CHAPTER II
PURIFICATION AND CHARACTERIZATION OF LECTIN FROM THE
MUSCLE OF VILLORITA CYPRINOIDES (GRAY, 1825)

2.1. Introduction

The phylum Mollusca is one of the most large, diversified and important groups in the animal kingdom which includes the familiar snails, slugs, clams, mussels, and octopus. The class bivalvia has about 7,500 species and was the second most diverse class of molluscs after gastropod, and some of them are of commercial importance, which comprised of animals unclosed in two shell valves, such as mussels, oysters, scallops and clams. In the long course of evolution, bivalves have developed an array of effective strategies to protect themselves from the attacks of various pathogens and environmental stresses. As invertebrates, bivalves rely exclusively on an innate, non lymphoid system of immune reactions. (Loker et al., 2004). The internal defense of bivalve is mediated by both cellular and humoral components. The cellular includes phagocytosis or encapsulation, with subsequent pathogen destruction via enzyme activity and oxygen metabolite release while the humoral includes various reactions mediated by series of molecules (Wootton et al., 2003). Among the many weapons in the chemical arsenal of bivalves are humoral defense factors such as agglutinins (lectins), ROS, antimicrobial peptides, and lysozymal enzymes (Canesi et al., 2002).

In bivalves, the first line of defense, among effectors, inhibitors of proteases and antimicrobial peptides have been studied. The inhibitors are known to target microorganism proteases to prevent host infection (Labreuche et al. 2006 a, b). Several of these molecules have been identified such as α2-macroglobulin (Ma et al.
2005), serine proteases inhibitor (serpin) (Gueguen et al. 2003, Tanguy et al. 2004) and metalloprotease inhibitor (Montagnani et al. 2001). The first antimicrobial peptides characterized were found in *Mytilus* (Charlet et al. 1996, Hubert et al. 1996) which include four families of peptides: defensin (Hubert et al. 1996, Mitta et al. 1999b), myticin (Mitta et al. 1999a), mytilin (Mitta et al. 2000b) and mytimicin (Mitta et al. 2000a). More recently, antimicrobial peptides were also isolated from other bivalves (Seo et al. 2005, Gueguen et al., 2006, Zhao et al. 2007, Gestal et al. 2007, Bettencourt et al. 2007). In addition, these effectors are present in mostly all phyla of the living kingdom.

Practically all classes and subclasses of invertebrate examined have lectins. These include crabs, snails, worms (helminths) (Greenhalgh et al., 1999; Hirabayashi et al., 1998), insects (Ingram and Molyneux, 1991; Kubo et al., 2001), molluscs and sponges (Müller et al., 1997). There are two types of soluble elements thought to play a role in defense and have been identified in the hemolymph of hard clams and many other marine bivalves: hemolysins and agglutinins (Chu, 1988). Lytic factors, generally protein in nature, have been demonstrated in the body fluids or tissue extract of sipunculids, annelids, arthropods, molluscs and echinoderms using vertebrate erythrocytes as experimental targets, and consequently named hemolysins (Weinheimer et al., 1969, 1970; Parrinello et al., 1979; Anderson et al., 1980; Parrinello and Rindone, 1981; Cenini, 1983; Tuan and Yoshino, 1984; Tuckova et al., 1986; Leippe and Renwrantz, 1988). Hemolysins, substances that lyse vertebrate erythrocytes, are present in the hemocytes, the cell-free hemolymph, and the shell fluid of hard clams (Anderson, 1981). In some cases, the killing properties against Gram-negative bacteria depend on proteins that also have hemolytic activity (Anderson and Chain, 1982).
Hemolytic lectin was reported in different invertebrates, such as echinoderm *Cucumaria echinata* (CEL-III), (Hatakeyama *et al*., 1995), mushroom *L. sulfureus* (Konska *et al*., 1994) and the seeds of *Croton tiglium* (Banerjee and Sen, 1981). In molluscs, different type of lectins are identified (Tripp, 1992; Dam *et al*., 1993; Bulgakov *et al*., 2004; Takahashi *et al*., 2008; Adhya *et al*., 2009; Zhang *et al*., 2009; Yang *et al*., 2010; Zhang *et al*., 2011; Kong *et al*., 2011; Chen *et al*., 2011; Li *et al*., 2011). However, except hemolysin, reports on the presence of hemolytic lectin in molluscan species are lacking. Consequently, in the beginning the significance of hemolysins to molluscs is unclear, but they may represent a lytic activity that could be activated against other non-self cells. Recently, the hemolytic lectin properties, mode of action, interaction studies and functiontional properties were well studied (Hatakeyama *et al*., 1995; Hatakeyama *et al*., 1996; Fujisawa *et al*., 1997; Oda *et al*., 1999; Nakano *et al*., 1999; Uchida *et al*., 2004; Yoshida *et al*., 2007). Now, the hemolytic lectins have emerged as significant molecules in all aspect of research especially in the cancer research. In this context, the present study has been carried out to screen the hemolytic lectin available in the bivalve mollusks. In the present chapter,

The major objectives of the investigation were

1. To detect and identify the occurrence of natural lectin from the muscle of *Villorita cyprinoides*

2. To characterize the physico chemical parameters of the identified lectin

3. To purify the lectin by affinity chromatography.

4. To analyze molecular weight by SDS- PAGE and MALDI-TOF
2.2. MATERIALS AND METHODS

2.2.1. Systematics

- **Kingdom**: Animalia
- **Phylum**: Mollusca
- **Class**: Bivalvia
- **Order**: Veneroida
- **Family**: Corbiculidae
- **Genus**: Villorita
- **Species**: *cyprinoides*
Figure 2.1. Fresh water clam *Villorita cyprinoides*
The black clam *V. cyprinoides* (Common Indian marsh clam), is the most important clam species found in estuaries, rivers, lagoons, man-made canals, marshes, and mangroves (Ravindran *et al.*, 2006). The black clams are located just below the surface of the soft bottom sediments. They are exploited primarily for the shell, while the meat is sold locally. The State of Kerala has been, by far, the leading producer of the species. Nearly all the landings, about 25,000 tons (t)/year are harvested in Vembanad Lake Kerala, The black clam shell is a valuable raw material required by various industries like limekilns, cement, carbide, medicines, pharmaceutical, fertilizers, pesticides etc. The biology and fishery of *V. cyprinoides* has been studied by Rasalam and Sebastian (1976), Sivankutty Nair (1975) Achary (1988), Kripa and Mathew (1993) and Laxmilatha and Appukuttan (2002). The black clam attains sexual maturity at a length of 11 to 15 mm (0.4 to 0.6 inch). It does not show sex reversal or hermaphroditism. It spawns twice a year, from May to August, and from January to late March.

### 2.2.2. Collection of Animals

The clams, *V. cyprinoides* were collected from the Anchalikadavu, a tributary of Thamirabharany River near Athencode, Vilavancode taluk, Kanyakumari district, Tamil Nadu. The collected animals were transported to the laboratory in plastic containers. In the laboratory, the animals were kept in plastic tanks with a continuous supply of fresh water.

### 2.2.3. Preparation of sample

The shell of the clam was cleaned with water and dried using absorbent paper. The muscle was dissected out by gently opening the shell and cleaned with distilled water. 100 mg of dissected muscle in 1ml of ice cold TBS buffer (pH 7.6) and homogenized in a tissue homogenizer, centrifuged at 4000 × g for 10min at
The clear supernatant was dialysed against TBS buffer and the dialyzed extract was stored at -20°C for further study.

2.2.4. Preparation of erythrocytes (Red blood cells)

Human and other blood samples from different animal species, obtained by venous or cardiac puncture, were collected in sterile Alsever’s solution (30 mM sodium citrate, 77 mM NaCl, 114 mM glucose, 100 µg/ml neomycin sulphate, 330 µg/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).

2.2.5. 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 RBC were washed with 0.9% saline by centrifugation for 400 × g in 5 min at room temperature and finally fixed by suspending the RBC pellet in TBS (pH 7.6) containing 10% formaldehyde for 24 h at 10°C. Fixed RBC were extensively washed in 0.9% saline and unless otherwise specified, resuspended in TBS.

