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**Degradation of λ -carrageenan by *Pseudoalteromonas carrageenovora*
 λ -carrageenase: a new family of glycoside hydrolases unrelated to κ -
and ι -carrageenases**

Marion GUIBET[§], Sébastien COLIN[§], Tristan BARBEYRON, Sabine
GENICOT, Bernard KLOAREG, Gurvan MICHEL and William HELBERT*

Centre National de la Recherche Scientifique; Université Pierre et Marie Curie-Paris6, Unité
Mixte de Recherche 7139 "Marine Plants and Biomolecules", Station Biologique, F-29682
Roscoff Cedex, Bretagne, France

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*Corresponding author: fax +33 298 292 324, E-mail: helbert@sb-roscoff.fr

[§]These authors have equally contributed to this work.

LIST OF ABBREVIATIONS

AMAC: 2-Aminoacridone

C-PAGE: Carbohydrate - polyacrylamide gel electrophoresis

DP: degree of polymerization

EDTA: ethylenediaminetetraacetic acid

GH: glycoside hydrolase family

¹H-NMR: proton nuclear magnetic resonance

HPAEC: high performance anion exchange chromatography

IPTG: isopropyl-1-thio- β -D-galactopyranoside

LC-MALLS: Liquid chromatography - Multi angle laser light scattering

ORF: open reading frame

PCR: polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SYNOPSIS

Carrageenans are sulphated galactans found in the cell walls of red seaweeds. They are classified according to the number and the position of sulphate ester groups. λ -Carrageenan is the most sulphated carrageenan and carries at least three sulphates per disaccharide unit. The sole known depolymerising enzyme of λ -carrageenan, the λ -carrageenase from *Pseudoalteromonas carrageenovora* has been purified, cloned and sequenced. Sequence analyses have revealed that the λ -carrageenase, referred to as CglA, is the first member of a new family of glycoside hydrolases, which is unrelated to families GH16, that contains κ -carrageenases, and GH82, that contains ι -carrageenases. This large enzyme (105 kDa) features a low complexity region, suggesting the presence of a linker connecting at least two independent modules. The N-terminal region is predicted to fold as a β -propeller. The main degradation products have been purified and characterized as neo- λ -carratetraose (DP4) and neo- λ -carrahexaose (DP6), indicating that CglA hydrolyzes the β -(1 \rightarrow 4) linkage of λ -carrageenan. LC-MALLS and $^1\text{H-NMR}$ monitoring of the enzymatic degradation of λ -carrageenan indicate that CglA proceeds according to an endolytic mode of action and a mechanism of inversion of the anomeric configuration. Using 2-aminoacridone-labelled neo- λ -carrabiose oligosaccharides, we demonstrate that the active site of CglA is constituted of at least 8 subsites (-4 to +4) and that a DP6 oligosaccharide binds in the subsites -4 to +2 and can be hydrolyzed into DP4 and DP2.

INTRODUCTION

Marine red algae (*Rhodophyta*) are characterized by an abundance of sulphated polysaccharides, which have no equivalent in land plants [1]. These anionic polymers, agars and carrageenans, are laid out in the cell wall at a high density and can constitute up to 50% of the dry mass of a seaweed [2]. This large family of hydrocolloids are well known for their gelling properties and are used in a variety of laboratory and industrial applications [3]. They are made up of linear chains of galactose with alternating α -(1 \rightarrow 3) and β -(1 \rightarrow 4) linkages. In all these galactans the β -linked galactose units are in the D configuration (G unit), but the α -linked galactose units are in the L configuration in agars (L unit), whereas they are in the D configuration in carrageenans (D unit). Carrageenans are further classified according to the number and the position of sulphated ester (S) and by the occurrence of 3,6-anhydro-bridges in the α -linked residues (DA unit) found in gelling carrageenans [4]. The three most industrially exploited carrageenans, namely, kappa- (κ , DA-G4S), iota- (ι , DA2S-G4S), and lambda- (λ , D2S6S-G2S) carrageenan, are distinguished by the presence of one, two, or three ester-sulphate groups per repeating disaccharide unit, respectively.

κ - and ι -Carrageenans form thermoreversible gels in aqueous solutions, their rigidity decreasing strongly with the degree of sulphation of the carrabiose unit. The gel properties of carrageenans are also strongly dependent on the presence of salts and on the ionic strength of the medium [5]. With three sulphate groups per carrabiose unit, λ -carrageenan is the most negatively charged galactan from red algae. Recently, it has been shown that some carrabiose units of the λ -carrageenan from *Gigartina skottsbergii* are even substituted with four sulphate groups (D2S,6S-G2S,4S) [6]. Since λ -carrageenans do not feature 3,6-anhydro bridges, they are more hydrophilic than κ - and ι -carrageenans and do not make physical gels but highly viscous solutions. This unique viscosity is due to the semi-rigidity of the chain, which is likely conferred by the high density of sulphate groups along the polymer.

Carrageenans constitute a crucial carbon source for a number of marine bacteria. These microorganisms, which belong mainly to the classes *Gammaproteobacteria*, *Flavobacteria* or *Sphingobacteria*, degrade the cell walls of marine red algae by secreting specific glycoside hydrolases (GH), referred to as carrageenases [7]. κ -Carrageenases belong to family 16 of the glycoside hydrolases (GH16) [8, 9], a polyspecific family which encompasses at least eight different enzymatic activities, including β -agarases (<http://afmb.cnrs-mrs.fr/CAZY/>) [10, 11]. Phylogenetic analysis and crystallographic investigations demonstrated that family GH16 enzymes have evolved from a common ancestor and that κ -carrageenases have likely emerged

from the β -agarase branch [9, 12, 13]. κ -Carrageenases hydrolyze β -(1 \rightarrow 4) glycosidic linkages with retention of the anomeric configuration [14]. The κ -carrageenase CgkA from *Pseudoalteromonas carrageenovora* adopts a jellyroll fold and displays a tunnel-shaped active site suggesting a processive mode of action [12]. The κ -carrageenan chain is composed of alternating neutral and negatively charged sugars (DA and G4S, respectively). To accommodate the dual nature of its substrate, CgkA features in its active site both conserved aromatic and basic residues which are predicted to interact with DA and G4S moieties, respectively [12]. ι -Carrageenases define a monospecific family of glycoside hydrolases (GH82) which is unrelated to that of κ -carrageenases [15]. The ι -carrageenase CgiA from "*Alteromonas fortis*" (ATCC 43554) folds as a right-handed β -helix and cleaves β -(1 \rightarrow 4) glycosidic bonds with an inverting mechanism [15, 16]. Electron microscopy analysis demonstrated that CgiA degrade ι -carrageenan fibres according to a processive mode of action, which is consistent with the tunnel topology of its active site [17]. ι -Carrageenan, which is constituted only by negatively charged sugars (DA2S and G4S) is recognized by CgiA essentially through ionic interactions between its sulphate groups and several conserved arginines of the protein [17].

These studies on κ - and ι -carrageenases have provided some insights into sulphated polysaccharide – protein interactions. But the chemical complexity of sulphated polysaccharides is a patent obstacle to such analyses and we need better structural characterization of most of these biopolymers. In this context, carrageenans appear to be a relatively well defined family of sulphated polysaccharides and they constitute an interesting model due to their linearly increasing degree of sulphation. Furthermore, we have recently described the complete structural characterization of λ -carrageenan [6]. Therefore to extend this study, we report here the cloning and mechanistic characterisation of the first λ -carrageenase. Up to now, the marine bacterium *P. carrageenovora* is the only microorganism known to degrade λ -carrageenan [18]. This activity was initially proposed to be due to an extracellular complex involving three hydrolases [19]. However, a single extracellular enzyme of 98 kDa has been purified and shown to degrade carrageenans of the lambda family. This enzyme has no activity on agarose, κ - or ι -carrageenans [20]. But, few details are available on this unique enzyme which is expected to largely differ from κ - and ι -carrageenases. Indeed the λ -carrageenase interacts with an oversulphated galactan, which is genuinely soluble in contrast to κ - and ι -carrageenans which form insoluble fibres.

Sequence analysis reveals that the λ -carrageenase, referred to as CglA, is the first member of a new family of glycoside hydrolases, which is unrelated to families GH16 and

GH82. The characterization of the hydrolysis products indicates that CglA cleaves the β -(1 \rightarrow 4) linkages of λ -carrageenan. LC-MALLS and $^1\text{H-NMR}$ monitoring of the enzymatic degradation of λ -carrageenan demonstrate that CglA proceeds according to an endolytic mode of action and a mechanism of inversion of the anomeric configuration. Finally, we have mapped the subsite organization of the active site, using AMAC-labelled neo- λ -carrabiose oligosaccharides.

