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Varuna litterata (Fabricius) Lectin (VLL) Purification and Characterization

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ABSTRACT: The carbohydrates are held together by the lectins which are glycoproteins. The apoptosis, involvement of pathogens, identification and tying of carbohydrates were some of the biological processes which happens. The adaptive and innate immune response, adaptive and innate immune response has an important part which is the binding features of universal ancient molecules called Sialic acid-binding lectins (SABLs). In this research, a novel sialic-binding lectin was isolated from the swimming crab *Varuna litterata* (Fabricius). The obstruction of growth and the abolishment of bacteria and fungi can be promoted by the accuracy of several carbohydrates present in the Lectins. The presence of reagents in biochemical research and the investigation of diagnosis were employed in the research of sialic acids for the treatment of pathogenic diseases and tumors which has attained a lot of interest in sialic acid-specific lectins. The molecular mass of the lectin was identified as 70 kDa on SDS-PAGE. In the current analysis, research was going on to isolate, identify and categorize the lectins from the haemolymph of agglutinin. To hinder agglutination, the potentiality of several glycoproteins and sugars (mono and oligosaccharides) was tested by making use of a hemagglutination inhibition (HAI) assay. After 1-hour, total inhibition of agglutination has been provided by the reciprocal of the lowest dilution of inhibitors which was shown by hemagglutination inhibition titer. The cross-adsorption assays have proceeded to identify whether there is the presence of single or multiple agglutinins in the hemolymph. Along with the desialylated rat erythrocytes, the agglutinin's preference for sialic acid is demonstrated by a decrease in hemagglutination activity. Moreover, a sepharose 4B column was connected with bovine submaxillary mucin which was triggered by CNBr to purify the lectin. Hemolymph agglutinin showed greater affinity towards the protease-treated rat erythrocytes and lesser affinity towards neuraminidase-treated rat erythrocytes than the native rat erythrocytes. As the O-acetyl sialic acid, a high preference was given for the inhibition of purified lectin by bovine submaxillary mucin and the non-inhibitory action of de-O-acetylated bovine submaxillary mucin. The results confirm the presence of an O-acetyl sialic acid-specific lectin in the hemolymph of the crab *Varuna litterata* respectively. The production of anti-tumor and antiviral drugs based on O-acetyl sialic acid-specific lectin in the hemolymph lectin of *Varuna litterata* may also have a significant utility in therapeutic industry.

Keywords: *Varuna litterata*, Sialic Acid, Lectin, Hemagglutination, Antigen, Agglutinin, Hemolymph, Glycoproteins, Rat Erythrocytes, Cross-Adsorption.

INTRODUCTION

Being a staple food consumed by people all over the world and a top environmental indicator, crabs are commercially significant crustaceans. Asia places great importance on seafood, particularly *Portunus reticulatus* a blue swimming crab. With the help of their innate immune system, invertebrates defend themselves from outside diseases, and lectins are crucial for identifying foreign substances (Ahmed *et al*., 2021). However, the varied species of contaminating microbes used in crab production, shipping, and storage are a factor in the colony structure of the crabs, which is diversified. Additionally, the loose connections between collagen fibres, free amino acids, and high moisture content offer a fertile habitat for the development of bacteria. Crabs' meat spoils quickly, even at frigid temperatures, which reduces the product's shelf life. High-throughput sequencing technology has recently advanced alongside molecular biology methods, enabling not only the detection of nonculturable microorganisms but also the estimation of the relative abundance of microorganisms (Pan *et al*., 2021). Environmental and genetic factors can be assessed concerning a crab's weight to see how they affect its morphometric data. Conversely, the condition factor provides data on the "well-being" of creatures and may indicate strategies for feeding and responding to the environment. *Varuna litterata* is a rare species, and it is growing harder to find it in acceptable sizes for human utilization. To guarantee a long-term supply of crab as a food source, a stable crab supply is aggressively pursued while ensuring the survival of

crab species in the wild (Jumawan *et al*., 2022). The grapsid crab *Varuna litterata* is a member of the Varunidae family. The fishing industry in Southeast Asia relies heavily on *V. litterata*. During their breeding season, *V. litterata* are particularly prevalent in markets because of their delectable ovaries. Fish sauce is typically used to preserve *V. litterata* (Suppapan, *et al*., 2017). In comparison to other marine species, the raw ingredient used to make pickle crabs in the Chaoshan region of China is a marine crab called *Varuna litterata*, and studies have shown that this crab's crude extract has a strong bacteriostatic influence on both gram-negative and gram-positive bacteria. To perform preliminary purification, the crude proteins of *Varuna litterata* were salted off, and they were then further purified using gel filtration or an anion exchange chromatographic column. With the rise in protein purity, there was a corresponding rise in antibacterial activity (Wang *et al*., 2021).

Lectins can bind precisely and reversibly to sugar and glycoproteins moieties, in particular the presence of carbohydrates in both glycoproteins and glycoconjugates. To identify and eliminate invasive bacteria, lectins were used which are either membrane-bound or soluble PRR. Numerous proteins make up lectins, which can bind and specifically identify sugars like lactose, mannose, galactose, N-acetyl galactosamine, and N-acetylglucosamine. The outcome is the non-covalent interactions. To identify the infection, lectin-carbohydrate interaction is considered an essential factor of immunity and several biological processes were carried out such as cell adhesion, complement activation, opsonization, agglutination, and phagocytosis (Abraham *et al*., 2022; Johnson *et al*., 2022). A straightforward two-step method which makes use of the solution of polar salt and purification method by column chromatography is utilized to extract lectins from marine materials. Lectins perform several immunomodulatory processes, such as pathogen identification, inflammatory responses, involvement in different hemocyte functions (such as agglutination), and phagocytic processes, among others. Additionally, lectins can regulate molecular transport, protein folding, RNA splicing, and cell proliferation. Lectins have drawn the interest of scientists and companies due to their alleged biological and pharmacological activity (Ahmmed *et al*., 2022; Jiang *et al*., 2021). In Tamilarasan *et al*. (2021) studied, from the grub of hemolymph of the banana pest *Odoiporus longicollis*, thelectin was purified, biochemically characterized, and functionally characterized. The lectin was purified by PEG precipitation and ion exchange chromatography using Q-Sepharose as a matrix. Hemagglutination (HA) activity against rat erythrocytes was present in the purified lectin. It was also cation-independent, heat-labile, and EDTA-insensitive.

