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Transglucosylation of cell wall polysaccharides in *Equisetum fluviatile*

Kyle Edward Mohler



A thesis prepared in fulfilment of the requirements for
the degree of Doctor of Philosophy (PhD),
The University of Edinburgh 2011

Declaration

This thesis has been composed by myself and the work, of which it is a record, has been carried out by myself. All sources of information have been specifically acknowledged by means of a reference.

Kyle Edward Mohler

Acknowledgments

Thank you to Steve Fry, my supervisor, who invited me to come to Edinburgh to pursue my PhD. I was never short of guidance and advice, for which I am grateful and appreciative. Thank you also to Andrew Hudson, my second supervisor, for giving me self-confidence and keeping me full of encouragement. I am deeply indebted to Professors Kenneth and Noreen Murray, and The Darwin Trust of Edinburgh for sponsoring my studentship which allowed me to come to Scotland.

The laboratory has been a great place to work, largely because of Janice Miller. Thank you for giving so much, for stopping your own work to help me at any time, and just for listening (not to mention all the advice). The lab wouldn't have been the same without Sandra, David, Harriet, John, Amjad, Ben, Sally, Airianah, Tom, Tim, Dimitra, Lenka, Claire, and Christina (hope I haven't forgotten anyone!), who were always good for a jolly laugh and sharing some tea and cakes.

My family and family-in-law deserve quite a few thank yous too, for all the support while I've been studying.

I cannot neglect the support staff at IMPS and the School of Biological Sciences, who seldom get the credit they're due.

Thanks to God as well, for wisdom and direction. Who am I, to have been brought so far?

To Jessica, with all my love

Abstract

Plant cell walls determine cellular shape and provide structural support for the entire plant. Polysaccharides, comprising the major components of the wall, are actively remodelled throughout development. Xyloglucan endotransglucosylase (XET) /hydrolase (XTH, EC 2.4.1.207) cleaves xyloglucan (XyG), the donor substrate, and attaches a portion to another XyG chain, the acceptor substrate. Recently, a novel transglucosylase called mixed-linkage β -glucan (MLG) : XyG endotransglucosylase (MXE) was discovered in horsetails (*Equisetum* spp.) that could attach a portion of MLG to XyG, resulting in a hetero-polymer product. My aims were to further investigate the nature of this activity, biochemically characterize the enzyme, and explore its physiological role. MXE activity was attributable to an enzyme unlike *Equisetum* XTHs. MXE had a pI of 4.1 (XTHs were 6.6–9), a pH optimum of 6.3 (XTHs preferred 5.5), and had higher activity using smaller oligosaccharide acceptor substrates like XXXGol (XTHs were more active using XLLGol). Importantly, the MXE protein was shown to utilize both MLG and XyG as donor substrates, and therefore have both MXE and XET activity. Also, the enzyme was capable of using various glucan oligosaccharides (O) as substrates, including MLGO, XyGO, and cello-O, but not laminari-O. By using a novel *ex vivo* approach, the proportion of

extractable MXE product to XET product was found to increase in older tissues. Transglucosylase products were localized in sclerenchyma and structural parenchyma by *in situ* assays, implying a strengthening function for MXE. Surprisingly, another novel activity was discovered that could covalently attach cellulose to XyG, and termed cellulose : xyloglucan endotransglucosylase (CXE). This activity was attributed to the MXE enzyme, implying that the protein is a promiscuous endotransglucosylase. The presence of CXE in other plants has not yet been tested. Besides being a novel discovery in plant cell biology, the modification of cellulose has applications in a number of industries.

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Abbreviations

| | |
|------|---|
| AAW | acetone : acetic acid : water |
| AIR | alcohol insoluble residue |
| Ara | arabinose |
| AS | ammonium sulfate |
| ASP | AS-precipitate |
| BAW | butanol : acetic acid : water |
| BD | blue dextran |
| BSA | bovine serum albumin |
| CB | chlorobutanol |
| Ces | cellulose synthase |
| CGA | charophycean green algae |
| ConA | Concanavalin A |
| cpm | counts per minute |
| Csl | cellulose synthase-like |
| CXE | cellulose : xyloglucan endotransglucosylase |
| DMA | dimethyl acetamide |
| DP | degree of polymerization |
| EFW | ethanol : formic acid : water |
| EtOH | ethanol |
| FA | formic acid |
| Fuc | fucose |
| Gal | galactose |
| GalA | galacturonic acid |
| GGM | galactoglucomannan |
| GH | glycoside hydrolase |
| Glc | glucose |
| GlcA | glucuronic acid |

| | |
|-----------|---|
| GlcM | glucomannan |
| GPC | gel permeation chromatography |
| HG | homogalacturonan |
| HOAc | acetic acid |
| IEC | ion exchange chromatography |
| IEF | isoelectric focussing |
| IP | isoprimeverose |
| mAb | monoclonal antibody |
| MALDI-TOF | matrix-assisted laser desorption ionization time of flight |
| Man | mannose |
| MLG | mixed-linkage β -glucan |
| MXE | mixed-linkage glucan : xyloglucan endotransglucosylase |
| O | oligosaccharide |
| PAGE | polyacrylamide gel electrophoresis |
| PC | paper chromatography |
| PL | poly-lysine |
| PME | pectin methylesterase |
| PPO | 2,5-diphenyloxazol |
| PrAW | propan-1-ol : acetic acid : water |
| PyAW | pyridine : acetic acid : water |
| RG | rhamnogalacturonan |
| Rha | rhamnose |
| SA | specific activity |
| SDS | sodium dodecyl sulfate |
| SES | sulfoethyl-Sephadex |
| SR | sulforhodamine |
| TFA | trifluoroacetic acid |
| TLC | thin layer chromatography |
| XEG | xyloglucan endoglucanase |
| XEH | xyloglucan endohydrolase (activity) |
| XET | xyloglucan endotransglucosylase (activity) |
| XGA | xylogalacturonan |
| XGO | xyloglucan oligosaccharide |
| XTH | xyloglucan endotransglucosylase/hydrolase (protein or gene) |
| XyG | xyloglucan |
| Xyl | xylose |

1 Introduction

1.1 Plant cell walls

The plant cell wall is a complex macromolecular structure made of polysaccharides, proteins (both structural and enzymic), polyphenols (lignin), and water. During the developmental processes, some cell walls must expand by many times its original size, all the while maintaining the structural integrity of the entire plant (Cosgrove, 2005). Other roles of the cell wall include energy storage (Albersheim et al., 2011), defence against pathogen attack, and potentially cell-to-cell communication (Caffall and Mohnen, 2009). Cell walls are composed mostly of polysaccharides, which are complex and diverse in nature.

Two types of cell walls are formed in plants, primary and secondary. The primary cell wall is laid down first, and has the capacity to yield to turgor pressure to allow for growth. Polysaccharides, including cellulose, hemicelluloses, and pectin, are synthesized during this process, maintaining the thickness of the wall (Taiz, 1984). At the end of growth, some cells produce a secondary cell wall, or even multiple layers of secondary walls. These walls contain more cellulose, and lignification of both the primary and secondary cell wall may occur at this stage (Albersheim et al., 2011). The structure, occurrence, and function of cell wall polysaccharides is discussed

below. Cells can change their cell wall composition either by *de novo* synthesis of different or modified polysaccharides, or by altering pre-existing apoplastic components. Enzymes play both direct and indirect roles in this process (Cosgrove, 2005). One such enzyme, xyloglucan endotransglucosylase/hydrolase (XTH), is thought to be of particular importance to cell wall development (Eklöf and Brumer, 2010; Mellerowicz et al., 2008; Campbell and Braam, 1999 a), and is also discussed.

Recently, a novel transglucosylase, termed mixed linkage glucan : xyloglucan endotransglucosylase (MXE) was discovered (Fry et al., 2008 a). High levels of MXE activity can be extracted from horsetails (*Equisetum*). Therefore, their physiology and growth are described in detail. Being evolutionarily far removed from seed plants, the horsetail's place in plant evolution is discussed. Also, the changes in plant cell walls through evolution are reviewed. Finally, a brief summary of the aims of this project is introduced.

1.2 Structure, occurrence and function of plant cell wall

polysaccharides

Polysaccharides comprise the largest component of the plant cell wall. The carbohydrates can be grouped into three main categories: cellulose, hemicellulose and pectin.

1.2.1 Cellulose

Cellulose is a homopolymer of β -(1 \rightarrow 4)-glucan. An individual chain of cellulose is quite rigid and regular, allowing multiple chains to bind to each other, forming a microfibril, through hydrogen bonds and van der Waals forces (Somerville, 2006). As a demonstration of their strong affinity for aggregation, cello-oligomers longer than about DP 6-8 are not soluble in water (Fry, personal communication). Individual cellulose chains are typically DP 2,500–4,500 in primary cell walls, but are usually larger in secondary cell walls, up to DP 14,000 (McNeil et al., 1984). About a third of the dry weight of primary cell walls is composed of cellulose. This value increases to about half of the dry weight in secondary cell walls (Vogel, 2008). Some specialized walls, such as cotton fibres, can be up to 95% cellulose (Kim and Triplett, 2001).

Multiple cellulose chains form a crystalline structure at the centre of microfibrils. In the peripheral regions of the microfibril, cellulose chains are less ordered and more amorphous. Some models of cellulose microfibrils allow for the possibility that hemicelluloses, such as xyloglucan, may become trapped in the amorphous regions during cellulose synthesis (Rose and Bennett, 1999).

Cellulose microfibrils function as the major load-bearing component of the cell wall. Microfibrils do not stretch; they can be likened to cables. Therefore, their orientation in the primary cell wall is important to their function (Taiz, 1984). Cellulose is produced by cellulose synthase (CesA) rosette complexes at the plasma membrane (Fig. 1.1). The net movement of these complexes is perpendicular to the direction of growth. As internal

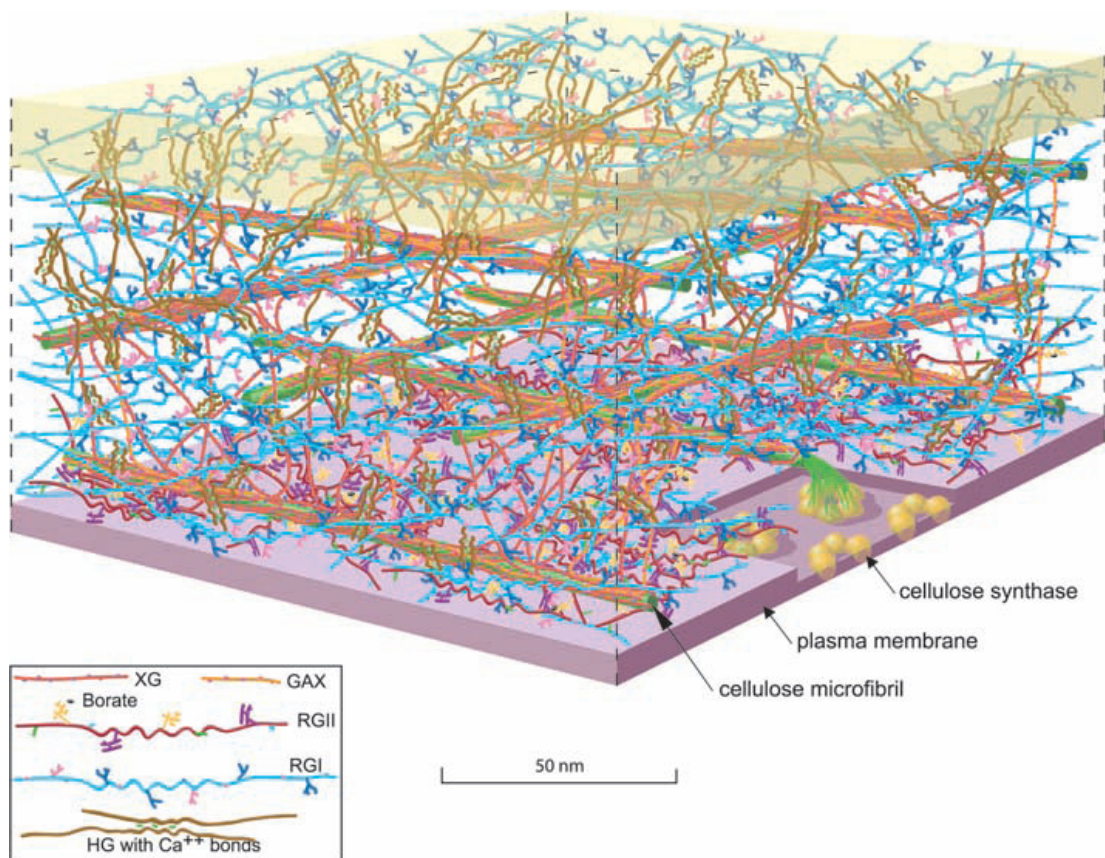


Figure 1.1 Structure of the primary plant cell wall

Cellulose is synthesized at the plasma membrane by CesA rosette complexes. Hemicelluloses may later bind to the cellulose microfibrils. Pectins are shown, including their capacity to be bound together through borate dimerization or Ca^{2+} . Image taken from Somerville et al., 2004.

turgor pressure increases and applies a force on the wall, parallel microfibrils separate from each other as the wall yields (Somerville et al., 2004).

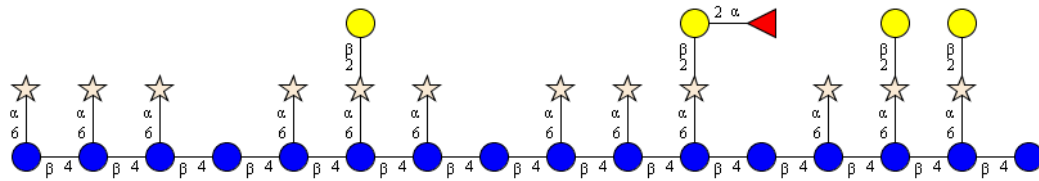
1.2.2 Hemicelluloses

The historical definition of hemicellulose was the material that required strong alkali for extraction from plant cell walls. A more recently proposed definition, ' β -(1→4)-glycans with an equatorial configuration' of hydroxyl groups on the sugar ring, may imply a functional role (Scheller and Ulvskov, 2010). Hemicelluloses can hydrogen bond to cellulose microfibrils, and are long enough to span the gap between neighbouring microfibrils (McCann et al, 1990). Thus, they may tether microfibrils together against turgor pressure-driven expansion forces. They may also coat microfibrils, preventing two microfibrils from binding to each other (McCann et al, 1990). Some hemicelluloses are found in large quantities in seed endosperm, presumably as an energy reserve (Fry, 2011). Four main types of hemicelluloses are found in land plants: xyloglucan, xylan, mannan, and mixed-linkage glucan (Fig. 1.2).

1.2.2.1 Xyloglucan (XyG)

XyG is a cell wall polysaccharide with a repeating pattern of Xyl residues along a Glc backbone. Typically, the first three of every four β -(1→4)-Glc residues is substituted by α -(1→6)-Xyl, from the non-reducing terminus. The second and third Xyl can be further substituted by other sugars; in

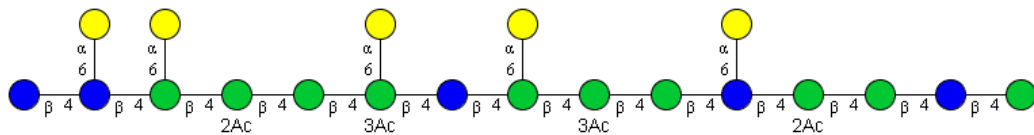
Xyloglucan from dicots



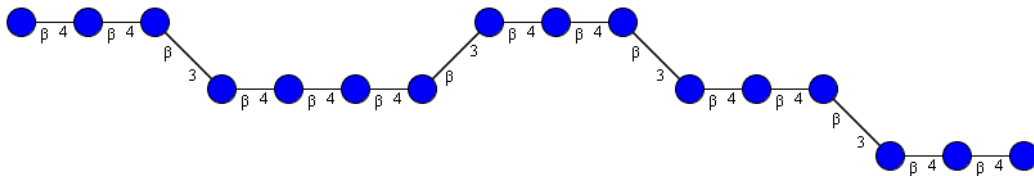
Xylan from grasses



Galactoglucomannan from gymnosperms



Mixed-linkage glucan from cereals










| Legend | | | |
|---|-----------|---|-----------------|
|  | glucose |  | arabinose |
|  | xylose |  | fucose |
|  | galactose |  | glucuronic acid |
|  | mannose | | |

Figure 1.2 Structure of hemicelluloses from various land plants
Models built using *GlycoWorkbench* (Ceroni et al., 2008).

angiosperms a further β -(1→2)-Gal residue is common. Single letter abbreviations each representing a single backbone Glc residue, with or without side-chain substitutions, provide unambiguous nomenclature. An unsubstituted Glc is represented by **G**, α -Xyl-(1→6)- β -Glc by **X**, β -Gal-(1→2)- α -Xyl-(1→6)- β -Glc by **L**, α -Fuc-(1→2)- β -Gal-(1→2)- α -Xyl-(1→6)- β -Glc by **F** (Fry et al., 1993). *Equisetum* has unusual side-chain substitutions that include α -L-Arap in the place of Gal. When Glc is substituted with α -L-Arap-(1→2)- α -Xyl-(1→6), the letter **D** is given, and when substituted with α -Fuc-(1→2)- α -L-Arap-(1→2)- α -Xyl-(1→6), **E** was designated (Peña et al., 2008).

XyG structure has been studied through the application of a XyG-specific hydrolase, xyloglucan endoglucanase (XEG). This enzyme cleaves the β -(1→4)-Glc bond in the sequence, **GX** (Pauly et al., 1999). XEG may be less active on fucosylated or acetylated XyG. Some endo- β -(1→4)-glucanases (cellulases) can cleave XyG, but also hydrolyse cellulose or other polymers, and are less suitable for XyG study (Pauly et al., 1999).

XyG is the most abundant hemicellulose in the primary cell walls of many types of land plants. It is present in all land plants including bryophytes, but not in charalean green algae (Popper and Fry, 2003). XyG is present in high concentrations in primary walls but low concentrations in secondary walls. Commelinid monocots have notably little XyG in their walls, and incorporate other hemicelluloses instead (Vogel, 2008).

The function of XyG is debated in parallel with the functions of all hemicelluloses. Most cell wall models predict that a hydrogen-bonded XyG–cellulose matrix is the load-bearing structure of the wall (Carpita and Gibeaut, 1993; Caffall and Mohnen, 2009; Albersheim et al., 2011). Cell wall loosening, or creep, occurs when hemicelluloses are modified by enzymes such as XyG endotransglucosylase/hydrolase (XTH). This enzyme and its action is described in more detail below. Supporting this hypothesis, Takeda et al. (2002) showed that incorporating full length XyG into expanding pea hypocotyls restricted growth. Also, addition of XyG oligosaccharides (XGO), effectually reducing the length of XyG through the XyG endotransglucosylation (XET) reaction, accelerated growth (McDougall and Fry, 1990; Takeda et al., 2002).

Not all evidence supports the idea that XyG contributes to the mechanical forces that resist creep. The generation of an *Arabidopsis* mutant without XyG might provide evidence against this paradigm (Cavalier et al., 2008). This mutant lacks two functional genes, xyloglucan xylosyltransferase 1 and 2 from T-DNA knockout. These gene products add xylose residues to the β -glucan backbone. Compared to wild type, no gross morphological phenotype was seen in these plants besides a root hair aberration, indicating that XyG is not necessary for normal cell wall function. The stiffness and ultimate stress of etiolated hypocotyls was significantly but not

catastrophically reduced (Cavalier et al., 2008). If the XyG–cellulose network was the load bearing structure of the cell wall, an elimination of XyG might have greatly weakened the wall, but this was not found. In support of the idea that a hydrogen-bonded network is not the growth-resisting structure, a calculation showed that the total potential XyG–cellulose hydrogen bond energy was not enough to resist the measured work done during wall growth (Thompson, 2005). While XyG may indeed provide some structural support for the cellulose microfibrils, it is not the sole mechanical network in the wall. Other polysaccharide linkages and interactions, or other forces should be included in models of the load-bearing network.

XyG is modified after deposition in the wall. As high energy substrates, like UDP-glucose or other activated sugars, are not present in the apoplast, few new bonds are synthesized (except by transglycosylases, like XET, which is discussed later). Rather, XyG can be hydrolysed down to its monomer constituents in the wall. At least five activities are necessary to complete the digestion of most dicots' XyG: xyloglucan endoglucanase (XEG), α -fucosidase, β -galactosidase, α -xylosidase, and β -glucosidase, all of which have been previously characterized. Augur et al. (1993) showed that α -fucosidase from pea epicotyls is specific for XGOs, not XyG or other wall polysaccharides. This protein was about twice as efficient at cleaving α -Fuc-(1→2)- β -Gal (1→ when Gal was substituted at 6-O with an acetyl group than

without (Augur et al., 1993). A β -galactosidase protein was purified from germinating nasturtium plants that acts on XyG polysaccharide (Edwards et al., 1988). Arabidopsis has only one functional XyG-active α -xylosidase, which can only remove the Xyl residue from the non-reducing terminal Glc, a property that is common in plant α -xylosidases (Sampedro et al., 2010). Apoplastic β -glucosidases are very active in Arabidopsis, and are not the limiting factor in XGO degradation (Iglesias et al., 2006). As mentioned previously, XyG forms a tight bond with cellulose. The activities listed above can modify this interaction through changing the sugar substitutions on XyG, which can change the affinity of XyG for other wall polymers (Minic and Jouanin, 2006).

1.2.2.2 Xylan

Xylans are composed of a β -(1 \rightarrow 4)-xylan backbone with non-ordered GlcA, Ara, acetyl, or methyl substitutions. GlcA residues are linked α -(1 \rightarrow 2) to the backbone and can be methylated at the O-4 position. Ara substitutions occur at the O-2 or O-3 positions, and sometimes both. Acetyl groups can also be found at O-2 and/or O-3 (Scheller and Ulvskov, 2010). Some xylans contain a disaccharide substitution, α -Ara-(1 \rightarrow 2)- α -Ara, attached to the O-3 position of a Xyl residue (Mazumder and York, 2010).

Xylans are found in secondary cell walls in vascular and supportive tissues. In these tissues, they are the most abundant non-cellulosic polysaccharide (Scheller and Ulvskov, 2010). The function of xylan in secondary walls is unknown, but its deficiency causes a loss in structural integrity of xylem vessels. Some of the xylan biosynthetic genes were discovered through an investigation of *Arabidopsis* mutants with collapsed xylem and dwarfed phenotype (Brown et al., 2007). GlcA and MeGlcA substitutions, found in the xylan of many eudicotyledonous plants, may not be necessary to the function of the polymer (Mortimer et al., 2010). Xylan can hydrogen bond to cellulose or the cellulose-like regions of MLG (Izydorczyk and MacGregor, 2000).

Grasses utilize xylans differently. Xylans are the most abundant non-cellulosic polysaccharide in their primary cell walls. Ara substitutions may be further substituted with ferulate, which themselves can be oxidatively coupled to other feruloyl-xylans or lignin (Wende and Fry, 1997; Vogel, 2008).

The occurrence of xylans is widespread in the tracheophytes. Xylans were labelled by monoclonal antibodies (mAb) in the vasculature and fibre tissues of lycopodiophytes, monilophytes, and spermatophytes. Xylans were also detected in the spores of hornworts, the closest relative of tracheophytes, but no xylans were found in mosses or liverworts (Carafa et al., 2005).

1.2.2.3 Mannan

Mannan polysaccharides are diverse in biochemistry and function. Some algae synthesize pure β -(1 \rightarrow 4)-mannan, which forms microfibrils similar to cellulose. Glucomannan (GlcM), a polymer of β -(1 \rightarrow 4)-mannan interspersed randomly with β -(1 \rightarrow 4)-glucose and partially acetylated, is a minor component of angiosperm primary walls and a major component of gymnosperm secondary walls (Schröder et al., 2009). Galactomannan or other mannans might function as a cellulose microfibril tether, similar to XyG, as shown in artificial cellulose composites (Whitney et al., 1998). Galactoglucomannan (GGM) has a GlcM backbone with single α -(1 \rightarrow 6)-Gal substitutions on either the mannose or glucose residue, and frequent acetyl groups at the O-2 or O-3 position (Moreira and Filho, 2008). This polymer is also found in primary cell walls. Although GGM is shorter than other mannans, it is the only mannan able to bind to paper cellulose made from cotton fibres (Schröder et al., 2004). GGM has been reported in all land plants (Popper, 2008). All mannans may play a role in hardening cellulose structures in seed endosperm, secondarily thickened epidermal walls, tracheids, etc., but can also be storage polysaccharides in certain situations (Schröder et al., 2009).

1.2.2.4 Mixed-linkage glucan (MLG)

MLG is a polymer of purely glucose residues without side-chain substitutions. Glucose residues linked by consecutive β -(1 \rightarrow 4) bonds are similar to cellulose. These cellulose-like regions, typically DP 3 or 4, are linked by single flexible β -(1 \rightarrow 3) bonds, depicted below:

...G3G4G4G3G4G4G4G3G4G4G3G4G4G3G...

where G represents a β -glucose residue and (1 \rightarrow 3) and (1 \rightarrow 4) bonds are represented by 3 and 4, respectively. Underlined regions are similar to cellulose. The 'kinked' polymer is soluble in water at low concentrations, but can form extremely viscous solutions (Stone and Clarke, 1992).

The structure of MLG is often characterized by an MLG-specific hydrolase, lichenase, which is commercially available from Megazyme, Inc. This enzyme cleaves the (1 \rightarrow 4) bond directly following (in the reducing-terminal direction) a (1 \rightarrow 3) bond, releasing unique and diagnostic oligoglucans (Planas, 2000).

MLG has been best studied in grasses. The DP3 : DP4 ratio of oligosaccharides released by lichenase digestion is variable between species or organs, but is usually about 2:1. Lower amounts of larger cello-like oligomers can also be found (Buckeridge et al., 2004). MLG is most abundant in seed endosperm walls, most likely functioning as energy storage, but is also found in growing plants (Carpita et al., 2001). MLG is synthesized in the

Golgi apparatus (Urbanowicz et al., 2004; Carpita and McCann, 2010), potentially by *Cs1F* or *Cs1H* gene products (Burton et al., 2006; Doblin et al., 2009; Burton and Fincher, 2009), at the onset of elongation. Its concentration reaches a maximum at the peak of cell growth, but the polymer subsequently becomes degraded by endo- and exo- β -glucan hydrolases at the cessation of growth (Buckeridge et al., 2004). Some Poalean species incorporate MLG into their mature vegetative tissues (Trethway et al., 2005), but the function of the polymer in these tissues is unknown.

MLG is synthesized in other organisms as well. Fungi, algae, lichens, and other non-plant species can synthesize the polymer. Lichenan, the name given to MLG from lichens, contains 86% cellotriose units, and the remaining 14% is comprised of other sized oligomers (Buckeridge et al., 2004). MLG from algae (Eder et al., 2008) and fungi (Burton and Fincher, 2009) similarly have a large proportion of cellotriose units in their MLG.

Interestingly, two groups discovered MLG independently in one other land plant genus, *Equisetum*. MLG was detected using mAb and enzyme-specific digestion, and was also confirmed by monosaccharide linkage analysis (Fry et al., 2008 b; Sørensen et al., 2008). MLG in *E. arvense* was not detected in primary walls, but was found in high abundance in secondary walls (Sørensen et al., 2008). Uniquely, the cellotetraose unit is more abundant than the cellotriose unit, and cellobiose units were detected (Fry et

al., 2008 b). The functions of MLG in *Equisetum* are unknown, but are likely to be different from those of poalean MLG, as its linkage structure and occurrence are strikingly dissimilar.

1.2.3 Pectin

Pectins are the third group of polysaccharides that make up the plant cell wall. They comprise a significant portion of dicot primary cell walls, about one third of their alcohol insoluble residue (AIR, Caffall and Mohnen, 2009). Pectins contribute to wall porosity (McCann et al., 1993), and also make up the middle lamella at the border between two cells. Pectic oligosaccharides released by fungal attack induce defence responses (McNeil et al., 1984).

Four main types of pectin, homogalacturonan, xylogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II are thought to be covalently linked to each other along their backbone (Fig. 1.3), but can also be attached by other types of linkages, which are detailed below.

1.2.3.1 Homogalacturonan (HG)

HG is composed of α -(1→4)-GalA, which can be methyl-esterified at C-6 carboxyl group or acetylated at O-2 or O-3. HG is the most abundant form of pectin, comprising more than 60% of cell wall pectins (Caffall and Mohnen,

2009). At physiological pH, unmethylesterified GalA residues are negatively charged. Ca²⁺ has been shown to bind to these negatively charged groups,

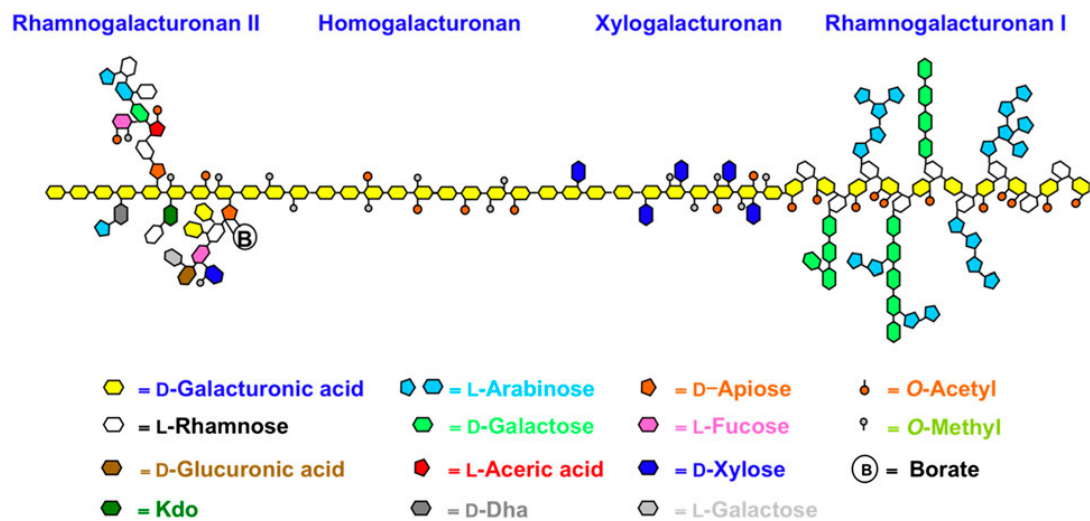


Figure 1.3 Common structures of pectin

Different regions or types of pectin can be linked along a shared backbone. Some side-chain structures are different in certain plants. Image taken from Scheller and Ulvskov, 2010.

and has the ability to bind together neighbouring HG polymers. This occurs when at least 10 consecutive residues are demethyl-esterified (Caffall and Mohnen, 2009). The pectin-rich middle lamella is rich in calcium-bound HG, which contributes to cell–cell adhesion. Calcium-bound HG also is involved in wall strengthening in green algae (Proseus and Boyer, 2006; Eder and Lutz-Meindl, 2008), and potentially in land plants as well (Stolle-Smits et al., 1999). HG is synthesized in the Golgi apparatus with a high degree of methylesterification (Pelloux et al., 2007) possibly to prevent aggregation prior to deposition in the wall. Some pectin methyl esterases (PME) remove

methyl groups from HG in a processive manner, which creates regions suitable for calcium binding (Willats et al., 2001).

1.2.3.2 Rhamnogalacturonan I (RG-I)

RG-I has a repeating backbone of [α -GalA-(1→2)- α -Rha-(1→4)-] with sidechain substitutions attached (1→4) to about half of the Rha residues (Caffall and Mohnen, 2009). The side chains are varied in linkage and branching pattern, and include but are not limited to α -(1→5)-arabinan, β -(1→4)-galactan, and β -(1→3)-galactan. The arabinan or galactan side-chains can be substituted with other sugars in different species (Naran et al., 2008). Backbone GalA residues may also be acetylated. The high degree of side-chain heterogeneity may indicate various functions, as they are developmentally and spatially regulated (Caffall and Mohnen, 2009). The arabinan and galactan side-chains, especially, of RG-I may confer flexibility to the cell wall (Harholt et al., 2010). Removal of arabinan side-branches of RG-I reduce stomatal guard cells' ability to respond to opening or closing signals, possibly due to a subsequent 'locking' by Ca-bonded HG (Jones et al., 2003; Jones et al., 2005). Unbranched RG-I from many plants is featured in seed mucilage. Degradation products of RG-I might be involved in allelopathy (Yamada et al., 1997).

1.2.3.3 Rhamnogalacturonan II (RG-II)

RG-II is a highly substituted polymer with a short (7–9 residues) α -(1→4)-GalA backbone. There are four well defined side-chains, composed of 12 different types of sugar residues, including many rare sugars. Two of the side-chains contain D-apiofuranose, which have the unique capability of diesterifying with borate. One borate molecule can bind two apiose residues, and in doing so covalently cross-links pectin chains (Albersheim et al., 2011). Boron is an essential nutrient for plants, and this cross-link could be integral to the function of the cell wall (O'Neill et al., 2004). Plants deficient in boron have thicker cell walls and a dwarfed phenotype, potentially because they are less able to succumb to turgor pressure (O'Neill et al., 2004).

RG-II occurs in all land plants, and in most tissue types. In fact, the structure of RG-II is highly conserved across all land plant species, with few exceptions (Matsunaga et al., 2004). One side-chain has small variations in some plant genera. Many pteridophytes and lycophodiophytes include 3-O-methyl-Rha into RG-II, but *Equisetum hyemale* RG-II resembles that of the angiosperms (Matsunaga et al., 2004). RG-II is found throughout the primary and secondary walls but not in the middle lamella (O'Neill et al., 2004). Functional RG-II dimers are necessary for pollen tube growth (Holdaway-Clarke et al., 2003). Generally, the dimerization of RG-II could play an integral part in providing strength to the plant cell wall.

1.2.3.4 Xylogalacturonan (XGA)

XGA is a polymer with an α -(1→4)-GalA backbone substituted with β -(1→3)- or, less frequently, β -(1→2)-Xyl residues. Sometimes the Xyl can be further substituted with one or more Xyl residues (Caffall and Mohnen, 2009). XGA has been found in seeds, but is also present in vegetative tissues, as shown by xylogalacturonan hydrolase digestions of pectic extracts (Zandleven et al., 2007). Levels of mRNA encoding a potential XGA-specific xylose transferase, XGD1, are increased in response to pathogen attack, implicating the polymer in plant defence mechanisms (Jensen et al., 2008). However, the functions of this polysaccharide have not yet been fully elucidated.

1.3 Xyloglucan endotransglucosylase/hydrolase (XTH)

XTHs (EC 2.4.1.207) are apoplastic-targeted proteins capable of two actions (Rose et al., 2002). The first, xyloglucan endotransglucosylase (XET), cleaves a β -1(→4)-Glc bond in a XyG chain at the same position as XEG, and forms a covalent intermediate between the substrate and enzyme. The first XyG substrate is termed the donor substrate, and it supplies the energy for the reaction. The reaction is completed when the β -(1→4) bond is reformed with the non-reducing terminus of another XyG polymer or oligomer, the acceptor substrate (Fry et al., 1992; Nishitani and Tominaga, 1992). Some XTH

proteins in the family are capable of hydrolysing the substrate by using water as the acceptor substrate (Farkaš et al., 1992, as referenced in Rose et al., 2002). This action is xyloglucan endohydrolase (XEH).



Figure 1.4 Phylogenetic tree of the XTH gene family. Bootstrap values shown. Taken from Baumann *et al.*, 2007.

XTH phylogeny has traditionally revealed three clades of XTH-related genes (Campbell and Braam, 1999). However, the increased knowledge of genome sequences, and thus XTH sequences, has blurred the lines between the classically named groups I and II (Yokoyama et al., 2004). These groups are now known as group I/II to reduce ambiguity with

previous literature. Concurrently, the third group has resolved into two distinct clades, namely groups III-A and III-B (Fig. 4). The archetypal XTH hydrolase, Tm-NXT1, resides in group III-A with structurally similar genes predicted to have hydrolytic activity. Hydrolases can be found in other groups as well, such as Sl-XTH5 in group III-B and Sl-XTH2 in group I/II (Saladié et al., 2006). An individual enzyme's endotransglucosylase or hydrolase activity cannot be predicted by the current XTH phylogenetic grouping (Baumann et al., 2007).

1.3.1 Function of XTH

Since the discovery of XET, its function has been linked with cellular expansion. The highest amount of activity per fresh weight was extracted from the elongation region of etiolated pea seedling stems (Fry et al., 1992). XET could cause expansion of cell walls by cleaving a XyG tether, under tension from turgor pressure, allowing for the separation of cellulose microfibrils. The molecular strength of the XyG tether would not be lost, as the bond is reformed after slippage, or creep, has occurred.

This model is supported by further experimental data. Onion epidermal peels under constant tension yielded more when incubated with purified XTH than without XTH (Van Sandt et al., 2007a). An artificial cellulose (from *Acetobacter xylinus*) and XyG composite exhibited creep

behaviour while being modified by XET action but not XEH, cellulase, or boiled XTH enzyme (Chanliaud et al., 2004). XET action was shown *in vivo* through the application of a fluorescently-labelled acceptor substrate. Active XTH was localized with XyG most strongly at or directly behind the elongation zone in *Arabidopsis* and tobacco roots, as well as at the sites of lateral root formation (Vissenburg et al., 2000). XET activity was also localized at the site of growth in many bryophytes, although XET activity was indiscriminate in some species. However, XET action was also detected in green algae (Van Sandt et al., 2007b), many species of which were previously shown to lack XyG (Popper and Fry, 2003). Indeed, the completion of the *Physcomitrella patens* genome allowed for data mining and the discovery of a large number of probable XTH genes. Many were implicated in tip growth in that moss (Yokoyama et al., 2010). Also, the reduction of XET by the constitutive expression of XTH antisense RNA in lettuce (*Lactuca sativa*) resulted in smaller leaves (Wagstaff et al., 2010). Similarly in *Arabidopsis*, the knockout of *AtXTH21* by T-DNA insertion caused dwarf plants, but its overexpression led to larger plants (Liu et al., 2007). Earlier, Osato et al. (2006) showed that reduction of a root-specific XTH (*AtXTH18*) by RNAi decreased root length, demonstrating that XTHs were involved in cell elongation. Lastly, the overexpression of a Brassica gene (*BcXTH1*) in *Arabidopsis* caused a stem elongation and large leaf

phenotype, and the authors showed that higher levels of the XTH gene product lead to larger cells in those plants (Shin et al., 2006).

However, not all experimental data support this model. Some research has shown that XET does not induce creep, or can even strengthen walls. McQueen-Mason et al. (1993) could not induce creep in cucumber hypocotyls using extracts with high XET activity, in direct contrast to the effects of expansin. While high levels of XET activity are found in root elongation zones, some XTH genes (e.g. *AtXTH17* and *AtXTH18*) are only expressed in mature root tissues, where elongation has ceased (Vissenberg et al., 2005). Anti-XTH antibodies revealed that XTH proteins are produced during wood secondary wall development, and XET activity in these tissues persisted for years after synthesis (Mellerowicz et al., 2008). The authors suggested that XTH could be involved in the strengthening of tissue fibres. Finally, the addition of *AtXTH26* or *AtXTH14* to onion epidermal peels during constant-load extensimetry decreased wall extensibility (Maris et al., 2009). Arabidopsis root growth was inhibited when incubated in solutions of the same proteins (Maris et al., 2009). *AtXTH26* and other XTHs could have a strengthening role in some tissues.

Other proteins may also play a role in cell growth. Expansin has been associated with plant growth through the relaxation of the wall during cell expansion. Two groups of proteins, α -expansin and β -expansin, cause

elongation of inactivated cucumber hypocotyls in creep assays (McQueen-Mason et al., 1992). α -Expansins and a few β -expansins are implicated in 'acid-growth,' or the rapid enlargement of cells after a drop in apoplastic pH. Other β -expansins are highly expressed in pollen tubes during fertilization (Sampedro and Cosgrove, 2005). No enzymatic activity has been shown, but the proteins are thought to break hydrogen bonds between cell wall polymers, which loosens the wall matrix (McQueen-Mason and Cosgrove, 1995).

As some XTHs are implicated in elongation, and others are implicated in wall strengthening, it is possible that XTH does not have a direct effect on cell wall mechanics. Rather, the properties of the XyG being modified (e.g. its M_r) could be more relevant to the effect of XET activity (Nishikubo et al., 2011). Perhaps, the abundance ratio of XyG to XET could also be a determining factor in the effects of XET action (Nishikubo et al., 2011). If so, the synthesis of XyG, its dynamics in the wall, i.e. *in situ* modification, and degradation, should all be included in a comprehensive model for the function of XTH.

1.3.2 Characteristics of XTH

Many isoforms of XTH have been isolated and recombinantly expressed for characterization.

The pH optimum of many XTHs is mildly acidic; however, exceptions occur. In a study of recombinant XTHs which are all expressed in root tissues, Maris et al. (2011) found that AtXTH12, AtXTH13, and AtXTH19 had the highest activity at pH 5, 6, and 7, respectively. Interestingly, another (AtXTH18) kept much of its activity across the pH range of 6.5–8.0 (Maris et al., 2011). Differentially expressed XTHs could be regulated in different tissues by pH. Expansins may also be regulated by pH, as their wall-loosening effects are increased by a change in apoplastic pH to 4.5–6.0 (Cosgrove, 2005).

XET activity is also dependent on the type of substrate available. Some XTHs have higher activity on different XyG or XGO acceptors, depending on the substrates' side-chain substitutions. For example, HvXET3 has almost 4-fold higher activity when using XLLGol compared to XXXGol as an acceptor, but HvXET6 has twice as much activity on XXXGol than XLLGol (Vaaje-Kolstad et al., 2010). At very low rates, some XTHs have been shown to use non-XGO acceptors, such as cello-oligos, but the rates on alternative acceptors was either not quantified (Ait Mohand and Farkaš, 2006; Kosík et al., 2010), or was very low (Stratilová et al., 2010) and could even be an artefact of the fluorescent label attached to the acceptor (Kosík et al., 2011). Transglucosylation products could not be found using SR-labelled cello-oligosaccharides in other plants (Vissenberg et al., 2000; Yokoyama et

al., 2010). Cellulosic substrates were not able to bind into the active site of *PttXET16–34 in vitro*, but the enzyme did utilize under-xylosylated substrates (Saura-Valls et al., 2008).

When heterologously produced and purified, some XTHs are able to use other β -glucans as donor substrates as well, including water-soluble cellulose acetate, hydroxyethyl cellulose, cellulose sulfate, and carboxymethyl cellulose (Hrmova et al., 2007; Maris et al., 2009; Maris et al., 2011). The discovery of a barley XTH, *HvXET5*, that was able to use MLG as a donor and cello-oligosaccharide acceptors in a transglucosylation reaction led to the theory that cellulose microfibrils could be covalently cross-linked by hemicelluloses such as MLG in grasses (Hrmova et al., 2007). However, neither $\text{MLG} \rightarrow \text{XGO}$ or $\text{XyG} \rightarrow \text{cello-oligosaccharide}$ transglucosylation activities were greater than 0.2% of the $\text{XyG} \rightarrow \text{XGO}$ rate, limiting the potential effectiveness of the enzyme to produce the bonds suggested.

Recently, a heterotransglucosylase called mixed-linkage glucan : xyloglucan endotransglucosylase (MXE) was discovered in a green alga and a pteridophyte genus, *Equisetum* (Fry et al., 2008 a). Crude extracts from *Equisetum* contain large amounts of MXE activity, up to 146% of the XET activity (Fry et al., 2008 a). The highest relative amounts of activity came in mature shoots, indicating that MXE activity could be used in a strengthening role. Also, the authors argued that MXE activity was not the result of an

XTH enzyme, as reaction rates using various acceptor substrates were different using MLG or XyG as the donor substrate, the reaction rate kinetics indicated that the two activities maintained different V_{\max} and K_m values, and extractable MXE activity was greater in mature than young shoots when compared with the XET activity (Fry et al., 2008 a). The concurrent discovery of MLG in Equisetum (Fry et al., 2008 b) indicated that the overall structure of the wall, the organization of polymers in the wall, and reorganization by endotransglucosylases could be very different in these plants than in any other plants studied to date (Fry et al., 2008 a).

1.4 Evolution of Viridiplantae and their walls

The colonization of land began with plants, adapting to a different environment and the challenges therein. Among those challenges were the effects of UV radiation, gas exchange, water translocation and prevention against desiccation. The cell walls of plants evolved to be able to survive in the presence of new stresses. However, prior to terrestrialization, some structural features were already present in what many now agree is the closest relative to land plants, green algae. Horsetails, being a focus of this project, are shown in their place among the evolution of plants in Fig. 1.5.

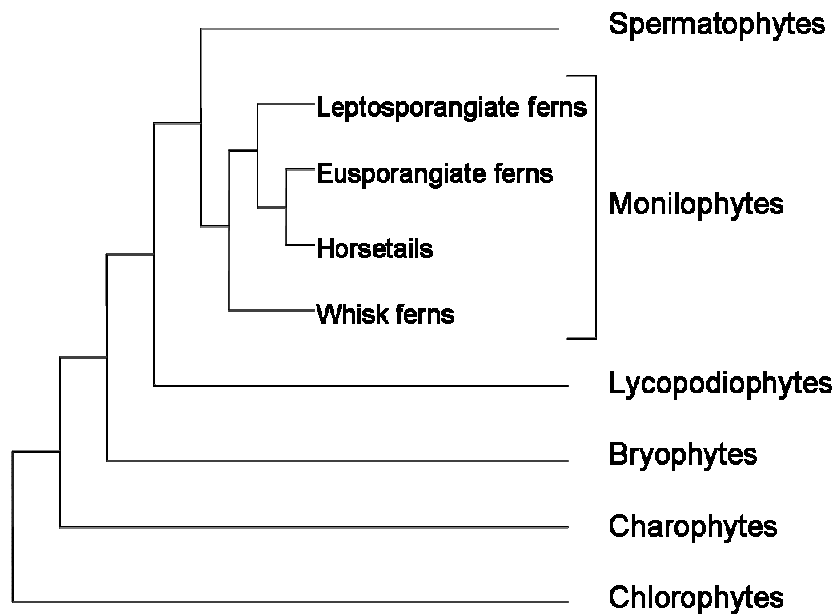


Figure 1.5 Diagram of plant evolution

Summarized from Qui et al. (2006) and Langdale (2008). Horsetails form a monophyletic group with true ferns and whisk ferns in the division of Monilophyta, which is sister to seed plants, the Spermatophyta.

