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A sialic acid-binding lectin from ovine placenta: purification, specificity and interaction with actin

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A sialic-acid-specific lectin from ovine placental cotyledons was purified by affinity chromatography on bovine submaxillary mucin-agarose followed by gel filtration, and it showed a molecular weight of 65 000 by sodium dodecylsulfate-polyacrylamide gel electrophoresis. This lectin has the capacity to interact with actin, since it binds to actin-F in a cosedimentation assay and it acts as a mediator in the binding of actin to the affinity column. The lectin agglutinated rabbit and rat erythrocytes, but not human A, B or O erythrocytes. Haemagglutination inhibition assays of different saccharides, glycoproteins and glycolipids indicate that this lectin has affinity for sialic acid, which is enhanced by its O-acetylation. The N-terminal sequence of the protein shows 92% identity with rabbit and porcine uterine calreticulin.

Keywords: lectin, actin, placental, ovine, actin-binding proteins, calreticulin

Introduction

Recognition of glycoconjugates is an important event in biological systems, and is frequently mediated by carbohydrate–protein interactions. The preferred location of sialic acids at terminal or side positions of the oligosaccharide chains renders them accessible to these interactions. Ahmed and Gabius [1] isolated a sialic acid-specific lectin from human placenta, with preferential affinity to O-acetyl sialic acids. Its molecular mass, determined by SDS-PAGE, was 53 kDa, and the hemagglutinating activity was Ca^{2+} independent.

Actin is present in all eukaryotic cells and in many cells it is the most abundant cytoplasmic protein. It consists of a single polypeptide chain of about 375 residues [2]. The cytoplasmic actins are present in all cells of lower eukaryotes, invertebrates, and in non-muscle cells of vertebrates, and the molecule was highly conserved throughout evolution [3–6].

Many classes of actin-binding proteins have been characterized and classified according to their *in vivo* and *in vitro* activities [7] and the number is still growing [8]. Recently, a soluble lectin that binds actin was isolated from human brain [9], and the authors suggested that this binding was mediated by non-carbohydrate interactions.

In this paper we describe the purification of a sialic acid-specific lectin from ovine placental cotyledons, its binding specificity and its capacity to bind actin. We also show that its amino-terminal sequence is virtually identical with that of calreticulin, an ubiquitous Ca^{2+}-binding protein [10].

Materials and methods

Bovine submaxillary gland mucin (BSM), asialomucin (from bovine submaxillary gland), rabbit muscle actin, Sepharose 4B, divinyl sulfone, phenylmethylsulfonyl fluoride (PMSF), aprotinin, bovine albumin, rabbit anti-actin affinity isolated antigen specific antibody, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, nitrocellulose membranes, dithiothreitol, β-mercaptoethanol, molecular weight markers, cyanogen bromide, D-lactose, D-glucose, D-fructose, D-galactose, maltose, melibiose, D-fucose, α-L-rhamnose, D-galactosamine,

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N-acetyl-D-galactosamine, N-acetylglucosamine-6-sulfate, D-trehalose, α-methyl-D-mannoside, sucrose, D-fructose-1,6-diphosphate, D-galactose-6-phosphate, D-mannose-6-phosphate, β-D-glycerophosphate, D-galacturonic acid, mannan, glycophorin (human, type MN), asialoglycophorin (human, type MN), thyroglobulin (bovine), heparin, fetuin, colominic acid, human orosomucoid, N-acetylneuraminic acid, N-glycolylneuraminic acid, thiodigalactoside, monosialogangliosides GM_1 and GM_3, disialogangliosides GD_3, GD_{1a} and GD_{1b}, trisialoganglioside GT_{1b}, and the cationic carbocyanine dye Stains-all (1-ethyl-2-[3-(1-ethylnaphto [1, 2d] thiazolin-2-ylidene)-2-methylpropenyl] naphto [1, 2d] thiazolium bromide) were purchased from Sigma Chemical Co., (St. Louis, MO, USA) and trifluoroacetic acid from Baker Chemical Co. (Phillipsburg, NJ, USA). Acetonitrile was HPLC grade and all other chemicals were AR grade.

Ovine placental tissue was surgically removed from ewes at a slaughterhouse, immediately frozen and stored at −70 °C prior to processing.

Rabbit muscle actin was dissolved in 10 mM Tris-HCl buffer, pH 8.5, 1 mM dithiothreitol, 0.2 mM $CaCl_2$, and ten-fold diluted with 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0 (Buffer A).

Base treatment of BSM was carried out as described by Ravindranath *et al.* [11]. A solution of 300 μl BSM (0.5 mg ml^{-1}) was added to 100 μl of 0.4 M NaOH, vortexed, incubated on ice for 45 min, and then neutralized with 400 μl 0.1 M HCl.

Ultracentrifugation was carried out at 80 000 × **g** for 3 h at 4 °C in a Beckman L8–55 Ultracentrifuge.

