 µL/well unless otherwise stated, after each step of the protocol the  
363 wells were rinsed with washing buffer (1X PBS, 0.05% Tween 20), and incubation, with the  
364 exception of the coating of the plates, was at ambient temperature. First, plates were coated  
365 with carrageenans (varying concentrations in 1X PBS, pH 7.5), covered with 96-well plate  
366 seal tape and incubated overnight at 37°C. This was followed by a brief shaking down of the  
367 overnight condensate and heating at 60°C for 25 min. Blocking was done with BSA (1% in  
368 1X PBS, pH 7.5), 90 min and shaking (400 rpm). Galectin 1 (12.5 ng/well or 50 µL of 18.66  
369 µM) and Galectin 3 (3.125 ng/well or 50 µL of 19.23 µM) were added in 1X PBS containing  
370 2 mM EDTA, pH 7.5 either with or without the inhibitor thiodigalactoside (TDG; 1.4 mM and  
371 incubated shaking for 2 h. BSA served as a negative control. The plate-bound Galectins 1 or 3  
372 were conjugated with biotin conjugated monoclonal Detector Galectin 1 (0.25 µg/mL) or  
373 Detector Galectin 3 (1 µg/mL) antibodies in blocking buffer [prior to this stage, unconjugated  
374 Detector antibody to Galectin 3 was biotinylated by the Biotinylation Kit / Biotin Conjugation  
375 Kit (Fast, Type B) - Lightning-Link® according to the manufacturer's instructions]. A  
376 streptavidin-peroxidase conjugate (0.5 µg/mL) was added with a TMB chromogenic substrate  
377 as an indicator at 450 nm to detect the plate-bound galectins bound to the Detector Galectins.  
378 The results are expressed as percentage mean value relative to the negative control (100 %).

## 379 **Statistical analysis**

380 All data are expressed as the arithmetic mean ± standard deviation. Activity data were  
381 tested for differences using one-way repeated measures analysis of variance (ANOVA) with  
382 Tukey post-hoc test. A probability value (P) less than 0.05 was considered significant.

383

## 384 **Results and discussion**

385 With growing understanding of the potential of biological responses originating from  
386 specific glycan interactions, tuning of the sugar code is important to achieving therapeutic  
387 benefit. Following the examples of polymers like heparin, which provides the basis for  
388 therapeutic oligosaccharides and glycomimetics (Courtois, 2009; Varki & Gagneux, 2015),  
389 we produced a library of oligo-galactans from the red algal carrageenans with varying degrees  
390 of sulfation. During the current investigation the focus was narrowed down to gelling type  
391 carrageenans (which contain high amounts of the 3,6-anhydro-D-galactose monosaccharide),

392 to examine the effect of the exolytic 3,6-anhydro-D-galactosidases after the action of the  
393 endolytic carrageenases.

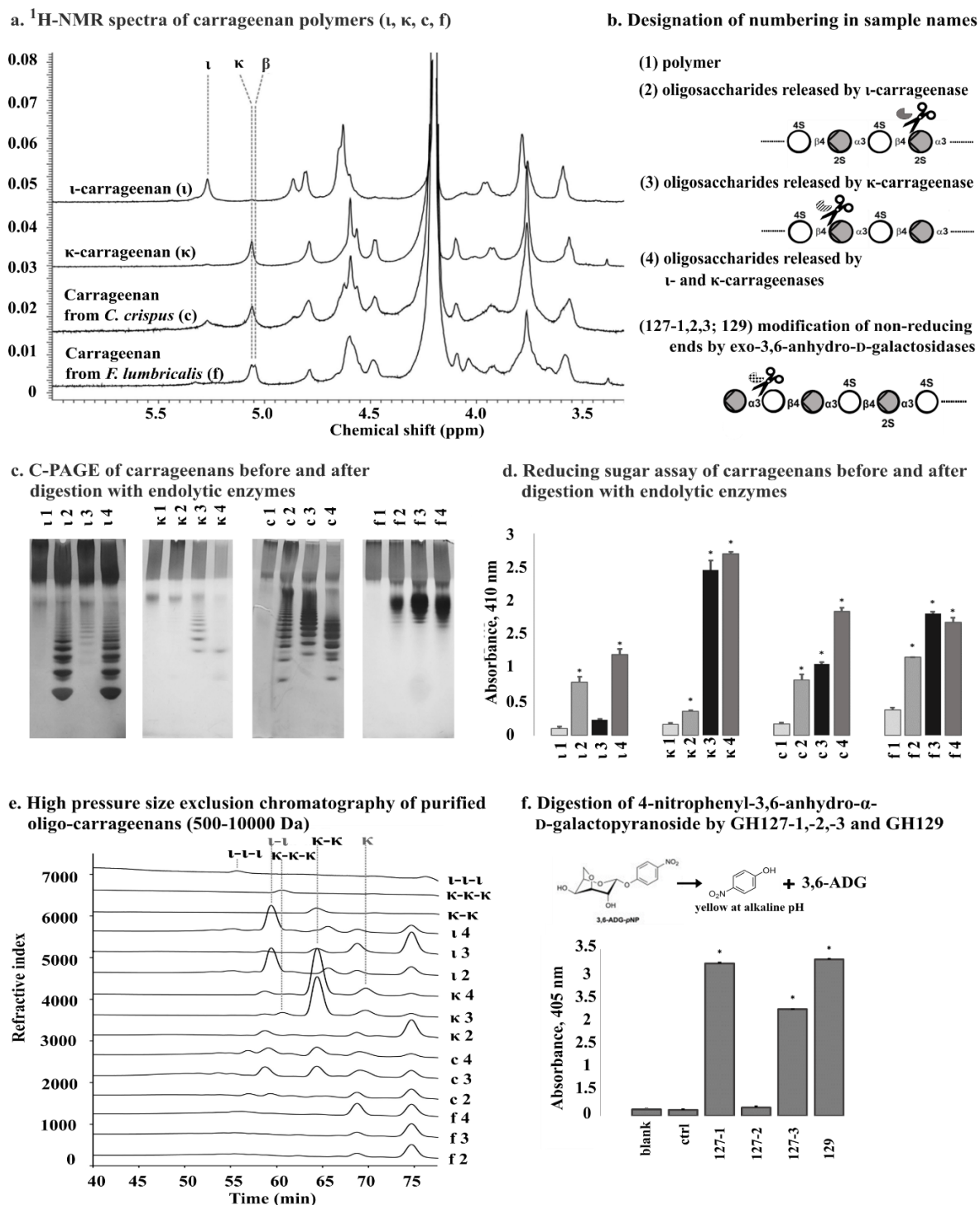
394 Four gelling carrageenan samples have been used in the current study. Commercial  $\kappa$ -  
395 and  $\iota$ -carrageenans were used as controls as well as two natural, hybrid carrageenans. Hybrid  
396 carrageenans have different sulfation motifs along the carrageenan chains which can affect  
397 their functional and rheological properties (Van de Velde, 2008) and are thus also of interest  
398 for bioactivity studies. The native carrageenans were gently extracted from the red algae *C.*  
399 *crispus* gametophyte and *F. lumbricalis* giving a yield after extraction, precipitation and  
400 dialysis of 9.52% and 16.44%, respectively. The structures of all carrageenan samples,  
401 including commercial, were determined by <sup>1</sup>H-NMR and their spectra are presented in Fig.  
402 1a. These data confirmed the attribution of commercial samples to  $\iota$ - and  $\kappa$ -types. Not  
403 unexpectedly, the native carrageenan structures found in the *C. crispus* gametophyte are  
404 mainly hybrid  $\iota/\kappa$ -motifs (Guibet et al., 2008) and the structures of the *F. lumbricalis* are  
405 mainly  $\kappa/\beta$ -motifs with a small portion of  $\iota$ -carrageenan (Correc et al., 2012; Rodriguez  
406 Sanchez, Matulewicz, & Ciancia, 2022).

