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Original Research

Role of serine proteases and agglutinins in the endotoxin-induced protein coagulation in *Archachatina marginata* hemolymph

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hemocyanin; serine proteases.

Abstract

Objective: In this study, we hypothesized that endotoxin-induced protein coagulation in the snail is mediated by lectins and serine proteases.

Methods: Experiments were carried out to determine the lectin activities of the hemolymph fractions and those of their coagulants and supernatants. The plasma was also exposed to 5000 EU/ml endotoxin, incubated for 1 h at 37 °C and the supernatant's protein profile was compared on SDS-PAGE to a non-treated plasma. In addition, LPS-agarose was used as an affinity column to isolate lipopolysaccharide binding protein(s). Eluted proteins were subjected to SDS-PAGE, mass spectrometry and proteomic analysis. Finally, endotoxin-induced coagulation was tested for Ca²⁺ ion- and serine protease-dependence.

Results: Results from this study indicated that lectins were involved in the coagulation process induced by endotoxin. There was a loss in total lectin activity from the plasma fraction on exposure to endotoxin. Several mono- and disaccharides inhibited the agglutination of the reconstituted lyophilized plasma by rabbit erythrocytes, indicating the possible presence of several types of lectins in the plasma. Endotoxin-absorbed plasma resulted in a reduction of proteins in the >250 kDa range, ~66 kDa and a 37 kDa protein based on SDS-PAGE analysis. Excision of those bands and subsequent proteomic analyses predicted the presence of hemocyanin (389-394 kDa), an allergen (69 kDa) and a Gram-negative binding protein (50 kDa). Endotoxin-induced protein coagulation was inhibited by a trypsin protease inhibitor. No coagulation was detected in the absence of Ca²⁺ ion.

Conclusions: From these results, we conclude that endotoxin-induced protein coagulation in *Archachatina marginata* hemolymph is lectin and serine protease-mediated and Ca²⁺ ion dependent.

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INTRODUCTION

The invertebrate protein coagulation systems have been shown to be important in the innate immunity of the invertebrates against micro-organisms. In crustaceans for example, processes of defense involve agglutination, encapsulation, and phagocytosis, mediated by glycoproteins and clottable proteins. All are induced by carbohydrate-driven recognition

patterns [1]. Coagulation systems are well studied in the limulus crab, freshwater crayfish, silkworm larvae and a number of insects. Haemocytes of *Limulus polyphemus*, the horseshoe crab found on the east coast of the United States of America, are highly sensitive to endotoxin, which induces rapid hemolytic clots in its presence [2]. A similar clotting response has been demonstrated for the Japanese horseshoe crab

Tachypleus tridentatus [3]. The *Limulus* and *Tachypleus* coagulation systems are characterised by a cascade of reactions involving serine proteinases leading to the activation of coagulation enzymes which in turn acts on a coagulogen found in the haemocyte lysate [2,4]. This system involves several serine proteinases terminating with the action of a transglutaminase [5]. The first reaction in this cascade is triggered by Gram-negative bacteria or the lipopolysaccharide derived from Gram-negative bacteria in doses as low as 0.2 ng/ml (1 Endotoxin Unit/ml = 1EU/ml). β -(1-3) D-glucans derived from fungi vegetative bodies also elicit similar reactions [6,7] but at concentrations in mg/ml.

The phenoloxidase cascade pathway associated with protein coagulation in invertebrates is common in insects and crustaceans and involves a reaction cascade leading to the activation of phenoloxidase (tyrosinase) [2]. This reaction cascade ultimately leads to formation of melanin and/or sclerotization of cuticles or egg shells. Analogous to the horseshoe crab coagulation system, the pro-phenoloxidase cascade also can be triggered by pathogen-associated molecular patterns (PAMPs) such as peptidoglycan, lipopolysaccharide or β -(1,3)-D glucans. Cascade activation also involves several serine proteinases associated with the pro-phenoloxidase cascade that eventually leads to a formation of cross-linked protein gel and the deposition of melanin that serves to bind the intruding Gram-negative bacteria or fungi through reactivity with their cell wall components. Lipopolysaccharides of Gram-negative bacteria (LPS, endotoxin) bind to a 'masquerade-like' host protein whose function was likened to an opsonin [8]. Interestingly, the crayfish phenoloxidase cascade is not provoked by Gram-positive bacteria [9], indicating that regulation of PO activation may depend on specific recognition signals.

Endotoxin-induced protein gel formation has been reported in the freshwater crayfish [10] and has been shown to be responsible for microbial clearance in infected crayfish [8]. Therefore, the phenoloxidase reaction cascade and the formation of melanin serves multiple physiological functions in these, and other arthropod species, including internal defense against bacterial and other microbial infections and reactions leading to cuticular or egg shell tanning/hardening of cuticles. Endotoxin-induced coagulation in the terrestrial snail *Archachatina marginata* has yet to be fully studied. In *A. marginata*, we reported that endotoxin induced a protein coagulation reaction in various hemolymph fractions including plasma, haemocyte lysate (HL), haemocyte lysate supernatant (HLS) and the hemocyte lysate debris (HLD) [11]. The endotoxin-induced protein coagulation in the plasma/HL mixture was endotoxin concentration-

dependent. However, the hemolymph proteome participation in the response to endotoxin remained unknown. The focus of the present study was to investigate the involvement of lectins (agglutinins), serine proteases and Ca^{2+} on the observed endotoxin-induced protein coagulation in *A. marginata*. We also separated lipopolysaccharide binding proteins from reconstituted lyophilized plasma using LPS-agarose as an affinity matrix in an attempt to isolate and identify possible LPS binding proteins involved in the coagulation reaction.

