Microscopic and Molecular Insights into Heterogeneous Phase Degradation of Agars and Carrageenans by Marine Bacterial Galactanases

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Summary: Agars and carrageenans are sulfated galactans and are the main matrix component of the cell wall of red algae. They form gels made of a 3D network of fibres built by the aggregation of polysaccharide double-helices. Agarases and carrageenase are enzymes produced by marine bacteria involved in the conversion of algal biomass. We have analyzed the structures and the modes of action of these enzymes adapted to the degradation of anionic polysaccharides in heterogeneous phase. We found that κ- and ι-carrageenases proceed *via* an endo-processive mode of action as it was already described for other enzymes active on neutral polysaccharides (cellulose, starch).

Keywords: agarases; carrageenases; degradation; gels; processivity

Introduction

Numerous polysaccharides, such as cellulose, starch and chitin, occur in their biological context in an insoluble, quasi crystalline form. The biodegradation of such recalcitrant polymers is a challenging task, and the proteins which attack these substrates have evolved specific features to increase their efficiency. Among the most efficient polysaccharidases, many have developed a non-random, processive behavior. After the initial attack, a processive hydrolase remains attached to one extremity of the polymer, thus performing the cleavage of several glycosidic bonds before it dissociates.

In the last decade, combination of electron microscopy and crystallographic approaches has given insight into the mode of action of several glycoside hydrolases acting on insoluble polysaccharides. The processive character of α-amylases, cellobiohydrolases, and chitinases was demonstrated by electron microscopy, in monitoring the enzymatic degradation of their solid substrate[1–4]. Crystallographic studies have established a number of structural bases for processivity. The cellobiohydrolases of families GH-6, GH-7 and GH-48, as well as the exo-chitinases of family GH-18, display different folds, but they all exhibit active site with a tunnel-topology[5–8]. This active site topology allows the protein to progress unidirectionally along the polysaccharide chain without dissociating from its substrate between two hydrolysis events. The presence of an additional carbohydrate binding module (CBM), which remains loosely associated to the crystalline substrate, can also promote the processive behavior of some enzymes with an open groove topology. Such a CBM was observed in the endo/exo cellulase E4 and the exochitinase ChiB[8,9].

Agars and carrageenans are the main components of the cell walls of red algae (Rhodophyta), where they are laid out as highly ordered molecules. This large family of hydrocolloids is made up of linear chains of galactose, with alternating $\alpha(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linkages. The red algal galactans are polyanionic polymers, highly sulfated.

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The ideal disaccharidic monomers of the main industrially used algal galactans are substituted by zero (agarose), one (κ-), two (ι-), or three (λ-carrageenan) sulfate groups. Due to the occurrence of 3,6-anhydro bridges in the α-linked galactose residues, agars, κ- and ι-carrageenans form thermoreversible gels in aqueous solutions, involving a superhelical type mechanism of gelation, which consists of the formation of helices followed by their aggregation. Carrageenan gelation is promoted by the presence of cations, such as potassium for κ-carrageenan and calcium for ι-carrageenan. Thus, in contrast to cellulose or chitin, which occur only as solid, neutral polysaccharides, the sulfated galactans constitute an original model of polyanionic polymers which can reversibly adopt either a soluble or a solid state.

The recycling of red algal biomass involves marine bacteria which produce enzymes specific for the degradation of agars and carrageenans. As enzymes which naturally attack concentrated gels, galactanases constitute an interesting model to study the heterogeneous phase degradation of sulfated polysaccharides. To this end, we have cloned several β-agarases, κ- and ι-carrageenases from marine bacteria[10–12]. β-Agarases and κ-carrageenases belong to family GH-16 of the glycoside hydrolases, while ι-carrageenases constitute a distinct structural family (family GH-82). We have determined the catalytic mechanisms and the crystal structures of the two paralog β-agarases AgaA and AgaB[13], the κ-carrageenase CgkA[14], and the ι-carrageenase CgiA[15]. Structures of AgaA and CgiA in complex with agarose and ι-carrageenan fragments also unravel the mode of recognition of these galactans[16,17]. We have also undertaken biochemical and electron microscopy analysis of the mode of action of these enzymes, using gels that mimic the physical state of their substrate *in vivo*[12,17,18]. This multi-faceted approach has thus provided the first molecular bases for the enzymatic degradation of agarose, κ- and ι-carrageenan in heterogeneous phase.

Mode of Action of Family GH-16 β-agarases and κ-carrageenases

The β-agarases AgaA and AgaB from *Zobellia galactanivorans*[13] and the κ-carrageenase CgkA from *Pseudoalteromonas carrageenovora*[15] adopt a similar jelly-roll fold, constituted by two curved, antiparallel β-sheets forming a β-sandwich. The catalytic machinery, which is localized at the surface of the inner β-sheet, is conserved in both types of galactanases and corresponds to the pattern ExDxxE, where the two glutamic acids are the nucleophile and acid/base catalysts, respectively. Despite their identical fold and catalytic machinery, β-agarases and κ-carrageenase display different active site topologies. The β-agarases AgaA and AgaB both feature an open groove topology, an active site typical of endo hydrolases. In contrast, the κ-carrageenase CgkA has a tunnel topology, reminiscent of the cellobiohydrolase active site. This latter topology suggests that the κ-carrageenase is a processive hydrolase[14].