407 The oligo-carrageenans produced after hydrolysis of the  $\beta$ -1,4-linkage by  $\kappa$ - and  $\iota$ -  
408 carrageenases (Fig. 1b) were characterized by carbohydrate polyacrylamide gel  
409 electrophoresis (C-PAGE) and the PAHBAH reducing sugar assay (Fig. 1c-d). The  
410 predominant electrophoretic pattern of oligo-carrageenan fragments in C-PAGE reflects  
411 separation by charge-to-mass ratio, with the less charged species migrating more slowly and  
412 the smaller species migrating more rapidly. Undigested (controls) or digested but still high-  
413 molecular-weight and/or less-charged carrageenans exhibit limited penetration into the  
414 polyacrylamide gel matrix and mostly remain in the stacking area. Our results demonstrate  
415 that after treatment of the commercial ( $\iota$  and  $\kappa$ ) and hybrid *C. crispus* and *F. lumbricalis* ( $\kappa/\iota$   
416 and  $\kappa/\beta$ ) carrageenans with either  $\kappa$ - or  $\iota$ -carrageenase alone, or both of the carrageenases  
417 sequentially ( $\iota$ -carrageenase first, followed by  $\kappa$ -carrageenase) it results in oligosaccharides  
418 with different migration patterns by C-PAGE. This is particularly evident with the hybrid  
419 carrageenans (Fig. 1c).

420 In some cases, the separation of the hybrid and commercial carrageenans is clearly  
421 visible in the gel, with the stacking area appearing emptier with the samples digested by  $\kappa$ -  
422 and  $\iota/\kappa$ -carrageenases relative to the polymer control (Fig 1c, samples k3, k4, c3, c4). The  
423  $\kappa/\beta$ -type oligo-carrageenans from *F. lumbricalis* poorly penetrate the gel matrix - probably  
424 due to their limited charge density. Since the C-PAGE technique depends largely on the

425 charge of the fragments to be qualitatively analyzed, we also evaluated the depth of the  
426 endolytic degradation by assaying the increase in free reducing ends by the PAHBAH  
427 reducing sugar method (Lever, 1972). This provided a quantitative method to monitor the  
428 substantial hydrolysis of low sulfated  $\kappa/\beta$ -carrageenan from *F. lumbricalis*. It was also  
429 confirmed that degradation of commercial  $\kappa$ - and  $\iota$ -carrageenans was highly specific to their  
430 appropriate enzymes,  $\kappa$ -carrageenase and  $\iota$ -carrageenase, respectively, though we did see  
431 some significant crossover activity of the  $\kappa$ -carrageenase on commercial  $\iota$ -carrageenan likely  
432 due to the presence of some  $\kappa$ -carrabiose motifs in the mainly  $\iota$ -carrageenan chain (Fig 1.  
433 ad), and vice versa for the  $\iota$ -carrageenase on commercial  $\kappa$ -carrageenan (Fig 1. ad).





