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BIOACTIVE POLYSACCHARIDES FROM PLANTS*

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Key Word Index—Polysaccharides; isolation; purification; structure elucidation; biological activity.

Abstract—Biologically active polysaccharides isolated from natural sources during the period January 1977 to December 1988 are reviewed. Besides biological activity, the recent techniques used in their isolation, purification and structure elucidation are discussed.

INTRODUCTION

Polysaccharides are naturally occurring polymers of aldoses and/or ketoses linked together through glycosidic linkages. These are called homoglycans when only one type of monosaccharide unit is present and heteroglycan when more than one kind of monosaccharide are the constituent units. Polysaccharides are essential constituents of all living organisms and are associated with a variety of vital functions which sustain life. They are found most abundantly in the seaweeds, fungi and higher terrestrial plants. In the recent years, polysaccharides of plant origin have emerged as an important class of bioactive natural products. Antitumour [1–5], immunological [6–9], anticomplementary [10], anti-inflammatory, anticoagulant, hypoglycemic and antiviral activities [11] have been observed in a wide range of polysaccharides and constitute the subject matter of several excellent reviews. The present review covers the available information on biologically active plant polysaccharides from January 1977 to December 1988 as given in Chemical Abstracts.

Polysaccharides possess complex structures because there are many types of inter-sugar linkages involving different monosaccharide residues. They can form secondary structures which depend on the conformation of component sugars, molecular weight, inter-and intra-chain hydrogen bonding. Since the biological activity of a polysaccharide has an imporatant bearing on its state of purity and chemical structure, it is appropriate to discuss various modern techniques employed for their isolation, purification and structure determination.

ISOLATION AND PURIFICATION

Polysaccharides are usually isolated by precipitation with alcohol from a non-dialysable fraction of water extract of the source material. The precipitate is digested with water, filtered, freeze-dried and subjected to dia-filtration. Usually extraction of neutral polysaccharide is carried out with water and that of uronic acid containing polysaccharides with dilute sodium hydroxide. Kato *et al.* [12, 13] have used zinc chloride for extraction of 6-branched β-1,3-glucan from *Grifola frondosa*. In the case of GZ polysaccharide of *Ganoderma applanatum* [14] fractionation by precipitation of polysaccharide with cetyl trimethyl ammonium hydroxide (CTA-OH) has been found to be specially effective in purifying the antitumour fraction. The same reagent has also been used to precipitate the active LC–11 and 'lentinan' polysaccharide fraction from *Lentinan edodes* while the inactive fractions remain in solution [15]. Fractional solubilization with acetic acid is used to free the polysaccharide from its salt followed by precipitation with ethanol. Complex formation with copper salts or boric acid has also been utilised for purification of polysaccharide fractions [16, 17].

The crude polysaccharide fractions obtained by precipitation are usually further purified by ion-exchange, gel permeation and affinity chromatography. Thus purification of the polysaccharide of *Padina pavonia* [18] has been successfully achieved by fractionation on DE-52 cellulose. Molecular weight of polysaccharides are determined on a Sepharose CL-2B column equilibrated with 0.2 M NaOH Sepharose CL-4B column (0.2 M NaOH–8 M urea) or by gel filtration over Sepharyl 16–200 or 5500 Sephadex G-150 and G-200 and ultracentrifugal analysis. The M_r of H_{11} isolated from *Poria cocos* Wolf [19] has been determined as *ca* 5×10^6 by gel filtration method using CPG-1000 A°.

STRUCTURE DETERMINATION

Methylation

In complex carbohydrates, glycosidic linkages are usually determined by methylation analysis [20–23], which is either performed by Hakomori's method [24] or a modification thereof [25]. Recently, a procedure has been developed by which the glycosidic linkages of complex carbohydrates can be determined at 1–5 μg level [26, 27]. In this technique, saccharides are reduced with sodium borodeuteride prior to per-*O*-methylation. Before

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permethylation, the polysaccharides containing uronic acid residues are passed through a cation exchange resin (H^+ form), in order to convert all the carboxyl groups into the protonated form, which permits a more complete permethylation of polysaccharides containing uronic acid residues. Recovery of the per-*O*-methylated products in good yields are achieved by the use of reverse phase liquid chromatography on Sep-Pak C_{18} cartridges. Identification of microscale partially-*O*-methylated alditol acetates by GC-MS in the multiple, selected ion-mode (stacked) electron-impact, mass chromatography [28] is found to be about seven times as sensitive as standard GC-MS.

