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Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass

Keith Gourlay [a], Jinguang Hu [a], Valdeir Arantes [a], Martina Andberg [b], Markku Saloheimo [b], Merja Penttilä [b], Jack Saddler [a,*]

[a] Forest Products Biotechnology/Bioenergy Group, Department of Wood Science, Faculty of Forestry, University of British Columbia, 2424 Main Mall, Vancouver, BC, Canada V6T 1Z4
[b] VTT Technical Research Centre of Finland, P.O. Box 1000, FIN-02044 VTT, Finland

HIGHLIGHTS

- Swollenin, a non-hydrolytic disruptive protein, is applied to pretreated corn stover.
- Low levels of monomeric and oligomeric sugars were solubilized.
- Swollenin acted synergistically with xylanases (~300% increase in xylose release).
- Disruption of the hemicellulosic fraction of the biomass enhances xylan hydrolysis.
- Xylan removal improves the overall efficiency of enzymatic biomass deconstruction.

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ABSTRACT

A key limitation in the overall hydrolysis process is the restricted access that the hydrolytic enzymes have due to the macro-and-micro structure of cellulose and its association with hemicellulose and lignin. Previous work has shown that several non-hydrolytic proteins can disrupt cellulose structure and boost the activity of hydrolytic enzymes when purer forms of cellulose are used. In the work reported here, Swollenin primarily disrupted the hemicellulosic fraction of pretreated corn stover, resulting in the solubilisation of monomeric and oligomeric sugars. Although Swollenin showed little synergism when combined with the cellulase monocomponents exoglucanase (CEL7A) and endoglucanase (CEL5A), it showed pronounced synergism with xylanase monocomponents Xylanase GH10 and Xylanase GH11, resulting in the release of significantly more xylose (>300%). It appears that Swollenin plays a role in amorphogenesis and that its primary action is enhancing access to the hemicellulose fraction that limits or masks accessibility to the cellulose component of lignocellulosic substrates.

1. Introduction

The innate recalcitrance of plant cell walls, stemming from their complex, heterogeneous nature, has hindered the cost-effective depolymerisation of cell wall carbohydrates to useable sugars (Himmel et al., 2007; Klein-Marcuschamer et al., 2012). Plant biomass is composed of a matrix of polymers and macromolecules, including cellulose, hemicelluloses, lignin and extractives (Himmel et al., 2007). The cellulose chains are typically organized into microfibrils, which are densely-packed bundles of cellulose encased within a sheath of hemicelluloses and lignin, in a manner which restricts access to the cellulose chains (Arantes and Saddler, 2010, 2011). In nature, these polymers are typically degraded by a consortium of micro-organisms, including bacteria and fungi, which produce a wide range of proteins/enzymes designed to efficiently break down biomass, primarily to access its component sugars as an energy source (Chundawat et al., 2011).

The biochemical based "biorefinery process" utilizes a suite of enzymes, commonly termed the "cellulase mixture" to break down biomass into its component sugars for subsequent conversion of these sugars into fuels and chemicals. This cellulase mixture includes a variety of well-studied glycosyl hydrolases, including cellulases and hemicellulases (Engel et al., 2012; Saloheimo and

Abbreviations: CBM, carbohydrate binding module; SPCS, steam pretreated corn stover; PACE, polyacrylamide carbohydrate electrophoresis; HMW, high molecular weight; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid.

* Corresponding author. Tel.: +1 604 822 9741; fax: +1 604 822 8157.
E-mail address: jack.saddler@ubc.ca (J. Saddler).

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Pakula, 2012). More recently it has been shown that some fungi, including *Hypocrea jecorina*, also produce a class of oxidative enzymes, known as polysaccharide mono-oxygenases, which directly cleave cellulose chains through an oxidative mechanism, and appear to act synergistically with the canonical hydrolytic enzymes (Harris et al., 2010; Quinlan et al., 2011; Vaaje-Kolstad et al., 2010).