1.4.1 Green Algae

In simplest form, green algae are unicellular, non-motile, autotrophic organisms (e.g. *Halosphaera*, *Osteococcus*, *Chlamydomonas*). Some species can, however, exhibit multicellularity and multi-dimensional growth (e.g. *Ulva*, *Coleochaete*, *Chara*; Bell and Hemsley, 2000). Reproduction is sexual or asexual, depending on the species and the life cycle stage. Biochemically, green algae are characterised by the presence of chlorophylls *a* and *b*, and β -carotene, giving them their overall green colour (Domozych, 2001). Also, they use starch as an energy storage polymer and have double membrane-surrounded plastids (Stewart, 1983; Lewis and McCourt, 2004). Of the two

major divisions of green algae, Chlorophyta and Charophyta, the latter are the closest relatives to land plants (Kenrick and Crane, 1997; Lewis and McCourt, 2004). Within the Charophyta, the division of Charales is likely to be sister to land plants (Karol et al., 2001; Becker and Marin, 2009).

The cell walls of charophycean green algae (CGA) contain many features found in embryophytes. Most green algae can produce cellulose (Niklas, 2004; Stewart, 1983), including members of both the charophytes and the chlorophytes (Popper and Tuohy, 2010) and some cellulose synthase complexes are arranged in a hexameric configuration, similar to land plants (Niklas, 2004). The AIR of CGA do not contain the hemicellulose xyloglucan (Popper and Fry, 2003). Eder et al. (2008) and Domozych et al. (2010) found a polysaccharide in CGA that was recognized by XyG antibodies, but subsequent characterization of the polysaccharide was either not performed or did not confirm the labelling results (Sørensen et al., 2010). *Micrasterias*, a charophycean desmid, was shown to contain another hemicellulose, mixed-linkage β -glucan. The ratio of tri- to tetra-saccharide units of its MLG closely resembles lichenan, and could only be immuno-localized to the secondary cell wall (Eder et al., 2008). Pectins are abundantly found in *Chara* (Proseus and Boyer, 2006), *Micrasterias* (Eder and Lutz-Meindl, 2008), and other species (Popper and Fry, 2003). *Penium*, another desmid, contains high levels of homogalacturonan, and possibly other types of pectins (Domozych et al.,

2007). Many green algae produce sporopollenin, a decay-resistant polymer, and phenols similar to lignin monomers (Graham, 1996).

1.4.2 Non-vascular land plants: bryophytes

The division of bryophytes include liverworts (e.g. *Marchantia*), hornworts (e.g. *Anthoceros*), and mosses (e.g. *Sphagnum*, *Polytricum*, *Physcomitrella*).

These, the simplest of land plants, have limited cell differentiation, lacking vasculature, true roots, and sporophytic shoot branching (Bell and Hemsley, 2000; Langdale, 2008). The main stage in the life cycle of all bryophytes is the gametophyte, with the sporophyte usually short-lived and completely dependent on the gametophyte for survival (Bell and Hemsley 2000).

Mosses, more complex than the liverworts, have multicellular, potentially branched, rhizoids and leafy gametophytes. Their sporophytes grow from an apical cell (Rashid, 1998). Although some specialized cells transport water and photosynthetic products, the potential absence of lignin in their cell walls separates bryophytes from tracheophytes (Langdale, 2008).

Among the bryophytes, hornworts may be sister to tracheophytes, but this point is still debated (Qiu et al., 2006).

The cell walls of bryophytes are distinctly different than algal walls.

The hemicellulose XyG was found in all land plants examined (Popper and Fry, 2003). However, the pattern of side-chain substitution was different in

liverworts and mosses than in angiosperms, having only two consecutive xylose substitutions, as well as other unique substitutions (Peña et al., 2008). Hornwort xyloglucan appears to be quite similar to vascular plants' xyloglucan, reinforcing the hornworts' position as sister to vascular plants (Peña et al., 2008). Monosaccharide analysis from total wall preparations of various bryophyte species showed an increased proportion of mannose compared to other sugars, but the origin of this monosaccharide could not be determined (Popper and Fry, 2003; Nothnagel and Nothnagel, 2007). GalA and GlcA were two other especially abundant sugars, possibly components of pectins (Popper and Fry, 2003). Mosses from many genera were labelled with multiple pectin-specific antibodies (Ligrone et al., 2002). Based on NMR data of partially purified polysaccharides, Ballance et al. (2006) suggested that *Sphagnum* could have RG-I. Also, RG-II was detected, albeit at low concentrations, in 13 species of liverworts, mosses, and hornworts (Matsunaga et al., 2004). The side-chain structures of RG-II were similar but not identical to angiosperm RG-II, having some terminal 3-O-methylrhamnose (Matsunaga et al., 2004). This unique sugar was detected in appreciable quantities (Popper and Fry, 2003), was a component of AGPs (Fu et al., 2007), and may be a component of other polysaccharides as well (Matsunaga, et al., 2004). Bryophytes do not synthesize lignin, but do make

lignan, which is a dimer of hydroxycinnamic acids (Weng and Chapple, 2010).

1.4.3 Basal vascular plants: pteridophytes

The lycopodiophyte branch of the pteridophytes include the genera *Lycopodium* and *Selaginella*. Both form microphyllous leaves on aerial stems, and roots that branch (Gifford and Foster, 1989). The remaining pteridophytes belong to the monilophyte group, including whisk ferns (e.g. *Psilotum*), eusporangiate ferns (including the Marattiaceae and Equisetaceae families), and the largest group, the leptosporangiate ferns (Smith et al., 2006). With the exception of the horsetails and whisk ferns, the transition to the ferns marks the appearance of megaphylls. Not to be confused with a size distinction, megaphylls contain branched venation (Gifford and Foster, 1989). Ferns complete their life cycle through the alternation of generations externally, as in bryophytes (Grounds, 1974). Mature sporophytes disperse spores that germinate to form prothalli. Egg cells and sperms combine to reinstate the diploid condition and grow into sporophytes (Grounds, 1974).

The pteridophytes' cell walls have not been extensively studied. The few pieces of information cast light into an otherwise unknown set of knowledge.

The hemicelluloses of the pteridophytes are different from the bryophytes'. Xyloglucan is prominent, but some of the side chain substitutions of some genera's XyG are similar to angiosperm XyG, but others' are quite dissimilar (Peña et al., 2008). β -Xylan was evident by antibody staining in the secondary cell walls of tracheophytes but not bryophytes (Carafa et al., 2005). The prevalence of mannose changed in this category; eusporangiate ferns maintained high levels of the sugar but leptosporangiate ferns significantly decreased the amount of mannose in their wall (Popper and Fry, 2004). Surprisingly, MLG was discovered in horsetails and confirmed by multiple methods (Fry et al., 2008 b; Sørensen et al., 2008).

Of the pectins, GalA continued to be a prevalent monosaccharide in the pteridophytes (Popper and Fry, 2004). High amounts of HG, sometimes methyl esterified, were found in *Equisetum arvense* by antibody staining (Sorensen et al., 2008). Relative amounts of RG-II were higher in tracheophytes than in bryophytes (Matsunaga et al., 2004).

Espiñeira et al. (2010) confirmed the presence of lignin in all pteridophytes examined, but reported very low levels of lignin in some species including *E. telmateia*. Lignin staining of internode sections revealed the polymer only in xylem elements, but not in thick-walled sclerenchyma tissue (Espiñeira et al., 2010). Other strategies could be employed for

strengthening the plant, possibly the deposition of silica (Currie and Perry, 2009).

1.4.4 Seed plants: spermatophytes

Seed plants have dominated our terrestrial environment in the past ~250 million years (Willis and McElwain, 2002). The morphology of spermatophytes is too varied and complex to be concisely summarized.

The vast majority of research in the field of plant cell walls has been accomplished in angiosperm, notably crop, species (Popper, 2008). The spermatophytes' wall compositions are as different from each other as they are different from all other plants. However, some generalizations can be made about the prevalence and occurrence of some hemicelluloses. XyG is regularly found in primary cell walls, and although in smaller proportions, in secondary walls as well, e.g. dicotyledonous angiosperms. GlcM and GGM make up a large majority of the hemicelluloses in gymnosperm wood, but only 3–5% of the hemicelluloses in angiosperms (Moreira and Filho, 2008). Xylans are the most abundant non-cellulosic polysaccharide in non-commelinid monocots (Vogel, 2008). Xylans are also an important component of secondary walls in many plants. MLG can only be found in one group of spermatophytes, the Poalean monocots. Although MLG is most

abundantly found in primary walls and seed endosperm, traces were detected in mature vegetative walls as well (Trethway et al., 2005).

Pectin varies as much as hemicelluloses in seed plants. In most plants, pectin is the most abundant non-cellulosic polysaccharide, of which HG is the most common domain (Mohnen, 2008). However, Poalean monocots incorporate very little pectin into their cell walls (Vogel, 2008). Some pectic polymers are found in tissue-specific regions. For example, XGA is abundant *Arabidopsis* leaves, but less common in the stems, and absent from roots and seeds (Zandleven et al., 2007). The study of pectins' distribution could elucidate their potentially very diverse roles in plants.

1.5 *Equisetum* physiology

1.5.1 Reproductive life cycle

The dominant phase of the bryophyte life cycle is the gametophyte (n). However, the sporophyte (2n) of pteridiophytes is larger and more complex (Sporne, 1975).

Sporophytes of *Equisetum* produce and release spores borne on sporangia, located terminally on aerial shoots (Sporne, 1975). The spores are unique in two ways: they are dense in chloroplasts and have four spoon-like appendages called elaters, which could be used to aid in dispersal. When

located on suitably moist medium, they quickly germinate (Gifford and Foster, 1989).

The mature gametophytes are small, usually a few mm but up to 1 cm. Prothallus-like gametophytes are anchored to the substratum by unicellular rhizoids (Gifford and Foster, 1989). They develop antheridia (containing spermatozoids), archegonia (containing a single egg), or both. Reports conflict on whether a single spore develops into completely male, completely female, or bisexual gametophyte, having both antheridia and archegonia on the same plant. Sex determination may depend on environmental conditions, and heterospory, homosporous, and bisexual-spore generation could be species-dependent. Mature spermatozoids are motile in water, and travel to the archegonia for fertilization (Gifford and Foster, 1989).

Zygotes develop into sporophytes out of but independent from the gametophyte. Mature sporophytes can, and commonly do, propagate vegetatively, forming new shoots from underground (e.g. *E. arvense*) or underwater (*E. fluviatile*) rhizomes (Bold, 1957).

1.5.2 Anatomy of the sporophyte

Zygotes develop two shoots, one aerial and the other rhizomatous, and a minor foot-like root. The two shoots are not dissimilar from each other. The apical meristem is protected by multiple layers of leaves, which are fused

together to form whorls around the stem. The microphyllous leaves are attached to nodes (Bold, 1957). The stem can be easily sectioned into nodes and internodes upon visual investigation. Most of the height of the stem comes from division of cells at basal intercalary meristems located at each node. Therefore, the oldest components of each internode are the leaves, followed by upper portions of the internode, with the middle and lower portions of each internode becoming increasingly younger. Still-developing tissue can be found at the base of most internodes (Gifford and Foster, 1989). Some species of *Equisetum* also produce lateral stems, as in *E. telmateia* and *E. arvense*, but others remain unbranched, as in *E. hyemale*. Roots and lateral branches are attached at nodes (Sporne, 1975).

1.5.3 *Equisetum* cell walls

Many land plants are benefited by silica, but it is not an essential element. Plants take up silica through their roots as silicic acid $[\text{Si}(\text{OH})_4]$, and deposit it in the cell wall (or other tissues) to precipitate as a polymer of amorphous silica ($\text{SiO}_2-n\text{H}_2\text{O}$), or opal (Richmond and Sussman, 2003). Si presence can alleviate biotic and abiotic stresses, including pathogen attack, drought, salinity, metal toxicity, and high temperature (Currie and Perry, 2007).

Horsetails are the only land plants that require silica for growth (Epstein, 1994). Silica was found in a thin epidermal layer around *E. hyemale*

stems, but the concentration of silica was especially high in knob regions at the tips of ridges (Sapei et al., 2007). Internal silica deposition correlated with thickened secondary walls and xylem, and was surmised to play a structural role in *Equisetum* (Laroche, 1968, as referenced in Hoffman and Hillson, 1979). In addition, when grown on media reduced in silicon, *E. arvense* stems drooped and fewer strobili were formed (Horrman and Hillson, 1979). As *Equisetum* has very little lignin (Españeira et al., 2010), epidermal and/or internal silica could provide mechanical strength to the plant.

Equisetum species have long stood out from other land plants as they require silica for growth, and deposit large amounts of silica in their cell wall (Epstein, 1994). Other than this fact, the cell walls of *Equisetum* and other non-angiosperm plant groups have not been well studied (Popper and Fry, 2004). However, recent studies using various techniques have revealed more information on the composition of *Equisetum* cell walls.

Pectins are abundant component of *Equisetum* walls. Monoclonal antibody labelling of *E. ramosissimum* revealed homogalacturonic acid, both esterified and de-esterified (Leroux et al., 2011). Galactans and arabinans, potential side-chain structures of RG I, were also detected (Leroux et al., 2011). RG II, extracted from *E. hyemale* and *E. variegatum*, was found in quantities similar to extracts from angiosperm species. The structure was

similar but very slightly different than previously described RG II structure (Matsunaga et al., 2004).

The hemicelluloses of *Equisetum* are also similar to the hemicelluloses of the most highly evolved plants. Popper and Fry (2004) discovered high levels of mannan after acid hydrolysis of AIR, similar to gymnosperms. Mannans, glucomannan, and galactoglucomannan epitopes were detected in *Equisetum* stems (Leroux et al., 2011). Xylans have been recently characterized in *E. hyemale* (Kulkarni et al., 2011). The structure is very similar to Arabidopsis xylan, which contains GlcA and 4-O-methyl-GlcA attached at O-2, but no monocot-like arabinoxylan was detected (Kulkarni et al., 2011). XyG in *E. hyemale* shares similarity with eudicot XyG, but the unique sugar, α -L-arabinopyranose, is a common substituent (Peña et al., 2008). An unexpected discovery of MLG in *Equisetum* proved the polysaccharide to not be restricted to Poalean monocots in land plants (Fry et al., 2008; Sørensen et al., 2008). MLG in *Equisetum* is different to grasses and cereals, in both the fine structure (a greater proportion of the cello-tetrasaccharide-like region is found in *Equisetum* MLG, Fry et al., 2008; Sørensen et al., 2008) and developmental regulation (MLG is abundant in *Equisetum* secondary walls but not primary, Sørensen et al., 2008; Leroux et al., 2011).

1.6 Project aims

1.6.1 Purification of MXE

Mixed-linkage glucan : xyloglucan endotransglucosylase (MXE) activity was recently discovered in green algae and a pteridophyte genus, *Equisetum* (Fry et al., 2008 a). However, this protein was not identified. One goal of this project was to purify the protein for sequence identification. This protein may be similar to XTHs, but not necessarily. Other transglycosylases are known to exist, including mannan endotransglucosylase/hydrolase (MTH). The protein with transglycosylase activity was found to be LeMAN4, an endo- β -mannanase, belonging to GH family 5 (Schröder et al., 2009). These enzymes have the same mechanism as XTHs (Eklof and Brumer, 2010). Purification is a useful step to determining whether MXE belongs in the XTH family or in another, potentially new, family.

1.6.2 Characterization of MXE

Purification of MXE would also allow for its characterization. Crude extracts frequently include not only the protein of interest, but also others that could directly affect the reaction studied. Many plant extracts contain glycan hydrolases or glycanases, which could degrade or compete for potential reactants of a transglucosylation reaction. For example, although the rate of MLG : MLG oligosaccharide (MLGO) or cello-oligosaccharide

transglucosylation was only 15% and 8% of the XET activity rate, respectively (Fry et al., 2008 a), the rate could be higher if freed of competing hydrolases. Also, XTHs could compete for the same substrates. Kinetics, pH optimum, exact substrate specificity, or other characteristics cannot be determined in a crude mixture. For example, Takeda et al. (1996) gave evidence supporting a sequential reaction mechanism for XET from crude sycamore enzyme extracts, only for others to conclude that XET works by a bi-bi ping-pong mechanism (Rose et al., 2002 and references therein; Saura-Valls et al., 2006; Hrmova et al., 2009).

1.6.3 *In vivo* MXE action

Vissenberg et al. (2000) introduced a technique that allowed for visualization of XET in tissues. A fluorescently labelled acceptor substrate was incubated with growing *Arabidopsis* or tobacco roots, and high XET activity was shown in the region of cellular elongation. Others have subsequently used this technique to show XET *in vivo* in different tissues and systems, including secondary wall formation in aspen (Bourquin et al., 2002), lettuce leaves (Wagstaff et al., 2010), and various moss tissues (Yokoyama et al., 2010).

Applied to horsetail sections, this technique can show the location of MXE or XET action. However, this technique will not distinguish between the two, since both activities use the same acceptor substrate. Therefore, to

distinguish between the two, a radiolabelled acceptor can be used in additional studies. If hemicelluloses can be extracted from the tissue sections, they can be distinguished from each other by digestion with specific endoglucanases, XEG or lichenase, and oligosaccharides produced can be separated by thin-layer chromatography.

2 Materials and Methods

2.1 Routine laboratory techniques

All chemicals, unless stated otherwise, were obtained from the Sigma-Aldrich Chemical Company, Poole, UK. All water used was deionised water, unless specifically stated otherwise. Buffers were made by adding NaOH or HCl to the weak acid or base and adjusted to the pH indicated, unless otherwise stated.

2.1.1 Plant material

Equisetum fluviatile was grown in an outdoor pond at Edinburgh University. Plant material was washed with tap water before either immediate use, in the case of green shoots, or storage in suitable medium, in the case of rhizomes. As all plants (and the resulting cell cultures) came from the same colony, it is likely that they were genetically identical, as very little sexual reproduction occurs. Lycopodiophytes were collected from the wild, and washed in tap water before homogenization. *Hordeum vulgare* plants were grown in glasshouses at Edinburgh University for 12 weeks before use.

2.1.2 Extraction of enzymes from plant material

Crude enzyme mixtures were extracted from fresh plant tissue in CaCl₂ (10 mM), succinic acid (0.3 M) and ascorbic acid (20 mM), made fresh to pH 5.5. Polyclar AT (3% w/v) was added to complex with phenolics. Fresh tissue was homogenized in a food blender with 5 ml of the extractant above per gram of fresh weight tissue. The tissue and extractant were stirred for 2.5 h on ice to slow proteins' denaturation by proteases. The extract was filtered through two layers of Miracloth and centrifuged in a Sorvall Evolution RC Centrifuge (10 min, 10,000 rpm, 4°C). The supernatant was collected and aliquotted, then frozen in liquid nitrogen and stored at -80°C.

2.1.3 Alcohol-insoluble residue (AIR)

Plant material was ground with a mortar and pestle or homogenized in a standard food blender, then transferred to a 15-ml tube. Excess Ethanol (to 75% v/v) was added, and tubes were rotated on a Stuart Rotator SB1, SB2, or SB3. After 2 h, the tubes were centrifuged in an MSE Centaur 2 (3700 rpm, 2 min). The ethanol wash was removed and replaced with fresh 75% ethanol. The centrifugation and replacement of ethanol was repeated at least three times or until no more chlorophyll came into solution. The remaining residue was dried at room temperature.

2.1.4 Saccharide chromatography

2.1.4.1 Thin layer chromatography (TLC)

Sugar (1–100 µg) was applied to a Merck TLC Silica Gel 60 (VWR) TLC Plate. The TLC was developed by ascending chromatography in a solvent of butanol : acetic acid (HOAc) : water (BAW), 2:1:1, made fresh, until the solvent front was <1 cm from the top of the plate. Some oligosaccharides became better separated after 2 or 3 developments in this solvent. Another common solvent used was propanol : HOAc : water (PrAW), 2:1:1; however, TLC plates developed in this solvent were first washed by slowly rocking in acetone : HOAc : water (AAW), 2:1:1, for 45 minutes.

2.1.4.2 Paper chromatography (PC)

Sugar (25 µg) was loaded as 15-µl spots on the origin of Whatman 3MM chromatography paper. When analysing monosaccharides, the chromatogram was developed using a descending mobile solvent of ethyl acetate : pyridine : water (8:2:1), for 24 h. Oligosaccharides were developed in a similar manner in ethyl acetate : HOAc : water (10:5:6).

2.1.4.3 Gel permeation chromatography (GPC)

Dry Bio-Gel P-series (Bio-Rad, Inc.) beads were swollen in pyridine : HOAc : water (PyAW, 1:1:98) + 0.5% chlorobutanol (CB) in a beaker overnight. The

solution was degassed using a water aspirator. The beads were poured into a glass column (1.5 cm diameter) to the desired height, and washed in excess buffer. To determine the void and included volumes, a solution of blue dextran (BD) and cobalt chloride (CoCl_2), 5% v/v of the bed volume, were passed through the column at the rate recommended by the manufacturer. Fractions were collected using a Bio-Rad Model 2110 automatic fraction collector, and analysed using a Cecil CE8020 spectrophotometer. The column was washed again in excess buffer and stored at room temperature.

2.1.5 Sugar detection

2.1.5.1 Thymol

A developed TLC was submerged in a tank with a solution of sulfuric acid (5% v/v) and thymol (0.5% w/v) in ethanol and dried in a fume hood. When dry, it was incubated at 105°C for 15 min, until spots became dark. A digital photograph was taken with an Epson GT-1500 scanner.

2.1.5.2 Silver nitrate

This detection method required the chromatogram to be dipped through three solutions. The first solution was made by adding silver nitrate (AgNO_3)-saturated water (2 ml) to acetone (400 ml), where the AgNO_3 precipitated. Water was added drop-wise until the precipitate re-dissolved.

The second solution consisted of NaOH (0.125 M) in ethanol. If sensitive detection was required for a smaller amount of sugars, the chromatogram could be dipped multiple times in the second solution, drying after each dip. Finally, the chromatogram was dipped through 10% sodium thiosulfate in water to fix the stain, then washed in running tap water and dried for long term storage.

2.1.5.3 Anthrone

Anthrone (0.2%) in concentrated sulfuric acid (H_2SO_4 , 1 ml) was added to 0.5 ml of a carbohydrate solution to measure hexoses. The solution was carefully mixed and incubated (5 min, 100°C), then cooled. Absorbance was measured at 620 nm. A standard curve was constructed with glucose.

2.1.6 Protein assays

2.1.6.1 Bradford assay

Bio-Rad Protein Assay Dye (0.2 ml) was added to a cuvette (Fisherbrand FB55147) with a protein solution (0.8 ml), covered with Parafilm, and mixed by inversion. The A_{595} was measured immediately. A standard curve with bovine serum albumin (BSA) was constructed. Three stock solutions of 1% BSA were made in water, then diluted to 5–30 $\mu\text{g}/\text{ml}$. A best fit line was calculated in Microsoft Excel excluding the origin.

2.1.6.2 Absorbance at 280 nm (A_{280})

In instances when the Bradford assay was impractical or impossible, protein content was assayed by A_{280} . A fresh solution of BSA (1% w/v) was diluted to a final concentration of 0.2–1.0 mg/ml. The standards were placed in a HEL quartz cuvette and their absorbance was measured. Unknown samples or fractions were placed in the same quartz cuvette and measured directly.

The advantages of this assay are that the unknown sample can be recovered in its original form. One disadvantage of this assay is that some other compounds absorb light at this wavelength, for example phenolics.

2.1.6.3 Ninhydrin stain for amino acids

Ninhydrin (0.5%) was dissolved in acetone. A series of samples or fractions (10 μ l) were dried on paper, then dipped through the stain. The paper was heated (105°C, \geq 5 min) until spots became visible. Most amino acids appear violet, but proline and hydroxyproline stain yellow.

2.1.7 Polysaccharide hydrolysis

2.1.7.1 Trifluoroacetic acid (TFA) hydrolysis

A solution or mixture of AIR, polysaccharides, or oligosaccharides was incubated in TFA (2 M, 120°C) for 1 h in a rubber-sealed screw-cap Sarstedt

tube. Crystalline cellulose was the only PCW polysaccharide not hydrolysed by this method, and was separated from the hydrolysate by centrifugation in an MSE Microcentaur (5 min, 3000 rpm).

2.1.7.2 Enzymic hydrolysis

2.1.7.2.1 Xyloglucan endoglucanase (XEG)

A lyophilised powder of xyloglucanase (SP566, REBU-2000-06677-01), also known as XEG (Pauly et al. 1999), a generous gift from Novo Nordisk A/S (Bagsværd, Denmark), was dissolved to 1 mg/ml by gentle inversion in PyAW (1:1:98) + 0.5% CB. This solution was aliquotted and stored at -80°C for future use. XyG (50 μg) was added to a tube with XEG (0.05%) in 20 μl . The reaction was stopped by the addition of an equal volume of formic acid (FA). The digestion mixture was usually dried with a Savant AES1010 SpeedVac, and re-dissolved in water for further analysis.

2.1.7.2.2 Lichenase ((1 \rightarrow 3),(1 \rightarrow 4)- β -glucan endoglucanase)

Lichenase was purchased from Megazyme, Inc. as a precipitate in a solution of ammonium sulfate. The enzyme (100 units (U)) was pelleted and redissolved in PyAW + 0.5% CB (10 ml). MLG (50 μg in 10 μl) was mixed with 10 μl (0.1 U) of lichenase, and stopped by FA (4 μl). The mixture was usually dried in a SpeedVac and redissolved in water for further analysis.

2.1.7.2.3 Driselase

Driselase (purified as described in Fry, 1982), a complex mixture of endo- and exo-glycan hydrolases, was dissolved in PyAW (0.5% w/v) and added to a solution or mixture of polysaccharides. The digest was incubated for 24 h at room temperature, and denatured in boiling water.

2.2 Radioactivity detection and measurement

2.2.1 Tritium in solution

Tritium in solution was mixed with ScintiSafe3 (Fisher Scientific) aqueous scintillant, 1:10 ratio, in a scintillation vial, and scintillation counted using a Beckman LS 6500 Multi-purpose scintillation counter. The average counts per minute (cpm) were reported.

2.2.2 Scintillation counting of TLC segments

A developed TLC was overlaid with Scotch tape to prevent crumbling of the silica. The TLC was cut into 0.5-cm segments along the running lane by hand, starting at 0.25 cm below the origin line. Aqueous scintillant (5 ml) was added to the segment in a scintillation vial, mixed well, and left overnight. The segment was assayed for ^3H twice for 10 min, with the cpm reported.

2.2.3 Scintillation counting of PC segments

A developed paper chromatogram was divided into lanes and cut into 2-cm segments, starting at 1 cm below the origin line. Paper strips were placed into scintillation vials with 2 ml of Optiscint 'Hisafe' (Fisher Scientific) paper scintillant. They were assayed twice for 4 min, with the average cpm reported.

2.2.4 Detection of radiolabelled compounds by fluorography

A developed TLC or PC was dipped through a solution of 7% 2,5-diphenyloxazol (PPO) in ether. The chromatogram was taped down in a Du Pont Cronex Lightning Plus developing book. [¹⁴C]Glucose, dissolved in black ink, was spotted asymmetrically in three corners in order to maintain the orientation of the film with the chromatogram. In a dark room, a piece of Kodak BioMax MR film was placed in the developing book and sealed tightly and wrapped in a black plastic bag. It was stored at -80°C for 1-10 weeks. Film was developed in a Konica SRX-101A developer.

2.2.5 Detection of radiolabelled compounds by autoradiography

This detection method was similar to fluorography with one omission. When ¹⁴C or a high level of ³H was used, a fluor was not necessary to amplify

the radioactive signal. Therefore, chromatograms were not dipped through PPO in ether, and the film was exposed to the chromatogram at room temperature.

2.3 Assay of transglucosylation activity

2.3.1 Radioactive transglucosylation assay

Tamarind XyG, MLG, or other donor substrates (1.0% w/v) were added to 1.5-ml Treff tubes. Acceptor substrate, usually but not always borotritiide-reduced ($[^3\text{H}]\text{NaBH}_3$ -reduced) heptasaccharide fragment of xyloglucan, $[^3\text{H}]\text{XXXGoI}$ (specific activity (SA) 100 MBq/ μmol), was added. Extract or fractions potentially containing activity was added to the mixture of donor and acceptor substrate. At specified time points, the reaction was stopped by the addition of formic acid (FA, 10 μl). For zero time points, FA was added before the enzyme.

2.3.1.1 Detection of radioactive product by water washing

Each reaction mixture was applied to a square of Whatman 3MM chromatography paper. The papers were dried and washed in tap water for 16–40 h, during which any unused oligosaccharide reactant washed off, leaving only polysaccharide product. The papers were again dried and

placed in scintillation vials with Optiscint 'Hisafe' paper scintillant (2 ml).

The papers were assayed for radioactivity as in Section 2.2.3.

2.3.1.2 Detection of radioactive product by paper chromatography

Endotransglucosylase products could also be detected using PC. The reaction mixture was applied to the PC origin of Whatman 3MM chromatography paper and dried. The PC was developed in ethyl acetate : HOAc : water (EAW), 10:5:6, for 40 hours. The acceptor substrate was mobile in this solvent, but the polysaccharide product remained at the origin. The origin of the dried chromatogram of each lane, plus and minus 1.5 cm, was cut out and assayed for radioactivity as in Section 2.2.3.

2.3.2 Fluorescent transglucosylation assay

2.3.2.1 Preparation of the assay papers

Dot-blot, or test, papers were made following Fry, 1997. Test papers were made with Whatman 1CHR chromatography paper. XET-test paper was made by dipping through 1.0% XyG, dried, then dipping through a conjugate of XXXG and sulforhodamine (SR). Another paper was dipped through 1.0% MLG, dried, then XXXG-SR to make MXE-test paper. Control paper was made, containing no supplemented polysaccharide donor

substrate, but included the acceptor substrate. The final acceptor substrate concentration for all test papers was ~125 pmol/cm².

2.3.2.2 Test paper assay

Test papers, cut to size, were used in two ways: either enzyme solutions were applied to the papers as small dots (dot blot assay), or the papers were applied in close contact with native PAGE-gels (zymogram assay). The assay was incubated in a humid environment between two sealed glass plates. The papers were then dried at room temperature and washed in ethanol : FA : water (EFW) 1:1:1 for one hour. The strips were dried, pressed under weight overnight, and photographed using a UVP Multi Doc-It Digital Imaging System. Positive transglucosylation was evident as fluorescence when excited under ultraviolet light at 254 nm.

2.3.3 Cellulose : xyloglucan endotransglucosylase assays

Whatman 1CHR chromatography paper (10 mg) was incubated with an enzyme extract or fraction, [³H]XXXGol (2 kBq), and citrate buffer (pH 6.3, up to 100 µl) for a designated time. The reaction was stopped by the addition of FA (30 µl), then washed by repeated additions of water, centrifugation, and removal of the supernatant, until the supernatant no longer contained radioactivity. The cellulose/reaction product usually required about six

washes to become free of soluble radioactivity. The remaining cellulose was suspended in 0.5 ml of water, transferred to a scintillation vial with 5 ml of aqueous scintillant, and assayed for radioactivity by scintillation counting.

2.4 Protein purification methods

2.4.1 Ammonium sulfate (AS) precipitation

All supplies were stored at 6°C overnight prior to use, and all work was carried out at 6°C. Crude *Equisetum* extract (50 ml) was allowed to thaw from -80°C gently by incubation at 6°C. To remove unfiltered cell wall material or other large cell fragments, the extract was spun in a Sorvall Evolution RC Centrifuge using a Sorvall SS34 rotor (10,000 rpm, 45 min). Solid AS was dissolved in the supernatant up to the desired percentage saturation under constant stirring. The extract was spun again as above. The pellet was dissolved in 0.3 M citrate (pH 6.3). The procedure was repeated, increasing the concentration of AS at intervals up to 90% saturated.

2.4.2 Polyacrylamide gel electrophoresis (PAGE)

2.4.2.1 Sodium dodecyl sulfate (SDS) PAGE

SDS PAGE protocols followed Laemmli (1970). Gels were prepared with a 4% stacking gel above a 12% resolving gel, using Bio-Rad 40% acrylamide/bis-acrylamide 29:1, in a BioRad Mini PROTEAN Tetra Cell

system, and electrophoresed at 150 V until the bromophenol blue marker completely moved out of the gel. Molecular weight marker from Sigma (catalogue number SDS7) contained bovine albumin (66 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α -lactalbumin (14.2 kDa).

2.4.2.2 SDS PAGE silver stain

An electrophoresed gel was carefully washed lightly in water, and fixed in a solution of 50% Ethanol, 12% HOAc, and 0.05% of 37% formaldehyde. The gel remained rocking in this solution for at least 90 min to 16 h. The gel was washed three times for 20 min in 50% Ethanol, followed by 0.01% sodium thiosulfate for exactly 60 s. It was washed three times for 20 s in water and stained in a solution of 0.1% silver nitrate and 0.075% of 37% formaldehyde, made fresh. After 20 min, the stain was removed and the gel was washed twice in water, then developed in a solution of 3% sodium bicarbonate, 0.5% of 37% formaldehyde, and 2 mg/l sodium thiosulfate. When the ideal intensity was achieved, 10 mM ethylenediaminetetraacetic acid (EDTA) was added. The gel was left in this solution up to 2 h, but was replaced with fresh solution if bands continued to darken after 10 min.

The gel was stored temporarily in water. For more permanence, the gel was first soaked in a solution of 20% ethanol and 10% glycerol and dried between two cellophane drying sheets.

2.4.2.3 Native PAGE

Native polyacrylamide gels were made similar to SDS PAGE, but with a few differences. The stacking gel was made to 4.3% acrylamide with tris(hydroxymethyl)aminomethane (Tris) (67 mM, pH 6.7 with H₃PO₄). The resolving gel concentration was 7.5% acrylamide with Tris (376 mM, pH 8.9). Running buffer contained Tris (5 mM) and glycine (38 mM). Gels were electrophoresed at 6°C for 25 min at 20 mA, then 3 h at 40 mA.

2.4.3 Gel permeation chromatography (GPC) on Bio-Gel P-100

Bio-Gel P-100 dry beads were prepared as described in the Bio-Gel P reference manual, and equilibrated in 0.3 M citrate (pH 6.3). The sample (up to 5% of the bed volume) was chromatographed at 6°C, and fractions 2% of the bed volume were collected. Fractions were frozen in liquid nitrogen and stored at -20°C overnight for further analysis.

2.4.4 Ion-exchange chromatography (IEC)

Sulfoethyl-Sephadex C50 (Pharmacia) beads were washed twice in NaCl (1 M), then in formate (10 mM, pH 3.7) twice. Beads were packed to 1 ml bed volume in a Poly-Prep column and washed with excess buffer. AS-precipitated (ASP) enzyme was dialysed against 10 mM formate (pH 3.7) overnight, then centrifuged (6°C, 10 min, 13,000 rpm) to remove precipitates. The dialysate (0.2 ml) was applied to the column and followed with 1.8 ml of the same buffer. Proteins were eluted with buffers of increasing pH or increasing salt concentration. Fractions were tested for transglucosylation activity by the radioactive method and for protein content.

2.4.5 Concanavalin A (Con A) lectin affinity chromatography

Con A (from jack bean)-Sepharose 4B beads (1 ml) were packed in a Poly-Prep (Bio-Rad) column and washed with excess 50 mM citrate (pH 6.3) containing CaCl₂, MnCl₂, MgCl₂ (1 mM each). ASP was diluted 1:5 in water, then centrifuged (6°C, 5 min, 13,000 rpm) to remove precipitates. Diluted enzyme was applied to the column and 0.5-ml fractions were collected. Wash buffer followed the enzyme to pass any non-glycosylated proteins. Glycoproteins were eluted by increasing concentrations of methyl α -mannopyranoside dissolved in the above buffer, up to 625 mM.

Each fraction was assayed for protein content by A_{280} . The buffer was also assayed, with and without methyl α -mannopyranoside. Each fraction was also assayed for MXE and XET activity, with controls including the initial diluted protein before application to the column mixed with the maximum level of methyl α -mannopyranoside to check for inhibition.

At the end of fraction collection, the column was prepared for storage according to the manufacturer's instructions. The column was first washed with five bed volumes of an alkaline solution of 0.1 M borate (pH 8.5) and 0.5 M NaCl. It was followed with five bed volumes of an acidic solution of 0.1 M acetate (pH 4.5) and 1.0 M NaCl. This alkaline and acidic wash was repeated three times, and finally stored in a solution containing 1.0 M NaCl and 5 mM each of CaCl₂, MgCl₂, and MnCl₂, in 20% ethanol at 6°C.

2.4.6 Rotofor isoelectric focusing (IEF)

A Bio-Rad Rotofor Cell was assembled and prepared according to the manufacturer's manual. The Rotofor was powered by a Bio-Rad PowerPac HV. Ampholytes were mixed with water and either a marker mixture containing phycocyanin, hemoglobins A and C, and cytochrome c, or a dialysed protein sample. The separation was conducted at 10 W constant power until the voltage stabilized, and fractions were collected according to

the manufacturer's methods. A Sartorius PB-11 pH meter was used to measure the pH of the fractions. Transglucosylase activity was also assayed.

2.4.7 MXE sequence analysis by mass spectrometry

2.4.7.1 Trypsin digestion of an SDS PAGE band

A protein sample was separated by SDS PAGE (12%) and stained with GelCode Blue Stain (Pierce). The band of interest, potentially MXE, was cut out tightly using a clean razor blade. Cut gel pieces were stored in microfuge tubes at -20°C .

The cut band was incubated in 300 μl of 0.2 M ammonium bicarbonate (NH_4HCO_3) in 50% acetonitrile (ACN) at 30°C for 30 min, repeated three times, to remove SDS. The gel piece was then incubated in 300 μl of 20 mM dithiothreitol (DTT), 0.2 M NH_4HCO_3 , in 50% ACN at 30°C for 1 h to reduce the protein. The band was then washed in 300 μl of 0.2 M NH_4HCO_3 in 50% ACN, repeated three times to remove the DTT. The band was incubated in the dark in 50 mM iodoacetamide and 0.2 M NH_4HCO_3 in 50% ACN for 20 min to alkylate cysteine residues. Using a clean razor blade, the gel was cut into approximately 1- mm^3 pieces. The pieces were covered in 100 μl ACN until they turned white. The ACN was removed and the gel pieces were allowed to dry at room temperature. The dry gel pieces were then swollen in 29 μl of 50 mM NH_4HCO_3 containing 1 μl porcine trypsin (Promega). The

solution was initially kept at 4°C until the gel swelled, then the tube was sealed with Nescofilm and incubated at 32°C for 16 h. Each solution used was made fresh throughout the procedure.

2.4.7.2 Analysis of tryptic digest with MALDI–TOF MS

The ionizing matrix used in matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry (MS) consisted of α -cyano-4-hydroxycinnamic acid (CHCA) (1%) in TFA (0.3%) and ACN (50%).

The tryptic peptide solution (0.5 μ l) was dried with the matrix (0.5 μ l) on the MALDI sample plate. Peptides were excited with a laser of 337 nm in a Voyager DE-STR MALDI–TOF MS (Applied Biosystems). Spectra were collected and analysed by calibrating against the commonly found 842.51-Da trypsin autolysis fragment using Data Explorer software (Applied Biosystems). The top (approx.) 100 peaks were selected and searched against the NCBI non-redundant (NCBIInr) database on MASCOT software (Matrix Science).

2.4.7.3 Analysis of tryptic digest by LC–MS

The LC–MS system consisted of an Agilent 1200 Series high performance liquid chromatography (HPLC) system. A Kasil guard column (~3 cm long), attached before the HPLC column, consisted of Pursuit C18 beads (5 μ m

particle size, Varian). The peptides, after passing through the guard column, eluted from a PicoTip Emitter analytical column (PF 360-75-15-N-5, New Objective) packed to a length of (approx.) 20 cm with Pursuit C18 beads (5 μm particle size).

The column was equilibrated with solvent A (0.1% formic acid in 2.5% ACN). Up to 8 μl of the tryptic digest was loaded and then eluted by a gradual change to solvent B (0.1% formic acid, 0.025% TFA in 90% ACN). Solvent A was changed to solvent B with a linear gradient of 0–10% over 8 min, of 10–60% over 8–35 min, of 60–100% over 35–40 min; then solvent B over 45 min at a flow rate of 5 $\mu\text{l}/\text{min}$.

Eluate from the HPLC column was continuously analysed on LTQ XL linear ion trap mass spectrometer (Thermo Scientific) fitted with a nanoLC ESI source. Data-dependent acquisition was controlled by Xcalibur software (Thermo Scientific). Spectra were analysed using in-house licensed MASCOT software (Matrix Science).

2.5 Endogenous transglucosylation products using exogenous acceptor substrates

2.5.1 *Ex Vivo*: extraction of transglucosylation products formed using endogenous enzyme and donor substrates

Equisetum fluviatile shoots were harvested from an outdoor pond on 11/10/07 and stored at -20°C. The shoots were slowly thawed, and cross-sections of the stems were cut <1 mm thick using a new razor blade. Slices (50 mg) were placed in water (250 µl) to prevent drying. [³H]XXXGol (0.1MBq/ml) was added and incubated overnight at room temperature. AIR of the tissues was prepared.

To extract hemicelluloses and the transglycosylase product, NaOH (6 M) was added to each homogenate. The extracts were incubated at 37°C overnight, and the supernatant was collected. The pellet was washed in water (2 ml) for 2 h. The supernatant was collected, and the pellet was washed once more in water. The NaOH extract and water washes were pooled. The pH was adjusted to <7 using HOAc, and dialysed against water in 12–14 kDa molecular weight cut off dialysis tubing (Medicell International, Ltd. (London, UK)), to remove unreacted [³H]XXXGol and salts.

The dialysate was collected and stored at -20°C. A portion (0.2 ml) of each sample was scintillation counted in 2 ml of Fisher Scientific ScintiSafe 3 aqueous liquid scintillant. The remaining extracted hemicellulose was dried

in a SpeedVac and resolubilized (0.5% w/v). Three replicates of 0.25 mg each were digested with XEG (0.01%, 15 μ l), lichenase (0.15 U in 15 μ l), or buffer only as a control for 90 min. The digests were stopped with FA, dried, and redissolved in water (10 μ l). Each digest (8 μ l) was applied to a TLC plate and developed in fresh BAW three times, or PrAW once. Plates were fluorographed or cut with scissors and scintillation-counted.

2.5.2 *In situ* transglucosylation action

Tissue-specific distribution of endogenous transglucosylase action was detected with the use of a sulforhodamine-labelled fragment of xyloglucan, XXXG-SR. Sections (0.5 mm thick) of fresh stems were made using a new razor blade by hand. Two sections were placed in 150 μ l of 8 μ M XXXG-SR in water. Two more sections were cut and placed in 150 μ l water. Ethanol (75%) was added after 2 h of incubation to wash out any unused reactant. Sections were washed three more times in 75% ethanol, then soaked in 90% ethanol overnight to prepare for resin embedding. Ethanol was removed and replaced with 90% ethanol : LR White resin, 1:1, for 45 min, then exchanged for ethanol : resin, 1:2, for 45 min, then ethanol : resin, 1:3, for another 45 min. Pure fresh resin was added three times for 60 min and incubated at 60°C for 24 h for solidification. Sections were made using glass blades with the assistance of Dr. Steve Mitchell. Sections incubated in water

instead of XXXG–SR were stained with toluidine blue, or nothing as an autofluorescence control. Fluorescence under UV light in cell walls result from a transglucosylation reaction involving endogenous enzyme and donor substrate, using the supplied acceptor substrate XXXG–SR. Photographs were taken using a Leica Fluorescent microscope with filter cube N2.1.

2.6 *Equisetum fluviatile* cell cultures

2.6.1 Cell culture initiation

Equisetum fluviatile shoots were excised from rhizomes, dipped in 75% ethanol for 5 minutes to sterilize the surface, and dried. Longitudinal and transverse sections from the shoots were placed on solid Murashige and Skoog basal salts (Sigma M5519) containing 2% glucose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1% agar. Initiated callus was sub-cultured to fresh medium monthly.

2.6.2 Optimization of the culture medium

Some of the cultures were split onto media containing supplemented silicate ($\text{SiO}_2\cdot\text{NaOH}$, 1 mM). Sodium silicate (27% w/v in water) was added to the basal salts drop-wise during the preparation of the medium, maintaining the pH <7 with HCl to prevent the precipitation of salts. The cultures did not grow significantly differently on increased levels of silica.

Table 2.1 Culture media containing various concentrations of 2,4-D and kinetin

| Label | 2,4-D (mg/L) | kinetin (mg/L) | |
|---------------|--------------|----------------|---|
| Initiated on: | 2 | 0.0 | Initiated callus cultures were divided onto solid media containing different amounts and types of hormones. |
| V | 0 | 1.0 | |
| W | 1 | 1.0 | |
| X | 1 | 0.5 | |
| Y | 2 | 1.0 | |
| Z | 2 | 0.5 | |

Cultures were also tested for optimal hormone conditions (Table 2.1).

Cultures grew optimally in hormone mixture 'X', and were maintained at room temperature in the dark for >3 years.

2.7 Radiolabelling of XGO

An XGO (0.6 mg in 75 μ l water) was added to 8 μ mol of [3 H]NaBH₃ (6.25 MBq/ μ mol) in a fume hood, where the terminal glucose was reduced to glucitol, and gained a tritium atom. Ammonia (NH₃, 2 M, 100 μ l) was added to maintain alkaline conditions during the 1–2 day labelling period. Xylose (0.5 M, 80 μ l) was added to oxidize any remaining [3 H]NaBH₃. After one more day, the cap was then removed for 24 h to allow NH₃ release. HOAc was added to neutralize the solution, then left for another 24 h, but small amounts of water were added to ensure that the solution never became dry. The solution was purified by streaking on two AAW-washed TLC plates, and developed in PrAW. After 10 days of autoradiography, silica containing radiolabelled XGO was scraped off into a 15-ml Greiner tube. Water (10 ml) was added to solubilize the XGO and rotated overnight. The tube was

centrifuged (4000 rpm), the supernatant was removed, then concentrated under vacuum to 2.45 ml. A portion was assayed to estimate the amount of successfully radiolabelled substrate.

2.8 Dissolution of cellulose in DMA/LiCl

This procedure was modified from Gurjanov et al. (2008). Molecular sieve (4Å) was activated (100°C, 3 h). Dimethylacetamide (DMA) was dried over the sieve for at least 5 d. LiCl (8 g) was dried (180°C, 4 h), and dissolved in dry DMA (100 ml). Pieces of Whatman 1CHR were hydrated in water for 1 h, then filtered on nylon mesh. The paper pieces were washed in acetone, then incubated in acetone for 1 h. The pieces were again filtered out using nylon mesh, and washed in DMA, and incubated overnight in dry DMA. The DMA was removed and replaced with 8% LiCl in DMA, so that the paper was 1% (w/v). The paper was dissolved by stirring at room temperature. An equal volume of dry DMA was added to reduce the viscosity of the cellulose solution. The solution was slowly added by a peristaltic pump to rapidly stirring 6 M NaOH, where the cellulose precipitated, but hemicelluloses from the paper were expected to remain in solution.

