Purification and characterization of a lectin from the coelomic fluid of the starfish, Protoreaster linckii (Blainville, 1830)

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Abstract—

Agglutinins from the coelomic fluid of the starfish, Protoreaster linckii was purified by affinity chromatography using PSM (Porcine Stomach Mucin) coupled to cyanogen bromide activated Sepharose 4B. The molecular weight of the lectin was 73 kDa on SDS–PAGE and showed a single protein band. Purified lectin agglutinated rabbit erythrocytes with greater avidity and was inhibited by the glycoproteins PSM, BSM, transferrin and sugars D- galactosamine, GalNAc and trehalose suggesting the affinity of the lectin to sialic acid. Reduction in HA with desialylated PSM confirms the sialic acid specificity of the lectin. Since the purified lectin is sialic acid specific, it has the capacity to bind to altered cell structures thus paving way for lectin targeted therapy.

Keywords: Coelomic fluid, affinity chromatography, glycoconjugates, sialylation

1. INTRODUCTION

Lectins are proteins/glycoproteins of non-immune origin, which have at least one non-catalytic domain that exhibit reversible binding to specific monosaccharides or oligosaccharides [1]. The lectin-monosaccharide interactions are relatively very weak and the dissociation constants lie in millimolar range. However, in nature for the multimeric sugars the dissociation constants are several folds higher, indicating that multiple protein-carbohydrate interactions are involved in the recognition and binding events [2]. Lectins are multivalent in nature and can bind to the carbohydrate moieties on the surface of erythrocytes and agglutinate them, without altering the properties of the carbohydrates [3]. They are invaluable tools for the detection, isolation and characterization of glycoconjugates, primarily of glycoprotein, for histochemistry of cells and tissues and for the examination of changes that occur on cell surface during physiological and pathological processes, from cell differentiation to cancer [4].

Purification of the agglutinin can be done effectively only if the sugar or glycoprotein specificity of the agglutinin was reasonably established. In affinity chromatography, the sugar, polysaccharide or glycoprotein was converted into an immobile and insoluble matrix by coupling to agarose activated with CNBr [5] [6]. The agglutinin, along with other proteins was passed through the affinity column to enable it to recognize and bind to the specific sugar on the affinity matrix. Factors promoting strong binding between the agglutinin and the sugar were often

considered while loading the agglutinin on the affinity matrix. Such factors may include pH, temperature and cations [7].

Literature reveals that a number of lectins have been most successfully isolated from the coelomic fluid of echinoderms by affinity chromatography, as it gave a higher fold of purification and percentage of recovery [8] [9] [10] [11] [12] [13] [14] [15] [16]. Based on the selective carbohydrate-binding properties, invertebrate lectins or agglutinins with defined specificities are being widely used as tools for purification and characterization of polysaccharides or glycoconjugates and as probes to localize and analyse the functions of cell surface carbohydrate moieties [17]. The purified lectins are used to specially target cancer cell and other pathogens that express specific carbohydrates on its surface. Hence, purification of a lectin from the coelomic fluid of starfish will be of use in identification of its therapeutic application.

2. MATERIALS AND METHODS

Collection of coelomic fluid

Coelomic fluid was collected by cutting the tip of the arm of starfish, P. linckii with scissors or knife and the fluid was drained into the centrifuge tubes placed on ice [18].

Purification of lectin from the coelomic fluid of Protoreaster linckii

The lectin was purified by affinity purification [19].

Characterization of purified lectin

Hemagglutination assay and Hemagglutination inhibition assay

Hemagglutination (HA) and Hemagglutination inhibition (HAI) assays were performed in 'U' bottom microtitre plates at room temperature (30 ± 2°C) [20].

Physico chemical characterization of lectin

The physicochemical properties were determined by hemagglutination assays with purified lectin samples under conditions of varying pH (5 to 10), temperature (0o to 100oC), bivalent cations calcium, magnesium and manganese (0.01 mM to 100 mM) and calcium chelators like di and tetra sodium EDTA and trisodium citrate (0.01 mM to 100 mM).

Sialidase treatment of rabbit erythrocytes and sialoglycoprotein

Sialidase treatment of erythrocytes and sialoglycoproteins of the lectin was carried out.

