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Enzymatically-derived oligo-carrageenans interact with α -Gal antibodies and Galectin-3

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

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 (Gabijs, 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 & Gabijs, 2019). Among glycan ligands, galactans stand out for their medical
46 potential, mostly because humans produce staggering amounts of galactosyl-binding
47 (glyco)proteins as anti-Gal 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 α Gal
50 epitope, α -Galp-(1→3)- β -Galp-(1→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- α Gal
53 antibodies (Macher & Galili, 2008). Maintenance of high levels of natural anti- α Gal antibodies
54 is believed to be due to continual environmental exposure, for instance, to members of the adult
55 human gut microbiome producing an α Gal epitope (e.g. *Escherichia coli*, *Haemophilus*
56 *influenza* and *Lactobacillus* species) (Montassier et al., 2019). It is estimated that the amount
57 of anti- α Gal antibodies reaches up to 1-3% of all circulating Ig produced by about 1% of all B
58 cells, ensuring a strong innate immunity reaction. Upon precipitation of surfaces marked with
59 the epitope, the constant Fc parts of Ig triggers the natural immunity into action to battle virus
60 and tumors or recruit and activate tissue macrophages for skin regeneration (Galili, 2013), thus
61 making the antibodies interesting targets for improved wound healing.

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

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

83 Epitopes for galectins and anti- α Gal antibodies contain β -galactose in their motifs and the
84 α Gal antigen has proven to be a ligand for galectins as well (Gal-1 and Gal-3) (Hirabayashi et
85 al., 2002). Seaweed matrix polysaccharides are potential epitope mimics as they have
86 regiospecific sulfations (Ficko-Blean, Hervé, & Michel, 2015) but differ structurally from
87 animal sulfated mucosal glycans and glycosaminoglycans. Biologically active substances
88 derived from seaweeds are often sulfated carbohydrates covering multiple biological properties
89 like anticoagulant, antitumor, immunomodulatory and many others (Deniaud-Bouet, Hardouin,
90 Potin, Kloareg, & Herve, 2017; Huang, Tan, & Nie, 2022). Among these algal sulfated
91 polysaccharides, carrageenans are a natural source of galactose-containing glycans with an
92 appealing array of biological properties (Bhattacharyya et al., 2010; Carlucci et al., 1997;
93 Cheong, Qiu, Du, Liu, & Khan, 2018; Cosenza, Navarro, Pujol, Damonte, & Stortz, 2015).
94 Notably, they exhibit strong antiviral properties when they are administered topically to the
95 mucosa of the nasal cavity and the upper respiratory tract (Graf et al., 2018; Pereira & Critchley,
96 2020). These linear polymers are composed of alternating 3-linked β -D-galactopyranosyl units
97 (G-unit) and 4-linked α -D-galactopyranosyl units (D-unit). In gelling carrageenans the latter is
98 mainly 4-linked 3,6-anhydro- α -D-galactopyranosyl (DA-unit) such as found in β - (G4-DA), κ -
99 (G4S-DA) or ι - (G4S-DA2S) carrageenan motifs. The structures of carrageenans are well
100 known and have been reviewed (Ciancia, Matulewicz, & Tuvikene, 2020; Ficko-Blean et al.,

101 2015). The designation of G-, D-, DA- is made in accordance to the nomenclature proposed by
102 Knutsen and co-authors (Knutsen, Myslabodski, Larsen, & Usov, 1994).

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

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

128 The exolytic 3,6-anhydro-D-galactosidase enzymes (ZgGH127-1, ZgGH127-2,
129 ZgGH127-3 and ZgGH129), were recently discovered in the first described carrageenan PUL
130 (Ficko-Blean et al., 2017). They are specific for the removal of the α -1,3-DA monosaccharide
131 residue from the non-reducing end of neo-carrageenan oligosaccharides, this results in a non-
132 reducing end β -1,4-G residue after exohydrolase treatment. One 3,6-anhydro-D-galactosidase,
133 ZgGH129, has undergone a thorough kinetic characterization (Wallace, Guee, et al., 2020) and

134 its fine specificity has also been examined, revealing a preference for unsulfated neo- β -
135 carrabiose motifs on the non-reducing end (Wallace, Ficko-Blean, & Stubbs, 2020; Wallace,
136 Guee, et al., 2020). While the catalytic activity of the other 3,6-anhydro-D-galactosidases from
137 the GH127 family has been confirmed, qualitative analyses indicate they may not all share the
138 same fine specificities; what is known is that they are able to remove the 3,6-anhydro-D-
139 galactose residue from natural neo- β - κ - κ DP6 oligosaccharides obtained from *Furcellaria*
140 *lumbricalis* (Ficko-Blean et al., 2017).

141 Since carrageenans have modified Gal α (1,3)Gal motifs and internal β -galactose in their
142 motifs, they are probably able to intervene in processes involving sugar codes. Thus, the aim of
143 the study is to explore the possibilities of carrageenan-based galactans to interact with the
144 human galactoside binding proteins Galectins 1 and 3 (Gal-1 and Gal-3) and human serum
145 glycan binding antibodies. Using carrageenan-specific marine bacterial enzymatic treatments
146 to change the carrageenan structures should enable us to postulate which galactan components,
147 either terminal or along the glycan chain itself, are responsible for binding to the galactoside-
148 specific proteins.

149

150 **Materials and methods**

151 κ -Carrageenan was a kind gift from Sanofi Bio-Industries.

152 *The reagents purchased from Sigma, St. Louis, USA.* ι -Carrageenan from *Euchema*
153 *denticulatum* (cat no. C1138, lot no. SLCC4631), Ampicillin sodium salt (cat no. A9518), R-
154 2A Agar (cat no. 17209, lot no. BCCB8737), sodium deoxycholate (cat no. D6750, lot no.
155 MKBS0373V), sucrose (cat no. S0389, lot no. SLCF6079), lysozyme (cat no. L6876),
156 Ammonium persulfate, for electrophoresis (cat no. A3678, lot no. 113K0656), silver nitrate (cat
157 no. 31630, lot no. STBJ5526), Human Serum (cat no. P2918, lot no. SLCC8367), Anti-Human
158 IgM (μ -chain specific)-Peroxidase antibody produced in goat (cat no. A6907, lot no.
159 SLCH6187), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (cat no. A 3219, lot no.
160 MKCM9914), Streptavidin-HRP Solution (ORO3L, lot no. 3792178), Thiodigalactoside (cat.
161 no. SML2310, lot no. 0000093539), Thrombin Inhibitor Screening Kit (Fluorometric) (cat. no.
162 MAK243), Factor Xa Inhibitor Screening Kit (Fluorometric) (cat. no. MAK239)

