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Iota-carrageenan hydrolysis by *Pseudoalteromonas carrageenovora* IFO12985

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We report *iota*-carrageenan hydrolysis by *Pseudoalteromonas carrageenovora* IFO 12985. *Kappa*-carrageenase and *lambda*-carrageenase were previously isolated from this organism, but *iota*-carrageenase activity had not been reported in the literature. *P. carrageenovora* was grown in *iota*-carrageenan-based liquid medium. Using the zone of depression assay, transfer of aliquots of the culture to solid medium with 2% *iota*- and *kappa*-carrageenan showed extensive hydrolysis of *iota*-carrageenan. Analysis of the hydrolysates by C-13 Nuclear Magnetic Resonance spectroscopy confirmed degradation of the *iota*-carrageenan. Hydrolytic activity of *P. carrageenovora* grown in *iota*-carrageenan was compared with that of the same organism grown in *kappa*-carrageenan. Cell-free supernatants from each yielded subtle differences in hydrolytic profiles, but showed degradation patterns consistent with hydrolysis to fragments smaller than 1.4 kDa, corresponding to six or fewer monosaccharide units. Different protein expression bands on SDS-PAGE were also observed for the cell-free supernatants of *P. carrageenovora* grown in *iota*- versus *kappa*-carrageenan, with lower *kappa*-carrageenase expression observed in the organism grown in *iota*-carrageenan.

Key Words: carrageenan oligosaccharides; enzymatic hydrolysis; *Eucheuma* polysaccharide*; iota*-carrageenase; polysaccharides;

INTRODUCTION

Carrageenans are sulfated galactopyranose polysaccharides which are widely used in both food and non-food products as thickeners and stabilizers. They belong to the family of galactan polysaccharides that also include the agars, and are produced by red algae (Rhodophyta). Carrageenans are linear sulfated galactans with alternating 3-linked beta-D-galactopyranosyl residue and 4 –linked 3,6-anhydro-alpha-D-galactopyranosyl residues. There are about 15 types of carrageenans which differ in terms of the number and position of the sulfate groups and the presence of the 3,6-anhydro bridge substructure (Knutsen et al. 1994). Figure 1 shows the idealized repeating units of *iota*-, *kappa*- and *lambda*-carrageenan. Carrageenans have heterogeneous structures, which can vary according to the algal species, stage in its life cycle, and processing procedure.

Both the 3,6-anhydro bridge and the sulfate groups are important in determining the physical properties (Therkelsen 1994). The gelation properties of these hydrocolloids depend on the degree of sulfation. For example, unsulfated agarose produces a stiff and brittle gel, *iota*-carrageenan gel is very soft and elastic, and *lambda*-carrageenan does not form gels at all.

In the native form, carrageenans are estimated to have molecular weights of over 100kDa. They are broken down into smaller fragments by heat, chemical hydrolysis, and specific enzymes. Most of the applications of carrageenans are associated with the high molecular weight polymeric

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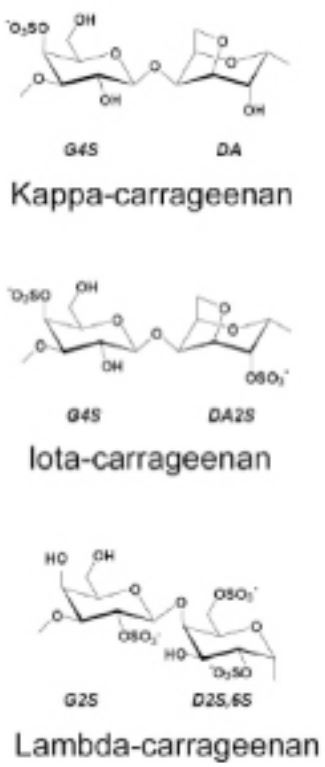

Figure 1. Idealized repeating unit structures of the *kappa*-, *iota*-, and *lambda*-carrageenans.

forms. Small carrageenan oligosaccharides have attracted interest due to their immunomodulation and anti-tumor activity, as described in animal studies (Yuan et al. 2006). On the other hand, carrageenans in the diet, especially degraded carrageenans with approximate molecular weights 20–40 kDa, have been associated with inflammation and cancer formation in animal studies (Tobacman 2001). Nevertheless, the latest toxicological assessment of the major world authorities classifies carrageenan and processed *Eucheuma* seaweed as safe, with no limits on acceptable daily intake for humans (Joint FAO/WHO Expert Committee on Food Additives 2007). The FAO/WHO report only advised against the use of carrageenans in infant formula.

