*Carbohydrate Research*, 213 (1991) 251–261
Elsevier Science Publishers B.V., Amsterdam



# A T-antigen-binding lectin from *Channa leucopunctatus* (Murrel) plasma*,†

Sunita R. Manihar, Rakha H. Das, and Hasi R. Das§
*C.S.I.R. Centre for Biochemicals, Delhi University Campus, Delhi-110007 (India)*

(Received January 26th, 1990; accepted for publication, in revised form, September 14th, 1990)

ABSTRACT

The plasma of *Channa leucopunctatus*, which agglutinates human A,B,O blood-group erthrocytes nonspecifically, contains three separate agglutinating activities that are distinguishable by hemagglutination with specific blood-group erythrocytes. Blood group A agglutinating activity of the plasma was separated from the other two hemagglutinating activities by DEAE-cellulose column chromatography and further purified to homogeneity by affinity chromatography on 2-acetamido-2-deoxy-D-galactose coupled to epoxy-activated Sepharose 6B. The apparent homogeneity of the lectin was established by poly(acrylamide) gel electrophoresis, isoelectric focusing, immunodiffusion, and cross-immunoelectrophoresis. The native protein has a mol. wt. of 140000 and two identical subunits. The isoelectric point of the affinity-purified lectin is 4.6. Amino acid analysis indicated high proportions of glycine, alanine, and aspartic acid. The lectin is a glycoprotein and it has a requirement for divalent cations, $Ca^{2+}$ and $Mg^{2+}$ or $Mn^{2+}$, for hemagglutinating activity. 2-Acetamido-2-deoxy-D-galactose, 4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside, T-antigenic disaccharide, and Forssman glycolipid are potent inhibitors.

## INTRODUCTION

Only very few species of fish[1–4] have been studied for the presence of lectin activity, even though the lectin specific for blood group O in eel fish sera[1] was reported as early as 1944. Lectins of various specificities from roe of some fish families, such as *Salmonidae* and *Clupeidae*[5–7], are known. Agglutinins were also found in some of the species belonging to the families, *Cyprinidae*[8–10] and *Percidae*[11]. The blood group B-specific lectins have been identified in roe of the Pawn[6] as well as Ayu fish[12]. It was suggested that these agglutinins play an important role in the egg fertilization and protection of the eggs from pathogens. In this report, we describe the isolation and characterization of a lectin from the plasma of *Channa leucopunctatus*, a fresh-water fish of the family *Channidae*.

* Dedicated to Professors Nathan Sharon and Toshiaki Osawa.
† This work was supported by the Council of Scientific and Industrial Research.
§ To whom correspondence should be addressed.

0008-6215/91/$03.50 

EXPERIMENTAL

*Collection of plasma.* — Blood samples were collected from the caudal vein of the fish, in citrated–phosphate buffered saline solution (PBS)[13]. Plasma was separated from the citrated blood by centrifugation at 1000*g* for 10 min at 4°.

*Protein and amino acid analysis.* — Qualitative protein determination in plasma, and DEAE-cellulose- and affinity-purified lectin preparations were carried out by the method of Lowry *et al.*[14] using bovine serum albumin as standard. For amino acid analysis, the purified lectin was dissolved in 0.1% trifluoroacetic acid, the solution dried, and the residue hydrolyzed in 6M HCl for 24 h at 110° in *vacuo*[15] and analyzed with a Hewlett–Packard amino acid analyzer. The neutral sugar content was determined by the phenol–sulfuric acid method[16] and the sialic acid content by the procedure of Warren[17].

*Metal-ion effect.* — The metal-ion effect was studied after dialysis of the lectin against normal saline solution (pH 6.8) at 4° for 48 h with four changes.

*Electrophoresis.* — Poly(acrylamide) (Sigma Chemical Co., U.S.A.) gel electrophoresis of the native lectin was performed following the method of Davis[18]. Sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli[19]. Isoelectric focusing was performed in 5% poly(acrylamide)-tube gel containing Ampholine (pH, 3.5–9.5) by use of a standard calibration kit following the manufacturer's (LKB, Sweden) instructions.

*Preparation of a specific-affinity column.* — 2-Acetamido-2-deoxy-D-galactose was coupled by the method of Vretblad[20] with water-swollen, epoxy-activated Sepharose 6B (Sigma) in 0.1M NaOH for 15 h at 45° with constant shaking. The product was washed extensively with water, 0.05M Tris·HCl (pH 8.0) containing 0.15M NaCl, and then with 0.15M NaCl in 0.05M sodium formate buffer, pH 4.0; it was finally washed and equilibrated with normal PBS[13].

