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Review

An Exopolysaccharide from the Cyanobacterium *Arthrospira platensis* May Utilize CH−π Bonding: A Review of the Isolation, Purification, and Chemical Structure of Calcium-Spirulan

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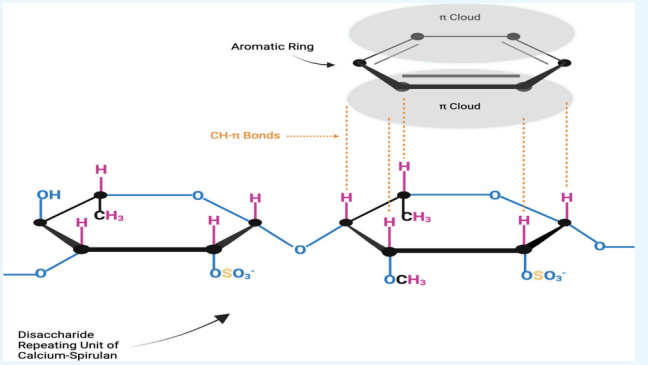
ABSTRACT: The CH−π bonding potential of a saccharide is determined primarily by the number of hydrogen atoms available for bonding and is reduced by side groups that interfere with the CH−π bond. Each hydrogen bond increases the total bond energy, while interfering hydroxyl groups and other side groups reduce the bond energy by repulsion. The disaccharide repeating units of Calcium-Spirulan (Ca-SP), a large exopolysaccharide sub fraction-ated from the supernatant of the cyanobacterium *Arthrospira platensis*, contain a unique monosaccharide that is completely devoid of hydroxyl groups and side groups on its entire beta surface, leaving five hydrogen atoms available for CH−π bonding in the planar conformation. While planar conformations of independent pyranose rings are rare-to-nonexistent, due to ring strain associated with that conformation, the binding site of a protein could provide the conformational energy needed to overcome that energy barrier. By enabling a planar conformation, a protein could also enable the sugar to form a novel 5-hydrogen CH−π bond configuration. One study of the anticoagulant property of Ca-SP shows that the molecule acts as an activator of Heparin Cofactor II (HC-II), boosting its anticoagulant kinetics by 10^4. In comparison, the longstanding anticoagulant drug Heparin boosts the HC-II kinetics by 10^3. The difference may be explained by this unique CH−π configuration. Here, we review current knowledge and experience on the isolation techniques, analytical methods, and chemical structures of Ca-SP. We emphasize a discussion of the CH−π bonding potential of this unique polysaccharide because it is a topic that has not yet been addressed. By introducing the topic of CH−π bonding to the cyanobacterial research community, this review may help to set the stage for further investigation of these unique molecules, their genetics, their biosynthetic pathways, their chemistry, and their biological functions.



INTRODUCTION

Photosynthetic microorganisms are being studied and used for multiple industrial applications: CO_2 sequestration, air purification, biofuels, bioplastics, human nutrition, farm feeds, fish feeds, pet feeds, fertilizers, soil amendments, cosmetics, textiles, color dyes, pharmaceutical manufacturing, medicinal chemistry, and wastewater remediation. These applications are reviewed in a number of publications.[1−12] The United States National Aeronautics and Space Administration (NASA) is studying them as a nutrition source for space missions.[13] The cyanobacterium *Arthrospira platensis* (commonly "Spirulina") is one of the best studied species in this field.

One of the unique characteristics of *A. platensis* is its abundant Extracellular Polymeric Substances (EPS), especially the EPS that are secreted completely to become free-floating constituents of aqueous media. Here, we use the fractionation nomenclature of Ahmed et al. (2014) whereby this free-floating fraction is referred to as the "Released Extracellular Polymeric Substances" (REPS). This distinguishes it from two other fractions of EPS: "Loosely bound Extracellular Polymeric Substances" (LEPS) and "Tightly bound Extracellular Polymeric Substances" (TEPS).[14] We also use the term Calcium-Spirulan (Ca-SP) to describe one particular sub-fraction of REPS, according to the nomenclature introduced by Hayashi et al. (1996).[15] Ca-SP is one of the subfractions of the REPS layer.

In terms of evolutionary function, the REPS of cyanobacteria have been described mostly as protective molecules that evolved to protect the species from various kinds of environmental stresses such as UV radiation, metals, salts,

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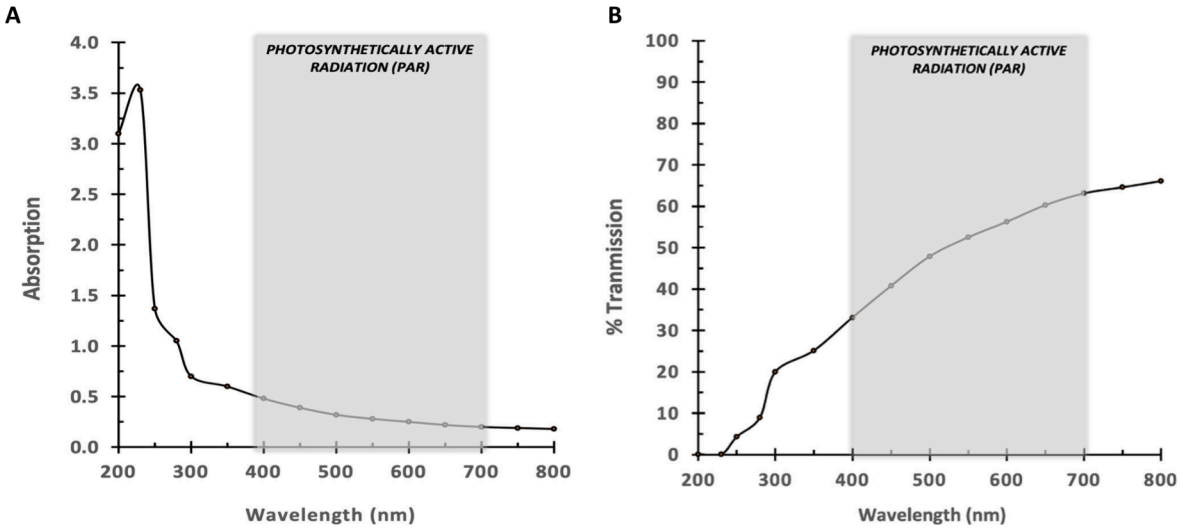

Figure 1. (A) Absorption spectrograph of the REPS of *A. platensis*. (B) Transmission spectrograph of REPS *A. platensis*. Adapted from ref 20. Copyright 2009 Springer Nature.

bacteriophages, and drought.[16−19] They have also been noted to act as an adherent or an extracellular web-like matrix that binds to solid surfaces and to other cells.[17] EPS isolated from the related cyanobacterium *Streptomyces* sp. were shown to have an affinity for iron ions that is almost as strong as the common iron chelator Na-EDTA.[19]

The EPS of *A. platensis* has also been studied for potential medical, nutritional, and industrial applications. The anticoagulant properties of a subfraction of REPS, called PUF2, was shown to increase thrombin inhibition of heparin 4-fold, suggesting that it might be useful as a rapid first response against ischemic stroke and/or cardiac arrest.[20] Based on their fibrous nature, EPS of microalgae have also been studied for their benefits as a dietary fiber, a topic that was reviewed by Raposo and Morais.[21] The Ca-SP subfraction of the EPS of *A. platensis* inhibits the invasion and metastases of three kinds of human tumor cell lines in vitro,[22] a property that might be helpful in the war on cancer. Ca-SP also inhibits the replication of several enveloped viruses: HSV-1, HCMV, Measles virus, Influenza virus, Polio virus, Coxsachie virus, and HIV-1.[23] In the food science industry, the REPS have also been studied as thickening agents for food and beverages, where their viscosity is a valuable property.[24]

The properties of REPS, however, are not always beneficial. EPS are also understood to play a role in the fouling of industrial equipment used to cultivate microorganisms, such as photobioreactors (PBRs), where their adhesive properties accelerate the fouling process and decelerate the cleaning process.[17] Fouling is a major source of production loss because it inhibits light penetration and requires significant downtime for cleaning. Moreover, to the extent that REPS molecules are released into the culture medium, they may also represent a significant loss of biomass production for producers who harvest only the bound cellular components of a culture. For producers interested strictly in protein, the use of cellular energy and resources for polysaccharide production might be considered wasteful.