While the actions and mechanisms of the hydrolytic and oxidative proteins are relatively well understood, there is evidence to suggest that lignocellulolytic organisms also produce a third class of biomass degrading proteins which have been termed "disrupting" or "amorphogenesis inducing" proteins (reviewed in Arantes and Saddler (2010)). Examples of these types of proteins include the Expansins (Cosgrove, 2000), bacterial Expansin-like proteins (Lee et al., 2010), fibril forming protein (Banka et al., 1998), certain carbohydrate binding modules (Din et al., 1991, 1994; Gao et al., 2001), and the fungal Expansin-like proteins, Loosenin (Quiroz-Castañeda et al., 2011) and Swollenin (Gourlay et al., 2012; Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013).

Although a direct catalytic mode of action of these proteins has not been identified, amorphogenesis-inducing proteins appear to disrupt and weaken the plant cell wall without releasing a significant amount of soluble sugars (Arantes and Saddler, 2010; Banka et al., 1998; Cosgrove, 2000; Din et al., 1991, 1994; Gao et al., 2001; Gourlay et al., 2012; Jäger et al., 2011; Lee et al., 2010; Quiroz-Castañeda et al., 2011; Saloheimo et al., 2002; Verma et al., 2013). The role of these proteins in biomass deconstruction appears to be to facilitate the action of the hydrolytic and oxidative enzymes by promoting amorphogenesis of the substrate, thereby opening up the plant cell wall and providing the catalytic enzymes with enhanced access to the glycosidic linkages within the sugar polymers (Arantes and Saddler, 2010, 2011).

One of the amorphogenesis-inducing proteins, Swollenin, was first discovered as a *H. jecorina* fungal protein homologous to the plant β-Expansin protein family (Saloheimo et al., 2002). Expansins are known to promote cell wall extension in plants, likely through a mechanism involving the disruption of hydrogen bonding between adjacent sugar chains (Cosgrove, 2000). The structure of Swollenin includes an N-terminal cellulose-specific carbohydrate binding module (CBM) connected via a linker region to a C-terminal expansin-like domain (Saloheimo et al., 2002). Additionally, this expansin-like domain contains mammalian fibronectin type III-like repeats, which have been shown to impart flexibility to proteins, and may be involved in the mechanism of action of Swollenin (Saloheimo et al., 2002). Previous work on the function of CBMs (Tomme et al., 1988) and expansins (Cosgrove, 2000) suggests that the primary role of the CBM is to direct the biomass-disrupting expansin-like domain to the target polymers within the biomass.

Swollenin has been shown to disrupt and swell cotton fibers and Valonia cell walls, weaken filter paper, reduce the crystallinity and particle size of filter paper, Avicel, and α-cellulose, release small particles from cotton fibers and enhance the hydrolysis of a range of model cellulolytic substrates, while causing negligible sugar release (Chen et al., 2010; Gourlay et al., 2012; Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013; Wang et al., 2011). These previous experiments have relied heavily on imaging techniques, including light microscopy (Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013) and Scanning Electron Microscopy (Gourlay et al., 2012).

Recent work has also shown that Swollenin is capable of sloughing off roughened patches of cellulose from mercerized cotton fibers while causing an increase in the amount of accessible amorphous cellulose and, in particular, an increase in the amount of accessible crystalline cellulose (Gourlay et al., 2012). This increase in the amount of available accessible crystalline cellulose suggested that Swollenin acts by separating relatively intact (crystalline) regions of cellulose from each other (i.e. by separating microfibrils) (Gourlay et al., 2012).

It has previously been suggested that Swollenin promotes the disruption of cellulosic biomass through a non-hydrolytic weakening of hydrogen bonding (Chen et al., 2010; Gourlay et al., 2012; Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013; Wang et al., 2011). Evidence to support this stems from the observation that Swollenin acts to disperse/disaggregate the fibers within filter paper. Since the predominant force between adjacent fibers in filter paper is hydrogen bonding, it stands to reason that Swollenin is capable of disaggregating the fibers through a weakening of hydrogen bonding (Saloheimo et al., 2002; Wang et al., 2011).

In the work reported here, the effects of Swollenin on a native pretreated lignocellulosic substrate were investigated. As will be described in more detail within the paper, Swollenin alone could solubilize oligomeric sugars from steam-pretreated corn stover in a weakly temperature-dependent manner. However, the main focus of the work was to assess whether Swollenin could act synergistically with cellulase and xylanase monocomponents and cocktails when acting on a "realistic biomass substrate" such as steam-pretreated corn stover. It was also hoped that the work would provide some insights into the mode of action of Swollenin when applied to a commercially-relevant pretreated lignocellulosic substrate.

