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Original Article

Biological Transmission of Bacteria Inhibit By Hemolymph Lectins of American Cockroach

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Abstract

Background: Although cockroaches are mechanical vector of many pathogens, still biological transmission of pathogens via these insects is controversial. More covenant immune defense in the hemolymph of cockroaches may take this advantage to block any adaptation of pathogens regarding to biological transmission. Lectin molecules in hemolymph of insects play as mediators of nonself recognition, in the innate immune response.

Methods: Initially, lectin-carbohydrate interactions in hemolymph of American cockroach, *Periplaneta, americana,* have been investigated and then as a model, non pathogenic soil bacterium, *Streptomyces griseus* was used to specify the cockroach hemolymph reaction.

Results: The results showed that the hemolymph lectin activities were inhibited with GlcNAc, galactose, mannose, arabinose, and fucose but not with fructose, lactose and glucose. Also, *S. griseus* cells were agglutinated with the cockroach hemolymph while in the presence of fructose, mannose and galactose, the agglutinin activities were inhibited. Introducing the soil bacterium, *S. griseus*, into the hemolymph increased both the lectin activities as well as the number of hemocytes from an average 101 to 2688 cells per milliliter hemolymph 6 days after injection. At the same time, the number of prohemocytes in the hemolymph declined while the granular cell numbers rapidly increased.

Conclusion: Generally injecting *S. griseus* into the cockroach body resulted in a positive correlation between an increase in hemolymph titers and enhancement of hemocyte numbers. The results tentatively support the idea that soluble and/or cell-associated lectins may be involved in immuno-recognition in insects and block biological transmission of pathogens.

Keywords: Lectins, *Hemolymph*, *Periplaneta americana*, *Streptomyces griseus*

Introduction

Unlike vertebrates, insects lack specific acquired nonself recognition systems such as anti-body, therefore, lectins molecules act as mediators of nonself recognition, in the innate immune response (1-2) Generally, in invertebrates, lectins may play important roles in several physiological progresses. These molecules are probably involved in various stages of the immune defense reactions, such as wound repair, phagocytosis, hemolymph coagulation, encapsulation, clearance of non-self substances from circulation and killing of bacteria and parasites (3-4). These molecules are mainly produced by immunocytes and by the fat body (4-5). Both proPO-dependent and proPO-independent mechanisms are involved in producing the lectin-induced level of phagocytosis in the hemolymph (6-7). In cockroaches, although antibacterial peptides are apparently absent (7), the endogenous lectins are also cable of enhancing the activation of the prophenoloxidase system, a melanization cascade that is important component of the insect immune defense reactions (8-9). In cockroaches both endogenous and exogenous lectins are capable of activating the proPO system (9). In the American cockroach, *Periplaneta americana*, two lectins, LPS-binding protein and the *Periplaneta* lectins have been purified and characterized from the hemolymph (5-10). These are lectins involved in the clearance

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of *Escherichia coli* from the hemolymph and are Ca^{2+} depended. In addition, in the hemolymph of the Indian cockroach, *Blaberus discoidalis,* four lectins have been recognized and purified, namely BDL1, BDL2, BDL3 and GSL (9). Also, it has been shown that multiple endogenous serum lectins involved in recognition and killing of microorganism (7).

The aim of present study was to show the presence of agglutinin activity in hemolymph of *P. americana* and interaction between the hemolymph lectins and a bacterium, *Streptomyces griseus,* in vitro.

Materials and Methods

Insect rearing and bleeding American cockroaches, *P. americana*, were maintained in an insectary at 25±2° C with a 12 h light/dark ratio, and fed on dried bread with water. Adults and final instar cockroaches of both sexes were used for bleeding and were anaesthetized with CO_2. The ventral surface of sternum of each cockroach was sterilized with 70% ethanol, and then pierced via the coax membrane using a needle. The hemolymph was collected with a hematocrit capillary tube from 10 adult insects and transferred into a sterile Eppendorf tube. The collected hemolymph was homogenized by freezing and thawing and then centrifuged at 4000 rpm. The supernatants were kept in -20° C until use. Subsequently, all samples were assayed for protein by the Bradford method (11) before use.

