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Enzymatic depolymerization of the GY785 exopolysaccharide produced by the deep-sea hydrothermal bacterium *Alteromonas infernus* : structural study and enzyme activity assessment

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Abstract :

Polysaccharides have attracted much attention due to their interesting physico-chemical and also biological properties that are explored in food, cosmetic and pharmaceutical industries. GY785 exopolysaccharide (EPS) presenting an unusual structure is secreted by the deep-sea hydrothermal bacterium, *Alteromonas infernus*. Low-molecular weight (LMW) derivatives obtained by chemical depolymerization of the native high molecular weight (HMW) EPS were previously shown to exhibit biological properties similar to glycosaminoglycans (GAG). In the present study, in order to generate well defined derivatives with a better control of the depolymerization, an enzymatic approach was applied for the first time. Various commercially available enzymes were firstly screened for their depolymerizing activities, however none of them was able to degrade the polysaccharide. Enzymatic assays performed with *A. infernus* protein extracts have shown that bacterium produces by itself endogenous enzymes able to depolymerize its own EPS. The oligosaccharides released by the enzymes were analyzed and their structures allowed to assess that the protein extract contains several depolymerizing activities.

Highlights

► Screening for depolymerizing enzymes was performed. ► The GY785 EPS was enzymatically depolymerized. ► Mass spectrometry was applied to assess the structure of released oligosaccharides. ► One lyase, one sulfatase and glycoside hydrolase activities were detected.

Keywords : exopolysaccharide, enzyme, depolymerization, oligosaccharides, mass spectrometry

1. Introduction

Microorganisms constitute an inexhaustible source of various and unique metabolites, such as polysaccharides, proteins, enzymes and other molecules that can find several

applications in food, cosmetic, pharmaceutical and medical industries. Among a large diversity of microorganisms, bacteria represent a class of living cells able to produce multiple macromolecules, in particular exopolysaccharides (EPS). Several EPS excreted by bacteria have already been described in term of their chemical composition and structure, *i.e*. xanthan, gellan, succinoglycan or glucans such as cellulose, curdlan and dextran (Freitas, Alves, & Reis, 2011). A few bacterial EPS have emerged as high-added value compounds due to their outstanding physico-chemical features, e.g. gelling, thickening and stabilizing properties, and are currently produced on a large scale for several industrial applications (xanthan, gellan). Besides their interesting physico-chemical properties, bacterial EPS may also exhibit some biological activities, which arise directly from their structures. Hyaluronic acid (HA), a polysaccharide naturally occurring in animal connective tissues and belonging to glycosaminoglycan family, is also produced by several bacterial strains (e.g. *Streptococcus equi*, *Streptococcus zooepidemicus*) (Rehm, 2009). Due to its biological activities, HA is explored as a biopolymer for biomedical applications in cartilage regeneration and wound healing, and is also widely exploited in cosmetic industry. The use of bacteria to produce EPS becomes highly advantageous over the traditional sources used for the polysaccharide extraction (seaweeds, plants or animals). The EPS production through fermentation allows a renewable and optimized polysaccharide recovery with no risks linked to raw material supply, and both the composition and the structure of the final polymer are conserved. Since the polysaccharide is produced by bacteria directly into the culture medium, no chemical extraction step is necessary to recover it, in contrast to the polysaccharides obtained from other sources. Moreover, a wide bacterial biodiversity ensures the isolation of a large diversity of polysaccharides with original and rare structures, especially for the discovery of new molecules endowed with novel functional, *i.e.* physico-chemical and biological properties.

The marine environment and in particular extreme habitats, such as deep-sea hydrothermal vents constitute a potential resource of unknown bacteria that can synthesize EPS with unique structures (Delbarre-Ladrat, Sinquin, Lebellenger, Zykwinska, & Colliec-Jouault, 2014; Delbarre-Ladrat, Leyva Salas, Sinquin, Zykwinska, & Colliec-Jouault, 2017). Indeed, several bacteria excreting EPS of peculiar structures have already been described, among which *Alteromonas macleodii* subsp. *fijiensis* biovar deepsane producing the bioactive HYD657 EPS (Cambon-Bonavita, Raguenes, Jean, Vincent, & Guézennec, 2002; Le Costaouëc *et al*., 2012; Lelchat et al., 2015), *Vibrio diabolicus* secreting HE800 EPS displaying some biological activities in skin and bone regeneration (Rougeaux, Kervarec, Pichon, & Guezennec, 1999; Zanchetta, Lagarde, & Guezennec, 2003; Senni *et al*., 2013), and *Alteromonas infernus* synthesizing GY785 EPS (Raguénès *et al*., 1997; Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004). Low molecular weight (LMW) oversulfated derivatives of GY785 EPS have shown some biological properties close to glycosaminoglycans (GAG). They possess anticoagulant properties similar to heparin (Colliec-Jouault *et al*., 2001) and their presence enhances the chondrogenic differentiation of mesenchymal stem cells, which are considered as an attractive source of cells for cartilage regeneration, through the specific interaction with growth factors (Merceron *et al*., 2012; Rederstorff *et al*., 2017). Recently, oversulfated GY785 EPS derivatives were shown to effectively inhibit both the migration and invasiveness of osteosarcoma cells *in vitro*, as well as the establishment of lung metastases *in vivo* (Heymann *et al*., 2016). All these studies put in evidence the importance of the molecular weight of the biopolymer since LMW GY785 derivatives obtained after chemical depolymerization present a better benefit/risk ratio than the high-molecular weight (HMW) EPS. Moreover, the biological properties were enhanced after chemical sulfation reaction applied to these LMW derivatives. Although chemical depolymerization using chemical hydrolysis or free radicals is efficient to decrease the