3 Results and Conclusions

3.1 Purification of MXE

The purification of MXE was necessary for proper characterization. In a crude extract, there could have been small molecule inhibitors which affected the kinetics of the enzyme. Also, other proteins could have acted on the substrates of MXE, degraded them or otherwise rendered them unusable. Another reason to purify MXE was to attempt amino acid sequence identification for alignment with other sequenced proteins of related type.

3.1.1 Ammonium sulfate (AS) sequential precipitation

Ammonium sulfate (AS) precipitation is a common technique used to concentrate proteins. More hydrophilic proteins precipitate in the presence of higher concentrations of AS, and hydrophobic proteins precipitate at lower concentrations (Skopes, 1987). The technique was applied to extracts of *Equisetum* to determine the optimal method for AS precipitation and purification.

3.1.1.1 AS precipitation from 30 to 90% saturation

Crude *Equisetum fluviatile* extract from mature shoots harvested in late September was subjected to ammonium sulfate sequential precipitation. The

Table 3.1.1 Ammonium sulfate sequential precipitation of crude *Equisetum* extract

| % (sat.) AS | Protein (mg) | Total Activity (CPM/min) | | | | Specific Activity (cpm/min/mg) | |
|------------------|--------------|--------------------------|------------|-------|------------|--------------------------------|------|
| | | MXE | % of total | XET | % of total | MXE | XET |
| Original extract | 41.2 | 20200 | (100) | 21900 | (100) | 490 | 532 |
| 0 | 8.48 | 352 | 1.74 | 378 | 1.73 | 41.5 | 44.6 |
| 30 | 10.5 | 22000 | 109 | 17600 | 80.4 | 2100 | 1680 |
| 40 | 5.30 | 268 | 1.33 | 836 | 3.82 | 50.6 | 158 |
| 50 | 2.94 | 21.6 | 0.11 | 151 | 0.69 | 7.35 | 51.4 |
| 60 | 2.88 | 0.08 | 0.00 | 29.6 | 0.14 | 0.03 | 10.3 |
| 70 | 1.80 | 15.4 | 0.08 | 36 | 0.16 | 8.56 | 20.0 |
| 80 | 1.30 | 5.44 | 0.03 | 15.6 | 0.07 | 4.18 | 12.0 |
| 90 | 2.20 | 2.24 | 0.01 | 16.8 | 0.08 | 1.02 | 7.64 |
| % Recovery: | 85.9% | 112% | | 87.0% | | | |

A portion of each fraction's MXE and XET activity was assayed by the radioactive method, and the total CPM of product formed per minute of incubation for the entire fraction was estimated. Rate was calculated as a best fit line of four time points 5–90 min. The original extract was assayed in a different buffer than the AS precipitated proteins. Protein content was measured by the Bradford assay.

pellets were redissolved in citrate buffer and assayed for protein, and for MXE and XET activity (Table 3.1.1).

One fraction was especially enriched in MXE activity. The proteins were also concentrated, improving the sample for other purification steps such as column chromatography or gel electrophoresis. The vast majority of MXE activity precipitated in the first AS fraction and was aliquotted for use in many other experiments.

3.1.1.2 AS Precipitation 2

A second AS precipitation was conducted to better separate the transglucosylase activities from each other or from other proteins. Another

portion of the same extract was AS precipitated starting with lower concentrations of AS and proceeding through smaller increases (Table 3.1.2).

Table 3.1.2 Ammonium sulfate low-concentration sequential precipitation

| % (sat.) AS | Protein (mg) | Total Activity (cpm/min) | | | | Specific Activity (cpm/min/mg) | |
|------------------|--------------|--------------------------|------------|-------|------------|--------------------------------|------|
| | | MXE | % of total | XET | % of total | MXE | XET |
| Original extract | 55.2 | 17400 | (100) | 21900 | (100) | 315 | 397 |
| 0 | 16.5 | 258 | 1.48 | 233 | 1.06 | 15.6 | 14.1 |
| 10 | 0.636 | 1210 | 6.95 | 795 | 3.63 | 1900 | 1250 |
| *15 | 0.817 | 5660 | 32.5 | 3000 | 13.7 | 6930 | 3670 |
| *20 | 1.25 | 13500 | 77.6 | 8540 | 39.0 | 10800 | 6830 |
| *25 | 1.75 | 9690 | 55.7 | 5280 | 24.1 | 5540 | 3020 |
| 30 | 4.04 | 1650 | 9.48 | 1820 | 8.31 | 408 | 450 |
| 40 | 3.85 | 20.0 | 0.11 | 642 | 2.93 | 5.19 | 167 |
| 90 | 12.4 | 2.51 | 0.01 | 125 | 0.57 | 0.20 | 10.1 |
| % Recovery: | 74.7% | 184% | | 93.3% | | | |

Activity and protein assayed as in Table 3.1.1. Rate was calculated as a best fit line through the linear range of four time points 0–3 h. *Fractions pooled for use in further experiments.

MXE and XET activities did not separate from each other in this purification method, but did become more purified than in the previous experiment. The most enriched fractions, indicated by '*' in Table 3.1.2, were pooled and stored at –80°C for future use. In those fractions, 6.9% of the total protein, 166% of the MXE activity, and 76.8% of the XET activity was recovered.

3.1.1.3 AS Precipitation 3

As stocks of enzyme were used in various experiments, fresh enzyme was extracted from mature shoots harvested in September. An equal ionic strength of citrate was used instead of succinate in this extraction, then

precipitated with AS (Table 3.1.3). Aliquots of the redissolved pellet precipitated by 20% saturated AS were stored at -80°C for further use.

Table 3.1.3 Bulk preparation of ammonium sulfate precipitated proteins

| % (sat.) AS | Protein (g) | Total Activity (kcpm/min) | | | | Specific Activity (cpm/min/g) | |
|------------------|-------------|---------------------------|------------|-----|------------|-------------------------------|------|
| | | MXE | % of total | XET | % of total | MXE | XET |
| Original extract | 2.25 | 1280 | (100) | 814 | (100) | 569 | 362 |
| 10 | 0.42 | 190 | 14.8 | 100 | 12.3 | 452 | 238 |
| 20 | 0.30 | 840 | 65.6 | 480 | 59.0 | 2800 | 1600 |
| 30 | 0.96 | 120 | 9.38 | 110 | 13.5 | 125 | 115 |
| 40 | 0.17 | 1 | 0.08 | 15 | 1.84 | 6 | 88 |
| % Recovery: | 82% | 90% | | 87% | | | |

Proteins were extracted in citrate extraction buffer (165 mM, pH 6.3) and tested for MXE and XET activity, and for protein as in Table 3.1.1. Rate was calculated as a best fit line of seven time points 0–3 h.

3.1.2 Native PAGE

As a possible purification technique, native PAGE gels were used. One gel was loaded with 10 lanes of crude extract, the other was loaded with ASP. One lane from each gel was excised and stained with GelCode Blue stain, and three others were used in dot blot assays for activity. The remaining lanes were sectioned for protein recovery.

For zymogram detection of transglucosylation activity, excised lanes were laid on MXE, XET, or control test paper strips, and incubated in a humid environment for 16 h. Fluorescent product remaining after washing was photographed under UV light (Figure 3.1.1).

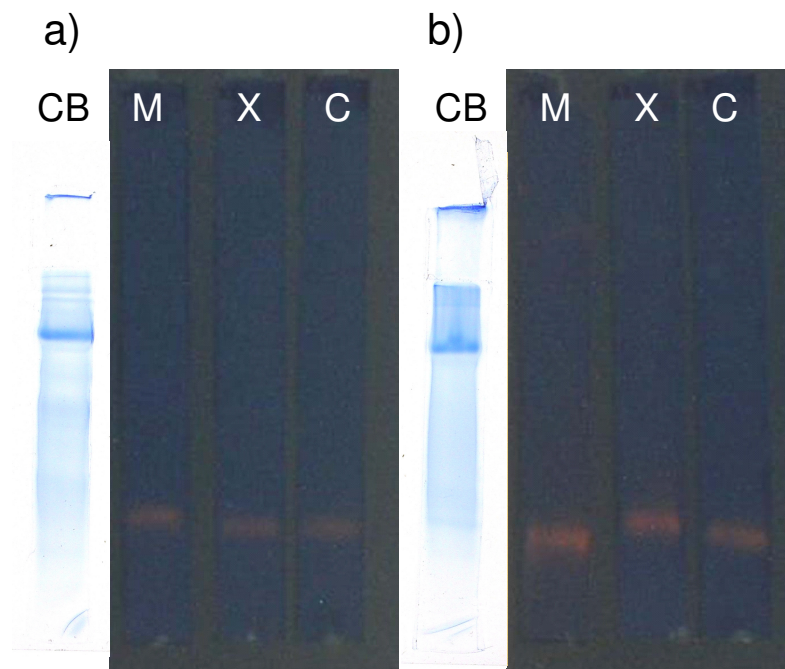


Figure 3.1.1 Zymogram of native PAGE

Crude extract (a) or ASP (b, pooled fractions from Table 3.1.1) was run on native PAGE. One lane was stained with Coomassie Blue (CB). Three lanes of the electrophoresed gels were excised and washed twice in 0.3 M citrate buffer (pH 6.3) for 15 min. Enzyme activities were detected by overlaying the lane with paper impregnated with MLG and XXXG-SR (M), XyG and XXXG-SR (X), or just XXXG-SR (C). Pink bands indicate polysaccharide-to-oligosaccharide transglucosylation.

Both MXE and XET were active after electrophoresis, evident by the pink bands of transglucosylation product in M and X. However, the transglucosylation in the control strip (C) was the result of neither activity. The only potential donor substrate was the paper itself, mainly cellulose. This observation was investigated further and will be discussed in a later chapter. Within some error for gel stretching or uneven running of the gel, all three activities migrated to roughly the same distance in the gel.

Proteins were extracted from unused portions of the gel. Fractions down the lane were excised and incubated with excess buffer at 6°C. No

activity was recovered. Because of the difficulty in recovering the protein, this method was not used preparatively to purify MXE.

3.1.3 Bio-Gel P-100

Gel-permeation chromatography (GPC), which separates proteins on the basis of molecular size, was another method employed to purify MXE. Bio-Gel P-100 beads were chosen based on their 5–100 kDa molecular weight cut-off. Closed arrows indicate the void volume and open arrows show the included volume throughout this chapter.

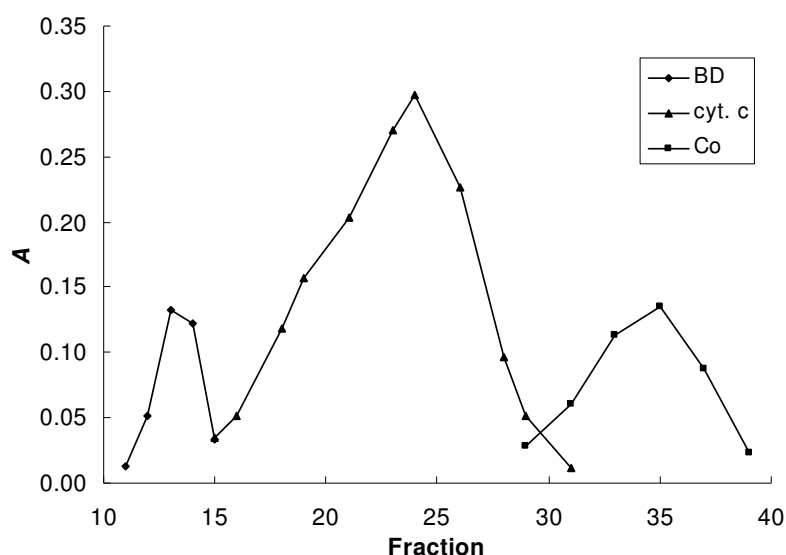


Figure 3.1.2 Calibration of BioGel P-100 column

The 87-ml bed-volume column was equilibrated in citrate buffer (0.3 M, pH 6.3). Fractions (2 ml) were collected at a rate of ~14 ml/h. BD, cytochrome c_{551} , and CoCl_2 were detected by spectrophotometry (600 nm, 410 nm, 510 nm, respectively).

3.1.3.1 Column Calibration

Beads were prepared according to the manufacturer instructions and poured in a column with a 1.5 cm diameter. The column was calibrated using Blue Dextran (BD) (void), cytochrome c_{551} (partially included), and cobalt chloride (CoCl_2) (included). The void volume is in the 13th fraction, while the totally included volume eluted in fraction 35 (Figure 3.1.2).

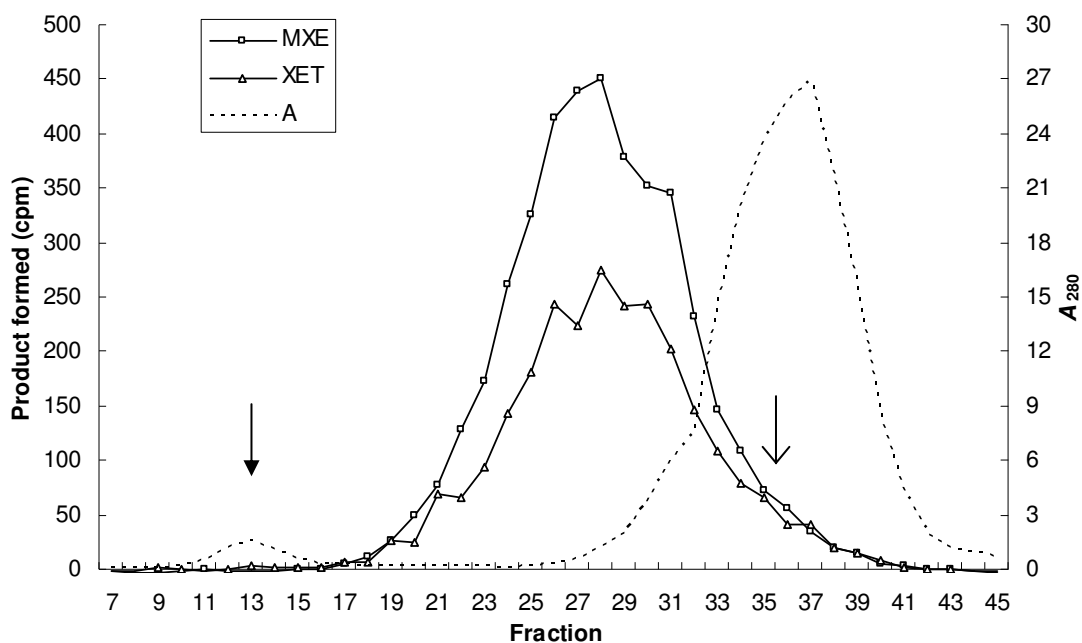


Figure 3.1.3 BioGel P-100 chromatograph of crude extract

Fractions (2 ml) were collected (flow rate ~14 ml/h). Each fraction was measured once for activity by the radioactive method; reaction mixture included each fraction (20 μl), [^3H]XXXGol (1 kBq), and MLG or XyG (0.16%); 2 h incubation. The undiluted (calculated) samples' A_{280} is reported. For determination of void and included volumes, refer to Figure 3.1.2.

3.1.3.2 Crude extract separated by Bio-Gel P-100

Crude *Equisetum* extract (4 ml) was centrifuged to remove cell debris, then fractionated on Bio-Gel P-100 (Figure 3.1.3). Protein measurement was attempted by A_{280} .

MXE and XET activity eluted in a broad peak from the Bio-Gel P-100 column with an k_{av} of 0.68. Each fraction was only assayed for activity once, resulting in an unknown degree of variability for each data point. Still, the amount of MXE and XET activities recovered was roughly 2.5 times more than the starting material. This increase in activity is mostly due to the buffer exchange, as MXE and XET activities from *Equisetum* are enhanced by citrate at pH 6.3.

3.1.3.3 Stable enzyme–substrate complex purification

Steele and Fry (1999) reported a simple method to purify XTHs based on the enzymes' stable intermediate form. Samples containing XET activity from a partially purified extract could be complexed with XyG (the donor substrate), and subjected to GPC. The enzyme complexed with XyG would elute in the void volume. Fractions containing activity were supplied with XXXG (the acceptor substrate) and could then complete the reaction, releasing the enzyme from its donor substrate. When run a second time on the same column, there was an M_r shift in the enzyme, which eluted much

later. This method was applied to transglucosylase enzymes of *Equisetum* extracts.

3.1.3.3.1 Crude extract complexed with MLG

Centrifuged crude *Equisetum* extract was given the opportunity to bind with its donor substrate at 4°C in 0.2% MLG for 30 min. The mixture was applied to a Bio-Gel P-100 column and followed by 0.2% MLG in citrate buffer, to allow any free MXE to bind or re-bind to the donor substrate. Citrate buffer

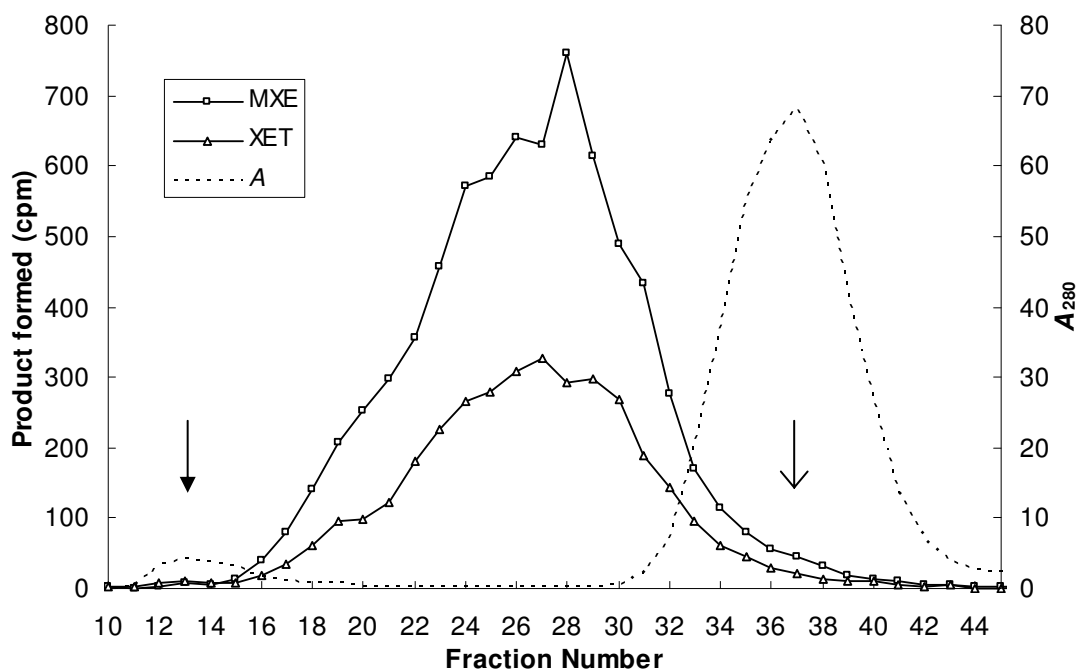


Figure 3.1.4 Elution of enzyme-MLG complex from Bio-Gel P-100
Fractions (2 ml) were eluted in citrate buffer (0.3 M, pH 6.3) in 6°C at a rate of ~12 ml/h. MXE and XET activities were assayed by incubating each fraction (20 µl) with [³H]XXXGol (1 kBq) and MLG or XyG (0.32%) for 2 h. The undiluted (calculated) samples' A₂₈₀ is reported.

was used as the remaining eluant. Fractions were collected and assayed for MXE and XET activity (Figure 3.1.4).

MLG added to the mixture prior to separation on the column most likely eluted in the void volume. The average M_r of the polysaccharide was not supplied by the manufacturer. If MLG from the fraction was mixed with XyG in an XET assay, results could be ambiguous. Therefore, each fraction was incubated in water alone, with no additional donor substrate. The results of this assay showed no activity attributable to the MLG present in the eluant, and was not shown in Figure 3.1.4.

MXE was expected to form a complex with MLG in a similar manner to how XTH formed a complex with XyG (Steele and Fry, 1999). However, this was not the case. Compared to the extract alone, MXE could have begun to elute earlier, evident by the leading shoulder in fractions ~18–22, but did not interact with its donor substrate to form a complex many times larger than the enzyme itself. The peak of activity still eluted with an k_{av} of 0.63, similar to the extract alone. As in Figure 3.1.3, both MXE and XET activities eluted in a similar pattern.

3.1.3.3.2 ASP complexed with XyG

MXE appears to not form a stable complex with MLG. However, XTH was attempted to be removed from MXE by the method described by Steele and Fry (1999).

Pooled ASP from Table 3.1.2 was incubated with XyG (0.2%), BD (0.13%) and glucose (0.25 g) for 30 min at 6°C. The sample was passed through a freshly prepared Bio-Gel P-100 column, followed by 0.2% XyG in citrate, then only citrate buffer. The fractions were assayed for MXE and XET activity (Figure 3.1.5).

Some XET activity had an apparent M_r shift, and eluted in the void volume instead of in the partially included fraction. Each fraction was incubated with no additional donor substrate to determine the activity attributable to the XyG in the eluant. The activity shown from this assay was XET, as the donor substrate was XyG, but was reported as 'background'. This activity, which resulted from XTH forming a stable, very high- M_r complex with XyG, was present in fractions 11-15.

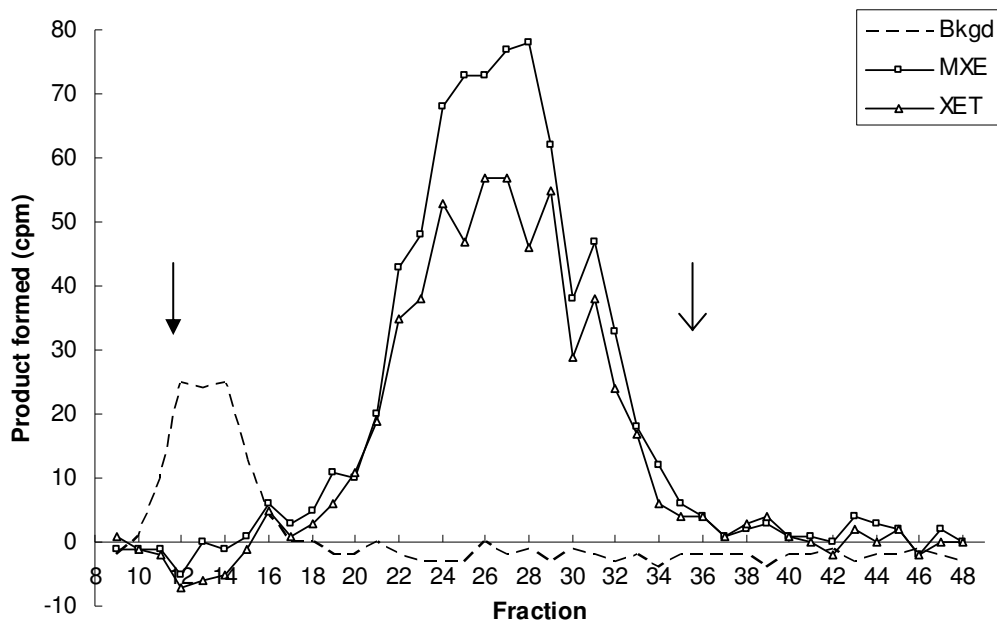


Figure 3.1.5 Elution of enzyme–XyG complex from Bio-Gel P-100

Pooled ASP from Table 3.1.2 was incubated with XyG (0.2%), BD (0.13%), and sucrose (33%) for 30 min at 6°C. Void volume was determined with BD, included volume was determined by silver-stained sucrose. Activity was assayed by incubating 20 µl of the fraction with [³H]XXXGol (1 kBq) and XyG (XET curve), MLG (MXE curve) (0.32%), or water (Bkgd curve). The background (Bkgd) values were subtracted from the XET and MXE values.

Some XET activity eluted in the void volume, the result of a stable enzyme–substrate intermediate, but the elution of the largest peak of XET activity did not change elution patterns after incubation with XyG. Most XET activity did not have a M_r shift, and eluted with a k_{av} of 0.63, similar to the original extract. This activity again co-eluted with MXE.

Low levels of activity eluted from the column. Only 13% of the MXE activity and 19% of the XET activity were recovered. Most of the activity was lost to inactivation or more likely, to non-specific binding to the column beads.

3.1.3.4 Prevention of enzyme binding to the column

Significant amounts of activity were not recovered from the column in Figure

3.1.5. The lost activity was presumed to have bound to the column beads.

Various additives were tested for their ability to prevent the binding or at least protect the activity.

3.1.3.4.1 Effect of poly-lysine (PL) and bovine serum albumin (BSA) on activity

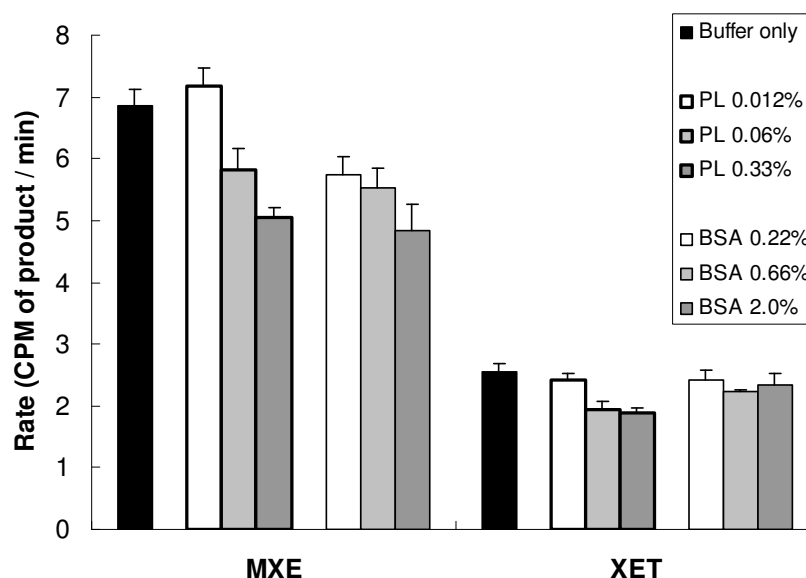


Figure 3.1.6 Effect of PL and BSA on activity

ASP (pooled fractions from Table 3.1.2) was diluted 1/10 in citrate buffer (0.3 M, pH 6.3) and was incubated with [³H]XXXGol (1 kBq), XyG or MLG (0.32%), and PL or BSA. Rate of activity was measured over a time course of 0–192 min. The gradient \pm SE of the best-fit curve is reported, n=7.

First, PL (12–24 kDa) and BSA were added at a range of concentrations in a simple *in-vitro* assay to test their effects on MXE and XET activity (Figure 3.1.6).

PL markedly inhibited both MXE and XET activity, but at low concentrations of PL, activity was unaffected. BSA at 0.66% inhibited the MXE rate by 19%. Subsequent purification steps could remove BSA, and presumably remove the inhibition as well. Both additives were tested for their ability to prevent the loss of activity during GPC purification.

3.1.3.4.2 Effect of Triton X-100 and BSA on activity

Triton X-100 is another additive that can be used to prevent non-specific binding of proteins to glassware or other materials, and was tested alone and in combination with BSA (Table 3.1.4).

Table 3.1.4 Activity rate in the presence of Triton X-100 and BSA

| Additive | MXE (cpm of product/min) | XET (cpm of product/min) |
|----------|-----------------------------|-----------------------------|
| None | 14.2 ±0.2** | 6.14 ±0.14* |
| BSA | 12.5 ±0.3* | 6.16 ±0.11 |
| Triton | 13.2 ±0.3* | 5.81 ±0.19* |
| Combined | 14.0 ±0.7** | 5.33 ±0.46 |

Activity was measured as in Figure 3.1.6 over the linear phase of a time course 0–180 min in the presence of 0.66% BSA, 0.05% Triton, or both; cpm of product / min reported, ±SE of the curve, n=7, * n=6, ** n=5.

As in the previous section, BSA slightly inhibited MXE activity but not XET activity. However, when combined with Triton X-100 the effect was opposite: MXE activity was maintained close to the original rate, while XET

activity was reduced by 13%. The combination of Triton and BSA affects the activities the least of any additives tested, and was therefore included in future running buffers for GPC.

3.1.3.4.3 Elution of MXE and XET activity with additives from Bio-Gel P-100

A small Bio-Gel P-100 column was equilibrated in 0.3 M citrate (pH 6.3). Between chromatographic runs, the column was washed in citrate buffer with 0.05% Triton X-100, then re-equilibrated in citrate.

ASP with no additives was first passed through the column to determine the baseline level of activity that can be recovered. Fractions were collected and assayed for MXE and XET activity (Figure 3.1.7 a).

PL (1 ml, 0.1% in water) was passed through the small Bio-Gel P-100 column, followed by citrate buffer. Ninhydrin staining of fractions showed that PL eluted close to the expected void volume (data not shown). It is possible that the 12–14 kDa PL had a non-globular secondary structure that forbade it entering the GPC beads, even though its M_r is lower than the 100 kDa cut-off. The column was then tested for the ability to separate and elute MXE and XET activity (Figure 3.1.7 b).

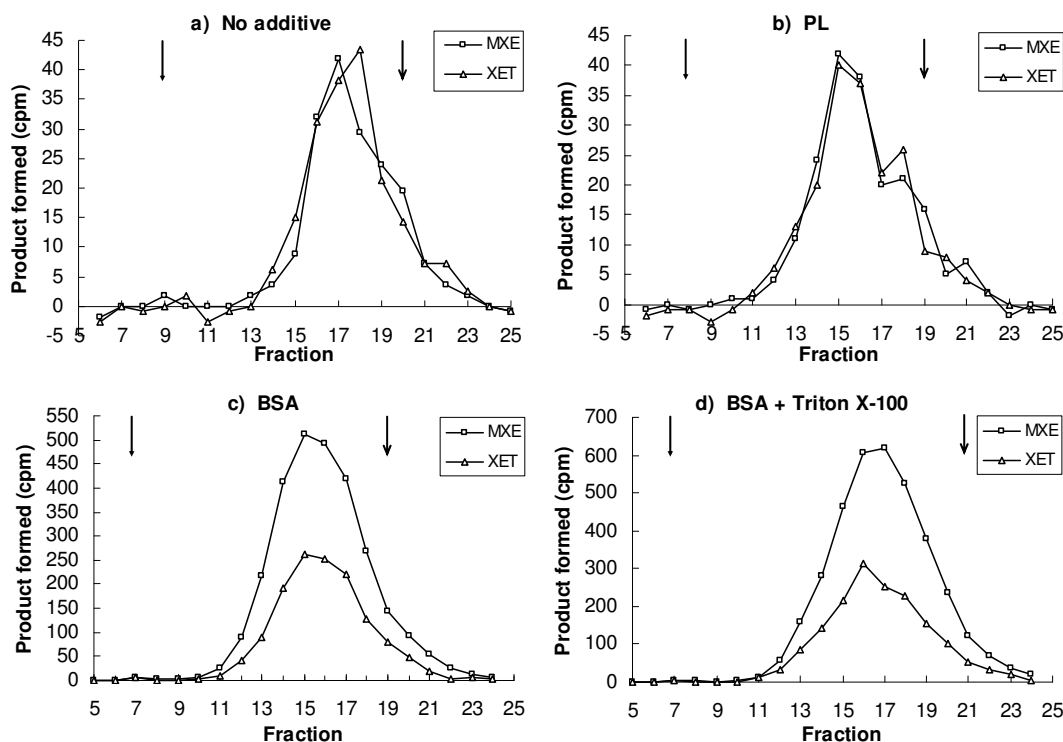


Figure 3.1.7 Elution of activity with additives

Pooled ASP from Table 3.1.2 (0.1 ml) was mixed with BD and fructose (internal markers) in citrate (0.3 M, pH 6.3), and separated on a Bio-Gel P-100 column (~20 ml, 0.5 cm diameter). Fractions (1 ml) were collected at 6°C at ~4 ml/h. First, no additive or protectant was added to the column (a). Next, PL (1 ml, 0.1%) was passed through the column to coat the beads, before ASP was fractionated (b). One bed volume of BSA (1%) was passed through the column before ASP (mixed with 1% BSA) was applied and eluted (c). Finally, one bed volume of 1% BSA + 0.05% Triton X-100 was passed through the column before ASP (in 1% BSA, and 0.05% Triton X-100) was applied and eluted (d). Each fraction was assayed for activity as in Figure 3.1.4. The k_{av} of the MXE activity peaks was 0.64–0.73.

When eluted with no additive (Figure 3.1.7 a) the peak of MXE and XET activity appeared to elute in different fractions. However, high variability levels are present because of the low rate of activity, and because only one replicate was tested. The general pattern of elution was overlapping and unchanged from previous GPC fractionations. As

previously observed, a lower amount of radioactive product was recovered than was expected. There was an estimated loss of ~90% of activity.

After the beads were coated with PL, MXE and XET activity eluted in a similar fashion as with no additive; only 11% of the expected activity was recovered (Figure 3.1.7 b). PL does not protect from the loss of MXE activity.

BSA protected from the loss of MXE and XET activity on the column (Figure 3.1.7 c). The amount of MXE recovered, considering 19% inhibition by BSA, was 96% of the initial activity. The goal of protecting MXE and XET activity from non-specific binding or other loss of activity was accomplished by the addition of BSA to the eluant.

Since BSA was successful in protecting MXE and XET activity from loss, BSA and Triton X-100 together were tested to see if even more activity could be recovered. The combination of BSA and Triton X-100 increased the recovery of MXE activity to 106% of the initial activity. Further BioGel P-100 purifications included 1.0% BSA and 0.05% Triton X-100 in the eluant.

3.1.4 Ion-Exchange Chromatography (IEC)

IEC was another method employed to purify MXE. Many stationary phase beads were tested using either anion-exchange or cation-exchange strategies.

3.1.4.1 Cation exchange

Four resins were chosen to test for their ability to bind and elute MXE and XET activity; Sulfooethyl-Sephadex (SES) C50 cation exchanger (Pharmacia), Sulfoethyl-Sephadex (SES) C50 cation exchanger (Pharmacia), Sulfoethyl-Sephadex (SES) C50 cation exchanger (Pharmacia), Carboxymethyl-Sephadex (CMS) C25 cation exchanger (Sigma), Carboxymethyl-Sephadex (CMS) C25 cation exchanger (Pharmacia), and Carboxymethyl-Cellulose (CMC) CM32 Series III (Whatman Column Chromedia).

Table 3.1.5 Activity eluted from cation-exchange columns

a) Control rate (cpm / min)

| | Buffer | MXE | XET |
|-------------------------|--------|------|------|
| FT (form., pH 3.7) | | 3.37 | 1.46 |
| E (phos. + NaCl pH 7.0) | | 6.29 | 2.63 |

b) Fraction rate (cpm / min)

| Fraction | SES | | SPS | | CMS | | CMC | |
|----------|------|------|-----|-----|------|------|------|------|
| | MXE | XET | MXE | XET | MXE | XET | MXE | XET |
| FT | 0 | 0 | 0 | 0 | 4.59 | 3.19 | 1.21 | 0.81 |
| E | 3.17 | 1.54 | 0 | 0 | 0.76 | 0.90 | 2.08 | 1.44 |

c) Rate / control rate

| Fraction | SES | | SPS | | CMS | | CMC | |
|----------|------|------|-----|-----|------|------|------|------|
| | MXE | XET | MXE | XET | MXE | XET | MXE | XET |
| FT | 0 | 0 | 0 | 0 | 1.36 | 2.18 | 0.36 | 0.55 |
| E | 0.50 | 0.59 | 0 | 0 | 0.12 | 0.34 | 0.33 | 0.55 |

Resins were prepared by washing in 1 M NaCl twice, followed by formate buffer (10 mM, pH 3.7). Columns were packed to 1 ml bed volume. ASP from Table 3.1.3 was dialysed in formate (10 mM, pH 3.7) overnight, then centrifuged at 6°C. A portion of the dialysate (200 µl) was applied to the column and followed with 1.8 ml formate (10 mM, pH 3.7). This fraction was termed flow-through (FT). Bound proteins were eluted with phosphate (10 mM, pH 7.0) in NaCl (1 M), and called eluant (E). a) The dialysed extract (2 µl) was simply mixed with the FT or E buffer (18 µl) and assayed in [³H]XXXGol (1 kBq) and MLG or XyG (0.32%) for 0-120 min (n=4). b) FT and E fractions from the columns were assayed (20 µl) with substrates as in (a). c) The activity rate for each fraction was calculated as a proportion of the theoretical maximum rate to account for buffer effects.

Each fraction was assayed for activity (Table 3.1.5). Because the FT and E were in different buffer conditions (pH and salt), a portion of the dialysate was diluted 1/10 in each buffer and assayed as control.

IEC purifies best by binding and eluting proteins with salt or pH. SES was the optimal resin tested, binding and eluting 50-60% of the MXE and XET activity. No activity was recovered from SPS, presumably the proteins were strongly bound to the resin. Proteins which flowed straight through the CMS column would not have been separated from other proteins, and was rejected for further use. MXE and XET probably bound weakly to the CMC column, and eluted partially in both the flow through and eluant. SES was the best column tested, and was optimized for the purification of MXE.

3.1.4.2 Anion exchange

Diethyl[2-hydroxypropyl]aminoethyl Sephadex (QAES) A25 Anion exchanger (Sigma) and Dowex 1X4-200 anion exchange resin (Sigma-Aldrich) were also tested for their ability to purify MXE and XET (Table 3.1.6).

MXE and XET activity were able to be bound and eluted from cation-exchange columns after dialysis in Tris buffer. Very little activity was recovered from QAES, but a larger proportion of the total possible activity was eluted from the Dowex column (Table 3.1.6 c). However, the majority of activity was not recovered. Neither of these columns was chosen as suitable purification method.

Table 3.1.6 Activity eluted from anion-exchange columns

a) Control rate (cpm / min)

| | Buffer | MXE | XET |
|------------------------|--------|------|------|
| FT (Tris pH 8.1) | | 5.96 | 3.72 |
| E (pyr. + NaCl pH 5.0) | | 15.7 | 6.35 |

b) Fraction rate (cpm / min)

| Fraction | QAES | | Dowex | |
|----------|------|------|-------|------|
| | MXE | XET | MXE | XET |
| FT | 0 | 0 | 0 | 0 |
| E | 0.37 | 0.23 | 5.29 | 2.65 |

c) Rate / control rate

| Fraction | QAES | | Dowex | |
|----------|------|------|-------|------|
| | MXE | XET | MXE | XET |
| FT | 0 | 0 | 0 | 0 |
| E | 0.02 | 0.04 | 0.34 | 0.42 |

The resins were prepared by washing in 1 M NaCl, followed by Tris HCl (10 mM, pH 8.1). The beads were packed in 1-ml columns and washed with more buffer. ASP from Table 3.1.3 was dialysed in Tris (10 mM, pH 8.1, HCl) overnight, then centrifuged at 6°C. The chromatography proceeded as in Table 3.1.5, but the FT buffer was Tris (10 mM, pH 8.1), and the E buffer was pyridine (10 mM, pH 5.0, HCl) + NaCl (1 M). a) The dialysed extract (2 µl) was mixed with the FT or E buffer (18 µl) and assayed in [³H]XXXGol (1 kBq) and MLG or XyG (0.32%) from 0–120 min (n=4). b) FT and E fractions were assayed (20 µl) with substrates as in (a). c) The activity rate for each fraction was calculated as a proportion of the theoretical maximum rate to account for buffer effects.

3.1.4.3 SES IEC, salt or pH change

Both pH change or an increase in salt concentration can elute proteins from an ion-exchange column. The efficiency of MXE and XET activity recovery was tested in response to a pH change, salt concentration increase, or both (Table 3.1.7).

Either an increase in NaCl concentration or pH caused MXE and XET activity to elute from the columns. More activity was not eluted by a second change in pH or NaCl concentration. A change in pH eluted more activity

than a change in salt concentration, and so was deemed the better method with which to elute activity from the SES column. However, only 25% of the possible MXE activity was recovered, so further optimization was necessary.

Table 3.1.7 Activity in fractions eluted with salt or pH change

| a) | | | b) | | | | | | |
|---------------------|-----|----------|----------------------|-----|---------------------|-----|----------|----------------------|-----|
| Fraction conditions | | | Activity | | Fraction conditions | | | Activity | |
| Fraction | pH | NaCl (M) | MXE (cpm of product) | XET | Fraction | pH | NaCl (M) | MXE (cpm of product) | XET |
| 1 | 3.7 | 0.0 | 2 | -2 | 1 | 3.7 | 0.0 | -3 | -5 |
| 2 | 3.7 | 0.1 | 126 | 89 | 2 | 5.0 | 0.0 | 208 | 164 |
| 3 | 3.7 | 0.3 | 35 | 32 | 3 | 6.0 | 0.0 | 97 | 81 |
| 4 | 3.7 | 1.0 | 9 | 14 | 4 | 7.0 | 0.0 | 24 | 33 |
| 5 | 5.0 | 1.0 | -2 | 0 | 5 | 7.0 | 0.1 | 6 | 20 |
| 6 | 6.0 | 1.0 | -1 | 4 | 6 | 7.0 | 0.3 | -3 | -2 |
| 7 | 7.0 | 1.0 | 0 | 1 | 7 | 7.0 | 1.0 | -5 | -6 |

SES columns (1 ml) were prepared as in Section 3.1.6.2, and 200 µl of dialysed ASP from Table 3.1.3 (in 10 mM formate, pH 3.7) was applied to each column. Fractions (2 ml) were collected at 6°C. Formate (10 mM) buffered at pH 3.7, while citrate (10 mM) was used at higher pHs. Activity in each fraction was assayed once for 3 h in citrate buffer (70 mM, pH 6.3), 0.5 M NaCl, [³H]XXXGol (1 kBq, 0.25 µM), and MLG or XyG (0.24%).

3.1.4.4 SES IEC, narrow pH change with Triton X-100

Triton X-100 was able to prevent non-specific binding of enzymes to Bio-Gel P-100, and was tested here as an additive in the eluant to assist in recovering activity. Table 3.1.7 b shows that MXE and XET activity can be eluted between pH 3.7–6.0. Two columns were tested to compare the effects of MXE and XET elution by small increases in pH using a citrate + formate buffer, with or without Triton X-100 (Figure 3.1.8).

MXE and XET activity are both highly enriched in the fractions eluted from SES columns with Triton. The largest peak of activity eluted with pH

4.1 buffer. No other peaks of activity were present in the dialysate, suggesting that there may be only one isoform of MXE in the extract. When compared to dialysate prior to IEC, 66% of MXE activity and 51% of XET activity were recovered by this procedure.

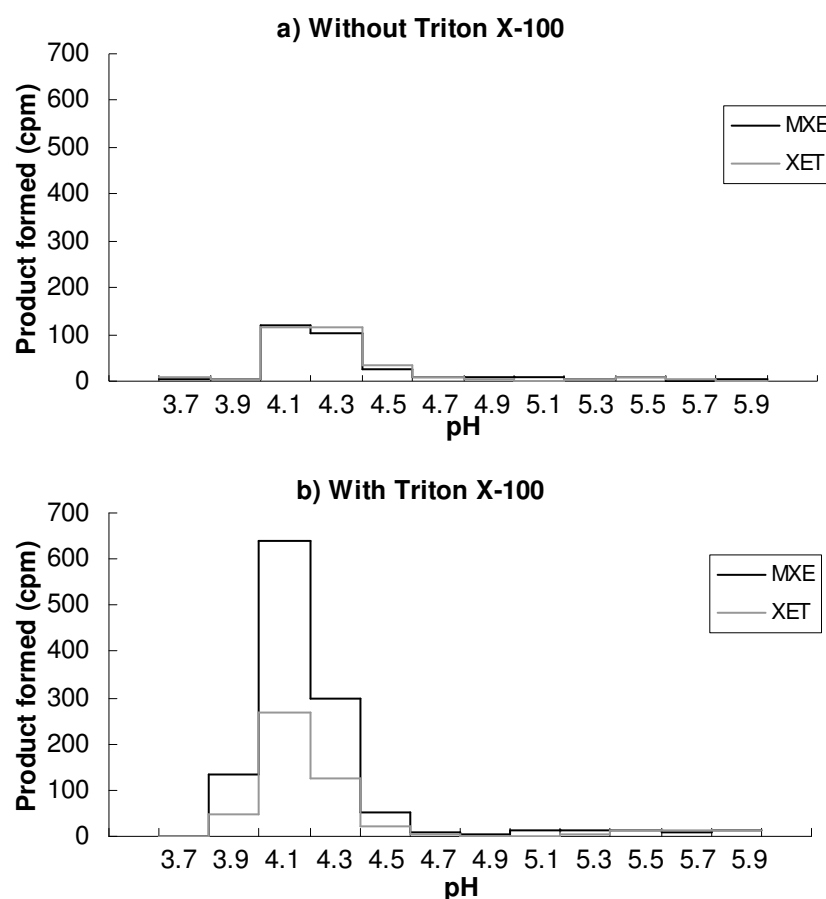


Figure 3.1.8 Histogram of activity elution from SES IEC in narrow-range pH

Dialysed ASP from Table 3.1.3 (in 10 mM formate + 10 mM citrate, pH 3.7) was applied to each column. Fractions (2 ml) were collected at 6°C. Enzymes were eluted with a) citrate + formate buffer (10 mM each) or b) with the same buffer and Triton X-100 (0.05%). Fractions (20 µl) were assayed for 3 h in citrate buffer (75 mM, pH 6.3), [³H]XXXGol (1 kBq), and MLG or XyG (0.24%).

3.1.5 Concanavalin A (ConA)

XTHs are glycoproteins; the glycosyl residues are located near the active site of the protein and may play a role in action (Campbell and Braam, 1999 b).

Due to the similarities in action, MXE was predicted to be a glycoprotein.

