PURIFICATION AND CHARACTERIZATION OF A NEW N-ACETYL NEURAMINIC ACID SPECIFIC LECTIN (TcLec) FROM THE MIDGUT GLAND OF THE MILLIPEDE, *TRIGONIULUS CORALLINUS*

[1]*Anitha C [2]Basil Rose M.R and [3]Viswambari Devi R [4]Josephine Priyatharshini C and [5]Arokya Glory P.T

[1,4,5]Assistant Professor, [2]Associate Professor, Department of Zoology, Holy Cross College (Autonomous), Nagercoil, affiliated to ManonmaniamSundaranar University, Tirunelveli-627 012, India.[3]Bioengineering and Drug Design Laboratory, Department of Biotechnology, Indian Institute of Technology, Chennai – 600036, India.
Email: anithajeyaraj85@gmail.com

Abstract

A sialic acid specific lectin with specific affinity for the **Gal β1→3**inter-residue glycosidic linkages of rabbit erythrocytes was purified from the extract of the midgut gland of the rusty millipede, *T. corallinus* by affinity chromatography using lactoferrin linked cyanogen bromide activated Sepharose-4B and biospecific adsorption using formalinized rabbit erythrocytes. While the lectin purified by biospecific adsorption showed 465-fold augment in specific activity, the affinity purified lectin showed 2925-fold augment in specific activity. The lectin recognized rabbit erythrocytes with greater avidity than the crude agglutinin and was inhibited by lactoferrin with great potency like the crude agglutinin and appeared as a single band with a molecular mass of 76 kDa(named TcLec) on SDS-PAGE. Experiments on hemagglutination with desialylated erythrocytes and hemagglutination inhibition using desialylatedlactoferrin confirmed the sialic acid specificity of the midgut gland lectin.

Key words: Lactoferrin**,** purification, specificity, adsorption, affinity chromatography

Introduction

All invertebrate species have mechanisms in place to prevent the invasion of bacteria, parasites, viruses, and even cells from other individuals of their own species. Precise identification systems in every known phylum enable differentiation between self and non-self, reducing the possibility of contamination and being mediated by innate immunity.The innate immune system, which includes humoral and cell-based immunity, is an animal's first line of defence against semi- or non-specific pathogen infection. It is strictly controlled by immunological molecules including prophenoloxidases, antimicrobial peptides, lectins, and others (Yang et al., 2012; Romo et al., 2016).

Lectins are structurally complex molecules with single/multi-carbohydrate recognition domains and therefore of potential multiple binding sites. They are an important component of the humoral immune mechanism of arthropods and the processes of self/non-self recognition (Dodd and Drickamer, 2001; Kurata et al., 2006; Vasta et al., 2007).They are able to exhibit antimicrobial and anti-tumor properties by recognising residues of glycoconjugates on the surface of the cells. They do this by recognising sequences of two or more saccharide moieties with specificity towards both inter-residue glycosidic linkages and also anomeric configuration (Richard et al., 2004; Simone et al., 2006).Relatively very few millipede lectins have been isolated and characterized (Basil-Rose et al., 2014; Ponrajet al., 2016). To fully understand the role that lectins play in the millipede innate immune system, further research is still needed to understand their structure, function, and carbohydrate-binding characteristics.Therefore, the present investigation aimed at the purification and characterization of the midgut gland lectin of the millipede, *Trigoniuluscorallinus*.

Materials and Methods

Millipede collection and maintenance

The Zoological Survey of India, Chennai, identified the millipede as *T. corallinus* after it was collected from swampy areas of homes and coconut groves in several diverse localities (Nattalam, Elavuvilai, Arumanai, Panachamoodu, and Nagercoil) in the Kanniyakumari District of Tamilnadu after the monsoon.They were given raw potatoes, cucumber, cabbage, and papaya while being kept in huge cement tanks filled with moist bricks, plantain tree trunks, or dry decomposing leaves. By molting, copulating, and depositing eggs, millipedes easily acclimated to the laboratory environment.

