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Binding characteristics and synergistic effects of bacterial expansins on cellulosic and hemicellulosic substrates

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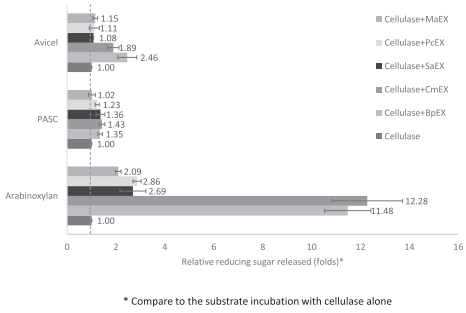
HIGHLIGHTS

- Binding kinetics of five bacterial expansins on polysaccharides was studied.
- Binding efficiency (B_{max}/K_d) was in the order of PASC > arabinoxylan > Avicel.
- All expansins showed varying synergy on hydrolysis of tested substrates.
- BpEX and CmEX showed the highest synergy of >11-fold on arabinoxylan.
- Binding and synergy were related to types and crystallinity of substrates.

GRAPHICAL ABSTRACT



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ABSTRACT

Expansins are non-catalytic proteins which loosen plant cell wall structure. In this study, binding kinetics and synergistic action of five bacterial expansins on cellulosic and hemicellulosic polysaccharides were studied. The expansins differed in binding capacity (B_{max}) and affinity (K_d) for different substrates. A common pattern of binding efficiency (B_{max}/K_d) was found among the expansins tested, in which efficiency was greatest for the phosphoric acid-swollen cellulose (PASC), then the hemicellulose arabinoxylan followed by the microcrystalline cellulose (Avicel PH101). The expansins enhanced the action of *Trichoderma reesei* cellulase/hemicellulase mixture for degrading all three substrates to varying degrees. Among the substrates and expansins tested, BpEX from *Bacillus pumilus* and CmEX from *Clavibacter michiganensis* showed the greatest enhancement effect on arabinoxylan with 11.4 and 12.2-fold greater reducing sugar yield than the reaction with enzyme alone. The work gives insights into the wider application of expansins on enhancing polysaccharide hydrolysis, particularly on hemicellulosic substrates.

1. Introduction

Degradation of lignocellulosic plant biomass is a key bio-geochemical process in the organic carbon cycle, and it also represents a challenging topic in biotechnology. Lignocelluloses possess a complex molecular structure comprising (i) cellulose, a homopolymer of D-glucose linked by a β-1,4 glycosidic linkage which is organized into a highly crystalline microfiber structure; (ii) hemicellulose, a branched amorphous heteropolymer of pentoses, hexoses, and sugar acids which acts as a interconnecting substance; and (iii) lignin, an aromatic polymer composed of three phenyl-propane subunits. Lignocellulose fibers are arranged into a complex three-dimensional network that provides strength to the plant cell (Howard et al., 2003; Van Dyk and Pletschke, 2012). This highly complex and ordered structure makes the plant cell wall recalcitrant to physical, chemical, and biological attack.

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In nature, the synergistic and cooperative action of cellulases and hemicellulases is essential for the efficient hydrolysis of lignocelluloses. The synergies among glycosyl hydrolases (GH) and auxiliary enzymes (AA) from bacterial and fungal origins either as free enzymes, or cellulosomal systems have been reported (Bayer et al., 2004; Doi and Kosugi, 2004; Wilson, 2008). Expansins are non-hydrolytic proteins which have been demonstrated to enhance the hydrolytic efficiency of lignocellulose by loosening the plant cell wall structure. They are small proteins of molecular mass approximately 25 kDa and consist of two domains (domain 1 and domain 2) connected by a peptide linker (Cosgrove, 2000b; Yennawar et al., 2006). Domain 1 shows structural and sequence similarity to endoglucanases in glycosyl hydrolase family 45 (GH45) (Cosgrove, 2000b, 1997; Saloheimo et al., 2002; Tatusova and Madden, 1999; Yennawar et al., 2006), but lacks catalytic activity (Cosgrove, 2005; McQueen-Mason et al., 1992). Domain 2 contains conserved aromatic and polar residues that form a polysaccharide binding site (Cosgrove, 1997; Yennawar et al., 2006).

Expansins act by loosening densely packed polysaccharides such as celluloses and hemicelluloses in the plant cell wall. Han and Chen (2007) demonstrated that the Zea h protein from corn stover synergises with cellulases and increased degradation of biomass, even though it did not have any detectable cellulase activity by itself. The expansin-like protein swollenin produced by fungi exhibits loosening activity on cotton fiber (Saloheimo et al., 2002). The BsEXLX1 protein from *Bacillus subtilis* enhances the action of cellulase on filter paper hydrolysis (Kim et al., 2009) and on degradation of alkali-pretreated rice straw (Suwannarangsee et al., 2012). A varying degree of synergy between *Trichoderma reesei* cellulase and different bacterial expansins identified by bioinformatics approach on degradation of lignocellulosic substrates has been recently reported (Bunterngsook et al., 2014).