average molecular weight of polysaccharides, the lack of specificity and undesired reactions that may affect the polymer structure by the loss of substituents and sugar residues, make that the depolymerization using chemicals is not enough reliable and reproducible. On the contrary, the use of enzymes to depolymerize polysaccharides constitutes an alternative method, which ensures both the specificity of glycosidic bond cleavage and the preservation of the integrity of the polysaccharide fine structure. In addition, replacing chemicals by enzymes represents a "green" environmentally-friendly method, which should be favored. However, since enzymes are specific towards a substrate, polysaccharides of atypical structures, such as GY785 EPS, may require a new screening step to find active enzymes. Indeed, GY785 EPS is a high molecular weight highly branched heteropolysaccharide with a nonasaccharide repeating unit presented on Fig. 1 (Roger *et al*., 2004). The main chain of the polysaccharide is composed of three sugar residues: glucose (Glc), galacturonic acid (GalA) and galactose (Gal) covalently linked in the following sequence: →4)-β-D-Glc*p*-(1→4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*-(1→. GalA residue of the main chain carries at *O*-2 one sulfate group and is substituted at *O*-3 by a short side chain constituted of two glucuronic acids (GlcA), Gal and Glc linked in the sequence: β-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→. The two GlcA of the side chain are each substituted by one Glc residue (Fig. 1).

In the present study, the screening for enzymes able to depolymerize the GY785 EPS and generate LMW derivatives that could advantageously replace the derivatives obtained through chemical depolymerization was performed. On the one hand, several commercially available enzymes were tested for their depolymerizing activities toward the native HMW EPS. On the other hand, *A. infernus* protein extracts were prepared to assess if the bacterium produces by itself enzymes depolymerizing its own EPS. The results obtained allowed to establish the

foundation for the development of a new enzymatic depolymerization process that could further lead to bioactive LMW derivatives.

2. Material and Methods

2.1. Production of the native GY785 EPS.

GY785 EPS is produced by *A. infernus*, a deep-sea, aerobic, mesophilic, and heterotrophic bacterium isolated from a sample of fluid collected among a dense population of *Riftia pachyptila* in the vicinity of an active hydrothermal vent of the Southern depression of the Guaymas Basin (Gulf of California). The production and isolation of the EPS were previously described (Raguénès *et al*., 1997). In the present study, GY785 EPS production was carried out at 25°C and pH 7.4 in a 30 L fermenter (Techfors 30 L INFORS, Switzerland). 20 L of Zobell medium, a marine culture medium prepared with 5 g/L of tryptone, 1 g/L of yeast extract, and 33.3 g/L of aquarium salts, were introduced in the fermenter before addition of 2 L of cell suspension inoculum. The carbohydrate source necessary for biosynthesis of the EPS was 30 g/L of glucose, autoclaved separately and added at the beginning of the batch. At the end of the fermentation process (48h), the EPS excreted in its soluble form and remaining in the culture medium was separated from bacterial cells by a centrifugation step. The supernatant was ultrafiltrated on a 100 kDa cut-off membrane to eliminate glucose and salts, and freeze-dried.

2.2. Enzymatic assays using commercial enzymes.

Commercial enzymes selected for screening of depolymerization activities are presented in Table S1 (Supplementary data). Enzymes were prepared at 10 mg/mL or twice diluted, when enzyme was supplied in a liquid form, in 100 mM phosphate buffer at pH 6. GY785 EPS was prepared at 2 mg/mL in the same buffer containing 0.02% NaN_3. Enzymatic assays

were performed by incubating 200 µL of the EPS solution with 50 µL of the enzyme solution at 37°C under continuous agitation. Aliquots were withdrawn at 0, 4, 24, 48 and 72h, heated at 100°C for 7 min, centrifuged at 10 000 g for 10 min and the supernatants were kept at -20°C until analysis.

2.3. Enzymatic assays using A. infernus protein extracts.

A. infernus protein extracts were prepared as follows. Firstly, 1L of Zobell medium 2 times concentrated containing 8 g/L of wheat peptone, 2 g/L of yeast extract, and 66.6 g/L of aquarium salts was prepared. 1 L of glucose solution at 60 g/L, autoclaved separately, was then added. 40 mL of cell suspension inoculum was finally introduced to the medium and the cells were incubated at 30°C for 96h under agitation (150 rpm). After centrifugation of the whole culture at 8000 g for 45 min at 10°C, the supernatant (S) was concentrated by ultrafiltration on a 10 kDa membrane cut-off and stored at 4°C for further experiments. The remaining pellet containing bacterial cells was suspended in 80 mL of 10 mM Tris HCl buffer at pH 8 containing 20 g/L NaCl and centrifuged at 10 000 g for 20 min at 10°C. The washed pellet was suspended in 80 mL of 10 mM Tris HCl buffer at pH 8 containing protease inhibitors (cOmplet EDTA-free, Roche). In order to recover the enzymes potentially localized in cell membranes, cell lysis was performed by sonication of 10 mL aliquots during 7 cycles of 1 min with 1 min pause. After centrifugation (10 000 g, 20 min), the supernatant called lysate (L) was separated from the insoluble pellet containing cell debris (D).