Preparation of midgut gland extract

The millipedes were dissected using a pair of sterile clean scissors and the midgut gland was taken out. The extract was prepared by grinding 100 mg of midgut gland in 1ml of cold saline (0.7%) using a mortar and pestle. The extracts were centrifuged at 4000 g for 10 minutes at 4°C and the supernatant was collected for subsequent use.

Purification of lectin

Affinity chromatography

The manufacturer's instructions were followed while making the CNBr-activated Sepharose 4B gel.The gel was poured into a 5 ml lactoferrin solution (4 mg/ml). At room

temperature, the mixture of swelling gel and lactoferrin was rotated end to end for two hours.The amount of lactoferrin (a protein) in the coupling media was used to gauge the degree of coupling. The Folin-Ciocalteau technique was used to quantify the protein content in the medium before and after coupling.Finally, 5 ml of ethanolamine 1 M HCl (pH 8) was added and gently stirred for a further hour in order to quench the surplus of activated group (if there).After coupling, excess uncoupled lactoferrin was eliminated by washing the adsorbant with pH 8.3 coupling buffer and pH 4 acetate buffer solutions alternately four or five times over a sintered glass funnel.Lactoferrin-agarose was kept refrigerated in TBS with a pH of 7.5 and 0.02% sodium azide. Most of the lactoferrin, or around 75%, was coupled.

The rusty millipede, *T. corallinus*, midgut gland extract (20 ml) was added to 3.5 ml of lactoferrin-agarose in a Bio-Rad econo-column that had been pre-equilibrated with TBS at 4°C.0.6 ml of eluant was taken in every minute.The column was washed with HSB until the effluent's A280 value fell < 0.002. The column was then washedwith heated LSB (32^oC) until the A280 of the effluent was below 0.002, then with cold LSB (4°C). To get homogeneous lectin, this procedure, which also eluted more inert proteins, was required.The buffers used in each of these processes included the calcium needed for the lectin to bind to lactoferrin-agarose. Elution buffer (EB), which contained the calcium chelator (10 mM disodium EDTA), was used to elute the lectin, and fractions of 1 ml were collected at a rate of 0.3 ml/min on ice in polypropylene tubes containing 100 µl of 100 mM $CaCl_2$.The collected fractions required calcium chloride since the lectin was incredibly unstable in the presence of EDTA. After being collected, the fractions were immediately vortexed and kept at 4°C.In order to identify the presence of lectin, using a 1.5% solution of rabbit erythrocytes, the effluents (fractions) obtained during adsorption, rinsing, re-equilibration, and elution were tested for HA activity.The fractions that eluted with the elution buffer included a considerable quantity of lectin were collected on the same day and dialyzed at 4°C against 10 mM $CaCl_2$for two hours, followed by fresh 1 mM $CaCl_2$ for another three hours.

Lectin purified by biospecific adsorption

Erythrocytes from rabbits were collected in Alsevier's solution. Rabbit erythrocytes were immediately centrifuged at 1000 × g for five minutes after being received and in 20 litres of PBS with a pH of 7.5, three times (75 mM NaCl, 75 mM Na_2HPO_4).The formalin solution (3% solution in PBS adjusted to pH 7.5 with 0.1M NaOH) was added in an equal amount after the

cells were suspended at an 8% PBS concentration by volume (pH 7.5).With gentle shaking, for 16 hours, the mixture was incubated at 37°C.The cells were then kept at 4°C as a 10% suspension in the same buffer after being washed four times with five litres of PBS (pH 7.5) per packed cell volume.Emphasis should be placed on the necessity of using relatively fresh erythrocytes for formalinization.Since cells stored for more than two weeks crenated throughout the procedure, they couldn't be used in the agglutination experiment.

The formalin-stored cells were cleaned six times in ten volumes of TBS to prepare them for use as an affinity reagent (50 mM Tris-HCl; 100 mM NaCl).The packed cells were then incubated for 2 hours at 4°C with gentle shaking in a plastic tube containing 20 volumes of cleared midgut gland extract, and rinsed three times with 20 volumes of TBS, pH 7.5, containing 10 mM $CaCl_2$.