163 *Reagents from other companies.* Celite 545 (Diatomeaceous silica for filtration) was from
164 PROLABO (cat no. 22552.290, lot no. 93103, Paris, France), tryptone (cat no. BP9727, lot no.
165 137-676, Fisher BioReagents, USA), yeast extract (cat no. 1349-9539, lot no. A1985, Fisher

166 Scientific, USA), DNase I (cat no. 18405J, Interchim, France), imidazole (cat no. G3H4N2, lot
167 no. 211276, ThermoScientific, Germany), N,N,N',N'-tetramethylethylenediamine (TEMED)
168 (cat no. 87689, Fluka, USA), acrylamide/bis-acrylamide (37.5:1) solution 30% (cat no.
169 1610158, Bio-Rad Inc., France), Alcian Blue (cat no. J60122, lot no. U19F032, AlfaAesar,
170 Germany), Carbo-bind plate (Costar, Corning Inc., NY, USA), 96-well tissue culture treated
171 sterilized polystyrene Imagelock microplate (cat no. 4379, Sartorius, Essen BioScience, Ann
172 Arbor, MI, USA). Human VEGF DuoSet ELISA (cat no. DY293B, R&D Systems, USA).
173 Gal α 1-3Gal β 1-4Glc carbohydrate sequence (cat no. OG44766, Biosynth Carbosynth)

174 *Reagents from Abcam.* Biotinylation Kit / Biotin Conjugation Kit (Fast, Type B) -
175 Lightning-Link® (ab201796) composed of Quencher reagent, ab273995, lot no. GR3401609-
176 1, Modifier reagent, ab273994, lot no. GR3404190-4; Biotin (Typ B) Conjugation Mix,
177 ab274078, lot no. GR3395269-1. Human Galectin 1 Matched Antibody Pair Kit (ab218170) or
178 Human Galectin 1 Detector antibody (Abcam, SNT-12499, lot no. 2101008040). Human
179 Galectin 3 Detector antibody (Abcam, cat no. ab 270341, lot no. GR3405992-1). Human
180 Galectin 3 Capture antibody (ab270340, lot no. GR3404762-1). human monoclonal antibodies
181 to Alpha Gal [m86] (ab281899, lot no. GR3422108-1). TMB ELISA Substrate (High
182 Sensitivity) (cat. no. ab171523, lot no. 2101010452). Recombinant human Galectin 1 protein
183 (ab50237, lot no. GR322443-22 and GR322443-23). Recombinant human Galectin 3 protein
184 (active) (ab89487, lot no. GR339273-2).

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

188 **Polysaccharide extraction**

189 *C. crispus* gametophytes and *F. lumbricalis* were harvested during low tide along the
190 shore of Roscoff (Brittany, France) on December 15th, 2020 and September 3rd, 2015,
191 respectively. After the harvest the algae were washed with water to remove impurities, dried in
192 an oven at 40°C for 24 h, ground in powder by Waring blender and weighed. The
193 polysaccharides from both algae were isolated by aqueous extraction in MilliQ water at 80°C
194 for 4 h (w:v = 30 g : 2 L). To remove the algal biomass residue, Celite 545 (20 g) was added to
195 the resultant solution followed by filtration by a paper filter or nylon membrane. The
196 carrageenan polymer was recovered by precipitation with 4 volumes of ethanol, washed with
197 ethanol and then dried overnight in a 45 °C oven. In order to remove excess salt and non-
198 polymeric materials, the carrageenans were dialyzed (12-14 kDa MWCO Spectra/Por ®)

199 against distilled water for 3 days at room temperature followed by precipitation with ethanol (v
200 : v = 1 : 4) and drying at 45°C overnight. The carrageenan yield was calculated from the
201 percentage of dried algae.

202 **Nuclear magnetic resonance**

203 One dimensional ¹H NMR spectra were recorded at 353K on a BRUKER AVANCE III
204 HD 500 spectrometer equipped with an indirect 5 mm probehead BBFO 1H/ [BB]. NMR
205 analyses were performed on samples dissolved in D₂O (10 mg/mL). The spectra were collected
206 according BRUKER's pulse programs with standard pulse sequences of 2 s of delay, a 30° pulse
207 and 64 scans. Chemical shifts were expressed in ppm relative to TMS as external reference.

208 **Media**

209 LB-media: Luria-Bertani medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g, 1 L H₂O,
210 autoclaved for 20 min at 115°C), LB/Amp-media: LB-media supplemented with ampicillin
211 sodium salt (0.01%, w : v = 0.1 g : 1 L), LB/ Amp/agar-media: LB-media supplemented with
212 agar (1.5 %) autoclaved for 15 min at 121°C after cooling down to 45-50°C ampicillin sodium
213 salt was added (0.01 % w/v). ZYP 5052 media: After autoclavation of ZY solution (10 g
214 tryptone, 5 g yeast extract, 925 mL water) the media was supplemented with the following
215 solutions 1 mL 1M MgSO₄, 1 mL 1000×trace metal mixture (1×conc = 50 μM Fe, 20 μM Ca,
216 10 μM Mn, 10 μM Zn, 2 μM Co, 2 μM Cu, 2 μM Ni, 2 μM Mo, 2 μM Se, 2 μM H₃BO₃), 20
217 mL 50×5052 (1×5052 = 0.5 % glycerol, 0.05% glucose, 0.2% α-lactose), 50 mL 20×NPS
218 (1×NPS = 100 mM PO₄, 25 mM SO₄, 50 mM NH₄, 100 mM Na, 50 mM K) + 1 mL ampicillin
219 stock (100 mg/mL).

220 **Cloning, enzyme production and purification**

221 All genes coding for endolytic and exolytic carrageenan-related enzymes were cloned
222 from genomic DNA extracted from the marine bacterium *Zobellia galactanivorans* (Barbeyron
223 et al., 2016) into the ampicillin resistant plasmid pFO4 as previously described (Groisillier et
224 al., 2010). The pFO4 plasmid incorporates a 6His tag on the N-terminus of the proteins for
225 immobilized metal affinity chromatography (IMAC). The plasmids were transformed into the
226 *E. coli* BL21 (DE3) strain for protein expression. The list of recombinant enzymes is the
227 following : κ-Carrageenase (Label: ZgGH16, locus ID: Zgal_236, MW: 33.5 kDa) (Matard-
228 Mann et al., 2017), ι-carrageenase (ZgGH82, Zgal_4265, MW: 51.0 kDa (Barbeyron et al.,
229 2000; Groisillier et al., 2010) and four 3,6-anhydro-D-galactosidases (3 GH127 enzymes:
230 ZGAL_3147, MW: 77.0 kDa; ZGAL_3148, MW: 75.0 kDa; ZGAL_3150, MW: 78.8 kDa; and

231 one GH129 enzyme: ZGAL_3152, MW: 77.8 kDa) (Ficko-Blean et al., 2017; Wallace, Ficko-
232 Blean, et al., 2020; Wallace, Guee, et al., 2020).