A number of studies have been reported on the bacterial degradation of *kappa*-, *iota*-, and *lambda*- carrageenans. Carrageenan-degrading microorganisms are believed to have hydrolytic enzymes that are specific to the different types of carrageenan. Table 1 summarizes the information from genetic, structural, and activity studies of the carrageenan hydrolases.

The most studied carrageenan-degrading microorganism is *Pseudoalteromonas carrageenovora* (Yaphe & Baxter 1955; Weigl & Yaphe 1966; Michel et al. 2006). To date, only *kappa*- and *lambda*- carrageenases have been isolated from *P. carrageenovora*, although other enzymes such as sulfatases have also been described. *Kappa*-carrageenase was isolated and described by McLean and Williamson (1979), while *lambda*-carrageenase was isolated and described by Greer and Yaphe (1984).

Iota-carrageenases have been isolated from other organisms, namely *Pseudoalteromonas fortis* and *Zobellia galactanivorans*, as shown in Table 1. Like the *kappa*-carrageenases described so far, *iota*-carrageenases are endohydrolases, breaking internal linkages rather than hydrolyzing units from the ends. Also, both enzymes are processive, hydrolyzing several units in succession. In contrast, *lambda*-carrageenase cleaves internal linkages randomly. Although they all hydrolyze carrageenan substrates, *kappa*-, *iota*-, and *lambda*-carrageenases do not share significant sequence or structure homology, and belong to different structural families as noted in Table 1.

Iota-carrageenan is intermediate between *kappa*- and *lambda*- carrageenans in number of attached sulfate groups. *Kappa*-carrageenase is capable of degrading *iota*-carrageenan, although with low efficiency. *Lambda*-carrageenase from *P. carrageenovora*, on the other hand, is active against highly sulfated carrageenans such as *lambda*-, *eta*-, and *mu*- carrageenans, but not against agarose, *kappa*- or *iota*- carrageenans (Michel et al. 2006; Guibet et al. 2007).

In this study, we report on the hydrolytic activity of *Pseudoalteromonas carrageenovora* using *iota*-carrageenan as substrate.

MATERIALS AND METHODS

Pseudoalteromonas carrageenovora IFO 12985 was obtained as a lyophilized culture from National Institute of Technology and Evaluation (NITE) NMRC, Japan (formerly Institute of Fermentation Osaka (IFO), Japan). *P. carrageenovora* was cultured in Bellion's solid or liquid medium as noted, containing 2% of *iota*- or *kappa*-carrageenan, or other polysaccharides, and grown at 27°C for two days (Bellion et al. 1982).

To determine the hydrolysis products, the cell-free extracts grown in *iota*-or *kappa*-carrageenan were inoculated into a medium containing 0.5% of the carrageenan. After two days of incubation at 40° C, the enzyme resistant fractions were separated from the degradation products by ethanol precipitation followed by centrifugation at 11,000 rpm, 4° C for 20 min. The supernatant, which contained the hydrolysates, was concentrated by rotary-evaporation (Hitec, RE-51) at 34° C and freeze-dried (Labconco, Freezone 4.5). Reducing sugar was assayed using the method described by Kidby and Davidson (1973).

Table 1. Comparison of *kappa*-, *iota*- and *lambda*-carrageenases.