By incubating the cells in 10 volumes of 10mM disodium EDTA in TBS, the cells' adsorbed hemagglutinin was eluted. In order to eliminate any remaining particle material, the elution mixture was centrifuged at a speed of 28000 ×g for 10 minutes.After being dialyzed, the resultant supernatant was examined for hemagglutination using 1.5% rabbit erythrocytes in TBS at pH 6.5.The Folin-Ciocalteau technique was used to determine the lectin's protein concentration.

Preparation of erythrocyte suspension

Numerous animals' blood samples were immediately obtained in a modified Alseivier's medium (pH 6.1). Erythrocytes were suspended, cleaned three times with Tris-Buffered Saline (TBS, pH 7.5), and resuspended in TBS as a 1.5% solution by centrifugation at 4000 × g (Mercy and Ravindranath, 1993).

Hemagglutination assay

The hemagglutination test was conducted as per Ravindranath and Paulson's instructions (1987). In microtitre wells, 25 µl of 1.5% erythrocyte suspension was combined with 25 µl of lectin (TcLec), which had been serially diluted with 25 µl of TBS (pH 5 to 11). One hour was spent incubating the microtitre plates at various temperatures (0 to 100°C). The maximum dilution of the sample that produced agglutination is the reciprocal of the hemagglutinationtitre.

Hemagglutination inhibition assay

The method used by Ravindranath et al. (1985) for performing the hemagglutination inhibition test was followed. We tested the efficacy of different sugars and glycoproteins to

prevent the agglutination of rabbit erythrocytes using hemagglutination inhibition (HAI) of the midgut gland lectin. In this experiment, in microtitre plates, 25 µl of known inhibitor concentration (sugar/glycoprotein) was serially diluted with 25 µl of TBS (pH 6.5).After that, each well received 25 µl of the midgut gland extract diluted to a sub-agglutination concentration in TBS (to give HA for one well), and each well was incubated for 60 minutes. 1.5% rabbit erythrocyte suspension was added, combined, and then incubated with 25 µl of the mixture. The maximum inhibitor dilution that completely inhibited agglutination after an hour was described as the hemagglutination inhibition titre.

Enzyme treatment

According to Pereira et al. (1981), rabbit erythrocytes were centrifuged at 4000 × g for 5 minutes at ambient temperature (30°C) and then resuspended in the same buffer after being rinsed five times with TBS (pH 7.5). Trypsin and neutral protease were added in an equal amount, mixed, and then incubated at 37°C for 60 minutes. Erythrocytes treated with the enzyme were utilized for the hemagglutination test after being washed five times in TBS.

A reaction mixture (1.0 ml) containing 10% washed rabbit erythrocytes in PBS-BSA (pH 7.0) and 140 milliunits of *Clostridium perfringens* neuraminidase (Sigma: Type X) was used to create sialidase-treated erythrocytes. This reaction mixture was incubated at 37°C for 4 hours. Cells that had been treated with neuraminidase underwent three PBS-BSA washes before being pelleted by low-speed centrifugation. HA tests were performed against these desialylated rabbit erythrocytes using TcLec.

Asialolactoferrin was made by combining 2 mg of glycoprotein (lactoferrin) with 0.1 unit of *Clostridium perfringens* sialidase (Type X: Sigma) and incubating the mixture for two hours at 37°C in 400 µl of a 5 mM acetate buffer with a pH of 5.5. Lactoferrin was handled similarly without sialidase as a control. Using sialidase-treated and untreated lactoferrin, the HAI test was performed against a 1.5% solution of rabbit erythrocytes.

Polyacrylamide gel electrophoresis

According to Laemmli(1970), sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out. In sample buffer, samples were heated for three minutes at 100°C. A solution comprising 50% methanol and 7% acetic acid was used to remove the stain from the gels after they had been fixed and stained with Coomassie blue R-250, 10% acetic acid, and 50% isopropyl alcohol in a solution at room temperature (30±2°C).

