New Lipopolysaccharide Binding Proteins from the Jellyfishes

Aurelia Aurita and Rhopilema Asamushi of Sea of Japan

G.A. Naberezhnykh*

Introduction

Invertebrates, in contrast of vertebrate animals, have no acquired immunity, therefore for protection against intussusceptions of foreign agents they use innate immunity system\(^1\). The part of ancient protective system forming the basis of non-specific immunity of invertebrates, has served as the precursor of the complicated immunity of vertebrate animals\(^2\). The antimicrobial function of the innate immunity is realized by using specific “recognition” proteins known as pattern recognition proteins (PRPs) that bind to specific molecules on the surface of pathogens also been called as pathogen-associated molecular patterns (PAMPs) to activate the entire immune system\(^3\). In Gram-negative bacteria, one of the PAMP compounds is the lipopolysaccharide (LPS). A LPS molecule has an amphiphilic nature and consists of three parts: lipid A, core oligosaccharide, and the O-polysaccharide chain. Proteins that “recognize” LPS are called LPS-binding proteins (LBP) which are capable interact with both lipid and the carbohydrate moieties of the LPS molecule\(^4,5\).

Currently known LPS-binding proteins were found in species that inhabit in the Southeast Asia tropical seas\(^5\). Such proteins have not been previously sought in invertebrates of the northern seas. Now LBP of rather small number of invertebrates species are studied, mainly on the crustaceans and mollusca of tropical seas. Basically, they are proteins with molecular weights of 40 to 175 kDa. It is determined that LBP are synthesized in various tissues of invertebrates, but mostly found in blood cells\(^8\). In experiences of pathogenic bacteria infection on crustaceans with have shown that LPS-binding proteins are inducible with expression increases greatly within 8-12 hours after infects animals with Gram-negative bacteria or LPS administration\(^9\). In invertebrates, binding of these proteins with LPS promote protective mechanisms such as haemolymph coagulation\(^10\) and activation of phenoloxidase system\(^11\).

Study of the proteins that play an important role in the protection against bacterial infections will contribute to a better understanding of the innate immune system function in invertebrates and thus would help to develop a new generation of antimicrobial preparations. The need for such preparations

Abstract

The commercial species of jellyfishes of Sea of Japan were studied as the sources of proteins rich in lipopolysaccharides (LPSs) of Gram-negative bacteria. LPS-binding proteins have been found in lysates of jelly fishes mesogloea by means of immunofluorescence assay using LPS labeled with fluorescein (F-LPS). It was shown that lysate from rhopilema demonstrate the major LPS-binding activity in comparison with extracts from aurelia. For isolation and purifying of LPS-binding proteins from lysate of jellyfishes, the cation-exchange chromatography was used. It was shown that fractions of jelly fishes lysate after cation-exchange chromatography contained several LPS-binding proteins with high-molecular weights. By ligand enzyme solid phase assay it was shown that isolated LBP of jellyfishes was bound to LPS directly by concentration-dependent manner in saturation process.

Keywords: Marine invertebrates; Innate immunity; Scyphozoan jellies; Lipopolysaccharides; Lipopolysaccharide binding proteins

Received date: February 02, 2017
Accepted date: March 30, 2017
Published date: April 06, 2017


DOI: 10.15436/2381-0750.17.1334

Copyrights: © 2017 Naberezhnykh, G.A. This is an Open access article distributed under the terms of Creative Commons Attribution 4.0 International License.
in invertebrates is currently important because of the development of mariculture. Moreover, LPS-binding proteins are of interest as potential agents for the treatment of endotoxemia and Gram-negative sepsis in humans[10].

From jellyfish Aurelia aurita, the cationic peptide possessing antimicrobial and membrane-bound properties potentially capable to interlink with LPS was isolated and described[11]. Recently we discovered variety of invertebrates of Sea of Okhotsk can be considered as new sources of high-molecular LBP[12].

The purpose of the present work is to study the presence of LPS-binding proteins in the jellyfishes Rhopilema asamushi (rhopilema) and Aurelia aurita (aurelia) in the Sea of Japan. Undertaken study will allow identifying of perspective jellyfishes species for isolation of these proteins for further study.

Materials & Methods

Raw material

Jellyfishes were collected in Ussuriisk bay of Sea of Japan in the autumn season (from September to October) and stored in refrigerator at 18°C. Lysates from R. asamushi and A. aurita were obtained by 3-fold freeze-thawing process. Insoluble fraction was removed by centrifugation and supernatant fluid concentrated by ultrafiltration on the membrane with exception limit of 3 kDa (Millipore, USA) followed by dialyzed against PBS. Concentration of the total protein in the lysates of jellyfishes was determined by the Bradford method[13].

Preparation of fluorescein and biotin labeled LPS[13]

A total of 30 mg of LPS E. coli 055 B5 (Fluka, Germany) were dissolved in 1 ml of sterile deionize water. For disaggregation of LPS, 2 µL of triethylenamine was added and solution was treated on ultrasonic bath for 10 min. For introduction of amino groups in polysaccharide part of LPS, 20 mg of cyanogen bromide in 50 µL of acetonitrile was added. The mixture was incubated for 10 minutes and ethylenediamine (30 mg in 0.02 M phosphate buffer at pH of 6.8), collecting fractions by 1 mL. Absorbance of fractions at 280 nm was determined and checked on LPS-binding activity.