In terms of photometric properties, the EPS also constitutes a radiation barrier, both in the culture medium and on the surface of cells. The focal point of the literature discussion about the spectrographic properties of REPS is its UV blocking capability. For example, Trabelsi et al. (2009) published a UV−vis spectrum of their REPS fraction of *A. platensis* (Figure 1A,B).

These spectrographs show the tremendous UV blocking ability of these molecules at a peak absorbance level of 3.5 at 230 nm and also a shoulder at 280 nm.[25] Little has been written, however, about the impact of EPS on the visible portion of the radiation spectrum 400 nm −700 nm, which is the photosynthetically active radiation (PAR).

It should also be noted that both the component fractions and the total mass of the EPS have been shown to vary considerably based on culture conditions. The relative proportion of the carbohydrate and protein constituents of the REPS layer of *A. platensis* varies by more than 100% depending on the nitrate level and light intensity under which they were grown.[26] The fraction of REPS relative to total cellular biomass changes significantly depending on the salinity of the growth medium.[27]

Still, there remain many unanswered questions, particularly with regard to detailed molecular biology. In the United States National Library of Medicine (NLM) National Center for Biotechnology Information (NCBI) data sets, the best annotated genome among the strains of *A. platensis* (Strain C1) includes 6057 putative coding sequences (CDS), of which 1885 (31%) are labeled either Domain of Unknown Function (DUF) or Hypothetical Protein (HP).[28,29] This translates to an Annotation Completion Ratio (ACR) of 69%, whereas the model prokaryote *E. coli* K-12 has an ACR of 100%.[29]

Here, we review the latest knowledge and experience on the methods used to isolate, analyze, and characterize the chemical structure of the exopolysaccharides of *A. platensis*. This review is intended to set the stage for further experimentation to elucidate new details on these macromolecules secreted by *A. platensis*, their properties and applications, and their genetic/biosynthetic pathways. This may help to find new annotations in the genomes of *A. platensis* or to discover novel carbohydrates, novel protein(s), protein domain(s), or regulatory mechanism(s), which have not been found in other organisms to date. We plan a second review covering the genetics and biosynthetic pathways of the EPS of *A. platensis*; thus, we do not cover these topics here.

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Table 1. Methods of Extraction, Separation, Purification, and Characterization of EPS of *A. platensis*[a]

author year	extr method	sep method	purif method	prot/NA elimination	characterization methods
Filali 1993[30]	centrifuge + heat	CTAB ppt + centrifuge	KCl wash, EtOH ppt	none	Blumenkrantz uronic acid assay; methylation; gas chromatography; acid hydrolysis; peracetylation; rheology
Hayashi 1993[31]	hot H_2O; centrifuge	none	none	none	Evaluation of Antiviral Properties
Hayashi 1996a[23]	hot H_2O; centrifuge	SEC; IEC; HPLC	dialysis	TCA ppt	Dubois sugar assay; optical rotation; UV−vis; FTIR; scanning electron microanalysis; desulfation
Hayashi 1996b[15]	hot H_2O; centrifuge	SEC; IEC; HPLC	dialysis	TCA ppt	Dubois sugar assay; optical rotation; UV−vis; FTIR; scanning electron microanalysis; desulfation
Lee 1998[32]	hot H_2O; centrifuge	SEC; IEC; HPLC	dialysis	TCA ppt	gas chromatography, mass spectroscopy, paper chromatography, NMR, m-hydroxydiphenyl method, X-ray analysis, FTIR, flask combustion method, optical rotation, methylation analysis, Dubois sugar assay
Mishima 1998[22]	hot H_2O; centrifuge	SEC; IEC; HPLC	dialysis	TCA ppt	evaluation of anti-cancer properties
Lee 2000[33]	hot H_2O; centrifuge	SEC; IEC; HPLC	dialysis	TCA ppt	gas chromatography, electrospray ionization mass spectroscopy, paper chromatography, NMR, m-hydroxydiphenyl method, X-ray analysis, FTIR, flask combustion method, optical rotation, methylation analysis, Dubois sugar assay
Trabelsi 2009[20]	filtration	concentrator	diH_2O Wash	none	flash elemental analysis; bicinchoninic acid method (protein); Dubois sugar assay; Blumenkrantz uronic acid assay; UV−vis; FTIR
Ahmed 2014[14]	centrifuge	filter; shaking; EDTA	centrifuge	none	Bradford protein assay; DuBois sugar assay; HP ion exchange chromatography; carbozole uronic acid assay; dimethyl methylene blue glycosaminoglycan assay; confocal laser
Depraetere 2015[34]	filtration	none	conc; dialysis	none	Dubois sugar assay; total organic carbon analyzer; HP ion exchange chromatography; Lowry protein

[a]Abbreviaions: TCA, trichloroacetic acid; SEC, size exclusion chromatography; HPLC, high performance liquid chromatography; IEC, ion exclusion chromatography; CTAB, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; UV−vis, ultraviolet/visible spectroscopy; FTIR, Fourier-transform infrared spectroscopy; HP-IEC, high performance ion exchange chromatography; NMR, nuclear magnetic resonance.

We begin with a summary table (Table 1) and then proceed with a more detailed description and discussion of the isolation, analysis, and characterization methods used to date. After presenting the unique disaccharide repeating units found in Calcium-Spirulan, this review then culminates in a discussion of the CH−π bonding capability of these molecules, a topic that has not yet been published by either cyanobacterial biologists or CH−π chemists.

ISOLATION AND PURIFICATION OF EXOPOLYSACCHARIDE CONSTITUENTS

Filali et al. (1993). They studied the EPS of *Spirulina platensis* (the historical name for *Arthrospira platensis*).[30] Two centrifuge steps were used to separate EPS from the cells: the resulting supernatant was then precipitated with cetyltrimethylammonium bromide (CTAB), followed by another centrifuge step to pellet the precipitate. To verify that all EPS had precipitated, the resulting supernatant was mixed with EtOH. No precipitate was found, which confirmed that all of the EPS had already pelleted in the prior step.[30] That EPS pellet was then purified by KCl washes, reprecipitated by Et−OH, and centrifuged. This final pellet was then dried, either by speed vac or lyophilization.[30]

The methods used here did not include any subfractionation of the EPS components, and therefore, the resulting samples probably contained more than one type of polysaccharide molecule. This limitation is acknowledged by the authors in the title of the paper, which describes their results as a "preliminary characterization" of the EPS.[30] CTAB is a cationic surfactant that precipitates anionic macromolecules, including polysaccharides, proteins, and nucleic acids.[35] It is not selective for polysaccharides alone, and it will not necessarily precipitate neutral polysaccharides.[35] Therefore, this method seems insufficient for studying EPS polysaccharides.