Polyacrylamide gel electrophoresis

3.RESULTS

Purification of Protoreaster linckii lectin by affinity column

The profile of a lectin isolated from the coelomic fluid of P. linckii by affinity chromatography is shown in Figure 1. The clarified sample which was passed through the affinity matrix and the effluent collected during subsequent washing of the matrix with tris buffer did not contain any detectable level of HA activity against rabbit erythrocytes. This indicated that all the hemagglutinins in the serum had been adsorbed to the affinity matrix. Further washing of the column with HSB and LSB helped to elute all inert proteins bound to the column.

These steps were necessary for obtaining the lectin in the homogeneous state. Further elution with the elution buffer (EB) containing 5 mM trisodium citrate, gave a sharp peak at 280 nm absorbance. The PSM affinity column purification of the lectin gave a 3354.76 fold increase in the specific activity of the clarified serum (Table 1).

Figure1. PSM – affinity elution profile

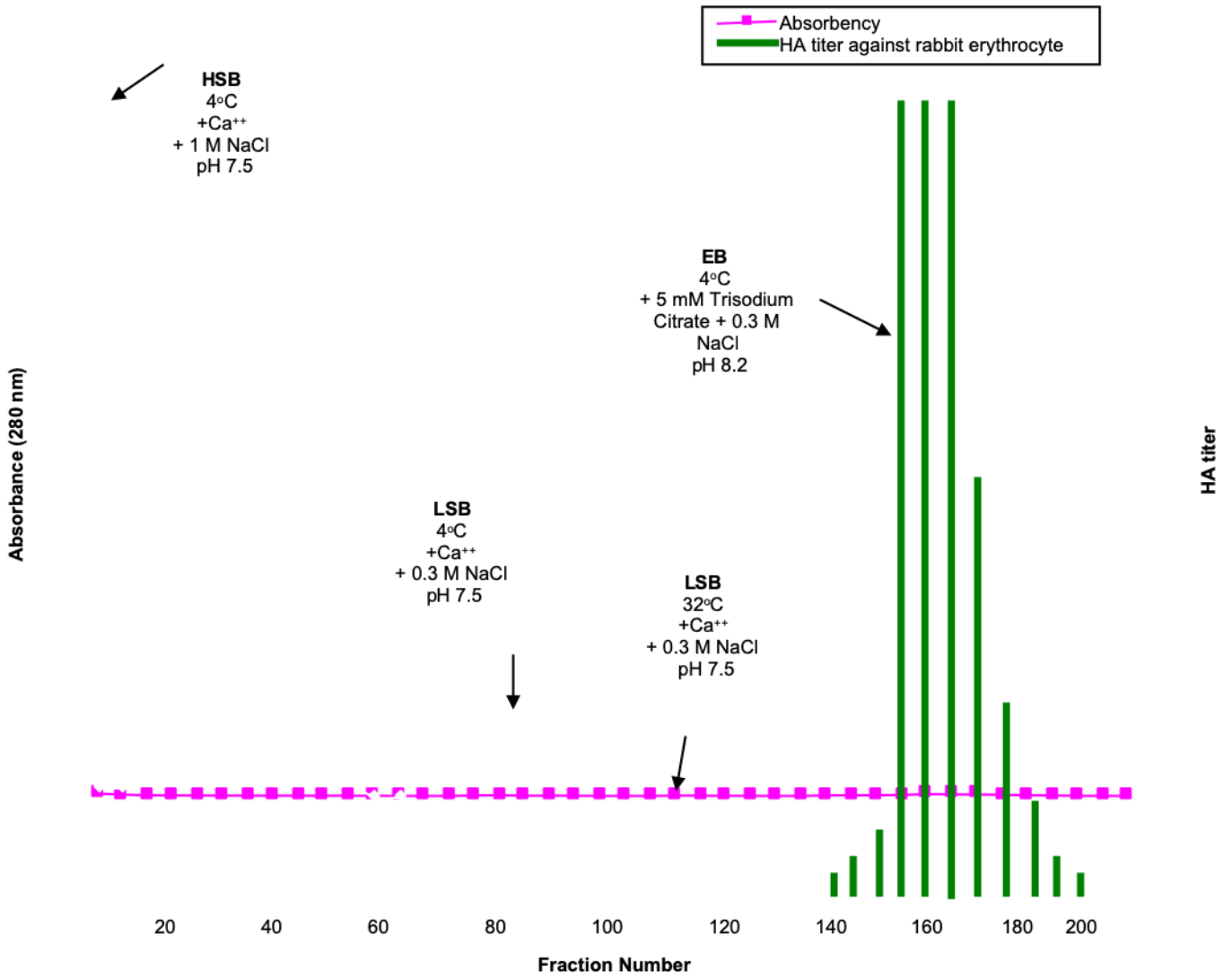


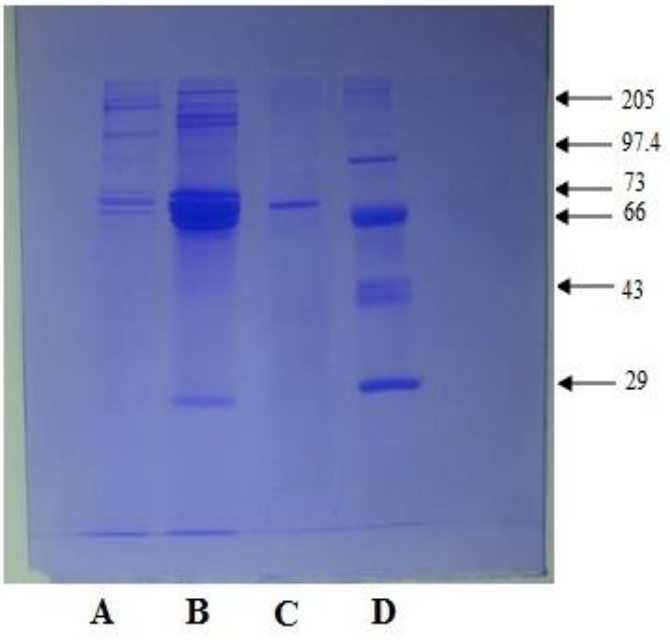
TABLE 1.

PURIFICATION OF THE COELOMIC FLUID LECTIN OF THE STARFISH, PROTOREASTER LINCKII

Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/ mg)	Purification fold
Crude coelomic fluid	50	2050	2.56 x105	124.87	1
Clarified coelomic fluid	30	84	1.53 x 105	1828	14.63
Lectin purified using PSM affinity column	9	0.11	4.6 x 104	4.1 x 105	3354.76

Electrophoretic analysis and molecular weight determination