EXPERIMENTALS

λ -carrageenase activity assay

λ -carrageenase activity was determined by the reducing sugar method adapted from Kidby and Davidson [21]. λ -carrageenan purified from tetrasporophytic plants of *G. skottsbergii* (CP-Kelco, GENU[®] 7055) was used as substrate. 0.5% λ -carrageenan in 0.1 M NaNO₃ (pH 7.5) was incubated with λ -carrageenase aliquots (0.7 μg , equivalent to 7 pmoles of enzyme per mL of substrate, i.e. 7 nM) at 30 °C. NaNO₃ solutions were not buffered, however, it was verified that pH of 7.5 remained constant throughout the degradation reaction. Inactivation of the enzyme was achieved by heating the medium in boiling water for 10 minutes. Initially we attempted to measure the production of reducing sugar according to the ferricyanide method previously applied for the enzymatic digestion of κ - and ι -carrageenans [14, 15]. However, the reducing ends of λ -carrageenan oligomers were less reactive than those of the κ - or ι -carrageenan oligosaccharides. By plotting a standard curve using various concentrations of glucose, neo- ι -carratetraose, neo- λ -carratetraose and neo- λ -carrahexaose, we found that the reactivity of neo- λ -oligocarrabiose reducing-ends toward the ferricyanide solution was about 9 times lower than that of the other oligosaccharides. Consequently, we modified the protocol of the ferricyanide method by using ten times more concentrated ferricyanide solution and by reacting this with the reducing sugars for 10 min rather than 7 min. Aliquots (900 μL) of the reaction medium were mixed with 100 μL of ferricyanide agent (3 g potassium hexacyanoferrate III, 24 g of Na₂CO₃, 10 mL NaOH 5M, QSP 1 L). The mixture was boiled for 10 min, cooled to room temperature and the absorbance was read at 420 nm. The specific activity is expressed in μmol of reducing end neo- λ -carratetraose equivalent produced per minute for 1 mg of protein.

Production and purification of λ -carrageenase from *P. carrageenovora*

P. carrageenovora was obtained from the American Type Culture Collection (ATCC 43555). This marine bacterium was grown in the presence of 10 g of λ -carrageenan purified from the tetrasporophytic plant of *G. skottsbergii* (CP-Kelco) at 20 °C for 48 h in 5 L of the Y-2 modified medium [18]. The culture medium was centrifuged (10 000 g, 30 min) and the cell-free supernatant was concentrated 5 times by tangential ultrafiltration (Pellicon system, 10 kDa, Millipore). The proteins were fractionated by slowly adding ammonium sulphate. The precipitate obtained between 30 and 70% ammonium sulphate saturation was recovered by centrifugation and resuspended in 100 mL sodium phosphate buffer (20 mM sodium phosphate buffer pH 7.7, 1 M NaCl, 30% ammonium sulphate). The solution was loaded onto a Phenyl Sepharose 6 Fast Flow column (200 mL, GE Healthcare) previously equilibrated in the same sodium phosphate buffer. Elution of the sample was performed using a linear decreasing gradient of ammonium sulphate starting from 30% to 0% over 440 min at 1 mL / min. The λ -carrageenase activity was observed after 375 min elution which corresponded to a concentration of 4.5% ammonium sulphate. Fractions containing λ -carrageenase activity were pooled and concentrated using an Amicon cell 8050 under pressurised nitrogen gas (1.5 bar) with a 10 kDa cut-off YM membrane (Millipore). The concentrate (4 ml) was loaded onto a preparative Superdex 200 column (GE Healthcare) and eluted at 1 mL/min with the following buffer, 20 mM sodium phosphate buffer pH 7.7, 1 M NaCl, 2% ammonium sulphate (buffer A). The Superdex 200 column was calibrated with the Gel Fitratio HMW kit (GE Healthcare). All the protein fractions were analyzed by SDS-PAGE under reducing conditions (1 mM DTT) at 20 mA in a 0.75 mm gel stained with Coomassie blue. The enzyme molecular mass was estimated using the SDS low range standard from Bio-Rad. The fractions containing pure λ -carrageenase were pooled and stored at 4 °C in buffer A for several months without noticeable loss of activity. The concentration of λ -carrageenase was estimated using the Bradford assay (Bio-Rad) and bovine serum albumin (Sigma) as a standard [22].

Isolation and analysis of λ -carrageenase clones

Pure λ -carrageenase was analyzed by SDS-PAGE under reducing conditions and acrylamide gels were stained with Amido Black 0.003 % w/v. Protein bands containing pure enzyme were excised and microsequenced by Edman degradation at the Pasteur Institute (Paris, France). One N-terminal and two internal peptide sequences were determined (A, B and C, respectively). Genomic DNA from *P. carrageenovora* was prepared as previously

described [23] and digested by the restriction endonuclease *Sau3AI*. DNA fragments of approximately 4-10 kb were purified using a 5-40 % sucrose gradient in 10 mM Tris-HCl pH 8, 10 mM NaCl and 5 mM EDTA, after a centrifugation at 85000 *g* for 23 h. DNA fragments were ligated into the *Bam*HI site of plasmid pAT153 and used to transform *Escherichia coli* competent cells (strain DH5 α). The genomic library contained approximately 6000 clones. From the amino acid sequence of the peptides A and B, degenerated DNA primers were designed and used in polymerase chain reaction (PCR) to obtain a 5' DNA probe of 943 nt. This probe was radioactively labelled (kit Megaprime DNA labelling systems, GE Healthcare) and used to screen the genomic library according to Sambrook and Russell [24]. Clones of interest were shown to be independent by restriction mapping. A Southern blot experiment was performed with the same labelled probe on genomic DNA from *P. carrageenovora* digested by several combinations of restriction endonuclease (*Sau3AI*, *Hin*DIII, *Hin*DIII/*Bam*HI, *Bam*HI, *Bam*HI/*Eco*RV, *Eco*RV, *Eco*RV/*Hin*DIII). Sequencing was carried out by gene walking using synthetic oligonucleotides as primers and a 3100 Genetic Analyser with BigDye Terminator V3.0 chemistry (Applied Biosystem). Sequences were verified at least 5 times and most of them more than 10 times.

Sequence analysis

The nucleotide sequence was searched for open reading frames which were translated according to the universal genetic code. They were investigated for Ribosomal Binding Sequences (RBS), promoters, and transcription terminator hairpins via the *mfold* server [25]. A signal peptide was predicted with SIGNALP 3.0 [26]. Searches for protein sequence similarities were performed using BLASTp [27] on the UniProt knowledgebase (trEMBL and SwissProt). Protein domains were searched with InterProScan [28]. Low complexity regions were identified with the program SEG [29].

Expression of recombinant λ -carrageenase

The construction of the expression vector was based on GATEWAY Cloning Technology according to the supplier's instructions (Invitrogen). The coding region of *P. carrageenovora* λ -carrageenase was amplified by PCR with high fidelity PLATINUM *Pfx* DNA polymerase (Invitrogen). PCR products were inserted into the pDONR201 vector. The sequence was verified, and plasmids carrying unmutated inserts were used to create an in-phase protein fusion with an N-terminal 6xHistidine tag under the control of a T7 promoter in the pDEST17 vector. These recombinant vectors were transformed in the BL21(DE3) and

Rosetta(DE3) *E. coli* strains, with or without the pLysS plasmid (Novagen). Various culture conditions were tested to obtain soluble protein expression: concentration of isopropyl-1-thio- β -D-galactopyranoside (IPTG) ranging from 0.1 mM to 1 mM, different culture media (LB and M9) and various induction temperatures (15, 20, 25, 30 and 37 °C). After centrifugation of the culture media (15000 g, 30 min), the cell pellets were disrupted using BugBuster Protein Extraction Reagent (Novagen). The cellular extracts were spun down (40000 g, 10 min) and the resulting insoluble and soluble fractions were analyzed by SDS-PAGE in reducing condition with Coomassie blue staining.

Inclusion bodies of recombinant λ -carrageenase were also obtained by expression in the BL21(DE3) *E. coli* strain incubated in LB medium at 37 °C for 6 h induction with 1 mM IPTG. These inclusion bodies were purified using BugBuster solution according to supplier protocol (Novagen) and were solubilized in Tris buffer 50 mM pH 8 with 8 M urea. The λ -carrageenase solution (500 μ g / ml) was diluted 50 times in a buffer containing 0.5% λ -carrageenan in 0.1 M NaNO₃, pH 7.5. The reaction medium was incubated for 96 h at 30 °C or for two weeks at 20 °C. The release of λ -oligosaccharides was visualised by Carbohydrate-PAGE [30].