Haemagglutination and haemagglutination inhibition assays

Blood from various species was obtained by venipuncture. Erythrocytes were treated with trypsin and glutaraldehyde [12]. Serial dilutions (two-fold) in buffer A of the protein solution (25 μl) were mixed with equal volumes of 1% bovine albumin and of 2.5% suspension of treated erythrocytes in the same buffer. After incubation for 1 h at room temperature, the agglutination was recorded visually. The reciprocal of the highest dilution of protein solution showing visible agglutination was recorded as the titer. The specific activity of the lectin was defined as the titer mg^{-1} of protein ml^{-1}. Rabbit erythrocytes were used for activity determinations during the isolation procedure and for inhibition assays. Various potential inhibitors were dissolved in buffer A and, when necessary, the pH was readjusted to 7.0. Lectin solution (titer 2) was preincubated with serial two-fold dilutions of these inhibitors in buffer A for 1 h, and then mixed with 25 μl of 1% bovine albumin and equal volume of 2.5% suspension of treated erythrocytes. In some cases intermediate dilutions of inhibitors were added. The lowest dilution of inhibitor that suppressed hemagglutination was determined.

Preparation of the affinity resin

BSM-Sepharose 4B was prepared as described by Ahmed and Gabius [1], except that the residual reactive groups were blocked with glycine buffer instead of β-mercaptoethanol. The coupling efficiency was 60%.

Isolation of actin-lectin complex from ovine placental cotyledons

All steps were performed at 4 °C unless otherwise stated. Ten g of frozen ovine placental cotyledons were homogenized in 50 ml of 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0, (buffer A), containing 1 mM PMSF and aprotinin (0.02 mg ml^{-1}), with an Omnimixer during 1 min. Insoluble material was removed by centrifugation at 10 000 × **g** for 30 min and the supernatant was passed through a Sepharose 4B column (2.8 × 11 cm) equilibrated and eluted with buffer A, in order to eliminate material that could be adsorbed non-specifically to this matrix. The eluate was then applied slowly to the BSM-Sepharose 4B column (2.8 × 10 cm) previously equilibrated with buffer A. After having been thoroughly washed with the same buffer, the column was eluted with 10 mM Tris-HCl buffer, pH 8.5. The fractions were monitored for protein content at 280 nm and for hemagglutinating activity.

Purification of lectin from ovine placental cotyledons

Ten g of frozen ovine placental cotyledons were homogenized in 50 ml of 2 mM Tris-HCl buffer, pH 7.2, 1 mM dithiothreitol, 0.2 mM $CaCl_2$, 0.2 mM ATP (buffer G) [13], containing 1 mM PMSF and aprotinin (0.02 mg ml^{-1}), with an Omnimixer during 1 min. Insoluble material was removed by centrifugation at 10 000 × **g** for 30 min and the supernatant was passed through a Sepharose 4B column (2.8 × 11 cm) equilibrated and eluted with buffer G. The eluate was then applied slowly to the BSM-Sepharose 4B column (2.8 × 10 cm) previously equilibrated with buffer G. After having been thoroughly washed with the same buffer, the column was washed with buffer A in order to eliminate the proteins retained due to the low ionic strength of buffer G. The column was eluted with 10 mM Tris-HCl buffer, pH 8.5. The fractions were monitored for protein content at 280 nm and for haemagglutinating activity. The active fractions were pooled, and the lectin was further purified by FPLC on a Superose 12 HR 10/30 column (Pharmacia LKB) equilibrated and eluted with buffer A. The flow rate was 0.5 ml min^{-1}, the effluent was monitored for protein content at 280 nm and for lectin activity by the haemagglutination assay.

Protein determination

Protein content was determined by the method of Bradford [14].

Polyacrylamide gel electrophoresis (PAGE), Western blot and staining with Stains-all

Dialysed and freeze-dried samples were subjected to PAGE in the presence of sodium dodecyl sulfate (SDS) at room temperature in slab gels as described by Laemmli [15]. The gels were stained with Coomassie Brilliant Blue R-250.

Separated proteins were electroblotted onto a nitrocellulose membrane and the membrane was blocked with 3% milk in 50 mM Tris-HCl buffer, pH 7.6, 0.15 M NaCl. The membrane was probed with rabbit anti-actin antibody and the blot developed with alkaline phosphatase-conjugated anti-rabbit immunoglobulin using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrate.

Staining with the cationic carbocyanine dye Stains-all was carried out as described [16].

Gel filtration analysis

Samples were submitted to FPLC on a Superose 12 HR 10/30 column (Pharmacia LKB) calibrated with standard proteins. The column was eluted with buffer A at a flow rate of 0.5 ml min^{-1}.

Actin cosedimentation assay

It was carried out essentially as described by Ervasti and Campbell [17]. Rabbit muscle actin was dissolved in buffer G and added to lectin solution (final concentration of actin: 5.2 μM). Actin polymerization was initiated by the addition of NaCl and $MgCl_2$ to the final concentration of 100 mM and 3 mM respectively. After incubation at room temperature for 1 h, the samples were centrifuged at 80 000 × **g** for 3 h at 4 °C [18]. A sample containing only lectin was simultaneously processed as a control. The supernatants were tested for protein content at 280 nm and for haemagglutinating activity.

Cyanogen bromide cleavage

One hundred μg of protein were dialysed, freeze-dried, and dissolved in 200 μl of 70% formic acid. CNBr (0.3 mg) was added and the mixture was incubated at room temperature for 20 h. Then the sample was diluted with water and taken to dryness in a Speed Vac rotatory dessicator (Savant Instruments). The fragmented protein was reduced with 45 mM dithiothreitol in 8 M urea, 0.4 M ammonium bicarbonate at 50 °C for 15 min and carbamidomethylated with 100 mM iodoacetamide at room temperature for 15 min.