	Kappa-carrageenase		**Iota-carrageenase**		**Lambda-carrageenase**	
Substrate	kappa-carrageenan		iota-carrageenan		lambda-carrageenan	
E.C. number	3.2.1.83		3.2.1.127		3.2.1.162	
Organism	*Pseudoalteromonas carrageenovora*	*Zobellia galactanivorans*	*Pseudoalteromonas fortis*	*Zobellia galactanivorans*	*Pseudoalteromonas carrageenovora*	*Pseudoalteromonas CL19*
Gene	cgkA	cgkA	cgiA	cgiA	cgl	
No. of residues	397	545	491	491	942	917
MW, kDa (sequence)	44.2	61.5	54.8	53.4	105.6	103
MW, kDa (SDS-PAGE)	32	40	57	50	97	
Family (Henrissat and Davies, 1997)	16		82		no assignment yet	
Site of hydrolysis	beta-1,4-linkage		beta-1,4-linkage		beta-1,4-linkage	
Mode of action	endo		endo		endo	
Mechanism	retaining		inverting		inverting	
Processivity	processive		processive		random	
UniProt ID	P43478	O84907	Q9F518	Q9F284	Q0JRK4	
3D structure	Yes (PDB ID: 1DYP)		Yes (PDB ID: 1H80, 1KTW)		none	
Reference	Potin et al. 1995; Barbeyron et al.1998; Michel et al. 2001a		Barbeyron et al. 2000; Michel et al. 2001b		Guibet et al. 2007	Ohta and Hatada 2006

Molecular weight profiles of the degradation products and intact carrageenan were estimated by size exclusion chromatography (Perkin Elmer 200 series). The lyophilized hydrolysates and intact carrageenan were resuspended in deionized water to final concentrations of 2 mg/mL and 1 mg/mL, respectively. The solutions were filtered thru a 0.22-µm filter disk (Acrodisc). Samples (20-µL) were loaded onto an aqueous column, PL Aquagel-OH 40 (300 mm x 7.5 mm), equilibrated with filtered, deionized water. The flow rate was set to 0.5 mL/min. Elution was monitored using a refractive index detector. The standards used were purchased from Polymer Laboratories with the following average molecular weights (Mp): polyethylene glycol (PEG) Mp = 1470; PEG Mp = 4120; polyethylene oxide (PEO) Mp = 10,000; PEO Mp = 50,000; PEO Mp = 645,000. The standards were dissolved in deionized water to give a final concentration of 1 mg/mL.

Both degraded and intact *iota*-carrageenan samples were analyzed by ^{13}C NMR spectroscopy (JEOL Lambda LA 400 NMR spectrometer). About 40 mg of the lyophilized hydrolysates and about 80 mg of the intact carrageenan were dissolved in D_2O in separate 10 mm NMR tubes and the spectra were obtained at 35° C and 90° C, respectively. Spectra were recorded using 16K data points using the following parameters: spectral width = 27,100 Hz, acquisition time = 605 msec, pulse angle = 9 µsec, number of scans = 500,000 times for the hydrolysates, and 50,000 times for the undegraded polymer, line broadening = 0.8 Hz. Trimethylsilyl propionic acid (TSP) was used as internal reference.

To determine the effect of different substrates on protein expression, *P. carrageenovora* was grown in Bellion's liquid medium containing 2% *iota*-carrageenan or 2% *kappa*-carrageenan. The cultures were grown at 27° C, 100 rpm for two days. Profiles of the extracellular proteins were determined by SDS-PAGE (Mini Protean 3, Bio-Rad) on 10% T acrylamide-bisacrylamide gels. Gels were stained with silver nitrate. Broad range protein molecular weight markers (Promega) were used to estimate the sizes of the proteins.

RESULTS

Extracts from *Pseudoalteromonas carrageenovora* IFO 12985 grown in Bellion's liquid medium with 2% *iota*-carrageenan were applied to Bellion's solid medium containing 2% *iota*-carrageenan or 2% *kappa*-carrageenan. Substantial liquefaction of *iota*-carrageenan was observed, as shown in Figure 2. Complete degradation of the *iota*-carrageenan was observed after five days (data not shown).

