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Production of sulfated oligosaccharides from the seaweed *Ulva* sp. using a new ulvan-degrading enzymatic bacterial crude extract

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ABSTRACT

Green macroalgae of the genus *Ulva* have complex and hardly degradable polysaccharidic extracellular matrix. One of its main components, the ulvan, is a 3-sulfated rhamnoglucuronan that has a wide range of properties and is a source of rare sugars. The production of mono- and oligo-saccharides from this polysaccharide could motivate its use to an industrial scale. Enzymatic tools to realize this process are still scarce. Here we describe the activity of an enzyme crude extract proceeding from a new *Alteromonas* species isolated from the gut of *Gammarus insensibilis*, an amphipod from southern Spanish salt marshes. HPTLC separation and NMR spectrometry allowed describing the formation of low molecular weight oligosaccharides formed principally of unsaturated 3-sulfated rhamnose and uronic acids. The control of hydrolysis kinetics allowed preparing different molecular weight fractions down to a 5 kDa oligosaccharides mix. These results highlight the presence of a potentially new β-lyase produced by these hitherto undescribed bacteria. This work identifies a new source of enzymes with potential application for the production of high added value compounds from *Ulva* sp.

1. Introduction

Marine green macroalgae are fast growing species with an important capacity to fix CO_2 and remove excess nutrients from the water. Although they are excellent biofilters and can be cultivated for that purpose the biomass generated is still largely underexploited mainly because of its low value [1]. Due to their unique composition, algal polysaccharides have been shown to exhibit a wide range of bioactivities. Green algal cell walls, especially of species of the genus *Ulva* are constituted by a long chain polymer called "ulvan" [2]. This heteropolysaccharide is interesting because it is mainly composed of rare sugars and is highly sulfated as are many polymers of animal origin [3]. It may contain between 16%–23.2% of sulfate, up to 45% of rhamnose, 2.1–12% xylose, 0.5–6% glucose and 6.5–19% of uronic acids. The main structural subunit ulvanobiuronic acid type A ($A3_S$) is composed of L-rhamnose sulfated on the third carbon and linked on its non-reducing end with a β-D-glucuronic acid with an α 1-3 linkage, the B type ulvanobiuronic acid contains iduronic acid instead of glucuronic acid ($B3_S$) [4–6]. Its molecular weight depends on the algal species and the extraction protocol; however it is generally high and can round up to 700–800 kDa [7]. This glycosaminoglycan-like structure has anticoagulant [8], antioxidant [9–11], immunomodulator [12,13], antihypercholesterolemic [14], antihyperlipidemic [15,16], antiviral [17], antitumoral [18,19] and plant defense elicitor activities [20–23]. It has been used in forming biomaterials such as nanofibers, nanofibrous membrane [24,25], microparticles [26], molecular sponges for cell culture [27], antiadhesive activity [28] or as ion exchanger hydrogel [29]. L-rhamnose as well as L-α-rhamnosyl oligosaccharides and glycoconjugates have been described to strongly interact with specific lectin domains in several proteins and organisms [30–38].

The importance of sugars in many biological processes has given rise to the study of new carbohydrate-based drugs able to mimic endogenous sugars and glycoconjugate behaviors and act as receptor antagonists. Sulfated oligosaccharides are implicated in biological events such as protein localization at cell surfaces [39]. These interesting features make ulvan an excellent candidate as a renewable and cheap compound for industrial purposes. Unfortunately its structure is difficult to define [7,40,41]. An enzymatic or chemical reduction of its molecular weight could lead to a more definable structure and improve the range of its activities, the efficiency and reduce the polydispersity of its molecular weight. The physical properties of carbohydrates mainly depend upon the sugar composition of the chain, the glucosidic linkages, the side groups and the molecular size. Their conformation in solution determines their affinity for receptors and their biological properties. Given that a change in molecular weight can have a great influence on the geometrical conformation of some carbohydrates [42], the bioactivity of carbohydrate can be enhanced by depolymerization and making more accessible the active sequences to ligand receptors [43,44]. In addition, *Ulva* and other seaweeds are viewed as promising candidates for the production of biofuels [45], however, as industrial microbes cannot currently realize the metabolization of the complex

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polysaccharide components, an efficient depolymerization step will increase their potential for biofuel production [46,47].

