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Chapter 8

Lichen Polysaccharides

Desiree Nedra Karunaratne, R.G.U. Jayalal and V. Karunaratne

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1. Introduction

Lichens are symbiotic organisms composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, which may be either a green alga or a cyanobacterium (Nash III, 1996; Wolseley and Aguirre-Hudson, 1994 and Yoshimura, 2004). The mycobiont in this combination is an ascomycete or a basidiomycete and the photobiont a green alga or a cyanobacterium. Some lichen species can contain more than one green algal species as photobionts (Friedl and Gärtner, 1988; Friedl, 1989; Ihda *et al*., 1993). At present, about 13,500 fungal species have been recognized to be involved in lichen symbiosis (Hawksworth *et al*. 1995; Kirk *et al*., 2001). However, Sipman and Aptroot (2001) stated that this number could be as high as 20,000 after including "orphaned" species; according to Lumbsch *et al*., (2011), about 10,000 of lichenized fungi have been estimated as undescribed species.

The hyphae of mycobionts are septate, branched, thin or thick walled, and the walls are colourless or variously coloured. It is necessary that the photobiont, which is essentially aquatic in nature, remains protected from desiccation in a lichenized terrestrial condition. The protection is provided by the mycobiont which forms the bulk of the thallus, by the development of specialized hyphal tissues in the form of a cover or cortex over the stratum of the photobiont. The development of the cortex is assumed to be stimulated by the photobiont (Ahmadjian, 1987). In some gelatinous lichens with cyanobacteria, the polysaccharidic sheath produced by the photobiont (cyanobacteria) contributes to water retention (Prieto *et al*., 2008). Lichens have been used for ecological, medicinal and other economic purposes for over 100 years and these beneficial effects have been correlated to some extent with their polysaccharide content. Amongst the identified lichens so far, about 100 species have been studied for their polysaccharides and their composition (Cordeiro *et al*., 2005).

2. Separation and characterization

The cell wall of fungi is composed mainly of polysaccharides such as lichenan, isolichenan, and galactomannan (fig.1) (Elix and Stocker-Wörgötter, 2008). Bernard and Latgé (2001)

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described that the fungal wall is a complex structure composed typically of chitin, 1,3-β- and 1,6-β-glucan, mannan and proteins, although wall composition frequently varies markedly between the species of fungi.