Sialic acids are crucial for several biological processes, comprising ageing, apoptosis, growth, ion transport, differentiation, and cancer. It is also possible to identify highly pathogenic bacterial strains using sialic acid-specific lectins. Arthropods and molluscs among the invertebrates have agglutinins in their hemolymph that can identify sialic acid. To prepare for their isolation, purification, and pharmacological research, (Prakash Shobaand Basil Rose 2016; Sivakamavalli *et al*., 2021)conducted a study to first characterize the lectins present in *Varuna litterata*'s serum. Additionally, the lectin of protozoal parasites like *Entamoeba histolytica* is connected to certain sugars on the surface of the host intestinal epithelial cells. Both host-host and host-parasite interactions are significantly impacted by this sugar-lectin molecule. As a result, lectins' ability to interact with carbohydrates on cell surfaces is crucial to their primary functions(Swamy*et al*., 2022). Specific polysaccharide binding is always dependent on the kind, metal ion, molecular size, and amino acid sequence. The analysis of lectin characterizations, isolations, and purifications is what accounts for the stark biological disparities (Al-Morshidy *et al*., 2021). On fetuin-agarose, affinity chromatography was used to partially purify the lectins. According to the lectins' characterization, the saccharides (mono-, di-, and tri-saccharides) will not affectthe ability to bind to hemagglutinin, which is solely blocked by the fetuin (González-Cruz *et al*., 2022). Consequently, in this study based on binding the Purification and Characterization of sialic acid carried out from *Varuna litterata* crab were discussed.

LITERATURE SURVEY

In an earlier study (Adebayo *et al*., 2022), the proteins of Malaysian *Tachypleus gigas* hemocytes that had been activated by lipopolysaccharides (LPS) and those that had not were analyzed using LC-MS/MS. In both types of samples, 154 distinct proteins were discovered. Over 77 proteins have also been present in both circumstances frequently, but only 52 and 25 proteins were detected exclusively in hemocytes triggered by LPS and those not stimulated by it. Finally, the molecules, biological processes, and activation of the innate immunity of *T. gigas* were all dependent on the proteins contained in the hemolymph of the organism.

The *Portunus trituberculatus* derived from type IIacrustin was cloned and given the name PtCrustin5. An essential antimicrobial peptide found in crustaceans is crusting. The PtCrustin5 comprised the characteristic features of crustin of type II, including a peptide signal, a cysteine-rich, whey-acidic protein and in the area of glycine-rich domain. After being challenged for 3 and 6 hours with *Vibrio alginolyticus*, PtCrustin5 demonstrated the greatest transcriptional in the hepatopancreas, as well as the level of expression, has been improved in the hepatopancreas, gills, and hemocytes. One of the most significant cytokines in both vertebrate and invertebrate biology is tumour necrosis factor (TNF), and several biological processes were carried out in this method. Huang *et al*. (2022) a new member has been identified in the TNF superfamily from the Chinese mitten crab (*Eriocheir sinensis*) known as EsTNFSF. The extracellular C-terminal TNF domain and a transmembrane region were both present in the deduced EsTNFSF protein. EsTNFSF was shown by phylogenetic analysis to be closely related to other TNFSFs from crustaceans. According to the findings, EsTNFSF, an inducible

immune response gene was essential for Es antibacterial immune defence.

I-type lectins known as siglecs have an amino-terminal and are selective for sialic acid. Silvester *et al*. (2022) discussed the lectins found in brachyuran and anomuran crabs. There is a presence of a high amount of sialic acid-specific lectin which has important medicinal potential in arthropods and molluscs. A deep investigation is made for one of the lectins of crabs especially brachyuran crabs which is a type of crab lectin and is sialic acid-specific. For each cancer cell, the cell surface is identified using Molecularly imprinted polymer particles (SA-MIPs) imprinted with sialic acid (SA) (Beyer *et al*., 2022). As per the adjourn and dispersion features, the previously employed core-shell SA-MIPs are better than the SA-MIPs. The findings demonstrate that the SA-MIPs had a variety of binding behaviours with the cancer cell lines chosen for this study. To prevent the binding of the SA-MIPs, two various pentavalent SA conjugates were used for breast, skin, and lung cancer cell lines thus establishing the flow of cytometry and confocal fluorescence microscopy in SA-MIPs.

However, the leftover corona still included enough sialic acids to cause off-target binding. For use in biosensing and diagnostics, polymer-tethered glycosylated gold nanoparticles had disclosed potential, but the effect of the glycoprotein corona hasn't yet been determined. As a consequence, Ahmad *et al*. (2022), explored how serum proteins associate with the polymer-tethered glycosylated gold nanoparticle, which shows how the protein corona generates additional glycans and therefore off-specific targeting potential. Galactose, a glycan placed on the polymer's chain end, was still accessible for binding in lectin-binding experiments despite the creation of the protein corona. Rat, mouse, and buffalo erythrocytes agglutinated very avidly in the hemolymph of the sea crab *Menippe rumphii*. HA ability was discovered in the hemolymph, hepatopancreas, and muscles of the *Travancoriana charu* when it came to the various tissues that were examined for the existence of agglutinins (Sheeja and Basil Rose 2018). Aspects of the hemolymph's biochemistry such as its water, protein, and calcium concentration had no observable effects on the HA titer.

