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Affinity purification and affinity characterization of carbohydrate-binding proteins in bovine kidney

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ABSTRACT

Ca^{2+}-dependent carbohydrate-binding proteins were purified from bovine kidney by two-step affinity chromatography on fetuin and heparin columns and subsequent anion-exchange high-performance liquid chromatography. On sodium dodecyl sulphate–polyacrylamide gel electrophoresis, the purified fraction gave two protein bands corresponding to proteins of relative molecular mass 33 000 (p33) and 41 000 (p41), respectively. Although the proteins had no haemogglutinating activities towards human and rabbit erythrocytes, their carbohydrate-binding activity was examined by a newly developed method using horseradish peroxidase (HRP) and/or biotin-labelled glycoconjugates as affinity probes. They could bind in a Ca^{2+}-dependent manner to labelled fetuin and heparin in a specific and dose-dependent manner by solid-phase assay after immobilization on plastic plate surface. Inhibition assay of the binding revealed that N-acetylneuraminic acid is the most potent inhibitor of the proteins among the monosaccharides tested. Fucoidin and heparan sulphate most strongly inhibited the binding of the proteins to labelled heparin. Direct binding assay to acidic glycolipids prepared from bovine kidney showed that the proteins react with the ganglioside fraction but not with sulphatide [Gal(3-SO_4)β1–1Cer]. These results indicated that the purified proteins have a significant affinity to charged oligosaccharides linking to glycoproteins, glycolipids and charged polysaccharides in a Ca^{2+}-dependent manner.

INTRODUCTION

The organs and tissues of mammals have been found to contain a number of carbohydrate-binding proteins or lectins which have wide sugar specificities. In particular, hepatic asialoglycoprotein receptor [1], mannan-binding protein from liver and plasma [2–4], mannose 6-phosphate receptor [5] and soluble β-D-galactoside-specific lectins from various tissues [6–8] have been most extensively studied. However, in spite of the broad distribution of sialic acid and sulphated polysaccharides, reports on lectins binding to glycoconjugates having negative charged residues have been limited [9–13].

Carbohydrate-binding properties of lectins have traditionally been determined by haemagglutination tests with red blood cells and precipitation with glycoconjugates and their inhibition assays. Enzyme (and other biochemical substances)-linked probes with specific affinities are useful tools for the sensitive and selective detection of biomolecules [14,15]. In recent studies, some applications of enzyme labelling have been reported for lectin detection [16–18]. We also proposed a method for characterizing the carbohydrate-binding activity of proteins by using horseradish peroxidase and/or biotin-labelled glycoconjugates [19]. In this paper, we report the affinity chromatographic purification and partial characterization of novel Ca^{2+}-dependent carbohydrate-binding proteins from bovine kidney. Although the purified proteins lacked haemagglutinating activities towards human and rabbit erythrocytes, the new methods were very useful for investigating the carbohydrate-binding properties of the proteins.

EXPERIMENTAL

Materials

Fresh bovine kidney was obtained from a local slaughterhouse. Calf fetuin (type III) and *N*-acetylneuraminic acid were purchased from Sigma (St.

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Louis, MO, USA). Asialofetuin was prepared from fetuin by desialylation with 25 m*M* sulphuric acid at 80°C for 1 h. Heparin and saccharides were obtained from Wako (Osaka, Japan). Heparan sulphate (porcine kidney) [20], dermatan sulphate (umbilical cord) [21], keratan sulphate (whale nasal cartilage) [22], chitin sulphate (tests of tunicate) [23], and fucoidin (brown seaweed *Pelvetia wrightii*) [24] were prepared in our laboratory as described previously. Horseradish peroxidase (HRP, I-C) (Toyobo, Osaka, Japan) and N-hydroxysuccinimide biotin (NHS-biotin) (Sigma) were used for the preparation of binding probes. HRP-conjugated lectins and chondroitin sulphate A (super special grade, whale cartilage), chondroitin sulphate C (super special grade, shark cartilage) and hyaluronic acid (umbilical cord) were obtained from Seikagaku Kogyo (Tokyo, Japan). Other reagents were of analytical-reagent grade from Wako.

Preparation of affinity gels

Fetuin was immobilized on formyl-Sepharose 4B by reductive amination as reported previously [25]. Heparin was coupled to amino-Sepharose 4B with the aid of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) [26,27].