Enzymatic assays were performed by incubating one volume of the GY785 EPS solution at 2 mg/mL in 10 mM Tris HCl buffer pH 8 containing 0.02% NaN_3 with one volume of the protein extract (S, L, D) at 37°C under continuous agitation. Aliquots were removed at 0, 2, 24, 48, 72 and 160h, heated at 100°C for 7 min, centrifuged at 10 000 g for 10 min and the supernatants were kept at -20°C until analysis.

2.4. Batch oligosaccharides preparation using A. infernus lysate (L).

Oligosaccharides were generated by incubating 10 mL of the GY785 EPS solution at 2 mg/mL in 10 mM Tris HCl buffer pH 8 containing 0.02% NaN_3 with 10 mL of lysate (L) for 72h at 37°C under continuous agitation. After the incubation, the solution was heated at 100°C for 7 min, centrifuged at 10 000 g for 10 min and the supernatant was fractionated on a Superdex 30 column (GE Healthcare 30®) at a flow rate of 2 mL/min with 0.1 M ammonium bicarbonate as eluent buffer. The recovered fractions were freeze-dried for further analysis.

2.5. Analyses

2.5.1. Sugar composition

Monosaccharide composition was determined according to Kamerling, Gerwig, Vliegenthart and Clamp (1975) method, modified by Montreuil *et al*. (1986). GY785 EPS and oligosaccharides were hydrolyzed for 4h at 100°C by 3 M MeOH/HCl with *myo*-inositol used as internal standard. After re-N-acetylation with acetic anhydride overnight at room temperature, the methyl glycosides were converted to their corresponding trimethylsilyl derivatives. Separation and quantification of the per-*O*-trimethylsilyl methyl glycosides were performed by gas chromatography (GC-FID, Agilent Technologies 6890N).

2.5.2. Molecular weight

The weight-average molecular weight was determined by high-performance size-exclusion chromatography (HPSEC) coupled with a multiangle light scattering (MALS, Dawn Heleos-II, Wyatt Technology) and a differential refractive index (RI) (Optilab Wyatt

technology) detectors. HPSEC system was composed of an HPLC system Prominence Shimadzu, a PL aquagel-OH mixed, 8 μm (Varian) guard column (U 7.5mm × L 50 mm), and a PL aquagel-OH mixed (Varian) separation column. Samples were eluted with 0.1 M ammonium acetate. The molecular weight was calculated using a refractive index increment dn/dc of 0.145 mL/g.

2.5.3. Electrophoresis in agarose and polyacrylamide (PAGE) gels.

Agarose gel (0.7 % w/v) was prepared in TAE buffer (0.04 M Tris-acetate; 0.01 M EDTA, pH 8.5). 30 µL of samples in native electrophoresis buffer (Bio-Rad) were loaded on gel wells and electrophoresis was run in TAE buffer for 2h in a refrigerated Maxi cuve (20 cm x 10 cm, Fisher Bioblock Scientific). Polyacrylamide separating gel (27 % w/v acrylamide) was prepared in 1.5 M Tris HCl buffer at pH 8.8 containing ammonium persulfate (10% w/v) and tetramethylethylenediamine (Temed). Polyacrylamide stacking gel (4 % w/v acrylamide) was prepared in 0.5 M Tris HCl at pH 6.8, ammonium persulfate (10 % w/v) and Temed. 40 µL of samples prepared in loading buffer (0.5 M Tris HCl pH 6.8, glycerol, 0.5 M EDTA, 0.5 % w/v bromophenol) was then loaded on polymerized acrylamide gels.

Gels were fixed for 4 h in 25% (v/v) isopropanol and then colored overnight in the dark by Stains All (3,3′-Diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine) solution at 0.005% prepared as follows: 5 mL of a mother 0.1% Stains All solution in dimethylformamide (w/v); 5 mL of 300 mM Tris–HCl pH 8.8; 5 mL of dimethylformamide; 25 mL of isopropanol; 60 mL of H_2O (Lee & Cowman, 1994). Gels were then destained for 2h under natural light.

2.5.4. Reducing sugar assays.

Reducing sugars were determined using Park method (Park & Johnson, 1949) based on the reduction of ferricyanide ions measured at 690 nm. Briefly, 70 µL of sample or

standard were mixed with 140 µL of AP reagent (potassium hexacyanoferrate 0.5 g/L; dipotassium hydrogenophosphate 35 g/L pH 10.6) and 70 µL of BP reagent (sodium carbonate 4.54 g/L; potassium cyanide 0.65 g/L). The mixture was heated at 100°C for 7 min. Samples were then cooled on ice for 4 min and 140 µL of CP reagent (chloride iron (III)-6H_2O 4.164 g/L; polyvinyl pyrrolidone 20 g/L, sulfuric acid 1.02 M final concentration) were added and mixed. After 15 min in the dark, the absorbance was read at 690 nm. Glucose was used as the standard.

2.5.5. Electrospray mass spectrometry.

Electrospray QTOF mass spectrometry (MS) experiments were performed in positive ionization mode on a Synapt G2Si HDMS (Waters, Manchester, UK). Each Superdex fraction from enzymatic reactions were diluted in MeOH/H_2O (1:1, v/v) at 100 µg/mL and infused at 5µL/min for MS analysis. Tandem MS experiments were performed on eight species selected in the fraction 6. For all acquisitions, the ion spray capillary voltage was tuned at 2.7 kV and the source temperature maintained at 120°C. MS mode acquisitions were performed in the m/z range 350 to 2000. For tandem MS experiments, argon was used as collision gas and collision energies were adjusted manually for each precursor based on the signal intensity observed for fragments. Data were recorded using MassLynx 4.1 (Waters, Manchester, UK) Raw data were transformed in mzML format using MSConvert (http://proteowizard.sourceforge.net/downloads.shtml) and processed using mMass 5.5.0 (Niedermeyer, & Strohalm, 2012). For fragment annotations the nomenclature of Domon & Costello (1988) was used.