To characterize the hydrolysis products, *P. carrageenovora* was grown in Bellion's liquid medium with either 0.5%

iota- or 0.5% *kappa*-carrageenan. The hydrolysis products from the two different carrageenans were analyzed by size exclusion chromatography. Figure 3 (a) shows the chromatograms for the intact carrageenans (1% solutions). Both are relatively high molecular weight polymers, with elution volumes consistent with molecular weights in the hundreds of thousands. Figure 3 (b) shows the profile of the hydrolysates of *iota*-carrageenan by *P. carrageenovora* cell-free extracts grown in either *iota*- or *kappa*-carrageenan. Both extracts show extensive hydrolysis activity, with the major peak eluting at about 8.0 mL. More subtle differences are observed in the chromatogram region between 4-7 mL. The *iota*-grown extract's profile is skewed to the right, towards lower molecular weight products. For comparison, the hydrolysis profiles for *kappa*-carrageenan are shown in Figure 3(c). Again the major peak elutes at close to 8.0 mL.

The major peaks elute later than 7.6 mL, the elution volume of the lowest molecular weight standard of 1.47 kDa. This precluded reliable molecular weight estimates. However, the results suggest that the major low-molecular weight hydrolysates are likely to be carrahexaoses (1.3 kDa) or carratetraoses (0.9 kDa), rather than units of 8 monosaccharide units or more. The results are consistent with the expected activity of the carrageenases as reviewed by Michel et al. (2006).

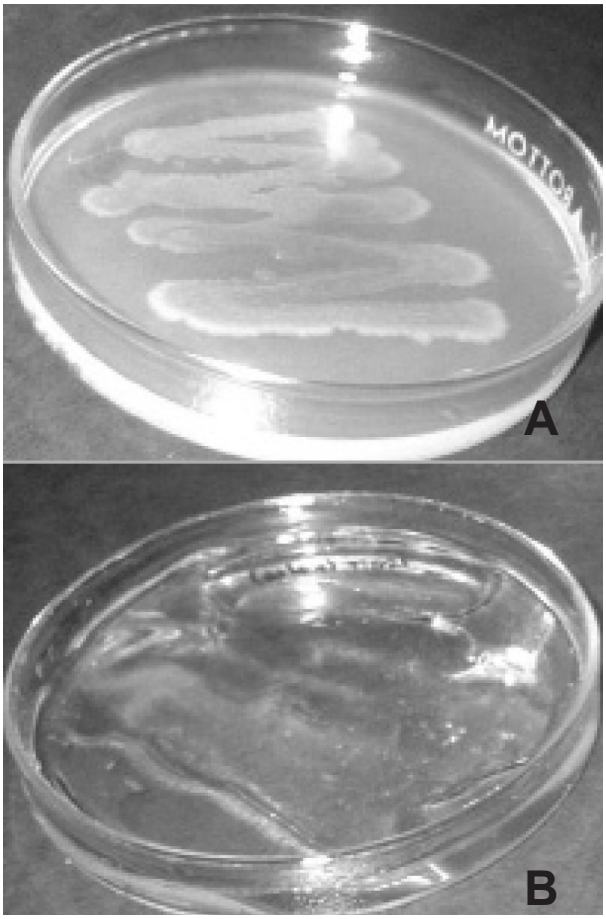

Figure 2. Zone of depression of filtered extracts of *Pseudoalteromonas carrageenovora* grown in the corresponding medium. The filtered extracts were placed on Bellion's solid medium with 2% carrageenan substrates: (a) *iota*-carrageenan; (b) *kappa*-carrageenan. Hydrolysis was observed as depression or liquefaction around the areas of application after two days of incubation at 27°C.