Due to the genus *Varuna* small number of individuals, the evolutionary position of the group has not been fully determined. To reassemble the phylogeny of the Varunidae, Zhang *et al*. (2022) created a unique mitogenome for this genus (*Varuna litterata*) and integrated the available mitogenomes. All 37 genes as well as a potential regulatory region were included in the 16 368-bp mitogenomes. All Varunidae species were grouped according to phylogenetic analysis, and they were members of the Macrophthalmidae sister clade. The study of the reorganization of genes and enhancement of molecules in Varunidae mitogenomes was improved by the phylogenetic studies of Brachyura and their respective information. Deyashiand Chakraborty (2022), employed nimbecidine plus and mahua oil cake (MOC) on the freshwater crab of haemocyte groups, *Varuna litterata*, followed by acute exposure to evaluate the immunotoxic effects of two biopesticides. During a 4-day static renewal bioassay test in the lab, 16 healthy adult male crabs were subjected to 96-h LC50 values of Nimbecidine Plus and MOC aqueous extract separately. The results of this experiment may offer important insight into the immunological reactions of *V. litterata* to biopesticide toxicity. Ramaraj*et al*., 2022aimed at the purification and bacterial agglutinating process of lectin generated from *P. reticulatus* serum. To evaluate and identify the species, the mitochondrial cytochrome oxidase of the region I is used whereas the NCBI and BOLD were also submitted. The lectin (PrLec) was further purified using a chitosan-based affinity column that was chosen after a preliminary assessment of its HA, cross-adsorption, specificity for binding carbohydrates, and physical features. The molecular mass of the PrLec was 126 kDa. The lectin was successful in binding both bacterial strains.

MATERIALS AND METHODS

After the gathering of freshwater crabs from the Hemolymph, in 96 well microtiter plates with 'U' bottom the Hemagglutination (HA) assays were carried out using mammalian erythrocytes. A positive agglutination is achieved by the reciprocal of highly diluted samples of the identified HA titer. The pH, temperature, divalent cations, especially calcium dependency of agglutinin activity was evaluated by hatching the serum at specific pH, temperature, cations and EDTA (a calcium chelator) for one hour. For every sample, the activity of HA is evaluated after the incubation. To inhibit the agglutination (Mercy and Ravindranath 1993), a Hemagglutination Inhibition (HAI) assay has been carried out to identify the capability of several glycoproteins and sugars (mono and oligosaccharides). After one hour, the inhibition of Hemagglutination is described as a reciprocal of the highest dilution of inhibitor which shows the total inhibition of agglutination. Along with the method of Hall and Rowlands (1974a); Mercy and Ravindranath (1992), the cross-adsorption assay was also performed.

(a) Animal Collection and Maintenance. In this research, the samples of *Varuna litterata* crabs were collected for investigation. The trained collectors from the local area were given training in the collection of samples using a fish net. According to the morphology of the abdomen, the gender of the crab was determined, in which for females it is wide and for males it is narrow.

Fig. 1. *Varuna litterata* (Fabricius).

In the Indo-West Pacific, the *Varuna litterata* (Fabricius, 1798) (type locality: east coast of India) is one of the most found species over a range of the

eastern coast of Africa to New Guinea and Polynesia. It is mostly found in the upstream connected freshwater areas as it is a species found in brackish water. In freshwater settings or brackish waters or slow-moving water areas, these species were found. The features of *Varuna litterata* has been shown in Table 1.

Table 1: Key Diagnostics Characteristics of *Varuna litterata.*

Character	*V. litterata*
Type	Euryhaline Species of Crab
Width	Narrow, width to ischium width 0.55~0.8 in males, 0.55~0.71 in females
Anterior Margin of Large Male Chelipedal Merus	Saw-Edged
Dorsal margin of a large male chela	Rounded
Environment	Marine, Brackish, Fresh
Parent	Varuna H. Milne
Body Size	Larger, G1 of similar-sized males (CL 7.4~11.8 mm)
Varunidae Species	*Helicetient sinensis*, *Eriocher sinesis*, etc
Locality	East Coast of India

(i) Collection of Hemolymph. From the intermoult male crabs with good health, the Hemolymph was gathered. From the third walking leg, the dactylus was cut to get the hemolymph of *V. litterata*. The sterile centrifuge tubes were placed on ice with less shake which the hemolymph will bleed into it. The serum was extracted from the hemolymph immediately after collection and kept in Eppendorf tubes at -20 °C after being centrifuged for 5 minutes at 1500 rpm.

(ii) Hemagglutination Assay (HA). In microtiter plates (Falcon 3910) (Ravindranath and Paulson 1987) with 'U' shaped bottom, the Hemagglutination assays were carried out with a two-fold dilution serum of about 25 µl or by extraction from the whole body or from the equal volume of TBS the tissue is generated. At room temperature, for about one hour 25 µl of erythrocyte suspension is incubated and 1.5% is added after the dilution. To calculate the hemagglutination titers, completely tested RBCs along with high dilution of the test samples were employed. After this method, to determine the carbohydrate specificity of the agglutinin, the concentration of glycoproteins and sugars was used by performing Hemagglutination Inhibition (HAI) experiment by Ravindranath *et al.* (1985).