3. Results and discussion

A. infernus bacterium produces a high-molecular weight (HMW) sulfated heteropolysaccharide called GY785 EPS (Raguénès *et al*. 1997). Osidic composition of the native polysaccharide is presented in Table 1. The main sugars constitutive of the EPS are Gal, Glc, GalA and GlcA. Some minor additional residues were also detected, such as rhamnose (Rha), fucose (Fuc) and mannose (Man). GY785 EPS is characterized by its HMW of 2,000,000 g/mol and its sulfur content of 3 % (w/w). In order to depolymerize this HMW polysaccharide and to produce LMW derivatives, the enzymatic approach was explored for the first time.

3.1. Screening of depolymerizing activities

A total of 26 commercially available enzymes (Table S1, Supplementary data) were selected for testing their ability to depolymerize the native HMW GY785 EPS. Several Carbohydrate Active enZymes (CAZymes), glycoside hydrolases and polysaccharide lyases with various substrate specificities were chosen, together with few proteases, such as papain, pronase and pepsin. Glycoside hydrolases catalyze the cleavage of the glycosidic bonds through addition of water molecule, while polysaccharide lyases act through β-elimination mechanism with the loss of water molecule. Indeed, the linkage between a neutral monosaccharide and the C4 of a uronic acid is cleaved with simultaneous introduction of a double bond between C4 and C5 of the uronic acid (Linhardt, Galliher, & Clooney, 1986; Sutherland, 1995, 1999; Yip & Withers, 2006).

Enzymatic assays performed with commercial enzymes revealed that none of selected enzymes was able to depolymerize the GY785 EPS (data not shown). No LMW bands

indicating the polysaccharide depolymerization were observed upon agarose gel electrophoresis, which constitutes a particularly well adapted method to rapidly confirm the polysaccharide degradation by the presence of typical migration patterns (Rigouin, Delbarre-Ladrat, Sinquin, Colliec-Jouault, & Dion, 2009). The absence of depolymerization after incubation with commercial enzymes suggests that their substrate specificity is too high to cleave the highly complex GY785 EPS bonds.

It was then assumed that *A. infernus* strain could produce by itself enzymes able to degrade its own EPS. The presence of polysaccharidases and polysaccharide lyases degrading the EPS synthesized by the same bacteria has already been reported for few other EPS producing bacterial strains (Sutherland 1995, 1999; Lelchat *et al*., 2015). To explore this hypothesis, three protein extracts were prepared and tested on the GY785 EPS: the culture supernatant (S), the soluble lysate (L) and the insoluble cell debris (D), obtained both after bacterial cell membrane lysis. Upon enzymatic incubation, all the samples were firstly analyzed by agarose gel electrophoresis and gels were subsequently stained by Stains All suitable for acidic polysaccharides (Fig. 2). The distance of migration of a polysaccharide within an agarose gel depends mainly on the charge as well as the molecular weight of the chains. No evolution of the migration pattern was observed for the EPS incubated with the culture supernatant (S). A broad, extended smear characteristic of HMW polydisperse EPS was visible (Fig. 2A), which indicates that no depolymerization occurred even with increasing incubation time (Fig. 2B). However, upon incubation with lysate (Fig. 2C), the broad smear observed at the beginning (0h) was shifted to lower molecular weight less polydisperse bands up to 160 hours of incubation, suggesting that the polysaccharide molecular weight decreased with increasing time of incubation. Similar migration pattern was observed upon incubation with cell debris (Fig. 2D). These observations emphasize that enzymes responsible for the EPS depolymerization are only present in bacterial cells; a part of them may be bound to

membranes. Similar observations were reported after the incubation of HYD657 EPS with protein extracts obtained from *A. macleodii* subsp. *fijiensis*. Active enzymes were only evidenced in bacterial cells (Lelchat *et al*., 2015).

It was previously evaluated that the lower limit in molecular weight resolution in migration on agarose gel (0.7%) is about 40,000 g/mol, which excludes analysis of small oligosaccharides (Rigouin *et al*., 2009). Therefore, in order to determine if such LMW oligosaccharides are also generated by *A. infernus* depolymerization enzymes, electrophoresis analysis was also performed on PAGE gels, which were subsequently stained with Stains All (Fig. 3). No migration pattern was observed for the native HMW GY785 EPS (Fig. 3A). In contrast, upon incubation with lysate (Fig. 3B) and cell debris (Fig. 3C), besides the band noticed on the top of the gel indicating the presence of HMW chains, several bands were noticed after 24h. The migration pattern of the lysate incubated in Tris HCl buffer (Fig. 3D) allowed to assign that these new bands correspond to LMW fragments generated by the EPS depolymerization.

Since electrophoresis was applied as a qualitative method to rapidly detect the eventual polysaccharide depolymerization, in order to quantify this phenomenon, Park assay, allowing the measurement of the amount of reducing sugars released during hydrolysis of glycosidic bonds, was performed. Fig. S1 (Supplementary data) presents the amount of reducing sugars measured upon incubation of the GY785 EPS with enzymes present in lysate (L) for different incubation times. An increase in reducing sugars was clearly observed with increasing incubation time with a plateau value reached after 72h of incubation. This observation confirms that depolymerizing enzyme activities are present in bacterial cells.

