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Antimicrobial properties and phenoloxidase activation of the lectin isolated from kadal shrimp (*Metapenaeus dobsoni*)

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ABSTRACT

The present study reveals purification and characterization of the lectin from the haemolymph of *Metapenaeus dobsoni*. The *Md*-Lec was purified by affinity chromatography with mannose coupled sepharose CL-4B column and it exhibits single band with a molecular weight of 68 kDa in SDS-PAGE. Furthermore, the molecular mass was confirmed by MALDI-TOF and functional groups present were analysed by FTIR. The surface morphology of purified *Md*-Lec displays the homogeneous nature of protein. The X-ray diffraction (XRD) analysis expresses three peaks at 10.7716̊, 21.6258̊ and 31.7523̊which indicate the crystalline nature of the protein and the retention time of 3.068 min evident from HPLC reveals the purity of the sample. Functional analysis of purified *Md*-Lec exhibits yeast agglutination activity against *Saccharomyces cerevisiae* and has the ability to agglutinate the human erythrocytes, which was observed by light microscopy. It also exhibited phenoloxidase activation, encapsulation and phagocytic activities. In addition, purified *Md*-Lec showed the broad spectrum of bacterial agglutination activity against Gram negative *Vibrio parahaemolyticus* and *Aeromonas hydrophila*, important fish pathogens.

1. Introduction

Lectins are glycoproteins which can bind to carbohydrates like mannose, galactose, lactose, N-acetyl glucosamine, N-acetyl galactosamine, fucose, and rhamnose with significant specificity [1]. They possess at least one carbohydrate recognition domain (CRD) that specifically and reversibly binds to different sugar moieties present on the surfaces of pathogens, and lectins are present in almost all organisms including plants, animals, viruses, bacteria, cyanobacteria and yeasts [2–5].

Lectins are involved in many biological functions including cell adhesion, phagocytosis, complement activation and innate immunity [6]. Based on their structure, binding specificities and calcium dependency, lectins are classified into different families like C-type lectins, F-type lectins, galectins, intelectins, rhamnose binding lectins, I-type lectins, Lily-type lectins etc. [7]. C-type lectin (CTL), one of major marine lectins, is characterized by Ca^{2+}-dependentbinding tomono and oligosaccharides, and they are classified in several groups; collectins, proteoglycan core proteins, selectins directly or indirectly involved in immune function [8]. Role of different types of lectins, their therapeutic applications and the tissue distribution of lectins in the fishes is described by our group [9].

In invertebrates like shrimp, based on structural differences, expression patterns and functions, there are seven groups of lectins: C-type, L-type, P-type, M-type, fibrinogen-like domain lectins, galectins, and calnexin/calreticulin [10]. The shrimp lectins take part in innate immune mechanisms by activating prophenoloxidase, encapsulation, melanization and promotion of phagocytosis [11–15].

Metapenaeus dobsoni is distributed along the Indian coast, off Sri Lanka, Malaysia, Indonesia to Philippines and New Guinea. In India it is common along the South west coast- Kerala, Goa, Karnataka and the south east coast-Orissa, Visakhapatnam, Tamil Nadu and Union territory of Puducherry [16]. Presently little information is available about the immune system and immune response in kadal shrimp. Therefore, the present study addressed to characterize the immune response in kadal shrimp, with focus on isolation and characterization of a lectin from haemolymph and substantiate its functional roles in agglutination, antimicrobial and anticancer properties.

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2. Materials and methods

2.1. Collection and preparation of samples

Live and healthy kadal shrimps (*Metapenaeus dobsoni*) were obtained from a hatchery at Varapuzha, Kerala, India. Haemolymph was collected and extracted in aseptic conditions using gauge 23 hypodermic needles and diluted with 1:1 anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 7.5). A total of 30 ml haemolymph was pooled for purification of lectin. Then it was centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was collected into fresh tubes and stored at −20 °C until use.

2.2. Purification of lectin from kadal shrimp haemolymph

Lectin was purified according to the method described by Jayanthi et al. [17] with slight modifications. Briefly, mannose coupled sepharose CL-4B column was used for the purification of *Md*-Lec. The column was extensively washed with TBS/$CaCl_2$ buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4). The haemolymph was equilibrated with an equal volume of TBS/$CaCl_2$ buffer. Followed by this equilibration, the equilibrated sample was gently applied into a mannose coupled sepharose CL-4B column. Purified fractions were eluted with elution buffer (10 mM TrisHCl, 140 mM NaCl, 3 mM EDTA, pH 8.0) containing EDTA.

2.3. Characterization of purified Md-Lec

2.3.1. Molecular weight determination

Polyacrylamide gel electrophoresis (12%) was performed on eluted fractions under reduced conditions as described by Laemmli [18]. After electrophoresis, the gel was stained with Coomassie brilliant blue (GE Healthcare Bio-Sciences, India) and image captured through ChemiDoc XRS + system (Bio-rad, USA). The molecular mass of the purified *Md*-Lec was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins (Takara BIO INC, Japan). Total protein concentration was determined by Lowry's method [19] using bovine serum albumin (BSA) as standard.

2.3.2. High performance liquid chromatography and X-ray diffraction analysis

HPLC separation was carried out using a reversed phase C18 column (7.8 mm × 30 cm) previously equilibrated with TBS-I at a flow rate of 0.8 ml min^{-1}. HPLC system (Zorbax Bio-series GF-250, Du Pont, Willington, DE, USA), was used for the homogeneity analysis. In order to determine the spatial distribution of atomic coordinates and the arrangement of atoms, an XRD analysis (XRD, Scintag-SDS 2000) was performed on purified *Md*-Lec at 40 kV/20 mA, using continuous scanning 2θ mode. The average grain size and shape of the purified *Md*-Lec was determined using Scherrer's formula [$d = (0.9\lambda/\beta\cos\theta)$, where d is the mean diameter of purified *Md*-Lec, λ is the wavelength of the X-ray radiation source, and β is the angular FWHM of the XRD peak at the diffraction angle (θ).

