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ORIGINAL RESEARCH PAPER

Xanthomonas campestris expansin-like X domain is a structurally disordered beta-sheet macromolecule capable of synergistically enhancing enzymatic efficiency of cellulose hydrolysis

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Abstract

Objectives   To biochemically characterize an expansin-like X protein domain from *Xanthomonas campestris* (XcEXLX1) and to study its synergy with cellulases in cellulose depolymerization.

Results   The protein was purified using a combination of ion exchange and size exclusion chromatography rendering about 30 mg pure protein/l culture medium. Circular dichroism spectroscopy and small-angle X-ray scattering studies of XcEXLX1 reveal that it is a strongly disordered β-sheet protein. Its low resolution envelope fits nicely the crystallographic structure of the homologous protein EXLX1 from *Bacillus subtillis*. Furthermore, we demonstrate that XcEXLX1 shows a synergistic, pH-dependent effect when combined with a commercial enzymatic preparation (Accellerase 1500), enhancing its hydrolytic activity on a cellulosic substrate. The strongest effect was observed in acid pHs with an increase in sugar release of up to 36 %.

Conclusion   The synergistic effect arising from the action of the expansin-like protein was considerable in the presence of significantly larger amounts of the commercial enzymatic cocktail then previously observed (0.35 FPU of Accellerase 1500/g substrate).

Keywords   Accellerase · Biofuel · Cellulase · Cellulose depolymerization · Expansin synergism · *Xanthomonas campestris*

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Introduction

Renewable fuels, especially biofuels from lignocellulosic biomass, are considered an important, sustainable alternative to fossil fuels. The conversion of lignocellulosic material, such as, agricultural and forest residues, into fermentable sugars can be achieved by a combination of physical or chemical pretreatment and enzymatic hydrolysis (Saha 2004).

Although enzymatic hydrolysis has been extensively studied until recently the role of auxiliary proteins has not received enough attention. Expansins are responsible for the loosening of the cellulosic cell walls thereby allowing the plants to grow (Cosgrove 2000).

Although expansins cause plant cells expansion and modify their cell wall mechanical properties, the

capacity of expansins to enhance efficiency of the cellulosic biomass remains controversial. Some reports have concluded that although lacking hydrolytic activity, expansins in general, including those from microbial origin, are capable of promoting improvements in the enzymatic hydrolysis of crystalline cellulose by synergizing with cellulases (Cosgrove 2000). Other studies have reached the opposite conclusions and suggested that microbial expansins do not act synergistically with selected cellulases for soluble sugar release from cellulose (Georgelis et al. 2014).

Here, we describe the cloning, expression and biophysical characterization of an expansin-like X domain from *Xanthomonas campestris* (XcEXLX1) and its synergistic effect on the hydrolysis of cellulosic substrates by a commercial cellulase mixture. We show that XcEXLX1 is a naturally disordered β-sheet protein, capable of boosting the enzymatic hydrolysis of cellulosic substrates by up to 36 %.

Materials and methods

Construction of the expression plasmid

The expansin-like X gene of *X. campestris* EXLX1Xc was amplified using PCR and inserted into the IPTG-inducible expression vector pSMT3 (provided by Prof. Lawrence Shapiro, Columbia University, NY-USA) (see supporting information).

Expression of the protein and its purification process

The culture of *E. coli* Rosetta (DE3) pLysS was grown in lysogeny broth and purified using Ni–NTA Agarose column (Qiagen) and ÄKTA purifier 10 system (GE Healthcare Life Sciences) (see supporting information).

Circular dichroism spectroscopy

CD spectra of XcEXLX1 in 20 mM NaCl, 10 mM Tris/HCl, pH 8 were recorded on a Jasco J-720 spectral polarimeter using 0.1 cm path length cuvettes (Jasco, Tokyo, Japan). The spectra were collected from 190 to 280 nm with 100 nm min^{-1} increment step, 10 s averaging time, 1 nm bandwidth and a response time of 0.5 s.