Purification of cyanogen bromide peptides

These fragments were separated by HPLC. A unit assembled with Laboratory Data Control components, a C4 Vydac column (0.46 × 25 cm) and a 100 μl fixed-volume Reodyne injector (COTATI, CA, USA) were used. The mobile phase consisted of 100 ml of a linear gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid, and the eluent was monitored at 220 nm.

Peptide sequencing

Selected CNBr peptides were applied to a polybrene-coated glass filter and sequenced in an Applied Biosystems Model 477 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA), run according to the manufacturers instructions.

Results

Isolation of an actin–lectin complex

An homogenate of ovine placental cotyledons, previously passed through a Sepharose 4B column, was applied to a BSM-Sepharose 4B column, taking advantage of the high sialic acid content of BSM. A single peak was obtained (fraction 1) when the affinity column was eluted with Tris-HCl buffer, pH 8.5, and the yield was approximately 1.5 mg of protein from 10 g of placental cotyledons.

The presence of a lectin in this fraction was detected by haemagglutination with rabbit erythrocytes, showing a specific activity of 180. It was not possible to calculate the recovery of haemagglutinating activity because the assay is not specific in the initial extract, where it detects other lectins as well.

Fraction 1 was examined by SDS-PAGE in the presence of β-mercaptoethanol (Fig. 1) showing a main band with an apparent molecular weight of 45 000 and other faint bands. This main band was not present in significant amounts in the flow-through from the affinity column (Fig. 1) and had the same migration position as muscle actin, which has a 42 000 molecular weight. In the absence of reducing agent the sample was unable to penetrate the gel, indicating a high molecular weight complex. Consistent with this result, when an attempt was made to estimate its molecular weight by gel filtration on a Superose 12 HR column, the total protein, together with the haemagglutinating activity, eluted in a peak corresponding to a molecular weight above 300 000.

The predominant protein was identified as actin in both reduced and non-reduced samples by Western blot, using anti-actin antibody (Fig. 2). A control of rabbit muscle actin was used.

When the main band from the SDS-PAGE was blotted onto a polyvinylidene difluoride membrane and applied to the automatic sequencer, no PTH-amino acid peaks were detected in significant amounts, thus indicating that the N-terminus was blocked.

In order to confirm the identification of the predominant protein, the fragments obtained by cyanogen bromide treatment were submitted to HPLC and the

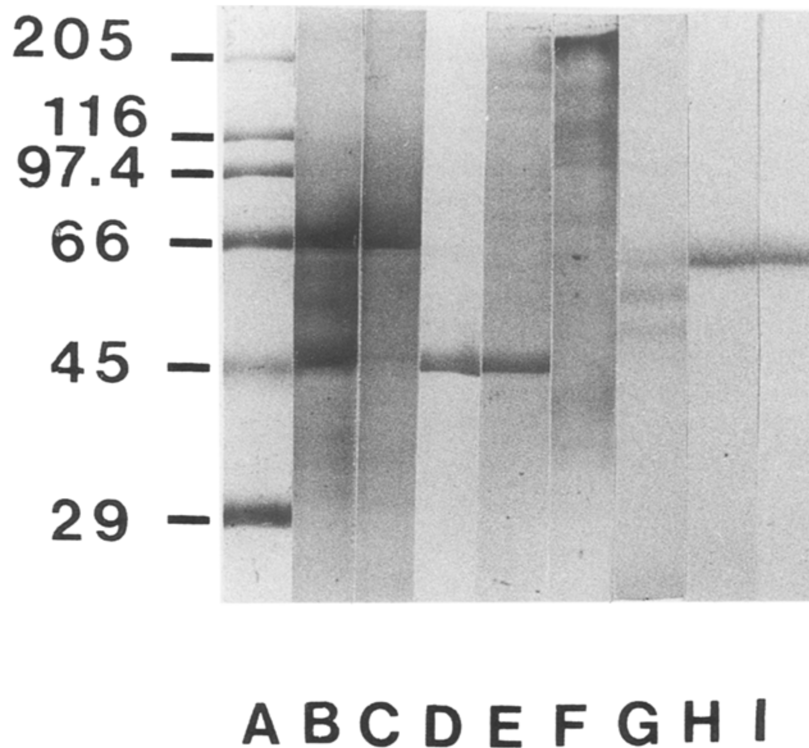

Figure 1. SDS-PAGE of fractions obtained by affinity chromatography on BSM-Sepharose. Electrophoresis was carried out on 10% acrylamide gels, as described in the Materials and methods section. Lane A contains 2 μg of each of the following molecular mass markers: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa); lane B, the sample applied to the affinity column (100 μg of total protein); lane C, the flow-through from the affinity column (50 μg of total protein); lane D, 3 μg rabbit muscle actin (42 kDa); lane E, 5 μg of reduced fraction 1 obtained by affinity chromatography; lane F, 5 μg of non-reduced fraction 1 obtained by affinity chromatography; lane G, 5 μg of reduced fraction 2 obtained by affinity chromomatography; lane H, 5 μg of reduced lectin purified by gel filtration; lane I, 5 μg of non-reduced lectin purified by gel filtration. The protein was visualized by Coomassie Blue staining. The numbers on the left indicate the position and mass of protein markers expressed in kDa.

results are shown on Fig. 3. Selected peptides were applied to the automatic sequencer, their sequences are shown on Fig. 4, and they are identical to those of β and γ mammalian cytoplasmic actins.

