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Molecules and Cells

An Expansin-Like Protein from *Hahella chejuensis* Binds Cellulose and Enhances Cellulase Activity

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Molecular function of the expansin superfamily has been highlighted for cellulosic biomass conversion. In this report, we identified a new bacterial expansin subfamily by analysis of related bacterial sequences and biochemically examined a member of this new subfamily from *Hahella chejuensis* (*Hc*EXLX2). Among the various complex polysaccharides tested, *Hc*EXLX2 bound most efficiently to cellulose. The relative binding constant (K_r) against Avicel was 2.1 L g^{-1} at pH 6.0 and 4°C. *Hc*EXLX2 enhanced the activity of cellulase, producing about 4.6 times more hydrolysis product after a 36 h reaction relative to when only cellulase was used. The extension strength test on filter paper indicated that *Hc*EXLX2 has a texture loosening effect on filter paper, which was 53% of that observed for 8 M urea treatment. These activities, compared with a cellulose binding domain from *Clostridium thermocellum*, implied that the synergistic effect of *Hc*EXLX2 comes from not only binding to cellulose but also disrupting the hydrogen bonds in cellulose. Based on these results, we suggest that the new bacterial expansin subfamily functions by binding to cell wall polysaccharides and increasing the accessibility of cell wall degrading enzymes.

INTRODUCTION

Plant expansin is a non-enzymatic protein that plays an important biological function in plant cell growth and development (Cosgrove et al., 2002; Sampedro and Cosgrove, 2005; Shin et al., 2005). Plant expansin is known to bind to complex polysaccharides (CPs) such as cellulose and hemicelluloses and loosen the plant cell wall components causing cell wall expansion (Cosgrove et al., 2002; Sampedro and Cosgrove, 2005). Although the exact mechanism of this process is not fully understood, binding to and loosening the cell wall components imply that it disrupts the hydrogen bonds in CPs and enhances the accessibility of cell wall degrading enzymes (McQueen-Mason and Cosgrove, 1995; Sampedro and Cosgrove, 2005). In addition to its biological role in plants, the use of the expansin superfamily for recalcitrant biomass deconstruction has been extensively examined because its known molecular functions (binding to CPs and expanding plant cell wall) provide a synergistic effect on the activities of cell wall degrading enzymes and thus reduces the amounts and costs of enzyme usage in the process (Kim et al., 2009). Since high loading of those enzymes is the major obstacle in developing cost-effective biomass deconstruction processes, recent biofuel production scenarios have shown that halving cellulase loading can substantially reduce the total processing costs associated with total process improvement (Lynd et al., 2008). As a result, although the reduction of enzyme loading can be achieved by improving the activity of the enzyme (Merino and Cherry, 2007) or by a synergistic combination of enzymes (Henrissat et al., 1985), the use of non-enzymatic proteins to enhance cellulase activity has also been considered an option to reduce enzyme loading (Kim et al., 2009; Merino and Cherry, 2007).

The members of the expansin superfamily mainly belong to plants but have also been found in fungi (Saloheimo et al., 2002) and animal (Qin et al., 2004). However, no prokaryotic homolog has been described until recently when the three-dimensional structure of the YoaJ protein of *Bacillus subtilis* (*Bs*YoaJ) was shown to have structural similarity to a plant expansin (Zea mays EXPB1) (Kerff et al., 2008; Kim et al., 2008). Due to this structural similarity, *Bs*YoaJ was annotated as an expansin-like protein (EXLX1) according to the nomenclature of the expansin superfamily (Kende et al., 2004). The molecular function of *Bs*EXLX1 was similar to that of plant expansin and was reported to bind to CPs such as cellulose and peptidoglycan and enhance cellulase activity (Kerff et al., 2008; Kim et al., 2008), indicating that this protein can be used to convert cellulosic biomass to sugars.

In order to screen for synergistic non-enzymatic proteins for biomass deconstruction, we searched for bacterial expansin homologs using the structure and sequence of *Bs*EXLX1 as a template and found several candidates in bacterial species. In this report, we analyzed the sequences of these candidate bacterial expansin-like proteins and identified a new subfamily of the expansin superfamily, designating as EXLX2. Among the members of this new subfamily, we identified a plant expansin-like protein (HCH_03669) in *Hahella chejuensis*, which is a marine bacteria belonging to gram-negative gamma proteobacteria that produce algicidal pigment and includes many enzymes and horizontally transferred genes showing metabolic diversity (Jeong et al., 2005). The molecular function of

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HCH_03669 was initially unknown but was later annotated as endoglucanase C-terminal domain/subunit and related protein (http://www.uniprot.org/uniprot/Q2SG15). We cloned *Hc*EXLX2, heterologously expressed it in *E. coli* and examined the molecular function of recombinant *Hc*EXLX2 in terms of binding to various CPs, enhancing cellulase activity and loosening the filter paper structure. Based on the results of these molecular function tests, we believe that *Hc*EXLX2 can be utilized as a potential biochemical agent in cellulosic biomass conversion for bioenergy production.