The lectin purified by PSM-Sepharose 4B column was analysed using SDS-PAGE. The purified lectin designated PLL gave a single protein band with a molecular weight of 73 kDa (Figure 2).



Lane A - clarified coelomic fluid; Lane B - crude coelomic fluid; Lane C- PSM- Sepharose 4B affinity chromatography purified lectin; Lane D - Protein molecular weight markers

Figure 2. SDS-PAGE of lectin purified from the coelomic fluid of starfish, P. linckii

Erythrocyte binding specificity

The lectin purified from the coelomic fluid of the starfish, P. linckii exhibited RBC-binding properties recognizes the sialyl epitopes of the erythrocytes. Table 2 shows the hemagglutination titre of the purified starfish lectin with various mammalian erythrocyte preparations.

TABLE 2.

HEMAGGLUTINATION TITRE OF THE PURIFIED LECTIN OF THE COELOMIC FLUID OF THESTARFISH, P. LINCKII

Erythrocytes (n=10)	HA Titre of purified lectin
Rabbit	128
Human A	64
Human B	64
Dog	16
Human O	32
Human AB	32
Buffalo	16
Rat	8

Physicochemical properties of hemolymph lectin

The purified lectin was pH and temperature sensitive. The HA was stable between pH 7.0 to 8.5 and temperature ranging from 0-40°C (Table 3). Addition of divalent cations (Ca2+, Mg2+ and Mn2+) increased the HA titer at 10 mM Ca2+ which on further increase showed a decrease in hemagglutinating activity (Table 4). Calcium chelators di and tetra sodium EDTA and trisodium citrate reduced the HA at 5 mM concentration and a decrease was noted with further increase in concentration (Table 5).

TABLE 3.