*DEAE-cellulose column chromatography.* — Whole plasma was dialyzed against 0.01M phoshpate buffer, pH 7.2, and then applied onto a DEAE-cellulose column (0.9 × 15 cm for 32 mg protein), which had been previously equilibrated with the same buffer. The column was extensively washed with the equilibration buffer until $A_{280}$ became zero. The bound proteins were eluted sequentially with the buffer containing 0.1, 0.3, 0.5, 0.75, and 1.0M NaCl. Protein content of the various salt-eluted fractions (2 mL each) was monitored by u.v. absorption spectroscopy at 280 nm. Hemagglutination activity of the eluted fractions was determined after dialysis against PBS. Protein fractions showing hemagglutinating activity with blood group A erthrocytes were pooled, dialyzed, and further purified by specific-affinity column chromatography.

*Affinity-column chromatography.* — The aforementioned fractions were loaded onto the affinity column (0.6 × 9 cm) which was washed at a flow rate of 15 mL/h with sufficient buffer[13] until $A_{280}$ of the effluent became zero. Affinity-column-bound protein was then eluted at the same rate with lactose (40 mg/mL) in saline solution by collecting 2-mL fractions. Fractions showing $A_{280}$ were pooled and dialyzed against PBS, and the hemagglutination activity was determined with blood group A erythrocytes.

*Hemagglutination (HA) and inhibition assays.* — Hemagglutinating activity was determined by a two-fold serial dilution of lectin using a 2% suspension (25 $\mu$L) of erythrocytes in PBS[13] for 20 min at 22° with plasma or purified lectin (25 $\mu$L). One hemagglutination unit (H.U.) is defined as the minimum amount of protein required for 100% agglutination under the assay conditions. A 2% suspension of erythrocytes was treated at 37° in phosphate buffered saline solution for 20 min and 80 min with trypsin (5 $\mu$g/mL) and neuraminidase (0.5 unit/mL; Gibco, U.S.A.), respectively, as described by Das *et al.*[21]. Inhibition of hemagglutination was done by two-fold serial dilution of the sugars (Sigma Chemical Co., U.S.A.) using a 2% suspension of blood group A erythrocytes and four hemagglutination doses of lectin.

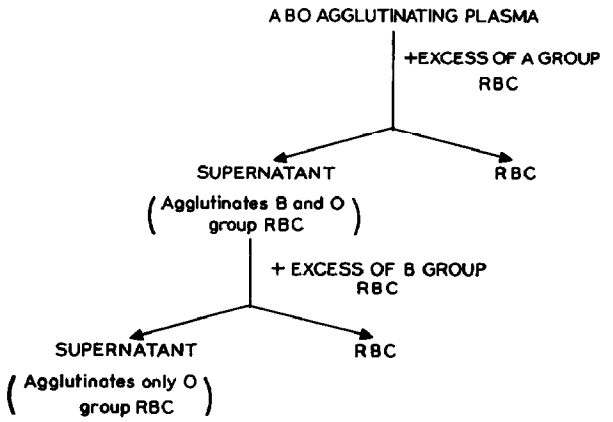
*Immunological methods.* — Rabbits were immunized by subcutaneous injections of whole plasma (1 mg) twice during the first week, followed by four injections of plasma with complete Freund's adjuvant. Finally, a booster injection was given with incomplete Freund's adjuvant. Antiserum was collected five days after the last injection

TABLE I

Agglutination of human erythrocyte by plasma of *Channa leucopunctatus*

| *Erythrocyte* | *Reciprocal of the hemagglutination titres*[a] | | |
|---|---|---|---|
| | *Untreated* | *Trypsin-treated* | *Neuraminidase-treated* |
| Human A | 16 | 16 | 64 |
| Human B | 8 | 8 | 32 |
| Human O | 16 | 16 | 32 |

[a] The hemagglutination titre is the highest dilution giving 100% agglutination under the assay condition.




Scheme 1. Identification of separate blood group A, B, and O agglutinating activities in the plasma of *C. leucopunctatus*. Agglutinations were carried out at 22° for 20 min with an excess of 30% erythrocyte suspension for complete adsorption of agglutinating activities with the same blood group. The supernatant of agglutination reactions was collected by centrifugation at 2000*g* for 10 min at 4°.

and was used in immunodiffusion and cross-immunoelectrophoresis[22] in 1.5% agarose and 0.1M sodium barbital buffer, pH 8.6.