LBP of jellyfishes after ion-exchange chromatography was eluted with linear gradient of 2 M NaCl in phosphate buffer (pH 6.8), collecting fractions by 1 mL. Absorbance of fractions at 280 nm was determined and checked on LPS-binding activity.

Detection of LPS-binding proteins by agglutination LPS - latex

Amino-LPS (5 mg.mL⁻¹ in deionize water) was bound with 200 µL activated polystyrene latex preliminarily flushed with 0.2 M NaHCO₃ pH 9.5. Latex was washed off with PBS-T from the not bounded LPS and suspended in 10 ml of PBS-T. Lysates of jellyfishes were 2-fold titrated in round bottomed dishes and 50 µL of LPS-latex was added. Results were registered by forming of “umbrella” – positive or “points” – negative results. The maximum dilution of extract with positive result was considered as extract titer.

Detection of LBP by the ligand-enzyme solid-phase assay

A total of 100 µL pooled fractions sample after cation-exchange chromatography (concentration of the total protein 20 µg.mL⁻¹) or polymyxin B in same concentration was placed into the wells of polystyrene plate PolySorp (Nunc, USA) and incubated for 16 hours at 37°C. Non-specific binding sites were blocked with PBS-T, containing 1% BSA. The plates filled with 100 µL of PBS-T were used as blank, and those with 100 µL PBS-T containing 100 µg.mL⁻¹ BSA were used as negative control. The plates were washed with PBS-T and wells were filled with 100 µL of B-LPS solution in different concentrations (0.08 – 2.0 mg.mL⁻¹) and incubated for 4 hours at 37°C. After incubation, the plates were washed with PBS-T, the conjugate of horse radish peroxidase and streptavidin in dilution of 1:1000 was added into the wells followed by incubated for 1 hour at 37°C. The amount of the bound B-LPS was determined by measuring the optical density of solution after addition of substrate to enzyme. O-phenylenediamine was used as a chromogen in the substrate mixture. The absorbance of reaction mixture was determined with µQuant Bio-TEK Instruments spectrophotometer (INC, USA) at a wavelength of 492 nm.

FPLC ion-exchange chromatography of jellyfishes extracts

Chromatography was carried out on FPLC-chromatography (Amersham Pharmacia Biotech, Sweden) on cation exchange column Sourse 15S. Sample of 5 mL of jellyfishes lysates were applied, not bounded protein was washed off with 0.02 M phosphate buffer at pH of 6.8, cationic proteins were eluted with linear gradient of 2 M NaCl in phosphate buffer (pH 6.8), collecting fractions by 1 mL. Absorbance of fractions at 280 nm was determined and checked on LPS-binding activity by means of F-LPS. The active fractions were combined and concentrated by ultrafiltration on the membrane with exception limit of 3 kDa (Millipore, USA) and dialyzed against PBS.

Polyacrylamide Gel Electrophoresis of Proteins Followed by Electrotransfer to Nitrocellulose Membrane

LBP of jellyfishes after ion-exchange chromatography were separated by SDS-PAGE-electrophoresis on 12 % PAAG.
according to the method of Laemmli[17]. Samples were prepared according to two methods: without warming-up at 100°C and prior heating of the sample (5 min at 100°C) in a buffer containing 2 % sodium dodecyl sulfate (SDS) and β-mercaptoethanol. The set of colored proteins (Fermentas, Lithuania) with molecular weights of 11, 17, 24, 33, 40, 55, 72, 100 and 130 kDa were used as markers. Proteins separated in gel were stained with solution of Coomassie R-250 in 10% acetic acid and 30% methanol.

For the Western blotting, proteins after electrophoresis were transferred on nitrocellulose (0.2 μm, Sartorius, Germany) followed standard procedure[18]. To block the nonspecific binding sites, membrane was treated with PBS containing 0.25% Tween-20 and 1% BSA. Detection of LPS-binding proteins on blots was performed using the F-LPS as described above the DOT analysis. Fluorescence of F-LPS-protein of complexes was registered by Versa Doc imaging system (Bio-Rad, USA).

Results and Discussion

Lysates from R. asamushi and A. aurita were obtained by 3-fold freeze-thawing. By means of the DOT analysis using F-LPS, LBP have been found in extracts of both species of jellyfishes (Figure 1).

Figure 1: Quantitative DOT analysis of binding of F-LPS, (A) with lysates of R. asamushi, (B) with lysates of A. aurita with 1 – 6 serial twofold dilutions of the samples. Concentration of the total protein lysates 2 mgmL⁻¹

It should be noted that extracts from rhopilema, in comparison with extracts from aurelia, showed the major LPS-binding activity that has been determined by intensity of fluorescence of complexes F-LPS-protein in the DOT analysis.