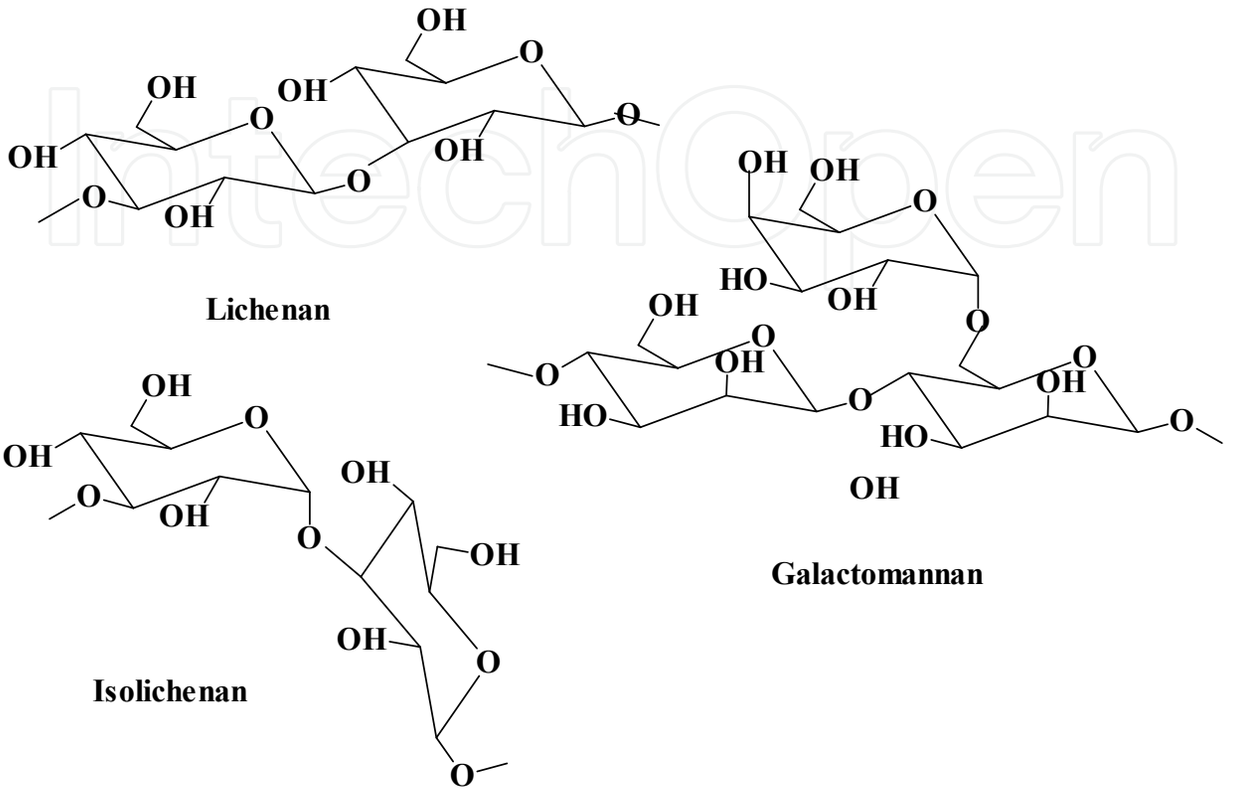


Figure 1. Structures of some polysaccharides contained in fungal cell wall

For the separation and isolation of lichen polysaccharides, traditional methods and modern techniques are used. Traditional methods basically involved freezing and thawing of the material originally extracted with hot water. Dialysis and ethanol precipitation has been employed for further purification. Also, alkali solutions have been used to extract these compounds. The polysaccharides present in the thalli of *Umbilicaria mammulata* were isolated by an exhaustive method starting with successive extraction with hot water at 100°C followed by extraction of the residue with hot 2% KOH at 100 °C. The alkaline extract was further treated with Fehlings solution to yield the 1,3-β- glucan from the supernantant and a 1,6-β- glucan was obtained from the precipitated Fehlings complex (Carbonero *et al*., 2006). In our laboratory, we have used a similar alkaline extraction procedure to isolate a polysaccharide from *Usnea* cf. *cornuta* Körb. Chromatographic techniques and filtration devices were utilized to separate lichen polysaccharides (Paulsen *et al*., 2002). However, different column chromatographic methods, including GP-HPLC, HPLC and ion exchange chromatography have been used recently to separate polysaccharides from lichens. Olafsdottir *et.al* (1999), fractionated the polysaccharides from *Cetraria islandica* on DEAE Sepharose CL-6B anion exchange columns with a 0 -1 M NaCl gradient. Further purification was performed by Sephacryl S-400 gel filtration. To determine homogeneity and M_r of these

compounds, electrophoretic methods and gel permeation chromatography are used. For the determination of monosaccharides, TLC and GC methods are used. The monosaccharides could be derivatized into alditol acetates or into trimethylsilyl (TMS) ethers for analysis by GC. The linkage analysis is performed by using NMR spectroscopy (1D proton, 2D-COSY, NOESY, 2D-TOCSY, 1H 13C-HSQC, HMBC, H2BC and HSQC-NOESY) (Paulsen *et al*., 2002 and Jensen *et al*., 2010) and methylation analysis (Omarsdottir *et.al*., 2005; Olafsdottir *et. al*., 1999; Ruthes *et al*. 2010).

3. Biological source of the polysaccharide fraction

Whether these polysaccharides are produced by the mycobiont or photobiont separately or in symbiosis has been debated for a long time. Takahashi *et al*. (1979) showed that aqueous extracts from cultivated myco and photobiont had different monosaccharide composition and physical properties. It was also found that while the extracts of the mycobiont had a similar composition to that of the parent intact lichen, the photobiont fractions were different from those of the symbiotic thalli and its mycobiont. Complete structural analysis by Cordeiro *et al*. (2004) confirmed Takahashi's results, and showed that the nigeran, laminaran and the galactomannan, found previously in the symbiotic thalli of *Ramalina peruviana* (Cordeiro *et al*. 2003) were also generated by the aposymbiotically cultured fungal partner.