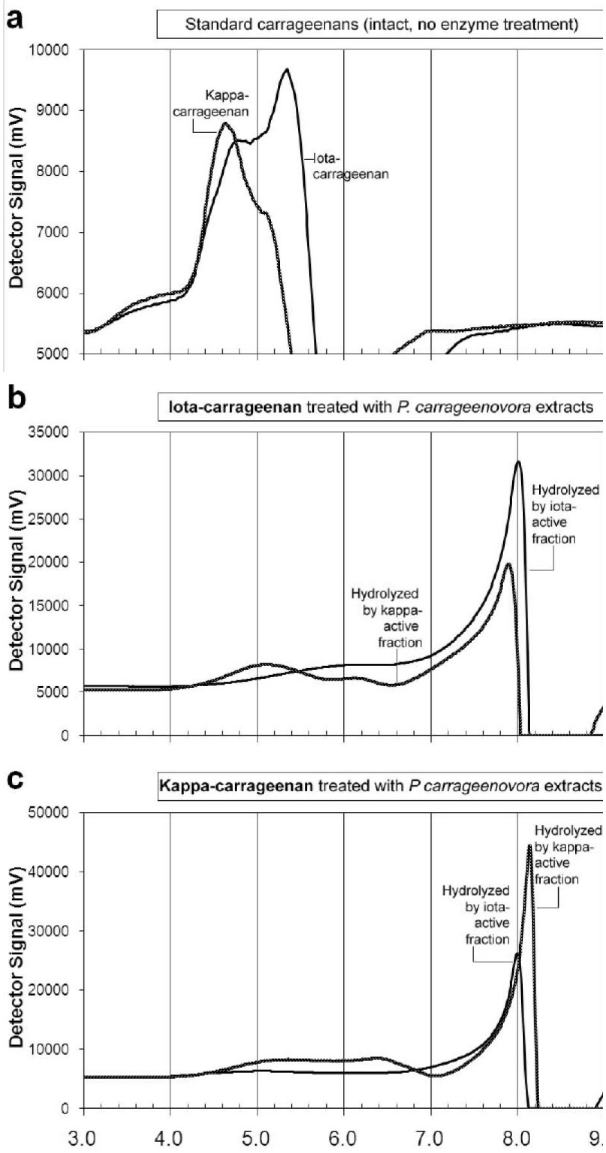

Figure 3. Analysis of carrageenan hydrolysates by size exclusion chromatography. (a) Intact *iota*- and *kappa*-carrageenan standards (0.1%); (b) hydrolysis products of *iota*-carrageenan after treatment with cell-free extracts of *Pseudoalteromonas carrageenovora* grown in *iota*-carrageenan medium or *kappa*-carrageenan medium; (c) hydrolysis products of *kappa*-carrageenan after treatment with cell-free extracts as noted in (b). Samples were analyzed using a PL Aquagel-OH 40 column equilibrated with deionized water at a flow rate of 0.5mL/min. Fractions were monitored by refractive index.

^{13}C NMR analysis of the hydrolyzed *iota*-carrageenan is most informative in the region of 105 to 90 ppm, where the anomeric carbon signals are observed. The standard *iota*-carrageenan displays the expected signals at around 103 ppm for the G4S and at around 93 ppm for the DA2S units (Figure 4a). In contrast, the hydrolyzed *iota*-carrageenan's spectrum displays new peaks at the regions of 97 ppm and 95 ppm, and a shift to 92.5 ppm (Figure 4b). The signals at around 97 ppm and 95 ppm can be attributed to the anomeric carbon of the galactopyranosyl-4-sulfate residue at the reducing end of the chain, which may have either alpha or beta conformations as noted (Greer et al. 1985). The large increase in this pair of peaks (relative to the other peaks in the anomeric region) indicates that degradation of *iota*-carrageenan took place mainly at this linkage. The more complex set of peaks centered at 92.5 ppm, on the other hand, are associated with the anomeric carbon of the 3,6-anhydrogalactose-2-sulfate unit, with more detailed assignments estimated based on Greer et al. (1985).

When grown in liquid medium containing 2% carrageenan for two days at 27°C, *P. carrageenovora* appreciably hydrolyzes *iota*-carrageenan. The bacterium grown in *kappa*-carrageenan hydrolyzes *kappa*-carrageenan more efficiently than *iota*-carrageenan (Table 2). On the other hand, *P. carrageenovora* grown in *iota*-carrageenan degrades both *iota*- and *kappa*-carrageenan equally well, although not as efficiently, with only approximately 10% hydrolysis observed under these conditions.

