Chapter III

PURIFICATION AND CHARACTERIZATION OF HEMOLYMPH LECTIN
FROM *BRADINOPYGA GEMINATA* (RAMBUR, 1842)

3.1. INTRODUCTION

Insects display a wide variety of humoral responses with rather specific recognition of self from nonself. Pathogens invading invertebrate organism often require opsonization before they can be recognized by specific receptors on phagocytic cells. In insects, there are several different classes of molecules which are opsonic. These include humoral lectins, lectin-like agglutinin molecules and prophenoloxidase (proPO) cascade system. Hemagglutinins, presently considered to be lectins, are non-catalytic carbohydrate binding proteins that have been reported from several insects such as the lepidopteran *Citheronia regalis* (Bernheimer *et al.*, 1952), the cockroach *Periplanata americana*, the locust *Schistocerca gregaria* (Lackie, 1981), *Blaberus craniifer* (Donlon and Wemyss, 1976), *Leucophaea maderae* (Amirante and Mazzalai, 1978), and the bug *Rhodnius prolixus* (Pereira *et al.*, 1981). Hemagglutinins of insect species differ in properties and electrophoretically slow moving fraction of hemolymph proteins such as heat labile, nondialyzable, euglobulin type protein (Lackie, 1981). Lectin play a role in insect immunity, they can act as a membrane bound receptors or as humoral opsonic factors or by acting independently of hemocytes and agglutinate invading bacteria (Lackie, 1981). Naturally occurring glycoprotein or proteins in the hemolymph of the locust and cockroach, agglutinate flagellate protozoa (Seaman and Robert, 1968; Ingram *et al.*, 1984).
In most species, lectins have been detected in hemolymph but are known to occur in other tissues. Agglutinin occurs in several insect species which recognize microorganism. In Diptera and the lepidopteran which agglutinate *Trypanosoma, Leishmania* and *Crithidia* (Ingram et al., 1983; Ingram et al., 1984; Ibrahim et al., 1984) and active against *Bacillus thurinaiensis* (El Moataz Bellah et al., 1988). In honeybees, agglutinins are synthesized in response to a bacterial pathogen (*Bacillus larvae*) (Gilliam and Jeter, 1970).

Among insects, the humoral lectin from the larvae of the flesh fly *Sarcophaga peregrina* (Komano et al., 1980) has dual function, operating both in immune defense and developmental processes. Other insect lectins share similarities with the well characterized *Sarcophaga* lectin system. A lectin from pupal hemolymph of the Chinese oak silkworm *Antheraea pernyi* also has dual function, such as in developmental and in immune defense (Qu et al., 1987). Another inducible lectin was detected from the larval hemolymph of velvet bean caterpillars *Anticarsia gemmatalis* (Pendland and Boucias, 1985). Insect hemagglutinins exhibit the broad range of specificities found in lectins from species in other kingdoms, a significant number of insect lectins appear to be specific for galactosyl residues. The galactose/glucose lectin exists in adult grasshoppers (Jurenka et al., 1982; Hapner, 1983; Stebbins and Hapner, 1985). The galactose-inhibitable lectin functions during pupation in recognition of effect of larval tissue and as a wound response protein in larvae (Komano et al., 1981).

In insects, lectins were detected in the hemolymph of only few of the Lepidoptera (Bernheimer, 1952; Pendland and Boucias, 1985) and Hemiptera (Feir and Walz, 1964). In addition, Ratcliffe and Rowley, (1983) examined the occurrence of agglutinins in hemolymph of several insects species and showed only few
members of orders with higher hemagglutination titer while certain species of Orthoptera, and Coleoptera reacted and showed low hemagglutination titer when assayed against sheep erythrocytes. But the order Odonata contained two species the *Anax imperator* and *Brachytron pretense*, both showed high hemagglutination activity.

Lectins from invertebrates, particularly insects’ lectin are studied in detail and most of the previous studies are restricted to orders such as Orthoptera, Phasmida, Dictyoptera, Dermaptera, Isoptera, Hemiptera, Lepidoptera, Diptera, Hymenoptera and Coleoptera. In the present study, a lectin molecule from the hemolymph of the dragon fly *Bradinopyga geminata* (Granite Ghost) (Rambur, 1842) has been purified. This is the first report on purification and characterization of a lectin from the order Odonata.

The main objectives of the present chapter is:

1. To screen and identify the hemagglutinin from the hemolymph of *B. geminata*
2. To identify the stage-specific occurrence of natural agglutinins in the dragon fly *B. geminata* nymph that include I instars, II instars, III instars and final molting stage and adult.
3. To characterize the physico chemical parameters of the lectin.
4. To develop appropriate strategy for purifying the lectin by affinity chromatography
5. To purify the isolated lectin by RP-HPLC
6. To analyze the molecular weight of the purified lectin using SDS-PAGE.
3.2. Materials and Methods

3.2.1. Systematics

Fig. 3.1. *Bradinopyga geminata*

Kingdom : Animalia
Phylum : Arthropoda
Class : Insecta
Order : Odonata
Family : Libellulidae
Genus : *Bradinopyga*
Species : *geminata* (Rambur, 1842)
Fig. 3.2. *Bradinopyga geminata* (Granite Ghost) and their larvae
*Bradinopyga geminata* (Granite Ghost) is a widespread species all over India (Mitra 1988, 2002), Sri Lanka (Tsuda 1991) and Thailand (Kiauta and Kiauta 1983). Adults (Fig 3.2) are common along small pools of water or small stagnant water bodies nearby to the agricultural fields (Kumar and Mitra 1998). They also breed in wells, tanks, drums, rainy hollows in the rocks and invariably settling with wings flattened on the granite face, with which their marbled grey coloured body harmonies to such an extent that they become practically invisible. Immature insects that go through incomplete metamorphosis are called nymphs in the case of dragonflies and damselflies as naiads. Nymphs are similar in form to the adult except for the presence of wings, which are not developed until adulthood. Wingless dragonfly nymphs live and grow in water. A nymph may go through 16 instars before the process is complete. Some nymphs complete the instar process in five weeks; other species may take as long as five years. The last instar nymph will emerge from the water, dry off and molt one more time, revealing wings and fully capable of flight. With each molt, nymphs grow larger and become more similar in appearance to adult insects. Skimmers (Family: Libellulidae) are the most diverse group of odonates.

**3.2.2. Sample collection**

The *B. geminata*, nymph with different instars of the larvae were collected from the house water tanks and brought to the laboratory. In the laboratory larvae were sort out to each stage and maintained.
3.2.3. **Hemolymph extraction**

The hemolymph was obtained from the below the region of the eye with hypodermic syringe provided with a 26 gauge needle and the hemolymph was cleared by centrifugation at $16000 \times g$ at $4^\circ C$ for 30 minutes. The supernatant was dialyzed against Tris-buffered saline (TBS: 50mM Tris-HCl, 0.15M NaCl, 10mM CaCl$_2$, pH 7.6) with several changes and stored at -80°C until further use.

3.2.4. **Preparation of erythrocytes (red blood cell)**

Human and other blood samples from different animal species, obtained by venous or cardiac puncture (in animal), were collected in sterile Alsever’s solution containing (30 mM sodium citrate, 77 mM NaCl, 114 mM glucose, 100Ug/ml neomycin sulphate, 330ug/ml chloramphenicol pH 6.1. Red blood cells were washed thrice with 0.9% saline and once with TBS and finally resuspended, unless otherwise specified in the same buffer as 1.5% suspension (v/v).

