Physical state of \(\kappa\)-carrageenan modulates the mode of action of \(\kappa\)-carrageenase from *Pseudoalteromonas carrageenovora*

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### INTRODUCTION

Agars and carrageenans are sulfated galactans widely used for industrial applications owing to their unique physicochemical properties [1–3]. They are the most abundant components of the red algal (Rhodophyta) cell wall and can represent up to 50% of algal dry weight. Agar and carrageenan are densely packed in the cell wall in a three-dimensional solid network of pseudo-crystalline fibres which assemble during the deposition of cell wall macromolecules [4,5]. These polysaccharides are made up of linear chains of galactose with alternating \(\alpha\)-(1 \(\rightarrow\) 3) and \(\beta\)-(1 \(\rightarrow\) 4) linkages. In these galactans, the \(\beta\)-linked galactose units are in the D configuration (G unit). However, whereas the \(\alpha\)-linked galactose units are in the L configuration in agars (L unit), they are in the D configuration in carrageenans (D unit). Carrageenans are classified further according to the number and position of sulfate esters (S) and by the occurrence of 3,6-anhydro-bridges in the \(\alpha\)-linked residues (DA unit) found in gelling carrageenans [6,7]. For example, \(\kappa\)-(DA-G4S), \(\iota\)-(DA2S-G4S) and \(\lambda\)-(D2S6S-G2S) carrageenans are distinguished by the presence of a single or three ester sulfate groups per repeating disaccharide unit respectively.

Agarose, \(\kappa\)- and \(\iota\)-carrageenans form thermo-reversible gels in aqueous solution, and their stiffness decreases with the degree of sulfation. Agarose and \(\kappa\)-carrageenan produce rigid and brittle gels, whereas \(\iota\)-carrageenan gels are elastic. \(\lambda\)-Carrageenan, which lacks anhydro-bridges, gives highly viscous solutions. The gelling properties of carrageenan depend on the ionic strength and the type of salts occurring in the medium. It is well-known that potassium chloride and calcium chloride induce gelation of \(\kappa\)- and \(\iota\)-carrageenans respectively [8,9]. At the molecular level, gelation is preceded by a disorder–order (helix) transition of polysaccharide conformation, followed by aggregation of helices which gives rise to semi-crystalline fibres [10,11].

X-ray crystallography analyses of oriented fibres of \(\iota\)-carrageenan suggest that double helices, probably parallel-stranded duplexes, are packed together in junction zones (i.e. the fibres) [10,12]. In the case of \(\kappa\)-carrageenan, the conformational state of the macromolecules in gel form is still a matter of debate. Experimental data provide support for a dimeric structure of polysaccharides in an ordered state, and interpretations lead either to duplexes of single helices [13–15] or, as for \(\iota\)-carrageenan, double helices (intertwined strands) [8,10,16]. The aggregation of agarose molecules in gels also occurs by association of single or double helices [17,18].

Agar- and carrageenan-degrading enzymes, namely agarases and carrageenases, have so far only been found in marine bacteria belonging mainly to the Gammaproteobacteria, Bacteroidetes or Sphingobacteria classes [19]. Known \(\kappa\)-carrageenases (EC 3.2.1.83) belong to the GH16 (glycoside hydrolase 16) family, a polyspecific family which encompasses at least eight different enzymatic activities, including, notably, \(\beta\)-agarases (http://www.cazy.org) [20]. Phylogenetic analysis and crystallographic investigations have demonstrated that the GH16 family of enzymes evolved from a common ancestor and that \(\kappa\)-carrageenases most likely emerged from the \(\beta\)-agarase branch [21]. \(\beta\)-Agarases and \(\kappa\)-carrageenases adopt a jellyroll fold and hydrolyse \(\beta\)-(1 \(\rightarrow\) 4) glycosidic linkages with retention of the anomeric configuration [22,23]. Analyses of the mode of action of \(\beta\)-agarases suggest that agarose molecules undergo random depolymerization. In the case of agarase A, in addition to the active catalytic site, a second strong binding site was observed on the catalytic module. It has been proposed that the co-occurrence of two binding sites facilitates heterogeneous phase degradation of agarose by unwinding or dissociating helices occurring in the gel state [24]. The mode of action of \(\kappa\)-carrageenase has not been investigated biochemically. However, considering the tunnel-shaped topology of the active site, it has been suggested that...
this enzyme proceeds via a non-random processive mechanism [25].