The possibility of simple coelution of actin and lectin was investigated by ultracentrifugation of fraction 1 at 80 000 × **g** for 3 h, conditions in which F-actin pellets [18]. The protein content of the supernatant was reduced to 41% of the initial content, closely paralleled by the decrease in haemagglutinating activity, thus showing binding of the lectin to F-actin.

To further examine the interaction of actin with the lectin present in ovine placental homogenate, we applied muscle actin and the homogenate to the BSM-column, at first separately, and then mixed. When muscle actin solution was passed through the affinity column it was not retained (Peak I, Fig. 5A). This solution was mixed with the homogenate obtained from 3 g of ovine placental cotyledons and passed once more through the affinity column (Fig. 5C). The absorbance of the material eluted with Tris-HCl buffer, pH 8.5, corresponded to the absorbance obtained when the same amount of homogenate was submitted to the identical procedure (Peak II, Fig. 5B), plus the absorbance of the actin solution (Peak I, Fig. 5A).

Purification of the lectin

When the initial homogenization was done in buffer G adjusted to pH 7.2, and the affinity column was washed with this buffer and buffer A, elution with Tris-HCl buffer, pH 8.5, yielded a protein peak (fraction 2) with haemagglutinating activity (Fig. 6). This fraction showed several bands by SDS-PAGE in the presence of β-mercaptoethanol (Fig. 1), and a specific activity of 320. The band of actin was absent under these conditions.

Further purification was achieved by gel filtration on a Superose 12 HR column (Fig. 7). The haemagglutinating activity eluted in a peak corresponding to a molecular weight above 300 000. The active fractions were pooled and analysed by SDS-PAGE, showing a single band

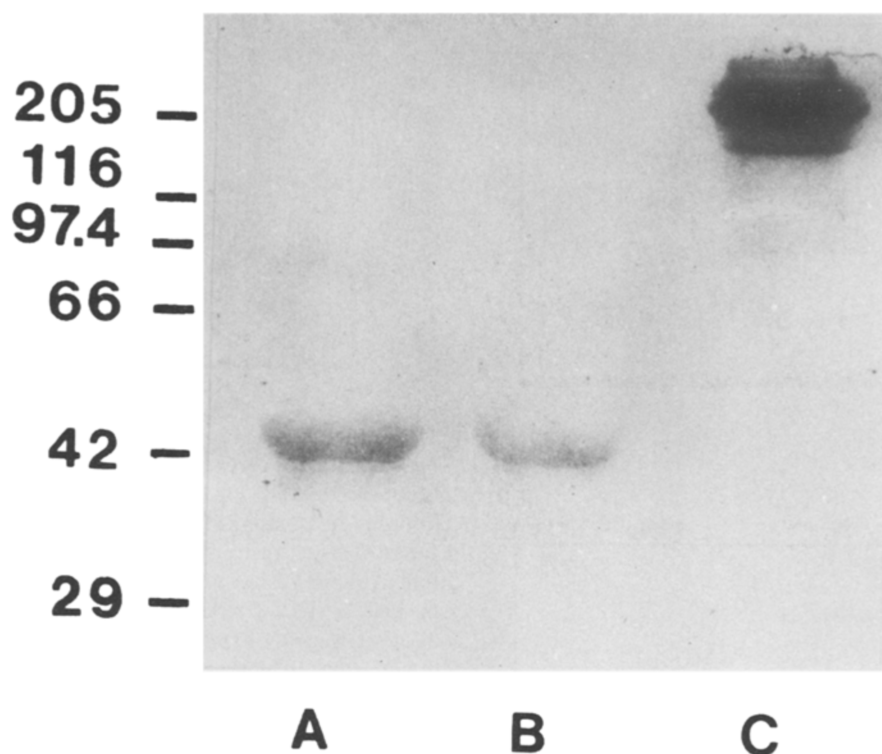

Figure 2. Western blot of the actin-lectin complex. Proteins were fractionated on SDS-polyacrylamide gel electrophoresis and submitted to Western blotting as described under Materials and methods. Lane A, reduced actin-lectin complex (fraction 1 from affinity chromatography); lane B, reduced rabbit muscle actin; lane C, non-reduced actin-lectin complex. The numbers on the left indicate the position and mass of protein markers expressed in kDa.

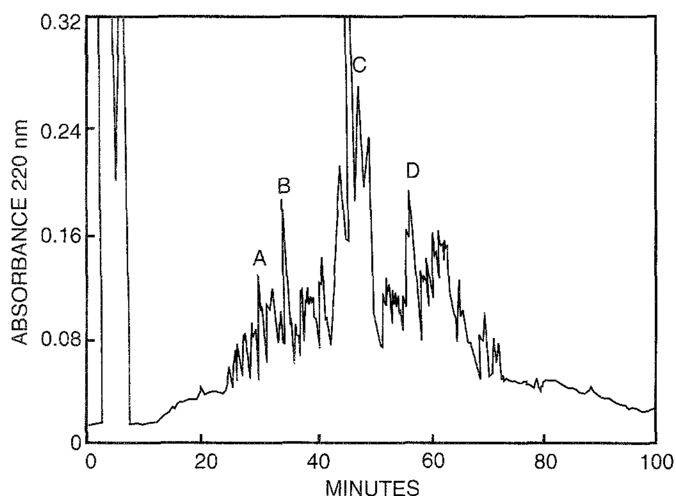

Figure 3. HPLC elution profile of cyanogen bromide fragments from fraction 1 (obtained by affinity chromatography). A linear gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid was used, the flow rate was 1 ml min^{-1}, and the eluent was monitored at 220 nm.

