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1 Physical state of κ -carrageenan modulates the mode of action of κ -
2 carrageenase from *Pseudoalteromonas carrageenovora*

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4 Maud Lemoine, Pi Nyvall Collén and William Helbert*

5

6 *Université Pierre et Marie Curie, Paris VI, CNRS, Marine Plants and Biomolecules, UMR*
7 *7139, Station Biologique, BP 74, F29680 Roscoff Cedex, France*

8

9

10 *Corresponding author; Fax: + 33 298 292 332, Email: helbert@sb-roscoff.fr

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15 **Abbreviation list**

16 DP: degree of polymerisation

17 GH: glycoside hydrolase

18 MW: molecular weight

19 HPAEC: high performance anion exchange chromatography

20 LC-MALLS: liquid chromatography-Multi angle laser light scattering

21 TEM: transmission electron microscopy

22 ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid

23 DMSO: dimethyl sulfoxide

24 FACE: fluorescence-assisted carbohydrate electrophoresis

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25 **Abstract**

26 *Pseudoalteromonas carageenovora* κ -carrageenase is a glycoside hydrolase involved
27 in the bioconversion of carrageenans. Carrageenans are sulphated galactans that are densely
28 packed in red algal cell walls. Previous crystallographic investigations revealed that the active
29 site of κ -carrageenase has a tunnel-shaped topology, suggesting a processive mode of action
30 for this enzyme. To biochemically characterise the enzymatic depolymerisation of κ -
31 carrageenan, soluble and solid substrates (in both gel and powder forms) were incubated with
32 *P. carageenovora* κ -carrageenase. The average molecular weight of soluble carrageenan
33 decreased rapidly and all possible degradation products were observed, suggesting random
34 degradation of κ -carrageenan. In contrast, as expected for a processive-type carrageenase, the
35 average molecular weight of solid carrageenan decreased very slowly and tetrasaccharide
36 production was high. Interestingly, experimentally determined processivity was very similar
37 for gel and powder, suggesting that, in addition to an adapted catalytic site, the substrate must
38 be in the solid state for κ -carrageenase processivity to operate, whatever the level of
39 carrageenan ordering.

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40 Introduction

41 Agars and carrageenans are sulphated galactans widely used for industrial applications
42 due to their unique physico-chemical properties [1-3]. They are the most abundant
43 components of the red algal (Rhodophyta) cell wall and can represent up to 50% of algal dry
44 weight. Agar and carrageenan are densely packed in the cell wall in a three-dimensional solid
45 network of pseudo-crystalline fibres which assemble during the deposition of cell wall
46 macromolecules [4, 5]. These polysaccharides are made up of linear chains of galactose with
47 alternating α -(1 \rightarrow 3) and β -(1 \rightarrow 4) linkages. In these galactans, the β -linked galactose units are
48 in the D configuration (G unit). However, while the α -linked galactose units are in the L
49 configuration in agars (L unit), they are in the D configuration in carrageenans (D unit).
50 Carrageenans are further classified according to the number and position of sulphate esters (S)
51 and by the occurrence of 3,6-anhydro-bridges in the α -linked residues (DA unit) found in
52 gelling carrageenans [6, 7]. For example, kappa- (κ , DA-G4S), iota- (ι , DA2S-G4S), and
53 lambda- (λ , D2S6S-G2S) carrageenans are distinguished by the presence of one, two, or three
54 ester-sulphate groups per repeating disaccharide unit, respectively.

55 Agarose, κ - and ι -carrageenans form thermo-reversible gels in aqueous solution, and
56 their stiffness decreases with the degree of sulphation. Agarose and κ -carrageenan produce
57 rigid and brittle gels, while ι -carrageenan gels are elastic. λ -Carrageenan, which lacks
58 anhydro-bridges, gives highly viscous solutions. The gelling properties of carrageenan depend
59 on the ionic strength and the type of salts occurring in the medium. It is well-known that
60 potassium chloride and calcium chloride induce gelation of κ - and ι -carrageenans,
61 respectively [8, 9]. At the molecular level, gelation is preceded by a disorder–order (helix)
62 transition of polysaccharide conformation, followed by aggregation of helices which gives
63 rise to semi-crystalline fibres [10, 11]. X-ray crystallography analyses of oriented fibres of ι -
64 carrageenan suggest that double helices—probably parallel-stranded duplexes—are packed
65 together in junction zones (i.e. the fibres) [10, 12]. In the case of κ -carrageenan, the
66 conformational state of the macromolecules in gel form is still a matter of debate.
67 Experimental data provide support for a dimeric structure of polysaccharides in an ordered
68 state, and interpretations lead either to duplexes of single helices [13-15] or, as for ι -
69 carrageenan, double helices (intertwined strands) [8, 10, 16]. The aggregation of agarose
70 molecules in gels also occurs by association of single or double helices [17, 18].

71 Agar- and carrageenan-degrading enzymes, namely agarases and carrageenases, have
72 so far only been found in marine bacteria belonging mainly to the *Gammaproteobacteria*,
73 *Bacteroides* or *Sphingobacteria* classes [19]. Known κ -carrageenases (E.C. 3.2.1.83) belong
74 to the GH16 glycoside hydrolase family, a polyspecific family which encompasses at least
75 eight different enzymatic activities, including, notably, β -agarases ([http://afmb.cnrs-](http://afmb.cnrs-mrs.fr/CAZY/)
76 [mrs.fr/CAZY/](http://afmb.cnrs-mrs.fr/CAZY/)) [20]. Phylogenetic analysis and crystallographic investigations have
77 demonstrated that the GH16 family of enzymes evolved from a common ancestor and that κ -
78 carrageenases most likely emerged from the β -agarase branch [21]. β -Agarases and κ -
79 carrageenases adopt a jelly-roll fold and hydrolyse β -(1 \rightarrow 4) glycosidic linkages with retention
80 of the anomeric configuration [22, 23]. Analyses of the mode of action of β -agarases suggest
81 that agarose molecules undergo random depolymerisation. In the case of agarase A, in
82 addition to the active catalytic site, a second strong binding site was observed on the catalytic
83 module. It has been proposed that the co-occurrence of two binding sites facilitates
84 heterogeneous phase degradation of agarose by unwinding or dissociating helices occurring in
85 the gel state [24]. The mode of action of κ -carrageenase has not been investigated
86 biochemically. However, considering the tunnel-shaped topology of the active site, it has been
87 suggested that this enzyme proceeds via a non-random, processive mechanism [25].

88 Processivity, also referred to as the multiple-attack mechanism, was first described in
89 α -amylases [26, 27]. In this mode of action, enzymes form a complex with the target
90 polysaccharide and remain attached to one end of the polymeric chain, thus cleaving several
91 glycosidic linkages before dissociating. This mechanism implies that the enzyme slides along
92 the polysaccharide chain. In the case of neutral polysaccharides, this involves intermediate
93 enzyme-substrate complexes stabilised by hydrophobic interactions between aromatic amino
94 acid and sugar residues [28, 29]. Although the sliding mechanism of processive enzymes on
95 polyanionic molecules is not well understood, several examples of processive enzymes acting
96 on anionic polysaccharides have been reported [30-32]. Interestingly, ι -carrageenases, which
97 form a monospecific family of glycoside hydrolases (GH82) unrelated to κ -carrageenases,
98 degrade carrageenan gels according to a processive mechanism [32]. This was demonstrated
99 both by observation of thinning carrageenan fibres using transmission electron microscopy
100 and by detection of a particular groove in the active site using X-ray crystallography. This
101 groove closes in the presence of substrate as a result of a shift in a protein domain and leads to
102 a tunnel-shaped topology. This conformation allows the enzyme to progress unidirectionally
103 without dissociating from the substrate.

