Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.2014JCLM-2014-0013

Assessment of antimicrobial activity of c-type lysozyme from Indian shrimp *Fenneropenaeus indicus*

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PEER REVIEW

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Comments

The authors are expressed, the antibacterial and antifungal activity of c-type lysozyme from Indian shrimp *F. indicus*. The work supports, shrimp will be used for antibacterial and antifungal activity. In this study the high sensitivity of *Vibrio alginolyticus* and *Aeromonas veronii* to rFi-Lyz than rHEWL underscores the scope of using this for disease control in aquaculture.
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ABSTRACT

Objective: To assess the multitudinal antimicrobial effects of recombinant lysozyme from *Fenneropenaeus indicus* (rFi-Lyz) in comparison with commercially available recombinant hen egg white lysozyme (rHEWL).
Methods: Antimicrobial activity of the recombinant rFi-Lyz using several Gram positive, Gram negative bacteria and fungi in comparison with rHEWL has been evaluated. rFi-Lyz was expressed and purified using Ni^{2+} affinity chromatography. The effect of rFi-Lyz in the growth of yeast *Candida krusei*, plant molds *Rhizoctonia solani* and *Fusarium solani* was assessed by well diffusion assay in petri plates with potato dextrose agar.
Results: rFi-Lyz exhibited high inhibitory activity on Gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. Among various Gram negative bacteria tested *Klebsiella pneumoniae* exhibited the highest inhibition followed by *Pseudomonas aeruginosa* and *Shigella dysenteriae*. rFi-Lyz also exhibited significant inhibition on two marine pathogens *Aeromonas veronii* and *Vibrio alginolyticus*. Among the various fungal strains tested, rFi-Lyz inhibited the growth of budding yeast *Candida krusei* significantly. Further the growth of two other plants fungus *Rhizoctonia solani* and *Fusarium oxysporum* were retarded by rFi-Lyz in the plate inhibition assay.
Conclusions: rFi-Lyz exhibits a broad spectrum of antimicrobial activity like a natural antibiotic on various pathogenic bacteria and fungal strains.

KEYWORDS
Lysozyme, *Fenneropenaeus indicus*, Antimicrobial activity

1. Introduction

Lysozyme is one of the most ubiquitous antibacterial molecules, widely distributed in vertebrates and invertebrates that exert broad spectrum of antimicrobial action. This is a relatively small enzyme that catalyzes the hydrolysis of specific kinds of polysaccharides comprising the cell walls of bacteria and also exhibits catalytically independent antimicrobial activity that depends on its internal peptides[1]. One of the natural antimicrobial proteins widely studied is lysozyme that is well known for its muramidase activity against Gram positive bacteria *Bacillus* and *Streptococcus*[2,3]. Lysozyme is an enzyme known for its unique ability to degrade the polysaccharide architecture of many kinds of cell walls, normally for the purpose of protection against bacterial and fungal infection by catalyzing the hydrolysis of 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-

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Foundation Project: Supported by a grant from the Department of Biotechnology, Government of India, New Delhi, India as a part of Indo Norwegian program for fish and shellfish vaccine development with Grant No: BT/AAQ/Indo-Norway/183204/2007.

Article history:
Received 11 Feb 2014
Received in revised form 25 Feb, 2nd revised form 15 Mar, 3rd revised form 10 Apr 2014
Accepted 20 Jun 2014
Available online 15 Sep 2014

D-glucosamine residues in peptidoglycan, and between N-acetyl-D-glucosamine residues in chitodextrins[4]. Hen egg white lysozyme (HEWL) also acts against Gram negative bacteria by mechanisms such as perturbation of DNA or RNA synthesis and membrane permeabilization or membrane disruption and this is a major mechanism by which antibacterial peptides and proteins act on both Gram negative and Gram positive bacteria[5-7]. Since lysozyme has been widely recognized for its antibacterial and antifungal properties, it has a wide variety of uses both in biochemical and pharmaceutical applications[8-10].