MATERIALS AND METHODS

Snails: Twenty *A. marginata* snails were purchased from the Ipata market, Ilorin and one specimen was submitted to the Department of Zoology, University of Ilorin for identification. The specimen was identified and retained as reference sample. Endotoxin test kits - Pyrosate[®] (0.25EU/ml sensitivity) LAL kits were purchased from Cape Cod Associates, (Knowsley Liverpool, UK). Lipopolysaccharide from *E. coli* 0111:B4 and Epoxy-activated agarose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in the preparation of buffers and reagents were of analytical grade and obtained from Sigma-Aldrich. All studies have been approved by the Postgraduate Studies committee of the Department of Biochemistry, University of Ilorin in charge of ethics and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Endotoxin handling

All the pyrogenic stock solutions were handled with sterile gloves and in a biological safety hood. Material Safety Data regarding toxicity and proper handling was available for these and other reagents used in this study.

Extraction of haemolymph

The snails *A. marginata* were thoroughly cleaned by washing their shells and head foot in distilled water. Haemolymph was obtained by the apical shell cracking method [12]. Between 20-25 ml of fresh haemolymph was obtained per animal. Haemolymph from all animals were pooled on ice at 4°C and an equal volume of 10 mM EDTA in 10 mM phosphate buffer, pH 7.4, containing 0.142 M NaCl and 0.18 M glucose was added. This was followed by centrifugation at room temperature, 800 x g for 10 min [5]. The supernatant was collected as plasma, while the hemocyte pellet was washed twice at 25 °C in 10 mM phosphate buffer pH 7.4 containing 0.1M CaCl_2 and centrifuged at 800 x g for 10 min. Isolated pellets were homogenized in 10 mM phosphate buffer, pH 7.4, containing 10 mM EDTA using ice-cold pestle and mortar. The

homogenate was suspended in the same buffer and adjusted to the initial volume of the haemolymph that was initially processed. The homogenate, designated the haemocyte lysate (HL), was centrifuged at 800 x g for 20 min and the supernatant collected as HL supernatant (HLS). The pellet, designated the haemocyte lysate debris (HLD) was washed twice in 1 ml 10 mM phosphate buffer, pH 7.4, and suspended in the same buffer made up to the initial hemolymph volume. The plasma was stored frozen or first freeze-dried and stored dried at 4°C.

For preparations meant for agglutination reactions, mammalian phosphate buffered saline (mPBS), pH 7.4, was used to dilute the fresh haemolymph 1:1, followed by centrifugation at 800 g, 25 ± 2 °C. The hemocytes (pellets) so harvested were fractionated as described above with the exception that all buffers used in the preparation of hemocytes for isolation and fractionation were devoid of 10 mM EDTA.

Coagulation reactions in the HL, HLS, HLD and plasma

Borosilicate glass tubes and pipettes previously depyrogenated by dry heating in an oven at 250°C for 30 min [13] were used in these experiments. LPS (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 CaCl_2 . The same procedure was repeated using HLS, HLD, plasma and a mixture of plasma:HL. Four replicates were prepared for each hemolymph fraction. A control was also prepared in quadruplicate for each fraction using endotoxin-free water (≤ 0.025 EU/mL). The reaction mixtures were incubated at 37 °C for 1 hr, followed by centrifugation 800 g, 20 min to pellet any coagulated proteins forming in the tubes [1]. After discarding the supernatants, the pellets were then solubilized in 0.9% w/v NaCl, in a volume corresponding to the original haemolymph volume prior to processing. To test for Ca^{++} dependence, CaCl_2 was replaced with mPBS. To test if plasma protease(s) may be required for endotoxin-mediated coagulation, the trypsin inhibitor 4-(2-aminoethyl) benzene sulfonylfluoride hydrochloride was added to test samples at a final concentration of 0.1 mM, while controls were incubated without endotoxin or inhibitor. The total protein contents of coagulates obtained from endotoxin-mediated reactions with each fraction and controls were determined using nanodrop Spectrophotometer, ND 1000 after sonication in mPBS for 3 mins.

Haemagglutination Assay

100 µl of PBS was delivered sequentially into the wells arranged in rows of 12 wells in a U-shaped micro-titre plate. 100 µl of the extract was added into the first well to obtain a 1:2 dilution. A serial dilution of the extract was performed by then transferring 100 µl of the

diluted sample in a particular well into the next well containing 100 µl of PBS. 50 µl aliquots of the 2% erythrocyte suspension was dispensed respectively into each well and the wells were left for about 1 hour in order to allow the agglutination of the red blood cells to occur after which the agglutination titres were recorded. The titre of the lectin was taken as the reciprocal of the highest dilution of the extract exhibiting visible haemagglutination and this was taken as one hemagglutinating unit.

