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Review

Chromatography and electrophoresis in separation and characterization of polysaccharides from lichens

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1. Introduction

Polysaccharides from lichens have been studied for many years, and a number of reports on crude structures can be found in the literature. However, many of these studies suffer from lack of purity and homogeneity of the polysaccharides involved, and it was not until recently that chromatographic methods have been used for purification and characterization of lichen polysaccharides. Structural studies have mainly served biochemical and taxonomical purposes, but have also been done in connection with investigations of biological activity.

The traditional method for separation and isolation of the polysaccharides has been the combination of freezing and thawing of the material originally extracted with hot water. During the freeze and thaw procedure, some polysaccharides precipitate while others are kept in solution. Extraction with alkali,

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e.g., NaOH and KOH, has also been performed, either directly or following successive extraction with organic solvents and hot water. Fractionation of lichen extracts prior to chromatography has often been performed in a similar manner to that used for polysaccharides from higher plants [1,2], with the addition of the freeze–thaw fractionation steps.

One of the earliest papers using chromatographic methods in the study of lichen polysaccharides is by Takahashi et al. [3] where different polysaccharide fractions from the lichen *Lobaria orientalis* were separated by gel filtration. Until recently, few other studies were reported using chromatographic methods for separation of the lichen polymers. Thus, in most cases, both chemical and biological investigations were performed on mixtures of polymers.

As discussed below, the polymers studied from lichens are mainly neutral polymers of the glucan type, i.e., β-(1→3)(1→4) lichenan-type, α-(1→3)(1→4) isolichenan-type and β-(1→6) pustulan. A few other lichen polysaccharides have been studied as well, such as galactomannans, glucogalactomannans and a few heteroglycans [4]. But in all surprisingly few studies have been performed.

Below we describe the different chromatographic and electrophoretic methods presented in the literature that have been used for isolation, separation and characterization of the lichen polysaccharides.

2. Isolation and purification

2.1. Ion-exchange chromatography

Crude polysaccharide preparations have been separated by anion-exchange chromatography on different DEAE materials like DEAE-cellulose, DEAE-sepharose 4B and 6B and DEAE-Sephacel. The size of the columns varies between 2.6 and 6.0 cm in diameter and 23 and 100 cm in height. The columns are either converted into the chloride or phosphate form. After application of the polymers, the columns are first eluted with water or a low concentration of phosphate buffer (pH 6.8), followed by a gradient or stepwise elution with either NaCl up to 1 *M* or phosphate buffer up to 0.1 *M*. The elution profiles are monitored by the following methods: UV at 206 nm, refractive index (RI) (although not a preferred method due to interaction with the gradient), polarimetry or by the phenol sulphuric acid assay [5] of the fractions obtained. Fractions containing carbohydrate polymers are pooled, dialysed and lyophilised [6–15].

2.2. Gel permeation chromatography (GPC)

GPC is often a method of choice for further separation and purification following anion exchange chromatography. Various gels have been used like Sephacryl S-400 HR, Sephadex G-200 and Sepharose 6B. The column sizes vary between 1.6 and 2.5 cm in diameter and 45 and 88 cm in height. The columns are eluted with H_2O, phosphate buffer and in some cases with dilute alkali for polymers being difficult to dissolve in water. The elution profiles are monitored as described above, and the part of the eluent containing carbohydrate material is pooled, dialysed and lyophilised [3,6–9,16,17]. Preparative GP-HPLC can be used if ordinary GPC is not sufficient to obtain a homogenic polymer. A Superose 6HR 10/30 column was used for preparative purification of the water-soluble lichen heteroglycan thamnolan [9].

3. Determination of homogeneity and M_r

3.1. Electrophoresis

Electrophoretic examination of isolated polysaccharides from lichens gives information of the homogeneity of the polymer. The polysaccharides are applied onto cellulose acetate as the electrophoretic support and the electrophoretic buffer 0.05 *M* borax–NaCl, pH 9, is used. After finishing the electrophoresis, the polysaccharide material is located with Procion blue [16,18,19].

3.2. Gel permeation chromatography

GPC is also used for the examination of the homogeneity and determination of M_r of the isolated polymers. Conventional GPC is done on the following media: Sephadex 200, Sepharose 4B, Sepharose CL-4B and -6B. The column sizes vary between 0.85 and 1.8 in diameter and between 25 and 61 cm in

height. Elution of the polymers is monitored as described above. For M_r determination different dextrans and pullulans are used as standards [10,11,16,19–21].

