Partial purification and characterization of lectin from serum of American cockroach, *Periplaneta americana*

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ABSTRACT

The partial purification and characterization of haemagglutinin (lectin) were carried out from the hemolymph of the adult American cockroach, *Periplaneta americana*. The hemolymph was drawn from cockroach and lectin was purified by a single-step method, using ammonium sulfate (NH₄)₂SO₄ salt fractionation and gel filtration. Gel filtration showed two peaks. The Hemagglutination Activity (HA) was observed in the 20th fraction of the second peak. The purified lectin showed a molecular weight of 26.8kDa on Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis. The purified lectin showed an increase in HA at pH 7.5 and, subsequently, a sharp decline at pH 8. This indicates that HA was specific to a certain pH level. Similarly, an increase in HA was observed until 30°C, followed by a decline at 40°C. This indicates the heat labile nature of lectin. The HA showed a higher specificity to divalent Ca²⁺ and showed no specificity for Ba²⁺. It also showed a higher inhibition for sugar D-galactose and a least inhibition for D-lactose. The HA to vertebrate blood group showed a highest activity to goat Red Blood Cells (RBCs). The study concludes that carbohydrate-binding-specific lectin is important for recognition of the cell surface carbohydrate of invading pathogens.

1. INTRODUCTION

Lectins have been purified from various insect species, such as fleshflies and cockroaches [1,2]. Some of them actively participate in self-defense by recognizing the polysaccharide chains on the surface of pathogens. Some insect lectins bind to microbial components such as lipopolysaccharide and activated prophenoloxidase (ProPO), and enhanced the cellular immune response by modulating hemocytes. The insect immune system activation mechanism was studied in *Drosophila melanogaster*, as a model organism [3,4]. *Drosophila* is estimated to have more than 30 C-type lectin genes [5,6]. However, very little is known about the participation of lectin in Drosophila immunity [7]. Undoubtedly, the presence of lectin in the hemolymph of insects appears to have an important function in insect immune defense system [8,9].

In invertebrates, including insects where they lack an antibody-based immunity, lectin probably plays a major role in nonself recognition, with several reports of endogenous serum lectin having opsonic activity, for invading pathogens [10,11]. Other than opsonization, lectin trigger ProPO activation pathway [12]. Arumugan, et al.[13] purified, characterized and identified humoral immune function on the induction of PO activity with the hemolymph lectin of *Periplaneta americana*. In this study, we examine in detail the hemolymph lectin from *P. americana* hemolymph, for its role in immune recognition and its properties.

2. MATERIALS AND METHODS

2.1. Insect Rearing

The cockroach, *P. americana* was collected from Godown’s and reared in the laboratory in a dark container. They were reared in room temperature 27 ± 1°C and 12:12 Total Light (LL): Total Dark (DD) cycle. All experiments were conducted at this room temperature unless specified.

2.2. Preparation of Hemolymph Sera

The insect hemolymph was collected from 40 cockroaches of the same sizes. Before collecting hemolymph, the individual cockroaches were first anesthetized by keeping them at low temperature for 20 minutes. Then, they were bled by severing

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hind legs with fine scissors, and hemolymph was collected in a sterile centrifuge tube containing phenyl thiourea crystals. Phenyl thiourea crystals were used to inhibit phenoloxidase activity. After 10 minutes, the tubes were centrifuged at 10,000 rpm at 4°C for 10 minutes to remove hemocytes. The supernatant was carefully removed and used as a serum for HA assay against goat’s Red Blood Cells (RBCs). The collected samples were tested immediately to prevent counterfeit negative results.

2.3. Isolation of Lectin: (Salting out of Serum Protein)
The lectin was isolated according to the procedure as described by Dorrah et al. [9].

2.4. Preparation of Erythrocyte Suspension and Assay of Hemagglutination Activity (HA)
Blood samples from goat and chicken were collected aseptically, each in an equal volume in heparin. Human blood (types A, B, and O) was collected in citrate/dextrose. The erythrocyte suspension and HA was determined by the procedure described in [14].

2.5. Effect of Divalent Cations
To study the effect of divalent cation for HA, the agglutination tests were performed as described by Richards et al. [15]. An aliquot of 100 μl of diluted serum was dialyzed for 24 hours at 4°C against 200 ml Tris Buffer Saline (TBS), pH 7, either without or with 20 mM Ca²⁺, 20 mM Ba²⁺, 20 mM Mg²⁺, 20 mM Zn²⁺, 20 mM Hg²⁺, or 10 mM Ethylene Di-amine Tetra Acetic acid (EDTA) with 2 changes of buffer. HA assays were carried out by using goat RBCs washed in dialysis buffer.