Hayashi et al. (1993). They began a series of studies of the EPS of *A. platensis*, which evolved into the most comprehensive, complete, and detailed characterization of these molecules to date.[31] At first, in 1993, there was no subfractionation of EPS constituents, and there was no identification of monosaccharide sequence(s).[31] What they *did* find was that a hot water extraction of EPS from *A. platensis* showed in vitro antiviral activity against Herpes Simplex Virus (HSV) in HeLa cells.[31] Spurred on by this biological activity, this group (with others) produced four more publications, which did include subfractionation as well as the first ever detailed structural characterization of the disaccharide repeating units of an extracellular polysaccharide isolated from *A. platensis*.[15,23,32,33]

Hayashi et al. (1996). They began with freeze-dried powder, rather than live filaments.[23] Methods of freezing and freeze-drying can break cells, causing release of intracellular components upon rehydration, and freezing can also damage various macromolecules of interest.[36] The freeze-dried powder was then mixed with with hot water and treated with 10% trichloroacetic acid (TCA).[23] TCA precipitates proteins and nucleic acids, but it does not precipitate polysaccharides.[37]

The resulting supernatant was then purified as polysaccharides without any protein or nucleic acids. The purified polysaccharide dissolved in TCA was then extracted by dialysis against distilled water and lyophilized to reveal a grayish-green powder.[23] The grayish-green coloration may reflect the presence of chlorophyll, its metabolites, or some other contaminant molecule other than the proteins and nucleic acids that were precipitated out by TCA.

To remove cations, this greyish-green powder was then resuspended in a saline-sodium citrate (SSC) buffer.[23] Size Exclusion Chromatography with Sepharose 6B (SEC-S6B) was then used to separate polysaccharides by size, and these

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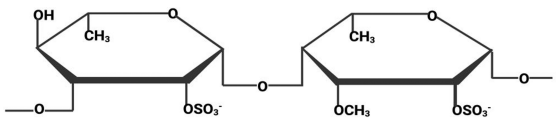
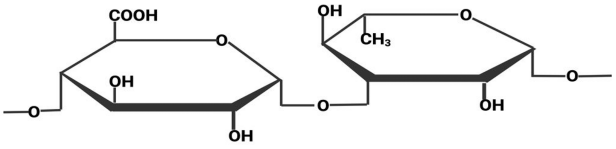
subfractions were observed using both 260 nm spectroscopy and 480 nm spectroscopy (the phenol-sulfuric acid method).[23] The 260 nm results showed two major absorption peaks, and the 480 nm results showed three overlapping absorption peaks.[23] The authors focused the rest of their study exclusively on the subfraction identified by the second absorption peak of the 480 nm results, labeled "SP-H-2", because that fraction alone showed the antiviral activity.[23] They assumed that this peak represents a single polysaccharide, and the molecule was named "Calcium Spirulan" (Ca-SP).[23] When dried, the Ca-SP subfraction was colorless, without any sign of green chlorophyll, white salts, or gray coloration; Ca-SP was also the fraction that was used to determine the chemical structure of the disaccharide repeats shown below (Figures 2 and 3).[32]



O-rhamnosyl-acofriose (sulfated)

Figure 2. 6-Deoxy-2-sulfa-α-L-rhamnose (1,2) α-6-deoxy-3-*O*-methyl-2-sulfa-α-L-rhamnose. Structural model of one the first disaccharide repeating unit of Calcium Spirulan first discovered by Hayashi et al. (1996) and later structurally identified by Lee et al. (2000), who used the label "O-rhamnosyl-acofriose" for this disaccharide.[15,33] The IUPAC name and the CAS number for the first monosaccharide are (2*R*,3*R*,4*R*,5*R*,6*S*)-6-methyloxane-2,3,4,5-tetrol and 6014-42-2, respectively. The IUPAC name and the CAS number for the second monosaccharide are (2*R*,3*R*,4*S*,5*S*)-2,4,5-trihydroxy-3-methoxyhexanal and 78185-80-5, respectively.[38]



O-hexuronosyl-rhamnose (aldobiuronic acid)

Figure 3. α-D-Glucuronic (1,3) α-6-deoxy-L-rhamnose. Structural model of the disaccharide repeating unit of Calcium Spirulan first discovered by Hayashi et al. (1996) and structurally identified by Lee et al. (2000), who used the label "O-hexuronosyl-rhamnose (aldobiuronic acid)" for this disaccharide.[23] The IUPAC name and the CAS number for the first monosaccharide are (2*S*,3*S*,4*S*,5*R*,6*S*)-3,4,5,6-tetrahydroxyoxane-2-carboxylic acid and 70021-34-0, respectively. The IUPAC name and the CAS number for the second monosaccharide are (2*S*,3*R*,4*R*,5*R*,6*S*)-6-methyloxane-2,3,4,5-tetrol and 73-34-7, respectively.[33,38]

For the purpose of reviewing and understanding *all* of the EPS components and pathways, it should be emphasized here that Ca-SP is not the only major polysaccharide to elute from this hot water extraction. The results clearly show that there was *at least* one other major polysaccharide constituent in their extract.[23] Furthermore, when a secondary separation method using DEAE-cellulose ion-exchange chromatography was applied, the results suggest that there may actually have been two different ionic subfractions within the size subfraction that was labeled as Ca-SP.[23] Later, in 1998, a related group of authors used a different type of DEAE-cellulose ion-exchange resin, which produced more definitive ionic subfractions, showing more conclusively that the Ca-SP subfraction contains at least two different species of polysaccharide.[32]

Trabelsi et al. (2009). They published a similar study on a different strain.[25] Instead of centrifugal separation of the EPS from the cells, however, this group used a multistep filtration process, using progressively smaller pore sizes.[25] That cell-free filtrate was then concentrated using tangential ultrafiltration cells, producing a cell-free concentrate of all molecules greater than the 30 kDa cutoff. The authors described this fraction as the "released EPS."[25] This fraction was then washed three times with deionized water, freeze-dried, and then oven-dried.[25] No further subfractionation of this sample was undertaken, which means that the product of this fractionation method was most likely a multimolecular sample.

This method resulted in substantial protein constituents (55% w/w), an uncharacterized fraction (32%w/w), and polysaccharides (13%w/w).[25] Moreover, each of these fractions contained more than one subfraction. The term "partial characterization" was used to denote the limitations of this study.[25] The large amounts of protein found in these samples are consistent with findings on *E. coli*, in which large amounts of protein have also been found in EPS samples.[39] Notably, the samples analyzed by Trabelsi were drawn directly from a live culture, rather than freeze-dried cells.[25]

Chaiklahan et al. (2013, 2014). They compared EPS extraction from dried *A. platensis* in different temperatures, different solvents, and with different Molecular Weight Cut-Off (MWCO) ultrafiltration membranes.[40,41] Taking pages from both Filali and Hayashi, this group used both CTAB and SSC.[40] They concluded that high temperature and a high ratio of water-to-solid optimizes the extraction yield.[41] They also used a Sepharose 6B (S6B) column to subfractionate two of their fractions by size.[40] The results showed substantial increases in concentration of extracted polysaccharides with increasing temperature.[40]

Three separation methods were evaluated (CTAB precipitation, Evaporator concentration, andultrafiltration concentration), and the results were mixed, with pros/cons for each method.[40] SEC-S6B defined two definitive, but overlapping, peaks; based on dextran standards, these peaks correspond to 212 and 12.6 kDa, respectively[40] Interestingly, Chaiklahan also found high levels of phenolic content,[41] using the method of Wu and Hansen with gallic acid as a standard.[42]

The molecular weight estimations (212 and 12.6 kDa) by Chaiklahan[40] differ dramatically from the molecular weight estimation for Ca-SP (75 kDa) found by Lee.[32] There are two possible explanations for this.