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

240 A chemical lysis procedure was used to lyse the cells. First, the harvested cells were
241 suspended in 10 mL of resuspension buffer (25 % sucrose, 50mM Tris pH 8.0). After 10 min
242 on ice with lysozyme (150 µL, 100 mg/mL), 20 mL of lysis buffer containing 100 mM NaCl,
243 1% Na deoxycholate, 1% Triton X-100, 20 mM Tris pH 8.0 was added and incubated 5 min on
244 ice. The final step of the chemical lysis consisted in the addition of MgCl₂ (to 5mM) and DNase
245 I (150 µL, 500 000 Units/mL in CaCl₂ 10 mM, MgCl₂ 10 mM, Tris 10 mM pH8) at room
246 temperature for 5 minutes. The lysed cells were centrifuged at 20 000 g for 45 min at 4°C and
247 the supernatants containing the soluble protein were passed through 0.2 µm filter.

248 Enzyme purification was performed by means of an ÄKTA Avant system with a 5 mL
249 HisTrap FF column immobilized with Ni²⁺. After loading the column with the filtered
250 supernatant, the column was washed with 20 mM Tris pH 8.0 and 100 mM NaCl until there
251 was no longer a change in the UV. The proteins were eluted in a linear imidazole gradient up
252 to 0.5M imidazole, 25 mM Tris pH 8, 50mM NaCl. A second purification step was performed
253 on a Sephadex-200 gel filtration column by isocratic elution with 50 mM HEPES pH 7.5 and
254 100 mM NaCl. Purity of the protein samples was analyzed by SDS-PAGE.

255 **Digestion of polymers by endolytic enzymes**

256 Endolytic carrageenases (κ - and ι -carrageenases) were incubated with the carrageenan
257 polymer samples to release oligosaccharides. The carrageenans were treated three different
258 ways: 1) digested with ι -carrageenase, 2) digested with κ -carrageenase or 3) digested with κ -
259 and ι -carrageenase enzymes sequentially. All samples were kept in identical conditions (100
260 rpm, 30°C, 0.5% carrageenan solutions (3 g/600 mL) in 50 mM NH₄HCO₃ at pH 7). The
261 sequential reactions were started by treatment with ι -carrageenase (1.2 µg/mL) over 5 days,
262 heat denaturing for 25 mins at 70°C, followed by treatment with κ -carrageenase (1.2 µg/mL)

263 over 4 days. The single enzymatic reactions were done by adding either ι -carrageenase (1.2
264 $\mu\text{g}/\text{mL}$) or κ -carrageenase (1.2 $\mu\text{g}/\text{mL}$). All the reactions were stopped by heating for 25 min at
265 70°C in a water bath.

266 **Carbohydrate polyacrylamide gel electrophoresis**

267 Carbohydrate polyacrylamide gel electrophoresis (C-PAGE) (Zablackis & Perez, 1990)
268 was used to qualitatively visualize the polydispersity of charged oligo-carrageenans after
269 digestion with endolytic enzymes (ι - and/or κ -carrageenases). The concentration of acrylamide
270 in the stacking and running gels was 6% and 27%, respectively. The upper and lower buffer
271 chambers contained 50 mM Tris, pH 8.7 with 1 mM EDTA. The samples (20 μL , 0.5 %) were
272 mixed with 60 μL of sucrose-phenol red solution (10 % sucrose, 0.1 % phenol red), after that
273 2-3 μL of the mixture were loaded, and the samples were electrophoresed at ambient
274 temperature and 200 V until 75% of the migration front of the gel length (visualized with the
275 phenol red). The gels were placed into a big petri dish. The carbohydrates were fixed and stained
276 in the gel with 0.5% alcian blue in MilliQ H_2O (10 minutes, with agitation) and destained in
277 MilliQ H_2O with agitation until the gel showed clear again. The silver staining of the glycans
278 was performed by silver nitrate (0.4%, 10 min, without agitation). After washing 3 times with
279 H_2O under agitation, a solution of sodium carbonate/formaldehyde (7 g sodium carbonate and
280 80 μL formaldehyde in 100 mL MilliQ H_2O) was added, manually agitated and the reaction
281 was stopped with 1 mL acetic acid added directly to the petri dish.

282 **Reducing sugar assay**

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

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

291 The oligosaccharides from *C. crispus* and *F. lumbricalis* (0.7 % solutions in H_2O)
292 produced after endolytic digestion by κ - and ι -carrageenases were treated with the 3,6-anhydro-
293 D-galactosidases or with the enzyme buffer as a control, otherwise all samples were treated the
294 same. The enzymes or enzyme buffer were added to their respective solutions two times at 24

295 h intervals and incubated a total of 72 h at 25 °C. After the first enzyme addition the
296 concentration of GH127-2, GH127-3, GH129 was 0.05 mg/mL and of GH127-1 was 0.005
297 mg/mL. After the second enzyme addition the final enzyme concentration was 0.1 mg/mL for
298 GH127-2, GH127-3, GH129 and 0.01 mg/mL for GH127-1. Carrageenan polymers were also
299 treated with a mixture of the 3,6-anhydro-D-galactosidases with final concentrations of 0.025
300 mg/mL for GH127-2, GH127-3, GH129 and 0.0025 mg/mL for GH127-1. Controls contained
301 only enzyme buffer solution in a volume equal to the volume of enzymes added.

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

303 The activity of the 3,6-anhydro-D-galactosidases was tested on an artificial substrate 4-
304 nitrophenyl 3,6-anhydro- α -D-galactopyranoside (3,6-ADG-pNP) (Wallace, Guee, et al., 2020).
305 The enzymes (ZgGH127-1, ZgGH127-2, ZgGH127-3 and ZgGH129, 5 μ g/mL, final) were
306 incubated with 2 mM 3,6-ADG-pNP in water in an endpoint digestion over 2 days at ambient
307 temperature, the development of the color reaction with the substrate was registered at 405 nm.
308 The blank was with water instead of the 3,6-anhydro-D-galactosidases and control was with
309 boiled mixture of enzymes.