Results

Purification of TcLec from the midgut gland of the millipede *T. corallinus*

The midgut gland lectin (TcLec) desorbed from formalinized rabbit erythrocytes and affinity matrix using TBS containing 10 mM disodium EDTA resulted in a tremendous increase in the specific activityof the purified lectin about from 2135 (crude extract) to 6241523 of hemagglutinin per mg of protein (Table 1). Figure 1 shows the column profile depicting the purification of the midgut gland agglutinin on lactoferrin-agarose. On SDS-PAGE, the lectin appears as a single band of 76 kDa (Figure 2).

Table 1: Purification of midgut gland lectin

Sample (n=5)	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/mg)	Purification Fold
Crude extract	20	3070	6553600	2135	1
Centrifuged extract	15	74.25	4915200	66197.97	31
Lectin purified using formalinized rabbit RBCs	10	3.3	3276800	992969.69	465
Lectin purified using lactoferrin affinity column	10	2.1	13107200	6241523.81	2925

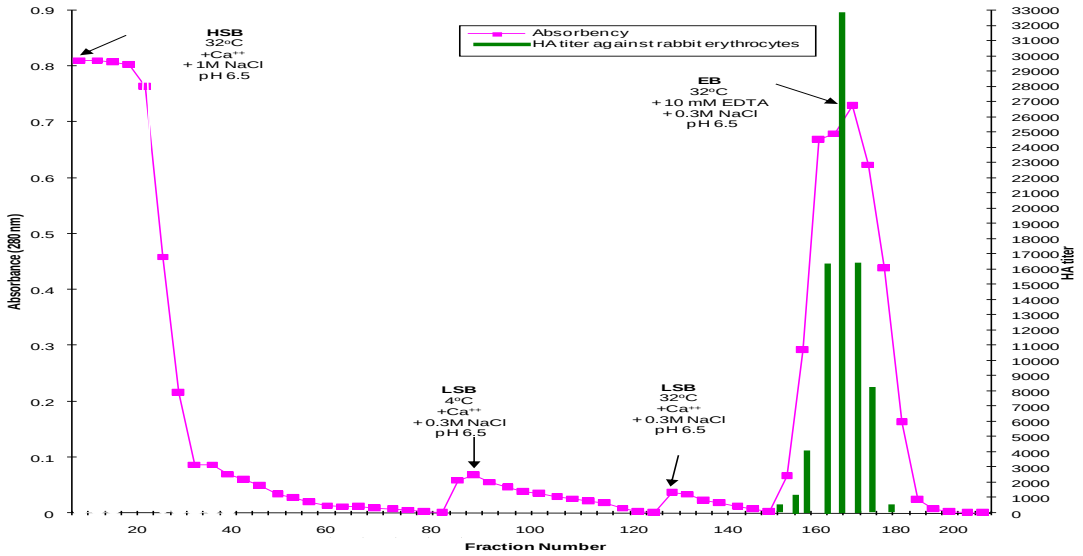

Figure 1: Lactoferrin-affinity elution profile

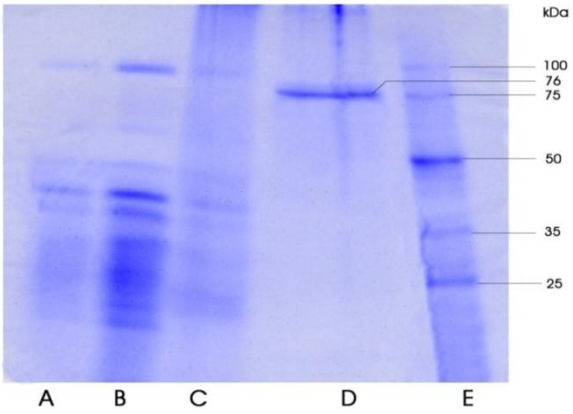

Figure 2 SDS-PAGE of TcLec

Erythrocytes specificity of TcLec

The TcLec could agglutinate only four erythrocyte types (Figure 3) out of the fifteen erythrocytes used for the assay. The lectin recognized erythrocytes differently with diverse agglutinability with rabbit (HA titer = 32768), rat and pig (HA titer = 2048) and human A (HA titer = 32) erythrocytes and at temperature 35^{o}C, pH 6.5 in the presence of calcium ions. Table 2 shows the sialoglycolinkages of different erythrocytes. The protease and sialidase (neuraminidase) treated rabbit erythrocytes were not agglutinated by the lectin (Figure 4).