104 The enzymatic digestion of κ -carrageenan, whose chemical structure is intermediate
105 between agarose and ι -carrageenan, has not yet been investigated. β -Agarases share a
106 common ancestor with κ -carrageenase, but the physico-chemical properties as well as the
107 anionic nature of κ -carrageenan chains closely resemble those of ι -carrageenan. The tunnel
108 topology found in both κ - and ι -carrageenase reinforces the hypothesis of convergent
109 evolution of these enzymes toward a mode of action adapted to substrates that share common
110 chemical and physical properties. In this context, we sought to characterise the mode of action
111 of the *P. carrageenovora* κ -carrageenase in order to consider the potential correlation
112 between the tunnel-shaped topology of the active site with the enzyme's mode of action. We
113 demonstrate that the κ -carrageenase is a processive enzyme when acting on solid substrates.
114 Nevertheless, κ -carrageenase has a random mode of action on soluble substrates. These
115 observations suggest that the conditions for processivity not only require that the active site
116 possess the adapted topology, but also that the substrate be in an appropriate physical and
117 conformational state.

118

119 **Experimental**

120 ***Purification of κ -carrageenan***

121 κ -Carrageenan extracted from *Kappaphycus alvarezii* (*Euचेuma cottonii*) was
122 supplied by CP Kelco (κ -carrageenan, cottonii X-6913 and low-gelling κ -/ μ -carrageenan,
123 cottonii X-6042). Before use, carrageenans were purified by isopropanol precipitation as
124 follows. Carrageenan powder (5 g) was suspended in 500 mL cold ultra-pure water (Millipore
125 Water Purifier). The suspension was heated at 70°C under slow stirring until the carrageenan
126 was completely dissolved. The polysaccharides in solution were precipitated by drop-wise
127 addition of 1 L of isopropanol under vigorous stirring. After centrifugation at 10,500 \times g, the
128 precipitate was collected and dissolved again in 500 mL ultra-pure water. This precipitation
129 protocol was repeated twice. Carrageenans were then dissolved in 500 mL water prior to
130 adding 29.22 g NaCl. This quantity of NaCl corresponds to a salt concentration 50 times
131 greater than that of the ester sulphate groups in carrageenan. After overnight stirring, the
132 solution was dialysed (6-8000 Da MWCO Spectra/Por®) against ultra-pure water in order to
133 remove excess salt and non-polymeric materials in the presence of 5 mM NaOH to prevent
134 acidification of the sample. Finally, the Na-carrageenan solution was filtered through a

135 0.45 μm filter (Pall Life Sciences, Acrodisc Syringe Filter, HT Tuffryn Membrane) and
136 freeze-dried in the presence of 20 mM ammonium carbonate.

137 ***Recombinant κ -carrageenase***

138 *Pseudoalteromonas carrageenovora* κ -carrageenase was recombinantly expressed in
139 *E. coli* as described by Michel *et al.* (1999) [33]. The over-expressed His-tagged fusion
140 protein was purified by metal affinity chromatography on a chemical Sepharose Fast Flow
141 column (GE Healthcare) loaded with NiSO_4 , followed by gel permeation chromatography on
142 a MonoS column (HR 5/5, GE Healthcare). A stock solution containing $60 \mu\text{g mL}^{-1}$ of κ -
143 carrageenase with an activity of $0.06 \text{ mM eq. glucose min}^{-1} \mu\text{g}^{-1}$ (0.125% κ -carrageenan in
144 0.2 M Tris) was stored at 4°C .

145 ***Enzymatic degradation***

146 A stock solution of 0.4% (w/v) carrageenan in 10 mM Tris, pH 8.0 was prepared by
147 mixing at room temperature under gentle stirring overnight. To ensure complete dissolution,
148 the solution was heated at least for 1 h at 70°C . It was stored at 4°C for 2 weeks at most. Salt
149 solutions containing 200 mM LiNO_3 , 2 mM citric acid (used as internal standard for
150 chromatography experiments) and various concentrations of KCl ranging from 0 to 80 mM
151 were prepared just prior to use.

152 For enzymatic degradation, the 0.4% carrageenan solution and the salt solutions were
153 heated separately at 70°C for 10 min before being mixed at equal volume resulting in final
154 concentrations of 0.2% carrageenan in 100 mM LiNO_3 , 1 mM citric acid and 0-40 mM KCl.
155 The mixture was kept at 70°C for at least 30 min before being transferred to room temperature
156 (22°C). Gels were stabilised for at least 12 h at room temperature prior to incubation with
157 enzyme. Given the range of salt concentrations used (from 10 to 40 mM KCl), gels were
158 vigorously stirred and pipette tip ends were cut so that gel suspensions could be pipetted.

159 Enzymatic digestions of κ -carrageenan were conducted at 30°C . At first, the tubes
160 were shaken vigorously and initial undigested samples were collected. κ -Carrageenase was
161 then added to the incubation medium: $0.12 \mu\text{g mL}^{-1}$ for carrageenan solutions and
162 $0.60 \mu\text{g mL}^{-1}$ for carrageenan gels. NaOH was added to a final concentration of 100 mM in

163 order to stop the enzymatic reaction. κ -Carrageenase is inactivated in alkaline solutions [34]
164 without any alteration of the chemical structure of κ -carrageenan.

165 ***Reducing-sugar assay***

166 The amount of reducing sugars produced during the enzymatic digestion of κ -
167 carrageenan was determined using a method adapted from Kidby and Davidson [35]. Aliquots
168 (100 μ l) of the reaction medium diluted five times were mixed with 1 mL ferricyanide
169 solution (300 mg potassium hexocyanoferrate III, 29 g Na_2CO_3 , 1 mL 5 M NaOH, completed
170 to 1 L with water). The mixture was boiled for 10 min, cooled to room temperature and its
171 absorbance was read at 420 nm. We defined 100% degradation as the amount of reducing
172 sugars obtained when soluble κ -carrageenan was completely converted to DP4 and DP2 and
173 DP6 (neo- κ -hexacarrabiose) could no longer be detected by HPAEC.

174 ***Liquid chromatography – multi-angle laser light scattering (LC-MALLS)***

175 Prior to LC-MALLS experiments, samples were extensively dialysed against ultra-
176 pure water containing 20 mM ammonium carbonate (to prevent depolymerisation which may
177 occur during freeze-drying) at 4°C using a 100 Da membrane (Spectra/Por® 100, cellulose
178 ester) to eliminate KCl salts that promote gelation of carrageenan. Dialysed specimens were
179 lyophilised. Samples were then dissolved in 100 mM LiNO_3 to a final concentration of about
180 0.3%. Soluble carrageenan (without KCl) and carrageenan gels (with KCl) were prepared in a
181 similar fashion.