MATERIALS AND METHODS

Strains and materials

We obtained the genomic DNA of *H. chejuensis* from Dr. J. F. Kim at Korea Research Institute of Biosciences and Biotechnology (Korea). We used *E. coli* DH5α and *E. coli* BL21 (DE3) as the cloning and expression host, respectively. Bacterial microcrystalline cellulose (BMCC) was prepared from *Acetobacter xylinum* ATCC 23769 using a previously described method (Gilkes et al., 1992). Other complex polysaccharides (Avicel, birch-wood xylan and oat spelts xylan) were purchased from Sigma Co. (USA). We used Celluclast 1.5 L™ (Novozyme, USA) as a cellulase source for testing the synergistic effect of cellulose hydrolysis by cellulase.

Identification of bacterial expansin homologs

In order to retrieve the bacterial expansin homologs, we used the sequence of the YoaJ protein from *B. subtilis* (*Bs*YoaJ) which structure showed a high similarity to the known fold of *Zea mays* expansin (*Zm*EXPB1). The procedure used for the nomenclature of bacterial expansins was based on the representative protein sequences of expansins as previously described (Kende et al., 2004). The sequence of *Bs*YoaJ was searched against a non-redundant protein database in the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) or position specific iterated (psi) BLAST. To avoid false positive selection by the transitivity of homology and to follow the rules for designating a sequence as the expansin superfamily, we further analyzed the domain organization of retrieved proteins using the Superfamily database (release 1.73) (Wilson et al., 2009) and motif conservation using the MEME software (Grundy et al., 1997). The multiple sequence alignment was generated by T-coffee (Notredame et al., 2000) and the phylogenetic tree was constructed using the Phylip package (Retief, 2000). The aligned sequences were bootstrapped 100 times by 'seqboot'. Then, we calculated the distance matrix using 'protdist', and built the tree using the neighbor joining method ('neighbor') and the consensus tree was chosen using 'consense' in the Phylip package.

Cloning, expression and purification of *Hc*EXLX2

For heterologous expression of *Hc*EXLX2 in *E. coli*, we cloned the gene in a modified pET21a vector using a ligation independent cloning method, which was designed at Berkeley Structural Genomics Center (BSGC) (Lee and Kim, 2009). In order to avoid the adverse effect of signal sequence during the overexpression of the gene, the potential signal peptide in amino terminus (1-23) was excluded from the entire gene when primers were designed. The final construct has a six-histidine tag in the carboxy terminus for affinity purification. The gene was amplified with polymerase chain reaction (PCR) and the oligomers used in the experiment were as followed: Hch-N, 5′-GAAAATCGAGTTTCTGCGACTC-3′; Hch-C, 5′-TTTGTCTGCCTGATTAATAACGCC-3′. The PCR product was purified with a PCR clean-up kit (Cosmo Genetech, Korea). The recombinant *Hc*EXLX2 was expressed in *E. coli* BL21 (DE3) using Luria Broth media containing ampicilin (100 μg/ml) at 37°C. For soluble expression of proteins, we decreased the growth temperature to 16°C after induction with a final concentration of 0.1 mM isopropyl thiogalactoside (IPTG) and allowed them to continue to grow for 16 h. After harvesting and breaking the cells in a lysis buffer (10 mM Tris Cl, pH 8.0) with sonication, the crude cell extract were subjected to histidine affinity chromatography using a HisTrap column (GE Healthcare, USA) and LP system (Bio-Rad, USA). The purity of proteins was examined by SDS-PAGE and the concentration was measured using a Bradford assay kit (Bio-Rad).

Cloning, expression and purification of cellulose binding domain from *Clostridium thermocellum* (*Ct*CBD)

In order to compare the activity and mode of carbohydrate binding, we designed a synthetic *Ct*CBD gene that is the known active carbohydrate binding domain region (368-522 region of Uniprot id: Q06851, 155 residues) as appeared in the structurally determined part of CipA (PDB id: 1NCB) (Tormo et al., 1996). We purchased a synthetic gene from IDT Inc. (USA) that was based on the nucleotide sequence of the original *Ct*CBD gene. The insert fragment was amplified by PCR from a cloned vector provided by the company (forward primer 5′-AATTTGAAGGTTGAATTCTACAACAG-3′; reverse primer 5′-GGTTCTTTACCCCATACAAGAAC-3′). All procedures used for cloning, expression and purification were the same as those described for *Hc*EXLX2.