Solvent contributions were subtracted from the original spectra curves. We deconvoluted the CD spectra using the Dichroweb webserver (Whitmore and Wallace 2004) to obtain the estimates of the protein secondary structure composition.

Small angle X-ray scattering (SAXS)

Small angle X-ray scattering experiments were conducted at the D02A-SAXS2 Laboratório Nacional de Luz Síncrontron (LNLS) using a bi-dimensional detector (MarResearch, EUA). A distance of 1000 mm between it by the sample and the detector was used, covering a range of momentum transfer 0.013 Å^{-1} < q < 0.33 Å^{-1} (q = 4π sin(θ)/λ), where 2θ is the scattering angle and λ = 0.148 nm is the X-ray wavelength. The protein samples were measured at 2 and 5 mg/ml in 10 mM Tris/HCl, 20 mM NaCl, pH 8 buffer.

The data was analyzed using a FIT2D program (Hammersley 1997), normalizing the data by the intensity of the beam and multiplying it by sample absorption. The scattering of the buffer was subtracted from the scattering curve of the protein's solution. The radius of gyration (Rg) was calculated using the slope of a Guinier plot.

The estimate of XcEXLX1 molecular mass was obtained with a SAXS MoW webtool (Fischer 2010). The distance distribution function p(r), the maximum diameter Dmax and Rg were computed from the entire scattering patterns using a GNOM package (Svergun 1992). These results were compared with the homologous structure of expansin-like protein X from *Bacillus subtilis* (Kerff et al. 2008);(PDB id 3d30). A theoretical SAXS curve from the model structure was generated and compared with the experimental curve using a Crysol program (Svergun et al. 1995). We also used the experimental curve to build dummy atom models (DAMs) of XcEXLX1 using the DAMMIN program (Svergun 1999).

Enhancement of enzymatic cellulose hydrolysis

The effect of XcEXLX1 addition on the cellulose enzymatic hydrolysis on filter paper Whatman No. 1 substrate was measured using the protocol described by Kim et al. (2009) with some adaptations. The commercial enzyme preparation, Accellerase 1500 (DuPont Industrial Biosciences), diluted to 0.35 FPU (Adney and Baker 1996) per g substrate was used,

whereas the XcEXLX1 was diluted to 0.7 mg/g of substrate, and the bovine serum albumin (BSA) was diluted to 0.23 mg/g substrate. The reaction time varied from 30 min to 24 h, and it was carried out at 50 °C in 50 mM sodium citrate buffer using 3.3 mg filter paper per reaction. The determination of pH optimum was conducted in the pH range from 3 to 6 was analyzed. The released sugars were measured using 3,5-dinitrosalicylic acid (DNS) method.

Results and discussion

Identification of the expansin-like x domain

An expansin-like X domain was identified within *X. campestris* Xcc 3535 gene sequence accession number AAM42805.1 using the Domain-Fish program (Contreras-Moreira and Bates 2002) and refined by Clustal program (Higgins and Sharp 1988) using amino acid sequence with both BsEXLX1 (expansin-like from *B. subtillis*) amino acid sequence and with the sequence of expansin from *Zea mays* (Kim et al. 2009). A specific region of the protein, corresponding to the amino acids 385–607 (Supplementary Fig. 1), displayed characteristics that matched two characteristic expansin domains: a rare lipoprotein domain and a pollen allergen domain.