To get further insight into the degradation process, the samples were analyzed by HPSEC-MALS. The elution profiles of the GY785 EPS incubated with lysate (L) for different incubation times are shown on Fig. 4. Two main peaks (at 7.5 and 8.3 min) corresponding to two HMW chain populations were observed on the elution profile at the beginning of incubation with enzymes (0h). Increasing the time of incubation led to a significant shift of eluted masses, with the loss of the first peak of HMW population at 7.5 min and an occurrence of a new peak (at 11 min) from 24h of incubation, indicating generation of LMW population. However, the depolymerization of the EPS was not complete even after 160h of incubation with enzymes. By taking into account the very high molecular weight of the native GY785 EPS (more than 2,000,000 g/mol), it cannot be excluded that the polysaccharide chains remain still associated/entangled to some extent even after initial bond cleavage, which prevents from efficient depolymerization. The evolution of the weight-average molar masses, *Mw* and gyration radii, *Rz* of the GY785 EPS populations released upon incubation with lysate (L) is presented in Table S2.

3.2. Characterization of the oligosaccharides generated by A. infernus depolymerizing enzymes.

In order to determine the structure of LMW fragments generated by *A. infernus* depolymerizing enzymes, the enzymatically depolymerized by cell lysate GY785 EPS was fractionated on a Superdex 30^{TM} column with fractionation range < 10,000 g/mol. Six main fractions were recovered and analysed in term of their monosaccharide composition. Sugar composition of each fraction, expressed as molar ratio compared to GalA, is presented in Table 1. The principal osidic residues characteristic of the native polysaccharide were detected in all the six fractions, however their molar ratios were slightly different in comparison to the molar ratios of osidic residues constitutive of the native GY785 EPS. Two

Glc and Gal residues were only detected, which indicates the loss of these residues from the polysaccharide repeat unit. GlcA molar ratio was also affected, as less than 2 residues per fraction were detected. Besides the typical residues constitutive of the native polysaccharide, other sugars, *i.e.* Rha, Fuc and Man were also quantified in all the fractions in high amounts (Table 1). To assess if these osidic residues originate from the soluble protein extract (L), sugar analysis of the lysate was performed. However, only Gal and Glc were detected with residual amounts of Rha and Man, which suggests that the majority of Rha, Fuc and Man residues originate from the GY785 EPS molecule.

To go further into analysis and to elucidate the structure of LMW oligosaccharides released by enzymes, all the fractions were analysed by MS with electrospray ionization in positive mode. Similar spectra were obtained for all the six fractions with several parent ions systematically present as shown on Fig. S2 (Supplementary data). Some of them were annotated and selected for further MS/MS experiment in the aim to define their structures and thus deduce the type of enzymes produced by *A. infernus*, involved in their formation. Indeed, these released species provide the information about the specificity of the enzymes in the total extract.

Table 2 summarizes the structures of the oligosaccharide fragments revealed by MS and tandem MS analysis. An example of a MS/MS spectrum of the precursor ion isolated at *m/z* 875.19 was shown on Figure 5A. This ion was assigned to a $[M+Na]^+$ of a compound containing two hexoses, three uronic acids and one unsaturation. Two isomers, which

constitute a part of the EPS and whose structures are depicted on Figure 5B and C can thus be proposed. For assignation, all the fragments were annotated with a specific colour (blue or red) on the spectrum, while the purple corresponds to CO_2 losses. On the structures deduced from the tandem MS data, red and blue arrows correspond to specific fragments of each isomer. Purple arrows were used for non-specific fragments. Due to the lack of intracyclic fragments, which provide crucial information about the branching pattern between the sub-units, the structures proposed were deduced from the known structure of the native GY785 EPS presented on Fig. 1 (Roger *et al*., 2004). Both isomers contain the core composed of three uronic acids with the unsaturated one systematically presents, corresponding most likely to GalA of the main chain. This observation reveals lyase activities in the protein soluble extract (L). Furthermore, the uronic acid core can either be branched with Gal residue attached to unsaturated uronic acid of the main chain (Fig. 5B) or with Gal and Glc residues attached to GlcA of the side chain (Fig. 5C). The tandem MS experiments performed on the other species present (Table 2) revealed that they constitute fragments of the non-sulfated nonasaccharide repeat unit of the native GY785 EPS. The seven validated structures containing uronic acid-rich oligosaccharides (Table 2, in bold) systematically showed the unsaturation only on one uronic acid, corresponding most likely to GalA of the main chain. However, this unsaturated GalA lacked its sulfated group initially present at *O*-2. Taken together, all these results emphasize that the protein soluble extract (L) constitutes an enzymatic cocktail containing not only lyase and hydrolase activities, but also sulfatase activity. It can be thought that these depolymerizing enzymes act in synergy, where sulfatase catalyses firstly the desulfation of the GalA residue, thus allowing better accessibility for lyase cleavage through β-elimination. Indeed, lyase activity was only detected on the uronic acid of the main chain, which remains crucial for the polysaccharide depolymerization. This sequential depolymerization may explain why this process does not lead to a rapid decrease in

the polysaccharide molecular weight (Fig. 4). Bacteria synthetizing polyanionic exopolysaccharides such as alginate, gellan and hyaluronan also produce lyases as constitutive enzymes (Sutherland, 1995, 1999). Moreover, the co-occurrence of glycoside hydrolases and polysaccharide lyases with sulfatases was also revealed by analysing genomes of some polysaccharide-degrading bacteria (Helbert, 2017).