Binding to various CPs and measuring the binding constant

The binding activity to various CPs was determined as follows. We prepared 1.5 ml eppendorf tubes containing 20 mg of various CPs such as Avicel, bacterial micro crystalline cellulose (BMCC), birch-wood xylan and oat spelts xylan in 250 μl of reaction buffer (0.1 M sodium phosphate, pH 7.0). We added 50 μl of bovine serum albumin (BSA) solution (10% w/v) as a blocking agent to prevent non-specific binding to CPs and adjusted the final volume to 500 μl using distilled water. The tubes were incubated for 1 h at 25°C and centrifuged at 15,000 *g* for 5 min. After discarding the supernatant, the pellets were washed three times with 500 μl of the reaction buffer and 62.5 μg (or 2.5 nmole) of purified *Hc*EXLX2 proteins in 500 μl of the same buffer were then added. The samples were incubated for additional 1 h at 25°C and centrifuged at 15,000 × *g* for 5 min. The unbound proteins in the supernatant were determined by measuring the absorbance at 280 nm using a spectrophotometer and using a molecular extinction coefficient of 36,245 M^{-1} cm^{-1}. The fraction of bound and unbound proteins was also verified by SDS-PAGE. The proteins bound to CPs were extracted with gel loading buffer by boiling for 5 min. The optimum pH for binding was determined using various buffers (pH 4-6 for sodium citrate, pH 7-8 for sodium phosphate, pH 9 for glycine-NaOH buffer) at 25°C. The optimum temperature was determined by varying the reaction temperature from 4 to 50°C in sodium phosphate buffer (50 mM, pH 7.0). The relative binding constant (K_r) against Avicel was calculated from the fraction of bound and unbound proteins at various concentrations of *Hc*EXLX2 under the optimum binding conditions (pH 6.0 and 4°C) using a previously described procedure (Gilkes et al., 1992).

Determination of the synergistic effect on cellulose hydrolysis by cellulase

The synergistic effect was determined using a protocol developed in a previous report (Kim et al., 2009). The synergistic

effect was calculated from the ratio of the amount of reducing sugars released by cellulase in the supernatant of a reaction mixture with or without target proteins (*Hc*EXLX2, BSA and CBD). We used Whatman No. 1 filter paper (a disc of 7 mm diameter; 2.5 mg) as the substrate. With appropriate amounts (0.06-0.6 Filter Paper Unit) of Celluclast 1.5 L™ and 15 nmole of each target protein in a reaction buffer (50 mM sodium citrate, pH 5.0), the hydrolysis reaction was carried out at 50°C for 2 days in a 96-well plate. We sampled the reaction at different time intervals (12 h) and measured the amount of reducing sugars released from the paper disc by the DNS method using glucose as a standard (Xiao et al., 2004).

The extension strength test on the filter paper

We used the Universal Testing Machine (UTM; Instron, USA) to measure the tensile strength of a strip of Whatman filter paper No. 3 (2.0 cm × 5.0 cm). The strip was immersed in sodium acetate buffer (50 mM, pH 4.8) with each protein sample for 1 h. We also used a strip immersed in the same volume of 8 M urea and 0.1 M BSA solution for 1 h as positive and negative controls, respectively. The tensile strength (σ_{max}) was calculated from the equation $\sigma_{max} = F_{max}/A$, where A = cross sectional area (cm^2) and F_{max} = the maximum load (kg). The crosshead speed for the UTM used in this experiment was 0.5 mm/min (Kwon et al., 2008).