This gene was classified as expansin-like X 1 from *X. campestris* (XcEXLX1) according to the nomenclature
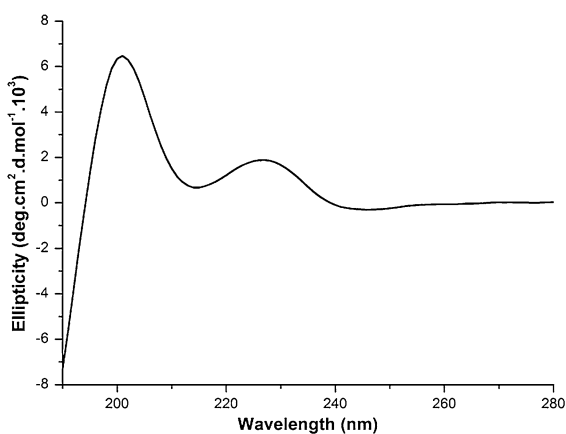
Fig. 1 Structural characterization of XcEXLX1 by CD spectroscopy. The far-UV spectrum was recorded in 20 mM Na_2HPO_4, 20 mM NaCl, pH 8, using an optical path length of 1 mm

for members of the expansin superfamily of genes and proteins (Kende et al. 2004).

Cloning, expression and purification of the protein

The ORF corresponding to XcEXLX1 was amplified from genomic DNA of *X. campestris* and cloned into the pSMT3 vector as described in "Materials and methods" section. The XcEXLX1-pSMT3 plasmid was sequenced and compared with the original XcEXLX1 sequence using the ContigExpress software (a component of Advance Vector NTI 11.0, Invitrogen Corporation), revealing no cloning errors or artifacts.

XcEXLX1 was expressed with a His_6-Sumo affinity tag to facilitate purification and the tag was subsequently removed via proteolytic cleavage with ULP1. The expression and purification protocol (see supporting information) rendered an average of 30 mg pure protein/l cell culture.

Circular dichroism spectroscopy

The CD spectra (Fig. 1) shows a negative minimum around 208 nm due to π–π* transitions and a positive maximum around 226 nm. Why the later peak appears in the CD spectra is not quite clear. However, one possible explanation of the appearance of the positive peak around 226 nm could be due to the presence of aromatic residues which contribute to the positive region of the spectrum between 215 and 230 nm. It is known that specific interaction between certain aromatic amino acids could result in a signal at far UV, which may affect the analysis of secondary structures by CD (Andersson et al. 2001).

The secondary structure composition, calculated from the XcEXLX1 spectra (Fig. 1) using the algorithm CDSSTR with the *SP175* dataset (Lees et al. 2006) resulted in 3 % α-helices, 47 % β-sheets, 9 % turns and 41 % disordered secondary structures. The RMSD of our analysis was 6.3 %. These results show that XcEXLX1 is a predominantly β-sheets protein with a considerable fraction of disordered regions.
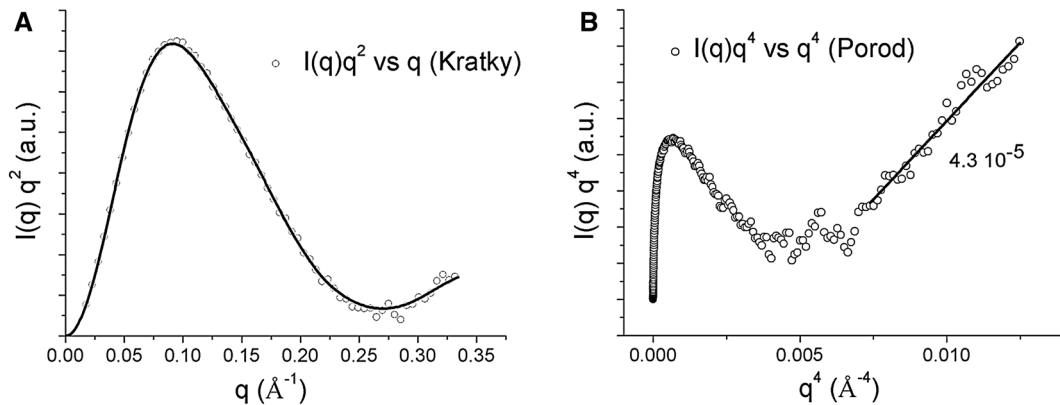
Small angle X-ray scattering (SAXS) studies

The experimental SAXS curves did not display any spatial correlation effects within the studied

concentration range (2 and 5 mg/ml). For this reason, subsequent analyses were performed using SAXS data collected from samples at 5 mg/ml.

