Developmental and Comparative Immunology 33 (2009) 932–938

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Identification and cloning of an invertebrate-type lysozyme from *Eisenia andrei*

Radka Josková, Marcela Šilerová, Petra Procházková, Martin Bilej *

Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, 142 20, Prague 4, Czech Republic

ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 9 March 2009
Accepted 12 March 2009
Available online 1 April 2009

Keywords:
Annelids
Invertebrates
Antimicrobial protein
Peptidoglycan
Isopeptidase activity
Real-time PCR

ABSTRACT

Lysozyme is a widely distributed antimicrobial protein having specificity for cleaving the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (GlcNAc) of peptidoglycan of the bacterial cell walls and thus efficiently contributes to protection against infections caused mainly by Gram-positive bacteria.

In the present study, we assembled a full-length cDNA of a novel invertebrate-type lysozyme from *Eisenia andrei* earthworm (EALys) by RT-PCR and RACE system. The primary structure of EALys shares high homology with other invertebrate lysozymes; however the highest, 72% identity, was shown for the destabilase I isolated from medicinal leech. Recombinant EALys expressed in *Escherichia coli* exhibited the lysozyme and isopeptidase activity. Moreover, real-time PCR revealed increased levels of lysozyme mRNA in coelomocytes of *E. andrei* after the challenge with both Gram-positive and Gram-negative bacteria.

1. Introduction

Lysozyme is a ubiquitous enzyme widely distributed within the animal and plant kingdoms [1]. Based on the differences in their structure, catalytic character and original source, lysozymes are classified into six groups: chicken-type lysozyme (c-lysozyme) present in many vertebrates and insects is the most extensively studied lysozyme (for review see [2]); goose-type lysozyme (g-lysozyme) identified mainly in vertebrates including mammals, birds and fish [3–6]; invertebrate-type lysozyme (i-lysozyme) [7–14], plant lysozyme [15], bacterial lysozyme [16] and phage lysozyme [17].

Lysozyme possesses the hydrolytic activity to specifically cleave β-1,4-glycosidic bonds between *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (NAM) of the peptidoglycan present in the bacterial cell walls and thus efficiently protects the host against infections caused by Gram-positive bacteria [1]. In addition, lysozyme was described as an important digestive enzyme in ruminants [18], leaf-eating monkeys, birds [19] and also fruit flies [20]. All the digestive lysozymes mentioned above belong to the c-type lysozymes. Recently, the digestive role was also suggested for the i-type lysozyme purified from a bivalve mollusc *Crassostrea virginica* [13].

In this study, we characterise the lysozyme isolated from an earthworm *Eisenia andrei* (formerly *E. fetida andrei*; Oligochaeta, Annelida) that shows high similarity with other i-type lysozymes of various invertebrate species. Recombinant EALys expressed in *Escherichia coli* BL21(DE3) cells showed the lysozyme as well as isopeptidase activities. Finally, we show up-regulation of EALys expression upon *E. coli* and *Bacillus subtilis* challenge.

2. Materials and methods

2.1. Isolation of coelomic fluid and coelomocytes

Coelomic fluid of non-stimulated or stimulated (approximately 10^8 CFU of *E. coli* K12 or *B. subtilis* W23, Institute of Microbiology ASCR, Prague) adult *Eisenia andrei* was obtained by puncturing post-clitellum segments of the coelomic cavity with Pasteur micropipette. Coelomocytes were isolated by centrifugation (500 × *g*, 10 min, 4 °C).

2.2. RNA isolation, cDNA synthesis, PCR and rapid amplification of cDNA ends (RACE)

Total RNA was isolated from coelomocytes using 1 ml of TRIzol® reagent (Invitrogen) according to manufacturer's protocol. DNAse I-treated total RNA (1 μg) was reverse-transcribed using Random Primers and SuperScript™ II Reverse Transcriptase (Invitrogen). A set of degenerated primers based on conserved regions of known i-type lysozyme sequences was designed using the CODEHOP programme [21]. Combination of primers Lydeg2

* Corresponding author. Tel.: +420 2 4106 2343; fax: +420 2 4172 1143.
E-mail address: mbilej@biomed.cas.cz (M. Bilej).

Abbreviations: EALys, lysozyme from *Eisenia andrei*; NAM, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; IPTG, isopropyl-β-D-thiogalactopyranoside.

0145-305X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2009.03.002

Table 1
List of primers used in this study.

Primer	Usage	Direction	Sequence
Lydeg2	Initial RT-PCR	Fwd	5′-TGYCGNATGGAYGTNGG-3′
Lydeg8	Initial RT-PCR	Rev	5′-CANCCNCGNGGNCCNCCRTT-3′
Lys2-5RACE	5′-RACE PCR	Rev	5′-GGCGTAATCCTGACAGGTG-3′
Lys3-3RACE	3′-RACE PCR	Fwd	5′-GCCATTCCAAATCAAGGAAC-3′
Lys4-5RACE	5′-RACE PCR	Rev	5′-TAGGTACCGTAGCGCTTCAT-3′
LysForBamH	PCR for cloning in pRSET B	Fwd	5′-ACAGGATCCACAAATCTCGGAAAACTGC-3′
LysRevEcoRI	PCR for cloning in pRSET B	Rev	5′-GCCGAATTCTTACTGACGATAGACGGT-3′
LysRT-3	Real-time PCR	Fwd	5′-GGAACAAGGTGAAGCAGTG-3′
LysRT-4	Real-time PCR	Rev	5′-GGCAGTCCAGGTGGTAGTGT-3′
28S-RT1	Real-time PCR	Fwd	5′-ACTATGCCCGAGTAGGACGA-3′
28S-RT2	Real-time PCR	Rev	5′-GCCTCCAGTCATTCGCTTTA-3′