RESULTS

Identification of domains and motifs in bacterial expansin-like proteins

Expansins are composed of two domains, the GH45-like domain (N-terminus) and the grass pollen group-2 allergen-like domain (C-terminus) (Kende et al., 2004), which correspond to Barwin-like endoglucanases and PHP pollen allergen domains in the Superfamily database (Wilson et al., 2009). It has been proposed that a protein can be designated as a member of the expansin superfamily only if the protein contains these two domains in the context of a single protein (Kende et al., 2004). There had been no known bacterial expansin homolog until the YoaJ protein from *Bacillus subtilis* (*Bs*EXLX1, PDB id: 2bh0) was shown to have high structural similarity to a plant expansin (EXPB1 of *Zea mays*, PDB id: 2hcz) (Kerff et al., 2008; Kim et al., 2009) despite its low sequence similarity. We used the sequence and structure of *Bs*EXLX1 as a template to search for other bacterial homologs in the public protein database. The BLAST and Psi-BLAST search of *Bs*EXLX1 against the non-redundant database in NCBI resulted in 48 bacterial protein hits with no archaeal proteins (E-value cutoff = 10^{-3}; restricted to bacterial sequences only). Unlike plant expansin, where the two domains are mostly found in a single protein, many bacterial expansin-like proteins contained different domains indicating that bacterial expansins occur as multi-domain proteins. Interestingly, the most popular domains occurring together were cellulases (endo-glucanase). Because many of the hit sequences were almost identical, we created a representative sequence set that excluded redundant sequences (> 90% sequence identity; >150 residues; representing single *Genus*). The remaining 21 proteins of the 48 hits were combined in the standard sets of expansins (41 proteins available for the nomenclature, http://www.bio.psu.edu/expansins/nomenclature.htm) (Kende et al., 2004) and the phylogenetic relationship among these proteins were analyzed. The phylogenetic tree clearly revealed the predefined expansin subfamilies (EXPA, EXPB, EXLA, EXLB and EXLX) and interestingly, the bacterial expansin homologs were divided into two distinct subfamilies (Fig. 1). Because HCH_03669 belonged to a different cluster, which was distinct from that of *Bs*EXLX1, we chose HCH_03669 for further biochemical examination of molecular function and the protein was designated as a member of the new subfamily of bacterial expansin-like proteins (EXLX2). We also performed a motif analysis of the bacterial expansin-like proteins (EXLX) using the MEME software (Grundy et al., 1997). As shown in Fig. 2, two motifs (M2 and M6) were identified as common motifs conserved in all members of the expansin-like proteins (See Supplementary Fig. S1 for the multiple sequence alignment of two motifs). It is worth noting that the M1 motif was conserved across all expansin proteins except one bacterial subfamily belonging to EXLX1, which had the M14 motif in the same position. By counting the motifs shared between bacterial EXLXs and other authentic expansin subfamilies, several subfamily-specific motifs were identified (Fig. 2).

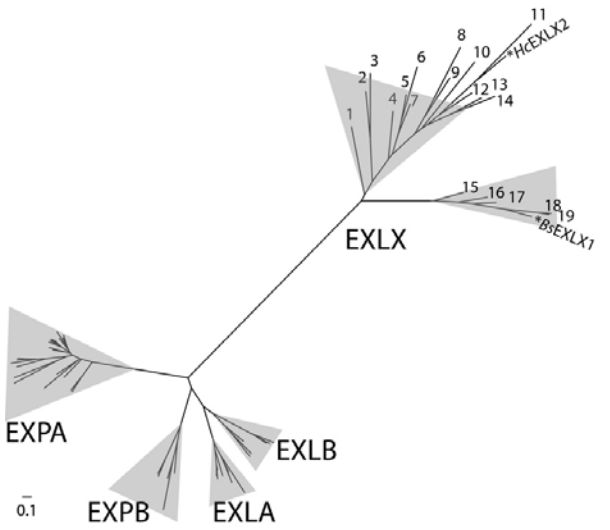

Fig. 1. Phylogenetic analysis of the expansin superfamily. Known subfamilies were represented as EXPA (α-expansin), EXPB (β-expansin), EXLA (expanin-like A), EXLB (expansin-like B) and EXLX (expansin-like group X), which is the same nomenclature used in Kende et al. (2004). The representative sequences (41 proteins) for the nomenclature used in (Kende et al., 2004) were combined with 21 representative bacterial proteins selected in this study. The phylogenetic tree was built using the Phylip package as described in the method. *Bs*EXLX1(gi|16078923) and *Hc*EXLX2 were indicated with asterisks (*) in the figure. The origin and Genbank accession number of bacterial proteins used in the analysis were shown as numbers in the figure as follows; 1, *Leptothrix cholodnii* (gi|171061047), *Lch*; 2, *Clavibacter michiganensis* (gi|148272660), *Cmi*; 3, *Nakamurella multipartite* (gi|229224499), *Nmu*; 4, *Frankia sp.* (gi|158317309), *Fsp*; 5, *Micromonospora sp.* (gi|238061663), *Msp*; 6, *Streptomyces sviceus* (gi|254404534), *Ssv*; 7, *Catenulispora acidiphila* (gi|229246366), *Cac*; 8, *Plesiocystis pacifica* (gi|149922302), *Ppa*; 10, *Herpetosiphon aurantiacus* (gi|159898342), *Hau*; 11, *Chloroflexus aurantiacus* (gi|163845817), *Cau*; 12, *Acidovorax avenae* (gi|120612050), *Aav* ; 13, *Roseiflexus sp.* (gi|148655687), *Rsp*; 14, *Myxococcus xanthus* (gi|108762346), *Mxa*; 15, *Stigmatella aurantiaca* (gi|115373847), *Sau*; 16, *Xanthomonas campestris* (gi|188990055), *Xca*; 17, *Ralstonia solanacearum* (gi|83747707), *Rso*; 18, *Xylella fastidiosa* (gi|71275113), *Xfa*; 19, *Pectobacterium atrosepticum* (gi|50121146), *Pat*; 20, *Dickeya zeae* (gi|251789717), *Dze*. The scale bar represents a sequence divergence equivalent to an expected 0.1 changes per site.