310 **Purification of the carrageenan oligosaccharides**

311 Carrageenan oligosaccharides, treated with the 3,6-anhydro-D-galactosidases and the
312 buffer controls, were ultrafiltrated via a 10 kDa CutOff membrane on Amicon® Ultra-15 10K
313 centrifugal filter devices for volumes up to 15 mL (cat no. UFC901008D, UFC901024D,
314 UFC901096D, Millipore, Sigma, USA) at 2500 g. Monosaccharides under 100 – 500 Da were
315 removed by dialysis with Spectra/Por dialysis tubes (MWCO 100 - 500 Da) against ultrapure
316 water.

317 **High performance size exclusion chromatography (HPSEC)**

318 Filtered samples (200 μ L, 0.22 μ m) were injected on analytical Superdex 200 and
319 Superdex peptide columns (Cytiva) connected in series. The elution was conducted in 0.1 M
320 LiNO₃ using an isocratic Thermo Scientific Ultimate 3000 pump working at a flow rate of 0.5
321 mL/min. The elution was monitored using a Wyatt Optilab Rex refractive index detector.
322 Chromeleon software (Thermo Scientific) was used for data acquisitions. For comparison, in-
323 house purified standards oligosaccharides were also injected (Guibet et al., 2008; Jouanneau,
324 Boulenguer, Mazoyer, & Helbert, 2010).

325 **Thrombin and Factor Xa inhibitor screening capacity of carrageenans**

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

337 **Binding human serum antibodies and monoclonal human antibodies to α Gal [m86]**

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

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

357 The galectin binding assay was done on CARBO-bind® 96-well microwell plates. All
358 reagents were added at 50 μ L/well unless otherwise stated, after each step of the protocol the

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

375 **Statistical analysis**

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

379

380 **Results and discussion**

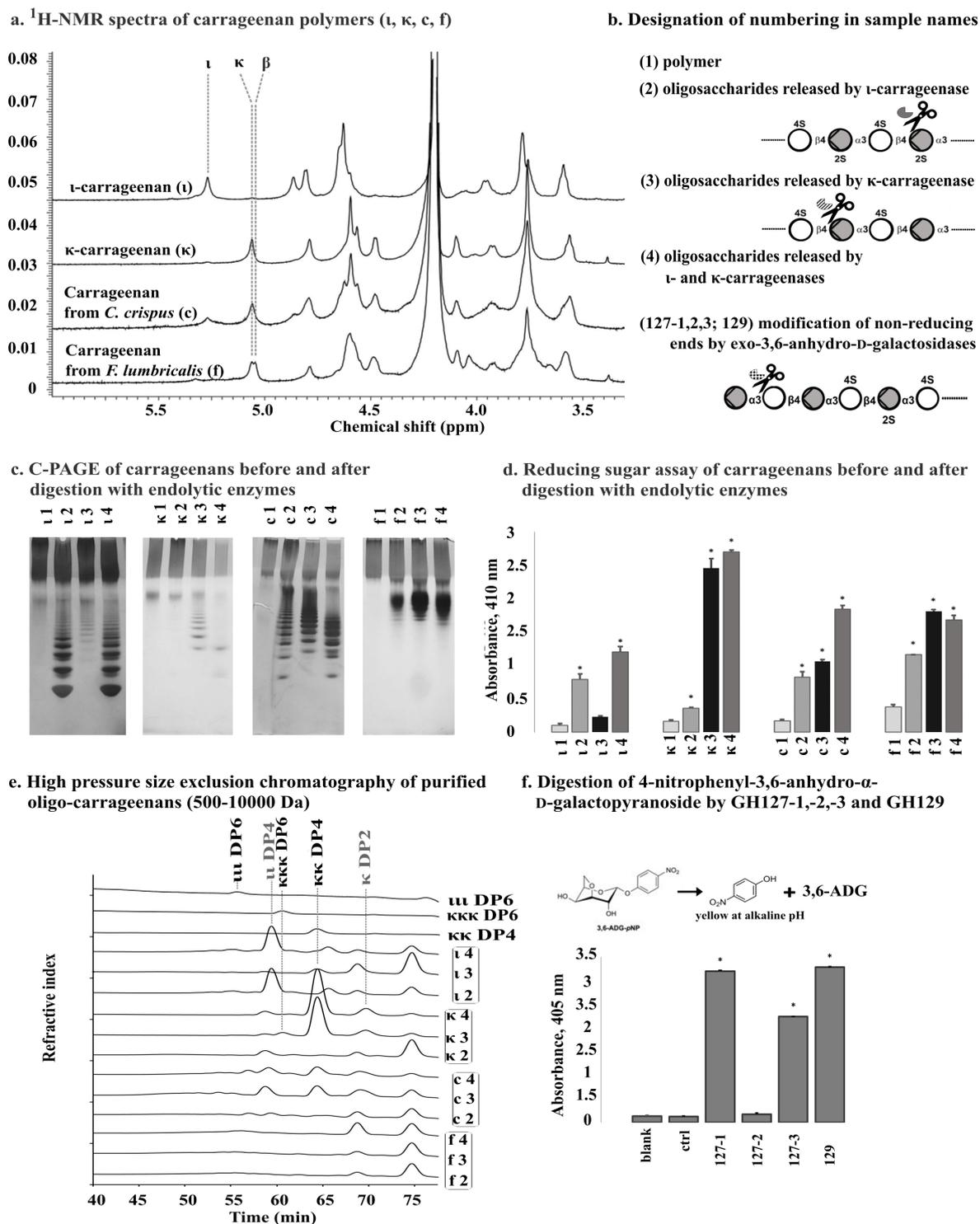
381 With growing understanding of the potential of biological responses originating from
382 specific glycan interactions, tuning of the sugar code is important to achieving therapeutic
383 benefit. Following the examples of polymers like heparin, which provides the basis for
384 therapeutic oligosaccharides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

390 Four gelling carrageenan samples have been used in the current study. Commercial κ -
391 and ι -carrageenans were used as controls as well as two natural, hybrid carrageenans. Hybrid
392 carrageenans have different sulfation motifs along the carrageenan chains which can affect their
393 functional and rheological properties (Van de Velde, 2008) and are thus also of interest for
394 bioactivity studies. The native carrageenans were gently extracted from the red algae *C. crispus*
395 gametophyte and *F. lumbricalis* giving a yield after extraction, precipitation and dialysis at 12-
396 14 kDa of 9.52% and 16.44%, respectively. The structures of all carrageenan samples, including
397 commercial, were determined by 1H-NMR (Fig. 1a, Supplementary Table 1) and the MWs
398 were also determined by MALS (Supplementary Table 2). The 1H-NMR data confirmed the
399 attribution of commercial samples to ι - and κ -types (Supplementary Table 1). Not unexpectedly,
400 the native carrageenan structures found in the *C. crispus* gametophyte are mainly hybrid ι/κ -
401 motifs (Guibet et al., 2008) and the structures of the *F. lumbricalis* are mainly κ/β -motifs with
402 a small portion of ι -carrageenan (Correc et al., 2012; Rodriguez Sanchez, Matulewicz, &
403 Ciancia, 2022).