Isolation of binding proteins

Unless indicated otherwise, all procedures were performed at 4°C. After removal of fat, fresh bovine kidney was homogenized in four volumes (v/w) of MTBS [10 m*M* Tris–HCl (pH 7.5)–150 m*M* NaCl–4 m*M* 2-mercaptoethanol–0.5 m*M* phenylmethanesulphonyl fluoride] containing 2 m*M* EDTA and shaken gently for 1 h. After centrifugation at 15 000 *g* for 60 min, the supernatant was removed and the pellet was re-extracted with two volumes of the homogenization buffer in a similar manner. After centrifugation at 30 000 *g* for 1 h, the combined supernatant was adjusted to 5 m*M* $CaCl_2$ by addition of 1 *M* $CaCl_2$, and used as a crude extract. Fetuin-Sepharose 4B suspended in 5 m*M* $CaCl_2$–MTBS was added to 10 volumes (v/w) of crude extract and incubated for 18 h. The gel was separated by centrifugation at 400 *g* for 10 min, washed three times with 5 m*M* $CaCl_2$–MTBS, then poured into a column, from which the adsorbed protein was eluted with 2 m*M* EDTA–MTBS. The protein fractions obtained were pooled and centrifuged at 400 *g* for 10 min. The supernatant was adjusted to 5 m*M* $CaCl_2$ and applied to a heparin-Sepharose 4B column (3 × 0.8 cm I.D.). The adsorbed protein was eluted with 2 m*M* EDTA (fraction I) and 0.3 *M* NaCl (fraction II) by stepwise elution. Fraction I was further applied to a DEAE ion-exchange high-performance liquid chromatographic (HPLC) column (Protein Pak G-DEAE; Nihon Waters, Tokyo, Japan), previously equilibrated with 10 m*M* Tris–HCl (pH 7.2), at a flow-rate of 1 ml/min. The column was then developed with a linear salt gradient buffer (0–0.3 *M* NaCl).

Preparation of binding probes

Fetuin was conjugated with HRP by the method reported previously [14]. Heparin and HRP were coupled with the aid of EEDQ [27]. Biotinylated fetuin was prepared using NHS-biotin as described previously [28].

Binding assay with affinity probes

The quantitative binding of the proteins and its inhibition were assayed after immobilization on polystyrene microtitre plates (Falcon Micro Test III; Becton Dickinson, Lincoln Park, NJ, USA) by use of biotinylated fetuin and HRP-heparin. A 50-μl volume of sample solution was placed in the well and allowed to stand for 4 h at 4°C. After washing with TBS [10 m*M* Tris–HCl (pH 7.5)–150 m*M* NaCl], 200 μl of 2% bovine serum albumin (BSA)–TBS were added to the well to block the remaining protein-binding sites. A 50-μl volume of biotinylated fetuin or HRP-heparin (10 μg/ml TBS containing 5 m*M* $CaCl_2$) was added in the presence or absence of inhibitor. After incubation for 1 h at 4°C, the wells were washed three times with TBS. In the binding assay with biotinylated fetuin, avidin–biotin–peroxidase complex (Amersham international, Amersham, UK) was added and incubated for 30 min, then washed out. Colour development was initiated by addition of 100 μl of the substrate solution [0.04% *o*-phenylenediamine and 0.007% hydrogen peroxide in 100 m*M* citrate–phosphate buffer (pH 5.0)]. After incubation for 5 min at room temperature, the reaction was stopped with 50 μl of 8 *M* H_2SO_4, and the colour developed was measured spectrophotometrically at 490 nm with a Model 3550 microplate reader (Bio-Rad Labs., Richmond, CA, USA).

Gel electrophoresis and electroblotting

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of 0.1% sodium dodecyl sulphate (SDS) on a 9.5% acrylamide running gel with a 3% stacking gel by the method of Laemmli [29]. Electroblotting and lectin staining were carried out as described previously [27].

Amino acid analysis and N-terminal sequencing

Purified proteins were separated by SDS-PAGE and transferred electrophoretically to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon, pore size 0.45 μm; Millipore, Bedford, MA, USA) by the method of Hirano [30]. Direct hydrolysis of protein bands on the PVDF membrane and subsequent amino acid analysis were carried out as described previously [19]. N-Terminal amino acid sequences of the protein bands on the PVDF membrane were analysed with an automated gas-phase Model 477A protein sequencer (Applied Biosystems Japan, Tokyo, Japan).