and Lydeg8 (Table 1) resulted in amplification of a specific product of approximately 230 bp. This product was ligated into pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The 3′end of lysozyme cDNA was obtained using 3′RACE System (Invitrogen). In brief, 1 μg of total RNA was reverse-transcribed using a supplied adapter primer while a universal amplification primer in combination with a lysozyme-specific internal sense primer (Lys3-3RACE; Table 1) were used in a subsequent PCR reaction. Similarly, the 5′end of lysozyme cDNA was obtained using 5′RACE System (Invitrogen). Reverse transcription was carried out using 1 μg of total RNA and an antisense lysozyme internal primer (Lys2-5RACE; Table 1). Terminal deoxyribonucleotidyl transferase was used to add homopolymeric oligo-dC tail to the 3′end of purified cDNA. A supplied sense abridged anchor primer (AAP) and an antisense lysozyme-specific primer (Lys4-5RACE; Table 1) were used in a subsequent PCR. Both 3′ and 5′RACE products were cloned in pCR2.1-TOPO and sequenced as described below.

2.3. Sequencing

Isolated and purified plasmid DNA was sequenced with ABI PRISM BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems). The chain termination reaction [22] was performed by cycle sequencing technique [23] according to manufacturer's protocol. Finally, sequences were determined using ABI PRISM 3100 DNA sequencer (Applied Biosystems).

2.4. Sequence analysis

The signal peptide of EALys was predicted using both neural network and hidden Markov model on a Signal IP 3.0 Server [24]. Molecular weight and isoelectric point (p*I*) were predicted using ProtParam on the ExPASY Server [25]. Multiple sequence alignment of selected invertebrate lysozymes was carried out using ClustalW [26]. The lysozyme of *E. andrei* (ABC68610) was aligned with other i-type lysozymes from bivalve molluscs *Mytilus edulis* (ABB76765), *Crassostrea virginica* (BAE47520), *Tapes japonica* (BAC15553), the nematode *Caenorhabditis elegans* (AAC19181), the medicinal leech *Hirudo medicinalis* (AAA96144), the starfish *Asterias rubens* (AAR29291) and the sea cucumber *Stichopus japonicus* (ABK34500).

2.5. Preparation of recombinant E. andrei lysozyme (rEALys)

A DNA fragment containing the entire coding region of *E. andrei* lysozyme without signal peptide was generated by PCR using primer pairs containing BamHI or EcoRI restriction sites (LysFor-BamH and LysRevEcoRI primers; Table 1) at the 5′terminal and 3′terminal ends, respectively. After digestion, PCR products were ligated into pRSET-B vector containing N-terminal 6x His affinity tag (Invitrogen). The BL21(DE3) *E. coli* strain was transformed, grown in the presence of ampicillin (100 μg/ml) and the expression of lysozyme was induced by incubation with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 2 h. The His-fused protein from bacterial lysates was purified by affinity chromatography using Ni-CAM HC Resin (Sigma–Aldrich) and eluted with 500 mM imidazole in 8 M urea solution. Recombinant EALys was renaturated by a dialysis against decreasing linear gradient of urea solution as described previously [27].

2.6. Lysozyme activity assay

A lysoplate assay was performed according to a modified protocol by Lie et al. [28]. A solution of 1% agarose in 50 mM phosphate buffer (monosodium phosphate monohydrate and disodium phosphate heptahydrate, pH 4.5–8.0) containing 1 mg/ml *Micrococcus lysodeicticus* was prepared. A drop (5 μl) of standard (hen egg white lysozyme, Roche), rEALys or coelomic fluid of *E. andrei* was placed on individual Petri dishes that were incubated at 37 °C. The diameter of lysed zone (mm) was measured after 24 h.

In order to quantitatively evaluate the influence of pH, ionic strength, time of incubation and temperature on lysozyme activity of rEALys (5–350 μg/ml), we resuspended *M. lysodeicticus* (0.6 mg/ml) in 50 mM phosphate buffer (pH 4.5–8.0) with adjusted ionic strength using NaCl (0.005–0.3 M) and incubated under different temperatures (25, 30 and 37 °C). The lysozyme activity was measured after 0–24 h by reading OD at 530 nm.

Enzyme activity of rEALys was quantitatively determined according to Ref. [29].

2.7. Isopeptidase activity assay

Isopeptidase activity of rEALys (5–350 μg/ml) was determined by the production of p-nitroanilide (p-NA) from L-γ-glutamine-p-nitroanilide (L-γ-Glu-pNA; 2 mg/ml) [12]. Recombinant lysozyme (50 μl) was mixed with the substrate (L-γ-Glu-pNA) dissolved in 50 mM 3-morpholinopropanesulfonic acid (MOPS; pH 5.0–9.0) with adjusted ionic strength using NaCl (0.005–0.3 M). The mixture was incubated up to 72 h at different temperatures (25, 30 and 37 °C) and then the absorbance was measured at 405 nm.

2.8. Chitinase activity assay

Chitinase activity of rEALys (5–350 μg/ml) was measured using chitin azure suspension (Sigma) in 0.1 M sodium acetate buffer pH 3.0–8.0 [13]. The release of Remazol Brilliant Blue R covalently linked to crab shell chitin was measured as an indicator of chitinase activity. The assay was carried out in 96-well plates—50 μl of recombinant lysozyme was mixed with 50 μl of chitin azure suspension (2 mg/ml). Chitinase from *Streptomyces griseus* (Sigma) was used as a positive control. After incubation at different