Mass spectrometry

Of late, mass spectrometry has emerged as a powerful tool for tackling structural problems relating to carbohydrates. Newer methods of ionisation have now made it possible to analyse the non-volatile, highly polar and large M_r materials with relative ease [29]. The chemical ionisation mass spectrometry has turned out to be the most flexible technique. Its use is, however, limited at present to the oligosaccharides. The unique feature introduced by the variability of the reagent gas provides ample opportunity for characterization of sample by specific gas phase chemical reactivity, adduct-ion formation and ion-identification by isotopic variations in the composition of the reagent gas. Methane, isobutane, ammonia or combinations thereof are mostly used as the reagent gases. CH_5^+, $C_4H_9^+$ and NH_4^+ are the reactive species of these gases in decreasing order of their protonating capability. Methane and isobutane CI mass spectra of saccharides generally carry a large number of fragment ions; on the other hand in the ammonia CI mass spectra, ammonia clusters and sample adduct ions are abundantly observed. Other important features of the ammonia CI mass spectra are the higher stability of molecular ion adduct. Improvements in sensitivity and specificity have been reported by using methane chemical ionisation mass spectrometry and selected ion-monitoring [30].

Field desorption mass spectrometry (FDMS) comprises one of the best soft ionisation techniques especially for highly polar organic compounds, including salts. In FDMS free sugars, di-and tri-saccharides give rise to $[M+H]^+$ and $[M+H-H_2O]^+$ ions with little fragmentation at lower emitter currents. Higher emitter current can be used to produce cleavage on either side of the glycosidic oxygens leading to sequence information. While the lower M_r oligosaccharides can be analysed without much difficulty, saccharides in the M_r range of 2500 can be handled after partial methylation [31]. Recently, the FDMS analysis of a 6-*O*-methylglucose polysaccharide isolated from *Mycobacterium smegmatis* [32] containing 20 hexose units have been successfully achieved.

Incessant search for the newer soft ionisation techniques have culminated in the development of secondary ion (SIMS), fast atom bombardment (FAB) and laser desorption (LD) mass spectromerty, which now find extensive use in the analyses of biopolymers. This approach utilises a beam of projectiles or photons to ionise and desorb the sample molecules deposited on a solid support, while secondary ion (SIMS), fast atom bombardment (FAB) and laser desorption (LD) mass spectrometry have already made significant contributions in the analyses of biopolymers. Heavy ion induced mass spectrometry and $^{252}C_f$ plasma desorption mass spectrometry [33–36] have displayed their potential to desorb the intact biopolymers with M_r upto 7000. The latter two techniques have yet to be commercialized.

In SIMS, an energetic beam of primary ions (e.g. Ar^+) is allowed to impinge on a sample coated on the metallic surface. This results in the sputtering of both positive and negative 'secondary ions' from the sample which are detected by conventional means [37]. FAB mass spectra differs from SIMS in that it has a primary beam consisting of fast atoms (e.g. Ar) rather than ions which strikes the sample dissolved in a liquid matrix, typically glycerol. Ion peaks arising from the cleavage at glycosidic bonds are the dominant features of both SIMS and FAB mass spectra. Under FAB conditions, however cleavage occurs preferentially on the terminal side of the glycosidic oxygen giving predominant peaks with sequential losses of monosaccharide units [38]. Both negative and positive FAB mass spectra can be recorded. This has made the structure determination of oligosaccharides much easier and faster. With partial methylation of the product, it is now possible to reach a polysaccharide of the 3500 M_r range. Thus a glucose polysaccharide containing twenty glucose residues when partially methylated exhibited intense peaks at m/z 3536 and 3554 for $[M+Na]^+$ and $[M+K]^+$ by which the number of hexose units and degree of methylation could be computed [32]. Conversion of the neutral molecules to ionic species can be utilised to increase the reactivity and improve the quality of FAB mass spectra [39]. The application of this method is under investigation with oligosaccharides [40]. LD mass spectrometry is presently limited to the analysis of smaller molecules.