3.2.5. **Trypsin treatment and fixation of red blood cells**

According to the method described by Maheswari *et al.* (1997), saline washed rabbit erythrocytes were resuspended in the TBS containing 5mg ml$^{-1}$ trypsin and incubated for 1h at 37°C with occasional shaking. Trypsinated red blood cells were washed with 0.9% saline by centrifugation for 400 X g in 5 min at room temperature and finally fixed by suspending the red blood cell pellet in TBS (pH 7.6) containing 10% formaldehyde for 24 h at 10°C. Fixed red blood cells were extensively washed in 0.9% saline and, unless otherwise specified, resuspended in TBS.

3.2.6. **Hemagglutination assay**

The hemagglutination assays were performed in V-bottomed microtitre plates by serial two – fold dilution of a 25µl serum sample with an equal volume of TBS-Ca. After dilution, 25 µl red blood cell suspensions was added to each well and incubated
for 1 h at room temperature. The hemagglutination titre was recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of red blood cells. Each experiment was performed in duplicate, and the hemagglutinating activities were expressed as the median hemagglutination titre. Finally the purified lectin was used for the hemagglutination assay.

The hemagglutinating activity (HA=HU/mL⁻¹) was defined as the hemagglutination Units per volume used. Specific hemagglutinating activity was expressed as the activity per mg of protein.

### 3.2.7. Cross adsorption tests

Hemolymph samples (300 µl) were mixed with an equal volume of washed and packed native cow, hen, pig, goat, mouse, rabbit and human (A, B, O) red blood cells and incubated for 1h with frequent shaking at R.T. The suspension was centrifuged (400g, 5min, R.T.); the supernatant was collected and adsorbed for a second and third time under the same conditions. The supernatant sample was adsorbed finally and tested for hemagglutinating activity against all the nine red blood cell types.

### 3.2.8. Effect of pH and thermal stability

Hemolymph sample and purified lectin (500 µl) were dialyzed against the buffers at pH ranging from 3.5 to 10, using acetate buffer, Tris-HCl and glycine NaOH. After dialysis, all the samples were finally equilibrated by dialysis against TBS-Ca. The dialysates were centrifuged and the supernatant was tested for hemagglutinating activity using rabbit red blood cells.

The thermal stability of hemolymph sample as well as purified lectin were examined by holding 100 µl of samples for 30 min at temperature ranging from 10-80°C. All
samples were centrifuged and the clear supernatant was used to determine agglutination activity using rabbit erythrocytes.

3.2.9. Effect of deproteinising agent, protease and periodate

Precipitation of hemagglutinin from hemolymph was attempted using ammonium sulphate ((NH₄)₂SO₄) and trichloroacetic acid (TCA) as described previously (Murali et al., 1994). Briefly, the hemolymph sample was mixed with ice-cold ((NH₄)₂SO₄) at 10-100 % saturation and incubated for 4 h at 4°C. The resulting precipitates were removed by centrifugation at 400g for 10 min, dissolved, and dialyzed extensively in TBS at 4°C and was used for agglutinating assay. Similarly TCA precipitation of hemagglutinin from hemolymph was performed by mixing 500 µl of hemolymph sample with an equal volume of ice-cold 20 % TCA solution and incubating for 1h at 4°C. The supernatant collected after centrifugation (400g, 10 min) was dialysed as described above. The hemagglutination titers for all the dialysates were determined using rabbit erythrocytes.

The hemolymph samples as well as purified lectin were mixed with equal volume of trypsin at a final concentration of 6 mg/ml as described by Maheswari et al. (1997). The mixture was incubated for 3 h at 25°C, centrifuged and the supernatant was tested for hemagglutinating activity.

The effect of oxidizing agent on hemagglutinating activity of sample was tested using potassium metaperiodate. For this 300 µl sample was dialysed against TBS and centrifuged. The resulting supernatant was mixed with equal volume of 0.08M potassium metaperiodate and incubated for 3 h at 25°C. The mixture was re-dialysed with TBS and centrifuged. The supernatant was used for hemagglutinating activity (Maheswari et al., 1997).
3.2.10. Divalent cation dependency and EDTA sensitivity

The initial hemagglutination activity of hemolymph samples (untreated) and purified sample was determined in TBS containing 10 mM CaCl$_2$. The hemolymph samples (each 500 µl) were dialyzed extensively against TBS (to test divalent cation dependency) or in TBS-EDTA (to examine EDTA sensitivity) at 15°C. The samples were dialysed against TBS-EDTA and re-equilibrated by subsequently dialysis in TBS. After centrifugation (400g, 5min, room temperature.), the supernatant was used to determine the hemagglutinating activity with human and animal red blood cell in the presence of TBS that did or did not contain different concentration of CaCl$_2$, MgCl$_2$, MnCl$_2$, HgCl$_2$, BrCl$_2$, MnSO$_4$ and MgSO$_4$ (pH 7.6).

3.2.11. Hemagglutination Inhibition Assay

Purified lectin was allowed to react with equal volume of several carbohydrates and glycoprotein (Sigma), such as galactose, galactosamine, glucosamine, arabinose, glucose, lactose, L-fucose, mannose, sucrose, fructose, ribose maltose, melibiose, trehalose, mannitol, xylose, raffinose, cellobiose, dextrose, and sorbitol. N-acetyl neuraminic acid, N-acetyl glucosamine, N-acetyl galactosamine, glucuronic acid, methyl-β-D-galactopyranoside, p-nitrophenyl-α-D-galactopyranoside, fetuin, asialofetuin, and mucin solutions at room temperature for 1 h. After incubation, 25 µl of preprepared 1.5% rabbit erythrocyte suspension was added to the mixture and after 1 h incubation the hemagglutination was examined. The results were expressed as the minimum concentration of the inhibitor required to completely terminate the agglutination.
3.3. Purification of hemolymph lectin from B. geminata.

All chromatographic procedures were performed at 25°C. Protein concentrations of the column eluents were monitored by $A_{280}$

Step 1

3.3.1. Ammonium sulphate fractionation

Hemagglutinin was precipitated from hemolymph submitted to ammonium sulphate fractionation (saturation of 40-50 %). A volume of hemolymph was gently stirred while an equal volume of an ammonium sulphate solution (40-50%) was slowly added and mixed well (Green and Hughes, 1955). The reaction mixture was set aside at room temperature for 4 h and then centrifuged at 400 $\times$ g for 10 min and to pack the precipitated protein. The supernatant fluid was removed and stored for later analysis. The precipitate was resuspended and dissolved in distilled water to a final volume equal to the original volume of hemolymph. For a second precipitation, the dissolved protein was gently stirred while an equal volume of an ammonium sulphate solution was slowly added. The mixture was immediately centrifuged at 400 $\times$ g for 10 min, the supernatant fluid was discarded. All fractions were dialyzed against frequent changes of pH 7.6, TBS solution until sulphate was no longer detected in the dialysate. A small volume of saturated barium chloride solution was added to an equal volume of well-mixed dialysate to check for the presence of sulphate. If no cloudiness resulted, the dialyzed fraction was considered substantially free of sulphate. The hemagglutination titers for all the dialysates were determined using rabbit erythrocytes prior to next step of purification.
Step 2

3.4. Affinity chromatography

3.4.1. Preparation of CNBr-activated fetuin Sepharose 4 fast flow

Commercially available CNBr-activated sepharose 4 Fast Flow was used for preparation of affinity matrix. 2.5 g freeze dried CNBr-activated sepharose 4 Fast Flow powder was swelled with the help of 1 mM HCl for ~15 min. at room temperature. The swelled matrix was transferred to sintered glass funnel and washed with 200 ml of 1 mM HCl. The slurry was stirred with glass rod for about 20 minutes until matrix was swollen, then applied to vacuum to remove liquid and the matrix was washed with 3 x 100 ml 1mM HCl. The matrix was washed to dryness in each time.