Size exclusion chromatography of neo- λ -carrabiose oligosaccharides

Analysis and fractionation of digested and undigested λ -carrageenan samples were performed according to a modified version of the protocol of Knusten and coworkers [31]. After filtration through a 0.22 μ m membrane (Millipore), 500 μ L of the sample (0.25% w/v) was injected onto a semi-preparative Superdex 30 column (600 x 16 mm i.d., GE Healthcare). Elution was performed with 50 mM (NH₄)₂CO₃ at a flow rate of 1 ml/min. Detection was achieved by differential refractometer (Spectra System RI-50) connected to a computer equipped with the Datalys acquisition software.

High performance anion exchange chromatography (HPAEC)

Oligosaccharide mixtures (0.25% w/v) were filtered through a 0.22 μ m membrane (Millipore) and injected onto an analytical AS11 column (20 μ l; 4 x 250 mm Ion Pac®; Dionex) coupled with an AS11 guard column. The elution was conducted at a flow rate of 0.5 mL/min with a NaOH (280 mM) step gradient (0-4 min: 3-5%, 4-6.5 min: 5-30%, 6.5-15 min: 30-57.5%, 15-26 min: 57.5-100%) monitored by a GP40 Gradient Pump (Dionex). Oligosaccharides were detected by conductimetry using an ASRS ultra-4mm (Dionex). Acquisition of the chromatogram was achieved with Chromeleon Peak Net software.

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

Filtered (0.22 μm , Millipore) hydrolysed samples (200 μL , 0.25% in 0.1 M NaNO_3) were injected (Waters 717 plus Autosampler, controlled by a Waters 6005 controller) onto a Superdex S200 HR column (300x10 mm i.d., GE Healthcare). Elution was performed with 0.1 M LiNO_3 (refractive index R.I. = 1.327) at a flow rate of 0.5 mL/min (Waters 626 pump) at 25°C. This HPLC system was coupled to a Waters 2414 refractive index detector, used as a mass sensitive detector, working at 890 nm at 35 °C. MALLS measurements were performed at 690 nm with a DAWN EOS system (Wyatt Technology, Santa Barbara, CA, USA) equipped with a 30 mW Ga-As linearly polarized laser. The intensity of scattered light was measured at 12 different angles, from 35° to 143°. Chromatographic data were collected and processed by the Astra software (Wyatt Technology, Santa Barbara, CA, USA). The Zimm fit method was used for molecular mass determinations. The calculated dn/dc was of 0.115 ml/g. Bovine serum albumin monomer (Sigma, St.Louis, MO) was used to normalize the signals recorded at various angle of detection, with the signal measured at 90°.

¹H-NMR spectroscopy analysis of hydrolysis mechanism

The experiments were performed with λ -carrageenan (0.5% w/v) and purified native λ -carrageenase (8.4 μg / mL) dialysed against 0.1 M NaNO_3 in 99.97 atom% D_2O at 10°C. The enzyme (500 μL) was added to 5 mL of substrate equilibrated at 30 °C. At chosen hydrolysis times, 500 μL of hydrolysate were transferred into a 5 mm NMR tube and ¹H NMR spectra were recorded at 70 °C. NMR analysis were achieved with a BRUKER Advance DRX 500 spectrometer equipped with an indirect 5 mm gradient probehead TXI ¹H/¹³C/³¹P. Spectra were recorded using 32K data points and the parameters were as follows: pulse angle, 30°; sweep width, 10330 Hz; acquisition time, 1.58 s; relaxation delay, 2 s. The number of scans was 64 for $t = 0$ and $t = 1$ min and 128 for each subsequent point of the time course, digital resolution being of 0.31 Hz/point. Chemical shifts are expressed in ppm based on an external reference TSP (trimethylsilylpropionic acid).

Synthesis and enzymatic digestion of fluorescent oligosaccharides

The protocol for the derivatisation of sugar reducing ends using 2-Aminoacridone (AMAC) from Goubet and co-workers was applied to the oligo- λ -carrageenan series [32]. Mixtures of DP4 and DP6 oligo- λ -carrageenans obtained after overnight enzymatic digestion (see previously) were labelled according to the protocol described by Goubet and co-workers [32]. After the grafting reaction had added the AMAC to the reducing ends, the samples were

freeze-dried, dissolved in 50 mM $(\text{NH}_4)_2\text{CO}_3$, filtered and then purified by size-exclusion chromatography (Superdex 30 preparative grade column, 600 x 26 i.d. mm, GE Healthcare). The elution was performed in 50 mM $(\text{NH}_4)_2\text{CO}_3$ running at 102 ml/h. The pure fluorescent oligosaccharides (DP4 and DP6) were collected, lyophilised and stored at 4 °C.

Fluorescent oligo- λ -carrabiose (DP2) was obtained using the protocol of Goubet and coworkers [32] using purified DP2 as a substrate [6]. Since DP2 was eluted with salts and other small molecules, it could not be purified by size-exclusion chromatography. Therefore, fluorescent DP2 was purified by carbohydrate polyacrylamide gel (27% w/v) electrophoresis (C-PAGE) [30]. The fluorescent band corresponding to the labelled DP2 was excised, ground and allowed to diffuse in a small amount of water at 4 °C. After 24h, the fluorescent DP2 was recovered, lyophilised and stored at 4 °C.

Enzymatic digestion of fluorescent oligo- λ -carrageenans was achieved by incubating about 1 mg of pure oligosaccharide in 50 μl 0.1 M NaNO_3 with 3 pmole of λ -carrageenase overnight at 30°C. The AMAC grafted onto the oligosaccharides does not substantially alter the mode of binding of substrates to the enzyme. The time course of digestion was monitored by C-PAGE (27% w/v) running at 20 mA for 30 min [30]. The migration front of the fluorescent oligosaccharides was visualised using an UV transilluminator BioDoct-It™ system (UVP) emitting light at 302 nm. An equimolar mixture of purified fluorescent DP2, DP4 and DP6 in 0.1M NaNO_3 was used as standard.

RESULTS

Purification of the extracellular λ -carrageenase from *P. carrageenovora*

P. carrageenovora was grown in the presence of λ -carrageenan in order to induce production of the enzyme [19]. The λ -carrageenase was secreted into the extracellular medium. As previously reported by Greer [20], optimal protein production occurred at the early stationary phase (48h). Purification to electrophoretic homogeneity of the enzyme from the concentrated culture supernatant (5L) was achieved in three steps: protein precipitation with ammonium sulphate at 30-70% saturation followed by hydrophobic interaction chromatography on Phenyl Sepharose and size-exclusion chromatography on Superdex 200 (total protein amount: 85 mg, initial specific activity: 1.75 $\mu\text{mol}/\text{min}/\text{mg}$, pure protein amount: 0.5 mg, final specific activity: 30 $\mu\text{mol}/\text{min}/\text{mg}$, recovery: 10%). During the last chromatography step, the enzyme eluted as a single peak, with an apparent molecular mass of 78 kDa. This protein fraction migrated as a single band in SDS-PAGE analysis, corresponding

to a higher apparent molecular mass of 97 kDa (Figure 1). This protein band was excised and one N-terminal and two internal peptide sequences were determined by Edman degradation, SQSAIKSIETNRTITK, YSYDMWK and LSAGYDNSDGIS. These are referred to as A, B and C, respectively (supplementary data).

