Red microalgal cell-wall polysaccharides: biotechnological aspects
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The area of sugars and glycosylation is not as well developed as other fields in cell biology owing to biotechnological constraints. However, the biotechnological potential of sugars, including polysaccharides, is the driving force pushing research efforts to meet the challenge. Algae produce cell-wall sulfated polysaccharides, with those of the red unicells, which dissolve into the medium, having unique characteristics—structure, composition, fluid dynamics, and extreme stability. These characteristics, combined with polysaccharide bioactivities, offer a vast range of potential applications. Research has thus been directed toward an in-depth understanding of the molecular structure, biosynthesis, and characteristics of the red microalgal sulfated polysaccharides and to the development of molecular-genetic tools, aiming at large-scale production for applications that can benefit humanity.

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Introduction
The biotechnological potential of natural polysaccharides is currently gaining increased recognition as a result of two parallel, seemingly unrelated, processes—the tendency of global markets to switch from synthetic to natural products and a growing understanding of the functions of sulfated sugars and of the importance of glycosylation in the post-genomic era. In this context, the polysaccharides of seaweeds have been under investigation for many years, but those of the microalgae remain almost unstudied and elucidating the role of these complex sugars in cell metabolism poses an exciting challenge. This review is thus dedicated to the information that is available on red microalgal cell-wall polysaccharides and their potential biotechnological applications.

Polysaccharides play significant roles in a variety of functions in the cells of different organisms. In algae, polysaccharides serve mainly as storage and structural molecules. In seaweeds, the structural cell-wall polysaccharides usually consist of an outer amorphous mucilage matrix, commonly made up of linear sulfated galactan polymers (carrageenans, agarans, and alginates) and an inner rigid component, cellulose fibrils. In the red microalgae, the cell walls lack this cellulose microfibrillar component; rather they are encapsulated within a sulfated polysaccharide in the form of a gel [1]. During growth in a liquid medium, the external part of the polysaccharide undergoes dissolution from the cell surface into the medium (soluble fraction) [2,3], whereas most of the polysaccharide (~50–70%) remains attached to the cell (bound fraction). A variety of functions have been suggested for the red algal cell wall: mechanical support [1], biorecognition [4], and ion-exchange capacity [5]; In the red microalgae, the polysaccharide supplies the cells with environmental protection: the gel structure protects against desiccation; its stability to temperature, pH, and salinity protects against environmental extremes, and its antioxidant activity is probably used as a free radical scavenger [7,8].

The Arad laboratory has taken a multidisciplinary approach to developing the biotechnology of red microalgae. This effort requires the coordination of various disciplines: chemistry (composition and structure), physiology (effect of environmental conditions), biochemistry (biosynthesis and sulfation pathways), biotechnology (applications and bioactivities) [9,10**,11**], and bioengineering a commercial/large-scale cultivation system [9,10**,12]. Since modern biotechnology demands molecular-genetic studies, we have made significant progress in the field of red microalgal genomics by establishing expressed sequence tag (EST) databases of two species of red microalgae, Porphyridium sp. and Dixoniella grisea [13], and in the development of molecular-biology tools [11**,13,14**].

Chemical characterization
The chemical characteristics of red algal cell walls have been investigated mainly in seaweeds, with knowledge about the chemical structure and characteristics of red microalgae being more limited. This lag is mainly due to the complexity of the red microalgal polysaccharides and
to the lack of specific enzymes that degrade them [6,9,15]. The studies that have been performed on the red unicells have been devoted mainly to four species from different habitats: Porphyridium sp. (seawater), P. cruentum (seawater), P. aerugineum (fresh water), and Dixoniella grisea (brackish water). The soluble cell-wall polysaccharides of the different species have a common structural feature—galactan heteropolymers (molar mass $2\times10^6$ g mol$^{-1}$) that contain sulfate residues [3,16–19]. The polysaccharides are anionic owing to the presence of GlcA and half-ester sulfate groups. In all the species studied, the main sugars of the polymers are xylose, glucose, and galactose, but in different ratios. Additional minor sugars—methylated sugars, mannose, arabinose, and ribose—have also been detected. The polymers have different sulfate contents (1–9%, w/w), with the sulfate groups being attached to glucose and galactose in the 6 or 3 position [20]. The soluble polysaccharides of the different species have a common basic building block composed of aldobiouronic acid 3-O-(α-D-glucopyranosyluronic acid)-L-galactopyranose disaccharide [21,22]. Recently, the Arad group has shown for Porphyridium sp. that this building block is part of a larger linear building block that contains (1→2 or 1→4)-linked xylopyranosyl, (1→3)-linked galactopyranosyl, and (1→3)-linked glucopyranosyl or glucopyranosyluronic acid residues [23]. One of the proposed structures of this larger building block is given in Figure 1.

