Identification and physico-chemical characterization of the hemagglutinin from the hepatopancreas of the freshwater crab, *Oziotelphusanaga*

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Abstract

The present investigation was carried out to identify and characterize the agglutinin from the hepatopancreas of the freshwater crab, *Oziotelphusanaga*. Specifically, the study aimed to determine the agglutinin / lectin specificity towards erythrocytes, glycoproteins and sugars; pH and thermal stability, and the effects of divalent cation and calcium chelators. A naturally occurring hemagglutinin with high hemagglutination titer of 2048 with rabbit erythrocytes was identified in the hepatopancreas of the crab, *O. naga*. The HA activity was stable between pH 7.5 and 8 and showed thermal stability between 20º C and 40º C. The hepatopancreas agglutinin was calcium dependent and HA activity was reduced when exposed to calcium chelators such as EDTA and trisodium citrate. Hemagglutination inhibition assay exhibited the strongest binding specificity towards thesugar GlcNAc and glycoprotein fetuin. The crossadsorption assay revealed that the hepatopancreas possesses single agglutinin.

Index terms: cation dependency, GlcNAc, hemagglutinin, hepatopancreas, *Oziotelphusa*.

Introduction

Agglutinins/lectins are proteins or glycoproteins usually without catalytic activity that have the ability to bind to specific carbohydrates expressed on different cell surfaces. Due to the fact that they are in general at least bivalent i.e. the molecule has at least two specific binding sites, they can bind cells and an agglutination reaction occurs (Marques and Barracco 2000). Lectins are structurally diverse oligomeric proteins composed of subunits varying in molecular size, amino acid composition, three dimensional structure and metal requirement (Sharon and Lis 1989).

Lectins have become the focus of intense interest forbiologists and in particular for the research and applications in medicine (Movafagh et al. 2013). These proteinswith unique characteristics have found use in diverse fields ofbiology and as more lectins are being isolated

and their role innature elucidated, they continue to occupy an important place intherapeutic areas of research.Among the various lectins studied for biomedical applications,sialic acid specific lectins have gained importance owing to their potential to target malignant cells (Ravindranthand Cooper 1984; Viswambari Deviet al. 2010; Sun et al. 2019). Crustaceans of phylum Arthropoda are extensively studied for the presence of agglutinin / lectin and has been reported from hemolymph and hepatopancreas(Mercy and Ravindranath 1993; Maghil Denis et al. 2003; Pereyra et al. 2012; Wang and Wang 2013; Sheeja and Basil Rose 2018; 2019).

In crustaceans, the epithelial cells of the midintestinal gland (hepatopancreas) are major sources of immune molecules such as lectins, hemocyanin, ferritin, antimicrobial and antiviral proteins, proteolytic enzymes and nitric oxide. There is interference between midintestinal gland cells and phagocytes, which aids the initiation of the immune response and the clearance of pathogens. The midintestinal gland is thereby an integrated organ of immunity and metabolism. Immunity was the primary functions of the midintestinal gland cells and their role in the intermediate metabolism has evolved during the course of their further specialization (Roszer 2014).Thus,hepatopancreas is considered an important immune related tissue in crustaceans and the place where most CTLs (C-type lectins) are generated (Sun et al. 2008; Sheeja and Basil Rose 2019). When the microorganisms invade the hepatopancreas, proteolytic pathways take place instantly, allowing elimination of invading microbes (Ratcliffe et al. 1985). The presence of agglutinins has been detected in the hepatopancreasof few crabs (Jin et al. 2013; Sheeja and Basil Rose 2019).

Hence an attempt was carried out in this study to identify and characterize hemagglutinin from the hepatopancreas of a commonly available freshwater crab of our locality*Oziotelphusanaga*.

Materials and methods

Animal collection

Freshwater crabs, *Oziotelphusanaga* were collected from the paddy field of Parakkai, Kanyakumari District, Tamilnadu, India(8.169° N and 77.452° E; 9 m altitude) and were maintained in the laboratory in plastic tubs with freshwater. The water was changed on alternate days and the crabs were fed with paddy grains and puffed rice.

Preparation of hepatopancreas extract

Hepatopancreas were dissected from the healthy crabs. They were cleansed well in 0.7% of saline and the extracts was prepared by homogenizing 100 mg in 1 ml of cold TBS (Tris Buffered Saline: Tris HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10mM, pH 7.5) using a homogenizer. The extracts were centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant was used for hemagglutination assay.