2.3.3. Fourier transform infrared spectroscopy (FTIR) and MALDI-TOF analysis

For FTIR spectra studies, purified *Md*-Lec (1–50 µl) was placed in a thermostated cell fitted with CaF_2 windows (with 6 µm Teflon spacer for measurements in water). The spectra of biological molecules were recorded at a resolution of 4 cm^{-1}.

For MALDI-TOF analysis, the purified 68 kDa protein was excised from SDS-PAGE gel and subjected to trypsin digestion. In brief, the gel bands corresponding to *Md*-Lec were finely cut and added to 1.5 ml eppendorf tubes containing stain removal solution (100 mM ammonium bicarbonate: 50% acetonitrile (1:1)) vortexed for 30 min. After complete removal of stain, the gel pieces were dehydrated with 100% acetonitrile (ACN). Then, the gelpieces were subjected to reduction and alkylation. Trypsin solution was formulated in 40 mM NH_4HCO_3 and added to each eppendorf tubes containing gel pieces and stored at ice cold condition for absorption of trypsin. After absorption, NH_4HCO_3 was added and incubated at 37 °C for 12–16 h. The digestion was stopped by adding 4% formic acid. The peptides were extracted by washing gel pieces 3 times with 4% FA in 50% ACN. The resultant peptides were analysed by MALDI-TOF/TOF MS (Shimadzu, Biotek Axima Performance) [30].

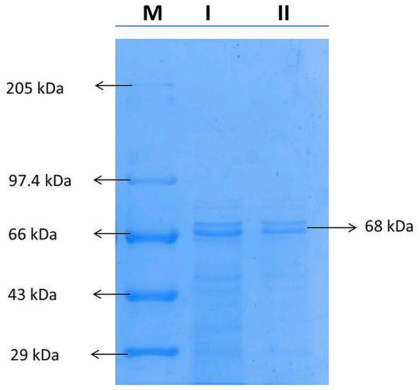

Fig. 1. Polyacrylamide gel electrophoresis (12%) in the presence of sodium dodecyl sulfate (SDS) of *Md*-Lec purified by Mannose-Sepharose CL 4B affinity chromatography. Lane M contains High molecular weight marker. Lane I contains reducing pattern and Lane II contains non-reducing pattern of purified *Md*-Lec from *Metapenaeus dobsoni* haemolymph. The protein bands were stained with Coomassie brilliant blue – R 250. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4. Functional analysis

2.4.1. Haemagglutination assay

The haemagglutination assay (HA) was performed in microtiter plates according to Correia and Coelho [20]. Lectin preparations (50 µl) were serially two fold diluted in PBS before addition of 50 µl 2% (v/v) suspension of human erythrocytes. In control, purified *Md*-Lec was replaced by BSA. The titer was expressed as the highest dilution exhibiting haemagglutination. The HA was defined as the inverse of the last dilution at which the sample still showed haemagglutination. Specific HA (SHA) was defined as the ratio between the titer and protein concentration (milligrams per milliliter). These haemagglutinated titres of *Md*-Lec were visualized by light microscopy at the magnification of 40X (Leica DMIL).

2.5. Phenoloxidase enhancing activity

The ability of *Md*-Lec in activating prophenoloxidase was studied by measuring the formation of dopachromes from L-DOPA. As per the method described by Iswarya et al. [21], 50 µg/ml of purified *Md*-Lec was mixed with equal volume of laminarin (1 mg ml^{-1}) and incubated at 25 °C for 15 min. Then 50 µl haemocyte lysate supernatant was introduced and re-incubated for 45 min in the presence of 5 mM $CaCl_2$. Subsequently, 50 µl L-DOPA (3 mg ml^{-1}) as enzyme substrate was added and incubated at 25 °C for different time intervals (30, 60, 90, 120 min). In control *Md*-Lec is replaced by TBS I buffer. After