2.6. Inhibition Assay
The binding of sugars to serum lectin was studied by competitively binding and measuring HA in the presence of goats RBCs. The method of inhibition followed in this study was as described by Ayyad et al. [14]. The sugars assessed in this study are monosaccharides (D-galactose, D-glucose, and D-fructose) and oligosaccharides (D-lactose). The minimum concentration of sugars for causing 50% inhibition (IC50) of HA was studied.

2.7. Effect of Temperature and pH
For the heat stability test, an aliquot of the protein sample was incubated in a heating block at different temperatures (10°C, 20°C, 30°C, and 40°C) for 30 minutes. The solution was cooled on ice immediately and then allowed to warm up to 27°C at room temperature, and thereafter the HA was assayed. The percentage of HA was calculated by dividing the activity of the sample with the activity of the control at temperature 27°C Room Temperature (RT) multiplied by 100.

For pH test, the lectin in buffer solutions with different pH values, i.e., 6–6.5 pH (0.2 M Citrate buffer) and 7–8 pH (0.2 M Tris-HCl buffer), was used. Equal volumes of protein sample and buffer solution were mixed and incubated at room temperature for 30 minutes. The solution was neutralized and the HA assay was performed. The hemagglutination titer of the protein sample was incubated in Phosphate Buffer Saline (PBS) (pH 7), which served as control. The percentage of HA was calculated by dividing the activity of the sample with the activity of the control of pH 7.5 multiplied by 100.

2.8. Determination of Protein Concentration
The total protein concentration was determined according to the method of Lowry et al. [16], using bovine serum albumin (BSA) dissolved in 0.15 M NaCl was used as a standard.

2.9. SDS-Poly Acrylamide Gel Electrophoresis
The SDS-PAGE of the isolated lectin was carried out according to the procedure described by Laemmli [17]. The electrophoresis was performed at a constant voltage of 200 V for 90 minutes. The molecular weight of the protein bands was determined using standard medium-range molecular markers: 97.4KDa-Phosphorylase-b; 66KDa-BSA; 43 kDa ovalbumin, 29kDa carbonic anhydrase, 20.1kDa soyabean trypsin inhibitor, 14.2kDa lysozyme.

3. RESULTS AND DISCUSSION

3.1. The Physico-Chemical Properties of the Isolated Lectins
In this study, a single-step purification procedure was used to purify lectin. The chromatographic step using column Sephadex G-75 was used to remove most of the impurities and the purification fold of 2.38 was observed (Table 1). The gel filtration showed two peaks and the second peaks fraction 20 showed HA (Fig. 1). The obtained data in the present study revealed that HA showed a decrease at 40°C (Table 2). On the other hand, a high HA was observed at 30°C. At 10°C the HA showed a least activity. This observation indicates that lectin from cockroach are heat-labile in nature, and shows the highest activity at certain temperatures only. The

| Step       | Volume (ml) | Total Protein (mg/ml) | Hemagglutinating Activity (%) | Yield (%) | Purification (fold) |
|------------|-------------|-----------------------|------------------------------|-----------|--------------------|
| HLS        | 5.3         | 1023                  | 0.25                         | 100       | 1                  |
| Aspt       | 4.8         | 672                   | 0.2                          | 65.68     | 1.21               |
| Dialysis   | 4.0         | 486                   | 0.15                         | 47.50     | 1.26               |
| Gel Filtration (F-20) | 3.0         | 366                   | 0.0375                       | 35.77     | 2.38               |

Figure 1: The profile of gel filtration on Sephadex G 75 showing two peaks. Only the second peaks fraction 20 showed hemagglutinating Activity.
heat instability is a characteristic of lectins of some other insects, e.g., the orthopteran, *Teleogryllus commodus* [18], *Melanoplus sanguinipes*, [19] and the dipteran, *Glossina fuscipes* [20], in phasmid *Exatosoma tiaratum* [15]. However, in contrast to this, in *Leptinotarsa decemlineata* [21], and in *Locusta migratoria* [22], the lectins were reported to be heat resistant. Similarly, we observed HA at different pH ranges of 6–8 pH with the highest activity at pH 7.5 (Table 3). Similar results were observed in *Pterockdiella capillacea* (pH 7.5) [23], and *P. americana* (pH 7.5) [13]. Ayaad [24] reported an HA at pH 6.2 for *Aedes caspius*.