In the animal kingdom, three major distinct lysozyme types have been identified, the c-type (chicken or conventional type), the g-type (goose-type) and the i-type (invertebrate type) lysozyme of which c-type lysozymes are predominantly present in the phylum Chordata that plays an important role as an antimicrobial peptide (AMP) in immune mechanism of invertebrates like shrimps[11]. We have previously cloned and characterized c-type lysozyme [lysozyme from *Fenneropenaeus indicu* (*F. indicus*) (rFi-Lyz)] from Indian shrimp *F. indicus* that exhibited inhibitory activity on *Salmonella typhimurium* (*S. typhimurium*)[12]. In this communication antimicrobial activity of the recombinant rFi-Lyz using several Gram positive, Gram negative bacteria and fungi in comparison with recombinat HEWL has been evaluated. The findings of this preliminary study may help to make progress for the next level of research using animal models, thus for the development of attractive novel therapeutic approaches using rFi-Lyz or its internal peptides to treat diseases of human and shrimps.

2. Materials and methods

2.1. Determination of antibacterial activity of rFi-Lyz

The lysozyme open reading frame (ORF) from *F. indicus* was cloned into pET-28a vector (Invitrogen, NY, USA) and transformed into *Escherichia coli* BL21 (DE3) (*E. coli*) as per standard protocol. rFi-Lyz was expressed, purified using Ni^{2+} affinity chromatography with 200 mmol/L imidazole and confirmed by Western blot analysis with monoclonal antibody against 6× His tag. Turbidimetric assay was performed according to the published protocol with minor modifications to assess the activity of purified rFi-Lyz and it was compared with HEWL[13].

Turbidimetric assay was performed to examine the lytic activity of rFi-Lyz against Gram positive bacteria, Gram negative bacteria and fungi. An initial experiment using *Micrococcus luteus* with 5 to 20 μg of rHEWL indicated that 10 μg was the lowest dosage at which the bacterial growth was inhibited. In these assays, suspensions of these organisms were prepared with 0.1 mol/L phosphate buffer (pH 6.5), adjusted to a final concentration (OD_{600}=0.6) and 10 μg of the purified rFi-Lyz was applied to each well containing 20 μL of the test organism and absorbance was measured at 600 nm after incubation at 37 °C for bacteria for 3 h.

2.2 Determination of antifungal activity of rFi-Lyz

The effect of rFi-Lyz in the growth of yeast *Candida krusei* (*C. krusei*), plant molds *Rhizoctonia solani* (*R. solani*) and *Fusarium solani* (*F. solani*) was examined. Suspension *C. krusei* (OD_{600}=0.6) was prepared with 0.1 mol/L phosphate buffer (pH 6.5), and 10 μg of the purified rFi-Lyz was added to 20 μL of the test organism, incubated at 28 °C for 24 h and absorbance was measured at 600 nm. The lysozyme activity on the plant mold was determined by well diffusion assay in petri plates with 20 mL of potato dextrose agar. Seven millimeter diameter discs from Whatman No.1 filter papers (stack of 3 filter papers) were prepared, sterilized at 121 °C for 15 min and dried. These discs were placed on the medium. Aliquots 10 and 20 μg of rHEWL/rFi-Lyz were pipetted onto the discs. The plates were incubated for 72 h at 28 °C and the formation of a zone of clearing around the discs was considered a positive indication of inhibitory activity and was compared with the controls.

1x phosphate buffered saline (1xPBS) was used as negative control and 10 μg and 20 μg of rHEWL served as the positive controls. The statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). All data are expressed as mean±SD of three independent experiments, and the significant differences between test and control groups were calculated using One-way ANOVA and *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Antimicrobial activity of rFi-Lyz

rFi-Lyz was expressed in bacteria and purified by immobilized metal-affinity chromatography (IMAC). As expected, Western blot analysis with monoclonal anti Histidine (α-His) and polyclonal anti rFi-Lyz showed a reactivity corresponding to 20 kDa (Figure 1a). The biochemical activity of purified rFi-Lyz was assessed by turbidimetric assay using *Micrococcus luteus* and exhibited a significant reactivity but in a lower degree when compared with rHEWL.