HP-GPC is also a method that has been used for determination of homogeneity and M_r. The columns μ-Bondagel E-500 and E-1000 and Superose 6HR 10/30 have been successfully used. A 10–20-μl sample of a 1% solution of the polymer is applied onto the column and the eluate is monitored by refractometry and UV detection at 206 nm [6–9].

4. Determination of the monosaccharide composition

4.1. Thin-layer chromatography (TLC)

Qualitative determination of the sugar composition in hydrolysates of lichen polysaccharides has been performed on silica gel 60 F_{254} TLC plates with butanol–acetone–acetic acid–H_2O (7:7:2.4) as the solvent, and aniline diphenylaminophosphoric acid (100 °C, 10 min) or naphthoresorcinol (100 °C, 10 min) as detection reagent [6–8].

4.2. Gas-liquid chromatography (GC)

4.2.1. Trimethyl silyl (TMS) derivatives of the methyl glycosides

The polymer is first methanolysed using 4 *M* HCl in methanol at 80 °C for 24 h. After removal of the reagents flushing with nitrogen, the TMS reagent (hexamethyldisilazane–trimethylchlorosilane–pyridine, 2:1:5) is added and the mixture injected directly into the GC. Quantitative determination of the monosaccharide composition has been performed on a DB 5 capillary column (30 m×0.32 mm I.D.) with a film thickness of 0.25 mm, fitted into a Carlo Erba 6000 Vega Series 2 gas chromatograph fitted with a FID (flame ionisation detector) using a Shimadzu C-R6A integrator. The injection temperature was 260 °C and detector temperature 310 °C. The initial column temperature was 140 °C, then increased by 1°/min to 170 °C followed by 6 °C/min to 250 °C. The carrier gas was helium [8,9].

4.2.2. Alditol acetates

Alditol acetates can be prepared using the following procedure: the polymer is hydrolysed using 2 *M* trifluoro acetic acid (TFA) at 100 °C for 2 h. The reagent is then removed by flushing with nitrogen, followed by reduction of the monomers to the corresponding alditols using sodium borohydride (freshly made 1 *M* in 2 *M* ammonium hydroxide). The reaction is stopped by addition of acetic acid and the reagents removed by thorough evaporation to dryness. The product is then acetylated using 1-methylimidazole and acetic anhydride for 10 min. The excess anhydride is destroyed by addition of water and the resulting alditol acetates extracted into dimethylchloride. The quantitative determination of the monosaccharide composition can then be determined by gas chromatography on a glass column (3 m×2 mm) packed with 3% OV225 on Chromosorb W-HP at 215 °C. Inositol is often used as internal standard. A glass column (0.4×200 cm) packed with ECSS-M 3% on Gas Chrom Q can also be used [6,11].

GC–MS of the alditol acetates was carried out on a capillary column (30 m) DB-210 coupled to an ion-trap mass spectrometer [16].

5. Linkage analysis

The polysaccharide is converted into the fully methylated polymer after first having deprotonized the secondary hydroxyl groups under alkaline conditions followed by addition of methyl iodide. After complete acid hydrolysis, the obtained partly methylated monosaccharides are converted to the corresponding alditol acetates as described above. The partly methylated alditol acetates thus obtained [21] are used for the linkage determination of different monomers present in the lichen polysaccharides. By GC alone the compounds were separated on a CP Sil 19 CB WCOT fused capillary column (2 m×0.23 mm I.D.) with a temperature gradient from 150 to 210 °C, and an increase of 4 °C/min, gas flow 1 ml/min and a FID detector. When analysed by GC–MS the column was a CP Sil 19 CB capillary column (25 m×0.23 mm I.D.), with a temperature gradient from 150 to 220 °C, with an increase of 5 °C/min [5].