### 3.2. Divalent Cation Requirement

The study revealed that isolated lectins are calcium-ion-dependent. The cations like Mg$^{2+}$ or Zn$^{2+}$ appear to have limited ability to replace Ca$^{2+}$; however, Ba$^{2+}$ showed no effect on HA (Table 4). When EDTA was added, complete inhibition of HA was observed. Similar observations were reported on insects having C-type lectin, *L. commodus*, *M. sanguinipes*, *E. tiaratum*, and adult *Schistocerca gregaria* [15,18,19,25]. The mechanism of carbohydrate binding of C-type lectin is suggested by Zelensky and Gready [26]. According to Zelensky and Gready [26], each of the C-type lectins possesses a carbohydrate recognition domain structure with conserved Ca$^{2+}$ binding sites, and a second site [27]. The lectin of *S. gregaria* [25] is Ca$^{2+}$ dependent. However, Yu and Ma [28] suggested that calcium is not required for lectin binding to the sugar, but is necessary to protect it from proteinase digestion. Several workers have reported that the C-type lectin possesses a common carbohydrate recognition domain [29,30] and a second domain [31] that may help in increasing their binding affinity to carbohydrate. The suggested mechanism may involve interactions with the conserved Ca$^{2+}$ and carbohydrate, which may possibly result in the formation of hydrogen bonding with acid and amide side groups [32]. A similar mechanism is carried out in this study.

We observed that the HA of *P. americana* was strong in the presence of calcium, followed by zinc, magnesium, and mercury. The results showed that cockroach HA may be condensed by heavy metal ions, possibly due to protein denaturation. The inhibition of HA of *P. americana* by Ba$^{2+}$ was important because it did not show any HA. The plausible reason for this may be that barium ions may be directly bound to the carbohydrate-binding sites, which may seriously affect its conformation modifications resulting in blocked active sites.

### 3.3. HA of Whole Serum to Erythrocytes

The HA of serum of *P. americana* against the tested RBCs in this study is shown in Table 5. The cockroach serum showed a relatively strong HA against goat RBCs, but less detectable activity against human (A, B, and O) and chicken RBCs. The results showed a

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**Table 2:** The HA of cockroach serum to different temperature.

| Temperature (°C) | HA     | %HA |
|------------------|--------|-----|
| 10               | 0.025 ± 0.00 | 68.3 |
| 20               | 0.143 ± 0.0037 | 39.07 |
| 30               | 0.366 ± 0.0023 | 100  |
| 40               | 0.122 ± 0.0038 | 33.33 |

Values are $n = 3$, $x \pm SD$

**Table 3:** The HA of cockroach serum to different pH.

| pH   | HA         |
|------|------------|
| 6.0  | 0.027 ± 0.0046 |
| 6.5  | 0.030 ± 0.0046 |
| 7.0  | 0.211 ± 0.025  |
| 7.5  | 1.001 ± 0.023  |
| 8.0  | 0.1 ± 0.00     |

Values are $n = 3$, $x \pm SD$

**Table 4:** The HA of cockroach serum to different divalent cations.

| Metal ion (20 mM) | HA     | %HA |
|------------------|--------|-----|
| Ba$^{2+}$        | ND     |     |
| Ca$^{2+}$        | 0.211 ± 0.0025 | 100  |
| Zn$^{2+}$        | 0.1 ± 0.00 | 47.39 |
| Mg$^{2+}$        | 0.122 ± 0.0032 | 57.81 |
| Hg$^{2+}$        | 0.03 ± 0.0046 | 14.21 |
| EDTA             | 0      | 0   |

Values are $n = 3$, $x \pm SD$, ND = Not detected.

**Table 5:** The HA of cockroach sera to different blood groups.

|                     | HA         |
|---------------------|------------|
| Goat RBCs           | 0.366 ± 0.023 |
| Chicken TBCs        | 0.233 ± 0.0023 |

**Human erythrocytes**

| Group | HA     |
|-------|--------|
| A     | 0.1 ± 0.00 |
| B     | 0.083 ± 0.00 |
| O     | 0.025 ± 0.00 |

Values are $n = 3$, $x \pm SD$.

Measured in a sample pool of 30 insects for each RBCs types. Standard assay conditions using TBS/Ca$^{2+}$–Mg$^{2+}$.
high specificity of serum lectin to goat RBCs when compared to that of chicken and human.