There may have been strain differences between these two studies. Strain NG was used by Hayashi;[15] it is not clear which strain was used by Chaiklahan.[40,41,43] It seems more likely, however, that the use of a lipid extraction step prior to polysaccharide extraction by Chaiklahan[40,41,43] was the cause of this significant difference in molecular size. In their series of related studies, neither Hayashi nor Lee used a lipid extraction step.[15,23,31−33]

Lipid extraction is disruptive to cell membranes and could cause several effects on subsequent polysaccharide extraction and characterization steps. For example, disrupting the cell membranes could release polysaccharide-degrading enzymes from the cytoplasm, which degraded large polysaccharides and reduced their molecular size estimates. Another possible explanation is that the solvents used during lipid extraction might have degraded the large polysaccharides, thus changing

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their size and molecular weight estimates. Furthermore, certain hydrophobic polysaccharide(s) may have separated out with the lipid fraction due to their hydrophobic nature, thus changing the molecular size estimates. It is also possible that some polysaccharides may have been covalently bound to lipids, causing them to separate with the lipid fraction to be degraded by the lipid extraction steps.

The study of the lipids of *A. platensis* is of interest for the production of biofuels, specialized nutrients (Omega-3 fatty acids), and/or other purposes, but we believe that an accurate study of the EPS is best done without a lipid extraction step in order to avoid cellular disruption and the release of intracellular components.

Ahmed et al. (2014). They published a paper on the EPS of *A. platensis*.[14] The terms Released EPS (REPS), Loosely bound EPS (LEPS), and Tightly bound EPS (TEPS) were formally introduced along with the methods used to separate these fractions.[14] The REPS was separated from the cell culture by centrifugation, and the supernatant was filtered and freeze-dried.[14] The pellet was then resuspended by vortex and placed in a shaking hot water bath to release the LEPS from the cells.[14] After causing release of the LEPS, the sample was then centrifuged, and that supernatant was filtered and freeze-dried.[14] The cell-containing pellet was then resuspended in EDTA and placed in a shaking water bath at room temperature to release the TEPS.[14] This sample was then centrifuged, and the resulting supernatant contained the TEPS, separated from the cell-containing pellet.[14] Throughout these extraction steps, cell samples were monitored under a microscope to ensure that no cell lysis had occurred along the way, because cell lysis would contaminate the fractions with unwanted intracellular materials.[14] Ahmed's samples also came from live cell cultures, and protein was found in these samples as well, albeit at a lower concentration (32%) than for Trabelsi (55%).[14]

The reason for the difference in protein content between the samples of Trabelsi and Ahmed is not entirely clear. Both used live cultures at plateau phase, and notable differences include strain differences, different culture conditions, and differences in protein quantification methods.[14,25] It is also possible that the high-power centrifugation used by Ahmed pelleted significant amounts of very large proteins with the cells, thus removing some of the larger and more dense proteins from the EPS supernatants; in contrast, Trabelsi's gravity filtration was a more gentle technique dependent on molecular size alone.[14,25]

Finally, it should also be emphasized that neither Ahmed nor Trabelsi undertook any subfractionation of their respective polysaccharide fractions.[14,25] Therefore, it is possible that more than one polysaccharide was present within each of their samples and that their respective results reflect multiple different polysaccharide component macromolecules rather than a single molecule.

Depraetere et al. (2015). They used a simplified version of the Trabelsi filtration method. Culture samples were filtered and concentrated with a molecular weight cutoff ultrafiltration membrane, followed by a dialysis step to remove salts.[34] This protocol also did not include any subfractionation to separate different polysaccharides or any steps to remove proteins and nucleic acids.[34] After using a 3 kDa ultrafiltration membrane to concentrate their filtrates, 12% protein (% w/w) was found.[34] This figure differs significantly from the 55% protein found by Trabelsi[25] and the 32% found by Ahmed.[14] Again, this discrepancy could be attributable to any of the following explanations: strain differences, culture conditions, protein quantification techniques, or extraction methods.

Wang et al. (2018). They systematically compared four different extraction methods for polysaccharides of *A. platensis*: hot-water extraction, alkali extraction, ultrasonic-assisted extraction, and freeze−thaw extraction.[44] It was determined that alkali extraction using NaOH followed by Et−OH precipitation was the optimum method, in terms of polysaccharide yield (% w/w).[44] They also used an enzymatic method for deproteination: acid-enzymatic treatment followed by boiling to inactivate the enzyme, then TCA.[44] Decolorization was achieved with 30% H_2O_2 followed by Et−OH precipitation.[44] Still, these methods did not include any subfractionation of the various polysaccharide constituents, characterization of monosaccharide components, or elaboration of the monosaccharide sequence(s). Moreover, this study used freeze-dried samples, which could've damaged both cells and macromolecules, thus distorting results.[36]

Color differences in the various solid extracts should be noted. The extracted solids produced by Ahmed were all white in color,[14] which suggests that no photoactive chlorophyll survived those extraction methods. However, the white color may also reflect the presence of common media salts, such as Sodium Bicarbonate (Na_2HCO_3). This white coloration stands in contrast to the grayish-green products extracted by Hayashi et al. (1993).[23] Trabelsi's REPS appeared as yellow-brown after freeze-drying.[25] Protein can appear as yellow-brown,[45] but chlorophyll degradation products, such as pheophytin a, can also appear as brownish colors.[46] The source of the yellow-brown color in Trabelsi is therefore not clear.

IMPORTANCE OF PRECISE STRUCTURAL CHARACTERIZATION

When thinking about large polysaccharides, some assume that their functionality is mostly that of an inert gel or matrix that provides steric hindrance. It is no surprise that the structure and gel-like properties of EPS are top-of-mind for many research scientists, because two widely used research gels, agar and agarose, are made up of polysaccharides.[47] The gel-like properties are also widely used in food sciences.[48] Polysaccharides are, however, much more complex than inert gels and molecular I-beams. Polysaccharides also exhibit highly specific protein binding sites, the specificity of which depends on their exact 3-D structure including their branching, side chains, conformations, and monosaccharide sequence.[49−56]

Carbohydrate Binding Modules (CBMs) are a large array of protein domains that exhibit specific binding capacity for particular sites on carbohydrates.[57] There are currently 475659 different modules categorized into 100 different families based on amino acid sequence similarity.[58] By definition, CBMs have a specific carbohydrate binding capability. Some CBMs also have a catalytic function, but catalytic function is not required for a module to be defined as a CBM.[58]

Monoclonal antibodies with specificity to certain epitopes on polysaccharides are used for research in molecular biology, a powerful tool for molecular biologists studying the expression, localization, and/or regulation of large polysaccharides, their building blocks, and/or their metabolites.[56,59,60] The first interaction between a human spermatozoa and a human egg is defined by a specific, noncovalent protein−carbohydrate bond, which begins the cascade of events known as fertilization.[61] Moreover, there is an entire class of proteins