Another GC–MS system has also been used, having the following specifications: the sample was injected into a split/splitless injector in the split mode onto a Supelco fused-silica capillary column (30 m×0.20 mm I.D.) with a film thickness of 0.20 mm. The column was inserted directly into the ion source of the mass spectrometer. The injector temperature was 250 °C and detector temperature 300 °C. The column temperature was 80 °C at the injection time, after 5 min, the temperature was increased at 30 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then 30 °C/min to 300 °C and kept for 25 min. Helium was used as carrier gas at a flow-rate of 0.9 ml/min. The gas chromatograph in the system was a Fisons Instrument GC 8000 series (8065) equipped with an MD 800 Mass Selective Detector 5970 [8,9,11]. Sone et al. [11] analysed the derived partly methylated partly acetylated alditols using a Shimadzu LKB 9000B GC–MS equipped with a column of 3% ECSS-M on Gas Chrom Q. GC–MS on capillary columns DB-210, OV-225, SP-2340 and SP2380 have also been used for linkage determination of lichen polymers [12,16].

Although outside the scope of this review, the importance of NMR in structure characterization of polysaccharides, e.g., linkage analysis, must be emphasized.

6. Other aspects

HPLC has been used to determine oligosaccharides produced by acetolysis. As an example, the use of a Delta-PAK C_{18} column, eluting with H_2O–CH_3CN (3:2), flow-rate 3 ml/min, can be referred to Ref. [20]. An HPLC Sensyu-Pak Aquasil SS-452 N column (25 cm×4.6 mm I.D.), has been used for the analysis of Smith-degradation products of polysaccharides. The method can apparently also be used for preparative separation [22].

Smith degradation products were also analysed by GC–MS on the OV-225 column [16]. Recently the isolation of glycolipids from a Brazilian lichen was reported using a column of silicic acid (particle size, 0.1 mm), using $CHCl_3$–MeOH as eluent. Structure elucidation was carried out using NMR and GC–MS [23].

Both the content and positions of acetyl groups have been determined by gas chromatography of polysaccharides from *Umbilicaria* spp. [24]. Determination of the content of acetyl groups in the polymers were performed on a column packed with 20% tetramethyl cyclobutanediol adipate–4% phosphoric acid on Chromosorb W (80–100 mesh) at 120 °C using propionic acid as internal standard [25].

7. Discussion

The polysaccharides in lichens are traditionally isolated via a process of freezing and thawing of hot water extracts. When thawed, a precipitate is formed, which often has been recognised as a pure polysaccharide. Polysaccharides can also be found in the supernatant after this procedure. These processes led in the early days to the isolation of lichenan and isolichenan, respectively, β- and α-(1→3)(1→4) glucans [26–28]. The polysaccharides are not always easily dissolved in water, neither cold nor hot, but are extractable with alkaline solutions [5,8]. In certain cases polysaccharides were isolated by precipitation with barium hydroxide and Fehling's solution giving rise to galactomannans and mannans [29–31].

These methods will normally give somewhat impure polysaccharides and the development of column chromatographic methods, especially ion-exchange chromatography and gel filtration has led to the development of new procedures for obtaining pure polysaccharides. As seen from the reference list of this review, relatively few projects have up to now been published using chromatographic methods for preparation and isolation of pure polysaccharides from lichens.

Various lichen polysaccharides exhibit different types of biological activities, and this has been the focus for numerous studies performed by Japanese groups [32–34]. However, chromatographic methods were not used for obtaining pure compounds for these studies.

On the other hand, chromatographic procedures have been included in some studies for the isolation and molecular size determination of different types of glucans, mannans and complex mannans and a group that can be classified as heteroglycans.

The glucans isolated and/or partly characterized

with column chromatographic methods include α-(1→3)(1→4) glucans from *Stereocaulon paschale* [15] and other stereocaulaceous lichens in a taxonomic study [3] and an immunologically active α-(1→3)(1→4) glucan from *Cetraria islandica* [8]. Furthermore, an α-(1→4)(1→6) glucan from *Xanthoria parietina* [13] has been isolated.

β-Glucans include a lichenan-type β-(1→3)(1→4) glucan from *Hypogymnia physodes* [17], a linear β-(1→6) and a branched β-(1→3) glucan from *Gyrophora esculenta* [11] and an acetylated β-(1→6) glucan from *Umbilicaria pustulata* [21].

The mannans found have an α-(1→6) mannose backbone with side chains containing either only galactose or both galactose and glucose. They vary in structure and the galactose units are present both in furanose and pyranose forms [6,7,11,12,16,20].