A conjugate of ConA-Sepharose 4B was purchased (Sigma C9017) and prepared and used according to the manufacturer's instructions. ASP was

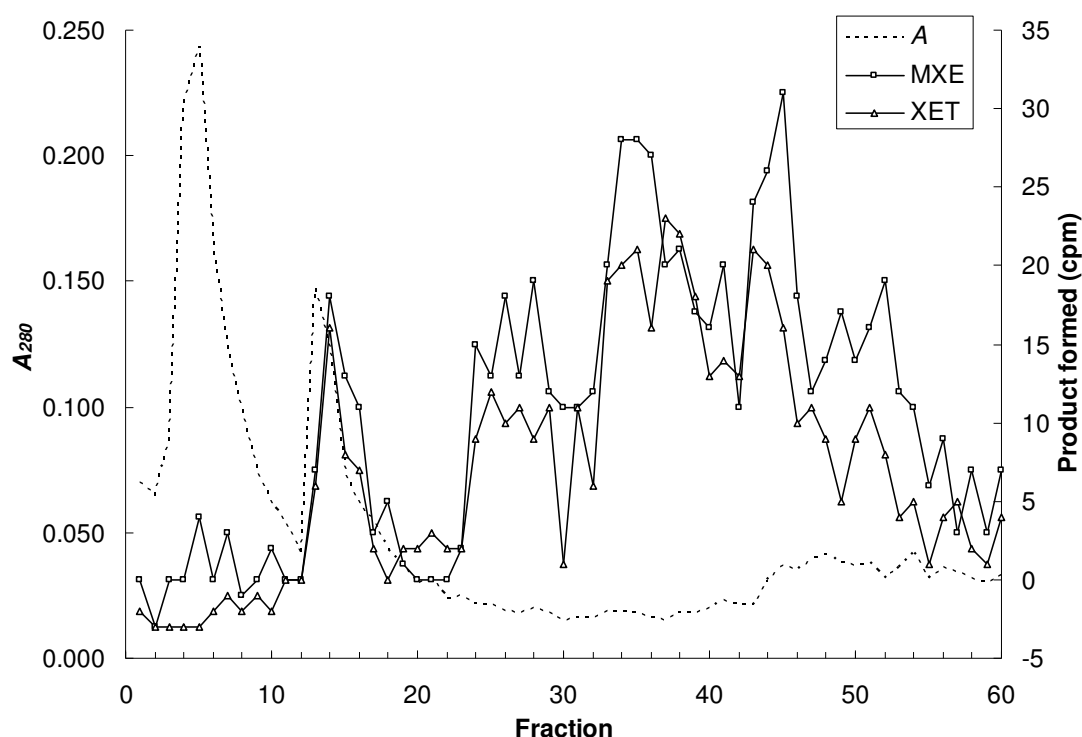


Figure 3.1.9 ConA fractionation of ASP

A 1-ml column was packed and washed in NaCl (1 M) + CaCl₂ + MnCl₂ + MgCl₂ (5 mM each). ASP from Table 3.1.3 was diluted 1:5 to reduce the buffer concentration to 50 mM citrate (pH 6.3) and applied to the column. ASP (0.86 ml) was followed by buffer (4.14 ml). Fractions (0.5 ml) were collected. An increasing concentration of methyl- α -mannopyranoside (5, 25, 125, 625 mM) in buffer was added (5 ml, except 10 ml of 625 mM) to elute proteins. Activity was assayed by incubating each fraction (20 μ l) in [³H]XXXGol (1 kBq) with XyG or MLG (0.32%) for 2 h.

applied to the column and eluted with increasing concentrations of methyl- α -mannoside. Activities and A_{280} of each fraction were assayed (Figure 3.1.9).

Absorbance data estimates protein content, but a contaminant in the methyl- α -mannopyranoside also absorbed at 280 nm. While this complicates the later eluting fractions, early eluting fractions appear to have large peaks of protein. Accounting for the A_{280} -contaminant in fractions 44–60, approximately 120% of the protein applied to the column was recovered. Therefore, the A_{280} data can only be used as an estimate of protein content.

MXE and XET were able to be purified by ConA affinity. MXE and XET were not able to be purified from each other, the two activities eluted in a similar manner in all fractions. The activities did not elute in one distinct peak, but eluted over a broad range of eluant concentrations. This might indicate that the proteins are not uniformly glycosylated, as some proteins were more firmly bound to the lectin than others. However, the majority of proteins eluted before MXE and XET activity, indicating that ConA chromatography could be used in a batch-purification of MXE. The addition of Triton X-100 could assist in the recovery of activity as in other columns.

3.1.6 Three successive columns

MXE and XET activity were able to be purified on three columns; Bio-Gel P-100, SES IEC, and Con A. MXE and XET activity were not separable by any

individual method, but could possibly be separated by a combination of techniques.

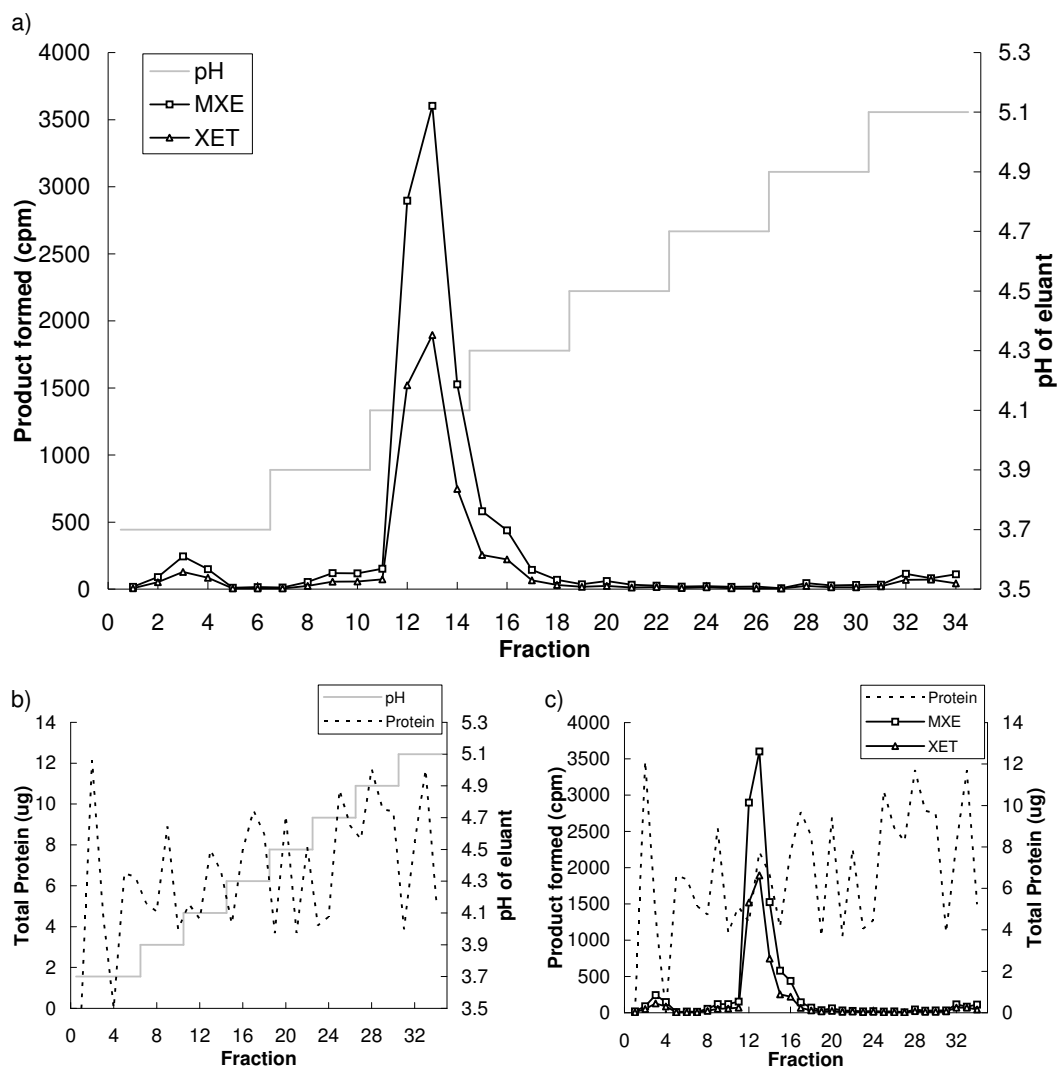


Figure 3.1.10 Batch elution of MXE and XET activity from SES

ASP from Table 3.1.3 was dialysed for 16 h at 6°C (citrate + formate, 10 mM each, pH 3.7) and centrifuged to removed precipitates. Fractions (2.5 ml) were collected at 6°C. Dialysate (5 ml) was applied to the column, followed by 10 ml portions of buffer with 0.05% Triton X-100. Activity was assayed by incubating 20 µl of each fraction in citrate (75 mM, pH 6.3), [³H]XXXGol (1 kBq), and MLG or XyG (0.24%) for 3 h. Protein was measured by the Bradford assay. Data displayed as activity with pH (a), protein with pH (b), or activity with protein (c).

First, ASP was purified by SES IEC (Figure 3.1.10). Most of the MXE and XET activity was eluted by a change in pH from 3.9 to 4.1. A small amount of activity was present in the flow through, about fraction 3. This might suggest that all the binding sites on the column were occupied, and a small amount of protein was unable to bind. Protein consistently eluted in a major peak 2–3 fractions after each change in buffer. Comparing the specific activity (SA) of fractions 12 and 13 with the dialysate, MXE and XET were purified about two-fold. However, a higher rate of activity might have been present. Over half of the acceptor substrate was consumed in the reaction, which would decrease the apparent reaction rate due to the lack of substrate. Still, activity was highest in these two fractions, and were pooled to continue purification.

A BioGel P-100 column was prepared and equilibrated in citrate + BSA + Triton X-100. The majority of fractions 12 and 13 from the SES column were mixed with BSA (1%) and Triton (0.05%), but no internal markers. The sample was applied to the column and eluted with citrate buffer + BSA + Triton (Figure 3.1.11).

Most of the activity applied to the column was eluted and recovered from the Bio-Gel P-100 column; ~90% of the MXE activity and ~70% of the XET activity. A large portion of fractions 41–44 (~50% of the total activity recovered) were pooled for further purification.

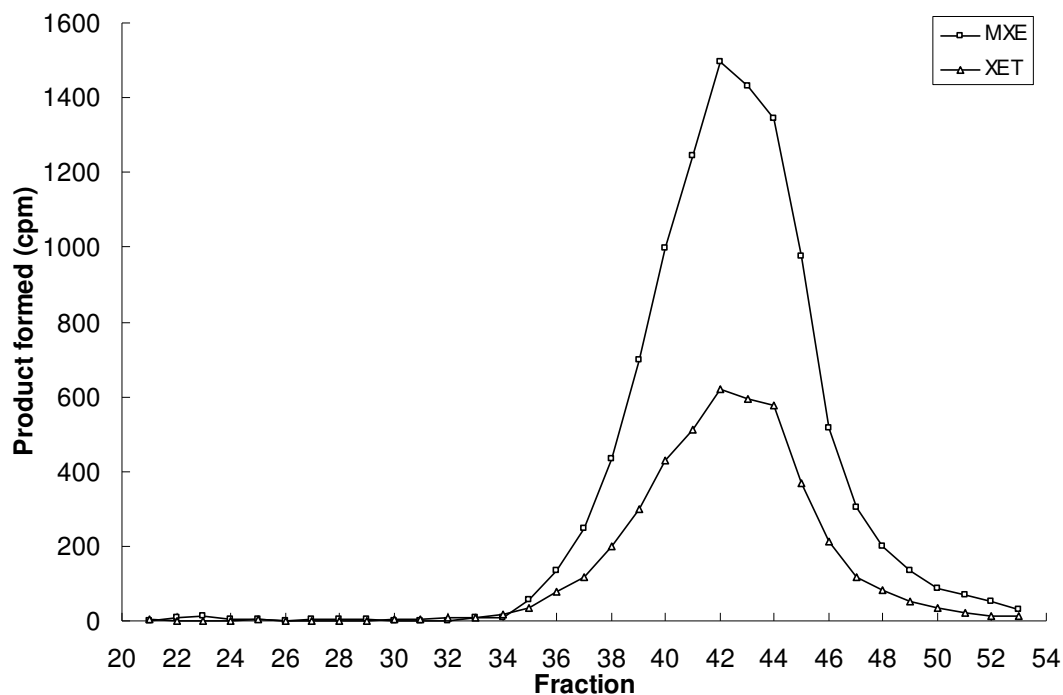


Figure 3.1.11 Batch elution of MXE and XET from Bio-Gel P-100

The 100-ml Bio-Gel P-100 column was equilibrated in citrate (0.3 M, pH 6.3) and BSA (1%) and Triton X-100 (0.05%) at 6°C. Fractions (1.8 ml) were collected at 4.4 ml/h. Activity was assayed for 3 h in [³H]XXXGdI (1 kBq) and MLG or XyG (0.32%). SA could not be determined due to the large amounts of BSA in the eluant. Void and included volumes are estimated from calibration prior to the run. $k_{av} = 0.79$

Con A beads were prepared and packed in a 1-ml column. Fractions 41-44 from Figure 3.1.11 were applied to the column and eluted with increasing concentrations of methyl- α -mannopyranoside. (Figure 3.1.12).

Protein was estimated by A_{280} , but contaminants in the eluant distort the data, as in Figure 3.1.9. The proper background was subtracted from the A_{280} values except in transition between two eluants where, due to mixing, the content of the eluant is intermediary. Therefore, the measurement of protein in each fraction is only an estimate.

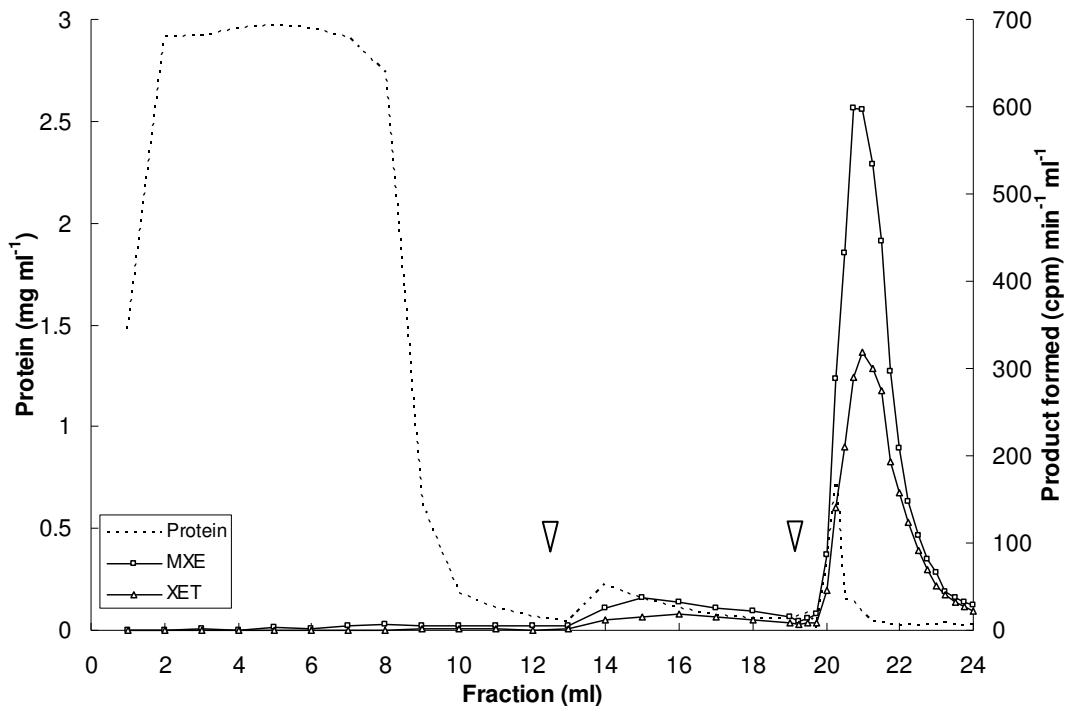


Figure 3.1.12 Batch elution of MXE and XET from Con A

Fractions 41-44 (1.7 ml each) from Figure 3.1.11 were combined with MgCl_2 , MnCl_2 , and CaCl_2 (each 1 mM), and applied to the column. Citrate buffer (0.3 M, pH 6.3) with MgCl_2 , MnCl_2 , and CaCl_2 (1 mM each) was applied to wash out any non-glycoproteins, including BSA. As fractions were eluting, their A_{280} was measured. When A_{280} was consistently low, the buffer was changed to citrate (0.3 M, pH 6.3) with MgCl_2 , MnCl_2 , CaCl_2 (1 mM each), and methyl- α -mannopyranoside (5 mM). The eluant was changed again when there was no more detectable protein eluting from the column. The final buffer consisted of citrate (0.3M, pH 6.3), methyl- α -mannopyranoside (0.6 M), and Triton (0.05%). Downward triangles denote a change in eluant. Fractions (1 ml for first two eluants, 0.25 ml for the last eluant) were collected at 6°C. Activity was assayed by incubating each fraction (20 μl) with [^3H]XXXGol (1 kBq) and XyG or MLG (0.32%). A portion of the original sample (2 μl) was also tested in each eluant (18 μl) to determine the effects of the additives on the rate. Activity was adjusted to reflect the rate without inhibition.

MXE and XET activity eluted as one major peak in the last eluant, and after the peak of protein. The peak of MXE activity eluted in fractions (ml) 20.75-21.00, while the peak of XET activity eluted in fractions (ml) 21.00-

21.25. MXE and XET have slightly shifted peaks of activity, but only slight.

The difference in peaks could be due to sampling error.

A portion of each fraction from the three columns was retained for SDS-PAGE to monitor protein purification. Fractions containing the peak of activity from each step were separated on SDS-PAGE (Figure 3.1.13).

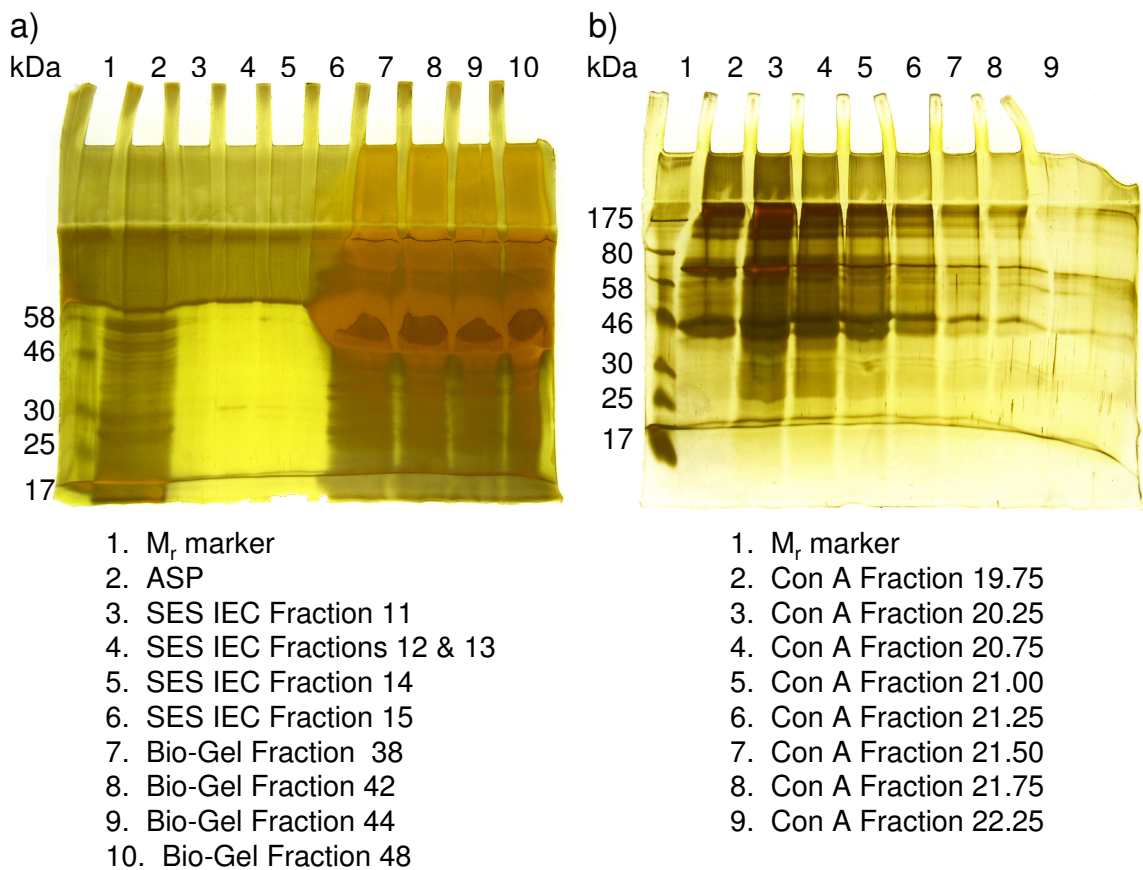


Figure 3.1.13 SDS-PAGE of fractions from 3 columns

SDS-PAGE gels (12% acrylamide) were electrophoresed until bromophenol blue reached the bottom of the gel. The gels were stained with silver nitrate.

In Figure 3.1.13 a), BSA from Bio-Gel fractions contaminated the running buffer and the entire gel, causing the dark brown stain above ~70 kDa. Still, information about the SES IEC fractions can be gleaned from this

gel. Very few proteins are present in these fractions, but the most prevalent protein (lane 4) migrates roughly with the 30 kDa marker. Most XTH proteins are 29-33 kDa, and MXE could be a similar size. The amount of protein in this band across the four fractions correlates with the amount of activity, and could be a candidate for MXE.

Subsequent purification steps did not simplify the mixture. BSA itself is not a glycoprotein, and should have been separated from MXE in the ConA column. However, BSA was only 96% pure. Other proteins were co-purified with MXE in the final step (Figure 3.1.13 b). In the SES fractions the major band in the fraction was ~30 kDa, many other proteins in the ConA fractions are present in higher amounts than any protein ~30 kDa.

The addition of BSA was detrimental to the purification of MXE, but the initial steps of dialysis and IEC were very efficient. The amount of purification was monitored by measuring the SA (Table 3.1.8).

Table 3.1.8 Summary of purification by 3 columns

| Sample | Specific Activity | | SA (product formed (cpm) min ⁻¹ μg ⁻¹) values calculated from: Table 3.1.3 (ASP), Figure 3.1.10 fractions 12 & 13 (SES IEC), and Figure 3.1.12 fraction 21.00 (ConA). |
|-----------|-------------------|-----|--|
| | MXE | XET | |
| ASP | 2.8 | 1.6 | |
| Dialysate | 195 | 101 | |
| SES | 372 | 198 | |
| Con A | 7.6 | 4.1 | |

Based on the summary of SA across all methods used, BioGel P-100 was not a beneficial step. Dialysis and IEC increased the purity of MXE and

XET better than any other step. Another method similar to IEC is isoelectric focusing (IEF), and was used in the purification of MXE.

3.1.7 Rotofor

Rotofor IEF purifies molecules based on isoelectric point in a horizontal liquid column. Twenty separated fractions, kept apart by porous membranes along the column, were collected simultaneously by vacuum pressure.

3.1.7.1 Rotofor Marker Separation

Coloured pI markers were separated using broad range ampholytes pH 3–10. Three coloured markers, phycocyanin, hemoglobin A&C, and cytochrome C were focused (Figure 3.1.14).

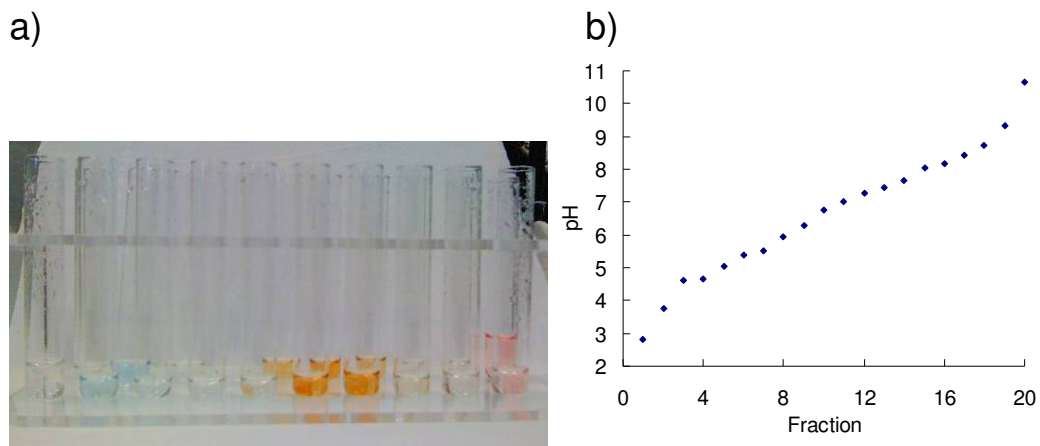


Figure 3.1.14 Rotofor isoelectric focusing of pI standards

The Rotofor Cell was electrophoresed at 10W constant power until the voltage stabilized, and fractions from the cell were collected into culture tubes. (a) Coloured markers were focused into a few fractions (phycocyanin, blue, fractions 3-4, pI 4.7; hemoglobin, orange, fractions 10-14, pI ~7.2; cytochrome C, pink, fractions 19-20, pI 9.6) based on their pI. (b) The pH of the fractions increased roughly linearly.

The separation of visual markers validates the Rotofor Cell system. Phycocyanin (blue) concentrated in two fractions near the anode, the hemoglobins (orange) focused to a few fractions in the middle fractions, and Cytochrome C (pink) was collected in fractions near the cathode. The pH across most fractions was roughly linear from pH ~4-9, with slight extremes at the initial and final fractions. The non-linear pH gradient that the extremities due to a leaking of the electrolytes (H_3PO_4 and NaOH) into the column.

3.1.7.2 Broad Range pH

MXE and XET from *Equisetum* were first separated over a broad range of pH. Fractions were assayed for pH, and MXE and XET activity.

Fractions containing activity were pooled and separated a second time, using the ampholytes which were already in those fractions. A second focusing, suggested by the manufacturer, could separate the same proteins even further, enhancing the purification. Each fraction was assayed for pH and activity, and the fractions containing activity were examined by SDS-PAGE (Figure 3.1.15).

High levels of activity were able to be recovered by this method. The exact amount is unknown, as the ampholytes' effect on activity was significant but unknown. Estimated recovery of activity was about 25% for

both MXE and XET. To determine exact activity, fractions could have been dialysed against citrate buffer. However, if the fractions were dialysed, they could not have been separated a second time in their own ampholytes.

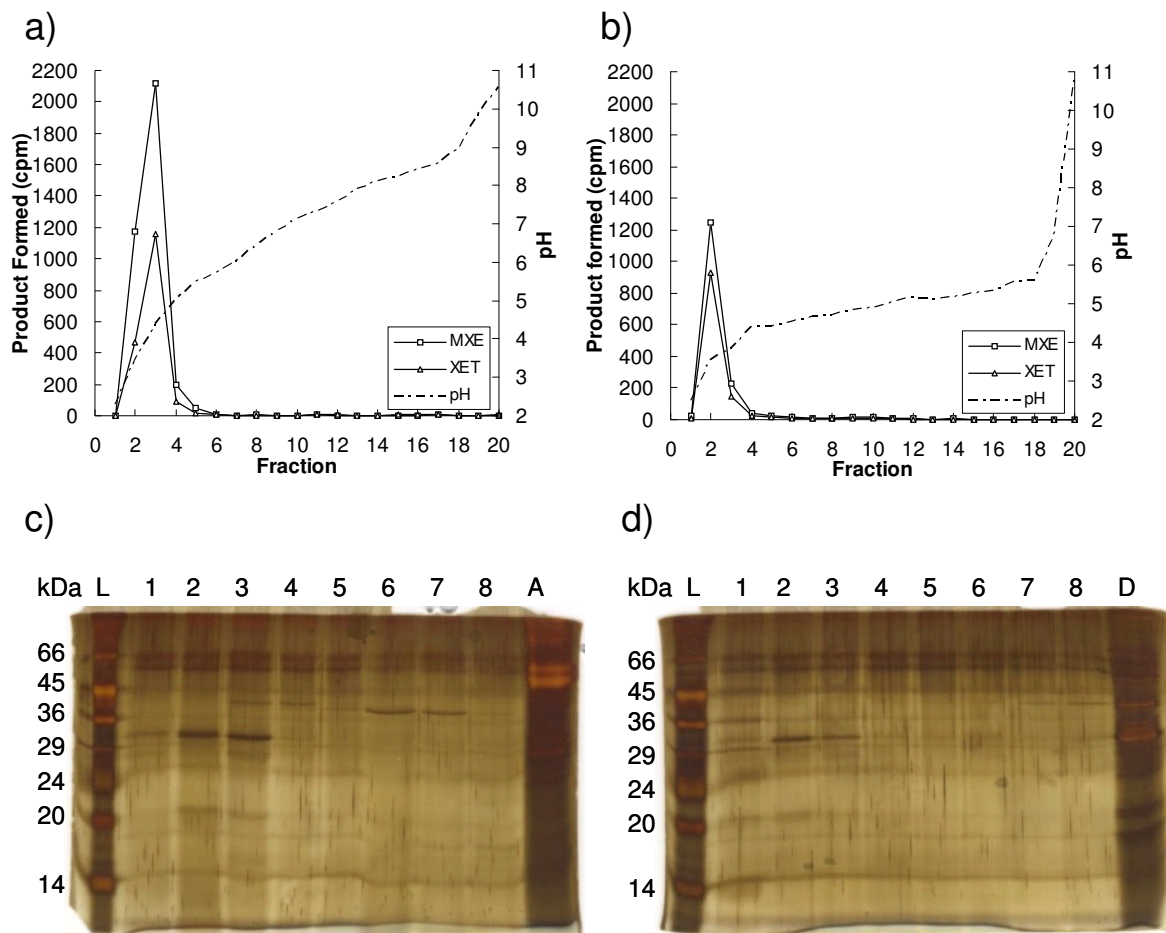


Figure 3.1.15 Rotofor separation of ASP over broad range pH

ASP from Table 3.1.3 was dialysed against formate buffer (10 mM, pH 3.7, 16 h) prior to electrophoresis. The dialysate (1 ml) was centrifuged at 6°C to remove precipitants, then separated by IEF with BioLyte ampholytes pH 3–10 (a). Activity was assayed by incubating the fraction (20 μ l) with citrate buffer (0.188 M, pH 6.3, to overwhelm the ampholytes' buffering effects), [3 H]XXXGol (1 kBq), and MLG or XyG (0.12%). The most active fractions (2–5) were pooled and separated a second time with the ampholytes present in those fractions (b). Triton X-100 (0.05%) was also included to maintain solubility. Fractions 1–8 from the first separation (c) and fractions 1–8 of the second separation (d) were also assayed by SDS-PAGE to monitor the purification of possible proteins of interest. L, ladder; A, ASP; D, dialysate.

The pattern of focusing in the second IEF does not exactly agree with the first. The major peak of activity focuses to a lower pH in the second separation than it did originally. The pH of the fraction with the most activity in the first instance was 4.37, but the second was 3.45. Also, significantly less activity was recovered in the second purification.

Fractions analysed by SDS-PAGE indicate that a relatively low number of proteins are present in each fraction after IEF. Proteins that are present in all fractions are most likely keratin or another contaminant of the gel (e.g. two bands ~60–70 kDa). A strong candidate for MXE follows the pattern of activity in fractions 2–3 of c) and in fraction 2 of d), running between the markers 29–36 kDa.

As predicted from IEC data, this method efficiently increases the purity of MXE and XET activity in two steps, dialysis and IEF.

3.1.7.3 Rotofor separation of MXE and XTH

IEF of MXE over a broad range of pH indicated that its pI was quite low, around 4. An extract from young stems, with more XET (Fry et al., 2008 a), was tested by IEF to determine if *Equisetum* has any XTHs with higher pIs.

Equisetum proteins were extracted from young spring shoots and separated by IEF in BioLyte ampholytes pH 3–10. MXE activity has a pH

optimum at 6.3, but XET has a reported pH optimum at 5.5. Each fraction was tested for MXE and XET activity in both pHs (Figure 3.1.16).

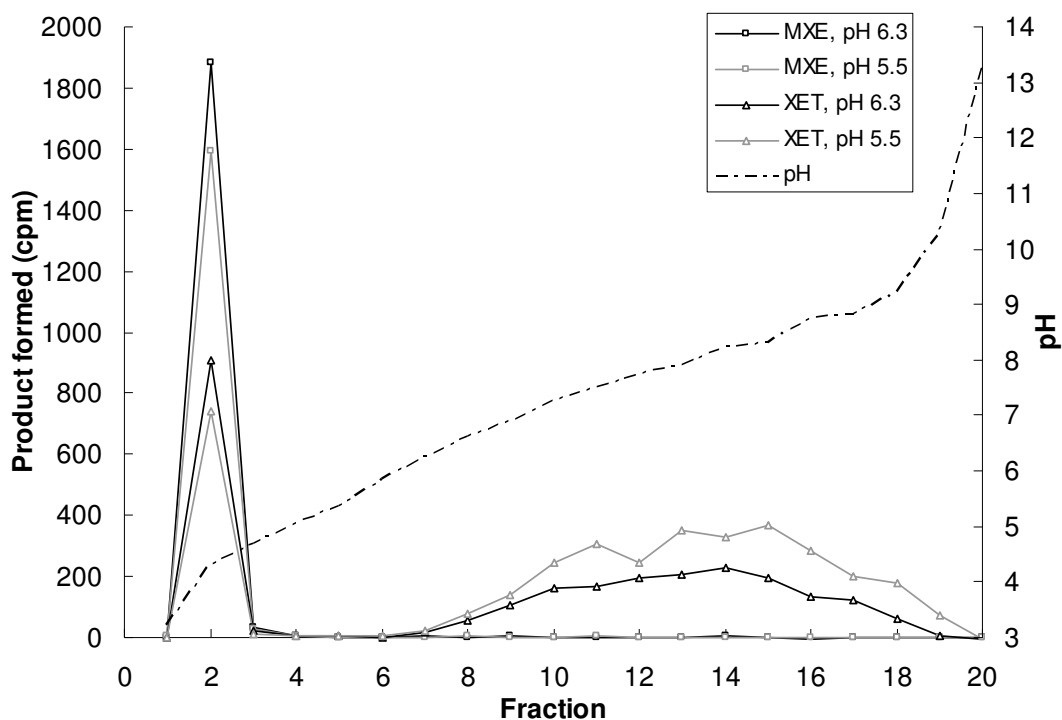


Figure 3.1.16 Separation of young stem MXE and XET over broad range pH

Equisetum extracts from shoots harvested in May were dialysed against succinate buffer (10 mM, pH 5.5). The dialysate was mixed with Bio-Lyte ampholytes (pH 3–10) and separated by Rotofor IEF. Activity was assayed by incubating the fraction (20 μ l) with enough buffer to overwhelm the ampholyte buffer effects, either citrate buffer (0.188 M, pH 6.3) or succinate buffer (0.188 M, pH 5.5), [3 H]XXXGol (1 kBq), and MLG or XyG (0.12%).

In young tissue there could have been only one isoform of MXE, focused at a low pH, about 4. However, there were many isoforms of XTH in this sample with a range of pIs. The activity exhibited in fractions 8–18, from enzymes with pI from 6.6–9.0, had similar characteristics of previously

reported XTHs. Their activity was greater when assayed in pH 5.5 than in pH 6.3, and they had no significant MXE activity.

The activities in fraction 2 could have been the result of a single enzyme. The MXE activity was higher when assayed in pH 6.3 than in pH 5.5. The XET activity in this fraction, which was higher when assayed in pH 6.3 than 5.5, had the same characteristics of the MXE activity. In the first report of MXE, the authors believed that MXE was a different enzyme than XTH based on other differences (Fry, et al. 2008 a). One of those differences was the enzymes' acceptor substrate specificities, which was tested here.

3.1.7.4 Acceptor specificity of Equisetum MXE and XTH

Fry et al. (2008 a) reported that one of the pieces of evidence that MXE and XTH were different enzymes was the difference in acceptor substrates. Three XGO acceptor substrates were tested here; XXXGol, XXLGol, and XLLGol. Four enzyme fractions from Figure 3.1.16 (2, 11, 13, 15) were tested for differences between their acceptor substrate specificities (Figure 3.1.17).

As previously shown, MXE activity was present only in fraction 2, not in other fractions. The rate of activity was best with XXXGol, lower with XXLGol, and still lower with XLLGol. XET activity of the 'classical' XTHs in fractions 11, 13, and 15 followed a pattern which agrees with previously

reported figures (Fry et al., 1992; Purugganan et al., 1997; Hrmova et al., 2009 data not shown). XLLGol was the best acceptor substrate for all XTHs.

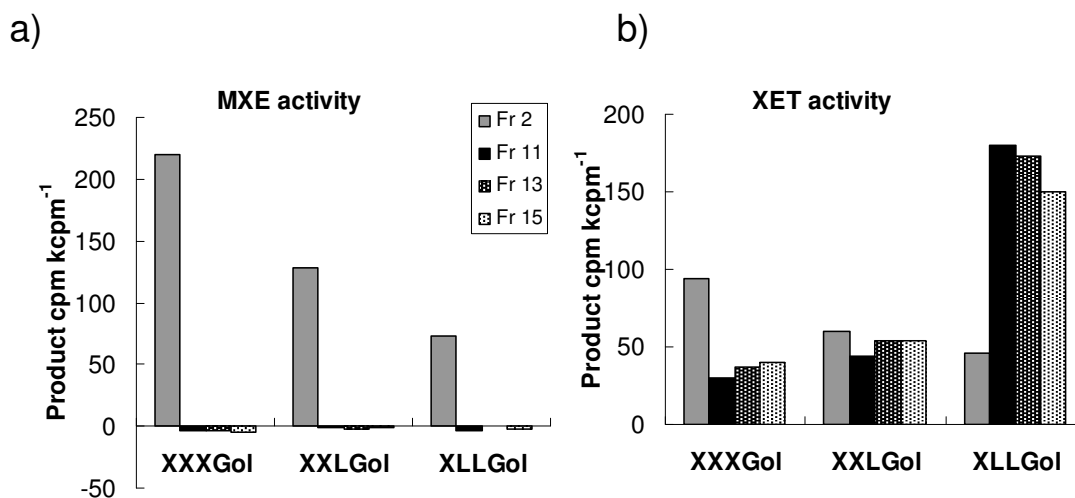


Figure 3.1.17 Acceptor substrate specificities of Rotofor separated MXE and XTH

Activity was tested by incubating fractions 2, 11, 13, or 15 (20 μ l) from Figure 3.1.16 with succinate buffer (0.2 M, pH 5.5), acceptor substrate, and MLG (a) or XyG (b) (0.11%) for 3 h. Results presented as product formed (cpm) per acceptor substrate (kcpm) supplied. Citrate buffer (0.2 M, pH 6.3) was also tested, with similar results.

The question posed with this experiment was, would the fraction 2 XET activity with the three acceptor substrates mimic the pattern of MXE activity or 'classical' XTHs? If it were the same pattern as the other XTHs, the XET activity in fraction 2 would most likely be the result of an XTH with a low pI, and be a different enzyme than MXE. However, the fraction 2 pattern of XET activity closely mirrored the pattern of MXE activity, suggesting that the two activities could be the result of a single enzyme.

3.1.7.5 Bulk preparation of MXE

To prepare for mass spectrophotometry, MXE was separated by IEF using Servalyt ampholytes pH 3–5 (Figure 3.1.18).

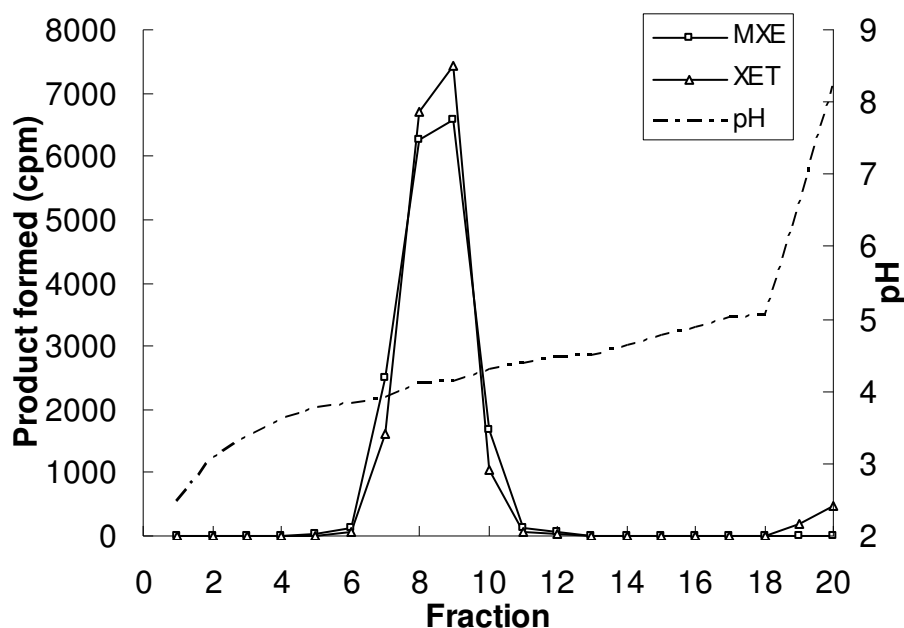


Figure 3.1.18 Bulk preparation of MXE

ASP (4.5 ml) from Table 3.1.3 was dialysed against 5 L of formate (10 mM, pH 3.7). The centrifuged dialysate was mixed with Servalyte (pH 3-5) ampholytes and Triton X-100 (0.05%). Activity of IEF fractions were measured by incubating each fraction (20 μ l) in citrate (0.188 M, pH 6.3) with [3 H]XXXGol (1 kBq) and MLG or XyG (0.12%) for 3 h.

The separation of MXE was successful, focusing to pH 4.09–4.14. The rate of activity was limited by the amount of acceptor substrate, and was likely much higher than the assay suggested.

3.1.8 Mass spectrometry

An attempt to identify Rotofor-purified MXE was made using two mass spectrometric methods. Fraction 9 of Rotofor separation in Figure 3.1.18 was separated in two 12% SDS-PAGE lanes (Figure 3.1.19), then excised and

digested with porcine trypsin overnight. The digests were either used immediately or stored at -80°C for further analysis.

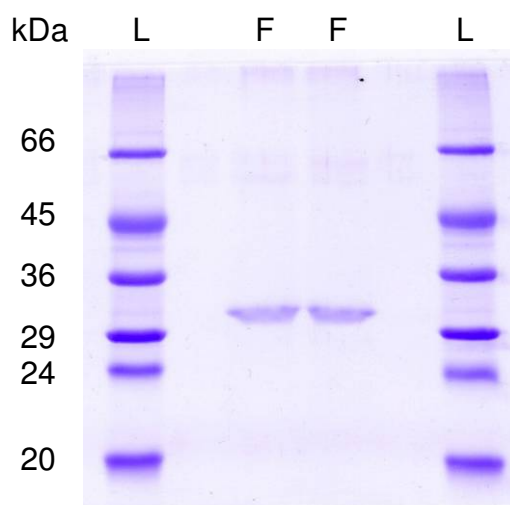


Figure 3.1.19 SDS-PAGE of bulk Rotofor-purified MXE

Protein ladders (L, Sigma prod. no. SDS7) flank two lanes of fraction 9 from Figure 3.1.21 (F). The gel was stained with GelCode Blue Stain (Pierce). The one major band in lane F was excised and stored at -20°C .

3.1.8.1 MALDI-TOF MS

An excised and digested MXE band similar to that described in the previous section was analysed by MALDI-TOF MS. A spectrum of the masses detected in the digest was collected and is shown in Figure 3.1.20.

One peak peptide fragment of about 964 kDa was especially abundant in this digest. The digest was expected to produce many fragments across the range of 600–3000 Da. In an attempt to produce more peaks of relatively equal abundance, another gel was loaded with the same Rotofor fraction, and was digested in a replicate experiment (Figure 3.1.21).

The replicate spectrum was quite similar to the first. Many of the most abundant peaks were found in both spectra. However, many minor peaks were different.

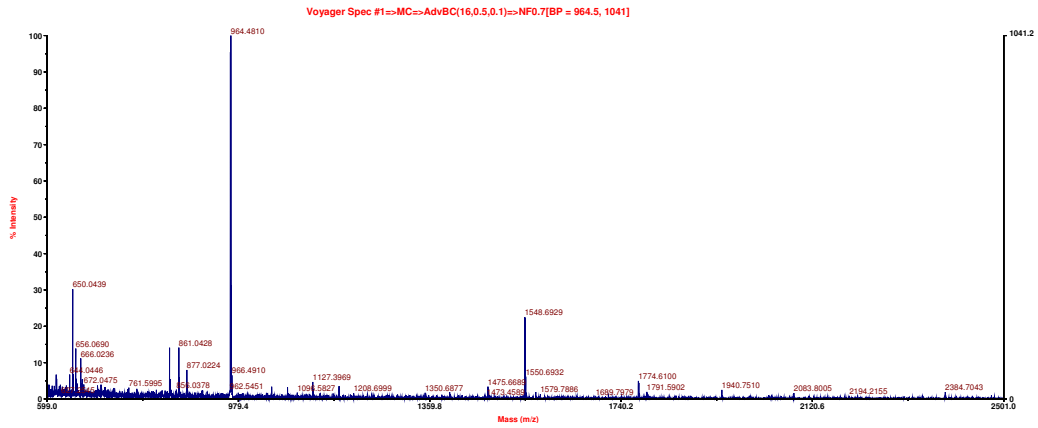


Figure 3.1.20 MXE MALDI-TOF spectrum

Rotofor-purified MXE was excised from a 12% SDS-PAGE gel and digested with trypsin. The figure shows masses of peptide fragments which were detected by the mass spectrometer, with some of the peaks' masses labelled.

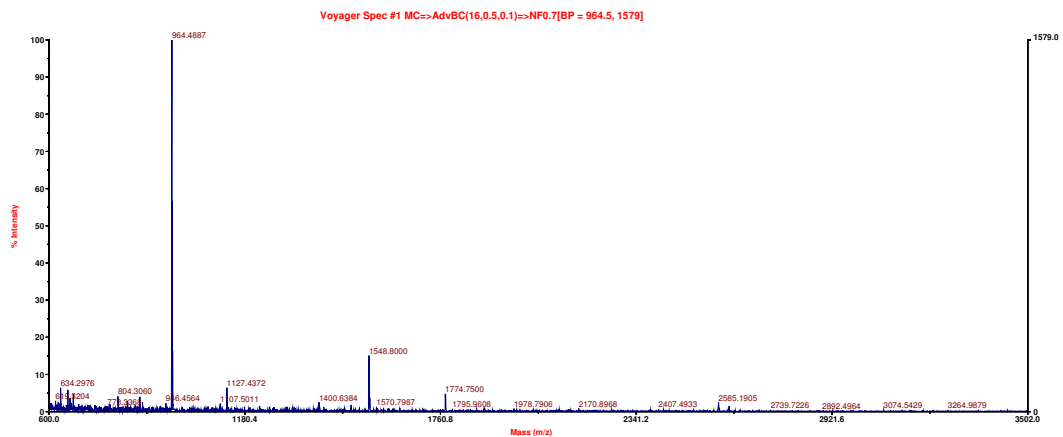


Figure 3.1.21 Replicate MXE MALDI-TOF spectrum

Data were collected as in Figure 3.1.20.