Bacteria A soil non pathogen bacterium, *Streptomyces griseus*, was used in order to trigger the immune system of the cockroaches. The bacteria were cultured in Sabouraud dextrose agar pH 6.9 at room temperature for 48 h. Then, the bacteria were harvested and suspended in 10mM PBS (containing 150mM NaCl and 10mM sodium phosphate at pH 7.4 and 380 mOsm). The number of suspended bacteria was compared with Mac Farland standards methods to achieve a concentration of 1×10^8 bacterium per ml.

Haemagglutination assay

In order to exam haemagglutination activities in the hemolymph of the American cockroach, *P. americana*, rabbit and human RBCs (O, A, B, AB groups inclusive Rh^+ and Rh^-) were used. All RBCs were washed three times in 10mM PBS (as above, but with 1mM $CaCl_2$ added) and the concentration of the RBCs suspensions adjusted to 5% in PBS. Prior to use in haemagglutination assay, the RBCs were further diluted with PBS to give a final concentration of 2%. Serial dilutions of the insect hemolymph, each 2µl in volume, were prepared with PBS in microtiter plates to give final dilution ranges of 2^{-1} to 2^{-10} prior to the addition of 2µl RBCs to each well. The plates were covered and kept at room temperature for two hours. The end points of agglutination were examined under a stereomicroscope and by the flow characteristics of the erythrocyte pellets when the plate was held at an angle. All experiments were replicated three times and the controls always included PBS plus RBCs alone.

Carbohydrate inhibition assays The sugar specificities of cockroach lectins were investigated by competitive binding using the following carbohydrates: D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, L-(-)-fucose, lactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and arabinose (all purchased from Sigma Co.). Stock solutions of sugars were prepared in PBS at 0.2 M and stored in -4 °C until use. Two folded serial dilutions of hemolymph (each of 2.5 µl) were prepared in PBS followed by addition of 2.5 µl of appropriate carbohydrate at the above initial concentration. The plates were incubated at room temperature for 120 min and then 2.5 µl of RBCs (rabbit blood cells) were added to the respective wells. The controls consisted of hemolymph or carbohydrate plus RBSs, and PBS plus RBCs alone. Inhibitory effects were recorded as those reducing agglutination in the wells. The end points of agglutination were examined under a stereomicroscope and by the flow characteristics of the erythrocyte pellets when the plate was held at an angle. This experiment was replicated three times.

Bacteria agglutination and inhibition assays Serial dilutions of the hemolymph, each 2µl in

volume, were prepared with PBS in microtiter plates to give final dilution ranges of 2^{-1} to 2^{-10}. Then, 2µl of the bacteria suspension, *Streptomyces griseus,* at a concentration 1×10^8 cells/ml, was added to each well and incubated at room temperature for 2 h. The agglutination titer was defined as the reciprocal of the highest dilution showing visual agglutination of the bacteria under a microscope. This experiment was repeated three times and the controls always included PBS plus BSA alone.

In order to find specificity of lectin binding carbohydrates, the sugar specificities of cockroach lectins were also investigated by competitive binding using the following carbohydrates: D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, L-(-)-fucose, lactose, fructose, N-acetyl-D-glucosamine, arabinose, and fructose (all from Sigma). Stock solutions of sugars were prepared in PBS at 0.2 M and stored in -4 °C until use. Serial two fold dilutions of 2µl aliquots of inhibitor were added in microtitre wells, followed by 2µl of hemolymph lectin adjusted to a titer of 1: 1024. These were mixed by incubated for 1 h at room temperature. Two microplates of 2% bacteria suspension was added to each well and the minimum concentration of inhibitors required to block agglutination was determined after 1h.

In vitro Bacteria assay The bacteria were washed with PBS twice and then injected (in what volume) into hemolymph of the cockroaches at a concentration of 10^8cells/ml. The injected insects were kept in an insectarium until bleeding. The hemolymph was collected daily from 10 cockroaches up to the 10^{th} day. The lectin activities of each group were then measured and the numbers and types of hemocytes were counted using a hemocytometer. The controls were hemolymph free bacteria.

Results

Haemagglutination assay The haemagglutination patterns of whole serum collected from cockroaches free of bacteria against a range of erythrocytes were determined (Fig. 1). The highest activities occurred against rabbit erythrocytes (titer≤ 128) followed by human AB (titer≤ 64). The lowest activity was observed against human O^- erythrocytes (titer≤ 16). Thus, rabbit erythrocytes were used for inhibition assays.