182 After filtration (0.22 μ m, Millipore), samples (200 μ L) were injected on a Pharmacia
183 Superdex 200 column (300 \times 10 mm i.d., GE Healthcare) followed by a Superdex peptide
184 10/300 column (Pharmacia GE Healthcare), mounted in series. Elution was performed with
185 0.1 M LiNO_3 filtered at 0.1 μ m (refractive index = 1.327) at a flow rate of 0.3 mL min^{-1}
186 (Waters 626 pump) at 25°C. Detection was monitored by a Waters 2414 refractive index
187 detector, used as a mass sensitive detector, set at 890 nm at 35°C. MALLS measurements
188 were performed at 690 nm with a DAWN EOS system (Wyatt Technology, Santa Barbara,
189 CA, USA) equipped with a 30 mW Ga-As linearly polarized laser. The intensity of scattered
190 light was measured at 12 different angles, from 35° to 143°. Chromatographic data were
191 collected and processed by Astra software (Wyatt Technology, Santa Barbara, CA, USA).
192 The Zimm fit method was applied for molecular weight determinations. The calculated dn/dc

193 was 0.115 ml g⁻¹. Bovine serum albumin monomer (Sigma, St.Louis, MO) was used to
194 normalize the signals recorded at various angles of detection, with the signal measured at 90°.

195 Below 100 kDa, scattering of carrageenan was too low to determine molecular weight.
196 Molecular weight was then measured by high performance steric chromatography using
197 dextran sulphate as a standard (1, 5, 8, 100, 250 kDa). The chromatographic system and the
198 elution conditions used were the same as described above.

199 *Liquid chromatography of oligosaccharides labelled with 8-aminonaphthalene-1,3,6-* 200 *trisulfonic acid (ANTS)*

201 Prior to labelling, the enzymatic reaction was stopped by adding 500 µL of ethanol to an
202 equivalent volume of sample. The mixture was maintained at 80°C for about 10 min to ensure
203 that the enzyme was denatured. The mixture was then dried under vacuum. Then, 40 µL
204 of 0.15 M the ANTS solution (ANTS disodium salt in 15% acetic acid) was added to the dried
205 sample and kept at 37°C for 30 min. Then, 75 µL of 1 M NaBH₄CN in DMSO was added and
206 the sample was kept at 37°C for 16 h (i.e. overnight). The sample was dried under vacuum
207 and dissolved in 2.5 mL of water, which corresponds to a 5X dilution of the initial sample
208 concentration. For solid substrates (gel and powder), the final concentration of KCl was
209 5 mM, a concentration at which carrageenan does not gel. At this stage, ANTS-labelled oligo-
210 carrageenans were visualised after migration in a 27% (w/v) carbohydrate polyacrylamide gel
211 (fluorescence-assisted carbohydrate electrophoresis, FACE) according to Guibet *et al.* (2007).

212 The sample was filtered (0.22 µm, Millipore) and 100 µL was injected on a Superdex
213 peptide 10/300 column (Pharmacia GE Healthcare) using the Dionex Ultimate 3000
214 chromatography system. Elution was performed with 50 mM ammonium carbonate at a flow
215 rate of 0.3 mL min⁻¹. The ANTS-labelled oligosaccharides were detected using a UV-visible
216 detector (VWD-3400RS, Dionex) operating at 365 nm. Acquisition data was achieved using
217 the Chromeleon software (Dionex) and the surface areas of the peaks were integrated using
218 PeakFit 4.12 software (Seasolve software Inc.). A standard curve using ANTS-labelled
219 maltose was used to determine oligo-carrageenan concentrations.

220 *High performance anion-exchange chromatography (HPAEC)*

221 Degradation products were analysed using a Dionex chromatograph DX 500 equipped
222 with a 20 µl injection loop, an AS 100XR automated injection system (Thermo Separation

223 Products) and an AS11 anion exchange column (4 mm × 250 mm, Dionex IonPac®) with a
224 AG11 pre-column (4 mm × 50 mm, Dionex IonPac®). The system was operated in
225 conductivity mode using a ED40 detector (Dionex) and a Dionex ASRS ultra-4 mm
226 suppressor with a current of 300 mA. Mobile phases were ultra-purified in water and 290 mM
227 NaOH. Elution was conducted at a flow rate of 0.5 mL min⁻¹ with GP40 Gradient Pump
228 (Dionex). A linear gradient starting from 0% to 100% (290 mM NaOH) was applied for
229 33 min. Between each injection, the column was equilibrated with the mobile phase for
230 10 min. The Chromeleon-Peak Net software (Dionex) program was used for data acquisition
231 and treatment. The area of the oligosaccharide peaks was integrated and normalised using the
232 peak of 1 mM citric acid which was systematically included in the carrageenan preparations
233 and used as an internal standard.

234 *Polarimetry*

235 Optical rotation measurements were performed using a Perkin-Elmer 341 polarimeter
236 operating at a wavelength of 365 nm produced by Hg lamp. Samples were kept at 25°C in a
237 thermostated jacketed cell having an optical path length of 10 cm.

238 *Transmission electron microscopy (TEM)*

239 One drop of each κ-carrageenan sample was deposited on carbon-coated copper grids.
240 After 30 s, the excess solution was removed using filter paper and the sample was allowed to
241 dry. Samples were negatively stained using 1% uranyl acetate solution. One drop of stain was
242 deposited on the grid and contact with the sample was maintained for 20 s. Excess stain was
243 removed with filter paper and the remaining thin film of uranyl acetate solution was allowed
244 to dry. Sample preparation was performed at room temperature and protected from direct
245 light. Electron micrographs were recorded with a JEOL 1200X transmission electron
246 microscope operating at 80 kV.

247 **Results**

248 In order to obtain soluble and gelled substrates, carrageenans were dissolved by
249 heating in solutions containing increasing amounts of KCl from 0 to 40 mM. In the absence of
250 KCl, the carrageenan solution remained in liquid form at room temperature, corresponding to
251 complete dissolution. Gelation was observed macroscopically at room temperature when the
252 mixture contained at least 10 mM KCl. The formation of carrageenan helices and the

253 occurrence of aggregation were confirmed by optical rotation (Figure 2B) and by light
254 scattering (not shown), respectively. At KCl concentrations of 10 mM, a fragile, elastic,
255 easily-broken gel was obtained. At KCl concentrations greater than 10 mM, gels increased in
256 strength but could still be broken by shaking. The transmission electron micrograph of
257 negatively stained κ -carrageenan gel prepared in 20 mM KCl showed that it was composed of
258 interconnected elongated fibres of about 10-20 nm wide (Figure 1A). The fibres were made
259 up of a thin substructure ascribed to helix duplexes or aggregated helices [36, 37].