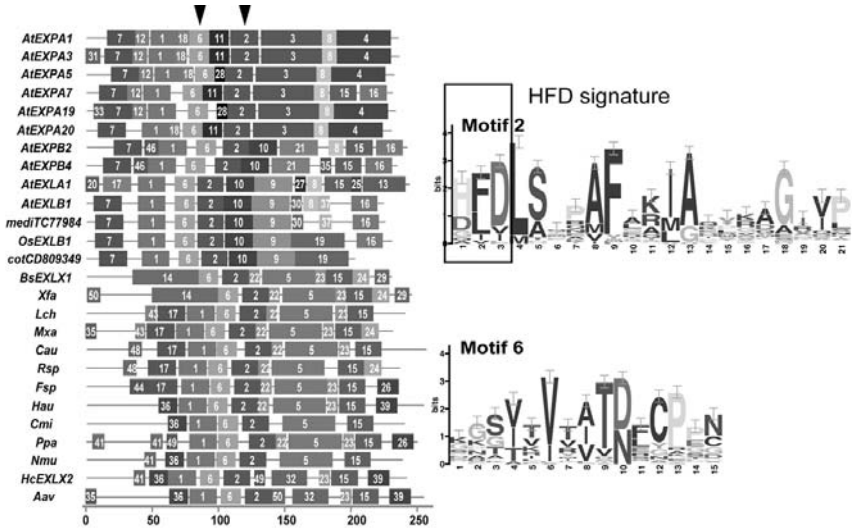

Fig. 2. Motif analysis of the expansin superfamily. The motifs were identified using the MEME software from representative sequences used in Fig. 1 (the default parameters used in the MEME software except the followings options; -nmotifs 50 -minsites 2 -minw 8). To determine the motif distribution in the expansin superfamily, proteins having the same motif organizations were omitted in the figure. Each subfamily is represented by EXPA (*At*EXPA1, *At*EXPA3, *At*EXPA5, *At*EXPA7, *At*EXPA19 and *At*EXPA22), EXPB (*At*EXPB1 and *At*EXPB4), EXLA (*At*EXLA1), EXLB (*At*EXLB1 and *Os*EXLB1), EXLX1 (*Bs*EXLX1, *Xfa*), EXLX2 (*Hc*EXLX2, *Aav, Cau, Cmi, Fsp, Hau, Mxa, Nmu, Ppa, Rsp*). The subfamily and sequence symbol used in the figure were the same as the standard set for nomenclature (Kende et al., 2004) in Fig. 1. The multiple sequence alignment made from selected members was shown in Supplementary Fig. S2.

Binding of HcEXLX2 to various complex polysaccharides

A known molecular function of the expansin superfamily is binding to complex CPs such as celluloses and hemicellulose (e.g. xylans) on the plant cell wall (Yennawar et al., 2006). We used various CPs (Avicel, BMCC, birch-wood xylan and oat spelts xylan) to test whether *Hc*EXLX2 can bind to these CPs. Two different types of cellulose, Avicel and BMCC represent the plant cell components but the degree of crystallinity is different (BMCC has higher crystallinity). Xylans represent a major component of the hemicellulose composition of the plant cell wall. As shown in Fig. 3, *Hc*EXLX2 bound to both celluloses and xylan but had more preference for binding to Avicel than BMCC, birchwood xylan or oat spelts xylan. The optimum temperature and pH for binding to Avicel were 4°C and pH 6.0, respectively. At these optimum conditions, the relative binding constant (K_r) to Avicel was determined to be 2.1 L g^{-1}.

