Biochemical and Biophysical Research Communications 521 (2020) 15–18

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Upregulation of cellulase activity and mRNA levels by bacterial challenge in the earthworm *Eisenia andrei*, supporting the involvement of cellulases in innate immunity

Seyoung Kim [a, 1], Donggu Jeon [a, 1], Ju-Young Lee [b, 1], Sung-Jin Cho [c], Younghyun Lim [a], Seong-il Eyun [a], Soon Cheol Park [a, **], Young-Jin Seo [a, *]

[a] Department of Life Science, Chung-Ang University, Seoul, Republic of Korea
[b] Department of Biomedical Engineering, College of Medical Convergence, Catholic Kwandong University, Gangneung, Republic of Korea
[c] School of Biological Sciences, Chungbuk National University, Cheongju, Republic of Korea

ARTICLE INFO

Article history:
Received 12 September 2019
Accepted 28 September 2019
Available online 19 October 2019

Keywords:
Earthworm
Bacterial challenge
Cellulase
Upregulation
Humoral effector

ABSTRACT

To investigate whether earthworm cellulases contribute to the innate immune system, the responsiveness of cellulase activity and mRNA expression to bacterial challenge was examined by zymography and RNA sequencing. A zymographic analysis revealed that the activity levels of earthworm cellulases were upregulated in response to either a bacterial (*Bacillus subtilis* or *Escherichia coli*) or LPS challenge. After the challenge, significant increases in cellulase 1 and cellulase 2 activity levels were observed within 8–16 and 16–24 h, respectively. In the coelomic fluid, both activities were significantly upregulated at 8 h post-injection with *B. subtilis*. Based on RNA sequencing, cellulase-related mRNAs encoding beta-1,4-endoglucanases were upregulated by 3-fold within 6 h after *B. subtilis* injection. Our results clearly demonstrated that earthworm cellulases are upregulated by bacterial challenge at the mRNA and protein levels. These results support the view that earthworm cellulases act as inducible humoral effectors of innate immunity against bacterial infection.

1. Introduction

Since the initial discovery of an animal cellulase [1], numerous cellulases in Ecdysozoa [2], Lophotrochozoa [3], and Deuterostomia [4] have been identified and characterized. Research on animal cellulases has focused on their molecular characteristics at the gene and protein levels [5,6], industrial applications for biofuel production [7], host-invasive process [8], and horizontal gene transfer in evolution [9].

In the earthworm, endogenous cellulase activity was identified primarily for the contribution to cellulose digestion [10]. Interestingly, earthworm cellulase mRNA was shown to be expressed not only in gut epithelial cells but also in non-digestive tissues, such as the epidermis and coelomic cell-aggregates. This suggests that in addition to digestion, earthworm cellulases may be involved in other processes including innate immunity [11]. However, the direct relationship of earthworm cellulases with innate immunity has not been identified yet. To investigate whether earthworm cellulases are involved in host defense and innate immunity, it is necessary to examine their responsiveness to bacterial challenge.

In this study, zymography and RNA sequencing were used to quantitatively evaluate changes in earthworm cellulase activity and mRNA expression levels in response to bacterial stimulation. Our findings strongly suggest that these enzymes act as an inducible humoral effectors of innate immunity against bacterial infection.

2. Materials and methods

2.1. Animals

Sexually mature earthworms *Eisenia andrei* with well-developed clitella were obtained from a commercial source (Seoul, Korea) and reared as described previously [11]. Before use,

* Corresponding author. Department of Life Science, Chung-Ang University, 84 Hukseok-ro, Dongjak-gu, Seoul, 06974, Republic of Korea.
** Corresponding author. Department of Life Science, Chung-Ang University, 84 Hukseok-ro, Dongjak-gu, Seoul, 06974, Republic of Korea.
E-mail addresses: scpark@cau.ac.kr (S.C. Park), yjseo@cau.ac.kr (Y.-J. Seo).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbrc.2019.09.134
0006-291X/

earthworms were placed on moistened filter paper in Petri dishes with earthworm saline for 48 h to purge the gut contents and avoid sample contamination.