(ii) Separation of Hemocytes. According to the method of Soderhall and Smith (1983), the hemocytes were separated. In an ice-cold (4°C) of about 1.35 ml, anticoagulant buffer, Citrate-EDTA: trisodium citrate with 30 mM, citric acid with 26 mM, glucose with 100 mM and disodium EDTA with 10 mM, the hemolymph was gathered by cutting the dactylus of the crab. Hemolymph and buffer were combined, gently mixed to ensure rapid mixing, and then centrifuged (200xg) for 2 minutes at 4°C. In 1.5 ml of iso-osmotic buffer (Tris-HCl), the hemocyte pellets were resuspended (50 mM),

PH and Thermal Stability of the Agglutinin. Before the addition of erythrocyte suspension, the effect of pH can be evaluated by the dilution of 25 µl of the crab serum eventually with the same amount of Tris Buffered Saline (TBS) in microtiter plates with varying pH levels of about 4.5 to 9.5 and for about one hour it was placed in an incubator at room temperature 30 ± 2°C. After that for one hour with a temperature of 0-60°C, a crab serum of about 300 µl is kept in an incubator with aliquots to evaluate the thermal stability of the agglutinin. After the incubation process, in the HA assay, the samples of serum that were collected were used.

Purification of Hemolymph Agglutinin by Affinity Chromatography . Two affinity matrices were used for the purification of serum agglutinin namely the BSM-Sepharose 4B affinity column and bovine thyroglobulin-Sepharose 4B affinity column since the capability of hemolymph is hemagglutinating in which the rabbit erythrocytes were hindered by the BSM and buffalo erythrocytes which has equivalent potency. Based on existing research (Schwyzer and Hill 1977; Mercy and Ravindranath 1993), the generation of optimal pH, temperature and the need for calcium ions have been evaluated under the strategies for adsorption. In an economic column (Bio-Rad) that had been previously equilibrated with TBS at 4°C, clarified serum (20 ml) has been added with 3.5 ml of BSM/bovine thyroglobulin-Sepharose 4B and at a rate of 0.6 ml per minute, the eluant was collected. To obtain a homogenous pool of lectin, the column was first washed with high salt buffer (1 M NaCl), low salt buffer (0.3 M NaCl) at 4°C and low salt buffer with calcium at room temperature (32 ºC) respectively until the A280 of the effluent was <0.002 each. In a buffer that contains 10 mM disodium EDTA, the lectin has been extracted at the rate of 0.3 ml/ minute, in polypropylene tubes with 10 µl of 100 mM calcium chloride, the fractions are gathered which were again kept in ice. After that, it was vortexed and then kept in ice for the determination of lectin by HA assay. Finally, for one hour or 30 minutes the collected fractions were polled and dialyzed at the same time in 10 mM $CaCl_2$, at 4℃, then it was aliquoted, lyophilised (speed-vac, Sawant) and preserved at - 20°C

Cross Adsorption Assay. According to the method of Hall and Rowlands (1974); Mercy and Ravindranath (1992), the cross-adsorption assay was performed.

Collection of Mammalian Erythrocytes. In an enhanced Alsevier's medium (sodium citrate 30 mM, NaCl 77 mM, glucose 114 mM, neomycin sulfate 100 µg/ml, chloramphenicol 330 µg/ml, pH 6.1), the blood samples of humans and other mammals were collected. From the rats, the erythrocytes were evaluated.

(b) Enzyme Treatment of Rat Erythrocytes. The molecules that were based on blood can be diffused into enzyme-loaded erythrocytes which flow in the body. By this approach congenital enzyme deficiencies can be treated due to the increase of pathological plasma

metabolite which passes through the erythrocyte membrane and metabolism is carried out in the normal product. Due to the presence of α-1,3galactosyl in the RBC of rats, it was selected for the experiment. Before performing the transfusion experiment, the ability to remove the Gal1, 3Gal epitope from RBC which had been treated with 1,3-galactosidase was evaluated to monitor the presence of RBC in the blood.

Protease Treatment. In the procedure of Pereira *et al.* (1981), the protease treatment was performed.

Neuraminidase Treatment. The process of asiago-erythrocytes was carried out in a method which was similar to the Mercy and Ravindranath (1993); Ravindranath *et al*. (1988).

Preparation of BSM-Sepharose Affinity Column. To make a gel, CNBr-activated Sepharose 4B (purchased from Sigma, Pvt. Ltd, Bangalore) has been employed. After that, the BSM-Sepharose was packaged and kept in cold TBS with a pH of 7.5 and 0.02% sodium azide. Along with 3.5 ml of BSM/bovine thyroglobulin-Sepharose 4B, 20 ml of clarified serum has been added in a Bio-Rad econo column and with the temperature of 4°C, the pre-titrated TSB has been added. At a temperature of 4°C, the column was initially cleaned with high salt buffer (1 M NaCl), and low salt buffer (0.3 M NaCl) to get the homogenous pool of lectin, and at a temperature of 32°C, the low salt buffer is obtained.

Preparation of Erythrocyte Suspension. Here, the erythrocytes were cleaned thrice along with ten times the amount of tris-buffered saline (TBS) pH 7.5 (Tris-HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM) for about 5 minutes at a rate of 1500 rpm and for the suspension process, it was resuspended with 1.5% of suspension. Human, rat, and rabbit erythrocytes under control and neuraminidase treatment were mixed with fresh serum. The results are presented as percent hemolysis, with 100% equivalent to full hemolysis.