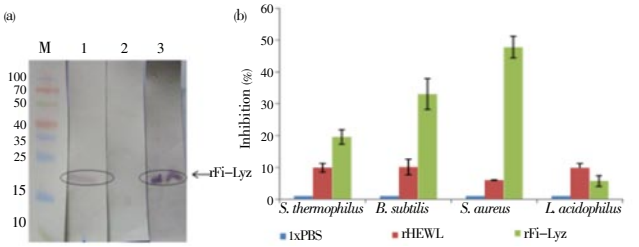

Figure 1. Western blot analysis of purified rFi-Lyz and its anitbacterial activity against Gram positive bacteria.

(a) Western blot analysis of purified rFi-Lyz with anti-HIS and anti lysozyme antibodies; (b) Antibacterial activity of rFi-Lyz against Gram positive bacteria. Results are expressed as mean±SD of three independent experiments.

In order to evaluate the antimicrobial effects of rFi-Lyz, Gram positive bacteria, Gram negative bacteria and fungi have been included. Among the Gram positive bacteria tested, *Staphylococcus aureus* (*S. aureus*) exhibited highest degree of inhibition (50%) followed by *Bacillus subtilis* (*B. subtilis*) with 40%. Interestingly, rFi-Lyz had no significant effect in the growth of *Streptococcus thermophilus* (*S. thermophilus*) and another non-pathogenic bacteria *Lactobacillus acidophilus* (*L. acidophilus*) (Figure 1b). With the Gram negative organisms, *Klebsiella pneumoniae* (*K. pneumoniae*) exhibited 80% inhibition when other pathogens *viz*. *Pseudomonas aeruginosa* (*P. aeruginosa*) (40%) and *Shigella dysenteriae* (*S. dysenteriae*) (30%), also exhibited significant inhibition but a lower percentage with $P<0.05$ and rHEWL did not show any reactivity. rFi-Lyz showed negligible inhibition in the growth of enteropathogenic *E. coli* when rHEWL exhibited 10% (Figure 2). Among marine pathogens tested, *Aeromonas veronii* (*A. veronii*) exhibits a higher inhibition of 60% when compared to *Vibrio alginolyticus* (*V. alginolyticus*) (30%). rFi-Lyz and rHEWL did not show any effect in the growth of *Vibrio harveyi* (*V. harveyi*) (Figure 3).

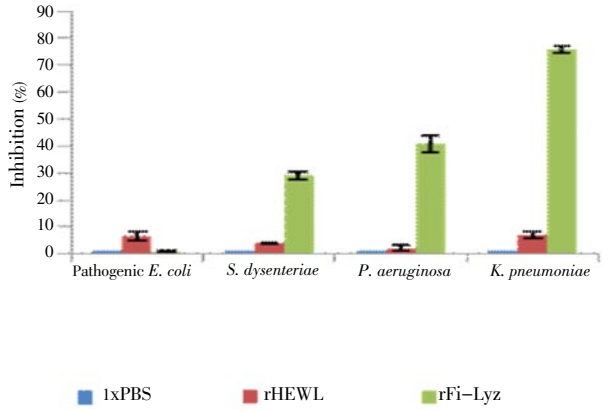

Figure 2. Antibacterial effects of rFi-Lyz against Gram negative bacteria. Results are expressed as mean±SD of three independent experiments.

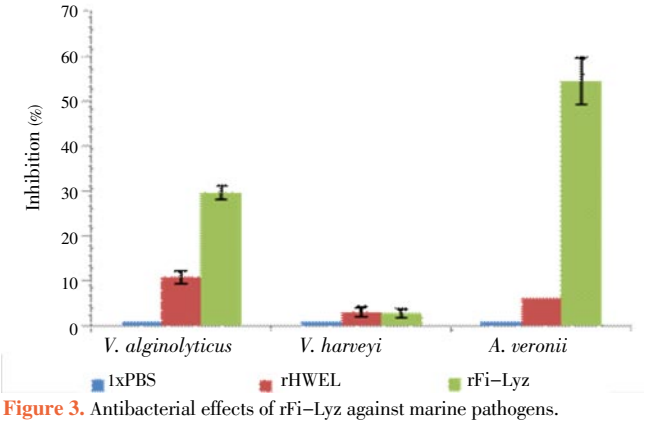

Figure 3. Antibacterial effects of rFi-Lyz against marine pathogens. Results are expressed as mean±SD of three independent experiments.