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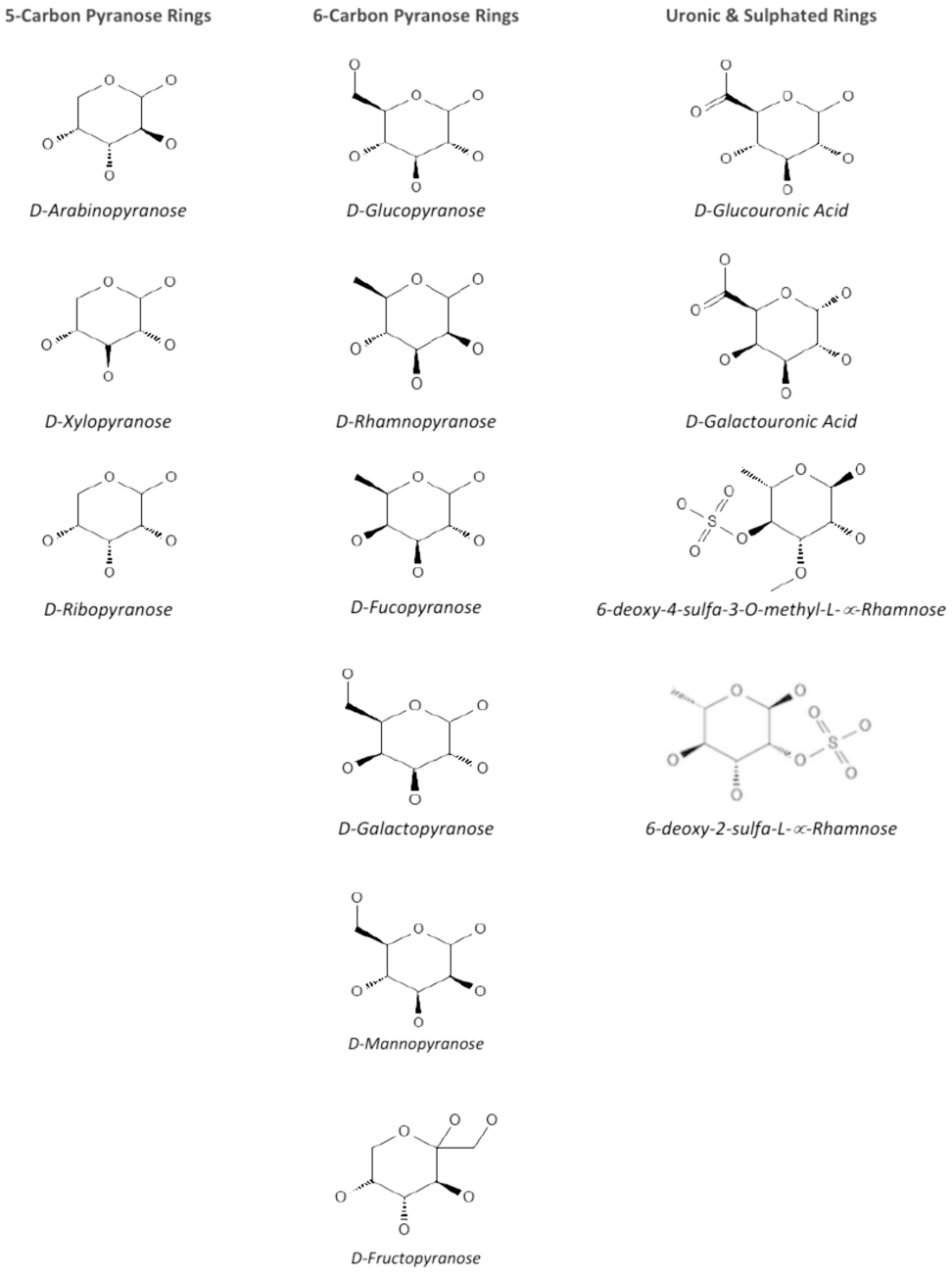

Figure 4. Structures of constituent monosaccharides of the REPS of *A. platensis*.[23,25]

(lectins) which is defined as proteins that interact with noncovalent specificity to polysaccharides.[62−64]

The detailed polysaccharide structure is also related to other biological functions in vivo. In human digestion, for example, the enzyme amylase hydrolyzes the alpha-1,4 glycosidic bonds that connect individual glucose molecules together.[65] The same enzyme cannot, however, hydrolyze the beta-1,4 glycosidic bonds of cellulose.[66] Starch and cellulose contain the exact same elemental components and the same sequence of monosaccharides. The only difference between the two is the orientation of the 1,4-glycosidic bonds that join the monosaccharides together in a chain. This difference in glycosidic orientation alone makes a difference in the enzyme reactivity. This seemingly minor difference also explains how and why cellulose is considered by dietitians to be a healthy "dietary fiber",[67] while starches are generally considered to be unhealthy polysaccharides that cause rapid spikes in blood sugar levels.[68] The orientation of the glycosidic bonds alone makes the difference between good health and severe, life-threatening metabolic disease.

Moreover, monosaccharide sequence is to polysaccharide 3-D structure as amino acid sequence is to protein 3-D structure. A particular sequence of monosaccharides with a particular arrangement of side groups produces a unique secondary, tertiary, and quaternary structure and biological activity. Much like proteins, three-dimensional polysaccharides can present certain highly specific immunogenic epitopes that can trigger immune responses and/or allergic reactions in humans.[69−71] A successful polysaccharide vaccine against *Haemophilus influenzae* Type B was developed during the 1980s[72,71−77] and reviewed by Robbins et al. (1987).[78] It is now part of the standard battery of vaccines given to infants around the world,

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Table 2. Monosaccharide Constituents (%) Identified in Studies of REPS of *A. platensis*[a]

Paper	Ara	Glc	Xyl	Rha	Fuc	Gal	Rib	Man	Fru	UA	$SO4^{-2}$	ND
Filali#1[b]	n/a	16	10	2	5	22	n/a	n/a	n/a	40	5%	n/a
Filali#2[b]	n/a	17	12	3	5	18	n/a	n/a	n/a	40	5	n/a
Ahmed[c]	n/a	17	8	41	15	19	0	0	0	31	n/a	n/a
Trabelsi[d]	1	13	14	4	13	15	n/a	0	n/a	15	n/a	25
Hayashi[e]	n/a	5	1	44	1	2	2	1	34	11	+	n/a

[a]Abbreviations: n/a - not assayed; Ara, arabinose; Glc, glucose; Xyl, xylose; Rha, rhamnose; Fuc, fucose; Gal, galactose; Rib, ribose; Man, mannose; Fru, fructose; UA, uronic acid. [b]Adapted from Filali et al. (1993).[30] [c]Adapted from Ahmed et al. (2014).[14] [d]Adapted from Trabelsi et al. (2009).[25] [e]Adapted from Hayashi et al. (1996b).[23]

and polysaccharide epitopes (not protein epitopes) were shown to be the immunogenic factors, according to reviews.[79,80]

METHODS OF 3-D STRUCTURAL CHARACTERIZATION

To date, the most advanced study of the 3-D structure and sequence of a particular polysaccharide subfraction of *A. platensis* (Calcium-Spirulan) concluded that the disaccharide repeating units of that polysaccharide are novel and unique, having never been seen before.[32] This suggests that there might also be novel gene(s), enzyme(s), or signaling mechanism(s) underlying the biosynthesis of these disaccharides.