An anionic polymer separated from the bound polysaccharide of Porphyridium sp. [24] was found to contain three major neutral monosaccharides, Xyl, Glc and Gal, and GlcA. Uronic degradation of this polymer yielded two oligosaccharides, which differ from the aldobiouronic acid unit described above.

Very intense research in the Arad laboratory revealed that there are several proteins bound non-covalently to the cell-wall polysaccharide of Porphyridium sp., the most prominent being a 66 kDa glycoprotein consisting of a polypeptide of approximately 58 kDa and a glycan moiety of approximately 8 kDa [25,26]. Sequencing of a cDNA clone encoding the 66-kDa glycoprotein revealed that this is a novel protein, with four potential N-glycan sites, which does not show similarity to any protein in public-domain databases or in our in-house EST database of D. grisea [25,26]. These findings differ from previous reports of a glycoprotein covalently bound to the cell-wall polysaccharide of P. cruentum [27,28]. The glycan structure of moieties attached to proteins in the cell-wall polysaccharides of various red microalgae are currently under investigation, indicating novel N-glycan structures that have not been shown in any other organisms (Oshrat Ontman, personal communication).

**Physicochemical characterization**

One of the main characteristics of the red microalgal polysaccharides that makes them suitable for industrial applications is their fluid-dynamic behavior [9,10,13,17,29–37]—highly viscous aqueous solutions at relatively low polymer concentrations, yielding rheological properties comparable with industrial polysaccharides, for example, xanthan [17,29–31]. Aqueous solutions of Porphyridium sp. polysaccharide were found to be stable (as reflected by their viscosity) when exposed to a wide range of pH values (2–9), temperatures (30–120°C), and salinities [6,32].

An intrinsic viscometry study of dilute solutions of the soluble fraction of Porphyridium sp. polysaccharide showed that the stiffness of the polymer chains is in the same range as that of xanthan gum and DNA [34]. It was suggested that the biopolymer chain molecules adopt an ordered conformation in solution and that the polysaccharide has the form of a double or triple helix [34]. X-ray diffraction studies revealed that the
polysaccharide is composed of an oriented, single, two-fold, helical structure of pitch 1.6 nm, with extended regions having a regular chemical repeat [35]. The polysaccharide solutions showed marked shear thinning behavior with no evidence of a Newtonian plateau. At higher concentrations (1%) heating caused visible gelation accompanied by syneresis, which was reversible upon cooling. It was also shown that transition upon heating of the solutions is reversible and sharp from a weak to a strong elastic gel network. This rare phenomenon appears to be mediated by hydrophobic interaction in addition to a strong elastic gel network. The biotechnological potential of the polysaccharide is further enhanced by findings that it was superior to hyaluronic acid as a lubricant in terms of friction reduction, adsorption, and stability [32–37].

**Cell-wall polysaccharide formation**

In contrast to the cell-wall polysaccharides of seaweeds, which are characterized by an organized structure, composed of repeating disaccharide blocks, the polysaccharide structure of the red microalgae is more complex. Various approaches have been taken to elucidate cell-wall formation in red microalgae; these include the use of synchronized cultures, cell-wall-modified mutants, inhibitors of polysaccharide formation, carbon partitioning and elucidation of sulfation pathways, and glyco-protein formation, as described below.

**Polysaccharide formation in synchronized cultures**

It was previously suggested that the polysaccharide is secreted to the cell surface by membrane-mediated exocytosis [38]. By following cell-wall formation during the cell cycle of *Porphyridium* sp. and *D. grisea* in synchronized cultures, an intermediate polysaccharide complex of 0.5 × 10^6 Da containing xylose was detected at the beginning of the cell cycle (hour 2) [39,40]. It appears that this sulfated poly-xylose molecule polymerizes further, incorporating other sugars to produce higher molecular weight intermediates (molecular masses in addition to 0.5–2 × 10^6 Da at hours 4–6), which finally polymerize extracellularly at hour 8 to produce the cell-wall polysaccharide [39,41].