3.2. Antifungal activity of rFi-Lyz

rFi-Lyz showed 80% inhibition in the growth of yeast *C. krusei* in Sabarose dextrose broth when rHEWL inhibited the growth by 20%. Moulds such as *R. solani* and *F. solani* were tested for anti-fungal activity using the plate assay. With *R. solani*, increasing effect in the inhibitory activity was observed using 10 and 20 μg/mL of rFi-Lyz as determined from the zone of clearance. Similar trend was observed in *F. solani* while rHEWL showed no significant effect (Figure 4).

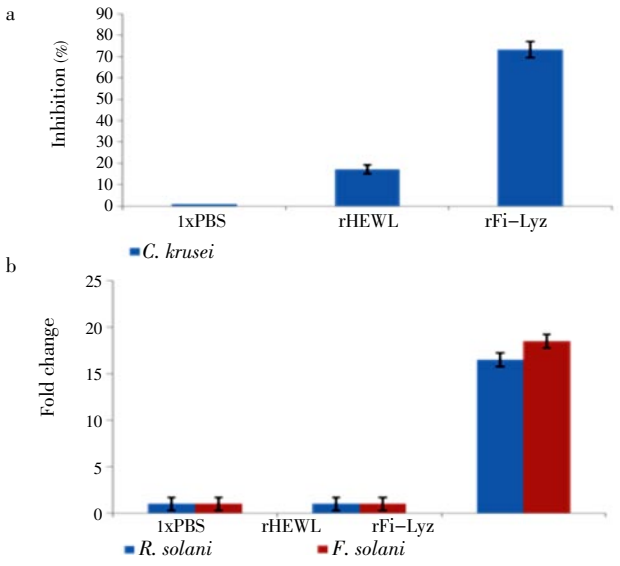

Figure 4. Antifungal activity of rFi-Lyz against Fungi.
(a) Antifungal activity of rFi-Lyz against Yeast *C. krusei*; (b) Antifungal activity of rFi-Lyz against plant mold *R. solani* and *F. solani*. Results are expressed as mean±SD of three independent experiments.

4. Discussion

In the main aspect of testing the multitudinous functions of lysozyme, an antimicrobial peptide bestows its use in clinical areas both for human as well as veterinary medicine and can pave way for novel and efficient methods of disease control not only in aquaculture, but also in pharmaceutical industry. In the present study, the high sensitivity of various microbes to rFi-Lyz compared with rHEWL emphasizes it as a better antimicrobial agent. Differential susceptibility of streptococci to various antibiotics has been reported[14]. Human pathogens *S. aureus* are reported to be resistant to HEWL and human lysozyme due to the O-acetylated at C6-OH in the muramic acid of peptidoglycan[15]. In another report, differential susceptibility *S. aureus* to HEWL is reported[16]. *S. thermophilus* and *L. acidophilus*, two probiotic bacteria did not exhibit significant growth inhibition in presence of rFi-Lyz but not with rHEWL and the differences in sensitivity seem to be the proportion of N-acetylamino sugars linked in a specific manner. This shows that the normal microbial floras remain unattached by the AMPs like rFi-Lyz thus narrowing down their activity mainly to pathogenic species.

K. pnemoniae, the major causative agent of pneumonia

resist the mucosal lysozyme and attack through enzyme mediated peptidoglycan modification[17]. *P. aeroginosa*, is reported to be sensitive to HEWL which is a causative agent of lung infections[18]. Here the significant inhibition of three pathogenic bacteria *viz*. *P. aeruginosa*, *K. pneumoniae*, *S. dysenteriae* in presence of rFi-Lyz augments as an efficient antimicrobial agent than rHEWL. Enteropathogenic *E. coli* is resistant to rFi-Lyz that could be attributed by specific resistance factor coded by plasmid as reported earlier[19]. However, the mechanism in lysozyme activity against Gram negative bacteria is still a hot topic of current researches. It has also been found that the chemical and thermal modification of lysozyme increases its antimicrobial properties towards Gram negative bacteria and such modified lysozyme exhibits a novel, but not completely defined antimicrobial action.