	306 312
Peptide A	Y P G I A D R
β-actin	Y P G I A D R
	124 131
Peptide B	F E T F N T P A
β-actin	F E T F N T P A
	191 200 209
Peptide C	K I L T E R G Y S F T T T A E X E I V . .
β-actin	K I L T E R G Y S F T T T A E R E I V . .
	154 160 168
Peptide D	D S G D G V T H T V X I Y E G . . .
β-actin	D S G D G V T H T V P I Y E G . . .

Figure 4. Sequence of the cyanogen bromide peptides from fraction 1. Peptides A, B, C and D were obtained by HPLC (Fig. 3). Peptides A and B were directly applied to the automatic sequencer, while peptides C and D were previously rechromatographed with a more shallow gradient. Their sequences are aligned with the sequence of human cytoplasmic β-actin [4] and the numbering corresponds to the latter, showing complete sequence identity. The X represent cycles in which there was ambiguity in clearly identifying the amino acid.

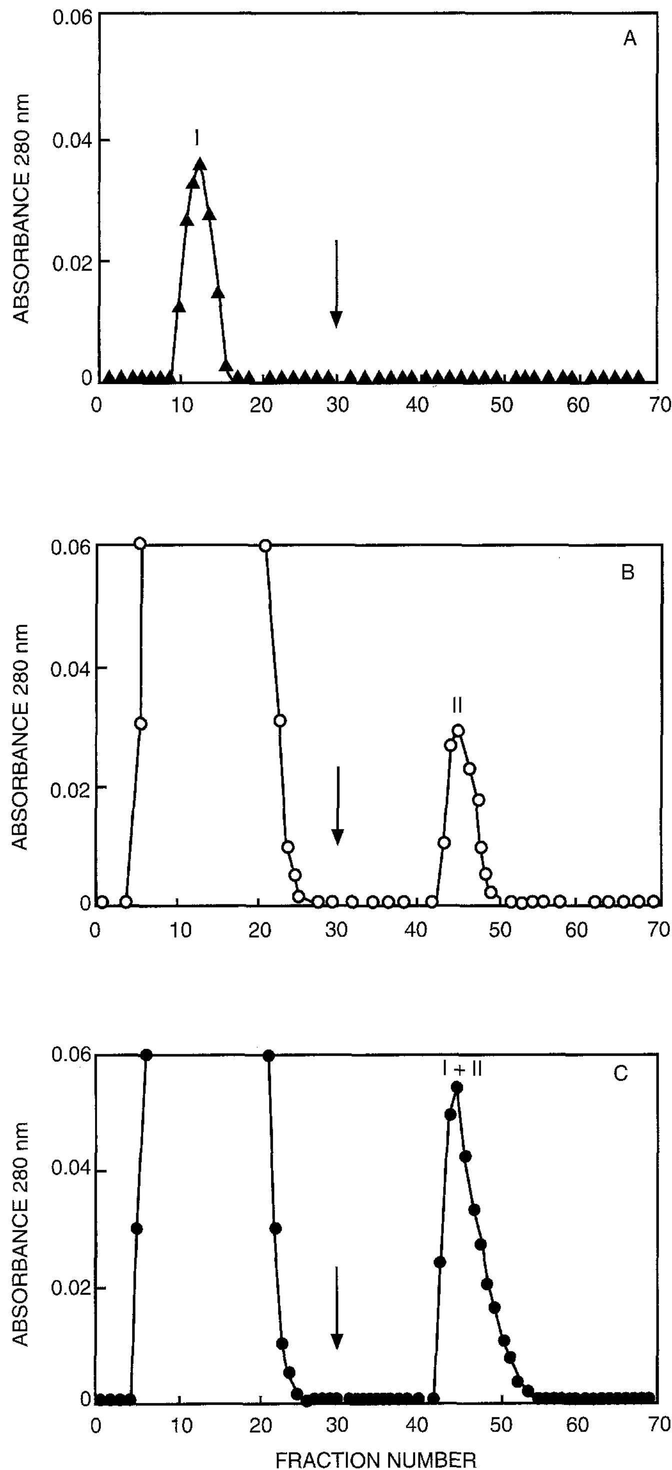

Figure 5. Affinity chromatography on a bovine submaxillary gland mucin-Sepharose 4B column. Samples in 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0 were first passed through a Sepharose 4B column. The affinity column (2.8 × 10 cm) was equilibrated and eluted with the same buffer and fractions of 3 ml were collected. The arrow indicates change to 10 mM Tris-HCl buffer, pH 8.5. (A) Rabbit muscle actin (50 μg/ml); (B) homogenate supernatant obtained from 3 g of ovine placental cotyledons; (C) homogenate supernatant obtained from 3 g of ovine placental cotyledons mixed with eluate from rabbit muscle actin.