Sugar Inhibition test

The sugar specificity of the extract was carried out by assaying for different sugars on the basis of the minimum concentration required to inhibit the agglutination of the red blood cells by the lectins [14]. This was carried out in two steps:

In the first step, lectin was diluted serially until the end-point dilution causing haemagglutination was obtained. 50 µl of 0.2M sugar solution was added to each well while the control well contained PBS instead of the sugar solution. 50 µl of erythrocyte suspension was added to each well and the titre of lectin activity was determined as previously described. Inhibitory sugars caused a reduction in the titre of the lectin activity shown by the PBS-control experiment.

In the second step, the minimum concentration of each sugar required to inhibit lectin-specific haemagglutination of blood cells by 50% was investigated [15] Two-fold serial dilution of sugar samples was prepared in PBS (0.2 M initial concentration). All the dilutions were mixed with an equal volume (50 µl) of the solution of the lectin with known haemagglutination units. The mixture was allowed to stand for 1 h at room temperature and then mixed with 50 µl of a 2% RBC suspension. The haemagglutination titres obtained were compared with a non-sugar containing blank.

Absorption of endotoxin activity in *A. marginata* reconstituted plasma

A 19:1 mixture of reconstituted plasma and LPS solution was prepared to give a final endotoxin concentration of 5.000 EU/ml and incubated at 37 °C for 1h; giving Endotoxin-Absorbed plasma (EAP). An endotoxin-free control was prepared using mPBS in place of endotoxin solution to give endotoxin free plasma (EFP). The mixtures were incubated at 37 °C for 1 h. Aliquots (50 µL each) of EAP and EFP was added to 50 µL of 1% suspension of RBC in mPBS containing 0.5% BSA (v/v). The mixtures were incubated at 25°C for 1 h, followed by centrifugation at 1000 x g for 10 min. The supernatants were collected and subjected to SDS-PAGE to determine the effect of endotoxin absorption on the plasma protein profile.

Sensitization of fixed rabbit erythrocytes with endotoxin

Equal volumes of 1% suspension of rabbit erythrocyte (RBC) in BSA (0.5% in mPBS, pH 7.4) and 100,000 EU/ml endotoxin were mixed in 1.5 ml tubes and incubated at 25°C for 1 h to form endotoxin-sensitized erythrocyte (ESR). Three replicates were prepared per tube. An endotoxin-free preparation also was made by substituting mPBS in place of endotoxin to form an endotoxin-free erythrocytes preparation. After incubation the mixtures were then used to agglutinate *A. marginata* plasma. A 1:1 mixture of the endotoxin sensitized erythrocyte (ESR) was centrifuged at 1000 x g for 1 min and the supernatants collected and subjected to SDS-PAGE along with Endotoxin Absorbed Plasma (EAP), Endotoxin-Free Plasma (EFP). This was to investigate the plasma proteins missing due to endotoxin exposure and red cell agglutination respectively. EFP served as a control and reference protein profile to compare EAP and Endotoxin sensitized Red blood cells (ESR) on the same SDS-PAGE gel.

Preparation of LPS-agarose column

One gramme of resin was hydrated in 10 mL of pyrogen-free deionized water for 30 min, centrifuged at 1000 x g for 1 min and re-suspended in 10 mL of pyrogen-free water. This washing procedure was repeated 3 times. The swollen resin was then coupled to LPS as described by Fox and Hechemy [16]. Briefly, 1.0 mg endotoxin was dissolved by sonication in 3 mL of 1.0 mM sodium bicarbonate buffer, pH 10.5, containing 100 mM sodium chloride and then added to the swollen resin in distilled water. The mixture was gently mixed in a closed tube and then incubated in a shaking water bath at 37 °C for 16 h. At the end of the incubation 10 volumes of the bicarbonate buffer, pH 9.5, was added and centrifuged at 1000 x g for 1 min. The supernatant was discarded and this procedure repeated thrice. Following washing, endotoxin-coupled resin were quenched by adding 10 mL of 1 M ethanolamine, pH 8.0, and incubating for 4 h at 40 °C. Thereafter, the resins were washed with 3 rounds of 10 volumes of alternating low/high pH buffers: Low pH wash buffer contained 0.1 M sodium acetate (pH 4.0) in 0.5 M NaCl, while the high pH wash buffer contained 0.1 M Tris buffer (pH 8.0) in 0.5 M NaCl. The affinity resin was finally suspended in the high pH buffer and loaded into a Bio-Rad spin column (2-ml capacity) by sterile transfer pipette and allowed to settle for 60 min. Buffer was allowed to flow out until just before the surface of the resins were exposed

RESULTS

As illustrated in Table 1 exposure of fresh plasma, HL, HLD HLS, their coagulates and supernatants to endotoxin resulted in a reduction in total lectin activity.

Figure 2 shows that there appears to be a reduction of the >250 kDa protein bands and an apparent merging of protein bands at approx 66-70 Kda region on the endotoxin absorbed plasma. The erythrocyte absorbed plasma (EAP) shows a thick band at 66 kDa most likely due to the presence of bovine serum albumin (BSA)) used to block fixed RBCs. However, there appears to be a protein of similar molecular weight in the *A. marginata* plasma responding to endotoxin.