C-type lysozyme from *Litopenaeus vannamei* was effective against marine bacteria *V. alginolyticus*, *Vibrio parahemolyticus* and *Vibrio cholera*[7]. In this study the high sensitivity of *V. alginolyticus* and *A. veronii* to rFi-Lyz than rHEWL underscores the scope of using this for disease control in aquaculture. However, it does not inhibit *V. harveyi*, a fish pathogen, thus underlining the specificity of the compound.

Considering the anti-fungal activity, *C. krusei* showed a remarkably high inhibitory response (as high as 75%) to rFi-Lyz and similar findings have been reported earlier in the case of *Saccharomyces cerevesiae* and *Pichia pastoris*[12]. Moreover, rFi-Lyz also shows inhibitory activity when tested with plant pathogenic fungi such as *R. solani* and *F. solani*, at a concentration of 20 μg and 10 μg respectively. There are few reports on the antifungal activity of lysozyme[21-22]. But the mechanism of degradation of the strong chitin layer present in these organisms by the AMPs is still unclear and needs more investigations.

In general, lysozyme monomer exhibits strong antibacterial activity against Gram positive organisms by attacking the peptidoglycan, also these AMPs somehow destabilise the outer membrane, weakening its architecture, thus paving way for its further encounter with the subsequent layers. The findings of the present study concluded that rFi-Lyz showed a varying activity against several Gram positive, Gram negative and fungi whose exact mechanism of action are not yet to be investigated and this could open new avenues of using shrimp lysozyme in combating infections of human, plants and aquatic species.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

This work is supported by a grant from the Department of Biotechnology, Government of India, New Delhi, India as a part of Indo Norwegian program for fish and shellfish vaccine development with Grant No: BT/AAQ/Indo-Norway/183204/2007. We thank Dr. Alavandi, Central Institute for Brackish water Aquaculture, Chennai for providing us with microorganisms for turbidimetric assays.

Comments

Background

Lysozyme is one of the most ubiquitous antibacterial molecules, widely distributed in vertebrates and invertebrates that exert broad spectrum of antimicrobial action. This is a relatively small enzyme that catalyzes the hydrolysis of specific kinds of polysaccharides comprising the cell walls of bacteria and also exhibits catalytically independent antimicrobial activity that depends on its internal peptides.

Research frontiers

In general, lysozyme monomer exhibits strong antibacterial activity against Gram positive organisms by attacking the peptidoglycan, also these AMP somehow destabilise the outer membrane, weakening its architecture, thus paving way for its further encounter with the subsequent layers. The findings of the present study concluded that rFi-Lyz showed a varying activity against several Gram positive, Gram negative and fungi whose exact mechanism of action are yet to be investigated and this could open new avenues of using shrimp lysozyme in combating infections of human, plants and aquatic species.

Related reports

Similar findings have been reported earlier in the case of *Saccharomyces cerevesiae* and *Pichia pastoris*[12].

Innovations and breakthroughs

In this paper discussed the antibacterial and antifungal activity of c-type lysozyme from Indian shrimp *F.*

indicus.

Applications

The findings of this preliminary study may help to progress to the next level of research using animal models, thus for the development of attractive novel therapeutic approaches using rFi-Lyz or its internal peptides to treat diseases of human and shrimps.

Peer review

The authors are expressed, the antibacterial and antifungal activity of c-type lysozyme from Indian shrimp *F. indicus*. The work supports that shrimp will be used for antibacterial and antifungal activity. In this study the high sensitivity of *V. alginolyticus* and *A. veronii* to rFi-Lyz than rHEWL underscores the scope of using this for disease control in aquaculture.

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