Three different polysaccharide structural types: β-glucans, α-glucans (linear or lightly substituted), and galactomannans (branched) (Carbonero *et al*, 2005a) are present in the fungal cell wall. According to Olafsdottir and Ingólfsdóttir (2001), all the polymers present in lichen thalli are categorized into glucan type [β-(1→3)(1→4)], lichenan type [α-(1→3)(1→4)] and pustulan [β-(1→6)]. Some of these types are depicted in Table 1. However, the recent discovery of a few additional complex heteroglycans, such as rhamnogalactofuranan (Olafsdottir, 1999), galactomannoglucans (Woranovicz-Barreira, 1999 and Carbonero *et al*., 2002) and thamnolan (Carbonero *et al*., 2005a) necessitated the closer examination of the origins of the polysaccharides. Studies carried out by Cordeiro *et al*., (2005), revealed that the algal partner (*Trebouxia* sp.) also consists of some carbohydrates such as β-galactofuranan heteropolysaccharide. Later in 2007, new polysaccharides xylorhamnogalactofuranan and Xylan in the photobiont *Asterochloris* sp. were recorded by Cordeiro *et al.* Thereafter, Jensen *et al*. (2010), isolated another heteropolysaccharide called colleman from a cyanobacteria present in the lichen *Collema flaccidum*.

In the past, lichen polysaccharides have been extracted from the whole thallus without giving consideration to the origin of components such as the fungal partner or the photobiont (Gorin and Iacomini, 1984, 1985; Gorin *et al*., 1993; Teixeira *et al*., 1995). Later Cordeiro *et al*. (2005, 2007, 2008), found that, dissimilarities between the polysaccharides extracted from the cultivated photobionts *Trebouxia* and *Asterochloris* with those from their respective lichens could indicate that the photobiont or mycobiont was responsible for these differences. Galactoglucomannans were isolated from the mycobiont of *Parmotrema* species including *Parmotrema austrosinense, Parmotrema delicatulum, Parmotrema mantiqueirense,*

Polysaccharide	**Main unit**	**Side chain**	**Lichen species**	**Mycobiont/ Photobiont**	**reference**
Lichenan	Homoglucan with β(1→3) (1→4) linkage		*Cetraria ilandica, C. nivaris, C. richardsonii, Usnea barbata, U. lingissima, U. bayleyi, Parmelia tinctorum, P. conspersa, P. hypotrypella, P. nikkoensis, Alectoria sulcata, A. sarmentosa*	Whole thallus	Shibata, 1973
Isolichenan	Homoglucan with α(1→3) (1→4) linkage		-do-	-do-	Shibata, 1973
Pustulan	Glucan with β(1→6) linkage		*Lasallia pustulata, L. papulosa, Umbilicaria hirsuta, U. angulata, U. caroliniana, U. polyphylla,Gyrophora esculenta*	-do-	Shibata, 1973
Everniin	Glucan with α(1→3) (1→4) linkage		*Evernia prunastri*	-do-	Shibata, 1973
Acroscyphan	Homoglucan with α(1→3) (1→4) (1→6) linkage		*Acroscyphus sphaerophoroides*	-do-	Shibata, 1973
Galacto-glucomannans	a (1→6)-linked main chain of ◎-Manp	O-2 and O-4 by α-Galp and β-Galp nonreducing end-units	*Parmotrema austrosinense, P. delicatulum, P. mantiqueirense, P. schindlerii, P. tinctorum and Rimelia (R. cetrata* and *R. reticulata)*	mycobiont	Carbonero *et al*., 2005b
Xylorhamno-galactofuranan	(1→3)-linked galactofuranosyl	galactofuranosyl units 5-*O* and 6-*O*-substituted, rhamnopyranosyl units 2-*O*, 3-*O* and 2,3-di-*O*-substituted in position 6.	*Cladina confusa*	Photobiont	Cordeiro *et al*., 2007