HEMAGGLUTINATION TITRE OF THE PURIFIED LECTIN OF THE COELOMIC FLUID OF P. LINCKII IN RELATION TO CHANGE IN PH AND TEMPERATURE

pH (n= 5)	HA Titre	Temperature (°C) (n = 5)	HA Titre
5.0	32	0	128
5.5	64	10	128
6.0	64	20	128
6.5	64	30	128
7.0	128	40	128
7.5	128	50	64

8.0	128	60	16
8.5	128	70	8
9.0	64	80	8
9.5	64	90	0
10.0	32	100	0

TABLE 4.

EFFECT OF DIVALENT CATIONS ON THE PURIFIED LECTIN OF THE COELOMIC FLUID OF P. LINCKII

Cation Concentration (mM) (n=5)	HA Titre		
	Ca2+	Mg2+	Mn2+
0	128	128	128
0.01	128	256	128
0.1	128	128	128
1	128	128	64
10	128	64	64
20	64	64	64
30	32	64	32
40	32	64	32
50	16	64	32
100	0	64	16

TABLE 5.

EFFECT OF CALCIUM CHELATORS ON THE PURIFIED LECTIN OF THE COELOMIC FLUID AGGLUTININ OF P. LINCKII

Chelator Concentration (mM) (n = 5)	EDTA		Trisodium citrate
	Disodium	Tetrasodium	
0	128	128	128
0.01	32	128	128
0.1	32	128	128
1	32	128	128
5	32	64	8
10	16	64	8
20	8	64	8
30	8	8	4
40	8	8	2
50	0	4	0
100	0	0	0

Hemagglutination inhibition assay of P. linckii lectin

To ascertain the nature of the binding specificity of the purified coelomic fluid lectin, hemagglutination inhibition assays were performed with different sialoglycoprotein and sugars.

The agglutinability of the purified lectin was inhibited by glycoproteins in the following order: PSM = BSM = transferrin > lactoferrin (Table 6). Sugars reduced the HA titre of the coelomic fluid lectin and N- acetyl D- galactosamine was the most effective sugar to produce complete inhibition at 0.09 mM concentration. D- galactosamine, trehalose, D-glucose 6- phosphate, sucrose, lactose, glucose, raffinose, L- fucose, D- fucose and melibiose were inhibitory at concentration up to 25 mM. The other sugars tested failed to inhibit the lectin (Table 7).

TABLE 6.

HEMAGGLUTINATION INHIBITION OF THE COELOMIC FLUID LECTIN OF THE STARFISH, P. LINCKII BY GLYCOPROTEINS

Sialoglycoprotein	HAI Titre	Minimum concentration required for inhibition (µg/ml)	Relative inhibitory Potency (%)
PSM	**1024**	**5.273**	**100**
BSM	**1024**	**5.273**	**100**
Transferrin	**1024**	**5.273**	**100**
Lactoferrin	**128**	**42.187**	**12.5**

TABLE 7.

HEMAGGLUTINATION INHIBITION OF THE COELOMIC FLUID LECTIN OF THE STARFISH, P. LINCKII BY SUGARS

Sugars	HAI Titre	Minimum concentration required for inhibition (mM)	Relative inhibitory potency (%)
N- acetyl D-galactosamine	**1024**	**0.097**	**100**
D- Galactosamine	**512**	**0.195**	**50**
Trehalose	**512**	**0.195**	**50**
D- Glucose- 6-phosphate	**128**	**0.78**	**12.5**
Sucrose	**128**	**0.78**	**12.5**
α- Lactose	**128**	**0.78**	**12.5**
Glucose	**32**	**3.12**	**6.25**
Raffinose	**16**	**6.25**	**3.12**
L- fucose	**16**	**6.25**	**3.12**
D- fucose	**8**	**12.5**	**1.56**
Melibiose	**4**	**25**	**0.781**

Effect of neuraminidase treatment on HA and HAI

The purified lectin showed a significant reduction in HA titre when treated with neuraminidase treated rabbit erythrocytes (Table 8). The neuraminidase treated PSM, the potent inhibitor of the coelomic fluid lectin of P. linckii showed a tremendous reduction in its ability to inhibit agglutination with rabbit erythrocytes (Table 9).

TABLE 8.