2.2. Microbial challenge and sample collection

After purging the gut contents, 20 μL of PBS containing lipopolysaccharides (LPS, 5 μg), gram-negative *Escherichia coli* (10^4 CFU), or gram-positive *Bacillus subtilis* (10^4 CFU) was administered via parenteral injection into the post-clitellum. Earthworms injected with PBS alone were used as controls. After the injection, earthworms were severed to obtain the middle region (about 15–20 segments of the post-clitellum) at various time points. Pooled samples (5 earthworms per sample) were prepared for non-reducing SDS-PAGE by a previously described procedure [11]. The coelomic fluid (CF) was prepared as described previously [12]. The CF samples from 5 earthworms were pooled and centrifuged (8000×*g*, 10 min). The supernatant was then stored at −80 °C until use. The protein concentration was determined using the SMART BCA Protein Assay Kit (iNtRON, Seongnam, Korea) according to the manufacturer's instructions.

2.3. Zymography and densitometry

A zymographic analysis of cellulase activity was performed as described previously [11,13], with slight modifications. Briefly, zymogram gels were copolymerized with carboxymethyl cellulose (CMC, 1 mg/mL). Either 0.5 μg (CF) or 5 μg (middle region) of protein per lane was loaded onto a non-reducing gel (SDS-PAGE, 10%). The gels were run at a constant voltage on ice, maintaining a running buffer temperature below 4 °C. After electrophoresis, for renaturation, the gels were washed five times for 30 min at room temperature with 0.1 M succinate buffer (pH 5.8) containing 10 mM DTT and then incubated at 37 °C for 1 h. After incubation, the gels were stained with 0.1% Congo red. The stain was then rinsed with 1 M NaCl. Areas with enzymatic activity appeared as clear bands over a dark reddish background. Densitometry of clear bands was performed with scanned images using ImageJ (NIH, Bethesda, MD, USA).

2.4. RNA sequencing and annotation

At 6 h post-injection (hpi) with *B. subtilis*, RNA samples were prepared by a previously described method [14] and sequenced using the Illumina HiSeq2500 System at Theragen Etex Bio Institute (Suwon, Korea). The Illumina reads that did not meet the minimum quality score (30 per base) across the whole read were removed using Trim Galore! (ver. 0.6.2). Unknown nucleotide 'Ns' were trimmed. After the filtering process, *de novo* assemblies of the transcriptomes were generated using Trinity (ver. 2.8.5) [15]. The transcripts were annotated by BLAST (standalone blastx ver. 2.8.1) searches against the non-redundant protein database of National Center for Biotechnology Information (NCBI). Putative endoglucanase genes were verified by blastp searches against the CAZy database [16]. Paired-end reads were mapped to our assembled transcriptomes using bowtie2 (ver. 2.3.5) and the expression values (FPKM) were calculated using Cufflinks (ver. 2.2.1) [17].

2.5. Statistical analyses

Comparisons between groups were performed using unpaired Student's *t*-tests. Results are presented as means ± standard error of the mean (SEM). Data are representative of at least three independent experiments.

3. Results and discussion

The activity profile of cellulases from *E. andrei* indicated two main enzymes with estimated molecular masses of 29.2 (cellulase 1) and 15.3 (cellulase 2) kDa (Fig. 1A). No cellulase activity was detected in the lysates obtained from two bacteria used in this study, indicating that cellulase activity in the zymogram was not affected by bacterial sources (Fig. 1B).