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

The enzymatic digestion of κ-carrageenan, whose chemical structure is intermediate between agarose and κ-carrageenan, has not yet been investigated. β-Agarases share a common ancestor with κ-carrageenases, but the physicochemical properties as well as the anionic nature of κ-carrageenan chains closely resemble those of κ-carrageenan. The tunnel topology found in both κ- and i-carrageenases reinforces the hypothesis of convergent evolution of these enzymes towards a mode of action adapted to substrates that share common chemical and physical properties. In this context, we sought to characterize the mode of action of the Pseudoalteromonas carrageenovora κ-carrageenase in order to consider the potential correlation between the tunnel-shaped topology of the active site with the enzyme’s mode of action. We demonstrate that the κ-carrageenase is a processive enzyme when acting on solid substrates. Nevertheless, κ-carrageenase has a random mode of action on soluble substrates. These observations suggest that the conditions for processivity not only require that the active site possesses the adapted topology, but also that the substrate be in an appropriate physical and conformational state.

**EXPERIMENTAL**

**Purification of κ-carrageenan**

κ-Carrageenan extracted from Kappaphycus alvarezii (Eucheuma cottonii) was supplied by CP Kelco (κ-carrageenan, cottonii X-6913 and low-gelling κ-μ-carrageenan, cottonii X-6042). Before use, carrageenans were purified by propan-2-ol precipitation as follows. Carrageenan powder (5 g) was suspended in 500 ml of cold ultrapure water (Millipore Water Purifier). The suspension was heated at 70 °C with slow stirring until the carrageenan was completely dissolved. The polysaccharides in solution were precipitated by dropwise addition of 1 litre of propan-2-ol with vigorous stirring. After centrifugation at 10500 g, the precipitate was collected and dissolved again in 500 ml of ultrapure water. This precipitation protocol was repeated twice. Carrageenans were then dialysed (6–8 kDa molecular mass cut-off Spectra/Por®) against ultrapure water in order to remove excess salt and non-polymeric materials in the presence of 5 mM NaOH to prevent acidification of the sample. Finally, the sodium carrageenan solution was filtered through a 0.45 μm pore-size filter (Pall Life Sciences, Acrodisc Syringe Filter, HT Tuffryn Membrane) and freeze-dried in the presence of 20 mM ammonium carbonate.

**Recombinant κ-carrageenase**

Pseudoalteromonas carrageenovora κ-carrageenase was recombinantly expressed in Escherichia coli as described by Michel et al. [33]. The overexpressed His-tagged fusion protein was purified by metal-affinity chromatography on a chemical Sepharose Fast Flow column (GE Healthcare) loaded with NiSO₄, followed by cation exchange chromatography on a MonoS column (HR 5/5, GE Healthcare). A stock solution containing 60 μg·ml⁻¹ of κ-carrageenase with an activity of 0.06 mM equiv. glucose min⁻¹·μg⁻¹ (0.125 % κ-carrageenan in 0.2 M Tris/HCl) was stored at 4 °C.

**Enzymatic degradation**

A stock solution of 0.4 % (w/v) carrageenan in 10 mM Tris/HCl (pH 8.0) was prepared by mixing at room temperature (22 °C) with gentle stirring overnight. To ensure complete dissolution, the solution was heated for at least 1 h at 70 °C. It was stored at 4 °C for 2 weeks at most. Salt solutions containing 200 mM LiNO₃, 2 mM citric acid (used as internal standard for chromatography experiments) and various concentrations of KCl ranging from 0 to 80 mM were prepared just before use.

For enzymatic degradation, the 0.4 % carrageenan solution and the salt solutions were heated separately at 70 °C for 10 min before being mixed at equal volume resulting in final concentrations of 0.2 % carrageenan in 100 mM LiNO₃, 1 mM citric acid and 0–40 mM KCl. The mixture was kept at 70 °C for at least 30 min before being transferred to room temperature. Gels were stabilized for at least 12 h at room temperature before incubation with enzyme. Given the range of salt concentrations used (from 10 to 40 mM KCl), gels were vigorously stirred and pipette tip ends were cut so that gel suspensions could be pipetted.