^1H and ^{13}C NMR spectrometry

The determination of anomeric configuration of the number of different sugars present in a repeating unit, the presence of amino and deoxy sugars, the identification of *O*-acetyl groups and other non-sugar substituents are now routinely performed by ^1H and ^{13}C NMR spectroscopy but ^1H NMR spectroscopy has limited utility [41], since the amount of information available from ^1H NMR spectra diminishes with the increasing size and complexity of the molecule. Moreover, signal broadening resulting from the decreased spin–spin relaxation time (T_2) further adds to the intractability of the spectra. An excellent review on the ^{13}C NMR spectroscopy of polysaccharides covering the literature up to 1978, has been published by Gorin [41]. The most valuable information that can be elicited from ^{13}C NMR spectra of polysaccharides is on structural sequences in simple polymers. $J_{C,H}$ values may provide significant information regarding glycosidic configuration. Molecular ratios and chemical structure may be probed by increasing the spin lattice relaxation time (T_1).

The assignments of signals in a polysaccharide can be made by comparison of the chemical shifts with those of the constituent monosaccharides having appropriate configurations and making an allowance for the strong downfield shift of the *O*-glycosylated carbon (α-effect) with a smaller upfield shift of the adjacent carbon signal (β-effect). More recently Saito *et al.* [42–49] examined the conformations of poly-and oligosaccharides such as lentinan, curdlan, cellulose, chitin, cyclodextrin by solid state

cross polarisation magic angle spinning (CP/MAS) ^{13}C NMR spectroscopy. They found that the chemical shift of several carbons of these polysaccharides in the solid are different from those in solution because of the difference in their ultrastructures. The ultrastructure of the antitumour polysaccharide fractions obtained from *Grifola frondosa* [50] were compared by means of cross polarisation magic angle spinning, which led to the conclusion that they possessed two types of conformations, helical and random coil. In the helical form the C-3 signal of β-glucosyl moiety appeared at δ89 whereas in the random coil structure C-3 signal resonated at a higher field (δ86) in the ^{13}C NMR spectrum. Curdlan which is known to exist as a helix in solid state or under physiological conditions, exhibited its C-3 signal at δ90. However, in 0.2 M NaOH or DMSO solution the C-3 signal appeared at δ 87 demonstrating the transformation of the curdlan helix into a random coil. On the other hand, laminarin has been found to possess a random coil conformation under physiological conditions. This technique has been utilised to probe the conformational transition of polysaccharides during extraction and purification with consequent alterations in the biological activity.

BIOLOGICAL ACTIVITY

It has been observed that a number of higher plants, fungi, algae, lichen and bacteria serve as rich sources of antitumour polysaccharides. The nature of these polysaccharides varies from glycans to heteroglycans, lipopolysaccharides and sulphated polysaccharides. Fungi belonging to Basidiomycetes have been reported to elaborate useful antitumour polysaccharides [51–65], viz. lentinan and schizophyllan which are already in clinical use for cancer immunotherapy [66–68]. From various species of *Grifola* active polysaccharides fractions have been obtained [54–59, 69–75]. In all cases the activity was due to a 6-branched (1→3) β-glucan. In general, the (1→3)-β-glucan and polysaccharide having long stretches of (1→3) β-linkages in the main chain are more active as compared to the polysaccharide that contains mainly (1→6) linkages [76, 77], possibly because β-configuration of the D-glucosyl residues affords a helical chain conformation while the molecules of (1→3) linked α-D-glucans have a ribbon like side chain conformation extending along the fibre axis [76, 77]. Besides homopolysaccharides [14, 21, 78–93], a few hetero-polysaccharides have also been isolated [55, 56, 59, 78, 83, 84, 94–106]. Protein bound active polysaccharides have been isolated from *Peziza vesiculosa* [81] and *Coriolus vesicolor* [79, 107–111].

Comparision of the above mentioned active homo- or hetero-polysaccharides, isolated from fungi presents a characteristic feature that they all had the same backbone and differ from each other in the degree and position of branching with a few exceptions [98, 112]. Singh *et al.* [113] in their article on non-cytotoxic antitumour polysaccharide have indicated a possible relationship between activity and structure of simpler glycans.