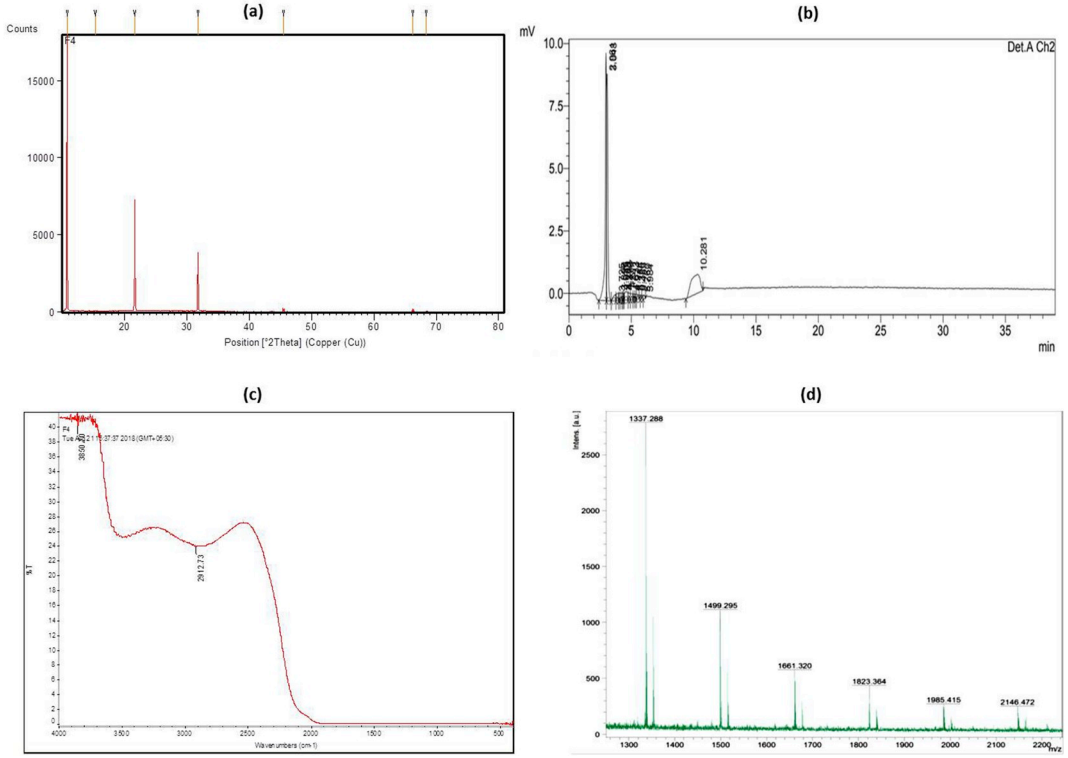

Fig. 2. Biochemical characterization of *Md*-Lec. **(a)** XRD analysis of purified *Md*-Lec showed diffraction peaks at 10.771$\mathring{0}$, 21.625$\mathring{8}$ and 31.752$\mathring{3}$ which indicate the crystalline nature of the protein **(b)** The HPLC analysis of purified *Md*-Lec showing a single peak with a retention time at 3.068 min which indicates the purity of the sample **(c)** FTIR analysis for functional group identification. The attributions of the main absorption characteristics of glycosidic structures are related to O–H stretching (3000–3500 cm^{-1}) **(d)** MALDI–TOF/TOF MS peptide analysis of 68 kDa Md-Lec.

incubation, the formation of dopachrome was spectrophotometrically measured in the wavelength of 490 nm and expressed as unit/min/mg/protein.

2.5.1. Encapsulation assay

To evaluate the encapsulation activity of *Md*-Lec was carried out by the method reported earlier [21], with slight modifications. The haemocyte suspension was mixed with sepharose beads suspension and *Md*-Lec (25, 50 and 100 μg/ml) in V- bottomed microtitre plate and allowed to incubate at 25 °C for 45 min with intermediate mixing at every 15 min. In control wells, *Md*-Lec was replaced by TBS I buffer. The entire volume from each suspension was spread on sterilized glass slide and kept undisturbed for 10 min. Then the slides were observed under light microscope (40X) (Leica DMIL).

2.5.2. Yeast agglutination assay

To determine the ability of agglutination of *Md*-Lec to yeast cells (*Saccharomyces cerevisiae*), 50 μl of different concentrations of the purified *Md*-Lec was added to a U-shaped 96-well microtitre plate containing equal volume of Tris buffer. The same volume of a suspension of yeast (10^6 cells ml^{-1})was then added to the wells and incubated for 4 h at 25° C. In control, purified *Md*-Lec was replaced by BSA. The pattern of agglutination was monitored with an inverted light microscope (40X) (Leica DMIL).

2.5.3. Bacterial agglutination assay

The bacterial agglutination by purified *Md*-Lec against *V. parahaemolyticus* and *A. hydrophila* was evaluated as described by Rubeena et al. [22]. Different concentrations of the purified *Md*-Lec were added to a 96-well microtitre plate and an equal volume of Tris buffer was added. In control, purified *Md*-Lec was replaced by BSA. The same volume of a suspension of bacterial cells (10^6 cells ml^{-1}) was then added to the wells and incubated for 30 min and then the pattern of agglutination was monitored with an inverted light microscope (10X) (Leica DMIL).

2.5.4. Antibiofilm assay

The effect of purified *Es*-Lec on biofilm-forming Gram-negative bacteria (*Aeromonas hydrophila* and *Vibrio parahaemolyticus*) was tested using 24-well polystyrene plates containing glass pieces immersed in Luria Bertani broth inoculated with the bacterial suspension of 1% inoculum from overnight cultures (10^7 CFU ml^{-1}). Different concentrations of *Md*-Lec were introduced into the wells and incubated at 37° C for 48 h. For examining the biofilm, the media were discarded and weakly adherent cells were removed by thorough washing with deionized water and allowed to air dry before staining. The biofilm were stained with 1 ml of 0.4% acridine orange (w/v) for 10 min. The biofilm inhibition in 3D view was observed by confocal laser scanning microscopy (Carl Zeiss LSM 710 Germany). In control, *Md*-Lec was replaced by BSA.

2.6. Statistical analysis

All experiments were performed in triplicates (n = 3). Data are presented as mean ± standard error mean (S.E.M.) of control and treated samples. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Tukey's HSD test and the significance accepted at $P^{<}$ 0.05.