Besides, LPS-binding activity of jellyfishes extracts was determined by means of agglutination of the latex loaded with LPS. It was shown that titers of rhopilema extracts were 1:8 which was more than of aurelia - 1:4.
Lipopolysaccharide Binding Proteins from the Jellyfishes

The active fractions were combined and concentrated by ultrafiltration on the membrane with exception limit of 3 kDa. Activity in these filtrates was not found that indicated lack of LPS-binding activity at low molecular mass peptides.

Proteins pooled fractions after cation-exchange chromatography of lysates in these species were separated by SDS-PAGE electrophoresis and LBP were detected by Western blotting after treatment by F-LPS. The explored fractions were differ against each other by both amount, and molecular masses of proteins (Figure 4A). From the electrophoregrams of aurelia, several bands slightly colored by Coomassie in gel top were observed (Figure 4A, track 1). Western blotting of this sample detected two intensive (100 and 120 kDa) and two weak bands (55 and 130 kDa) of the proteins associated with a fluorescently labeled LPS (Figure 4B, tracks 1,2). Heating of the sample at 100°C in 2 % SDS, LPS-binding proteins were manifested in the not form of new polypeptides (Figure 4B, tracks 1,2).

From the electrophoregram and Western blotting of rhopilema proteins we observed a different pattern of polypeptide distribution by size and activity. At the non-denaturing conditions of Western blotting, predominant polypeptides with molecular weights of 130,120 and 110 kDa and a minor band at 55 kDa demonstrated LPS-binding activity (Figure 4B, track 3) were observed. After heating to 100°C, LPS-binding proteins appeared as the predominant polypeptide as dominant band at 110 kDa and a minor band at 45 kDa (Figure 1A, track 4). Changes of the profiles of LPS binding rhopilema proteins in Western blot analysis after boiling in 2% SDS lysates can be a result of either dissociation or denaturation of proteins. The small difference in the apparent molecular weights of LBP before and after boiling supports their possible denaturation.

According to the literature data, marine invertebrates contain different LPS-binding proteins of high and low molecular weights, as oligomers and monomers. Thus, a protein with a molecular weight of 420 kDa that was capable of binding LPS was detected in the haemolymph of the shrimp *Penaeus japonicus*; during SDS-PAGE electrophoresis under denaturing conditions the protein dissociated to a monomer with a molecular weight of 32 kDa[19]. However, Luo et al. isolated LPS-binding protein with a low molecular weight of 18 kDa from *Penaeus monodon* and found that the weight did not change under denaturing electrophoresis conditions[20].

We used a ligand enzyme solid phase assay to study the interaction of LPS with proteins after cation-exchange chromatography. LBP of jellyfishes after chromatography with the same total protein concentrations were adsorbed on the surface of a polystyrol plate and titrated with biotin labeled LPS. A streptavidin–horseradish peroxidase conjugate was used to determine the resulting complexes. As it appears from figure 5, binding of proteins with LPS has a specific character, as saturation of binding sites on the proteins can be reached by the ligand. At the saturation point, the amount of LPS that was bound by the proteins was significantly smaller than that of polymyxin B (Figure 5, line 1). The difference in the binding activity of the proteins and polymyxin B might be due to a high constant of binding of polymyxin with LPS. This was in agreement with Vaara (1992) highlighted that PmB binding point can reaches to 2.5 x 10^7 M[^21].

Figure 3: LPS-binding activity of fractions after cation-exchange chromatography of aurelia (A) and rhopilema (B) lysates.

Figure 4: Electrophoregram of jellyfishes proteins after cation-exchange chromatography. (A) - electrophoresis in PAAG; (B) - Western blot analysis of samples identified with fluorescein-labeled LPS. 1, 3 - samples were prepared without warming-up at 100°C; 2, 4 - prior heating of the sample at 100°C in a buffer containing 2 % sodium dodecyl sulfate (SDS) and β-mercaptoethanol 1, 2, A. aurita. 3, 4- R. asamushi. Left - molecular weights of proteins marker.

Figure 5: Binding of biotin-labeled LPS with immobilized on polystyrene plates: (1) polymyxin B, (1), proteins of rhopilema (2) and aurelia (3). Concentration of the total protein lysates and polymyxin B 20 µg.mL^-1.
Lipopolysaccharide Binding Proteins from the Jellyfishes

Conclusion

The LPS-binding proteins are present in lysates of two jellyfishes tissues; the rhopilema in comparison with aurelia showed the major LPS-binding activity that has been determined by intensity of fluorescence of complexes F-LPS-protein in the DOT analysis. High-molecular weights LBP specifically binding with LPS was isolated by cation-exchange chromatography. These species of invertebrates are widely available and are of interest as a new source of LPS-binding proteins which are potential antimicrobial agents. In addition, we developed a highly sensitive DOT analysis for LPS-binding proteins determination in the extracts of invertebrates using fluorescently labeled LPS. This is a fairly quick method that does not require any sophisticated equipment and applicable in field conditions.

Acknowledgments: The work completed at partial financial support of Russian Science Foundation (RSF) and Far Eastern federal university (FEFU) grant «Time-lapse technology and rational use of marine biological resources» № 14-50-00034 at the Direction № 3 «Development of innovative pharmaceutical drugs and functional foodstuff».

References