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Biochemical analysis of expansin-like proteins from microbes

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ABSTRACT

Expansins cause plant cell wall loosening and are present primarily in the plant kingdom. Gene sequence analysis suggests that expansins are present in several plant-colonizing or plant-pathogenic bacteria and fungi. However, experimental evidence of microbial expansin activity is largely lacking. Here we provide evidence that expansins from three plant pathogenic bacteria and one fungus cause extension of cell walls *in vitro* and weaken filter paper networks, without lytic activity. Since expansins were able to weaken cellulose networks, we tested whether they synergistically enhanced the activity of several cellulases in hydrolysis of cellulose. The microbial expansins did not show such synergism beyond the nonspecific effect of bovine serum albumin. Our results show that the expansins present in several pathogenic microbes have weak wall-loosening activity and we infer a role for these expansins in plant pathogenesis. Additionally, the convenient expression of several expansins in *Escherichia coli* makes a future comparative structure–function analysis among expansins possible in order to understand their activity at the molecular level.

1. Introduction

Primary cell walls provide mechanical support and determine the shape of plant cells and at the same time are able to expand in response to wall-loosening agents, enabling cells to enlarge and thereby plants to grow (Cosgrove, 2005). Expansins comprise a family of plant proteins that mediate plant cell wall loosening and stress relaxation, with multiple roles in plant physiology and development, including cell expansion, fruit softening, organ abscission and pollen tube penetration of the stigma (Cosgrove, 2000; McQueen-Mason, Durachko, & Cosgrove, 1992; Sampedro & Cosgrove, 2005). They lack lytic activity against cell walls and are believed to break non-covalent interactions between cell wall components, allowing polymer slippage (creep) and cell wall loosening, but the molecular details of their action and their specific targets are not yet well defined, in part because of the structural complexity of the cell wall and in part because of the lack of any detectable enzymatic activity by expansins (Cosgrove, 2000; McQueen-Mason & Cosgrove, 1995; Tabuchi, Li, & Cosgrove, 2011). Structurally, canonical expansins consist of two tightly packed domains (D1, D2) with a polysaccharide binding surface spanning the two domains (Yennawar, Li, Dudzinski, Tabuchi, & Cosgrove, 2006). Domain D1 is a double-ψ beta barrel resembling family-45 endoglucanases, but lacks key residues necessary for hydrolytic activity; domain D2 has an immunoglobulin-like beta-sandwhich fold and is the principle determinant of expansin binding to cell wall components (Georgelis, Tabuchi, Nikolaidis, & Cosgrove, 2011; Georgelis, Yennawar, & Cosgrove, 2012).

Looking beyond the plant kingdom, expansin-like proteins are evidently employed by a few, diverse organisms. *Globodera rostochiensis*, a plant-parasitic nematode, contains a functional expansin-like protein, presumably for invading plant roots (Qin et al., 2004) and expansin-like genes or domains are found in the genomes of the slime mold *Dictyostelium discoidium* (which makes cellulose in some life stages) and in some fungi (e.g., 'swollenins') (Bouzarelou, Billini, Roumelioti, & Sophianopoulou, 2008; Darley, Li, Schaap, & McQueen-Mason, 2003; Li et al., 2002; Saloheimo et al., 2002). Recently it was shown by X-ray crystallography that a protein (EXLX1) from the plant-colonizing bacterium *Bacillus subtilis* is structurally homologous to the maize pollen expansin EXPB1 (Kerff et al., 2008). Biomechanical assays showed that BsEXLX1 could induce plant cell wall creep *in vitro*, indicating that it has classical expansin activity, notwithstanding its low sequence identity to EXPB1 (~15%) and its lack of motifs conserved in plant expansins (Kerff et al., 2008). Gene database searches identified other predicted proteins or protein domains with significant sequence similarity to EXLX1 in several plant pathogenic bacteria, providing evidence that expansins may be present in many, though not all, plant pathogens (Kerff et al., 2008; Li et al., 2002).