Erythrocyte collection

Erythrocytes from several mammals were collected for hemagglutination assay. Blood for this purpose was obtained by heart puncture (rat and guinea pig), venipuncture of the ear (rabbit), fore arm (human and dog), neck (buffalo and ox) and from the slaughter house (pig, cow and goat). Erythrocytes were collected directly in modified Alsevier's medium containing sodium citrate (30 mM, pH 7.1), sodium chloride (77 mM), glucose (114 mM), neomycin sulfate (100 mg/ml) and chloramphenicol (330 mg/ml). They were suspended and washed three times with ten volumes of Tris-Buffered Saline TBS, pH 7.5 and resuspended in the same as 1.5% suspension.

Identification of agglutinin

Hemagglutination assay

Hemagglutination assays were carried out as described by Ravindranath and Paulson (1987) to find out the presence of hemagglutinin and to know the erythrocytes specificity.

Physico-chemical characterization

pH and thermal stability

pH and temperature dependence of agglutinin was measured by pre-incubating the hepatopancreasextract at specific pH (5.5-11.5) and temperature (0ºC-100°C) for 1 hour before adding erythrocyte suspension for hemagglutination assay.

Cations and EDTA treatment

To study divalent metal cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) dependence on hemagglutination, HA assays were performed in TBS (pH 7.5) with and without these ions at varying concentrations. To study the effect of calcium chelators (EDTAand trisodium citrate) on the agglutinin, the hepatopancreasextract was pre-incubated at different concentrations (0.01 to 100 mM) of EDTA and trisodium citrate for 1 hour before adding erythrocyte suspension for HA assay.

Hemagglutination inhibition assay

The hemagglutination inhibition (HAI) assay was carried out following the procedure of Ravindranath et al. (1985).

Nature of hemagglutinin

Cross adsorption test

To test whether the hepatopancreas contain single or multiple agglutinin, thecrossadsorption assays were carried out following the method of Hall and Rowlands (1974) and Mercy and Ravindranath (1992).

Results

Hemagglutination assay

The hepatopancreas extract of *Oziotelphusanaga*agglutinated rabbit erythrocytes with a high HA titer of 2048 and also agglutinated rat erythrocytes (HA = 64) dog and pig erythrocytes (HA = 2). The agglutinin failed to agglutinate other mammalian erythrocytes tested (Table 1). The results revealed the presence of a natural agglutinin in the hepatopancreas of the experimental crab.

Table 1 Hemagglutination titer of hepatopancreas extract against different mammalian erythrocytes

Erythrocytes (n=10)	HA titer
Rabbit	**2048**
Rat	64
Dog	2
Pig	2
Goat	0
Buffalo	0
Cow	0
Human A	0
B	0
O	0
Ox	0
Guinea pig	0

n = No. of crabs tested

pH and thermal stability

The hemagglutinin of the hepatopancreas extract of *Oziotelphusanaga*was noted to be stable at pH 7.5-8 (Table 2). pH below 7.5 and above 8 gradually reduced the hemagglutinability of the agglutinin. The extract was tested for the thermo stability of its hemagglutinating activity after incubation at temperature ranging from 0 to 100ºC. Optimum activity was reported at a temperature ranging from 20°C to 40°C and the complete loss of activity at 90°C was observed (Table 2).

Table 2 Hemagglutination titer of the hepatopancreas extract of the freshwater crab,*Oziotelphusanaga*in relation to pH and temperature

pH (n=10)	HA titer	Temperature(n=10)	HA titer
5.5	128	0	128
6	128	10	128
6.5	256	20	**2048**
7	512	30	**2048**
7.5	**2048**	40	**2048**
8	**2048**	50	512
8.5	1024	60	256
9	512	70	64
9.5	256	80	4
10	128	90	0
10.5	32	100	0
11	8		
11.5	8		

n = No. of crabs tested

Effect of divalent cations and calcium chelators on HA

Hepatopancreas agglutinin of *Oziotelphusanaga*requires divalent cations for their hemagglutinating activity as shown in table 3. Enhanced activity was noted upto 20 mM andhigh concentration of calcium, magnesium and manganese ions reduced the HA activity.

