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RESEARCH ARTICLE

Identification and expression pattern of a new digestive invertebrate-type lysozyme from the earthworm

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Abstract

Background The invertebrate type (i-type) lysozyme not showing a clear homology with the known types of lysozyme was first demonstrated from a marine bivalve, conch and earthworm by N-terminal sequence. An i-type lysozyme isolated from the earthworm found to be up-regulated upon bacterial challenge, suggesting this lysozyme to function as an inducible immune factor. However, information on the i-type lysozyme related with digestive function is very limited in the earthworm.

Objective The objective of this study is to investigate the molecular characteristics and function of the new i-type lysozyme from the earthworm.

Methods To identify a new i-type lysozyme, multiple amino acid sequence alignment and phylogenetic analyses were employed. Its mRNA expression pattern was observed by fluorescent in situ hybridization (FISH).

Results A new i-type lysozyme (*Ea-iLys*) from an earthworm, *Eisenia andrei* with the open reading frame of 678 bp (226 amino acid residues) appeared to comprise conserved 14 cysteine residues for disulfide bridges and amino acid residues for the enzyme activities of lysozyme and isopeptidase, of which mRNA expression is mainly localized in the lining of midgut epithelium. No significant expression signal was detected in immune competent sites such as chloragogue tissue, typhlosole region, body coelom and muscle layers.

Conclusion Our results suggest that this enzyme primarily acts as a digestive enzyme rather than an innate immune factor.

Keywords Earthworm · Invertebrate-type lysozyme · Expression pattern · Digestion

Introduction

Lysozymes (EC 3.2.1.17) are antibacterial enzymes widely distributed within the animal and plant kingdoms (Jolles 1996). Besides well-known types of lysozyme (chicken-type and goose-type), a typical invertebrate type (i-type) lysozyme was first proposed in Jolles and Jolles (1975). This type lysozyme including as many as 14 cysteine residues and not showing a clear homology with the known types of lysozymes was first demonstrated from a marine bivalve, conch and earthworm by N-terminal sequence (Ito et al. 1999). The i-type lysozymes identified in the digestive glands of eastern oyster, *C. virginica* (cv-lysozyme 2, 3) appeared as a digestive enzyme unlike cv-lysozyme 1 purified from plasma, which supports the fact that lysozymes from the same species may exhibit different functions depending on their molecular and biochemical characteristics (Xue et al. 2007, 2010). In addition, it has been proposed that the lysozyme plays a dual role in insects: digestion of ingested bacteria in the gut and host defense against

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pathogens that enter the haemocoel (Ursic Bedoya et al. 2005), suggesting that some i-type lysozymes originally bacterolytic are probably in an evolutionary pathway from host defense to digestion (Xue et al. 2010).

It has also been reported that an i-type lysozyme isolated from the earthworm, *Eisenia andrei* found to be up-regulated upon bacterial challenge, suggesting this lysozyme to function as an inducible immune factor (Joskova et al. 2009). More recently several forms of lysozyme have been reported in other earthworm species, *Dendrobaena veneta*, displaying a strong expression in epidermis and midgut cells (Fiołka et al. 2012). In the earthworm, due to its habitat with active bacterial activity and the hydrolytic activity of lysozymes on the peptidoglycan present in the bacterial cell walls, the primary function of these enzymes is believed to be associated with the innate immunity to efficiently protect the host against bacterial infections (Cotuk and Dales 1984). However, since microorganisms including bacteria and fungi constitute an important nutrient element of the earthworm diet (Edwards and Fletcher 1988), it would be rational to anticipate the presence of i-type lysozymes directly involved in the digestive process. Several lysozymes belonging to the chicken-type (c-type) have been described as a digestive enzyme in vertebrates as well as invertebrates (Lemos and Terra 1991). More recently the digestive role of i-type lysozymes expressed mainly in digestive glands has been also reported from a mollusc (Xue et al. 2010), suggesting their adaptive evolution from host defense to digestion. To our knowledge, information on the i-type lysozyme related with digestive function is very limited in the earthworm.

In this study, we report a new digestive i-type lysozyme from an earthworm, *Eisenia andrei*, comprising conserved 14 cysteine residues for disulfide bridges, and amino acid residues for the enzyme activities of lysozyme and isopeptidase, of which mRNA expression is mainly localized in midgut epithelium, providing the direct evidence that its function would be primarily associated with digestive process.