**Cell-wall modified mutants**

The herbicide 2,6-dichlorobenzonitrile (DCB), which is known to inhibit cellulose biosynthesis, was found to inhibit growth and polysaccharide production in *Porphyridium* sp. [40,42*] and *D. grisea* [43]. Spontaneous DCB-resistant *D. grisea* mutants with modified cell-wall compositions and modified characteristics, but with sulfate and protein contents similar to those of the wild type, were isolated [43]. In *Porphyridium* sp., it was shown that DCB inhibits cell-wall regeneration in protoplasts, and a number of *Porphyridium* sp. mutants have been isolated, all with modified cell-wall compositions that differ from one another [40,42*].

**Formation of the sulfated polysaccharide in the Golgi apparatus**

Using the inhibitor brefeldin A (BFA) it was shown that polysaccharide biosynthesis takes place through the Golgi system [44].

**Carbon partitioning**

By adding 14C-floridoside (a disaccharide composed of Gal and glucose) to a culture of whole cells or to a crude extract of a cell-free system, it was shown that *Porphyridium* cells assimilate and metabolize florisidoide. It was therefore suggested that the carbon metabolic pathway in *Porphyridium* sp. passes through the photoassimilatory product florisidoide, which, in turn, channels the fixed carbon toward the synthesis of sulfated cell-wall polysaccharide according to physiological conditions, for example, nitrogen starvation [45,46*]. Thus, florisidoide functions as a dynamic carbon pool used by the cells as a carbon precursor in the biosynthesis of starch and the cell-wall polysaccharide [45,46*]. It was further suggested that the polysaccharide synthesized inside the cells might be transferred outside the cells into the medium via two different pathways: directly or by solubilization of the cell wall into the medium [10**,44].

**Sulfation**

Sulfation of polysaccharides is limited to red and brown algae and to mammals [47]. Since it has been hypothesized that sulfate is the bioactive group of the red microalgal polysaccharides, focus was directed toward understanding the process. The mode of sulfation of the cell-wall polysaccharide of *Porphyridium* sp. was studied by incorporation of sulfur from Na_2^35SO_4 or [35S]cysteine into the cell-wall polysaccharide [48**]. The findings implied that in addition to the commonly accepted ‘inorganic’ sulfation pathway, there might be another sulfation pathway in which cysteine can serve as the sulfur donor [48**]. This study was followed up with the characterization of the enzyme sulfotransferase, which is responsible for the attachment of sulfur to the cell-wall polysaccharide. The sulfotransferase gene was identified (in the ESTs), and a biochemical assay was developed to facilitate its functional characterization [49].

**66-kDa glycoprotein**

Sequencing of a cDNA clone encoding the 66-kDa glycoprotein attached to *Porphyridium* sp. polysaccharide revealed that the glycoprotein shows some structural similarities to protein superfamilies in the SCOP databases, within the carbohydrate-binding domain (CBD), namely, glycosyltransferases, pectin lyase-like, xylanases, and conA-like lectins/gluconases, indicating a possible role of the 66-kDa glycoprotein in synthesis/modification of the cell-wall polysaccharide [25**,26]. In addition, the N-terminal and one internal peptide of the 66-kDa protein shows homology to endo-β-1,4-xylanase [26]. Moreover, the 66-kDa protein was found in the intermediate cell-wall
polysaccharide complex of 0.5 × 10⁶ Da mentioned above [39,40] and in the cell-wall modified mutants [26]. These observations and the strong association of the glycoprotein with the cell-wall polysaccharide suggest that the 66-kDa protein is involved in the initial stages of cell-wall polysaccharide formation [25**,26].

**Biotechnological aspects**
The increasing market demand for natural polysaccharides for the food, cosmetics, and pharmaceutical industries cannot be met by currently available conventional sources—red and brown macroalgae. These traditional sources of polysaccharides, which are usually harvested from their natural habitats [50,51], are being depleted by intensive harvesting and detrimental environmental conditions. An attractive alternative may be found in the sulfated polysaccharides of the red microalga, which offer a vast range of potential applications for a variety of industries [3,9,10**,11**,13,52,53]. Moreover, various significant applications are under development.

The unique rheological properties of red microalgal polysaccharides (viscoelasticity) may be exploited for a variety of applications. Thus, owing to the superiority of red microalgal sulfated polysaccharides over hyaluronic acid, that is, stability in the body (resistance to hyaluronidase), their adsorption properties and their superior rheological characteristics, they make good candidates as water-soluble lubricants and other biolubricating fluids and additives to the synovial fluid of joints [32**,36]. New developments of these applications are underway. In parallel, the fluid-dynamic behavior of the polysaccharides can be exploited in tertiary oil recovery: the polysaccharides have been used as thickening agents for driving fluids to enhance recovery of petroleum trapped in the pores of reservoir rocks [29–31].