434

435 **Figure 1. Characterization of carrageenans and enzymatically-produced oligocarrageenans. (a).**

436  $^1\text{H-NMR}$  spectra of the carrageenans used in this study. The designation of samples is assembled by

437 numbers (explained in section **b**) and letters: “ $\iota$ ” stands for commercial  $\iota$ -carrageenan “ $\kappa$ ” -  $\kappa$ -

438 carrageenan, “c” - carrageenan from *C. crispus*, and “f” - carrageenan from *F. lumbricalis*. (**b**) A

439 representative scheme of the nomenclature used to describe the polymers and oligosaccharides

440 released by endolytic enzymes and modified by exolytic ones: **1** - polymer, **2** -  $\iota$ -carrageenase, **3** -  $\kappa$ -

441 carrageenase, **4** - sequential double digestion with both endolytic enzymes; **127-1**, **-2**, **-3** or **129** -

442 modification by 3,6-anhydro-D-galactosidases GH127-1, GH127-2, GH127-3 or GH129. (**c-d**) C-

443 PAGE and reducing sugar assay (PAHBAH) of carrageenans before (**c**) and after (**d**) digestion with

444 endolytic enzymes. (**e**) High pressure size exclusion chromatography (HPSEC) of purified oligo-

445 carrageenans (500 - 10 000 Da). (**f**) Digestion of artificial substrate 4-nitrophenyl-3,6-anhydro- $\alpha$ -D-

446 galactopyranoside (3,6-ADG-pNP) by 3,6-anhydro-D-galactosidases GH127-1, GH127-2, GH127-3  
447 and GH129 where blank was with buffer instead of enzyme and control (ctrl) was with a boiled  
448 mixture of the enzymes. Statistical significance difference ( $P < 0.05$ ) of degradation in comparison to  
449 each initial polymer is marked with an asterisk (\*). Bars represent mean values of 2 independent  
450 experiments with each of them done in triplicates  $\pm$ SD.

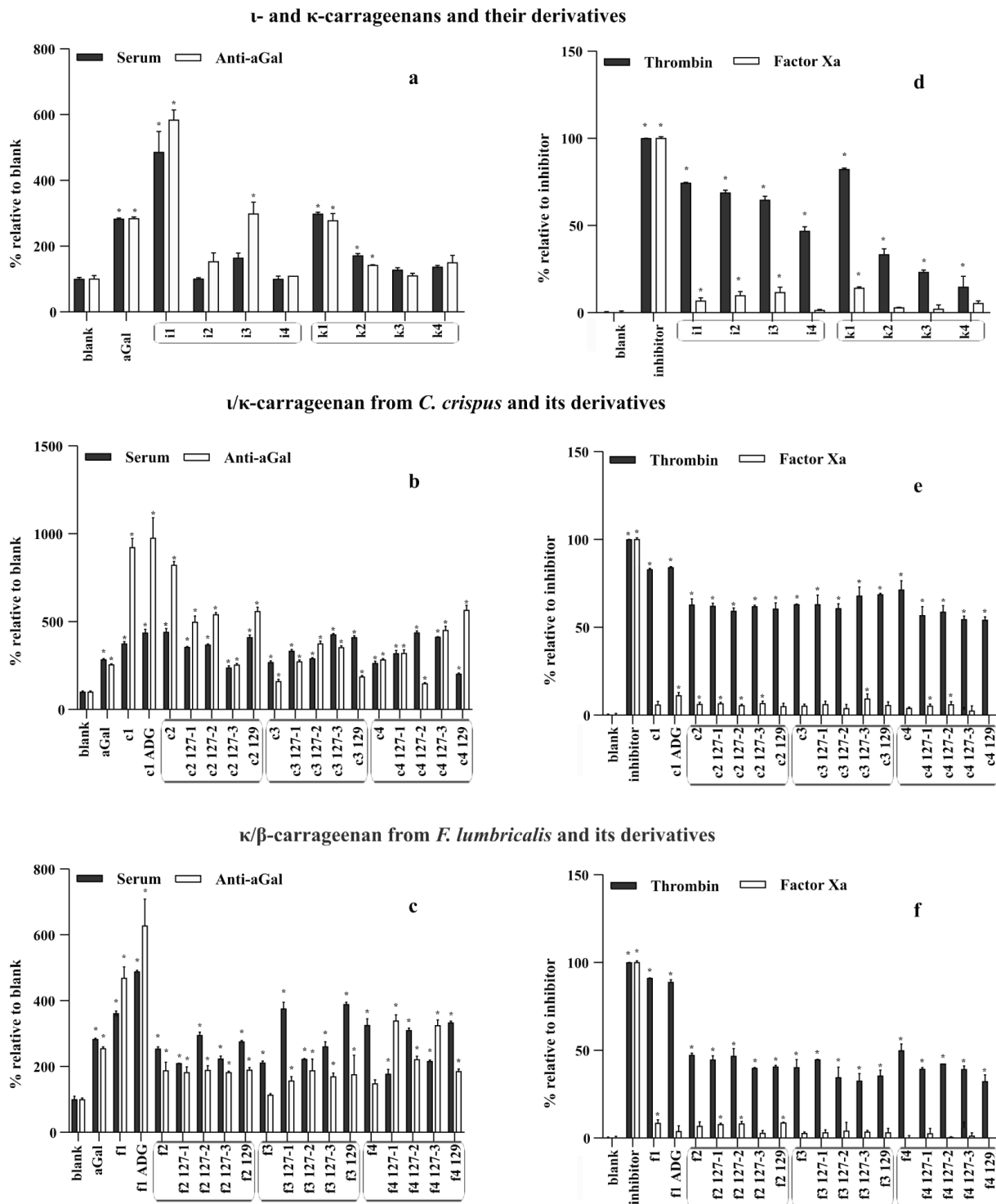
451 HPSEC analysis (Fig. 1e) confirms the production of oligosaccharides mixtures  
452 containing at least  $\kappa$  – di, tetra- and hexa-saccharides, and  $\iota$  – tetra and hexa-saccharides.  $\kappa$ -  
453 carrageenan hydrolyzed by  $\kappa$ -carrageenase or by both enzymes contained  $\kappa$ -tetrasaccharide as  
454 a major product, while  $\iota$ -carrageenan hydrolyzed by  $\iota$ -carrageenase or by both enzymes  
455 contained  $\iota$ -tetrasaccharide as a main product. Hydrolysates of carrageenans from *C. crispus*  
456 and *F. lumbricalis* gave more complex mixtures, containing hybrid oligosaccharides for  
457 which standards were not available.