## RESULTS

Plasma of *Channa leucopunctatus* agglutinated human A,B,O erythrocytes non-specifically with or without trypsin, or neuraminidase treatment (Table I). Neuraminidase but not trypsin treatment increased the susceptibility of these erythrocytes to agglutination. The nonspecific agglutinating activity of the plasma is due to three, separately identifiable, hemagglutinating activities as shown in Scheme 1. The selective adsorption of one agglutinating activity with specific erythrocyte did not affect the activity of the others (data not shown). DEAE-cellulose chromatography of the dialyzed plasma showed both blood groups B and O agglutinating activity in fractions eluted with 0.1M sodium chloride, but none of these fractions agglutinated blood group A erythrocyte (Fig. 1a). Subsequent elution of the column with 0.3 and 0.5M sodium chloride did not show any hemagglutinating activity. Protein fractions obtained with 0.75M sodium chloride elution were found to agglutinate blood group A erythrocytes. Further washing of the column with M sodium chloride did not show any agglutinating activity.

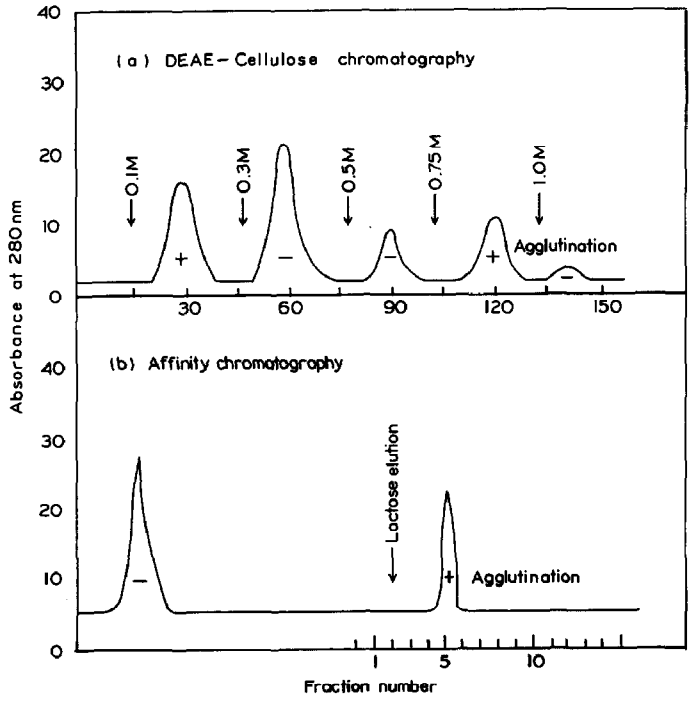



Fig. 1. (a) Chromatography of *C. leucopunctatus* plasma on a DEAE-cellulose column. Fractions obtained with 0.1M and 0.75M NaCl agglutinated blood group erythrocytes *O*, B, and A, respectively. (b) Elution profile of the affinity column.

TABLE II

Purification of blood group A agglutinating activity from *Channa leucopunctatus* plasma

| *Purification step* | *Total (mg)* | *Specific activity[a] (H.U./mg of protein)* | *Total activity[a] (H.U.)* | *Purification (-fold)* | *Recovery (%)* |
|---|---|---|---|---|---|
| Whole plasma | 32.00 | 9 | 288 | 1 | |
| DEAE-cellulose column (0.75M fractions) | 0.32 | 806 | 258 | 89 | 89 |
| Affinity chromatography | 0.10 | 2000 | 200 | 222 | 69 |

[a] One hemagglutination unit (H.U.) is expressed as the minimum amount of protein required for complete agglutination of an equal volume of 2% blood group A erythrocytes suspension at 22° in 20 min as described in the Experimental section.