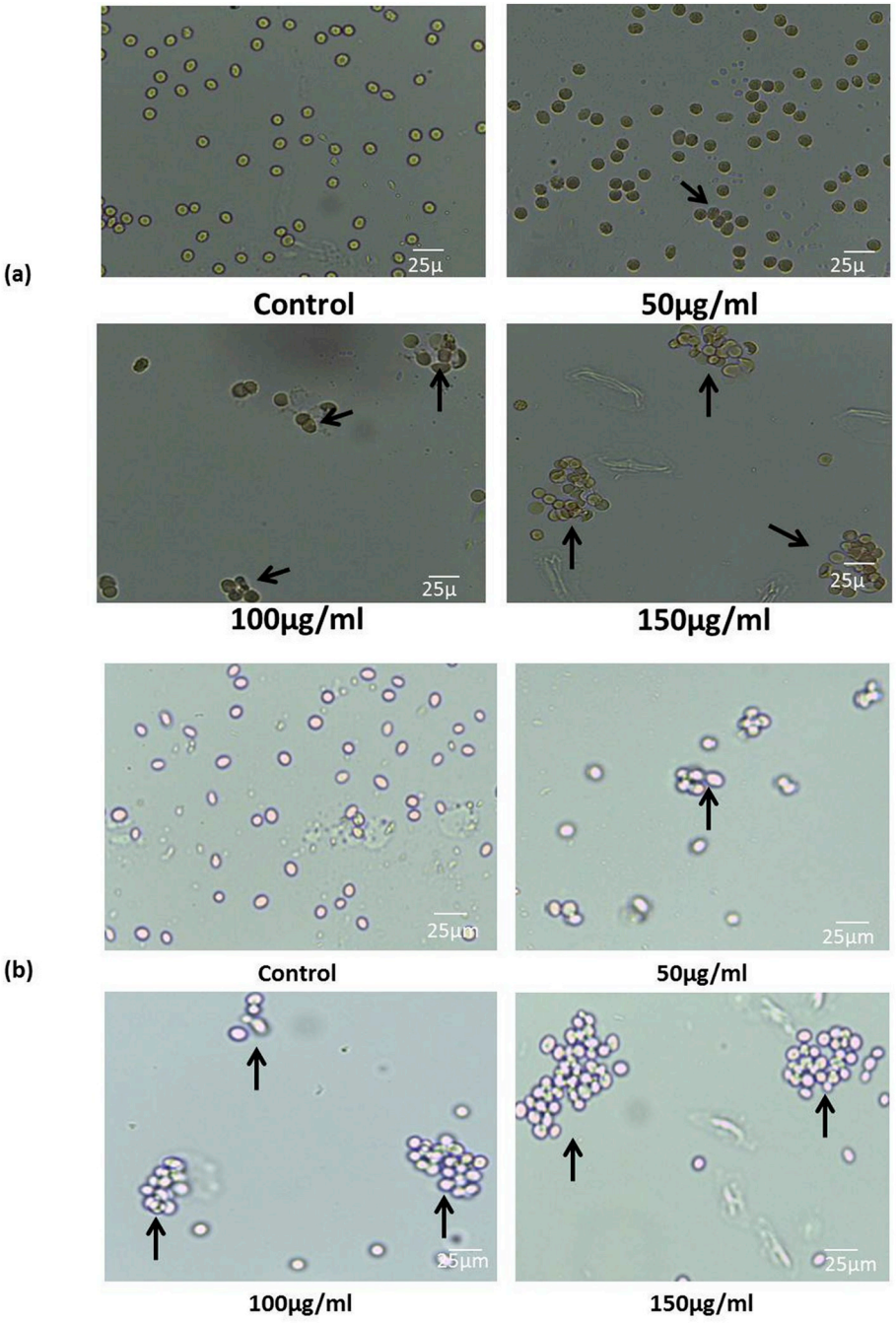

Fig. 3. (a) Haemagglutination assay with against 2% human RBC suspension and **(b)**yeast agglutination assay of *Md*-Lec at different concentrations. Highest concentration (150 µg/ml) showed maximum agglutination. Black arrows indicate the agglutination.

3. Results

3.1. Purification of lectin from kadal shrimp

Lectin from haemolymph of kadal shrimp was purified by affinity chromatography and the SDS-PAGE analysis revealed a doublet band of approximately 68 kDa in 12% SDS-PAGE under reducing conditions (Fig. 1).

3.2. HPLC and X-ray diffraction analysis

The XRD analysis of purified *Md*-Lec showed one major diffraction peak at 10.771Å and other peaks at 21.625Å and 31.752Å (Fig. 2a) which shows the lattice arrangement and crystalline nature of protein. The HPLC analysis revealed a single peak of retention time of 3.068 min (Fig. 2b).

3.3. FTIR and MALDI-TOF analysis

FTIR analysis was done for functional group identification. The attributions of the main absorption characteristics of glycosidic structures are related to O–H stretching (3000–3500 cm^{-1}) (Fig. 2c). The intensity of the peaks obtained in MALDI-TOF analysis refers to the 68 kDa protein (Fig. 2d) and the mass measurement was done following Henzel et al. [36].

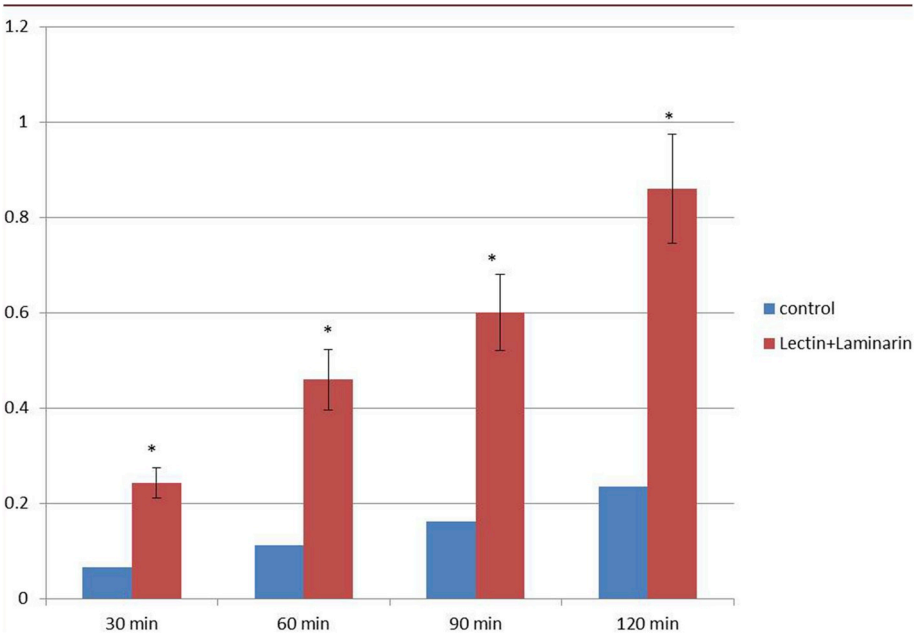

Fig. 4. Specific PO activity of *Md*-Lec at different time intervals of incubation. PO activity increased with time of incubation.

