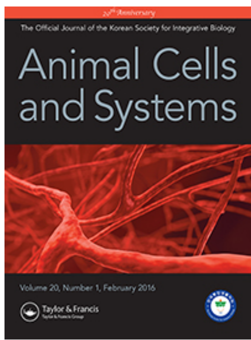

Animal Cells and Systems

ISSN: 1976-8354 (Print) 2151-2485 (Online) Journal homepage: https://www.tandfonline.com/loi/tacs20

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To cite this article: Daehwan Kim, Jin-Se Kim, In-Yong Park, Hee-Jin Kwak, Dong Ho Lee, Sung-Jin Cho & Soon Cheol Park (2016) A novel chitinase from the earthworm, *Eisenia andrei*, Animal Cells and Systems, 20:1, 48-51, DOI: 10.1080/19768354.2015.1119193

To link to this article: https://doi.org/10.1080/19768354.2015.1119193

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Published online: 05 Jan 2016.

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ANIMAL CELLS AND SYSTEMS, 2016
VOL. 20, NO. 1, 48–51
http://dx.doi.org/10.1080/19768354.2015.1119193

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A novel chitinase from the earthworm, *Eisenia andrei*

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ABSTRACT
A novel chitinase gene, *EaChi*, and its expression pattern from the earthworm, *E. andrei* are demonstrated. Based on a deduced amino acid sequence, in *EaChi*, two specific domains for GH family 18 are well conserved with two essential amino acid residues for enzyme activity. The phylogenetic analysis shows that earthworm chitinase, *EaChi*, is evolutionarily close to other lophotrochozoan chitinases. The expression pattern analysis of *EaChi* indicates that the major expression is localized at intestinal epithelium and epidermis, possibly suggesting that the prime functions of the chitinase activity could be related to not only digestive process but also self-defending immunity as a biochemical barrier to protect the invasion of chitin-containing pathogens, including fungi, nematodes and protozoa.

ARTICLE HISTORY
Received 25 October 2015
Revised 5 November 2015
Accepted 9 November 2015

KEYWORDS
Earthworm; novel chitinase; phylogenetic tree; expression pattern; self-defense

The chitinase (EC 3.2.1.14) catalyzes the hydrolysis of β-1, 4-linkage between *N*-acetylglucosamines (GlcNAc) in chitin, which is one of the most abundant polysaccharides in nature next to cellulose and starch (Garcia-Fraga et al. 2014). This enzyme has widely different functions involved in digestion, molting processes, defense/immunity and growth/development (Arakane & Muthukrishnan 2010). In general, in the chitinous animal, the function of chitinase is mainly associated with growth and pattern formations, such as insect molting. On the other hand, chitinases expressed in the non-chitinous pathogens are likely to be related to nutrition or innate immunity against chitinous pathogens (Mali et al. 2004). In the earthworm, since among microorganisms in surrounding environment, chitinous organisms including fungi, protozoa and algae are major sources of nutrients and/or pathogens (Edward & Fletcher 1988), and it is commonly supposed that the chitinase is a necessary enzyme to mainly support functions not only of a digestive enzyme for the hydrolysis of dietary but also of a host defense factor against chitin-containing pathogens.

Since the basic knowledge of the earthworm chitinase activity regarding its presence and distribution was reported in the mid-twentieth century, in which earthworm gut might possess the chitinase activity mainly in the posterior part of intestine probably originated from the earthworm itself rather than symbiotic microbes (Tracy 1951), very little information has been accumulated on the molecular level characteristics of the earthworm chitinase, enabling us to better understand its biological significances in view of earthworm nutrition and self-defense.

Here, we report a novel earthworm chitinase gene, *EaChi,* from the earthworm, *E. andrei* with providing its full-length cDNA sequence and multiple alignments analysis as well as histological expression pattern.

Sexually mature *E. andrei* were reared by the method of Cho et al. (2009) with the supply of powdered cow manure as a food material. Sequence and bioinformatics analyses were carried out by the methods described by Tak et al. (2015). To obtain the full-length cDNA of earthworm chitinase gene, *EaChi*, 3′- and 5′-rapid amplification of cDNA ends (RACE) PCR was performed according to manufacturer's manual (Clonetech) with specific primer sets: 3′Race [5′ GACTTCAACGGTGCCTGGGACACT 3′], 5′ Race [5′ GCACCGTCCATAGGTGGCAGTACC 3′], full-length (forward) [5′ ATGAGGGTCGCCCTCTGCT TAATCGTG 3′], full-length (reverse) [5′ TTATAGACCG CAGGCTGCCTTCCGTTC 3′]. For *in-situ* hybridization (ISH) of *EaChi*, specific primers (forward: ACTTC GATCTGCTGAACCAG; reverse: TAGCCGGATTGACGTA GAGTC) were designed. Riboprobes (1116 bp) were amplified from *E. andrei* cDNA, gel extracted, cloned into pGEM-T Easy (Promega) and labeled with digoxygenin using the MEGAscript (Ambion) kit, according to the manufacturer's instructions.