A list of about 100 of the most abundant peaks from both spectra were searched against the NCBItr and SwissProt databases to attempt to match the tryptic fragments to proteins on the databases. Unfortunately, no

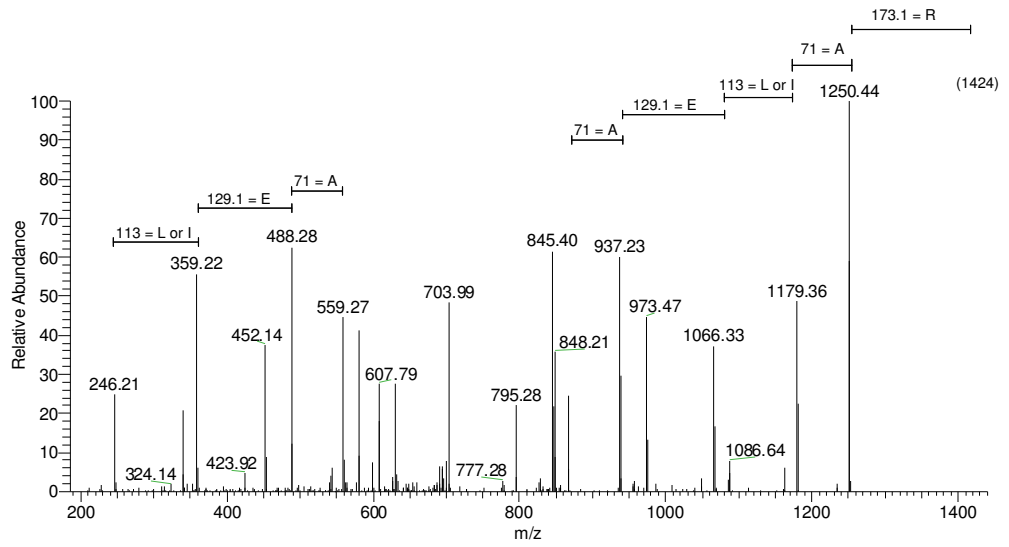
proteins were matched. Hits were found for contaminants, e.g. keratin, which confirmed that the spectra had been accurately calibrated.

3.1.8.2 Liquid chromatography–mass spectrometry (LC–MS)

Another instrument was utilized to gather more information about the tryptic fragments. The first step was to separate tryptic fragments on a C18 HPLC. As peptides eluted off the column, they were fed into an LTQ XL machine continuously. The most abundant peptides identified in the first MS mode, were trapped and fragmented a second time. The fragment masses were measured and collected (MS2). The resulting spectrum of fragments could be used to piece together a sequence of the original peptide fragments by searching against known protein databases. Examples of the MS2 fragmentation pattern and the calculated/predicted amino acid sequence of the original fragment are shown (Figure 3.1.22).

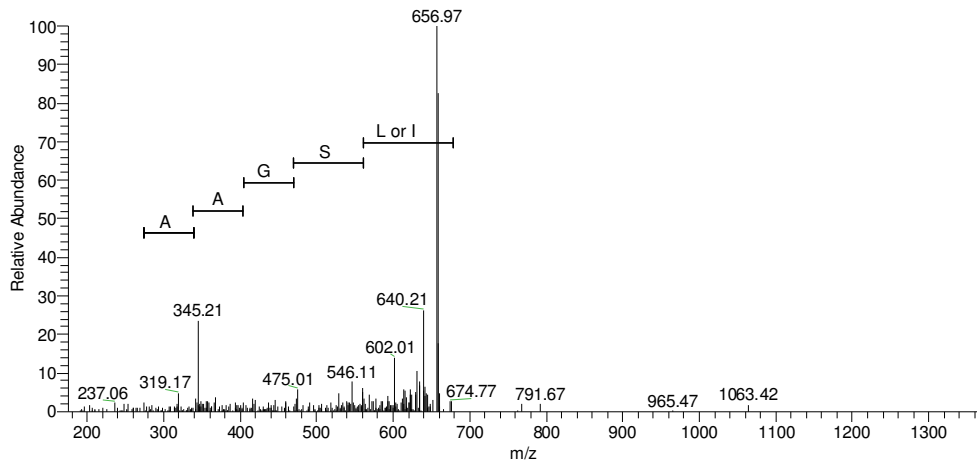
Gathering this information might have yielded a sequence which was similar to the conserved active site of an XTH, but unfortunately this was not found.

a)



| # | a | a ⁺ | b | b ⁺ | Seq. | y | y ⁺ | y [*] | y ^{***} | # |
|----|-----------|----------------|-----------|----------------|------|-----------|----------------|----------------|------------------|----|
| 1 | 88.0847 | 44.5460 | 116.0948 | 58.5511 | D | | | | | 11 |
| 2 | 185.1999 | 93.1036 | 213.2100 | 107.1087 | P | 1309.4690 | 655.2382 | 1292.4385 | 646.7229 | 10 |
| 3 | 371.4098 | 186.2086 | 399.4199 | 200.2136 | W | 1212.3538 | 606.6806 | 1195.3233 | 598.1653 | 9 |
| 4 | 531.6040 | 266.3057 | 559.6141 | 280.3107 | C | 1026.1439 | 513.5756 | 1009.1134 | 505.0604 | 8 |
| 5 | 694.7773 | 347.8923 | 722.7874 | 361.8974 | Y | 865.9497 | 433.4785 | 848.9192 | 424.9633 | 7 |
| 6 | 809.8647 | 405.4360 | 837.8748 | 419.4411 | D | 702.7764 | 351.8919 | 685.7459 | 343.3766 | 6 |
| 7 | 908.9958 | 455.0016 | 937.0059 | 469.0066 | V | 587.6890 | 294.3482 | 570.6585 | 285.8329 | 5 |
| 8 | 1038.1098 | 519.5586 | 1066.1199 | 533.5636 | E | 488.5579 | 244.7826 | 471.5274 | 236.2674 | 4 |
| 9 | 1151.2674 | 576.1374 | 1179.2775 | 590.1424 | L | 359.4439 | 180.2256 | 342.4134 | 171.7104 | 3 |
| 10 | 1222.3453 | 611.6763 | 1250.3554 | 625.6814 | A | 246.2863 | 123.6468 | 229.2558 | 115.1316 | 2 |
| 11 | | | | | R | 175.2084 | 88.1079 | 158.1779 | 79.5926 | 1 |

b)



| # | a | b | Seq. | y | y [*] | # |
|---|----------|----------|------|----------|----------------|---|
| 1 | 86.1549 | 114.1650 | I | | | 7 |
| 2 | 173.2322 | 201.2423 | S | 560.6239 | 543.5934 | 6 |
| 3 | 230.2835 | 258.2936 | G | 473.5466 | 456.5161 | 5 |
| 4 | 301.3614 | 329.3715 | A | 416.4953 | 399.4648 | 4 |
| 5 | 372.4393 | 400.4494 | A | 345.4174 | 328.3869 | 3 |
| 6 | 471.5704 | 499.5805 | V | 274.3395 | 257.3090 | 2 |
| 7 | | | R | 175.2084 | 158.1779 | 1 |

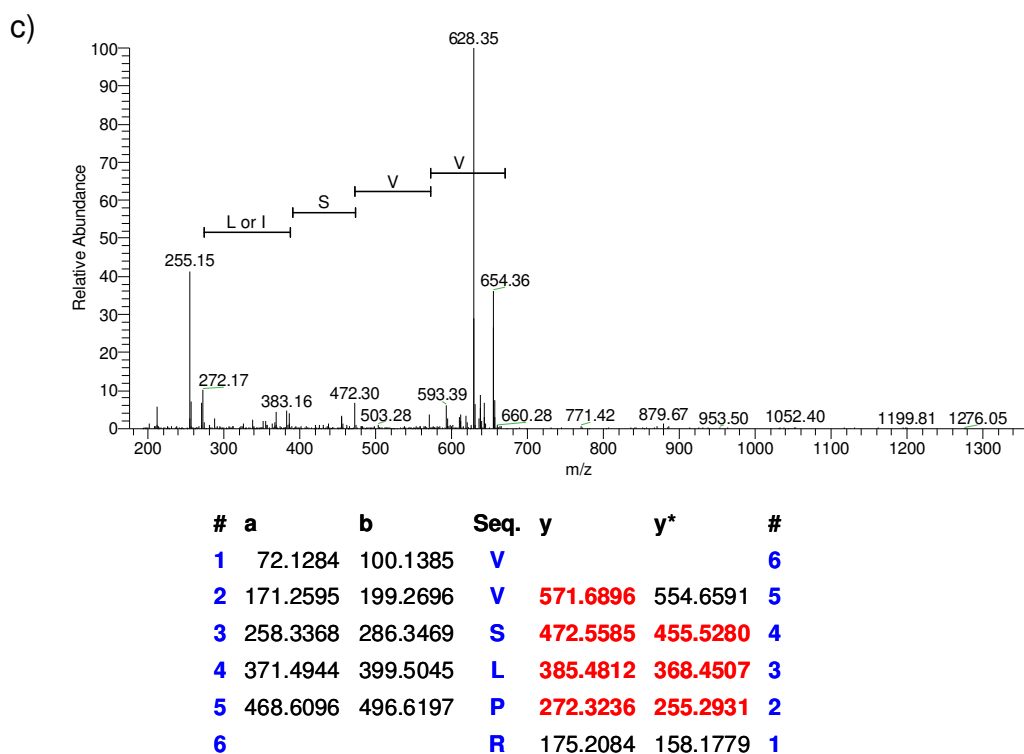


Figure 3.1.22 Example of MS/MS spectra and predicted sequence of the peptide

Data were collected and analysed by Xcalibur software, then aligned with known protein sequences to produce the possible amino acid sequence of the fragment. Masses highlighted in red indicate that a fragment was found that matches the predicted mass for a known peptide sequence. The headings of the tables indicate how the peptide has been fragmented. Fragments which contain the original peptide's N-terminus are labelled b-ions, and the fragments containing the original C-terminus are y-ions. a-ions contain the original N-terminus but have also lost a side-chain. If a fragment's y ion breaks a second time on a side chain, its termed a y*-ion. The symbol '++' is a prediction of the mass/charge ratio (m/z) of a doubly charged fragment.

The sequences predicted by the software, combined with other possible sequences in the data set, were compared with known proteins from the NCBI Inr and SwissProt databases. Again, the best matches of full proteins aligned with expected contaminants, but some individual fragments were aligned with a sequence with relative accuracy. These fragments, listed

below, are possible tryptic fragments of MXE (parentheses indicate an unconfirmed amino acid, where there was no MS peak to correspond with the predicted amino acid in the sequence), written N→C-terminus:

ISGAAVR (Figure 3.1.22 b)

ENYLK (data not shown)

VVSLPR (Figure 3.1.22 c)

FHYGITRR (data not shown)

VV(S)VEDG(I)R (data not shown)

Some MS/MS fragmentations resulted in strong, regularly spaced peaks across a range of m/z , but the software could not align the peaks with a known protein. In these cases, manual interpretation of the results could be attempted by simple addition and subtraction. Peaks which differed by masses corresponding to one amino acid could be traced and hence the sequence tentatively identified. Sequences produced or extended manually are listed below:

AELAR (Figure 3.1.22 a)

LEGNE (data not shown)

MXE could not be identified by this method, nor could it be matched to any known XTH or other similar enzyme. If the DNA sequence of *Equisetum* becomes available in the future or when the NCBI database increases in size, this data set could be re-analysed to determine the sequence of the protein purified with absolute certainty. The quality of the data collected is of a sufficiently high standard to allow this to be done.

3.2 Functional Analysis and Enzymology of MXE

3.2.1 Donor and Acceptor substrate specificity

Extant plants such as horsetails contain biochemically different polysaccharides in their cell walls. The structural components may also be connected by unique enzymes or activities. To search for such enzymes, extracts from *Equisetum* were tested for the presence of transglucosylase activities other than MXE and XET. Various donor and acceptor substrates were tested using crude extracts or partially purified transglucosylases.

3.2.1.1 Lichenan as donor substrate and laminarin as acceptor

MLG from *Cetraria islandica* (lichenan) and laminarin (from *Laminaria hyperborea*) were tested as possible donor and acceptor substrates in a transglucosylation reaction (Table 3.2.1).

Table 3.2.1 Transglucosylation rate (cpm / min) with lichenan and laminarin

| Donor substrate | Rate with acceptor substrate of: | |
|---------------------|----------------------------------|-------------------|
| | XXXGol | Laminaritetraitol |
| Tamarind xyloglucan | 4.23 ± 0.09 | 0.006 ± 0.0075 |
| Barley MLG | 3.51 ± 0.17 | 0.028 ± 0.008 |
| Lichenan | 0.046 ± 0.005 | 0.011 ± 0.006 |
| None added | 0.014 ± 0.007 | 0.015 ± 0.009 |

Crude *Equisetum* extract was incubated for 0–180 min (7 time points) in succinate buffer (0.1 M, pH 5.5), donor substrate (0.17%), and acceptor substrate (1 kBq per time point). Rate expressed as cpm of product per min of reaction, ± SE of the gradient.

Lichenan was not a significant donor substrate for any transglucosylase tested for in this extract. With XXXGol as the acceptor

substrate, lichenan was only able to be used at 1% of the XET rate. Cereal MLG is composed of more cellotriose units than cellotetraose units, but in *Equisetum* the latter prevail. Lichenan is composed of mostly cellotriose units. It is possible that MXE recognizes the cellotetraose structure as a site for donor substrate cleavage.

Laminaritetraose was not a suitable acceptor substrate when paired with any donor substrate tested. Although low, MLG was the donor substrate used at the highest efficiency with this acceptor substrate. When compared with the MXE rate, the MLG : laminaritetraitol transglucosylation rate was only about 1% as high. Laminarin is unlikely to be a suitable or significant acceptor substrate in *Equisetum* tissues.

3.2.1.2 Laminarin as a donor substrate

Laminaritetraose had been tested as a possible acceptor substrate, but laminarin was not tested in the same experiment as a donor substrate.

Unlike MLG or XyG, laminarin dried on paper would have dissolved in

Table 3.2.2 Transglucosylation rate with laminarin as a donor substrate

| Donor | Rate (cpm / min) | ASP as described in Table 3.1.1 was incubated with citrate (40 mM, pH 6.3), donor substrate (0.32%), and [³ H]XXXGol (1 kBq) for 0–120 min (n=7). Products analysed by PC method. ± SE of the curve reported. |
|-----------|------------------|---|
| XyG | 6.94 ± 0.59 | |
| MLG | 10.5 ± 0.33 | |
| Laminarin | -0.023 ± 0.033 | |

water. PC was necessary to separate the products from the oligosaccharide acceptor substrate (Table 3.2.2). Laminarin was not able to be used in a transglucosylation reaction with XXXGol.

3.2.1.3 Mannan or glucomannan as donor substrates

Mannans and glucomannans are β -1 \rightarrow 4 linked hexans, with similar backbone structures as XyG or the cellulose-like regions of MLG.

Glucomannan (konjac, low viscosity; partially acetylated) and β -1 \rightarrow 4 mannan ($[^3\text{H}]\text{NaBH}_3$ reduced, DP ~15) were tested for their ability to be a donor substrate in a transglycosylation reaction (Table 3.2.3).

Table 3.2.3 Transglycosylation rate with mannan or glucomannan as donor substrates

| Donor | Rate (cpm / min) | |
|-------------|---------------------|---|
| XyG | *10.4 | Mannan was solubilized as per manufacturer's instructions in 2.5 M NaOH, followed by neutralization with HOAc. Mannan precipitated again in a few hours. ASP as described in Table 3.1.3 was incubated with citrate (0.2 M, pH 6.3), donor substrate (0.32%), and $[^3\text{H}]\text{XXXGol}$ (1 kBq) for 0–209 min (4 time points, * 1 time point). Products were analysed by the PC method. \pm SE of the curve reported. |
| MLG | *21.5 | |
| Mannan | 0.0085 \pm 0.0213 | |
| Glucomannan | 0.159 \pm 0.009 | |

Neither glucomannan nor mannan was able to be used at an appreciable rate in a transglycosylation reaction. The glucomannan rate was only 1.5% of the XyG rate. Although the rate was low, this type of heterotransglycosylation has not yet been described previously. The glucomannan was composed of 60/40 mannose/glucose, and was partially acetylated, which increases solubility. Due to the random order of sugar

units along the glucomannan backbone, 4 glucoses could have been positioned together, and could have been the site of transglycosylation on the donor substrate.

Mannan was not a suitable donor substrate for transglycosylation. High levels of NaOAc (necessary to neutralize the NaOH used to solubilize the substrate), or the relatively small M_r of mannan, could have reduced or inhibited the rate of mannan transglycosylation. Still, very low or no transglycosylase activity indicates that neither mannan nor glucomannan are likely *in vivo* transglycosylation targets.

3.2.1.4 Acceptor specificity with MLG as the donor

Equisetum produces an enzyme that can attach MLG to XyG, but this enzyme might not be specific to this combination. Many XGO and other glycan oligosaccharides were tested with partially purified ASP using MLG as the donor substrate (Table 3.2.4). Larger XGOs are heterogeneous oligosaccharides with a backbone of either 8 (G_8) or 12 glucose residues (G_{12}). Most acceptor substrates were reduced with [3H]NaBH₃, except XXFG, which contained 3H in its fucose residue.

The non-XGO acceptor substrates, fragments of galacturonan, galactan, and arabinan, were not suitable for transglycosylation. The XGOs

tested were all able to be used, although some were preferred. XXXGol, with the fewest number of substitutions, was the optimal substrate tested, with

Table 3.2.4 XGO and other glycan acceptor substrates for MXE

| Acceptor | Rate \pm SE (cpm / min) | substrate added (cpm) | % of total produced per h |
|-----------------------|------------------------------|--------------------------|------------------------------|
| XXXGol | 10.3 \pm 0.3 | 2264 | 27.3% |
| XXLGol | 13.8 \pm 0.4 | 5274 | 15.7% |
| XLLGol | 1.66 \pm 0.11 | 2631 | 3.8% |
| XXFG | 1.40 \pm 0.06 | 2421 | 3.5% |
| G ₈ XGO | 3.59 \pm 0.11 | 2068 | 10.4% |
| G ₁₂ XGO | 1.83 \pm 0.23 | 3347 | 3.3% |
| GalA ₅ -ol | 0.00 \pm 0.02 | 1822 | 0.0% |
| Gal ₇ -ol | 0.00 \pm 0.01 | 5277 | 0.0% |
| Ara ₉ -ol | 0.00 \pm 0.02 | 3301 | 0.0% |

Various acceptor substrates were incubated with ASP from Table 3.1.2 in citrate buffer (0.1 M, pH 6.3) and MLG (0.33%) for 0–180 min (n=5–7), \pm SE of the curve.

the octasaccharide, XXLGol, second best. The nonasaccharides, XLLGol and XXFG, were less able to be used as acceptor substrates. Larger XGOs were increasingly less suitable substrates. These larger XGOs were presumed to have a random non-reducing terminal structure, possibly XXXG, XXLG, or XLLG. The unknown nature of these XGOs limits the interpretation of these substrates.

3.2.1.5 Oligo-glucan acceptor substrates of partially purified MXE

Glucose oligomers (MLGO, cello-oligosaccharides (Cello), and laminari-oligosaccharides (LamO)) were tested as acceptor substrates for MXE, but were partially degraded by hydrolases in crude extracts, thus competing

with concurrent transglucosylation reactions (Fry et al., 2008 a). Potentially free of these hydrolases, a partially purified Rotofor fraction containing MXE and XET activity was tested for its ability to use the previously mentioned acceptor substrates (Table 3.2.5).

Table 3.2.5 Oligo-glucan acceptor substrates of MXE

| Donor | cpm of product formed with reduced acceptor of: | | | | |
|-------|---|------------|----------|-----------|------|
| | MLGO (7-10) | MLGO (7-8) | LamO (4) | CellO (6) | XXXG |
| MLG | 196 | 280 | -2 | 298 | 2329 |
| XyG | 43 | 104 | -12 | 49 | 1239 |

| Donor | Relative product as % of XXXG | | | | |
|-------|-------------------------------|------------|----------|-----------|------|
| | MLGO (7-10) | MLGO (7-8) | LamO (4) | CellO (6) | XXXG |
| MLG | 8.4 | 12 | - | 13 | 100 |
| XyG | 3.5 | 8.4 | - | 4.0 | 100 |

Substrates were prepared by [³H]NaBH₃ reduction. Parentheses indicate DP. MLG with DP 7-10 was made by partial digestion with lichenase, while MLG 7-8 was made by partial digestion with cellulase. Rotofor-purified enzyme (fraction 8 from Figure 3.1.18) with 0.2% BSA was dialysed against citrate (10 mM, pH 6.3) for 19 h. The dialysate was incubated with the acceptor substrate indicated (2 kBq per 30 µl) and donor substrate (0.33%) for 3.1 h. The reactions were stopped with FA (5 µl) and applied to a TLC plate, which was developed 6 times in 70% Ethanol to remove unused acceptor substrate. The origins containing the polysaccharide transglucosylation product were excised and scintillation counted. Replicate mixtures with no donor substrate were applied to another TLC plate and developed in PBAW 1:1:1:1 to assay for degradation of the acceptors.

The optimal acceptor substrate for MXE tested in this assay was XXXGol, but other glucan oligos were also able to be used. Most notably, cello-hexaitol was able to be used as an acceptor substrate 13% as well as XXXGol when MLG was the donor. Also, MLG to MLGO transglucosylation was observed. MLG was a better donor substrate than XyG with every acceptor. As previously noted (Table 3.2.1), laminari-oligos were inefficient acceptor substrates.

reducing half of the DP 14 acceptor molecule by MXE. If MXE has the potential to attach MLG to a xylose residue or to any of the (non-reducing

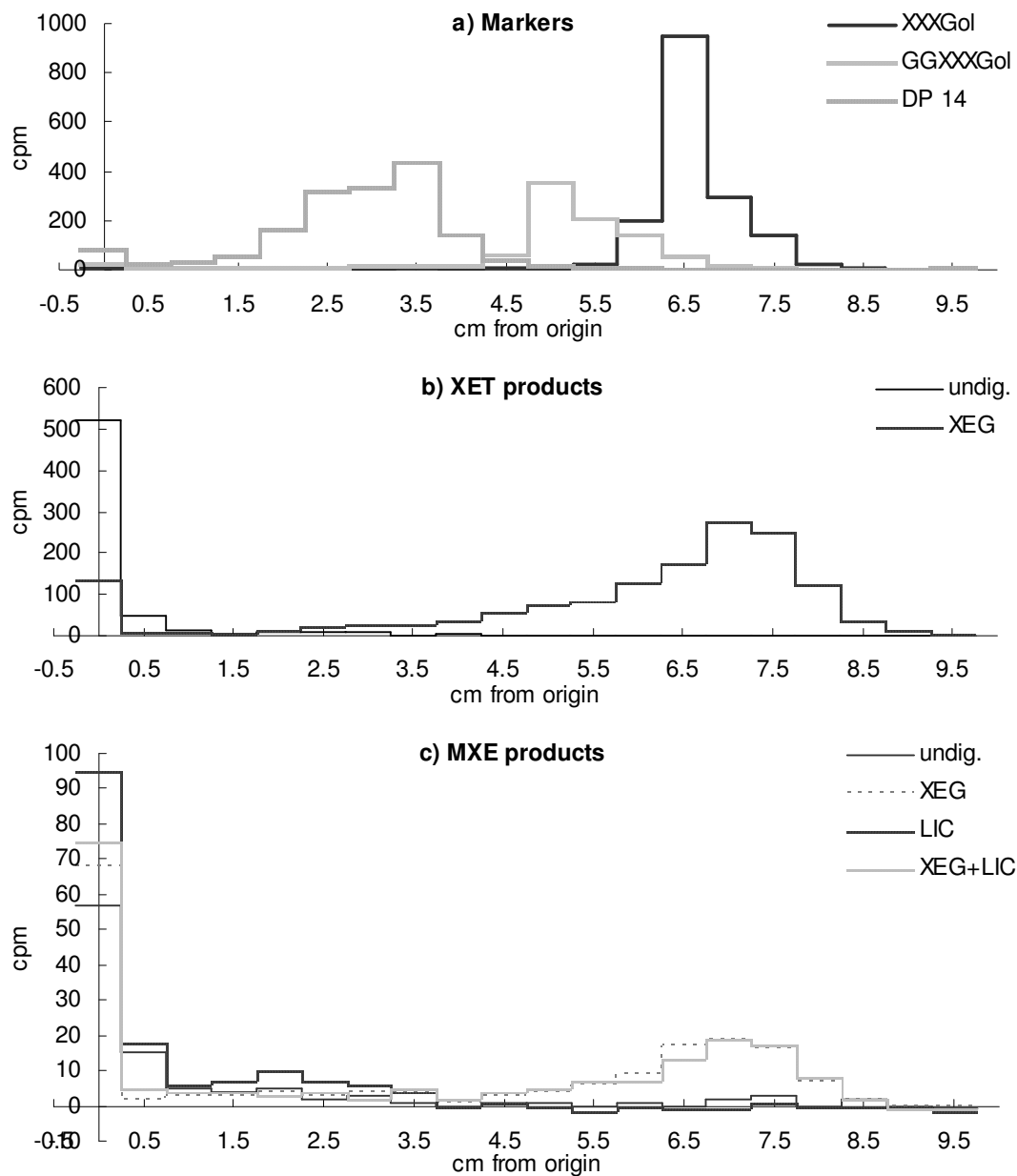


Figure 3.2.2 TLC histogram of digested MLG–DP 14 products

XXXGXXXG (Megazyme, Inc.) was tritiated with $[^3\text{H}]\text{NaBH}_3$, then 0.1 MBq was incubated with XyG or MLG (0.15%) and crude *Equisetum* extract (91 mM succinate, pH 5.5) overnight. The products were precipitated in 75% Ethanol and ammonium formate (1.5%) and washed free of unused acceptor substrate. They were redissolved in water (0.1%) and digested with XEG (0.002%), lichenase (2 U/ml), or with both simultaneously. The digestion

products were analysed by TLC, followed by cutting of segments and scintillation counting. a) Markers were in adjacent lanes. For description of GGXXXGol synthesis, refer to Figure 3.3.3. b) XET product was undigested, or digested with XEG. c) MXE product was undigested, or digested with XEG, lichenase (LIC), or both (XEG+LIC).

terminal) backbone-glucoses of the DP 14 acceptor, it could attach MLG to the reducing half of the molecule. However, if MXE attaches MLG only to the non-reducing terminus, MLG must be attached to the non-radioactive half of the DP 14 acceptor. A schematic diagram explains how digestion of the MXE–DP14 product with lichenase and XEG can elucidate the nature of the newly-formed bond (Figure 3.2.1).

Crude *Equisetum* extract was incubated with XyG or MLG and the DP 14 substrate. The products were precipitated and washed free of oligosaccharide starting material in 75% Ethanol. Purified MXE product was redissolved in water and digested. As a control, purified XET product was digested with XEG (Figure 3.2.2).

Upon digestion with XEG, the XET product migrated only slightly faster than the marker, XXXGol (Figure 3.2.2 b). The crude enzyme mixture extracted from *Equisetum* could have included a glycosidase, such as α -xylosidase, which degraded the oligosaccharide to a substrate smaller than XXXGol. Incomplete digestion of the polysaccharide, evident by the radioactive product which remained at the origin, may have been due to the lack of total re-dissolution after drying.

MXE appears to attach MLG to the non-reducing terminus of the XGO acceptor (Figure 3.2.2 c). Similarly to the XET product, MXE product did not completely digest under any conditions. However, enough product was released to sufficiently analyse the result of the experiment. As expected, the lichenase only digest (LIC) yielded an oligosaccharide larger than DP 14, which migrated 2 cm from the origin. The purified MXE product was hydrolysed to a substrate similar to XXXGol when digested with both XEG and lichenase. No products were detected migrating with the marker, GGXXXGol, indicating that all the purified MXE product had formed a bond between MLG and the non-radiolabelled (non-reducing) half of the DP 14 substrate.

3.2.3 MXE reaction kinetics

The mechanism of the XET reaction has been debated since the discovery of the enzyme. Takeda et al. (1996) reported that the enzyme has a sequential mechanism with multiple binding sites for the donor and acceptor substrates. Other reports later showed that purified XTH enzymes react with a ping-pong mechanism (Rose et al., 2002; Saura-Valls et al., 2006; Hrmova et al., 2009), requiring the donor substrate to bind and be cleaved before the acceptor substrate can interact with the active site. The kinetics of MXE were tested by independently changing the donor and acceptor substrate

concentrations (Figure 3.2.3). Analysis of the reaction kinetics could determine the reaction mechanism.

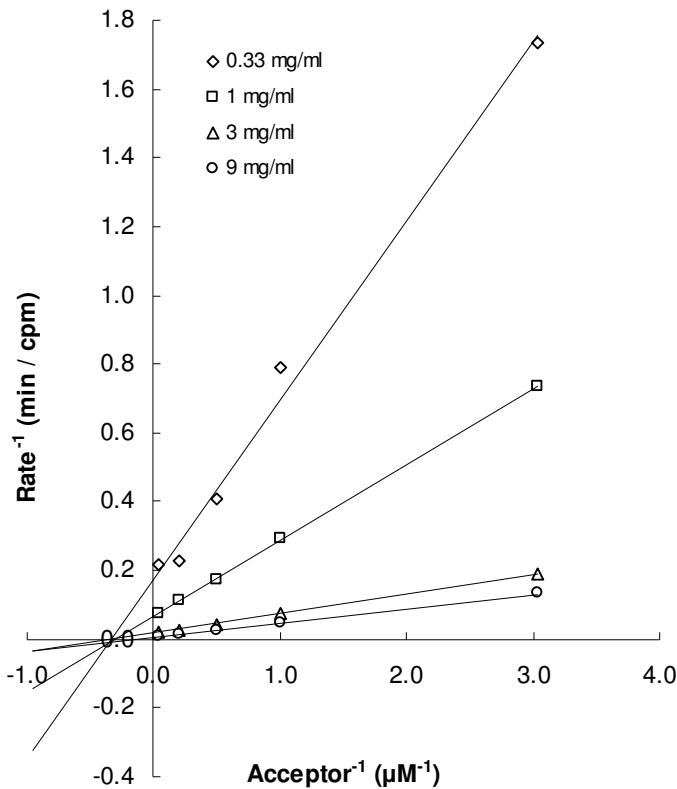
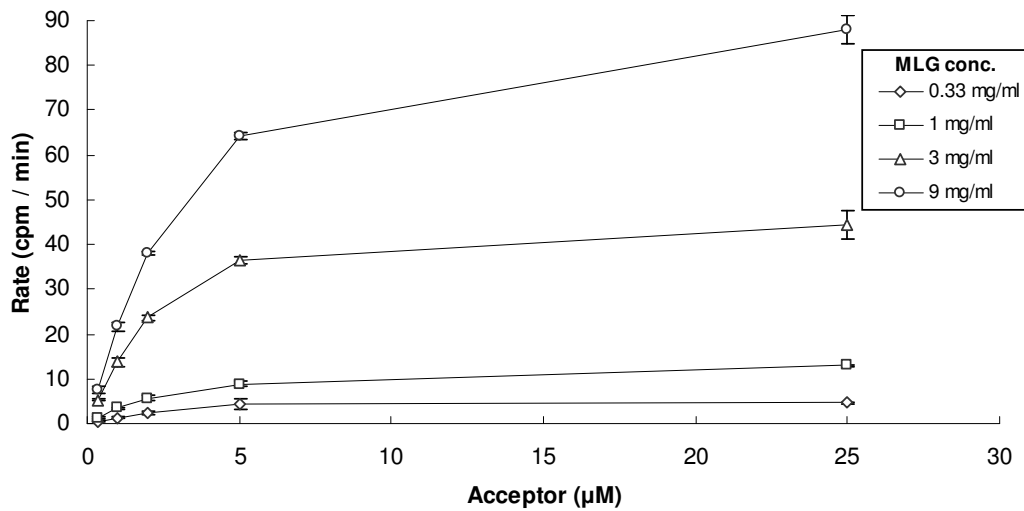


Figure 3.2.3 MXE reaction kinetics
Crude *Equisetum* extract was incubated in succinate buffer (0.1 M, pH 5.5), MLG (varied concentrations), and [³H]XXXGol (1 kBq, plus non-radioactive XXXGol) for 0–3 h. Each data point represents the average rate over 4 time points. Above, ± SE of the curve reported.

The pattern of best fit lines through data points with the same donor substrate concentration on a double reciprocal (Lineweaver-Burk) plot indicate the mechanism of reaction. Data produced by an enzyme with the

ping-pong mechanism form parallel lines, but with the sequential mechanism form lines that converge above the x -axis and to the left of the origin. In the presence of inhibitors, data produced with enzymes of either mechanism can diverge from these two standard models (Fromm, 1979).

The mechanism of MXE cannot be determined in this experiment. As best fit lines of the double reciprocal plot converge below the x -axis, an inhibitor must be present in the crude reaction mixture. The inhibitor could have been glucosidases or other hydrolases, which would have degraded products or reactants. Proteases could have denatured MXE itself. Perhaps small molecules, such as cello-oligosaccharides or others, could have inhibited the reaction. MXE needed purification to determine its mechanism by this method.

3.2.4 Inhibitors of MXE

In an attempt to inhibit β -glucosidase or other enzymes which may have degraded potential acceptor substrates such as MLGOs or CellOs, cellobiose or gluconolactone were first incorporated into a transglucosylation reaction mixture to determine whether these small molecules could inhibit MXE or XET.

3.2.4.1 Gluconolactone

Solid gluconolactone was dissolved in an MXE reaction mixture with crude enzyme extract and incubated for 0–3 h (Figure 3.2.4). Gluconolactone might inhibit β -glucosidase, but it also inhibits MXE activity.

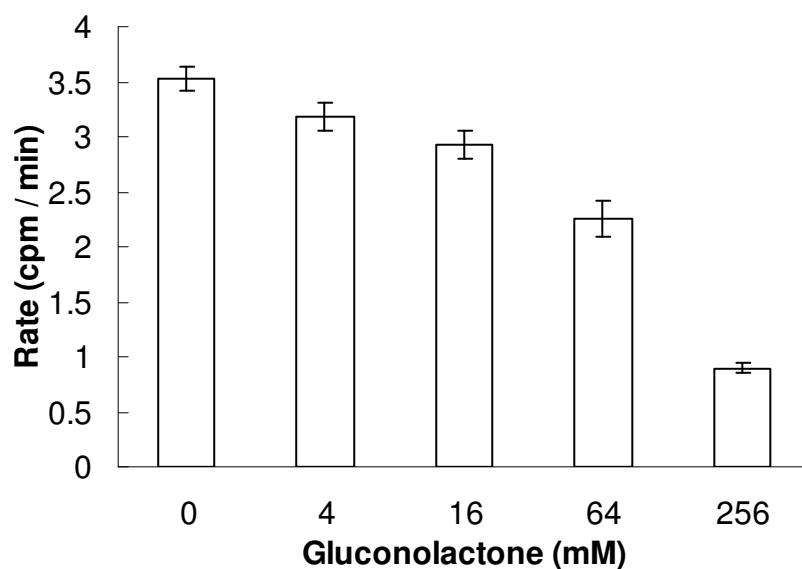


Figure 3.2.4 Inhibition of MXE by gluconolactone

Dried gluconolactone was redissolved in crude enzyme extract (0.1 M succinate, pH 5.5), MLG (0.17%), and XXXGol (1 kBq) and assayed for 0–180 min (n=7 time points). \pm SE of the curve shown.

3.2.4.2 Cellobiose

Solid cellobiose was dissolved in crude enzyme extract, then assayed for MXE and XET activity (Figure 3.2.5).

Cellobiose was previously shown to be only a slight inhibitor of XET up to 100 mM (Fry, 1997). However, in this experiment cellobiose was an effective inhibitor of both MXE and XET activity. At 64 mM almost all of the MXE activity was inhibited, but about 25% of the XET activity remained.

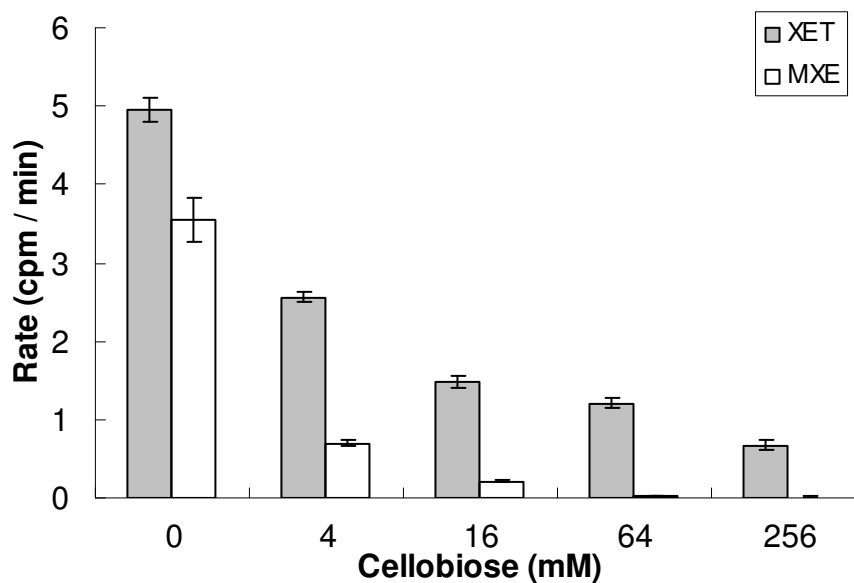


Figure 3.2.5 Inhibition of XET and MXE by cellobiose
 Activity assayed as in Figure 3.2.1. \pm SE of the curve reported.

One enzyme might have been responsible for both MXE and XET activity. Another enzyme (or more than one) might have had only XET activity. The MXE containing enzyme may have been inhibited by cellobiose, causing a decrease in both MXE and XET activity. However, other enzymes with only XET may have been less affected by cellobiose, retaining some activity even at high concentrations of the disaccharide.

Both cellobiose and gluconolactone inhibited MXE and XET activity, and therefore neither could be used to 'protect' potential acceptor substrates from β -glucosidase degradation.

3.2.5 pH optimum of MXE

MXE activity was firstly tested over a broad range of pH to determine the optimal pH conditions for the assay. Five ions, buffered between 3.7 and 7.7, were tested (data not shown). Neither extreme was optimal, MXE activity was better between 4.7 and 6.7. Each set of assays were not directly comparable because each ion may have had different effects on the activity. Therefore, three buffer ions were tested across the same range of pH, and both MXE and XET were assayed (Figure 3.2.6).

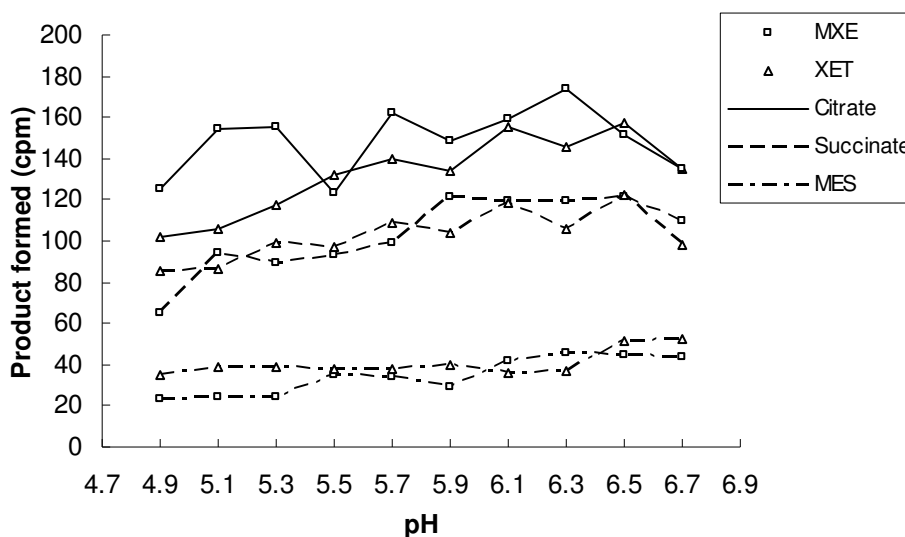


Figure 3.2.6 pH optimum of MXE and XET activity from crude extracts MXE and XET were assayed twice per pH (average shown) in the presence of buffer (295 mM), MLG or XyG (0.1%), and XXXGol (1 kBq) for 30 min.

The highest amount of product was formed at pH 6.3 in citrate buffer.

Citrate enhances both MXE and XET activity across the range of pH tested.

Citrate is a trivalent ion, succinate is a divalent ion, and MES is monovalent.

The perceived enhancement by citrate could be due to the higher amount of

Na⁺ in the buffer. Whether or not the higher rate of activity was attributable to the concentration of Na⁺, the optimal condition for MXE was to be assayed in citrate buffer.

3.2.6 pI of MXE

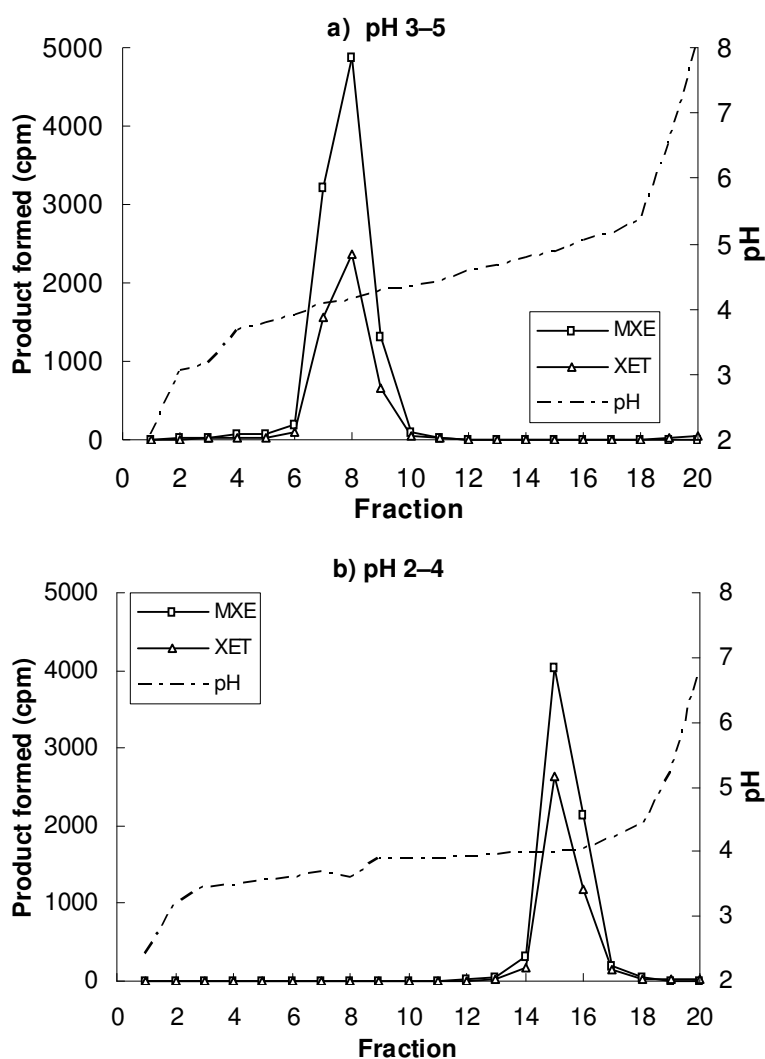


Figure 3.2.7 pI of MXE focused in two ampholyte ranges

Dialysed (in formate, 10 mM, pH 3.7) ASP was Rotofor separated in ampholytes with (a) pH 3–5 or (b) pH 2–4. Triton X-100 (0.05%) was included to improve the recovery of protein. Activity was assayed by incubating each fraction (20 μ l) with citrate buffer (188 mM, pH 6.3), MLG or XyG (0.12%), and XXXGol (1 kBq) for 3 h.

The isoelectric point of MXE was measured using the Rotofor Cell IEF system. ASP was separated twice in Servalyt ampholytes pH 3–5 or pH 2–4 (Serva, Figure 3.2.7).

When separated between pH 3 and 5 (Figure 3.2.5 a), MXE activity was found mostly in two fractions with pH 4.07–4.15. In the second separation (Figure 3.2.5 b) between pH 2 and 4, the activity moved to two fractions with pH 3.98–4.04. Thus, the pI of MXE was 4.0–4.1. Only one peak of activity is present in both separations. Therefore, only one isoform of MXE may be present in mature shoots.

3.2.7 Presence of MXE in lycopodiophytes

Following the discovery of MXE in *Equisetum*, two other species of early-diverging plants were examined for the presence of MXE activity. Proteins were extracted from four plant species, *Equisetum fluviatile*, *Holcus lanatus* (a common grass), *Lycopodium clavatum* (stag's-horn clubmoss), and *Diaphasiastrum alpinum* (alpine clubmoss) and tested for MXE and XET activity (Table 3.2.5). As potential polysaccharide donor substrates could have been extracted as well, a control with no added polysaccharide was included.

Previous data have shown that MXE was highly active in *Equisetum*, but negligible MXE activity can be extracted from grasses. These observations were confirmed here. Two other land plants, tested for the first

Table 3.2.5 MXE activity in lycophodiophytes

| Genus | Rate (cpm / min) with donor substrate of: | | |
|-----------------------|---|-----------------|------------------|
| | XyG | MLG | None added |
| <i>Equisetum</i> | 4.45 ± 0.1193 | 3.53 ± 0.2549 | 0.0357 ± 0.00665 |
| <i>Holcus</i> | 7.03 ± 0.3199 | 0.0655 ± 0.0124 | 0.0536 ± 0.0095 |
| <i>Lycopodium</i> | 0.118 ± 0.0101 | 0.0607 ± 0.0064 | 0.0095 ± 0.0045 |
| <i>Diaphasiastrum</i> | 0.206 ± 0.0132 | 0.0238 ± 0.006 | 0.0100 ± 0.0056 |

Proteins were extracted and assayed in succinate buffer (pH 5.5) for XET activity (with XyG), MXE activity (with MLG), or for 'background' (with no added polysaccharide). Activity was assayed at seven time points between 0 and 180 min. ± SE of the curve reported.

time, also have MXE activity. The ratio of XET/MXE activity in *Lycopodium* was 0.51, and in *Diaphasiastrum* the ratio was 0.12. Even though MXE activity in *Diaphasiastrum* was low, it was statistically above background (*t*-test *P* value < 0.02).

3.3 Action of MXE in *Equisetum* tissues

While XTH has been implicated in cell expansion, MXE may or may not play a similar physiological function in horsetail cell walls. Extractable MXE activity was greater in mature shoots than in young shoots, implying a role in mature cell walls, possibly to strengthen or repair the wall (Fry et al., 2008b). The physiological role of MXE was further investigated by a variety of techniques.