458 After treatment with the specific endohydrolases, the oligosaccharides produced are  
459 from the neo-series, with 3,6-anhydro-D-galactose on the non-reducing end, though the  
460 sulfation pattern may vary. These oligosaccharides are thus predisposed to modification by  
461 the exolytic 3,6-anhydro-D-galactosidases. We first verified the activity of the 3,6-anhydro-D-  
462 galactosidases (ZgGH127-1, ZgGH127-2, ZgGH127-3, ZgGH129) on the artificial  
463 colorimetric substrate 3,6-ADG-pNP by endpoint reaction (Fig. 1f) (Wallace, Guee, et al.,  
464 2020). The data show that 3,6-ADG-pNP substrate was hydrolyzed by all the 3,6-anhydro-D-  
465 galactosidases except GH127-2 under the conditions assayed. In order to select an upper size  
466 limit of oligosaccharides, the samples were ultrafiltrated at 10 kDa. To ensure removal of the  
467 monosaccharide 3,6-anhydro-D-galactose (DA), which could alone have bioactive properties  
468 (Lee et al., 2019; Yun et al., 2013), a dialysis into water with a 100-500 Da membrane was  
469 also performed after treatment with the 3,6-anhydro-D-galactosidases.

470 In the present investigation two types of carrageenan-specific glycoside hydrolases were  
471 used: the depolymerizing endo-acting carrageenases, with preference for internal hydrolysis  
472 of the polysaccharide chain, and the exo-acting galactosidases, with specificity for hydrolysis  
473 of 3,6-anhydro-D-galactose found on the non-reducing (nr) end of oligosaccharides released  
474 by the endo-carrageenases (Fig. 1b). Glycan-protein interactions were assayed in a system  
475 where the reducing ends of sugars were covalently attached to the surface via a reaction of the  
476 open chain aldehydes with the hydrazide coated surface. The methods used to detect  
477 interactions between covalent surface-adhered glycans and proteins were ELISA-based and  
478 thus efficiently able to profile a large number of samples (Wolfenden, Cousin, Nangia-  
479 Makker, Raz, & Cloninger, 2015). The orientation in space and elimination of access to  
480 reducing ends gave us an opportunity to delve into the biochemistry of binding favored by

481 eukaryotic galactose-binding proteins important to communication using the sugar code. The  
482 tested proteins were polymorphic antibodies of total human serum, a monoclonal IgM anti-  
483  $\alpha$ Gal antibody (m86 mAb) and Galectins 1 and 3 (Gal-1 and Gal-3). The usefulness of using  
484 the CARBO-bind® plates is that they provide a multivalent surface for the antibody and lectin  
485 binding, which likely contributes to the avid binding of the protein partners.

486 Total human serum antibodies and monoclonal antibodies against Gal $\alpha$ (1,3)Gal, a  
487 minimal  $\alpha$ Gal epitope (Cunningham et al., 2013; Milland et al., 2007), (m86 mAb (Galili,  
488 LaTemple, & Radic, 1998)) were analyzed for capacity to bind carrageenans (Fig. 2a-c). As a  
489 positive control we used the trisaccharide Gal $\alpha$ 1-3Gal $\beta$ 1-4Glc covalently linked to the plate  
490 surface and leaving free the minimal  $\alpha$ Gal epitope on the non-reducing end. M86 mAb were  
491 IgM type and, out of human serum antibodies, only IgM variety was selected for  
492 determination because IgM human serum was shown to be far more reactive to the  $\alpha$ Gal  
493 epitope than IgG (Galili, Macher, Buehler, & Shohet, 1985; Sandrin, Vaughan, Dabkowski, &  
494 McKenzie, 1993). Our results demonstrated that IgM of both natural human serum and mAb  
495 were able to bind carrageenans (Fig. 2a-c). Similar binding intensities to natural antibodies  
496 and mAb were detected only for  $\iota$ - and  $\kappa$ -commercial carrageenans and their derivatives. Less  
497 signal was detected on the oligosaccharides, suggesting that the antibodies have enhanced  
498 binding to the carrageenan polymer 3D structure rather than the non-reducing ends of the  
499 carrageenan oligosaccharides (Fig. 2a). This endo-binding could be intra- or intermolecular.  
500 The signal intensity for binding is similar between the trisaccharide of the positive control and  
501  $\kappa$ -carrageenan polymer, but the signal with  $\iota$ -carrageenan polymer is twofold higher, thus  
502 sulfation degree may also affect antibody binding (Fig. 2a). The primary theory of binding by  
503 antibodies to the  $\alpha$ Gal epitope is end-on insertion (Milland et al., 2007), but the groove type  
504 binding of Gal $\alpha$ (1,3)Gal for some lectins has already been reported and the possibility of  
505 similar binding type in antibodies is implied (Yuriev et al., 2009). Our results support the  
506 possibility of groove type antibody binding, at least for the carrageenan-polymer based  
507 substrates.