Adult earthworms were fixed at 4°C overnight (4% paraformaldehyde in 1 × PBS) and dehydrated in methanol series, equilibrated in 30% sucrose and mounted in

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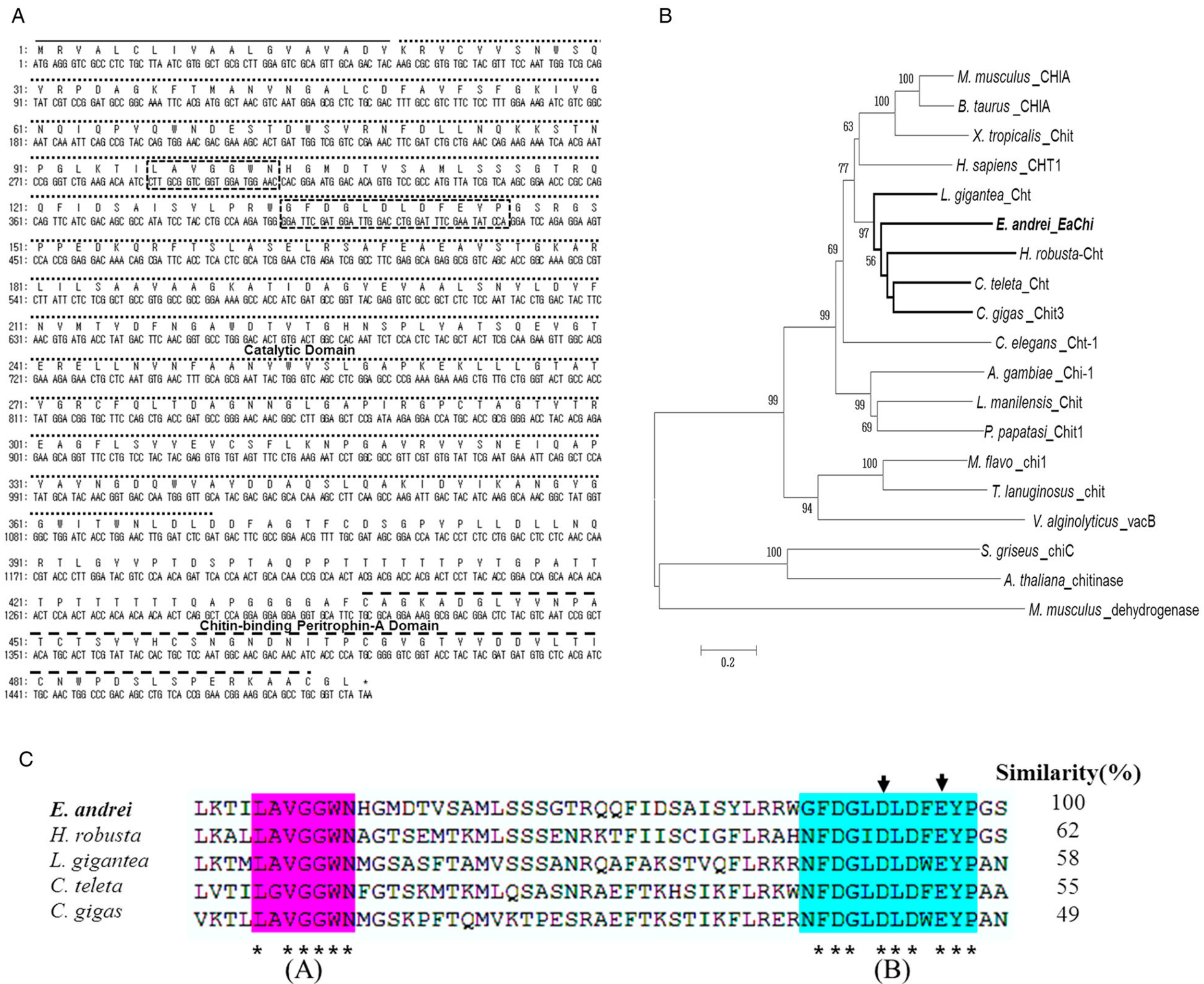