260 Carrageenan powder, obtained by freeze-drying Na-carrageenan solubilised in hot
261 water, was suspended in 20 mM KCl without heating to avoid the formation of extended
262 helices that induce gelation. As expected, TEM revealed that the specimen was mainly
263 composed of poorly organised or amorphous carrageenan aggregates. However, as illustrated
264 in Figure 1B, very thin fibre-like structures 2-5 nm wide were observed. The thin fibres were
265 probably already present in the carrageenan powder but may also result from the
266 rearrangement of carrageenan molecules when the lyophilised powder was re-hydrated in the
267 KCl solution. In what follows, we considered gel and powder forms of carrageenan as
268 organised and poorly organised solid substrates, respectively.

269 Hydrolysis was quantified by measuring the amount of reducing sugars produced
270 during the incubation of the κ -carrageenan with the recombinant *P. carrageenovora* κ -
271 carrageenase. The degradation kinetics of κ -carrageenan allowed to gel in various
272 concentrations of KCl is shown in Figure 2A. In the absence of KCl, the degradation appeared
273 to follow an exponential increase in cleavage which reached a maximum after 360 min. At
274 this stage, no more reducing ends could be obtained after addition of more enzyme. For
275 increasing amounts of KCl, trends were similar with rapid increases at very short incubation
276 times that levelled off later in the experiment with only marginal increases in digestion. For
277 low KCl concentrations (0-10 mM), degradation was rapid and 100% degradation was
278 attained in 360 min. At concentrations greater than 15 mM, i.e. conditions at which gelation
279 was observed in the test tube and by microscopy, the kinetics and yield of hydrolysis were
280 greatly reduced. For example, only 35% degradation was observed after 360 min for 20 mM
281 KCl gel.

282 Hydrolysis velocities recorded at the beginning of incubation (< 15 min) are given in
283 Figure 2B. As observed for the degradation kinetics (Figure 2A), velocity decreased with
284 increasing amounts of KCl, with the minimum velocity being measured when KCl

285 concentrations reached 30 mM. Kinetic experiments were also conducted with κ -carrageenan
286 containing about 15% μ -carrabiose units, which is a carrageenan well known for its low
287 gelling capacity. The overall kinetic curves were very similar to that of pure κ -carrageenan
288 and 100% degradation was also attained in 360 min (data not shown). However, in contrast to
289 pure κ -carrageenan, the amount KCl in the medium had less effect on the apparent activity of
290 the enzyme. As illustrated in Figure 2B, the initial velocity decreased slowly and linearly as a
291 function of KCl concentrations and about 20% loss of activity was observed in presence of
292 30 mM KCl.

293 The depolymerisation of κ -carrageenan induced by κ -carrageenase activity was
294 followed by gel permeation chromatography coupled with a MALLS detector. In order to
295 analyse all components occurring in the incubation medium, including gelled and soluble
296 fractions of carrageenan, the samples were dialysed against water until buffer and KCl salts
297 were removed. This procedure led to the complete dissolution of all carrageenan molecules
298 and released any carrageenan oligosaccharides still entrapped in gel networks. As a
299 consequence, the chromatography experiments presented hereafter are pictures of the overall
300 molecular distribution of the incubation medium. In Figures 3A and 3B, the time courses of
301 depolymerisation of soluble (0 mM KCl) and gelled κ -carrageenan (20 mM KCl),
302 respectively, are presented. The chromatogram corresponding to undigested samples showed
303 a single peak eluting in the exclusion volume (at 16 mL retention volume) attributed to a
304 750 kDa molecular weight polysaccharide (Figure 3). As the degradation of soluble substrate
305 proceeded (Figure 3A), the signal corresponding to the polymer fraction shifted toward higher
306 elution volumes indicating a decrease in molecular weight. This was correlated to the MALLS
307 measurements shown in Figure 4A which shows the drop in the molecular weight to 40 kDa
308 after 10% hydrolysis. In addition, the shape of the peak became broader indicating an increase
309 in polydispersity. Similar observations were done with low gelling κ -carrageenan incubated in
310 0 and 20 mM KCl. Permeation gel chromatograms as well as variation in molecular weight
311 quantified by MALLS followed the same patterns (data not shown) whatever the KCl
312 concentration. The decrease in molecular weight of low-gelling carrageenan in KCl shown in
313 Figure 4 clearly demonstrates that depolymerisation followed a pattern similar to that of
314 soluble carrageenan incubated without KCl. Recorded after 15% hydrolysis, the gel
315 permeation chromatogram (Figure 3A) shows well-resolved signals that were ascribed to neo-
316 oligocarrabioses from DP2 to DP12 (DP, degree of polymerisation, where DP2 indicates a

317 disaccharide). The rapid depolymerisation, the increase of polydispersity and the production
318 of all possible oligosaccharides are features of an endo mode of action.

319 In contrast to soluble substrates, the degradation pattern of the 20 mM KCl
320 carrageenan gel showed a bimodal distribution of high and low molecular weight degradation
321 products (Figure 3B). The high molecular weight fraction eluting at 16 mL remained in the
322 exclusion volume, with signal intensity decreasing with the extent of digestion. MALLS
323 analyses of this fraction indicated that the molecular weight decreased very slowly (Figure 4)
324 and that polydispersity was constant (data not shown). In the low molecular weight fraction,
325 only neo-tetracarrabiose (DP4) and neo-carrabiose (DP2) were observed at 33 and 36 mL
326 elution volumes, and not other oligosaccharides such as neo-hexacarrabiose (DP6). Finally,
327 no carrageenan fragments of intermediate molecular weight eluting between 18 and 32 mL
328 were detected. Similar chromatograms were recorded for κ -carrageenan powder suspensions
329 in 20 mM KCl suggesting that the enzyme adopts a similar mode of action.

330 The time course of oligosaccharide production was determined after labelling the
331 reducing ends with the ANTS fluorophore. Using this approach, it was possible to directly
332 measure the signal intensity of DP2 which eluted too close to salts (Figure 3) to directly
333 determine its abundance by size-exclusion chromatography. Although HPAEC made it
334 possible to visualise all the other oligosaccharides produced, again DP2 co-eluted with salts
335 (not shown) hindering direct estimation of its production. The ANTS-labelling method offers
336 the additional advantage of making it possible to quantitatively determine the amount of all
337 the oligosaccharides produced. As expected, at low percentages of degradation of soluble
338 carrageenan, a wide range of fluorescent oligosaccharides could be visualised by
339 electrophoresis (Figure 5A). For example, after 15% degradation, it was possible to
340 distinguish oligosaccharides from DP2 to DP26 which were converted to only DP4 and DP2
341 at the end of the kinetics experiment. Precise determination of oligosaccharide concentration
342 from DP2 to DP8 was achieved by gel permeation chromatography coupled to a UV detector
343 allowing the measurement of signal intensity corresponding to labelled oligosaccharides
344 (Figure 6). The time-course production of the intermediate products DP6 and DP8 had a bell
345 shape, while the amount of end-products DP2 and DP4 increased as the degradation
346 proceeded. After complete degradation, the DP4:DP2 ratio was calculated to be, on average,
347 0.576 ± 0.005 ($n = 6$) Similarly, chromatograms of low-gelling κ -/ μ -carrageenan incubated
348 without KCl and with 20 mM KCl showed a wide distribution of oligosaccharides with

349 abundances comparable to those observed with pure soluble κ -carrageenan, but hybrid κ -/ μ -
350 carrageenan oligosaccharides were also present in the digest (results not shown).