The flexibility of the protein can be evaluated using the Kratky plot (Fig. 2a). XcEXLX1 small-angle scattering curve is consistent with a partially folded protein (Rambo and Tainer 2011). In this case the initial parabolic peak is followed by an elevated baseline at high q that is typical for random coil structures. These data are coherent with our CD spectroscopy results that also showed a large portion of disordered regions in the structure of the protein. This behavior can also be evaluated using a Porod plot (Fig. 2b), in which the slope at high q_s give us a Porod

constant equal to 4.3×10^{-5}. A non-flexible system would give a slope close to zero (constant).

The radius of gyration (R_g) value was calculated using the Guinier plot (Fig. 3a, insert) and its value is consistent with a monomeric state of XcEXLX1 in solution. An independent R_g estimate was obtained from the analysis of the distance distribution function (p(r)) analyses (Fig. 3b; Table 1), which also demonstrates that there is no interference among the protein particles in solution. The R_g and D_{max} values were calculated using the GNOM program. A new value of $q_{max} = 0.29$ Å^{-1} was adjusted to eliminate regions with weak scattering signal. (Fig. 3a; Table 1). The SAXS-based estimate of XcEXLX1 molecular mass,

Fig. 2 **a** Small-angle X-ray scattering curve of XcEXLX1. A Kratky plot of scattering data illustrating changes in the behavior of the curve for partially folded protein. **b** Porod plot of same scattering data
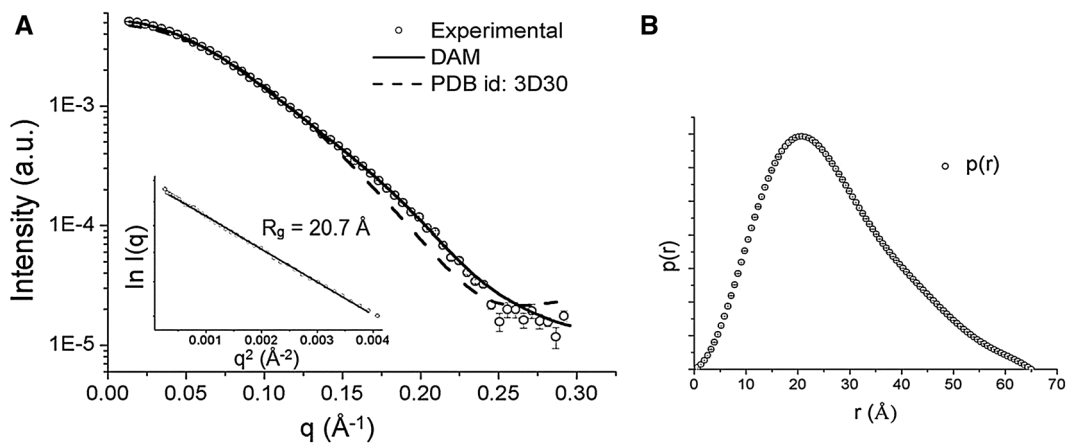
Fig. 3 **a** Small-angle X-ray scattering curve for XcEXLX1 (log I (q) × q) results of fitting procedure: experimental curve (*circle*), the high resolution model of the pdb id 3D30 (*dashed line*) and the simulated scattering DAM (Dammin) (*solid line*). The *insert* shows the Guinier adjustment for obtaining the R_g. **b** The p(r) plot

Table 1 Structural parameters derived from SAXS for XcEXLX1

Parameters	Exp.*	PDB†	DAM.‡
$D_{máx}$ (Å)	65 ± 5	66.03	66.46
R_g (Å)	20.7 (Guinier)		
	20.68 ± 0.01 (Gnom)	18.98	20.63
MW (kDa)	22.6		
Resolution (Å)$^\epsilon$	21.24	–	21.24

* Exp., calculated from the experimental data 3 mg/ml

† PDB id: PDB 3D3O 60 % sequence identity with XcEXLX1

‡ DAM, parameters of the (DAM) without symmetry obligation in the program Dammin

ϵ Resolution $2\pi/q_{max}$

obtained using the SAXS MoW server, was 22.6 kDa with a relative error of 1.2 %. This value is very close to a theoretical molecular weight of XcEXLX1 and confirms that XcEXLX1 exists in solution predominantly in a monomeric state.