Given the differences observed in activity between *iota*-grown and *kappa*-grown *P. carrageenovora*, we checked for differential expression of proteins in the cell-free extract.

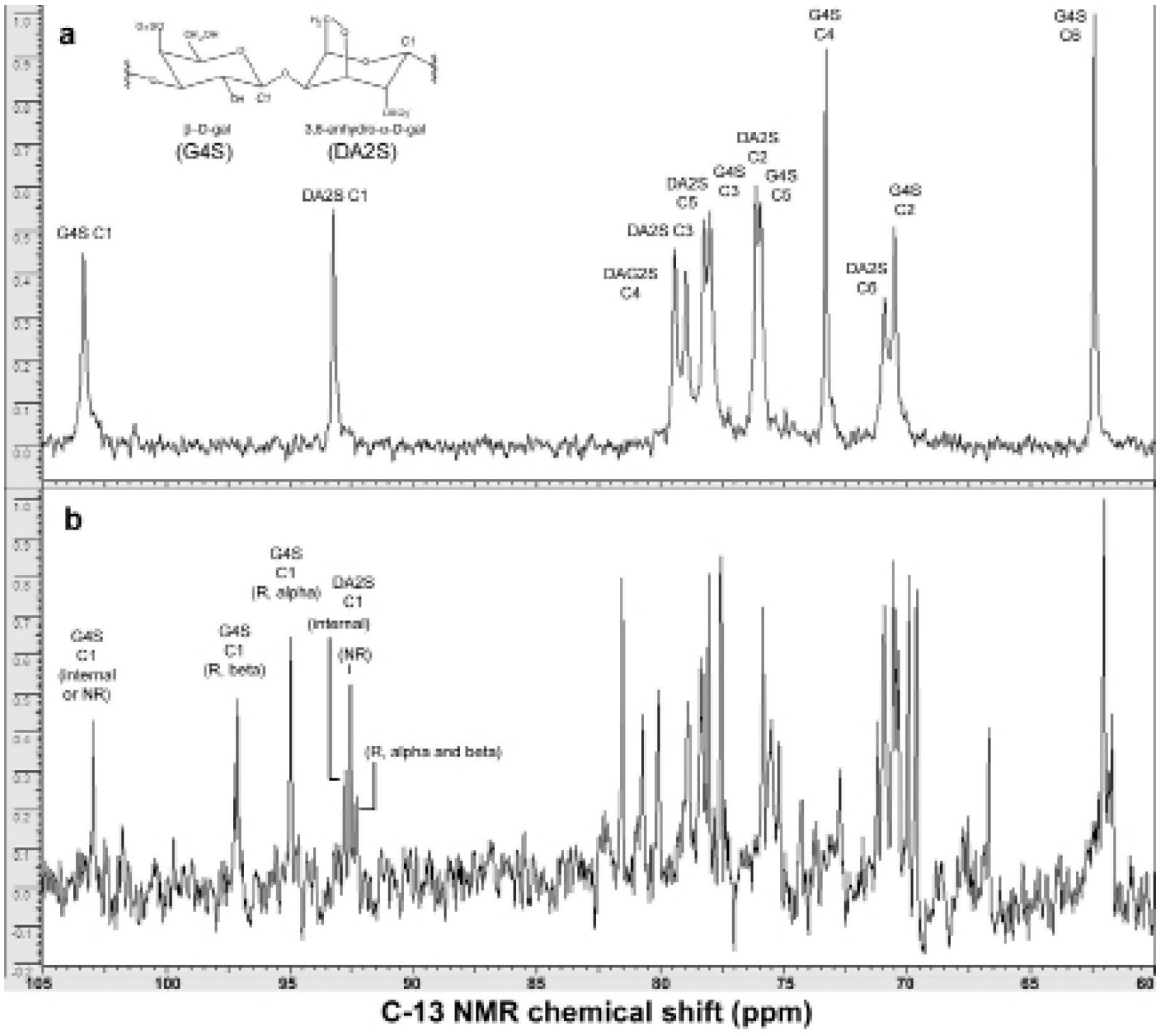

Figure 4. Analysis of the *iota*-carrageenan hydrolysates by ^{13}C Nuclear Magnetic Resonance spectroscopy. (a) ^{13}C NMR of intact *iota*-carrageenan. NMR spectrum was acquired at 90° C. (b) ^{13}C NMR of *iota*-carrageenan hydrolysates after treatment with cell-free *Pseudoalteromonas carrageenovora* extract. NMR spectrum was acquired at 35° C. For (a) and (b), D_2O was the solvent and trimethylsilyl propionic acid (TSP) was used as internal reference (NR-nonreducing end, R-reducing end). Assignments are based on Greer et al. 1985.

Figure 5 shows the SDS-PAGE profile of proteins in the cell-free extract of *P. carrageenovora* grown in either *iota*- or *kappa*-carrageenan. The arrow indicates the *kappa*-carrageenase band at about 32 kDa, which was determined from previous purification of this enzyme in our laboratory (Henares and Aguilan, unpublished data). The enzyme, as expected, is present in large quantities in the *kappa*-carrageenan-grown extract but is at low concentration in the *iota*-carrageenan-grown extract.

DISCUSSION

Pseudoalteromonas carrageenovora is the most well-studied carrageenan-modifying organism. It hydrolyzes *kappa*- and *lambda*-carrageenan through specific carrageenases. Both *kappa*- and *lambda*-carrageenase have been isolated and characterized in detail from this organism. On the other hand, *iota*-carrageenase activity had not been described for this organism (Michel et al. 2006), although the enzyme was isolated from other organisms such as *Pseudoalteromonas fortis*.

