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An unexpectedly lichenase-stable hexasaccharide from cereal, horsetail and lichen mixed-linkage β-glucans (MLGs): Implications for MLG subunit distribution

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ABSTRACT

Mixed-linkage (1→3),(1→4)-β-D-glucan (MLG) is a biologically and technologically important hemicellulose, known to occur in three widely separated lineages: the Poales (including grasses and cereals), *Equisetum* (fern-allies), and some lichens e.g. Iceland moss (*Cetraria islandica*). Lichenase (E.C. 3.2.1.73) is widely assumed to hydrolyse all (1→4) bonds that immediately follow (1→3) bonds in MLG, generating predominantly the tetrasaccharide β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-D-Glc (G4G4G3G; MLG4), the corresponding trisaccharide (G4G3G; MLG3), and sometimes also laminaribiose (G3G; MLG2). The ratio of the oligosaccharides produced characterises each polysaccharide. We report here that digestion of MLG from barley (*Hordeum vulgare*), *Equisetum arvense* and *C. islandica* by *Bacillus subtilis* lichenase also yields the unexpectedly stable hexasaccharide, β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-D-Glc (G3G4G4G4G3G, i.e. MLG2–MLG4), identified by thin-layer chromatography, gel-permeation chromatography, HPLC (HPAEC), β-glucosidase digestion, $^{1}H/^{13}C$-NMR spectroscopy and mass spectrometry. On HPLC, G3G4G4G4G3G is the major constituent of a peak previously ascribed solely to the nonasaccharide G4G4G4G4G4G4G3G. Because it was widely presumed that lichenase would cleave G3G4G4G4G3G to MLG2 + MLG4, our data both redefine the substrate specificity of *Bacillus* lichenase and show previous attempts to characterise MLGs by HPLC of lichenase-digests to be flawed. MLG2 subunits are particularly underestimated; often reported as negligible, they are here shown to be an appreciable constituent of MLGs from all three lineages. We also show that there is no appreciable yield of water-soluble lichenase products with DP > 9; potential identities of products previously labelled DP > 9 are suggested. Finally, this discovery also provides a opportunity to investigate the spatial distribution of subunits along the MLG chain. We show that MLG2 subunits in barley and *Cetraria* MLG are not randomly distributed, but predominantly found at the non-reducing end of MLG4 subunits.

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1. Introduction

1.1. Structure of MLG

Mixed-linkage (1→3),(1→4)-β-D-glucan (MLG) is a linear homopolysaccharide composed of β-D-glucopyranose residues (abbreviated 'G') linked by (1→3) and (1→4) bonds. The MLG chain typically comprises sequences such as

. . . G3G4G4G4G3G4G4G3G4G4G3G4G4G4G4G3G4G3G4G4G3G . . .

where 3 is a (1→3) bond and 4 is a (1→4) bond, the sugar residues being listed from non-reducing to reducing end. Each underlined segment is a cello-oligosaccharide subunit: these are mainly of degree of polymerisation (DP) 3 or 4, and are interconnected by single

Abbreviations: 6x, MLG hexasaccharide not conforming to the sequence type $(G4)_nG3G$; AIR, alcohol-insoluble residue; COSY, correlation spectroscopy; CSSF, chemical-shift-selective; DP, degree of polymerisation; G4G3G etc., β-D-glucose residues interlinked by (1→4) or (1→3) bonds; Glcol, glucitol; GPC, gel-permeation chromatography; HSQC, heteronuclear single quantum spectroscopy; K_{av}, elution volume on GPC relative to glucose ($K_{av} = 1$) and dextran ($K_{av} = 0$); MLG, mixed-linkage (1→3),(1→4)-β-D-glucan; MLG2, $(G4)_nG3G$ where $n = 0$; MLG3, $(G4)_nG3G$ where $n = 1$; MLG4, $(G4)_nG3G$ where $n = 2$; MLGO, MLG oligosaccharide; NOESY, nuclear Overhauser spectroscopy; PAD, pulsed amperometric detector; PyAW/CB, pyridine/acetic acid/water (1:1:98 by volume, pH ~4.7) containing 0.5% (w/v) chlorobutanol; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; XEG, xyloglucan endoglucanase.