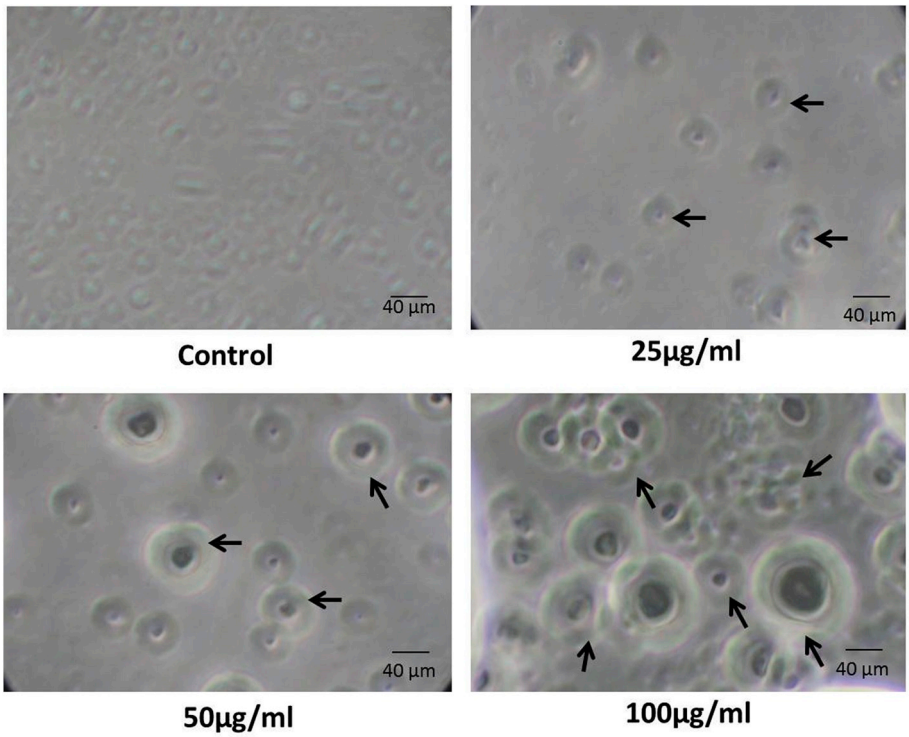

Fig. 5. Light microscopic images showed encapsulation of Sepharose CL4B beads with *Md*-Lec. The arrow indicates the encapsulation of beads.

3.4. Haemagglutination assay

Purified *Md*-Lec had the ability to agglutinate human erythrocytes, and agglutination was highest in 150 µg/ml of purified *Md*-Lec *vs.* the lower concentration (50 µg/ml). No agglutination occurred when purified *Md*-Lec was replaced by BSA (Fig. 3a).

3.5. Phenoloxidase enhancing activity

PO assay indicates that purified *Md*-Lec could enhance phenoloxidase activity together with laminarin and it strongly depends on time as it was proved that longer the incubation time higher the PO activity (Fig. 4).

3.6. Encapsulation assay

Encapsulation ability of the haemocytes from *M. dobsoni* was evaluated using sepharose beads *in vitro*. The haemocytes extensively encapsulated sepharose beads up on the addition of *Md*-Lec. High encapsulation activity was observed with 100 µg/ml of purified *Md*-Lec. In control, encapsulation activity was not observed due to the absence of lectin (Fig. 5).