The Dammin program was used to calculate ten independent low-resolution protein models, consistently generating similar molecular envelopes. The average χ value for the simulated 10 models was 2.30.

An experimental X-ray scattering curve and two theoretical SAXS curves (one simulated from an average DAM and another one computed from the homology model [(Kerff et al. 2008); PDB id: 3D3O] overlap closely (Fig. 3a). The small difference between the experimental curve and the simulated curve derived from the PDB id 3D3O could be attributed to the flexibility effect yielding a curve that represents an average of different molecular conformations in solution. As a following step we used the Subcomb program to superpose the DAM with a high-resolution structure of the homologous protein BsEXLX1 [(Kerff et al. 2008); PDB id 3D3O] (Fig. 4). The high-resolution X-ray structure fits nicely into the low-resolution SAXS envelope reconstructed for XcEXLX1.

Activity tests: enhancement of the enzymatic hydrolysis of cellulose

To evaluate whether XcEXLX1 is capable of boosting the efficiency of cellulolytic enzymatic preparations, we applied it in a combination with Accellerase 1500 (DuPont Industrial Biosciences) to filter paper enzymatic hydrolysis. The hydrolysis reaction was performed in a sodium citrate buffer using 0.7 mg XcEXLX1 per g substrate and 0.35 FPU of Accellerase 1500/g substrate in the pH range of 3–6 (Fig. 5a). The concentration of the substrate was kept at 3.3 mg per reaction and the reaction volume was kept at 60 μl. The mass ratio between the total protein mass of Accellerase 1500 and XcEXLX1 was 4 (i.e., there were four times more cellulase enzymes in terms of their weight). Since XcEXLX1 is much smaller than a typical cellulase and, assuming that the average molecular weight of the cellulase in the Accellerase 1500 is equal to the molecular mass of *Trichoderma reesei* Cel7A (the major enzymatic component of the *T. reesei* supernatant which corresponds to approximately 60 % of the total secreted protein), the molar relation between the cellulases and the XcEXLX1 was 1.7 (i.e. there were 1.7 times more cellulase enzymes in terms of their molarity). Our results clearly demonstrate the synergistic effect between Accellerase 1500 and the XcEXLX1 protein, which resulted in an increase of the relative activity of the commercial enzymatic preparation.

Most of the fungal-derived industrial enzymatic mixtures which are available commercially such as Accellerase 1500 function best at acidic pHs between pH 4 and 6. The optimal pH for the mixture of XcEXLX1 with Accellerase 1500 was found to be 5 and at this pH, the addition of XcEXLX1 increased the enzymatic hydrolysis efficiency by approximately 36 % after 30 min of reaction (Fig. 5a) and by 20 % after 24 h of reaction performed at pH 5 (Fig. 5b). Furthermore, our experimental results show that the synergistic effect of XcEXLX1 is pH-dependent with a tendency to become stronger at acidic pHs (Fig. 5a) and less relevant or non-existent at pHs equal or higher than 6. This might indicate that XcEXLX1 could synergize even better with more acidic enzymatic preparation with optimum pHs between 4 and 5 (Fig. 5a). However, we also cannot exclude a hypothesis that XcEXLX1 might have some stabilizing effect on the enzymes which are typically non-active under very acid conditions. It is important to note that under optimum conditions XcEXLX1 had stronger effect on the enhancing of cellulase activity than a commonly used test protein, BSA (Fig. 5a).