Carbohydrate inhibition assay As shown in Fig. 2, the haemagglutination inhibition of sugars for whole serum was dissimilar. The hemolymph lectin activity was inhibited with mannose, arabinose, galactose, fucose and N-acetyl-D-galactosamine but not by fructose, lactose and glucose.

Bacteria agglutination and inhibition assay: The hemolymph lectins agglutinated bacteria cells completely at titer 1064 but lactose, glucose and fucose reduced agglutinin activity slightly (Table 1). The highest inhibition reduction was occurred in the presence of fructose, galactose and mannose at titer of ≤64. No activity was observed in both controls.

In vitro bacteria assay The number of hemocytes in injected cockroaches rapidly increased and from an average of 101 cells reached to maximum 2688 cells/ml after 6 days and then renamed constant until the end of the experiment (Fig. 3). The immune system of the control group was also trigged by injecting sterile PBS and the number of hemocytes rapidly increased but consequently declined after 5 days and finally returned back to normal by day 10^{th}. However, the presence of *S. griseus* in the hemolymph reduced the number of prohemocytes from approximately 55 to 15 cells per ml. In contrast, the number of granular cells increased about 15 times. The normal number of granular cells, and plasmotocyte were about 60 to 115 cells per ml before bacteria injection, but they totally reached to maximum 1950 cells/ml approximately after injection. The lectin activity of whole hemolymph was also amplified after bacteria injection. A positive correlation between the lectin activity and the increasing number of hemocytes was observed (Fig. 4).

Table 1: Effects of inhibitory sugars on agglutinin activities of *Periplaneta americana* hemolymph treated with bacteria *Streptomyces griseus*

Carbohydrates / **Proteins**	**Arb**	**Frc**	**Lac**	**Glu**	**Gal**	**Man**	**Fuc**	**GlcNAc**	**Hemolymph free of sugars**
Hemolymph incubated with the bacteriam	7 (128)	6 (64)	9 (512)	9 (512)	6 (64)	6 (64)	9 (512)	7 (128)	10 (1024)
BSA control	0	0	0	0	0	0	0	0	0
PBS free hemolymph	0	0	0	0	0	0	0	0	0

The number represents the number of wells that bacteria agglutination was seen and the numbers in parentheses show end-point titers expressed as the reciprocals of the dilutions (1/n). All samples were prepared three times and the assay repeated each time with 2 replicates. Arb(arabinose), Frc (fructose) Lac (lactose), Glu (glucose), Gal (galactose), Man (mannose), Fuc (fucose), GlaNAc (N-acetyl-D-glucosamine