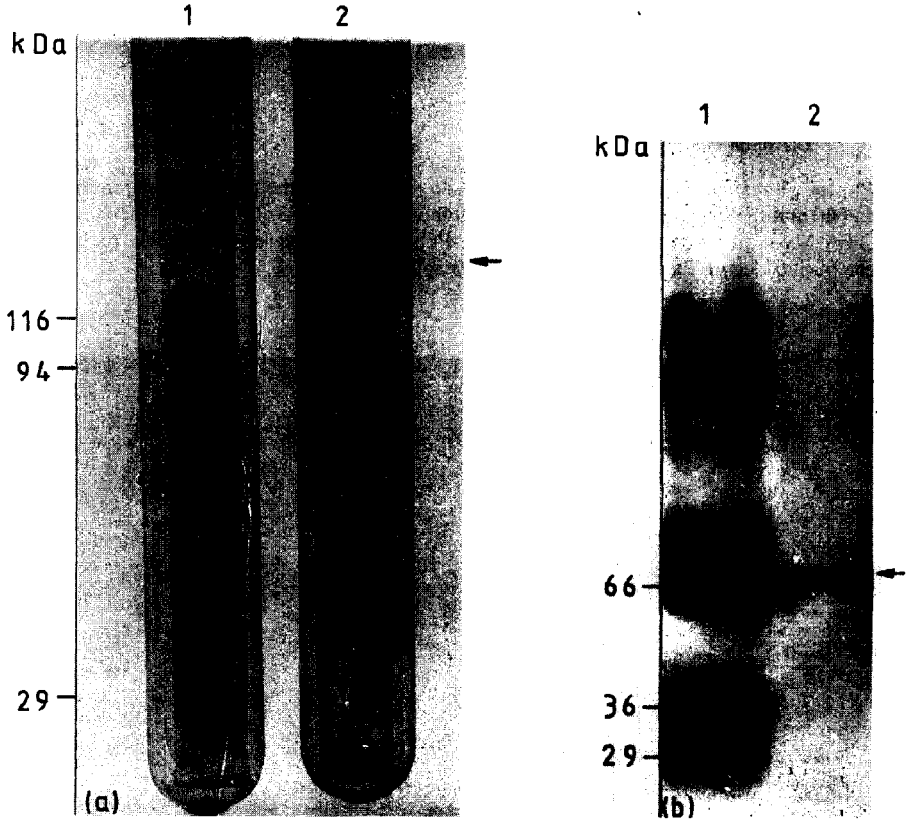



Fig. 2. Poly(acrylamide) gel electrophoresis (PAGE) of purified lectin: (a) On native gel (7.5%). Protein mol. wt. markers and purified lectin (20 μg) in tubes 1 and 2, respectively, were subjected to electrophoresis in Tris–glycine buffer (pH 8.53). (b) SDS–PAGE (10%). Mol. wt. markers and purified lectin (10 μg), respectively, in lanes 1 and 2 of the slab gel, were subjected to electrophoresis in Tris–glycine buffer (pH 8.53) containing 0.1% sodium dodecyl sulfate. Gels were stained with Coomassie Blue.

Agglutination of blood group A erythrocytes by 0.75M sodium chloride-eluted fractions required both $Ca^{2+}$ and $Mg^{2+}$ ions, and the hemagglutination was inhibited by 2-acetamido-2-deoxy-D-galactose (data not shown). Fractions eluted from DEAE-cellulose and showing agglutination towards blood group A erythrocyte bound to 2-acetamido-2-deoxy-D-galactose coupled to epoxy-activated Sepharose 6B in the presence of metal ions, $Ca^{2+}$ and $Mg^{2+}$. Fig. 1b shows the binding of blood group A

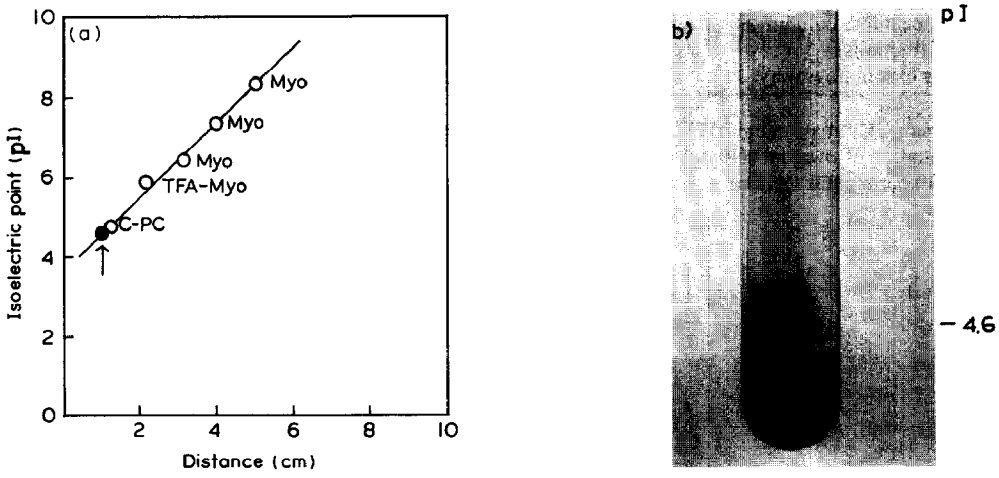



Fig. 3. (a) Plot of pI against the distance from the anode to determine the pI of affinity-purified lectin. Purified lectin (●) and the standard proteins (○) [myoglobin (MyO), pI 8.3, 7.3, and 6.45; trifluoroacetyl-myoglobin (TFA-Myo), pI 5.92, and C-phycocyanin (C-PC), pI 4.85] were run in parallel. (b) Isoelectric focusing of the purified lectin. Purified lectin (20 μg) was applied on the gel.

