Separation of bovine heart galactose lectin from endogenous glycoproteins co-purified with the lectin during affinity chromatography

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During affinity chromatographic purification of bovine heart 14 kDa galactose-binding lectin (galectin 1) on lactose-Sepharose, several high molecular weight non-lectin glycoproteins were co-purified with the lectin. Glycoprotein binding to the affinity matrix was neither hydrophobic nor ionic, but galactose-dependent since lactose abolished binding. Purification of galectin from the co-purified glycoproteins by affinity electrophoresis in presence of the specific sugar lactose increased agglutination activity about 65-fold, indicating that a complex containing galectin molecules bound sugar specifically to endogenous glycoproteins with sugar binding sites still available had been retained on lactose-Sepharose.

1. Introduction

Structure-specific recognition between cognate biomolecules is being increasingly proved to be the initiator of events that mark major biological processes, be they normal such as fertilization, growth and differentiation or pathological such as infection or cancer. Lectins which are proteins of non-immune origin that recognize and bind to corresponding sugar residues without altering the structure of the latter have been detected in most plants and animals and are believed to be major participants in biological recognition (Drickamer 1988; Sharon and Lis 1989; Jessell *et al* 1990; Barondes *et al* 1994). Lectins are particularly suited for this role as their cognate biomolecules, the glycoconjugates, are largely available on cell surfaces (Frazer and Glaser 1979; Paulson 1989).

Galectin-1, a galactose binding lectin with subunit molecular weight around 14 kDa is nearly ubiquitous in animal tissues (Barondes 1984; Barondes *et al* 1994). This fact, combined with its remarkable evolutionary conservation (human and rat brain proteins share 87% amino acid homology) suggests a major role for this lectin in the physiology of the mammalian body (Meromsky *et al* 1986). Moreover, a 14 kDa lectin has been shown to be increasingly synthesized in tissues in experimental tumours (Raz *et al* 1986). Elucidation of the properties of this lectin including its interaction with endogenous or foreign glycoconjugates required purification of the lectin. This paper describes glycoproteins co-purified with bovine heart galectin 1 during affinity chromatography on lactose-Sepharose as well as a procedure to remove them. Biochemical and clinical significance of co-purified glycoproteins are discussed.

2. Materials and methods

Horse radish peroxidase (HRP) type II, acrylamide, divinyl sulfone and fetuin were purchased from Sigma Chemical Co. (USA). Fetuin was desialylated by heating a solution in 0·1 N H_2SO_4 at 80°C for 1 h. Adult bovine heart muscle tissue obtained from the local slaughter house was kept frozen at – 20°C and thawed before use. Lactose-Sepharose was prepared by coupling lactose to Sepharose 4B using divinyl sulfone (Dean *et al* 1985) and asialofetuin-Sepharose by coupling asialofetuin to Sepharose 4B after cyanogen bromide activation of the gel (Lowe 1979).

Keywords. Galectin; endogenous glycoprotein; affinity chromatography; bovine heart

2.1 *Preparation of bovine heart lectin (BHL-T) by affinity chromatography*

All procedures were performed at 4°C. Thawed and minced bovine heart tissue (70 g) was homogenized in 250 ml 20 mM potassium phosphate buffer pH 7·4 containing 150 mM NaCl (PBS) containing 50 mM lactose, 0·2 mM phenyl methyl sulfonyl fluoride, 2 mM benzamidine hydrochloride, 2 mM EDTA and 5 mM 2 ME using a POLYTRON homogenizer and stirred for 1 h at 4°C. After centrifugation at 16,000 *g* for 20 min, the supernatant was subjected to 70% ammonium sulphate precipitation followed by stirring for 1 h. After a similar centrifugation, the precipitated proteins were redissolved in 30 ml PBS-2 ME and dialyzed against the same buffer to remove lactose. After another centrifugation as above, the supernatant was applied to lactose-Sepharose or asialofetuin-Sepharose (40 ml) equilibrated in PBS-2 ME. Unbound proteins were removed by thorough washing using PBS-2 ME containing 1 M NaCl and bound proteins eluted using a solution of 150 mM lactose in PBS-2 ME in fractions of 2 ml. Protein-rich fractions were pooled and concentrated by ultrafiltration (Amicon PMIO membrane) and dialysed against PBS-2 ME to remove lactose, if necessary.

To obtain BHL-T by Triton extraction, 1% Triton X-100 was incorporated in the extraction buffer after homogenization, and the supernatant was directly loaded to the affinity column without ammonium sulphate precipitation.

