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First isolation of polysaccharidic ulvans from the cell walls of freshwater algae

R. Pankiewicz [a,*], B. Łęska [a], B. Messyasz [b], J. Fabrowska [a], M. Sołoducha [a], M. Pikosz [b]

[a] Adam Mickiewicz University in Poznan, Faculty of Chemistry, Umultowska 89b, 61-614 Poznań, Poland
[b] Adam Mickiewicz University in Poznan, Faculty of Biology, Department of Hydrobiology, Umultowska 89, 61-614 Poznań, Poland

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ABSTRACT

For the first time polysaccharidic ulvans have been isolated from freshwater green macroalgae *Ulva flexuosa* and *Cladophora glomerata*. One of the main components of ulvan is 3-sulfated rhamnoglucuronan showing a wide range of interesting properties and being a source of rare sugars. The production of mono- and oligosaccharides from this polysaccharide can potentially be applied in cosmetic, pharmaceutical and food industry. In this paper a new and effective method for the isolation of ulvans from freshwater algae is described. The substance has been characterized after enzymatic hydrolysis by Fourier Transform Infrared (FT-IR) spectroscopy and elemental analysis. FT-IR spectroscopy permits identification of the band characteristic of the sulfonic acid groups, specific for this kind of polysaccharides. With increasing size of the thalli, the amount of ulvans in the algae cells increases. Hence, freshwater green macroalgae *U. flexuosa* and *C. glomerata* were found as a new source of polysaccharidic ulvans that can be considered to be potentially used in medicine, pharmacy, food and cosmetic industry.

1. Introduction

Recently, in phycological, biochemical and medical literature a lot of information about bioactive compounds in marine algae has appeared [1,2]. Many authors have been interested in the marine *Ulva* genus and in particular in ulvans — a group of complex sulfated polysaccharides (SPs) composed of sulfate ester, uronic acids, xylose, rhamnose and glucose [2]. These compounds have been widely investigated, however, their chemical structure, occurrence and properties are not fully understood. Ulvans are water and alkali soluble [3], semi-crystalline compounds with hydroscopic properties [4]. These natural polymers are specific of green algae and occur in *Ulva* genus. So far, ulvans have been found only in the following marine species of *Ulva*: *Ulva lactuca* [5,6], *Ulva rigida* [7,8], *Ulva armoricana*, *Ulva rotundata*, *Ulva scandinavica*, *Ulva olivascens*, *Ulva gigantea* [9], *Ulva clathrata* [10], *Ulva conglobate* [11], *Ulva fasciata* [12], *Ulva pertusa* [13] and *Ulva flexuosa* [14].There are no reports in literature about the occurrence of ulvans in freshwater *Ulva* species. Polysaccharides are very interesting bioactive compounds, because of their physical, chemical properties and their potential for therapeutic application (i.e., antiviral activity, immuno-inflammatory activity). Sulfated polysaccharides (SPs) isolated from algae include agars and alginates which are produced in red algae (i.e., *Chondrus ocellatus*), fucoidanes isolated from brown algae (i.e., *Laminaria japonica*) and ulvans from green algae (i.e., *Ulva linza*) [15]. Polysaccharides may occur in cell walls or as food reserves, and more specifically ulvans are widespread in the intercellular space and fibrillar walls of the two-cell layer-thick thallus [16]. Kylin [17], in 1913, used for the first time the term "ulvan" to define different fractions of SPs in the cell wall of *U. lactuca*. Nowadays SPs have been only prepared from marine algae belonging to the genera *Ulva* (38%), *Enteromorpha* (14%), *Monostroma* (14%) and the others (34%) [18]. Ulvan extracted from the cell wall of *Ulva*, *Enteromorpha* represents about 8–29% of the algae dry weight [1].