Enzymatic digestions of κ-carrageenan were conducted at 30 °C. At first, the tubes were shaken vigorously and initial undigested samples were collected. κ-Carrageenase was then added to the incubation medium: 0.12 μg·ml⁻¹ for carrageenan solutions and 0.24 μg·ml⁻¹ for carrageenan gels. NaOH was added to a final concentration of 100 mM in order to stop the enzymatic reaction. κ-Carrageenase is inactivated in alkaline solutions [34] without any alteration of the chemical structure of κ-carrageenan.

**Reducing-sugar assay**

The amount of reducing sugars produced during the enzymatic digestion of κ-carrageenan was determined using a method adapted from that of Kidby and Davidson [35]. Aliquots (100 μl) of the reaction medium diluted five times were mixed with 1 ml of ferricyanide solution (300 mg of potassium hexacyanoferrate III, 29 g of Na₂CO₃, and 1 ml of 5 M NaOH, made up to 1 litre with water). The mixture was boiled for 10 min and cooled to room temperature, and its absorbance was read at 420 nm. We defined 100 % degradation as the amount of reducing sugars obtained when soluble κ-carrageenan was completely converted into DP4 (where DP is the degree of polymerization) and DP2, and DP6 (neo-κ-hexacarrabioside) could no longer be detected by HPAEC (high-performance anion-exchange chromatography).
LC (liquid chromatography)–MALLS (multi-angle laser light scattering)

Before LC–MALLS experiments, samples were dialysed extensively against ultrapure water containing 20 mM ammonium carbonate (to prevent depolymerization which may occur during freeze-drying) at 4°C using a 100 Da membrane (Spectra/Por® 100, cellulose ester) to eliminate KCl salts that promote gelation of carrageenan. Dialysed specimens were freeze-dried. Samples were then dissolved in 100 mM LiNO₃, to a final concentration of approx. 0.3%, Soluble carrageenan (without KCl) and carrageenan gels (with KCl) were prepared in a similar fashion.

After filtration (0.22 µm pore-size filter; Millipore), samples (200 µl) were injected on a Superdex 200 column (300 length × 10 mm internal diameter; GE Healthcare) followed by a Superdex 200 carrageenan gels (with KCl) were prepared in a similar fashion. By adding, and the sample was kept at 37°C. The signals recorded at various angles of detection, with the signal measured at 90°C. Refractive index detector, used as a mass-sensitive detector, set at 890 nm at 35°C. MALLS measurements were performed at 690 nm with a DAWN EOS system (Wyatt Technology) equipped with a 30 mW GaAs linearly polarized laser. The intensity of scattered light was measured at 12 different angles, from 35° to 143°. Chromatographic data were collected and processed using Astra software (Wyatt Technology). The Zimm fit method was applied for molecular mass determinations. The calculated dn/dc was 0.115 ml · g⁻¹. BSA monomer (Sigma) was used to normalize the signals recorded at various angles of detection, with the signal measured at 90°C.

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

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

Before labelling, the enzymatic reaction was stopped by adding 500 µl of ethanol to an equivalent volume of sample. The mixture was maintained at 80°C for approx. 10 min to ensure that the enzyme was denatured. The mixture was then dried under vacuum. Then, 40 µl of 0.15 M ANTS solution (ANTS disodium salt in 15% acetic acid) was added to the dried sample and kept at 37°C for 30 min. Then, 75 µl of 1 M NaBH₄CN in DMSO was added, and the sample was kept at 37°C for 16 h (i.e. overnight). The sample was dried under vacuum and dissolved in 2.5 ml of water, which corresponds to a 5x dilution of the initial sample concentration. For solid substrates (gel and powder), the final concentration of KCl was 5 mM, a concentration at which carrageenan does not gel. At this stage, ANTS-labelled oligo-carrageenans were visualized after migration in a 27% (w/v) carbohydrate polyacrylamide gel [FACE (fluorescence-assisted carbohydrate electrophoresis)] using the method of Guibet et al. [36].

