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Putative Role of Cellulosomal Protease Inhibitors in *Clostridium cellulovorans* Based on Gene Expression and Measurement of Activities▽

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This study is the first to demonstrate the activity of putative cellulosomal protease/peptidase inhibitors (named cyspins) of *Clostridium cellulovorans*, using the *Saccharomyces cerevisiae* display system. Cyspins exhibited inhibitory activities against several representative plant proteases. This suggests that these inhibitors protect their microbe and cellulosome from external attack by plant proteases.

Some species of the Gram-positive anaerobic bacterium *Clostridium* can efficiently degrade polysaccharides in plant cell walls. Their efficient degradation is attributed to the cellulosome, which is the cellulolytic complex consisting of many cellulose-degrading enzymes. The cellulosome is composed of two major components: (i) a cell surface-attached nonenzymatic scaffolding protein with cohesion domains and (ii) a variety of cellulosomal enzymes with dockerin domains. Through an interaction between cohesin and dockerin, these cellulosomal proteins can assemble on cell surface-attached scaffolding protein. Assembly of enzymes in the cellulosome leads to effective and conjugative reactions (9). To understand the molecular mechanism underlying saccharification by the cellulosome, genomic analysis of several cellulosome-producing microorganisms was performed (*Clostridium thermocellum* and *Clostridium cellulolyticum*, analyzed by the Joint Genome Institute in 2007 and 2009, respectively). Whole-genome sequence analysis of *Clostridium cellulovorans*, which was suggested to have protoplast formation activity (1, 16), identified putative cellulosomal genes (14). Compared to genomes of other cellulosomal clostridia, *C. cellulovorans* has the smallest number of cellulosomal genes (15). The cellulosome of *C. cellulovorans* was indicated to be the most simple and suitable for the mechanistic analysis of the cellulosome. Genome analysis revealed 57 cellulosomal genes, including 4 scaffolding proteins and 53 cellulosomal enzymes. Most cellulosomal enzymes belong to the glycoside hydrolase family; there is, however, an interesting characteristic. Three protease/peptidase inhibitors are predicted in *C. cellulovorans*. Putative protease/peptidase inhibitors have been predicted in other cellulosome-producing microorganisms, such as *C. thermocellum* (20), *C. cellulolyticum* (3), and *Piromyces* sp. strain E2 (13). However, the role of protease inhibitors in cellulosomes has yet to be clarified. The prediction of gene function on the basis of genome analysis does not always correspond to protein profiling; therefore, gene function analysis at the level of protein production and activity is important to understand the true role of the gene. Because it is difficult to genetically manipulate *C. cellulovorans*, measurement of cellulosomal protein activity was examined with a *Saccharomyces cerevisiae* display system (17, 18), a heterologous gene expression system. This system is convenient to measure the activity of proteins because the displayed heterologous proteins can be quickly and directly analyzed using intact cells without the need for purification and concentration (17, 18). Furthermore, the proteins produced by the yeast display system are located on the yeast cell surface as well as cellulosomal enzymes that were naturally located on the cell surface of *Clostridium* species. In this study, using the yeast display system, we identified the function of the protease/peptidase inhibitors in *C. cellulovorans* cellulosomes and suggest their role in several cellulosome-producing microorganisms.

The regions encoding the protease/peptidase inhibitors cyspin 1 (putative *cys*teine *p*eptidase *in*hibitor *1*), cyspin 2, and cyspin 3 were amplified from the *C. cellulovorans* genome by PCR using one of three primer pairs, respectively: cyspin 1, 2-forward and cyspin 1-reverse; cyspin 1, 2-forward and cyspin 2-reverse; or cyspin 3-forward and cyspin 3-reverse (Table 1). By subcloning the resulting PCR product into the BglII-XhoI restriction site of pULD1 (8), three plasmids, pULD1-cyspin 1, pULD1-cyspin 2, and pULD1-cyspin 3, were constructed for display on the yeast cell surface by fusion with α-agglutinin and FLAG tag for detection (17, 18) (Fig. 1a). The constructed plasmids were introduced into *Saccharomyces cerevisiae* strain BY4741/*sed1*Δ (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 YDR077w*::*KanMX4*) by the lithium acetate method (5) using the EZ-Yeast transformation kit (BIO 101, CA). To detect displayed cyspins, cells were analyzed by immunofluorescence staining. After washing with phosphate-buffered saline (PBS) (pH 7.4), yeast cells were incubated in PBS (pH 7.4) containing 1% bovine serum albumin for 30 min. Mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO) was added as the primary antibody at a dilution factor of 1:300, and then the mixture of cells and antibody was incubated on a