Polysaccharide	Main unit	Side chain	Lichen species	Mycobiont/ Photobiont	reference
laminaran and pustulan and galacto-furanomannan	(1→3)-and (1→6)-linked β-glucans	(1→6)-linked α-mannopyranosyl	*Umbilicaria mammulata*	Mycobiont	Carbonero *et al*., 2006
Hetero-polysaccharide	(1→5)-linked galactofuranosyl units	Complex	*Ramalina gracilis*	Photobiont	Cordeiro *et al*., 2008
O-methylated mannogalactan	(1→6)-linked β-galactopyranose	at O-3 by β-Gal*p*, 3-OMe-α-Man*p* or α-Man*p* units	*Peltigera aphthosa*	Photobiont	Cordeiro *et al*., 2010
Colleman like	Complex heteroglycan	2-OMe Man*p*, 2-OMe-Arap, Xyl*p* and Gl*cpA*	*Collema flaccidum*	Whole thallus (cyanobacteria)	Jensen *et al*., 2010
	(1→4)-linked β-D-xylan		*Peltigera canina*	Mycobiont	Ruthes *et al*., 2010

Table 1. Summarized data of lichen polysaccharides and their origin

Parmotrema schindlerii, Parmotrema tinctorum and *Rimelia cetrata* and *Rimelia reticulata* by Carbonero *et al*., in 2005b. These galactoglucomannans consisted of (1→6)-linked main chain of α-Man*p* units, which were substituted preferentially at O-2 and O-4 by α-Gal*p* and β-Gal*p* nonreducing end-units, respectively. Further, they also isolated two galactomannan fractions from the lichen, *Roccella decipiens*. One galactomannan fraction had a main chain with (1→4)-linked α-D-Man*p* units, substituted at O-2 with side chains containing a nonreducing end, 2-O- and 6-O-substituted α-Man*p* units. The other fraction had a similar α-D-Man*p* core structure, but with side chains containing nonreducing end, 5-O-, 6-O-, and 5,6-di-O-substituted β-D-Gal*f* units (Cordeiro *et al*., 2005). Another heteropolysaccharide xylorhamnogalactofuranan was isolated from the lichen *Cladina confusa.* It consisted of (1→3)-linked galactofuranosyl units with side chains of galactofuranosyl units 5-*O* and 6-*O*-substituted, as well rhamnopyranosyl units 2-*O*, 3-*O* and 2,3-di-*O*-substituted at position 6. Nonreducing end units were composed of Xylose (Cordeiro *et al*., 2007). In 2006, (1→3)-and (1→6)-linked β-glucans, namely laminaran and pustulan and galactofuranomannan which have a main chain of (1→6)-linked α-mannopyranosyl residues, partially substituted at O-2, O-4 were isolated from another lichen *Umbilicaria mammulata* (Carbonero *et al*., 2006). Later in 2008, Cordeiro *et al*. found Galactofuranose-rich heteropolysaccharide from the lichen *Ramalina gracilis*. This polysaccharide has (1→5)-linked galactofuranosyl units at the main chain with very complex branched structures of side chains in position 6. They found that this polysaccharide arose from the algal symbiont *Trebouxia sp.* of the lichen *Ramalina gracilis*. An O-methylated mannogalactan was isolated from *Peltigera aphthosa* by Cordeiro *et al*. in 2010. This consisted of (1→6)-linked β-galactopyranose main chain partially substituted at O-3 by β-Gal*p*, 3-OMe-α-Man*p* or α-Man*p* units. The algal symbiont *Coccomyxa mucigena* of the lichen *Peltigera aphthosa* was thought to be the origin of this heteropolysaccharide since the lichen thallus yielded a polysaccharide of different structure. A colleman like heteropolysaccharide was isolated from the cyano lichen *Collema flaccidum*.