3.4.2. Coupling of Fetuin with CNBr-activated sepharose 4 fast flow

After washing, with 3 x 100 ml 1mM HCl, the activated matrix was again washed with 50 ml coupling buffer (Coupling buffer, pH 9.0, 0.1 M NaHCO₃, 0.5 M NaCl). Then 5 mg of ligand (Fetuin) was dissolved in 15 ml coupling buffer and quickly transferred to the activated Sepharose to a flask containing the ligand solution and shake it very gently overnight at 4°C. 100 µl of the aliquot was collected from coupling medium and the degree of coupling was checked by reduction of fetuin for later analysis. The coupled matrix was filtered in the sintered glass funnel and collected in the flow through. 10 µl of the matrix was taken for analysis for comparing to 10 µl of starting solution to estimate binding efficiency of whether the coupling reaction was worked. The coupled matrix was washed with 100 ml coupling buffer on sintered glass filter. The matrix was transferred back to the flask and incubated with 50 ml freshly prepared blocking buffer at room temperature for two hours or at 4°C for overnight (Blocking buffer, pH 8.0 – ie, 1 M ethanolamine in coupling buffer (sterile filtered). The adsorbent was washed thoroughly on a sintered
glass funnel with 0.2 M NaCl and finally washed with distilled water. The processed CNBr-activated fetuin sepharose 4 Fast Flow was stored in cold TBS (pH 7.6) containing 0.02% sodium azide at 4°C until further use.

### 3.4.3. Purification of B. geminata hemolymph lectin by affinity chromatography using CNBr-activated fetuin Sepharose 4 fast flow.

2 ml of the processed active fraction of ammonium sulphate precipitation (40%) was used for affinity chromatography purification and applied to CNBr-activated fetuin sepharose 4 fast flow glass column, previously equilibrated with TBS (pH 7.6) at 4°C. The column was washed with TBS until 0.002 OD at 280 nm at attained. This is to remove unwanted or unbinding proteins from the column, so as to obtain homogenous lectin protein. The elution was done with elution buffer containing 0.1M N-acetyl neuraminic acid and the fractions were collected in 2 ml polypropylene tubes at the rate of 0.4 ml/min. Fractions were immediately collected and stored at 4°C. Each fraction was taken OD at 280 nm in spectrophotometer. The fractions having high protein content were identified using hemagglutination assay. After identification of active fraction, it was dialyzed against distilled water for overnight at 4°C. The resulting fractions were the aliquoted and stored at -20°C. The protein content was estimated by Lowry et al., (1951) method.

**Step 3**

### 3.5. Gel filtration chromatography using Sephacryl S-300 HR

#### 3.5.1. Preparation of gel filtration matrix Sephacryl S-300 HR

Sephacryl™ HR, the purification matrix that was gently shaked in the bottle to make even slurry and measured out the required volume of medium slurry. The medium slurry was diluted in eluent buffer and stirred it well with a glass rod to make a homogeneous suspension that was free from aggregates. The slurry was equilibrated
with 50 mM TBS buffer (pH 7.6) stirred well, allowed to settle and supernatant was
decanted by suction. The swelled gel was washed with elution buffer (TBS with
50mM Tris 140mM NaCl pH 7.6) for 4 or 5 times to settled the gel. The matrix
Sephacryl S-300 HR was gently stirred and allowed to settle and fine parts was
removed by suction. The same processes were repeated for three times. The
equilibrated gel was kept at 2°C-4°C overnight before the column was packed.

The prepared and equilibrated, Sephacryl S-300 HR gel was taken from the
freezer and allowed to attain the room temperature. The column was checked for any
damages and air bubbles. After the buffer was degassed, the gel was packed in the
glass column (0.6 × 100 cm). Sephacryl S-300 HR gel was uniformly packed with out
air bubbles. Then the gel was equilibrated with elution buffer. After equilibration, the
diazyed affinity purified active sample was applied to the column. Elution was done
with the help of elution buffer, at the flow rate of 0.1ml/min and the fractions were
collected in 2 ml propylene tubes. The OD of the fractions was observed at 280 nm in
a spectrophotometer. Active fractions were identified through the hemagglutination
assay and its protein content was estimated using Lowry et al., (1951) method.

3.6. Lectin purification by RP-HPLC

The gel filtration purified lectin of B. geminata was applied to the HPLC C18
column (250 × 4.6mm) Varian (Lake forest, CA, USA, Cyberlab, USA). The lectin
was then applied to the analytical reverse phase HPLC and equilibrated with buffer A
(0.1% trifluoroacetic acetic acid; TFA) for 15 minutes. Elution of the protein was
subsequently conducted using a buffer B (65% acetonitrile and 35% water in buffer
A), and the chromatographic run was monitored at 280 nm of absorbance. Fractions
were carried out at the flow rate of 1ml/min. After elution, the active peak was
collected and the fraction was concentrated using Speed Vac, lyophilized and stored at –40°C. The concentrated sample was subjected to native PAGE and SDS-PAGE.

3.7. Protein concentration determination

Protein concentrations were determined according to the Lowry method with bovine serum albumin as the standard (Lowry et al., 1951) method.

3.8. Protein denaturation

For analytical SDS/PAGE, samples were diluted to 1:4 with a solubilizer (1 % SDS; 0.02 % bromophenol; 1 % betamercaptoethanol in running buffer) and boiled for 3 min before electrophoresis. Separation gels were 10 % acrylamide/0.01 % SDS in 0.5 M Tris HCl pH 8.8. Stacking gels were 5.5 % of acrylamide in 1.5 M TrisHCl, pH 6.8. A conventional buffer system (Laemmli, 1970) was used with a 10X running buffer (2 M-glycine/0.1 % SDS/0.4 M Tris, pH 8.3). Electrophoresis was carried out at 50 V for 3 h 30 min in a mini vertical gel electrophoresis system (GeNei™). Visualization of protein band was performed by silver staining.

3.9. Results

3.9.1. Screening and identification of hemolymph agglutinin from B. geminata

Fig 3.3. Hemagglutination titers of the hemolymph from the nymph of dragon fly (Granite Ghost) B. geminata
The hemolymph sample was tested for hemagglutination activity using a battery of human and animal erythrocytes (Fig 3.3). The hemolymph of *B. geminata* agglutinates most of the erythrocytes. From the results the highest HA activity titre of 2048 was obtained with rabbit erythrocytes. The agglutinin agglutinated human erythrocytes, cow, mouse, hen, squirrel, pig, dog, goat and turkey. In human erythrocytes, human’O’group showed highest activity and A and B group showed similar activity in the titre of 8. Interestingly the human blood group A1 showed highest activity than that of human A group erythrocytes. Among human blood group erythrocytes, A1 and O group erythrocyte showed highest activity and especially human A1 erythrocytes activity was three fold higher activities than human O group. Squirrel, pig and turkey showed similar hemagglutination activity and other animal erythrocytes such as cow, dog and goat gave lower activity. The mouse erythrocytes showed much lower activity among erythrocytes used. Based on the HA titers obtained, the erythrocytes could be graded as rabbit > human O >squirrel = pig = turkey > human A = hen = human B = cow= goat > dog > mouse.