Preparation of acidic glycosphingolipids

Total lipids were extracted from lyophilized bovine kidney and acidic glycosphingolipids were prepared from total lipids by DEAE ion-exchange chromatography as described previously [31]. Sulphoglycolipids and gangliosides were separated by silica gel column chromatography with a gradient system of chloroform–methanol–water (55:45:2 to 25:85:2) as eluent. Sialic acid was determined by ion-exclusion HPLC as reported previously [32].

Production of specific antiserum

Polyclonal antiserum against the proteins [mixture of proteins of relative molecular mass (M_r) 33 000 and 41 000] was generated by immunization of rabbit [33]. Polyclonal rabbit antiserum was purified on a protein A immobilized column (Ampure PA kit, Amersham), and stored at −20°C.

Binding to acidic glycosphingolipids

Lipids dissolved in methanol were added to microtitre wells and adsorbed on the polystyrene surface by drying. To saturate non-specific protein-binding sites, 200 μl of 2% BSA–TBS were applied to the wells for 1 h, then immobilized lipids were incubated with the purified proteins (5 μg per 50 μl TBS) for 18 h at 4°C in the presence of 5 m*M* $CaCl_2$ or 2 m*M* EDTA. The wells were washed and blocked again with 2% BSA–TBS before incubation with rabbit polyclonal antibody to purified proteins. After 1 h, the wells were washed and HRP-conjugated anti-rabbit IgG antibody (Kirkegaard & Perry Labs., Gaithersburg, MA, USA) was added and left for 30 min. Colour development was performed by use of *o*-phenylenediamine.

RESULTS

Purification of proteins

Carbohydrate-binding proteins were purified from bovine kidney extract by two steps of affinity chromatography and successive ion-exchange HPLC. Typical profiles of the affinity chromatographic purification of the proteins are shown in Fig. 1. After incubation of kidney extract with fe-

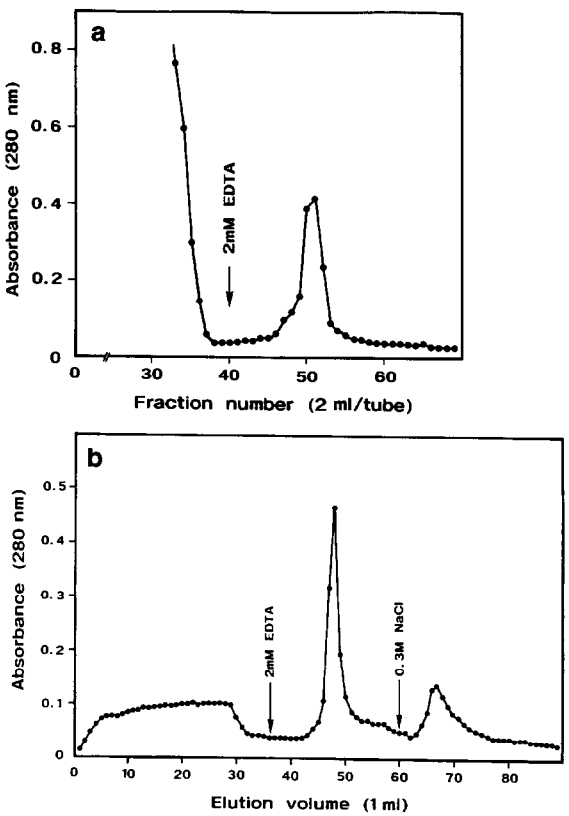

Fig. 1. Profiles of affinity chromatographic purification of bovine kidney proteins. Carbohydrate-binding proteins were purified by two-step affinity chromatography on (a) fetuin and (b) heparin columns in the presence of Ca^{2+} as described under Experimental.

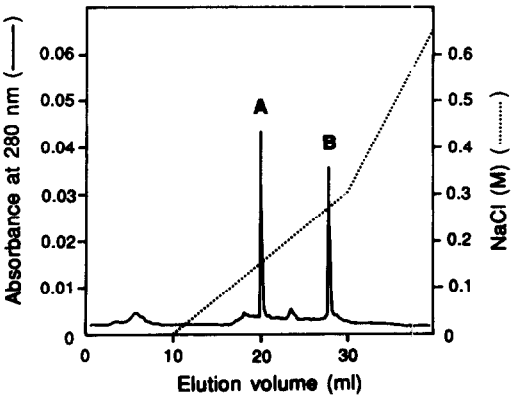

Fig. 2. Purification of proteins by DEAE ion-exchange HPLC. Affinity-purified proteins were applied to a DEAE column as described under Experimental.

tuin-Sepharose gel, the proteins adsorbed on the gel were eluted with 2 m*M* EDTA–MTBS (Fig. 1a). The eluted fractions were then subjected to affinity chromatography on a heparin-Sepharose column in the presence of 5 m*M* $CaCl_2$ (Fig. 1b). Proteins eluted from the heparin column with 2 m*M* EDTA were further purified by DEAE ion-exchange HPLC. As shown in Fig. 2, two fractions, A and B, were obtained by elution with a linear salt gradient. Neither fraction at a protein concentration of 0.5 mg/ml had haemagglutinating activities towards intact and trypsinized human and rabbit erythrocytes in the presence of Ca^{2+}.