In Figure 1, the areas in the boxes show the regions of observed differences between the plasma P, endotoxin Absorbed Plasma (EAP) and Red blood cell Absorbed Plasma (RAP). The EAP has reduction in the intensity of protein bands in the 250-100 kDa molecular weight range, as well as at 75-70 KDa and 50-40 KDa range. Such reductions are seen in RAP but quantitatively less. Endotoxin could therefore cause some proteins to be depleted from the plasma.

Figure 2 indicates that endotoxin caused alteration of protein banding in the EAP at approx 250 and 70 kDa region.

In Figure 3, it appears that Endotoxin Sensitized Red blood cells caused proteins of large molecular weights (> 250 KDa) in the reconstituted plasma to degrade and lower molecular proteins to form as shown by thick band at approximately 70, 37 and 15 KDa regions.

Figure 4 shows the eluted LBP on SDS-PAGE gels achieved by pooling of repeated binding and elutions and enhancement of certain bands at >250 KDa and at approx 37 KDa. However there were several other protein bands similar to the plasma banding in the eluted proteins.

Figure 5 shows that LPS-agarose eluate (LBP) as a band > 250 KDa (at low protein loading of the gel); the LBP (loaded after concentration by centrifugation) with enhanced bands at > 250 KDa and 37 KDa regions.

Table 2 indicate that some mono saccharides, disaccharides and polysaccharides inhibit lectin activity in the plasma (reconstituted after lyophilization). The inhibition result may give clues about the types of lectins present in the plasma. As shown in Table 3 subjecting plasma to LBP-affinity chromatography resulted in an enhanced agglutination titre (~23-fold enrichment) over that of the original plasma sample, indicating that the Agarose LPS has enriched for proteins with lectin activity (agglutinins).

Table 1. Agglutination titres of *plasma, HL, HLS, HLD and their corresponding endotoxin-induced coagulates and their supernatants

Haemolymph fraction	*Agglutination Titre with rabbit erythrocytes	Loss of activity due to endotoxin binding
Whole plasma	2^{13}	
plasma coagulate	2^4	
plasma supernatant	2^0	2^9
Haemocyte lysate (HL)	2^{13}	
HL coagulate	2^4	
HL supernatant	2^4	2^5
HLS	2^4	
HLS coagulate	2^2	
HLS supernatant	2^1	2
HLD	2^9	

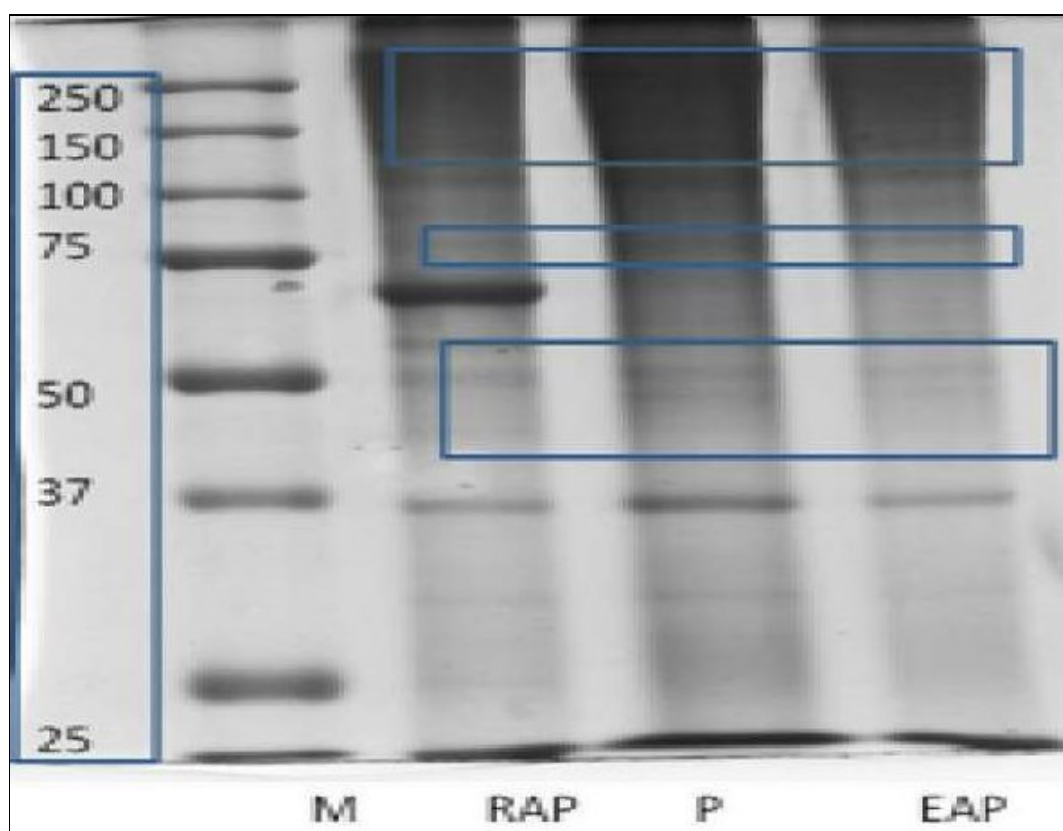


Figure 1. Protein profiles of endotoxin-absorbed plasma (EAP), plasma (P) and rabbit erythrocyte-absorbed plasma (RAP) of *A. marginata*