Figure 1. (a) Nucleotide and deduced amino acid sequences of the earthworm chitinase, *EaChi*. The open reading frame of earthworm chitinase gene, *EaChi*, consists of 1494 nucleotides encoding 497 amino acids. The solid line delineates putative signal peptide sequence. Black dotted and broken lines delimit two conserved domains for glycosyl hydrolases (GH) family 18, catalytic and chitin-binding Peritrophin-A domain, respectively. Two boxed sequences in catalytic domain indicate the chitin-binding and chitin-catalyzing domain, respectively from N-terminal. This sequence information has been deposited in GenBank with accession number, KR259353. (b) Phylogenetic analysis constructed using the neighbor-joining method based on amino acid sequences of the GH18 family chitinases. The bootstrap analysis was performed with 1000 replications. All chitinases from the lophotrochozoa can be grouped into a clade, in which earthworm *EaChi* appeared to be close to a chitinase from the leech, *H. robusta*. The following sequences were employed in phylogenetic analyses [(GeneBank accession or *Protein ID numbers, JGI Genome Website; *H. robusta* (http://genome.jgi-psf.org/Helro1/Helro1.home.html), *C. teleta* (http://genome.jgipsf.org/Capca1/Capca1.home.html)]: Lophotrochozoa; Polychaeta, *C. teleta_Cht* (*205081); Clitellata, *H. Robusta_Cht* (*173643); *E. andrei_Cht* (KR259353); Mollusca; Bivalvia, *C. gigas_Chit3* (CAI96027); Gastropoda, *L. gigantea_Cht* (*130278). Ecdysozoa; Nematoda, *C. elegans _Cht-1* (AAA83586); Arthropoda, *A. gambiae_Chi-1* (AAB87764); *L. manilensis_Chit* (ABK76337); *P. papatasi_Chit1* (AAV49322). Vertebrata; mouse, *M. musculus_CHIA* (ABK78778), *M. musculus_dehydrogenase* (S39807); frog, *X. tropicalis_Chit* (AAH91095); human *H. sapiens_CHT1* (AAI04971); cow *B. taurus_CHIA* (AAI02932). Bacteria; Proteobacteria, *V. alginolyticus_vacB* (CAC29091); Actinobacteria *S. griseus_chiC* (BAA23739). Fungi; *T. lanuginosus _chit* (AAY99632); *M. flavoviride _chi1* (CAB44709). Plant; *A. thaliana_chitinase* (BAB03157). (c) Multiple sequence alignment of the catalytic domain center of *EaChi* with other GH18 family chitinases from lophotrochozoans. All these chitinases show highly conserved chitin-binding (a) and chitin-catalyzing (b) domain. *EaChi* appeared to exhibit the highest sequence similarity (62%) to chitinase from the leech. The asterisks refer to the conserved amino acid residues and arrows indicate the positions of essential amino acid residues (d and e) for catalyzing activity of the chitinase.

OCT (Tissue-Tek) and stored frozen before sectioning on a cryostat (8-μm sections). Prepared sections were dried (50°C, 30 min) and stored at −70°C until further use. The specimens were incubated at 37°C for 10 min in 20 μg/ml pronase E in PBT (PBS plus 0.1% Tween20, pH 7.4), then rinsed three times in PBT and incubated in 0.3% H_2O_2 in methanol at room temperature for 20 min. Sections were then postfixed for 20 min in 4%