2.3. Screening and identification of lectin from V. cyprinoides

2.3.1. Determination of hemolytic lectin

2.3.1.1. Hemagglutination assay

Hemagglutination assay was performed using different human and animal erythrocytes such as human A, A₁, B, and O, rabbit, goat, dog, pig, cow, hen, turkey, squirrel and mouse. Twenty five microliter aliquots of serial twofold dilutions of the sample in TBS containing 10 mm CaCl₂ were mixed with same volume of 1.5 % (v/v) suspension of erythrocytes in TBS. After incubation for 1h at room temperature, the
extent of agglutination or hemolysis was examined visually. The hemagglutination activity was expressed as a titer, i.e., the reciprocal of the highest dilution giving detectable agglutination.

2.3.1.2. Determination of hemolytic activity

According to the method described by Hatakeyama et al. (1994), hemolytic activity was determined either by visual examination of lysis of the erythrocytes under the same condition as for the hemagglutination assay or by measurement of the absorbance of 550 nm due to hemoglobin released from the erythrocytes.

2.3.1.3. Erythrocyte agarose diffusion test

Hemolytic assay was carried out as described by Mohrig et al. (1996). The hemolytic activity was detected using a 1mm thick agarose layer containing 1.5% erythrocytes in 0.9% NaCl. Wells of 4 mm diameter were cutout from the agarose and filled with 5µl of sample. The agarose wells were incubated for 16 hours at room temperature. After the period of incubation, the clear zones of hemolysis if any around the wells were noted. The diameter of plaques were measured and used as a degree of hemolytic activity.

2.3.1.4. Photometric determination of erythrocyte lysis- Scanning experiments

According to the method described by Eschbach et al. (2001) the Photometric scans were performed using completely lysed rabbit erythrocytes with purified lectin (1.5 % cells ml⁻¹) and controls as purified samples (V. cyprinoides). Absorption was scanned from 200 to 700 nm with ELICO-SL-159, UV/Visible spectrophotometer in a standard cuvette with an effective light path of 10 mm.

2.3.1.5. Cross adsorption tests

Muscle samples (300µl) 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 at 400 × g for 5 min at 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 hemolytic activity against all the nine red blood cells.

2.3.1.6. Effect of pH and thermal stability

The pH stability of the lectin was determined by extensive dialysis of the lectin (1 mg/mL) against buffers of different pH values ranging from pH 3.5-10.0 using acetate buffer, Tris-HCl and glycine NaOH. The pH of the lectin solution was adjusted to pH 7.6 by the addition of 0.1 N HCl or 0.1 N NaOH before hemagglutination activity was determined. The dialysates were centrifuged and the supernatant was tested for hemagglutinating activity using rabbit RBC.

The thermal stability of hemolytic lectin was examined by holding 100 µl of muscle sample and purified hemolytic lectin for 30 min at the temperatures ranging from 10 to 80°C. All the samples were centrifuged at 400 × g for 10 min at R.T and the clear supernatant was used to determine the hemolytic activity using rabbit erythrocytes.

2.3.1.7. Divalent cation dependency and EDTA sensitivity

The initial hemolytic activity of muscle sample (untreated) and the purified lectin was determined in Tris buffered saline (TBS) containing 10 mM CaCl$_2$. The muscle sample (each 500µl) and purified lectin were dialyzed extensively against TBS (to test divalent cation dependency) or in TBS - EDTA (to examine EDTA sensitivity) at 15°C. The samples were dialyzed against TBS - EDTA and were re-equilibrated by dialysis in TBS. After centrifugation at 400 × g for 5 min at room temperature, the supernatant was used to determine the hemolytic activity using 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).

### 2.3.1.8. Hemolytic Inhibition Assay

The sugars were prepared as 0.2 M solution in TBS. Purified lectin (2048 HU) 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, trehelose, mannitol, xylose, raffinose, cellobiose, dextrose, 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 and incubated at room temperature for 1 h. After incubation, 25 µl of preprepared 1.5% rabbit erythrocyte suspension was added to the mixture and incubated for 1 h. After incubation, the hemolytic activity was examined. The results were expressed as the minimum concentration of the inhibitor required to terminate the hemolytic activity.

### 2.3.1.9. Assay of hemolytic activity

This was carried out according to Kabat and Mayer (1967) and Banerjee and Sen, (1981). Rabbit erythrocytes were washed 3–4 times with TBS buffer containing 0.1 M NaCl, pH 7.6 and was used to prepare 0.5, 1, 1.5, 2, 2.5 and 3% of erythrocyte suspension (v/v). 25 µl of this each suspension was incubated at 25°C with an equal volume of lectin solution containing 18 mg - 0.07 mg/ml of lectin (kinetics of hemolysis). Aliquots of reaction mixture were withdrawn at definite time intervals, diluted to 2 ml with chilled TBS. The absorbance of the erythrocyte lysed mixture was read at 541 nm against a blank without lectin. Hemolytic activity was expressed as percentage of red cells lysed under the conditions of assay. Hemolytic activity was
determined either by visual examination of lysis of the erythrocytes under the same condition as for the hemolytic assay or by measurement of the absorbance of 541nm using ELISA reader (Cyber Lab).

2.4. Purification of lectin from *V. cyprinoides*

2.4.1. Preparation of DEAE-cellulose purification matrix

5 g DEAE cellulose powder was taken and mixed with TBS buffer and allowed to settle and decant the supernatant. The slurry of the matrix was washed with distilled water for 5 min under agitation for two times and allowed to settled and discard the supernatant. Washed matrix was treated with 1N NaOH solution (300 ml). DEAE-cellulose matrix was slowly added to 1N sodium hydroxide with gentle stirrering for 30 min till the pH reached to 13. Discard the sodium hydroxide solution and washed the matrix with double distilled water until pH reached 8.0. Then the fine particles were removed after settling. Then the matrix was replaced with 1 N hydrochloric acid with gentle stirrering for 30 min, till their pH reached to 1.0. Resin was washed with double distilled water until pH reached up to 3.0. After discarding the distilled water and the replaced it with 10 x buffers (500 mM Tris HCl pH 7.6) and gave gentle stirring for 30 min. After that discarded the 10x buffer and the matrix was equilibrated with 50 mM Tris - HCl pH 7.6 and the fine particle were removed. The suspension of DEAE-cellulose resin was transferred onto a glass column for immediate use or the equilibrated DEAE-cellulose slurry was resuspended in buffer containing sodium azide and stored it in 2-8°C for further use.

2.4.2. DEAE-cellulose column chromatography

Swollen DEAE-cellulose resin (Himedia) was pretreated in appropriate buffer. The resin was packed into a glass column (1 x 30 cm) to reach approximately 15 cm height, then equilibrated with 3 volumes of 50 mM Tris-HCl, pH 7.6 at 6-8 °C
with flow rate of 0.7-0.8 ml/min using peristaltic pump (Enter Tech Express, ENPD-100). The dialyzed crude extract was applied to a column of DEAE-cellulose column (HiMedia). Proteins were eluted with 100 ml of 50 mM Tris-HCl, pH 7.6 and followed with 100 ml of a stepwise gradient of 0.1-3M NaCl in 50 mM Tris-HCl, pH 7.6. The column effluent was collected, as 2 ml per fraction. All fractions were monitored at 280 nm using spectrophotometer (ELICO-SL-159, UV/Visible). The samples were then dialyzed against 25 mM Tris-HCl, pH 7.6 and again concentrated and freeze-dried (-70°C) until used.

2.5. Affinity chromatography

2.5.1. Sample preparation

The DEAE-cellulose purified active fraction was used for affinity chromatography purification. The sample was dialyzed by using cold TBS (pH 7.6), overnight at 4°C. The dialyzed sample was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was stored at -80°C.

2.5.2. Preparation of CNBr-activated fetuin Sepharose 4 Fast Flow

2.5 g freeze dried CNBr-activated sepharose 4 Fast Flow powder was swelled with 1 mM HCl for ~15 min. at room temperature. Transferred the gel to sintered glass funnel and washed with 200 ml of 1 mM HCl. The slurry was stirred with help of glass rod for about 20 minutes until matrix was swollen, then applied vacuum to remove all liquid. The matrix was washed with 100 ml 1mM HCl at three times and the matrix was dried each time.