3.3.1 *Equisetum* tissue culture and plant growth

Equisetum can be found commonly in nature: along roads or train-tracks, and in gardens, forests, and ponds. Mature tissue can be collected in abundance. However, developing stems, or young, rapidly dividing and expanding tissue was more difficult to acquire. *E. fluviatile* tissue cultures were initiated to be able to compare tissues of different age. The cultures grew as a soft, spongy cluster of undifferentiated cells for at least three years. Cells were subcultured monthly, in which time they roughly tripled in size. Old sections of tissue became brown and ceased growth. Fresh, rapidly expanding shoots were grown from outdoor-grown rhizomes in a glasshouse.

3.3.1.1 XyG and MLG in culture cells

The presence and nature of the hemicelluloses XyG and MLG in *Equisetum* cell cultures was investigated. AIR was digested with the enzymes XEG or lichenase, and compared with AIR of mature shoot (Figure 3.3.1).

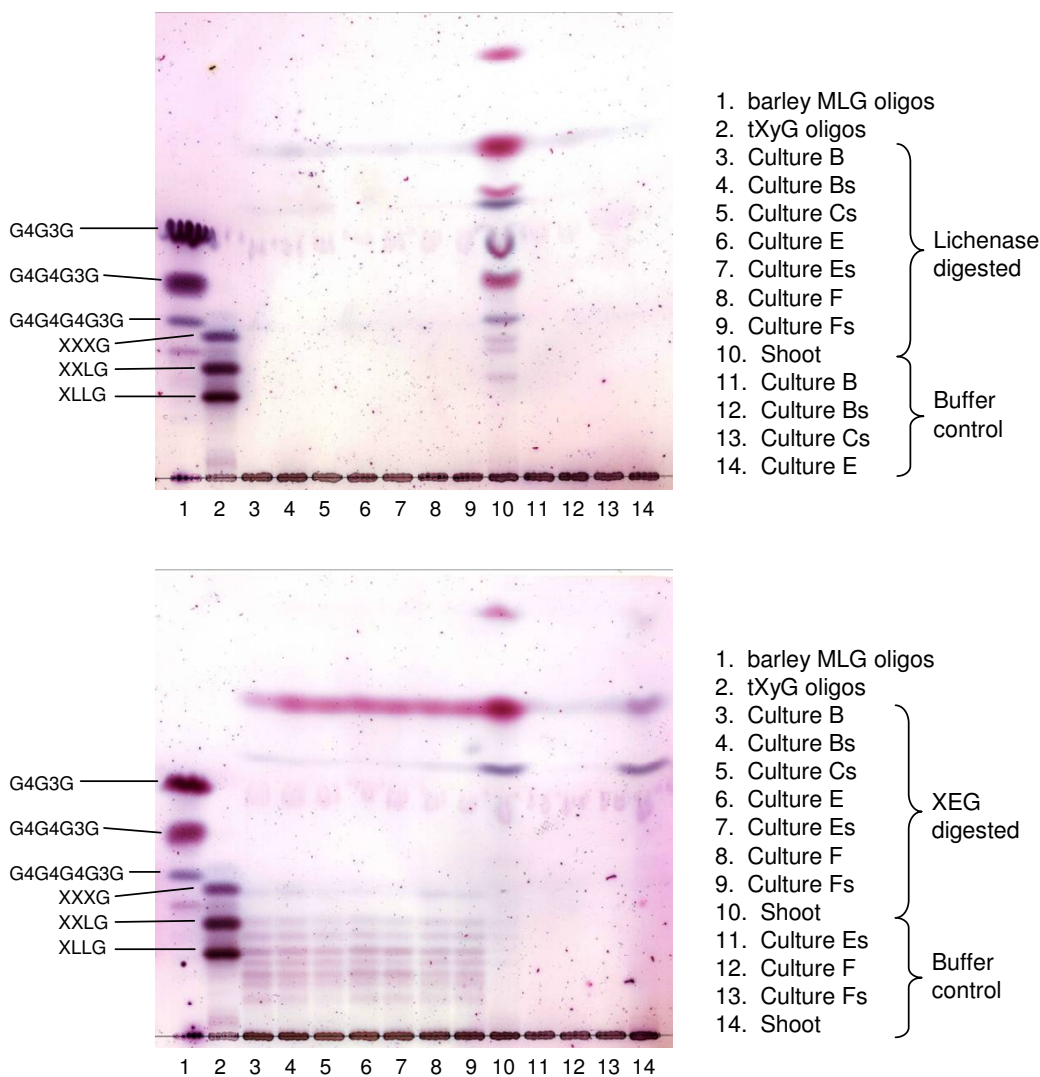


Figure 3.3.1 TLC of digested culture XyG and MLG

Pestle and mortar-ground AIR (20 mg) of culture cells or stem was incubated with XEG (0.004%), lichenase (0.008 U), or buffer only (PyAW 1:1:98 + 0.5% CB) overnight. A portion (40%) of the supernatant was concentrated and developed on TLC with BAW twice, then stained with thymol. Cultures grown on supplemented silica are indicated with an 's' following their label.

No MLG was detected in cultured cells by this method. As a positive control, lichenase-digested AIR from mature shoots formed a pattern of MLGO typical of *Equisetum* MLG.

XyG, however, was found in abundance in cultured cell AIR. Compared with the XEG-digested shoot AIR, a higher proportion of the AIR was converted to XGOs. At least 8 individual XGOs can be detected by this method with a backbone of four glucoses. While fewer XGOs can be detected in digests of shoot AIR, others could be present at lower concentrations, and may not be structurally different from the XGOs of culture cells. Peña et al. (2008) detected 10 distinct XyG subunits from *E. hyemale*, and described in detail the unique structures of *Equisetum* XyG. No differences were detected in cultures grown in the presence of supplemented silica in this work.

3.3.1.2 Transglucosylation activity of culture cells

Crude extracts of culture cells were tested for the presence of MXE and XET activity (Figure 3.3.2). As some polysaccharide donor substrate can also be extracted by this method, the extracts were tested without supplemented donor substrate as a 'background' control.

All cultures contained XET activity, but no culture exhibited a high amount of MXE activity above background. Culture 'Bs' was the only extract

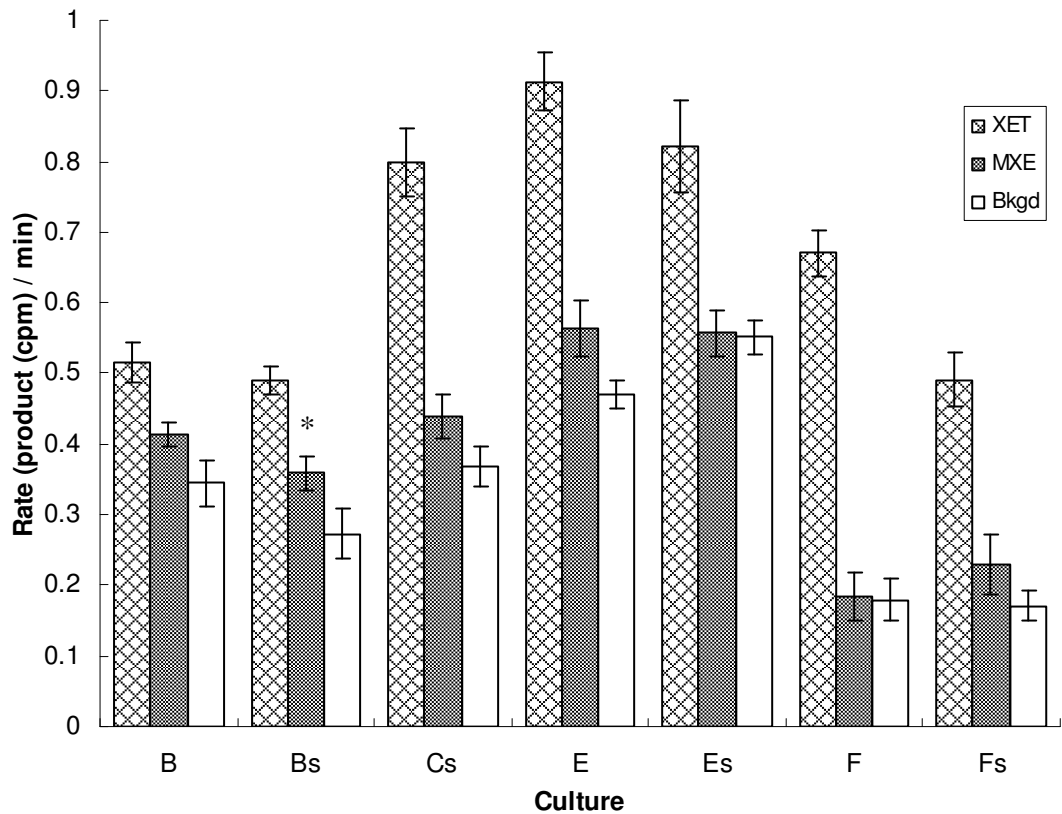


Figure 3.3.2 XET and MXE of culture cells

Proteins were extracted and assayed in citrate buffer (pH 6.3) for XET activity (with XyG, 0.32%), MXE activity (with MLG, 0.32%), or for 'background' (with no added polysaccharide). The reaction was assayed between 0 and 120 min, \pm SE of the curve reported, n=7. *MXE rate was statistically above background at 95% confidence interval.

with MXE activity above background when compared by the *t*-test at a 95% confidence interval. This evidence supports the hypothesis that XTH is active during cell expansion, but MXE could be used in strengthening cell walls.

3.3.1.3 *E. fluviatile* glasshouse growth

Rapidly growing *Equisetum* tissue was readily accessible in the natural environment in spring, but not in other seasons. Therefore, rhizomes were cut and stored in simulated winter conditions (low light, 4–6°C) from which to attempt to grow new shoots at any time. Multiple experiments, designed empirically, were executed until a suitable method was developed (Table 3.3.1).

Table 3.3.1 Attempt to grow *E. fluviatile* in controlled conditions

| Experiment description | Results |
|--|---|
| Shoots and rhizomes, including new buds, were cut and placed in trays with pond water or autoclaved pond water. | Maintenance of shoots, but no new growth. |
| Rhizomes cut and stored at 6°C in pond water, then surface sterilized with 75% EtOH. Rhizomes were cut to contain 1-2 nodes and fit in small jars containing sterile nutrients, some jars contained 0.8% agar. | No growth before fungal contamination. |
| Rhizomes cut mid-Jan, stored at 6°C, surrounded by damp paper. End of March, ~10 cm sections were placed in peat-free soil, under small stones, or nothing, with pond or tap water. Placed in a glasshouse under natural light and fixed 19-21 °C. | Rhizomes in the pond water all grew new shoots and rhizomes sooner than in tap water. Soil was the best medium for new growth. However, the natural cycle of dormancy and growth was unchanged from natural conditions. |
| Rhizomes cut mid-winter, stored at 6°C, under sand. Mid-summer, ~30 cm sections were placed in a tray of peat-free soil in pond water. Placed in a glasshouse under natural light and fixed 19-21 °C. | Rhizomes grew new shoots and rhizomes within 1 week. The new shoots were monitored to ensure continued growth. These shoots were used in Section 3.3.3.2. |

3.3.2 *Ex vivo*: Extraction of transglucosylation products formed from endogenous enzymes and donor substrates

3.3.2.1 Characterization of MXE product

Purified MLG-XXXGol (MXE product) was biochemically analysed using lichenase and XEG (Figure 3.3.3). The type of digestion products recovered may reveal the nature of the type of bond formed between the polymer and the reduced oligosaccharide. XET product was also purified and analysed as a positive control. The expected result of digesting XET product with XEG was the formation of the starting material, [³H]XXXGol.

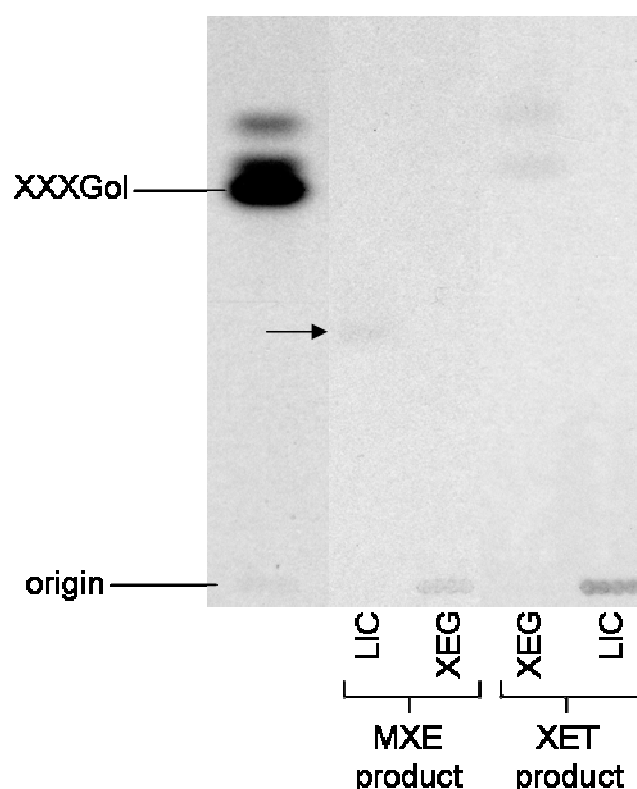


Figure 3.3.3 Digested MXE marker

MXE and XET products were made by incubating crude *Equisetum* or *Holcus lanatus* extracts with MLG or XyG (0.2%), respectively, and [³H]XXXGol (0.1 MBq). The products were precipitated with ammonium formate (0.2 g) in ethanol (75%) and washed free of oligosaccharide. The product was redissolved in water, and portions were digested with lichenase or XEG. The digestion products were analysed by TLC, developed in BAW thrice, and fluorographed. Lichenase digestion (LIC) of MXE product results in a larger oligosaccharide, indicated by the arrow, than the acceptor substrate, XXXGol, alone.

Lichenase digested MXE product to an oligosaccharide which was larger than the acceptor substrate alone, XXXGol. Further studies (T. Simmons, personal communication) revealed that two extra glucoses were attached to the backbone of XXXGol; most likely GGXXXGol was the final product of lichenase digestion. XEG did not digest MXE product; the polysaccharide product remained at the origin of the chromatogram. As expected, XET product was completely digested by XEG to an oligosaccharide closely resembling the starting material. Also, lichenase did not digest XET product.

3.3.2.2 *Ex vivo* transglucosylation products of barley and *Equisetum*

Endotransglucosylase activity can be shown in extracts *in vitro*, but enzymes may not necessarily act *in vivo* because of inhibition or physical separation between the enzyme and its substrates. Some XTHs can use MLG as a donor substrate, but this heterotransglucosylation action was not shown *in planta* (Hrmova et al., 2007; Stratilova et al., 2010). A method was developed to assay apoplastic transglucosylation, and to detect potential heterotransglucosylation products in barley.

Barley (*Hordeum vulgare*) stems and leaf blades, and *Equisetum fluviatile* stems were cut <1 mm thick and incubated in water with [³H]XXXGol. Overnight, the supplied substrate permeated the apoplast of still-living

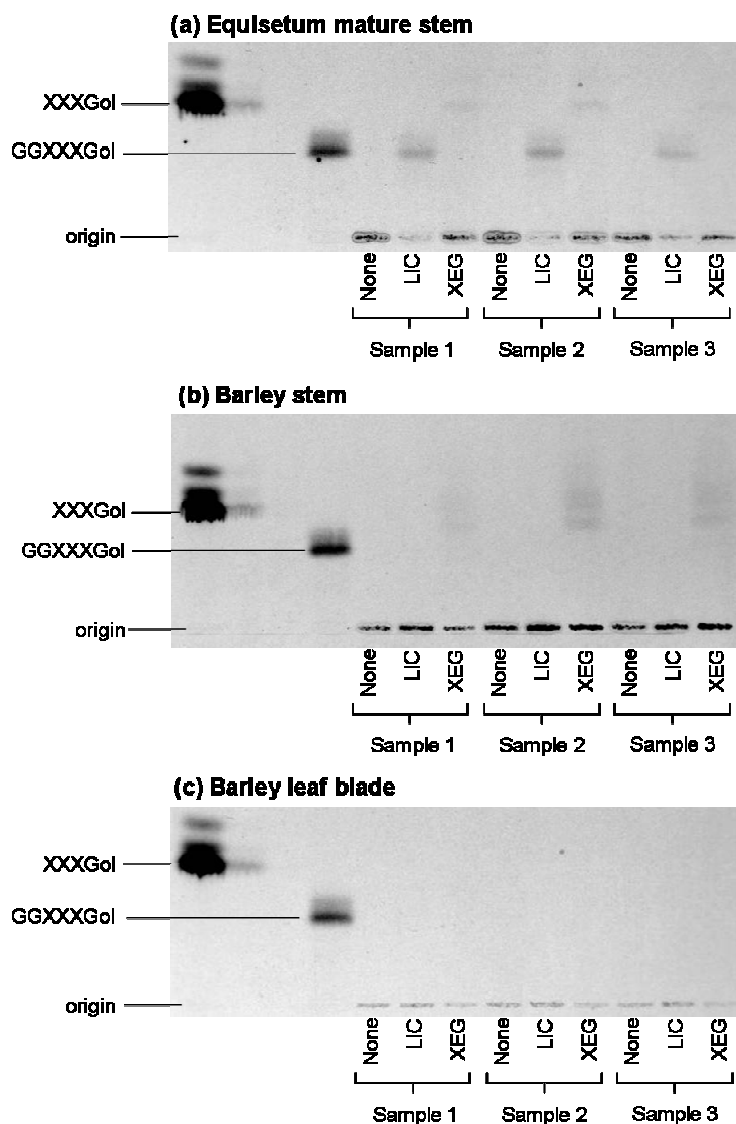


Figure 3.3.4 TLC fluorogram of ex vivo MXE and XET products from *Equisetum* and barley

(Figure 3.3.4 caption) Barley (*Hordeum vulgare*) was grown in shelter under natural light for 12 weeks. Cross-sections of *Equisetum fluviatile* stems (a) (grown outdoor in natural conditions), barley stems with sheath (b), and leaf blades (c) were cut by hand with a razor blade. Three replicates of slices (50 mg) were immediately mixed with 0.1 MBq/ml [^3H]XXXGol in 250 μl of water and incubated overnight at room temperature. AIR of the tissues was prepared and homogenized. The residue was incubated in NaOH (6 M, 37°C, 16 h) to extract hemicelluloses. The remaining solids were washed twice in water (2 ml) and pooled with the NaOH supernatant, followed by neutralization and dialysis to remove unreacted [^3H]XXXGol and salts. The dried dialysate was weighed and redissolved to 0.5% in water. A portion (0.2 mg) was incubated with buffer only, lichenase (LIC), or XEG. The products of digestion were concentrated and analysed by TLC (developed in BAW twice) and fluorography.

tissues. AIR was prepared, and ^3H -labelled hemicelluloses were extracted, but MXE and XET products were as yet indistinguishable from each other. The extract was digested with XEG, lichenase, or with nothing (Figure 3.3.4). As shown in Figure 3.3.3, XEG digested XET product only to XXXGol. Lichenase digested MXE product to a larger product, which migrated slower than XXXGol. The lichenase digestion product co-migrated with the *in vitro*-formed and digested product, GGXXXGol (Figure 3.3.3).

In *Equisetum* stems (Figure 3.3.4 a), the amount of MXE product (mobile after lichenase digestion but with lower R_f than XXXGol) exceeded the amount of XET product (mobile after XEG digestion and co-migrating with XXXGol) in all three replicate samples. The amount of MXE product plus the lichenase-indigestible product which remained at the origin was roughly equal (by visual inspection) to the buffer-only control in all three replicates.

Barley stems incorporated much of the supplied [^3H]XXXGol, evident by the dark band at the origin of all samples (Figure 3.3.4 b). No radioactive polysaccharide was digested by lichenase, indicating that MLG was not a significant donor substrate for transglucosylation. A portion of the *ex vivo* product from barley stems was hydrolysed by XEG. Poalean XyG has fewer Xyl substitutions, having some backbone AcGlc substitutions instead

(Gibeaut et al., 2005). NaOH extraction of hemicelluloses removes Ac groups, which may otherwise confer solubility. The extracted barley XyG may have precipitated and become inaccessible to XEG. Blurred bands which do not have the exact mobility of the marker XXXGol most likely result from overloading of invisible, nonradioactive XGOs. However, the majority of the *ex vivo* product remained at the origin.

Little of the radioactive substrate was incorporated into barley leaf blade hemicellulose (Figure 3.3.4 c). No [³H]XXXGol was detected after digestion with XEG, but less radioactivity was detected at the origin after digestion. This implies that the product was XET product, similar to that in barley stem.

3.3.2.3 Quantification of *Equisetum ex vivo* products

The *ex vivo* procedure was repeated to examine the profile of MXE and XET action in tissue types that represent a range of developmental stages of *Equisetum* stems and leaves. Culture cells were also used as a simulation of meristematic tissue. Results were analysed not by fluorography, but by scintillation counting of segments of each TLC lane (Table 3.3.2).

XET product was extracted from meristematic tissue and callus cultures, but little or no MXE product was detected in those samples. No

Table 3.3.2 Quantitative TLC analysis of MXE and XET from various *Equisetum* tissues

| Tissue | Portion (%) of radioactivity detected co-migrating with the marker indicated ¹ after digestion with the glucanase indicated ² | | | cpm |
|--------------------------|---|---|--|--|
| | XXXGol ¹ XEG ² | GGXXXGol ¹ lichenase ² | origin ¹ none ² | origin ¹ none ² |
| callus culture | 91 ± 3 | 2 ± 2 | 79 ± 8 | 249 ± 129 |
| intercalary meristem | 89 ± 4 | 2 ± 2 | 98 ± 2 | 201 ± 17 |
| immature green leaf | 62 ± 10 | 41 ± 9 | 90 ± 3 | 345 ± 59 |
| mature green stem | 29 ± 7 | 64 ± 3 | 100 ± 5 | 131 ± 16 |
| brown over-wintered stem | 26 ± 5 | 60 ± 6 | 94 ± 4 | 163 ± 55 |
| brown over-wintered leaf | 15 ± 4 | 79 ± 6 | 97 ± 2 | 116 ± 14 |

Hemicellulose was extracted from AIR of XXXGol-fed tissues and digested with XEG, lichenase, or with nothing. The amount of radioactive product which co-migrated with the marker (XXXGol for XET product, GGXXXGol for MXE product, or remained at the origin when undigested) after digestion (or not) is reported as the percent of the total radioactivity in the lane. Tissues are listed in order of youngest to oldest. \pm SD, n=3.

MXE product was detected in the absence of XET product. In increasingly mature tissues, the amount of MXE action became greater than XET action.

The greatest ratio of MXE:XET product was extracted from the oldest tissue, brown over-wintered leaves. As a general trend, the ratio of MXE:XET increased from youngest to oldest tissues.

The method used here detects transglucosylase product when endogenous enzyme is able attach endogenous donor substrate to the supplied acceptor substrate. This method does not, however, elucidate the difference between the absence of enzyme or the absence of available substrate when no product is detected. Not surprisingly, no MXE product is detected in meristematic tissues. Culture cells, rapidly dividing and undifferentiated cells most similar to meristem tissue, have been shown in

Figure 3.3.1 to lack both MLG and extractable MXE activity. The occurrence of MXE product in more developed tissues must be the result of both the synthesis of MLG and of a protein with MXE activity. Older, over-wintered tissues, with an increased MXE:XET product ratio, could have either a higher MLG:XyG ratio or a higher MXE:XET activity, or both. It is also possible that these older tissues simply have less XyG or XTH, resulting in the lower ratio.

3.3.3 Localization of transglucosylation activity

3.3.3.1 Transglucosylation localization using sulforhodamine-labelled XXXGoI (XXXG-SR)

Radioactive labelling of transglucosylation products can quantify MXE and XET action, but fluorescent labelling of the products can more closely pinpoint the sub-cellular location of these actions.

Thin sections of *E. fluviatile* stems were cut and incubated in XXXG-SR overnight, followed by washing in Ethanol. Resin-embedded tissues were sliced and photographed using a fluorescent microscope (Figure 3.3.5). Replicate sections were stained with toluidine blue to assist in tissue identification.

Transglucosylation product, evident by pink fluorescence, was found in the cell walls of all tissue types in the intercalary meristem (Figure 3.3.5 b). Slightly less fluorescence can be seen in the vascular bundles. XET activity in

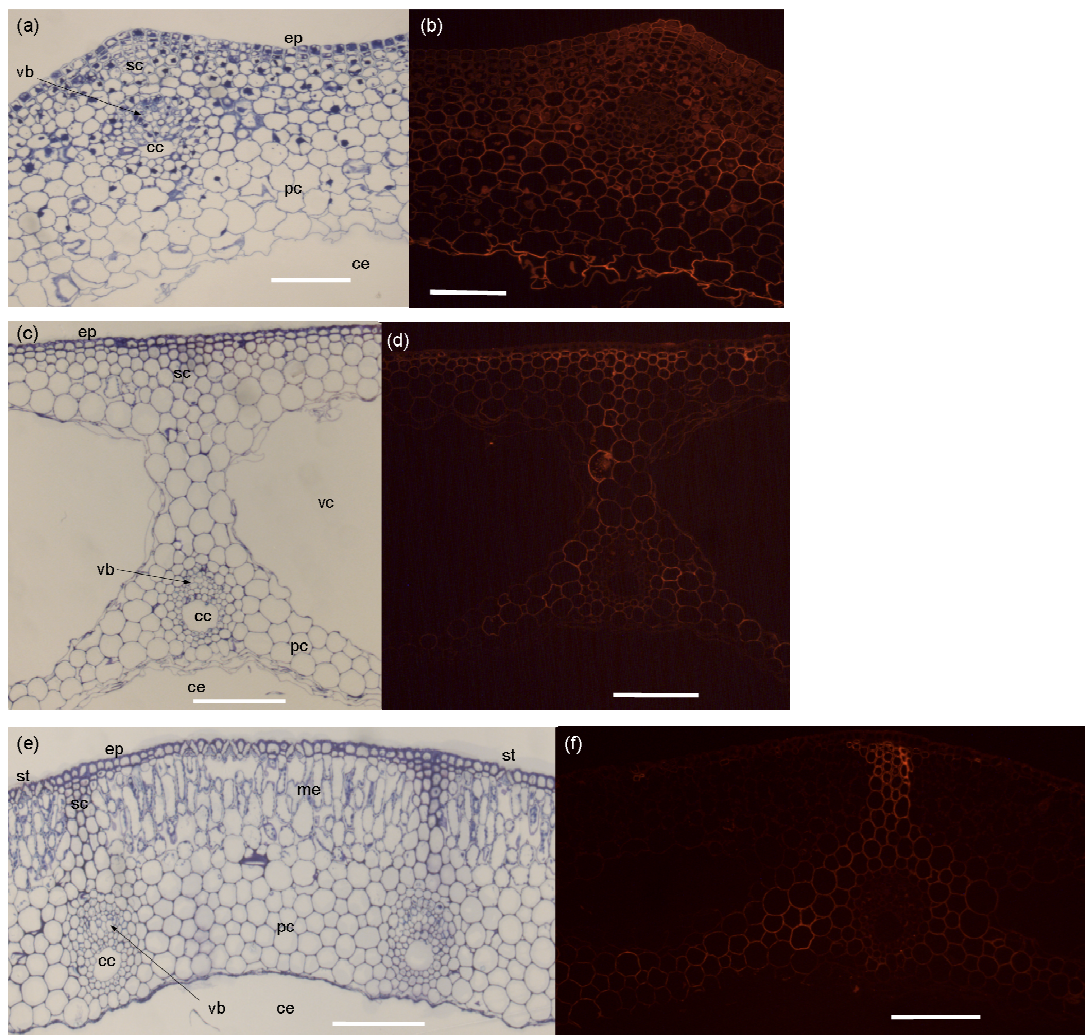


Figure 3.3.5 Localization of transglucosylation products in *Equisetum*

Mature green stems and intercalary meristems were harvested from glasshouse-grown shoots (described in Table 3.3.2), and over-wintered stem tissues were collected from outdoor-grown plants. *E. fluviatile* expanding intercalary meristems (a, b), over-wintered brown stems (c, d), and mature green stems (e, f) were cut by hand and incubated in XXXG-SR (8 μ M) overnight (b, d, f), followed by washing in Ethanol (75%). Replicate sections were incubated in water and stained with toluidine blue (a, c, e). Tissues sections were embedded in resin, sliced with a microtome, and photographed using a Lieca fluorescent microscope. Scale bars equal 100 μ m in a, b; 200 μ m in c, d, e, f. Abbreviations: vb, vascular bundle; ep, epidermis; sc, sclerenchyma; cc, carinal canal; pc, parenchyma; ce, central canal; vc, vallecular canal; st, stoma; me, mesophyll.

primary cell walls is thought to assist in cell wall loosening, allowing for cell expansion. Meristematic tissue is rich in XET activity but lacks MXE activity (Table 3.3.2). Therefore, the fluorescent product seen in the photograph of meristematic tissue is likely to be solely the result of XET action.

Autumn-initiated stems preserve their strength and rigidity through difficult weather conditions, such as freezing temperatures and high winds or underwater turbulence. The cell walls of these old, over-wintered stems are therefore responsible for maintaining their integrity for many months. MXE and XET activity were both present in such tissues, but the majority of the extractable transglucosylase product was shown in Table 3.3.2 to be MXE product, at a ratio with XET product approx. 2:1. Most tissue types shown in Figure 3.3.5 d exhibited some transglucosylase activity, but activity was especially robust in sclerenchyma and parts of the parenchyma. While this procedure cannot distinguish between MXE and XET action, the majority was known to be MXE. MXE could play a role in strengthening *Equisetum* cell walls, since it is absent from growing cell walls and present in tissues responsible for maintaining the structural integrity of the whole plant.

The MXE and XET action in mature green stems is similar to that in over-wintered stems, in both ratio (Table 3.3.2) and tissue distribution (Figure 3.3.5 f). The highest concentration of transglucosylation product can be seen in the sclerenchyma, but is also seen in distinct parts of the

parenchyma. Transglucosylation by MXE and XET is not distributed across all tissue types in mature stems, but focussed to the areas which form the structural elements of the plant.

The epidermal cell layer, rich in silica deposits, draws attention for its distinct lack of transglucosylation activity (Figure 3.3.5 d, f).

Immunofluorescence labelling studies in *E. arvense* have shown that the epidermis is rich in MLG (Sørensen et al., 2008). However, despite the abundance of MLG in *Equisetum* epidermal tissues, MXE action is restricted. Besides the epidermis, silica is also heavily deposited in the sclerenchyma, concurrent with transglucosylation action — likely to be MXE. While it has been proposed that MLG may play a role in silica deposition (Fry et al., 2008 b), there seems to be no correlation between the location of silica deposits and MXE action.

3.3.3.2 Differentiation between MXE and XET in tissue sections

The *in situ* transglucosylation experiment of Section 3.3.3.1 did not distinguish between MXE and XET products. Determining whether MXE and XTH were expressed in separate or overlapping tissues could elucidate a functional difference between the two enzymes.

E. fluviatile stems were sectioned and incubated in a solution of XXXG-SR, then washed free of unused reactant. Some sections were then incubated

overnight in either a solution of XEG (thereby removing XET products), or a solution of lichenase (removing MXE products). The enzymes were denatured and oligosaccharides were washed out. Some of the sections were embedded in resin, sliced, and analysed with a fluorescent microscope (images not shown).

The sections should have been free of MLG if incubated in lichenase, or free of XyG if incubated in XEG. However, the cell wall matrix could have restricted large enzymes from accessing all of the polysaccharides. To determine whether the endogenous polysaccharides were indeed digested, hemicelluloses were NaOH-extracted from replicate sections. The extracted hemicellulose was analysed for MLG and XyG by digestion with lichenase and XEG (Table 3.3.3).

The digestion of XyG from sections of tissue was not complete, evident by analysis of the extracted hemicellulose after the initial digestion. XEG does not usually extract all XyG from plant tissue (Pauly et al., 1999). Therefore, the fluorescent sections digested by XEG were not completely rid of XET product. The experimental aim of distinguishing between MXE and XET products was not achieved. Also, the relative intensities of transglucosylation products was highly variable between sections of the same type of tissue. Further experimentation must address this variability, and improve the digestion of endogenous XyG.

Table 3.3.3 Hemicellulose monitoring of digested sections

| Section incubated in: | Hemicellulose extracted then digested in: | Expected TLC results: | Actual TLC results: |
|-----------------------|---|-----------------------|---------------------|
| lichenase | lichenase | No bands | No bands |
| lichenase | XEG | XGOs | XGOs |
| XEG | lichenase | MLGOs | MLGOs |
| XEG | XEG | No bands | XGOs |

E. fluviatile stems were sectioned as thin as possible by hand with a razor blade. The sections were incubated in XXXG-SR (10 μ M), then washed free of unused reactant. Sections were incubated for ~10 h in either XEG (0.01%), or lichenase (10 U/ml), or in buffer only. The enzymes were denatured and oligosaccharides were washed out in excess 75% Ethanol, followed by 80% acetone. Some of the sections incubated in glucanases were ground and incubated in 6 M NaOH to extract remaining hemicelluloses. Extracts were dialysed, digested with either lichenase or XEG, dried, and analysed by TLC.

3.4 Cellulose as a donor substrate for MXE

Included as a negative control in Figure 3.1.1, the paper strip with no added polysaccharide donor substrate was not expected to retain any fluorescent substrate after washing. However, a band of apparent transglucosylase activity was mirrored on all three XET, MXE, and control test papers. As cellulose was the only known polysaccharide present in the controls, the possibility of β -1 \rightarrow 4-glucan to act as a donor substrate in a transglucosylation reaction was investigated.

3.4.1 Test papers impregnated with XXXG–SR

First, the apparent transglucosylation product formation with no (other than paper) donor substrate observation was repeated in a slightly different experiment. Partially purified enzyme, rich in MXE and XET, was applied as a dilution series to three test papers, impregnated with MLG, XyG, or no added polysaccharide and XXXG–SR. The papers were maintained in a humid environment for 1 h, then washed free of XXXG–SR, and photographed under UV light to show fluorescent transglucosylation product (Figure 3.4.1 a). The three papers were then incubated in 6 M NaOH to remove hemicelluloses from the paper and photographed again (Figure 3.4.1 b).

The initial observation that paper alone, with no added donor substrate, can be a substrate for a transglucosylation was indeed replicated here. The three test papers all show transglucosylation product, as seen by the fluorescent spots, even when the enzyme is diluted 16-fold in buffer. Hemicelluloses, including MXE and XET products, would have been washed out in 6 M NaOH. As expected, the MXE and XET test strips have significantly less product, possibly none, remaining on the paper. The control paper retains product after the NaOH wash, although less remains. Cellulose and some mannans do not dissolve in aqueous NaOH (Moreira and Filho, 2008), and were likely candidates for the donor substrate.

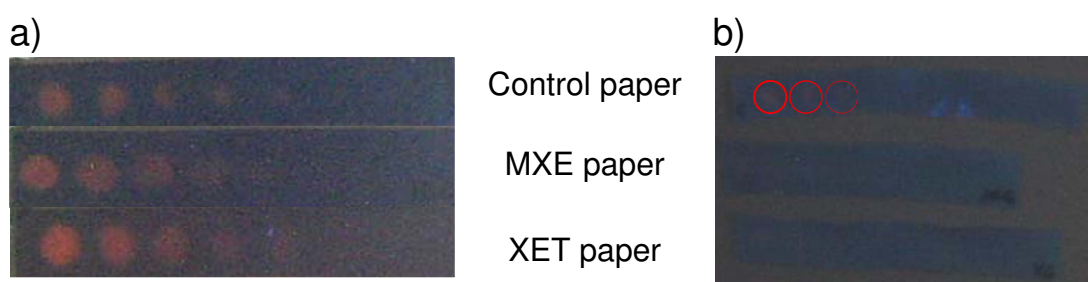


Figure 3.4.1 Dot blot paper

a) Three test paper strips were loaded (3 μ l each, 8 spots) with a 2-fold dilution series of ASP enzyme (Table 3.1.3) in citrate (0.3 M, pH 6.3). The strips were incubated in humid conditions for 1 h, then dried at room temperature. The strips were washed in EFW and photographed. b) The strips were washed in 6 M NaOH at 37°C overnight, rinsed in water, dried, and photographed again. The papers shrank in size during the wash. Red circles show the remaining firmly bound endotransglucosylase product.

Whatman 1CHR chromatography paper was used in this experiment.

It is made from cotton, but the manufacturer did not disclose information about the treatment of the material in the process of making the paper. The

most abundant polysaccharide present which could donate the energy required for a transglycosylation reaction was undoubtedly cellulose, but the presence of other polysaccharides could not be eliminated.

3.4.2 Whatman 1CHR paper analysis

Whatman 1CHR, a component of which was able to be the donor substrate in a transglycosylation reaction, was analysed for saccharide content by TFA and Driselase hydrolysis. While TFA hydrolyses polysaccharides (other than cellulose) to monosaccharides, Driselase is a mixture of hydrolytic enzymes capable of breaking almost all bonds present in plant cell walls, but lacks α -xylosidase. The presence of the disaccharide, α -Xyl-(1 \rightarrow 6)- β -Glc, also called isoprimeverose (IP), after Driselase digestion indicates the presence of XyG. Also, Driselase does not digest β -Xyl-(1 \rightarrow 4)- β -Xyl. Xylobiose is indicative of xylans.

Whatman 1CHR was subjected to sequential extraction, first in aqueous NaOH, followed by aqueous NaOH with borate. Borate may separate mannans from cellulose. The original paper, both extractants (dialysed), and the remaining solids after both incubations were hydrolysed with TFA and Driselase, and analysed by PC (Figure 3.4.2).

Overall, both TFA and Driselase hydrolysis showed that Whatman 1CHR is composed mostly of glucose, most likely from cellulose. TFA

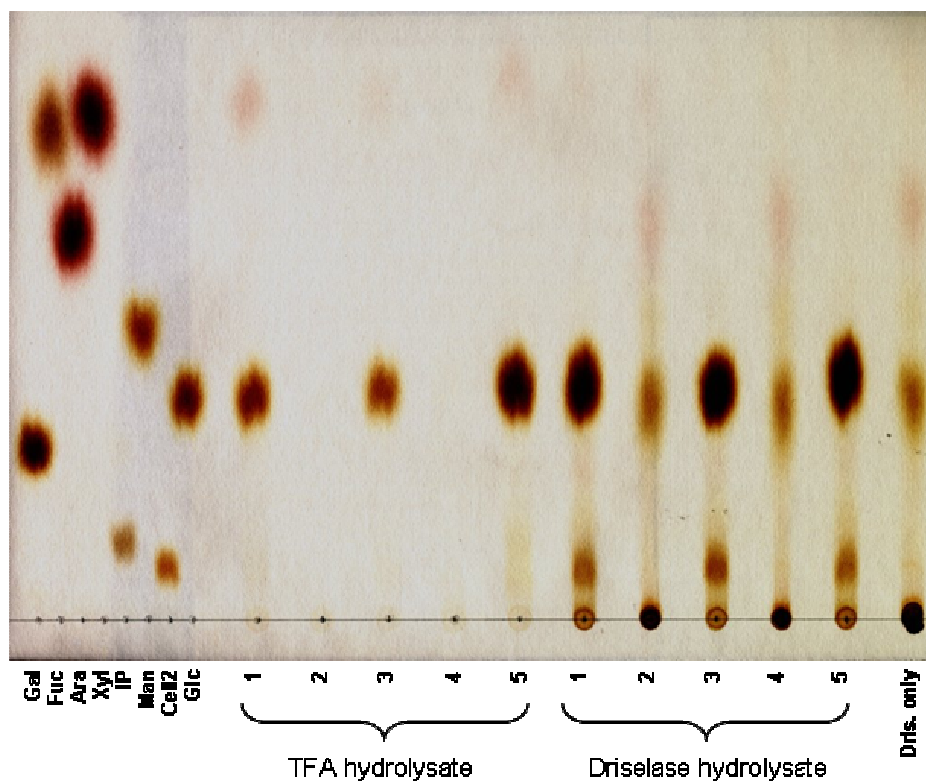


Figure 3.4.2 PC analysis of paper saccharides

Whatman 1CHR was washed in 6 M NaOH at 37°C overnight. The supernatant was removed and dialysed (5 ml). The remaining solids were washed in excess 0.5% CB, and some was kept aside. Most was washed again in 6 M NaOH + 4% boric acid at 37°C overnight. The supernatant was removed and dialysed (5 ml). The remnants were washed in 0.5% CB many times. The original paper (1), the first supernatant (2), the intermediate solids (3), the second supernatant (4), and the final solids (5) were subjected to TFA hydrolysis (2 M, 120°C, 1 h) or Driselase digestion (0.5%, 24 h, stopped by incubation at 100°C for 5 min). The concentrated hydrolysates (from 1 ml of dialysates, 0.2 ml of the TFA-hydrolysed solids, or 0.1 ml of the Driselase-digested solids) were developed on Whatman 3MM paper in EPW and stained with silver nitrate. Uncommon abbreviations: IP, isoprimeverose; Cell2, cellobiose.

hydrolysis also showed traces of xylose. Similarly, Driselase digestion

produced xylose as the most abundant sugar after glucose and cellobiose.

Also, the digestion did not show traces of isoprimeverose, indicating an

absence of XyG. Driselase is a mixture of enzymes, some of them are glycoproteins which autolyse during the incubation, producing Glc and Ara.

Another glucose homopolymer, callose, would give similar results upon TFA and Driselase hydrolysis. These methods would not distinguish between callose and cellulose. However, callose is not a suitable transglucosylase donor substrate, and callose oligosaccharides are not suitable acceptor substrates, as shown in Tables 3.2.1 and 3.2.2.

3.4.3 Potential cellulose : xyloglucan endotransglucosylase (CXE) radioactive assays

To assay the new potential transglucosylase activity with cellulose as the donor substrate, tentatively termed 'CXE', the radioactive acceptor [³H]XXXGol was used.

3.4.3.1 Natural cellulose as donor

To determine whether this potential activity was relevant to the growth of *Equisetum* plants, plant material was used as a potential donor substrate.

First, AIR of callus culture cells and mature plant stems was prepared. The residue was incubated in 6 M NaOH to remove hemicelluloses, some of which would also be donor substrates. AIR and NaOH-washed residue were tested as potential donor substrates (Figure 3.4.3).

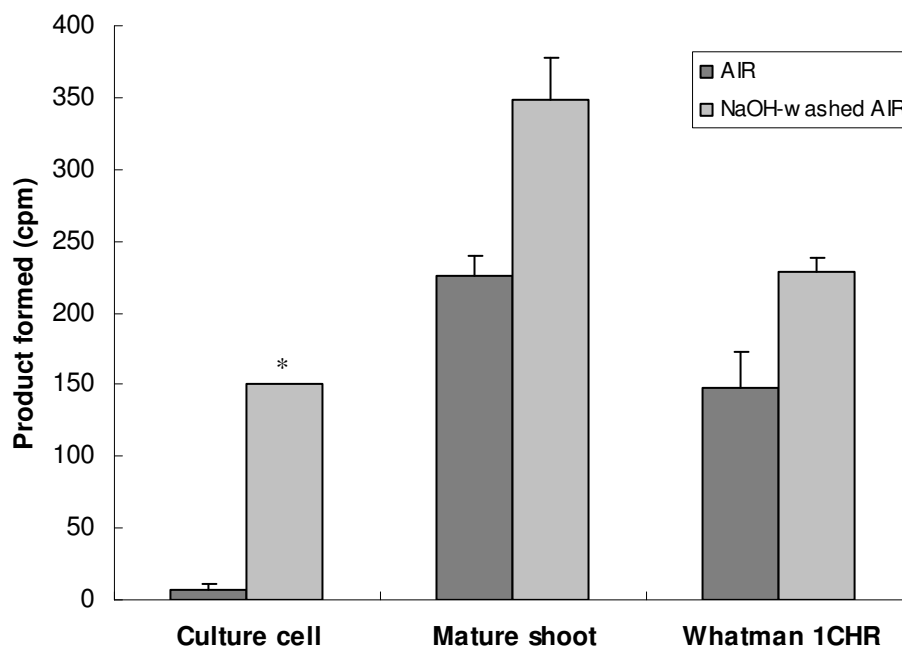


Figure 3.4.3 Natural cellulose as donor for CXE

AIR was prepared from mature *E. fluviatile* shoots and a mixture of cell culture tissue, harvested at the end of the subculturing cycle. A portion of the AIR was incubated in 6 M NaOH at 37°C overnight, then washed in water to remove the salt, lyophilized, and stored. Each substrate (10 mg) was rehydrated overnight, and excess liquid was removed prior to assay. The solid substrate was mixed with [³H]XXXGol (2 kBq), ASP (2 µl, from Table 3.1.3), and citrate buffer (0.3 M, pH 6.3, 97 µl). After 2 h, the reaction was stopped with FA (30 µl), and the solids were washed in water until void of ³H. The solids (in 1 ml water) were transferred to scintillation vials and incubated with scintillant overnight before ³H testing. Each sample tested in triplicate, +SD shown. * n=1.

As was shown previously, Whatman paper was able to be a donor substrate for a transglucosylation reaction. Culture cells are rich in XyG but lack MLG (Figure 3.3.1), and were expected to supply the donor substrate for XET. Mature shoots, rich in both MLG and XyG, contained the substrates for MXE, XET, and CXE. Interestingly, though, all samples washed in NaOH incorporated more acceptor substrate than unwashed paper or AIR. If 6 M NaOH removes all hemicelluloses covering the cellulose microfibrils, and if it

can reduce the crystallinity of the microfibrils, it is possible that cellulose was a better substrate for the dominant transglucosylase than any other substrate.

Mannans are potential hemicelluloses not separated from cellulose by 6 M NaOH. Cellulose enriched samples from Figure 3.4.3 were recovered and subjected to further fractionation with NaOH and borate, which may extract mannans from cellulose (Figure 3.4.4).