Materials and methods

Animals and computational sequence analysis

Sexually mature *Eisenia andrei* obtained from a commercial source (Seoul, Korea) were reared by the method previously described (Park et al. 2017). Before being used, earthworms were placed in petri dishes lined with filter paper moistened with earthworm saline for 48 h to purge gut contents possibly interfering cryosection. Molecular weight, trypsin cleavage sites and deduced amino acid sequence were analyzed using ProtParam, PeptideCutter and translate tool on the ExPASy Server, respectively. Prediction of signal peptide was performed by PSORT II. The open reading frame was determined using ORF finder on the server of National Center of Biotechnology Information (NCBI).

Fluorescent in situ hybridization (FISH)

FISH on cryosections was performed as previously described (Cho et al. 2014) using riboprobe of 462 bp with 4,6-diamidino-2-phenylindole (DAPI, Sigma) to visualize cell nuclei. Briefly, prehybridization buffer was replaced with fresh hybridization buffer (50% formamide, 5 × SSC, 1 × Denhardt's solution, 0.1% CHAPS, 100 mg/ml Heparin, 0.1% Tween20, 100 mg/ml tRNA) containing 2 ng/ml of the corresponding riboprobe 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. For fluorescent in situ hybridization (FISH), we used the NEN Tyramide Signal Amplification (TSA) Plus Kit (PerkinElmer, Wellesley, MA). After washing with PBT, we pre-incubated the specimens in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 15 min, then specimens were blocked in 1% blocking reagent for nucleic acids during 2 h at room temperature, and incubated at 4 °C for 16 h with 1/1000 Anti-DIG/ POD in 1% blocking reagent. After incubation, specimens were rinsed twice with TNT buffer (0.1 M Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% Tween20). Subsequent washes with TNT at room temperature were followed by a single rinse with NEN TSA Plus amplification solution. The color reaction was initiated by adding a 1:50 dilution of reconstituted Cyanine-3 Tyramide in NEN amplification solution. Stained specimens were dehydrated in ethanol, mounted in Fluoromount-G (Southern Biotech), and examined by a microscopy.

Comparative and phylogenetic analyses

The nucleotide sequences of i-type lysozymes were retrieved from the GenBank Database via BLASTX. Amino acid sequences and alignment was carried out using the ClustalW software (http://clustalw.genome.jp). Phylogenetic analysis was performed by the neighbor joining (NJ) method, using the MEGA 4.0 program (http://www.megasoftware.net). Bootstrap analysis was performed with 1000 replications. The phylogenetic tree was built with lysozyme orthologs in metazoan animal models [Lophotrochozoa: *Eisenia andrei* (EALys: ABC68610; Ea-iLys: MH499641), *Hirudo medicinalis* (Hm-Des: AAA96144), *Crassostrea gigas* (Cg-iLys: Q6L6Q6), *Ruditapes philippinarum* (Rp-iLys: Q8IU26) and *Crasostrea virginica* (Cv-iLys: P83673); Ecdoysozoa: *Drosophila melanogaster* (Dm-iLys: AAL49382) and *Apis mellifera* (Am-iLys: XP_393161); Deuterostomia: *Mus musculus*

(Mm-cLys: NP_083643; Mm-gLys: NP_034809), *Xenopus laevis* (Xl-gLys: NP_001088153) and *Homo sapiens* (Hs-cLys: NP_000230; Hs-gLys: NP_002280)].

Results and discussion

Sequence analyses

Nucleotide and predicted amino acid sequences of the i-type lysozyme found in the earthworm *E. andrei* (*Ea-iLys*) were shown in Fig. 1a. The open reading frame (ORF) of *Ea-iLys* consisted of 678 bp corresponding to a polypeptide of 226 amino acid residues with a calculated molecular mass of 22.2 kDa except signal peptides. Figure 1b shows the multiple alignments of the amino acid sequences of Ea-iLys with other animal i-type lysozymes. Compared to other i-type lysozymes derived from lophotrochozoan species, Ea-iLys showed the highest amino acid sequence similarity of 58% to that from a leech i-type lysozyme (Hm-iLys) and more than 50% similarity to those from other lophotrochozoan i-lysozymes. In addition, Ea-iLys appeared to comprise conserved 14 cysteine residues for disulfide bonds, which are possibly involved in high number of disulfide bridges distinguishing i-type lysozymes from other types. Besides the conserved cysteine residues, the Ea-iLys sequence contains three amino acid residues (arrows) for lysozyme activity and two residues (arrow heads) for isopeptidase activity. Of amino acid residues for isopeptidase activity at the position of 166 and 197, whereas histidine residue (197) is relatively well conserved, serine residue at the

