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RESEARCH ARTICLE

Effect of *Escherichia coli* endotoxin on *Archachatina marginata* hemolymph coagulation system

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Abstract

Context: *Archachatina marginata* Swainson (Achatinidae) is found in Nigeria, West Africa. Its hemolymph is applied as a disinfectant to blades and fresh cuts of circumcision in Yorubaland. The hemolymph is also used in traditional medicine practice. Investigation into its anti-endotoxin response is being studied for the first time.

Objective: This study determined whether endotoxin causes measurable and concentration-dependent protein coagulation in the separate hemolymph fractions and in hemocyte lysate (HL)/plasma mixtures.

Materials and methods: Endotoxin was prepared by inoculating 5% w/v dextrose with locally isolated *Escherichia coli* cells and incubated for 48 h before sterilization. Pyrogenicity was determined by rabbit test method and use the of LAL kit. Hemolymph fractions were exposed to endotoxin while controls were exposed to endotoxin-free water (0.025 EU/ml). HL/plasma (1:1 v/v) was exposed to varied endotoxin concentrations.

Results: Data indicated significantly higher protein coagulates induced by endotoxin in all the hemolymph fractions ($P < 0.05$). Maximum protein coagulation in mixture of HL/plasma 1:1 was recorded. Exposure of HL/plasma at optimal ratio to varied endotoxin caused linear protein coagulation up to 1.0 EU/ml, beyond which it dropped significantly and unresponsive to further increase in endotoxin doses.

Discussion and conclusion: There was endotoxin-induced protein coagulation, which is endotoxin concentration-dependent. The optimal coagulation observed for 1:1 HL/plasma mixture suggests stronger interaction between the hemocytes and the plasma in response to endotoxin. There are LPS-binding proteins in the plasma and hemocytes of *A. marginata*. This finding may be employed in detection and quantification of endotoxin in future.

Keywords: *Escherichia coli*, *Archachatina marginata*, invertebrate protein coagulation systems, animal lectins, endotoxin

Introduction

Innate immunity in invertebrates is a nonclonal defense mechanism which depends upon the ability of the organisms to respond to pathogen-associated molecular patterns (PAMPs), through the use of pattern recognition receptors (PRRs) on their hemocyte cell membranes. The recognition of PAMPs leads to generation of responses leading to the destruction of the invading pathogen. Such responses lead to recognition of self and nonself antigens and help protect the organism against pathogens responses elicited sometimes include protein

coagulation and/or melanization or synthesis of antimicrobial peptides. PAMPs are generally preserved patterns associated with microorganisms such as the peptidoglycan in all bacteria, lipid A of the lipopolysaccharides of Gram negative bacteria and β -1-3-D-glucans in fungi (Medzhitov and Janeway, 1997).

The invertebrate protein coagulation systems are important in the defense of invertebrates against microorganisms. In crustaceans for example, processes of defense involve agglutination, encapsulation, phagocytosis, glycoproteins and clottable proteins. All are induced

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by carbohydrate-driven recognition patterns (Pereyra et al., 2009; Vazquez et al., 2009). The invertebrate coagulation systems are well studied in the *Limulus* crab (Tamura et al., 1996). The *Limulus* endotoxin-induced coagulation is the basis of limulus amoebocyte lysate (LAL) test of bacterial endotoxin in medical preparations in use to date.

Archachatina marginata Swainson (Achatinidae) is a gastropod found in the forest and savannah areas of West Africa (Raut and Barker, 2002). It is a good source of protein, eaten in soups and stew in Nigeria. *A. marginata*'s meat has 12–60% protein, 0.05–0.8% fat and 45–50 mg/kg iron (Cobbinah, 1997). Its hemolymph is made up of 5.6 g/dl proteins. Triglycerides and cholesterol have been detected in the hemolymph. Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate transaminase are some enzymes with activity found in the snail's hemolymph. Na⁺, K⁺, Ca²⁺, Mg²⁺, Zn²⁺ and bicarbonate ions are some of the minerals found in the hemolymph of both the wild and captive snails. The hemolymph biochemical parameters have been reported to contain, albumin/globulin ratios similar to those of some mammals like the pangolin and sheep (Ogunsanmi et al., 2003).