Figure 2. Protein profiles of plasma coagulates and endotoxin-induced plasma coagulates as separated on 10% SDS-PAGE gel

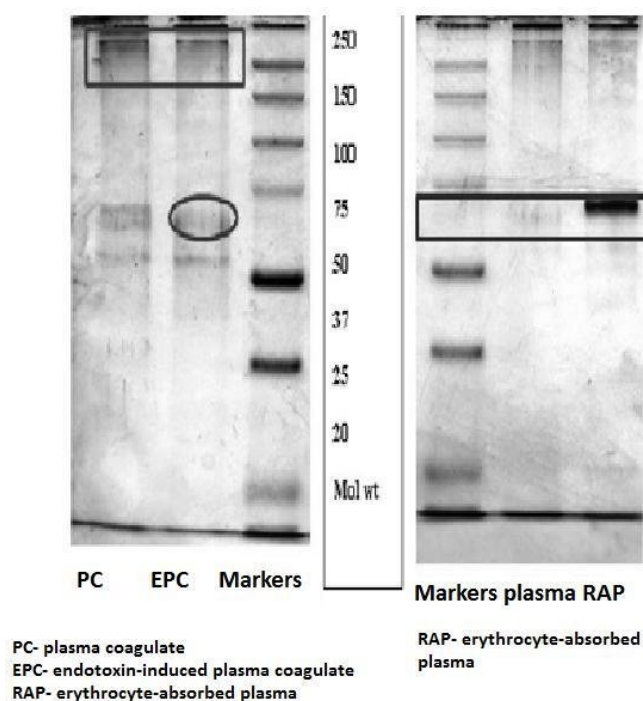
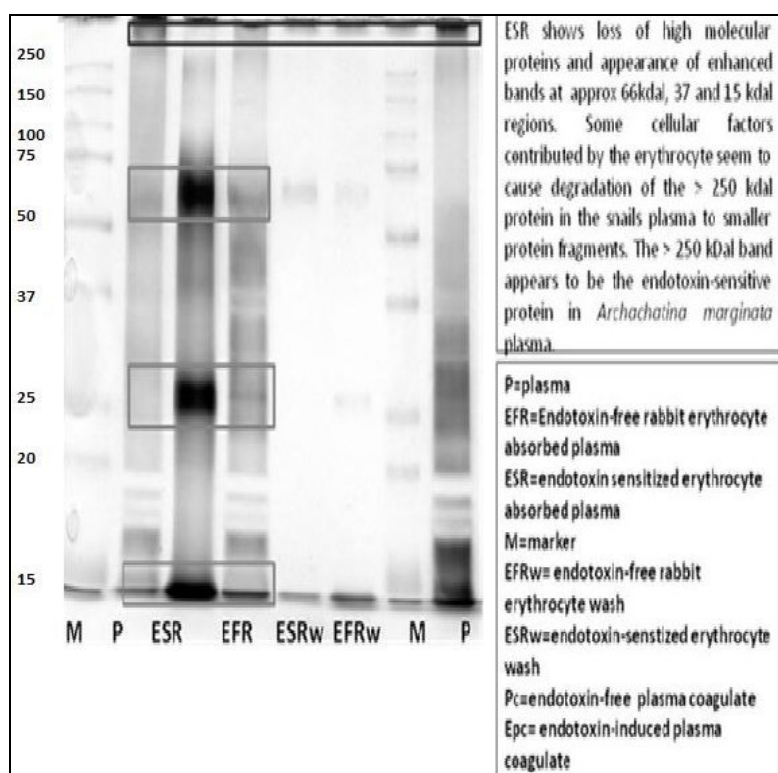


Figure 3. Protein profiles of supernatants of absorbed plasma, ESR and EFR as separated on 10% SDS-PAGE