Table 2. Extent of hydrolysis of carrageenans by *Pseudoalteromonas carrageenovora* extracts. The organism was cultured in Bellion's liquid medium containing either iota- or kappa-carrageenan (0.5%). The unhydrolyzed fractions were precipitated in ethanol, centrifuged, and dried, while the hydrolysates in the supernatant were lyophilized.

***P. carrageenovora* extract**	Carrageenan substrate	% Carrageenan hydrolyzed (wt/wt)
P. carrageenovora grown in iota-carrageenan	Iota-carrageenan	10%
	Kappa-carrageenan	10%
P. carrageenovora grown in kappa-carrageenan	Iota-carrageenan	50%
	Kappa-carrageenan	99%

In this study, we observed that *Pseudoalteromonas carrageenovora* is capable of hydrolyzing *iota*-carrageenan. NMR spectroscopy suggests that the beta-1,4-linkage of the D-galactose-4-sulfate unit is hydrolyzed, which is consistent with the mechanisms of the known carrageenases.

We carried out a comparison of the hydrolytic activity of *Pseudoalteromonas carrageenovora* extracts that differ on whether the organism is first grown in *kappa*- or in *iota*-carrageenan. The culture medium was expected to bias the organism's expressed enzyme profile towards digesting the specific substrate present. As expected, when grown in *kappa*-carrageenan, the organism efficiently degraded *kappa*-carrageenan, with 99% by weight hydrolyzed (Table 2). Under these conditions, the organism also degraded *iota*-carrageenan, albeit by only about half its efficiency. In the original isolation and purification of *kappa*-carrageenase, McLean and Williamson (1979) showed that *kappa*-carrageenase is far more selective for *kappa*- over *iota*-carrageenan, as measured by reducing sugar and viscometric assays.