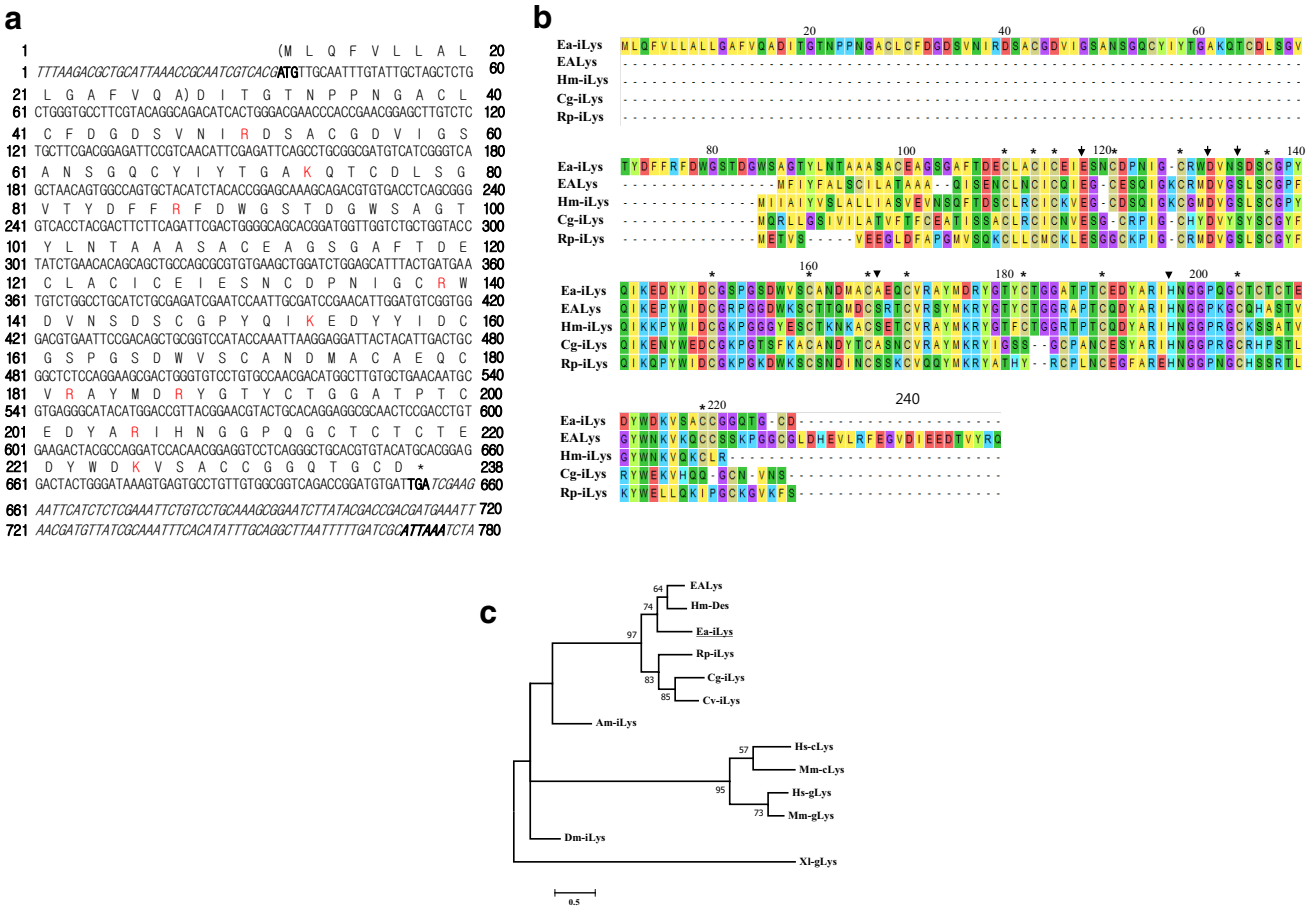

Fig. 1 Primary sequence analyses of earthworm i-type lysozyme (*Ea-iLys*) and phylogenetic analysis. **a** Nucleotide and deduced amino acid sequences of earthworm i-type lysozyme, *Ea-iLys* (GenBank accession no. MH499641). Parentheses indicates the putative signal peptide for secretion. The amino acid residues shown in red represent predicted trypsin cleavage sites. Asterisk marks stop codon and polyadenylation signal (ATTAAA) is shown in bold. **b** Deduced amino acid sequence alignment of Ea-iLys with those from other lophotrochozoans. The asterisks indicate conserved 14 cysteine residues. Arrows and arrow heads designate possible active site residues of lysozyme and isopeptidase activity, respectively. **c** Phylogenetic analysis indicates that underlined Ea-iLys could be clustered with other i-type lysozymes from the annelid (EALys and Hm-Des). *Ea, Eisenia andrei; Hm, Hirudo medicinalis; Rp, Ruditapes philippinarum; Cg, Crassostrea gigas; Cv, Crassostrea virginica; Am, Apis mellifera; Hs, Homo sapiens; Mm, Mus musculus; Dm, Drosohpila melanogaster; Xl, Xenopus laevis*