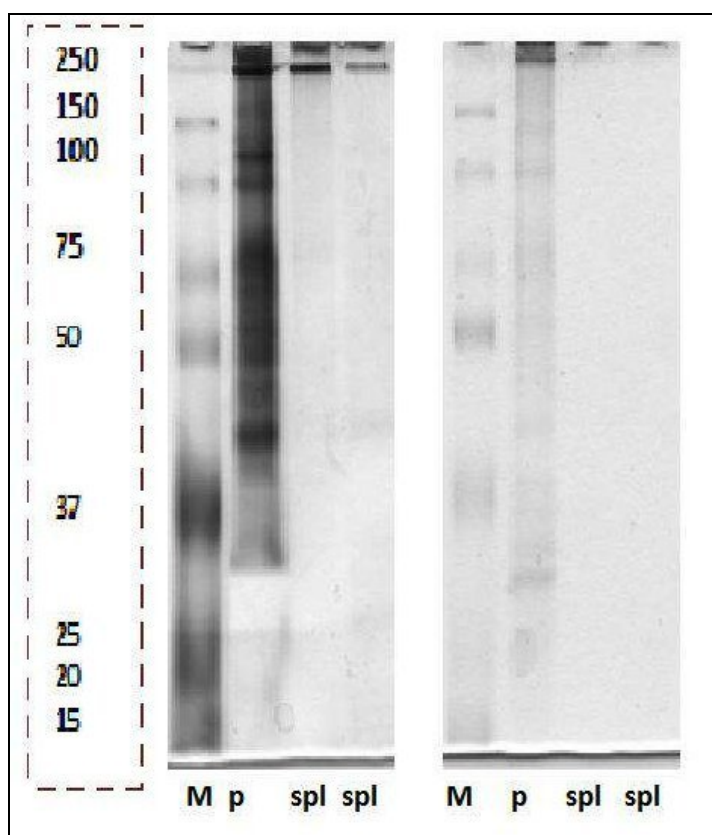


Figure 4. LBP on Native PAGE stained with Coomassie blue (left) and silver stain (right)

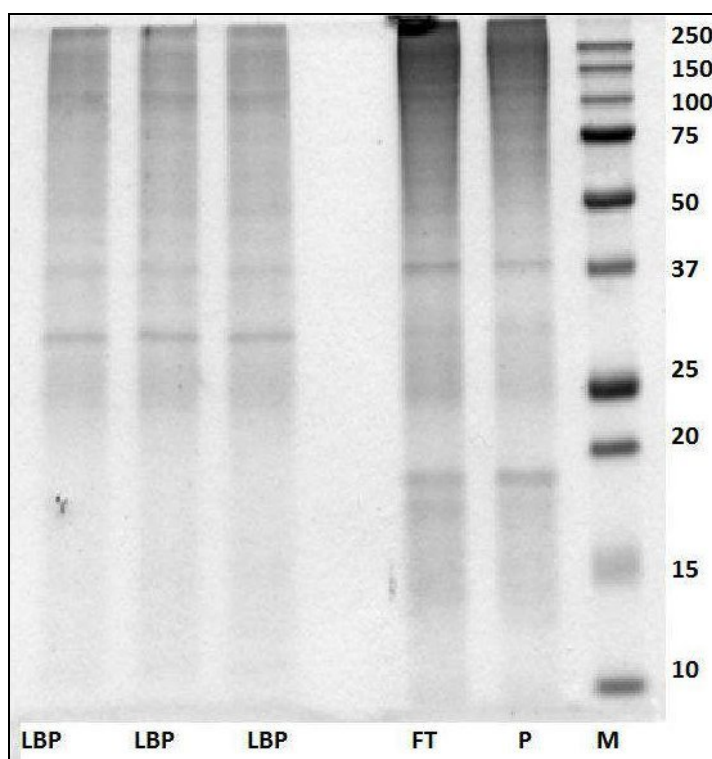


Figure 5. Concentrated Lipopolysaccharide Binding Proteins (LBP) on SDS-PAGE gel (Coomassie-stained)

Figure 6. Endotoxin concentration-dependence of level of protein coagulation in the plasma of *A. marginata*

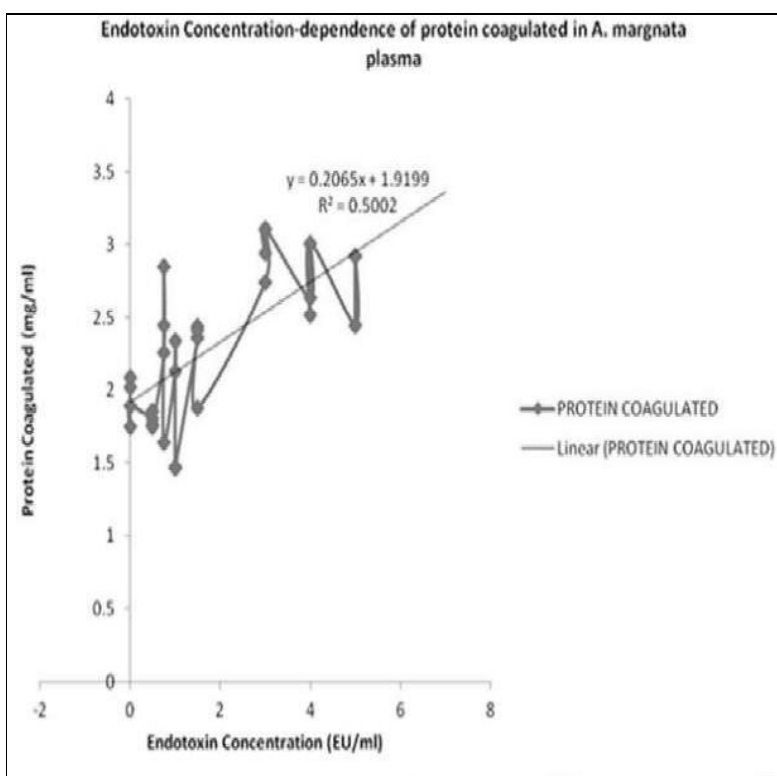
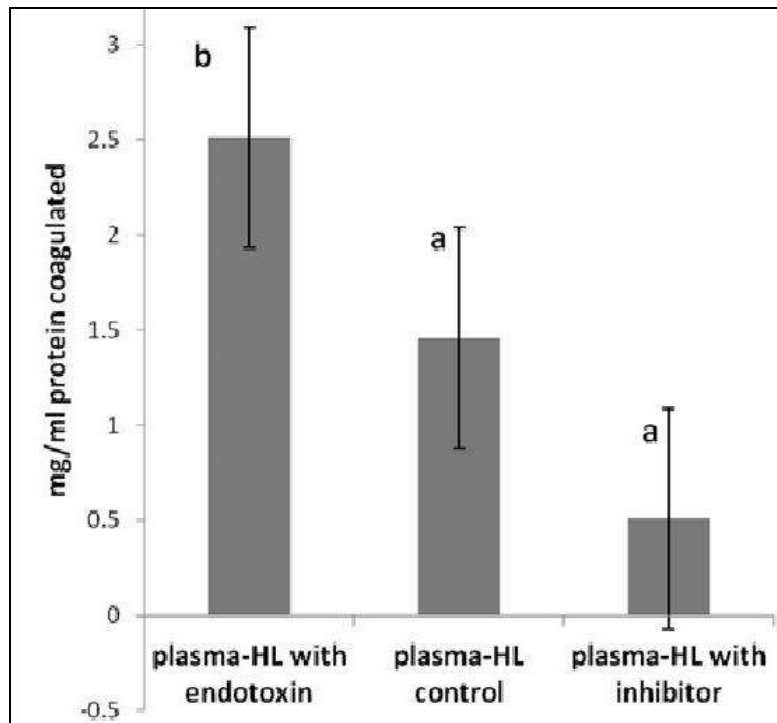