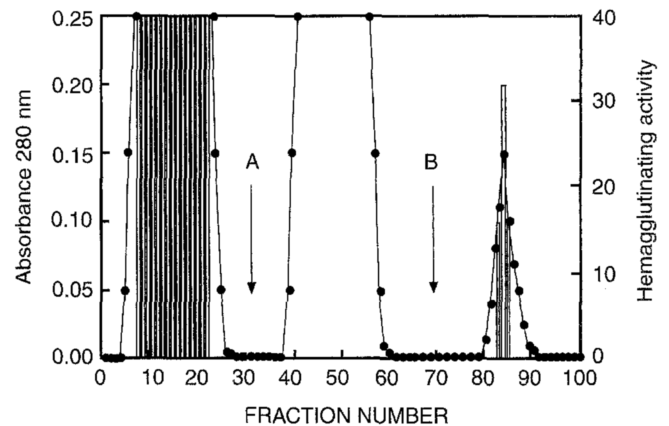

Figure 6. Affinity chromatography on a bovine submaxillary gland mucin-Sepharose 4B column. Homogenate supernatant obtained in 2 mM Tris-HCl buffer, pH 7.2, 1 mM dithiothreitol, 0.2 mM $CaCl_2$, 0.2 mM ATP was first passed through a Sepharose 4B column. The affinity column (2.8 × 10 cm) was equilibrated and eluted with the same buffer and fractions of 3 ml were collected. Arrow A indicates change to 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0 and arrow B indicates change to 10 mM Tris-HCl buffer, pH 8.5. Haemagglutinating activity is indicated by bars (fraction 2).

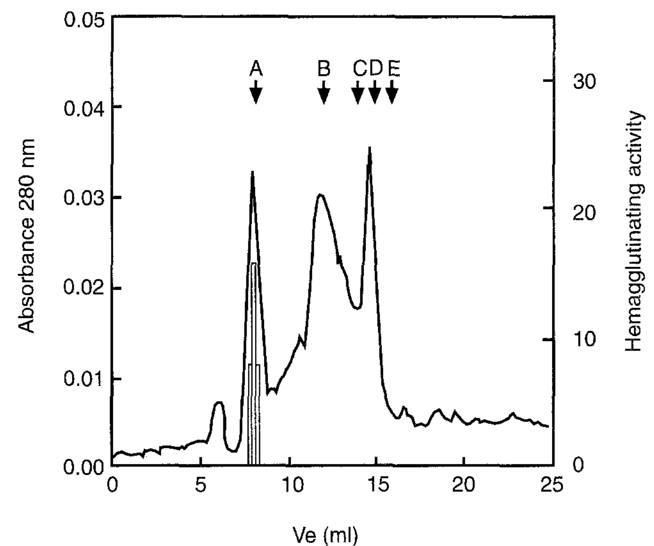

Figure 7. Gel filtration of fraction 2 (Fig. 6) on a Superose 12 HR 10/30 column equilibrated and eluted with 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0. The flow rate was 0.5 ml min^{-1}, and the effluent was monitored for protein content at 280 nm. Haemagglutinating activity is indicated by bars. The column was calibrated with the following markers, shown by arrows: (A) Blue Dextran (2000 kDa); (B) β-amylase (200 kDa); (C) ovalbumin (45 kDa); (D) carbonic anhydrase (29 kDa); (E) cytochrome c (12.4 kDa).

corresponding to an apparent molecular weight of 65 000 (Fig. 1) both in the presence or in the absence of the reducing agent. The yield was approximately 30 μg of protein obtained from 10 g of placental cotyledons, with a specific activity of 4000.

	1												13
(a)	E	P	T	V	Y	F	K	E	Q	F	L	D	G...
(b)	E	P	V	V	Y	F	K	E	Q	F	L	D	G...
(c)	E	P	T	I	Y	F	K	E	Q	F	L	D	G...

Figure 8. Amino-terminal sequence of sialic acid-binding lectin from ovine placenta and of rabbit and porcine uterine calreticulin. The purified protein (a) was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane and applied to the automatic sequencer. The sequence is aligned with those from (b) rabbit uterine calreticulin and (c) porcine uterine calreticulin (22).

When the band from the SDS-PAGE was blotted onto a polyvinylidene difluoride membrane and applied to the automatic sequencer, the amino-terminal sequence obtained showed a high degree of similarity to that of calreticulin (Fig. 8).

Besides, this band from SDS-PAGE stained blue with Stains-all (data not shown), which is characteristic of Ca^{2+}-binding proteins such as calreticulin [16].

Actin cosedimentation assay

To test whether the isolated lectin is capable of interacting with actin, a cosedimentation assay was performed, which resulted in the loss of 50% of the haemagglutinating activity in the actin pellet. When only lectin was subjected to the same procedure, there was no activity loss. These results confirm that the lectin is capable of binding F-actin.

Binding specificity of the lectin

To characterize the binding specificity of the lectin, several mammalian erythrocytes were used, which differ in their O-acetyl sialic acid content (Table 1). Those erythrocytes with no acetylation of sialic acid (Human A, B, and O groups) were not reactive with the lectin, whereas rabbit and rat erythrocytes, which contain O-acetyl sialic acid, were agglutinated. These observations suggested that the specificity of the lectin for sialic acid is greatly enhanced by the presence of O-acetyl groups. Both the actin-lectin complex and the purified lectin showed the same specificity.