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

416 In some cases, the separation of the carrageenans is clearly visible in the C-PAGE gel,
417 with the stacking area appearing emptier with the samples digested by κ - and ι/κ -carrageenases
418 relative to the polymer control (Fig 1c, samples k3, k4, c3, c4). The κ/β -type oligo-carrageenans
419 from *F. lumbricalis* poorly penetrate the gel matrix - probably due to their limited charge
420 density. Since the C-PAGE technique depends largely on the charge of the fragments to be
421 qualitatively analyzed, we also evaluated the depth of the endolytic degradation by assaying the
422 increase in free reducing ends by the PAHBAH reducing sugar method (Lever, 1972). This

423 provided a quantitative method to monitor the substantial hydrolysis of low sulfated κ/β -
424 carrageenan from *F. lumbricalis*. It was also confirmed that degradation of commercial κ - and
425 ι -carrageenans was highly specific to their appropriate enzymes, κ -carrageenase and ι -
426 carrageenase, respectively, though we did see some significant crossover activity of the κ -
427 carrageenase on commercial ι -carrageenan likely due to the presence of some κ -carrabiose
428 motifs in the mainly ι -carrageenan chain (Fig 1. acd), and vice versa for the ι -carrageenase on
429 commercial κ -carrageenan (Fig 1. ad). None of the carrageenans were treated chemically, they
430 were only treated enzymatically to modify their length.



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Figure 1. Characterization of carrageenans and enzymatically-produced oligocarrageenans. (a). $^1\text{H-NMR}$ spectra of the carrageenans used in this study. The designation of samples is assembled by numbers (visualized in section **b**) and letters: “ ι ” stands for commercial ι -carrageenan “ κ ” - κ -carrageenan, “c” - carrageenan from *C. crispus*, and “f” - carrageenan from *F. lumbricalis*. (**b**) A scheme to visualize the nomenclature used to describe the polymers and oligosaccharides released by endolytic enzymes and modified by exolytic ones: 1 - polymer, 2 - ι -carrageenase, 3 - κ -carrageenase, 4 - sequential double digestion with both endolytic enzymes; 127-1, -2, -3 or 129 -modification by 3,6-anhydro-D-galactosidases GH127-1, GH127-2, GH127-3 or GH129. (**c-d**) C-PAGE (**c**) and reducing sugar assay (PAHBAH) (**d**) of carrageenans before and after digestion with endolytic enzymes. (**e**) High performance size exclusion chromatography (HPSEC) of purified oligo-carrageenans (500 -

442 10 000 Da). (f). Digestion of artificial substrate 4-nitrophenyl-3,6-anhydro- α -D-galactopyranoside
443 (3,6-ADG-pNP) by 3,6-anhydro-D-galactosidases GH127-1, GH127-2, GH127-3 and GH129 where
444 blank was with buffer instead of enzyme and control (ctrl) was with a boiled mixture of the enzymes.
445 Statistical significance difference ($P < 0.05$) of degradation in comparison to each initial polymer is
446 marked with an asterisk (*). Bars represent mean values of 2 independent experiments with each of
447 them done in triplicates \pm SD.

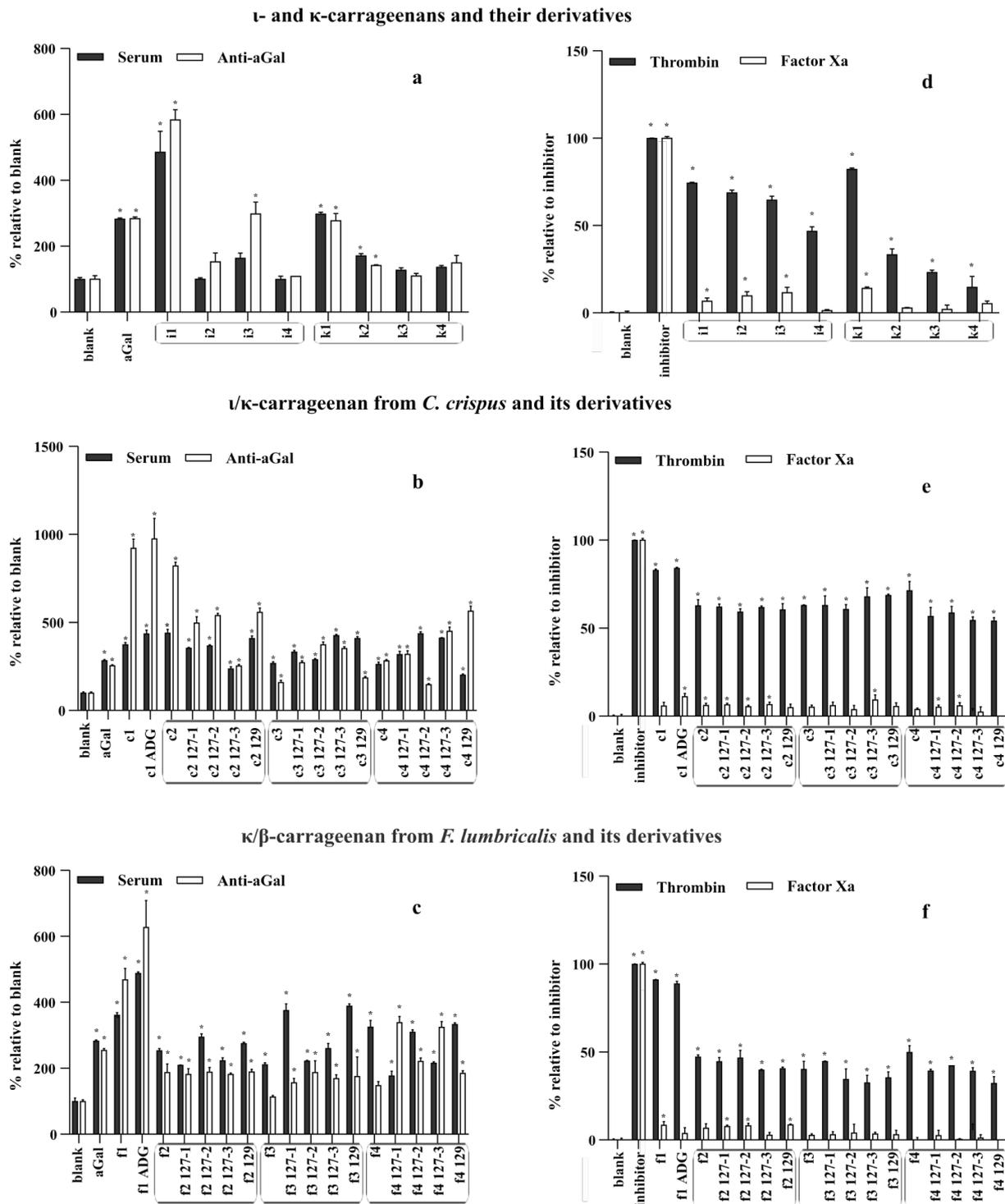
448 HPSEC analysis (Fig. 1e) confirms the production of oligosaccharides mixtures
449 containing at least κ – di, tetra- and hexa-saccharides, and ι – tetra and hexa-saccharides. κ -
450 carrageenan hydrolyzed by κ -carrageenase or by both enzymes contained κ -tetrasaccharide as
451 a major product,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