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TABLE 1. Primers used in this study

Primer	Sequence[a]
cyspin 1, 2-F	GAAGATCTATGTTAAAGAAAAAAAGAACATTTATTAGC (BglII)
cyspin 1-R	CCGCTCGAGCCTTAAAAGAGCCATTTTTAATAAGTTAAC (XhoI)
cyspin 2-R	CCGCTCGAGAGTTAAAAGAACCTTTCTTAATAATGCAAC (XhoI)
cyspin 3-F	GAAGATCTATGTTAAGGAAGAAAAAGAAATTTATCAG (BglII)
cyspin 3-R	CCGCTCGAGAGCTAAAAGAGCCTTTTTTAATAATGCAG (XhoI)

[a] Underlines indicate the restriction sites of the enzymes shown in parentheses.

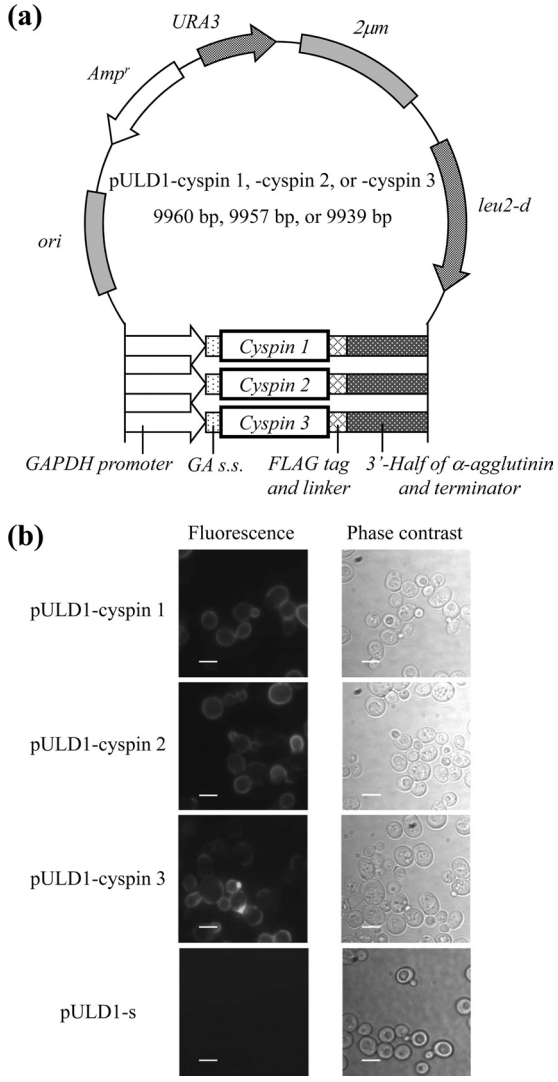

FIG. 1. Constructed plasmids and immunofluorescence staining of cyspin-displaying yeast cells. (a) Plasmid map of the pULD1-cyspin 1, 2, and 3 plasmids for display of cyspins on the yeast cell surface. Amp^r, ampicillin resistance gene for bacterial selection; 2μm, replication origin of yeast; *ori*, replication origin of *Escherichia coli*; *URA3*, uracil marker gene; *leu2-d*, leucine marker gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GA s.s., glucoamylase secretion signal. (b) Immunofluorescence staining for cell surface display of cyspins with mouse monoclonal anti-FLAG M2 antibody as the primary antibody and Alexa Fluor 488 goat anti-mouse IgG as the secondary antibody. Scale bar, 5 μm.

rotator at room temperature for 1.5 h. The cells were washed with PBS (pH 7.4) and incubated with the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, CA), diluted at 1:300 at room temperature for 1.5 h on a rotator. After washing with PBS (pH 7.4), cells were suspended in 20 μl PBS (pH 7.4) and then observed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) through a U-MNIBA2 mirror unit with a BP470-490 excitation filter, a DM505 dichroic mirror, and a BA510-550 emission filter (Olympus). Live images were obtained using AquaCosmos 2.0 software (Hamamatsu Photonics, Hamamatsu, Japan) controlling a digital charge-coupled device camera (C4742-95-12ER; Hamamatsu Photonics). Fluorescence was observed on the cell surface of yeast cells harboring pULD1-cyspin 1, pULD1-cyspin 2, and pULD1-cyspin 3, while pULD1-s-harboring cells (8), which displayed only a Strep-tag epitope, did not fluoresce (Fig. 1b).