When the hemagglutinating activity of the hepatopancreas extract were tested with different concentrations of the calcium chelators like EDTA (Di and Tetra) and tri sodium citrate, no changes were observed in HA up to 1 mM concentration but above 1 mM a decline in activity of the hepatopancreas extract of *Oziotelphusanaga* (Table4) was noticed. This confirms the calcium dependency of the agglutinin.

Table 3 Effect of cations on the hemagglutinating activity of the hepatopancreas agglutinin of the freshwater crab,*Oziotelphusanaga*

Cation conc. in mM (n=10)	HA titer		
	Ca^{2+}	Mg^{2+}	Mn^{2+}
0	1024	1024	1024
0.01	1024	2048	2048
0.10	2048	2048	1024
1	2048	2048	1024
10	2048	2048	2048
20	2048	1024	2048
30	128	512	128
40	64	128	64
50	32	128	32
100	4	128	8

n = No. of crabs tested

Table 4 Effect of calcium chelators on the hemagglutinating activity of the naturally occurring agglutinin in the hepatopancreas of the freshwater crab,*Oziotelphusanaga*

Concentration in mM (n=10)	HA titer		
	EDTA		Trisodium citrate
	Disodium	Tetrasodium	
0	1024	1024	1024
0.01	2048	2048	2048
0.1	2048	2048	2048
1.0	2048	1024	2048
5	1024	1024	1024
10	128	512	1024
20	64	256	256
30	64	256	256
40	16	256	256
50	16	128	256
100	4	128	256

n = No. of crabs tested

Hemagglutination inhibition assay

Agglutination by hepatopancreas extract was inhibited by all glycoproteins tested.Fetuin strongly inhibited the hepatopancreas agglutinin. Transferrin, BSM and PSM weakly inhibited the agglutinin activity (Table 5).

All the 8 sugars tested, inhibited the agglutinating activity of the hepatopancreas agglutinin at varying capacities against rabbit erythrocytes as shown in table 6. Simple sugars like D-fucose and D-glucose 6 phosphates inhibitedmoderately and α-lactose, GlcNAc and Trehalosehighly inhibited the hemagglutinating activity of the hepatopancreas against rabbit erythrocytes. N-acetyl neuraminic acid and N-glycolyl neuraminic acid, a nine carbon sialic acid also inhibited the agglutination against rabbit erythrocytes.

Table 5 Hemagglutination inhibition of the hepatopancreas agglutinin of the freshwater crab, *Oziotelphusanaga*by glycoproteins

Glycoproteins (n=5)	HAI titer	Minimum Concentration Required (µg/ml)	Relative Inhibitory Potency (%)
PSM	4	1250	12.5
BSM	8	625	25
Transferrin	8	625	25
Fetuin	**32**	**62.5**	**100**

n = No. of crabs tested

Table 6 Hemagglutination inhibition of the hepatopancreas agglutinin of the freshwater crab,*Oziotelphusanaga*by various sugars

Sugars (n=5)	HAI titer	Minimum Concentration Required (mM)	Relative Inhibitory Potency (%)
D-glucosamine	2	50	6.25
L-fucose	2	50	6.25
N-glycolyl neuraminic acid	2	50	6.25
N-acetyl-neuraminic acid	4	25	12.5
D-glucuronic acid	4	25	12.5
N-acetyl-neuraminic acid	8	12.5	25
D-fucose	8	12.5	25
D-glucose 6 phosphate	8	12.5	25
Trehalose	16	6.25	3.12
GlcNAc	16	6.25	3.12
α-lactose	**32**	**3.12**	**100**

n = No. of crabs tested

Cross adsorption assay

Cross adsorption assay was carried out to identify the nature of the agglutinin (i.e) whether the agglutinin is single or multiple (Table 7). Agglutinating activity was removed within a single step for other erythrocytes tested by adsorption of the sample with rabbit and rat erythrocytes. These results revealed that the hepatopancreas of *Oziotelphusanaga* contain a single agglutinin.