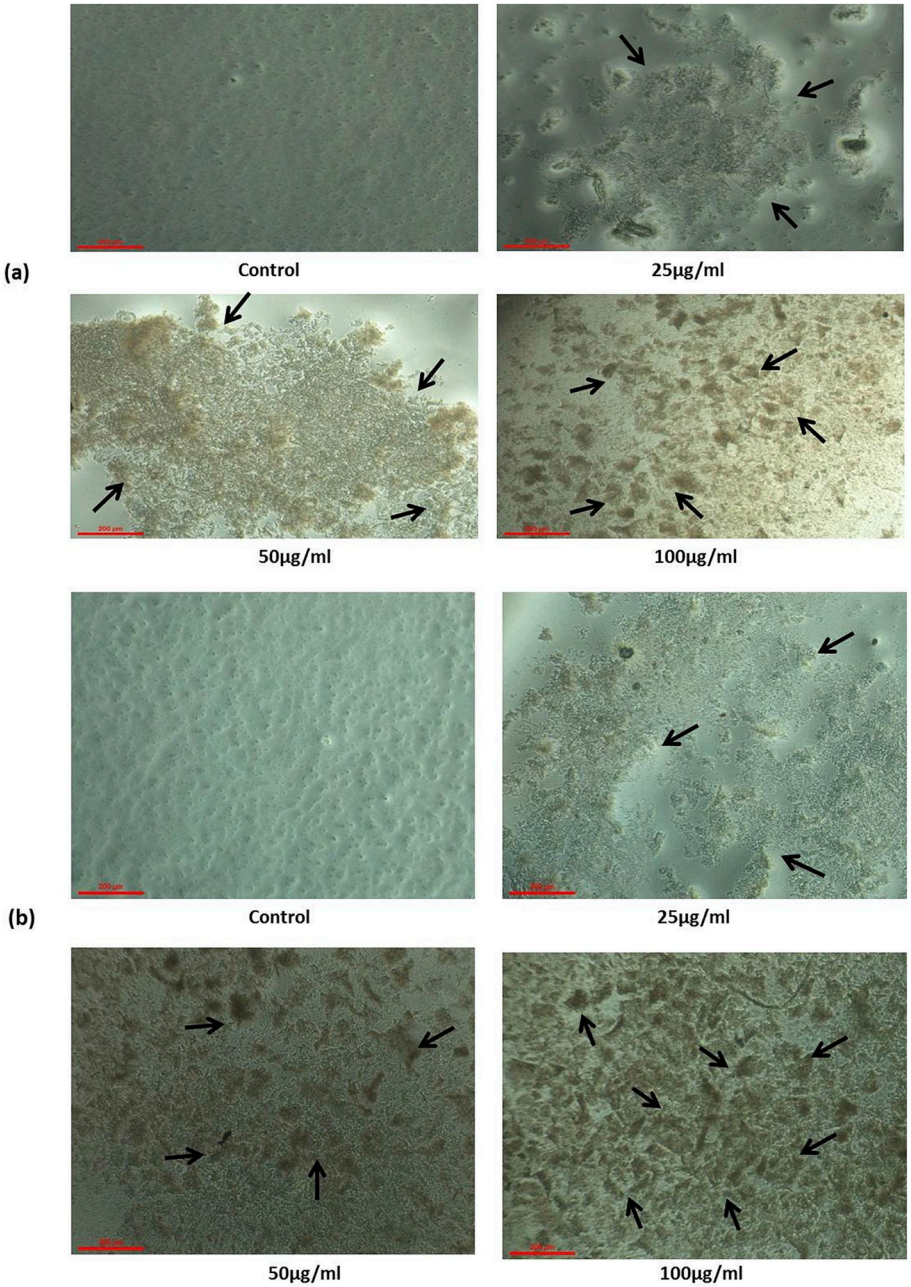

Fig. 6. Bacterial agglutination effect of *Md*-Lec against clinically important **(a)** Gram negative *A. hydrophila* and **(b)** Gram negative *V. parahaemolyticus* demonstrated by light microscopy. Higher concentration of lectin showed maximum agglutination activity. Black arrows indicate the agglutination.

3.7. Yeast agglutination assay

The purified *Md*-Lec showed ability to agglutinate *S. cerevisiae* and highest agglutination was revealed in 150µg/ml of purified *Md*-Lec compared to the lowest concentration (50 µg/ml). No agglutination occurred when purified *Md*-Lec was replaced by BSA (Fig. 3b).

3.8. Bacterial agglutination assay

The purified *Md*-Lec could bind and agglutinate *A. hydrophila,* and *V. parahaemolyticus* and agglutination was evident as clumps of bacteria as revealed in 100µg/ml of purified *Md*-Lec *vs*. the lowest concentration (25 µg/ml). No agglutination occurred when purified *Md*-Lec was replaced by BSA (Fig. 6a and b).

3.9. Antibiofilm assay

The biofilm architecture was interrupted the increased concentrations of *Md*-Lec which was evident by decreased bacterial count of *A. hydrophila* and *V. parahaemolyticus* observed by confocal laser scanning microscopy (Carl Zeiss LSM 710 Germany) (Fig. 7).

4. Discussion

Till date several lectins are purified from crustaceans with a higher range of molecular weights of 452 kDa from *Penaeus japonicas* (Kuruma shrimp) [23] and 220 kDa *Litopenaeus schmitti* (White shrimp) [24] to a lower range of molecular weights of 14 kDa from *Tachypleus tridentatus* (Tri-spine horse-shoe crab) [25] and 20 kDa from *Scylla serrata* (Mud crab) [26]. In the present study, we isolated and purified a mannose binding lectin of 68 kDa from the haemolymph of Kadal shrimp which

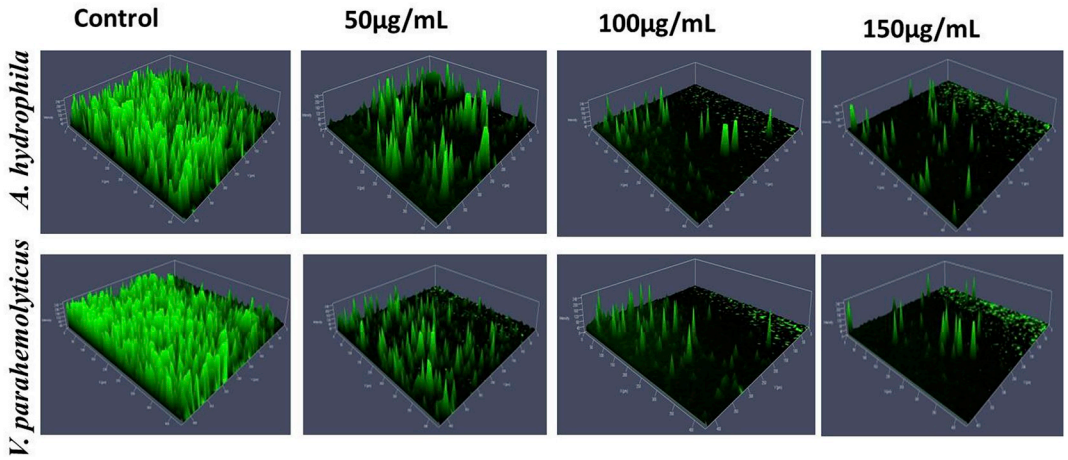

Fig. 7. Antibiofilm effect of *Md*-Lec on biofilm inhibition against clinically important Gram negative *A. hydrophila* and Gram negative *V. parahaemolyticus* with the stain of acridine orange demonstrated by confocal microscopy. Higher concentration of lectin showed maximum activity against biofilm formation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was confirmed by SDS-PAGE. The doublet band indicates the post translational modifications (phosphorylation) [35]. Previously, a lectin with a molecular weight of 66 kDa was reported in *Ictalurus punctatus* (Ictalurid catfish) [27]. The mannose coupled sepharose CL-4B affinity column was used for the purification of lectin from Kadal shrimp haemolymph. The calcium ions in the buffer facilitated the binding of lectin on to the mannose ligands in the column, while during elution of lectin from the column, the EDTA present in the elution buffer chelated the calcium ions and hence the lectin was released. Furthermore, the purity and crystalline nature was confirmed by HPLC and XRD analysis respectively, as reported by Jayanthi et al. [28] previously. The functional group analysis and confirmation of molecular mass was done by FTIR analysis and MALDI-TOF analysis which goes in parallel with those reported by Divya et al. [29].