The λ -carrageenase gene *cgIA*

A gene probe of 943 nt was synthesized by PCR using degenerate oligonucleotide primers designed from the peptide sequences A and B. Southern Blot experiments with this probe showed that *P. carrageenovora* contains only one copy of the λ -carrageenase gene. The same probe was used to screen a genomic library prepared with *P. carrageenovora* total DNA. Of the 6000 clones in the library, 14 clones hybridized with the probe, with an insert size ranging from 4 to 20 kb. Among them, three clones, referred to as pAT153la38, pAT153la45 and pAT153la53, were chosen for the moderate size of their inserts (3.8, 6.1 and 5 kb, respectively) and were subjected to physical mapping. Restriction analyses indicated that the three inserts corresponded to the same genomic DNA fragment, with a common region of 3.7 kb. The insert of plasmid pAT153la45 was completely sequenced, yielding a nucleotide sequence of 5665 nt. Three open reading frames (ORF) are predicted in this sequence, ORF1 and ORF2 on the direct strand and ORF3 on the reverse strand. Whereas ORF1 corresponded only to the 3' end of a gene, ORF2 and ORF3 were complete (2826 and 891 nucleotides, respectively), The amino acid sequence deduced from ORF2 contains the peptide microsequences A, B and C, indicating that this ORF corresponds to the gene coding the λ -carrageenase (supplementary data). To be consistent with the κ - and ι -carrageenase gene names, *cgkA* and *cgiA*, respectively [9, 15], the λ -carrageenase gene is referred to as *cgIA*. Two hexamers separated by 17 nucleotides, TTGACg and TAaAcT, are found in the 5' non coding region of *cgIA*. These sequences, located 68 nt upstream the start codon (ATG²¹⁷), are reminiscent of the -35 and -10 consensus promoter sequences in *E. coli* [33]. The hexamer AGGAat located 5 nt upstream of the start codon is likely to be a Shine-Dalgarno ribosome-binding site [34]. In the 3' non coding region two inverted-repeats were detected by the *mfold* program. These repeats could constitute a transcription terminator hairpin (supplementary data). The stem region shows a high GC content and displays the main characteristics of *E. coli* rho-independent terminators [35].

Sequence analyses of the CglA gene product

Translation of the λ -carrageenase gene *cglA* yielded a preprotein of 942 amino acids with a theoretical molecular mass of 105 kDa. The program SIGNALP v.3.0 predicted a clear signal peptide with cleavage between Ala25 and Ser26, using both the Neural Networks and Hidden Markov Models algorithms [26]. This prediction is consistent with the N-terminal sequence of the extracellular λ -carrageenase.

A sequence similarity search using the program BLASTp [27] indicated that there was no protein in the UniProt database homologous to the full length λ -carrageenase. About 75% of the sequence has no significant similarity with known proteins. Only the N-terminal part of CglA displays low sequence similarity with conserved hypothetical proteins with various lengths and annotations, such as bacterial quinoprotein (trEMBL code: Q6M0H6, 282 residues), YxaL protein (P42111: 410 residues), or cell surface protein MA_0850 (Q8TSE8, 2275 residues). The length of the matches with CglA varies between 100 and 260 residues, with 25-30% pairwise sequence identities. In all these proteins, the homologous region corresponds to several consecutive repeats belonging to various Pfam families (WD40, FG-GAP, BNR or PQQ repeats), which are included in the Pfam Beta propeller clan [36]. A domain search with InterProScan [28] identified an N-terminal Quinonprotein alcohol dehydrogenase-like domain (164 residues, E-value: 2.8 E-13), which adopts a 8-bladed beta-propeller fold (SCOP classification, [37]).

Using the SEG program [29], a low complexity region was identified in the middle of the CglA sequence, between Arg467 and Asn475. An Hydrophobic Cluster Analysis [38] confirmed the absence of secondary structure in this region, which can even be extended to Ala461-Asn475.

Attempts to overexpress the λ -carrageenase CglA

Despite the numerous conditions attempted to obtain a soluble λ -carrageenase in *E. coli*, the recombinant protein was systematically expressed as insoluble inclusion bodies (Figure 2A). These inclusion bodies were purified and solubilized in 8 M urea. The recombinant, unfolded λ -carrageenase (500 μ g / ml) was diluted 50-fold in the presence of its substrate (0.5% λ -carrageenan in 0.1 M NaNO₃, pH 7.5). After 96 h incubation at 30 °C, C-PAGE analysis revealed the release of λ -carrageenan oligosaccharides, including the main terminal products, neo- λ -carratetraose and neo- λ -carrahexaose (Figure 2B, lane 3). The reaction appeared complete after two weeks incubation at 20 °C (Figure 2B, lane 4). This pattern of

degradation is identical to that obtained with pure, native λ -carrageenase (0.7 $\mu\text{g} / \text{ml}$) after 24 h incubation at 30 °C (Figure 2B, lane 1). Therefore, this refolding experiment succeeded in restoring a fraction of active, recombinant λ -carrageenase, even though the yield was very low.

Kinetics of degradation of λ -carrageenan by CglA

Digestion kinetics were assayed by measuring the production of reducing ends occurring as a function of time. The curves have a classical shape, having a single exponential form with the asymptote proportional to the amount of substrate (data not shown). The kinetic parameters (K_m , k_{cat}) of the enzyme were tentatively estimated. However the low reactivity of the reducing ends prevented kinetic experiments at low substrate concentration, as did the high viscosity of the λ -carrageenan at high concentrations (above 1% w/v). In addition, applying the test of Selwin [39], we found that the enzyme activity decreased during kinetic experiments. Therefore, we were not able to calculate unambiguously the Michaelis constants.

The time course of λ -carrageenan depolymerisation was monitored by LC-MALLS (Figure 3). As soon as the enzymatic digestion began the molecular mass of λ -carrageenan rapidly decreased. The initial molecular mass, which was estimated to be 1430 kDa, diminished to about 180 kDa after 5% degradation (Figure 3A). For the same amount of cleaved linkages, the index of polydispersity increased more than three times (Figure 3B). Furthermore, a linear correlation was obtained between the reciprocal of the molecular mass ($1/M_n$) and the incubation time (Figure 3B). This corresponds to a first-order random depolymerisation which can be described by the equation (1) [40, 41].

$$(1) \quad 1/M_t = 1/M_0 + kt$$

In this expression M_t and M_0 are the molecular masses at time t and time zero, respectively. This linear relation between inverse molecular mass and degradation time has also been used to describe the acid hydrolysis of carrageenans [41-43].

Analysis of the cleavage of glycosidic bonds

The oligo- λ -carrageenans produced by various degrees of enzymatic degradation were observed by size exclusion chromatography (Superdex 30). The high molecular mass polymeric fraction (60-70 min retention times) was converted into oligosaccharides (95-135 min) as the degradation proceeded (Figure 4). These oligosaccharides, which were purified and analyzed by ^1H and ^{13}C NMR spectroscopy [6], belonged to the neo-carrabiose series. During the first period of digestion (0-3 h), only oligosaccharides ranging from the DP4

(G2S-D2S,6S)₂ to DP8 (G2S-D2S,6S)₄ were detected. Oligosaccharides of a higher degree of polymerization were detected only in very low amounts, when observed by SEC or by HPAEC. The main digestion products were DP6 and DP4, the signal of DP6 being the most intense throughout the depolymerisation reaction. After digestion of the entire high molecular mass carrageenan fraction (22-50 h), the amount of DP4 continued to increase while the level of DP6 seemed to decrease in parallel with a slow production of DP2. This suggests that the enzyme can slowly cleave DP6 into DP4 and DP2.

The enzymatic hydrolysis of λ -carrageenan was also monitored by ¹H NMR spectroscopy (Figure 5). ¹H NMR spectrum of the undigested polysaccharide presented very broad signals attributed to the high viscosity of the macromolecules. After 10 min of enzymatic degradation the viscosity diminished, resulting in an improvement of the spectrum resolution. New signals appeared, assignable to the newly formed reducing and non-reducing ends. The broad peak assignable to undigested substrate (5.55 ppm) was split into three distinct signals which were ascribed to the anomeric protons in α -configuration at the reducing end (G2Sr α -H₁, 5.52 ppm), non-reducing end (D2S,6Snr-H1, 5.48 ppm) and internal (D2S,6S-H1, 5.56 ppm). Integration of G2Sr α -H₁ and D2S,6Snr-H1 revealed that the intensity of these signals increased at the same rate for the first 20 min. The G2Sr α -H₁/D2S,6Snr-H1 ratio was displaced in favour of the non-reducing end signal, this being compensated by the apparition of the G2Sr β -H₁ signal. When the anomeric equilibrium was reached, the α -/ β -anomer ratio was about 75%, as previously determined [6].

Characterisation of the active site organization of CglA

To identify the subsite architecture of the active site, the mode of cleavage of neo- λ -carrabiose oligosaccharides was investigated. Purified DP4 (Figure 6A) and DP2 (not shown) were not further degraded by λ -carrageenase demonstrating that these oligosaccharides are end-products of the enzyme. DP8 was only split into DP4 (Figure 7C) suggesting that the active site of CglA consists of at least 8 subsites, numbered from -4 to +4 according to Davies and co-workers' nomenclature [44] (Figure 7). As expected from size exclusion chromatography analysis, DP6 was slowly converted into DP4 and DP2 (Figure 6B). The cleavage position was determined using fluorescent DP6 labelled by AMAC on its reducing end. Degradation of fluorescent DP6 gave rise to the production of fluorescent DP4 at the same concentration, indicating that the DP6 bound to the subsites -2 to +4 leading to the cleavage of the glycosidic bond between the fourth and the fifth galactose moieties starting from the reducing end (Figure 7).