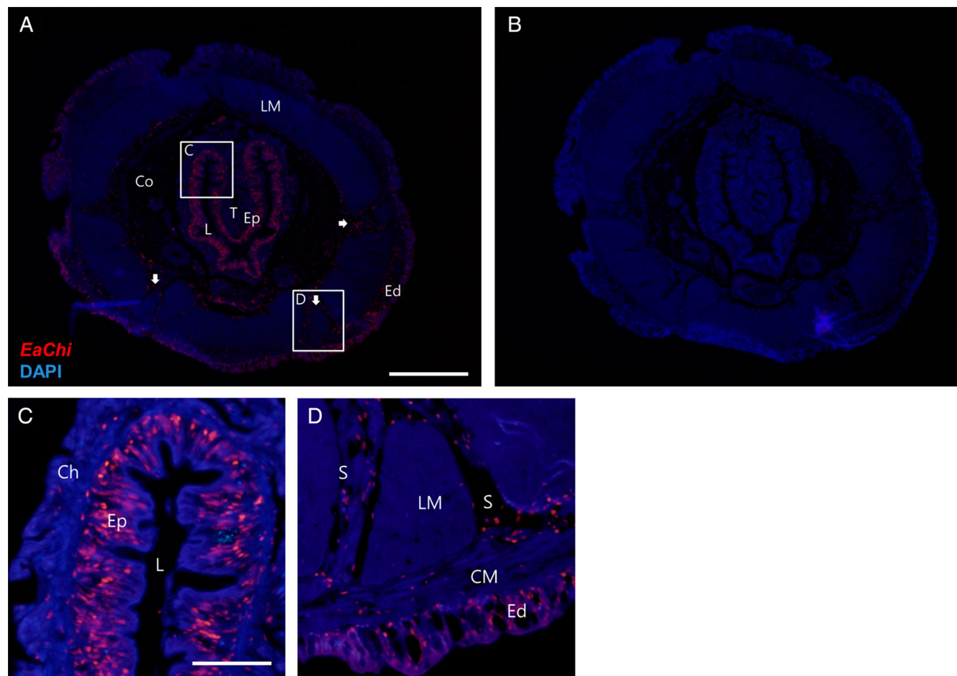

Figure 2. Expression of *EaChi* mRNA in earthworm tissues. The major expression of *EaChi* appeared to be localized in the lining of intestinal epithelial cells (Ep) and epidermal layer (Ed) (Figure 2(a) and (c)) with minor expression around setae areas (arrows, Figure 2(a) and (d)). The higher magnification of boxed areas in Figure 2(a) is shown in Figure 2(c) and 2(d). Control using sense-probe is represented in Figure 2(b). Ch: Chloragogue tissue; CM: Circular muscle; Co: Coelum; L: Lumen of intestine; LM: Longitudinal muscle; T: Typhlosole. Scale bars: A and B 500 µm; C and D 100 µm.

paraformaldehyde and finally rinsed twice in PBT. Prehybridization was carried out at 67°C for 30 min in hybridization buffer (50% deionized formamide, 5 × SSC, 1 × Denhardt's solution, 0.1% CHAPS, 100 µg/ml heparin, 0.1% Tween 20, 100 µg/ml tRNA). The prehybridization buffer was replaced with fresh hybridization buffer containing 2 ng/ml of the corresponding probe (*EaChi*) and specimens were incubated at 67°C overnight. Washed specimens were incubated at room temperature for 1.5 h in 1% blocking reagents (Roche) in PBT, then incubated at 4°C for 16 h with 1/1000 Anti-DIG/POD antibody (Roche) in 1% blocking reagents. Subsequent washes (3 × 20 min) in TNT at room temperature were followed by a single 1 × 30 min rinse in the NEN TSA Plus amplification solution. The color reaction was initiated by adding a 1:50 dilution of reconstituted cyanine-3 in the NEN amplification solution. Specimens were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma) to visualize cell nuclei. Stained specimens were dehydrated in ethanol, mounted in Fluoromount-G (SouthernBiotech), and examined by microscopy.

The open reading frame of earthworm chitinase gene, *EaChi*, from the midgut of *E. andrei*, consists of 1494 nucleotides encoding 497 amino acids with a signal peptide for extracellular localization (Figure 1(a)). In the deduced amino acid sequence of *EaChi*, two domains for glycosyl hydrolases (GH) family 18, catalytic and chitin-binding Peritrophin-A domain, are well conserved in the same way which is diagnosed D-X-X-D-X-D-X-E and S/AxGG motifs, respectively (Figure 1(a) and (c)) (Barariotti et al. 2011; Junges et al. 2014). The phylogenetic analysis (Figure 1(b)) as well as multiple sequence alignment of catalytic center (Figure 1(c)) indicates that earthworm chitinase, *EaChi*, is evolutionarily close to other lophotrochozoan chitinases, with the closest relationship to leech chitinase. In addition, two essential amino acid residues, glutamic and aspartic acid, for enzyme activity are present in the chitin-catalyzing domain (Figure 1(c)) (Watanabe et al. 1993).

In order to initially comprehend the physiological role of this chitinase, the transcriptional expression pattern analysis by *in situ* hybridization has been carried out. The major expression of *EaChi* appeared to be localized in the lining of intestinal epithelial cells and epidermal layer (Figure 2(a) and (c)) with minor expression around setae areas (Figure 2(a) and (d)). These results could allow us to suggest that in this earthworm species, the prime functions of the chitinase activity could be related to not only digestive process but also self-defending immunity as a biochemical barrier to protect the invasion of chitin-containing pathogens, including fungi, nematodes and protozoa of surrounding environment. Concerned the chitinase with immune-related function, a cnidarian chitinase has been reported to

exhibit a double role in pattern formation and immunity, based on its expression pattern in polyps mostly restricted to their basal portion where most pathogens are likely to be originated (Mali et al. 2004). Moreover, in the oyster, another lophotrochozoa evolutionally close to the earthworm, GH 18 chitinase could be transcriptionally induced in hemocytes by challenging of bacteria and lipopolysaccharides, indicating that this enzyme could play a significant role as an immunity effector (Barariotti et al. 2007). The physiological significance of *EaChi* expression around setae areas could not be clearly demonstrated only with the present data. However, considered that in the earthworm, altered self-structure such as setae, a chitinous structure related with locomotion, and necrotic muscle cells are degraded and secreted by the formation of brown body in coelomic cavity (Valembois et al. 1992), it cannot be completely excluded that the earthworm chitinase would contribute the degradation process of waste self-structure composed of chitin.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (PJ011661)” Rural Development Administration, Republic of Korea.

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