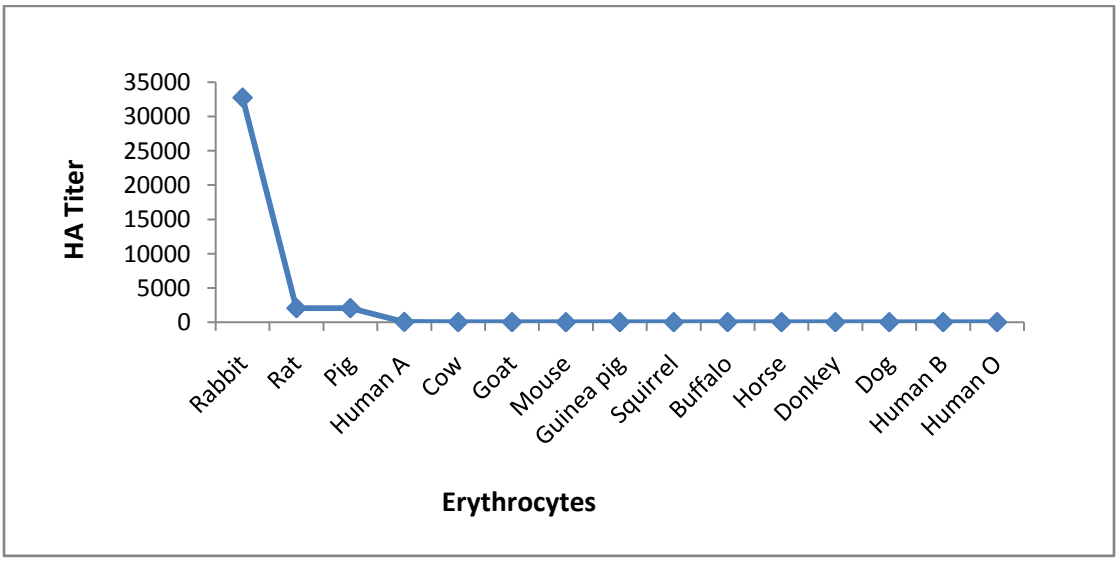

Figure 3 Glycocalyx recognition of TcLec using different mammalian erythrocytes

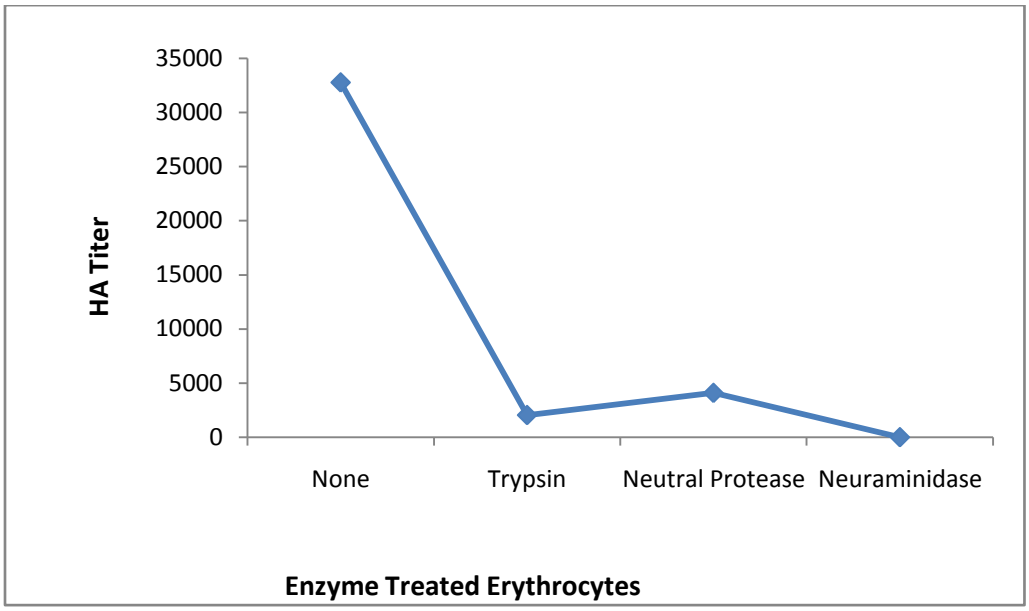

Figure 4 Sialic acid specificity of the TcLec

Table 2 Inter-residue sialo-glycolinkages of different erythrocytes

Erythrocytes	**Sialoglycoconjugates of erythrocytes**	**References**
Rabbit	NeuAc α 2 →3**Gal β1→3**GalNAc- / NeuAc α 2→6GalNAc- / Gal α, 1→3**Gal β1→3** / 4 GlcNAc β1→3 Gal β1→4 Glu β1-	Krotkiewski 1988; Aoki 2017; Yamakawa, 2005
Rat	NeuAc α 2→3**Galβ1→3**GalNAc- / NeuAc α 2→6GalNAc- / NeuAc α 2→3Galβ1→4GlcNAcβ1→6GalNAc-	Krotkiewski 1988; Aoki 2017
Pig	NeuGc α2→6GalNAc / Galβ1→3Galβ1→4GlcNAcβ1→3**Galβ1→3**GalNAc-	Krotkiewski 1988; Aoki 2017
Human A	NeuGc α2→3Galβ1→GalNAc→ / NeuAc α2→3**Galβ1→3**GalNAc / NeuAc α2→6GalNAc→	Krotkiewski 1988; Aoki 2017; Mo *et al*., 1994

The binding specificity of TcLec

Hemagglutination inhibition studies with diverse sugars and glycoproteins revealed the binding specificity of the TcLec.Among the various sugars tested against TcLec, 0.097mM was the minimum concentration needed by α-lactose to inhibit agglutination against rabbit erythrocytes. Agglutinability was also inhibited by D-galactosamine, dextrose, GluNAc relatively at below 1mM concentration. Of the two free sialic acidsNeuAc and NeuGc tested, NeuGc failed to inhibit the agglutinating activity. Similarly of the various glycoproteins tested,