The heteroglycans isolated have little in common from a structural point of view. Various antitumor active polysaccharides were isolated from several lichens belonging to the Stictaceae, and those from *Lobaria orientalis* have been studied most extensively. They are of a complex nature, and contain the sugars rhamnose, mannose, arabinose, galactose, xylose, glucose and glucosamine in different amounts in the fractions isolated [3]. From *Sticta* spp. Da Silva et al. [10] isolated a polysaccharide, rich in β-(1→4) Xyl*p* units, also containing the sugars arabinose, mannose and glucose. Recently a novel polysaccharide with an unusual structure has been isolated from *Thamnolia subuliformis*. This polymer consists basically of β-(1→3) linked galactofuranosyl units, with branches dominated by (1→2) linked rhamnosyl units [9].

Isolation of the lichen polysaccharides is not an easy task, and due to the nature of them, it is not possible to apply an extract directly onto a column and perform separation in the manner that can be done for polymers that are easily soluble in ordinary solvents. A combination of the traditional freeze and thaw procedure of water-soluble polymers should be combined with anion-exchange chromatography followed by gel filtration. From the experience the authors have with isolation of lichen polymers from different species, the procedures described in Figs. 1 and 2 could be used as a guide for other scientists being interested in the isolation of polysaccharides from lichens.

As mentioned above, GPC is used for determination of the homogeneity of the polymer. This is a good method, and when combined with the electrophoresis of the borate complexes, a good impression of the purity of the material is obtained. GPC is often the method of choice for the determination of the molecular size. But as the standards available, dextrans and pullulans, have normally a different hydrodynamic volume than the polymers that are analysed, the obtained M_r is only indicative of the size, not absolute. This is a major problem for the determination of the size of polysaccharides in general. In future, the laser light scattering as a method for determination of the absolute molecular mass will be of importance.

The monosaccharide composition can be determined by different methods. Thin-layer chromatography will give the composition of the polymer, with only rough indication of the quantities. The more rewarding method of gas chromatographic analysis of derivatives obtained after hydrolysis of the lichen polysaccharide will give both identity and quantity of the monomers present. Both gas chromatographic methods mentioned above are relevant to use. The advantage of using the method based on the alditol acetates, is that each monosaccharide will only be present as one peak and if combined with a mass spectrometer, ether-linked substituents to any of the compounds present can be identified. This method will give problems concerning the determination of uronic acid, which can be present in polysaccharides. The other method described is based on the formation of methyl glycosides of the monomers. The uronic acids will form the methyl ester as well, and in some cases also some internal lactones. But when methanolysis is performed until equilibrium between the different form have been achieved, the GC pattern gives a good finger print for proper identification, and as the ratio will be the same between the peaks each time, the major peaks can be used for quantification of the uronic acids. One drawback with this procedure is that all monosaccharides will give rise to several peaks. Depending on the monomer they can vary between two and five for one monosaccharide. This must be taken into account when preparing the standard curves. The advantage of this is that the patterns of the peaks will function as a fingerprint for each sugar and as such be a good system for identification of the different sugars. As mentioned the uronic acids can also be determined in