### 3.9.2. Stage specific agglutinin from *B. geminata* larvae

Titer of agglutinins in different stages of *B. geminata* larvae and adults were greatly comparable, indicating a stage related decrease in hemagglutinin content in the hemolymph, and individual variations in the hemagglutination titer (Fig 3.4). Results also revealed that the third stage larvae was found to be having high hemagglutination titer while the next stage (stage –IV), reduced the agglutination titer as well as in the adult stage assayed against the rabbit and human A1 erythrocytes. The stage I and II were found to have moderately active hemagglutination titer. This pattern of hemolymph agglutination was consistently reduced and not reproducibly observed over the successive developmental stages after the third stage. The reduced titer,
indicates that a variation had occurred independently in each developmental stage during their metamorphosis.

**Fig 3.4.** Agglutination titer of hemolymph pooled from different stages of *B. geminata* against the rabbit and human A1 type erythrocytes.

3.9.3. *Enzyme treatment on erythrocytes*

The enzyme treated erythrocytes were also tested for hemagglutination activity as shown in the figure 3.5. From the results, trypsin and papain treated rabbit and human ABO erythrocytes showed a two fold increase in the activity as compared with untreated erythrocytes. Whereas, neuraminidase treated erythrocytes reduced the hemagglutination titer as low as half of original activity. The trypsinated and papain treated rabbit erythrocytes also showed similar activity. The papain treated B erythrocyte showed the titer value of 256 that was higher than the trysin treated erythrocytes. Similarly, papain treated human O erythrocytes showed higher activity. But the trypsin treated O erythrocytes were devoid of activity.
**Fig 3.5.** Effects of enzyme treatment of erythrocytes on hemagglutination titer of the hemolymph lectin from the nymph of dragon fly (Granite Ghost) *B. geminata*

![Enzyme Treatment Graph](image)

3.10. Characterization of hemolymph agglutinin from *B. geminata* (Rambur, 1842)

3.10.1. *Effect of temperature and pH*

When the sample was pre incubated at different temperatures (Fig.3.6 and 3.7), the highest agglutination activity was recorded at 30°C. After 30°C, the activity was gradually reduced and the activity lost at 74°C. The optimal activity of the hemolymph of *B. geminata* was between 25 and 30°C. The titer of hemolymph and purified lectin heated at 45°C for 30 min decreased 5 fold in comparison to samples that were kept for the same time at 25-30°C. The titer of hemolymph and purified lectin heated at 65-70°C for 30 min decreased 2 fold in comparison to samples that were kept for the same time at 55-60°C. Incubation at 74°C for 30 min completely removed the hemagglutination activity of the *B. geminata* lectin. All assays were carried out in trypsinated rabbit erythrocytes.
Fig 3.6. Effect of temperature on hemagglutination titer of the hemolymph lectin from the nymph of dragon fly (Granite Ghost) *B. geminata*

![Graph showing hemagglutination titer vs. temperature](image)

Fig 3.7. Effects of temperature on hemagglutination titer of the hemolymph lectin from the nymph of dragon fly (Granite Ghost) *B. geminata*

![Graph showing hemagglutination titer vs. temperature](image)
3.10.2. Effect of pH on hemagglutination titer of the hemolymph lectin

The effect of dialyzing of whole samples against buffers at different pH is shown in the figure 3.8. The hemolymph and purified lectin of *B. geminata* showed hemagglutination activity over wide range of pH (6.0 to 9.5) and the activity was high between 7.5 and 8 (table 3). The activity was completely lost at pH 10 and at 5.5. The hemolymph and purified lectin appeared to stable for pH shifts in the range of 7.5-8.0. The activity was reduced at the extremes of pH and both were tolerant to alkaline rather than acid condition. Complete loss in activity occurred at pH 5.5 with 80% of the activity reduction at pH 9.0. The exact high activity was observed in the pH 7.6 with in the stable range of 7.5-8.0.

**Fig. 3.8.** Effect of change of pH on hemagglutination titer of the hemolymph lectin from the nymph of dragon flies (Granite Ghost) *B. geminata*
3.10.3. Cross adsorption test

Cross adsorption studies were carried out to assess whether the hemagglutination activity is due to single agglutinin or due to multiple agglutinins in the hemolymph investigated (Table 14). Absorption of the samples with any one of the erythrocytes used was sufficient to remove the hemagglutination activity for other erythrocytes. Human ABO, rabbit, cow, hen, pig and goat were used for cross adsorption test. As shown in Table 6, adsorption of hemolymph thrice with any one of the red blood cells types resulted in a complete removal of the agglutinating activity against red blood cell types. In this adsorption test each red blood cell types were found to completely or partly adsorb to other types, and the efficiency of adsorption differ among the eight RBC types.

The sample was adsorbed to rabbit erythrocytes, when the supernatant did not cross react with the other red blood cell type. But adsorption with human ABO erythrocytes and cross reacting with other erythrocytes especially in rabbit erythrocytes recorded the titer value of 64, 8, and 16 respectively, while the animal erythrocytes showed low hemagglutination activity. When pig erythrocytes was adsorbed with lectin and cross react with human O and rabbit red blood cells showed higher activity but others red blood cell types showed lower activity. When goat erythrocytes was adsorbed with lectin and it cross react with mostly all red blood cell types. The hen erythrocytes also showed cross adsorb with mostly all red blood cell types and showed higher activity titer in rabbit erythrocytes. In general the hemolymph lectin was highly specific to the rabbit erythrocytes.
Table 14. Hemagglutination titers of the hemolymph lectin from the nymph of dragon fly (Granite Ghost) *B. geminata* after adsorption with different erythrocytes.

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<thead>
<tr>
<th>Hemolymph adsorbed with red blood cells</th>
<th>HA titre against red blood cell types</th>
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<tbody>
<tr>
<td></td>
<td>Human A</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>Human A</td>
<td>0</td>
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<td>Human B</td>
<td>0</td>
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<tr>
<td>Human O</td>
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<td>Goat</td>
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3.10.4. Effect of metal ion on hemagglutination activity

In this experiment, the hemagglutination activity of the hemolymph sample was tested with different concentration of divalent cations such as CaCl₂, MgCl₂, ZnCl₂, MnCl₂, FeCl₂, HgCl₂, BaCl₂, MnSO₄, and MgSO₄ (Fig. 3.9). The higher activity was observed with addition of 50, 25 and 12.5 mm MgCl₂ and CaCl₂. The hemagglutination activity of the lectin was also high when treated with 25 and 12.5 nm of BaCl₂. The MgSO₄ observed similar hemagglutination activity with the titer value of 256 at the concentration of 100, 50 and 25 mM, but low concentration reduced the titer value. The addition of 100 mM concentration of MnCl₂ showed higher activity, but by the addition of 6.25 mM showed lower hemagglutination
activity. In the case of ZnCl$_2$, 25 mM concentration showed higher titer value of 128. The FeCl$_2$ and HgCl$_2$ showed no activity even at lower concentration.