DISCUSSION

The λ -carrageenase CglA is the first representative of a new glycoside hydrolase family

The λ -carrageenase from *P. carrageenovora* was purified to electrophoretic homogeneity. Size exclusion chromatography indicated that this enzyme exists as a monomer in solution. The gene coding the λ -carrageenase was cloned and sequenced using information from the peptides A and B that were sequenced from the purified enzyme. Several lines of evidence indicate that the correct gene has been cloned: i) the sequence of the *cglA* gene product features the peptide sequences found in the wild-type protein, including the peptide C which was not used for the cloning (A: S26-K41, B: Y338-K345, C: L487-S498) ; ii) despite a low refolding yield, λ -carrageenase activity was restored from the insoluble, recombinant protein CglA ; based on C-PAGE analysis, this fraction of active, recombinant enzyme released an oligosaccharide pattern identical to that of the wild-type λ -carrageenase. In the presence of λ -carrageenan, the Gram-negative bacterium *P. carrageenovora* exports the λ -carrageenase CglA into the extracellular medium. The N-terminal sequencing of CglA and the analysis of its sequence by SignalP 3.0 [26] are consistent with the cleavage of the precursor enzyme by the signal peptidase I and an initial targeting to the periplasm.

No proteins similar to CglA were found in the sequence databases. The low complexity region Ala461-Asn475 is likely to be a flexible linker connecting at least two independent structural domains. The C-terminal region of CglA (Y476-L942) displays no significant similarity with any known protein. The N-terminal region exhibits low, but significant sequence similarities with numerous conserved proteins that adopt the β -propeller fold. These observations are consistent with the identification of a N-terminal β -propeller domain by the server InterProScan [28]. According to the number of blades (repeats of four-stranded antiparallel β -sheet), the length of this domain in λ -carrageenase may vary between ~200 residues (4-bladed beta-propeller) and ~400 residues (8-bladed beta-propeller) [45]. Several families of glycoside hydrolases possess a β -propeller fold: the clan GH-E sialidases / neuraminidases (families GH33, 34 and 83), the xylanases and arabinases of the clan GH-F (families GH43 and 62) and the clan GH-J (families GH32 and 68) which includes invertases and fructosidases (<http://afmb.cnrs-mrs.fr/CAZY/>, [11]). However, proteins with a β -propeller fold are not limited to carbohydrate metabolism. Many are involved in other types of enzymatic reactions (proteolysis, oxido-reduction, etc.), but also in non-catalytic functions, such as ligand transport and protein-protein interaction [45]. Therefore, it is difficult to predict if this β -propeller domain is the catalytic domain of the λ -carrageenase. In order to define the

limits of the domain, we have attempted mild proteolysis of the purified λ -carrageenase with trypsin and pepsine. Unfortunately, we did not succeed in isolating defined domains (data not shown).

Altogether, the λ -carrageenase from *P. carrageenovora* defines a new family of glycoside hydrolases, unrelated to the GH16 κ -carrageenase and the GH82 ι -carrageenase families. This enzyme most likely has a modular architecture with at least two domains connected by a linker, including a β -propeller domain at the N-terminus. Nevertheless, the catalytic domain of the λ -carrageenase remains to be identified.

The λ -carrageenase CglA is a β -(1 \rightarrow 4) endo-galactanase

As shown by NMR analyses [6], the oligosaccharides released by the λ -carrageenase belong to the neo-carrabiose series, indicating that this enzyme cleaves the β -(1 \rightarrow 4) linkages of λ -carrageenan. $^1\text{H-NMR}$ monitoring of the enzymatic degradation of λ -carrageenan revealed that α -anomeric signals were initially produced and progressively gave rise to β -anomers when mutarotation took place. Therefore, the λ -carrageenase proceeds according to a mechanism of inversion of the anomeric configuration. The LC-MALLS experiments showed that CglA catalyses a rapid decrease in the molecular mass of λ -carrageenan, as well as an increase in the polydispersity. Furthermore, the linear decrease of the reciprocal M_n as a function of time strongly supports a reaction model involving random depolymerisation of polysaccharide chains, as described for the acid hydrolysis of λ -carrageenan. Therefore, the λ -carrageenase CglA follows a random, endolytic mode of action.

Size-exclusion chromatography revealed a high production of DP6 from the onset of hydrolysis. This production of DP6 is an indication of the non-processive character of CglA. Indeed, the alternation of β -(1 \rightarrow 4) and α -(1 \rightarrow 3) linkages in carrageenans results in two successive β -(1 \rightarrow 4) linkages alternatively pointing up and down. A processive carrageenase, which slides along the polysaccharide chain, would encounter a β -(1 \rightarrow 4) linkage in the correct orientation for the cleavage only every two disaccharide units. Therefore, processive carrageenases should release neo-carratetraoses (DP4) or oligo-carrageenans multiples of DP4 [12]. This is reminiscent of processive cellulases which release only cellobioses, since the cellulose chain adopts a 2-fold screw axis which exposes cleavable β -(1 \rightarrow 4) linkages only every two glucose units. In this context, the release of DP6 by λ -carrageenase requires the dissociation of the enzyme and is necessarily produced by a strict, endolytic mode of action.

However, it was difficult to observe oligosaccharides with a degree of polymerization higher than eight using SEC or HPAEC. We interpret the very low abundance of medium-size

fragments of λ -carrageenan as being a result of the physicochemical properties of this biopolymer. λ -Carrageenan chains have fairly rigid conformation, which are due to the repulsive interaction of the sulphate ester groups, thus forming a highly viscous solution. The smaller-size molecules are more flexible and more diffusible in viscous solution than higher molecular mass molecules. Therefore, as soon as they are produced, intermediate or low molecular mass fragments are likely to be more accessible to the enzyme than larger chains. According to this scheme, the λ -carrageenase would discriminate between the high and low molecular mass carrageenan molecules on the basis of their ability to diffuse in the medium.

Insights into the evolution of carrageenase families

Even if κ -, ι -, and λ -carrageenans are related sulphated galactans, our current knowledge indicates that the enzymes specific for their degradation are dissimilar and have emerged as three independent families of glycoside hydrolases. More generally, we have now six families of glycoside hydrolases encompassing enzymes degrading sulphated polysaccharides: families GH16 (κ -carrageenases, keratanases), GH79 (heparanases), GH82 (ι -carrageenases), a new GH family specific for the degradation of sulphated fucans [46] and two unclassified enzymes, *B. circulans* keratanase II [47] and *P. carrageenovora* λ -carrageenase (this work). With the exception of the GH16 family, all these families encompass enzymes that interact exclusively with anionic polysaccharides. In contrast, κ -carrageenases and keratan-sulphate endo- β -(1 \rightarrow 4)-galactosidase [48] fall into the GH16 family, which mainly encompasses enzymes acting on neutral polysaccharides [11] with a common ancestor that is likely to be a laminarinase [9, 12]. However, keratan-sulphate galactosidase [49] and κ -carrageenases are specific for moderately negatively charged patterns, with one sulphate group only per disaccharide unit. Moreover in κ -carrageenase, the nucleophilic attack on the C1 anomeric carbon occurs on the neutral face of the G4S bound in subsite -1 [12]. Thus, the catalytic machinery is not strongly influenced by the presence of the sulphate group of G4S. Altogether, the current data suggest that enzymes specific for sulphated polysaccharides could emerge from within GH families more adapted to neutral polysaccharides, if they degrade moderately sulphated polymers which do not affect the ancestral catalytic machinery. In contrast, enzymes acting on highly sulphated polysaccharides are more likely to define new GH families.

Like κ - and ι -carrageenases, λ -carrageenase cleaves the β -(1 \rightarrow 4) glycosidic linkage of its substrate. No enzyme hydrolyzing the α -(1 \rightarrow 3) linkage of carrageenans has been found yet. This might be due to intrinsic properties of carrageenans; for instance, a lower energy is

perhaps needed for the hydrolysis of the β -(1 \rightarrow 4) linkage in these sulphated galactans. Another possibility is that the sulphate groups which flank the α -(1 \rightarrow 3) linkage in κ -, ι - and λ -carrageenans are a steric obstacle to the hydrolysis of this glycosidic bond.