Figure 7. Reduction Of Level Of Protein Coagulation by 0.1mM 4-(2-Aminoethyl) Benzene Sulfonyl Fluoride Hydrochloride (Trypsin Inhibitor)



DISCUSSION

In the previous study, the exposure of HL, HLS, HLD and plasma of *Archachatina marginata* to endotoxin caused significantly higher ($p < 0.05$) level of protein coagulated in each fraction [11]. This observation suggests the presence of endotoxin-binding proteins [10] in the plasma as well as in the soluble fraction (HLS) of Haemocyte Lysate (HL) and on the haemocyte membrane contained in the Haemocyte Lysate Debris (HLD). The evidence implies that the plasma membranes are rich in receptors which bind endotoxin [17]. Higher protein coagulation was recorded in the HLD and the plasma. This finding is contrary to the coagulation reactions in *Pacifastacus leniusculus*, which requires the soluble cell lysate fraction and the plasma coming together for clotting to take place. The clotting reaction in the freshwater crayfish is serine protease-mediated and involves the cross-linking enzyme—TGase (transglutaminase) [5].

The differential protein coagulation in the plasma and HL were significantly higher ($p < 0.05$). A combination of both fractions in various ratios was exposed to endotoxin at 1 EU/ml concentration. The differential coagulation as against those of mixtures of plasma/HL exposed to endotoxin-free water (< 0.025 EU/ml) showed a gradual increase in quantity of coagulates obtained until an optimal ratio of 1:1 was attained. At ratios higher than 1. There was a sharp decline and no significant increase as the ratios of HL:plasma increased [11]. The evidence revealed that the haemocytes as well as the plasma are possibly involved in endotoxin-induced protein coagulation in *Archachatina marginata*. This is consistent with the type of coagulation reactions reported in crustaceans [18] and especially in *Sudanonautes africanus* [19].

In the previous study, the concentration-dependence of coagulation on endotoxin became evident that mixtures of HL:plasma of *A. marginata* exposed to varied endotoxin concentration from 0 to 5.0 EU/ml resulted in increasing and linear protein coagulation response from 0 to 1.0 EU/ml beyond which there was a sharp decline and a flattened out response. This may be as a result of a 'prozone-like effect' whereby the binding of a ligand (antigen) to its receptor (antibody) is maximal only at optimal receptor-ligand ratios (http://www.ehow.com/facts_7194064_prozone-effect-definition.html). This suggests that the proposed lectin-binding process can be sustained only at endotoxin levels of up to 0.2 ng endotoxin/ml (1 EU/ml). Higher doses tend to overwhelm the lectin-mediated response and the organism may be in danger and may have to resort to other protective means. This finding however shows a linear relationship up to 1 EU/ml. This may find application in the determination of endotoxin in solutions having less than 1.0 EU/ml. In Large Volume

Parenterals –LVPs the permissible limit of endotoxin is 0.5 EU/ml [20, 21].

In this study, the exposure of the plasma alone to varied concentration of endotoxin from 0-5 EU/ml resulting in a linear relationship, was also concentration-dependent (Figure 6). The ability of the plasma alone to clot in the presence of endotoxin is not in agreement with the type of endotoxin-induced coagulation in *Tachyplesus tridentatus* [2] which requires factors from the hemocytes and the plasma for clots to form. It suggests that some proteins in the *A. marginata* plasma are capable of binding to endotoxin and possibly coagulating out of the plasma.