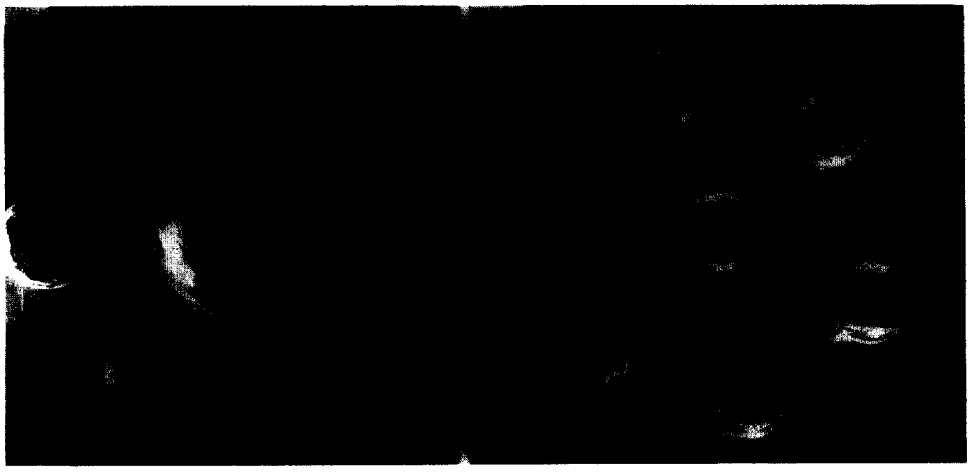


Fig. 4. Immunodiffusion pattern of *C. leucopunctatus* plasma (a) and purified lectin (b). Rabbit antiserum (20 μL) raised against whole plasma was applied in the center well and the same plasma (40 μg of protein) and purified lectin (5 μg) were applied in the outer wells. Double diffusion was carried out in 1.5% agarose gel for 48–72 h in a moist chamber at 4°.

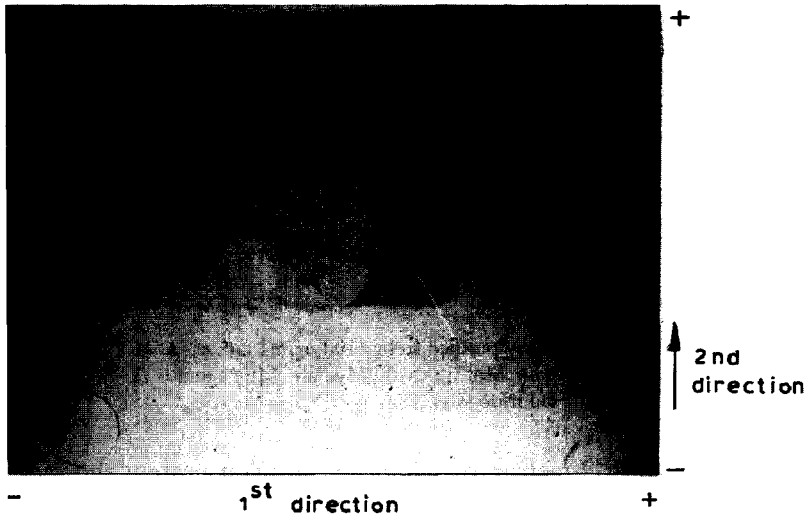



Fig. 5. Cross-immunoelectrophoresis pattern. Purified lectin (5 μg) was subjected to electrophoresis in 1.5% agarose gel in barbital buffer (pH 8.6) in the first direction, then the upper half of the gel was replaced with fresh 1.5% agarose containing antiserum against the plasma (500 μL) and the gel was subjected to electrophoresis in the second direction at 100 V for 16 h. The gel was dried, washed, and stained with Coomassie Blue.

agglutinating activity of the plasma (DEAE fractions) and its elution with lactose in the absence of metal ions. The purification profile in Table II indicated that an 89-fold purification of agglutinating activity had been achieved with 90% recovery after DEAE-cellulose chromatography. A further two-fold purification was obtained by specific-affinity column chromatography.