EFFECT OF NEURAMINIDASE ON HEMAGGLUTINATION OF THE COELOMIC FLUID LECTIN OF THE STARFISH, P. LINCKII

Enzyme used	Site of enzyme activity	HA titre
None	-	128
Neuraminidase (C. perfringens)	Neu Ac-D-GalNeuAc-D-GalNAc	8

TABLE 9.

HEMAGGLUTINATION INHIBITION OF THE COELOMIC FLUID LECTIN OF THE STARFISH, P. LINCKII BY DESIALYLATED GLYCOPROTEIN (PSM)

Glycoprotein treatment	HAI titre
PSM untreated	1024
PSM + Neuraminidase	128

4. DISCUSSION

A sialic acid specific natural lectin (Protoreaster linckii lectin- PLL) was purified by affinity column chromatography using PSM-linked Sepharose 4B. The purification of lectin from the starfish, P. linckii resulted in 3354.76 fold increase in specific activity. Affinity purification is considered as the best method to purify echinoderm lectins, since it could yield 99% pure lectins. A number of lectins from starfish have been purified by affinity purification. The purified lectin agglutinated rabbit, human A, B and O erythrocytes with great avidity.

Electrophoretic studies revealed the presence of a single lectin with a molecular mass of 73 kDa on SDS-PAGE. Single homogenous lectins were also reported in a number of echinoderms including 13 kDa in Paracentrotus lividus [23], 200 kDa in seminal plasma of sea urchin, P. lividus [24], 44 kDa in Cucumaria japonica [25].

HAI studies recorded a drastic increase in the inhibitory potential of PLL. The agglutinability of the pure lectin was inhibited by GalNAc > D- galactosamine = trehalose > D-glucose- 6-phosphate = sucrose = lactose > glucose > raffinose = L- fucose > D- fucose > melibiose. The hapten inhibition study shows that the agglutinin of the P. linckii is inhibited by GalNAc like that of Asterina pectinifera, Toxopneustes pileolus, Cucumaria echinata, Stichopus japonicas, Holothuria grisea [26] and Holothuria atra [27].

Inhibition study with glycoproteins provides valuable information pertaining to the sialyl oligosaccharide preference of the lectin from the starfish of P. linckii. The sialyl oligosaccharides of inhibitory glycoproteins are presented in Table 6. PSM containing 90% N- acetyl neuraminic acid [28] is proved to be a potent inhibitor. The presence of PSM specific lectin was also reported in some echinoderms.

Purified lectin obtained from the coelomic fluid of P. linckii showed the affinity towards erythrocytes indicating that the purified agglutinin is a lectin [29]. Though capable of agglutinating a variety of erythrocytes, the agglutinin showed high affinity for rabbit, human A, B and O. As the common receptor component of the glycocalyx of rabbit and human erythrocytes is NeuAc [30] [31] [32] [33] and the specific inhibitor, PSM has NeuAc it can be considered that starfish lectin may have preference for NeuAc as reported in the starfish Asterias amurensis.

Sialidase treatment of rabbit erythrocytes reduced their hemagglutination ability thereby revealing the affinity of the lectin to sialic acid on the surface of the rabbit erythrocytes which is further confirmed by the fact that the inhibitory potency of the desialylated PSM is greatly reduced than the native PSM. So the starfish, P. linckii has a lectin in the coelomic fluid with affinity for sialic acid that can be used in the detection of sialic acid related glycosidic linkages in cell- surface glycoproteins and glycolipids. Sialic acids play an important role as ligands in cell sociology. Sialylation of glycoproteins changes under pathological conditions as well as during developmental stages and altered sialylation often has significant implications in the physiological role of glycoproteins [34] [35]. Malignant transformation is associated with alterations in cell surface carbohydrate architecture [36]. Among the various types of sugar residues, sialic acid is extremely important as increased sialylation in the cellular glycoconjugates has been found to be associated with tumour progression and metastasis [37]. Over expression of sialylated antigens at the surface of lung cancer cells [38] [39] and presence of 9-O-acetyl-NeuAc [40] and N-glycolyl neuraminic acid [41] as well as α 2, 6-linked sialic acids [42] in human colon carcinoma have been reported. Lectins endowed with the ability to bind to carbohydrate residues of specific structure and configuration has emerged as a powerful tool to detect such changes [43].