Purification of Lectin from the Hemolymph of *V. litterata.* Along with the 3.5 ml of BSM Sepharose, an 20ml of Clarified serum has been added in an Econo-column (Bio-Rad) which has been already balanced with TSM at a temperature of 4°C. At a rate of 0.6 ml/min, the eluent was gathered. The column was then cleaned with HSB and LSB (cold) at 4°C to 32°C (warm) respectively, until the A_{280} of the effluent was <0.002 and tested the effluent was for HA activity. The buffers used for washing contained calcium which was required for the binding of lectin to BSM-Sepharose. The elution of lectin was done with elution buffer (EB) that contained 100 mM GluNAc and collected 1 ml fractions on ice in polypropylene tubes at a rate of 0.3ml/minute till the A_{280} of the effluent reached<0.002. After the collection of fractions, it was stored at a temperature of 4^{o}C. The fractions were combined on the same day and dialyzed for 2 hours at 10°C in distilled water, followed by another 3 hours in freshwater. After that the dialyzate has been aliquoted, lyophilized (Speed-Vac, Sawant), and kept at -20°C.

Quantitation of Sialic Acids. According to the periodate-resorcinol method of Jourdian *et al*. (1971) the evaluation of the presence of sialic acid in the glycoproteins was performed. As mentioned in Schauer (1970), the orcinol method was used to determine the sialic acids which were bounded glycosidically.

Sialidase treatment of Sialoglyco protein. The glycoprotein (fetuin) of about 2 mg was combined with *Clostridium perfringens* sialidase (Sigma type X) of about 0.1 unit of 400 µl is held in an acetate buffer of 5 mM to prepare Asialo fetuin with pH 5.5 for about 2 hours at temperature 37°C. Without the presence of sialidase, fetuin has been treated. The purified lectin was used in the HAI assay against a 1.5% dog erythrocyte suspension for both sialidase-treated and untreated fetuin.

Polyacrylamide Gel Electrophoresis, As per Laemmli (1970), the process of SDS-polyacrylamide with 12.5% in a slab gel electrophoresis was performed.

De-O-acetylated Preparation of Glycoproteins. Based onthe steps mentioned in Sarris and Palade (1979); Schauer (1982), DeO-acetylation of sialic acids takes place.

Determination of Molecular Weight by SDS-PAGE. Following Laemmli (1970), the process of SDS-polyacrylamide with 12.5% in a slab gel electrophoresis was performed. The Coomassie Brilliant Blue R-250 or silver staining is used to stain the gels. Electroblotting was used to transfer electrophoresed proteins to a nitrocellulose membrane for immunoblotting. The membrane was then incubated with a rabbit polyclonal antibody towards TL-1 and detected.

Estimation of Protein. In the process of Lowry *et al.* (1951), the concentration of protein has been defined.

RESULTS AND DISCUSSION

In the movement of protease-treated rat erythrocytes, neuraminidase-treated rat erythrocytes and native rat erythrocytes, the Neuraminidase plays an important role in the impact of electrophore. The decreased percentage is found to be high in humans and low in rats. The elimination of sialic acid and the changes found in the movement of the electrophore has high interaction. In the erythrocytes, its filtration time is not affected by Neuraminidase.

(a) Hemagglutination Properties of Hemolymph of the Crab *V. litterata.* A large number of erythrocytes is agglutinated in the hemolymph of the crab *V. litterata* that is rat > rabbit = guinea pig = horse = buffalo = human A = human B > human O > dog > donkey = goat. The hemolymph agglutinin was inhibited by sugars such as GluNAc, NeuAc, ManNAc, and GalNAc and glycoprotein Bovine submaxillary mucin. There was a stable level of pH from 7.5 to 9.5 in the hemagglutinin with a temperature from 0°C-35°C.

Cross Adsorption Test. According to the adsorption experiments, the hemolymph of the crab *V. litterata*has single agglutinin.

(i) Purification of *Varuna litterata* Lectin (VLL). The affinity column chromatography is used to isolate the lectin obtained from the hemolymph of the crab *Varuna litterata* which makes use of BSM-Sepharose affinity matrix and some activities performed which increases to 2446-fold as compared to serum with 44 fold for some activities.

Table 2: Purification of Lectin from the Native Hemolymph of *Varuna litterata.*

Sample	Volume (ml)	Protein (mg)	Total Activity (HA units)	Specific Activity (HA units/mg)	Purification Fold
Crude Hemolymph	20	560	1.02 × 105	182	1
Clarified Serum	20	6.5	5.12 × 104	7876	44
Purified Using BSM-Sepharose Affinity Column	20	0.24	1.02 × 105	4.4 × 105	2446

Table 2 illustrates the lection purification from the native hemolymph of *Varuna litterata*. The samples are crude hemolymph, Clarified serum and BSM-Sepharose affinity column. By employing SDS-PAGE, one protein band at a size of around 70 kDa has been generated to verify the generated enzyme's purity in the last buffer. From this, the clarified serum enzyme was purified 44-fold and the BSM-Sepharose affinity column was purified with 2446-fold.

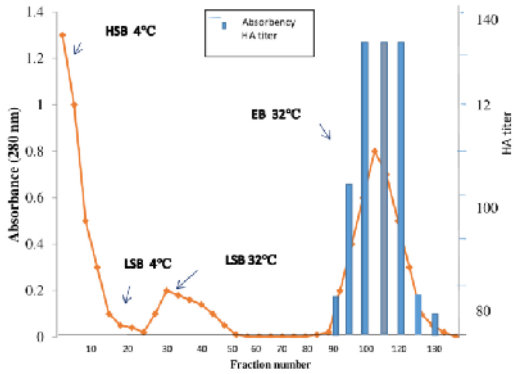

Fig. 2. BSM Affinity Column Profile.