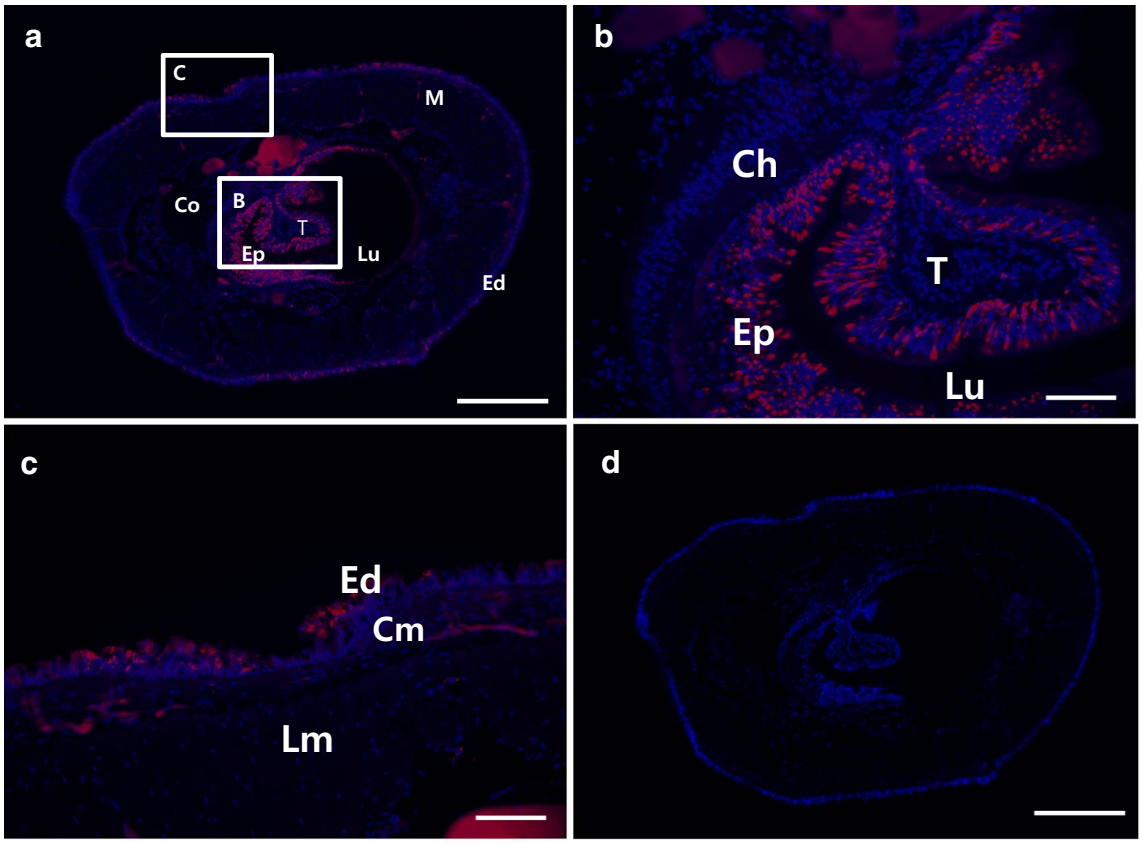

Fig. 2 Expression analysis of *Ea-iLys* mRNA using FISH on cryosections. **a** Expression pattern of *Ea-iLys* mRNA in a midgut region. Intensive expression signal was detected in the lining of epithelial cells (Ep) of midgut. Although much lesser positive signals could also be observed in epidermis (Ed) in part, no significant expression signal was detected in chloragogue tissue (Ch), typhlosole region (T), coelomocyte in body coelom (Co) and muscle layer (M). **b, c** Higher magnification views of the white boxed areas in **a**, which shows more clearly that the mRNA expression of this enzyme is mostly present in the epithelial cells of the midgut. **d** The DAPI staining in the same region using sense probe. *Ch* chloragogue tissue, *Cm* circular muscle, *Co* coelom of body, *Ed* epidermis, *Ep* epithelial cells, *Lm* longitudinal muscle, *Lu* lumen of midgut, *M* muscle layer, *T* typhlosole. Scale bar in **a, d**: 500 μm and in **b, c**: 100 μm

position of 166 is frequently substituted to alanine in several i-type lysozymes from molluscs, *Mytilus edulis* (Me-iLys-BM, Me-iLys2), *Mytilus galloprovincialis* (Mg-iLys1, Mg-iLys2), *Crassostrea gigas* (Cg-iLys1, Cg-iLys2), *Crassostrea virginica* (Cv-iLys2, Cv-iLys3), and insect, *Aedes aegypti* (Ae-iLys1, Ae-iLys2) (Van Herreweghe and Michiels 2012). In phylogenetic analysis, i-type lysozymes of lophotrochozoan could be clustered together distinguished from ecdysozoan i-lysozyme (Am-iLys), which are sub-clustered into two groups, annelid and molluscs types with a node value of 97 (Fig. 1c), indicating that Ea-iLys could be categorized as a member of annelid type i-lysozymes.

Expression analysis using FISH