To examine the property of each cyspin, their inhibitory activities were measured against several representative plant proteases, including papain, bromelain, and ficin. Cyspin-displaying yeasts were precultivated in SDC-HLM (0.67% [wt/vol] yeast nitrogen base without amino acids, 2% glucose, 2% Casamino Acids, 0.002% L-histidine-HCl, 0.003% L-methionine, 0.003% L-leucine) for 24 h, transferred into fresh medium to obtain an optical density at 600 nm (OD_{600}) of 0.1, and cultivated for 24 h. After centrifugation at 3,000 × *g*, yeast cells were washed with PBS (pH 7.4) or 20 mM Tris-HCl (pH 7.8) and diluted to an OD_{600} of 40 with the same buffer. Similar display efficiencies were observed for cyspins 1, 2, and 3 by measuring the fluorescent intensity of cells after immunofluorescence labeling (data not shown). Fifty microliters of the yeast suspension was then added to a solution containing 130 μl of PBS (pH 7.4) or 20 mM Tris-HCl (pH 7.8) and 10 μl of protease (1 mg/ml papain [Wako Pure Chemical Industries, Ltd., Osaka, Japan] and ficin [Sigma-Aldrich] in PBS [pH 7.4] or bromelain [Wako Pure Chemical Industries] in Tris-HCl [pH 7.8]), and the solution was stirred gently for 10 min. Subsequently, 10 μl of benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-4-methyl coumarylamide (Z-Phe-Arg-MCA) (100 μM; saturated condition) (Peptide Institute, Osaka, Japan) was added, and fluorescence was immediately measured with a Fluoroskan Ascent fluorometer (Labsystems OY, Helsinki, Finland) using a 96-well tissue culture plate (353072; Becton Dickinson Labware, NJ). A filter pair with excitation and emission at 355 nm and 460 nm, respectively, was used to detect the fluorescence of 7-amino-4-methyl-coumarin (AMC) liberated from Z-Phe-Arg-MCA. The relative protease activity was calculated by the following equation: relative protease activity = fluorescence intensity of the reaction solution by cyspin-displaying yeast per cell/fluorescence intensity by control yeast per cell.

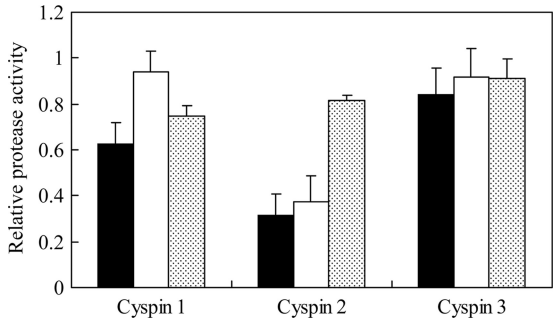

FIG. 2. Inhibition activity of cyspin-displaying yeast cells against papain, ficin, and bromelain. Closed, open, and stippled bars indicate the remaining protease activity rates against papain, ficin, and bromelain, respectively. Z-Phe-Arg-MCA was used as the substrate. A value of 1 for relative protease activity (in relative fluorescence units [RFU]/min) means that there was no inhibitory activity. The initial rates of activity against papain, ficin, and bromelain were 1.80, 0.715, and 0.739 RFU/min. Each data set represents averages and standard deviations from three independent experiments.