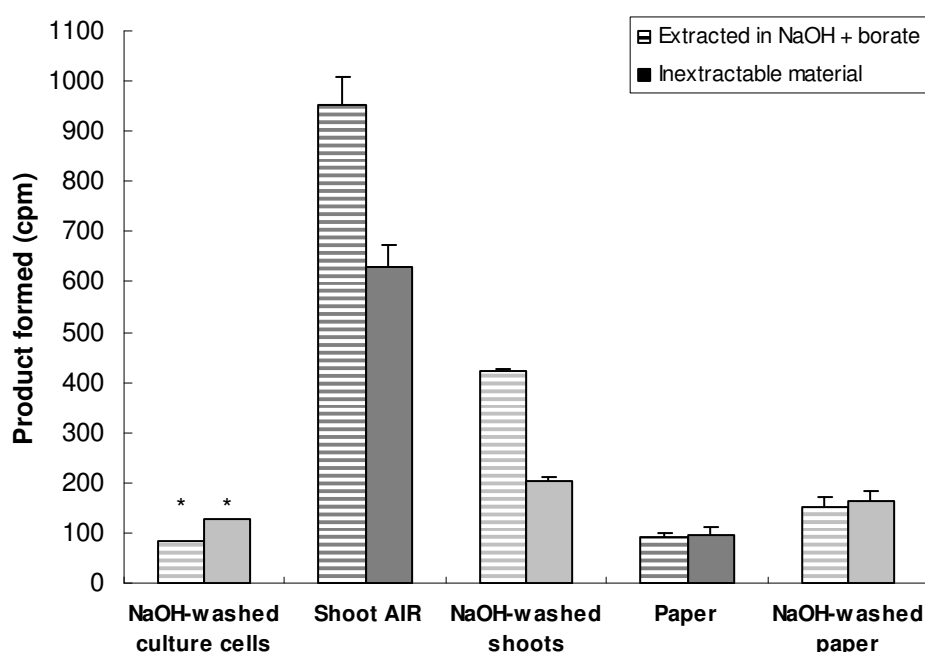


Figure 3.4.4 NaOH + borate extraction of natural substrates for CXE
 Samples from Figure 3.4.3 which contained significant levels of radioactivity were recovered from the scintillant by washing in acetone. The samples were incubated in 6 M NaOH + 4% H₃BO₃ at room temperature overnight. The supernatant was separated from the solids, and both were neutralized with HOAc, then assayed for radioactivity. Each sample tested in triplicate, +SD shown. * n=1.

The sum of extracted and inextricable cpm after incubation in NaOH + borate exceeded the initial measurement in every sample. For example, the first assay of radioactivity in the NaOH-washed culture cells was 150 cpm,

but after incubation, the supernatant measured 84 cpm, and the remaining solids measured 128 cpm, for a total of 212 cpm. Tritium in solution can be assayed with higher efficiency than as a solid. The scintillant comes in closer contact with molecules in solution, and scintillations are not 'masked' by solid matter. Therefore, this observation was not surprising.

The portion of radioactivity that remains with the paper sample after incubation in NaOH + borate may indeed be covalently attached to cellulose. However, if cellulose was the donor substrate for the transglucosylation reaction, no radioactive product should have been soluble in NaOH + borate. The nature of the radioactivity which became soluble after incubation was investigated further.

3.4.3.2 Analysis of the extractable material from CXE product

XET is not specific in choosing the reducing or non-reducing end of a XyG polymer for its donor substrate (Rose et al., 2002). If cellulose was in fact the transglucosylation donor substrate, it is possible that the acceptor substrate had become attached to either a long or a short cellulose fragment. Short fragments of cellulose attached to XXXGol may have been soluble in NaOH + borate. The CXE product soluble in this solution was analysed by GPC.

Surprisingly, the extracted ^3H product eluted not with the void volume (indicating a polysaccharide) or the partially included volume

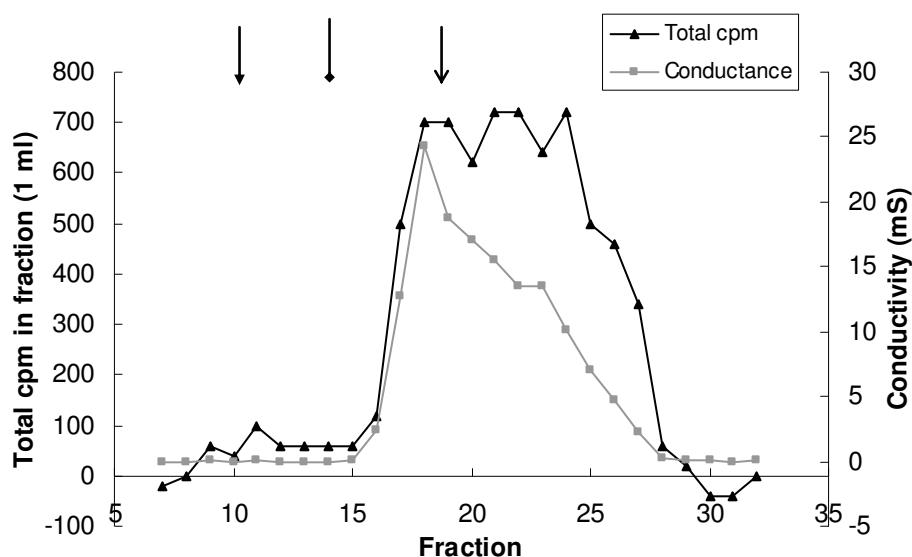


Figure 3.4.4 GPC of material solubilized from CXE product

A disc of Whatman 1CHR was incubated in 6 M NaOH overnight at 37°C, then washed in distilled water and dried. CXE product was made by incubating this paper in [³H]XXXGol (50 kBq) and ASP (from Table 3.1.3, 85 µl in 765 µl of 0.3 M citrate, pH 6.3) for 3 h. The paper was dried in mild heat, and washed under running tap-water for 3 d. A portion of the paper (5%) was cut out and scintillation counted (597 cpm). The remaining paper was incubated in 6 M NaOH + 4% H₃BO₃ for 24 h. The supernatant was neutralized with HOAc, and measured for radioactivity. The solid paper was stored at -20°C for further use. A portion of the supernatant (2 ml of 6 ml total) was separated on a Bio-Gel P-2 column (~21 ml bed volume). Immediately prior to the run, markers were analysed to determine the void volume (→), the elution of XXXGol (→◆), and the included volume (→). Fractions (1 ml) were assayed for radioactivity and conductivity (elution of the salts).

(indicating XXXGol), but with the included volume, indicating a

monosaccharide or smaller molecule. Alternately, the product could have an

affinity to the Bio-Gel P-2 column, but this is unlikely because the product

eluted in the same fractions as the salt. All (106%) of the amount of ³H

applied to the column was recovered, excluding the possibility that

polysaccharides precipitated in the column. To obtain further information,

two fractions (24 and 26) were dried and redissolved in water. A portion of

the fraction was assayed for radioactivity. A portion (44%) of the radioactivity was lost after the drying, but no more was after a repeated drying. Therefore, about half of the ^3H had been transferred to a volatile substance, possibly water, and the other half was transferred to a small molecule.

3.4.3.3 Degradation of [^3H]XXXGol in NaOH + borate

Reducing sugars can be peeled off the terminus in severe alkaline environments. However, reduced sugars, such as glucitol, are theoretically stable in NaOH + borate. To examine the potential breakdown products of XXXGol, and attempt to determine if CXE product was stable under the conditions applied, [^3H]XXXGol was incubated in 6 M NaOH + 4% H_3BO_3 , then neutralized and analysed three ways. First, a portion of the incubation products were dried and redissolved to determine if volatiles were produced. Second, a portion was analysed by PC. Lastly, a portion was separated by GPC (Table 3.4.1).

Contradictory to results obtained in Section 3.4.3.2, no radioactivity became volatile after incubation in NaOH + borate from [^3H]XXXGol (data not shown). PC of the incubated substrate indicated that some of the ^3H -heptasaccharide was converted to a ^3H -labelled product that migrated with

glucitol. GPC also agreed with the conclusion that some of the substrate was degraded into a smaller molecule during the incubation in NaOH + borate.

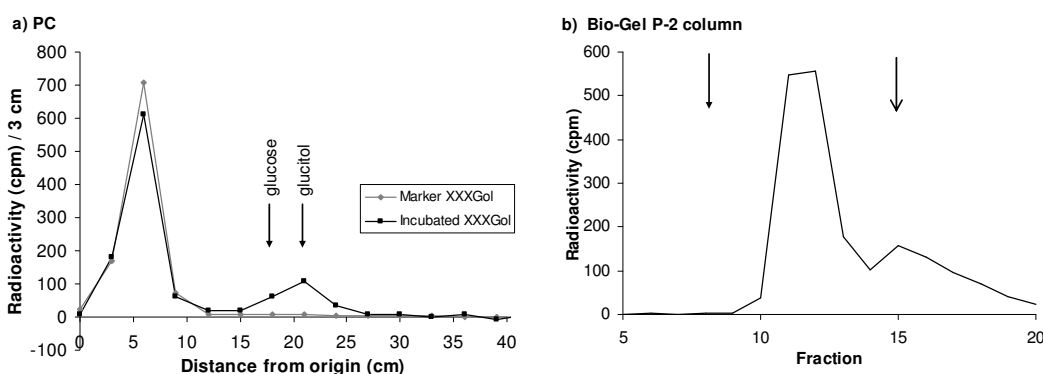


Figure 3.4.1 GPC and PC of $[^3\text{H}]\text{XXXGol}$

$[^3\text{H}]\text{XXXGol}$ (10 kBq) was incubated in 6 M NaOH + 4% H_3BO_3 at 37°C overnight, then neutralized with HOAc. One tenth of the solution was placed into four scintillation vials, two of which were left to evaporate overnight. The dried solutions' water was replaced, and all four vials were scintillation counted. a) Another portion was applied to Whatman 3MM PC next to a sample of untreated $[^3\text{H}]\text{XXXGol}$ and non-radioactive markers, and developed in EAW. b) Two tenths of the solution was mixed with BD and CoCl_2 and separated on a ~21 ml-bed volume Bio-Gel P-2 column, and the fractions were assayed for ^3H .

While the results from Figure 3.4.4 remained partially unclear, some of the CXE product could have been converted to a monosaccharide or other non-volatile compound during the incubation in NaOH + borate. However, simple incubation of XXXGol in aqueous NaOH and borate did not produce volatile products.

3.4.4 Cellulose and CXE product solubilization and reconstitution

It has been proposed that hemicelluloses may be trapped within amorphous regions of cellulose microfibrils (Rose and Bennet, 1999). Such 'trapped'

hemicelluloses may be more tightly connected to cellulose, remaining bound to microfibrils in warm alkali. One could argue that these hypothetical hemicelluloses were the true donor substrate for the observed transglycosylation with paper.

Another method of confirming that [³H]XXXGol was covalently linked to cellulose was to dissolve the cellulosic product. If cellulose microfibrils were reconstituted in alkali, hemicelluloses would no longer be trapped within a microfibril and would remain soluble.

Table 3.4.2 Reconstitution of CXE product

| | |
|------------------------|---------------------------|
| Soluble in 6 M NaOH | Precipitated cellulose |
| 6500 cpm | 14000 cpm |

CXE product (40 mg, produced as in Figure 3.4.4) was soaked in water for 1 h, followed by solvent exchange to acetone. The paper was soaked in acetone for 1 h, then exchanged for DMA freed of H₂O (over Sigma molecular sieve 4Å for 5 d), and rotated for 16 h. The DMA was removed, and the CXE product was incubated in dry DMA (4 ml) with 8% (w/v) LiCl for 16 h. An additional 4 ml of DMA was then added to reduce viscosity. The solution of cellulose was slowly added to stirring 6 M NaOH (80 ml) through a peristaltic pump at the rate of 3.2 ml/h. The resultant mixture was stirred for 48 h. A portion of the mixture was removed and centrifuged. The supernatant was separated from the precipitants; both were neutralized with HOAc and scintillation counted. The cpm of the total supernatant and the total precipitates is reported.

Cellulose was solubilized using lithium chloride (LiCl) in dimethylacetamide (DMA). A solution of 8% LiCl in DMA dissolved CXE product. The viscous solution was slowly transferred to a large volume of 6 M NaOH, where the cellulose re-precipitated. The solid cellulose was

separated from the supernatant, and the radioactive product was monitored in each fraction (Table 3.4.2).

Because much of the ^3H product precipitated, cellulose might have indeed been the true substrate for the transglucosylation reaction with paper. The majority of ^3H followed the expected pattern of cellulose precipitation in 6 M NaOH after dissolution in LiCl and DMA. While the measured ratio of tritium in the precipitate to tritium in the supernatant was 2.2:1, this ratio might have been higher still on a Bq basis since solid particles are counted with a lower efficiency than a solution.

The radioactivity that remained in solution might have been breakdown products of XXXGol, or could have been short pieces of β -(1 \rightarrow 4)-glucan attached to XXXGol with increased solubility because of the XGO.

3.4.5 CXE was an activity of the enzyme MXE

Table 3.4.3 CXE activity from partially purified MXE

| Enzyme | Product formed (cpm) |
|-------------|----------------------|
| pp MXE | 1825 |
| pp XET | 18 |
| ASP | 1217 |
| buffer only | 13 |

Partially purified (pp) MXE (from Figure 3.1.18, fraction 8, 5 μl), pp XET (from Figure 3.1.16, fraction 16, 5 μl), ASP (from Table 3.1.3, 5 μl), or buffer only (0.3 M citrate, pH 6.3) was incubated with [^3H]XXXGol (2 kBq), citrate buffer (up to 100 μl), and 10 mg of Whatman 1CHR paper (untreated) for 3.3 h. The reaction was stopped with FA (30 μl), and unused reactant was washed out with water. The paper (in 5 ml water) was incubated in scintillant and assayed for ^3H .

As shown in Figures 3.1.16 and 3.1.17, *Equisetum* had multiple proteins capable of transglucosylation, some of them displaying XET activity, and at least one other enzyme, MXE, capable of using either MLG or XyG as a donor substrate. Rotofor fractions containing the two enzymes were tested for their ability to use cellulose as a donor substrate (Table 3.4.3).

The partially purified MXE fraction contained high levels of CXE activity, but the fraction with only XET activity did not use cellulose as a donor substrate. While the MXE fraction was not one pure protein, it contained only a few and was highly enriched in one protein (Figure 3.1.19). This enzyme may be a relatively indiscriminate transglucosylase, able to use β -(1 \rightarrow 4)-glucans irrespective of side-chains or other backbone linkages.

4 Discussion

MXE is a novel enzyme, it catalyses the attachment of two different polymers. While this activity has been hypothesized and shown to occur in a heterologously expressed XTH from barley (Hrmova et al., 2007), no MXE products were detected from barley tissues. The high amounts of MXE product extracted from *Equisetum* indicates that a distinctly different organization of polysaccharides exists in horsetail walls.

MXE was characterized in this work and shown that MXE and XTH proteins are quite dissimilar. The protein was purified for sequencing, but its sequence was not determined here. During its characterization, another interesting activity was discovered, CXE. If CXE activity is shown in other plant classes, our understanding of how the cell wall is knitted together to perform its many functions could drastically change.

4.1 Characterization of MXE

4.1.1 Hydrophobicity

MXE precipitated in a lower concentration of AS than other proteins with XET activity from *Equisetum* (Tables 3.1.1, 2, 3), and relative to XTHs from other plant species (Steele and Fry, 1999). Less hydrophilic proteins precipitate in lower concentrations of salts than more hydrophilic proteins

(Skopes, 1987), indicating that MXE was more hydrophobic than XTH of *Equisetum*.

Recovery of active protein from both GPC and IEC was assisted by the addition of low levels of Triton X-100 (Figures 3.1.7 and 8, respectively). The loss of XET activity was previously documented in low salt preparations from cauliflower extracts (Takeda and Fry, 2004). High amounts of Triton X-100 denature proteins (Skopes, 1987), but the detergent might have assisted in maintaining MXE solubility in low ionic environments and at low protein concentrations through an association with more hydrophobic regions of the protein surface.

4.1.2 Glycosylation of MXE

MXE reversibly bound to the lectin, ConA (Figures 3.1.9 and 12), indicating that the protein was glycosylated. Whether or not all XTHs require glycosylation for any or enhanced activity is debated among protein biochemists (Campbell and Braam, 1999 a). Treatment of heterologously expressed XTH proteins with PNGase F causes a loss in activity (Campbell and Braam, 1998; Campbell and Braam, 1999 b). However, the XET activity of *Pichia*-expressed PttXTH16 was relatively unaffected (80% of the activity remained) after incubation in endo H (which cleaves off the majority of N-

linked glycans, Kallas et al., 2005). A similar result was shown using heterologously expressed BobXTH16 (Henriksson et al., 2003).

Deglycosylated MXE was not assayed for activity. Speculation cannot be made as to whether MXE requires glycosylation for activity.

4.1.3 Acidity of MXE

The pI of MXE was determined by Rotofor IEF to be 4.0–4.1 (Figure 3.2.7).

Similarly, the largest peak of MXE activity eluted from an SES IEC column in pH 4.1 buffer (Figure 3.1.8).

XTHs from Equisetum (Figure 3.1.16) and other plants focussed to a higher pI. Many isoforms of XTH were extracted from nasturtium and focused to pIs ranging from 4.4 to 9.6, and extracts from leek leaves in the same study contained an XTH with a pI of less than 4.0 (Farkaš et al., 2005). XTHs from nasturtium seeds focussed from pI 4.5 to 6.3, and an isoform from epicotyls focussed to pI 7.3 (Mohand and Farkaš, 2006). A crude extract of barley contained at least four isoforms of XTH, focussing to pI 5.1, 6.7 (later shown to be HvXET6), 7.2, and 8.5 (later shown to be HvXET5, Hrmova et al., 2007; Hrmova et al., 2009). The pI of MXE was lower than the most XTHs studied to date.

While the pI was low, the pH optimum for activity was more neutral, at pH 6.3 (Figure 3.2.6). Interestingly, the activity did not peak sharply at or

around a particular pH. Activity levels were only slightly changed between pH 5.1 and 6.5. This indicates an ability of MXE to act in various pH conditions. MXE activity is unlikely to be regulated *in vivo* by pH.

Some XTHs similarly maintain high levels of activity across a range of pH. For example, SkXTH1 is active across the pH range of 4.5–7.5 (Van Sandt et al., 2006). Also, a partially purified fraction of mung bean XTH was highly active from pH 5.0 to 7.0 (Steele and Fry 2000). However, other XTHs have more specific pH optima. The activity of AtTCH4 (later renamed AtXTH22) was highest at pH 6.0–6.5 (Purugganan et al., 1997), of BobXTH16A peaked at pH 5.5 (Henriksson et al., 2003), of HvXET5 at pH 6.0 (Hrmova et al., 2007), of partially purified XTH from mung bean and cauliflower was highest at pH 5.0–5.5 and 6.5, respectively (Steele and Fry, 2000).

4.1.4 Lack of stable enzyme–substrate complex

A simple XTH purification strategy was published by Steele and Fry (1999) utilizing the ability of XTH to form a stable enzyme–XyG intermediate.

Equisetum XTH may be able to be purified this way, but MXE appears to not form a similar stable complex with either MLG or XyG (Figures 3.1.4 and 5).

This demonstrates another difference between the two enzymes (or groups of enzymes).

4.1.5 MXE, a promiscuous enzyme different from XTH

Results may suggest that *Equisetum* maintains conventional XTHs similar to previously studied XTHs, but also has a novel enzyme, MXE, with the capability of using a number of β -(1 \rightarrow 4)-glucan-based substrates. Fry et al. (2008 a) described how MXE and XET activities were due to different enzymes based on a number of observations. However, crude enzyme mixtures can distort and confuse results. Hydrolases or other glycan-acting enzymes can degrade or otherwise compete for substrates. Small molecules, such as cellobiose or metabolites, could directly or indirectly affect activity. Six results, outlined below, describe the differences between an MXE with multiple attributable activities, and XTH.

First, MXE and XTH had different pIs. Rotofor IEF of crude enzyme extracts from young tissue showed that *Equisetum* may have multiple isoforms of XTH, focussing to a range of pIs, from 6.6 to 9.0, with no MXE activity (Figure 3.1.16). A single peak of MXE, containing both MXE and XET activity, focused to a much lower pI.

Second, the activities of MXE were higher at pH 6.3 than pH 5.5, in contrast to the activity of the XTHs from *E. fluviatile* (Figure 3.1.16). All XTHs extracted from *E. fluviatile* had higher activity at pH 5.5, and although this is

not true of all XTHs, it remains a difference between MXE and XTHs in this plant.

Third, the acceptor substrate specificity of both MXE activities (MXE and XET) tested were similar to each other, but different from those of XTHs (Figure 3.1.17). Both activities of MXE preferred de-galactosylated XGO acceptor substrates, in the order of XXXGol>XXLGol>XLLGol. Three fractions of XTH from *Equisetum*, with pI 7.5, 7.9, and 8.3, preferred the same XGO substrates in the opposite order, XLLGol>XXLGol>XXXGol. XTHs from other plant species vary in their acceptor substrate specificity, but usually prefer galactosylated XGOs (Rose et al., 2002), similar to *Equisetum* XTH.

Fourth, XTH had the ability to form a stable enzyme–substrate intermediate (Steele and Fry, 1999; this work), but MXE did not (Figure 3.1.5). When previously incubated with XyG, a portion of XET activity, possibly attributable to XTH, eluted from GPC in the void volume. A portion of XET activity, possibly attributable to MXE, eluted in the partially included volume. The protein, XTH, was separable from MXE, but XET activity was not.

Fifth, cellobiose completely inhibited MXE but not XTH. XTH was shown to be only slightly inhibited in 50 mM (Fry et al., 1992) or 100 mM cellobiose (Fry, 1997). Cellobiose was an effective inhibitor of both MXE and XET activity in crude extracts, but some XET activity remained at 256 mM

cellobiose (Figure 3.2.5). The MXE protein, and its MXE and XET activity, could have been inhibited by cellobiose, but XTH, and its XET activity, might not. Whereas the XTH was not inhibited by cellobiose (Fry et al., 1992; Fry, 1997), both activities of MXE were severely inhibited, implying that cellulose-like structures can bind to the active site of MXE. If cellobiose was able to bind to the active site of MXE, it was also likely that cellulose (and other β -(1 \rightarrow 4)-glucans) could bind to the same active site to be a donor or acceptor substrate. Cellobiose was not tested as an acceptor substrate, but cellohexose was a viable acceptor substrate (Table 3.2.5).

Finally, MXE was able to use cellulose as a donor substrate but an *E. fluviatile* XTH was not (Table 3.4.3). While the Rotofor-purified MXE fraction was not one pure enzyme, it contained only a few and was highly enriched in one protein (Figure 3.1.19). The XTH tested was unable to use either MLG or cellulose as a donor, similar to conventional XTHs. In another experiment, a series of Rotofor-purified fractions containing MXE were tested for CXE activity, and patterns of high CXE activity directly correlated with patterns of high MXE and XET activity (T. Simmons, personal communication). This enzyme may be an indiscriminate transglucosylase, able to use various β -(1 \rightarrow 4)-glucans irrespective of xylose side-chains or interspersed β -(1 \rightarrow 3) backbone linkages.

MXE was characteristically different than XTH in many ways.

Current evidence supports the hypothesis of a promiscuous MXE capable of using multiple donor and multiple acceptor substrates. Even still, absolute proof has yet to be shown attributing XET, MXE, and CXE activity to the same enzyme. Molecular biological techniques could be used to express the recombinant MXE gene, to purify the protein, and then to test for the activities to show that one enzyme is undoubtedly responsible for all activities.

4.2 Functional analysis of MXE

4.2.1 Summary of MXE activity using various donor and acceptor substrates

MXE was able to use cellulose, MLG, or XyG as donor substrates with many acceptor substrates (Table 4.2.1). While MLG was a better donor substrate than XyG, direct comparison of activity rates with cellulose as a donor substrate was difficult. The concentration of a polysaccharide in solution, such as MLG or XyG, cannot be compared with a similar concentration of a solid in water. In addition, tritium embedded in or on a solid substrate such as cellulose was counted with lower efficiency than tritium in solution, reducing the ability to detect CXE product. Therefore, MXE and XET activity can be directly compared, but only roughly compared with CXE activity.

Table 4.2.1 Summary of MXE activity using various donor and acceptor substrates

| Donor | Relative reaction rate with the acceptor of: | | | | | |
|-----------|--|-------|-----------------------|--------|--------|------|
| | XXXGol | MLGOs | Cello ₆ ol | XXLGol | XLLGol | XXFG |
| XyG | +++ | ± | ± | + | + | |
| MLG | ++++ | + | + | ++ | + | + |
| Cellulose | ++ | | | | | |
| Lichenan | ± | | | | | |
| Laminarin | - | | | | | |
| Mannan | - | | | | | |
| GM | ± | | | | | |

MXE was able to use a variety of donor and acceptor substrates *in vitro*. Not all possible combinations were tested, but further study would be of interest. For example, to which different acceptor substrates can cellulose be attached? Cellulose has thus far only been shown to be able to be attached to XXXGol. Cellulose and XyG may not co-exist in all tissues of *Equisetum*, as XyG was not labelled by mAb outside the vascular tissue (Sørensen et al., 2008). Possibly more relevant to the plant's physiology would be an activity that could graft cellulose to MLG, as this hemicellulose was present (Sørensen et al., 2008) and transglucosylase activity were both observed in the sclerenchyma and parenchyma (Figure 3.3.5).

Equisetum XyG contains many unique repeat units which were not tested, including XGO with α -L-arabinopyranose. According to Peña et al. (2008), α -L-arabinopyranose was a prominent side-chain XyG substituent of *E. hyemale*, and was a component of 78% of the XGOs. The group of XXXG, XLG/XLXG, XLLG, and XXFG only account for ~20% of the XyG fragments

of *E. hyemale*. The subunits unique to *Equisetum* could be more suitable acceptor substrates for MXE or other *Equisetum* transglucosylases.

4.2.2 Donor and acceptor attached terminus to terminus

MXE reformed the cut β -(1 \rightarrow 4) glucose bond from MLG with the non-reducing terminal glucose residue of the DP 14 XGO acceptor substrate (Figure 3.2.2). Therefore, only one donor molecule could be attached to one acceptor molecule. If the donor could be attached as a side chain to the acceptor, one could envision a backbone molecule (e.g. XyG) with very large side-chain polysaccharides (e.g. MLG) formed in the apoplastic matrix.

However, no such cross-links were formed between MLG and the DP 14 XGO. This result was similar to the action of XET (Fry et al., 1992; Nishitani and Tominaga, 1992).

4.3 MXE action in *Equisetum* tissues

The novel radioactive *ex vivo* method used shows that *Equisetum* transglucosylases were active in tissues and were co-localized with MLG and/or XyG (Figure 3.3.4 and Table 3.3.2). The products were NaOH-extractable and migrated on TLC similar to *in vitro* products after digestion with endoglucanases. In other experiments, extracts and heterologously expressed proteins show activity *in vitro*. However, these proteins may be

regulated *in vivo* through compartmentalization or small molecule activators/inhibitors, and the true action of the enzymes may not reflect the *in vitro* activity.

The *ex vivo* method also shows the relative amounts of MXE and XET product formed in different tissue types. More MXE activity was extractable from mature tissues than from younger tissues (Fry et al., 2008 a), and this work showed that the corresponding actions are present in the same tissues as the extractable activity. Results also show that MXE action was absent from meristematic tissues, and confirmed that MXE action was a feature of mature tissues (Table 3.3.2).

4.3.1 Potential caveat of the *ex vivo* method

Sørensen et al. (2008) suggested that MLG and XyG are not co-localized in *Equisetum* tissues. By antibody staining, they found MLG in most mature tissues, except the vasculature, and XyG only in the vasculature. It is possible that antibodies directed against XyG could not detect the unique XyG structures found in other tissues. Also, the XyG-specific antibody used, LM15, can be 'masked' by the presence of pectins (Marcus et al., 2008). HG was present in most tissues examined (Sørensen et al., 2008). XyG may have gone undetected in other tissues because of masking by HG.

However, if the conclusions proposed by Sørensen et al. (2008) are indeed true, then the *ex vivo* method (Sections 2.4.1 and 2) might supply exogenous XGOs to tissues that do not have XyG. While the method assays for endogenous enzyme and donor substrate, the supplied acceptor substrate could create an unnatural modification to the reaction *in vivo*. The extracted products cannot be assumed to be the same as natural transglucosylation products, nor can the natural acceptor substrate be assumed to be an XGO. The true action of MXE has not yet been detected.

4.3.2 CXE activity in *ex vivo* assays

Owing to the nature of the *ex vivo* method, only hemicellulosic transglucosylase products were detected (Figure 3.3.4 and Table 3.3.2). CXE products, being mostly cellulose with a very short fragment of XyG at the reducing terminus, might not have been extracted in 6 M NaOH, and not detected. The existence and proportion of CXE product made was unknown.

4.3.3 CXE activity *in situ*

CXE action was likely detected by incubating XXXG–SR in tissue sections (Figure 3.3.5). MXE, XET, and CXE products would have all been detected by fluorescence. As these activities all use the same acceptor substrate, they would not have been distinguished using this method.

4.4 CXE activity

4.4.1 Summary of evidence for CXE

A multitude of observations lead to the confirmation of cellulose : XyG endotransglucosylase activity.

Firstly, CXE activity was observed initially in control transglucosylation test papers. Control papers were not supplemented with hemicellulosic donor substrate. A dilution series of crude enzyme was applied to MXE, XET, and control papers, and transglucosylation was observed in all three cases (Figure 3.4.1). The XET and MXE products were removable from the paper support, but some CXE product on the control paper was not. In a separate experiment, native PAGE separation of *Equisetum* proteins revealed a similar result. Zymogram test papers showed transglucosylase activity in control papers with no supplemented hemicellulosic donor substrate (Figure 3.1.1).

Secondly, the monosaccharide composition of Whatman chromatography paper was determined to be almost completely glucose, with only minor levels of xylose also present (Figure 3.4.2). While callose could have been the source of the glucose, callose was shown to not be a donor substrate for a transglucosylation reaction (Table 3.2.2), and MLG has not been reported in cotton fibres.

Thirdly, both *Equisetum* AIR and paper were better substrates for transglucosylation after a wash in alkali and after hemicelluloses were removed (Figure 3.4.3). Cellulose incubated in 6 M NaOH was a better substrate than plain paper possibly because the treatment reduced the crystallinity of the cellulose microfibrils, creating more amorphous cellulose and increasing the accessibility of individual cellulose chains to the enzyme. The products formed remained attached to the cellulose after another wash in NaOH + borate (Figure 3.4.4), indicating a covalent attachment to cellulose.

Finally, CXE product dissolved by LiCl in DMA precipitated upon reconstitution of the cellulose in 6 M NaOH (Table 3.4.2), indicating that the transglucosylation product was not a hemicellulose.

In summary, cellulose was shown to be covalently linked to [³H]XXXGol by an *Equisetum* enzyme. This activity was termed cellulose : xyloglucan endotransglucosylase (CXE).

4.4.2 Proposed role of CXE

Fry et al., (2008 a) hypothesized that MXE could strengthen cell walls. It is possible that the CXE activity of MXE is even more relevant to the strengthening of covalently reinforcing the cellulose–hemicellulosic network.

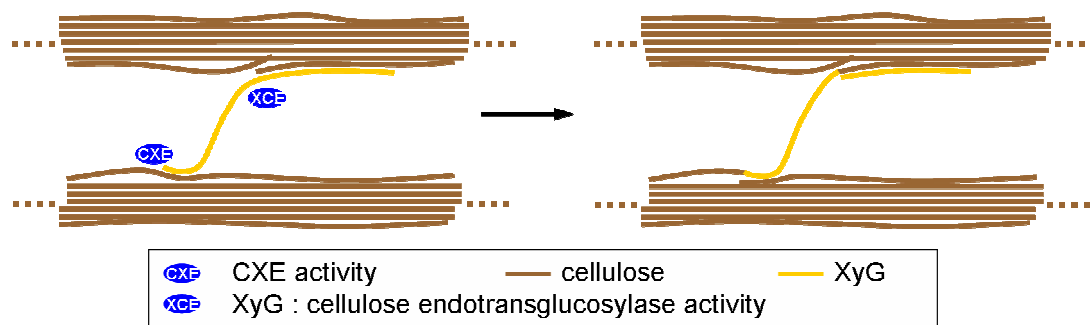


Figure 4.4.1 Potential of activities to covalently link cellulose microfibrils

It was shown that cellulose can be bound to xyloglucan (CXE activity, reducing terminus of cellulose attached to the non-reducing terminus of XyG), and that xyloglucan can be attached to cello-oligosaccharides (xyloglucan : cellulose endotransglucosylase, XCE activity, reducing terminus of XyG attached to the non-reducing terminus of β -(1 \rightarrow 4) glucan, Table 3.2.5). If the same xyloglucan molecule can be attached to two neighbouring cellulose microfibrils, the microfibrils themselves could become covalently attached through the XyG intermediate (Figure 4.4.1). Similarly, MLG could be the tethering glucan in the place of XyG, although cellulose : MLG endotransglucosylase activity has not been shown. A covalently linked cellulose–hemicellulose network could be stronger than a hydrogen bonded network.

4.5 Future studies

4.5.1 Other activities

The MXE protein may be active on other donor : acceptor substrate combinations not tested (see Table 4.2.1). The most interesting activities not yet tested could be cellulose : MLG endotransglucosylase and other activities with cellulose as the donor. If cellulose could be attached to MLG, the tethering hemicellulose depicted in Figure 4.4.1 could be either XyG or MLG, leading to a greater impact of this enzyme on the overall structure of the cell wall.

4.5.2 CXE in plants other than *Equisetum*

MXE activity was shown in charophytic algae (Fry et al., 2008 a) and also shown to be present in lycopodiophytes (Table 3.2.5). Although they have MXE activity, no MLG could be detected in these plants (Fry et al., 2008 b, Popper and Fry, 2004). However, all land plants and charophytic algae contain cellulose. The β -(1→4)-glucose regions of MLG may mimic cellulose enough to be recognized by enzymes whose *in vivo* substrates are cellulose. The MXE activity observed in algae and lycopodiophytes may actually be CXE activity *in vivo*. This has not yet been tested, but could be tested in the future. The presence of CXE activity in other land plants has also not yet been conclusively tested.

4.5.3 Extensibility assays

The proposed tethering role of CXE activity could be tested by extensibility assays using paper as the solid substrate using methods described by Chanliaud et al. (2004). The paper could be impregnated with (and controls without) xyloglucan or MLG, and incubated with active or denatured enzyme. The amount of force required to stretch or break the paper could afterwards be measured. If the amount of force required to stretch the paper increased after incubation with enzyme, CXE (and the corresponding XCE activity) could be shown to strengthen the artificial cellulose–hemicellulose network. Similar studies could be undertaken using methods described by McQueen-Mason et al. (1992).

4.5.4 Sequencing MXE

The transcriptome of *Equisetum fluviatile* will be investigated and elucidated in the near future. This line of inquiry should provide information about the number of candidate genes with MXE or CXE activity, and information about putative XTHs. Comparison of this information to the LC–MS MXE fragmentation data (Figure 3.1.22) should elucidate the gene or genes responsible for the MXE protein, the knowledge of which will impart the ability to heterologously express the gene or genes and characterize the

protein once purified. Only after heterologous expression and purification will there be proof that multiple activities (XET, MXE, CXE, etc.) come from the same enzyme.

4.5.5 Commercial application of the protein

Expression of the MXE gene in another plant, such as a grass or cereal, would be of considerable interest. The enzyme would be expected to act on polysaccharides native to their cell walls, cross-linking MLG, XyG, and/or cellulose. An analysis of the structural changes imposed by the protein could indicate the role of the protein in *Equisetum*.

The application of any potential changes could be valuable to crop growers, depending on the nature of the changes. For example, the expression of MXE activity in a cereal plant may covalently bind MLG to other wall components. This, in turn, could prevent its hydrolysis, leaving a greater amount of the polymer in plant residue. An increase in easily extractable glucose could be valuable to biofuel production (Pauly and Keegstra, 2008). MXE may cause structural changes to the cell wall that result in an increase in rigidity and strength. These properties could help maintain structural integrity of stems or stalks to resist the effects of lodging due to wind, pests, or high grain weight.

Expression of the protein in a bioreactor could have commercial interests as an enzyme which could modify cellulose. Cellulose is ubiquitous in human lives, being the largest part of cotton in clothing, wood fibre in paper, lumber, etc. The applications of modified cellulose are as far-reaching as the increased dye-ability of cotton, anti-bacterial medical bandages, increased strength of timber, specialized papers, as well as enhancing the strength of living plants.

The study of plant cell walls is far from complete. The mechanisms of cell wall relaxation that leads to cell and plant growth, and of maturation that leads to rigidity, are not well understood. The transglucosylating activities discovered and discussed in this work contribute to our understanding of wall dynamics, and the changes that are associated with physiological processes. However, these initial discoveries need further research to determine the full impact of this information on our understanding of the plant cell wall.

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Mixed-linkage β -glucan : xyloglucan endotransglucosylase, a novel wall-remodelling enzyme from *Equisetum* (horsetails) and charophytic algae

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Received 2 January 2008; revised 25 February 2008; accepted 11 March 2008.
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Summary

Mixed-linkage (1 \rightarrow 3,1 \rightarrow 4)- β -D-glucan (MLG), a hemicellulose long thought to be confined to certain Poales, was recently also found in *Equisetum*; xyloglucan occurs in all land plants. We now report that *Equisetum* possesses MLG:xyloglucan endotransglucosylase (MXE), which is a unique enzyme that grafts MLG to xyloglucan oligosaccharides (e.g. the heptasaccharide XXXGol). MXE occurs in all *Equisetum* species tested (*Equisetum arvense*, *Equisetum fluviatile*, *Equisetum hyemale*, *Equisetum scirpoides*, *Equisetum telmateia* and *Equisetum variegatum*), sometimes exceeding xyloglucan endotransglucosylase (XET) activity. Charophytic algae, especially *Coleochaete*, also possess MXE, which may therefore have been a primordial feature of plant cell walls. However, MXE was negligible in XET-rich extracts from grasses, dicotyledons, ferns, *Selaginella* and bryophytes. This and the following four additional observations indicate that MXE activity is not the result of a conventional xyloglucan endotransglucosylase/hydrolase (XTH): (i) XET, but not MXE, activity correlates with the reaction rate on water-soluble cellulose acetate, hydroxyethylcellulose and carboxymethylcellulose, (ii) MXE and XET activities peak in old and young *Equisetum* stems, respectively, (iii) MXE has a higher affinity for XXXGol ($K_m \sim 4 \mu\text{M}$) than any known XTH, (iv) MXE and XET activities differ in their oligosaccharide acceptor-substrate preferences. High-molecular-weight (M_r) xyloglucan strongly competes with [^3H]XXXGol as the acceptor-substrate of MXE, whereas MLG oligosaccharides are poor acceptor-substrates. Thus, MLG-to-xyloglucan grafting appears to be the favoured activity of MXE. In conclusion, *Equisetum* has evolved MLG plus MXE, potentially a unique cell wall remodelling mechanism. The prominence of MXE in mature stems suggests a strengthening/repairing role. We propose that cereals, which possess MLG but lack MXE, might be engineered to express this *Equisetum* enzyme, thereby enhancing the crop mechanical properties.

Keywords: mixed-linkage β -glucan, xyloglucan, transglycosylation, *Equisetum*, *Coleochaete*, cell wall.

Introduction

Xyloglucan is a ubiquitous structural polysaccharide thought to tether microfibrils in the cell walls of land plants (embryophytes) but not algae (Hayashi, 1989; Popper and Fry, 2003). Xyloglucan chains are remodelled during plant development by enzymes called xyloglucan endotransglucosylase/hydrolases (XTHs) (Rose *et al.*, 2002). XTHs are carbohydrate-acting enzymes of class GH16b (Strohmeier *et al.*, 2004) that exhibit one or both of two enzymic activities: (i) xyloglucan endotransglucosylase (XET; EC 2.4.1.207), which involves the breaking and reformation of glycosidic bonds in the backbone of xyloglucan by transglycosylation (Figure 1), and (ii) xyloglucan endohy-

drolase (XEH; EC 3.2.1.151), which simply involves hydrolysis of the backbone of xyloglucan. During XET action, one polysaccharide chain (the donor-substrate) is cleaved, and a portion of it is then grafted on to an acceptor-substrate [which can be either another xyloglucan chain or a xyloglucan oligosaccharide (XGO)] (Figure 1).

Interpolymeric transglycosylation (i.e. with both the donor and the acceptor-substrate of high M_r) has been demonstrated *in vivo*, establishing enzyme action as distinct from *in-vitro* activity (Fry, 2004; Thompson and Fry, 2001; Thompson *et al.*, 1997). Xyloglucan remodelling by XET action could (i) restructure existing wall-bound xyloglucan

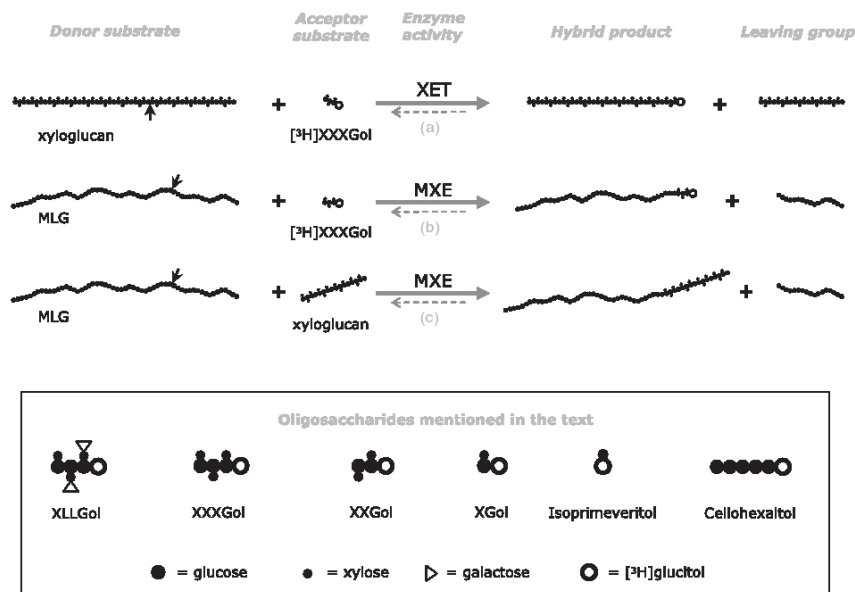


Figure 1. Enzymic reactions and oligosaccharide structures.

Xyloglucan endotransglucosylase (XET) activity cleaves xyloglucan (donor-substrate; ↑ = bond cleaved) and grafts a portion on to an acceptor-substrate, which can be a xyloglucan oligosaccharide [e.g. [³H]XXXGol; reaction (a)] or another xyloglucan chain (not shown). MLG:xyloglucan endotransglucosylase (MXE), the novel *Equisetum* enzyme, cleaves mixed-linkage (1 → 3,1 → 4)-β-D-glucan (MLG) (donor-substrate; ↓ = bond cleaved), and grafts a portion on to an acceptor-substrate, which can be a xyloglucan oligosaccharide [reaction (b)] or a high-molecular-weight xyloglucan chain [reaction (c)], the latter being favoured. The reactions would be freely reversible, but the reverse reactions (dashed arrows) would be undetectable by the methods used.

molecules, thereby contributing to the reversible wall loosening required for cell expansion (Fry *et al.*, 1992; Nishitani, 1997; Nishitani and Tominaga, 1992; Thompson and Fry, 2001; Van Sandt *et al.*, 2007b), (ii) reconnect xyloglucan chains to enable the shrinkage of tension wood fibres during gravitropism in trees (Nishikubo *et al.*, 2007), and (iii) help to integrate newly secreted xyloglucan chains into the cell-wall matrix (Ito and Nishitani, 1999; Thompson *et al.*, 1997).

Other taxonomically widespread hemicelluloses are (1 → 4)-β-D-xylans, (1 → 4)-β-D-mannans and glucuronomannans (Brett and Waldron, 1996; Fry, 2001; Obel *et al.*, 2006). However, besides xyloglucans, only one other class of cell-wall polysaccharide – (1 → 4)-β-D-mannans – is a known target of endotransglycosylases (Schröder *et al.*, 2004, 2006). No known endotransglycosylases target pectins (García-Romera and Fry, 1994) or xylans.

Xyloglucan endotransglucosylase/hydrolases can also attack some soluble artificial substrates. For example, Ait Mohand and Farkaš (2006) showed that *Tropaeolum* XTHs can catalyse transglucosylation, not only with xyloglucan as

the donor, but also (more weakly) with hydroxyethylcellulose (HEC) and carboxymethylcellulose (CMC) as donors, when ³H- or sulphorhodamine-tagged XGOs are offered as the acceptor-substrate. Transglucosylation was also detectable with xyloglucan as the donor when the acceptors were sulphorhodamine-tagged cello-oligosaccharides, or even when the acceptors were laminari-oligosaccharides. Following up on this observation, Hrmová *et al.* (2007) found that a pure XTH isoenzyme from barley could utilize not only xyloglucan but also HEC (at 44% of the xyloglucan rate), water-soluble cellulose sulphate (5% rate), and CMC (0.4%) as donor-substrates. No transglucosylation was observed, with xyloglucan as donor and either oligo-(1 → 4)-β-D-galactans or oligo-(1 → 5)-α-L-arabinans as the acceptor, in the presence of any of several XTHs *in vitro* or *in vivo* (Popper and Fry, 2008).

Class GH16b contains not only XTHs but also lichenases, which hydrolyse mixed-linkage (1 → 3,1 → 4)-β-D-glucan (MLG) (Strohmeier *et al.*, 2004). The sequence similarity between XTHs and lichenases suggested the possibility that

XTHs might be able to use MLG as an alternative donor-substrate. In agreement with this prediction, Hrmová *et al.* (2007) showed that a barley XTH can catalyse transglucosylation with MLG as the donor-substrate and a sulphorhodamine-tagged XGO as the acceptor-substrate. The rate of MLG-to-XGO transglucosylation catalysed by the barley enzyme was approximately 0.2% that of the classic XET reaction: xyloglucan-to-XGO transglucosylation.

Hrmová *et al.* (2007) used only a single, pure XTH – an approach that does not test the existence of novel enzymes with a greater propensity to act on MLG. As an alternative approach, we therefore tested the total buffer-extractable protein from diverse land plants, and algae, for enzymes that may preferentially catalyse transglucosylation of several naturally occurring wall polysaccharides. This work showed that *Equisetum*, a very early diverging pteridophyte genus with 15 extant species (Des Marais *et al.*, 2003; Smith *et al.*, 2006), has an endotransglucosylase acting on MLG. We term this enzyme MLG:xyloglucan endotransglucosylase (MXE). This discovery in *Equisetum* was initially surprising because MLG was thought to be confined to certain Poales, e.g. grasses, cereals and reeds (Trethewey *et al.*, 2005). The enzyme data prompted us to re-examine the taxonomic distribution of MLG, leading to the finding that it is also a major hemicellulose of all five *Equisetum* species tested (Fry *et al.*, 2008). Sørensen *et al.* (2008) independently detected MLG in *Equisetum arvense*.