508

509 **Figure 2. Activity of carrageenans towards human antibodies and anticoagulant factors. (a, b, c).**

510 Carrageenans ability to bind antibodies of human serum and m86 (monoclonal anti-αGal Ab) with a  
 511 trisaccharide α-Gal sequence (Galα1-3Galβ1-4Glc) as positive control. (d, e, f). Inhibition of  
 512 anticoagulant Factor Xa and Thrombin by carrageenans. Irreversible inhibitors GGACK  
 513 Dihydrochloride (10 mM) and PPACK Dihydrochloride (2 mM) were used for Factor Xa and  
 514 Thrombin, respectively. (a) and (d) corresponds to commercial ι and κ-carrageenans and their  
 515 enzymatic derivatives; (b) and (e) corresponds to *C. crispus* ι/κ-carrageenans and their enzymatic  
 516 derivatives; (c) and (f) corresponds to *F. lumbricalis* κ/β-carrageenans and their enzymatic derivatives.

517 The designation of the samples is assembled by letters and figures: “ι” stands for commercial ι-  
 518 carrageenan, “κ” - commercial κ-carrageenan, “c” - carrageenan from *C. crispus*, “f” - carrageenan

519 from *F. lumbricalis*. The numbering attributes to the initial polymer and after treatment with endolytic  
520 enzymes: **1** - polymer, **2** -  $\iota$ -Carrageenase, **3** -  $\kappa$ -carrageenase, **4**- digestion with both endolytic  
521 enzymes, 127-1, 127-2, 127-3, 129 correspond to the 3,6-anhydro-D-galactosidases ZgGH127-1,  
522 ZgGH127-2, ZgGH127-3 and ZgGH129, respectively; ADG – mixture of the four 3,6-anhydro-D-  
523 galactosidases. The asterisk (\*) indicates probability value  $P < 0.05$  relative to blank (100%) where no  
524 sugars were bound to the plate. The data are representative of triplicates from 2-4 independent  
525 experiments.

526 As with the commercial carrageenan polymers, the anti  $\alpha$ Gal mAb interactions showed  
527 more signal intensity with the sulfated *C. crispus*  $\kappa/\iota$ -carrageenans relative to the *F.*  
528 *lumbricalis*  $\kappa/\beta$ -carrageenans, for the serum antibodies it is less clear. The nuances of  
529 antibody exo-binding to the hybrid oligo-carrageenans were more profoundly studied by using  
530 3,6-anhydro-D-galactosidases to remove non-reducing 3,6-anhydro-D-galactose. *C. crispus*  
531 derivatives released by  $\iota$ -carrageenase (c2, c2 127-1, c2 127-2, and c2 129) and the double  
532 digest (c4 129) showed substantially more signal intensity for the monoclonal antibodies  
533 relative to the natural serum antibodies (Fig. 2b); however, the *C. crispus* oligosaccharide  
534 with the strongest signal is the c2 oligosaccharide, only treated with  $\iota$ -carrageenase, not with  
535 the 3,6-anhydro-D-galactosidases. This leads us to suggest that the increased signal for the c2  
536 oligosaccharide with mAb appears when there is a DANr, possibly sulfated in 2S since it was  
537 prepared with the  $\iota$ -carrageenase. On the other hand, treatment of the double digest c4 129  
538 sample is particularly interesting because there seems to be a much stronger selectivity for the  
539 mAb relative to the serum antibodies. Furthermore, the signal intensity of c4 gh129 is higher  
540 relative to the c4 for the mAb interaction. This leads us to suggest that the double digest  
541 followed by the GH129 3,6-anhydro-D-galactosidase treatment exposes a motif which has  
542 higher specificity for the mAb relative to the serum antibodies.

543 The *F. lumbricalis* carrageenan and its derivatives released by  $\iota$ -carrageenase (f2, f2  
544 127-1, f2 127-2, f2 127-3 and f2 129) and  $\kappa$ -carrageenase (f3, f3 127-1, f3 127-3, and f3 129)  
545 showed more signal intensity with serum antibodies relative to anti  $\alpha$ Gal mAb (Fig. 2c).  
546 Interestingly, this trend was not evident for the double digested f4 samples which gave a  
547 mixed preference for either serum antibodies (f4, f4 127-2, f4 129) or the anti  $\alpha$ Gal mAb (f4  
548 127-1, f4 127-3) (Fig. 2c). The highest signal for the *F. lumbricalis* serum antibodies with  
549 oligosaccharide was seen on f3 127-1 and f3 129 (treated with  $\kappa$ -carrageenase followed by the  
550 3,6-anhydro-D-galactosidases), suggesting a preference for Gnr or G4Snr. This contradictory  
551 result between mAb and the serum Abs is possibly related to the different specificities found  
552 in human serum antibodies. The monoclonal antibodies are specific to a minimal  $\alpha$ Gal epitope  
553 and this pivotal specificity is shared by some of the natural antibodies (Milland et al., 2007).  
554 However, the fine specificity to the full epitope is conferred to the natural antibodies by sugar

555 moieties downstream to the minimal recognition unit, such as Glc or GlcNAc residues  
556 (Milland et al., 2007). Carrageenans do not contain either Glc or GlcNAc and the galactose  
557 units themselves are often modified: firstly, by negatively charged sulfate groups and,  
558 secondly, by a hydrophobic 3,6-anhydro-bridge which sterically forces an inversion of the  
559 galactopyranose conformation (from  ${}^4C_1$  to  ${}^1C_4$ ) (Ciancia, Nosedá, Matulewicz, & Cerezo,  
560 1993; Ficko-Blean et al., 2015). These structural differences in the carrageenan derivatives  
561 may contribute to the differences in preference between the monoclonal and natural  
562 antibodies.