2. Methods

2.1. Materials

SPCS was prepared by steam pretreating corn stover at 190 °C for 5 min with 3% SO_2, followed by oven-drying, grinding, and passing through a 40-mesh sieve. All SPCS was washed thoroughly in 50 mM Sodium Acetate buffer, pH 5, prior to running the reactions.

Swollenin was expressed in *Trichoderma reesei* under the *cbh1* promoter with a C-terminal His_6-tag, and purified using immobilized metal ion affinity chromatography followed by anion exchange chromatography (DEAE Sepharose) following the procedure that we used previously with CEL61A (Karlsson et al., 2001).

The monocomponent hydrolytic enzymes CEL7A, CEL5A, XYN10A and XYN11A were purified as we described previously (Hu et al., 2011).

The effects of Swollenin on SPCS were studied by incubating 10 mg SPCS (dry weight), with 100 μg Swollenin or BSA protein control (Sigma). Reactions were carried out in 2 mL microcentrifuge tubes in 50 mM sodium acetate buffer (Fisher), pH 5.0, in a total volume of 1.5 mL. Samples were incubated in a FinepcrCombi SV12 hybridization incubator at 30 rpm for 18 h, and centrifuged for 10 min at 13,000 rpm.

2.2. High performance liquid chromatography

The supernatants from Swollenin-treated SPCS were analyzed for the presence of oligomeric and monomeric sugars. Monomeric sugars were determined by High Performance Liquid Chromatography (HPLC) on a Dionex DX-3000 HPLC system fitted with an AS3500 autosampler, a UV detector and a GP40 gradient pump. Oligomeric sugars were determined by acid hydrolyzing the supernatant to convert all oligomers into monomers. The total monomers (monomers released by Swollenin and monomers produced from acid-hydrolyzing the oligomers) in the supernatant were then quantified by HPLC. The amount of oligomers released by Swollenin was quantified by subtracting the monomers present in the

unhydrolyzed supernatant from the total monomers observed in the acid-hydrolyzed supernatant.

The concentration of acetic acid in the SPCS supernatants was determined using HPLC (ICS-500) fitted with an AS3500 autosampler, a UV detector and a GP40 gradient pump. The compounds were separated on an Aminex HPX-87H column (Biorad) at a temperature of 50 °C using 5 mM H_2SO_4 at 0.6 ml/min as the eluent, and detected by UV absorbance.

2.3. Polyacrylamide carbohydrate electrophoresis (PACE)

PACE was carried out by conjugating the solubilized monomers and oligomers to a fluorescent probe and separating these sugars by size on a polyacrylamide gel as described previously (Goubet et al., 2011). In brief, 500 μL of supernatants from the Swollenin and control treated SPCS were lyophilized. The dried sugars were conjugated to the fluorescent probe, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) (Invitrogen) by dissolving the sugars in 10 μL 0.1 M ANTS in 3/17 (v/v) acetic acid/water and 10 μL 0.1 M $NaCNBH_3$ (Acros Organics) in DMSO (Fisher) and incubating overnight at 60 °C. Tagged sugars were then lyophilized and dissolved in 100 μL 6 M Urea (BioRad) and stored at −20 °C. Polyacrylamide gels (19.5% w/w) were poured and sugar samples were run for 45 min at 200 V and imaged on an AlphaImager 2200 (Alpha Innotech). Densitometry was performed using AlphaImager 2200 Software, with manual peak selection.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of SPCS was carried out to determine the degree of synergy of Swollenin with isolated hydrolytic components. In brief, hydrolysis was performed using 10 μg hydrolytic enzyme plus 10 μg Swollenin or BSA per mg dry SPCS, as well as 2 μg β-glucosidase (Novo 188, Novozymes) per mg dry SPCS. Hydrolysis was carried out using 10 mg SPCS (dry weight) in 1 mL 50 mM Na-Acetate buffer, pH 5.0 (Fisher).