455 After treatment with the specific endohydrolases, the oligosaccharides produced are from
456 the neo-series, with 3,6-anhydro-D-galactose (DA) on the non-reducing end, though the
457 sulfation pattern may vary. These oligosaccharides were then further modified by the exolytic
458 3,6-anhydro-D-galactosidases. We first verified the activity of the 3,6-anhydro-D-
459 galactosidases (ZgGH127-1, ZgGH127-2, ZgGH127-3, ZgGH129) on the artificial
460 colorimetric substrate 3,6-ADG-pNP by endpoint reaction (Fig. 1f) (Wallace, Guee, et al.,
461 2020). The data show that 3,6-ADG-pNP substrate was hydrolyzed by all the 3,6-anhydro-D-
462 galactosidases except GH127-2 under assay conditions. In order to select an upper size limit of
463 oligosaccharides, the samples were ultrafiltrated at 10 kDa. To ensure removal of the DA
464 monosaccharide, which could alone have bioactive properties (Lee et al., 2019; Yun et al.,
465 2013), a dialysis into water with a 100-500 Da membrane was also performed and the yields
466 were calculated after lyophilization (Supplementary Table 3). All oligosaccharide samples
467 were analyzed using HPAEC (Supplementary Figure 1) to help inform on exolytic enzymatic
468 activity in the highly complex carrageenan oligosaccharide mixtures. The polymers also
469 underwent a treatment with the mix of 3,6-anhydro-D-galactosidases, followed by dialysis (100-
470 500 MWCO) into water for use as controls in the bioactivity experiments and their yields were
471 also calculated (Supplementary Table 4).

472 Glycan-protein interactions were assayed in a system where the reducing ends of sugars
473 were covalently attached to the surface via a reaction of the open chain aldehydes with the
474 hydrazide coated surface. The methods used to detect interactions between covalent surface-
475 adhered glycans and proteins were ELISA-based and thus efficiently able to profile a large
476 number of samples (Wolfenden, Cousin, Nangia-Makker, Raz, & Cloninger, 2015). The

477 orientation in space and elimination of access to reducing ends gave us an opportunity to delve
478 into the biochemistry of binding favored by eukaryotic galactose-binding proteins. The tested
479 proteins were polymorphic antibodies of total human serum, a monoclonal IgM anti- α Gal
480 antibody (m86 mAb) and Galectins 1 and 3 (Gal-1 and Gal-3). The usefulness of using the
481 CARBO-bind® plates is that they provide a multivalent surface for the antibody and lectin
482 binding, which likely contributes to the avid binding of the protein partners.

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



504

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

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

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

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

522 As with the commercial carrageenan polymers, the anti- α Gal mAb interactions showed
523 more signal intensity with the sulfated *C. crispus* κ/ι -carrageenans relative to the *F. lumbricalis*
524 κ/β -carrageenans; for the serum antibodies the preference is less clear. The nuances of antibody
525 exo-binding to the hybrid oligo-carrageenans were more profoundly studied by using 3,6-
526 anhydro-D-galactosidases to remove non-reducing 3,6-anhydro-D-galactose. *C. crispus*
527 derivatives released by ι -carrageenase (c2, c2 127-1, c2 127-2, and c2 129) and the double
528 digest (c4 129) showed substantially more signal intensity for the anti- α Gal mAb relative to the
529 natural serum antibodies (Fig. 2b); however, the *C. crispus* oligosaccharide with the strongest
530 signal is the c2 oligosaccharide, only treated with ι -carrageenase, not with the 3,6-anhydro-D-
531 galactosidases. This leads us to suggest that the increased signal for the c2 oligosaccharide with
532 mAb appears when there is a DANr, possibly sulfated in 2S since it was prepared with the ι -
533 carrageenase. On the other hand, treatment of the double digest c4 129 sample is particularly
534 interesting because there seems to be a much stronger selectivity for the mAb relative to the
535 serum antibodies. Furthermore, the signal intensity of c4 129 is higher relative to the c4 for the
536 mAb interaction. This leads us to suggest that the double digest followed by the ZgGH129 3,6-
537 anhydro-D-galactosidase treatment exposes a motif which has higher specificity for the mAb
538 relative to the serum antibodies.

539 The *F. lumbricalis* carrageenan and its derivatives released by ι -carrageenase (f2, f2 127-
540 1, f2 127-2, f2 127-3 and f2 129) and κ -carrageenase (f3, f3 127-1, f3 127-3, and f3 129) showed
541 more signal intensity with serum antibodies relative to anti- α Gal mAb (Fig. 2c). Interestingly,
542 this trend was not evident for the double digested f4 samples which gave a mixed preference
543 for either serum antibodies (f4, f4 127-2, f4 129) or the anti- α Gal mAb (f4 127-1, f4 127-3)
544 (Fig. 2c). The highest signal for the *F. lumbricalis* serum antibodies with oligosaccharide was
545 seen on f3 127-1 and f3 129 (treated with κ -carrageenase followed by the 3,6-anhydro-D-
546 galactosidases), suggesting a preference for Gnr or G4Snr. This contradictory result between
547 mAb and the serum Abs is possibly related to the different specificities found in human serum
548 antibodies. The monoclonal antibodies are specific to a minimal α Gal epitope and this pivotal
549 specificity is shared by some of the natural antibodies (Milland et al., 2007). However, the fine
550 specificity to the full epitope is conferred to the natural antibodies by sugar moieties