The functional properties of *Md*-Lec were evaluated by haemagglutination and yeast agglutination assay in which agglutination of RBC and yeast cells occurred in a dose-dependent manner. This suggests that when more lectin was available the more they could bind to the sugar moieties present on the cell surfaces. The haemagglutination activity reported by Silva et al. [30] agrees with the findings of the present study. In addition to the role of *Md*-Lec in agglutination, it also plays vital role in encapsulation and phagocytosis. *Md*-Lec was able to facilitate encapsulation against the sepharose beads and this property is previously reported in some other invertebrates also [28,31,32]. Prophenoloxidase (ProPO) mechanism is actively involved in the extermination of pathogens. Sivakamavalli et al. [31] reported that the complex formed between PAMPs and lectin could activate the ProPO (inactive form) to PO (active form) as a result of the degranulation of haemocytes [33]. In the present study, the PO system was triggered by *Md*-Lec along with laminarin and the PO activity was more efficient over time.

Furthermore, the antimicrobial property of *Md*-Lec was studied. *Md*-Lec could agglutinate the Gram negative aquatic pathogens tested in a dose-dependent manner and inhibited the biofilm formation which in turn prevented the pathogens from colonizing and proliferating on host surface as reported by Jayanthi et al. [28]. The intensity of biofilm disruption was studied by confocal microscopy [29]. To conclude, in this present study, we report the purification of immune related lectin *Md*-Lec with a molecular mass of 68 kDa from the haemolymph of *Metapenaeus dobsoni* by affinity column chromatography technique which was confirmed by SDS-PAGE and strongly establish the agglutination, encapsulation, PO mechanism and antibiofilm properties which were not extensively studied as evident by the available literature. Furthermore, the biochemical properties of *Md*-Lec were studied by MALDI-TOF, HPLC, XRD and FTIR. A significant antibacterial and antibiofilm activity against the most important aquatic pathogens-Gram-negative bacteria *V. parahaemolyticus* and *A. hydrophila* was exhibited by *Md*-Lec indicating its therapeutic potential which demands further molecular studies to develop this molecule into a reliable biological tool in the field of aquaculture.

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References

[1] C.L. Nilsson, Chapter 1 - lectins: analytical tools from nature, in: C.L. Nilsson (Ed.), In Lectins, Elsevier Science B.V, Amsterdam, 2007, pp. 1–13.
[2] R. Loris, Principles of structures of animal and plant lectins, Biochim. Biophys. Acta 1572 (2002) 198–208.
[3] M. Veelders, S. Brückner, D. Ott, C. Unverzagt, H.U. Mösch, L.O. Essen, Structural basis of flocculin-mediated social behavior in yeast, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 22511–22516.
[4] R.S. Ferreira, T.H. Napoleao, A.F.S. Santos, R.A. Sa, M.G. Carneiro-da-Cunha, M.M.C. Morais, R.A. Silva-Lucca, M.L.V. Oliva, L.C.B.B. Coelho, P.M.G. Paiva, Coagulant and antibacterial activities of the water-soluble seed lectin from *Moringa oleifera*, Lett. Appl. Microbiol. 53 (2011) 186–192.
[5] E.S. Nunes, M.A.A. Souza, A.F.M. Vaz, T.G. Silva, J.S. Aguiar, A.M. Batista, M.M.P. Guerra, M.C. Guarnieri, L.C.B.B. Coelho, M.T.S. Correia, Cytotoxic effect and apoptosis induction by *Bothrops leucurus* venom lectin on tumor cell lines, Toxicon 59 (2012) 667–671.
[6] M.A. Lino, R.F. Bezerra, C.D.C. da Silva, E.V.M.M. Carvalho, L.C.B.B. Coelho, Fish lectins: a brief review, Advances in Zoology Research, vol. 5, Nova Science Publishers, Inc., 2013, pp. 95–114.
[7] T. Ogawa, M. Watanabe, T. Naganuma, K. Muramoto, Diversified carbohydrate-binding lectins from marine resources, J. Amino Acids (2011) 838–914 2011.
[8] G.R. Vasta, H. Ahmed, Animal Lectins: A Functional View, CRC Press, 2008 2008.
[9] Elumalai Preetham, Abdul Salam Rubeena, Ratree Wongpanya, Matteo Cammarata, Einar Ringo, Baskaralingam Vaseeharan, Lectins in finfishes: a review, Reviews in Fisheries Sci. Aquacult. 27 (2019) 152–169.
[10] Xian-Wei Wang, Jin-Xing Wang, Diversity and multiple functions of lectins in shrimp immunity, Dev. Comp. Immunol. 39 (2013) 27–38.
[11] X.Q. Yu, M. R. Kanost Immulectin, An inducible C-type lectin from an insect, Manduca sexta, stimulates activation of plasma prophenol oxidase, Insect Biochem. Mol. Biol. 29 (1999) 585–597.
[12] X.Q. Yu, M.R. Kanost, Immulectin-2, a lipopolysaccharide-specific lectin from an insect, Manduca sexta, is induced in response to gram-negative bacteria, J. Biol. Chem. 275 (2000) 37373–37381.
[13] T. Luo, H. Yang, F. Li, X. Zhang, X. Xu, Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*, Dev. Comp. Immunol. 30 (2006) 607–617.
[14] N. Koizumi, M. Imamura, T. Kadotani, K. Yaoi, H. Iwahana, R. Sato, The lipopolysaccharide-binding protein participating in haemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains, FEBS Lett. 443 (1999) 139–143.
[15] T. Jomori, S. Natori, Function of the lipopolysaccharide-binding protein of