Most of the EPS characterization studies done to date were "partial characterizations" based on multicomponent fractions of EPS, without any subfractionation (Figure 4).[14,23,25,30,34] These studies are based on long-proven analytical chemistry, and they produce informative results. However, these results do not provide sufficient detail for the elucidation of precise biosynthetic pathways or specific binding sites. The studies of Lee et al. (1998, 2000) are the only studies that go beyond these partial characterizations, where a combination of physicochemical methods were used to reveal greater structural detail: Electrospray Ionization (ESI), Gas Chromatography (GC), Mass Spectroscopy (MS), and Nuclear Magnetic Resonance (NMR).[32,33]

Filali et al. (1993). They used a method developed by Blumenkrantz and Asboe-Hansen (1973) to detect uronic acids in the REPS using m-hydroxydiphenyl and galacturonic acid as a standard.[30,81] This method is specific for uronic acids, which are commonly found in the mucopolysaccharides of prokaryotes.[81] This method does not require prior hydrolysis of polysaccharides; it can be applied to whole polysaccharides with uronic acid moieties. Monosaccharide components were detected separately using the method of Montreuil et al. (1986), whereby neutral sugars were converted to trimethylsilylated and alditol acetate derivatives and then detected with gas chromatography.[30]

Ahmed et al. (2014). They used heat and sulfuric acid to hydrolyze the polysaccharides into monosaccharides; High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) was then used to separate the component monosaccharides from each other.[14] They used the phenol-sulfuric acid method of Dubois et al. (1956) to quantify the relative amounts of each component.[14,82] Total protein was measured with the Bradford Protein Assay.[14,83] Uronic acid content was determined with carbozole, sulfuric acid, and sodium borate, according to the method of Taylor and Buchanan-Smith (1992).[14,84] Glycosaminoglycan concentration was quantified with the dimethyl methylene blue method of Chandrasekhar et al. (1987).[14,85]

Trabelsi et al. (2009). They measured total mass of their EPS fraction using dry weight (DW) and volatile dry weight (VDW) methods at 105 and 550 °C, respectively, and elemental component analysis was conducted using a Flash Elemental Analyzer.[25] To quantify protein content, they used the bicinchoninic acid (BCA) method, according to Sigma-Aldrich, with bovine serum albumin (BSA) as a standard.[25,86] Neutral sugar content was measured by the method of Dubois et al. (1956), and uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen (1973).[25,81,82] This group also included UV−vis and FTIR spectroscopy on their EPS fraction.[25]

Table 2 summarizes the preliminary studies on the monosaccharide composition of REPS from each of the four papers mentioned above.

Certain qualitative observations about these data can be made. For example, glucose and rhamnose are consistently present, as are galactose and uronic acids. Sulfate groups are found in all of the studies that looked for them. Xylose and fucose are also present. However, detailed structural, biosynthetic, and/or genetic comparisons between and among these different studies requires more detailed structural studies that elucidate side groups, glycosidic bond orientations, and monosaccharide sequence.

It should also be noted that strains, media, life cycle, extraction techniques, and measurement methods are not the same among these studies. Any one, or more, of these differences could explain the seemingly incongruent results in Table 2.

Lee et al. (1998). They extended Hayashi's line of research by describing the side chain modifications on the monosaccharides in the Ca-SP subfraction.[32] Total sugar content was determined by the phenol-sulfuric acid method of Dubois et al. (1956).[82] Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen (1973).[81] Sugar analysis of the Ca-SP subfraction was performed by Gas Chromatography (GC), Gas Chromatography−Mass Spectrometry (GC-MS), and methylation analysis.[32] This combination of techniques—cross-checked with Paper Chromatography (PC) and Nuclear Magnetic Resonance (NMR)—elaborated detail on the side groups present in Ca-SP, including the presence of the following monosaccharides as components of the Ca-SP subfraction: 3-*O*-methylrhamnose (acofriose), 2,3-di-*O*-methylrhamnose, and 3-*O*-methylxylose.[32] Sulfur was detected by X-ray analysis, identified as sulfate groups by FT-IR, and then cross-checked by the flask combustion method. Optical rotation then indicated that Ca-SP has an alpha-L-configuration.[32]

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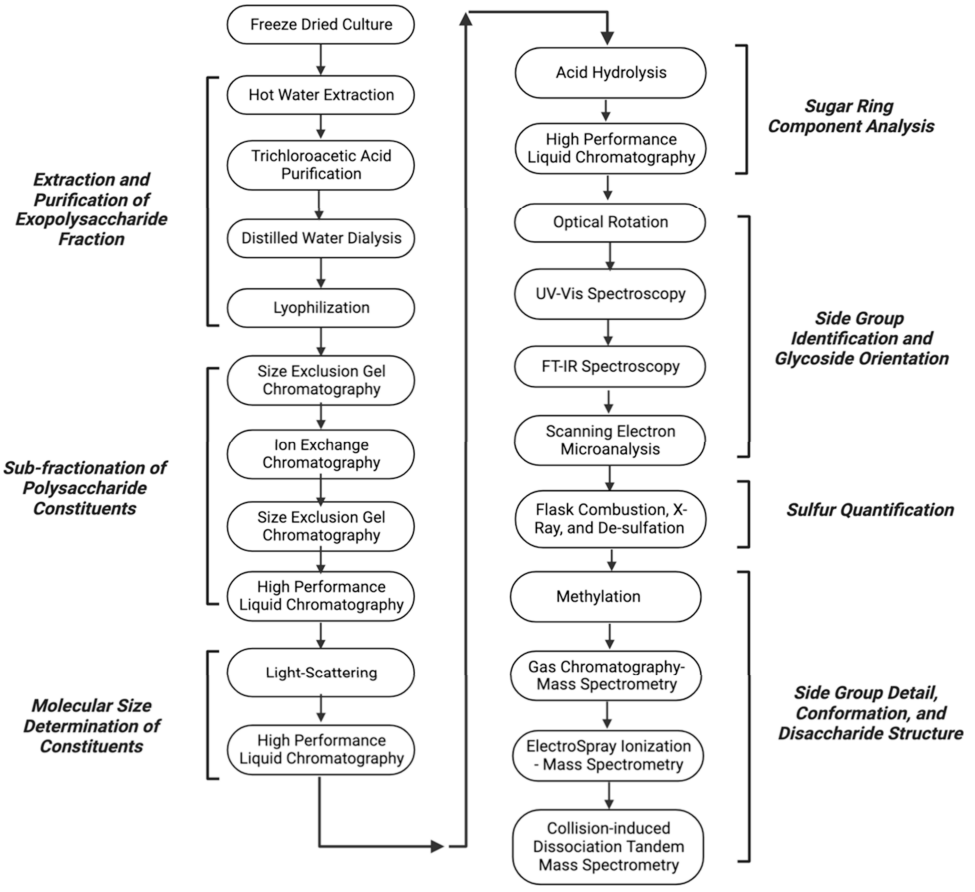

Figure 5. Combined methods of Hayashi, Lee et al. (1996−2000).[23,32,33]