Several methods to reduce the molecular weight of polysaccharides have been described. Mild acid hydrolysis reduces the molecular weight but sulfate groups are lost during the process. Furthermore, the alpha 1-4 linkage of the aldobiuronic acid moiety is resistant to this reaction [4]. Enzymatic cleavage of polysaccharides generally does not have these disadvantages. Intestinal microbiota associated with animals feeding on *Ulva* sp. can be a source of specific enzymes. Recently a new bacteria species *Persicivirga ulvanivorans* [48] was isolated from *Aplysia* feces and was found capable of degrading ulvan in to disaccharidic subunits with an extracellular ulvan-lyase [49]. Such an enzyme has already been described in *Ochrobactrum tritici* [50]. The genus *Persicivirga* has recently been merged in the genus *Nonlabens* [51] and the newly named *Nonlabens ulvanivorans* has been described to produce a β-glucuronyl hydrolase implicated in the degradation of ulvan [52]. Other authors have described the degradation of ulvan by enzymatic means but with alginate-degrading commercial enzymes or without further characterization of the bacteria used to obtain the enzyme [7,19,53]. The main challenge of enzymatic degradation is to maintain the basic structure of repetitive subunits with the sulfate functional groups while controlling the kinetics of degradation to obtain oligomers with a constant yield and molecular size.

The objective of this work was to obtain oligosaccharides from the large polysaccharides produced by the green alga *Ulva ohnoi* Hiraoka et Shimada by enzymatic means in order to assay their biological activity in further studies. In this paper we characterize the degradation products and the kinetics of an enzymatic degradation of ulvan. The crude enzyme mix has been extracted from a culture of a newly identified species of bacteria belonging to the genus *Alteromonas* isolated from the gut of the amphipod *Gammarus insensibilis*. Among several animal species proceeding from different taxonomic groups these amphipods were identified in a preliminary experiment as efficient grazers of *Ulva* spp. The degradation level has been assayed by static light scattering (SLS), thin layer chromatography (TLC) and subsequently characterized by ^{1}H-NMR.

2. Materials and methods

2.1. U. ohnoi cultivation

U. ohnoi Hiraoka and Shimada strain UOHN120810 was isolated from the outlet channel "Caño de Agua del Pino" close to the mouth of the Rio Piedras tidal inlet in SW Spain (37°12′57.39″ N, 7°5′5.29″ E) on 12 August 2010 and has been maintained in culture since then in the aquaculture centers of the IFAPA. Identification was confirmed by the analyses of the rbcL and ITS2 sequences (GenBank submission pending, Malta et al. in prep.). To obtain the biomass needed for ulvan analyses, stock cultures were upscaled to 15 L cylinders under constant light (150 μmol photons·m^{-2}·s^{-1}) and temperature (20 °C) in filtered (0.2 μm) natural seawater enriched with f/2 medium. After two weeks of cultivation sufficient biomass was obtained. Algae were harvested, rinsed with tap water, freeze-dried and kept in a dry place until the extraction.

2.2. Ulvan extraction and purification

Approximately 10 g of freeze dried algae was milled to a fine powder (around 8 μm particle diameter) using a coffee mill (Jata, Spain). Ulvan was extracted as described by Robic [54] with some further purification steps. Briefly, 6.7 g·l^{-1} of alga dry weight was in hot water (80 °C) with 0.05 M sodium oxalate (Sigma Aldrich, USA) during 2 h. The extract was centrifuged for 20 min at 14,000 rpm (Beckman, USA) filtered at 0.45 μm and a secondary extraction was made on the residue. Both supernatants were merged together and concentrated by ultrafiltration using a 10 kDa membrane mounted on a Centramate Ultrafiltration Unit (PALL, USA). Subsequently, pH was lowered to 2 by adding phosphoric acid and centrifuged in order to precipitate possible glucuronan. The extract was neutralized by adding NaOH, autoclaved for 21 min at 121 °C and centrifuged for 30 min at 30,000 *g* to remove proteins and contaminating molecules. The supernatant was purified by diafiltration with a 10 kDa cut-off membrane until the filtrate conductivity reached that of distilled water. Finally, the supernatant was freeze-dried and the resultant purified ulvan extract was kept in a dry and dark place.