The degree of synergy (DS) was calculated by dividing the amount of sugar released by Swollenin in combination with the various hydrolytic enzymes by the sum of the amount of sugar released by Swollenin alone and each hydrolytic enzyme in the presence of a BSA control.

3. Results and discussion

In this work we have shown that Swollenin, which had previously been shown to promote amorphogenesis of model cellulosic substrates (Gourlay et al., 2012; Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013) was also capable of disrupting a more realistic biomass substrate, steam pretreated corn stover (SPCS).

3.1. Quantification of oligomer release by Swollenin

3.1.1. HPLC analysis of solubilized sugars

Earlier work (Gourlay et al., 2012) has shown that when Swollenin is incubated with steam pretreated corn stover (SPCS), the turbidity of the supernatant, as measured at OD_{600}, was lower than the supernatant of the control-treated SPCS. This implied that the Swollenin acted by solubilizing the small particles present in the SPCS supernatant. To try to better quantify this proposed mechanism we collected the supernatants of the control and Swollenin-treated SPCS to see if any monomeric or oligomeric sugars had been released.

It was apparent (Fig. 1) that Swollenin was capable of releasing significant amounts of oligomers, as well as low amounts of glucose and xylose, from the SPCS substrate at 50 °C. The oligomers were predominantly composed of approximately equal amounts of glucose and xylose (Fig. 1A), but also appeared to contain trace amounts of mannose, arabinose and galactose (Fig. 1B), implying that Swollenin might also play a role in hemicellulose dissolution as well as cellulose degradation. The solubilisation of the hemicellulose fraction was particularly apparent when expressed as a percent conversion of the starting biomass (Fig. 1C). Swollenin activity was found to be temperature dependant with incubation at 20, 35, and 50 °C releasing slightly increasing amounts of sugar from the SPCS (Fig. 2). A temperature increase from 20 °C to 50 °C resulted in slightly more than a 2-fold increase in sugar release.

The release of low levels of xylo-oligomers by BSA may be due to the fact that BSA contains both hydrophilic and hydrophobic regions, and has previously been observed to act in a similar manner to surfactants (Tween 20) when applied to pretreated biomass (Eriksson et al., 2002). This apparent surfactant effect of BSA could explain the release of xylo-oligomers observed here.

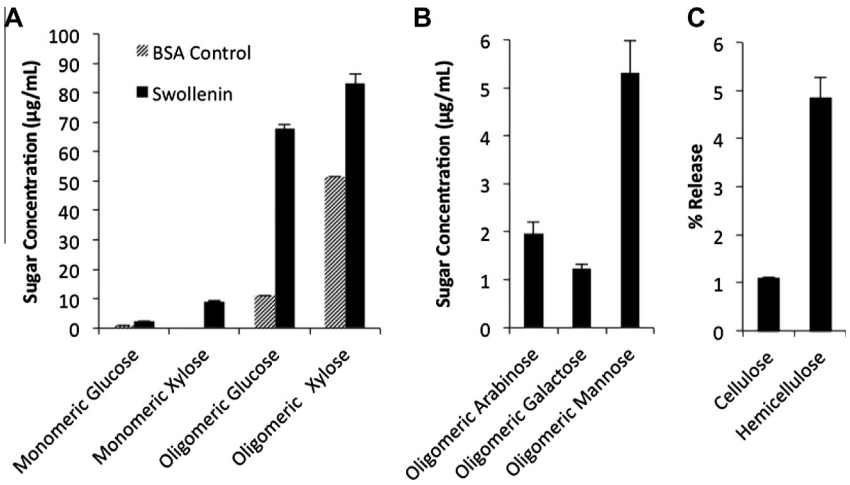

Fig. 1. Sugars detected after incubation of SPCS with either Swollenin or a BSA control. (A) Oligomeric and monomeric glucose and xylose detected after incubation with either Swollenin or a BSA control. (B) Amount of oligomeric arabinose, galactose and mannose detected after incubation with Swollenin. (These sugars were not detected in monomeric form, and oligomers were undetectable in the BSA-treated samples.) (C) Percent release of cellulosic and hemicellulosic sugars in a soluble form after incubation with Swollenin, relative to the original glucan and total hemicellulose content of the pretreated substrate. Values are relative to a BSA control. All reactions were run in triplicate and error bars represent one standard deviation from the mean.