In contrast to κ -carrageenases, ι - and λ -carrageenases proceed according to a mechanism of inversion of the anomeric configuration. Most likely these similar inverting mechanisms are fortuitous. But, it is also possible that high density of sulphate groups constrains the catalysis, particularly the retaining mechanism. Indeed a retaining mechanism usually involves large conformational changes of the substrate between the relaxed state, the transition state and the covalent glycosyl-enzyme intermediate [50]. Such large conformational changes are unlikely to occur in highly sulphated polysaccharides. Indeed, these biopolymers are fairly rigid, as a result of the repulsive interactions between the sulphate groups and of the steric hindrance of these bulky substituents. A sulphate group in the vicinity of the glycosidic bond may also preclude the formation of a covalent intermediate with the nucleophile catalytic residue for steric reasons. This is likely the case for λ -carrageenan whose the β -linked galactose moiety is 2-sulphated (D2S,6S-G2S). For all these reasons, the inverting mechanism might be more favourable for glycoside hydrolases acting on highly sulphated polysaccharides.

Finally, the mode of action of *P. carrageenovora* λ -carrageenase is not processive, in contrast to that already demonstrated for the “*A. fortis*” ι -carrageenase [17] and strongly suggested for the *P. carrageenovora* κ -carrageenase on the basis of its tunnel shaped active site topology [12]. Interestingly, the substrates of κ - and ι -carrageenase are gels made of crystalline fibres. These two enzymes seemed to have adopted a processive mode of action as an efficient strategy to digest solid fibres. In contrast, the endo-character of the λ -carrageenase seems more appropriate than a processive mode of action, in order to rapidly liquefy a viscous solution of polysaccharides.

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NUCLEOTIDE ACCESSION NUMBERS

The sequence of the *cglA* gene has been deposited in the EMBL Nucleotide Sequence Database under the accession number AM397269.

FIGURE LEGENDS

Figure 1: SDS-PAGE analysis under reducing conditions of the purified native the λ -carrageenase from *Pseudoalteromonas carrageenovora* (A). For comparison, the recombinant ι -carrageenase from *Alteromonas fortis* (B) and the recombinant κ -carrageenase from *P. carrageenovora* (C) were also loaded onto the gel.

Figure 2: Analysis of the recombinant λ -carrageenase. (A) SDS-PAGE analysis under reducing condition of λ -carrageenase expression in *E. coli* as a function of induction time. The same volume of each sample was loaded onto the gel (15 μ l). (B) Analyses by Carbohydrate-PAGE of λ -carrageenase activities. λ -carrageenan oligosaccharides released by the purified wild-type enzyme (lane 1), by the control, refolding buffer (lane 2), by the refolded recombinant enzyme after 96 h at 30 °C (lane 3) and two weeks at 20 °C (lane 4). The main final products, the purified neo- λ -carratetraose and neo- λ -carrahexaose, were loaded on lane 5 as references.

Figure 3: (A) Variation in the the molecular mass (Mn) and the polydispersity determined by LC-MALLS of the λ -carrageenan as a function of the percentage of degradation. (B) Linear increase of the reciprocal molecular mass (1/Mn) versus the time of hydrolysis.

Figure 4: Size exclusion chromatography of the digestion product of the λ -carrageenan following incubation with the λ -carrageenase CglA. The profiles were recorded at several time of incubation which were correlated with percentages of degradation (in bracket) determined by the reducing sugar assay. DP2 to DP8 correspond to the peaks of the neo- λ -carrabiose to the neo- λ -carraoctaose respectively, RF labelled the resistant fraction.

Figure 5: $^1\text{H-NMR}$ monitoring of the hydrolysis of λ -carrageenan by the λ -carrageenase CglA. The region of the spectra containing the protons assigned to the α - and β - anomer is detailed. Note that the intensity of the G2Sr α -H1 and D2S,6Snr-H1 increase with a similar rate at the beginning of the digestion until the peak assigned to G2Sr β -H1 appears.

Figure 6: High performance anion exchange chromatography of purified neo- λ -carratetraose (A), neo- λ -carrahexaose (B) and neo- λ -carraoctaose (C) incubated with the λ -carrageenase. (D) FACE experiment conducted on fluorescent neo- λ -carratetraose and neo- λ -carrahexaose labelled at their reducing end with AMAC. A mixture of labelled DP2, DP4 and DP6 were used as a migration standard.

Figure 7: Subsite organization of the λ -carrageenase active site. The subsites are labelled from -4 to +4. The black arrow indicates the position of the glycosidic bond cleavage. The circles represent the D-galactose moieties. The black circle represents the AMAC labelling of the reducing ends.

SUPPLEMENTARY MATERIAL

Nucleotide and deduced amino acid sequences of the λ -carrageenase CglA. The putative -35 and -10 boxes in the promoter region and the potential ribosomal binding site are shown in bold. The potential hairpin loops are indicated by arrows with their own respective minimum free energy below. The open reading frame starts at nucleotide 217. A 25-aa signal peptide is proposed in bold. The underlined amino acids regions corresponds the microsequenced peptides A, B, and C.

