The JSFS 85th Anniversary-Commemorative International Symposium
"Fisheries Science for Future Generations"
Symposium Proceedings, No. 06006

Original article

Lysozyme from Placozoa, an early diverging metazoan, lyses both gram-positive and negative bacteria

Munetsugu Bam [1,*] and Hiroaki Nakano [2]

[1] *Department of Anatomy and Structural Biology, Graduate School of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan*
[2] *Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka 415-0025, Japan*
* Correspondence: mban@yamanashi.ac.jp; Tel.: +81-55-273-6734

Keywords: Placozoa; Lysozyme; Lytic activity; Recombinant expression.

Received: 17 July 2017 / Accepted: 2 September 2017

Introduction

Hen egg white lysozyme (HEWL) is used as a food additive and in medicines, but its application range remains limited for various reasons. For example, the enzyme shows little lytic activity against Gram-negative bacteria and possibly causes allergies [1]. Although its activity against Gram-negative bacteria can be improved by adding EDTA, such addition may be restricted to avoid harm to the environment [2]. Thus, lysozymes that show lytic activity for Gram-negative bacteria are sought.

Placozoans are flat amoeba-like marine invertebrates about 1-2 mm in size. They lack neurons and muscles but are not sessile or parasitic, making them the simplest extant free-living animals [3,4]. We previously observed placozoans feeding on Gram-negative bacteria, *Spirulina* sp., in lab cultures and hypothesized that placozoan lysozymes (PLys) have activity against Gram-negative bacteria. In the present study, we produced recombinant PLys (rPLys) and modified recombinant PLys (mrPLys) with deletion of a cysteine-rich region. The PLys enzyme had higher enzyme activity than HEWL and showed lytic activity against Gram-negative bacteria. These results suggest that PLys can be used as natural antimicrobial agents against Gram-negative bacteria in various applications for which HEWL cannot.

Materials and methods

cDNA cloning of Placozoan lysozyme

Trichoplax sp. were collected at Shimoda Marine Research Center, University of Tsukuba [5] and proliferated by asexual reproduction for use in experiments. Total RNA was extracted from 100 animals using RNeasy mini kit (QIAGEN). First strand cDNA was synthesized from purified total RNA. To amplify PLys cDNA (Accession number LC311016), PCR reactions were performed using primers shown in Table 1 as follows: 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 5 min. The PCR products were cloned into expression vector pCold TF (Takara) and sequenced.

Table 1. Primers used in this study

Primer	Sequence
SacI-rPLys F	5'- GGGGAGCTCGCACTTAATGACGACTGTAA -3'
HindIII-rPLys R	5'- GGGAAGCTTTTAGTTGAAGCCATTATTAT -3'
SacI-mrPLys F	5'- GGGGAGCTCATGAGAATCAACCCCAGTGG -3'
HindIII-mrPLys R	5'- GGGAAGCTTTTAGTTGAAGCCATTATTAT -3'

Expression and purification of rPLys and mrPLys

Recombinant vectors were transformed to BL21 (DE3) competent cells for recombinant protein expression. The host cell culture was transfected with vectors and grown at 37°C overnight with shaking in 250 ml of LB medium containing 100 μg/ml of ampicillin. The culture was incubated at 16°C for 45 min, followed by addition of isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. The culture was then incubated overnight with shaking at 18°C. The cells were harvested and suspended in 5 ml of binding buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM imidazole). The solution was loaded on a HisTALON Gravity Column (Takara) pre-equilibrated with 5 ml of binding buffer and washed with 10 ml of binding buffer. Recombinant proteins were eluted with 5 ml of elution buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 300 mM imidazole). The elution fraction was desalted and concentrated using Amicon Ultra-0.5 50K (Merck). The solution was adjusted to reaction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl) and treated overnight with 5 μl HRV 3C protease (Funakoshi) at 4°C. To remove the HRV 3C protease and 6×His-trigger factor tag from the solution, 200 μl of TALON Metal Affinity Resin (Takara) was added followed by incubation at 4°C for 1 hr with shaking. The supernatant was diluted with 4 ml of 20 mM Tris-HCl (pH 8.0) and applied to a HiTrap Q HP column (GE Healthcare) that was equilibrated with 5 ml

of 20 mM Tris-HCl (pH 8.0) followed by elution with 1 ml of 20 mM Tris-HCl (pH 8.0). The eluted solution was desalted and concentrated using Amicon Ultra-0.5 10K (Merck) before use in assays.