Injection with LPS, gram-negative bacteria, or gram-positive bacteria induced the upregulation of cellulase 1 activity (Fig. 2A). After the challenge with gram-positive *B. subtilis,* cellulase 1 activity was upregulated within 8 hpi, reached a maximum level (>2 times higher than levels in the control) at 12 hpi, and then returned to levels in controls at 48 hpi. Stimulation by gram-negative *E. coli* resulted in a similar pattern, with upregulation starting at 8 hpi and maximum activity levels detected at 12 hpi. The effect of LPS was maintained until 16–48 hpi. As shown in Fig. 2B, cellulase 2 activity was significantly upregulated 8 h after stimulation with *B. subtilis*. Although *E. coli* challenge had a significant effect on cellulase 2 activity at 16 hpi, the magnitude of the effect was a slightly less than that of *B. subtilis*. In addition, activity levels in response to LPS

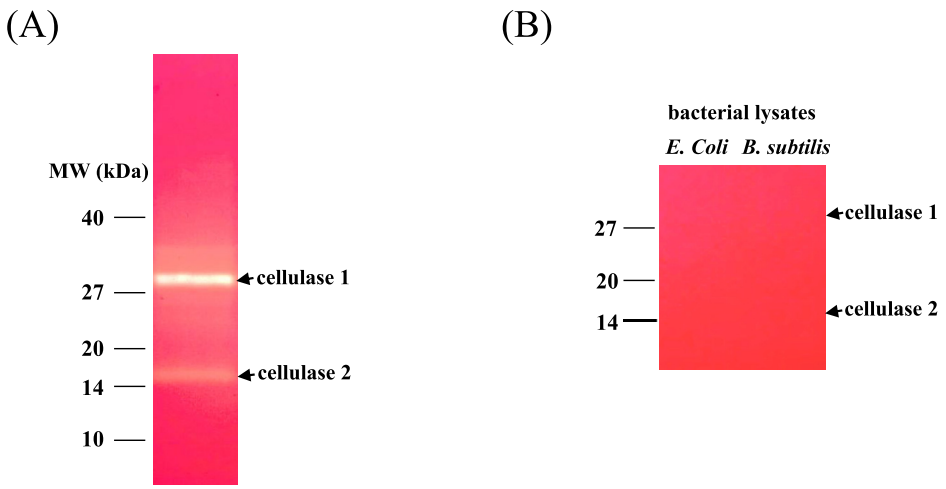

Fig. 1. Cellulase activity profile for the earthworm middle region (A) and lysates of bacteria injected into earthworms (B). (A) Two main bands were detected with estimated molecular masses of 29.2 (cellulase 1) and 15.3 (cellulase 2) kDa. (B) Lysates of *E. coli* and *B. subtilis* showed no cellulase activity.