**Fig 3.9.** Effects of metal cation on the hemolymph lectin from the nymph of dragon fly (Granite Ghost) *B. geminata.*

![Graph showing the effects of metal cations on hemagglutination titer](image)

3.10.5. *Effect of deproteinising agent, protease and periodate*

40-50% ammonium sulphate (Table 15) and 10% TCA precipitated all proteins having hemagglutinating activity suggesting that the proteinaceous nature of hemolymph lectin. The purified lectin was treated with trypsin at 25°C for 3h reduced the agglutinating activity. Similarly hemolymph samples treated with potassium metaperiodate and then dialyzed against TBS, reduced destroyed agglutinating activity.
Table 15. Ammonium sulphate precipitation of hemolymph of *B. geminata*

<table>
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<tr>
<th>Precipitation</th>
<th>Hemagglutination titer</th>
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<tr>
<td></td>
<td>10% 20% 30% 40% 50% 60% 70% 80% 90% 100%</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>0 0 4 4096 4096 4 0 0 0</td>
</tr>
</tbody>
</table>

3.10.6. Cation dependency and EDTA sensitivity

The agglutination activity of the lectin was Ca\(^{2+}\) dependent (it requires free calcium ions in the buffer). Removal of Ca\(^{2+}\) by dialysis against Ca\(^{2+}\) free TBS or TBS contain different concentration of EDTA resulted in complete loss of activity but the effect was not reversible and the activity could not be restored, in both cases, by returning Ca\(^{2+}\). In general, CaCl\(_2\) was required for the hemagglutination activity of this lectin (Table 16).

Table 16. Effect of cation dependency (CaCl\(_2\) and without CaCl\(_2\)) and EDTA sensitivity on hemagglutination activity of the hemolymph lectin from the nymph of dragon fly (Granite Ghost) *B. geminata*.

<table>
<thead>
<tr>
<th>Concentration of EDTA(mM)</th>
<th>HL titer</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDTA</td>
<td>CaCl(_2)</td>
<td>Without CaCl(_2)</td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>0</td>
<td>512</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12.5mM</td>
<td>0</td>
<td>4096</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>1024</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>512</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>512</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
3.10.7. Hemagglutination Inhibition

The sugar specificity of agglutinins in the hemolymph of *B. geminata* was assessed by hemagglutination inhibition efficiency by various sugar and glycoproteins (Table 17 and 18). The hemagglutinating activity of the hemolymph of *B. geminata* was weakly inhibited (HA 1 liter=1) by the arabinose and the galactose showed the lower inhibitory activity (HAI liter=2). The N-acetyl neuraminic acid showed higher inhibitory activity (HAI liter=8) among the other sugars used (i.e. 25 mM concentration of N-acetyl neuraminic acid). Except N-acetyl neuraminic acid, galactose and arabinose, other sugars such as glucose, lactose, mannose, sucrose, fructose, fucose, ribose, maltose, mannitol, xylose, melibiose, trehalose, rhamnose, raffinose, cellobiose, dextrose, sorbitol, galactosamine, glucosamine, N-acetyl glucosamine, n-acetyl galactosamine, glucuronic acid methyl, β-D galactopyranoside, and p-nitrophenyl-α-D galactopyranoside, has no inhibitory potency against the hemolymph agglutinin of *B. geminata*.

In the case of glycoproteins fetuin was one of the potent inhibitor of hemolymph agglutinin with the HA titer of 16 (i.e. 0.625 mM concentration of fetuin) while, the bovine sub maxillary mucin did not inhibit at to that extent. The desialilated fetuin (asialofetuin) completely abolished the inhibitory efficiency. Results clearly indicated that the isolated lectin was a N-acetyl neuraminic acid specific lectin (Table 17 and 18). Hemolytic inhibition potency of the sugars are graded as Fetuin > N-acetyl neuraminic acid >galactose > arabinose.
Table 17. Hemagglutination inhibition (HAI) activities of the hemolymph lectin of nymph of dragon fly (Granite Ghost) *B. geminata* by various sugars.

<table>
<thead>
<tr>
<th>Inhibitor tested (n=10)</th>
<th>Maximum concentration tested (mM)</th>
<th>Hemagglutination inhibition (HAI)</th>
<th>Minimum inhibitory concentration (mM)</th>
<th>Relative inhibitory potency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl neuraminic acid</td>
<td>200</td>
<td>8</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Galactose</td>
<td>200</td>
<td>2</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Arabinose</td>
<td>200</td>
<td>1</td>
<td>200</td>
<td>12.5</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Glucose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Lactose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Mannose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Fructose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Ribose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Maltose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Mannitol</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Xylose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Melibiose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Trehelose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Raffinose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Dextrose</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>N-acetyl glucosamine</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>N-acetyl galactosamine</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>Methyl β-D galactopyranoside</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
<tr>
<td>p-nitrophenyl α-D-galactopyranoside</td>
<td>200</td>
<td>-b</td>
<td>-b</td>
<td>-b</td>
</tr>
</tbody>
</table>

*a* The assay was repeated more than five times for each carbohydrate with identical results using samples from different preparations.

*b* No inhibition
Table 18. Hemagglutination inhibition (HAI) activities of the hemolymph lectin of nymph of dragon fly (Granite Ghost) *B. geminata* by glycoproteins.

<table>
<thead>
<tr>
<th>Inhibitor tested (n = 10)</th>
<th>Maximum concentration tested (mM)</th>
<th>Hemagglutination inhibition (HAI)</th>
<th>Minimum inhibitory concentration (mM)</th>
<th>Relative inhibitory potency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin</td>
<td>10</td>
<td>16</td>
<td>0.625</td>
<td>100</td>
</tr>
<tr>
<td>Asialo fetuin</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mucin (BSM)</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* The assay was repeated more than five times for each carbohydrate with identical results using samples from different preparations.

*b* No inhibition

3.11. Ammonium sulphate precipitation

Ammonium sulphate precipitation of crude hemolymph of *B. geminata* sample resulted in maximum activity 40-50% of ammonium sulphate saturation. High activity was observed in the 40% of saturation (Table 15). This fraction was applied to the CNBr-activated fetuin Sepharose 4 fast flow affinity column.

3.12. Purification of lectin using CNBr-activated fetuin Sepharose fast flow

The dialyzed and neutralized 40% of ammonium sulphate precipitated sample of hemolymph of *B. geminata* fraction was introduced into the CNBr-activated fetuin Sepharose 4 Fast Flow Affinity chromatography column. Ammonium sulphate precipitation, upon affinity chromatography yielded three peaks. One major peak and two minor peak. The adsorbed fraction that is 57th fraction had high hemolytic activity and 58 and 59th fractions also showed the hemolytic activity. Furthermore one minor peak also appeared but devoid of activity (Fig 3.10).
Fig. 3.10. Purification of *B. geminata* hemolymph lectin by affinity chromatography using CNBr-activated fetuin Sepharose fast flow

3.13. Purification of lectin by using Sephacryl S-300 HR

Affinity purified active fraction was chromatographed on gel filtration chromatography using Sephacryl S-300 HR (Fig 3.11). From this result, one major peak that was a high active fraction and one minor peak devoid of hemagglutination activity were obtained. Fraction number 11 to 15 gave hemagglutination activity and these fractions were analyzed in native PAGE and SDS-PAGE. The results were summarized in table 19, where the degree of purification is shown.
Fig. 3.11. Purification of *B. geminata* Lectin by Gel filtration chromatography using Sephacryl S-300 HR

![Graph showing A280 and hemagglutination activity vs fraction number.](image)