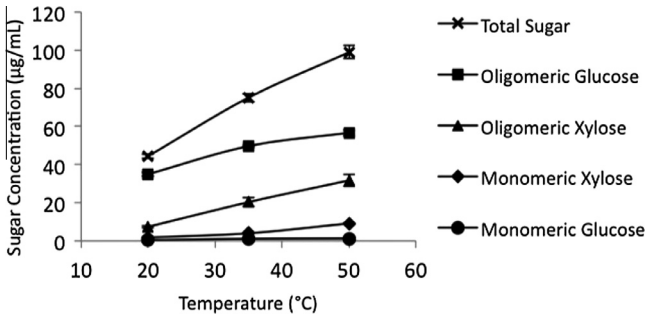

Fig. 2. Amount of monomeric and oligomeric glucose and xylose detected after incubation of Swollenin with SPCS between 20 °C and 50 °C. Total sugar represents the sum of all oligomeric and monomeric glucose and xylose. Values are relative to a BSA control, reactions were run in triplicate, and error bars represent one standard deviation from the mean.

3.1.2. Polyacrylamide carbohydrate electrophoresis

As previous work had shown that the size and composition of the solubilized oligomers could be quantified by polyacrylamide carbohydrate electrophoresis (PACE) (Goubet et al., 2011), the supernatants from Swollenin or protein control (BSA) treated SPCS were compared to a series of sugar standards, including glucose, xylose, xylobiose, xylotriose, xylotetraose, and xylohexaose (Fig. 3A). This was followed by a semi-quantitative densitometry analysis of the samples (Fig. 3B), and the percent increase in the release of the various sugars was subsequently estimated (Fig. 3C). Swollenin treatment resulted in a pronounced increase in the amount of glucose, xylose, and xylotriose released as well as an increase in cellobiose, xylobiose and xylotetraose. Swollenin treatment also caused a marked increase in the amount of higher molecular weight compounds that were detected in the supernatant. These higher molecular weight (HMW) compounds likely represent longer-chain (>4 monomers in length), substituted xylan fractions. The lack of distinct bands in this HMW region suggested that these longer xylan chains were probably substituted by arabinose, glucuronic acid and acetyl groups, as they are predominantly present within the glucuronoarabinoxylan of commelinid monocots such as maize.

Swollenin treatment also resulted in the release of a low molecular weight compound that was absent in the control-treated sample. Although we postulated that this band might result from the release of acetyl groups, subsequent HPLC analysis of the Swollenin and control-treated supernatants did not show any increase in the amount of free acetic acid detected in the Swollenin-treated supernatants.

3.1.3. Significance of Swollenin-induced oligomer release

The disruption or "opening up" of the lignocellulosic matrix has been suggested to be one of the key process limiting steps involved in the rapid depolymerizing of the carbohydrates within biomass into its component sugars (Arantes and Saddler, 2011; Lee et al., 1996). Although there has been a considerable amount of work carried out in the past on the little-understood family of proteins involved in promoting the disruption, or amorphogenesis, of lignocellulosic substrates such as Expansins, CBMs, etc., little is known about their actual action or how they might interact with the hydrolytic enzymes that constitute the majority of the proteins in a "cellulase mixture". In the work described here we have shown that the non-hydrolytic Swollenin protein, which had previously been shown to promote amorphogenesis of model cellulosic substrates (Gourlay et al., 2012; Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013) was also capable of disrupting a more realistic biomass substrate such as steam pretreated corn stover (SPCS).

Previous work had shown that Swollenin did not have significant hydrolytic activity on cellulose or on purified birchwood xylan (Jäger et al., 2011; Saloheimo et al., 2002). In the work reported here Swollenin alone was able to solubilize low levels of both the cellulose and hemicellulose components present in the SPCS. This suggested that the release of oligomers from SPCS observed here was due to the Swollenin-induced release of pre-existing oligomers bound within the substrate. It is possible that this solubilization was due to the non-hydrolytic weakening of hydrogen bonding between the xylo- and cello-oligomers and the cellulose and/or xylan within the substrate in a manner similar to that observed for the related Expansin family of proteins (Cosgrove, 2000). Although the exact mechanism of action is unresolved, previous work suggested that the flexible fibronectin type III repeats within the Swollenin protein may be involved in facilitating cellulose disruption (Saloheimo et al., 2002).