The sample was filtered (0.22 µm, Millipore) and 100 µl was injected on a Superdex peptide 10/300 column (GE Healthcare) followed by a Superdex 200 column (300 length × 10 mm internal diameter; GE Healthcare), mounted in series. Elution was performed with 0.1 M LiNO₃ filtered at 0.1 µm (refractive index = 1.327) at a flow rate of 0.3 ml · min⁻¹ (Waters 626 pump) at 25°C. Detection was monitored by a Waters 2414 refractive index detector, used as a mass-sensitive detector, set at 890 nm at 35°C. MALLS measurements were performed at 690 nm with a DAWN EOS system (Wyatt Technology) equipped with a 30 mW GaAs linearly polarized laser. The intensity of scattered light was measured at 12 different angles, from 35° to 143°. Chromatographic data were collected and processed using Astra software (Wyatt Technology). The Zimm fit method was applied for molecular mass determinations. The calculated dn/dc was 0.115 ml · g⁻¹. BSA monomer (Sigma) was used to normalize the signals recorded at various angles of detection, with the signal measured at 90°C.

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HsPAC

Degradation products were analysed using a Dionex chromatograph DX 500 equipped with a 20 µl injection loop, an AS 100XR automated injection system (Thermo Separation Products) and an AS11 anion-exchange column (4 mm × 250 mm, Dionex IonPac®) with a AG11 pre-column (4 mm × 50 mm, Dionex IonPac®). The system was operated in conductivity mode using a ED40 detector (Dionex) and a Dionex ASRS ultra-4 mm suppressor with a current of 300 mA. Mobile phases were ultrapurified water and 290 mM NaOH. Elution was conducted at a flow rate of 0.5 ml · min⁻¹ with GP40 Gradient Pump (Dionex). A linear gradient of 0 to 100% (290 mM) NaOH was applied for 33 min. Between each injection, the column was equilibrated with the mobile phase for 10 min. The Chromelon-Peak net software ( Dionex) program was used for data acquisition and treatment. The area of the oligosaccharide peaks was integrated and normalized using the peak of 1 mM citric acid which was systematically included in the carrageenan preparations and used as an internal standard.

Polarimetry

Optical rotation measurements were performed using a PerkinElmer 341 polarimeter operating at a wavelength of 365 nm produced by mercury lamp. Samples were kept at 25°C in a thermostatically controlled jacketed cell having an optical pathlength of 10 cm.

TEM

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

RESULTS

In order to obtain soluble and gelled substrates, carrageenans were dissolved by heating solutions containing increasing amounts of KCl from 0 to 40 mM. In the absence of KCl, the carrageenan solution remained in liquid form at room temperature, corresponding to complete dissolution. Gelation was observed macroscopically at room temperature when the mixture contained at least 10 mM KCl. The formation of carrageenan helices and the occurrence of aggregation were confirmed by optical rotation (see Figure 2B) and by light scattering (not shown) respectively. At KCl concentrations of 10 mM, a fragile, elastic, easily broken gel was obtained. At KCl concentrations greater than 10 mM, gels increased in strength, but could still be broken by shaking. The transmission electron micrograph of negatively stained κ-carrageenan gel prepared in 20 mM KCl showed that it was composed of interconnected elongated fibres approx. 10–20 nm

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wide (Figure 1A). The fibres were made up of a thin substructure ascribed to helix duplexes or aggregated helices [37,38].

Carrageenan powder, obtained by freeze-drying sodium carrageenan solubilized in hot water, was suspended in 20 mM KCl without heating to avoid the formation of extended helices that induce gelation. As expected, TEM revealed that the specimen was mainly composed of poorly organized or amorphous carrageenan aggregates. However, as illustrated in Figure 1(B), very thin fibre-like structures 2–5 nm wide were observed. The thin fibres were probably already present in the carrageenan powder, but may also result from the rearrangement of carrageenan molecules when the freeze-dried powder was rehydrated in the KCl solution. Hereafter, we considered gel and powder forms of carrageenan as organized and poorly organized solid substrates respectively.

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

Hydrolysis velocities recorded at the beginning of incubation (<15 min) are given in Figure 2(B). As observed for the degradation kinetics (Figure 2A), velocity decreased with increasing amounts of KCl, with the minimum velocity being measured when KCl concentrations reached 30 mM. Kinetic experiments were also conducted with κ-carrageenan containing approx. 15% μ-carrabiose units, which is a carrageenan well known for its low gelling capacity. The overall kinetic curves were very similar to that of pure κ-carrageenan, and 100% degradation was also attained in 360 min (results not shown). However, in contrast with pure κ-carrageenan, the amount of KCl in the medium had less effect on the apparent activity of the enzyme. As illustrated in Figure 2(B), the initial velocity decreased slowly and linearly as a function of KCl concentrations, and approx. 20% loss of activity was observed in the presence of 30 mM KCl.