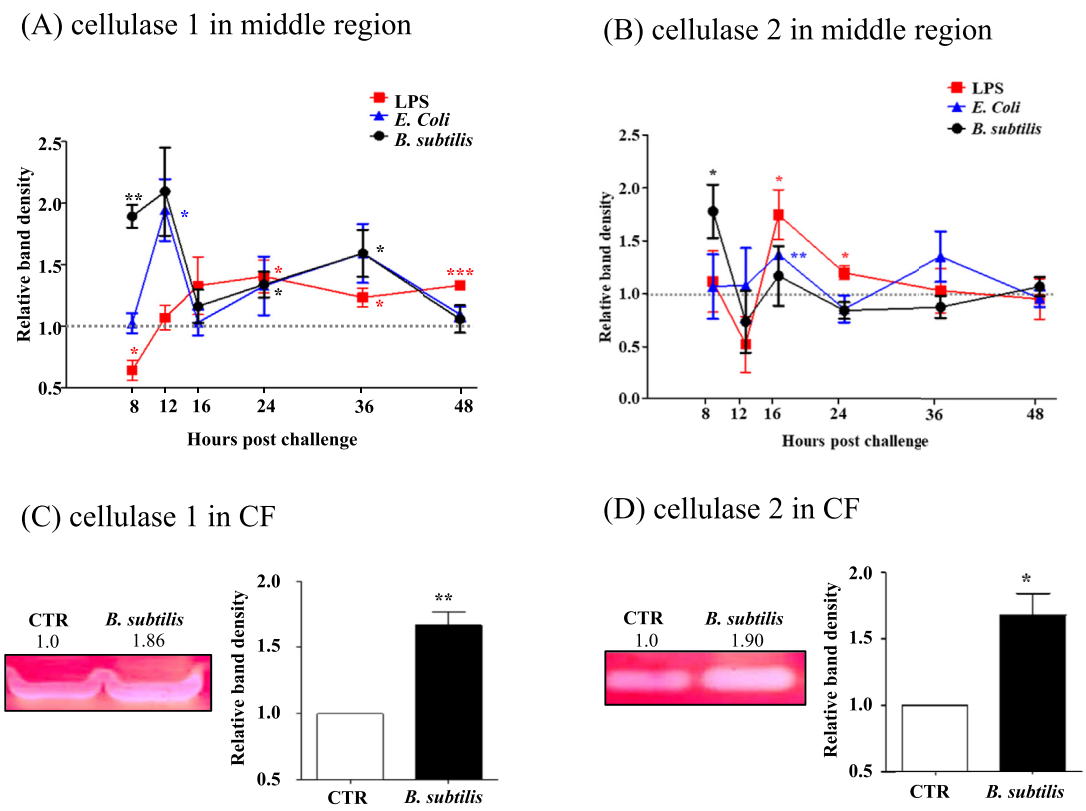

Fig. 2. Cellulase activity levels following bacterial or LPS challenges in the middle region and CF of the earthworm *E. andrei*. The responsiveness of cellulase activities in the middle region (A, B) and in the CF to *B. subtilis* (C, D) is shown. (A, B) Changes in the density of the zymographic bands relative to those of controls in the PBS alone-injected group are presented. Error bars represent means ± SEM of at least three independent experiments. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) The injection with gram-negative or gram-positive bacteria induced the upregulation of cellulase 1 activity to a maximum of two-fold higher than that of the non-stimulated control. Cellulase 2 activity was significantly upregulated by *B. subtilis* at 8 h after stimulation. (C, D) In CF, the activities of both cellulase 1 and 2 were significantly upregulated by approximately 2-fold at 8 hpi with *B. subtilis*.

gradually decreased to control levels after the peak at 16 hpi. In a previous study, high mRNA expression of a gene encoding an earthworm cellulase was observed in the coelom [11], where the innate immune response by parenteral bacterial challenges is expected. Thus, we examined the responsiveness of cellulase activity in CF following a *B. subtilis* challenge. The activities of both cellulase 1 and 2 were significantly upregulated by approximately 2-fold at 8 hpi (Fig. 2C and D).

In addition, we found that the expression levels of several cellulase-related mRNAs are upregulated by the *B. subtilis* challenge; the five most highly upregulated mRNAs are listed in Table 1. All five mRNAs were annotated as beta-1,4-endoglucanases and had open reading frames (ORF) of 1368 or 1116 bp, with significantly upregulated expression (by approximately 3-fold). Based on amino acid sequence similarity (maximally 98.25%) with previously reported earthworm proteins (Supplemental Fig. 1), they were identified as genes encoding cellulases. The deduced amino acid sequence of the two genes with short ORFs (1116 bp) showed only two amino acid residue differences (shown in red) with conserved C-terminal catalytic residues of GHF9 (Supplemental Fig. 1A) and the three genes with long ORFs (1368 bp) showed about 90% amino acid similarities with additional N-terminal catalytic residues (Supplemental Fig. 1B). These results indicate that the earthworm genome contains different types of cellulase genes encoding various isozymes that are stimulated by bacterial invasion.

These results clearly demonstrated that earthworm cellulases are upregulated by bacterial challenge at both the mRNA and enzymatic activity levels. These findings support the view that earthworm cellulases act as inducible humoral effectors of innate immunity against bacterial infection.