In Fig. 2, the column profile of the affinity chromatography has been shown. Here the process used to segregate the ectin from the hemolymph of *Varuna litterata* by employing BSM-coupled Sepharose 4B is also defined. By employing polypropylene Econo Column (0.8×4 cm), the affinity column has been processed. At a temperature of 4℃, and with 10 mm $CaCl_2$ there is a balance among BSM–agarose and NaCl/Tris. Again with the same temperature of 4℃ with 10 mm $CaCl_2$, a clear serum of about 10 ml along with NaCl/Tris that contains 1 M NaCl is cleaned till the effluent of A280 reaches 0.002. After that, in a water bath of about 32℃, the column is relinquished and then cleaned with NaCl/Tris with the presence of 300 mm NaCl till the effluent of A280 reaches 0.002. With the presence of NaCl/Tris with 10 mm EDTA, and at 32℃ the elution process is performed. Horse erythrocyte suspension at 1.5% in NaCl/Tris with 0.5% BSA was employed to evaluate the hemagglutination of a one-millilitre sample.

(ii) Electrophoretic Analysis. Based on reducing conditions of SDS-PAGE with mercaptoethanol, for the hemolymph lectin of the crab *Varuna litterata*, its molecular mass is isolated by affinity column chromatography and biospecific assimilation on dried rat erythrocytes was calculated to be 70 kDa. Fig. 3.

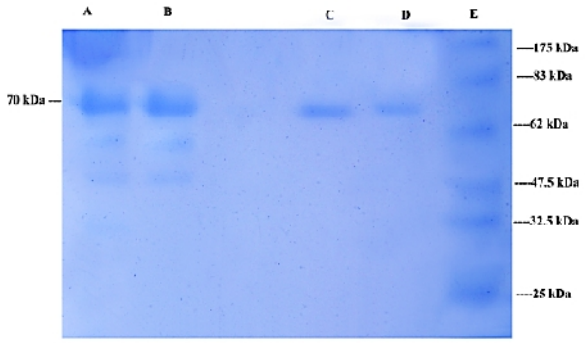

Fig. 3. SDS-PAGE of Purified Lectin VLL.

(iii) Enzyme Treatment on HA. The HA titer has been gradually decreased due to the Neuraminidase diagnosis of rats, hence for the hemolymph agglutinin the HA titer is increased by treating the rat erythrocytes using trypsin and neutral protease.

Table 3: Effect of Neuraminidase Treatment of Erythrocytes on the HA titer of the Hemolymph Lectin of the Crab *V. Litterata.*

Erythrocytes	HA titer
Untreated rat RBC	128
Neuraminidase-treated rat RBC	0

Table 3 illustrates the effect of rat erythrocytes neuraminidase treatment on the HA titer with the hemolymph lectin of the Crab *V. litterata*. The untreated rat RBC of the HA titer is about 128 and the neuraminidase-treated rat RBC of the HA titer is zero.

(iv) Neuraminidase Treatment. The ability of the lectin to agglutinate desialylated rat erythrocytes was tremendously reduced suggesting its ability to recognize sialic acid on rat erythrocyte membrane (Table 4).

Table 4: Effect of Enzyme Treatment of Erythrocytes on HA assay of Hemolymph Agglutinin of *V. litterata.*

Enzymes	Site of Enzyme Activity	HA titer
None		128
Neuraminidase (*Clostridium Perfringens* (Type X))	NeuAc-D-Gal; NeuAc-D-GalNAc	32
Trypsin (1 mg/ml)	Arg-Lys-S	256
Neutral protease (0.25 mg/ml)	All peptide links	256

(v) Binding Specificity of *Varuna litterata* Lectin (VLL). The nature of the binding specificity of purified *Varuna litterata* lectin (VLL) was assessed by hemagglutination inhibition assay by using various sugars and glycoproteins. Of all the sugars, agglutinability was hampered by N-Acetyl neuraminic acid = N-Acetyl-D-Glucosamine = N-Acetyl-D-Mannosamine> N-Acetyl-D-Galactosamine > Glucose = Mannose.

Table 5: Hemagglutination Inhibition of *V. litterata* Lectin by Sugars and Glycoproteins.

Sugars / Glycoproteins (N=10)	HAI Titer	Minimum concentration for inhibition (mM)	Relative inhibitory potency (%)
N-Acetyl-D-Glucosamine	16	6.25	100
N-Acetyl-D-Mannosamine	16	6.25	100
N-Acetyl-Neuraminic acid	16	6.25	100
N-Acetyl-D-Galactosamine	8	12.5	50
Glucose	2	50	12.5
Mannose	2	50	12.5
Bovine submaxillary mucin	128	39.06	100
Lactoferrin	32	156.25	25
Holotransferrin	16	312.5	12.5
α-acid glycoprotein	16	312.5	12.5
Porcine stomach mucin	8	625	6.25
Fetuin	2	2500	1.56
Apotransferrin	2	2500	1.56

Table 5 shows the glycoproteins estimation with relative inhibitory potency. Of the various glycoproteins tested, hemagglutin ability was inhibited by Bovine submaxillary mucin > Lactoferrin >Holotransferrin = α-acid glycoprotein > Porcine stomach mucin > Fetuin = Apotransferrin.