Why does BSA, which is not secreted by plant biomass-degrading fungi, enhance cellulose degradation by cellulases? BSA is known to bind to

Fig. 4 Superposition of the crystallographic model of BsEXLX1 [(Kerff et al. 2008); PDB id 3D30] with the low-resolution model generated by DAMMIN. Center and right models were rotated 90° around *y*-axis and 90° around *x*-axis from the orientation shown on the *left panel*
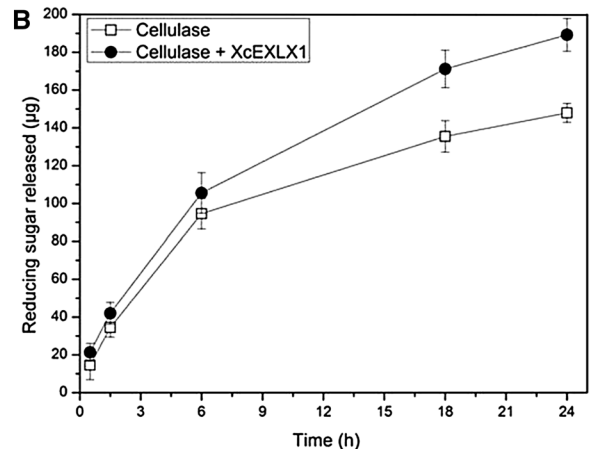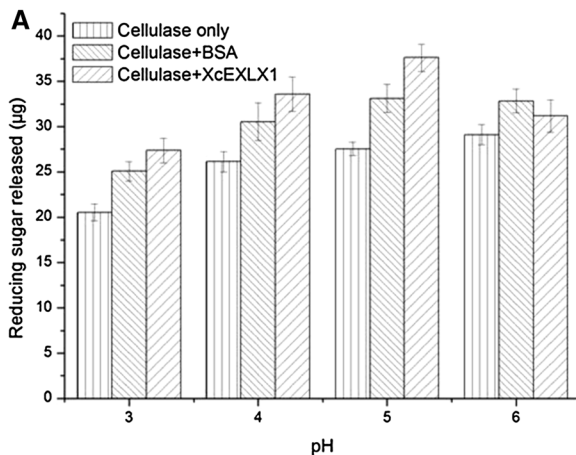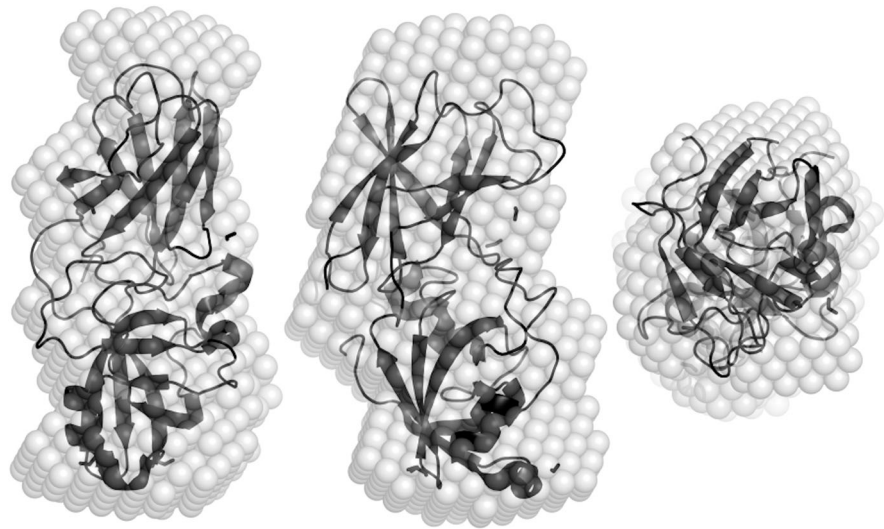
Fig. 5 **a** Evaluation of effect of XcEXLX1 on the apparent enzymatic activity of Accellerase 1500 measured in sodium citrate buffer, the range of pHs 3–6 and a temperature of 50 °C. In *gray* the activity of cellulase (Accellerase 1500) and in *black* the activity of cellulase supplemented with XcEXLX1. **b** Effect