In this study, the erythrocytes from human, goat, and chicken showed a non-specific HA with cockroach lectin. The lectin agglutinated all these erythrocytes with varying degrees of HA titers. The serum agglutinating activity is rather not highly specific since all the erythrocytes tested showed agglutination and it might be common as naturally occurring agglutinins are specific toward a wide range of vertebrate erythrocytes. The presence of lectin sugar on the surface of erythrocytes may result in an interaction with the lectin resulting in agglutination. The lectin showed a significantly higher HA for goat compared to chicken erythrocytes. The general specificity is influenced by the limited number of contacts with carbohydrates and the depth of the sugar-binding sites [30]. In addition, any modification or substitutions to a binding site can influence the binding specificity [33]. The observed difference in lectin activity with different blood groups may be due to the difference in carbohydrate lectin binding interactions, which can be attributed to differences in carbohydrates present in the cell surface of the different blood groups. The carbohydrates on the erythrocyte cell surface are differently distributed in various animal groups [34]. In the presence of lectin, sugar on the surface of erythrocytes results in an interaction with the lectin, leading to agglutination, or the carbohydrate unit structure and positions may be more specific with higher affinity for binding the lectin. It is possible that a similar mechanism may be responsible for increased goat erythrocyte agglutination. Moreover, lectin characteristics, such as multi-valency, may determine cross-linking interactions in binding recognition spatial distribution of multivalency among lectin structures, which, in turn, produce a higher level of specificity [35,36]. A high HA observed for goat erythrocytes might be due to the occurrence of specific carbohydrate moiety on the surface of goat erythrocytes other than RBCs types [13]. Unfortunately, several erythrocyte types were not examined for the HA activity in the previous reports [37,38].

3.4. Sugar specificity and inhibitions to HA

The inhibition of HA by sugar is shown in Table 6. The carbohydrate specificity of agglutinin is detected in the serum of cockroach and is called as the HA inhibition assay [39,40]. The HA of these lectins was preferentially inhibited by D-galactose, followed by D-lactose, D-glucose, and D-fructose. These results suggest that the carbohydrate-binding and carbohydrate recognition domains prefer α linked D-galactosides rather than free D-galactose. The affinity of the cockroach sera lectins toward α-linked galactosides is reported in L. migratoria [22] and in adult of S. gregaria [25]. The β-D-galactosides, as lectin-specific ligands, are found in insects, coleopteran Alomyrina dichotoma [41]. Barondes [42] also reported β-galactosides, as the lectin specificity in vertebrates, and in orthopterans like T. commodus [18] and the dipteran P. duboscqui [40] also possess lectins with amino sugars binding affinity. In many species of insects, especially lepidopteran, hemolymph lectins show an affinity for galactose and lactose [43] or to glucosides [21,44]. Some insects also show a binding affinity to mannose, e.g., the dictyopteran, Blaberus discoidalis [45] and lepidopteran, Heliothis virescens pupae [46]. Sugars such as galactose and lactose have been reported as inhibitors of the agglutinating activity of hemolymph of P Americana [37,38]. Volf et al. [40] and McGreal et al. [47] reported that sugars binding specificity is significant for the recognition of carbohydrate moieties on the surface, which are essential and responsible for the ability of the insect as a vector in pathogen transmission. A similar property has been reported in lectins of other insect, B. discoidalis [45], Philbotomus duboscqui [40], and A. caspius [24].

3.5. SDS-PAGE

SDS-PAGE showed a single band of molecular weight of 26.8 KDa. This molecular weight was near to 30 KDa lectin of P. americana [38], and 20KD lectin of Saracophaga peregrina [48].

4. CONCLUSION

In conclusion, we say that the lectin purified from cockroach has the highest HA at temperature 30°C and at pH 7.5. The cockroach serum lectin contains a C-type lectin and shows inhibition to D-galactose sugar. Furthermore, the insect’s C-type lectin appears to play an important role in pathogen recognition and cellular interactions. This ability is achieved due to binding with the carbohydrate component of the surface molecular patterns. The SDS-PAGE of lectin showed a molecular weight of 26.8kDa. The isolated lectin was highly specific to goat RBCs than for those of other vertebrates. Accordingly, we can say that the carbohydrate-binding specificity by lectin is necessary for the recognition of the cell surface carbohydrate including those of the invading pathogens.

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