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This idea is strengthened by tests showing that bacterial mutants lacking expansin genes or genes with expansin modules are crippled in their ability to colonize plant surfaces or to cause symptoms of bacterial wilts (Jahr, Dreier, Meletzus, Bahro, & Eichenlaub, 2000; Kerff et al., 2008; Laine et al., 2000); in addition, ectopic expression of swollenin in the fungus *Trichoderma asperellum* enhanced its ability to colonize cucumber roots (Brotman, Briff, Viterbo, & Chet, 2008). However, the potential cell-wall activities of these expansin-like proteins have not been examined; currently the only bacterial protein demonstrated to have expansin activity is EXLX1 from *B. subtilis* (Georgelis et al., 2011; Kerff et al., 2008).

Here, we tested the ability of three expansin-like proteins or modular domains from plant pathogenic bacteria *Xanthomonas campestris*, *Clavibacter michiganensis* and *Ralstonia solanacearum* and one from the plant pathogenic fungus *Aspergillus niger* to cause loosening (creep) of plant cell walls. We also tested the ability of the proteins to hydrolyze wall polysaccharides and to weaken pure cellulose networks, to gain knowledge into their exact target. Finally, we examined their potential synergistic effects with cellulases for soluble sugar release from cellulose.

2. Material and methods

2.1. Materials

Filter paper #3 (GE Healthcare, Waukesha, WI, USA) was used as cellulose substrate. Dye-coupled cross-linked (AZCL-) polysaccharides were purchased from Megazyme (Bray, Ireland). Wheat coleoptiles (*Triticum aestivum* L. cv Pennmore) were prepared for creep assays as described in Li, Bedinger, Volk, Jones, and Cosgrove (2003). *Gluconacetobacter xylinus* was grown as described in Whitney, Gidley, and McQueen-Mason (2000) and pellicle strips (10 mm × 0.5 mm × 0.5 mm) were prepared for extension assays as described in Georgelis et al. (2011).

2.2. Cloning and expression of microbial proteins in Escherichia coli

A full length protein and an expansin domain (Xantho-FL and Xantho-EXP, respectively) were PCR-amplified from *Xanthomonas campestris* pv. *campestris* str. ATCC 33913 genomic DNA (NCBI reference sequence: NP_638881.1) with primers 5′CAGCATATGCTTGTCGATGCAACAGGTCAGACGCTG3′-5′CAGGCGGCCGCCTACGGAAACTGCACATG3′ and 5′CAGCATATGACGAAGAATGCGAGTGGGAAGAAG3′-5′CAGGCGGCCGCCTACGGAAACTGCACATG3′, respectively. Ralstonia-EXP was PCR-amplified from *Ralstonia solanacearum* str. UW551 genomic DNA (NCBI reference sequence: ZP_00944742.1) with primers 5′CAGCATATGGCCTGGGACAGCACGTTC3′-5′CAGCTCGAGTTACTCGGGAAACTGCAC3′. Clavi-FL, Clavi-GH5, Clavi-GH5-CBM and Clavi-EXP were PCR-amplified from *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 382 plasmid DNA (NCBI reference sequence: YP_001220664.1) with primers 5′GCGATGGCCATGGCGACCGTAGCGGGGCCCGTT3′-5′CCGCTCGAGTCAGTGCACAGGGTAGAA3′, 5′GCGATGGCCATGGCGACCGTAGCGGGGCCCGTT3′-5′CCGCTCGAGCTACAAGATCGGCGCCAGGGCGT3′, 5′GCGATGGCCATGGCGACCGTAGCGGGGCCCGTT3′-5′CCGCTCGAGCTAGGCACGGCCATGCGTCGTG3′ and 5′GCGATGGCCATGACGACGCATGGCCGTGCCACGC3′-5′CCGCTCGAGTCAGTGCACAGGGTAGAA3′, respectively. The cDNA encoding Asper-EXP (NCBI protein accession: EHA21207.1) was synthesized by Life Technologies (Carlsbad, CA, USA) from amino acid sequence (M)ALSSEYSG to TASSNFE(HHHHHH) (a 6 His-tag was added at the C-terminus) and cloned between NcoI and XhoI sites of the pET22b vector (Novagen). Xantho-FL and Xantho-EXP were cloned between the NdeI and NotI sites of the pET22b vector while Ralstonia-EXP was cloned between the NdeI and XhoI of sites of the same vector. All Clavi proteins were cloned between the MscI and XhoI sites of the pET22b vector. A methionine was added at the N-terminus of all proteins. The proteins were expressed in *E. coli* strain BL21 (DE3-pLys). Cells were grown to OD600 = 0.6 at 37 °C and induced with 0.1 mM IPTG for 6 h at room temperature.