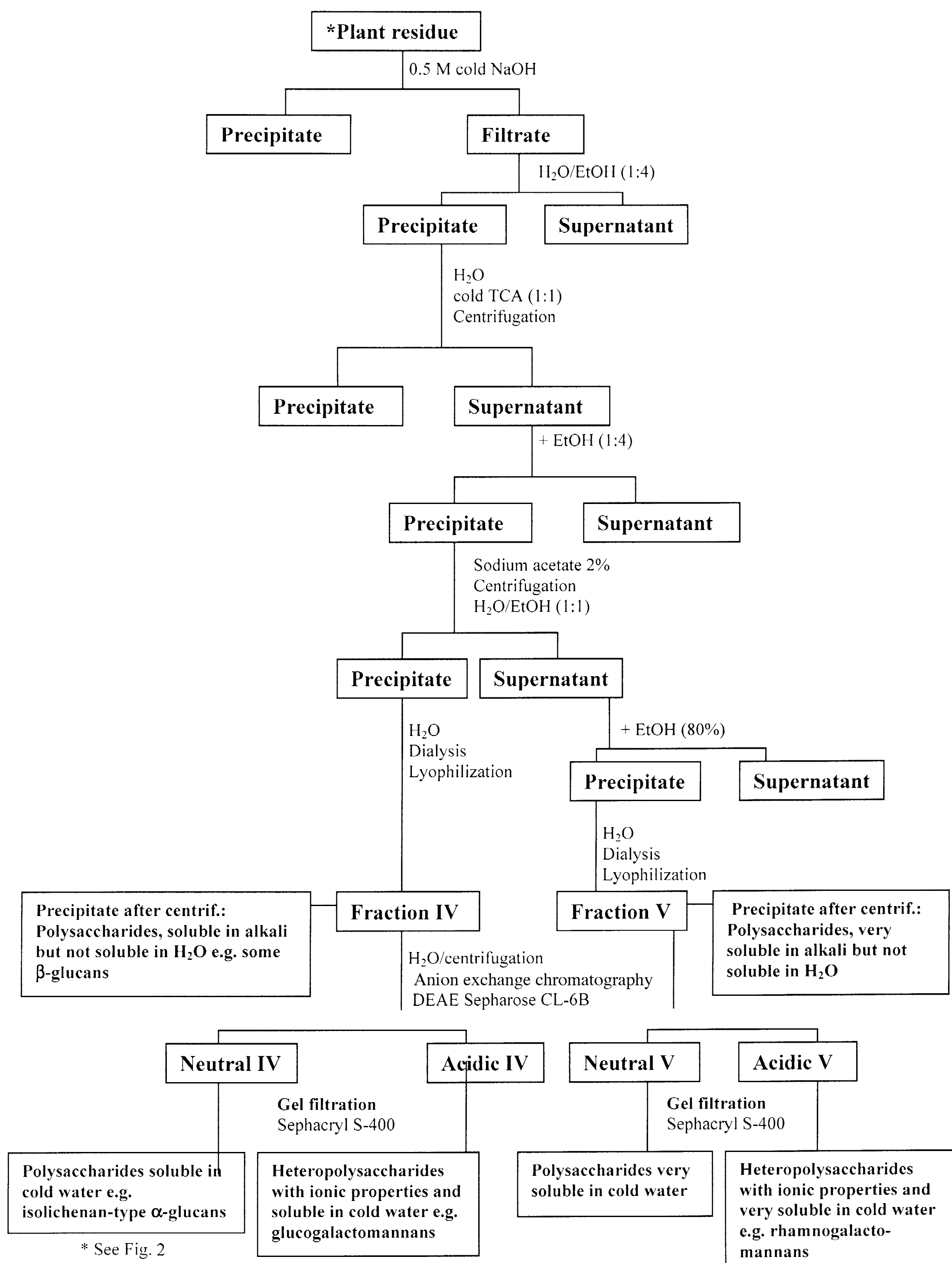

Fig. 1. Useful scheme for isolation, fractionation and purification of lichen polysaccharides extractable with water based on previously described procedures [7,35].