The depolymerization of κ-carrageenan induced by κ-carrageenase activity was followed by gel-permeation chromatography coupled with a MALLS detector. In order to analyse all components occurring in the incubation medium, including gelled and soluble fractions of carrageenan, the samples were dialysed against water until buffer and KCl salts were removed. This procedure led to the complete dissolution of all carrageenan molecules and released any carrageenan oligosaccharides still
Processivity of κ-carrageenase from Pseudoalteromonas carrageenovora

Figure 3 Degradation kinetics of soluble (A) and 20 mM KCl gelled κ-carrageenan (B) monitored by size-exclusion chromatography as a function of the percentage of degradation

entraped in gel networks. As a consequence, the chromatography experiments presented hereafter are pictures of the overall molecular distribution of the incubation medium. In Figures 3(A) and 3(B) respectively, the time courses of depolymerization of soluble (0 mM KCl) and gelled κ-carrageenan (20 mM KCl) are presented. The chromatogram corresponding to undigested samples showed a single peak eluting in the exclusion volume (at 16 ml retention volume) attributed to a 750 kDa polysaccharide (Figure 3). As the degradation of soluble substrate proceeded (Figure 3A), the signal corresponding to the polymer fraction shifted towards higher elution volumes, indicating a decrease in molecular mass. This was correlated to the MALLS measurements shown in Figure 4(A) which shows the fall in the molecular mass to 40 kDa after 10% hydrolysis. In addition, the shape of the peak became broader, indicating an increase in polydispersity. Similar observations were made with low-gelling κ-carrageenan incubated in 0 and 20 mM KCl. Permeation gel chromatograms as well as variation in molecular mass quantified by MALLS followed the same patterns (results not shown), whatever the KCl concentration. The decrease in molecular mass of low-gelling carrageenan in KCl shown in Figure 4 demonstrates clearly that depolymerization followed a pattern similar to that of soluble carrageenan incubated without KCl. Recorded after 15% hydrolysis, the gel-permeation chromatogram (Figure 3A) shows well-resolved signals that were ascribed to neo-oligocarrabioses from DP2 to DP12. The rapid depolymerization, the increase of polydispersity and the production of all possible oligosaccharides are features of an endolytic mode of action.

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

The time course of oligosaccharide production was determined after labelling the reducing ends with the ANTS fluorophore. Using this approach, it was possible to measure directly the signal intensity of DP2 which was eluted too close to salts (Figure 3) to determine its abundance directly using size-exclusion chromatography. Although HPAEC made it possible to visualize all the other oligosaccharides produced, again DP2 co-eluted with salts (not shown), hindering direct estimation of its production. The ANTS-labelling method offers the additional advantage of making it possible to quantitatively determine the amount of all of the oligosaccharides produced. As expected, at low percentages of degradation of soluble carrageenan, a wide range of fluorescent oligosaccharides could be visualized by electrophoresis (Figure 5A). For example, after 15% degradation, it was possible...
to distinguish oligosaccharides from DP2 to DP26 which were converted into only DP4 and DP2 at the end of the kinetics experiment. Precise determination of oligosaccharide concentration from DP2 to DP8 was achieved by gel-permeation chromatography coupled to a UV detector, allowing the measurement of signal intensity corresponding to labelled oligosaccharides (Figure 6). The time-course production of the intermediate products DP6 and DP8 had a bell shape, while the amount of end-products DP2 and DP4 increased as the degradation proceeded. After complete degradation, the DP4/DP2 ratio was calculated to be, on average, $0.576 \pm 0.005$ ($n = 6$). Similarly, chromatograms of low-gelling $\kappa/\mu$-carrageenan incubated without KCl and with 20 mM KCl showed a wide distribution of oligosaccharides with abundances comparable with those observed with pure soluble $\kappa$-carrageenan, but hybrid $\kappa/\mu$-carrageenan oligosaccharides were also present in the digest (results not shown).

As observed previously with unlabelled degradation products of carrageenan gels and powder, electrophoresis profiles revealed that only DP2 and DP4 were produced during incubation with $\kappa$-carrageenase (Figures 5B and 5C). As illustrated in Figure 6(B), the amount of DP2 and DP4 increased linearly with the extent of reaction for both gel and powder forms. The DP4/DP2 ratio calculated for gel and powder forms had similar values: $0.72 \pm 0.04$ and $0.69 \pm 0.03$ respectively.