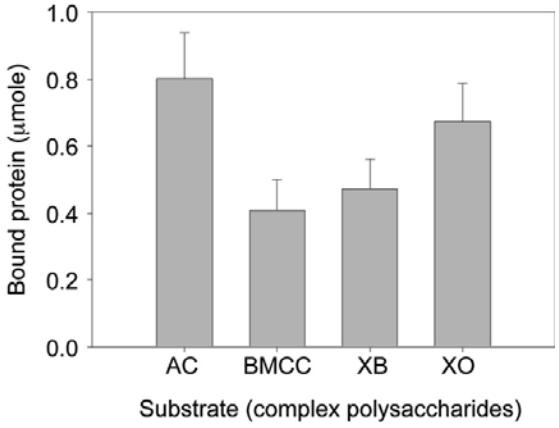

Fig. 3. Binding affinity of *Hc*EXLX2 to cell wall polysaccharides. *Hc*EXLX2 bound to Avicel (AC), bacterial micro-crystalline cellulose (BMCC), birchwood xylan (XB) and oat spelts xylan (XO). The bound protein was calculated by subtracting the amount of unbound protein from the amount initially added (2.5 nmole). All experiments were performed in triplicate and data were shown as mean ± standard error of mean.

Synergistic effect of *Hc*EXLX2 on the hydrolysis of cellulose by cellulase

Bacterial expansin has been proposed for use as a biochemical agent to reduce the cost of bioenergy production by enhancing cellulase activity (Kim et al., 2008; 2009). We tested the synergistic effect of *Hc*EXLX2 with cellulose for applications in biomass deconstruction. Since a standardized protocol for measuring cellulase activity was developed by the National Renewable Energy Laboratory (NREL) (Adney and Baker, 1996), we used this same protocol with a slight modification. The synergistic effect was determined by measuring the release of reducing sugars from cellulosic materials such as filter paper by cellulase in the presence of absence of *Hc*EXLX2. Because the cellulose binding domain (CBD) is known to have a synergistic effect by increasing the affinity of cellulase to the substrate, we used the CBD from *C. thermocellum* (*Ct*CBD) as a positive control to assess synergistic cellulase activity. As shown in Fig. 4, after a 36 h reaction, *Hc*EXLX2 significantly promoted cellulase activity, which produced about 4.6 times more reducing sugars (61.2 μg) than when only cellulase was used (13.2 μg). Surprisingly, the cellulase activity in the presence of *Hc*EXLX2 was approximately 2 time great than in the presence of *Ct*CBD, which is known to enhance cellulase activity (Carrard et al., 2000). It is worth noting that BSA also seemed to promote cellulase activity; however, we believe this enhancement was a non-specific effect of BSA (e.g. increasing enzyme stability) (Kerff et al., 2008; Kim et al., 2009).

Extension strength test on the filter paper

Since the proposed mechanism of the molecular function of plant expansin is the disruption of hydrogen bonds in CPs (McQueen-Mason and Cosgrove, 1994), we carried out the extension strength test on filter paper immersed with *Hc*EXLX2 and *Ct*CBD to determine whether these proteins have a texture-loosening effect on the filter paper. Treatment with 8 M Urea was used as a positive control because of its known capability to loosen the texture of filter paper and BSA was used as a negative control. As shown in Fig. 5, only *Hc*EXLX2, among all the tested proteins, showed a texture-loosening effect and this effect was 53% of that observed for 8 M Urea. However, *Ct*CBD treatment did not change the extension strength of

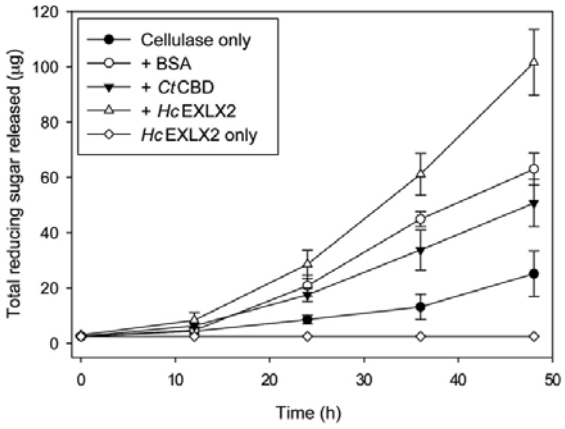

Fig. 4. Effect of *Hc*EXLX2 on the synergism of cellulose hydrolysis. According to NREL standard protocol for measuring the cellulase activity (Adney and Baker, 1996), filter paper disc was incubated with 0.06 FPU of cellulase with or without 50 nmole of BSA, *Ct*CBD or *Hc*EXLX2 per g of filter paper in a citrate buffer solution (pH 4.8) at 50°C. All experiments were performed in triplicate and data were shown as mean ± standard error of mean.