2.3. Protein purification

All proteins except for Asper-EXP and Clavi-GH5 were purified as described by Kerff et al. (2008), except 25 mM NaOAc (pH 5.5) was used instead of 50 mM Tris–HCl (pH 8.0). Clavi-GH5 was loaded onto a Q-Sepharose column using 25 mM HEPES pH 7.5 as buffer. Fifty ml of flow-through was collected, added to 25 ml of 3.5 M ammonium sulfate and mixed for 15 min. The mixture was centrifuged at 18,000 × *g* for 15 min and 75 ml of supernatant was mixed with 27.5 ml of 3.5 M ammonium sulfate for 15 min. The mixture was centrifuged at 18,000 × *g* for 15 min and the pellet was resuspended in 2 mL of 25 mM NaOAc (pH 5.5). The sample was loaded onto a Hiprep Sephacryl S-100 column (GE Healthcare) with 25 mM NaOAc (pH 5.5) + 0.15 M NaCl as mobile phase at 0.5 ml/min and fractions containing Clavi-GH5 were collected, concentrated and exchanged into 25 mM NaOAc (pH 5.5) with an Amicon 10-kD filter (EMD Millipore, Billerica, MA). Clavi-FL, Clavi-GH5-CBM and Xaca-FL were also loaded onto Hiprep Sephacryl S-100 after the initial purification steps for additional protein purification, as described for Clavi-GH5. Asper-EXP was purified with Ni-NTA agarose (Qiagen, Valencia, CA, USA) following the instructions in the product manual. All purification steps were done at 4 °C. The purity of 15-μg samples was checked on 12% SDS-PAGE stained with Coomassie Brilliant Blue R-250. For endoglucanase assays, Xantho-EXP and Ralstonia-EXP were further purified by HPLC on a reverse-phase column (Discovery C8, Supelco, Bellefonte, PA, USA) preequilibrated with 10% methanol containing 0.1% trifluoroacetic acid. Bound protein was eluted at 1 ml/min with a linear gradient of methanol at a flow rate of 1 ml/min at 25 °C. We confirmed creep activity of Xantho-EXP and Ralstonia-EXP purified in this way (data not shown).

2.4. Creep assay and paper strength assay

Creep assays of alkali-pretreated wheat coleoptiles and *G. xylinus* pellicle strips and paper strength assays were done as described by Georgelis et al. (2011).

2.5. Protein alignment

Proteins were aligned with ClustalW as implemented by MEGA software (Kumar, Tamura, & Nei, 2004) using default parameters except the BLOSUM substitution matrix. The alignment was visualized by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).

2.6. Test for synergism between expansins and cellulases

One disk of filter paper #1 (GE Healthcare) (2.5 mg) was incubated in 250 μl of 25 mM NaOAc (pH 5.5) in the presence of 1 μg of Celluclast 1.5L (CL) (Novozymes, Bagsvaerd, Denmark) or exoglucanase CBHI (Megazyme) or endoglucanase EGI (Cat#: E-CELTR, Megazyme) and 5 μg of expansin or bovine serum album (BSA, a negative control) at 30 °C. The total soluble sugars in the supernatant were measured at 12 and 24 h by phenol/sulfuric acid assay as described by Dubois, Gilles, Hamilton, Rebers, and Smith (1956) and modified by Hanson and Phillips (1981).