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PRELIMINARY CHARACTERIZATION OF HEMAGGLUTININ FROM THE SERUM OF A FRESHWATER FISH *CATLA CATLA*.

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ABSTRACT

A naturally occurring hemagglutinin (HA) was detected in the serum of freshwater fish *Catla catla* using mammalian erythrocytes (RBC) as indicator cells. The serum gave the highest HA titer with human B RBC. In cross adsorption tests, this RBC type completely adsorbed all HA activities from fish serum. An analysis of the physico-chemical properties of HA showed it to be specifically dependent on the presence of Ca^{2+} for its activity, irreversibly sensitive to EDTA, stable between pH 6.0 to 8.0 and the thermal stability stable between 10 to 30 ^{0}C. Further studies demonstrated that the HA is precipitable by Ammonium sulphate (25, 50 and 75%) but 10% TCA did not show precipitate. The Hemagglutinin - inhibition (HAI) assays performed with 25 carbohydrates revealed that the serum HA was specific for inhibited by GlcNAc, GalNAc, laminarin and lipopolysaccharide (*Escherichia coli*). Thus this agglutinin to be unique among all the known fish agglutinins.

KEY WORDS: hemagglutinin; serum; freshwater fish; *Catla catla*.

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INTRODUCTION

The immune system of fishes consist of humoral and cellular components. The cellular component include the freely circulating and fixed macrophages, lymphocytes and neutrophils which interact with a wide range of invading foreign pathogens leading to various types of cellular immune responses such as phagocytosis, encapsulation and extracellular cytoxicity. The humoral immune components encompass a multiplicity of naturally occurring or inducible serum factor such as immunoglobulin (IgM), agglutinins, lysins, complement system, macroglobulins, precipitins, C-reactive proteins and anti-microbial factors [1-2]. These factors, apart from direct interaction with the pathogen, also appear to interact and facilitate the cellular immune responses. Agglutinins (=Lectins) are multivalent proteins or glycoproteins with the ability to recognize specific carbohydrate determinants [3]. Agglutinins are known to specifically recognize the whole sugar [4] a specific part of a sugar [5] a sequence of sugars or their glycosidic linkages [6]. Thus the binding of lectins to these specific determinants associated either on the cell surface or with polysaccharides and glyco-conjugates in solution results in cell agglutination or precipitation of carbohydrate containing polymers [7-8]. The agglutinins are a class of soluble substances known to cause agglutination of particulates, as a consequence of binding of these agglutinins to the carbohydrate determinants on the cell surface. This phenomenon is commonly used to detect the presence of agglutinins using erythrocytes and bacteria as indicator cells and the agglutinin which mediated the agglutination of these foreign materials is referred as hemagglutinin and bacterial agglutinin, respectively. These agglutinin molecules are known to occur ubiquitously among plants [7,9-11], micro-organisms [12-13], invertebrates [14-17] and vertebrates [16,18-20]. Although, there are several reports on the occurrence and functions of these soluble factors in different groups of fishes, the informations available in fishes, the masters of the aquatic environment, are limited and not much detail to really understand the status of immunity [21]. Further, until recently, most of the thinking on the role of soluble serum factors in immune mechanisms in fish has somewhat been inherited from knowledge of mouse and humans. Vertebrates and Invertebrates, the sera of fishes contain naturally occurring agglutinins, which react with a wide range of cellular antigens [22] and these agglutinins have been reported to react with a variety of mammalian blood group antigens [23]. A perusal of literature revealed that these hemagglutinins are present in different body fluids such as serum [24]. The sugar binding specificity is predominantly to D-glucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl- D-galactosamine [25-33] and several reports on fish agglutinins, a systematic study of the specificities and functions of natural serum hemagglutins in fish is limited [29-30,32,34,35-39]. This study describes RBC binding activities, physico-chemical properties and carbohydrate specificity of a naturally occurring agglutinin in the serum of the freshwater fish *Catla catla*.

MATERIALS AND METHODS

Experimental animals and laboratory maintenance

The freshwater fish *Catla catla* were obtained from the Government fisheries departments' pond at Poondi reservoir near Chennai. They were first acclimated to the laboratory condition for 2 days and were maintained in plastic troughs. The water was aerated continuously and changed twice a week.