351 As previously observed with unlabelled degradation products of carrageenan gels and
352 powder, electrophoresis profiles revealed that only DP2 and DP4 were produced during
353 incubation with κ -carrageenase (Figures 5A and 5B). As illustrated in Figure 6B, the amount
354 of DP2 and DP4 increased linearly with the extent of reaction for both gel and powder forms.
355 The DP4:DP2 ratio calculated for gel and powder forms had similar values: 0.72 ± 0.04 and
356 0.69 ± 0.03 , respectively.

357 Discussion

358 The yields as well as the velocities of degradation of κ -carrageenan by *P.*
359 *carrageenovora* κ -carrageenase decreased with increasing amounts of KCl in the incubation
360 medium (Figure 2). This apparent decrease in activity was correlated with the aggregation of
361 carrageenan molecules promoted by KCl ions. This aggregation led to the formation of a
362 three-dimensional network of fibres visualised by TEM (Figure 1). In the solid state, fewer
363 glycosidic linkages are exposed to the enzyme and, moreover, the network of fibres reduces
364 the ability of the enzyme to diffuse. In low-gelling κ -/ μ -carrageenan, the velocity of
365 degradation decreased very little with increasing KCl concentrations (Figure 2B). μ -
366 Carrabiose units are known to hinder gelation of κ -carrageenan because of the irregularities or
367 kinks in the chain that they induce [6, 38]. Nevertheless, addition of KCl stiffened the κ -
368 carrabiose segments occurring in the hybrid chains, and as a consequence, increased the
369 viscosity of the medium thereby reducing enzyme and substrate diffusion. When KCl
370 concentrations were greater than 200 mM, concentrations at which κ -/ μ -carrageenan starts to
371 form a gel, we observed a decrease in the apparent activity of κ -carrageenase, equivalent to
372 that observed for pure κ -carrageenan in 30 mM (data not shown). In addition, in both soluble
373 carrageenan and low-gelling carrageenan incubated in 0 and 20 mM KCl, an endo mode of
374 action was revealed, given the rapid decrease in molecular weight and slow production of di-
375 and tetrasaccharides as a function of catalytic events. Consequently, it is likely that KCl ions
376 themselves do not play a direct role in the mechanism of action of κ -carrageenase but rather
377 strongly modulate the physico-chemical properties of κ -carrageenan as observed by optical
378 rotation and light-scattering experiments. The apparent decrease in enzymatic activity on pure
379 κ -carrageenan should be considered as a decrease in substrate accessibility and reduced
380 diffusion of the enzyme.

381 Analysis of the mode of action of glycoside hydrolases incubated with substrates in
382 different conformational or physical states can bring the different characteristics of an enzyme
383 to light. Soluble or poorly organised solid substrates are known to facilitate the detection of an
384 endo mode of action of an enzyme. For example, endo-acting cellulases are more efficient on
385 carboxymethylcellulose or phosphoric acid swollen cellulose (PASC) than on crystalline
386 cellulose (e.g. ref. [39-41]). Similarly, chitosan (partly deacetylated chitin) is preferred over
387 solid and unmodified chitin for identifying endo-acting chitinases [42, 43]. Although
388 processivity can be observed on soluble substrates [26, 43], it is more straightforward to
389 demonstrate in well-organised or crystalline material observed by electron microscopy [44-
390 47] or by chromatography methods [41]. By adopting a similar strategy, we prepared soluble
391 carrageenan as well as poorly (powder) and well-organised (gel) carrageenan in the solid
392 state. In solution, κ -carrageenan adopted a flexible rod conformation and enzymatic
393 depolymerisation led to the production of all likely neo-oligo-carrageenans (Figure 3A and
394 5A). This wide range of products was accompanied by a rapid decrease in molecular weight
395 and an increase of polydispersity (Figure 4). Consequently, in these conditions, *P.*
396 *carrageenovora* κ -carrageenase can be defined as an endo-acting hydrolase. The same
397 conclusions were drawn from analyses of enzymatic degradation products of the low-gelling
398 κ -/ μ -carrageenan (results not shown).

399 Chemical modification of polysaccharides can introduce a bias in the interpretation
400 and comparison of the mode of action of enzymes in homogenous and heterogeneous phases.
401 Consequently, carrageenan gels and powder were prepared with polysaccharides having the
402 same chemical structure and same macromolecular parameters (molecular weight,
403 polydispersity) as those studied in the soluble state. Since these carrageenans were not
404 subjected to any chemical modification, our observations were directly correlated to the
405 conformational and physical states of the substrate. When carrageenan was incubated in a
406 solid state (gel and powder), we only observed the end products DP4 and DP2. We assume
407 that any solubilised intermediate oligosaccharides were quickly degraded into DP4 and DP2.
408 Furthermore, these intermediate oligosaccharides were probably cleaved faster than more
409 recalcitrant carrageenan chains located in the solid polysaccharide network. As a
410 consequence, although degradation of carrageenan powder was faster than that of the gel, the
411 production of DP4 and DP2 increased linearly in both cases. The production of DP4 was
412 greater when the substrate was solid (gel or powder) than when the substrate was soluble.
413 Moreover, when carrageenan was incubated in the solid state, the molecular weight of κ -

414 carrageenan and its polydispersity did not change much between 0 and 30% degradation
415 (Figure 4).

416 We interpreted these features as evidence for a processive mode of action of κ -
417 carrageenase. Due to the alternation of β -(1 \rightarrow 4) and α -(1 \rightarrow 3) linkages in carrageenan, two
418 successive β -(1 \rightarrow 4) linkages are in opposite orientations, pointing up or down. Consequently,
419 as depicted in Figure 7, processive κ -carrageenase, as it slides along the polysaccharide chain,
420 would encounter a β -(1 \rightarrow 4) linkage in the correct orientation for cleavage only every two
421 disaccharide units. Therefore, in order to processively digest long-chain polysaccharides, the
422 κ -carrageenase must dissociate from the substrate molecule thereby releasing DP4 or oligo-
423 carrageenan multiples of DP4 [32]. In this context, the DP4 to DP2 ratio made it possible to
424 depict variation in the processivity of κ -carrageenase since DP4 is produced by random and
425 processive modes of action while DP2 is exclusively produced by random depolymerisation.
426 A comparison of DP4 to DP2 ratios revealed that the processivity of κ -carrageenase was
427 nearly identical when incubated with carrageenan gel or powder (0.72 ± 0.04 and 0.69 ± 0.03
428 respectively), and higher than when incubated with soluble carrageenan (0.576 ± 0.005). We
429 have thus demonstrated that κ -carrageenase acts in an endo manner in soluble substrates
430 (with and without KCl) but in a processive manner in solid substrates. Consequently, it
431 appears that *P. carrageenovora* κ -carrageenase is an endo-processive enzyme.