Table 7 Hemagglutination of hepatopancreas of the freshwater crab,*Oziotelphusanaga*after adsorption with different erythrocytes

Erythrocytes absorbed (n=5)	HA titer			
	Rabbit	**Rat**	**Pig**	**Dog**
None	2048	64	2	2
Rabbit	0	0	0	0
Rat	0	2(0)	0	0
Pig	0	0	0	0
Dog	0	0	0	0

n = No. of crabs tested

Discussion

The hepatopancreas extract of *Oziotelphusanaga* agglutinated various mammalian erythrocytes, among them the extract showed high agglutinating titer against rabbit erythrocytes with the titer value of 2048. This result argues for the specificity of the sugar residues constituting the glycocalyx of rabbit erythrocytes (pericellular matrix) which is a glycoprotein and glycolipid covering serving as a receptor to ligands (Hakomori 1973). Glycocalyx, allows the immune system to recognize and also target the foreign materials and changes in the glycocalyx of cancerous cells allow the immune system to recognize and eliminate the cells (Saladin 2014). The rabbit erythrocytes may contain carbohydrate units in a convenient structure and position which facilitates more affinity with higher specificity for the binding of hepatopancreas agglutinin. Spatial distribution of multivalence among lectin conformation may produce a higher specificity (Moreira et al. 1998; Adenikeand Eretan 2004).

pH dependence is a result of protein composition and it is effectively obtained in all enzyme reaction. Surface of the protein molecules have many ionizable groups and active component, having ability to react with hydrogen and hydroxylins (Adolph and Lorenz 1982).At high pH the

activity was almost reduced, which confirms the proteinaceous nature of the agglutinin, as high alteration in the pH denatures the agglutinin. The extract of hepatopancreas was thermo labile and the agglutinin was stable from 20 to 40°C. This result would indicate that, the dissociation or conformation changes in the hemagglutinin molecule may be responsible for either the inhibitory effect at low and high temperature on agglutinating activity (Kaneko et al. 1973).

Most of the lectins in crustaceans are divalent cation dependent especially calcium and they are sensitive to calcium chelators (Nalini et al. 1994). C-type lectins are calcium dependent glycoproteins which share primary and secondary structural homology in their carbohydrate recognition domain (CRDs). C-type lectins differ slightly in the type of glycans they identify with high affinity.The hemagglutinating activity of the hepatopancreas extract of *Oziotelphusanaga* required divalent cation such as Ca^{2+}, Mg^{2+} and Mn^{2+} to a minimum level. This suggests that intrinsic calcium is sufficient for agglutinationbut an addition of extrinsic calcium, the activity was increased. to a certain level. However high concentration of calcium inturn reduced the activity. This may be due to the stress caused by overloading of calcium ions, which inturn alter the ionic balance and reduce the HA activity. The sensitivity to calcium chelators, EDTA and trisodium citrate confirmed the calcium dependency of the agglutinin.In crustaceans, the hepatopancreas are the main source of C-type lectin (Gross et al. 2001).

The hemagglutination activity of the hepatopancreas of *Oziotelphusanaga* was inhibited by sugars such as α-lactose, GlcNAc, D-glucose 6 phosphate,D-fucose,D-glucuronic acid andN-acetyl-neuraminic acid and glycoprotein like fetuin. In crustaceans, lectins unique for various carbohydrates such as glucose and galactose (Umetsu et al. 1991), N-acetylated amino sugar (Sun et al. 2008) or sialic acids like Neu5Ac and 4 and 9-O acetyl neuraminic acid (Ravindranathet al. 1985; Sudhakar et al. 2012) and amino sugar (Sivakamavalli and Vaseeharan 2014) have been identified. The dominant inhibitor of the agglutinin from the hepatopancreas of *Oziotelphusanaga* is fetuin, a glycoprotein that contains galactose, mannose, glucosamine, galactosamine and sialic acids (sias) (Odin 1955). Siasare mainly present in the form of N-acetyl. Almost 7% of the total siasrepresenting 1 residue per mole of fetuin has an N-glycolyl group according to Klenk and Uhlenbruck (1957), who observed the presence of glycolic acid in fetuin. This result would suggest that, the hepatopancreas agglutinin was specific for sias.

The cross adsorption assay revealed the presence of single agglutinin; this may the reason for the complete removal of agglutinating activity when adsorbed with rabbit and rat erythrocytes.

Conclusion

The presence of sialic acid specific agglutinin in the crab, *Oziotelphusanaga* which is lacking the ability to synthesize sialic acids indicate that these agglutinins may be involved in the innate immunity of these animals and the purification of this agglutinin would be of great contribution to medicine.This study furnished all the information required for the purification of the agglutinin by affinity chromatography.

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