The snail's hemolymph is used in folk medicine. From orally given information, it is used in the traditional management of hypertension. The fresh hemolymph is swallowed in tablespoonfuls by the hypertensives. During circumcision, fresh hemolymph obtained by apical cracking of the shells of the snails is applied to fresh cut of the foreskin. This was believed to aid quick healing and prevent infection of the wound. However, no scientific evidence has been shown to support this practice.

There is dearth of information about the coagulation system in molluscs. This is the first report of the presence of LPS-binding protein in *A. marginata*. A study of the mollusc's hemolymph coagulation responses to *Escherichia coli* endotoxin may provide information on whether its hemolymph may serve as an alternative test material for endotoxin. This study was designed to examine the presence of endotoxin-induced protein coagulation reactions in *A. marginata* hemolymph fractions and the endotoxin concentration dependence of the reaction.

Methods

Snails collection and identification

Twenty *A. marginata* snails were purchased from Ipata Market, Ilorin, Kwara State, Nigeria, weighing 435 ± 35 g each with shell lengths of 165 ± 10 mm and largest whorl circumference of 145 ± 15 mm. A sample of the snails was submitted to and identified by Mr. Bolarinwa on October 3, 2008 and issued a collection number in the Department of Zoology, University of Ilorin. The sample was deposited with the department as reference sample. Dextrose monohydrate powder and *E. coli* laboratory isolates were obtained from the Quality Control/

Assurance Department of Biomedical Limited, Ilorin, Nigeria. All chemicals used were of analytical grade and obtained from the chemical store of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. The use of animals was approved by the Ethics Committee of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria.

Preparation of endotoxin from *E. coli*

E. coli endotoxin stock was prepared as reported earlier (Salawu et al., 2010). Briefly, 5% weight per volume (w/v) dextrose solution was prepared using sterilized endotoxin-free distilled water and dextrose monohydrate powder. The solution was inoculated with a strain of laboratory isolate of *E. coli* and incubated at 37°C for 48 h and then autoclaved for 35 min. A 5% w/v dextrose control solution was prepared, but sterilized immediately. The microbial load of both solutions was determined by using the pour plate methods. The pyrogen (endotoxin) content was determined using the standard rabbit test method as described in the (Radhakrishna, 2010). The dextrose solution inoculated with *E. coli* and sterilized by autoclaving after 48 h and tested for pyrogen. In anticipation of high endotoxin content, the solution was diluted with pyrogen-free 0.9% w/v sodium chloride (1:9). The control dextrose solution that was sterilized immediately was also tested for pyrogen using the rabbit test method (Precautions taken: The animals used for the tests have not failed a product in the previous 2 weeks. Animals used maintained their initial body temperatures to within 0.2°C within the 60 min before the injection of the dextrose solutions, three animals per sample were used weighing not less than 1.5 kg each).

Endotoxin handling

Pyrogenic stock solution was handled carefully with sterile surgical gloves. All other reagents used were common laboratory analytical reagents. The Material Safety Data sheets of the common reagents used are available from literature. None of the material/chemicals used required additional information in terms of its toxicity/pathological side effects.