Carbohydrate-binding activities

The carbohydrate-binding activities of the kidney proteins were confirmed by binding assays with HRP and/or biotin-labelled fetuin and heparin and these inhibition assays. Fractions A and B obtained by DEAE ion-exchange HPLC were immobilized at various concentrations on polystyrene microtitre plates and allowed to react with biotinylated fetuin and HRP-heparin. As shown in Fig. 3, fraction A reacted with labelled fetuin and heparin in a dose-dependent manner at concentrations from 10 to 100 μg/ml of the proteins, but fraction B did not. These results indicate that fraction A has carbohydrate-binding activity but the protein in fraction B does not. SDS-PAGE analyses (Fig. 4) showed that fraction A gave two bands corresponding to proteins of M_r 33 000 and 41 000 under non-reduced conditions. The purified proteins were termed to p33 and p41, respectively. Starting from 100 g of bovine kidney, *ca.* 100 μg each of protein were obtained, and their relative amounts differed from batch to batch.

To characterize the carbohydrate specificities of the proteins, inhibition assays of the binding of the proteins to the labelled glycoconjugates were performed with various saccharides and glycoproteins as inhibitors. The results of inhibition assays are

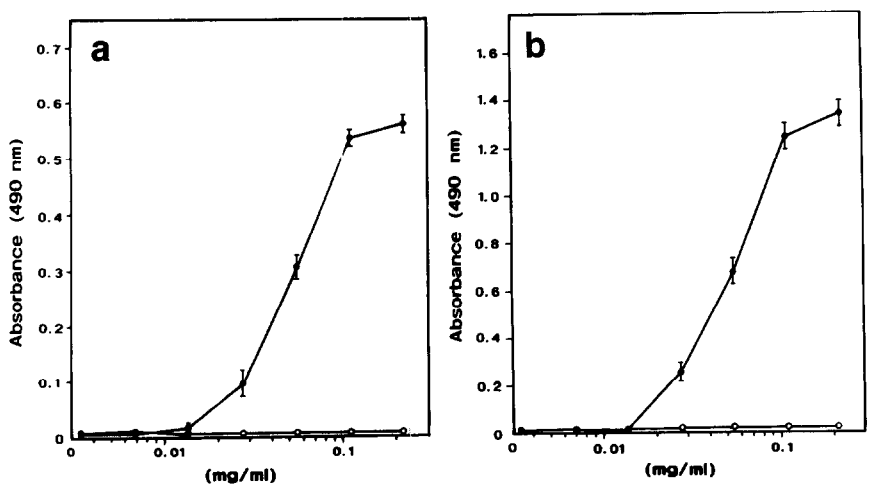

Fig. 3. Reactivities of the proteins with biotinylated fetuin and HRP-heparin. Fractions, A (●) and B (○), eluted from the DEAE column were immobilized at various concentrations on plastic titre plates. The panels show the reactivities with (a) biotinylated fetuin and (b) HRP-heparin. Determinations were done in triplicate.

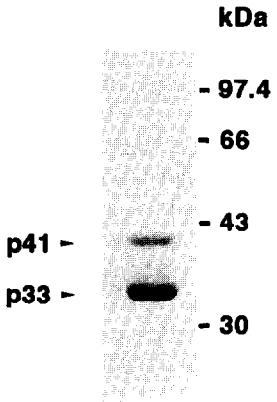

Fig. 4. SDS-PAGE of the purified proteins. The EDTA-eluted fraction from a heparin-column was further purified by DEAE ion-exchange HPLC and separated into two fractions. Fraction A, which was active in binding assays with labelled glycoconjugates, was analysed by SDS-PAGE. Values of molecular mass markers are indicated on the right. kDa = kilodalton.

summarized in Table I. N-Acetylneuraminic acid was the most potent inhibitor among the monosaccharides tested, but other saccharides also had some inhibitory potency at a concentration of 100 m*M*. Heparin and calf fetuin were good inhibitors. As the same substances were inhibitory to the binding of both probes, it appeared that the proteins have only one carbohydrate-binding site specific for both ligands.