The reduction in the endotoxin-induced protein coagulation in the plasma:HL mixtures of *Archachatina marginata* hemolymph by the trypsin inhibitor, indicates that the reaction is serine protease-mediated (Figure 7). This observation agrees with similar reactions reported in several other invertebrates, especially in the crustaceans. Serine protease mediated reaction characterise endotoxin-induced coagulation and pro-phenoloxidase activation by peptidoglycans in many invertebrates including *Tachyplesus tridentatus* and *Limulus polyphemus* [2].

Table 1 indicates that the lectin activities of whole haemolymph, whole plasma and plasma coagulate; the HL and the HL coagulate, without exposure to endotoxin agglutinates fixed rabbit erythrocytes to a titre of 2^{13} . Same for the whole plasma and the HLD. Their endotoxin-induced coagulates resulted in lower titres ranging from 2^2 to 2^9 . The loss in activity could be attributable to the binding of the lectins to endotoxin thereby leading to the coagulation of the proteins. This finding suggests that some of the proteins in the coagulates were likely to be lectins since they have agglutination titres which became affected by exposure to endotoxin (Table 1).

The study also reveals part of the plasma proteins which interact with endotoxin. By exposing it directly to endotoxin and also through endotoxin-sensitized rabbit erythrocyte as evidenced in this study, the presence of lipopolysaccharide binding proteins (LBP) (Fig. 1 to Fig. 4) was revealed. These Figures indicated that some high molecular weight protein bands (> 250 KDa) were reduced as a result of endotoxin exposure while some lower molecular weight bands were enhanced apparently as a result of the degradation of the high molecular weight bands (Fig. 1 to Fig. 3). The extraction of LBP from the plasma using LPS-agarose was done successfully (Fig. 4 and Fig. 5) and the eluate from the affinity column (LBP) had an agglutination titre 23 folds higher than that of the plasma from which it was extracted. This further supports the speculation of the presence of Lpopolysaccharide binding proteins

(LBP) in the plasma.

The nature of the agglutinins present in the plasma was further investigated by carrying out inhibition of agglutination with various sugars; Man, Man-6-P, Glc, GlcNAc, GalNAc, sialic acid, dextran sulphate, fucoidan, D- and L- Ara, D- and L- Fuc and endotoxin (Table 2). The evidence from the present study indicates that there are several types of lectins (agglutinins) present in *A. marginata* haemolymphs which might include: Mannose Binding Lectins (MBL) have been reported to be able to bind Man, Glc and Fuc and related sugars; P-type proteins recognise phosphate-linked sugars like Man 6-phosphate, which may account for a possible recognition of the *E. coli* LPS by the lectins because of the presence of two GlcN-P residues on the lipopolysaccharide structure; -

S-Type Lectins recognise Sulphate groups containing sugars and binds to them and Sialic acid binding Lectins [22]. This is probably present as the agglutination was inhibited by Dextran Sulphate.

The information from the extraction of the plasma with LPS-agarose and subjection to SDS-PAGE (Fig. 5 and Fig. 6) followed by agglutination reactions and proteomic analysis indicates the presence of haemocyanin, mol wt 389-392 kDa. (data not shown); Gram negative bacteria binding protein, mol wt 50 kDa and an 'allergen', a 69 kDa mol wt. It is therefore evident that agglutinin (lectin)-mediated coagulation is a possible defence response of *Achachatina marginata* to exposure to endotoxin.

Table 2. Sugar Inhibition of Agglutination of Rabbit Red Blood Cells By *A. marginata* Plasma

Sugar	Effect	Minimum inhibitory concentration mM
Man-6-phosphate	+	1.16
Man	+	3.33
Glc	+	1.67
GlcNAc	+	1.36
GalNAc	+	1.36
Fucoidan	-	NA
Dextran sulphate	+	0.97
NANA (sialic acid)	+	1.62
L (-) Lac	-	NA
L (-) Fuc	+	3.66
D (+)Fuc	+	3.66
L (-) Ara	+	4.0
D (+) Ara	-	NA

Keys: + Inhibition , - No inhibition, NA means not applicable.
Values are mean of 3 determinations

Table 3. Agglutination titre of the eluted Lipopolysaccharide Binding Protein

Sample	Agglutination titre	Protein content (mg/ml)	Specific activity
Plasma	2 ⁴	45.0	0.36
Flow through	2 ²	28.38	0.14
LPS-agarose extract	2 ²	0.17	23.52

CONCLUSION

The evidence from this study suggests that the endotoxin-induced protein coagulation in the haemolymph fractions of *Archachatina marginata* is mediated by plasma agglutinins, serine proteases and Ca^{2+} -dependent. The consistent effect of exposure of plasma to endotoxin affected the >250 Kdal band corresponding to haemocyanins; and subsequent extraction with Agarose-LPS caused this band to be extracted and eluted from the column suggests that the molluscan hemocyanin might be involved in the endotoxin-induced coagulation by yet to be known mechanism. The Lipopolysaccharide Binding Protein(s) extracted contain agglutinins.

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DECLARATION OF INTEREST

The authors declare that they have no conflict of interest.

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