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(1→3)-bonds; the (1→3)-bonds are never found consecutively (Peat et al., 1957; Parrish et al., 1960). Less frequent subunits are reported to include cello-oligosaccharides of DP2 and 5 and a small proportion of considerably longer subunits, e.g. of DP12 (Wood et al., 1994; Izydorczyk et al., 1998; Vaikousi et al., 2004; Papageorgiou et al., 2005; Sørenson et al. 2008; Liu and White, 2011).

1.2. Lichenase's presumed site of cleavage

Lichenase (EC 3.2.1.73) is an MLG endohydrolase, found both in microbes and plants (Fincher et al., 1986; Henrissat, 1991; Henrissat and Bairoch, 1993), and often used analytically to characterise the subunit composition of MLGs (e.g. Lazaridou et al., 2004; Sørenson et al. 2007; Xue and Fry, 2012). Lichenase's target site is widely assumed to be all (1→4) bonds immediately following (1→3) bonds (Planas, 2000). It does not hydrolyse pure (1→3)-β-D-glucans or (1→4)-β-D-glucans. The result of lichenase action on MLG would thus be purely a range of oligosaccharides in which the reducing end is always G3G and any other G residues are 4-linked as extensions at the non-reducing end. Such oligosaccharides are described here as standard MLGOs, or specifically MLG2, MLG3, MLG4 etc. according to their DP, MLG3 being G4G3G. The yield of MLG3 in a lichenase digest is taken to indicate the abundance of cellotriosyl units in the polysaccharide prior to digestion.

1.3. MLG found in different lineages of organisms

MLG occurs in at least three widely separated lineages: the Poales (grasses, cereals, reeds and their relatives; Smith and Harris, 1999; Popper and Fry, 2004), *Equisetum* (horsetails; an evolutionarily isolated genus of non-flowering vascular plants), and some lichens e.g. *Cetraria islandica* (Iceland 'moss', whose MLG is called lichenan; Perlin and Suzuki, 1962). MLG does not occur in the majority of plants, algae or fungi. Its presence in *Equisetum* was an unexpected recent discovery (Fry et al., 2008a,b; Sørensen et al., 2008; Xue and Fry, 2012); the Poales had long been assumed to be the only vascular plants possessing it (Stone and Clarke, 1992), though an MLG-related polysaccharide was found in the leafy liverwort *Lophocolea bidentata* (Popper and Fry, 2003).

In the vegetative tissues of the Poales and *Equisetum*, MLG is regarded as a hemicellulose because it is extractable from cell walls with alkali (especially 6 M NaOH at 37 °C), and because MLG chains are thought to hydrogen-bond to cellulosic surfaces, possibly tethering adjacent microfibrils and contributing to wall architecture (Fry, 1989; Sakurai, 1991; Fry et al., 2008a,b). In the Poales, most MLG is found in the endosperm and in rapidly expanding vegetative tissues; it is often metabolically labile, being hydrolysed to glucose after germination and after the completion of cell expansion (Buckeridge et al., 2004), and may thus also serve as a carbohydrate reserve (Inouhe and Nevins, 1991; Hatfield and Nevins, 1987). There is no evidence for MLG turnover in *Equisetum*, whose MLG is abundant in both young and senescing tissues. The presence of the enzyme MLG:xyloglucan endotransglucosylase (MXE) in *Equisetum*, but not in the Poales (Fry et al., 2008a,b; Mohler et al., 2013), provides further evidence for distinct roles for MLG in these two taxa.