432 We observed that purified DP4 can be slowly converted to DP2 (results not shown),
433 but the time and enzyme concentration required were greater than those used in the
434 experimental conditions for the complete degradation of soluble substrate. In the case of solid
435 substrates, enzyme concentrations used for degradation kinetics were twice as great as those
436 used for the soluble substrate and degradation only reached 65% and 70% for gel and powder
437 forms, respectively. After increasing enzyme concentrations four-fold, we were able to almost
438 completely degrade carrageenan powder. At this concentration, the DP4:DP2 ratio was $0.65 \pm$
439 0.02 , a value that is slightly lower than when enzyme concentration was only doubled. With
440 the four-fold enzyme concentration, we obtained about 75% degradation of the gel with a
441 DP4:DP2 ratio of 0.64 ± 0.02 . In both cases, the ratio of DP4 to DP2 in gel and powder forms
442 was higher than with soluble substrate, which provides additional support in favour of the
443 processive character of κ -carrageenase. The lower ratio of DP4 to DP2 at a higher enzyme
444 concentration can be explained by a low rate of conversion of DP4 to DP2. Unlike DP2, DP4
445 can be produced processively; thus the conversion of DP4 to DP2 would result in a

446 underestimation the processive character of κ -carrageenase. Therefore, all our investigations
447 on solid carrageenan may have underestimated the processive character of κ -carrageenase.

448 The processive mode of action of *P. carrageenovora* κ -carrageenase in heterogeneous
449 phases is in accordance with the prediction of Michel *et al* (2001) [25] who suggested this
450 type of mode of action after observing the tunnel or closed-groove topology of the active site.
451 In homogenous phases, we demonstrated that the κ -carrageenan chain is internally cleaved,
452 implying that the tunnel opens and, as a consequence, that the binding loop is flexible enough
453 to allow for repeated binding and dissociation with carrageenan chains. This is consistent with
454 crystallography data which revealed that amino acids constituting the loop presented higher
455 temperature factors than the core of the enzyme [25]. Opening of a tunnel has already been
456 observed in other endo-processive enzymes such as cellulases [48, 49], chitinases [43] and ι -
457 carrageenase [32]. The acquisition of the tunnel-shaped topology of the active site may be
458 attributed to convergent evolution of processive glycoside hydrolases acting on neutral and
459 anionic polysaccharides. Although the exact role of the binding loop is not well-understood, it
460 is supposed that it facilitates the extraction of polysaccharides from solid material [39, 50]. In
461 addition, and as for ι -carrageenase, the tunnel topology, and more specifically the loop, of κ -
462 carrageenase may be involved in unwinding the κ -carrageenan double helix [32].

463 κ -Carrageenan forms physical gels *in vitro* that mimic the conformational and physical
464 properties of algal cell walls [51, 52]. By definition, gels are not as well organised as highly
465 crystalline materials such as cellulose, chitin or starch. In addition, the chemical structure of
466 carrageenans *in vivo* are not regular, but usually hybrid or co-polymeric structures [2, 5]. This
467 chemical heterogeneity, assumed to play a role in regulating functional properties in the cell
468 wall as well as allowing for dense packing of macromolecules, creates defects which hinder
469 the formation of a highly organised structure [5]. Consequently, in this context, the flexibility
470 of the loop that opens the catalytic site, enabling it to accommodate one carrageenan chain,
471 may allow it to better extract polysaccharide chains from poorly organised regions which are
472 probably more abundant in cell walls than in gels prepared *in vitro*. Nevertheless, in the algal
473 cell wall, carrageenan is found in the solid state. Sliding along carrageenan chains would
474 increase enzyme efficiency by reducing its three-dimensional diffusion in the medium to find
475 glycosidic bonds. This may explain why carrageenan gels and powders were degraded in a
476 very similar processivity mechanism despite differences in molecular ordering of the
477 carrageenan macromolecule. Therefore, conditions required for the κ -carrageenase

478 processivity depend not only on the adapted configuration of the active site (amino acids,
479 loop), but also on the physical state of the substrate. Based on our results, we cannot rule out
480 the possibility that κ -carrageenase also processively degrades soluble substrates. However
481 determining the processivity index of κ -carrageenase—or other depolymerising enzymes—
482 using soluble substrates probably greatly underestimates their true processivity on solid
483 substrate and, as a consequence, *in vivo*.

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488 **Figure Legends**

489 **Figure 1:** Transmission electron micrograph of negatively stained κ -carrageenan gels in
490 20 mM KCl (A) and κ -carrageenan powder suspended in 20 mM KCl (B).

491 **Figure 2:** (A) Degradation kinetics of 0.2% carrageenan by *P. carrageenovora* carrageenase
492 subjected to gelation in increasing concentrations of KCl. (B) Initial velocity of degradation
493 of κ -carrageenan (\square) and low-gelling κ -carrageenan (containing 15% μ -carrabiose units) (\circ)
494 with respect to KCl concentration. Optical rotation recorded on κ -carrageenan preparation as
495 a function of KCl concentration (\blacksquare).

496 **Figure 3:** Degradation kinetics of soluble (A) and 20 mM KCl gelled κ -carrageenan (B)
497 monitored by size-exclusion chromatography as a function of the percentage of degradation as
498 defined in the Experimental section. DP: degree of polymerisation (i.e. DP4 indicates
499 tetrasaccharides). RF: Resistant fraction. * hybrid oligosaccharides

500 **Figure 4:** Variation in molecular mass of κ -carrageenan with respect to the percentage of
501 degradation as monitored by LC-MALLS. Variations in molecular weight are reported for gel
502 (\blacksquare), powder (\bullet), soluble (0 mM KCl, \circ) and low-gelling carrageenan (20 mM KCl, \square).

503 **Figure 5:** FACE of oligosaccharides released during the digestion of soluble (A), gelled (B)
504 and powder (C) forms of carrageenan at various percentages of degradation. Oligosaccharides
505 were labelled with fluorescent ANTS. * Hybrid κ -/ ι -oligosaccharides [53].

506 **Figure 6:** Amount of oligosaccharides produced during incubation of soluble (κ -carrageenan
507 in 0 mM KCl) and solid (20 mM KCl) forms of carrageenan. Oligosaccharide concentrations
508 were determined by ANTS labelling and integration of the signal from gel permeation
509 chromatography.

510 **Figure 7:** A) Schematic representation of the endolytic mode of action of *P. carrageenovora*
511 κ -carrageenase which produces all possible oligosaccharides. B) The processive degradation
512 of κ -carrageenase which slides from the reducing (R) to the non-reducing end (NR) and leads
513 to the production of DP4. The enzyme has six sub-sites and arrowheads indicate position of
514 catalytic amino acids (25).