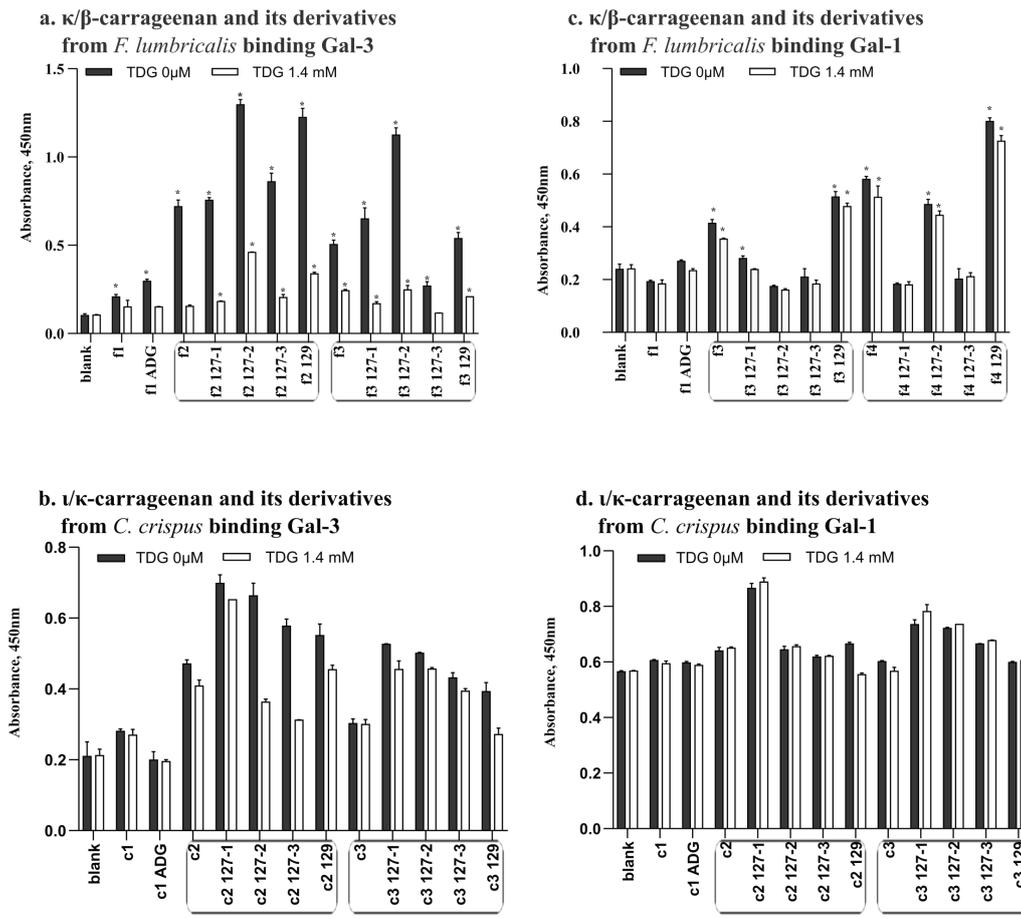
551 downstream to the minimal recognition unit, such as Glc or GlcNAc residues (Milland et al.,
552 2007). Carrageenans do not contain either Glc or GlcNAc and the galactose units themselves
553 are often modified: firstly, by negatively charged sulfate groups and, secondly, by a
554 hydrophobic 3,6-anhydro-bridge which sterically forces an inversion of the galactopyranose
555 conformation (from 4C_1 to 1C_4) (Ciancia, Nosedá, Matulewicz, & Cerezo, 1993; Ficko-Blean et
556 al., 2015). These structural differences in the carrageenan derivatives may contribute to the
557 differences in preference between the monoclonal and natural antibodies.

558 Strikingly, carrageenans can modulate blood protease cascades, with their sulfation
559 patterns helping to determine their pro- or anti-coagulant properties (Pomin, 2012).
560 Regiosulfation on the C2 of the anhydro- α -D-galactose and the C6 of the β -D-galactose has
561 been shown to increase the carrageenan's ability to inhibit thrombin activity (de Araujo et al.,
562 2013), however the importance of the 3,6-anhydro-D-galactoside has never been evaluated for
563 anticoagulant activity. To this end, we evaluated the effect of the carrageenans and their
564 potential for anticoagulation properties on thrombin and Factor Xa through an inhibition assay.
565 For commercial ι - and κ -carrageenan polymer we see a similar inhibition of thrombin and poor
566 Factor Xa inhibition (Fig 2d). Decreasing the MW reduced the thrombin inhibition more
567 significantly for the κ -oligosaccharides than the ι -oligosaccharides (Fig 2d). For *C. crispus*
568 carrageenans the decrease in MW by the endohydrolases (c2, c3, c4) and modification of the
569 non-reducing ends by the 3,6-anhydro-D-galactosidases (GH127-1, -2, -3, and GH129) did not
570 drastically affect the thrombin inhibitory activity relative to the polymer (c1) (Fig. 2e). For κ -
571 commercial and *F. lumbricalis* carrageenans and their oligosaccharide derivatives, decreasing
572 the MW reduced the thrombin inhibition relative to the polymer (Fig. 2d,f). This may also be
573 related to sulfation degree as both the κ -carrageenan and *F. lumbricalis* carrageenans (mainly
574 hybrid κ/β -motifs (Correc et al., 2012)) are significantly less sulfated than ι -carrageenan. In all
575 cases, the inhibitory potential of carrageenans towards Factor Xa was not substantial in
576 comparison to the positive irreversible inhibitor control (Fig. 2d-f).

577 The carrageenans were further tested with regard to their interaction with galectins. In
578 general, galectins are a large family of glycan-binding proteins with preference for β -
579 galactosides. Their subfamilies are classified by fine-tuned specificity for longer glycans of
580 saccharides linked on either side of the β -galactosides (Nielsen et al., 2018). Galectins bind
581 through two major types of interactions: a large hydrogen-bond network and hydrophobic CH-
582 π interactions between the glycan and aromatic amino acid sidechains in the CRD (Modenutti,
583 Capurro, Di Lella, & Marti, 2019). Gal-1 and Gal-3 have been chosen as target galectins

584 because inhibitors selectively favoring one of them would be promising since both galectins
585 have an assortment of functions (Nabi et al., 2015). Gal-3 has a binding affinity to
586 oligosaccharide structures bearing the lacNAc motif internally while Gal-1 prefers terminal
587 disaccharide lacNAc sequences (Laaf et al., 2019). Carrageenans contain endo- β -D-galactose
588 but no N-acetyl groups or nitrogen atoms. The α Gal epitope has been listed among galectin
589 inhibitors (Hirabayashi et al., 2002) and since carrageenans bind the mAb anti- α Gal, they may
590 also have potential for binding the galectins' CRD region. We thus chose to evaluate the
591 inhibition potential of the carrageenans and their derivatives with Gal-1 and Gal-3. The design
592 of the experiment included a competitive inhibition assay with thiodigalactoside (TDG), a CRD
593 inhibitor of Gal-1 ($K_d = 24 \mu\text{M}$) and Gal-3 ($K_d = 49 \mu\text{M}$) since binding may be within the CRD
594 or elsewhere on the galectins, such as observed with modified citrus pectin and galactomannan
595 polysaccharides (Stegmayr et al., 2016).