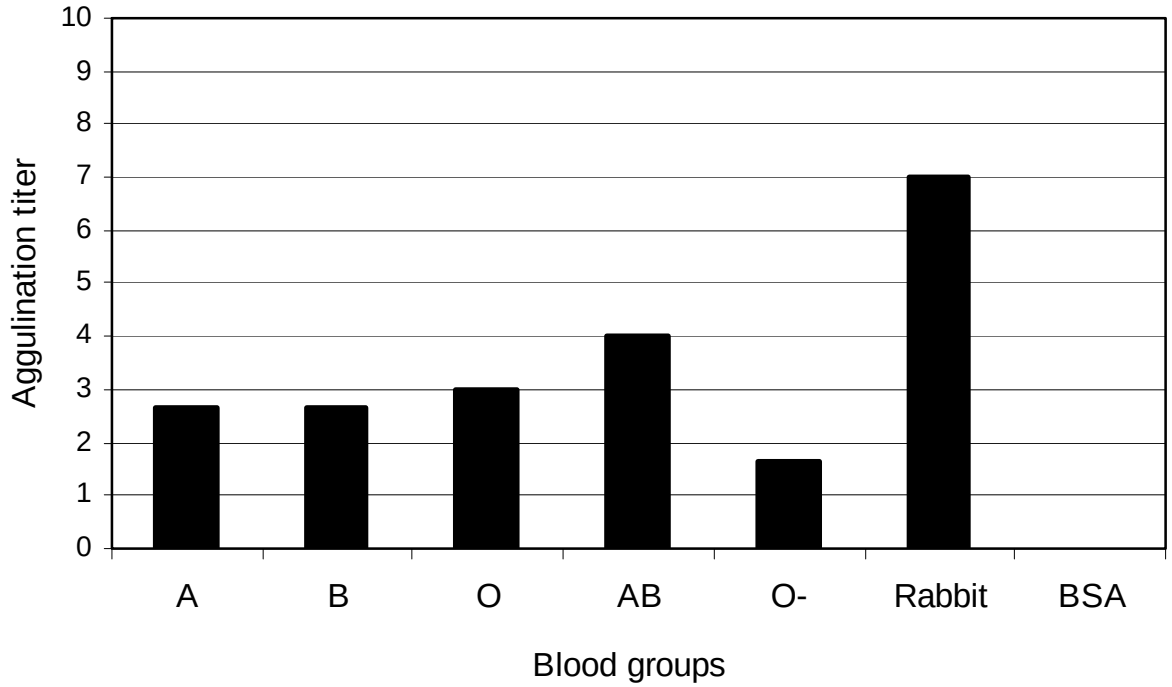

Fig. 1: Titer of agglutination activities in hemolymph of the American cockroach, *Perpelaneta americana*, against different red blood cells. All experiments were replicated three times and the controls always included PBS plus RBCs alone. BAS used as a control.

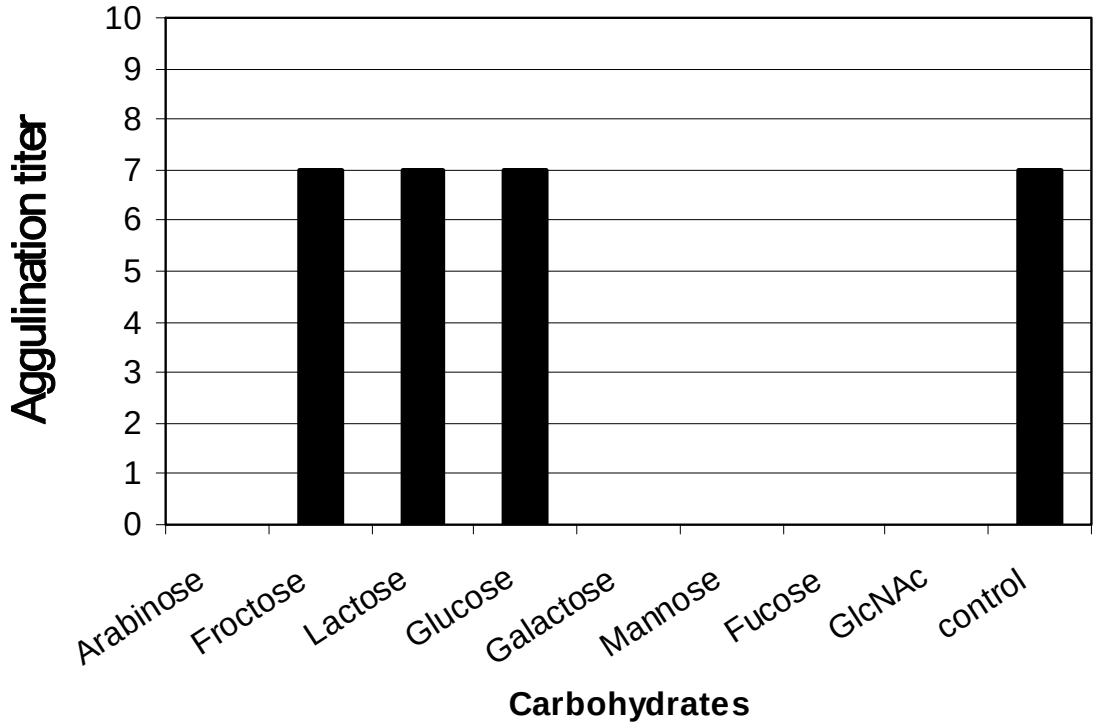

Fig. 2: Inhibition effects of various sugars on hemolymph lectin(s) activity of the American cockroach, *Perpelaneta americana*. All experiments were replicated three times.