Preparation of fish serum

The blood from the freshwater fish, *Catla catla* was collected by cardiac puncture using a sterilized syringe, in clean polystyrene plastic tubes held on ice and allowed to clot. Serum was separated by centrifugation (400 x g; 10 min; 20°C) and the resulting clear supernatant (= serum) was used immediately.

Preparation of erythrocyte (RBC) suspension

Human and other mammalian blood samples were obtained by venous or cardiac puncture and collected in sterile Alsever,s solution [40] containing 10 µg/ml of streptomycin. Prior to use, the RBC was washed thrice with 0.9% saline and once with (TBS-II: 50 mM tris-HCl, 115 mM NaCl, 10 mM $CaCl_2$ 300 mOsm) by centrifugation (400 X g, 5 min, RT). Unless specified, the RBC pellet was finally resuspended in TBS-II as 1.5% suspension (v/v).

Hemagglutination (HA) assay

HA assays were performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany) by serial two-fold dilution of a 25 µl of serum sample with an equal volume of TBS-II. After dilution, 25 µl RBC suspensions was added to each well and incubated for 45 min at RT. The HA titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC [40]. Controls for all assays consisted of the substitution of the sample by TBS-II. All the HA assays were performed in duplicate serum samples.

Cross adsorption tests

Serum samples (each 300 µl) were mixed with an equal volume of washed and packed human A, human B, mice and rabbit RBC and incubated for 1 h at RT with occasional shaking. The suspension was centrifuged, Supernatant collected and adsorbed for a second and third time under the same conditions. The serum adsorbed thrice was subsequently tested for HA activity against all the four RBC types mentioned above.

Cation dependency and EDTA sensitivity

Serum samples (each 600 µl) were dialysed (MW exclusion limit < 10,000) extensively against (TBS-I: 50mM tris-HCl, 135 mM NaCl 300 mOsm), to test cation dependency or (TBS-III : 50 mM tris-HCl, 115 mM NaCl, 50 mM EDTA 300 mOsm), to examine EDTA sensitivity at 15°C. The samples dialysed against TBS-III were subsequently re-equilibrated by dialysis against TBS-I. The resulting dialysates were centrifuged and the remaining HA activity in the supernatant was determined using human B RBC in the presence of TBS that did or did not contain 10 mM $CaCl_2$, $MgCl_2$ or $MnCl_2$. A serum sample, concurrently dialysed against TBS-II, was also tested for it's HA activity against human B RBC in TBS-II.

pH and thermal stability

The stability of HA activity in different pH was examined by dialyzing (24 h, 4°C) 300 µl serum samples against the following buffers at pH ranging from 3 to 12, 0.2 M acetate buffer [41-42] (pH 3 to 6), 0.2 M tris-HCl buffer (pH 7 to 9) and 0.1 M glycine-NaOH buffer (pH 10 to 12). After dialysis, all the samples were finally re-equilibrated by dialysis against TBS-II and the HA titer was determined with human B RBC. In another experiment designed to study the thermal stability of HA, 300 µl serum samples were held for 30 min at temperatures ranging from 10 to 100 °C, centrifuged and tested for the remaining HA activity with human B RBC.

Precipitation of Ammonium Sulphate and Trichloro acetic Acid.

Precipitation of HA activity from serum was attempted using 25%, 50% and 75% ammonium sulphate ($(NH_4)_2$ SO_4) solution as well as 10% trichloroacetic acid (TCA) as described previously [43]. The HA activity was finally measured using human B RBC.

HA -inhibition assays

Several carbohydrates were tested for their ability to inhibit serum HA activity. They were dissolved in (TBS-IV: 50 mM tris-HCl, 5 mM NaCl, 30 mM $CaCl_2$ 135 mOsm) and if necessary, the pH was adjusted to 7.5 using concentrated NaOH. Serum samples were diluted with TBS-IV to a HA titer of 4 against human B RBC suspended in (TBS- V: 50 mM tris-HCl, 72 mM NaCl, 40 mM $CaCl_2$ 300 mOsm). The inhibitor to be tested (25 µl) was serially diluted two-fold with an equal volume of diluted sample in microtiter plates and incubated for 1 h at RT. Human B RBC suspension (25 µl) was added to each well and kept for 3 h at RT. The minimal concentration of carbohydrate that completely inhibited HA activity was recorded.

Protein determination

Total protein concentration was measured according to[44] using bovine serum albumin (BSA) as a standard.