The solubilisation of xylo-oligomers by Swollenin suggests that it may play an important role in the metabolism of *H. jecorina*. Previous work has shown that Swollenin is generally regulated in much the same manner as other biomass-degrading enzymes (Ilmén et al., 1997). However, Swollenin is constitutively expressed at low levels, unlike the more tightly repressed major cellulase enzymes (Ilmén et al., 1997). As Swollenin treatment of SPCS results in the release of xylobiose and other xylo-oligomers which are

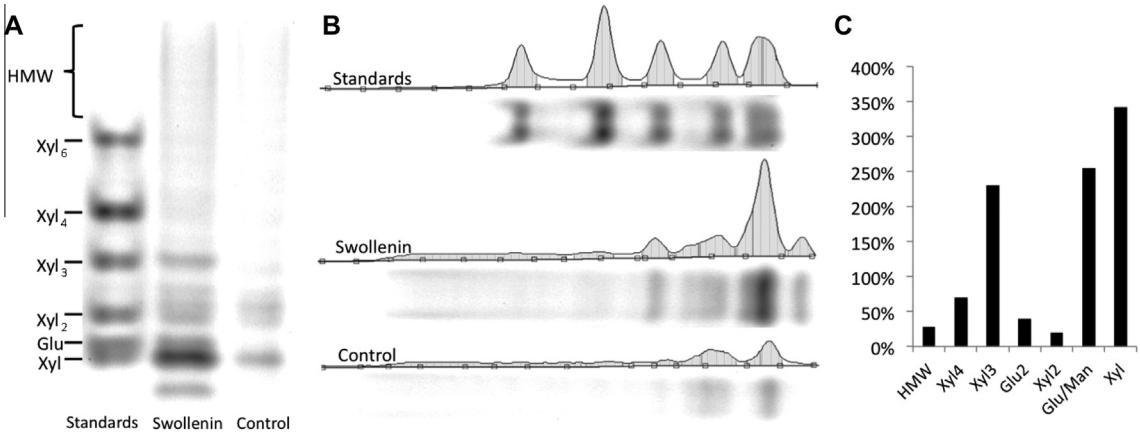

Fig. 3. PACE analysis of released carbohydrates. (A) Carbohydrates released after incubation of Swollenin and a BSA control with SPCS. Standards are labeled, HMW represents the higher molecular weight fraction. (B) Densitometry analysis and peak selection. (C) Quantification of the relative increase in sugars released by Swollenin compared to the control. Values represent the percent increase in peak area for each sugar.

known inducers of the major cellulases and xylanases (Aro et al., 2005), another possible function of Swollenin may be to release soluble inducers upon contact with a lignocellulosic substrate, thereby leading to increased genetic transcription and subsequent up-regulation of the enzymes required for biomass degradation.

3.2. Synergism between Swollenin and hydrolytic cellulase and xylanase monocomponents

To try to further elucidate the role that Swollenin might play in SPCS hydrolysis, several purified cellulase and xylanase enzymes were incubated with Swollenin and their possible synergistic interactions were assessed. β-glucosidase was also added to these combinations, to decrease possible end-product inhibition that might arise from cellobiose accumulation (Murphy et al., 2013). When Swollenin was incubated with either an exoglucanase (CEL7A), an endoglucanase (CEL5A), or two endoxylanases (XYN10A and XYN11A) which were isolated and purified from *H. jecorina*, little or no synergism occurred as assessed by the amount of glucose released compared to the hydrolytic enzymes alone (Fig. 4A).

However, it was apparent that there was a strong, synergistic interaction between Swollenin and CEL5A, XYN10A, and XYN11A resulting in considerably more xylose release (Fig. 4B). When the degree of synergism between Swollenin and the various hydrolytic enzymes were compared (Table 1), the most pronounced synergistic interaction occurred between Swollenin and XYN11A where a more than 300% increase in xylose release was obtained when compared to the xylose released when the two proteins were added separately. It was apparent that Swollenin exhibited strong synergistic interaction with endoxylanases.