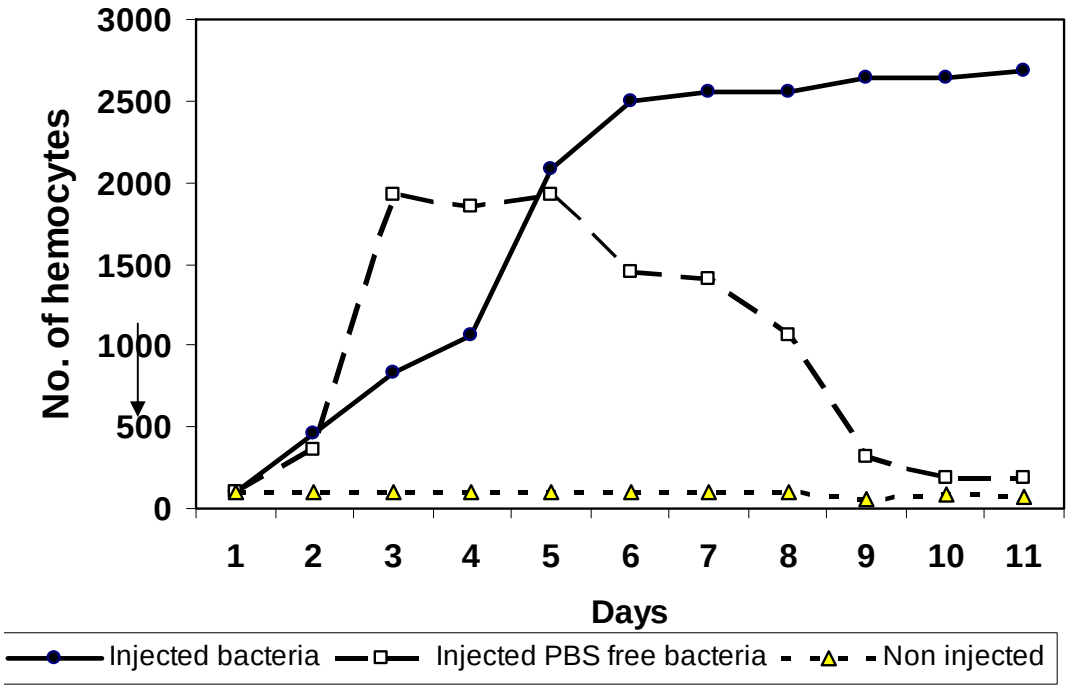

Fig. 3: Response of hemocytes of the American cockroach, *Perpelaneta americana* to the presence of bacterium *Streptomyces griscus*. The experiment was performed three times

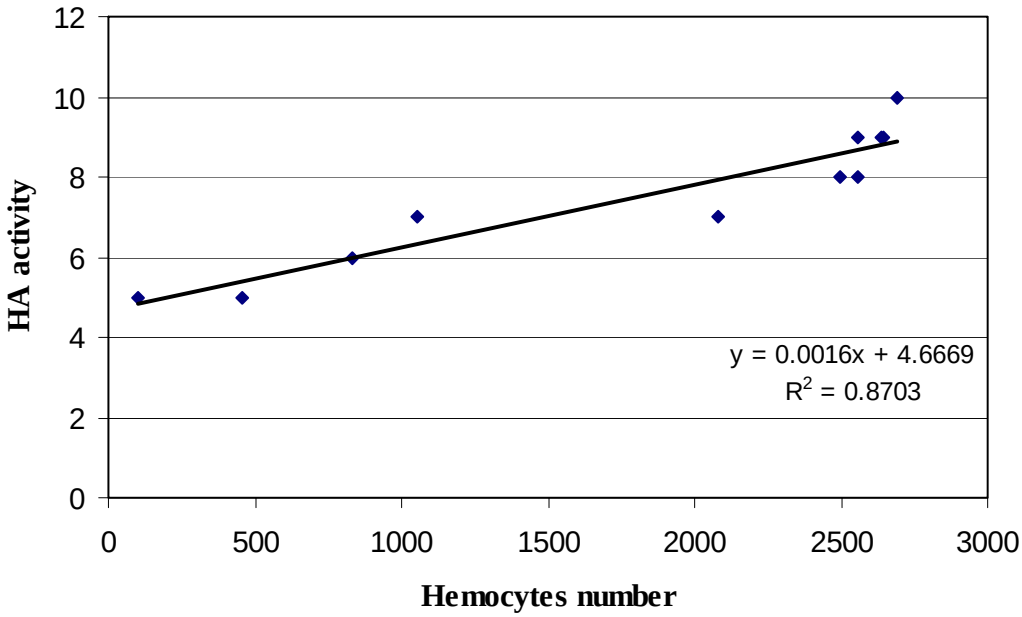

Fig. 4: Correlation between heamaglutinin activity of hemolymph and the number of hemocytes in *Periplaneta americana* . The bacteria, *Streptomyces griseus* triggered the immune system of cockroach and the lectin activity dependently was amplified while the number of hemocytes increased