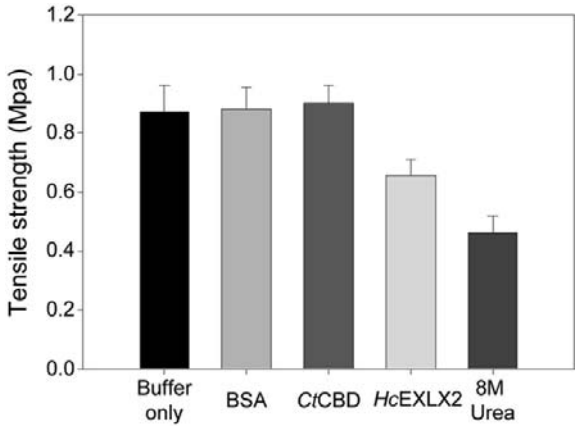

Fig. 5. Texture-loosening effect of *Hc*EXLX2 on filter paper. The effect was measured as the tensile strength of Whatman filter paper No. 3 after incubating in buffer (50 mM sodium acetate, pH 4.8) containing samples (50 μmole of *Hc*EXLX2 and *Ct*CBD) at 25°C for 1 h. 0.1 M BSA and 8 M urea were used as the negative and positive controls, respectively. All experiments were performed in triplicate and data were shown as mean ± standard error of mean.

the filter paper.

DISCUSSION

The expansin superfamily has been mainly divided into 5 subfamilies; α-expansin (EXPA), β-expansin (EXPB), expanin-like A (EXLA), expansin-like B (EXLB) and expansin-like group X (EXLX) in previous study (Kende et al., 2004; Sampedro and Cosgrove, 2005). More specifically, EXLX represents all other non-plant expansin-related proteins that are not classified into the 4 major subcategories (EXPA, EXPB, EXLA and EXLB) including bacterial homologs. In this report, we showed that bacterial expansins (EXLX) were clearly divided into two distinct subfamilies with a monophylectic mode (Fig. 1). These bacterial expansins were found in only a narrow range of bacterial taxa (mainly in Proteobacteria and Actinobacteria) with no archaeal homolog. Most of these bacteria possessing expansin-like proteins have been known to interact with plants in terms of pathogenic or symbiotic relationships (Kerff et al., 2008). Since two bacterial expansin subfamilies appeared as monophylectic in the phylogentic tree, these subfamilies might have evolved independently in a small range of taxa or had horizontally transferred from the plant. Although the members of EXLX1 mainly belong to Gram (-) bacteria, except *Bs*EXLX1, the EXLX2 subfamily retained both Gram (+) and Gram (-) bacteria. However, it is still not clear whether this limited taxonomic distribution is due to insufficient sequence information such as biases in the microbial genome sequencing projects. In addition to the phylogenetic analysis, we also carried out a motif profile analysis, which often provides information on the evolutionary conserved regions in the protein. Because these regions play functionally important roles in the superfamily or in a specific subfamily, these motifs can be employed to predict the family specific molecular function (e.g. expansin signatures) once we understand the relationship between motifs and molecular function. The highly conserved motifs across the entire expansin superfamily were determined to be M2 and M6 in the amino terminal domain (GH45 domain). However, M1 was also a highly conserved motif across all subfamilies except for the EXLX1 subfamily. More specifically, the M2 motif included parts of the catalytic residues (HFD) of GH45 proteins, which was previously reported as the signature sequence of EXPA and EXPB (Sampedro and Cosgrove, 2005). Subfamily-specific motifs were also identified, as shown in Fig. 2. These motifs will be helpful in classifying expansin-like proteins that have very low sequence similarity or recognizing functionally important residues in a hypothetical protein.