The competitive inhibition of charged polysaccharides on the binding of the lectins to HRP-heparin is summarized in Table II. At a concentration of 2 mg/ml, heparan sulphate, chondroitin sulphate A, chondroitin sulphate C, hyaluronic acid, and fucoidin completely inhibited the binding, whereas dermatan sulphate and keratan sulphate were less effective. Chitin sulphate and chondroitin showed little or no inhibitory activity. These results demonstrated that the sulphate content of the various polysaccharides tested is not correlated directly with inhibitory efficiency.

To confirm whether the purified proteins react with charged oligosaccharides attaching to lipids, the direct binding activity of the proteins with acidic glycolipids, sulphatide and gangliosides was examined. As shown in Fig. 5, the proteins were found to bind in a Ca^{2+}-dependent manner to gangliosides, but not to sulphatide [Gal(3-SO_4)β1–1Cer]. Mn^{2+} had a greater effect than Ca^{2+} on the binding to gangliosides (data not shown), whereas Mg^{2+} had no activation effect.

TABLE I

INHIBITION OF THE BINDING BETWEEN THE PROTEINS AND LABELLED FETUIN AND HEPARIN BY VARIOUS SACCHARIDES AND GLYCOPROTEINS

The values indicating inhibition activities of inhibitors are expressed as percentage inhibition, which was calculated by subtracting the percentage binding in the presence of inhibitor from 100%. Determinations were done in duplicate.

Inhibitor	Concentration (m*M*)	Inhibition (%)	
		Fetuin ligand	Heparin ligand
D-Galactose	100	0	30
D-Glucose	100	40	50
N-Acetyl-D-galactosamine	100	50	60
N-Acetyl-D-glucosamine	100	50	60
L-Fucose	100	50	60
D-Mannose	100	40	60
D-Glucuronic acid	100	50	50
N-Acetylneuraminic acid	100	80	80
	200	90	100
Lactose	100	30	50
Heparin	5 mg/ml	90	100
Calf fetuin	100	100	100
Calf asialofetuin	100	100	100

TABLE II

INHIBITORY ACTIVITIES OF CHARGED POLYSACCHARIDES

Each polysaccharide was used at a concentration of 2 mg/ml, and determinations were done in duplicate.

Polysaccharide	Sulphate content. (%)	Inhibition (%)
Heparin	30.3	100
Heparan sulphate	10.7	100
Hyarulonic acid	0	94.5
Chondroitin sulphate A	19.1	92.5
Chondroitin sulphate C	19.7	91.7
Dermatan sulphate	17.2	77.0
Keratan sulphate	14.7	69.4
Fucoidin	36.8	100
Chitin sulphate	26.5	7.4
Chondroitin	0	0

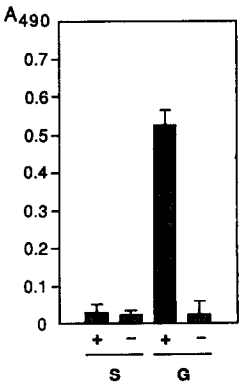

Fig. 5. Reactivities of bovine kidney proteins with acidic glycolipids. Acidic glycolipids were prepared from bovine kidney as described under Experimental (+) 5 m*M* $CaCl_2$; (−) 2 m*M* EDTA; (S) sulphatide [Gal(3-SO_4)β1–1Cer] (G) gangliosides.

Chemical compositions

Amino acid analyses were performed on the acid hydrolysates of protein bands electroblotted on to a PVDF membrane. As shown in Table III, p33 and p41 had similar amino acid compositions; Asx, Glx, Gly and Leu are the predominant amino acids. Neither p33 and p41 yielded PTH-amino acids during seven cycles of automated Edman degradation, indicating that the kidney proteins are N-terminally blocked. Therefore, their N-terminal amino acid sequences could not be elucidated.

TABLE III

AMINO ACID COMPOSITIONS

Amino acid	Concentration (mol%)	
	p33	p41
Asx	12.9	12.6
Ser	6.3	6.0
Thr	6.9	7.7
Glx	13.1	12.8
Pro	0.3	0.5
Gly	8.7	10.2
Ala	9.2	9.5
1/2Cys	N.D.[a]	N.D.
Val	4.8	6.7
Met	1.3	N.D.
Ile	5.9	5.3
Leu	11.7	9.8
Tyr	2.2	N.D.
Phe	3.5	4.0
Lys	5.9	7.2
His	0.5	0.9
Arg	6.9	6.7

[a] N.D., not detected.