Table 1. Haemagglutination activity of ovine placental lectin on mammalian erythrocytes.

	O-acetyl sialic acid content[b]	Haemagglutination titer	
Erythrocytes[a]	Total (%)	Actin–lectin complex	Lectin
Human A	0	0	0
Huamn B	0	0	0
Human O	0	0	0
Rabbit	20	32	32
Rat	25	16	16

[a]Trypsin and glutaraldehyde treated.
[b]Taken from Ahmed and Gabius [1].

The minimum concentration for complete inhibition at two haemagglutinating doses of lectin was determined for various substances (Table 2). Neither uncharged sugars (lactose, glucose, fructose, galactose, maltose, melibiose, fucose, rhamnose, galactosamine, trehalose, α-methyl-mannoside, sucrose, N-acetyl-D-galactosamine) nor charged substances (fructose-1,6-diphosphate, galactose-6-phosphate, mannose-6-phosphate, galacturonic acid, β-D-glycerophosphate) were inhibitory at 100 mM, neither was heparin (20 mg ml^{-1}). The lack of inhibition by charged substances excludes the possibility that the affinity is due only to ionic interactions. Besides, mannan (10 mg ml^{-1}) was not inhibitory, even in the presence of 10 mM Ca^{2+}.

All the substances which acted as inhibitors (N-acetylneuraminic acid, N-glycolylneuraminic acid, gangliosides GM_1, GM_3, GD_3, GD_{1a}, GD_{1b}, GT_{1b}, BSM, glycophorin, thyroglobulin and fetuin) (Tables 2 and 3) contained sialic acid. The fact that glycoproteins were stronger inhibitors than free sialic acid indicated enhancement of affinity by α-linked extension beyond the sialic acid, as was shown for human placental lectin [1]. Also N-glycolylneuraminic acid was a more potent inhibitor than N-acetylneuraminic acid, showing that the N-glycolyl group increases the binding to the lectin. Colominic acid, which is a homopolymer (Neu5Acα-(2–8) Neu5Ac), had no inhibitory effect at the concentrations used. This indicated that subterminal sialic acids have less affinity towards the lectin than terminal residues. The different inhibitory potency of gangliosides can be attributed to the number of terminal sialic acids, while the nature of the linkage appears not to cause any significant difference. Human orosomucoid, which has only N-glycosidically linked carbohydrate chains, did not inhibit, while thyroglobulin, glycophorin and fetuin did, suggesting a preference of the lectin for O-linked carbohydrate chains. The strongest inhibitor was BSM, which contains 9-O-acetyl groups [19]. Base treatment, specific for hydrolysis of the O-acetyl groups of sialic acids without cleavage of peptide bonds [19], diminished but did not abolish the inhibitory potency of BSM, while asialo BSM was not an inhibitor.

The specificity of the inhibition of haemagglutination was the same for the isolated lectin as for the actin–lectin complex, although the lectin was sensitive to lower concentrations of the inhibitors. Haemagglutination was not affected by the presence of 100 mM EDTA, showing that Ca^{2+} has no effect on this activity.

Table 2. Haemagglutination inhibition assay of ovine placental lectin and its actin–lectin complex by sialic acid derivatives and glycoproteins.

Inhibitor	Actin–lectin complex	Lectin
	Minimum inhibitory concentration[a]	
N-Acetylneuraminic acid	50 mM	1.2 mM
N-Glycolylneuraminic acid	6.25 mM	0.08 mM
Ganglioside GM_1	0.05 mM	0.003 mM
Bovine submaxillary gland mucin	$15.6\ \mu g\ ml^{-1}$ ($6\ \mu M$)[b]	$0.18\ \mu g\ ml^{-1}$ ($0.07\ \mu M$)[b]
Base-treated mucin	$95\ \mu g\ ml^{-1}$ ($38\ \mu M$)[b]	$1\ \mu g\ ml^{-1}$ ($0.4\ \mu M$)[b]
Asialomucin ($10\ mg\ ml^{-1}$)	n.i.[c]	n.i.
Human glycophorin (type MN)	$230\ \mu g\ ml^{-1}$ (0.24 mM)[b]	$3\ \mu g\ ml^{-1}$ ($3.2\ \mu M$[b]
Asialoglycophorin ($10\ mg\ ml^{-1}$)	n.i.	n.i.
Thyroglobulin	$5\ mg\ ml^{-1}$ (0.23 mM)[b]	$125\ \mu g\ ml^{-1}$ ($5.6\ \mu M$)[b]
Fetuin	$>10\ mg\ ml^{-1}$ (>1.3 mM)[b]	$1.25\ mg\ ml^{-1}$ (0.16 mM)[b]
Colominic acid ($10\ mg\ ml^{-1}$, 8.4 mM[b])	n.i.	n.i.
Human orosomucoid ($10\ mg\ ml^{-1}$, 8.4 mM[b])	n.i.	n.i.

[a]Concentration for complete inhibition of two haemagglutinating doses of lectin.
[b]Denotes concentration in terms of sialic acid, taken from literature (1, 28, 29).
[c]n.i., no inhibition.

Table 3. Haemagglutination inhibition assay of ovine placental lectin by sialic acid derivates.