In addition to their biolubricant activities, the red microalgal polysaccharides exhibit various bioactivities that have nutritional, medicinal, and cosmetic significance. Animal feeding experiments have shown that rodents whose diets are supplemented with low concentrations of red microalgal polysaccharides have considerably lowered levels of serum cholesterol, triglycerides, and very low-density lipoprotein levels [54–56,57*] with no evidence of toxic side effects. It was thus suggested that the polysaccharides act as dietary fibers [54–56] and can be valuable in a functional food [56].

*Porphyridium* sp. cell-wall sulfated polysaccharide was shown to have antitumor and antiviral activities. The antitumor activity was demonstrated against sarcoma inoculated in the peritoneal cavity of mice [58] and against myeloid Graffi tumor, both *in vitro* and *in vivo* in hamsters [59]. Sulfated polysaccharides were shown to have antiviral activities [60–62]. The antiviral activity of red microalgal polysaccharides has been demonstrated against a variety of animal viruses [63–67,68**,69]. The *Porphyridium* sp. sulfated polysaccharide exhibited antiviral activity against *Herpes simplex* viruses (type 1 and 2) and *Varicella zoster* virus [66,67,68**] and displayed *in vitro* inhibition of the replication of hemorrhagic septicemia virus (VHSV) and African swine fever virus (ASFV) [69]. The fact that the *Porphyridium* sp. polysaccharide is not toxic [68**], as compared with other sulfated sugars, makes it a valuable candidate for further pharmacological developments, especially for topical applications.

The Arad group has hypothesized that the bioactive group is the sulfate group, based on the following observations: Among the various red microalgal species, the highest antiviral activity was found in the polysaccharide having the highest sulfate content (highest in *Porphyridium* sp., 9% sulfate and the lowest in *P. aerugineum* with <1% sulfate). This hypothesis was indeed validated by experimental findings: a direct correlation was shown between the polysaccharide’s sulfate content and its antiviral activity against herpes viruses: Increasing the sulfate content of the polysaccharide by chemical methods significantly enhanced its antiviral activities (Arad, S, personal communication). While blocking the sulfate groups (by quaternization) [70], significantly decreased antiviral activity.

The polysaccharide of *Porphyridium* sp. was found to have anti-inflammatory, anti-irritating [71**], and antioxidant [7] activities. Owing to these bioactivities, this sulfated polysaccharide has already been introduced into a wide range of cosmetic products of a leading global cosmetics company, and additional developments are underway.

**Figure 2**

> Large-scale facility of red microalgae for polysaccharide production.
To facilitate commercial scale production of the polysaccharide, it was necessary to develop the biotechnology that is specifically directed for red microalgae that secrete cell-wall polysaccharides into the growth medium. The polyethylene vertical bioreactors [9,10,11,12] that were developed by the Arad group are currently in use for large-scale production of *Porphyridium* sp polysaccharide (Figure 2) by the multinational company Frutarom, which is situated in the Negev area of Israel.

The bioactivities described above bear witness to the potential of novel red microalgal polysaccharides, with their unique characteristics, in the development of biotechnological applications [52], mainly for the healthcare and pharmaceutical industries. For most of the potential applications, the market is still developing.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


Additional information on sugar-sequence in the complex polysaccharide is presented.


25. Heaney-Kieras J, Roden L, Chapman DJ: The covalent linkage of protein to carbohydrate in the extracellular protein-
The main characteristic of the red microalgal polysaccharide — its resistance — is demonstrated vis-à-vis that of hyaluronic acid.


Production of cell-wall modified mutants resistant to DCB is a valuable tool for understanding of cell-wall biosynthesis.


The paper shows the involvement of the disaccharide floridoside in cell-wall biosynthesis.


The authors show that there might be an alternative pathway for assimilation of sulfur into the polysaccharide, through cysteine.


The paper indicates the effect of feeding with algal biomass — including the polysaccharides — on lipid metabolism.


This review describes the potential of the antiviral activity of sulfated polysaccharides for the development of pharmaceutical applications.


The anti-inflammatory activities of the sulfated polysaccharides have opened the door to large cosmetic and pharmaceutical applications.