Extraction of hemolymph

The snails were washed in distilled water to remove dirt from their shells and foot by gently rubbing with hands. The hemolymphs were obtained by apical cracking method (Ogunsanmi et al., 2003). The fresh hemolymphs obtained per animal was between 20 and 25 ml in volume and pooled together. Equal volumes of 10 mM EDTA in 10 mM phosphate buffer pH 7.0 containing 0.142 M NaCl and 0.18 M glucose were added and centrifuged at room temperature, 800g for 10 min (Kopacek et al., 1993). The supernatant was collected as *A. marginata* plasma. The pellets were washed in 10 mM phosphate buffer pH 7.0 containing 0.1 M CaCl₂ and centrifuged at 800g for 10 min. The supernatants were discarded and the pellets harvested as hemocytes and then homogenized in

a 10-mM phosphate buffer pH 7.0 containing 10mM EDTA. The homogenate was suspended in the same buffer and adjusted to the initial actual volume of the hemolymph that was processed. The homogenate was the hemocyte lysate (HL). HL (10ml) was centrifuged at 800g for 20min and the supernatant collected as HL supernatant (HLS). The pellet cytosol free hemocyte lysate debris (HLD) contained the hemocyte membranes were washed twice in 1 ml 10-mM phosphate buffer pH 7.0 and suspended in the same buffer made up to the initial hemolymph volume that yielded the HLD. The fractions were stored frozen.

Coagulation reactions in the HL, HLS, HLD and plasma

Borosilicate glass tubes and pipettes previously deproteinized by dry heating in an oven at 250°C for 30 min (Radhakrishna, 2010) were used for this experiment. 0.1 EU in 0.1 ml distilled water was added to 0.8 ml of HL and 0.1 ml of 0.1 M CaCl₂. Same procedure was repeated using HLS, HLD and plasma, respectively. Four replicates were prepared for each hemolymph fraction. A control was also prepared in quadruplicates for each fraction using endotoxin-free water (≤ 0.025 EU/ml). The reaction mixtures were incubated at 37°C for 1 h. Coagulants were observed in each tube and then fixed to the bottom of the tubes by centrifugation (Vazquez et al., 2009) while supernatants were discarded. The pellets were then dissolved in 0.9% w/v sodium chloride solution.

Determination of protein content in the coagulants obtained from HL, HLS, HLD, plasma and the controls

The total protein contents of each fraction and its control were determined using biuret reaction (Gornall et al., 1949). The characterization of the protein coagulants was intended for future studies.

Determination of the optimum HL:plasma ratio required for endotoxin-induced coagulation in *A. marginata* hemolymph fractions

HL and plasma were mixed in various ratios and exposed to a final endotoxin concentration of 0.1 endotoxin unit per milliliter (EU/ml). The total volume of reaction mixture was 1.0 ml containing 0.1 ml 0.1 M CaCl₂. A control was prepared for each reaction mixture prepared in quadruplicates. The samples were then incubated at 37°C for 1 h centrifuged at 800g to fix the coagulants to bottom of test tubes. The supernatants were discarded and the coagulants dissolved in 1 ml 0.9% w/v NaCl and the protein content estimated by biuret reaction method (Gornall et al., 1949).

Determination of concentration dependence of endotoxin-induced protein coagulation using HL:plasma mixture

After the determination of the optimal ratio of HL:plasma, the ratio that gave maximum protein coagulation was utilized to study the concentration dependence of the

protein coagulation in *A. marginata* hemolymph. A reaction mixture containing equal volume of HL and plasma, 0.1 ml CaCl₂ and 0.1 ml of endotoxin was used (endotoxin concentrations of 0.0, 0.125, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 EU/ml). A blank was prepared containing endotoxin-free water (endotoxin concentration ≤ 0.025 EU/ml). Reaction mixtures were prepared in quadruplicates for 1 h, centrifuged at 800g and supernatants discarded. The coagulants were dissolved in 0.9% w/v NaCl and protein content estimated by biuret reaction (Gornall et al., 1949). Values were corrected with blanks prepared with endotoxin-free water (endotoxin concentration ≤ 0.025 EU/ml) incubated with each HL:plasma mixture.