2.5.3. Coupling of Fetuin with CNBr-activated sepharose 4 Fast Flow

After washed 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 shook 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.

2.5.4. CNBr-activated fetuin Sepharose 4 Fast Flow Affinity chromatography

2 ml of DEAE-Cellulose purified active fraction was used for affinity chromatography purification and applied to 2.5g of CNBr-activated fetuin sepharose 4 fast flow glass columns previously equilibrated with TBS (pH 7.6) at 4°C. After column packing, it was washed with TBS until it attains an OD of 0.002 at 280 nm. This is to remove the unwanted or unbinding proteins from the column, so as to obtain homogenous lectin protein. The elution was done with elution buffer containing 0.1M galactose 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. The fractions were taken OD at spectrophotometer of each fraction. The fractions having high protein content were identified using hemolytic assay. After the identification of
active fraction, it was dialyzed against distilled water for overnight at 4°C. The protein content was estimated using Lowry et al., (1951) method. The dialysates was aliquoted and stored at -20°C.

2.6. Gel filtration chromatography

2.6.1. Preparation of gel filtration matrix Sephadex G-75

The gel filtration column Sephadex G-75 was used to initially fractionate the galactose elution. 2g of Sephadex G-75 powder was swelled using distilled water and stirred it well and allowed to settle. The fine particles were removed from the supernatant by suction. The slurry was equilibrated with 50 mM TBS buffer pH 7.6 and stirred it well and allowed to settle and decanted the supernatant. The elution buffer (TBS with 50 mM Tris 140 mM NaCl pH 7.6) was added to the gel at 4 or 5 times to settle the gel volume. The Sephadex gel was gently stirred and allowed to settle and fine particles were removed. Buffer addition, gel settling, and decantation were repeated at three times. The equilibrated gel was kept at 2°C-4°C overnight before packing the columns.

2.6.2. Purification of clam lectin by Sephadex G-75

The swelled gel was degassed and packed in the glass column. (0.6 × 100 cm). Sephadex gel was uniformly packed without air bubbles. Then the gel was equilibrated with elution buffer. After equilibration, the dialyzed affinity purified active sample was applied to the column. Elution was done with the help of elution buffer (TBS) and the fractions were collected at the flow rate of 2 ml/20 min in propylene tubes. The OD value of each fraction was observed at 280 nm. Active fractions were identified through the hemolytic assay and protein content was estimated using Lowry et al., (1951) method.
2.7. HPLC purification

The purity of the already purified sample was checked in RP-HPLC. Analysis was carried out in a Reverse Phase HPLC (Cyberlab, USA) using C\textsubscript{18} column (250 × 4.6mm) equipped with a C\textsubscript{18} guard column. The gel had an average pore size of 300Å and its particle size was 5 µm. In order to prepare the column for chromatographic run, the following procedure was carried out. The HPLC grade 65% of acetonitrile and 35 % of dH\textsubscript{2}O with 0.1% of TFA was run for 8 min at 1ml/min. The eluent was run until the column had been equilibrated, as indicated by the baseline stability. Protein (20µl) of Sephadex G-75 purified fraction was filtered through the 0.22 mm syringe filter and loaded for each chromatographic run and the column was run with isocratic elution. Fractions were eluted out at the flow rate of 1ml/min. The active peak was collected and concentrated using Speed Vac. The concentrated samples were used for native PAGE and SDS-PAGE analysis.

2.8. Protein quantification

Protein concentration was determined following the method of Lowry et al., (1951) using the Protein assay Kit (Xpression Biotek) and bovine serum albumin as the standard. The reagents were mixed with specified ratio to make a working reagent. Albumin standard (2 mg/ml) was diluted in the series (2,000, 1500, 1000, 750, 500, 250, 125, 50, 25 and 5 µg/ml). In a 96- well round-bottom microtiter plate (Torson India), 10µl of the standard dilution and samples were applied into each well. Ten µl of the diluents was used for blank wells. The working reagents (200µl per well) were applied and the microtiter plate was shaken on a plate shaker for 30 secs. The microplate was covered and incubated at 36°C for 30-45 min. The microplate was kept at room temperature for 5 min and inserted into the ELISA reader (Cyberlab, USA). The absorbance was measured at 560 nm. The standard curve was documented
and the protein concentration of the samples was calculated from the standard curve. The protein content was also measured at 280 nm with the NanoDrop™ 8000 Spectrophotometer (Nano Drop Tech. Inc., USA).

2.9. Molecular weight determination

2.9.1. Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of *V. cyprinoides* hemolytic lectin was carried out by the method of Laemmli, (1970) using a 5% stacking gel (pH 6.8) and a 10% separating gel (pH 8.8) in Tris-glycine buffer (pH 8.3). Prior to SDS electrophoresis, hemolytic lectin (3.5 mg protein) was dissolved in sample buffer (0.06 M Tris-HCl buffer, 4% SDS, pH 6.8) that did contain 5% L-mercaptoethanol. A mixture of standard medium range molecular weight marker proteins was also prepared identically. All samples were heated (10 min, 100°C), cooled to room temperature and electrophoresed (5 mA per sample, 20°C in a vertical gel measuring 90× 90 × 0.7 mm). The proteins were silver stained.

2.9.2. Molecular weight determination by MALDI-TOF

Molecular mass of the purified lectin was determined by matrix assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry using Shimadzu Biotek AXIMA™ series systems CFR plus system equipped with a 337-nm nitrogen laser. Mass spectra were acquired in reflectron mode under 8-keV acceleration voltage and positive detection. Samples were prepared by mixing directly onto the target 1 µl of the reaction products and 1 µl of a 2, 5-dihydroxybenzoic acid matrix and allowing the mixture to crystallize at room temperature.
2.10. RESULTS

2.10.1. Hemolytic activity of the muscle extract of *V. cyprinoides*

The muscle extract of *V. cyprinoides* showed the hemolytic activity against the erythrocytes such as, human ABO, rabbit, goat, dog, pig, hen, turkey, squirrel and mouse (Fig 2.2). The muscle extract showed the maximum hemolytic activity titre with rabbit erythrocytes. The extract showed better activity against mouse erythrocytes than others. Among human erythrocytes, the B group erythrocytes gave strong hemolytic titre and the low activity was recorded in the A and O group erythrocytes. The hemolytic activity titer was relatively higher in human A1 erythrocytes as compared with human A erythrocytes. The turkey erythrocytes showed less activity. Based on hemolytic activity titre obtained, the erythrocytes could be graded as rabbit > mouse > pig>human B> squirrel = goat = hen > dog > human O = turkey = cow > human A.

**Fig 2.2.** Hemolytic titers of the muscle extract of Indian marsh clam

*V. cyprinoides*
2.10.2. Hemolytic assay (EADT)

The hemolytic assay showed clear hemolysis plaques in the erythrocytes treated with crude as well as purified lectins. The plaques measured 6 mm in diameter (Fig. 2.3). This result showed that the purified lectin had hemolytic property of erythrocytes.