The affinity-purified lectin showed a single band (Fig. 2a) of molecular weight 140 000 in 7.5% poly(acrylamide) gel. On denaturation by heating for 5 min at 100° in

TABLE III

Amino acid composition of purified lectin[a]

| *Amino acid* | *Residues per molecule*[b] | *Amino acid* | *Residues per molecule*[b] |
|---|---|---|---|
| Aspartic acid | 48 | Tyrosine | 7 |
| Glutamic acid | 37 | Alanine | 63 |
| Lysine | 13 | Valine | 30 |
| Arginine | 13 | Leucine | 36 |
| Histidine | 8 | Isoleucine | 20 |
| Glycine | 119 | Proline | 30 |
| Serine | 20 | Phenylalanine | 12 |
| Threonine | 13 | Methionine | 6 |
| Cystine | 58 | Tryptophan | [c] |

[a] The carbohydrate content of the lectin was 20%, measured as hexose. The lectin was also found to contain 1% of sialic acid. [b] The results are expressed as the number of residues per molecule based on a subunit mol. wt. of 68 000, and the values for the nearest integer are presented. [c] Not determined.

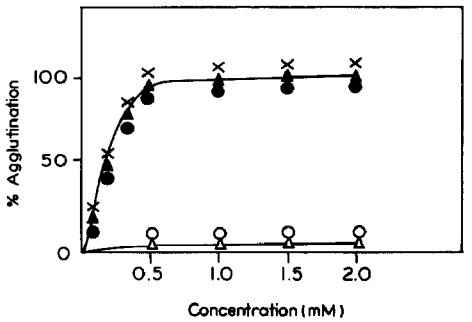



Fig. 6. Effect of metal ions on hemagglutination by purified lectin (100 $\mu$g/mL): (-▲-▲-)$Ca^{2+}$ + $Mg^{2+}$; (-●-●-)$Ca^{2+}$ + $Mn^{2+}$; (-×-×-)$Ca^{2+}$ + $Mg^{2+}$ + $Mn^{2+}$; (-o-o-)$Mg^{2+}$ + $Mn^{2+}$; and (-△-△-)$Ca^{2+}$ or $Mg^{2+}$ or $Mn^{2+}$. All the metal ions were as the chloride salts.

2% sodium dodecyl sulfate with 2-mercaptoethanol, the purified protein showed a single band of molecular weight 68 000 on SDS–PAGE (10% slab gel), as shown in Fig. 2b. Isoelectric focusing of the purified lectin in poly(acrylamide) gel also showed a single band (Fig. 3b) at the isoelectric point of 4.6 (Fig. 3a).

In the immunodiffusion pattern of the purified lectin (Fig. 4), a single precipitin line was observed with the purified lectin (b), whereas whole plasma showed several bands (a). Cross-immunoelectrophoresis of the purified material also gave a single peak for the affinity-purified lectin (Fig. 5).

Affinity-purified lectin agglutinates human A erythrocytes at a 10-$\mu$g/mL protein concentration under standard assay conditions, and no agglutination of blood group B or O erythrocytes was observed with twelve-fold more lectin. However, both blood groups B and O became susceptible to agglutination after neuraminidase treatment, at 120- and 80-$\mu$g/mL lectin concentration, respectively.

The amino acid composition of the affinity-purified lectin indicated that it is rich in glycine, alanine, cystine, and aspartic acid but poor in methionine, tyrosine, and histidine. Total carbohydrate content was estimated as 20% by the phenol–sulfuric acid method and 1% sialic acid content by the modified Warren's method.

Effect of divalent metal ions, such as $Ca^{2+}$, $Mg^{2+}$, or $Mn^{2+}$ is shown in Fig. 6. Both metal ions $Ca^{2+}$ and $Mg^{2+}$ (or $Mn^{2+}$) are required for hemagglutination activity. Optimal concentration of each metal ion is 0.5mM. The inhibitory concentration of sugars for 50% inhibition of hemagglutination is shown in Table IV. In the study of inhibitory potency by comparison with 2-acetamido-2-deoxy-D-galactose, 4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside and the T-antigenic disaccharide were the best inhibitory mono- and di-saccharide, respectively. Asialofetuin (0.15mM) was also a strong inhibitor, but the Forssman glycolipid inhibited the hemagglutination better than any other sugar tested. 2-Amino-2-deoxy-D-galactose, D-galactose, O-substituted D-galactose and methyl D-galactopyranoside derivatives, and di-D-galactose-containing disaccharides were poor inhibitors. D-Glucose, L-fucose, and 2-acetamido-2-deoxy-D-glucose did not exhibit any inhibitory effect at 0.1M concentration.