Results

The protein contents of the coagulants determined as A 550 nm are shown in Figure 1. The protein contents obtained with the presence of 1.0 EU/ml in each hemolymph fraction was significantly higher than those of the controls ($P < 0.05$). The introduction of endotoxin caused higher quantities of protein to coagulate from the hemolymph fractions. There was maximum protein coagulation at HL:plasma ratio of 1:1. Therefore, maximum anti-endotoxin response in terms of protein coagulation was obtained as an additive increase between the HL and the plasma (Figure 2). There was progressive increase in protein coagulation from endotoxin contents of 0–1.0 EU per reaction mixture corresponding to concentrations of 0–1.0 EU/ml solution of endotoxin incubated with the HL:plasma mixture. At higher concentrations, there was a drop in coagulation and a loss of concentration-dependent response (Figure 3).

Discussion

Protein coagulation systems in invertebrates serve as an important process for limiting infection by pathogenic

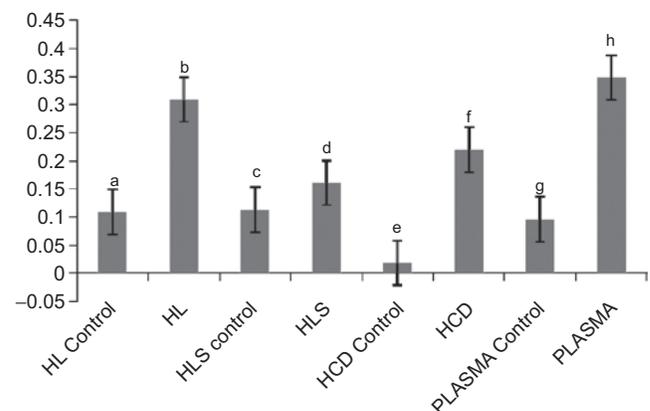


Figure 1. Endotoxin-induced protein coagulation in *Archachatina marginata* hemolymph fractions. Values are Mean \pm SEM of 4 determinations. Different superscripts on the test and control for each hemolymph fraction indicate significantly different values at $p < 0.05$.

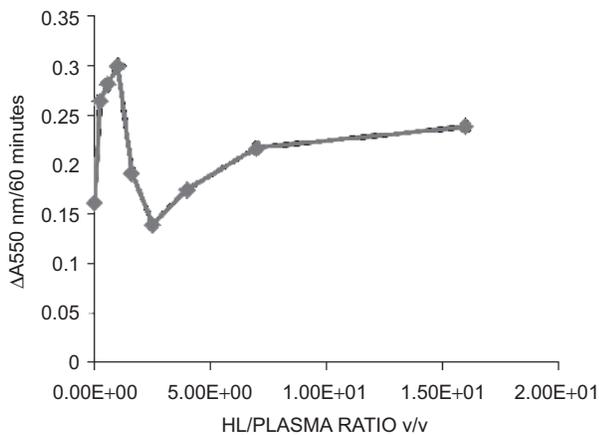


Figure 2. Determination of optimal ratio of HL: plasma required for endotoxin-induced protein coagulation in *A. marginata* hemolymph. Each point represents the average value for four determinations.

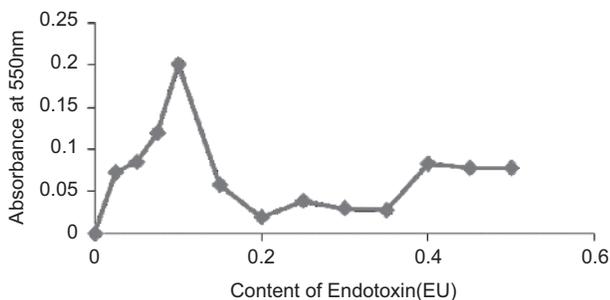


Figure 3. Determination of concentration dependence of endotoxin-induced protein coagulation in HL:plasma mixtures of *Archachatina marginata* hemolymph fractions.

organisms. In crustaceans (crabs and crayfish), coagulogen and coagulating enzymes (transglutaminases), which exist normally as zymogens, are involved in a cascade of reactions leading to protein coagulation, in order to protect the integrity of the organisms (Kawabata et al., 1996; Vazquez et al., 2009). The prophenoloxidase cascade also leads to melanization in protein meshwork in insects and other invertebrates at the sites of injury. It was associated with cuticle formation in crustaceans such as *Pacifastacus leniusculus* Dana (Astacidae) (Kopacek et al., 1993). The presence of opsonin-related proteins was discovered in the freshwater crayfish (Lee and Söderhäll, 2001) which helps in clearance of *E. coli* from the crayfish hemolymph *in vivo*.