RESULTS

Serum HA profile

The serum samples from individual *Catla catla* agglutinated eight RBC and failed to goat and horse erythrocytes (Table I). The serum gave the highest HA titer of 256 for human B RBC whereas it agglutinated human A, O and rabbit with a titer of 128. Mice, ox and buffalo RBC types were agglutinated (titer 64) and the serum weakly agglutinated sheep RBC (titer 2) type tested.

Cross adsorption tests

Since serum agglutinated human A, B, O and rabbit RBC at high titers, only these RBC types were used in cross adsorption tests. Adsorption of serum thrice with any one of these four RBC types was sufficient to completely remove the HA activity for that RBC type (Table 2). When the serum was adsorbed with human A, B or O RBC types. Serum with RBC types (human A and O) failed to agglutinate against rabbit RBC. All the three human RBC types tested and used for adsorption of serum HA activity, human B RBC appears to be more efficient in adsorbing completely agglutinating activity from serum. Since serum gave the highest HA titer for human B RBC and only this human B RBC type completely adsorbed all HA activity from serum, human B RBC were used as suitable indicator cells in all subsequent studies.

Cation dependency and EDT A sensitivity

The HA titer of serum samples, previously dialysed against divalent cation-free (TBS-I) and tested in the absence of any of these three cations, decreased significantly from 128 to 4 (Table 3). But the HA titer in these samples was completely regained only upon the addition of Ca^{2+}, and neither Mg^{2+} nor Mn^{2+} could substitute for Ca^{2+}. In addition, the HA titer of serum was not affected by dialysis against TBS containing Ca^{2+} (TBS-II). The serum samples, dialysed against TBS containing 50 mM EDTA (TBS- III) and tested in the absence of cations, showed a significant reduction in the HA titer.

pH and thermal stability

The HA activity was stable between pH 6.0 and 8.0 and reduced at pH below or above this range and completely lost at pH 2 and 11 (Table 4). The activity remained stable between 10 and 30°C, decreased at higher temperatures and completely lost at or above 60°C (Table 5).

Precipitation of Ammonium sulphate

Ammonium sulphate (25, 50 and 75 %) was incubated with serum for 3 h. The 50% ammonium sulphate completely precipitated highest HA with serum and ammonium sulphate (25 and 75 %) agglutinated low HA and the 10% TCA did not show HA activity against serum of *Catla catla* (Table 6).

Carbohydrate binding specificity

Of 25 Carbohydrates tested for inhibition, as many as 4 inhibited the serum HA activity of *Catla catla* against human B RBC (Table 7 & 8). Since the HA activity was inhibited by many different carbohydrates and their derivatives with diverse structures, no simple generalizations are evident concerning the structure of carbohydrates involved in HA inhibition. Nevertheless, acetylated aminosugars such as *N*-acetyl D-glucosamine (GlcNAc) and *N*-acetyl D-galactosamine (GalNAc) showed inhibited at 100 or 50 mM concentration respectively. However *N*-acetyl mannosamine (ManNAc), simple sugars, deoxy sugars, amino sugars and glycosides did not show any inhibition. The microbial cell wall components tested, laminarin, an algal cell wall material (*Laminaria digitata*) and lipopolysaccharides (LPS) from *E.coli* inhibited the agglutination of human B RBC. But other LPS did not show any serum hemagglutination inhibitory activity. Among all the inhibitory carbohydrates, laminarin was found to be the most potent inhibitor.

Table 1
Hemagglutinating (HA) activity of serum of freshwater fish Catla catla against various mammalian erythrocyte (RBC) types

RBC Types Tested	HA Titer*
Human B	256
Human A	128
Human O	128
Rabbit	128
Mice	64
Ox	64
Buffalo	64
Sheep	2
Goat	0
Horse	0

**** Based on 20 determinations for each RBC type.***

Table 2
Cross adsorption of serum hemagglutinin (HA) of serum of freshwater fish Catla catla with different types of mammalian RBC

Serum adsorbed with @	HA titer against RBC types tested *			
	Human B	Human A	Human O	Rabbit
None	256	128	128	128
Human B	0	0	0	0
Human A	0	0	0	32
Human O	0	0	0	128
Rabbit	256	128	128	0

@ The 60 min adsorption at Room Temperature.
**** Based on 10 determinations for each RBC types.***

Table 3
Effect of Divalent Cations and EDTA on the Hemagglutinating (HA) activity of serum of Freshwater fish Catla catla