It is not clear why *A. infernus* produces depolymerizing enzymes toward its proper EPS, which remain associated with cell membranes. By taking into account the hydrothermal origin of the strain, it can be thought that the release of enzymes could be induced by cell lyses due to cell death caused by different deleterious factors (low pH, high temperature, the presence of heavy metals). Released enzymes would then degrade the EPS biofilm, thus allowing the escape and colonization of new environments by remaining living cells. It can also be hypothesized that *A. infernus* produces depolymerizing enzymes to degrade its own EPS to oligosaccharides that may constitute a carbon source for bacterium, when other sources are absent. This could be possible in the case of exogenous enzymes, which are excreted outside bacterial membranes. However, *A. infernus* produces only endogenous depolymerizing enzymes, which makes this hypothesis very unlikely. It can also be thought that in the case of inactive EPS export system, the depolymerizing enzymes might protect the bacterial cells from the EPS intracellular accumulation.

Furthermore, tandem MS analysis of the precursor ion isolated at *m/z* 963.30 revealed the presence of a new family of oligosaccharides rich in methyl-pentoses (Table 2). Indeed, this ion was assigned to a $[M+Na]^+$ of a compound, which contained four hexoses, two methyl pentoses and one unsaturation (Fig. 6). Series of fragments of methyl-pentose contained oligosaccharide were annotated with specific colours. This type of oligosaccharides does not correspond to the native GY785 EPS but they are in accordance with osidic composition

presented in Table 1, which shows that fractions were unusually rich in Rha and Fuc monosaccharides. The spectrum presented on Fig. 6 shows that the specie isolated at *m/z* 963.30 contains four hexoses and two methyl-pentoses with one unsaturation. The doublet at *m/z* 835.26 and *m/z* 817.25 indicates that this unsaturation is located on a methyl-pentose subunit. It is not clear if these hexose and methyl-pentose-rich oligosaccharides are constitutive of the native EPS. It can be thought that *A. infernus* bacterium produces these oligosaccharides in parallel with the GY785 EPS or they are associated with cell membranes and are thus recovered during cell lysis.

4. Conclusions.

A. infernus, a deep-sea hydrothermal bacterium, produces HMW highly branched and naturally sulfated heteropolysaccharide, namely GY785 EPS. Previous studies have shown that LMW GY785 derivatives, obtained by chemical depolymerization of the native polysaccharide, exhibit some biological properties close to those described for GAG from mammalian origin. The present study describes for the first time the potential of the enzymatic approach that could be developed to obtain such LMW derivatives. Because of the particularly complex structure of the EPS, none of the commercially available enzyme was able to depolymerize the polysaccharide. Therefore, different protein extracts were prepared from *A. infernus* cells in order to investigate if the bacterium produces by itself enzymes that could depolymerize its own EPS. By combining several analytical techniques, it was evidenced that *A. infernus* produces a number of endogenous depolymerizing enzymes, which activities lead to various oligosaccharides. From the structure of the released oligosaccharides determined from MS/MS spectra, one lyase and one sulfatase as well as glycoside hydrolase activities were deduced. These depolymerizing enzymes may act in synergy to degrade the GY785 EPS. Further studies are however required to confirm this hypothesis. The purification of the crude protein extract and enzyme characterizations are currently being performed to get

further insight into their mechanism of action. These steps are crucial to optimize the enzymatic approach and to obtain LMW derivatives of defined molecular weight. In parallel, identification of the genes coding for depolymerizing enzymes in *A. infernus* genome and their cloning will also be undertaken.

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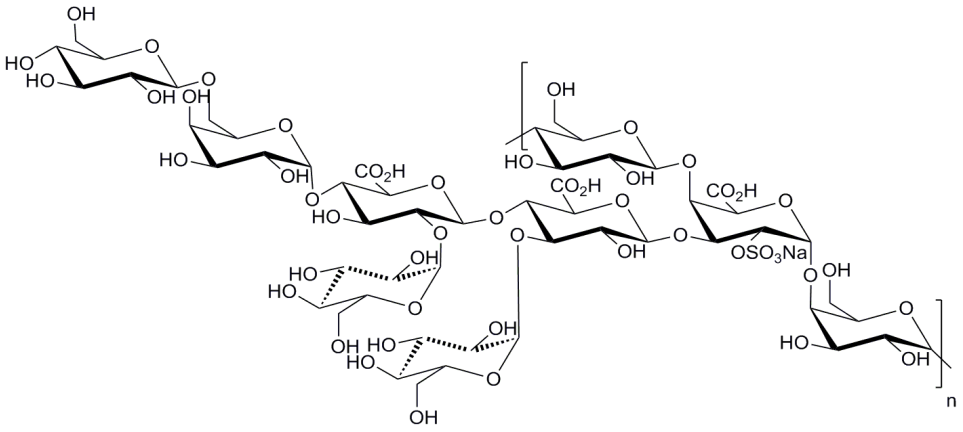

Fig. 1. Structure of the monosulfated nonasaccharide repeating unit of the GY785 EPS.