![Hemolysis of rabbit erythrocyte (In blood agar) by the lectin from of clam V. cyprinoides](image)

**Fig. 2.3.** Hemolysis of rabbit erythrocyte (In blood agar) by the lectin from of clam *V. cyprinoides* A: Hemolysis of rabbit erythrocytes by crude sample, resulting in hemolysis of the erythrocytes and formation of hemolytic circle (→). B: Hemolysis of rabbit erythrocytes by purified hemolytic lectin, resulting in hemolysis of the erythrocytes and formation of hemolytic circle (→)

2.10.3. Determination of hemolytic activity

In this assay, the rabbit erythrocytes showed higher OD value after treated with muscle extract (Table 8). Among human erythrocytes, ‘B’ erythrocytes recorded highest OD value of 0.236 and the ‘A’ group with lowest value of 0.201. In the ‘O’ group erythrocytes also recorded lowest OD value of 0.218, but relatively high OD value as compared with ‘A’ group erythrocytes. In the control, the OD value ranged between 0.070, 0.084, 0.079 and 0.069 in rabbit and human erythrocytes.
Table 8. Hemolytic activity of the muscle extract of Indian marsh clam *V. cyprinoides* using spectrophotometer and absorbance at 550 nm

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Erythrocyte suspension</th>
<th>Control OD</th>
<th>Experiment OD (550 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>1.5%</td>
<td>0.070</td>
<td>0.401</td>
</tr>
<tr>
<td>human A</td>
<td></td>
<td>0.069</td>
<td>0.201</td>
</tr>
<tr>
<td>human B</td>
<td></td>
<td>0.084</td>
<td>0.236</td>
</tr>
<tr>
<td>human O</td>
<td></td>
<td>0.079</td>
<td>0.218</td>
</tr>
</tbody>
</table>

2.10.4. Hemolytic activity of lectin on enzyme treated erythrocytes

Results on the hemolytic activity of trypsin treated rabbit and human ABO erythrocytes showed higher titer value of 2048, 64, 128 and 16 and very well augmented the hemolytic titer as compared with the original activity or untreated red blood cells. The papain treated rabbit and human ABO erythrocytes showed lower titer value of 32, 2, 16 and 4 and as low as half of the original activity. The neuraminidase treated rabbit and human ABO erythrocytes showed very low titer value (Fig 2.4).

Fig 2.4. Effect of enzyme treatment of erythrocytes on hemolytic activity of the muscle extract of Indian marsh clam *V. cyprinoides*
2.10.5. Scanning experiments

The spectrum of completely lysed rabbit erythrocytes from 200 – 700 nm showed the absorption peaks at 238, 264, 364, 444, 542, 578 respectively (Fig 2.5). The maximum absorption at 238 nm. Absorbance of this extract at 238 nm did not interfere with the absorbance of lysed erythrocytes at the same wavelength. But below the absorbance 600 nm interfere lysed erythrocytes and the purified lectin.

![Absorption Spectra](image)

**Fig.2.5.** The spectrum of completely lysed erythrocytes with hemolytic lectin and a purified hemolytic lectin. Absorption (y-axis) was scanned from 200 to 700 nm (x-axis).
Fig. 2.6. Comparative measurement of light absorption (y-axis) of different concentrations of lysed erythrocytes (x-axis) at 238 and 542 nm. Formulae indicate respective equations and coefficient of correlation ($r^2$ values) for the resulting curves. Parallel measurements of increasing concentrations of completely lysed rabbit erythrocytes at 238 and 542 nm (Fig. 2.6) showed, for both wavelengths, a linear correlation of absorption and cell concentration up to the maximum of 1.5%. Absorptions measured at 238 nm were many folds higher than the respective absorptions measured at 542 nm. Absorption at 600 nm can be used as a reference to eliminate non-specific absorption of microtitre plates, because neither lysed erythrocytes nor lectin fragments showed considerable absorption at this wavelength.
2.10.6. Effect of temperature on activity of clam lectin

The activity of the muscle extract and purified lectin was stable between 25-30°C. The hemolytic activity showed a gradual decrease after incubating above 30°C. The activity was completely lost when incubated above 62°C (Fig 2.7). In general, results of the present study showed that the lectin was heat sensitive.

Fig 2.7. Effect of temperature on hemolytic activity of the lectin of clam

V. cyprinoides

2.10.7. Effect of pH on clam lectin

The activity of the muscle extract and purified lectin was stable between pH 7.5-7.8. The hemolytic activity showed a gradual decrease after pH 8. The activity was completely lost when incubated above pH 9.8 and below pH 5.5 (Fig 2.8). In general, results of the present study showed that the lectin was active in between pH 7 to 8.
Fig 2.8. Effect of pH on hemolytic activity of the lectin of clam *V. cyprinoides*

![Graph showing effect of pH on hemolytic activity](image)

2.10.8. Cross adsorption test

Absorption of the samples with any one of the erythrocytes used was sufficient to remove the hemolytic activity for other erythrocytes used. Human ABO, rabbit, cow, hen, pig and goat were used for cross adsorption test. In this cross adsorption tests, each red blood cell types were found to completely or partly adsorb red blood cell types and the efficiency of adsorption differs among the eight red blood cell types tested. 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 group erythrocytes and cross react with other erythrocytes especially in rabbit erythrocytes recorded the titer value of 128, the animal erythrocytes showed low hemolytic activity. When pig erythrocyte was adsorbed with lectin and it cross adsorb with human B and O red blood cell. When goat erythrocyte was adsorbed with lectin and it cross adsorb with mostly all red blood cell types. In the case of cow and hen erythrocytes, they showed cross adsorb with mostly all red blood cell types (Table 9).
The hemolytic lectin was highly specific to the rabbit erythrocytes surface carbohydrates.

**Table 9.** Hemolytic titer of clam *V. cyprinoides* hemolytic lectin after adsorption with different erythrocytes.

<table>
<thead>
<tr>
<th>Muscle extract sample adsorbed with red blood cells of</th>
<th>Hemolytic titre against red blood cell types tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human A</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
</tr>
<tr>
<td>Human A</td>
<td>0</td>
</tr>
<tr>
<td>Human B</td>
<td>0</td>
</tr>
<tr>
<td>Human O</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0</td>
</tr>
<tr>
<td>Cow</td>
<td>2</td>
</tr>
<tr>
<td>Hen</td>
<td>4</td>
</tr>
<tr>
<td>Pig</td>
<td>0</td>
</tr>
<tr>
<td>Goat</td>
<td>2</td>
</tr>
</tbody>
</table>

### 2.10.9. Effect of metal cation on hemolytic activity

In this experiment, the hemolytic activity of the 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$_4$. The higher activity was observed with addition of 50 and 25 mm MgCl$_2$. The hemolytic activity of the lectin was also high when treated with 100, 50 and 25 nm of CaCl$_2$ and BaCl$_2$. The MnSO$_4$ gave low hemolytic activity in at higher concentration, but lower concentrations of 12.5 and 6.24 mm showed higher activity. Addition of 100 and 50 mm concentration of MnCl$_2$ showed higher activity, but the lower concentration gave lower hemolytic activity. In the case of ZnCl$_2$, the concentration of 12.5 to 100 mm gave same hemolytic titer. The addition of different concentration of FeCl$_2$ and HgCl$_2$ showed no activity even at lower concentration also (Fig 2.9).
**Fig 2.9.** Effect of metal cation on hemolytic activity of the muscle extract of Indian marsh clam *V. cyprinoides*.

![Graph showing effect of metal cation on hemolytic activity]

**2.10.10. Effect of cation dependency (CaCl$_2$ and without CaCl$_2$) and EDTA sensitivity**

When the hemolytic activity of the sample was tested in the presence of varying concentration of EDTA, there was great change in the hemolytic titre. The activity was very low when treated with low concentration of EDTA. CaCl$_2$ enhanced the hemolytic activity. In general, CaCl$_2$ was required for the hemolytic activity of this lectin (Table 10).