One possible explanation for this more marked effect on the hemicellulose component is that Swollenin is able to promote amorphogenesis within the relatively loosely ordered xylan structure, but less able to disrupt the more highly-ordered structures of the cellulose. Related work has shown how significant a role xylan "masking" can play in hindering cellulose hydrolysis, and how beneficial the addition of xylanase can be in enhancing fiber swelling and overall cellulose accessibility (Hu et al., 2011; Liao et al., 2005).

3.3. Importance of Swollenin in biomass deconstruction

Non-hydrolytic, non-oxidative proteins such as Swollenin have been shown to promote the disruption, or amorphogenesis, of lignocellulosic substrates during the enzymatic deconstruction of biomass (Arantes and Saddler, 2010). Previous work has shown that Swollenin can aid in the disaggregation of model cellulosic substrates (Gourlay et al., 2012; Jäger et al., 2011; Saloheimo et al., 2002; Verma et al., 2013). The work reported here suggests that, when more commercially relevant substrates such as steam pretreated corn stover are used, the greater influence of Swollenin addition to a cellulase mixture may be in enhancing accessibility to the hemicellulose fraction that limits or masks accessibility to the cellulose component of a lignocellulosic substrate.

Table 1
The degree of synergism (DS) between various purified hydrolytic enzymes and Swollenin on SPCS.

	Glucose		Xylose	
	DS	σDS	DS	σDS
CEL7A	1.09	0.17	1.11	0.25
CEL5A	0.77	0.08	1.75	0.21
XYN10A	1.15	0.10	2.75	0.17
XYN11A	0.91	0.10	3.34	0.34

4. Conclusions

The addition of Swollenin alone resulted in the release of low levels of soluble monomers and oligomers from steam pretreated corn stover. When various protein cocktails were assessed Swollenin showed strong synergism with the xylan degrading enzymes Xylanase GH10 and Xylanase GH11. Swollenin's inability to directly hydrolyze pure cellulose or xylan, combined with the Swollenin-induced oligomer release, suggests that Swollenin weakens and disrupts the substrate by promoting amorphogenesis. This disrupting activity primarily facilitated the enzymatic hydrolysis of xylan, likely enhancing enzyme access to the cellulose component of pretreated corn stover.

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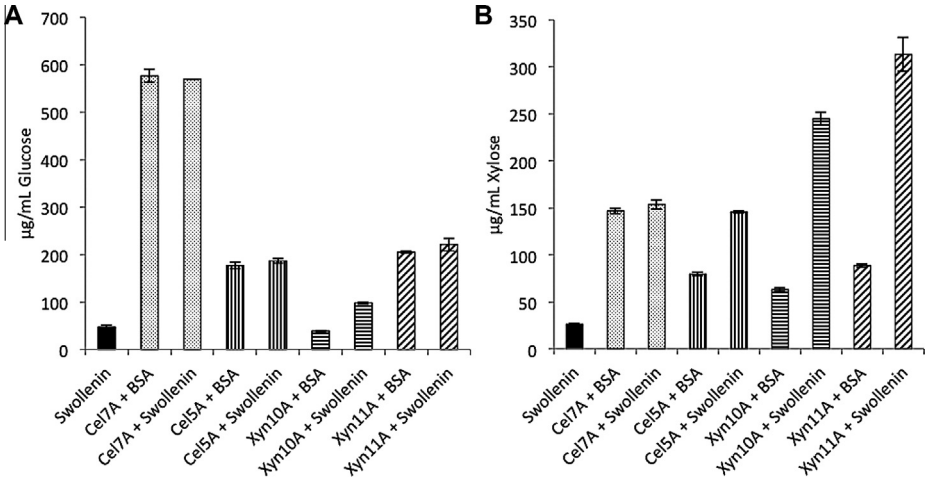

Fig. 4. The amount of glucose (A) and xylose (B) released from SPCS after incubation of various purified hydrolytic enzymes with either a BSA control or Swollenin. Reactions were run in triplicate and error bars represent one standard deviation from the mean.

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