596 Preliminary tests were done on all the carrageenans and their derivatives to determine the
597 fractions that showed binding signal with Gal-1 and Gal-3. The carrageenans that showed strong
598 signal intensity with the galectins were chosen for inhibition studies (Fig 3). The derivatives of
599 commercial κ - and ι -carrageenans did not exhibit binding (data not shown). Our results indicate
600 that carrageenans can be naturally-sourced glycan partners of Gal-1 and Gal-3 and they function
601 as such when some structural requirements are met (Fig. 3a-d). Molecular mass, the structure
602 of the non-reducing ends of oligo-carrageenans, and sulfation degree are features affecting
603 galectin-binding properties of carrageenans. Thus, a decrease in molecular mass relative to the
604 polymer improved binding signal intensity significantly with Gal-3 for all of the carrageenan
605 derivatives tested and, to a lesser degree, with Gal-1 with only some of the carrageenan
606 derivatives (Fig. 3a-d). This might be due to increased exo-binding as there are less endo-motifs
607 in the shorter chain oligo-carrageenans. Unlike the oligo-carrageenans, with varied and
608 significant capacities to bind Gal-1 and Gal-3 depending on sulfation degree and the treatment
609 with the 3,6-anhydro-D-galactosidases, the polymers demonstrated less interactions with the
610 galectins. Of the hybrid oligosaccharides tested, the oligo-carrageenans from *F. lumbricalis*
611 were particularly interesting as the concentration used to show active binding was 0.5 mg/mL,
612 whereas for the *C. crispus* oligo-carrageenans it was necessary to increase the concentration to
613 4 mg/mL. This is a striking difference between active concentrations of the f2 and c2
614 oligosaccharides for Gal-3 which is tempting to relate to the degree of sulfation on the
615 oligosaccharides (Fig. 3 a,b).



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

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There appears to be two modes of binding to Gal-3. The first mode of binding is via the CRD region as seen with the inhibition by TDG and the κ/β-oligo-carrageenans from *F. lumbricalis* (Fig. 3a). Further hydrolysis with the 3,6-anhydro-D-galactosidases improves the binding signal significantly in some cases (f2 127-2, f2 129, f3 127-2). In general, the interaction with Gal-3 CRD was improved after 3,6-anhydro-D-galactosidase treatment of the *F. lumbricalis* oligosaccharides (Fig 1a). This suggests that a non-reducing β-galactose moiety is preferable for binding the Gal-3 CRD. ZgGH129 requires neo-β-carrabiose on the non-

636 reducing end, but can tolerate a subsequent ι - or κ -motif during hydrolysis (Wallace, Ficko-
637 Blean, et al., 2020; Wallace, Guee, et al., 2020). Hence, the products after reaction would
638 contain a neutral G on the nr end but the subsequent disaccharide motif may be sulfated. The
639 tangible increase in binding might also be due to improved avidity to Gal-3 after hydrolysis of
640 DANr. The second mode of binding seems to be non-CRD binding-site mediated as in many
641 cases the TDG shows poor to no inhibition with Gal-3 when incubated with the *C. crispus* oligo-
642 carrageenans (Fig 3b). Notably, their active concentration was also 8-fold higher than for the
643 *F. lumbricalis* κ/β -carrageenan derivatives. In any case, these κ/ι -derivatives demonstrated
644 decreased interaction with Gal-3 relative to the κ/β -carrageenan derivatives using this binding
645 methodology. Overall, the *F. lumbricalis* κ/β -oligo-carrageenans have strong potential as
646 saccharide-based ligands selective and specific for the Gal-3 CRD.

647 Gal-1 binding to the carrageenans and oligosaccharide derivatives was not inhibited by
648 TDG, suggesting that all the interactions with the carrageenans are distal to the CRD
649 carbohydrate-binding region (Fig 3cd). Still some of the hybrid κ/β -oligo-carrageenans from
650 *F. lumbricalis* showed improved signal intensity (Fig 3c, f3 129, f4, f4 127-2, f4 129). The *C.*
651 *crispus* κ/ι -oligo-carrageenans showed poor binding in general to Gal-1 (Fig 3d) with a signal
652 similar to that of the blank, with the exception of the c2 and c3 samples treated with ZgGH127-
653 1.

654

655 **Conclusion**

656 The binding of carrageenan polymers relative to the enzymatically produced
657 oligosaccharides suggests anti- α Gal antibody binding through both groove-type and end-on
658 insertion. Gal-3 CRD revealed exo-binding as the CRD demonstrated a preference for the oligo-
659 carrageenans. Furthermore, treatment with one of the four 3,6-anhydro-D-galactosidases,
660 ZgGH127-1, ZgGH127-2, ZgGH127-3, or ZgGH129, inflicted a substantial impact on Gal-3
661 CRD binding to the oligosaccharides and, thus, the structure of the non-reducing ends of oligo-
662 carrageenans are a molecular determinant to binding the Gal-3 CRD. Our findings make
663 carrageenans alluring glycomimetics for future exploration as multivalent ligands for human
664 serum anti- α Gal IgM antibodies and as specific ligands selective for Gal-3 over Gal-1. These
665 revelations could suggest a molecular basis for widely studied oligo-carrageenans' antitumor
666 properties and provide more prospective potential of these molecules to anti-tumor research
667 (Calvo et al., 2019; Torres, Florez-Fernandez, & Dominguez, 2021). One of the mechanisms to
668 turn cold tumors with low responsiveness to immunotherapy into so-called hot or inflamed

669 tumors is by increasing local inflammation (Ochoa de Olza, Navarro Rodrigo, Zimmermann,
670 & Coukos, 2020). Local inflammation is a natural outcome of such potent inducers of
671 complement as the Gal α (1,3)Gal reactive antibodies. Carrageenans and their derivatives are
672 also prospective therapeutic agents in wound healing since they have the potential to affect both
673 immune response and the activity of coagulation cascade components.

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682 The authors declare no competing interest.

683 **Declaration of generative AI in scientific writing**

684 The authors have not use any artificial intelligence (AI) and AI-assisted technologies in
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686 **Author contributions**

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