2.3. Bacterial isolation

Approximately ten individuals of the marine amphipod *G. insensibilis* were collected from an earthen pond in the salt marsh ecosystem of the Rio San Pedro tidal inlet next to the centre IFAPA El Toruño (36°34′44.6″N, 6°12′25.0″W). The amphipods were maintained on a diet of freshly collected *Ulva* sp. from the same locality during 3 days in natural seawater. Animals were then externally sterilized by submerging them during 5 s in a 10% H_2O_2 solution. The gut was immediately extracted in sterile conditions and kept in 3 ml of sterilized seawater at a salinity of 35 ppm. A pool of five *G. insensibilis* intestines was homogenized and 5 μl was spread on a sterile ulvan gel prepared with autoclaved 10 mg·ml^{-1} ulvan in 35 ppm seawater (not containing agar). After one night of incubation the gel had been entirely liquefied and the supernatant was frozen for 24 h with 15% glycerol at −80 °C to eliminate helminths. The resulting bacterial culture was sterilely plated on a marine agar petri plate enriched with 4% ulvan. After 24 h several colonies had formed and among them some had formed small craters. The well separated colonies were individually picked up from the plate and newly spread on gelificated ulvan. No growth could be observed in liquid bacterial culture. The bacteria that degraded the gel were considered as ulvanolytic. Only one strain showed such activity and was conserved in glycerol 15% at −80 °C for further uses.

2.4. Crude enzyme extract isolation

50 ml ulvan gel (10 mg·ml^{-1} in 35 ppm sterilized natural seawater) was incubated with 100 μl of the bacteria strain in the dark at 25 °C. When the gel was completely liquefied the culture batch was centrifuged for 10 min at 10,000 *g* at 4 °C after which the supernatant was filtered twice with over 0.22 μm pore cellulose filters (Millipore, MA, USA) to remove bacteria. Proteins were then precipitated by salting out with to 60% and 80% ammonium sulfate (Panreac, Spain). The pellet was recovered in a 100 mM Tris–HCl buffer at pH = 8 and 200 mM NaCl and dialyzed with a 14 kDa membrane (Sigma-Aldrich, USA) during 24 h against the same buffer in order to remove all the salts and small molecules. Protein was concentrated 4-fold by centrifugal frontal ultrafiltration with a 5 kDa Amicon Ultra-4 tubes (Millipore, USA). Proteins in these crude enzymes mix were then quantified following the Bradford method [55] with Bovine Serum Albumin (Sigma-Aldrich, USA) as standard.

2.5. Bacterial identification

For the molecular identification of the bacterial isolate, DNA extraction was performed from 40 mg of frozen pellet using the FastDNA kit for 40 s at speed setting 5 in the Fastprep FG120 instrument (Bio101, Inc., Vista, CA). Primers used for PCR amplification of 16S rDNA were 63f and 1387r [56]. PCR reactions were carried out in 25 μl of reaction volume: 1 μl DNA template (20 ng) was added to 24 μl PCR mix consisting of 17.25 μl of sterile distilled water, 2.5 μl dNTP mix 10 mM, 2.5 μl of 10× buffer, 1 μl $MgCl_2$ 50 mM, 0.25 μl (1.25 U) BioTaq™ DNA polymerase (Bioline, London, UK), and 0.5 μl of each primer (10 mM). The thermal cycle profile was an initial denaturation step of 96 °C for 2 min was followed by 30 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min. PCR products were examined by electrophoresis on ethidium bromide-stained 2.0% agarose gel and visualized by UV