Pioneering investigation of ulvans was conducted on *U. lactuca* by Brading et al. in 1954, who proposed the structure of the sulfated polysaccharides and reported that they could be linked to glucose or rhamnose [5]. Afterwards, it was proven that also uronic acids, arabinose, xylose and glucuronic acids could be the components of the polysaccharides occurring in *U. lactuca* [6]. The name "ulvan" was proposed for the first time in 1993 by Lahaye and Axelos [19]. In later studies of *U. rigida* it was assumed that ulvans are mainly composed of glucuronic acid and sulfated rhamnose [7,19]. In further studies, the presence of iduronic acid as a constituent of ulvans has been shown [7]. Ulvans also have been found in other species of marine *Ulva* genus, such as: *U. armoricana*, *U. rotundata*, *U. scandinavica*, *U. olivascens* and *U. gigantea* by Lahaye et al. [9]. Polysaccharides in these algae were mainly composed of rhamnose, glucuronic acid, sulfate and traces of galactose. The major repeating units in the

* Corresponding author.
E-mail address: radek@px.pl (R. Pankiewicz).

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polysaccharide chain are ulvanobiuronic acid 3-sulfate A and B together with 1,4-linked-D-glucuronan as blocks within ulvan or as a separate glucuronan [9]. An important aspect of the ulvan study is the ultrastructure of these compounds. Haug has discovered that ulvans are able to create gels in the presence of calcium ions and boric acid [20]. It has been found that the SPs can also bind other metal ions, such as Al, Pb, Zn, Zn, Mn and Cu [1]. Robic et al. have proposed a three-dimensional spherical structure of ulvans, which may be formed in the presence of bivalent cations and boric acid [21,22]. Ulvan nanospheres are able to aggregate creating 'raspberry-like' structures connected by fiber-like constituents, which can be made of proteins, glucuronan and/or extended ulvan segments [22].

Cladophora glomerata (*Chlorophyta*, *Ulvophyceae*) is common in all types of freshwater ecosystems and can produce with time an important amount of biomass forming dense, seasonal blooms in lakes in nutrient-enriched waters [23]. *C. glomerata*, like other *Chlorophyta* species, has a two-layered polysaccharide cell wall, the inner one is made of cellulose and the outer one is chitinous/pectin, though cellulose is the main structural polysaccharide in the *Cladophora* [24]. For *C. glomerata* from lakes, in one cell the surface sorption area is 17.85 mm^3 per cell and for a filament of 1 cm in length this area amounts to 119 cm^3 [25].

Nowadays, biological properties and applications of ulvans are a very important topic. Ulvans exhibit a wide range of activities as bioactive compounds. What is more, these compounds are cytocompatible and are considered as non-toxic [26]. Ulvans have been found to show antioxidant properties [27–32], thus they are able to act as free-radical inhibitors or scavengers. The scavenging activity of ulvan towards superoxide and hydroxyl radicals, as well as metal chelating activity has been shown in vitro [27,30,33]. Moreover, the latest investigation of Qui and Sun on rats [32] has demonstrated the antioxidant activity in vivo of high sulfate content derivative of polysaccharides from *U. pertusa*. Another important property of ulvan is anticancer activity. There are different mechanisms of anticancer agent activity, including antioxidant, antiproliferative and antitumor [34]. The antiproliferative activity of ulvan polysaccharides has been investigated by Ahmed et al. [35]. Results from his group indicated antiproliferative and antitumor cytotoxic effects against EAC-cells, hepatoma (HepG2) and colon carcinoma (HCT116) human cell line [35]. Other authors have demonstrated the antiproliferative activities of ulvan towards human cancer cells, particularly to the breast adenocarcinoma cells [36]. SPs isolated from *Ulva* species exhibit immune stimulating properties [37–39]. This property of ulvan can be applied in therapy for diseases in which the immune system is impaired. Furthermore, anticoagulant activity of ulvan has been detected [11,36,40,41]. Ulvans have acted as heparin-like anticoagulant agents, and thus, could be potentially used in a therapy of cardiovascular and cerebrovascular diseases, for example, as a cure for hemophilia [18,34,42]. Antihyperlipidemic activity is another example of beneficial properties of ulvan in medical treatment. It has been shown that SPs from *U. pertusa* are able to regulate lipid metabolism by reduction of plasma low-density lipoprotein (LDL) cholesterol [43,44]. Also derivatives of ulvans from *U. pertusa*, such as acetylated ulvans and high sulfate content ulvans, show antihyperlipidemic activity [45]. Ulvan also has been found to show antiviral activity [36,46] towards herpes simplex virus type 1 (HSV-1) [47], and antiadhesive properties towards bacteria *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* [48]. All ulvan properties described above are mainly important for medical applications, in particular for the production of certain elements of medical devices. The gelling ability of ulvans [20–22] and specifically their ability to uptake water [49], make these compounds a potential biomaterial for biomedical applications, such as wound dressing and drug release systems [49–53]. What is more, the antioxidant activity of SPs may be applied in the cosmetic industry. Due to the ulvan's ability to enhance skin tissue regeneration [53] they may also be considered as bioactive compounds in the production of novel anti-aging and regenerating types of cosmetics. As natural anionic polymers, ulvans may be used as surfactants in cosmetics and detergent production [54]. Ulvans can also constitute a raw material for the production of diet supplements or a functional food due to their antioxidant, immunostimulating, as well as antihyperlipidemic properties. To sum up, ulvans isolated from different *Ulva* species show lots of interesting properties as novel bioactive compounds for several applications in medicine, pharmacy, as well as in food and cosmetic industry.