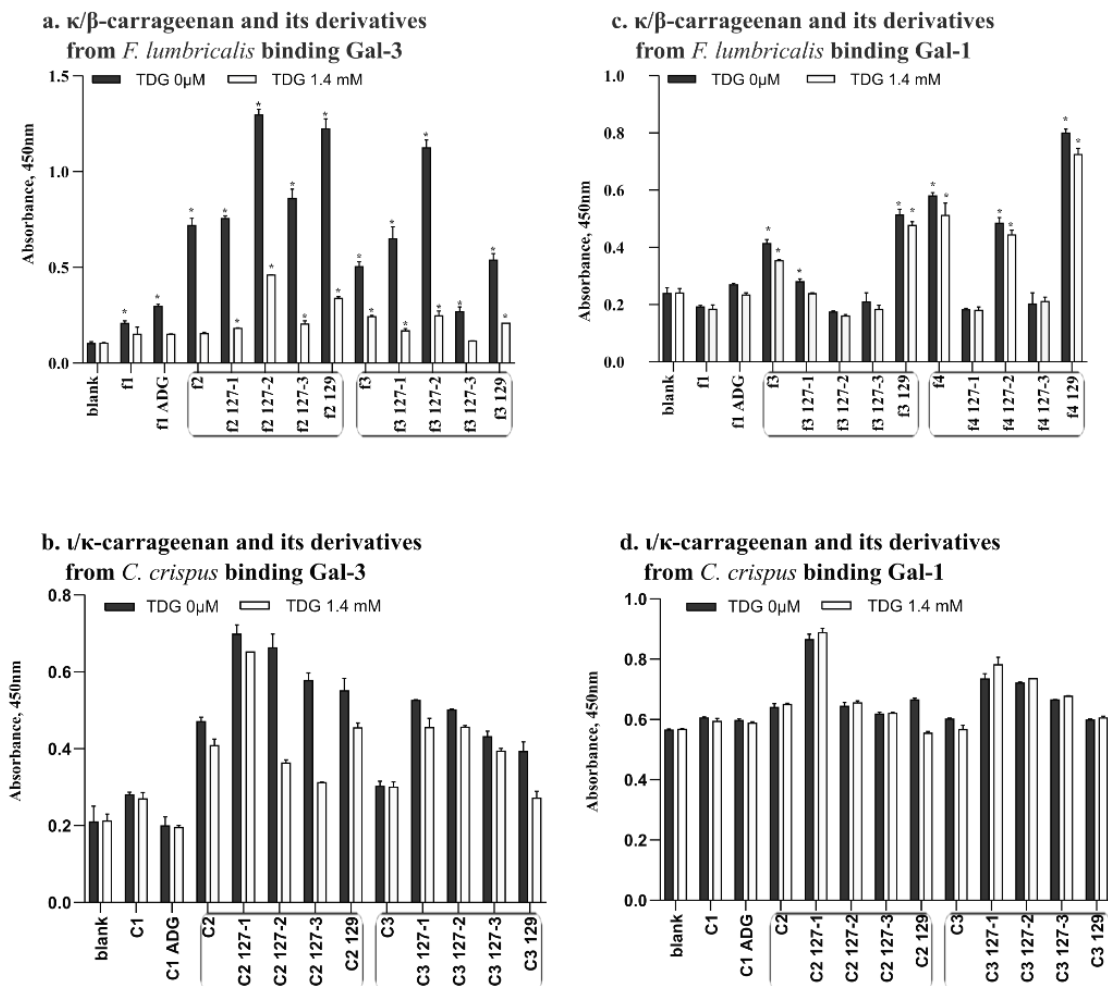
563 Strikingly, carrageenans can modulate blood protease cascades, with their sulfation  
564 patterns helping to determine their pro- or anti-coagulant properties (Pomin, 2012).  
565 Regiosulfation on the C2 of the anhydro- $\alpha$ -D-galactose and the C6 of the  $\beta$ -D-galactose has  
566 been shown to increase the carrageenan's ability to inhibit thrombin activity (de Araujo et al.,  
567 2013), however the importance of the 3,6-anhydro-D-galactoside has never been evaluated for  
568 anticoagulant activity. To this end, we evaluated the effect of the carrageenans and their  
569 potential for anticoagulation properties on thrombin and Factor Xa through an inhibition  
570 assay. For commercial  $\iota$ - and  $\kappa$ -carrageenan polymer we see a similar inhibition of thrombin  
571 and poor Factor Xa inhibition (Fig 2d). Decreasing the molecular weight reduced the  
572 thrombin inhibition more significantly for the  $\kappa$ -oligosaccharides than the  $\iota$ -oligosaccharides  
573 (Fig 2d). For *C. crispus* carrageenans the decrease in molecular weight by the endohydrolases  
574 (c2, c3, c4) and modification of the non-reducing ends by the 3,6-anhydro-D-galactosidases  
575 (GH127-1, -2, -3, and GH129) did not drastically affect the thrombin inhibitory activity  
576 relative to the polymer (c1) (Fig. 2e). For  $\kappa$ -commercial and *F. lumbricalis* carrageenans and  
577 their oligosaccharide derivatives, decreasing the molecular weight reduced the thrombin  
578 inhibition relative to the polymer (Fig. 2d,f). This may also be related to sulfation degree as  
579 both the  $\kappa$ -carrageenan and *F. lumbricalis* carrageenans (mainly hybrid  $\kappa/\beta$ -motifs (Correc et  
580 al., 2012)) are significantly less sulfated than  $\iota$ -carrageenan. In all cases, the inhibitory  
581 potential of carrageenans towards Factor Xa was not substantial in comparison to the positive  
582 irreversible inhibitor control (Fig. 2d-f).