0.3051µg/ml was the minimum inhibitory concentration of lactoferrin (Table 3). Following sialidase treatment, lactoferrin showed 512-fold decrease in HAI titre after 2 hours of incubation and 8192-fold decrease in HAI titre after 4 hours of incubation (Table 4).

Table 3 Rival effect of sugars and sialo-glycoproteins on agglutinating activity of the purified TcLec against rabbit red blood cells

Inhibitors (n=5)	**HAI Titre (2^n)**	**Minimum concentration required for inhibition (µg/ml) / (mM)**	**Relative inhibitory potency (%)**
Glycoproteins			
Lactoferrin	2^{14}	0.3051	100
PSM	2^6	0.39	0.39
BSM	2^6	0.39	0.39
Apotransferrin	2^6	0.39	0.39
Thyroglobulin	2^3	625	0.05
Fetuin	2^2	1250	0.02
Transferrin	0	0	0
Sugars			
α-lactose	2^{10}	0.097	100
D-galactosamine	2^9	0.19	50
Dextrose	2^8	0.39	25
GluNAc	2^7	0.78	12.5
NeuAc	2^6	1.56	6.25
D-galactose	2^4	6.25	1.56
L-fucose	2^2	25	0.39
ManNAc	2^1	50	0.19
NeuGc	0	0	0
D-fucose	0	0	0
Glu. 3 phosphate	0	0	0

Table 4 Effect of desialylation of lactoferrin

Glycoprotein (n=5)	**HAI Titre**
Lactoferrin (without sialidase)	16384
Lactoferrin + Sialidase (2 hrs)	64
Lactoferrin + Sialidase (4 hrs)	4