**Table 19.** Summary of purification of *B. geminata* hemolymph lectin and its specific activity

<table>
<thead>
<tr>
<th>Steps</th>
<th>Vol (ml)</th>
<th>Protein mg/ml</th>
<th>HA Activity</th>
<th>Total activity (HAU)</th>
<th>Specific Activity (HAU/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude hemolymph</td>
<td>5</td>
<td>6.35</td>
<td>2048</td>
<td>10240</td>
<td>322</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>2</td>
<td>3.92</td>
<td>4096</td>
<td>8192</td>
<td>1044</td>
<td>3.24</td>
<td>80</td>
</tr>
<tr>
<td>CNBr-activated Fetuin Sepharose</td>
<td>6</td>
<td>0.624</td>
<td>1024</td>
<td>6144</td>
<td>1641</td>
<td>5.09</td>
<td>60</td>
</tr>
<tr>
<td>Sephacryl S-300 HR</td>
<td>10</td>
<td>0.310</td>
<td>512</td>
<td>5120</td>
<td>1651.6</td>
<td>5.12</td>
<td>50</td>
</tr>
</tbody>
</table>
The dragon flies hemolymph lectins were purified by three steps (Table 19), Ammonium sulphate precipitation, CNBr-activated Fetuin Sepharose, and Sephacryl S-300 HR. In the first step of purification, obtained 3.92 mg/ml protein. This is the 3.24 fold of purification and gave obtained 80 % of yield. The second step of purification obtained 0.624 mg/ml protein. This is the 5.09 fold of purification to gave 60 % of yield. The final step of purification, gave 0.310 mg/ml of protein. This is the 5.12 fold purification and gave 50 % yield and showed higher hemagglutination activity.

3.14. Purification by RP-HPLC.

The protein was purified using a non-linear gradient concentration of aqueous acetonitrile (buffer B). The elution of the protein was monitored at 280 nm (Fig 3.12). The purity was checked by RP-HPLC. The single peak appeared at the retention time of 2.01 min and this peak contains 95 % of the whole protein.

Fig 3.12. RP-HPLC purification profile of purified lectin
3.15. SDS-PAGE analysis

The purified lectin had molecular mass of 146 kDa protein with two bands were observed, and the subunit mass estimates on SDS-PAGE 89.848 and 56.316 kDa (Fig 3.13 and 3.14).

![SDS-PAGE analysis](image)

**Fig 3.13.** A. Native PAGE and B. SDS-PAGE analysis of crude and purified lectin from *B. geminata*

A. C. Crude sample; 1-6. Purified fractions

B. M. Molecular Marker Protein; 1-2. Purified lectin subunits
Fig 3.14. Native PAGE and SDS-PAGE analysis of crude and purified lectin from *B. geminata*

A. Lane 1. Crude sample 2. Purified Lectin

B. Lane 1. Molecular Marker Protein used were: Phosphorylase b (97,400), Bovine Serum Albumin (66,000), Ovalbumin (43,000), Carbonic Anhydrase (29,000), Lactoglobulin (18,400), Aprotinin (6,500), Lane 2. Purified lectin subunits
3.16. Discussion

Insects belongs to one of the major invertebrate super phyla, the protostomia which divergent over 500 million years ago during Precambrian and included the annelids, molluscs and arthropods. Because of the recent success in demonstrating the presence of agglutinins in many of these invertebrates (Ozeki et al., 1997; Kawsar et al., 2009; Dorrah et al., 2009; Cao et al., 2010; Fujii et al., 2011; Jing et al., 2011; Kong et al., 2011; Chen et al., 2011; Zhang et al., 2011 and Matsumoto et al., 2011), it was decided to extent investigations to insects, particularly concerned with some physicochemical parameters that govern the hemagglutination activity, stage specific agglutination activity, purification of the lectin and applications.

In the present investigation, cell free hemolymph of B. geminata contained the naturally occurring hemagglutinin against various mammalian red blood cells. The hemolymph sample as well as purified lectin was tested for hemagglutination activity using a battery of human and animal erythrocytes. The hemolymph of B. geminata agglutinates most of the erythrocytes. From the result, the highest and most consistent end point (titer) was achieved with rabbit erythrocytes which produced a titre as high as 2048 when fresh rabbit erythrocytes were used (Fig 3.3). This result was corroborated by the earlier reports, such as the lectin from the giant stick insect Extatosoma tiaratum (Richards et al., 1988). The hemagglutinin from the serum of the desert locust Schistocerca gregaria strongly agglutinates rabbit erythrocytes (Ayaad et al., 2009). The hemolymph lectin from S. littoralis showed highest activity on rabbit erythrocytes (EL Deep et al., 1990). Similarly the hemolymph lectin from the grasshopper Melanoplus sanguinipes showed high activity was recorded on rabbit erythrocytes (Stebbins and Hapner, 1985). The larval stage of the insect C. vomitoria hemolymph lectin showed highest activity on rabbit and rat erythrocytes (Mckenzie...
and Preston, 1992). The hemolymph lectin from the larvae of *S. exigua* showed highest activity on rabbit and human O erythrocytes (Pendland and Boucias, 1986). In contrast, the hemagglutinin from the hemolymph of *T. commodus* showed no activity with rabbit erythrocytes, but trypsin treated rabbit erythrocytes or trypsinated rabbit erythrocytes showed the activity with a titre of 16 (Hapner and Jermyn, 1981). A lectin (Dorin M) from the plasma of soft tick *O. moubata* showed highest activity on mouse erythrocytes (Kovár et al., 2000). The hemagglutination activity of hemolymph lectin from *Anticarsia gemmatatis* showed highest activity on human O, rabbit and sheep erythrocytes, when to agglutinate with the fungus *Nomuraea rileyi* (Pendland and Boucias, 1985).

In the present experiment, mouse erythrocytes showed very much lower activity among erythrocytes used. Similar results were obtained in the lectin from the larvae of a moth *Phalera flavescens*. In the human A1 erythrocytes the activity titre was 64, but in the enzyme (sialidase) treated A1 erythrocytes the titre was 4096 (Umetsu et al., 1993). In contrast, the hemagglutinating activity of larval hemolymph of *Leptinotarsa decemlineata* in human A erythrocyte gave higher activity than A⁺ erythrocytes (Stynen et al., 1982). The hemagglutinin titer against rabbit erythrocytes is clearly higher than the others. Obviously rabbit red blood cells might have more accessible or more reactive receptors on their membranes. The enzymes such as trypsin, papain and neuraminidase treated erythrocytes were also tested against the hemagglutination activity of *B. geminata* lectin (Fig 3.5). Trypsin and papain treated rabbit and human ABO erythrocytes showed increase in the activity as compared with untreated erythrocytes. Whereas, neuraminidase treated erythrocytes reduced the hemagglutination titer as low as untreated or original activity. Very high titres were found against trypsin-treated rabbit erythrocytes.
Similar results were recorded in Humoral lectin from *Heterometrus granulomanus* (Ahmed *et al.*, 1986) and sialic acid binding lectin from American spiders (Vasta and Cohen, 1984d). Trypsinated erythrocytes were, therefore used as hemagglutination indicator particles in all subsequent experiments. In fact, the protease treatment of red blood cells might have exposed more hidden carbohydrate receptors for the hemagglutinins. This indicates that the carbohydrate determinant of these RBCs became accessible only after partial removal of the glycoprotein coat from erythrocytes surface. Agglutination of erythrocytes of all species were found to be strong after trypsin and papain treatment owing to the removal of charged groups from the erythrocyte surfaces, and rendered zero by neuraminidase. This indicates that *B. geminata* agglutinin showed specificity for sialic acid. Changes of titers against formol-fixed erythrocytes were also described by Tripp (1966).