The purified proteins, p33 and p41, electroblotted on to a PVDF membrane were allowed to react with HRP-labelled concanavalin A, peanut agglutinin, *Ricinus communis* agglutinin, *Lens culinaris* agglutinin, wheat germ agglutinin, *Ulex europeus* agglutinin-I, *Dolichos biflorus* agglutinin and *Phaseolus vulgaris* agglutinin. None of them stained the protein bands, suggesting that the proteins are not glycosylated.

DISCUSSION

To isolate Ca^{2+}-dependent carbohydrate-binding protein having affinity to charged (sialylated and sulphated) glycoconjugates, we selected fetuin and heparin as affinity ligands and examined Ca^{2+}-dependent affinity chromatography. Fetuin is a sialoglycoprotein having six carbohydrate chains, three O-linked and three N-linked to complex-type oligosaccharide chains [34], and heparin is a highly sulphated polysaccharide. When the bovine kidney extract supplemented with 5 m*M* $CaCl_2$ was applied directly to a column of fetuin-Sepharose gel, no protein could adsorb on the column. However, when the supplemented extract was incubated with fetuin-Sepharose gel for 18 h at 4°C, some proteins were adsorbed and eluted with 2 m*M* EDTA. The results suggest that batchwise and long-term incubations of the proteins with the affinity gel are essential for complete affinity adsorption in this instance. After another affinity chromatography on a heparin column and subsequent ion-exchange HPLC, we succeeded in purifying two kinds of carbohydrate-binding proteins. These proteins have molecular masses of 33 000 and 41 000 on SDS-PAGE and similar amino acid compositions. Only one band was detected in fraction A on native PAGE (data not shown), suggesting that p33 and p41 associate under native conditions.

Lectin staining of glycoproteins electroblotted on to a PVDF membrane is a sensitive, rapid and convenient method to detect carbohydrate modification. The results of the staining of each band by use of various labelled lectins of plant origin suggested that both proteins are not glycosylated. Conse-

quently, the difference in molecular mass between p33 and p41 seemed to be due to a difference in the length of polypeptide, not a difference in glycosylation.

The newly developed method for detecting and characterizing the carbohydrate-binding proteins with labelled glycoconjugates was useful for tracing the kidney proteins, as they had no haemagglutinating activity towards human and rabbit erythrocytes. It is well known that not all carbohydrate-binding proteins and lectins have haemagglutinating activities, even though they bind with oligosaccharide receptors on the cell surface. Sialic acid-specific lectins purified from the eggs of frogs, *Rana catesbeiana* and *Rana japonica*, strongly agglutinate certain transformed cells, but they cannot agglutinate human erythrocytes despite the high content of N-acetylneuraminyl residues on the cell surface [35]. The kidney proteins may have agglutinating activities towards cells other than human and rabbit erythrocytes, but these are yet to be confirmed.

While N-acetylneuraminic acid inhibited the binding of the proteins to labelled fetuin and heparin most strongly, other saccharides and asialoglycoprotein were also highly inhibitory. It is not possible to draw a definite conclusion concerning the ligand structure bound by the proteins. Such broad or multi-carbohydrate specificities have been also observed in the sialic acid-binding proteins from mammals [10], mannan-binding proteins from serum and liver [36,37] and the core protein of proteoglycans [38].

The purified proteins bound to gangliosides obtained from bovine kidney, but not to sulphatide [Gal(3-SO_4)β1–1Cer]. Ca^{2+} and Mn^{2+} enhanced the carbohydrate-binding activities of the proteins, but Mg^{2+} did not. These results indicate that the binding of the proteins to the ligands is due to a specific interaction between the proteins and carbohydrates moieties of the ligands, not to non-specific electrostatic interaction between positively charged groups on the proteins and negatively charged groups on the carbohydrates, or to metal ions cross-linking the negatively charged groups of the proteins and the carbohydrates.

The purified proteins are different from other sialic acid-binding proteins of mammalian origin in molecular mass and Ca^{2+} requirement for their activity. They also differ from cell adhesive proteins or heparin-binding proteins, such as selectin family proteins [13], fibronectin [39], laminin, thrombospondin [40] and vitronectin [41], and fibroblast growth factor [42] in molecular mass and in their affinity to sulphated glycoconjugates.

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