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FIGURE 1

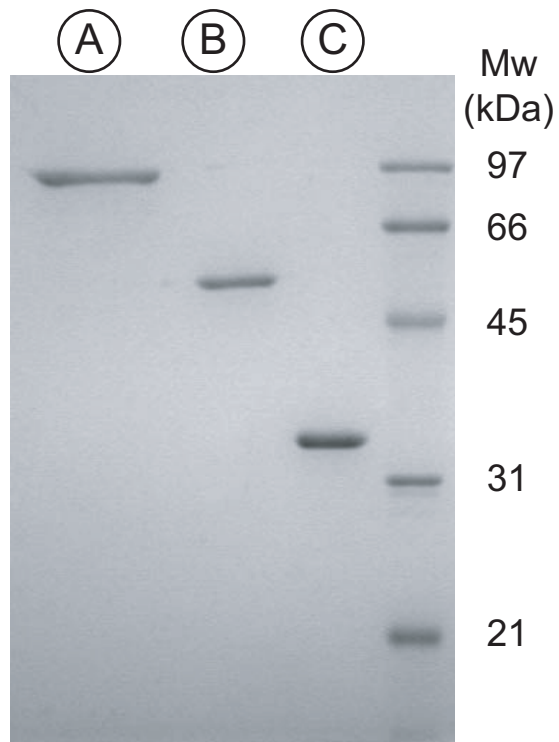


FIGURE 2

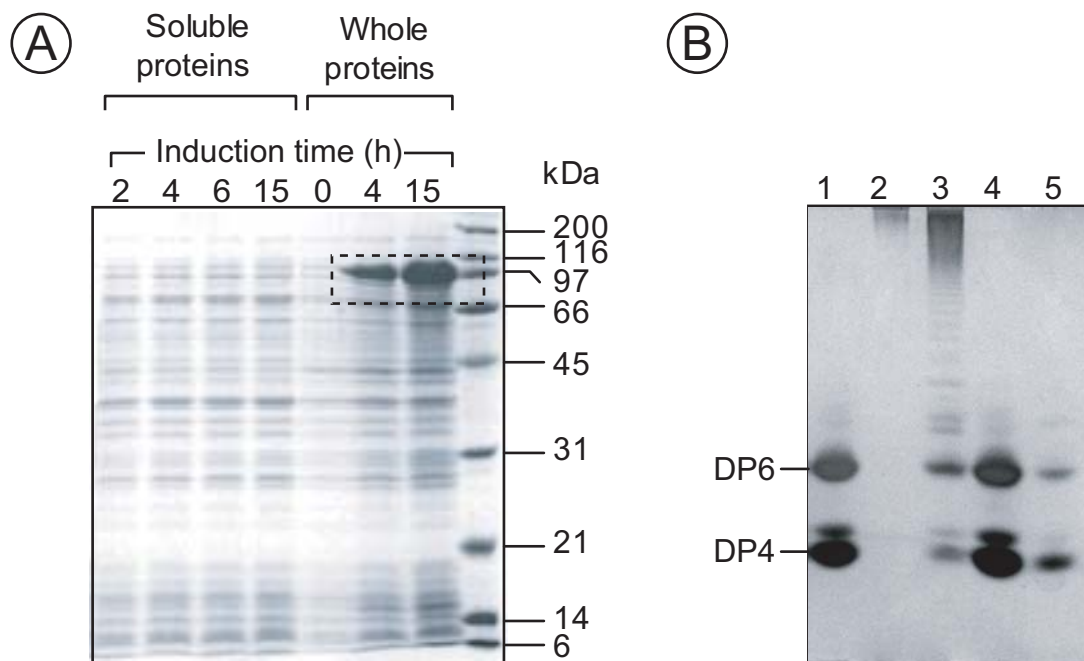


FIGURE 3

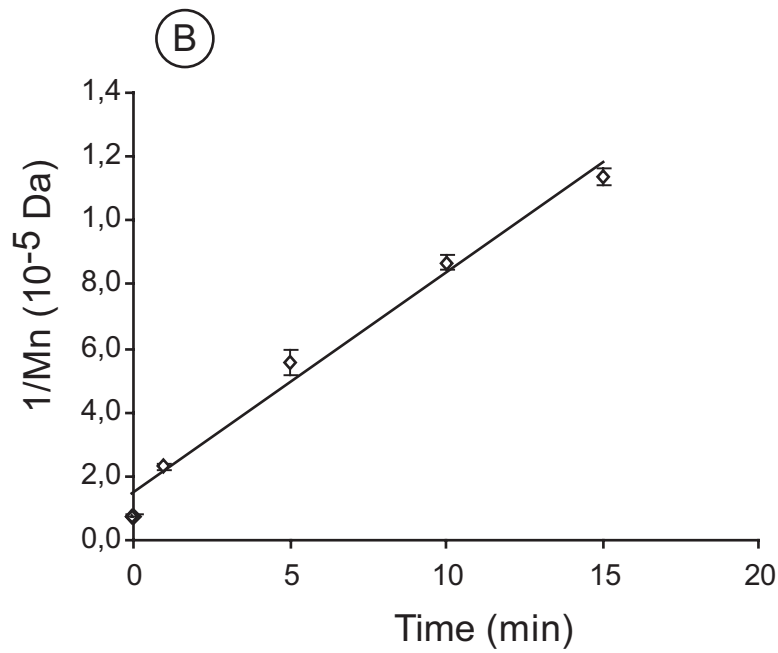
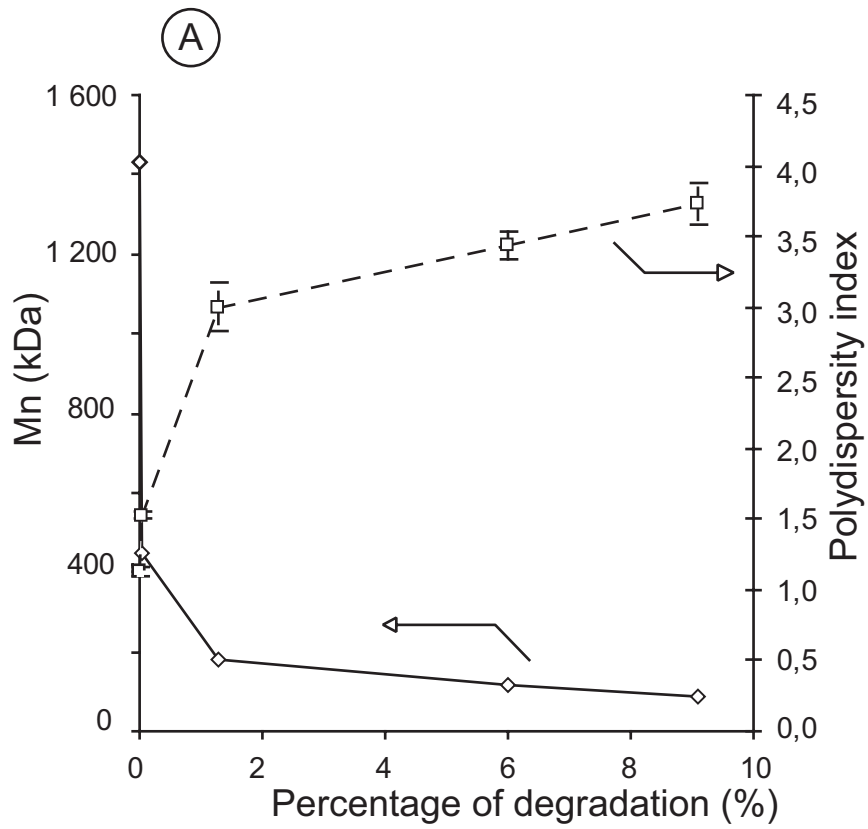


FIGURE 4

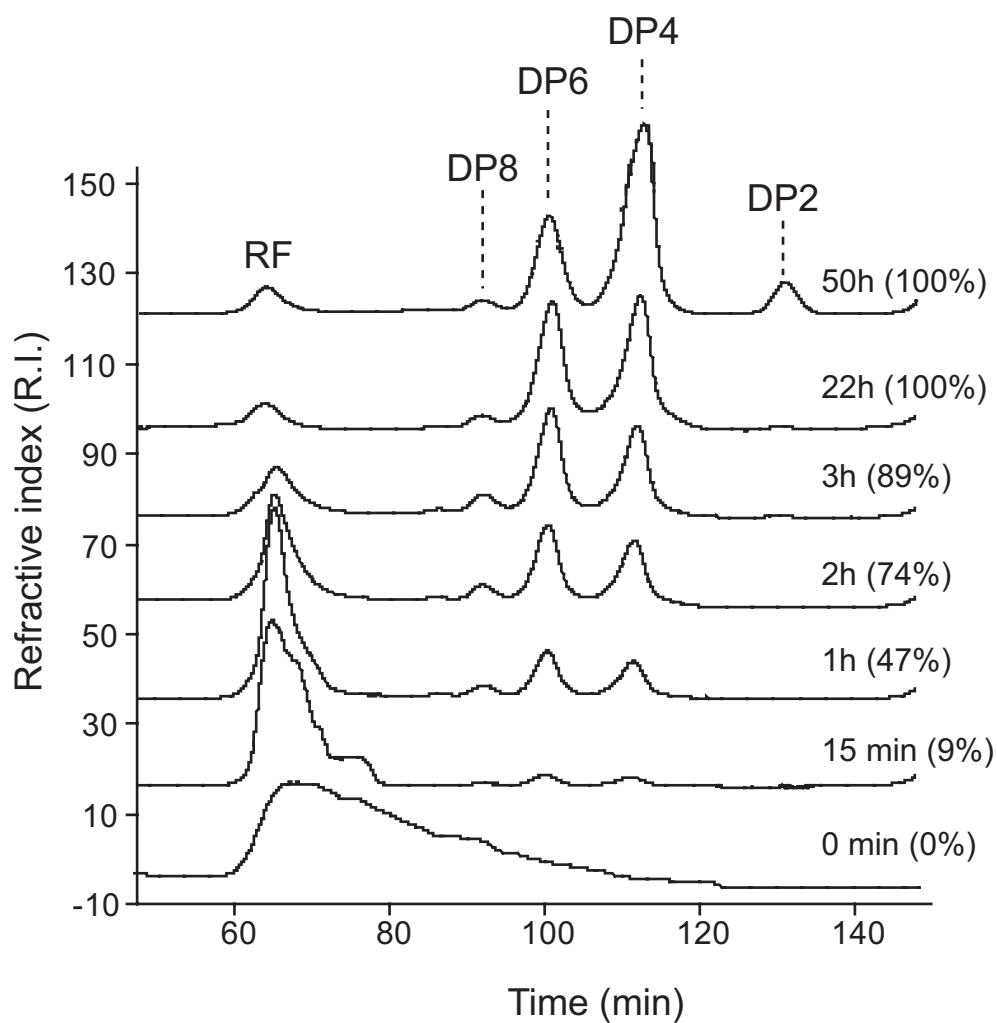
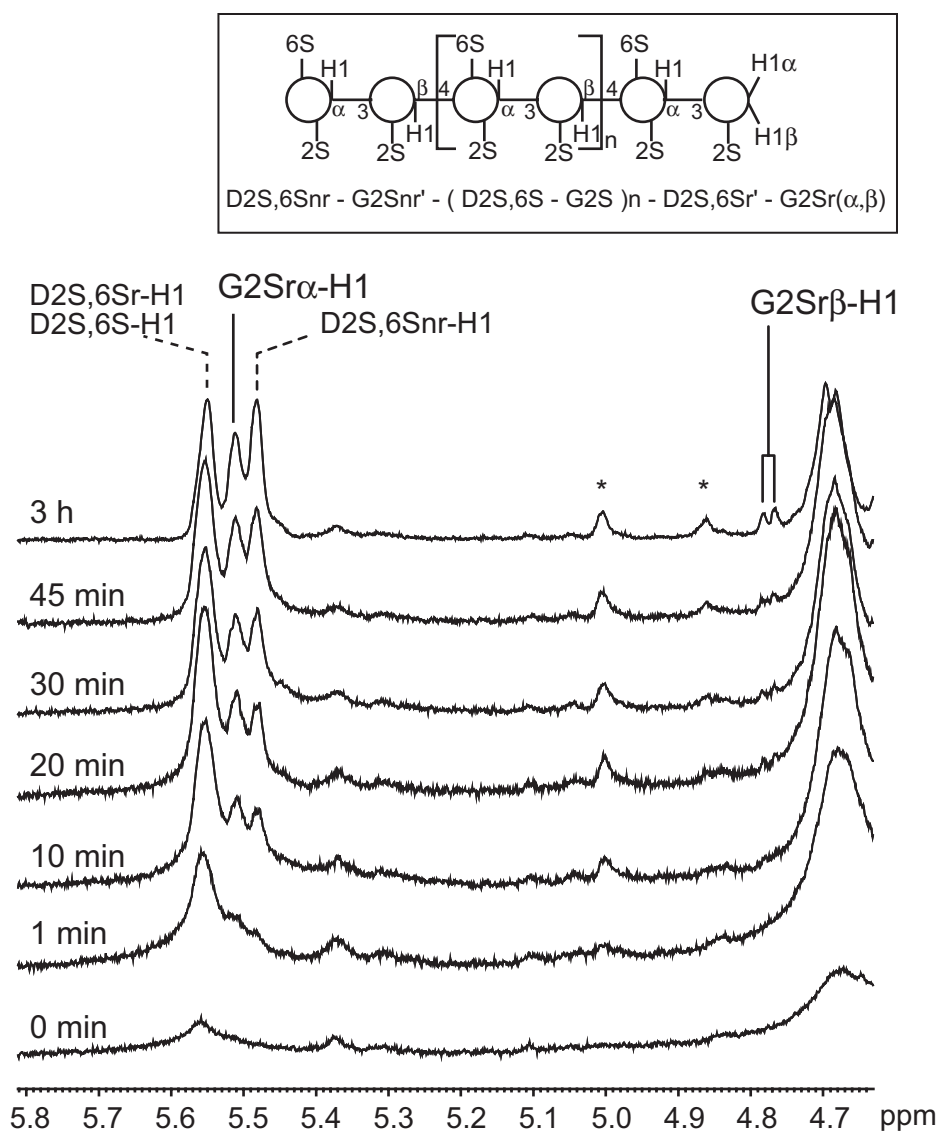