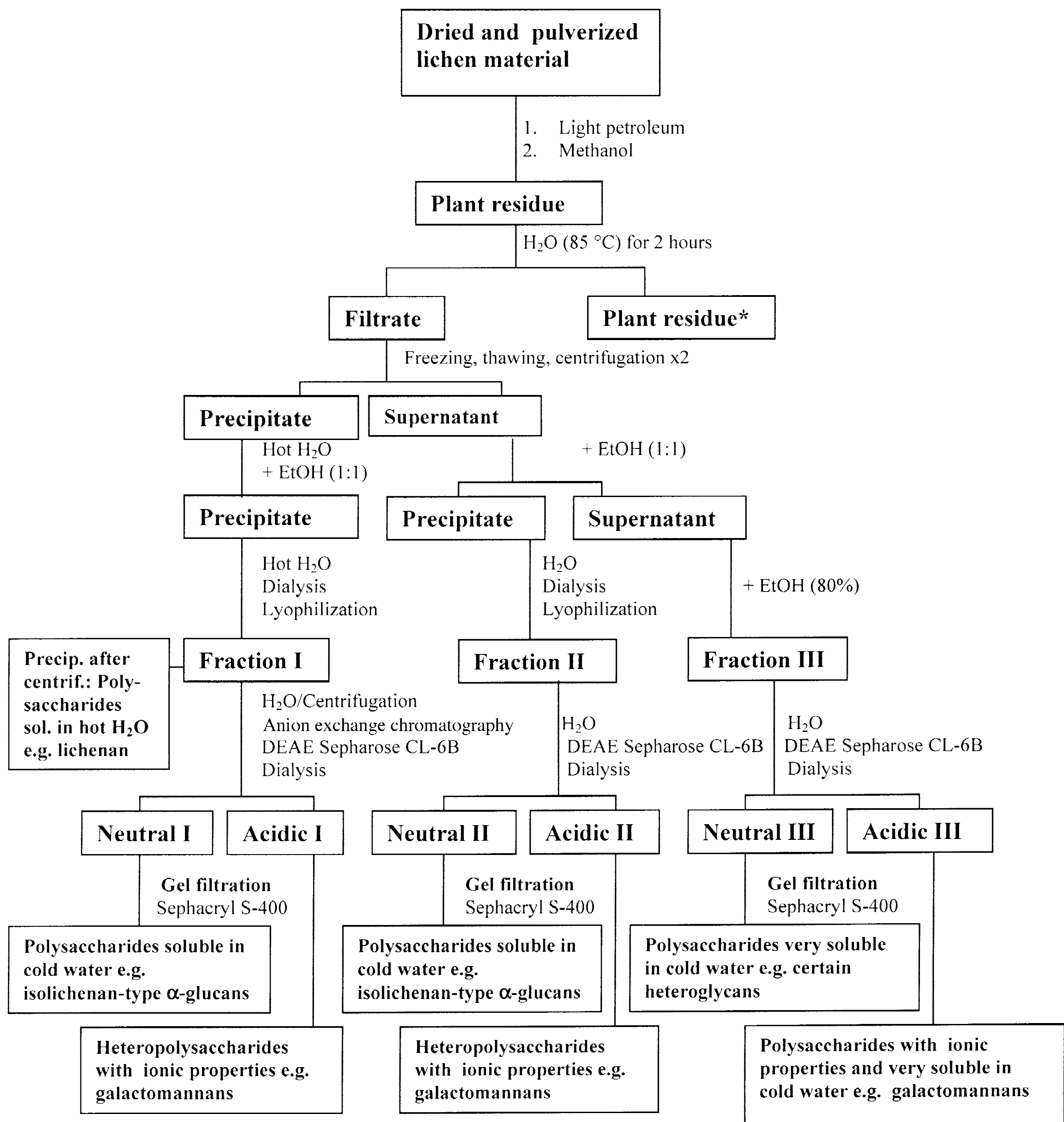

Fig. 2. Useful scheme for isolation, fractionation and purification of lichen polysaccharides extractable with alkali, based on previously described procedures [1,2,6].

the same run as all the other components present from the polysaccharide.

For linkage analyses, combined GC–MS is a must. This is performed on the partly methylated alditol acetates obtained from the original polysaccharide. These compounds can be prepared via different methods, but the principles behind all methods are the same. The hydroxyl groups not involved in a linkage are deprotonated in alkaline medium and methyl derivatives are prepared. After hydrolysis, the aldose products are reduced to the corresponding alditol and converted into the acetates. These are then subjected to GC–MS. Different GC columns can be used, and depending on the nature of the polymer being analysed, only one column can be enough, but in certain cases it is advisable to record the data from the sample run on two different columns. Mass spectrometry will give the answer to the position of the original linkages between the monomers, and if sodium borodeuteride is used as the reduction reagent, it is possible to distinguish between derivatives that otherwise would have been symmetrical around the central carbons of the alditol chain.

Depending on the degree of details that one wishes to obtain regarding the total structure of the polymer, partial acid hydrolysis and periodate oxidation followed by weak acid hydrolysis can give information on smaller parts of the polymer. Certain sequences can be found, and in order to do so, a separation on certain gel permeation media can be used, and as reported above HPLC is also a method used for identification and isolation of oligomers obtained after different types of degradation of the polymers.

8. Conclusion

Taking into consideration the established biological activity of many lichen polysaccharides [4], it can be expected that these compounds will attract further interest with subsequent requirements for adequate purification processes. The bioactivities reported are antitumor and immunological activities, antiviral activity and hippocampal potentiation [4]. Chromatography in the study of polysaccharides from lichens has not been used to a great extent, but in future studies the use of different column chromatographic procedures, including GP-HPLC, should be amongst the methods of choice. For further analyses both GC and HPLC, in each case combined with MS, will also be of importance. For homogeneity studies of the preparations, capillary electrophoresis (CE) will most certainly be a valuable tool in the future and will probably replace the electrophoresis of the borate complexes on cellulose acetate matrices. Capillary electrophoresis has not yet been reported as having been used for analyses of lichen polymers.

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