**DISCUSSION**

The yields as well as the velocities of degradation of $\kappa$-carrageenan by *P. carrageenovora* $\kappa$-carrageenase decreased with increasing amounts of KCl in the incubation medium (Figure 2). This apparent decrease in activity correlated with the aggregation of carrageenan molecules promoted by $K^+$ and $Cl^-$ ions. This aggregation led to the formation of a three-dimensional network of fibres visualized by TEM (Figure 1). In the solid state, fewer glycosidic linkages are exposed to the enzyme and, moreover, the network of fibres reduces the ability of the enzyme to diffuse.

In low-gelling $\kappa/\mu$-carrageenan, the velocity of degradation decreased very little with increasing KCl concentrations (Figure 2B). $\mu$-Carrabiose units are known to hinder gelation of $\kappa$-carrageenan because of the irregularities or kinks in the chain that they induce [6,39]. Nevertheless, addition of KCl stiffened the $\kappa$-carrabiose segments occurring in the hybrid chains and, as a consequence, increased the viscosity of the medium, thereby reducing enzyme and substrate diffusion. When KCl concentrations were greater than 200 mM, concentrations at which $\kappa/\mu$-carrageenan starts to form a gel, we observed a decrease in the apparent activity of $\kappa$-carrageenase, equivalent...
to that observed for pure \( \kappa \)-carrageenan in 30 mM (results not shown). In addition, in both soluble carrageenan and low-gelling carrageenan incubated in 0 and 20 mM KCl, an endolytic mode of action was revealed, given the rapid decrease in molecular mass and slow production of di- and tetra-saccharides as a function of catalytic events. Consequently, it is likely that \( \text{K}^+ \) and \( \text{Cl}^- \) ions themselves do not play a direct role in the mechanism of action of \( \kappa \)-carrageenase, but rather strongly modulate the physicochemical properties of \( \kappa \)-carrageenan as observed by optical rotation and light-scattering experiments. The apparent decrease in enzymatic activity on pure \( \kappa \)-carrageenan should be considered as a decrease in substrate accessibility and reduced diffusion of the enzyme.

Analysis of the mode of action of glycoside hydrolases incubated with substrates in different conformational or physical states can bring the different characteristics of an enzyme to light. Soluble or poorly organized solid substrates are known to facilitate the detection of an endolytic mode of action of an enzyme. For example, endo-acting cellulases are more efficient on carboxymethylcellulose or PASC (phosphoric acid-swollen cellulose) than on crystalline cellulose (e.g. [40–42]). Similarly, chitosan (partly deacetylated chitin) is preferred over solid and unmodified chitin for identifying endo-acting chitinases [43,44]. Although processivity can be observed on soluble substrates [26,44], it is more straightforward to demonstrate in well-organized or crystalline material observed by electron microscopy [45–48] or by chromatography methods [42]. By adopting a similar strategy, we prepared soluble carrageenan as well as poorly (powder) and well-organized (gel) carrageenan in the solid state. In solution, \( \kappa \)-carrageenan adopted a flexible rod conformation and enzymatic depolymerization led to the production of all likely neo-oligo-carrageenans (Figures 3A and 5A). This wide range of products was accompanied by a rapid decrease in molecular mass and an increase of polydispersity (Figure 4). Consequently, under these conditions, \( P. \) carrageenovora \( \kappa \)-carrageenase can be defined as an endo-acting hydrolase. The same conclusions were drawn from analyses of enzymatic degradation products of the low-gelling \( \kappa \)-//carrageenan (results not shown).

Chemical modification of polysaccharides can introduce a bias in the interpretation and comparison of the mode of action of enzymes in homogenous and heterogeneous phases. Consequently, carrageenan gels and powder were prepared with poly-saccharides having the same chemical structure and same macromolecular parameters (molecular mass, polydispersity) as those studied in the soluble state. Since these carrageenans were not subjected to any chemical modification, our observations were correlated directly with the conformational and physical states of the substrate. When carrageenan was incubated in a solid state (gel and powder), we only observed the end products DP4 and DP2. We assume that any solubilized intermediate oligosaccharides were quickly degraded into DP4 and DP2. Furthermore, these intermediate oligosaccharides were probably cleaved faster than more recalcitrant carrageenan chains located in the solid polysaccharide network. As a consequence, although degradation of carrageenan powder was faster than that of the gel, the production of DP4 and DP2 increased linearly in both cases. The production of DP4 was greater when the substrate was solid (gel or powder) than when the substrate was soluble. Moreover, when carrageenan was incubated in the solid state, the molecular mass of \( \kappa \)-carrageenan and its polydispersity did not change much between 0 and 30 % degradation (Figure 4).