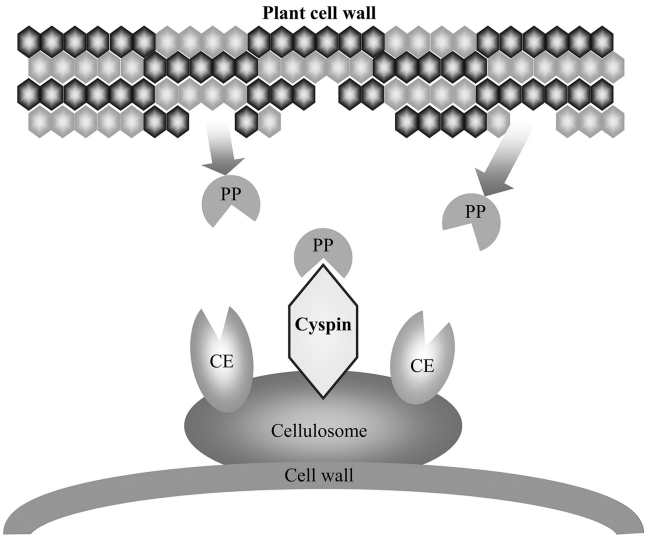

FIG. 3. Putative role of cellulosomal cyspins in *C. cellulovorans*. Cellulosomal protease/peptidase inhibitors protect their microbe and cellulosome. PP, plant proteases; CE, various cellulosomal enzymes.

Proteolytic enzymes in plants are involved in almost all aspects of growth and development, including germination, circadian rhythms, senescence, and programmed cell death (2). One function of a protease is to protect plant cells against outside attack. Proteases also appear to play key roles in regulating biological processes in plants, such as recognition of pathogens and pests and induction of effective defense systems. For example, papain, found in the latex of papaya trees, is excreted from wounds and contributes to defense response.

Cyspin 1 inhibited papain and bromelain, and cyspin 2 inhibited papain and ficin. In contrast, cyspin 3 inhibited all proteases, but its activity was lower than that of cyspins 1 and 2 (Fig. 2). These results indicate that these inhibitors exhibited cysteine-based activity against plant proteases, and of these, cyspins 1 and 2 were uniquely specific. This is the first report demonstrating the true inhibitory activity of cellulosomal protease/peptidase inhibitors against plant proteases. Several articles have proposed that cellulosomal protease/peptidase inhibitors could protect their respective microbes and cellulosomes from external proteases (3, 11). Our results with the inhibitory activity of cyspins actually demonstrated this hypothesis by measuring their inhibitory activity.

Genome analysis of *C. cellulovorans* suggested that the cellulosome included three kinds of protease/peptidase inhibitors (15). The actual presence of protease/peptidase inhibitors in *C. thermocellum* and *C. cellulolyticum*, in which their presence was previously predicted, has been suggested only by transcriptional and proteomic analysis (3, 6). Our study first demonstrated that protease/peptidase inhibitors can exhibit their activity in cellulosomes from *C. cellulovorans* and may play a vital role.

Furthermore, cyspins 1 and 2 were regulated by the same promoter on the basis of genome sequencing results, suggesting the possibility of simultaneous expression. Analysis of amino acid homology indicated that three cyspins have two conserved chagasin family peptidase inhibitor I42 domains, initially found in *Trypanosoma cruzi* (10). The first chagasin domain showed a high degree of amino acid homology (76 to 77%) among the three cyspins. Despite little difference in the first chagasin domain among the three cyspins, cyspin 3 had wide substrate specificity and low activity, unlike cyspins 1 and 2. Therefore, the second chagasin domain could be important for the inhibitory activity specific to cyspins 1 and 2. In fact, the second chagasin domain of cyspin 3 had 40% and 46% amino acid homologies with that of cyspins 1 and 2, respectively, although there was 57% amino acid homology between cyspins 1 and 2, suggesting a difference in the activity between cyspins 1 and 2 and cyspin 3. There is the possibility that cyspin 3 might specifically inhibit other types of proteases and play a role similar to cyspins 1 and 2.

In latex, some plant cysteine proteases show strong toxicity and growth inhibition against insects (7). In addition, cysteine proteases are involved in the defense response in *Arabidopsis thaliana* (19). These studies indicate that plant cysteine proteases could play an important role in self-defense. *C. cellulovorans* was isolated from the wood chip of the poplar tree (12), whose protease gene family is similar to that of *A. thaliana* (4). Therefore, the poplar could have the same protection system, using proteases for self-defense. Considering the defense systems in plants and cyspin function, it is possible that cyspins in cellulosomes could contribute to the protection of *C. cellulovorans* against attacks by plant proteases (Fig. 3).

In conclusion, we demonstrated, for the first time, the true function of cellulosomal protease/peptidase inhibitors against plant cysteine proteases using three kinds of cyspin-displaying yeast cells. Our study suggests that cellulosomal inhibitors might protect their microbe and cellulosome from plant protease attack.

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