(b) Key Findings

- The study performed lectin purification with Hemagglutination Inhibition of *V. litterata* Lectin by Sugars and Glycoproteins. The binding specificity has a higher inhibitory potency rate with 2446-fold purification.
- The binding specificity of purified *Varuna litterata* lectin (VLL) was assessed by hemagglutination inhibition assay using various sugars and glycoproteins. Of all the sugars, agglutinability was hampered by N-Acetyl neuraminic acid = N-Acetyl-D-Glucosamine = N-Acetyl-D-Mannosamine> N-Acetyl-D-Galactosamine > Glucose = Mannose.
- SDS-PAGE revealed one protein band at a location of around 70 kDa, confirming the purity of the enzyme recovered from the peak eluted with the final buffer.
- As mentioned in the procedures section, samples for electrophoresis contained roughly 10 g of protein from crude serum (lane A), 20 g of protein clarified serum (lane B), 10 g of BSM-Sepharose purified lectin (lane C), and 5 g of purified lectin (lane D).
- *Varuna litterata* hemolymph lectin isolated by affinity column chromatography and biospecific adsorption on dried rat erythrocytes was calculated to have a molecular mass of 70 kDa on SDS-PAGE in reducing conditions with mercaptoethanol.
- The presence of samples of about 10 µg of protein from crude serum (lane A) and 20 µg of protein clarified serum (lane B), 10 µg of BSM-Sepharose purified lectin (lane C), 5µg of purified lectin (lane D) were generated for electrophoresis.
- A standard (lane E) was compared with the molecular weight of about 70 kDa for *Varuna litterata* lectin with already mentioned molecular weight (pre-stained, broad range marker (Bio-Rad).

According to the study of Ramaraj *et al*. (2022), a maximum value of about 64 is shown in human A and high affinity is attained for the buffalo erythrocytes towards N-acetyl-D-glucosamine as shown by the purification and bacterial properties of lectin derived from the serum of *P. reticulatus.* The lectin agglutinated both bacterial strains efficiently. By employing the Mammalian erythrocytes as indicator cells to identify three different species of brachuran crabs and to discover agglutinins (Sheejaand Basil Rose, 2018). As the size of the animal is high, hemagglutinating activity has been processed in female crabs. The hemolymph's protein, calcium, and other biochemical components will not affect the HA titer. The Gram + bacterial pathogens were introduced by Guillermo Landa *et al.* (2021), which was determined by the alternate co-culture of *S. Aureus* and

Pseudomonas aeruginosa. As compared with urine, the presence of bacteria in similar levels is unable to identify, to achieve optical pathogen identification statistically, a high amount of bacterial loads is needed by the human serum. As the pure MmHc has been displayed by Dilna *et al.* (2022), Lewis B and Lewis Y tetrasaccharides were found to significantly block the hemagglutination activity. Along with the oxygen carrier function, the result of several sequential alignments and detailed research of molecular structures has been predicted by AutoDock. Following the purified Hp-Lec (25 and 50 µg/ml), the fungal strains were examined using microbial virulent pathogens based on research of Jeyachandran Sivakamavalli *et al.* (2021). The *Aspergillus niger* and *A. flavus* show the antifungal activity of the examined fungal pathogens.

DISCUSSIONS

For various invertebrates (Yeaton, 1981; Vasta and Marchalonis 1983; Ratcliffe *et al*., 1983; Renwrantz, 1986; Rittidach *et al*., 2007), carbohydrate-binding proteins like Lectins have identified in the hemolymph and other tissues of the body. The presence of sugar is determined (Ravindranath *et al*., 1985), whole sugar (Bretting and Kabat 1976), their glycosidic linkage (Shibuya *et al.*, 1987; Wang and Cummings 1988) or a sequence of sugars (Kobiler and Mirelman 1980; Mauchamp, 1982). According to Sherwani *et al.* (2003), Lectins have been considered an important factor in the cancer research field. For the tumour cells, the metastatic potentials have been enhanced as the level of α, 2-3-linked sialylation also increased (Dennis *et al.*, 1986). As the branching of glycans is increased, the α, 2-3-linked sialic acids also get increased to attain hepatocarcinoma (Montreuil *et al.*, 1997). The hemagglutinin in the hemolymph of *V. litterata* was stable from pH 7.5 to 9.5. pH sensitivity of agglutinin activity is reported in some crabs (Mercy and Ravindranath 1993). The polymeric hemocyanin molecules are in equilibrium with agglutinin molecules and with the rise in pH above 7.5, the hemocyanin molecules may dissociate and release the agglutinin (Ellerton *et al*., 1983). This could be the cause for the increase in HA activity above pH 7.5 which remained stable up to pH 9.5. Since the boost was stable at and above pH 7.5, pH 7.5 was considered optimum. The HA activity was maximum at a temperature ranging from 0-35°C. The agglutinin activity of the hemolymph will be decreased significantly above 35°C and abolished at 60°C conforming to its proteinaceous nature.A similar pattern in activity was also reported in *Sarcophaga peregrine* (Komano, 1980), *Limulus* and *Asterias* (Cooper, 1982) and many crustaceans (Hall and Rowlands 1974 a; Imai *et al*., 1994; Ji *et al*., 2001; Rittidach *et al*., 2007). On the surface of the hemocyte, several hemagglutinins have been identified which will act as receptors to tie up with the surface sugars of foreign particles.