Enzymatic activity of lysozyme

Lysozyme activity was measured using *Micrococcus luteus* (Wako) [6]. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} [7].

Lytic activity against bacteria

Lysoplate assay was conducted on 1% agarose gel plates in 20 mM HEPES (pH 6.7) containing *M. luteus* (Wako) or *E. coli* DH5α as previously described [8] but with minor modifications. The bacteria concentration in the plates was adjusted to 0.4 absorbance at 600 nm. Wells (4 mm in diameter) were formed in the gel to which rPLys, mrPLys and HEWL (Wako) were added at 20 μg for the *M. luteus* plates and 30 μg for the *E. coli* plates. The plates were incubated at 37°C for 30 hr.

Results

Recombinant protein expression

rPLys and mrPLys with the deletion of a cysteine-rich region (amino acid residues: 22-93) as shown in Fig. 1 were characterized here.

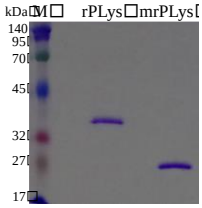

Fig. 1 SDS-PAGE analysis and detection of recombinant proteins. The purified recombinant proteins were applied to 10% SDS-PAGE under reducing conditions. Single bands were visualized by CBB staining. Lane M: molecular marker.

Comparison of enzyme activity of lysozymes

Enzymatic activity of lysozymes rPLys, mrPLys, and HEWL was quantified as a decrease in absorbance (Fig. 2) and found to be 1.6×10^6, 2.7×10^6, and 1.2×10^5 units/mg, respectively.

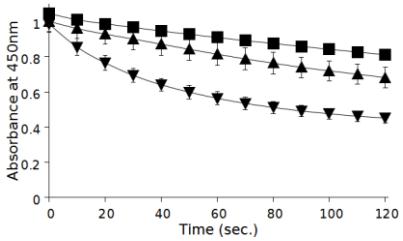

Fig. 2. Enzymatic activity of the lysozymes in 20 mM HEPES (pH 6.7, ionic strength 0.006) at 37°C. Enzymatic activity in reactions with rPLys (0.1 μg, ▲), mrPLys (0.1 μg, ▼), and HEWL (1 μg, ■) were monitored for 2 min. Experiments were performed in triplicate, and enzymatic activity (mean ± SD) was reported as units per milligram.

Lytic activity of lysozymes against bacteria

The lytic activity of the lysozymes rPLys and mrPLys with HEWL as a control were observed against both *M. luteus* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria). Based on the clear zone formed by lysis activity, the three lysozymes were shown to lyse *M. luteus* (Fig. 3a, b, c). rPLys also lysed *E. coli* (Fig. 3e), while the lytic activity of mrPLys was relatively weak (Fig. 3f) and HEWL showed no lytic activity toward *E. coli* (Fig. 3g). Negative controls showed no lytic activity against both types of bacteria (Fig. 3d, h).

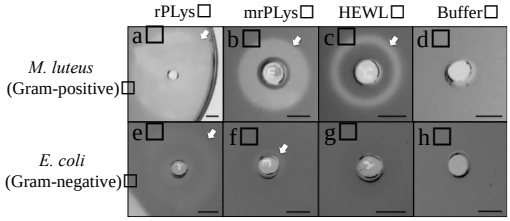

Fig. 3. Lytic activity of lysozymes against bacteria. 1% agarose plates with cultured *M. luteus or E. coli* were prepared and incubated with lysozymes. The clear zone (arrow) indicates lytic activity against bacteria. Scale bar: 5 mm.

Discussion

We showed that rPLys and mrPLys have lytic activity against both Gram-negative and positive bacteria with enzymatic activity more than 10 times that of HEWL. For marine animals, which are constantly exposed to various bacteria in the environment, lysozymes aid in the prevention of infection and in the digestion of bacteria as a nutrient source [12]. In addition, some lysozymes from marine invertebrates have been reported to exhibit enzymatic activity for Gram-negative bacteria; however, these lysozymes show lower activity against Gram-positive bacteria than HEWL [13-15]. In contrast, PLys showed higher enzymatic activity against not only Gram-negative bacteria but also Gram-positive bacteria than HEWL. These findings suggest that PLys could be an effective antibacterial agent against both types of bacteria and could be used in applications to which HEWL has not been applied.

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