of synergism between the XcEXLX1 and cellulase (Accellerase 1500) at pH 5. The experiments were performed in triplicate with 0.35 FPU of Accellerase 1500 and 0.7 mg of XcEXLX1 per g of substrate

hydrophobic surfaces (Jeyachandran et al. 2009) and thus might prevent unspecific binding between cellulolytic enzymes and hydrophobic parts of the substrates such as lignin (for example, see Brethauer et al. 2011). It can also stabilize cellulases in solution thus reducing the enzyme denaturation during hydrolysis (Eriksson et al. 2002) and also relieve sugar inhibition of enzymatic hydrolysis of biomass materials (Wang et al. 2015).

The most plausible explanation comes from the consideration of the molecular mechanism of cellulose

degradation by cellobiohydrolases. Two cellobiohydrolases (Cel7A and Cel6A) of *T. reesei*-based enzymatic mixtures, such as Accellerase 1500, are the major components of the cellulose preparate and correspond to approx. 60 and 20 % of the total protein mass secreted by *T. reesei*, respectively (Zhang and Lynd 2004). Both of them consist of a catalytic domain (CD) connected through a linker peptide to the carbohydrate-binding module (CBM) ((Jung et al. 2013; Kurašin and Väljamäe 2011; Lehtiö et al. 2003). Cel7A binds to the hydrophobic (110) face of cellulose

(Lehtiö et al. 2003; Liu et al. 2011). Cel7A and Cel6A hydrolyze glycosidic bonds, respectively, from the reducing and none-reducing ends of the cellulose chain in a processive manner (Igarashi et al. 2009). Cel7A typically performs 61 ± 14 catalytic acts before dissociating from the cellulose (Kurašin and Väljamäe 2011). The enzyme is only able to begin to cleave cellulose if the catalytic domain is at the hydrophobic surface of the cellulose and its active site recognized the free end of the cellulose chain and is in the proper conditions for the catalysis (Igarashi et al. 2009; Jalak and Valjamae 2014; Jung et al. 2013). However, exoglucanases are guided to the hydrophobic surface of cellulose by CBMs and their interaction with cellulose is mediated by both the CBMs and CDs. The CBM can bind to the hydrophobic surface of cellulose without the CD productively interacting with the available free chain ends, thus engaging in a non-productive binding (Igarashi et al. 2009; Jalak and Valjamae 2014; Jung et al. 2013). This would deplete active cellulase pool available in solution, without them effectively being involved in cellulose hydrolysis. BSA binding to the same hydrophobic surface of cellulose crystals (Jeyachandran et al. 2009) would hinder none-productive binding of cellulases hence avoiding their unproductive interactions with the substrate, increasing concentration of free cellulases and enabling a larger number of cellulases to get involved in the processive enzymatic cleavage of the substrate. Consequently, the BSA mechanism of action might somewhat resembles that of surfactants that also have been widely employed to increase efficiency of cellulase hydrolysis of cellulosic substrates (Eriksson et al. 2002). Molecular mechanisms by which expansins enhance cellulose hydrolysis deserve further investigations.

Thus our results contrast with a previous study in which expansin-like proteins from microbes have been reported to have no effect on the enhancement of the soluble sugars release from filter paper by cellulases (Georgelis et al. 2014) but confirm several other studies that found a boosting effect of expansins on cellulase activities (Kim et al. 2009; Lin et al. 2013).

Supporting information Supplementary methods. Construction of the expression plasmid, Expression of the protein and its purification process.

Supplementary Fig. 1—Aligned sequences of gene (1) Xcc3535, (2) 2BH0 (BsEXLX1) and (3) 2HCZ (expansin of Zea mays), using the multiple alignment Clustal program (Higgins and Sharp 1988).

Supplementary Fig. 2—Size-exclusion chromatogram of XcEXLX1.

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