Aberrant sialylation in cell surface carbohydrate architecture is a characteristic feature of cancer cells. Over expression of sialyl-lewisx (SLex) and sialyl-lewisa (SLea) in lung carcinoma was found to be associated with increased metastatic ability and poor survival of the patients. Furthermore the sialylation status of the serum glycans has been found to be altered in NSCLC (Non Small Cell Lung Cancer) [44]. It has been suggested that the increased level of sialic acid in the sera of cancer patients might be caused by spontaneous shedding of aberrant sialic acid rich glycoproteins and glycolipids from the cell surface into the extracellular milieu and profound sialylation of the serum microenvironment might interrupt adhesion and influence metastasis [45] [46]. Thus lectins that can detect such altered pattern of sialylation may have the diagnostic potential for NSCLC.

Thus the result of the present investigation strongly suggest that P. linckii has a sialic acid specific lectin in the coelomic fluid thus rendering it a powerful tool to study sialic acid containing structures associated with pathological process. The sialic acid recognizing lectin would be of immense value in identifying and discriminating sialic acids on the surface of the cancer cells as well as in distinguishing highly pathogenic strains of bacteria.

REFERENCES

[1] *Lis, H. and Sharon, N. (1986). Lectins as molecules and as tools. Annual Review of Biochemistry, 55: 35-67.*

[2] *Ambrosi, M., Cameron,N.R. and Davis, B.G.(2005).Ambrosi, M., Cameron, N.R. and Davis, B.G. (2005). Lectins: tools for the molecular understanding of the glycode. Org. Biomol. Chem.,3: 1593-1608.*

[3] *Lam, S.K. and Ng, T.B. (2011). Lectins production and practical applications. Applied Microbiology and Biotechnology, 89(1): 45-55.*

[4] *Sharon, N. and Lis, H. (2004). History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology, 14: 53-62.*

[5] *Shishikura, F. and Sekiguchi, K. (1984). Studies on perivitelline fluid of horseshoe crab embryo: 2. Purification of agglutinin binding substance from the perivitelline fluid of Tachypleus gigas embryo. Journal of Biochemistry, 96: 629-636.*

[6] *Ravindranath, M.H., Higa, H.H., Cooper, E.L. and Paulson, J.C. (1985). Purification and characterization of an O-acetyl sialic acid specific lectin from a marine crab, Cancer antennarius. Journal of Biological Chemistry, 260(15): 8850-8856.*

[7] *Schwyzer, M. and Hill, R.L. (1977). Porcine blood group specific N-acetyl galactosaminyl transferase: 1. Purification from porcine submaxillary glands. Journal of Biological Chemistry, 252: 2338-2345.*

[8] *Hatakeyama, T., Himeshima, T., Komatsu, A. and Yamasaki, N. (1993). Purification and characterization of two lectins from the sea cucumber Stichopus japonicus. Bioscience Biotechnology and Biochemistry, 57(10): 1736-1739.*

[9] *Hatakeyama, T., Kohzaki, H., Nagatomo, H. and Yamasaki, N. (1994). Purification and characterization of four Ca2+-dependent lectins from the marine invertebrates Cucumaria echinata. Journal of Biochemistry, 116: 209-214.*

[10] *Matsui, T., Ozeki, Y., Suzuki, M., Hino, A. and Titani, K. (1994). Purification and characterization of two Ca2+ -dependent lectins from coelomic plasma of sea cucumber, Stichopus japonicus. Journal of Biochemistry, 116: 1127-1133.*

[11] *Kakiuchi, M., Okino, N., Sueyoshi, N., Ichinose, S., Omori, A., Kawabata, S., Yamaguchi, K. and Ito, M. (2002). Purification, characterization and cDNA cloning of α-N-acetylgalactosamine-specific lectin from starfish, Asterina pectinifera. Glycobiology, 12(2): 85-94.*

[12] *Matsumoto, R., Shibata, T.F., Kohtsuka, H., Sekifuji, M., Sugii, N., Nakajima, H., Kojima, N., Fujii, Y., Kawsar, S.M., Yasumitsu, H., Hamako, J. and Ozeki, Y. (2011). Glycomics of a novel type-2 N-acetyl lactosamine-specific lectin purified from the feather star, Oxycomanthus japonicus (Pelmatozoa: Crinoidea). Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology, 158(4): 266-273.*