The biological functions of bacterial expansins are still unknown but one potential molecular function of *Bs*EXLX1 was previously examined; its ability to bind to cell-wall polysaccharides such as cellulose or peptidoglycan (Kerff et al., 2008) and its synergistic effect on cellulase activity (Kim et al., 2009). However, no member of EXLX2 has been cloned or functionally characterized. Thus, in this study, one member of the EXLX2 subfamily (*Hc*EXLX2) was cloned and overexpressed in *E. coli* for biochemical characterization. *Hc*EXLX2 was shown to bind to cell wall polysaccharides such as cellulose and xylans but had a preference for crystalline cellulose (Avicel) (Fig. 3). The relative binding constant (K_r = 2.1 L g^{-1}) against Avicel was comparable to known cellulose binding domains (CBDs) such as CfCBD (K_r = 2.7) from *Cellulomonas fimi* (Tomme et al., 1996) and *Ct*CBD (K_r = 1.4) from *C. thermocellum* (Morag et al., 1995). The optimum pH for binding was acidic (< pH 6) but the proteins were unstable and precipitated below pH 6. Interestingly, unlike *Bs*EXLX1, which is a basic protein (predicted pI = 9.53), the calculated pI value of *Hc*EXLX2 was 4.9. Expansins were initially identified as active components while studying acid-induced growth of plant cells and their optimum pH for cell-wall loosening activity was mostly reported to be acidic (McQueen-Mason and Cosgrove, 1995; Sampedro and Cosgrove, 2005). In addition, it was suggested that the basic pI of *Bs*EXLX1 was the reason why *Bs*EXLX1 had the capacity to bind to acidic sugars (Kerff et al., 2008). We examined the pI distribution of expansins by calculating the pI values of all representative members used in the phylogenetic analysis (see, Supplementary Table S1). While most plant expansins (EXPA, EXPB and EXLA) had basic pI values (> pH 8), the EXLB subfamily had acidic pI (< pH 7) values and the pI values of the bacterial expansins (EXLX1 and EXLX2) ranged from pH 4-10. Thus, the biological function of expansins may be dependent on their

environmental or physiological pH but the molecular function (e.g. binding to CPs) does not necessarily require a basic pI.

Another molecular function of bacterial expansin is enhancing cellulase activity (Kerff et al., 2008; Kim et al., 2008). Based on the domain organization in bacterial expanin-like proteins, it is plausible that the bacterial expansion-like proteins may still enhance the activity of cellulase even though they are multiple-domain proteins and the co-occuring domains may be mainly cellulase (endo-glucanase). *Hc*EXLX2 displayed a synergistic effect on cellulase activity as shown in Fig. 4. However, this synergistic effect was only observed when low amounts of cellulase (0.06 FPU per g filterpaper) were loaded into the reaction mixture. When a high load of either cellulase or *Hc*EXLX2 was used no synergistic effect was observed. Since the cellulase (Celluclast™) used in this study has a cellulose binding domain, there might be no synergistic effect when the cellulose substrate and *Hc*EXLX2 are both competing for the same cellulase binding site. Thus, the optimal dose for producing a synergistic effect and the binding mechanism of bacterial expansin should be examined in more details prior to its use in biomass deconstruction or being tested with a catalytic core of cellulase.

The proposed molecular mechanism for promoting cellulase activity by expansins is disruption of hydrogen bonding in recalcitrant cellulosic materials along with binding to cell-wall polysaccharides (Kerff et al., 2008; Kim et al., 2009). In general, cellulose binding domains have been known to result in a synergistic effect on cellulase activity by increasing the accessibility of cellulase to substrates (Carrard et al., 2000; Merino and Cherry, 2007) but their hydrogen bond breaking activity has not yet been fully examined (Din et al., 1991). In terms of the texture-loosening effect measured in the extension strength test, *Hc*EXLX2 was capable of disrupting hydrogen bonds in filter paper and this ability might also contribute to its synergistic effect on cellulase activity. In contrast to *Hc*EXLX2, *Ct*CBD did not show any texture-loosening effect, inferring that the synergistic effect of *Ct*CBD might come mainly from cellulose binding or the hydrogen bond breaking activity of *Ct*CBD is negligible. Although we could not estimate how much cellulose binding or hydrogen bonding disruption contributes to the synergistic effect on cellulase activity, it is plausible that both molecular functions of *Hc*EXLX2 play a role in this synergistic effect. Based on this comparison, the molecular mechanism by which *Hc*EXLX2 enhances cellulase activity might be different than the mechanism by which *Ct*CBD achieves this, since *Ct*CBD only displayed cellulose-binding activity.

Currently, one of the largest hurdles in producing bioenergy from cellulosic biomass is the high cost of the biomass deconstruction process (Lynd et al., 2008). Although we still need to have a better understanding of the biological or molecular function of bacterial expansins, most recent studies, including this report, suggest that bacterial expansins, which enhance cellulase activity, can be utilized as a biochemical agent in cellulosic biomass conversion to reduce the cost of bioenergy production.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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