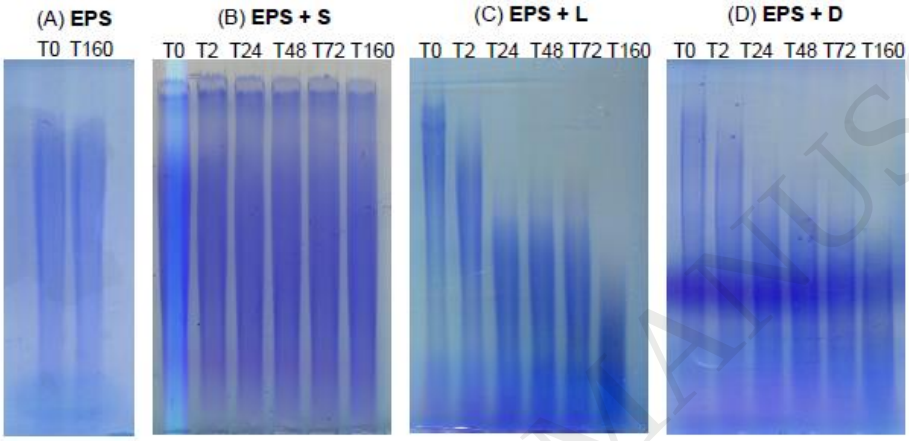

Fig. 2. Electrophoretic analysis on agarose gel of the native HMW GY785 EPS after incubation with (A) Tris HCl buffer (EPS), (B) culture supernatant (EPS+S), (C) soluble cell lysate (EPS+L) and (D) insoluble cell debris (EPS+D).

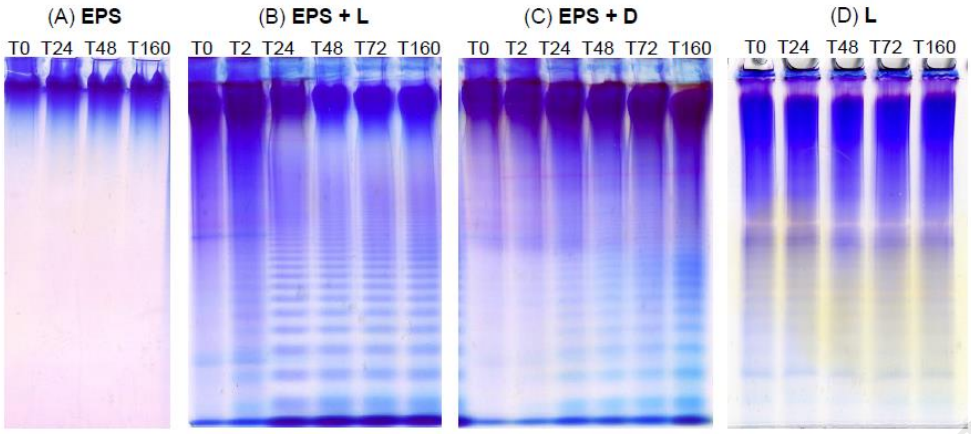

Fig. 3. Electrophoretic analysis on PAGE gel of the native HMW GY785 EPS after incubation with (A) Tris HCl buffer (EPS), (B) soluble cell lysate (EPS+L) and (C) insoluble cell debris (EPS+D). (D) Electrophoretic analysis on PAGE gel of lysate (L) incubated in Tris HCl buffer (control).

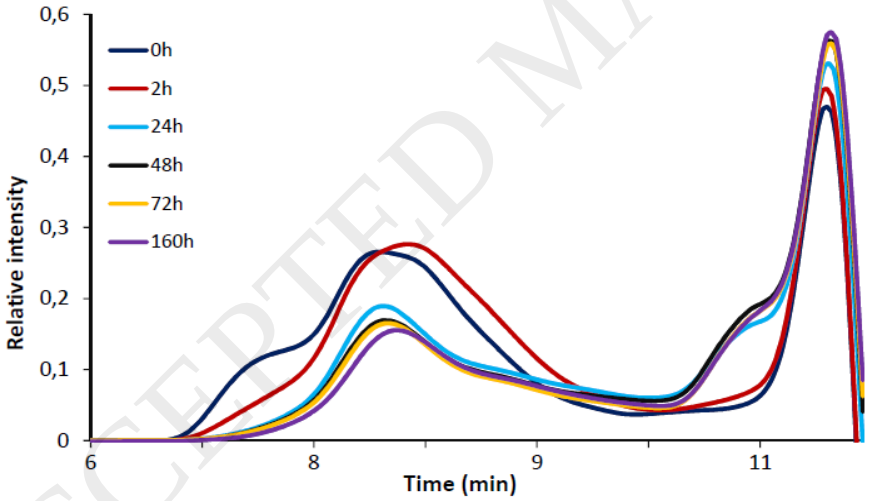

Fig. 4. HPSEC-MALS profiles of the GY785 EPS incubated with lysate (L) for 0, 2, 24, 48, 72 and 160h.