TABLE IV

Inhibition of the hemagglutinating activity of purified lectin

| *Inhibitors* | *Concentration*[a] *(mM)* | *Relative inhibitory potency*[b] |
|---|---|---|
| 2-Acetamido-2-deoxy-D-galactose | 0.78 | 1.00 |
| 2-Amino-2-deoxy-D-galactose | 12.50 | 0.06 |
| D-Galactose | 50.00 | 0.01 |
| D-Fucose | 100.00 | <0.01 |
| D-Glucose | 100.00[c] | <0.007 |
| 2-Acetamido-2-deoxy-D-glucose | 100.00[c] | <0.007 |
| L-Fucose | 100.00[c] | <0.007 |
| Methyl α-D-galactopyranoside | 50.00 | 0.01 |
| Methyl β-D-galactopyranoside | 50.00 | 0.01 |
| 6-*O*-Methyl-D-galactopyranose | 50.00 | 0.01 |
| 4-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside | 0.39 | 2.00 |
| 4-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside | 12.50 | 0.06 |
| 4-*O*-β-D-Glucopyranosyl-D-galactose | 100.00 | <0.01 |
| 6-*O*-β-D-Galactopyranosyl-D-galactose | 12.50 | 0.06 |
| 4-*O*-α-D-Galactopyranosyl-D-galactose | 50.00[c] | <0.01 |
| Methyl 3-*O*-α-D-galactopyranosyl-α-D-galactopyranoside | 50.00[c] | <0.01 |
| 2-Acetamido-2-deoxy-4-*O*-β-D-glucopyranosyl-D-galactose | 25.00 | 0.03 |
| 2-Acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactose | 0.09 | 8.66 |
| Forssman glycolipid | 0.03 | 26.00 |
| Asialofetuin | 0.15 | 5.20 |

[a] Minimal concentration required for 50% inhibition of four HA units of purified lectin with blood group A erythrocytes. [b] Relative to the potency of 2-acetamido-2-deoxy-D-galactose taken as 1.00. [c] Noninhibitory.

## DISCUSSION

Whole plasma of *C. leucopunctatus* nonspecifically agglutinating human blood groups A, B, and O appears to contain three separately identifiable lectins. The blood groups A and O agglutinating activities of the plasma were found to be the same when tested with normal erythrocytes, whereas the agglutinating activity towards blood group A was two-fold that with blood group O when tested with neuraminidase-treated erythrocytes. This increase may be due to some unknown factor(s) present in the whole plasma. The blood group A agglutinating activity of the *C. leucopunctatus* plasma was separated from the blood groups B and O agglutinating activities by DEAE-cellulose column chromatography. The active component was finally purified to apparent homogeneity by use of an affinity matrix consisting of 2-acetamido-2-deoxy-D-galactose coupled to epoxy-activated Sepharose 6B. The affinity-purified lectin is a dimer of two identical subunits and its homogeneity was evidenced by isoelectric-focusing gel, immunodiffusion, and cross-immunoelectrophoresis.

Amino acid composition and carbohydrate content of this acidic lectin are in agreement with its experimentally determined molecular weight. Both metal ions, $Ca^{2+}$ and $Mg^{2+}$, were necessary for hemagglutinating activity; however, $Mg^{2+}$ could be replaced by $Mn^{2+}$, but the requirement for $Ca^{2+}$ was obligatory. Sugar inhibition

studies indicated that 2-acetamido-2-deoxy-D-galactose is the minimum structural determinant required for sugar–lectin interaction. The acetamido group at C-2 appeared to be an important locus for lectin interaction as D-galactose and 2-amino-2-deoxy-D-galactose were less potent than 2-acetamido-2-deoxy-D-galactose. The configuration at C-4 position also influenced the lectin binding since neither D-glucose nor 2-acetamido-2-deoxy-D-glucose were inhibitory, even at a 100mM concentration. The α anomer of 2-acetamido-2-deoxy-D-galactose was preferred over the β anomer, as 4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside is 33-fold more potent than the corresponding β anomer. This is also apparent as the Forssman glycolipid is a strong inhibitor.