Some i-type lysozymes derived from molluscs have been suggested to be adaptively evolved for nutrition, of which expression was primarily detected in digestive gland tissues (Xue et al. 2007) whereas other i-type lysozyme identified from a shrimp species was mainly expressed in hepatopancreas, an immune-competent tissue, but not detectable in other tissues (Liu et al. 2016). It is also reported that an i-type lysozyme isolated from the same earthworm species exhibited muraminidase and isopeptidase activities of which expression is increased by the challenge of Gram-positive and -negative bacteria, strongly suggesting its involvement in host defense mechanism (Joskova et al. 2009). In the present study, it is revealed by mRNA in situ hybridization that *Ea-iLys* mRNA expression appeared to be strongly expressed in the lining of gut epithelium (Fig. 2a, b), providing the first direct evidence that this i-type lysozyme could be related to the digestive function in this earthworm species. It is likely that 14 cysteine residues capable of forming 7 disulfide bridges and few number of trypsin cleavage sites (< 10) possibly make this enzyme resistant to digestive proteinases secreted from gut glands. Although much lesser positive signals could be also observed in epidermis in part,

no significant expression signal was detected in immune competent sites such as chloragogue tissue (Ch), typhlosole region (T), body coelom (Co) and muscle layers (Cm, Lm) (Fig. 2a, c), suggesting that this enzyme primarily acts as a digestive enzyme rather than an innate immune factor. Very recently, it has been reported using proteomic analysis that earthworm lysozyme was up-regulated in response to exposure to *E. coli* and fly ash as an innate immune factor (Markad et al. 2016; Wang et al. 2018). However, our finding that earthworm lysozyme could function as a digestive enzyme suggests that more careful consideration is needed as to whether the increased lysozyme expression is associated with the innate immunity or the digestive process.

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