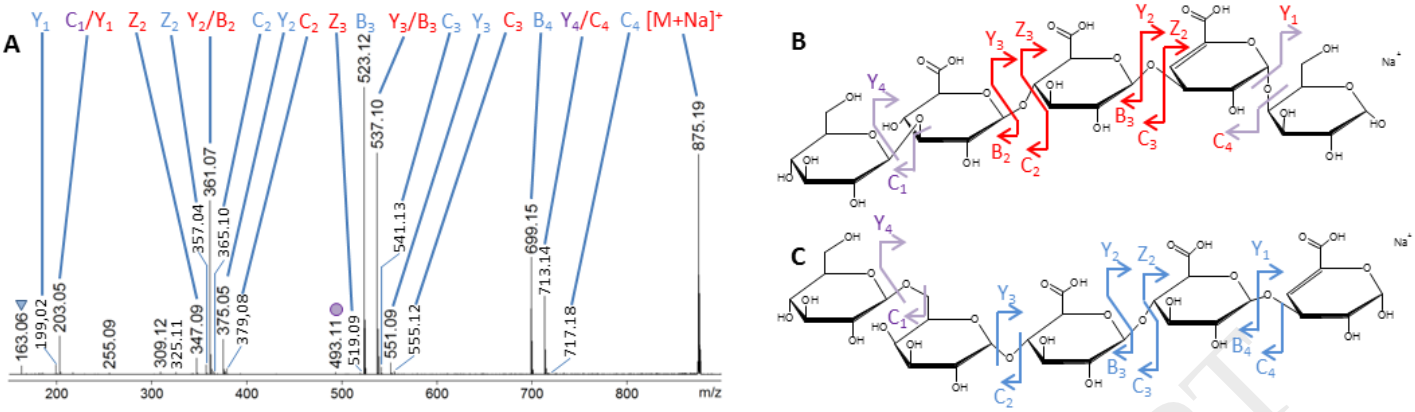

Fig. 5. (A) ESI-CID-MS/MS spectrum obtained in positive ionization mode of an uronic acid contained oligosaccharide at *m/z* 875.19 as $[M+Na]^+$ specie. (B and C) Structures of the two isomers assigned.

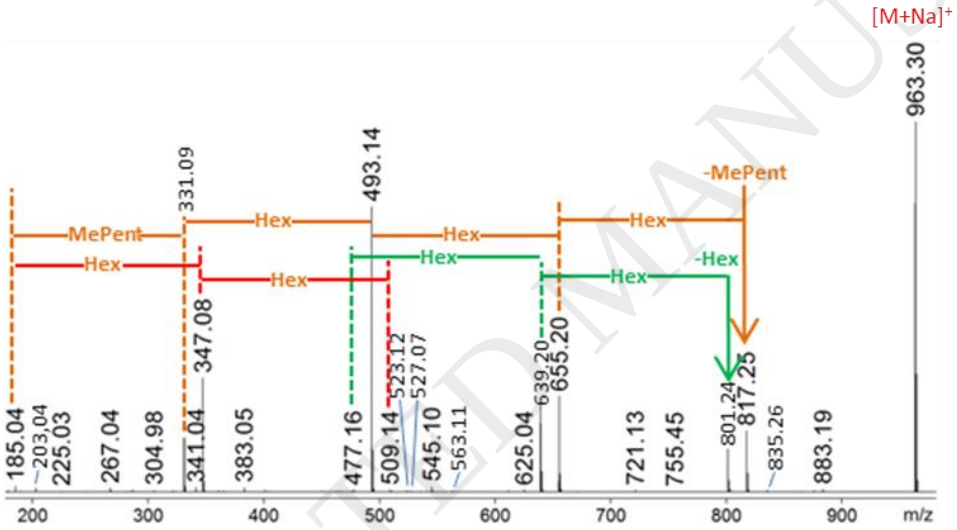

Fig. 6. ESI-CID-MS/MS spectrum obtained in positive ionization mode of a methyl-pentose contained oligosaccharide isolated at *m/z* 963.30 as $[M+Na]^+$ specie.

Table 1. Osidic composition of the native GY785 EPS, fractions recovered after the incubation of the native polysaccharide with soluble cell lysate (L) for 72h at 37°C and cell lysate (L).

Osidic composition (molar ratio)	**Rha**	**Fuc**	**Man**	**Gal**	**Glc**	**GalA**	**GlcA**
GY785 EPS	0.2	0.1	0.4	3.6	4.7	1.0	2.0
F1	1.5	0.7	0.4	1.5	2.0	1.0	1.4
F2	2.5	1.6	1.5	1.3	1.5	1.0	1.6
F3	1.3	0.5	0.7	1.9	1.8	1.0	1.2
F4	1.3	0.6	0.5	1.9	1.6	1.0	1.6
F5	1.7	0.4	0.6	2.1	1.9	1.0	1.0
F6	0.9	0.4	0.5	1.9	1.4	1.0	1.1
Lysate (L)	0.1	0	0.1	0.9	1.0	0	0

Rha: rhamnose, Fuc: fucose, Man: mannose, Gal: galactose, Glc: glucose, GalA: galacturonic acid, GlcA: glucuronic acid.

Table 2. Specie compositions attributed based on their mass measurements and structures validated in tandem MS (underlined in bold).

m/z	**Composition**	**m/z**	**Composition**
493.16	2.Hex; 1MePent; 1 //	**861.23**	3.Hex; 2.UA ; 1 //
509.16	3.Hex; 1 //	**875.21**	2.Hex; 3.UA ; 1 //
523.14	2.Hex; 1.UA; 1 //	963.33	4.Hex; 2MePent; 1 //
537.11	1.Hex; 2.UA ; 1 //	**1037.26**	3.Hex; 3.UA ; 1 //
655.22	3.Hex; 1MePent; 1 //	1125.39	5.Hex; 2MePent; 1 //
699.17	2.Hex; 2.UA ; 1 //	**1199.31**	4.Hex; 3.UA ; 1 //
713.15	1.Hex; 3.UA ; 1 //	1287.44	6.Hex; 2MePent; 1 //
801.29	3.Hex; 2MePent; 1 //	**1361.38**	**5.Hex ; 3.UA ; 1 //**
817.28	4.Hex; 1MePent; 1 //	1433.50	6.Hex; 3MePent; 1 //

Hex: Hexose; MePent: Methyl Pentose; UA: Uronic Acid; // unsaturation