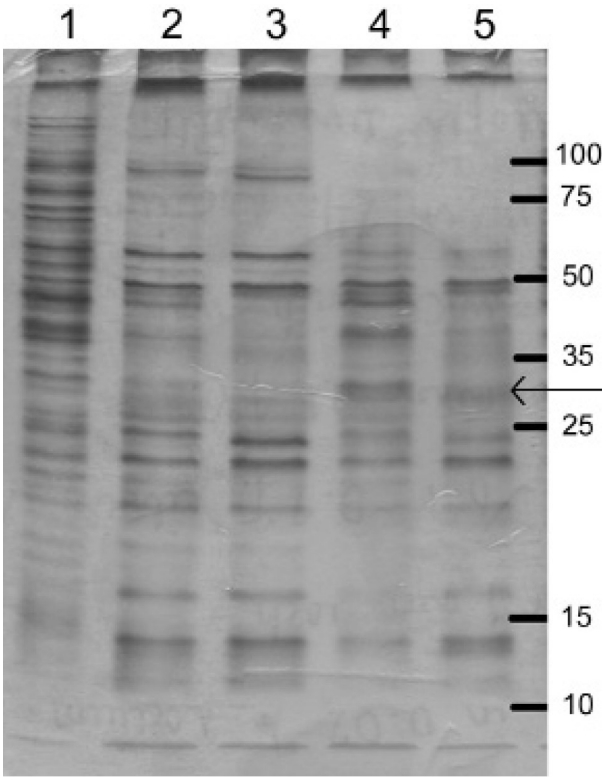

Figure 5. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *P. carrageenovora* grown in Bellion's liquid medium containing 0.2% carrageenan. The gels were 10% polyacrylamide and stained with silver nitrate. L1: *P. carrageenovora* in R708 medium; L2: cell-free extract from *iota*-carrageenan (with dithiothreitol (DTT) as reducing agent); L3: cell-free extract from *iota*-carrageenan without DTT; L4: cell-free extract from *kappa*-carrageenan with DTT; L5: cell-free extract from *kappa*-carrageenan without DTT. The positions of the molecular weight markers are noted (Promega Broad Range Markers). The arrow indicates the band corresponding to *kappa*-carrageenase.

It was hypothesized that if *iota*-carrageenan hydrolysis was not entirely due to *kappa*-carrageenase, growing *P. carrageenovora* in *iota*-carrageenan rather than *kappa*-carrageenan might induce increased hydrolytic activity for *iota*-carrageenan. Our results show that the selectivity for *kappa*-carrageenan hydrolysis disappears when the organism is grown in *iota*-carrageenan. Under these conditions, the overall hydrolysis was not very efficient, but it may be possible to optimize selective *iota*-

carrageenan hydrolysis by adjusting further the culture conditions of *P. carrageenovora*.

Whether *P. carrageenovora* is grown in *kappa*-carrageenan or in *iota*-carrageenan, the hydrolysis products are relatively low-molecular-weight oligosaccharides. The results from size exclusion chromatography (Figure 3) are consistent with the formation of neocarrahexaose-sulfate or neocarratetraose-sulfate units. Pure *kappa*-carrageenase is expected to yield neocarratetraose-sulfate and neocarrabiose-sulfate (McLean & Williamson 1979), while pure *iota*-carrageenase from *P. fortis* yields neocarrahexaose-sulfate and neocarratetraose-sulfate (Michel et al. 2006).

The change in hydrolytic activity associated with culture conditions may be associated with differential secretion of various proteins by *P. carrageenovora,* as shown by the SDS-PAGE profiles in Figure 5. In particular, the amount of *kappa*-carrageenase varies depending on whether or not the organism is cultured in *kappa*-carrageenan. Together with the lack of selectivity for *kappa*- carrageenan of this extract, the SDS-PAGE profile suggests that the hydrolytic activity of the *iota*-grown *P. carrageenovora* is not primarily due to its *kappa*-carrageenase. It is of further interest to determine whether the *iota*-carrageenan hydrolysis by *P. carrageenovora* is due to the combined action of known enzymes of this organism, or whether there is still an uncharacterized *iota*-specific enzyme or a set of enzymes present in this organism.

CONCLUSION

Although these questions remain, our results indicate that *P. carrageenovora* is more versatile than previously reported, and may be used for hydrolysis of *iota*-carrageenan as well as for *kappa*- and *lambda*-carrageenan under appropriate culture conditions.

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