To test those assumptions, the enzymatic degradation of agarose and κ-carrageenan was monitored by HPAEC (High Performance anion exchange chromatography)[12] and SEC (Size exclusion chromatography)[18], respectively. On melted agarose, the β-agarases AgaA and AgaB first produced high-molecular mass oligosaccharides, which were converted progressively into smaller oligosaccharides[12]. Such a digestion profile is typical of random, endolytic glycoside hydrolases, a mode of action consistent with the groove topology of the β-agarases[13]. Using liquid-phase agarose, both β-agarases degraded ~100% of the substrate at completion (24 h). However, the two enzymes differ markedly in their degradation of solid phase agarose gels. Based on the reducing-sugar assay, AgaA degrades at completion 42 % of a 0.125 % (w/v) agarose gel, whereas AgaB was capable of degrading only 21 % of a gel[12]. The presence of a secondary agarose-binding site located at the outer, non-catalytic face of AgaA[16]

likely explains the higher efficiency of this enzyme in the degradation of agarose gels. This additional, parallel agarose binding site, which is absent in the β-agarase AgaB, was proposed to either unwind the agarose double helices[16] or to disrupt the crystalline aggregates of helices constituting the agarose fibers[15].

The degradation profile of κ-carrageenase on liquid-phase κ-carrageenan is surprisingly similar to that of β-agarases (Figure 1A)[18]. The κ-carrageenase CgkA thus acts in solution as a random hydrolase, indicating that conformational changes should occur in the tunnel-forming loops to allow the endo-attack of the polysaccharide chain. However, the degradation profile drastically differs when the enzyme acts on κ-carrageenan gels. On soluble κ-carrageenan, CgkA produces only large oligosaccharides at ~50% of degradation (Figure 1A). In contrast, at the same level of degradation in solid phase, the κ-carrageenase essentially generates terminal products, the κ-neocarrabiose (DP2) and the κ-neocarratetraose (DP4) (Figure 1B). Therefore, CgkA behaves as a processive enzyme on κ-carrageenan crystalline fibers [18], as suggested by the tunnel topology of its active site[14]. Interestingly, we observed that the mode of action of this enzyme is strongly modulated by the physical state (soluble, solid) of its substrate.

Mode of Action of ι-carrageenases in Heterogeneous Phase

The degradation mechanism of ι-carrageenan gels by the ι-carrageenase CgiA from *Alteromonas fortis* was investigated by transmission electron microscopy (Figure 2)[17]. On the basis of the kinetics of release of reducing ends, when about 20% of the ι-carrageenan gel was digested, the initial structure of the fiber network was still apparent. However, the size of the fibers was deeply modified, with their length decreased down to 125–150 nm and their diameter reduced at least by half. Again, when 35% of the substrate had been solubilized, the gel-like structure was still there, but the thinning of the crystalline fibers was strongly amplified. As in the case of the cellobiohydrolases and the chitanases[2–4], this fiber thinning is indicative of a highly processive mode of action[17].

The molecular bases of this processivity were established by crystallographic studies of the ι-carrageenase CgiA. This enzyme, representative of the family GH-82, adopts a right-handed parallel β-helix fold with

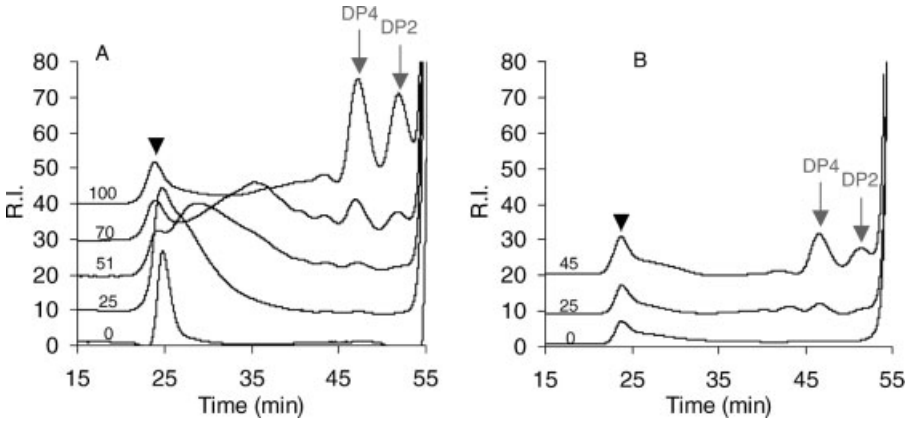

Figure 1.
Size exclusion chromatography of degradation products of κ-carrageenan in A. soluble state (150 mM Tris, 5 mM KCl) and B. gel state (150 mM Tris, 15 mM KCl). The numbers at the beginning of each chromatogram signifies the % of degradation as determined by reducing sugar assay. The arrow indicates the position of the initial polymeric substrate.