515

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FIGURE 1

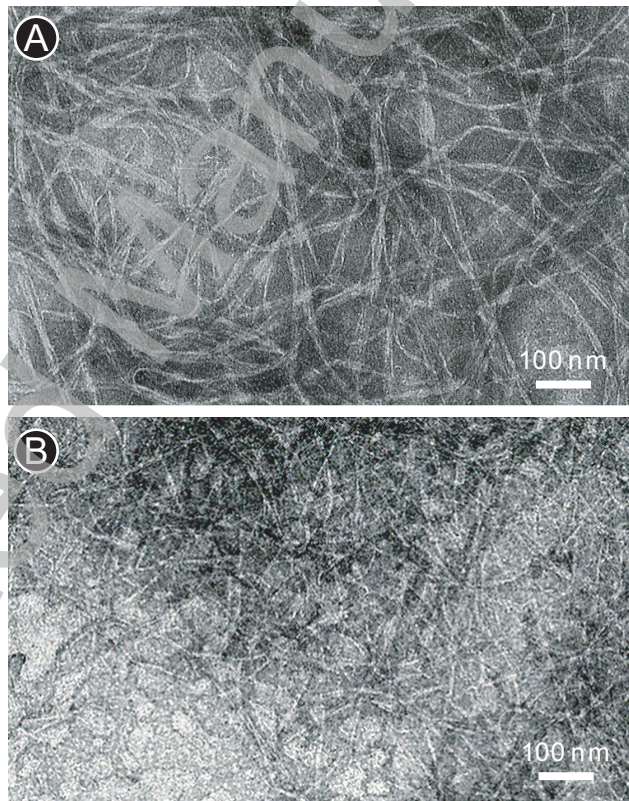


FIGURE 2

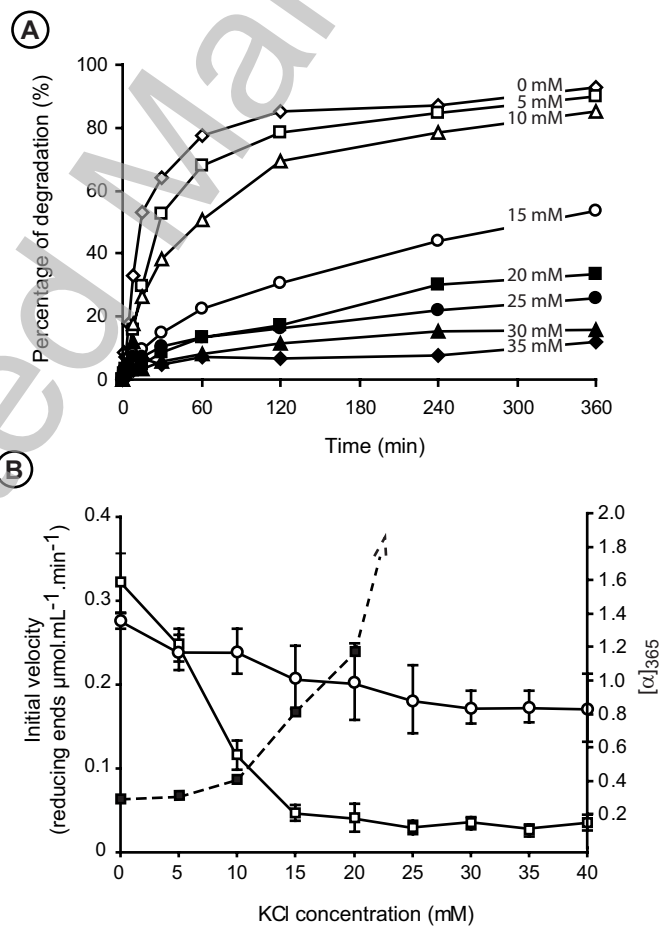


FIGURE 3

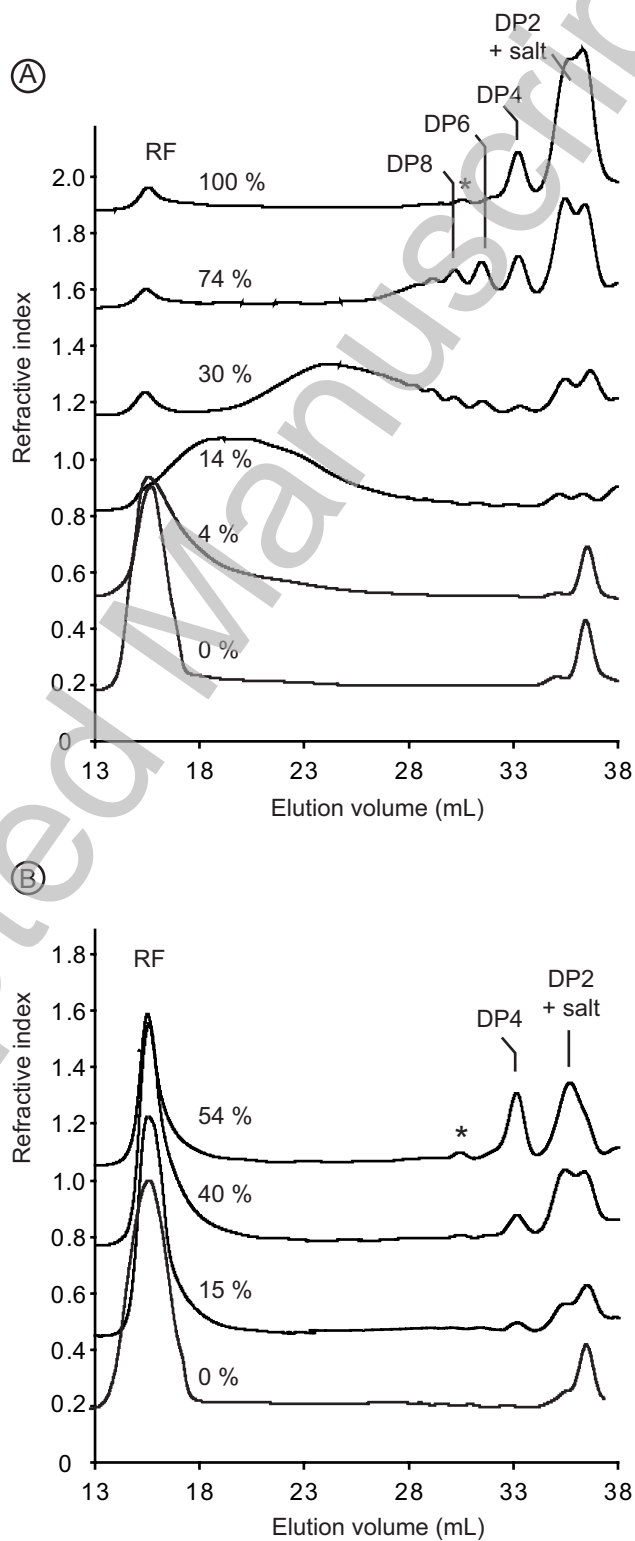


FIGURE 4

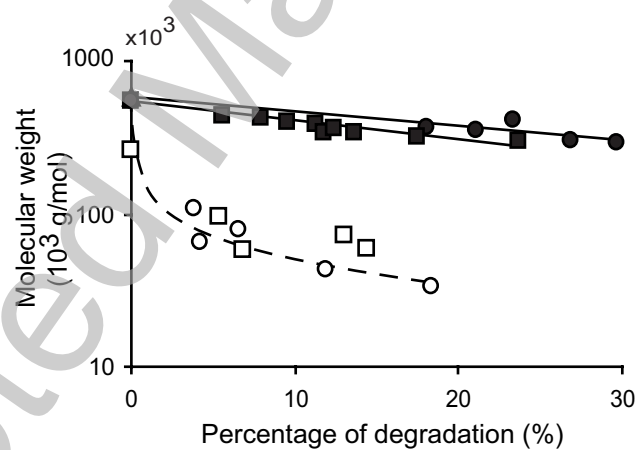