The disaccharides having a terminal, nonreducing α- or β-D-galactopyranosyl group linked (1→3), (1→4), and (1→6) to any subterminal sugar residue, except 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactopyranose (T-disaccharide) poorly inhibited the hemagglutination, indicating that the axial conformation of OH-4 and the 2-acetamido-2-deoxy group of 2-acetamido-2-deoxy-D-galactose in the T-disaccharide are important loci for interaction with the lectin. The T-disaccharide was four-fold more potent as inhibitor than 4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside, which was the best among the tested monosaccharide inhibitors. The increased susceptibility to agglutination of the neuraminidase-treated erythrocytes by the lectin and the inhibition of its activity by asialofetuin were likely due to exposure of T-antigenic disaccharide molecules. 2-Acetamido-2-deoxy-D-galactose was reported to be the best monosaccharide inhibitor of several T-antigen-binding lectins[23–25]. The relative inhibitory potencies of 2-acetamido-2-deoxy-D-galactose and the T-antigen disaccharide for the plasma lectin are comparable with those of the winged bean lectin[23]. In regard to disaccharide specificity, the plasma lectin resembles jackfruit[24] lectin and the lectin from the seeds of *Amaranthus caudatus*[25] (amaranthin). Peanut agglutinin (PNA), a true anti-T lectin, is different from the plasma lectin with respect to monosaccharide specificity, as PNA is inhibited by D-galactose but not by 2-acetamido-2-deoxy-D-galactose[26]. The D-galactosyl group of the T-disaccharide is the primary binding ligand for PNA[25], whereas the plasma lectin appears to recognize the 2-acetamido-2-deoxy-D-galactose residue as its primary binding ligand. An additional residue linked at O-3 of the 2-acetamido-2-deoxy-D-galactosyl residue probably increases the binding affinity by increasing the number of complimentary interactions. Comparative inhibitory potencies of asialofetuin, T-disaccharide, and Forssman glycolipid indicated that the sugar inhibition is not only influenced by substitution at O-3 of the 2-acetamido-2-deoxy-D-galactose unit but also affected by the extended linkage. Further studies with various other sugars are necessary to establish the unique sugar specificity of this plasma lectin.

ACKNOWLEDGMENTS

The authors thank Prof. B. K. Bachhawat for his valuable suggestions during the course of this investigation and Prof. Subhash Basu, University of Notre Dame, U.S.A., for the generous gift of Forssman glycolipid.

REFERENCES

1 B. Jonsson, *Acta Pathol. Microbiol. Scand. Suppl.*, 54 (1944) 456–464.
2 R. T. Kubo, B. Zimmerman, and H. M. Grey, in M. Sela (Ed.) *The Antigens*, Vol. 1, Academic Press, New York, 1973, pp. 417–471.
3 I. Matsumoto and T. Osawa, *Biochemistry*, 13 (1974) 582–588.
4 C. Kelly, *Biochem. J.*, 220 (1984) 221–226.
5 G. I. Pardoe, G. Uhlenbruck, D. J. Anstee, and U. Reifenberg, *Z. Immunitaetsforsch. Exp. Ther.*, 139 (1970) 468–485.
6 A. Krajhanzl, V. Hořejší, and J. Kocourek, *Biochim. Biophys. Acta*, 532 (1978) 209–214.
7 H. Kothbauer and H. Schenkel-Brunner, *Comp. Biochem. Physiol., A*50 (1975) 27–29.
8 S. Schnitzler, G. Muller, and O. Prokop, *Z. Immunitaetsforsch.*, 134 (1967) 45–49.
9 A. Krajhanzl, V. Hořejší, and J. Kocourek, *Biochim. Biophys. Acta*, 532 (1978) 215–224.
10 D. J. Anstee, P. D. J. Holt, and G. I. Pardoe, *Vox Sang.*, 25 (1973) 347–360.
11 H. Schenkel-Brunner, H. Splechtna, and H. Kothbauer, *Experientia*, 28 (1972) 1479.
12 F. Sakakibara, H. Kawauchi, and G. Takayanagi, *Biochim. Biophys. Acta*, 841 (1985) 103–111.
13 R. Dulbecco and M. Vogt, *J. Exp. Med.*, 99 (1954) 167–182.
14 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
15 S. Moore and W. H. Stein, *Methods Enzymol.*, 6 (1963) 819–831.
16 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
17 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
18 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404–427.
19 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
20 P. Vretblad, *Biochim. Biophys. Acta*, 434 (1976) 169–176.
21 H. R. Das, J. F. Codington, M. D. Maxfield, R. W. Jeanloz, S. Miller, G. Klein, and T. Dalianis, *Carbohydr. Res.*, 120 (1983) 303–314.
22 O. Ouchterlony and C. A. Nilson, in D. M. Weir (Ed.), *Handbook of Experimental Immunology*, Vol. 1 Blackwell Scientific, Oxford, 1978, pp. 1–19.
23 S. R. Patanjali, S. V. Sajjan, and A. Surolia, *Biochem. J.*, 252 (1988) 625–631.
24 M. M. V. Krishnashastry, P. Banarjee, S. R. Patanjali, M. J. Swamy, G. V. Swarnalatha, and A. Surolia, *J. Biol. Chem.*, 261 (1986) 11 726–11 733.
25 S. J. Rinderle, I. J. Goldstein, K. L. Matta, and R. M. Ratcliffe, *J. Biol. Chem.*, 264 (1989) 16 123–16 131.
26 R. Lotan, E. Skutelsky, D. Danon, and N. Sharon, *J. Biol. Chem.*, 250 (1975) 8518–8523.