In general, it has been observed that the soluble D-glucans are active antitumour agents [14, 78, 114–119]. Introduction of stearoyl and phosphoric ester groups into yeast mannan [120] and palmitoyl and phosphoric ester groups into dextran [121] have been reported to increase the antitumour activity. An effect of polyhydroxylation of a highly branched (1→3)-β-D-glucan on antitumour activity has been reported by Misaki *et al.* [122]. Besides, a polysaccharide requires certain ultrastructure for inducing antitumour activity. Thus lentinan, schizophyllan, curdlan possess triple helical structures and polysaccharide fractions (AHW, LLFD, LELFD) of *Grifola frondosa* possess native conformation, which is a prerequisite for the activity [123, 124]. Similarly, it has been found that aggregated molecular structure and small amount of branching is necessary for antitumour activity of *Grifolan* NMF-5N [54].

Immunostimulating activity

Plant polysaccharides seem to offer an important source of immunostimulants [125–135]. It has been noticed that in order to elicit an immune response, a high M_r structure is required. Polysaccharides containing uronic acid have been found to be better stimulants than uronic acid free polysaccharides. Complex polysaccharides of higher plants show a better immunostimulation in lower concentration as compared to simpler fungal glyucans. AIP, a water soluble heteropolymer consisting of uronic acid, hexose and peptide isolated from *Angelica acutiloba*, is a potent adjuvant. The primary antibody response to sheep erythrocytes was markedly augmented by an intra peritoneal injection of AIP [136]. There is a great diversity of polysaccharide components of Siberian ginseng which depends on source, growth condition, climate and nature of the soil [137, 138].

Anticoagulating activity

Sulphated heteropolysaccharides isolated from algae [18, 139–142] showed higher anticoagulating activity than heparin. *Artemisia herba* showed strongest actvity out of the 25 medicinal plant species tested by plasma recalcification test [143]. The active compound was an acidic polysaccharide composed of galacturonic acid and rhamnose with an average M_r of 10 000. Apart from this one exception, all other active polysaccharides are of algal origin. Heparin and dextran sulphate were found to be potent reverse transcriptase (RT) inhibitors [144]. The nonsulphated polysaccharides, e.g. dextran, xylofuranan, ribofuranan, lentinan and curdlan have no anti-HIV activity and do not inhibit RT, but after sulphonation these compounds not only inhibit cell free, but also cell to cell infection of HIV at 10–100-fold lower dose level that inhibited cell growth. It appears that the sulphate group plays an important role in inhibiting growth of HTLV-III [145]. Besides this, the structure of the polysaccharide is also important as homopolysaccharides, (e.g. fucoidan and dextransulphate) are more potent anit-HTLV-III agents than heteropolysaccharides (e.g. heparin and heparin sulphate). Neutral polysaccharides have no effect [146].

Anticomplementary activity

Polysaccharides possessing anticomplementary activity have been isolated from bacteria [147, 148], fungi [149, 150] and higher plants [151–169]. Some of these are also interferon inducers [154]. Anticomplementary polysaccharides like lentinan and pachymaran are water-insoluble and activate the alternative complement pathway [170, 171].

Anti-inflammatory activity. In recent years, polysaccharides preparations from some bacteria [172–176], fungi [177–180] and higher terrestrial plants [139, 181–186] are reported to possess anti-inflammatory activity.

Hypoglycemic activity. A non-sucrose portion of the juice from the stems of *Saccharum officinarum* containing saccharans A–F prominently decreases blood sugar level in mice, saccharan B and E being more effective than other saccharans [81]. Polysaccharides of *Atractylodes japonicum*, *Atractylodes ovala* [187], *Ganoderma lucidian* [188], *Eleutherococcus senticosus* [189] and *Panax gingseng* [168, 190] were found to have significant hypoglycemic activity.

From the above, it is apparent that the polysaccharides isolated from fungi usually show antitumour activity, while higher plant polysaccharides possess immunostimulatory activity and algal polysaccharides, which often contain sulphate, are good anticoagulants.

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