Colleman is a complex heteroglycan containing the unusual monosaccharides 2-OMe Man*p* and 2-OMe-Arap as well as Xyl*p* and Gl*cpA* (Jensen *et al*., 2010). The presence of uronic acids has been reported previously from cyanobacterial polysaccharides. Since the structural features and sugar content of colleman is representative of polysaccharides of cyanobacterial origin, it is proposed that colleman originates from the cyanobacterial partner. Ruthes *et al*. (2010) were able to isolate (1→4)-linked β-D-xylan (an EPS) and heteropolysaccharide with a complex structure of β-L-Ara*p* and β-D-Xyl*p*-(1→4)-linked units from *Peltigera canina*. Again it was opined that the photobiont, *Nostoc muscorum* was the source of this heteropolysaccharide.

4. Polysaccharides as a taxonomic tool

The identification and classification is generally based on morphology of the organism. Taxonomy of lichen species have been corroborated by phylogenetic applications with the advances in DNA technology. Lichen polysaccharides have been used as a taxonomic tool and chemotaxonomic classification has resulted in clarification of conflicting taxonomic data.

The lichen-forming ascomycete order Lichinales comprises around 250 species and is distributed among 52 genera and four families (Eriksson, 2006). Earlier molecular studies (Wedin *et al*., 2005) did not confirm its phylogenetic relationships, although the order was treated as a separate class, *Lichinomycetes* (Hibbett *et al*., 2007). Since alkali and water-soluble polysaccharides from *Lichina pygmaea* and *L. confinis* reflect phylogeny in other ascomycetes (Prieto *et al*. 2008), an isolated polysaccharide was purified to investigate whether such polysaccharide compounds in the Lichinomycetes are distinctive. Results support molecular studies showing that lichen species are remote from Lecanoromycetes as the galactofuranose residues are in the α-configuration. That the Lichinomycetes were part of an ancestral lichenized group cannot be established from the present data because the extracted polysaccharide does not have the galactofuranose residue in the β configuration; however, the data suggests that an ancestor of the Lichinomycetes contained a mannan and was part of an early radiation in the ascomycetes. Polysaccharides present support the molecular data obtained recently that the Lichinales are distinct from other ascomycete groups and should be treated as a separate order in the separate class Lichenomycetes (Reeb *et al*., 2004). However, more representatives in the order must be subjected to molecular studies and more polysaccharides be investigated before confirming this hypothesis. Interestingly, the relative basal position of *Lichina* with respect to the water soluble polysaccharides agrees with the suggestion that ancestral lichens contained cyanobacteria as the photosynthetic partner (Hawksworth, 1982).

Investigation of mannose containing polysaccharides as a taxonomic tool centers around the structural diversity of the galactomannans isolated from several lichenized fungi. The taxonomic value of these galactomannans depends on the side-chain substituents on (1 →6)-linked α-D-mannopyranosyl main chains (Gorin and Lacomini, 1985).

Although classical taxonomy regarded *Cladina* as sub-species of *Cladonia,* lichenologists considered them to be distinct species. It was shown that galactomannans are important chemotypes in determining the taxonomy of *Clodonia* spp. and other related genera

(Woranovicz-Barreira *et al*., 1999). However, based on molecular phylogentic results *Cladina* and *Cladoni*a were confirmed as synonyms (Ahti and Depriest, 2001).

Lichen polysaccharides, can also be used as a taxonomic tool to differentiate some lichen species, since some of the heteropolysaccharides and their chemical characters are unique to certain groups of lichens (Carbonero *et al*., 2006 and Cordeiro *et al*., 2007). Further, the polysaccharides content of the lichen photobiont may be used as a marker in algal symbiont taxonomy (Cordeiro *et al*., 2007).

5. Biological activities of lichen polysaccharides

Many lichens are known to have immunomodulating properties, potent antibiotic, antitumour, antiviral as well as antioxidant properties which are mostly attributed to the secondary metabolites (Malhotra *et al*.,2008, Behera, *et al*., 2007). According to Yanaki *et al*., (1986) and Bohn and BeMiller, (1995), functional activity of polysaccharides mainly depends on molecular weight, degree of branching, water solubility, structure and configuration. Hence they have different uses in different fields. The biological activities of lichen polysaccharides reported have been limited to anti-tumor, anti-inflamatory or immunomodulatory activity (Omarsdottir *et al*., 2007).