[13] *Bulgakov, A.A., Eliseikina, M.G., Kovalchuk, S.N., Petrova, I.Y., Likhatskaya, G.N., Shamshurina, E.V. and Rasskazov, V.A. (2013). Mannan-binding lectin of the sea urchin Strongylocentrotus nudus. Marine Biotechnology, 15(1): 73-86.*

[14] *Yoshitaka, I. and Yoshihiro, Y. (2013). Purification and characterization of a lectin from the starfish, Asterias amurensis. Fisheries Science, 79(6): 1007-1013.*

[15] *Santhiya, M., Jansi, M. and Vincent, S.G.P. (2013). First report on purification of a novel lectin from star fish Linckia laevigata a defence molecule that target the HeLa cells. Asian Academic Research Journal of Multidisciplinary, 1(12).*

[16]Edo, K. (2014). Study on a pedicellarial venom lectin from the sea urchin Toxopneustes pileolus in the coast of Tokushima Prefecture, Japan. Graduate school of Integrated Arts and Sciences, The University of Tokushima, 1-43.

[17]Gabius, H.J. (1997). Animal lectins. European Journal of Biochemistry, 243: 543-576.

[18]Sharlaimova, N.S. and Petukhova, O.A. (2012). Characteristics of populations of the coelomic fluid and coelomic epithelium cells from the starfish Asterias rubens L., able attach to and spread on various substrates. Cell and Tissue Biology, 6(2): 176-188.

[19]Mercy, P.D. and Ravindranath, M.H. (1993). Purification and characterization of N-glycolyl neuraminic acid specific lectin from Scylla serrata. European Journal of Biochemistry, 215: 697-704.

[20]Ravindranath, M.H. and Paulson, J.C. (1987). O-acetyl sialic acid specific lectin from the crab, Cancer antennarius. Methods in Enzymology, 138: 520-527.

[21]Lowry, O.H, Rosebrough, H.Y., Farr, A.L and Randal, R.G. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry, 193: 265-275.

[22]Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

[23]Drago, F., Malagoli, D., Pezzino, F.M., D'Urso, V. and Sammartano, F. (2009). Presence of a low molecular weight lectin in the coelomic fluid of the sea urchin Paracentrotus lividus. Invertebrate Survival Journal, 6: 15-20

[24]Stabili, L., Pagliara, P. and Canicatti, C. (1993). Characterization and isolation of an agglutinin from seminal plasma of the sea urchin. Canadian Journal of Zoology, 71: 2042-2046.

[25]Bulgakov, A.A., Nazarenko, E.L., Petrova, I. Y., Eliseikina, M.G., Vakhrusheva, N.M. and Zubkov, V.A. (2000). Isolation and properties of a mannan-binding lectin from the coelomic fluid of the holothurians Cucumaria japonica. Biochemistry (Moscow), 65(8): 933-939.

[26]de Melo, A.A., Carneiro, R.F., de Melo Silva, W., Moura Rda, M., Silva, G.C., de sousa, O.V., de Sousa Saboya, J.P., Nascimento, K.S., Saker-Sampain, S., Nagano, C.S., Cavada, B.S. and Sampaio, A.H. (2014). HGA-2, a novel galactoside-binding lectin from the sea cucumber Holothuria grisea binds to bacterial cells. International Journal of Biological Macromolecules, 64: 435-442.

[27]Mojica, E.R.E. and Merca, F.E. (2004). Lectin from the body walls of black sea cucumber (Holothuria scabra Jaeger). Philippine Journal of Science, 133(2): 77-85.

[28]Schoop, H.J. and Faillard, H. (1967). Contribution to the biosynthesis of the glycolyl group of N- glycolyl neuraminic acid. Hoppe Seylers Zeitschrift Fur Physiologische Chemie, 348(11): 1518-1524.

[29]Goldstein, I.J., Hayes, R.C., Monsugny, M., Osawa, T. and Sharon, N. (1980). What should be called a lectin? Nature, 285: 66.

[30]Bhavanandan, V.P., Buddecke, E., Carubelli, R. and Gottschalk, A. (1964). A complete enzymic degradation of glycopeptides containing O-seryl and O-threonyl linked carbohydrate. Biochemical and Biophysical Research Communications, 16: 353-357.