So far, ulvans have been isolated from marine species of *Ulva* [5–14]. To the best of our knowledge the isolation of these sulphated polysaccharides from freshwater macroalgae species as their new source has not been reported so far. Thus, the first aim of the study was to determine whether the freshwater green macroalgae (*U. flexuosa*, *C. glomerata*) contain ulvans — polysaccharides, which have been isolated so far only from marine algae. After their successful extraction an attempt was made to modify the process of isolation to obtain the highest performance. The general aim of the study was to carry out the ulvan isolation process from macroalgae of the class of freshwater green algae *U. flexuosa* and *C. glomerata*. The biomass of algae used in the study came from Wielkopolska (Poland, Europe) reservoirs.

2. Materials and methods

2.1. Freshwater algae and environmental conditions

The freshwater macrophytic algae (*Ulvophyceae*, *Chlorophyta*) came from the west part of Poland (Central Europe; Fig. 1). *C. glomerata* (L.) Kütz. was collected from Lake Oporzynskie (N52°55′, E17°9′) and *U. flexuosa* subsp. *pilifera* (Kütz.) M.J. Wynne from the Nielba river (N52°48′, E17°12′). Lake Oporzynskie is a shallow, eutrophic reservoir (average depth = 1.2 m) that is in the overgrowing phase, mostly by *Ceratophyllum demersum* L. Every year, both in the littoral and pelagic zone, the thalli of *C. glomerata* (L.) Kütz. develop massively, tightly covering the whole surface of the water [25]. This systematic appearance of macroalgae in the form of mats in Lake Oporzynskie drew our attention to the possibility of using the readily available biomass as raw material in various sectors of economy and industry.

2.2. Microscopic analyses

Microscopic images were taken using a camera (ProgRes® SpeedXT core 3, Jenoptik) connected to a light microscope (Axioskop 2 MOT Zeiss). Measurements of the length and width of cells, cell wall, pyrenoids and nuclei were made at ×20, ×40 and ×100 magnification. Pyrenoids in the chloroplasts were stained with Lugol's solutions and the nuclei by 1% acetocarmine.

2.3. Macroalgal biomass — harvesting area of U. flexuosa and C. glomerata

The biomass of algae *U. flexuosa* and *C. glomerata* used in the study was originally obtained from Wielkopolska Region (North Poland, Europe) reservoirs (Fig. 1). The biomass of algae *U. flexuosa* was harvested in the summer of 2014. Mats of filamentous green alga *C. glomerata* (L.) Kütz of macroscopic size formed a large surface mat, which freely floated on the water surface (macroalgae coating); mat formation was regularly repeated every year. Freshwater green algae (*C. glomerata*) were collected week by week, starting from the first week of May 2015 to the middle of June 2015, when dense mats of *Cladophora* tightly covered the water column of the shallow parts of Lake Oporzynskie. Samples of green algae were collected manually from the middle of the mats floating on the surface of the lake water. As the mats were also observed in the pelagic zone, the biomass was harvested using boats applying a strip, a cable, or a special rake. After the collection, fresh algal biomass (FM) was weighed immediately per 1 m^2 of the water surface. Algae material was washed and dried at 40 °C, for 40 min after harvesting. Then, the material was dried in a special drying oven until a dry matter (DM) of water content of <15% was obtained.

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