Cordeiro *et al*., (2008) reported that one of the β-galactofuranan polysaccharides isolated from *Trebouxia* sp., the algal symbiont of the lichen *Ramalina gracilis* expressed *in vitro* activity on peritoneal macrophages. Further studies carried out on 4 lichen polysaccharides by Omarsdottir, *et al*., (2006) showed that, three heteroglycans namely Ths-4, Ths-5 and thamnolan and a β-glucan (Th-2) isolated from the lichen *Thamnolia vermicularis* var. subuliformis showed an effect on the human immune system. Thamnolan, a galactofuranorhamnan had less mitogenic effect than Ths-5 and Ths-2 indicating that its unusual galactofuranorhamnan structure may be responsible for its different immunomodulatory activity. In a study on the immunomodulatory activities of an aqueous lichen extract from *Cetraria islandica* , Freysdottir *et al.,* (2007) discovered that the extract was able to upregulate IL-10 secretion. Interestingly, when the individual components of this extract (lichenan and isolichenan and secondary metabolites protolichesterinic and fumarprotocetraric acids) were subjected to the same assay, only lichenan displayed antiinflammatory effects (Freysdottir, *et al.,* 2008).

The cytotoxic activity, phagocytic activity and antitumor activity of an α-D glucan from *Ramalina celastri* has been reported (Leão *et al.,* 1997 and Stuelp-Campelo *et al*., 2002). Based on this, De Araújo *et al.,* (2011) found that a sulphated α-D glucan lichen polysaccharide extracted from *Ramalina celastri* exhibited antischistosomal activity. These α-D glucans with α(1–3) and α(1–4) linkages are linear and water soluble and are known for their ability to stimulate the mononuclear phagocyte system and improve host resistance to viral, bacterial and parasitic infections.

According to Nishikawa *et al*., (1970), O-acetylated pustulan isolated from three species of *Umbilicaria* showed a significant antitumor effect against the implanted Sarcoma-180 in mice. The polysaccharide fractions isolated from six species of lichens were studied for

antitumor activity (Nishikawa *et al*., 1974). The active polysaccharide present in *Lasallia pensylvanica,* was the partially O-acetylated (1-6) β-glucan, pustulan while that of *Usnea rubescens,* was identified as a lichenan type polysaccharide. The other 4 polysaccharides were from the *Cladonia* sp.; *Cladonia crispata, Cladonia rangiferina* subsp. *grisea, Cladonia mitis* and *Cladonia squamosa* all of which showed significant antitumor activity. These *Cladonia* polysaccharides which were complex heteroglycans of the galactomannan type were found to exert moderate antitumor activity. However, previous studies have shown that the isolichenin type polysaccharides were not as effective in their antitumour effect. In another study, isolichenin isolated from the lichen *Usnea fasciata* showed moderate activity against Ehrlich tumor cells (Periera *et al*., 1994). In 1989, Hirabayashi *et al*., showed inhibitory effect of a lichen polysaccharide sulfate (GE-3-S), isolated from the lichen *Umbilicaria esculenta* on the replication of human immunodeficiency virus (HIV) in vitro.

Behera *et al*., (2007) showed correlation between lichen protein/polysaccharide ratio and their antioxidant properties. Since cultured lichen extracts were used in the study, the effect of secondary metabolites as well as polyphenols present in the extract have not been evaluated. Thus the antioxidant activity cannot be solely attributed to the polysaccharide specially since polyphenols are known antioxidants. In addition, the mechanism of scavenging activity of polysaccharides on free radicals is not fully understood yet.

6. Conclusion

Polysaccharides from lichens unlike bacterial polysaccharides do not show a wide range of variation in sugar content. The predominant sugars are limited to glucose, galactose and mannose, with arabinose and xylose present in minor proportions in addition to others such as rhamnose. These polysaccharides however have been useful in chemotaxonomic studies due to this conservation of sugar structures and regular structural patterns. As far as biological activity is concerned, very few studies have been reported and it would be worthwhile further investigating the immunomodulating effects of the polysaccharides.

Author details

Desiree Nedra Karunaratne, R.G.U. Jayalal and Veranja Karunaratne
University of Peradeniya, Peradeniya, Sri Lanka

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