Discussion

The rusty millipede *T. corallinu*s' midgut gland was used in the current investigation to purify a new lectin called TcLec. According to the literature, affinity chromatography was the most effective approach for removing lectin from arthropods because it provided a better level of purity and recovery than biospecific adsorption and other methods (Mercy and Ravindranath 1993; Kamiya et al. 1994).On SDS-PAGE theTcLecappeared as a single band of 76 kDa molecular mass.The molecular mass of the arthropodan lectins varied based on the structure of the native lectin. Hemagglutination assay of the TcLec showed differential affinity with four different erythrocytes. The TcLec preferentially agglutinated rabbit erythrocytes suggesting the specificity of the TcLec to the glycosidic linkages expressed on the glycocalyx of rabbit erythrocytes. Lectin may specifically identify the whole sugar (Brettin and Kabat, 1976), a specific site of the sugar (Shimuzuet al., 1977), a sequence of sugars (Kobiler and Mirelman, 1980) or their glycosidic linkages (Koch et al., 1972). The core structure of erythrocyte membrane is NeuAc α 2 →3**Galβ1→3**GalNAc- / NeuAc α 2→6GalNAc- (rabbit), NeuAc α 2→3**Galβ1→3**GalNAc- / NeuAc α 2→6GalNAc- / NeuAc α →3Galβ1→4GlcNAcβ1→6GalNAc- (rat), NeuGc α2→6GalNAc / Galβ1→3Galβ1→4GlcNAcβ1→3**Galβ1→3**GalNAc- (pig) and NeuGc α2→3Galβ1→GalNAc→/ NeuAc α2→3**Galβ1→3**GalNAc / NeuAc α2→6GalNAc→ (human) (Table 2) (Krotkiewski 1988; Yamakawa, 2005; Aoki 2017; Mo et al., 1994). These observations suggest thatTcLecmay bind to **Galβ1→3** glycosidic linkages as reported in TcL, a hemolymphlectin of the freshwater crab, *Travancorianacharu* that has specific affinity to Galβ1→4GluNAc (Sheeja, 2017), hemolymphlectin of the crab,*Scyllaserrata* and serum of the crab, *Portunussanguinolentus*that expressed specificity to α 1-2 glucosidic linkages (Jayarajet al.,

2010; Meena et al., 2011), the hemolymph of the mangrove crab, *Episesarmatetragonum*contains lectins specific for NeuGcα 2, 3 Gal β1-4 GluNAc β1 linkage and O-acetyl sialic acids(Devi et al., 2013) and a hemolymph lectin of red palm weevil,*Rhynchophorusferrugineus*that is specific for Gal β1-3 linkages (Glory, 2017).

In arthropods, many lectins have been reported most of them familiar with the N-acetyl groups of carbohydrates such as NeuAc, GalNAc, GlcNAc and/or their adjacent sugar or glycosidic linkages like NeuAc α 2-3 Gal; NeuAc α 2-6; NeuAc α 2-8, even though they vary in molecular mass, structure and responsibility of divalent cations suggesting that, in spite of their structural variability, the binding properties that make a decision of their biological functions.TcLec was best inhibited by lactoferrin, PSM, BSM, apotransferrin, transferrin, α-lactose, D-galactosamine, Dextrose,GluNAc,NeuAc and D-galactose with high potency. PSM and BSM consists several Gal β1-3 GalNAc α1 O-glycans (Varki, 2007)and lactoferrinpossess NeuAc andNeuGc (Le Parcet al., 2017).

The derivatives of sialic acids (NeuAc/NeuGc) are extremely important components of the cell surface. Neuraminidases are glycoside hydrolase enzymes that cleave the glycosidic linkages of neuraminic acids. The inability of the lectin to agglutinate the protease and neuraminidase treated rabbit erythrocytes could be due to the removal of sialic acids by the sialidases thathydrolyze sialic acids from glycoconjugates or the enzyme may cleave/digest the glycosidic bond/sialyl residueor carbohydrate recognition domain resulting in theremoval of the lectinreceptor.Taken together, the red blood cell agglutination, hapten inhibition specificity and sialidase assay, it is clear that TcLec is sialic acid specific. So, the Gal β1-3 specific TcLec can be used as a valuable diagnostic tool in cancer research.

Conclusion

Consequently, sialic acid-specific TcLec may be of great utility in recognizing and differentiating sialic acids on the surface of cancer cells as well as in separating highly pathogenic bacterial strains.

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