Periplaneta americana as an opsonin, FEBS Lett. 296 (1992) 283–286.
[16] Pillai Lakshmi, P. Thirumilu, Fishery and stock assessment of *Metapenaeus dobsoni* (Miers, 1878) off Chennai, Indian J. Geo-Marine Sci. 42 (2013) 448–452.
[17] S. Jayanthi, R. Ishwarya, M. Anjugam, A. Iswarya, S. Karthikeyan, B. Vaseeharan, Purification, characterization and functional analysis of the immune molecule lectin from the haemolymph of blue swimmer crab *Portunus pelagicus* and their antibiofilm properties, Fish Shellfish Immunol. 62 (2017) 227–237.
[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *P*rotein measurement with the Folin phenol reagent, *J*. Biol. Chem. *1*93 (1951) 265–275.
[20] M.T.S. Correia, L.C.B.B. Coelho, Purification of a glucose/mannose specific lectin, isoform 1, from seeds of *Cratylia mollis* mart. (Camaratu Bean), Appl. Biochem. Biotechnol. 55 (1995) 261–273.
[21] A. Iswarya, M. Anjugam, B. Vaseeharan, Role of purified β-1, 3 glucan binding protein (β-GBP) from *Paratelphusa hydrodromus* and their anti-inflammatory, antioxidant and antibiofilm properties, Fish Shellfish Immunol. 68 (2017) 54–64.
[22] Abdul Salam Rubeena, Mani Divya, Baskaralingam Vaseeharan, Sivashanmugam Karthikeyan, Einar Ringø, Elumalai Preetham, Antimicrobial and biochemical characterization of a C-type lectin isolated from pearl spot (*Etroplus suratensis*), Fish Shellfish Immunol. 87 (2019) 202–211.
[23] H. Yang, T. Luo, F. Li, S. Li, X. Xu, Purification and characterization of a calcium-independent lectin (PjLec) from the haemolymph of the shrimp *Penaeus japonicas*, Fish Shellfish Immunol. 22 (2007) 88–97.
[24] M.R. Cominettia, M.R.F. Marquesb, D.M. Lorenzinib, S.E. Löfgrena, S. Daffrec, M.A. Barracco, Characterization and partial purification of a lectin from the hemolymph of the white shrimp *Litopenaeus schmitti*, Dev. Comp. Immunol. 26 (2002) 715–721.
[25] K. Inamori, T. Saito, D. Iwaki, T. Nagira, S. Iwanaga, F. Arisaka, S. Kawabata, A newly identified horseshoe crab lectin with specificity for blood group A antigen recognizes specific O Antigens of bacterial lipopolysaccharides, J. Biol. Chem. 274 (1999) 3272–3278.
[26] V. Jayasankar, T. Subramonian, Antibacterial activity of seminalplasma of the mud crab *Scylla serrata* (Forskal), J. Exp. Mar. Biol. Ecol. 236 (1999) 253–259.
[27] D.O. Donald, M.R. Wendy, Purification, characterization and seasonal variation of mannosebinding C-type lectin in Ictalurid catfish, Aquaculture 321 (2011) 191–196.
[28] S. Jayanthi, R. Ishwarya, M. Anjugam, A. Iswarya, S. Karthikeyan, B. Vaseeharan, Purification, characterization and functional analysis of the immune molecule lectin from the haemolymph of blue swimmer crab *Portunus pelagicus* and their antibiofilm properties, Fish Shellfish Immunol. 62 (2017) 227–237.
[29] Mani Divya, Baskaralingam Vaseeharan, Mahalingam Anjugam, Arokiadhas Iswarya, Sivashanmugam Karthikeyan, Palaniyandi Velusamy, Marimuthu Govindarajan, Naiyf S. Alharbi, Shine Kadaikunnan, Jamal M. Khaled, Csaba Vagvolgyi, Phenoloxidase activation, antimicrobial, and antibiofilm properties of β-glucan binding protein from *Scylla serrata* crab hemolymph, Int. J. Biol. Macromol. 114 (2018) 864–873.
[30] C.D.C. da Silva, et al., Purification and characterization of a mannose recognition lectin from *Oreochromis niloticus* (tilapia fish): Cytokine production in mice splenocytes, Appl. Biochem. Biotechnol. 166 (2012) 424–435.
[31] J. Sivakamavalli, B. Vaseeharan, Bifunctional role of a pattern recognition molecule β- 1, 3 glucan-binding protein purified from mangrove crab *Episesarma tetragonum*, J. Invertebr. Pathol. 119 (2014) 25–31.
[32] T. Sminia, E. Borghart-Reinders, A.W. Van, Encapsulation of foreign materials experimentally introduced into the fresh water snail *Lymnacea stargnalis*, Cell Tissue Res. 153 (1974) 307–326.
[33] K. Junkunlo, A. Prachumwat, A. Tangprasittipap, S. Senapin, S. Borwornpinyo, T.W. Flegel, K. Sritunyalucksana, A novel lectin domain-containing protein (LvCTLD) associated with response of the whiteleg shrimp *Penaeus (Litopenaeus) vannamei* to yellow head virus (YHV), Dev. Comp. Immunol. 37 (2012) 334–341.
[35] Natasha T. Snider, M. Bishr Omary, Assays for post-translational modifications of intermediate filament proteins, Methods Enzymol. 568 (2016) 113–138.
[36] William J. Henzel, Todd M. Billeci, John T. Stults, Susan C. Wong, Christopher Grimley, Colin Watanabe, Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 5011–5015.