In addition to cellulases, another carbohydrate-polymer hydrolase believed to play a role in host defense in cnidarians, the chitinase *HyChit1*, is expressed only in the stolon ectoderm and lower part of the polyp [18]. A chitinase from the oyster *Crassostrea gigas* is primarily expressed in non-digestive tissues and hemocytes; it is stimulated at the transcriptional level in response to either

Table 1
Top 5 cellulase-related mRNAs upregulated by *B. subtilis* challenge in *E. andrei*.

Trinity ID[a]	Size (bp)	Putative function	Matching organism (accession No.)	Fold Change (log_2)	*P*-value/E-value
DN2700_c0_g1_i11	1368	beta-1,4-endoglucanase	*Eisenia fetida* (BAM14716.1)	1.60	<0.03/0.00
DN2700_c0_g1_i1	1116	beta-1,4-endoglucanase	*Eisenia andrei* (ACE75511.1)	1.58	<0.01/0.00
DN2700_c0_g1_i5	1116	beta-1,4-endoglucanase	*Eisenia andrei* (ACE75511.1)	1.56	<0.01/0.00
DN2700_c0_g1_i3	1368	beta-1,4-endoglucanase	*Eisenia fetida* (BAM14716.1)	1.55	<0.02/0.00
DN2700_c0_g1_i10	1368	beta-1,4-endoglucanase	*Eisenia andrei* (ACE75511.1)	1.54	<0.03/0.00

[a] The deduced amino acid sequences are shown in Supplemental Fig. 1.

bacterial or LPS challenge, indicating an important role as an immune effector in mollusks [19]. Earthworm cellulases likely function as humoral effectors. In general, to form protective envelopes, most bacteria synthesize extracellular polysaccharides, including cellulose, which is a critical determinant of viability [20]. In addition, the enzymatic hydrolysis of capsular polysaccharides by a carbohydrate-degrading enzyme renders the bacterium vulnerable to host defense by increasing susceptibility to phagocytosis by macrophages [21].

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by the Chung-Ang University research grant in 2013 and by the National Research Foundation of Korea grant funded by the Korea government (MSIT) (No. NRF-2018R1A5A1025077).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.134.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.134.