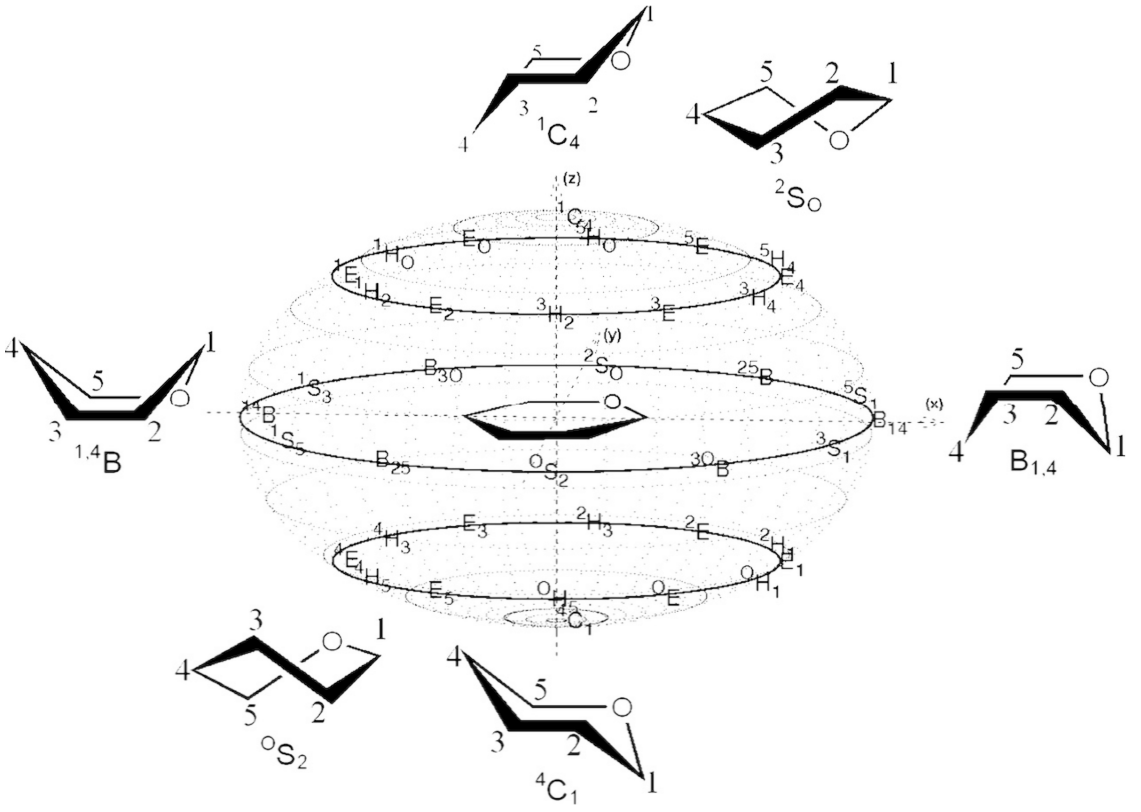

Figure 6. Seven different conformations of a pyranose ring.[88]

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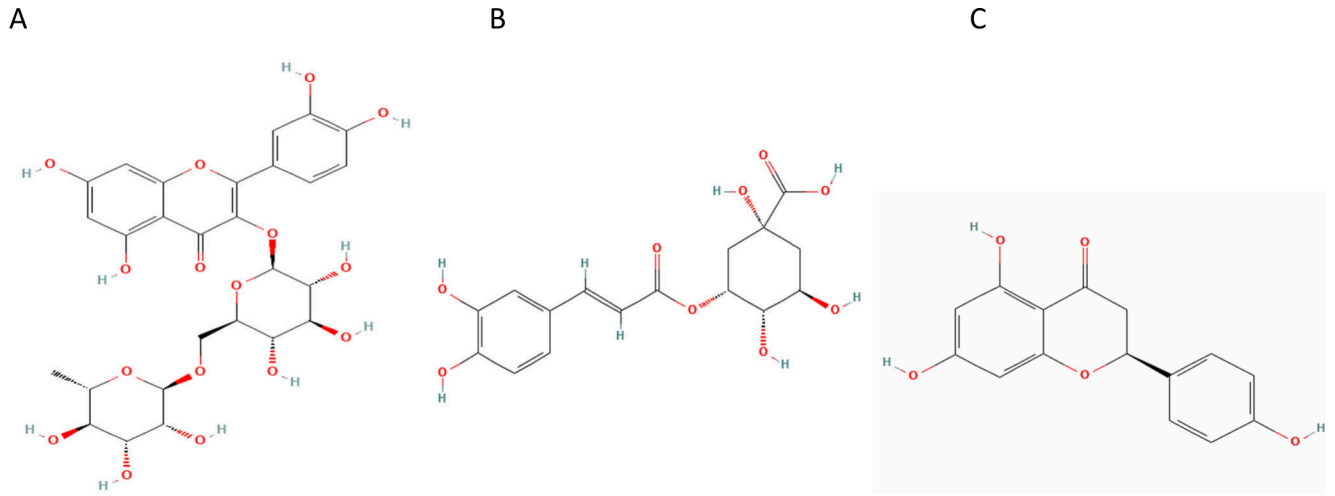

Figure 7. Examples of polyphenols found in *A. platensis* culture, which have π clouds that are well-suited for CH−π bonding to the sulfated disaccharide *O*-rhamnosyl-acrofriose: (A) rutin;[99] (B) neochlorogenic acid;[100] (C) naringenin.[101]

Lee et al. (2000). They then extended their own 1998 analysis of Ca-SP by using two variations of MS called Electrospray Ionization Mass Spectrometry (ESI-MS) and Tandem Mass Spectrometry (MS-MS) with Collision-Induced Dissociation (CID).[33] These methods finally produced some insight into the saccharide sequence of Ca-SP. Samples of the Ca-SP subfraction were first broken into oligosaccharides by partial hydrolysis with sulfuric acid and barium carbonate. The hydrolysates were subfractionated with a Dowex 1X8 resin into a neutral subfraction and an acidic subfraction, labeled as N and A, respectively. The authors concluded that Ca-SP is composed of two types of disaccharide repeating units (Figures 2 and 3).

Characterization of these disaccharide repeating units was a major step toward the sequencing of complete Ca-SP molecules, but there remains more work to be done. The complete sequence of saccharides has not yet been determined, and there are also other subfractions that have not been characterized to this level of detail. Moreover, the study of the biological and chemical behavior of these molecules has only just begun.

DISCUSSION

To date, the line of research started in 1993 by Hayashi et al. and culminating in 2000 with the Lee et al. description of the dissarcharide repeating units of Calcium-Spirulan (Figures 2 and 3) is the most complete and detailed research available on the structure of the REPS of *Arthrospira platensis* and its Ca-SP subfraction.[23,31−33] Figure 5 presents a graphic detailing the sequence of methods and techniques used to arrive at these key molecular structures.

Some interesting observations can be noted based on the currently available data and information. For example, in the first disaccharide unit (Figure 2), both monosaccharide elements display flat, hydrophobic regions on their beta surfaces because their side groups, and their glycosidic bond, face downward, in the alpha direction. This leaves flat, hydrophobic surfaces, facing upward in the beta direction, which might make a binding site for aromatic, hydrophobic amino acids, hydrophobic nucleic acids, or other kinds of molecules with hydrophobic moieties, such as polyphenols.

Pyranose rings can adopt more than 30 different conformational structures, such as "chairs" and "boats".[87,88] They can shift between different conformations as a result of pressures, either physical or chemical, in their local environment.[88] Therefore, a disaccharide such as *O*-rhamnosyl-acofriose (Figure 2) can adopt a large number of different conformations. The multiplicity of pyranose ring conformations increases its specific binding capabilities, because different conformations create different shaped binding sites.[88] Figure 6 shows 7 different conformations of pyranose rings, as presented by Ionescu et al. (2005).[88]

CH−π bonding is an underappreciated theme in chemical biology, which involves noncovalent bonds between an aromatic π-cloud and hydrogen atoms bound covalently to carbon atoms.[89] The π-cloud of an aromatic ring can act as a hydrogen acceptor, while the −CH moiety can act as a hydrogen donor, thus creating a type of hydrogen bond between −CH groups and π-clouds.[90] The total bonding energy of an aromatic ring with a single pyranose ring by this mechanism was calculated to be 3−6 kcal/mol.[90]

For comparison, the hydrogen bond energy between two water molecules has been estimated at 5 kcal/mol.[91] The binding energy of a monoclonal antibody against dextran (a sulfated polysaccharide that bears some resemblance to Ca-SP) to its specific epitope on dextran has been estimated at 4 kcal/mol.[92] The energy required to denature double-stranded DNA is reported to be approximately 1 kcal/(mol bp).[93] Finally, the study of RNA Polymerase binding to DNA (one of the stronger noncovalent interactions) has shown that the viral T7 RNA Polymerase (T7 RNAP) binds to the consensus 23-bp T7 DNA promoter region with a free energy of 16−16.5 kcal/mol.[94] From these comparisons, the CH−π interactions that bind pyranose rings to aromatic compounds are reasonably strong and perhaps even more so when these binding energies are normalized by the molecular surface area of the respective binding sites. The RNAP complex spans 23 bp DNA, whereas the CH−π bond area occupies only a single pyranose ring.