[31]Jeanloz, R.W. (1966). In: "Glycoproteins" (Gottschalk, A., ed). Elsevier publishing company, Amsterdam.

[32]Murayama, J., Takeshita, K., Tomita, M. and Hamada, A. (1981). Isolation and characterization of two glycophorins from horse erythrocyte membranes. Journal of Biological Chemistry, 89(5): 1593-1598.

[33]Berman, E. (1984). Structural and conformational analysis of sialo-glycosaccharides using carbon-13 nuclear magnetic resonance spectroscopy. Biochemistry, 23(16): 3754-3759.

[34]Roth, J. (1996). Poly sialic acids of vertebrates: biosynthesis, structural diversity, tissue expression and function. In. glycoproteins and Disease, (Montreuil, J., Vliegenthart, J.F.G. and Schachter, H. eds) New York, Oxford, Shannon, Tokyo, Elsevier Press, 413-418.

[35]Varki, A. (1997). Sialic acids as ligands in recognition phenomena. Federation of American Socities for experimental biology, 11: 248-255.

[36]Reis, C.A., Osorio, H., Silva, L., Gomes, C. and David, L. (2010). Alterations in glycosylation as biomarkers for cancer detection. Journal of Clinical Pathology, 63: 322-329.

[37]Lopez-Morales, D., Reyes Leyva, J., Santos- Lopez, G., Zenteno, E. and Vallejo - Ruiz, V. (2010). Increased expression of sialic acid in cervical biopsies with squamous intraepithelial lesions. Diagnostic Pathology, 5: 74-78.

[38]Ogawa, J., Inoue, H. and Koide, S. (1997). α-2,3-sialyltransferase type 3N and α-1,3-fucosyltransferase type VII are related to sialyl Lewisxsynthesis and patient survival from lung carcinoma. Cancer, 79: 1678-1685.

[39]Wang, Q.Y. Wu, S.L., Chen, J.H., Liu, F. and Chen, H.L. (2003). Expression of Lewis antigens in human non-small cell pulmonary cancer and primary liver cancer with different pathological conditions. Journal of Experimental and Clinical Cancer Research, 22: 431-440.

[40]Cheresh, D.H., Reisfeld, R.A. and Varki, A.P. (1984). O-acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. Science, 225: 844-846.

[41]Higiashi, H., Hirabayashi, Y., Fukui, Y., Naiki, M., Matsumoto, M., Ueda, S. and Kato, S. (1985). Characterization of N-glycolyl neuraminic acid containing gangliosides as tumor associated Hanganutziu-Deicher antigen in human colon cancer. Cancer Research, 45: 3796-3802.

[42]Saito, H., Yoshioka, Y., Uehara, N., Aketagawa, J., Tanaka, S. and Shibata, Y. (1991). Relationship between conformation and biological response of (1-3) β-D glucans in the activation of coagulation factor G from Limulus amoebocyte lysate and host-mediated anti-tumour activity. Demonstration of single-helix conformation as a stimulant. Carbohydrate Research, 217: 181-190.

[43]Lei, H.Y. and Chang, C.P. (2009). Lectin of Concanavalin A as an anti-hepatoma therapeutic agent. Journal of Biomedical Science, 16: 10-21.

[44]Arnold, J.N., Saldova, R., Galligan, M.C., Murphy, T.B., Mimura-Kimura, Y., Telford, J.E., Godwin, A.K. and Rudd, P.M. (2011). Novel glycan biomarkers for the detection of lung cancer. Journal of Proteome Research, 10: 1755-1764.

[45]Zhao, J., Simeone, D.M., Heidt, D., Anderson, M.A. and Lubman, D.M. (2006). Comparative serum glycoproteomics using lectin selected sialic acid glycoproteins with mass spectrometric analysis: application to pancreatic cancer serum. Journal of Proteome Research, 5: 1792-1802f

[46]Perez-Garay, M., Arteta, B., Pages, L., de Llorens, R., di Bolos, C., Vidal-Vanactoche, F., and Peracaula, R. (2010). α-2,3- sialyltransferase ST3 Gal III modulates pancreatic cancer cell motility and adhesion in vitro and enhances its metastatic potential in vivo. PLoS One, 5(9): e12524.