Serum sample tested	Cation (10 mM) in sample diluting and RBC suspension	HA titer* Human B
1. Before dialysis	$CaCl_2$	256
2. After dialysis against divalent cation free TBS (TBS-I).	None	16
	$CaCl_2$	128
	$MgCl_2$	64
3. After dialysis against TBS+10mM $Cacl_2$ (TBS-II)	$MnCl_2$	16
	$CaCl_2$	128
4. After dialysis against TBS+ 50 mM EDTA (TBS-V) followed by dialysis against TBS-I	None	8
	$CaCl_2$	64
	$MgCl_2$	4
	$MnCl_2$	4

**** Determination using human B RBC and the results based on 6 determinations.***

Table 4
pH stability of hemagglutinating activity of serum of freshwater fish Catla catla

S.no	pH	HA Titer* Serum
1.	1	0
2.	2	0
3.	3	8
4.	4	32
5.	5	64
6.	6	128
7.	7	128
8.	8	128
9.	9	64
10.	10	32
11.	11	4
12.	12	0

**** Based on 10 determinations for pH stability Human B RBC against Serum.***

Table 5
Thermal pH stability of hemagglutinating activity of serum of freshwater fish Catla catla

S.no	Temperature	HA Titer* Serum
1.	10	128
2.	20	128
3.	30	128
4.	40	64
5.	50	8
6.	60	0
7.	70	0
8.	80	0
9.	90	0
10.	100	0

**** Based on 10 determinations for thermal stability Human B RBC against Serum.***

Table 6
Ammonium Sulphate and TCA Precipitation of Hemagglutination (HA) activity against human B RBC from the serum of Catla catla

Ammonium sulphate and TCA	(%) Saturation	HA titer* Human B
Untreated Sample		256
25%		16
50%		256
75%		32
TCA 10 %		0

**** Determination using human B RBC and the results based on 6 determinations.***

Table 7

Carbohydrates tested for inhibition of serum hemagglutinating (HA: titer = 4) activity of Catla catla. If not otherwise stated, the starting concentration of each carbohydrate was 200mM

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM)*
Monosaccharides		
Simple sugars		
D-Arabinose	200	NI
L-Arabinose	200	NI
D-Xylose	200	NI
D-Galactose	200	NI
D-Mannose	200	NI
β-D-Allose	200	NI
L-Sorbose	200	NI
D-Fructose	200	NI
Deoxy sugars		
2-Deoxy - D - Glucose	200	NI
2-deoxy-D-galactose	200	NI
Amino sugars		
D-glucosamine (GlcN)	200	NI
D-galactosamine (GalN)	200	NI
Mannosamine (ManN)	200	NI
***N*-acetyl sugars**		
cetyl-D-glucosamine (GlcNAc)	200	100
cetyl-D-galactosamine (GalNAc)	200	50
cetyl-D-mannosamine (ManNAc)	200	NI
cosides		
nyl-α- D-glueopyranoside	200	NI
nyl-β- D-glucopyranoside	200	NI
-α-D-glueopyranoside	200	NI
-β-D-glueopyranoside	200	NI

**** The assay was performed three times for each carbohydrate with identical results using samples from different preparations.***
NI: No inhibition.

Table 8

Hemagglutination–inhibition of Catla catla serum (Hemagglutination titer = 4) by polysaccharides against human B RBC

Polysaccharides tested	Maximum concentration tested (mg. ml^{-1})	Minimum inhibitory concentration (mg. ml^{-1})*
Laminarin	1	0.06
Lipopolysaccharides *Escherichia coli*	2	0.25
Salmonella abortus equi	2	NI
Serratia marcescens	2	NI
Pseudomonas aeruginosa	2	NI

**** The assay was repeated three times for each polysaccharide with identical results using samples from different preparations. NI : No inhibitory.***