An attempt to gain insight concerning the mechanisms of the stage dependent hemagglutination titer level changes was as follows, in the first stage of the nymph hemagglutination titre were 512, but the second stage of the nymph hemagglutination titre of 1024 was recorded. In the third stage the hemagglutination titre increased 2048. Interestingly, the last stage, ie, just before metamorphosis, hemagglutination titre was found to be 4, which was more than 90% lesser titer as compared to the third stage. In the case of the adult dragonfly, hemolymph agglutination titre was found to be 1 (Fig 3.4). In the adult dragonfly the hemagglutination titre was almost lost as compared to the third stage of nymph. These results corroborated the earlier reports of Suzuki and Natori (1983), in which the *B. mori* agglutinin titer was rhythmic pattern of change during larval-pupal and pupal-adult ecdysis and suggested that agglutinins may be absorbed to the surface of decomposed larval tissues and may be functional in the immune surveillance of old tissue. Komano *et al.*, (1980, 1981)
reported that *S. peregrina* agglutinin changes during larval and pupation and suggest that lectin may be responsible for immuno surveillance, playing role in scavenging both invading foreign substances and decomposed self tissue fragments on pupation. El Moataz Bellah *et al.* (1988) studied agglutinin variation in relation to the larval stages of the larvae *Philosamia ricini*. In contrast, in *Halys dentata* hemagglutinin activity was found to decrease during apolysis-ecdysis in each instars and increased in intervening period. The titers increased with each developmental stage and were highest in adults (Pathak, 1991). This report was totally different from earlier proposed concept of Komano *et al.*, (1980, 1981), Suzuki and Natori, (1983) described that in holometabolous insects, complete reconstruction occurs during metamorphosis and lectin may be absorbed by degenerating larval tissues. Normally the lectin content is substantially variable during the developmental, individual, sexual, population, geographic and seasonal levels has been observed and although less frequently in carbohydrate specificity. In fact, the lectin content of tissue and body fluids vary during certain developmental stages and it may be related to cell-cell and cell-substrate recognition and tissue organization and are developmentally regulated.

In the present study, the purified lectin from hemolymph of *B. geminata* was incubated at different temperatures (Fig 3.6 and 3.7); the optimal activity was between 25°C and 30°C. Almost similar activity was observed in the Dorin M, a lectin from plasma of the soft tick *O. moubata* (Kovár *et al.*, 2000). The hemagglutinating activity of *Musca domestica* lectin was stable at temperatures up to 65°C (Cao *et al.*, 2010). In contrast, lectin from midgut of *Culex quinquefasciatus*, when incubated at 100°C, the 70% of the activity was lost (Ayaad, 2009). However, heat labile lectins were observed in some insects, such as, the orthopterans *Teleogryllus commodus*, (Hapner
and Jermyn, 1981), *M. sanguinipes* (Stebbins and Hapner 1985), the dipteran *Glossina fuscipes* (Ingram and Molyneux, 1990) and the orthopteran *S. gregaria* (Ayaad, 2004 and Dorrah *et al*., 2009). In the case of effect of pH on the hemagglutination activity, the activity was reduced at the extremes of pH and both were tolerant to alkaline rather than acid condition. Complete loss in activity occurred at pH 5.5 with 80% of the activity reduction at pH 9.0. The exact high activity was observed in the pH 7.6 with is in the stable range 7.5-8.0 (Fig 3.8). Similar results were observed in the hemolymph lectin from *G. fuscipes fuscipes* (Ingram and Molyneux, 1990) and a lectin from plasma of the soft tick *O. moubata* (Kovár *et al*., 2000). Serum lectin from the giant stick insect *E. tiaratum* showed an optimal range in the pH range of 7.2-10 (Richards *et al*., 1988). Meanwhile, *M. domestica* lectin showed a stable activity at a pH range of 4 - 8 (Cao *et al*., 2010).

The hemagglutination activity of the hemolymph sample was tested with different concentration of divalent cations such as CaCl$_2$, MgCl$_2$, ZnCl$_2$, MnCl$_2$, FeCl$_2$, HgCl$_2$, BaCl$_2$, MnSO$_4$ and MgSO$_4$. In this experiment MgCl$_2$ and BaCl$_2$ gave higher activity at 25 mM and 12.5 mM. FeCl$_2$ and HgCl$_2$ have no activity indicating that these ions were not involved during hemagglutination. MnSO$_4$ and MnCl$_2$ recorded very low activity (Fig 3.9). The requirements of divalent cations were very much involved in the hemagglutination activity. Increasing concentration of MgSO$_4$ showed the highest activity but the activity was low in lower concentration. The 25 mM and 12.5 mM concentration of CaCl$_2$ gave higher activity and decreasing concentration showed lower activity. The similar activity was observed in *M. domestica* lectin, that was also distinctive in that its hemagglutinating activity could not be inhibited by a variety of metal chlorides, except MnCl$_2$ and FeCl$_3$ (Cao *et al*., 2010). Interestingly, A novel lectin from *Cicada flammata*, the
hemagglutination activity was not affected in the presence of Mn$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, Fe$^{3+}$, Cu$^{2+}$ and Pb$^{2+}$ ions (Ye and Ng, 2010). The $G. fuscipes fuscipes$ agglutinin levels was lowered in the addition of heavy metal ions (Pb$^{2+}$ and Fe$^{2+}$) in the buffer (Ingram and Molyneux, 1990). The $B. geminata$ lectin activity was not affected in most of the metals whereas FeCl$_2$ and HgCl$_2$ affect the activities, indicating that the lectin was relatively stable.

The agglutination activity of the $B. geminata$ lectin was Ca$^{2+}$ dependent (it requires free calcium ions in the buffer) (Table 16). Similar result was observed in the Centruroides lectin which showed a decreased agglutination in the presence of EDTA and recovery of the activity after addition of Ca$^{2+}$ indicates divalent cation requirement (Vasta and Cohen, 1982a). The hemagglutination activity decreased in the absence of calcium and magnesium ions as observed in the agglutinin from the hemolymph of $L. migratoria$ (Drif and Brehelin, 1994). Ratcliffe et al., (1985) reported that most of the insect lectins activity was completely lost in the presence of EDTA. The calcium free or in presence of EDTA, complete loss of activity was observed in serum lectin of $E. tiaratum$ (Richards et al., 1988) and similar results was recorded in the hemolymph lectin from $M. sanguitipes$ (Stebbins and Hapner, 1985), hemolymph lectin from $T. commodus$ (Hapner and Jermyn, 1981). Ingram and Molyneux (1993) reported the three Glossina species of Testse fly showed the calcium and magnesium ion dependency. Similarly the hemolymph lectin from the larval stages of insect $C. vomitoria$ showed calcium ion dependency (Mckenzie and Preston, 1992) and also the lectin from Diamondback moth $Plutella xylostella$ (Madanagopal and Kim, 2007). In contrast, the soft tick $O. moubata$ lectin binding ability was not inhibited in the presence of EDTA. A lectin from hemolymph of $G. fuscipes fuscipes$ did not require the presence of divalent cations (Ca$^{2+}$, Mn$^{2+}$ or
Cu$^{2+}$ ions) for activity although an elevated concentration of Mg$^{2+}$ ions resulted in increased endpoint titers (Ingram and Molyneux, 1990). The erythroagglutinin from the hemolymph of scorpion, *Heterometrus bengalensis* has no divalent cation requirement for erythroagglutination (Basu *et al.*, 1984). The isolated agglutinin of *C. quinquefasciatus* lectin (Cqlec) as well as its crude form did not require Ca$^{2+}$ for expression of activity. When the final concentration of EDTA in the reaction mixture reached 50 mM, the hemagglutination activity was unaffected (Ayaad, 2009).