The isolated fraction takes chargeof the activity of HA found in the crude hemolymph as demonstrated by the lectin of pure hemolymph which is similar to the hemagglutinability to crude serum against a variety of mammalian erythrocytes. Both the crude hemolymph agglutinin and affinity-purified lectin gave the greatest titer of HA with rat erythrocytes. The report of Nowak *et al.* (1986) reveals the contiguity of NeuAc and O-acetyl sialic acid on the rat erythrocyte membrane. The ability of the lectin to agglutinate sialic acid-containing rat erythrocytes with great avidity suggests the possibility of the sialic acid specificity of the lectin. The presence of glycoproteins such as sialic acids N-acetyl neuraminic acid (85.5%), N-glycolyl neuraminic acid (14.5%) and N-acetyl, 9-O-acetyl neuraminic acid, 8, 9-di-O-acetyl neuraminic acid has been established by BSM to inhibit agglutination. The sialic acid specificity of the *V. litterata*, later the lectin was confirmed by the incompetence of the lectin to agglutinate desialylated rat erythrocytes and the desialylated and de-O-acetylated BSM to inhibit hemagglutination. A strong affinity for the *V. litterata* lectin may be assisted by the development and verification of sialic acid on rat erythrocyte sialo conjugates. *Cancer antennarius* has also been connected to O-acetyl sialic acid-specific lectins (Ravindranath *et al*., 1985). In addition to N-acetyl neuraminic acid and sialic acid-containing glycoproteins, the hemagglutinability was also inhibited by acetylated aminosugars GluNAc, GalNAc and ManNAc. The presence of serum agglutinins inhibited by acetylated aminosugars was also reported in lobster (Hall and Rowlands 1974b), prawn (Ratanapo and Chulavatanol 1990; Vargas Albores, 1993; Fragkiadakis and Stratakis 1995) and crab (Cassels *et al*., 1986).

The purified lectin showed avid binding to rat erythrocytes rich in neuraminic acid and was inhibited by BSM which also contains neuraminic acid. Sialidase treatment and de-O-acetylation of BSM reduced its composure potency. In addition to BSM, the crab lectin also binds to GluNAc, NeuAc and ManNAc with great potency and GalNAc with less potency. This goes along with the suggestion of Sharon and Lis (1989) who stated that in rare cases, lectins may bind to unrelated sugars as in the case of wheat germ agglutinin which reacts with GluNAc (including oligomers), NeuAc and GalNAc. Similar results were also reported in the freshwater prawn, *Macrobarachium rosenbergii*, (Agundis *et al*., 2000) and the shrimp *Litopenaeus vannamei* (Sun *et al*., 2007) where agglutinin was inhibited by N-acetylated sugar residues, such as N-acetyl-D-glucosamine, N-acetyl-D-neuraminic acid and N-acetyl-D-galactosamine. O-acetyl sialic acid-specific lectins have also been purified from brachyuran crabs such as *Cancer antennarius* (Ravindranath *et al.*, 1985), *Paratelphusa jacquemontii* (Denis *et al;* 2003) and *Episesarma tetragonum* (Viswambari Devi *et al.*, 2013). As compared to α2-3 and/or α2-6 linkage of the terminal sialic acid along with N-and/or O-type glycosylation the presence of sialic acid is high in glycoprotein as it was inhibited. Fetuin and acid glycoprotein are found to be powerful inhibitors of an agglutinin in the current experiment. Sialic acid-binding lectins are valuable diagnostic tools for identifying bacterial strains and tumour-associated sialylated antigens (Ravindranath and Cooper 1984; Ravindranath

et al., 1985; Ravindranath *et al*., 1988). They can also be used to assess the epitope specificity of the antibodies directed against salic antigens (Ravindranath *et al*., 1988). Based upon the features of lectins it may be employed. In the process of controlling pathogens, the antimicrobial and anti-viral activities of lectins can be employed. In the therapeutic industry, a large amount of anti-tumour and antiviral drugs were applied (Rabia Hamid *et al*., 2013).

CONCLUSION

The hemolymph of the freshwater crab *Varuna literatta* was purified lectin with a molecular weight of 70 kDa. The hemolymph lectin agglutinated with great avidity, the erythrocytes that exposed N-glycolyl neuraminic acid as the predominant epitope determinant. The isolated lectin was specific for the sugars D-galactose, N-acetyl–D-glucosamine, N-acetyl-Dmannosamine, trehalose and the sialoglycoproteins thyroglobulin, fetuin, and BSM. Sialic acid lectins or antibodies may be used as specialized sensors to examine the function of cell surface sugars during cellular development, differentiation, and malignant transformation. The purified hemolymph lectin against various mammalian erythrocytes was essentially the same as that of the crude serum; the ability of the lectin to agglutinate sialic acid-containing rat erythrocytes with great avidity suggests the possibility of the sialic acid specificity of the lectin. The hemolymph agglutinin was inhibited by N-acetyl sugars such as GluNAc, ManNAc, NeuAc and GalNAc, and sialic acid-containing glycoproteins, Bovine Submaxillary mucine> lactoferrin >holotransferrin = α-acid glycoprotein > PSM > fetuin = apotransferrin. The outcome of this research strongly implies that *V. literatta* possesses a sialic acid-specific lectin in the hemolymph with a significant affinity for the O-acetyl group. This lectin could be employed as a useful diagnostic tool for determining O-acetyl NeuGc on the cell surfaces of microorganisms and tumour cells. The sialic acid specificity of the *V. litterata* lectin was confirmed by the inability of the lectin to agglutinate desialylated rat erythrocytes and the desialylated and de-O-acetylated BSM to inhibit hemagglutination. The specific binding property of salic acid and their respective functions since the identification of the binding site of the pathogens will be useful in medical and therapeutic research because of the complexity of glycoconjugates on the cell surface of pathogens.

FUTURE SCOPE

During the study of cancer biology and research on lectins, the salic acids and their respective derivatives on the cell surfaces were identified. The hemolymph of the freshwater crab *Varuna literatta* lectin site of synthesis can be identified and was cloned. Application of lectins is possible depending on their properties. The antimicrobial and anti-viral activities of lectins can be made use of in the control of pathogens. The production of anti-tumor and antiviral drugs based on lectins may also have a significant utility in therapeutic industry

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