Inhibitor	Minimum inhibitory concentration[a]
Ganglioside GM_1	$3.0\ \mu M$ ($3\ \mu M$[b])
Ganglioside GM_3	$2.3\ \mu M$ ($2.3\ \mu M$[b])
Diganglioside GD_3	$1.5\ \mu M$ ($3\ \mu M$[b])
Diganglioside GD_{1a}	$1.5\ \mu M$ ($3\ \mu M$[b])
Diganglioside GD_{1b}	$1.2\ \mu M$ ($2.4\ \mu M$[b])
Triganglioside GT_{1b}	$0.8\ \mu M$ ($2.4\ \mu M$[b])

[a]Concentration for complete inhibition of two haemagglutinating doses of lectin.
[b]Denotes concentration in terms of sialic acid.

Discussion

The main protein present in the eluted fraction from affinity chromatography on BSM-Sepharose was characterized as actin by its molecular weight in SDS-PAGE (Fig. 1, lane E), by Western blot analysis (Fig. 2) and by sequencing of cyanogen bromide derived peptides (Fig. 4). On the other hand, the haemagglutinating activity, as well as the binding to the affinity column, indicate the presence of a lectin in this fraction, although there is not sufficient quantity to show more than a faint band in SDS-PAGE when 5 μg of total protein are applied (Fig. 1, lane E).

The actin loaded on the affinity column does not bind non-specifically since it is not retained by the first Sepharose column and rabbit muscle actin alone is not bound to the BSM-Sepharose column when applied under the same conditions.

The lectin appears to behave as an actin-binding protein. This hypothesis is supported by the fact that muscle actin was not retained by the affinity column when it was applied in buffer A (Fig. 5A), while it was bound to the column when mixed with the homogenate (Fig. 5C), suggesting that the lectin contained in the placental homogenate is capable of mediating the binding of muscle actin to the affinity column. Another evidence in favour of this hypothesis is the cosedimentation of the lectin with F-actin. It is possible that the binding of the lectin stabilizes the actin F form.

Even though actin has a possible site of glycosylation, it has never been reported to be glycosylated [20], but lectins may have carbohydrate-independent binding sites in addition to those that bind carbohydrates [21]. In fact, a human brain soluble lectin that binds actin has been

described [9], and it appears to bind actin at a non-carbohydrate-binding site.

Actin does not polymerize in buffer G, that is, it remains as actin G [13]. Apparently the lectin does not bind to actin G since, when buffer G was used to prepare the homogenate and load the affinity column, no actin was bound, thus allowing the purification of the lectin. The lectin obtained under these conditions (fraction 2) was purified by gel filtration, showing a single band with an apparent molecular weight of 65 000 on SDS-PAGE. This band is not seen in significant amounts on SDS-PAGE of the actin-lectin complex, thus excluding the possibility of a 1:1 complex. The recovery of 1.5 mg of actin-lectin complex and 30 μg of lectin from 10 g of placental cotyledons does not allow an accurate estimate of the stoichiometry of the complex, since their purification procedures are different and their recoveries may not be the same. Although it would be possible to calculate this stoichiometry on the basis of the specific activity, probably the binding of actin alters the haemagglutinating activity of the lectin, as shown by the different sensitivity to inhibitors of the actin-lectin complex with respect to lectin alone. This change could be due to a stabilization effect caused by the increase in molecular weight.

More detailed information on the structural basis of the actin-lectin interaction should help to elucidate the possible role of this lectin in the dynamic regulation of actin organization in placenta.

The species-specific agglutination of erythrocytes by the lectin is similar to human placental lectin [1]. Its lack of requirement for Ca^{2+} suggests that the carbohydrate recognition domain is not affected by the putative Ca^{2+} binding to the protein. This lack of requirement for Ca^{2+} and its preference for O-acetylsialic acids also resemble the human placental lectin [1]. Its specificity for sialic acid is shown by the lack of inhibitory capacity of the asialomucin and the asialoglycophorin, as well as of all the saccharides tested. Its binding does not depend on the presence of the O-acetyl groups, as judged by the inhibitory capacity of N-acetylneuraminic acid, N-glycolylneuraminic acid and gangliosides. However, its affinity for the ligand is apparently enhanced by its O-acetylation, since BSM, which is rich in such groups, shows a greater inhibitory capacity on the basis of sialic acid concentration than other sialoproteins, and the removal of O-acetyl groups by base treatment decreases this inhibitory capacity.

The amino-terminal sequence of the lectin proved to be very similar to that of calreticulin (Fig. 8) [22]. Thus, from 13 amino acids identified, 12 were identical to those of rabbit uterine calreticulin, with only one substitution in position 3, while also 12 were identical with those of porcine uterine calreticulin but the substitution was in position 4. Its molecular weight is also in the same range and, as for calreticulin [23], it stains blue with the cationic carbocyanine dye Stains-all. Calreticulin is a highly conserved, ubiquitously expressed, intracellular Ca^{2+}-binding protein [10]. It has been recently identified as a putative mannoside lectin [24], while the haemagglutinating activity observed for the placental lectin was not inhibited by mannan, even in the presence of Ca^{2+}. Besides, it has been suggested that calreticulin may be involved in protein-protein interactions [23]. Other animal sialic acid-binding proteins have been shown to interact with peptides, e.g. selectins [25], sarcolectin [26] and calcyclin [27].

Further structural studies are needed to consider the possibility that this placental lectin is a protein related to calreticulin, sharing an identical amino-terminus, or that it is the same protein.

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