At present, the molecular mechanism of how expansins enhance cellulose degradation is unclear and limited study has been performed on their substrate binding characteristics and interaction with cellulase-degrading enzymes. In this study, the binding characteristics of five recently identified expansins (Bunterngsook et al., 2014) originating from different groups of bacteria on cellulosic and hemicellulosic polysaccharides is reported. The synergistic action of these expansins with *T. reesei* cellulase on different types of polysaccharides is demonstrated. The work provides an insight into the biological functions of expansins on plant cell wall degradation with the potential uses on enhancing degradation and modification of plant lignocellulosic polysaccharides in bio-industries.

2. Methods

2.1. Cellulosic and hemicellulosic substrates

The microcrystalline cellulose Avicel PH101 was purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). Phosphoric acid-swollen cellulose was prepared as described by Zhang et al. (2009). Insoluble arabinoxylan from wheat flour was purchased from Megazyme (Wicklow, Ireland). All chemicals and reagents were of analytical grade and purchased from major suppliers (Sigma–Aldrich, St. Louis, MO, USA; Merck, Darmstadt, Germany; and Fluka, Milwaukee, WI, USA).

2.2. Bacterial strains and plasmids

Expression vectors pET28a (+) and pET32a (+) (Novagen, Darmstadt, Germany) were used for expression of the expansin genes. The genes encoding bacterial expansins BpEX from *Bacillus pumilus* (gi_194016885), CmEX from *Clavibacter michiganensis* (gi_147830590), SaEX from *Stigmatella aurantiaca* (gi_115369113), PcEX from *Pectobacterium carotovorum* (gi_251755056), and MaEX from *Micromonospora aurantiaca* (gi_302865685) were synthesized by GenScript (Piscataway, NJ, USA) with codon optimization for expression in *Escherichia coli* (Bunterngsook et al., 2014). *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) was used as the host strain for DNA cloning. *E. coli* Rosetta™ (DE3) pLysS (Novagen, Darmstadt, Germany) was used as an expression strain. Bacteria were grown at 37 °C in Luria–Bertani (LB) medium (1% bactotryptone, 0.5% bacto yeast extract, 1% NaCl) or on 1.5% agar plates supplemented with appropriate antibiotics.

2.3. Expression and purification of bacterial expansins

Genes encoding BpEX and CmEX were cloned into pET28a expression vector whereas genes encoding SaEX, PcEX, and MaEX from *M. aurantiaca* were cloned into pET32a expression vector as described previously (Bunterngsook et al., 2014). Transformants were grown in LB medium supplemented with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C with rotary shaking at 200 rpm until the OD_{600} reached 0.5. Target gene expression was induced by adding isopropyl-thio-α-D-galactopyranoside (IPTG) to a final concentration of 1 mM and the culture was incubated for an additional 6 h at 25 °C with shaking at 200 rpm. The recombinant cells were harvested by centrifugation at 7000*g* for 10 min and resuspended with 1 ml of 100 mM Tris–HCl pH 7.0. The cells were disrupted by sonication (Ultrasonic processor VCX750, Sonics and Materials, Inc, Newtown, CT, USA) on ice at sonicator amplitude of 60% for 4 min. The cell debris was separated by centrifugation at 7000*g* at 4 °C for 10 min. The supernatant was collected and clarified by centrifugation at 13,000*g* at 4 °C for 30 min and then filtered through a 0.2 μm membrane (Pall, Port Washington, NY).

Recombinant expansins were purified from the soluble fraction using a 5 ml HisTrap FF affinity column containing nickel–nitrilotriacetic acid matrix following the manufacturer's recommendations (GE Healthcare, Uppsala, Sweden). The column was pre-equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4) at a flow rate of 1 ml/min sample loading. The column was washed with 5 column volumes of binding buffer followed by 10 column volumes of stringent wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole, pH 7.4) to remove nonspecific proteins. His_6-tag recombinant proteins were eluted from the column with the elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 200 mM imidazole, pH 7.4). Protein purity was assessed by 12% SDS–PAGE stained with Coomassie Blue R-250. The eluted fraction was concentrated and desalted using a 10 kDa MWCO Amicon Ultrafiltration Unit (Millipore, Darmstadt, Germany). The concentration of purified protein was determined using a Bradford assay kit (Biorad, Hercules CA).

2.4. Binding assay to insoluble substrates

The binding activity of putative expansins to various insoluble substrates was determined by the method modified from Lee et al. (2010). The substrates tested included 10 mg/ml suspension of Avicel PH101, phosphoric acid swollen cellulose (PASC) and 1 mg/ml arabinoxylan. To prevent non-specific binding, substrates were blocked with bovine serum albumin (BSA). Substrate blocking was performed by mixing substrates with 50 mg/ml BSA. The final volume was adjusted to 500 μl with 50 mM sodium acetate buffer pH 5.0. The mixture was incubated at 25 °C for 1 h and then centrifuged at 13,000*g* for 5 min. The pellet was washed twice with 500 μl of 50 mM sodium acetate buffer pH 5.0. The binding reaction was studied by adding the purified expansin to the washed polysaccharide pellet to the final expansin protein concentration