Discussion

Unlike vertebrates, insects lack the Ab-based nonself recognition system; therefore, hemolymph lectins play an important role as mediators of innate immunity in these animals (2). The ability of a panel of lectins to potentiate the uptake of a variety of microorganisms is especially important in terms of invertebrate nonself recognition (12). Like mammals, in which hemolymph lectins activate the complement system and enhance phagocytosis as well as mediator of innate immunity (7). It has been also shown the multiple lectins in *Blaberus discoidalis* hemolymph are capable of enhancing nonself recognition and ingestion of foreign invaders by activating the proPO system and killing mechanisms such as those based on superoxide generation (13-14).

In this study, specificity of protein binding carbohydrates to the hemolymph lectins of the cockroaches were GlcNAc, galactose, mannose, arabinose, and fucose but not fructose, lactose and glucose. The galactose binding protein in hemolymph of *P. americana* have been previously studied in order to show the hemocytic cellular response (15). On the other hand, *S. griseus* cells were agglutinated with the cockroaches hemolymph while at the presence of fructose, mannose and galactose, the agglutinin activities inhibited. Thus, mannose and galactose binding proteins both were active against rabbit red blood and the bacterium cells. This results indicate that mannose and galactose binding lectins which are present in the cockroach hemolymph, be involved in immuno-recognition. We observed that entrance of a soil nonpathogenic bacterium such as *S. griseus* into the hemolymph of american cockroach, *P. americana* triggered the immune system of insect by increasing granulocyte and plasmotocyte cells as well as lectin activities. When the bacterium was introduced into the hemolymph of *P. americana,* the number of prohemocytes decreased which may indicated that these cells rapidly shift mostly to granulocytes or less plasmotocytes.

Generally, any response by hemocytes to an invasion of the hemocoel by an introducing organism necessitates that the cell distinguish the invader from the insect's own tissues and in this

scenario some molecules such as glycoproteins and mucoproteins may play as main receptors (2). The biological importance of multiple lectins in the hemolymph of insects is not well understood. However, diversification in physiochemical properties of hemolymph lectins may possibly reflect diversification in functions. In fact, in addition to the complex carbohydrate-protein interactions, there are possibly other binding sites, such as those that interact with the component of the prophenoloxidase cascade (7-9) which also may play as signal for trigger the rest of immune system in insects. However, we found a correlation between the number of hemocytes and agglutinin activities in hemolymph (Fig. 4). It is not clear that the ratio of lectin activities in hemolymph in what way can trigger hemocytes formation but we realized that by decreasing hemolymph lectin activities (after inducing the bacteria), prohemocytes shifted to granulocytes. Therefore, hemolymph lectins may play as signal for consequent reaction of immune system against foreign microorganism in insects. Similarity, in vertebrates, antibody triggers the rest of immune systems against microorganism invasion (16-17). In general, granulocytes may arise from prohemocytes and they release the granules which thought to be glycoproteins and mucopolysaccharides. The precise function of granulocytes is unproven, but some researchers have suggested that they serve storage and possibly secretory functions. They may be involved in cellular defensive functions in various insects and may be phagocytic in some insects (18).

Carboxylic residues in chitosanase in bacteria such as *Streptomyces* sp. would be expected to play an important role in interactions between bacteria and their host. A bacteria enzyme, chitinase has the highest affinity for the GlcNAc residue in which the binding free energy change of oligosaccharide binding is assumed to be equal to the sum of the values for the individual subsites occupied by the substrate sugar residues (19). Therefore, chitosanase of *Streptomyces* sp. participates in the binding of the sugar residue most probably through both electrostatic and hydrogen bonding interactions. However, different sugar specifics were present in Periplaneta hemolymph and purification, characterization and comparisons of them are essential for understanding their biological roles.

In conclusion, the results of this study give more details regarding to the role of immune system impacts on biological transmission and mechanism of the defense facing using hemocyctes and increasing agglutinin activities at the present of an unknown microorganisms.

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