**Table 10.** Effect of cation dependency (CaCl$_2$ and without CaCl$_2$) and EDTA sensitivity on hemolytic activity of the muscle hemolytic lectin of clam *V. cyprinoides*

<table>
<thead>
<tr>
<th>Concentration of metals mM</th>
<th>EDTA</th>
<th>CaCl$_2$</th>
<th>Without CaCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
<td>1024</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>1024</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>1024</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>1024</td>
<td>1</td>
</tr>
</tbody>
</table>
2.10.11. Hemolytic inhibition (HLI) activity

The sugar specificity of the hemolytic lectin of muscle of *Villorita cyprinoides* were assessed by the hemolytic inhibitor efficiency of various sugar and glycoproteins of the 27 carbohydrates (mono-di-oligo and polysaccharide) and glycoprotein (Fig 13). The hemolytic activity of the purified lectin was tested against trypsinized rabbit erythrocytes. In this experiment low inhibitory titer was recorded by the sugar arabinose at the concentration of 200 mM and their relative inhibitory potency was 25. The simple hexose, galactose and their amino derivative such as galactosamine were moderately inhibiting hemolytic activity at the concentration of 50 mM, 50 mM their relative inhibitory potency was 100. But their N-acetylated derivatives such as N-acetyl galactosamine (GalNAc) as well as methyl β-D galactopyranoside, p-Nitrophenyl α-D-galactopyranoside did not inhibit the hemolytic activity at any of the concentration even at 200mM. The hemolytic inhibition titer of 2 was observed by the sugar glucosamine at the concentration of 100 mM and their relative inhibitory potency was 50. Similarly, the hemolytic activity was not inhibited by the N-acetylated simple sugars such as N-acetyl neuraminic acid (NeuAc). Such that other carbohydrates such as glucose, lactose, L-fucose, mannose, sucrose, fructose, ribose, maltose, melibiose, trehalose, mannitol, xylose, raffinose, cellobiose, dextrose, sorbitol did not inhibit even at low concentrations (Table 11). The higher inhibitory titer was recorded by glycoprotein and fetuin at the concentration of 0.625 mg and the relative potency was 100. Similarly asialofetuin, inhibit at low titer at the concentration of 5 mg and their relative potency was 12.5%. Bovine sub maxillary mucin did not inhibiting hemolytic activity even at a concentration of 10 mg ml⁻¹ (Table 12). Results clearly indicated that the isolated lectin was a galactose specific lectin.
Hemolytic inhibition potency of the sugars are graded as Fetuin > galactose > galactosamine > glucosamine = asialofetin > arabinose.

**Table 11.** Hemolytic inhibition (HLI) activity of the muscle hemolytic lectin of clam *V. cyprinoides* by various sugars.

<table>
<thead>
<tr>
<th>Inhibitor tested</th>
<th>Maximum concentration tested (mM)</th>
<th>Hemolytic inhibition (HLI)</th>
<th>Minimum inhibitory concentration (mM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative inhibitory potency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>200</td>
<td>4</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>200</td>
<td>4</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>200</td>
<td>2</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Arabinose</td>
<td>200</td>
<td>1</td>
<td>200</td>
<td>25</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>N-acetyl neuraminic acid</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>

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

<sup>b</sup> No inhibition
Table 12. Hemolytic inhibition (HLI) activity of the muscle hemolytic lectin of Indian marsh clam *V. cyprinoides* by various glycoproteins.

<table>
<thead>
<tr>
<th>Inhibitor tested</th>
<th>Hemolytic inhibition (HLI)</th>
<th>Minimum conc: for inhibition (mg)</th>
<th>Relative inhibitory potency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin (10mg)</td>
<td>16</td>
<td>0.625</td>
<td>100</td>
</tr>
<tr>
<td>Asialo fetuin</td>
<td>2</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>Mucin (BSM)</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

![Figure 2.10](image)

**Fig.2.10.** Hemolytic inhibition assay 2048 hemolytic activity titer of lectins from the clam *V. cyprinoides*.

Each well of the microtiter plate contained 25 μl of lectin, 25 μl of a 1.5 % suspension of rabbit erythrocytes and 25 μl of 0.2 M sugar solution in Tris buffered saline, pH 7.6. The hemolysis of erythrocytes form a pale red coloured liquid that covers the
whole well; where no hemolysis occurs, the cells form a button at the bottom of the well. Picture taken after 2 hours at room temperature (Fig 2.10).

2.10.12. Kinetics of hemolysis

Kinetics of hemolysis of red blood cells (RBC) in the presence of different concentration of hemolytic lectin was measured spectrophotometrically. The hemolytic process in a rapid phase obeys a first order rate law. To gain a better understanding of the mechanism of hemolysis in the presence of hemolytic lectin, the hemolysis of a 0.5 to 3% suspension of rabbit erythrocytes was tested with different lectin concentrations in the range of 18 mg – 0.07 mg/ ml. It revealed that from the figure 2.11. The ‘hemolysis vs. time’ course is sigmoidal and is characterized by three distinct regions. one is a prelytic period or lag phase during which there is relatively little or no hemolysis, a period of rapid hemolysis during which a linear relation between per cent hemolysis and time and a static phase during which there is no further lysis. The sigmoidal nature of binding of the lectin molecule to rabbit erythrocyte surface and the final act of hemolysis. In the experiment, hemolysis was rapid when the addition of high lectin concentration, but the turbidity decreased whereas high percentage (3%) erythrocyte suspension produces lengthy prelytic period and partial lysis occurred. In contrast 1.5% erythrocyte suspension produces short prelytic period and complete lysis was occurred.
Fig 2.11. (a - f) Kinetics of hemolysis at different concentration of lectin with different percentage of rabbit erythrocytes.
2.11. DEAE-cellulose column chromatography purification

When the clam *V. cyprinoides* muscle extract sample was passed through a DEAE-cellulose column, three major peaks were observed (Fig 2.12). Among these, one major first peak had the hemolytic activity and is found in fractions of 2, 4, 5, 6, 7th and that was in unadsorbed fraction and another two peaks did not have hemolytic activity (eluted with NaCl by stepwise gradient) and that was adsorbed fraction. In this chromatographic purification, the desired protein did not adsorbed in this purification matrix. In the unadsorbed fraction number 3rd had very high activity. The other fractions 2, 4, 5, 6, 7th also showed lytic activity. The remaining fractions did not showed any activity. The high active fraction was identified through hemolytic assay. The protein content was estimated by Lowry *et al.*, (1951)

![Fraction Number vs A280, Hemolytic activity, NaCl](image)

**Fig 2.12.** Analysis of hemolytic lectin of *V. cyprinoides* by anion exchange chromatography in a DEAE-cellulose
2.12. CNBr-activated fetuin Sepharose 4 fast flow affinity chromatography

The galactose binding lectin was initially isolated from CNBr activated fetuin Sepharose 4 Fast Flow column. The protein (hemolytic lectin) was bound to the matrix and eluted primarily with 100mM galactose. The protein in the 100mm galactose elution fraction (hereafter called galactose elution) was visualized with SDS-PAGE. The galactose elution was used for the majority of further purification. From this CNBr-activated fetuin Sepharose 4 Fast Flow Affinity chromatography column yielded, three peaks, one major peak and two minor peaks. The adsorbed fraction (ie 59\textsuperscript{th} fraction) had high hemolytic activity and 57, 58, 61, 62\textsuperscript{nd} fractions also showed the hemolytic activity (Fig 2.13). Further more two minor peaks were also observed without any activity.

![Graph](image)

**Fig 2.13.** Elution profile of the clam *V. cyprinoides* muscle hemolytic lectin on CNBr-activated fetuin Sepharose fast flow column by light absorption at 280 nm.
2.13. Purification of clam hemolytic lectin by gel filtration chromatography using Sephadex G-75

Separation performed on the Sephadex G-75 column had a one major peak with additional minor two peaks at 280 nm (Fig 17). From this, the major peak showed the hemolytic activity. Fraction number 13 showed higher hemolytic activity and 12, 14, 15, and 16\textsuperscript{th} fractions also showed the hemolytic activity. Subsequently the 13\textsuperscript{th} fraction with high hemolytic activity was used in further characterization studies. The major broad peak resolved with the Sephadex G-75 contained 36.6 and 26.52 kDa band when fractions were examined with reducing SDS-PAGE (Figure 2.14)

![Elution profile of clam V. cyprinoides muscle hemolytic lectin on Sephadex G-75 column by light absorption at 280 nm.](image)

**Fig 2.14.** Elution profile of clam *V. cyprinoides* muscle hemolytic lectin on Sephadex G-75 column by light absorption at 280 nm.
Table 13. Summary of purification profile of *V. cyprinoides* hemolytic lectin

<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</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>10</td>
<td>25.55</td>
<td>1024</td>
<td>10240</td>
<td>40.078</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>12</td>
<td>2.9</td>
<td>512</td>
<td>6604</td>
<td>176.5</td>
<td>4.4</td>
<td>64.49</td>
</tr>
<tr>
<td>CNBr-activated Fetuin Sepharose</td>
<td>12</td>
<td>1.87</td>
<td>512</td>
<td>1536</td>
<td>273.79</td>
<td>6.84</td>
<td>15</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>10</td>
<td>0.35</td>
<td>128</td>
<td>1280</td>
<td>365.7</td>
<td>9.14</td>
<td>12.5</td>
</tr>
</tbody>
</table>