583 The carrageenans were further tested with regard to their interaction with galectins. In  
584 general, galectins are a large family of glycan-binding proteins with preference for  $\beta$ -  
585 galactosides. Their subfamilies are classified by fine-tuned specificity for longer glycans of  
586 saccharides linked on either side of the  $\beta$ -galactosides (Nielsen et al., 2018). Galectins bind  
587 through two major types of interactions: a large hydrogen-bond network and hydrophobic

588 CH- $\pi$  interactions between the glycan and aromatic amino acid sidechains in the CRD  
589 (Modenutti, Capurro, Di Lella, & Marti, 2019). Gal-1 and Gal-3 have been chosen as target  
590 galectins because inhibitors selectively favoring one of them would be promising since both  
591 galectins have an assortment of functions (Nabi et al., 2015). Gal-3 has a binding affinity to  
592 oligosaccharide structures bearing the lacNAc motif internally while Gal-1 prefers terminal  
593 disaccharide lacNAc sequences (Laaf et al., 2019). Carrageenans contain endo- $\beta$ -D-galactose  
594 but no N-acetyl groups or any nitrogen atoms. The  $\alpha$ Gal epitope has been listed among  
595 galectin inhibitors (Hirabayashi et al., 2002) and since carrageenans bind the mAb anti  $\alpha$ Gal,  
596 they may also have potential for binding the galectins' CRD region. We thus chose to  
597 evaluate the inhibition potential of the carrageenans and their derivatives with Gal-1 and Gal-  
598 3. Since binding to the galectins may be within the CRD or elsewhere on the galectins, such  
599 as observed with modified citrus pectin and galactomannan polysaccharides (Stegmayr et al.,  
600 2016), the design of the experiment included a competitive inhibition assay with  
601 thiodigalactoside (TDG), a CRD inhibitor of Gal-1 ( $K_d = 24 \mu\text{M}$ ) and Gal-3 ( $K_d = 49 \mu\text{M}$ ).

602 Preliminary tests were done on all the carrageenans and their derivatives to determine  
603 the fractions that showed binding signal with Gal-1 and Gal-3. The carrageenans that showed  
604 strong signal intensity with the galectins were chosen for inhibition studies (Fig 3). The  
605 derivatives of commercial  $\kappa$ - and  $\iota$ -carrageenans did not exhibit binding (data not shown). Our  
606 results indicate that carrageenans can be naturally-sourced glycan partners of Gal-1 and Gal-3  
607 and they function as such when some structural requirements are met (Fig. 3a-d). Molecular  
608 mass, the structure of the non-reducing ends of oligo-carrageenans, and sulfation degree are  
609 features affecting galectin-binding properties of carrageenans. Thus, a decrease in molecular  
610 mass relative to the polymer improved binding signal intensity significantly with Gal-3 for all  
611 of the carrageenan derivatives tested and, to a lesser degree, with Gal-1 with only some of the  
612 carrageenan derivatives (Fig. 3a-d). This might be due to increased exo-binding as there are  
613 less endo-motifs in the shorter chain oligo-carrageenans. Unlike the oligo-carrageenans, with  
614 varied and significant capacities to bind Gal-1 and Gal-3 depending on sulfation degree and  
615 the treatment with the 3,6-anhydro-D-galactosidases, the polymers demonstrated less  
616 interactions with the galectins. Of the hybrid oligosaccharides tested, the oligo-carrageenans  
617 from *F. lumbricalis* were particularly interesting as the concentration used to show active  
618 binding was 0.5 mg/mL, whereas for the *C. crispus* oligo-carrageenans it was necessary to  
619 increase the concentration to 4 mg/mL. This is a striking difference between active

620 concentrations of the f2 and c2 oligosaccharides for Gal-3 which is tempting to relate to the  
 621 degree of sulfation on the oligosaccharides (Fig. 3 a,b).



622  
 623 **Figure 3. Binding of carrageenans and their derivatives from *C. crispus* (4 mg/mL) and *F.***  
 624 ***lumbricalis* (0.5 mg/mL) to Galectin 1 and 3 in the presence or absence of TDG (1396.3 µM).** Data  
 625 are representative of three replicates in three independent experiments (Mean ± Standard deviation).  
 626 The designation of the samples is assembled by letters and figures: “*ι*” stands for commercial *ι*-  
 627 carrageenan, “*κ*” - commercial *κ*-carrageenan. “*c*” - carrageenan from *C. crispus*, “*f*” - carrageenan  
 628 from *F. lumbricalis*. The numbering attributes to the initial polymer and after treatment with endolytic  
 629 enzymes: 1 - polymer, 2 - *ι*-carrageenase, 3 - *κ*-carrageenase, 4 - digestion with both endolytic  
 630 enzymes, 127-1, 127-2, 127-3, 129 correspond to the 3,6-anhydro-D-galactosidases ZgGH127-1,  
 631 ZgGH127-2, ZgGH127-3 and ZgGH129, respectively; ADG – mixture of the four 3,6-anhydro-D-  
 632 galactosidases. The asterisk (\*) indicates significant differences <0.05 relative to blank, where no  
 633 sugars were bound to the plate. The data are representative of triplicates from 2-4 independent  
 634 experiments.