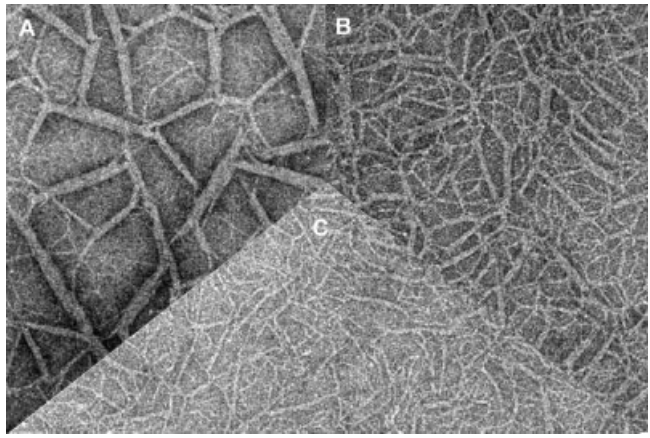

Figure 2.
A. Negative staining of ι-carrageenan undigested gel observed by transmission electron microscopy. Thinning of the fibers observed after 20% (B) and 35% (C) enzymatic degradation.

two additional C-terminal domains, A and B. In the native structure the domain A is highly flexible and the enzyme displays an open groove topology [15]. In the presence of ι-carrageenan oligosaccharides, domain A shifts toward the β-helix groove, forming a tunnel which holds a ι-carrageenan tetrasaccharide, while the N-terminal region binds a disaccharide. Thus, from an open conformation, which allows for the initial endo-attack of ι-carrageenan chains, the enzyme switches to a closed-tunnel form, consistent with its highly processive character[17].

Conclusion

We have functionally and structurally characterized three families of marine bacterial enzymes degrading sulfated galactans from red algae (Table 1). The β-agarases and the κ-carrageenases from family GH-16 have diverged from a common ancestor to acquire different substrate specificities. In contrast, the ι-carrageenases constitute a distinct family of glycoside hydrolases (family GH-82), unrelated in fold and catalytic mechanism.

However, these enzymes have in common to degrade gel-forming sulfated galactans. To efficiently degrade such recalcitrant substrates, all theses galactanases have developed additional features to increase their capacity to bind their crystalline substrates. The higher efficiency of the β-agarase AgaA to degrade agarose gels is likely due to the secondary agarose-binding site located at its outer, non-catalytic surface (Figure 3A)[16]. This additional substrate binding site plays a function similar to that of the cellulose binding module in the processive endo/exo cellulase E4[9]. Both κ- and ι-carrageenases display a tunnel-like active site (Figure 3B and C), explaining their endo-processive behavior[14,17,18]. It is

Table 1.
Structural and functional characteristic of the overexpressed galactanases

	Link disrupted	Mechanism	GH-family	fold	Active site topology	Mode of action
β-agarase A	β(1-4)	retaining	16	β-sandwich	Open groove	Endo
β-agarase B	β(1-4)	retaining	16	β-sandwich	Open groove	Endo
κ-carrageenase	β(1-4)	retaining	16	β-sandwich	Tunnel	Endo-processive
ι-carrageenase	β(1-4)	inverting	82	β-helix	Open and tunnel	Endo-processive

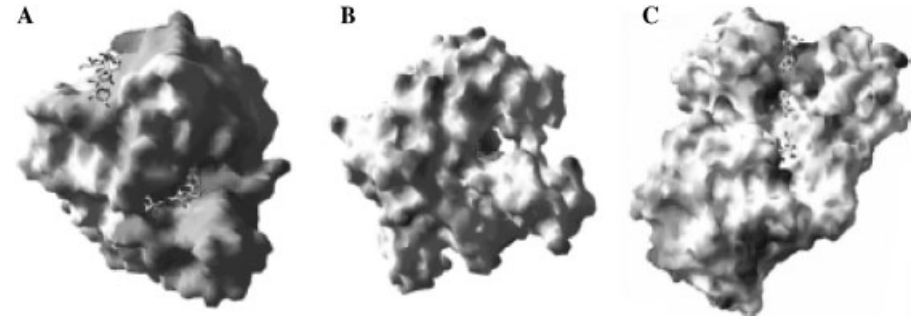

Figure 3.
Molecular surface of (A) the β-agarase AgaA complexed to agarose oligosaccharides, (B) the κ-carrageenase CgkA, and (C) the ι-carrageenase CgiA complexed to ι-carrageenan oligosaccharides.

noteworthy that this tunnel topology was acquired by convergent evolution.

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