The clam hemolytic lectin was purified by three steps, DEAE-cellulose, CNBr-activated fetuin Sepharose and Sephadex G-75. The first step of purification, gave 2.9 mg/ml protein through the unadsorbed fraction. This is 4.4 fold of purification to obtain 64.49 % of yield. The affinity purification, gave 1.87 mg/ml protein and had 6.84 fold of purification. From this 15 % yield was obtained. The final step of purification, obtained 0.35 mg/ml of protein. This is the 9.14 fold purification and gave 12.5 % yield. The higher hemolytic activity is shown (Table 13).
2.14. RP-HPLC purification

Separation of RP-HPLC with the C\textsubscript{18} column showed a major single peak at 280 nm in 2.01 min (retention time of 2.01) and obtained 98% of total protein concentration during an isocratic elution run of 0.1% TFA in 65% acetonitrile and 35% dH\textsubscript{2}O. This peak eluted from the column before the start of the acetonitrile gradient and this was true for numerous other attempts under a variety of conditions (varying the concentration of acetonitrile and water). No further resolution was obtained with this column and fraction collected from the column C\textsubscript{18} did not have any substantial bands with any gel preparation or stain (not shown). This showed the purity of the single hemolytic lectin (Fig 2.15).

**Fig 2.15.** Chromatography of the RP-HPLC purification hemolytic lectin

2.15. Molecular weight determination

The galactose elution from the CNBr-activated fetuin Sepharose and Sephadex G-75 and HPLC column comprised of 36.36 and 26.52 kDa bands under reducing condition that was visible with only silver stains (Figure 2.16, 2.17). Effort to make the silver stain over development did not show any other band. The periodic acid/Schiff staining method demonstrated that no bands were observed.
**Fig 2.16.** Reducing 10% SDS-PAGE of hemolytic lectin (silver stained). (A. Native PAGE (Left) and B. SDS-PAGE (Right)).

A. **C.** Crude sample; **F11-F16.** Purified Lectin

B. **M.** Molecular Marker Protein;

**F13-F14.** Purified lectin subunits
**Fig 2.17**. Native PAGE (A) and SDS-PAGE (B)

A. Lane 1. Crude sample 2. Purified Lectin

B. Lane 1. Molecular Marker Protein used were: Phosphorylase b (97400), Bovine Serum Albumin (66,000), Ovalbumin (43,000), Carbonic Anhydrase (29,000), Lactoglobulin (18,400), Aprotinin (6,500), Lane 2. Purified lectin subunits
2.16. Molecular weight determined by MALDI-TOF

The molecular mass of lectin was also determined by MALDI - TOF, which showed a peak corresponding to m/z 91.56 kDa (Fig 2.18). The molecular mass estimate of 91.56 kDa and was composed of two non-identical subunits (36.36 and 26.52 kDa) (fig 2.16, 2.17).

![Lectin molecular weight chromatogram by MALDI-TOF](image)

Fig 2.18. Lectin molecular weight chromatogram by MALDI-TOF

2.17. Protein estimation

Purified protein (lectin) was estimated Lowry et al., (1951) and assessed by nanodrop. The nanodrop results showed the 18 absorbance units.

The clam muscle extract contained approximately 25 mg of protein per ml. Once the unbound fraction from the column was washed, the equilibration buffer contained 77.89% of the total protein, the galactose elution contained 11.23%, and the gel filtration elution contained 5.28% protein (Table 13).
2.18. Discussion

Lectins have been implicated as recognition molecules in the invertebrate immune response, yet they have capacity to recognize potentially invasive microorganisms. A substance which agglutinates or lyses human or other mammalian erythrocytes occurs naturally in the hemolymph and in extract from tissues of molluscs. The results obtained clearly indicate the presence of naturally occurring hemolytic lectin in the clam *V. cyprinoides*. Among the different erythrocytes tested, rabbit erythrocyte showed relatively high hemolytic activity (Fig.2.2). Similar result was observed in the seed of *Croton tiglium* (Banerjee and Sen, 1983). Likewise the hemolytic activity of *Cucumaria echinata* lectin (CEL-III) was strong towards the rabbit and human erythrocytes, but did not lyse for chicken or horse erythrocytes (Hatakeyama et al., 1994).

Michelson and Dubois (1977) noted that hemagglutinin and hemolysins for human erythrocytes are not uniformly present in the members of the family Planorbidae and differences observed between genera and species as well as between populations of the single species. In the present study, the hemolytic activity was higher in the rabbit erythrocytes, followed by the mouse erythrocytes. In the case of human erythrocytes, the ‘B’ group has highest activity followed by, human ‘O’ group showed high activity. The human ‘A’ group has lowest activity, however, the human ‘A1’ group observed highest activity. Since it was established that this lectin induces hemolysis by binding to the red blood cell surface carbohydrates receptors, it is interesting to consider how the interaction of the lectin with cell surface glycoprotein or glycolipids can trigger off the events which lead ultimately to cell lysis. Although lectins are known to affect cell surface morphology and to induce complex biochemical events like agglutination and mitosis by binding specifically to
carbohydrate receptors (Goldstein and Hayes, 1978), no lectin has been shown to have cytolytic activity. But Hatakeyama (1995) describe *C. echinata* lectin (CEL-III), with strong hemolytic activity and cytolytic activities by forming ion-permeable pores composed of its oligomers in the target cell membrane, after binding to cell surface carbohydrate structures, likewise the clam hemolytic lectin might be bind by the surface carbohydrates and induce the hemolytic activity and cytolytic activities.

The density of antigen receptors on erythrocytes strongly affects the number and nature of the plaques in localized hemolysis. The present study emphasizes the importance of antigen-receptors density in the hemolysis of red blood cells because rabbit erythrocytes showed highest activity that may due to the presence of high receptors density and resulted in even very low lectin concentration showed hemolysis, in contrast human ‘A’ group showed very low titer value, and they may be very low antigen-receptors (lectin receptor) density on the surface of erythrocyte.

In the present work, trypsin treated rabbit and human ABO erythrocytes showed higher titer than non-treated (Fig 2.4). The difference in the hemolysis ability of enzyme treated and non-treated cells were shown when the number of hemolytic lectin epitopes on red blood cells were high in the trypsin treated cells and lower in the non-treated cells or that may be due to unveiling of hidden receptors that is specific for trypsin, similar results were observed in earlier work with erythrocyte antigens (Banerjee and Sen, 1983; Hatakeyama et al., 1995). In contrast, in the present work papain-treated red blood cell showed lower activity. Actually Papain is a proteolytic enzyme able to break peptide bonds (Komatsu et al., 1986) and significant reduction of the peripheral protein layer. The nanomechanical AFM analysis confirmed that the reduction of the hydrodynamic permeability of external soft glycoprotein layer of the cell and loss of cell surface integrity or rigidity under the
action of papain (Hyono et al., 2009). The neuraminidase treated erythrocytes also showed low hemolytic activity as compared with non-treated cells, that may be the low epitope density of the treated red blood cell or removing specific receptor that fit on lectin or not enough receptors on the treated red blood cells for binding of the lectin and reduce the hemolytic activity titers. Actually neuraminidase is a glycosyl hydrolases enzyme able to cleave the terminal sialic acid from substrate such as glycoprotein (Davies and Henrissat, 1995). Moreover the neuraminidase action leads to an important decrease in the interphase charge density by removing sialic acid from the cell soft layer (Hyono et al., 2009).