FIGURE 5



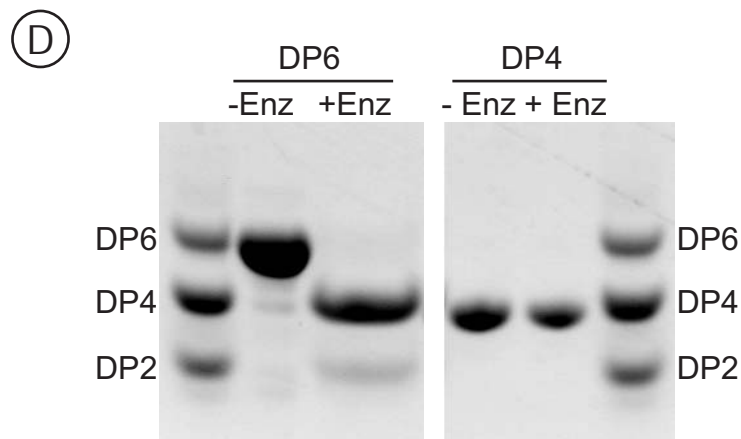
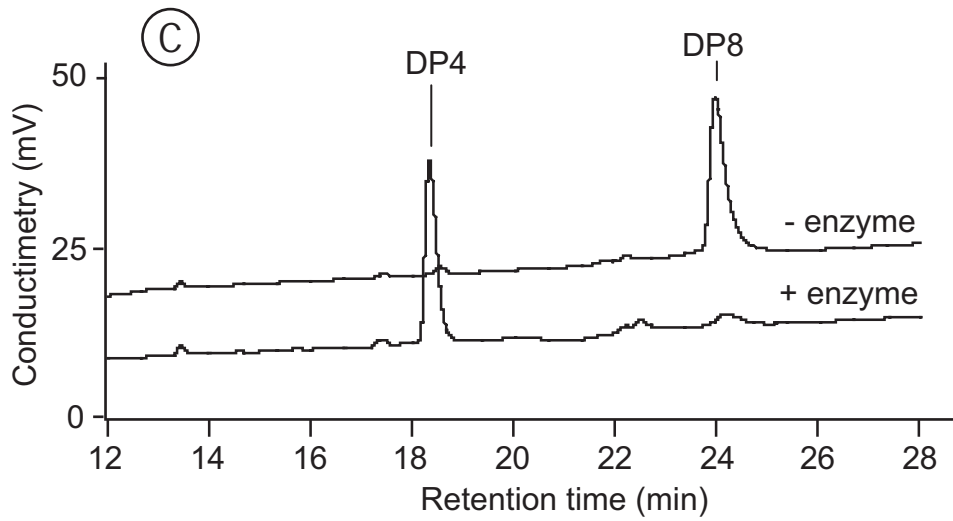
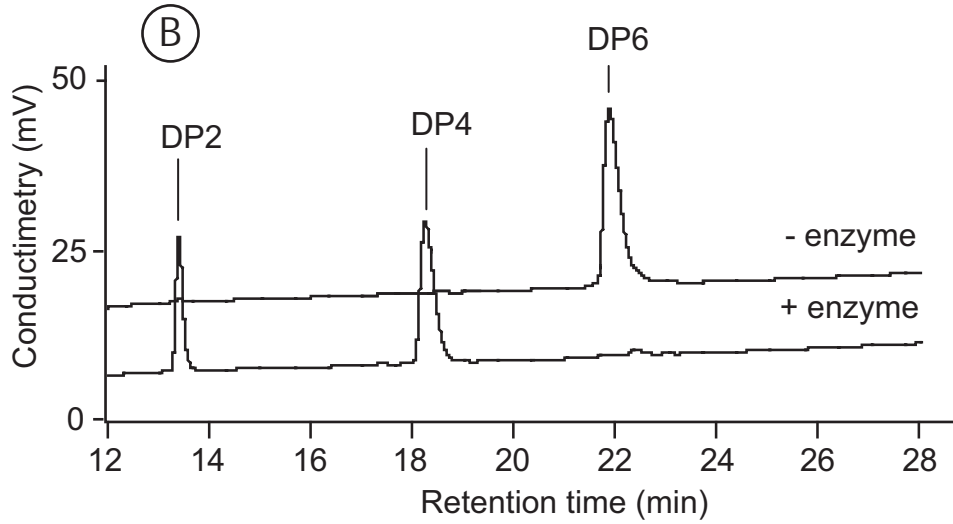
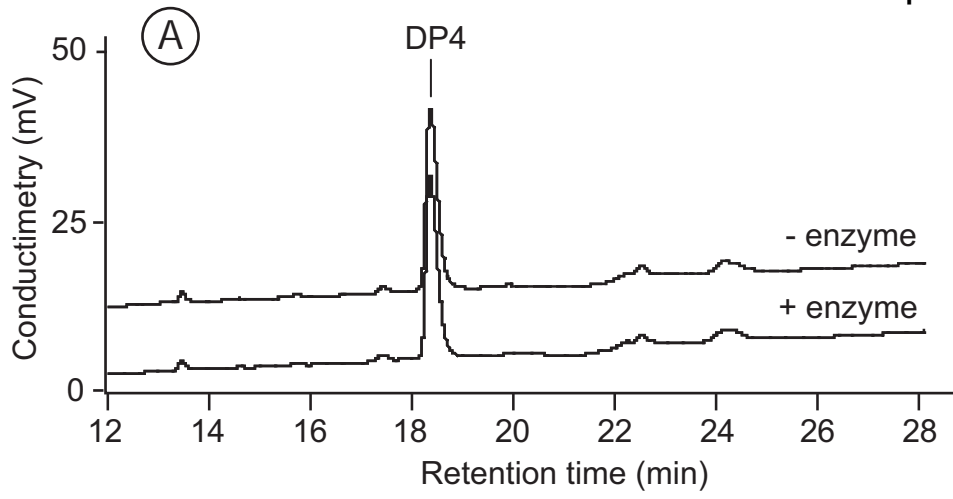


FIGURE 7