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

We observed that purified DP4 can be slowly converted into DP2 (results not shown), but the time and enzyme concentration required were greater than those used in the experimental conditions for the complete degradation of soluble substrate. In the case of solid substrates, enzyme concentrations used for degradation kinetics were twice as great as those used for the soluble substrate, and degradation only reached 65 and 70 % for gel and powder forms respectively. After increasing enzyme concentrations 4-fold, we were able to degrade carrageenan powder almost completely. At this concentration, the DP4/DP2 ratio was 0.65 ± 0.02, a value that is slightly lower than when enzyme concentration was only doubled. With the 4-fold enzyme concentration, we obtained approx. 75 % degradation of the gel with a DP4/DP2 ratio of 0.64 ± 0.02. In both cases, the DP4/DP2 ratio in gel and powder forms was higher than with soluble...
substrate, which provides additional support in favour of the processive character of κ-carrageenase. The lower DP4/DP2 ratio at a higher enzyme concentration can be explained by a low rate of conversion of DP4 into DP2. Unlike DP2, DP4 can be produced processively; thus the conversion of DP4 into DP2 would result in an underestimation of the processive character of κ-carrageenase. Therefore all our investigations on solid carrageenan may have underestimated the processive character of κ-carrageenase.

The processive mode of action of *P. carrageenovora* κ-carrageenase in heterogeneous phases is in accordance with the prediction of Michel et al. [25], who suggested this type of mode of action after observing the tunnel or closed-groove topology of the active site. In homogenous phases, we demonstrated that the κ-carrageenan chain is internally cleaved, implying that the tunnel opens and, as a consequence, that the binding loop is flexible enough to allow for repeated binding and dissociation with carrageenan chains. This is consistent with crystallography data which revealed that amino acids constituting the loop presented higher temperature factors than the core of the enzyme [25]. Opening of a tunnel has already been observed in other endo-processive enzymes such as cellulases [49,50], chitinases [44] and ι-carrageenase [32]. The acquisition of the tunnel-shaped topology of the active site may be attributed to convergent evolution of processive glycoside hydrolases acting on neutral and anionic polysaccharides. Although the exact role of the binding loop is not well understood, it is supposed that it facilitates the extraction of polysaccharides from solid material [40,51]. In addition, and as for ι-carrageenase, the tunnel topology, and more specifically the loop, of κ-carrageenase may be involved in unwinding the κ-carrageenan double helix [32].

κ-Carrageenan forms physical gels in vitro that mimic the conformational and physical properties of algal cell walls [52,53]. By definition, gels are not as well organized as highly crystalline materials such as cellulose, chitin or starch. In addition, the chemical structure of carrageenans in vivo are not regular, but are usually hybrid or co-polymeric structures [2,5]. This chemical heterogeneity, assumed to play a role in regulating functional properties in the cell wall as well as allowing for dense packing of macromolecules, creates defects which hinder the formation of a highly organized structure [5]. Consequently, in this context, the flexibility of the loop that opens the catalytic site, enabling it to accommodate one carrageenan chain, may allow it to better extract polysaccharide chains from poorly organized regions which are probably more abundant in cell walls than in gels prepared in vitro. Nevertheless, in the algal cell wall, carrageenan is found in the solid state. Sliding along carrageenan chains would increase enzyme efficiency by reducing its three-dimensional diffusion in the medium to find glycosidic bonds. This may explain why carrageenan gels and powders were degraded by a very similar processivity mechanism despite differences in molecular ordering of the carrageenan macromolecule. Therefore conditions required for the κ-carrageenase processivity depend not only on the adapted configuration of the active site (amino acids and loop), but also on the physical state of the substrate. On the basis of our results, we cannot rule out the possibility that κ-carrageenase also processively degrades soluble substrates. However, determining the processivity index of κ-carrageenase, or other depolymerizing enzymes, using soluble substrates probably greatly underestimates their true processivity on solid substrate and, as a consequence, in vivo.

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