In this study, *A. marginata* hemolymph fractions: the HL, HLS, HLD and the plasma were exposed to endotoxin and distilled water (endotoxin content ≤ 0.025 EU/ml), respectively. The fractions after incubation produced protein coagulates that were significantly different in quantity ($P < 0.05$). The fractions exposed to endotoxin actually produced higher quantity of endotoxin than those exposed to distilled water (≤ 0.025 EU/ml). A combination of various ratios of HL/plasma was also exposed to equal quantity of endotoxin. The 1:1 HL/plasma mixture produced optimal quantity of endotoxin. The exposure of optimal HL:plasma mixture

to endotoxin ranging from 0 EU/ml to 5.0 EU/ml produced a linearly increased coagulation up to endotoxin concentration of 1.0 EU/ml, beyond which there was a drop in response.

The data generated suggest that LPS-binding proteins were present in the hemolymph fractions of the snail—the plasma, HLS, HLD (Figure 1). The protein coagulation induced by endotoxin is a joint response by the LPS-binding proteins in the plasma as well as those produced by the hemocytes as shown in the response in the HL. A synergistic response was produced by the HL and the plasma when mixed in equal ratio (Figure 2). There was a progressive increase in protein coagulation in a mixture of HL/plasma until equal volume mixture of the fractions was attained (Figure 2). This suggests that there is a cellular response in terms of protein degranulation as well as LPS-binding of some plasma proteins simultaneously in response to the presence of endotoxin in the hemolymph. This binding and coagulation may serve a purpose in encapsulating and restricting an invading Gram negative pathogen, prior to hemocyte engulfing of the bacteria and endocytosis. Exposure of HL/plasma mixture at optimal ratio to varying concentrations of endotoxin causes quantitative response in terms of protein coagulation to *E. coli* endotoxin (Figure 3). There was progressive increase in the quantity of protein coagulates as endotoxin concentration increased from 0 to 1.0 EU/ml. At higher concentrations, there was a drop in the quantities of protein coagulated and then no further significant change with increasing concentration of endotoxin.

The coagulated proteins are likely to be lectins. Such proteins were discovered in *Achatina fulica*; achatinin-H (Sen and Mandal, 1995) and achatinin (Biswas, 2000). In *A. fulica*, a related gastropod to *A. marginata*, achatinin-H was isolated and characterized (Sen and Mandal, 1995), achatinin was also isolated from the hemolymph of the same gastropod and characterized (Biswas, 2000). The two proteins were lectins. On SDS-PAGE electrophoresis, achatinin showed 16 subunits each 15 kdal. Achatinin-H was shown to be homologous to that of achatinin. Both lectins were shown to be of importance in the innate immunity of *A. fulica* as they respectively bind (*E. coli* O55: B5) lipopolysaccharide's 9-*O*-acetyl sialic acid and coagulate out of solution. This reaction explains why *E. coli* cells are agglutinated by these lectins in *A. fulica*.

Further studies on the endotoxin-induced protein coagulates from *A. marginata* hemolymph fractions may provide information on the constituent proteins and their structure.

Conclusions

From this study, we conclude that lipopolysaccharide-binding proteins likely exist in the hemolymphs of *A. marginata*. There is a quantitative response to exposure to endotoxin at concentration range between 0 and 1.0 EU/ml by hemolymph fractions of *A. marginata*. This

can be employed in the future for detection and quantification of bacterial endotoxin in medications meant for parenteral use.

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Declarations of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the article.

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