Poalean MLGs are unbranched polysaccharides with sequences such as:



where 'G' is β -D-glucopyranose, and '3' and '4' indicate (1 \rightarrow 3)- and (1 \rightarrow 4)- bonds, respectively. The underlined

domains are effectively cello-oligosaccharide repeat units, with trisaccharides outnumbering tetrasaccharides (Meikle *et al.*, 1994). *Equisetum* MLG generally resembles poalean MLG, except that the cellotetraose units substantially outnumber the cellotriose units, and that appreciable proportions of cellobiose units also occur (Fry *et al.*, 2008).

Results

Taxonomic distribution of MXE activity

Although interpolymeric (xyloglucan-to-xyloglucan) grafting is the principal XET reaction occurring *in vivo*, it is simpler to use a radiolabelled xyloglucan oligosaccharide (XGO) as the acceptor-substrate *in vitro* (Figure 1). By transglucosylation, the radioactive moiety undergoes a readily detectable size increase (Fry, 2004; Rose *et al.*, 2002).

Using such radiometric assays, we surveyed taxonomically diverse plants and algae for endotransglucosylases acting on xyloglucan, MLG or xylan, with the heptasaccharide [3 H]XXXGol as the acceptor-substrate. Extracts from all species acted on xyloglucan (Figure 2; Table 1), confirming XET activity. Traces of 3 H-polymer were formed in controls with no added polysaccharide, probably indicating the presence of traces of xyloglucan co-extracted with the enzymes. Relative to these control rates, no extracts showed high activity on xylan (Table 1). Most land plants tested (bryophytes, a lycopodiophyte, pteridophytes and angiosperms) showed negligible MXE activity: for example, in extracts of *Holcus lanatus* (Yorkshire fog grass), the MLG rate was 0.17% of the xyloglucan rate (Table 1) – similar to

Figure 2. Transglucosylation catalysed by eight plant and algal extracts on two donor-substrates. Added polysaccharides were xyloglucan (●), mixed-linkage (1 \rightarrow 3,1 \rightarrow 4)- β -D-glucan (MLG) (■), or none (○). The acceptor-substrate was 4 kBq [3 H]XXXGol. Formation of radioactive polymeric products (kcpm = 10^3 counts per minute) was assayed by the running-water method. Error bars indicate the counting errors (95% confidence limits) of individual samples.

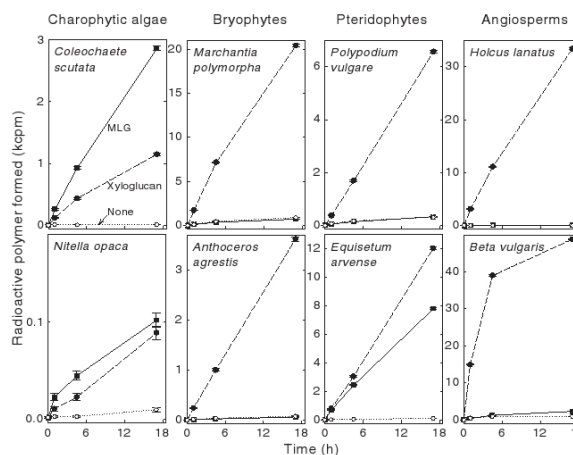


Table 1 Transglycosylation rates catalysed by plant and algal extracts on three donor-substrates

| Species | Classification | Rate of formation of radioactive polymer (cpm h ⁻¹ ± SE) with | | | | Ratio of corrected rates (Xyloglucan:MLG:Xylan) |
|---|-----------------------------|--|--------------------|-----------------|-----------------|---|
| | | No donor | Xyloglucan | MLG | Xylan | |
| <i>Spirogyra varians</i> | Charophyta: Zygnematales | 0.54 ± 0.24 | 3.08 ± 0.61 | 2.24 ± 0.58 | 0.20 ± 0.32 | 100 : 67 : -13 |
| <i>Klebsormidium subtile</i> | Charophyta: Klebsormidiales | 0.49 ± 0.22 | 4.81 ± 0.33 | 4.80 ± 0.35 | 0.37 ± 0.33 | 100 : 100 : -3 |
| <i>Klebsormidium fluitans</i> | Charophyta: Klebsormidiales | 0.62 ± 0.14 | 2.50 ± 0.28 | 2.53 ± 0.24 | 0.36 ± 0.26 | 100 : 102 : -14 |
| <i>Nitella opaca</i> | Charophyta: Charales | 0.45 ± 0.21 | 5.1 ± 0.3 | 5.5 ± 0.8 | 0.56 ± 0.53 | 100 : 109 : 2 |
| <i>Chara vulgaris</i> | Charophyta: Charales | 0.53 ± 0.24 | 16.0 ± 0.7 | 3.84 ± 0.47 | 0.92 ± 0.46 | 100 : 21 : 3 |
| <i>Coleochaete scutata</i> ^a | Charophyta: Coleochaetales | 0.24 ± 0.33 | 65 ± 5 | 165 ± 7 | 0.24 ± 0.26 | 100 : 254 : 0 |
| <i>Coleochaete scutata</i> ^b | Charophyta: Coleochaetales | 0.58 ± 0.35 | 88 ± 2 | 144 ± 5 | 0.55 ± 0.22 | 100 : 164 : 0 |
| <i>Marchantia polymorpha</i> | Bryophyte (liverwort) | 49 ± 10 | 1177 ± 74 | 40 ± 8 | 45 ± 6 | 100 : -0.8 : -0.4 |
| <i>Anthoceros agrestis</i> | Bryophyte (hornwort) | 40 ± 5 | 219 ± 17 | 31 ± 3 | 44 ± 5 | 100 : -5.0 : 2.2 |
| <i>Selaginella apoda</i> | Lycopodiophyte | 63 ± 4 | 1786 ^c | 87 ^c | 74 ^c | 100 : 1.4 : 0.6 |
| <i>Equisetum arvense</i> | Pteridophyte (horsetail) | 5.3 ± 0.8 | 708 ± 9 | 451 ± 16 | 6.2 ± 0.8 | 100 : 63 : 0.1 |
| <i>Osmunda regalis</i> | Pteridophyte (fern) | 6.8 ± 0.7 | 72 ± 3 | 3.4 ± 0.4 | 11.1 ± 1.2 | 100 : -5 : 7 |
| <i>Polypodium vulgare</i> | Pteridophyte (fern) | 17 ± 3 | 387 ± 2 | 18 ± 2 | 19 ± 3 | 100 : 0.3 : 0.5 |
| <i>Holcus lanatus</i> | Angiosperm (Poales) | 2.1 ± 0.4 | 1925 ± 93 | 5.3 ± 0.7 | 8.6 ± 0.8 | 100 : 0.2 : 0.3 |
| <i>Beta vulgaris</i> (spinach-beet) | Angiosperm (dicotyledon) | 169 ± 72 | 14900 ^c | 266 ± 25 | 344 ± 41 | 100 : 0.7 : 1.2 |

Polysaccharides tested as potential donor-substrates (2 mg ml⁻¹) were tamarind xyloglucan, barley mixed-linkage (1 → 3,1 → 4)-β-D-glucan (MLG), birchwood xylan, or no (added) donor. The acceptor-substrate in all cases was 4 kBq [³H]XXXGol. Time courses (as in Figure 2) were recorded for 0–17 h, and the mean reaction-rates (± standard error; n = 4) were calculated over the linear portion. Formation of radioactive polymeric products was assayed by the running-water method. The ratio of rates include a few negative values because the data were corrected for the appropriate zero-added-polysaccharide control.

^aGrown in artificial medium.

^bGrown in medium containing soil extract.

^cSingle determination at 1 h incubation time; the result is typical of two or more similar experiments.

the percentage reported for a purified barley XTH (Hrmová *et al.*, 2007).

Remarkably, however, *E. arvense* extracts showed very high activity on MLG (65% of the xyloglucan rate in this experiment). The data shown (Figure 2) are representative of five similar experiments with *E. arvense*.

MXE was also found in all charophytes tested, especially *Coleochaete scutata* (Figure 2; Table 1), in which MXE activity exceeded that of XET.

In subsequent surveys of six *Equisetum* species, gathered at different times of year according to availability, extractable MXE values as a percentage of XET activity were as follows: *E. arvense*, 146% (gathered in October); *Equisetum fluviatile*, 46% (April) or 72% (September); *Equisetum hyemale*, 29% (January); *Equisetum scirpoides*, 16% (January); *Equisetum telmateia*, 59% (November); *Equisetum variegatum*, 9% (January). Therefore, MXE is general to *Equisetum*, and the MXE:XET ratio varies between species, and seasonally, suggesting that the two activities result from different proteins. The finding that MXE activity can exceed XET activity indicates that *Equisetum* possesses enzyme(s) in which the favoured donor-substrate is MLG.

Developmental regulation of MXE activity

Xyloglucan endotransglucosylase activity often peaks in young, fast-growing tissues (Fry, 2004; Rose *et al.*, 2002),

where it contributes to wall assembly and restructuring. *Equisetum* XET follows this trend, whereas MXE activity peaks in older tissues (Table 2).

Donor-substrate specificity of grass and horsetail endotransglucosylases

Relative activities on various artificial donor-substrates further distinguished MXE from XET. A consistently effective alternative to xyloglucan was water-soluble cellulose acetate (WSCA; Figure 3), tested here for the first time, which structurally resembles grass xyloglucans (Gibeau *et al.*, 2005) in carrying an *O*-acetyl group on carbon 6 of the (1 → 4)-β-D-glucan backbone. Enzyme extracts from young shoots of *Equisetum* and the grass *H. lanatus* share similar donor-substrate profiles on artificial soluble β-glucans, with relative activities on xyloglucan:WSCA:HEC:CMC being 100:20–24:11–17:0.4–1.5, suggesting that these four (1 → 4)-β-D-glucans are attacked by common XTHs. However, the MLG-to-XXXGol transglycosylation rate was 38, 9 and <1% of the xyloglucan-to-XXXGol rate for *E. fluviatile*, *E. arvense* and *Holcus*, respectively. This indicates that MXE activity is not a side reaction of conventional XTHs.

Equisetum extracts did not act on lichenan (Table 3), which is an MLG built almost entirely of cellotriase repeat units.

Table 2 Transglucosylation rates on mixed-linkage (1 → 3,1 → 4)- β -D-glucan (MLG) and xyloglucan catalysed by extracts from *Equisetum* stems at different developmental stages

| Species | Collection date, 2007 | Source of enzyme ^a | Yield of ³ H-polymers (cpm) ^b with | | MLG rate as % of xyloglucan rate |
|----------------------|-----------------------|-----------------------------------|--|------------|----------------------------------|
| | | | Xyloglucan | MLG | |
| <i>E. arvense</i> | 4 May | Young early shoots ^c | 1383 ± 15 | 18 ± 2 | 1.3 |
| <i>E. arvense</i> | 22 August | Late shoots ^c | 530 ± 5 | 84 ± 2 | 15.9 |
| <i>E. arvense</i> | 22 August | Lateral stems on old early shoots | 308 ± 2 | 140 ± 1 | 45.3 |
| <i>E. arvense</i> | 22 August | Main stems on old early shoots | 974 ± 5 | 308 ± 4 | 31.6 |
| <i>E. fluviatile</i> | 4 May | Young main stems ^d | 701 ± 12 | 113 ± 3 | 16.1 |
| <i>E. fluviatile</i> | 22 August | Old laterals | 497 ± 12 | 420 ± 26 | 84.5 |
| <i>E. fluviatile</i> | 22 August | Old main stems | 507 ± 5 | 425 ± 15 | 83.9 |
| – | – | No enzyme | –0.2 ± 0.2 | –0.4 ± 0.1 | – |

Each assay contained the enzymes extractable from 1.6 mg fresh weight of material. Reaction mixtures contained either no added polysaccharide or 2 mg ml⁻¹ xyloglucan or barley MLG; the acceptor-substrate tested was [³H]XXXGol (1.35 kBq per assay). Reaction products were assayed after 85 min (linear reaction) by the running-water method. The yield of radioactive polymers formed in the absence of added polysaccharides was very low (data not shown), and has been subtracted from the data presented here.

^a*Equisetum arvense* produced large, tender, vigorous, highly branched shoots in May, described as 'early' shoots. Shoots that had fully grown in May but were not collected until August are described as 'old, early'. Smaller, weaker shoots sprouted from the *E. arvense* rhizomes during August, and are described as 'late' shoots. The leaves of *Equisetum* are extremely small and were not removed.

^bMean of three determinations ± SE.

^cMain stems + laterals, not separated.

^d*Equisetum fluviatile* had no lateral stems in May.

Acceptor-substrate specificity of XET and MXE activity

Xyloglucan endotransglucosylase and MXE also differ in acceptor-substrate specificity (Figure 4; Table 3). XET activities tend to be fairly conservative in their acceptor-substrate preferences. Thus, in plants as diverse as the pteridophyte *Equisetum* and the bryophyte *Anthoceros*, the XET acceptor-substrate preferences resemble those of dicotyledons (Rose *et al.*, 2002; Steele and Fry, 2000): XLLGol (a galactosylated xyloglucan-nonasaccharide) is the best acceptor-substrate tested, followed by the heptasaccharide XXXGol, and then by the pentasaccharide XXGol. Xyloglucan tri- and disaccharide are ineffective: celohexaitol and an MLG-heptasaccharide preparation (containing G4G4G3G4G4G3-glucitol) are very poor acceptor-substrates.

However, for *Equisetum* MXE activity (i.e. with MLG as the donor-substrate), the acceptor-substrate profile is distinctly different from that of XET. By far the best acceptor-substrate tested is XXXGol (both XLLGol and XXGol being much less effective), and there is low but significant activity on MLG-heptasaccharides and on celohexaitol, although not on laminaritetraitol (Figure 4; Table 3). The data again emphasize enzymological differences between the proteins catalysing MXE and XET activities.

MXE activities in *Holcus* and *Anthoceros* were too low to generate accurate data, but the MXE acceptor-substrate profile for the *Coleochaete* extract broadly resembles that for *Equisetum* (Figure 4). However, *Coleochaete* is more active on celohexaitol, and can even act on the trisaccharide XGol. *Coleochaete* XET and *Coleochaete* MXE activities resemble each other in acceptor-substrate profile, indicating

that in algae the same enzyme may have both activities. Two other charophytes tested, *Klebsormidium* and *Chara*, resemble *Coleochaete* in this respect (data not shown).

Enzyme kinetics of MXE and XET

Plots of reaction rate versus donor-substrate concentration gave good fits to rectangular hyperbolae for both *Equisetum* XET and MXE, as expected from Michaelis-Menten kinetics. XET and MXE activities have apparent K_m values of 0.35 and approximately 4 mg ml⁻¹ for their respective donor-substrates (Figure 5), indicating a moderate affinity of *Equisetum* MXE for barley MLG. Therefore, under the reaction conditions routinely used in the other experiments reported in this paper (2 mg ml⁻¹ donor-substrate; carrier-free radio-labelled acceptor-substrate), XET and MXE activities would be operating at about 85 and 33% of the respective maxima. It follows that the MXE:XET ratios quoted earlier for various *Equisetum* extracts can legitimately be multiplied by approximately 2.6. For example, in the *E. arvense* extract gathered in October, MXE would have been 146 × 2.6 = 370% of the XET activity, if both activities had been assayed at saturating donor-substrate concentrations.

In the presence of 1.4 μ M XXXGol, the apparent V_{max} (rate at saturating donor concentration) of XET activity was approximately 2 pmol h⁻¹ (Figure 5a). With 200 μ M XXXGol, its apparent V_{max} was much higher (26 pmol h⁻¹; Figure 5b), as expected for an enzyme with two substrates. However, the apparent V_{max} of MXE with 1.4 μ M XXXGol as acceptor-substrate (approximately 7 pmol h⁻¹; Figure 5a) was not enhanced by a 140-fold increase in acceptor-

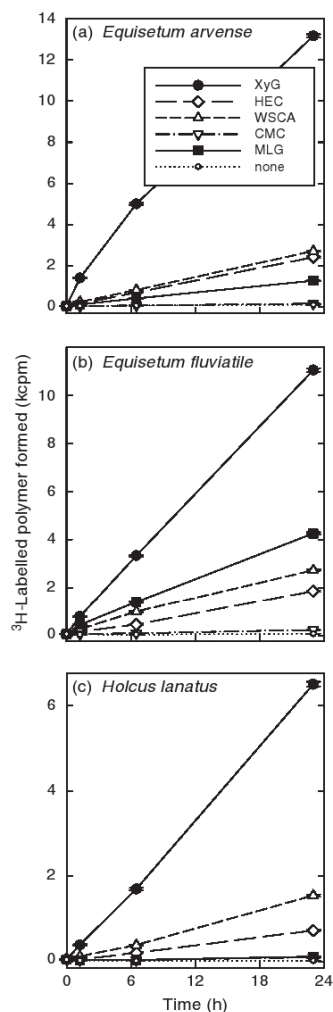


Figure 3. Transglucosylation catalysed by extracts from two horsetails and a grass with five water-soluble β -glucans as donor-substrates. Conditions were as for Table 1, but reaction mixtures contained either no added polysaccharide (\circ) or 2 mg ml^{-1} of tamarind xyloglucan (\bullet), hydroxyethylcellulose (HEC) (\diamond), water-soluble cellulose acetate (WSCA) (\triangle), carboxymethylcellulose (CMC) (∇) or barley mixed-linkage (1 \rightarrow 3, 1 \rightarrow 4)- β -D-glucan (MLG) (\blacksquare). Enzyme extracts were from shoots of (a) *Equisetum arvense*, (b) *E. fluviatile* and (c) *Holcus lanatus* all harvested in May. Reactions were stopped with ammonia solution and products were assayed by paper chromatography.

substrate concentration (Figure 5b), suggesting an extremely high affinity of MXE for XXXGol.

Figure 6 confirms that MXE has a much higher affinity for XXXGol than does XET. Considering only the four lowest acceptor concentrations tested in Figure 6(b), we estimate the apparent K_m of MXE for XXXGol to be $3.8 \pm 1.5 \mu\text{M}$. There was a decrease in rate at higher XXXGol concentrations. In contrast, the apparent K_m of *Equisetum* XET for XXXGol is approximately $80 \mu\text{M}$ (Figure 6a), indicating a much lower affinity.

Competition between xyloglucan and XXXGol as acceptor-substrates of MXE

In the presence of 2 mg ml^{-1} MLG ($\approx \frac{1}{2}K_m$) as donor-substrate and $1.4 \mu\text{M}$ [^3H]XXXGol as acceptor, an additional 6 mg ml^{-1} ($\approx 6 \mu\text{M}$) xyloglucan diminished the rate of total polysaccharide-to-[^3H]XXXGol transglucosylation, decreasing it almost to the XET rate obtained with that concentration of xyloglucan alone (Figure 5a). These data strongly suggest that *Equisetum* extracts contain two distinct enzymes: MXE and XET, with xyloglucan serving as donor-substrate for XET and as a competing acceptor-substrate for MXE.

High concentrations of MLG (unlike xyloglucan) only enhanced total polysaccharide-to-[^3H]XXXGol transglucosylation (Figure 5), indicating that MLG itself, at up to 8 mg ml^{-1} ($\approx 10\text{--}40 \mu\text{M}$; Burkus and Temelli, 2003), cannot appreciably compete with $1.4 \mu\text{M}$ [^3H]XXXGol as acceptor-substrate for MXE. This corroborates the finding that [^3H]heptasaccharides of MLG are only weak acceptor-substrates (Table 3). Thus, although MLG is the preferred donor-substrate of *Equisetum* MXE, its preferred acceptor-substrate is xyloglucan (or oligosaccharides thereof).

Discussion

Reaction catalysed by MXE

In this paper we show that *Equisetum* possesses extractable enzyme(s) with MXE activity: i.e., able to graft MLG to XGOs (and very probably also MLG to xyloglucan). Since energy-rich substrates such as ATP and UDP-Glc were not added in our *in-vitro* assays, the newly formed MLG-XGO bonds could only plausibly arise by transglucosylation reactions in which an existing bond in the donor-substrate is broken and its energy conserved in forming the new bond. Also, since the XGO used was [^3H]labelled at its reducing terminus, and yet the [^3H] became integrated into a high- M_r (paper-binding, chromatographically immobile) product during the reported reaction, we conclude that the XGO was the acceptor-substrate, and that MLG was the donor. If the XGO had been the donor, the [^3H]labelled moiety would have been released as part of a smaller leaving group (Figure 1). Direct evidence for the postulated MLG-XXXGol bond in a 'hybrid product' has

Table 3 Substrate specificity of *Equisetum* endotransglucosylases

| Donor-substrate | Transglycosylation rate (cpm h ⁻¹) with, as acceptor-substrate | | | |
|--------------------------|--|--|-----------------------------------|--|
| | [³ H]XXXGol | [³ H]laminar itetraitol ^a | [³ H]MLG ^b | [³ H]cello hexaitol ^c |
| Experiment A | | | | |
| Tamarind xyloglucan | 254 ± 5 | 0.35 ± 0.45 | | |
| Barley MLG ^d | 211 ± 10 | 1.70 ± 0.50 | | |
| <i>Cetraria</i> lichenan | 2.75 ± 0.27 | 0.65 ± 0.38 | | |
| None added | 0.82 ± 0.45 | 0.88 ± 0.52 | | |
| Experiment B | | | | |
| Tamarind xyloglucan | 172 ± 4 | 0.2 ± 0.9 | 4.5 ± 1.5 | 3.2 ± 0.6 |
| Barley MLG | 85 ± 7 | 0.7 ± 1.0 | 12.9 ± 1.5 | 6.9 ± 1.8 |
| None added | 0.2 ± 1.2 | -0.4 ± 1.0 | -1.4 ± 1.3 | -0.9 ± 1.5 |

Experiment A: the donor-substrates were supplied at 2 mg ml⁻¹, and the acceptors were supplied at 1 kBq per assay. The enzyme extract was from *Equisetum fluviatile* harvested in September. The reaction was conducted for 0–3 h, during which period the reaction was nearly linear; a regression line ± SE ($n = 4-6$) was fitted, giving the rate. Reaction products were assayed by the running-water method.

Experiment B: the enzyme extract, donor-substrates and protocol were similar to those in Experiment A, but the reaction products were dried on to a silica-gel TLC plate, and were assayed after washing with ethanol.

^aG3G3G3-glucitol.

^bMixed-linkage glucan heptasaccharides; a mixture of G4G4G3G4G4G3-glucitol and G4G3G4G4G4G3-glucitol.

^cG4G4G4G4G4-glucitol.

^dMixed-linkage (1 → 3,1 → 4)- β -D-glucan.

been presented in the case of a barley XTH acting weakly on MLG (Hrmová *et al.*, 2007). By analogy with the XET reaction (Rose *et al.*, 2002), the new bond may be formed to the 4-position of the non-reducing terminal Glc residue of the XGO. However, this remains to be tested in the case of MXE.

It might be expected that MLG-to-MLG transglucosylation also occurs. When MXE cleaves its donor-substrate (MLG) it will release a segment of MLG (the leaving group), the non-reducing terminus of which might be assumed capable of fitting back into the active site and serving as the acceptor-substrate in a reversible transglucosylation reaction. However, this assumption is not necessarily true: alternatively, MXE could resemble starch phosphorylase, which is a transglucosylase that obligatorily uses two different aglycones (acceptors). Starch phosphorylase reversibly transfers a Glc residue between amylose and phosphate, whereas it cannot transfer a Glc residue from one amylose chain to another, or from one phosphate ion to another (Cohn and Cori, 1948) – in contrast to the situation with sucrose phosphorylase (Doudoroff *et al.*, 1947). Our preliminary data suggest that MXE-rich extracts act only weakly with MLG oligosaccharides as acceptor-substrates (Table 3). In addition, there is no indication that MLG competes, even at high concentrations, with 1.4 μ M [³H]XXXGol as the acceptor-substrate (Figure 5). [The effects of such polysaccharide/oligosaccharide competition in the case of an XTH are illustrated in Figure 4(c) of Purugganan *et al.* (1997).] Our evidence thus indicates the existence of an *Equisetum* enzyme that mainly, or only, catalyses MLG-to-XGO (and MLG-to-xyloglucan) 'hetero-transglucosylation' (Figure 1).

Endogenous substrates of MXE in *Equisetum*

Equisetum is a taxonomically isolated genus, the ancestors of which diverged from those of all other living plants, including eusporangiate ferns, at least 370 million years ago (Bell and Hemsley, 2000; Smith *et al.*, 2006). *Equisetum* certainly has no close relationship with the Poales, the only other vascular plants reported to possess MLG.

For MXE to act *in vivo*, *Equisetum* would need to contain both xyloglucan and a polysaccharide resembling MLG. The Poales were long thought to be the only vascular plants possessing MLG, although an arabinosylated MLG-like polysaccharide occurs in a liverwort (Popper and Fry, 2003). However, the recent discovery of MLG in *Equisetum* (Fry *et al.*, 2008; Sørensen *et al.*, 2008) renders MXE action credible *in vivo*. For example, *E. fluviatile* stem polymers (alcohol-insoluble residue) consist of approximately 3.2% MLG (dry-mass basis). MLG is present in all five horsetail species tested (*E. arvense*, *E. fluviatile*, *E. scirpoides*, *E. sylvaticum* and *E. × trachyodon*; Fry *et al.*, 2008). We also confirmed that *Equisetum* contains xyloglucan, albeit with distinct structural differences from the xyloglucans of other land plants (Fry *et al.*, 2008).

MXE and XET activities are due to different enzymes

We confirmed that conventional XTHs (e.g. those present in crude homogenates of land plants other than horsetails) exhibit slight MXE activity, as reported previously (Hrmová

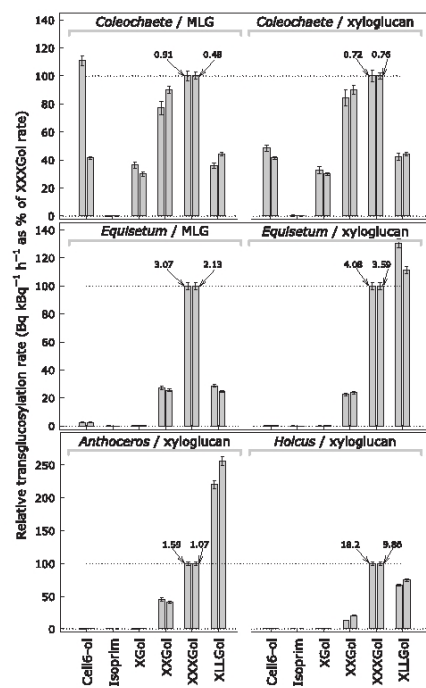


Figure 4. Acceptor-substrate profiles of endotransglucosylases from three plants and a charophytic alga. Donor-substrates were mixed-linkage (1 → 3,1 → 4)-β-D-glucan (MLG) or xyloglucan (indicated above each histogram). Potential acceptor-substrates tested were NaB³H₄-reduced oligosaccharides (left to right: cellobiose, isoprimeverose, xyloglucan-trisaccharide, -pentasaccharide, -heptasaccharide and galactosylated -nonasaccharide). The reaction time was 9 or 33 h (left and right bar, respectively). Data are corrected for the (very low) rates observed with no added polysaccharide. Error bars show 95% confidence limits of the counting ratios. Absolute rates (Bq kg⁻¹ h⁻¹) are indicated for the XXXGol data.

et al., 2007), but exceeded 100- to 1000-fold by XET activity, even in the MLG-rich Poales. This and the four following observations indicate that *Equisetum* MXE activity is not due to conventional XTHs.

- (i) XET but not MXE activity correlates with the reaction rate on three artificial water-soluble (1 → 4)-β-D-glucans (WSCA, HEC and CMC).
- (ii) MXE and XET activities peak in old and young *Equisetum* stems, respectively.
- (iii) MXE and XET activities have different acceptor-substrate preferences.

(iv) MXE has a higher affinity for XXXGol as acceptor-substrate ($K_m \approx 4 \mu\text{M}$) than any known XTH [the published K_m range being 33–320 μM (Fry *et al.*, 1992; Schröder *et al.*, 1998; Takeda *et al.*, 1996)].

Affinity of MXE for its donor-substrate

In *Equisetum* extracts, MXE and XET activities had apparent K_m values for barley MLG and tamarind xyloglucan (as donor-substrates) of approximately 4 and 0.35 mg ml⁻¹ respectively. A high affinity ($K_m = 0.62 \text{ mg ml}^{-1}$) was also reported in *Arabidopsis* XTH22 for fucosylated xyloglucan (Purugganan *et al.*, 1997). Thus, XTHs have a higher affinity (lower K_m) for their donor-substrate, xyloglucan, than MXE does for barley MLG. However, our negative data with lichenan suggest that MXE recognizes the tetrasaccharide repeat units of MLG, which are absent in lichenan. *Equisetum* MXE's only modern affinity for barley MLG may be attributable to barley MLG having about three times more trisaccharide than tetrasaccharide repeat units (Li *et al.*, 2006; Stone and Clarke, 1992), unlike *Equisetum* MLG, which is composed mainly of tetrasaccharide repeats (Fry *et al.*, 2008). In any case, the endogenous concentration of MLG in a fresh *Equisetum* cell wall is estimated to be approximately 10 mg ml⁻¹ (Fry *et al.*, 2008), well above the K_m of MXE for even barley MLG.

Water-soluble cellulose acetate and other artificial donor-substrates for XTHs

Water-soluble cellulose acetate had not previously been tested as a donor-substrate. Cellulose acetate with approximately 0.5–1.0 acetyl groups per Glc residue is water soluble (Gomez-Bujedo *et al.*, 2004). Because of the method of preparation, most of the –CO₂CH₃ groups in our WSCA would be attached to position 6 of the Glc residues, like the xylose residues of xyloglucan. We therefore suggest that WSCA mimics xyloglucan sufficiently well to act as a donor-substrate for conventional XTHs. Indeed, WSCA resembles the natural xyloglucans of the Poaceae, in which acetyl groups replace some of the xylose residues typical of dicot xyloglucans (Gibeaut *et al.*, 2005).

Hydroxyethylcellulose (HEC) also resembles xyloglucan, but with –CH₂CH₂OH groups in place of the xylose residues, and acts as an XTH donor-substrate (Ait Mohand and Farkaš, 2006; Hrmová *et al.*, 2007), although it acts more weakly than WSCA (Figure 3). CMC, which has –CH₂COO⁻ groups in place of xylose side chains, was a poor substrate for all enzyme extracts tested. Another polyanion, xyloglucan with its galactose and non-xylosylated Glc residues oxidized to galacturonate and glucuronate residues, respectively, is not a donor-substrate (Takeda *et al.*, 2008).

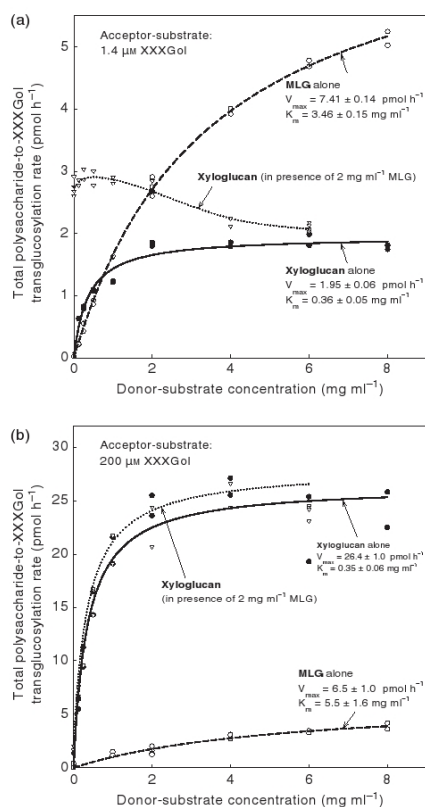


Figure 5. Effect of donor-substrate concentration on transglucosylation rate catalysed by *Equisetum* extract. The donor-substrate(s) tested were tamarind xyloglucan (●), barley MLG (○), or tamarind xyloglucan in the presence of 2 mg ml⁻¹ MLG (∇; x-axis indicates xyloglucan concentration). Acceptor-substrate: [³H]XXXGol at 1.4 μM (a) or 200 μM (b). The 100-μl assay mixture contained 10 μl extract of *Equisetum fluviatile* stems gathered in August (= 1.5 mg fresh weight). Products were assayed while the reaction was still linear (0–5 h). Rectangular hyperbolae are fitted to scattergrams of the two pure polysaccharides; apparent K_m and V_{max} estimates (± SE, determined by SIGMAPLOT) are indicated.

Acceptor-substrate specificity of XTHs and MXE

Equisetum MXE and *Equisetum* XET activities had markedly different acceptor-substrate preferences (Figure 4), which again supports the view that MXE activity is not due to an XTH.

The XET acceptor-substrate preferences of *Equisetum* (Figure 4) resemble those of many other non-polean

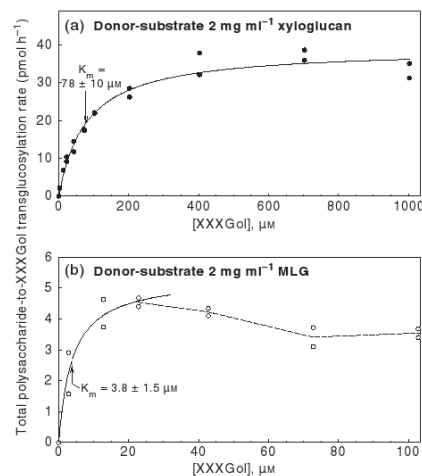


Figure 6. Effect of acceptor-substrate concentration on transglucosylation rate catalysed by *Equisetum* enzymes. The donor-substrate (2 mg ml⁻¹) was xyloglucan (a) or mixed-linkage (1 → 3,1 → 4)- β -D-glucan (MLG) (b). Note the different x-axis scales. The hyperbola in (a) has been fitted to the whole xyloglucan scattergram, giving a K_m estimate (± SE, as in Figure 5). In (b) the hyperbola is derived from the 0–20 μM XXXGol data only. Reaction products were assayed by the water-washing method; similar data (not shown) were obtained in an independent experiment by the paper chromatography method. The enzyme extract was as described in Figure 5.

plants. For example, in pea seedling extracts, the preferred acceptor-substrates are (with K_m values in parentheses): XLLG (19 μM) > XXXG (33 μM) > XXFG (50 μM) > XXG (approximately 200 μM); pea XET activity cannot act on GXG, XGG, XG, XGol or cello-oligosaccharides (Fry *et al.*, 1992; Lorences and Fry, 1993). *Tropaeolum* XTHs are also unable to use NaB³H₄-reduced cello-oligosaccharides as acceptors (Ait Mohand and Farkaš, 2006). For eight XTH isoenzymes from *Vigna* and *Brassica*, the order of preference is consistently XLLGol > XXLGol > XXXGol > XXGol (XGol was usually ineffective), their K_m values for XLLGol ranging from 16 to 130 μM (Steele and Fry, 2000). XET activities from cultured poplar cells and ripening kiwifruit have K_m values for XXXGol of 320 and 100 μM, respectively (Schröder *et al.*, 1998; Takeda *et al.* 1996). *Arabidopsis* XTH22 has a K_m for XLLGol of 73 μM, although it has a much higher affinity (K_m = 0.3 μM) for the non-reducing terminus of high-M_r xyloglucan as acceptor substrate (Purugganan *et al.*, 1997).

Extractable *Equisetum* XET activity had an apparent K_m for XXXGol (approximately 80 μM) typical of many other land plants. However, the apparent K_m of MXE for XXXGol

was approximately 4 μM , indicating an exceptionally high affinity, unequalled by any known XTHs. This again supports the idea that MXE is not a conventional XTH. The decrease in MXE rate at higher XXXGol concentrations (Figure 6b) may indicate that occupancy of the enzyme's acceptor-substrate subsite blocks the binding of MLG to the enzyme. Furthermore, competition experiments suggested that MXE uses high-*M*, xyloglucan as a favoured acceptor-substrate.

The very high affinity of MXE for xyloglucan-oligosaccharides supports the idea that 'hetero-transglucosylation', with MLG as donor-substrate and xyloglucan as the acceptor-substrate, is the main reaction catalysed by MXE *in vivo*.

Physiological roles of MLG transglucosylation in *Equisetum*

Extractable MXE and XET activities were highest in old and young *Equisetum* stems, respectively. This observation again supports the idea that MXE and XTHs are different proteins. It may also point to a role for MXE in wall strengthening during maturation, rather than in wall loosening during cell expansion. XTHs, in contrast, usually peak in young, rapidly growing tissue, where they may play a role in wall assembly (Ito and Nishitani, 1999; Thompson *et al.*, 1997) and/or the restructuring of existing wall material to enable reversible wall loosening (Thompson and Fry, 2001). However, some XTHs may contribute to wall strengthening and contraction (e.g. in tension wood), fulfilling this role in dead xylem cells for several years (Nishikubo *et al.*, 2007).

In angiosperms, MLG and xyloglucan are both very firmly hydrogen-bonded to cellulose microfibrils (Carpita *et al.*, 2001), possibly tethering them (Fry, 1989; Hayashi, 1989; Wada and Ray, 1978), and the same seems to apply in *Equisetum* (Fry *et al.*, 2008). We therefore speculate that MXE plays a role in older *Equisetum* tissues comparable to that of XTHs in tension wood: the linking of one hemicellulose chain to another, with the possibility of wall strengthening and repair.

MXE in grasses and cereals?

XTHs resemble MLG-hydrolases in their amino acid sequences (Strohmeier *et al.*, 2004), so the evolutionary recruitment of an XTH to function as an MXE seems feasible. However, no dedicated MXE appears to have emerged in the Poaceae during their approximately 65-million-year history. This may seem surprising because the Poaceae, like *Equisetum*, are rich in MLG. A purified XTH from barley has extremely low MXE activity relative to its XET activity (Hrmová *et al.*, 2007). We report the same for total grass proteins extractable by 300 mM succinate, which should include all unbound and many ionically wall-bound enzymes. It remains possible that non-extractable MXE is present in grass cell walls. However, most XTHs and *Equi-*

setum's MXE are freely extractable. Current evidence therefore suggests that grasses and cereals have indeed not (yet) evolved MXE, unlike *Equisetum*, which has probably been living with MLG in its walls for four or five times longer than the Poaceae. If the Poaceae do indeed lack MXE, then the possibility of engineering this *Equisetum* enzyme into them is potentially a valuable new strategy to improve the mechanical properties of the world's most important crops.

MXE as a primordial feature of plant cell walls

The finding of MXE activity in the Charophyta, the closest living algal relatives of land plants, suggests that such enzymes were a primordial feature of plant cell walls. In addition, the existence of XET activity in some charophytes was recently reported, and a candidate gene for an early endotransglucosylase has been identified in *Chara* (Van Sandt *et al.*, 2007a). The closest algal relatives of land plants have been variously considered to be the Coleochaetales (Graham, 1993; Petersen *et al.*, 2003) or the Charales (McCourt *et al.*, 2004). Our data show much higher extractable MXE (and XET) activity in the Coleochaetales than in the Charales (Table 1), possibly favouring the former as the closest living relatives of land plants. Native charophytic substrates, however, await identification.

Experimental procedures

Commercial polysaccharides

Tamarind seed xyloglucan was a generous gift from Dr K. Yamatoya, Dainippon Pharmaceutical Co. (<http://www.ds-pharma.co.jp>); CMC (Na⁺ salt, low viscosity), barley MLG, birchwood xylan and *Cetraria islandica* lichenan were from Sigma-Aldrich (<http://www.sigmaaldrich.com>); HEC was from Fluka (<http://www.sigmaaldrich.com>); and cellulose acetate was from Sigma-Aldrich.

Preparation of WSCA

Water-soluble cellulose acetate was synthesized by a modification of the method described by Gomez-Bujedo *et al.* (2004). Cellulose acetate (1 g) was dissolved in 10 ml of acetic acid/methanol (2.8:1, v/v) with shaking for 16 h at 20°C. Concentrated H₂SO₄ (500 μl) was then added and the mixture was incubated at 72°C for 45 min. This removes some acetyl groups from the polysaccharide by transesterification to methanol, accompanied by limited depolymerization. The mixture was then diluted into 40 ml of ice-cold water. After bench-centrifugation to pellet any remaining water-insoluble material, the supernatant was adjusted to 90% (v/v) acetone, and the pelleted WSCA was washed in acetone and dried. Complete de-esterification of a sample of the WSCA in 1 M NaOH at 20°C quickly precipitated all carbohydrate as material (cellulose) with $R_f = 0.00$ on silica gel TLC in butan-1-ol/acetic acid/water (2:1:1) (data not shown). Because of the method of preparation, most of the acetyl groups in our WSCA would be attached to position 6 of the

glucan backbone, thus resembling poalean xyloglucans (Gibeaut *et al.*, 2005).

Oligosaccharides

Details of the abbreviated nomenclature used for xyloglucan oligosaccharides are given by Fry *et al.* (1993); the main oligosaccharides used here are also summarized in Figure 1. Non-radioactive XXXGol was from Megazyme (<http://www.megazyme.com>). Specific cello-oligosaccharides and laminari-oligosaccharides were from Sigma or Megazyme. Tritiated XLLGol, XXXGol, XXGol, XGol, isoprimeveritol, celohexaitol and laminaritetrailol (typically approximately 7 MBq μmol^{-1}) were prepared by NaB^3H_4 reduction of the corresponding reducing oligosaccharides (Hetherington and Fry, 1993).

Mixed-linkage (1 \rightarrow 3,1 \rightarrow 4)- β -D-glucan oligosaccharides were prepared by partial digestion of barley MLG with lichenase; the heptasaccharide fraction was isolated by gel-permeation chromatography on Bio-Gel P-4 and reduced with NaB^3H_4 ; the products were purified by TLC on silica-gel in butanol/acetic acid/water (2:1:1). The preparation comprises a mixture of G4G4G3G4G4G3- $[\text{}^3\text{H}]\text{glucitol}$ and G4G3G4G4G4G3- $[\text{}^3\text{H}]\text{glucitol}$.

Plant materials

Cell-suspension cultures of the hornwort *Anthoceros agrestis* (Vogelsang *et al.*, 2006) were a generous gift of Dr Maik Peteresen, University of Marburg, Germany. *Marchantia polymorpha*, *Osmunda regalis*, *Polypodium vulgare*, *Holcus lanatus*, *Beta vulgaris*, *E. fluviale* and *E. arvense* were grown in a private garden in Edinburgh. *Chara vulgaris* and *Nitella opaca* (order Charales) were grown in a garden pond in Edinburgh. *Equisetum hyemale*, *E. scirpoides*, *E. variegatum* and *E. \times trachydodon* were generous gifts of the Royal Botanic Garden, Edinburgh. *Selaginella apoda* was grown in a greenhouse. *Equisetum sylvaticum* and *E. telmateia* were collected from the wild. The following algae were purchased from the Culture Collection of Algae and Protozoa (CCAP, <http://www.ccap.ac.uk>), and were maintained on 3N-BBM+V medium (http://www.ccap.ac.uk/media/recipes/3N_BBM_V.htm): *Spirogyra varians*, *Klebsormidium subtile*, *Klebsormidium fluitans* and *Coleochaete scutata*. *Coleochaete* was also grown in a medium that contained soil extract (JM:SE2, 7:3; http://www.ccap.ac.uk/media/documents/JM_SE.pdf), which produced paler green cells.

Enzyme extraction

Plant or algal tissue (1.5 g) was vigorously ground by pestle and mortar in 8 ml of an ice-cold extractant [10 mM CaCl_2 , 300 mM succinate (Na^+ , pH 5.5), 20 mM ascorbate, 15% (v/v) glycerol and 3% (w/v) polyvinylpyrrolidone] with a pinch of sand. The homogenate was kept at 0°C for 1–2 h to facilitate desorption of ionically bound enzymes, filtered through Miracloth (Calbiochem, <http://www.emdbiosciences.com>) and centrifuged at 12 000g for 5 min; the supernatant was used immediately or stored at -20°C (MXE and XET activity were both retained for at least a year).

Endotransglucosylase assays

Endotransglucosylase assays were based on the method of Fry *et al.* (1992). Unless otherwise stated, reaction mixtures typically contained a polysaccharide (2 mg ml^{-1} ; donor-substrate), a carrier-

free tritiated oligosaccharide (2–5 μM ; acceptor-substrate), 4 mM CaCl_2 , 120 mM succinate (Na^+ , pH 5.5), 8 mM ascorbate, 6% (v/v) glycerol and the enzymes extracted from 1.6 to 3.2 mg fresh weight of plant tissue, in a final volume of 30–50 μl , and were incubated at 20°C. The reaction was usually stopped by the addition of 20 μl of formic acid; however, when WSCA was the donor-substrate, we used 20 μl of 35% NH_3 so that the WSCA was converted to cellulose.

In assays to determine the effect of oligosaccharide concentration, the dose of $[\text{}^3\text{H}]\text{XXXGol}$ was held constant and various proportions of non-radioactive XXXGol were added, giving different specific radioactivities, from which we calculated absolute reaction rates.

High-*M*, tritiated products were separated from unreacted oligosaccharides by one of four methods: (i) products were dried on to Whatman 3MM paper (4 \times 4-cm; <http://www.whatman.com>), which was then washed in running tap water for 1–2 days, and then redried and assayed for ^3H , (ii) products were chromatographed on 3MM paper by the descending method in ethyl acetate/acetic acid/water (10:5:6 by vol.) for 48 h, after which the origin was assayed for ^3H , (iii) ethanol was added to a final concentration of 75% (v/v), and the precipitated polysaccharides were then washed by repeated resuspension and pelleting in 75% ethanol, until the supernatants were non-radioactive, after which the final pellet was redissolved in water and assayed for ^3H , (iv) products were dried on to squares pencilled on a silica-gel TLC plate, which was then washed twice overnight in 1 l of ethanol/acetic acid/water (6:3:1 by vol.), after which the plate was dried, and the squares were cut out and assayed for ^3H . Method (i) was used unless otherwise stated. Method (ii) was tested in case some polysaccharides did not remain firmly paper-bound in running water; however, we obtained essentially identical yields of ^3H -polymers with these two methods for both xyloglucan and MLG (data not shown), and the simpler method (i) was therefore preferred. Methods (iii) or (iv) were used for celohexaitol or MLG-derived heptasaccharides, because these are difficult to elute from paper.

Radioactivity on dry paper or silica gel was assayed by scintillation counting in Wallac OptiScint (PerkinElmer, <http://www.perkinelmer.co.uk>). For aqueous solutions, we used Wallac OptiPhase (PerkinElmer).

Acknowledgements

We thank Janice Miller for technical assistance and the BBSRC (UK) for funding this research. KEM thanks the Darwin Trust of Edinburgh for a studentship.

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