The trypsin treated erythrocytes may express enough quantities of antigen receptor on the cell surface. The above evidences show obviously prove the lytic property of the molecule and confirmed as hemolytic lectin, because their action is if, only their available specific receptors (sugars), if this receptor number was reduced, the hemolytic activity was also reduced. The hemolytic activity was confirmed by erythrocyte agarose plate assay and the activity was determined by the spectrophotometric assay (Fig 2.3). The erythrocyte agarose plate assay showed the clear hemolytic plaques and in the spectrophotometric assay, the 1.5% erythrocyte suspension gave higher hemolytic activity. This findings were supported the previous observation like Eisenia fetida hemolytic lectin (Eue et al., 1998) and Incilaria fruhstorferi contain water soluble fraction that causes hemolysis (Furuta et al., 1995). In the present study the scanning of completely lysed rabbit erythrocytes from 200 - 700 revealed peaks of absorption at 238, 264, 364, 444, 542, 578 respectively (Fig 2.5). The scanning of the spectrum of absorption of completely lysed rabbit erythrocytes from 200 to 700 nm revealed a maximum at 264 nm, being many fold larger compared with absorption at 541 nm. Additionally, a scan of a purified lytic
lectin was showed to be compared. In previous investigations of hemolytic activity were analyzed photometrically by measuring the amount of released hemoglobin at a wavelength of 540 nm (Simonsen and Moestrup, 1997; Arzul et al., 1994). Eschbach et al. (2001) reported the scanning spectrum of absorption of completely lysed carp erythrocytes from 350 to 700 nm revealed a maximum at 414 nm, being 10-fold larger as compared with absorption at 540 nm.

Studies on molluscan agglutinins or lectins are lacky where the effects of exogenous environmental factors on their ability to recognize and bind have been elucidated. Among the classical agglutinins, several have been shown to be affected by certain environmental factors under the laboratory conditions. Results of the present study shows that clam *V. cyprinoides* hemolytic lectin is in heat sensitive. Its activity was stable between 25 and 30°C. The hemolytic activity was completely lost after treatment with 62°C (Fig 2.7). Similar result was observed in hemolytic activity of venom from the jellyfish *Rhopilema esculentum* Kishinouye. Hemolytic activity was temperature-sensitive and when RFV (*R. esculentum* full venom) was pre-incubated at 20 and 30°C, the hemolytic activity was preserved and at temperatures over 40°C, it was sharply reduced (Yu et al., 2007). Similar activity was also observed in hemolysin from *Treponema hyodysenteriae* by (Saheb et al., 1980). The hemolytic activity of the nematocyst venom from the jellyfish *Cyanea nozakii* Kishinouye was stable up to 45°C, and at 100°C the activity was completely lost (Feng et al., 2010). The *Pleurotus eryngii* hemolysin (eryngeolysin) was stable between 0 and 30°C and at 50°C, the activity was lost (Ngai and Ng, 2006). The *Plasmodium berghei* hemolytic activity was temperature-dependent process, maximum activity was observed at 37°C (Gupta and Saxena, 1980). The *V. cholera* O139 hemolysin and the Eltor hemolysin lost their hemolytic activity completely by heating at 70°C for 60 and 10 min (Pal et
The tolaasin, a pore-forming peptide toxin produced by *Pseudomonas tolaasii* which is poor binding at 4°C observed by Cho *et al.* (2010). In contrast, the thermostability of lebbeckalysin, hemolysin from *Albizia lebbeck* was stable at 0-100°C and same as that of *V. parahemolyticus* hemolysin (Sakurai *et al*., 1973), which was stable at 100°C.

The fact could be that loss of hemolytic activity in the present lectin might be due to changes in molecular structure, heat denaturation of the hemolytic protein and temperature-induced changes in molecular structure. In the case of pH, *V. cyprinoides*, the hemolytic activity was high at 7.5-8.5. The hemolytic activities gradually reduced at pH 8.5 and completely lose its activity above pH 9.5 (Fig 2.8). These results corroborated earlier reports (Takada *et al.*, 2003; Haider *et al.*, 1992 and Bodade *et al.*, 2009) which showed that strong hemolytic activity in hemolysin from *Prevotella intermedia* at alkaline pH. RFV (*Rhopilema esculentum* full venom) hemolysin had strong hemolytic activity at stable between pH 6–10. The hemolytic activity was lost below pH 2–3 and above pH 11 (Yu *et al.*, 2007). The hemolytic activity of *Carybdea marsupialis* was preserved at pH 5.5–6.3 and 8.3–9.0 (Rottini *et al.*, 1995). Almost similar activity was observed in the hemolysin of *Fusobacterium necrophorum*, it had decreased hemolytic activity at the acidic pH and highest value recorded at pH 7 (Amoako *et al.*, 1994) In contrast, the *Trypanosoma cruzi* hemolysin was optimally active at pH 5.5, with negligible activity at neutral pH (Andrews and Whitlow, 1989). Alvarez *et al.* (2001) reported Sticholysin II (St II), the two isocytolysins purified from the Caribbean Sea anemone *Stichodactyla helianthus*. The cytolyisins were stable between at pH 2.2 and 9.0 and above pH 11.5 it drastically reduce the hemolytic activity. In fact, this indicates that the extreme alkaline and acidic condition of proteins are inactive and might be due to irreversible
conformational changes and mask the available receptors that notably reduce its biological activity, associated with partial defolding of the protein.

The requirements of some proteins for metal ions to retain their biological activity are well established. Lectins are usually metalloproteins containing cations such as Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$ (Borrebaeck et al., 1981). In the present study, the hemolytic activity of the 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$_4$. Lectin treated with 50 mm and 25m mol MgCl$_2$ showed higher activity. But BaCl$_2$ and CaCl$_2$ showed similar activity from 25 mM to 100 mM, but one fold decrease in activity. The FeCl$_2$ and HgCl$_2$ showed no activity and was not involved or required during hemolytic processes. MnSO$_4$ and MnCl$_2$ showed very low activity (Fig 2.9).

The requirements of divalent cations were very much involved in the hemolytic activity. The hemolysin of *Serpulina hyodysenteriae* was examined to have the effect of Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ on hemolytic activity. 0.1 to 1mM of either CuSO$_4$ or ZnSO$_4$ resulted in a two fold decrease or inhibited the hemolysin activity (Dupont *et al.*, 1994). The hemolytic activity of RFV (*R. esculentum* full venom) was reduced in the presence of Mg$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Ca$^{2+}$ ($\geq$ 2 mM), Mn$^{2+}$($\geq$ 1 mM) (Yu *et al.*, 2007). In the present experiments, the kinetics of hemolysis was tested in 0.5 % of rabbit erythrocyte suspension to 3 % and the result show that very rapid activity was present at the higher concentration of lectin molecule (18 mg/ml or 1:1 dilution) with 0.5% of rabbit erythrocytes. But with lower concentration of lectin molecule (0.0703 mg/ml or 1:256 dilution), the hemolysis show long prelytic period as in the course of hemolysis vs time. The hemoglobin content in the 0.5% erythrocyte suspension was little low as compared with 1.5% to
2.5\% erythrocyte suspension when absorbance at 541 nm (Fig 2.11). Banerjee and Sen, (1981) described the differential response of trypsinized rabbit red cells to *Croton tiglium* lectin-induced hemagglutination and hemolysis as well as by the different temperature-sensitivity of the two processes. The kinetics of hemolysis indicates that there is a finite time interval (lag phase) between the binding of the lectin molecules and the escape of hemoglobin. Further the length of this lag phase which is a measure of the kinetics of the secondary membrane events yet to be defined is a function of the concentration of lectin-receptor complex.