In the adsorption test each RBC types were found to completely or partly adsorb the red blood cell types and the efficiency of adsorption differ among the eight red blood cell types tested. When the sample was adsorbed to rabbit erythrocytes, these can’t cross react or adsorbed to other types that has the complete removal of agglutinating activity. These results indicate the involvement of more than one lectin in response of the RBC used in the study (Table 14). Similar results were observed in diverse hemolymph lectin in the cotton caterpillar *S. littoralis* (EL Deep *et al.*, 1990). Conforming results have been reported in invertebrates (Parrinello and Canicatti, 1982; Wright and Cooper, 1982, 1984) and in insects (Amirante, 1976; Pendland and Boucias, 1985; Madanagopal and Kim, 2007).

The sugar specificity of agglutinins in the hemolymph of *B. geminata* was assessed by hemagglutination inhibition efficiency by various sugar and glycoproteins. The hemagglutinating activity of the hemolymph of *B. geminata* was weakly inhibited (HA 1 liter=1) by the arabinose and the galactose showed lower inhibitory lectin activity (HAI liter=2). The N-acetyl neuraminic acid produced higher inhibitory activity (HAI liter=8) among used other sugars (Table 17). Expressed in terms of sialic acid content, BSM, fetuin exhibited much higher inhibitory power that the free sialic acid indicating that probably not only the terminal sugar is important in
the binding, but also subterminal sugars and conformational aspects of the oligosaccharide chains (Table 18).

In the present study remarkable similarities of the specificity of lectin enhanced inhibition of agglutination by protein bound NeuNAc have been observed in hemolymph of *T. commodus* (Hapner and Jermyn, 1981). The American spider *Aphonopelma sps* lectin inhibited sialic acid and their related compounds (Vasta and Cohen, 1984d). The naturally occurring hemagglutinins are found in the hemolymph of the scorpion *Paruroctonus mesaensis* Stahnke (Vasta and Cohen, 1983b), the lectin of *L. polyphemus* and scorpion *Androctonus australis* and *Centruroids sculpturatus* (Vasta and Cohen, 1982a; Vasta et al., 1982b). Similar specificity were also found by Shimizu et al., (1979) for the Japanese crab *T. tridentatus*, and by Bishayee and Dorai, (1980) for Indian horseshoe crab *C. rotundicauda*.

The serum lectin from the scorpion *Hadrurus arizonensis* stahnke was specific to sialic acid, since they were inhibited only by sialic acid, sialoconjucates or structurally related molecules (Vasta and Cohen, 1984b). In contrast most of the insect are galactose specific such that, lectin have been purified from the flesh fly, *S. peregrina* (Komano et al., 1980,1981), from *Hyalophora cecropia* ( Castro et al., 1987), from *Antheraea pernyi* (Qu et al., 1987), from *E. tiaratum* (Richards and Ratcliffe, 1990) and serum of the desert locust *S. gregaria* (Ayaad et al., 2009). But *Cicada flammata* lectin was inhibited by glucuronic acid and raffinose (Ye and Ng, 2010). In this respect, *B. geminata* lectin exhibited a rather restricted specificity when compared to a *L. polyphemus* and other chelicerate lectins which bind, although with different degree of affinity to sialic acids (Cohen, 1968; Roche and Monsigny, 1979), uronic acids (Nowak and Barondes, 1975), other 2-keto-3-deoxyacids (Pistole, 1979), Phosphorylcholine (Robey and Liu, 1981) and galactans (Cohen et al.,1975).
Purification of multiple lectin from serum of the desert locust *S. gregaria* by three pools, each containing one of the lectins, were obtained through (NH$_4$)$_2$SO$_4$ fractionation, ion-exchange chromatography on DEAE-cellulose and affinity chromatography on CNBr-activated Sepharose 4B (Dorrah *et al.*, 2009). The β-1,3-glucan-specific lectin from *B. discoidalis* was purified by gel filtration on a Bio-gel P300 column and affinity chromatography on a blue Sepharose CL-6B column (Chen *et al.*, 1999). An erythroagglutinin was found from the hemolymph of scorpion *H. bengalensis* and purified by gel filtration chromatography and ion-exchange chromatography (Basu *et al.*, 1984). The serum of the insect *Exatosoma tiaratum* contains heteroagglutinin and has been isolated with two-step procedure utilizing gel filtration and affinity chromatography using asialofetuin-sepharose 4B and Sepharose 4B (Richards *et al.*, 1988). A novel lectin was purified from *C. flammata* adsorbed on Q-Sepharose and unadsorbed on Affi-Gel blue gel and Superdex 75 gel filtration column (Ye and Ng, 2010). In the present study the *B. geminata* hemolymph lectin was purified through ammonium sulphate precipitation, affinity chromatography using fetuin sepharose and gel filtration using Sephacryl S-300HR (Fig 3.10 and 3.11).

The purified lectin from the insect *B. geminata* have the molecular mass of 146 kDa with two subunits. The subunit mass estimated on SDS-PAGE showed the two subunits 89.848 and 56.316 kDa (Fig 3.13 and 3.14). Results of the present study show remarkable similarities in the purified erythroagglutinin from the scorpion *H. bengalensis* which appears to be monomeric protein having a possible molecular weight between 146 and 148 kDa (Basu *et al.*, 1984). The lectin of the flesh fly has a molecular weight of 190kDa and consists of α (32kDa) and β (30kDa) subunits (Komano *et al.*, 1980). The purified β-1, 3-glucan-specific lectins from the sera of
several insect species have molecular masses of 520 kDa by gel filtration and subunit mass estimates of 80–82 kDa by SDS-PAGE (Chen et al., 1999). The *T. commodus* hemolymph lectin has the high molecular mass protein composed of 31,000 and 53,000 molecular weight polypeptide chains (Hapner and Jermyn, 1981). A dimeric lectin from *C. flammata* with a molecular weight of 60 kDa appeared as a single 30-kDa band in SDS-PAGE (Ye and Ng, 2010). The insect larvae *Pieris brassicae* lectin molecular weight was estimated to be 43kDa lectin, which constituted by two subunits which were estimated to be 23kDa molecular weight polypeptide chains. (Mauchamp, 1982). Three lectins were identified in the serum of the desert locust *Schistocerca gregaria* and the SDS/PAGE subunits had approximate molecular weights of 21.5, 44.5, and 43.9 kDa, respectively (Dorrah et al., 2009). The serum lectin from the giant stick insect *E. tiaratum* showed the molecular mass of 72-76 kDa and was probably composed of 6 non covalently linked subunits estimates of 12-14kDa (Richards et al., 1988). The purified lectin from the plasma of soft tick *O. moubata* has a molecular mass about 640 kDa with two noncovalently bound subunits closely around 37 kDa (Kovar et al., 2000). The *S. exigua* galactose binding lectin was comprised of large molecular weight aggregates of 100-700 kDa and it contain two subunits of 33.2 and 34.4 kDa (Boucias and Pendland, 1993). A lectin from the larvae of a moth *Phalera flavescens* showed the molecular mass of about 74 kDa and it consists of two different subunits glycosylated 18 kDa and unglycosylated 17 kDa subunits. (Umetsu et al., 1993).