DISCUSSION

The serum of freshwater fish *Catla catla* was found to possess naturally occurring agglutinating activity which showed the highest reactivity with human B RBC among other RBC types tested[29,32]. This serological specificity suggests that the receptor determine prequentially recognized by serum agglutinin is abundant or more

accessible on human B RBC rather than other RBC types tested. In cross adsorption tests performed with four types of mammalian RBC, human A, B and O RBC adsorbed HA activities from serum thereby indicating that the HA molecules exhibit relatively a higher affinity for human B RBC. These results also suggest that the RBC types agglutinated by the serum of *Catla catla* probably share a common surface receptor but with a quantitative difference in its HA binding sites[14]. The serum lost most of its HA activity after dialysis against cation-free TBS and tested in the absence of cations. However, the activity in this sample completely regained only upon addition of Ca^{2+} and the HA titer of serum did not change after dialysis against TBS containing Ca^{2+}. These observations demonstrated that the serum agglutinin of *C.catla* specifically requires Ca^{2+} for its HA activity. Hence the agglutinin detected in the serum *C.catla* could be classified under C-type lectin[45]. The C-type lectins are calcium dependent mosaic molecules, are known to be directly involved in innate defense mechanisms[46]. Supportingly, several earlier workers have been described that the serum agglutinin of freshwater fishes are divalent cation dependent for their activity [29-30,32,36]. Furthermore, the activity was sensitive to EDTA treatment since dialysis of serum against TBS containing EDTA resulted in a significant reduction in the HA activity. None of the cations tested could restore the HA activity in these samples thereby indicating that the HA of *C.catla* is irreversibly sensitive to EDTA which is in contrast with other fish agglutinins [29, 32, 36, 47]. The HA activity of *Catla catla* was found to be stable between pH 6.0 and 8.0 and it appeared to be more tolerant to acidic and alkaline conditions compared to their counterparts such as *Clarias batrachus* and *Heteropneustes fossilis* [29,32]. Further analysis revealed that the HA activity in thermal stability was found to be stable between 10 to 30 ^{0}C. The completely precipitated agglutinin molecules from serum with ammonium sulphate (50% saturation) but low agglutinin in 25 and 75% of ammonium sulphate. The 10% TCA did not show any HA activity. Here the serum agglutinin indicates that may be a protein or glycoprotein. Fish serum agglutinins were shown to be specific for L-Rhamnose [33], fructose [37] and galactose [32]. In contrast, the serum HA activity of *C.catla* was not inhibited by ManNAc tested. But it was inhibited by *N*-acetyl D-glucosamine (GlcNAc) and *N*-acetyl D-galactosamine (GalNAc). However, in contrast, their N-acetyl derivatives ManNAc failed to inhibit the HA activity.

Furthermore, the serum HA activity was also inhibited by *N*-acetyle-D-glucosamine (GluNAc) and *N*-acetyl-D-galactosamine (GalNAc) and failed to inhibit by *N*-acetyle-D-mannosamine (ManNAc) by *N*-acetyle D-glucosamine (GluNAc) and failed to inhibit by *N*- acetyl-D-mannosamine (ManNAc). The simple sugars, Deoxy sugars, amino sugars and glycosides did not inhibit *C.catla* serum. By contrast their *N*-acetyl derivatives (GlcNAc and GalNAc), which contain the acetyl group on C-2 position, inhibited the serum agglutinating activity at 100 and 50 mM concentration respectively. These observations clearly demonstrate that an acetyle group linkage at c-2 position arranged in equatorial orientation with glucose or galactose is essential for agglutinin ligand interaction. Further *N*-acetyle D-Mannosamine which is structurally similar to GlcNAc and GalNAc and possess acetyle group on C-2 position in an axial orientation did not show inhibition of serum HA activity. Here the necessity for having *N*-acetyle group at C-2 position (GluNAc and GalNAc) for agglutinin ligand interaction. The serum HA activity was also inhibited by the presence of laminarin and LPS (*E.coli*). The inhibition of serum HA activity by laminarin specific agglutinin molecules in the serum of *C.catla* [48-49].The hemagglutinins in the serum of Cat fish *C.batrachus* and *H.fossilis* selectively agglutinated certain species of Gram positive and Gram negative bacteria [30,32,39] and the hemagglutinin to interact with LPS

components of Gram negative bacteria. This observation clearly indicates the possible defensive role of serum hemagglutinin in the host defense of the freshwater fish *C.catla*. Thus, this study demonstrates the presence of an agglutinin specific for *N*-acetyl D-glucosamine (GlcNAc), *N*-acetyl D-galactosamine (GalNAc), laminarin (β-1, 3 glucan) and lipopolysaccharide (*Escherichia coli*) in the serum of the freshwater fish *C.catla*. This agglutinin appears to be unique among all the known fishes agglutinins. Further work needs to be undertaken into the purification of this novel agglutinin molecule of *Catla catla* as a prerequisite to elucidate the immunological roles of fishes agglutinins.

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