Most of the invertebrate’s lectin showed Ca$^{2+}$ dependency with some exception such as that from haemolymph of the shrimp *P. japonicus* (Yang et al., 2007). In the present study, the hemolytic activity of the sample was tested in the presence of varying concentration of EDTA; there was great change in hemolytic titre with the low activity in low concentration of EDTA. CaCl$_2$ enhanced the hemolytic activity of the lectin. This shows that this hemolytic lectin was highly calcium dependent activity and is called as C-type lectins (Table 10). Similar results were reported by Hatakeyama et al., (1994) *C. echinata*, Himeshima et al., (1994) *Stichopus japonicus* lectin, Canicatti and Roch, (1993) *Spirographis spallanzani* coelomic fluid, Parrinello and Rindone, (1981) *Spirographis spallanzanii viviani* coelomic fluid, Canicatti and Ciulla, (1987) *Holothuria polii* coelomocyte lysate. Chung et al. (2001) reported that the hemolytic activity of crude venom from *Carybdea alata* was dependent on divalent cations Ca$^{2+}$ or Mg$^{2+}$ were necessary for the hemolytic activity, while hemolytic activity was irreversibly eliminated when crude venom was dialyzed against buffer containing EDTA (20mM). Rottini et al., (1995) reported that when Ca$^{2+}$ concentration were increased to 10 mM, inhibition was observed in the hemolytic activity of venom of *Carybdea marsupialis*. In
addition, the hemolytic activity of venom from the cnidaria *Aiptasia pallid* (Hessinger and Lenhoff, 1973) and *Actinia equine* (Macek *et al.*., 1994) was found to be enhanced in the presence of Ca\(^{2+}\). All Ca\(^{2+}\) dependent invertebrate lectins showed aminoacid sequence that belongs to the C-type lectin family which share apparent homology. Therefore *V. cyprinoides* hemolytic lectin can also is expected to exhibit some extent of homology with the C-type lectin family. Cross adsorption studies were carried out to assess whether the hemolytic activity is due to single hemolytic lectin or due to multiple hemolytic lectin in the sample. In the cross adsorption tests, each red blood cell types was found to completely or partly adsorb the other red blood cell types and the efficiency of adsorption differ among the eight red blood cell types tested. It is interesting to note that rabbit erythrocytes completely lost its activity. This shows that the hemolytic lectin was highly specific to the rabbit erythrocytes surface carbohydrates (Table 9). These observations together with differential affinity of lectin for erythrocytes suggest that erythrocytes may share some common glycoconjugates on their membrane surface, and density of surface receptor may also differ. Shanmughavalli and Arumugam, (2011) reported that multiple specificity of the hemolysin in the serum of a hermit crab *Clibanarius longitarsus* and the complete removal of hemolytic activity against human A, B, O and sheep red blood cells.

Lectins with specificity to different human blood cell types are more strongly inhibited by saccharides present in the immune determinant glycoproteins. In the present study, the hemolytic lectin from *V. cyprinoides*, exhibit low inhibitory activity by the addition of sugar arabinose at 200 mM concentration. The simple hexose 25 mM galactose and their amino derivative such as 25 mM galactosamine and 50 mM glucosamine were moderately inhibiting hemolytic activity. By contrast their N-
acetylated derivatives such as N-acetyl galactosamine (GalNAc) as well as N-acetyl glucosamine (GlcNAc) did not inhibit the hemolytic activity at the concentration up to 200 mM. The N-acetylated simple sugars such as N-acetyl neuraminic acid (NeuAc) and other carbohydrates except galactose, galactosamine, glucosamine, and arabinose also did not inhibited the hemolytic activity even at low concentrations (Table 11). The fetuin was one of the potent inhibitor of the hemolytic activity at the concentration of 0.625 mg. Furthermore desialylation of these glycoproteins either slightly reduced or completely abolished the inhibitory potency. Bovine sub maxillary mucin did not inhibit any hemolytic activity even at a concentration of 10mg/ml (Table 12). From the results, it was obvious that the isolated lectin might be galactose specific lectin. Similar result was also observed in hemolytic lectin CEL-III from the marine invertebrate C. echinata (Hatakeyama et al., 1994). The hemolytic lectin from the parasitic mushroom L. sulfureus was highly specific to galactose containing carbohydrates such as N-acetyllactosamine (Konska et al., 1994). In contrast, the hemolytic lectin from seeds of C. tiglum, the saccharide moiety of the complex glycopeptides is primarily responsible for inhibition of hemagglutination and hemolysis. But glycoproteins like ovalbumin, human IgG, and fetuin are non-inhibitory (Banerjee and Sen, 1981).

Inhibition of hemolysis by carbohydrates has been observed in cnidarian hemolysins, Hemolysin from Hawaiian box jellyfish (Carybdea alata), exhibited lectin-like properties. Hemolysis was inhibited by D-lactulose and other sugars such as p-Nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside and N-acetyl-D-galactosamine (Chung et al., 2001). The hemolytic activity of Rhopilema nomadica was potently inhibited by N-acetylneuraminic acid (Gusmani et al., 1997). The hemolytic activity observed in the venom of the closely related Carybdea
marsupialis was completely inhibited by methyl-β-D-galactopyranoside and N-acetyleneuraminic acid (Rottini et al., 1995). The hemolysis of hemolytic lectin from *Villorita cyprinoides* was found to be dependent on the erythrocyte species, and inhibited by galactose and N-acetyl galactosamine, suggesting that the hemolysis is mediated by binding of hemolytic lectin from *V. cyprinoides* to the carbohydrate on the surface of the erythrocytes. High susceptibility of the hemolytic action of this hemolytic lectin was observed for rabbit and followed by mouse, pig, human B, squirrel, goat, hen, dog, human O, turkey, cow and human A. This suggests that the possible hemolysis proceeds through its binding to galactose and N-acetyl galactosamine chains on the erythrocyte surface and partial destruction of the membrane structure and formation of ion-permeable pores in the membrane, and like that of other invertebrates and bacterial hemolysins (Hatakeyama et al., 1994; Konska et al., 1994; Rottini et al., 1995; Gusmani et al., 1997 and Chung et al., 2001).

In DEAE-cellulose chromatographic purification, the desired protein was not adsorbed on the purification matrix as well as on CM-cellulose. The unadsorbed fraction number 3rd was the highly active one. The other fractions, such as 2, 4, 5, 6, and 7th showed lytic property. The remaining fractions did not show any activity. In the present study had corroborated with earlier works, such as Li et al., (2008) who reported that edible mushroom *Pleurotus citrinopileatus* lectin was showed unadsorbance on DEAE-cellulose. Liu et al. (2004) reported that novel lectin from the wild mushroom *Xerocomus spadiceus* showed unadsorbance on DEAE-cellulose. Wang and Ng, (2006) reported that lectin from samta tomato was purified through ion exchange chromatography on DEAE-cellulose; lectin was showed unadsorbance on DEAE-cellulose but adsorbed on Affi-gel blue gel and CM-cellulose. In the present study DEAE-cellulose isolated protein fractions were subjected to the CNBr-activated

The *Prevotella intermedia* hemolysin was purified by ammonium sulfate and polyethylene glycol precipitations and ion exchange chromatographies on DEAE-Sephael and CM-Sepharose (Takada et al., 2003), Isolation of erylysin A and B from *Pleurotus eryngii* was done passing through the first DE52 column was further fractionated by a second DE52 column (Shibata et al., 2010), *E. fetida* hemolysin was isolated by elution after PAGE separation (Eue et al., 1998). Isolation of the hemolysin from *Porphyromonas gingivalis* by nickel-nitrilotriacetic acid chromatography was carried out (Deshpande and Khan, 1999). Isolation of hemolysin from *Holothuria polii* through overlaying agarose-SRBC-gel (Canicatti and Ciulla, 1988). Hemolysin of *Vibrio fluvialis* (VFH) was purified by ammonium sulfate precipitation and successive column chromatographies on DEAE-cellulose and Mono-Q (Han et al., 2002).

The purified hemolytic lectin was further characterized by SDS-PAGE and MALDI-TOF analysis. SDS-PAGE showed that the lectin had a molecular mass of 91.56 kDa, and consisted of two sub units with 36.36 and 26.52 kDa (Fig 2.16 and 2.17). The molecular weight of the purified hemolytic lectin was confirmed by
MALDI-TOF and it was 91.56 kDa proteins (Fig 2.18). The hemolytic lectin from *C. echinata* (CEL-III) had a molecular weight of 45 kDa (Hatakeyama et al., 1994). The hemolytic lectin from *L. sulfureus* had the molecular weight in non-denaturing conditions and is about 190,000 Da. Its structure is tetrameric, with two distinct types of subunits about 60,000 and 36,000 Da (Konska *et al.*, 1994). The *E. fetida* hemolysin was three proteins complex, H1, H2, H3 with molecular weight of 46,43 and 40kDa (Eue *et al.*, 1998). The *C. tiglium* hemolytic lectin has been shown to consist of noncovalently bound subunits of molecular weight 55,000 by SDS-polyacrylamide gel electrophoresis in the presence and absence of 2-Me (Banerjee and Sen, 1981).