It is likely that MLG evolved independently in the Poales, *Equisetum* and lichens, and given their wide phylogenetic separation, it is understandable that their MLGO subunit ratios differ. The MLG3:MLG4 ratio of MLG is typically >24 in *Cetraria* (Lazaridou et al., 2004; Tosh et al., 2004a), ~1.5–4.5 in various species of the Poales (Li et al. 2006; Lazaridou and Biliaderis, 2007; Fry et al., 2008a,b), and <0.25 in *Equisetum* (Fry et al., 2008a,b; Sørensen et al., 2008; Xue and Fry, 2012).

While MLG2 (aka laminaribiose) is a clearly established constituent of *Equisetum* MLG (Fry et al., 2008a,b; Sørensen et al., 2008; Xue and Fry, 2012) and some have reported its presence in poalean MLG (e.g. Roubroeks et al., 2000), many investigators have failed to report it in the latter and in Iceland moss MLG (Wood et al., 1994; Izydorczyk et al., 1998, 2004; Wood et al., 2003; Tosh et al., 2004a,b; Vaikousi et al. 2004; Papageorgiou et al., 2005; Liu and White, 2011).

1.4. Technological importance of MLG

MLGs are industrially important, and their technologically exploitable properties are thought to be a product of their concentration, molecular weight and subunit composition. To better understand this, numerous studies have been aimed at characterising MLGs from different (typically poalean) sources, particularly with respect to the ratio of MLG3:MLG4 subunits. For example, Tosh et al. (2004b) showed that differences in the MLG3:MLG4 ratio affect the gelation characteristics and elasticity of MLG systems, lichen MLG (high DP3:DP4 ratios) forming gels at a quicker rate and with a higher 'melting' point than cereal MLGs. Likewise, cereal MLGs with the highest MLG3:MLG4 subunit ratios form gels the quickest (Lazaridou and Biliaderis, 2007).

MLGs are also important components of the human diet, affecting blood glucose and cholesterol concentrations (Battilana et al., 2001; Bell et al., 1999; Bourdon et al., 1999; Dikeman and Fahey, 2006; Kahlon et al., 1993; Lazaridou and Biliaderis, 2007; Wood, 1994, 2007), alleviating constipation by increasing faecal bulk (Malkki and Virtanen, 2001; Lazaridou and Biliaderis, 2007) and having beneficial effects on the immune system (Porter et al., 2006). For all these reasons, a better understanding of MLGs, and the enzymes that cleave them, is desirable.

1.5. Subunit arrangements of MLGs

Few studies have attempted to characterise the distribution of MLGO subunits along the intact MLG chain, probably because of the lack of suitable techniques for doing so. Staudte et al. (1983) attempted this by mathematically modelling the production of the four main penultimate products of lichenase digestion, i.e. DP6–8 oligosaccharides with two (1→3) linkages, namely G4G3G4G4G3G, G4G3G4G4G4G3G and/or G4G4G3G4G4G3G, and G4G4G3G4G4G4G3G. They concluded that MLG3 and MLG4 subunits are distributed randomly throughout the polysaccharide, but did not discuss the distribution of other, less predominant, units.

In the present work, we have reinvestigated the action of lichenase on MLGs, and report the discovery of a novel lichenase-resistant dimer of previously known repeat units that redefines lichenase's substrate specificity as well as the subunit composition and distribution of MLGs.

2. Results

2.1. Purification of Equisetum arvense MLG

MLG was purified from *E. arvense* alcohol-insoluble residue (AIR) and analysed for MLG and possible contaminants by four hydrolytic methods (Fig. 1). Trifluoroacetic acid (TFA) and Driselase yielded only glucose; xyloglucan endoglucanase (XEG) had no effect under conditions which completely hydrolysed tamarind xyloglucan; and lichenase digested the great majority of the polysaccharide, yielding a range of typical MLGOs, mainly of DP2–6. The results demonstrate a solution rich in *Eq*MLG with no detectable contaminating polysaccharides. The preparation was stored at −20 °C in an aqueous solution and thoroughly boiled before further use.

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