References

[1] H. Watanabe, H. Noda, G. Tokuda, N. Lo, A cellulase gene of termite origin, Nature 394 (1998) 330–331.
[2] T. Kikuchi, H. Shibuya, J.T. Jones, Molecular and biochemical characterization of an endo-beta-1,3-glucanase from the pinewood nematode Bursaphelenchus xylophilus acquired by horizontal gene transfer from bacteria, Biochem. J. 389 (2005) 117–125.
[3] Y. Li, Q. Yin, M. Ding, F. Zhao, Purification, characterization and molecular cloning of a novel endo-beta-1,4-glucanase AC-EG65 from the mollusc Ampullariacrossean, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 153 (2009) 149–156.
[4] Y. Nishida, K. Suzuki, Y. Kumagai, H. Tanaka, A. Inoue, T. Ojima, Isolation and primary structure of a cellulase from the Japanese sea urchin Strongylocentrotus nudus, Biochimie 89 (2007) 1002–1011.
[5] K. Nakashima, H. Watanabe, H. Saitoh, G. Tokuda, J.I. Azuma, Dual cellulose-digesting system of the wood-feeding termite, Coptotermes formosanus Shiraki, Insect Biochem. Mol. Biol. 32 (2002) 777–784.
[6] N. Tachibana, A. Saitoh, H. Shibata, M. Saitoh, S. Fujita, T. Ohmachi, Y. Kato, K. Takagaki, T. Yoshida, Carboxymethyl cellulase from mid-gut gland of marine mollusc, Patinopecten yessoensis. J. Appl. Glycosci. 52 (2005) 107–113.
[7] M. Ueda, A. Ito, M. Nakazawa, K. Miyatake, M. Sakaguchi, K. Inouye, Cloning and expression of the cold-adapted endo-1,4-beta-glucanase gene from Eisenia fetida, Carbohydr. Polym. 101 (2014) 511–516.
[8] L. Hu, R. Cui, L. Sun, B. Lin, K. Zhuo, J. Liao, Molecular and biochemical characterization of the beta-1,4-endoglucanase gene Mj-eng-3 in the root-knot nematode Meloidogyne javanica, Exp. Parasitol. 135 (2013) 15–23.
[9] W.E. Mayer, L.N. Schuster, G. Bartelmes, C. Dieterich, R.J. Sommer, Horizontal gene transfer of microbial cellulases into nematode genomes is associated with functional assimilation and gene turnover, BMC Evol. Biol. 11 (2011) 13.
[10] M. Nozaki, C. Miura, Y. Tozawa, T. Miura, The contribution of endogenous cellulase to the cellulose digestion in the gut of earthworm (Pheretima hilgendorfi: Megascolecidae), Soil Biol. Biochem. 41 (2009) 762–769.
[11] I.Y. Park, J.R. Cha, S.M. Ok, C. Shin, J.S. Kim, H.J. Kwak, Y.S. Yu, Y.K. Kim, B. Medina, S.J. Cho, S.C. Park, A new earthworm cellulase and its possible role in the innate immunity, Dev. Comp. Immunol. 67 (2017) 476–480.
[12] P. Kohlerova, A. Beschin, M. Silerova, P. De Baetselier, M. Bilej, Effect of experimental microbial challenge on the expression of defense molecules in Eisenia foetida earthworm, Dev. Comp. Immunol. 28 (2004) 701–711.
[13] P. Rishi, R. Thakur, U.J. Kaur, H. Singh, K.K. Bhasin, Potential of 2, 2'-dipyridyl diselane as an adjunct to antibiotics to manage cadmium-induced antibiotic resistance in Salmonella enterica serovar Typhi Ty2 strain, J. Microbiol. 55 (2017) 737–744.
[14] A. Bhambri, N. Dhaunta, S.S. Patel, M. Hardikar, A. Bhatt, N. Srikakulam, S. Shridhar, S. Vellarikkal, R. Pandey, R. Jayarajan, A. Verma, V. Kumar, P. Gautam, Y. Khanna, J.A. Khan, B. Fromm, K.J. Peterson, V. Scaria, S. Sivasubbu, B. Pillai, Large scale changes in the transcriptome of Eisenia fetida during regeneration, PLoS One 13 (2018), e0204234.
[15] B.J. Haas, A. Papanicolaou, M. Yassour, M. Grabherr, P.D. Blood, J. Bowden, M.B. Couger, D. Eccles, B. Li, M. Lieber, M.D. MacManes, M. Ott, J. Orvis, N. Pochet, F. Strozzi, N. Weeks, R. Westerman, T. William, C.N. Dewey, R. Henschel, R.D. LeDuc, N. Friedman, A. Regev, De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis, Nat. Protoc. 8 (2013) 1494–1512.
[16] B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics, Nucleic Acids Res. 37 (2009) D233–D238.
[17] C. Trapnell, B.A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M.J. van Baren, S.L. Salzberg, B.J. Wold, L. Pachter, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, Nat. Biotechnol. 28 (2010) 511–515.
[18] B. Mali, F. Mohrlen, M. Frohme, U. Frank, A putative double role of a chitinase in a cnidarian: pattern formation and immunity, Dev. Comp. Immunol. 28 (2004) 973–981.
[19] F. Badariotti, R. Thuau, C. Lelong, M.P. Dubos, P. Favrel, Characterization of an atypical family 18 chitinase from the oyster Crassostrea gigas: evidence for a role in early development and immunity, Dev. Comp. Immunol. 31 (2007) 559–570.
[20] S. Bielecki, M. Krystynowicz, H. Kalinowska, Bacterial Cellulose, Biopolymers Online, 2005.
[21] D.R. Middleton, A.V. Paschall, J.A. Duke, F.Y. Avci, Enzymatic hydrolysis of pneumococcal capsular polysaccharide renders the bacterium vulnerable to host defense, Infect. Immun. 86 (2018) e00316-18.