Kiessling and Diehl (2021) also reviewed CH−π interactions.[95] The authors indicated that carbohydrate residues with several C−H bonds facing the same direction—such as the beta surface of disaccharide O-rhamnosyl-acofriose (Figure 2)—have a tendency to form strong CH−π interactions with

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aromatic rings.[95] By this paradigm, the disaccharide *O*-rhamnosyl-acofriose (Figure 2) should exhibit a particularly strong CH−π bond energy because it displays a beta surface with five C−H bonds and no side groups to interfere with CH−π binding on that surface.

It should be emphasized, however, that a flat conformation is required to enable all five of the beta-surface hydrogen atoms for CH−π bonding. In a chair conformation or a boat conformation, fewer hydrogen atoms are enabled due to changes in their spatial positioning. While flat conformations are rare in free-floating polysaccharides, it is possible that a protein binding site could force the molecule into a flat conformation using other amino acid residues that influence the conformation of the ring. This is a topic that has not yet been addressed by the Spirulina research community and may help to explain the biological function(s) of the molecule.

The study of Carbohydrate Binding Domains (formerly known as Cellulose Binding Domains) also reveals that nonpolar, aromatic side chains of amino acids (e.g., tyrosine, tryptophan) play a pivotal role in the specific binding of proteins to the cellulose molecule, so this surface might also be well suited to interface with aromatic side chains of amino acids.[20,96] Interestingly, cellulose monosaccharides do not display the same sort of flat beta surface as that of *O*-rhamnosyl-acofriose (Figure 2). They have side groups that interfere with their CH−π bond energy. This suggests, again, that the CH−π bond strength in *O*-rhamnosyl-acofriose (Figure 2) might be considerably stronger than the CH−π bond strength in cellulose because it has fewer side groups to interfere with the CH−π bonding surface.

Another molecular family with aromatic rings that might be suitable for CH−π binding to polysaccharides is the polyphenols, which have been found in cultures of *A. platensis*, both intracellularly and extracellularly.[97] Phenolic acids such a neochlorogenic acid are also found in *A. platensis*.[98]

In some cases, phenolic acids or polyphenols are bound to sugar rings covalently (e.g., rutin and neochlorogenic acid in Figure 7A,B).[98] In other cases, polyphenols are not covalently attached to sugar rings (e.g., naringenin in Figure 7C).[98] Either way, CH−π bonds are likely to play a role in the shuttling, localization, and/or alignment of polyphenols in *A. platensis*, both intracellularly and extracellularly. Given that polyphenols exhibit strong UV absorptive and protective capabilities, the exopolysaccharides may act as their chaperones for extracellular secretion, where they can form multiple layers of UV-blocking functionality that protects the cells and filaments from UV radiation.

Nucleic acids also contain aromatic rings, although they differ in being nitrogenous. Nitrogenous bases can also interact with the disaccharides (Figures 2 and 3) by CH−π bonding.[102] In fact, some authors contend that the CH−π bonding energy is actually stronger for nitrogenous bases than for pure aromatics like benzene.[103] And, nitrogenous bases are also well-known to exhibit "ring stacking" when they form DNA or RNA polymers,[104] a topic that was reviewed in 1996 by Sponer et al.[105] Therefore, it seems reasonable that such interactions between exopolysaccharides and nucleic acids could also be involved with horizontal gene transfer, both ingoing and outgoing, a phenomenon that has been reviewed many times.[106,107]

To the extent that enzymes and/or antibodies do bind with high specificity and affinity to these two-sided polysaccharides, it stands to reason that some sort of amphiphilic clamp domain or beta sandwich with one side being hydrophobic and the other side being hydrophilic might be involved. CBM Family 3 is known to form a beta sandwich, one configuration of which might display this sort of amphiphilic binding.[108] The study of the interactions of Ca-SP with molecules of biological importance is ongoing. This topic was reviewed recently by Mazur-Marzec et al. (2021).[109]

The Ca-SP subfraction was shown, for example, to enhance the antithrombin activity of heparan cofactor II (HC II) by more than 10^4-fold.[110] Certain mutated versions of HC II were also enhanced by a similar amount, showing that Ca-SP's does not bind to HC II in a manner that involves either of those mutated amino acids.[110] Without calcium or sodium ions in solution, however, the booster effect was lost.[110] Moreover, the addition of certain protein-degrading enzymes interfered with the antithrombin effect, while other enzymes did not.[110] These results point toward very specific and unique binding sites.[110]

Calcium-Spirulan has also been the subject of a line of research on its antiviral activities. In HeLa cells, dose-dependent inhibition of herpes simplex virus type 1 (HSV-1) replication and dose-dependent inhibition of HSV-1 specific protein synthesis, without suppressing host cell protein synthesis, were found.[31] In live hamsters, food containing Ca-SP prolonged the survival time of HSV-1 corneally challenged animals.[31] Ca-SP was compared to dextran sulfate (a comparable sulfated polysaccharide) by three different in vitro assays.[15] Ca-SP was also shown to inhibit replication at different levels of inhibition for an array of enveloped viruses: HSV-1, HCMV, Measles virus, Mumps virus, Influenza virus, Polio virus, Coxsackie virus, and HIV-1.[23] The mechanism mediating this functionality is not entirely clear, but it does appear to be mediated by specific binding rather than general steric hindrance. Indeed, analogous results have been shown by using monoclonal antibodies against the glycoprotein D (gD) protein of HSV-1, which supports the notion that specific binding sites, not generalized steric hindrance, is involved with the anti-HSV-1 functionality of Ca-SP.[111]

Ca-SP has also shown potential as an anticancer treatment. B16-BL6 melanoma cells, colon 26 M3.1 carcinoma cells, and HT-fibrosarcoma cells were all tested in vitro with Ca-SP. It inhibited the invasion of these cells through Matrigel/fibronectin-coated filters.[22] Lung metastasis of the B16-BL6 melanoma cells was also reduced by Ca-SP.[22] In the same study, Ca-SP showed very specific inhibition of the functionality of purified heparinase, which points again to highly specific interactions rather than generalized steric hindrance.[22]

CONCLUDING REMARKS

Great strides toward a better understanding of Ca-SP and the other components of the exopolysaccharide fraction of *A. platensis* have been made by many different researchers around the world. As the tools of chemistry and molecular biology have improved, so has our capability to find clear and concise answers to application questions and our ability to fill in the basic science gaps in our understanding of *A. platensis* and its exopolysaccharides. Still, even with the advantages of new technologies, progress is made only by the focused long-term efforts of dedicated research scientists.

With great appreciation and respect for the work done by the hundreds of researchers referenced herein and others, we conclude by listing some important work that still lies ahead in this field of study.

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(1) the complete sequence of monosaccharides in Ca-SP
(2) the secondary, tertiary, and quaternary structures of Ca-SP
(3) the size, constituents, and sequences of the other subfractions of exopolysaccharides from *A. platensis*
(4) complete and detailed description of the genetics and biosynthetic pathways of these molecules
(5) identification, function, and bond strength of the specific binding sites of these exopolysaccharides, their counterparts, and their functions

These future studies will elucidate new functions of the exopolysaccharides of *A. platensis*, fill annotation gaps in the genome of *A. platensis*, and possibly lead to the discovery of novel biosynthetic enzymes in the biosynthetic pathways of these molecules.

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Notes

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