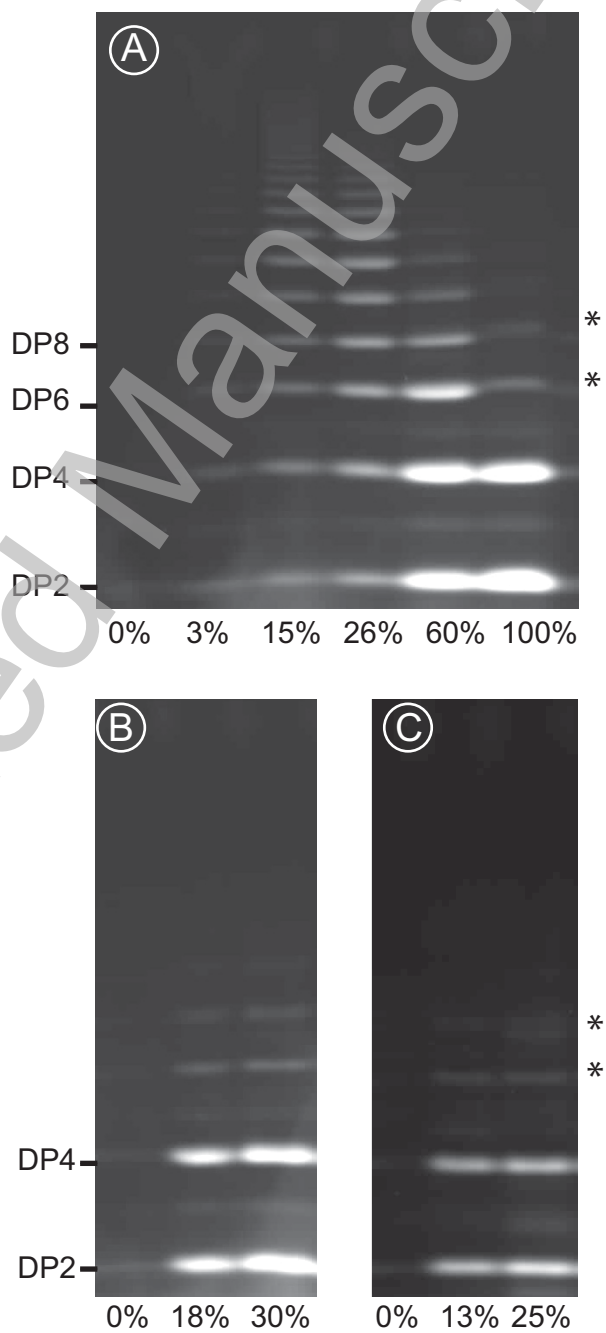


FIGURE 5



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FIGURE 6

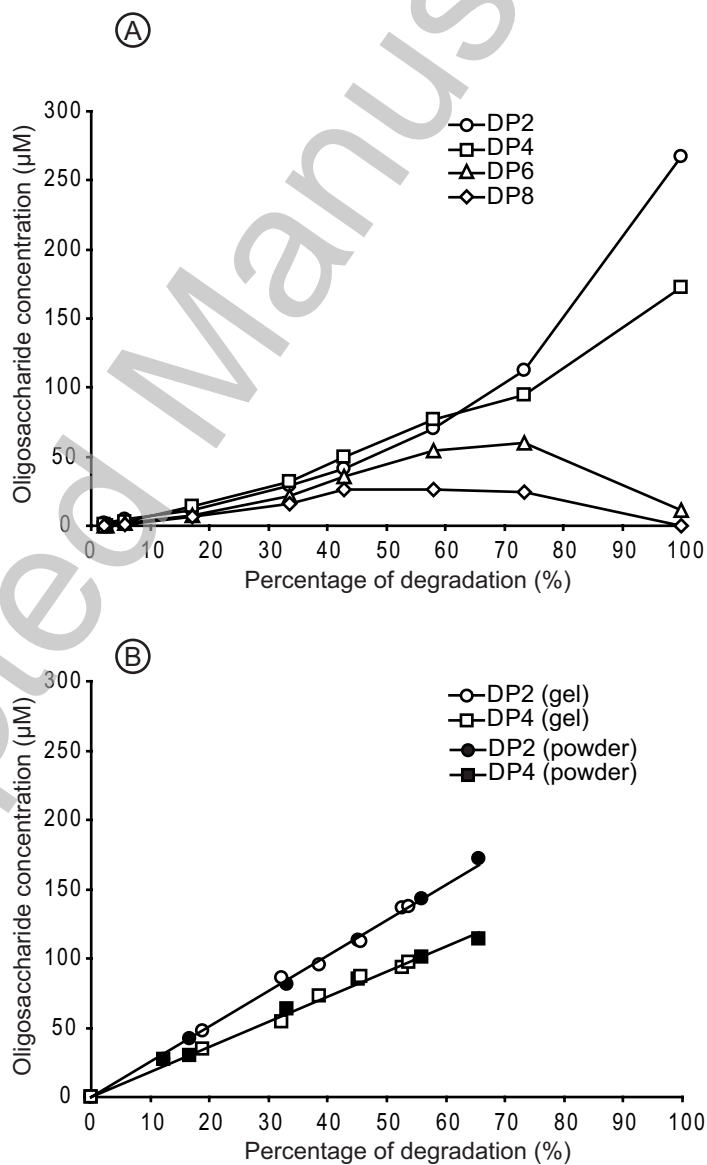
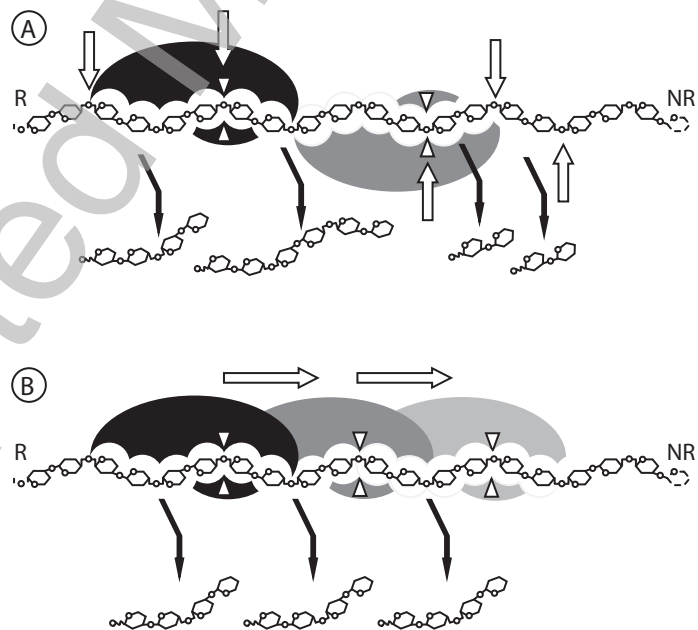


FIGURE 7



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