635 There appears to be two modes of binding to Gal-3. The first mode of binding is via the  
 636 CRD region as seen with the inhibition by TDG and the *κ/β*-oligo-carrageenans from *F.*  
 637 *lumbricalis* (Fig. 3a). Further hydrolysis with the 3,6-anhydro-D-galactosidases improves the



638 binding signal significantly in some cases (f2 GH127-2, f2 GH129, f3 GH127-2). In general,  
639 the interaction with Gal-3 CRD was improved after 3,6-anhydro-D-galactosidase treatment of  
640 the *F. lumbricalis* oligosaccharides (Fig 1a). This suggests that a non-reducing  $\beta$ -galactose  
641 moiety is preferable for binding the Gal-3 CRD. ZgGH129 requires neo- $\beta$ -carrabiose on the  
642 non-reducing end, but can tolerate a subsequent  $\iota$ - or  $\kappa$ -motif during hydrolysis (Wallace,  
643 Ficko-Blean, et al., 2020; Wallace, Guee, et al., 2020). Hence, the products after reaction  
644 would contain a neutral G on the nr end but the subsequent disaccharide motif may be  
645 sulfated. The tangible increase in binding might also be due to improved avidity to Gal-3 after  
646 hydrolysis of DANr. The second mode of binding seems to be non-CRD binding-site mediated  
647 as in many cases the TDG shows poor to no inhibition with Gal-3 when incubated with the *C.*  
648 *crispus* oligo-carrageenans (Fig 3b). Notably, their active concentration was also 8-fold higher  
649 than for the *F. lumbricalis*  $\kappa/\beta$ -carrageenan derivatives. In any case, these  $\kappa/\iota$ -derivatives  
650 demonstrated decreased interaction with Gal-3 relative to the  $\kappa/\beta$ -carrageenan derivatives  
651 using this binding methodology. Overall, the *F. lumbricalis*  $\kappa/\beta$ -oligo-carrageenans have  
652 strong potential as saccharide-based ligands selective and specific for the Gal-3 CRD.

653 None of the Gal-1 binding to the carrageenans and its derivatives was inhibited by  
654 TDG, suggesting that all the interactions with the carrageenans are distal to the CRD  
655 carbohydrate-binding region (Fig 3cd). Still some of the hybrid  $\kappa/\beta$ -oligo-carrageenans from  
656 *F. lumbricalis* showed improved signal intensity (Fig 3c, f3 129, f4, f4 127-2, f4 129). The *C.*  
657 *crispus*  $\kappa/\iota$ -oligo-carrageenans showed poor binding in general (Fig 3d).

658

## 659 **Conclusion**

660 While details of the fine enzymatic specificities remain to be determined, the current  
661 research opens up possibilities for exploitation of carrageenan-specific enzymes to fine-tune  
662 the structure of carrageenan oligosaccharides for molecular selectivity of biologically  
663 important protein-carbohydrate interactions. The binding of carrageenan polymers relative to  
664 the enzymatically produced oligosaccharides suggests antibody binding through both groove-  
665 type and end-on insertion. Gal-3 CRD revealed exo-binding as the CRD demonstrated a  
666 preference for the oligo-carrageenans. Furthermore, treatment with one of the four 3,6-  
667 anhydro-D-galactosidases, ZgGH127-1, ZgGH127-2, ZgGH127-3, or ZgGH129, inflicted a  
668 substantial impact on Gal-3 CRD binding to the oligosaccharides and, thus, the structure of  
669 the non-reducing ends of oligo-carrageenans are a molecular determinant to binding the Gal-3  
670 CRD. Our findings make carrageenans alluring glycomimetics for future exploration as

671 multivalent ligands for human serum anti- $\alpha$ Gal IgM antibodies and as specific ligands  
672 selective for Gal-3 over Gal-1. These revelations could suggest a molecular basis for widely  
673 studied oligo-carrageenans' antitumor properties and provide more prospective potential of  
674 these molecules to anti-tumor research (Calvo et al., 2019; Torres, Florez-Fernandez, &  
675 Dominguez, 2021). One of the mechanisms to turn cold tumors with low responsiveness to  
676 immunotherapy into so-called hot or inflamed tumors is by increasing local inflammation  
677 (Ochoa de Olza, Navarro Rodrigo, Zimmermann, & Coukos, 2020). Local inflammation is a  
678 natural outcome of such potent inducers of complement as the Gal $\alpha$ (1,3)Gal reactive  
679 antibodies. Carrageenans and their derivatives are also prospective therapeutic agents in  
680 wound healing since they have the potential to affect both immune response and the activity  
681 of coagulation cascade components.

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690 The authors declare no competing interest.

## 691 **Declaration of generative AI in scientific writing**

692 The authors have not use any artificial intelligence (AI) and AI-assisted technologies in  
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## 694 **Author contributions**

695 **Ekatarina Sokolova:** Conceptualization, Investigation, Original Draft, Review & Editing

696 **Diane Jouanneau:** Investigation, Review & Editing

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699 **Nathalie Desban:** Investigation, Review & Editing

700 **Pierre Colas:** Conceptualization, Review & Editing

- 701 **Elizabeth Ficko-Blean:** Conceptualization, Supervision, Review & Editing
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