2.2 *Preparation of purified galectin (BHL-L) from BHL-T by affinity electrophoresis and electroelution*

Tube gels (2 ml gel in 9 cm × 0·5 cm tube) for electrophoresis at pH 8·2 according to Davis (1964) was prepared incorporating 100 mM lactose by dissolving appropriate amount of the sugar in the catalyst solution prior to mixing with acrylamide. After electrophoresis at 4°C, one tube was immediately fixed in 12·5% trichloroacetic acid and stained with Coomassie brilliant blue R250 for reference. The gel portions from all gels corresponding to each band in the reference gels were cut, pooled and crushed before electroelution at 4°C according to the method of Odgen and Adams (1987). Briefly, the crushed gel particles were kept in a dialysis bag in Tris-acetate buffer (5 mM Tris, 2·5 mM acetic acid, pH 8·0) and immersed in the same buffer in a horizontal electrophoresis chamber. A current of 100 V was applied across the bag for 3 h. At the end the current was reversed for 10 min to detach proteins adhering to the sides of the bag. After centrifuging the contents of the bag at 1000 *g*, the supernatant containing proteins was recovered.

2.3 *Agglutination assay in the presence of detergent*

Agglutination titres of protein samples were determined using polystyrene 96 well microtitre plates with U bottom wells (Laxbro). Wells were first blocked by 1 h contact with PBS containing 5 mM 2-mercaptoethanol (PBS-2 ME) containing 0·05% Tween 20 (PBS-T). Trypsinized rabbit erythrocytes as well as protein samples were brought to the same medium. In the drained wells serial 2-fold dilutions of protein samples were made in a volume of 100 μl. Erythrocytes (25 μl of 1% suspension) were then added to each well and mixed by tapping. The agglutination titre was scored after 2 h.

2.4 *Cross-over immunoelectrophoresis*

The procedure described by Jurd (1981) was adapted. Agarose gel (1%) in PBS containing 50 mM galactose was layered on slides. BHL-T or BHL-L (purified galectin) was loaded in a well on one side and antibody to bovine brain galectin in PBS in the well at the opposite end. Electrophoresis was conducted in a horizontal electrophoresis apparatus. Antibody to purified bovine heart galectin (BHL-L) was produced in rabbits and purified as described by Hudson and Hay (1976).

2.5 *Western blot*

SDS-PAGE in 7·5% or 10% gel was done as described by Laemmli (1970). Electrophoretic transfer of proteins thus separated into nitrocellulose paper (Immobilon NC, Millipore) was achieved essentially as described by Towbin *et al* (1979). To check proteins on nitrocellulose for concanavalin A-specific sugar groups on them, nitrocellulose strips containing protein bands were blocked by 2 h treatment in 3% BSA and then immersed for 1 h in 1 mg/ml solution of concanavalin A in washing buffer (20 mM Tris, 150 mM NaCl containing 1% BSA). After three washes in the washing buffer, the strip was dipped in 100 μg/ml solution of HRP in PBS for 1 h. Strips were again washed in PBS and treated with 4-chloronaphthol substrate for colour development (Mastroianni *et al* 1991).

2.6 *BHL recognition of co-purified glycoproteins on dot blots*

To prepare HRP conjugate of electroeluted BHL-L, 16 μl 1% glutaraldehyde was added to a mixture of 200 μg each of the two proteins in PBS containing 50 mM lactose. After 2 h incubation at 25°C, the solution was diluted to 1 ml with and dialysed against PBS. BHL-T solution (2 mg/ml) was inactivated by 1 min heating in a boiling water bath and 4 μg samples dotted on Millipore NC nitrocellulose membrane strips. After

2 h blocking in 3% BSA, the strips were incubated for 2 h in 5 times dilution of the above conjugate with or without 100 mM lactose in PBS containing 0·05% Tween-20. After washing out the unbound conjugate, strips were stained with 4-chloronaphthol as in § 2.5.

3. Results and discussion

Out of the 70% ammonium sulphate precipitate from bovine heart tissue, those proteins that were retained by the lactose-Sepharose affinity column in the presence of 1 M NaCl and sugar-specifically eluted contained several large subunit proteins in addition to the 14 kDa subunit galectin, as shown by SDS-PAGE (figure 1A). Resistance of binding of the non-14 kDa proteins to washing by 1 M NaCl ruled out ionic interactions as the reason for their binding to lactose-Sepharose. Contribution of any chemical group (produced during divinyl sulfone-mediated coupling of lactose to Sepharose) towards attachment of larger proteins to lactose-Sepharose was also ruled out since use of asialofetuin-Sepharose (in which both the affinity ligand and the mode of attachment are entirely different) as affinity matrix instead of lactose-Sepharose yielded identical protein bands (figure 1B). Further, hydrophobic attachment to affinity matrix also could not have caused binding of non-14 kDa proteins since affinity chromatography in the presence of 1% Triton X-100 did not alter the pattern of bound proteins (figure 1C).

On the other hand, when 50 mM lactose was added to the 70% ammonium sulphate proteins before affinity chromatography as above, no detectable amount of protein was bound to the column or eluted with lactose indicating that sugar-specific binding to lactose was responsible for the retention of non-14 kDa proteins to lactose-Sephrose.

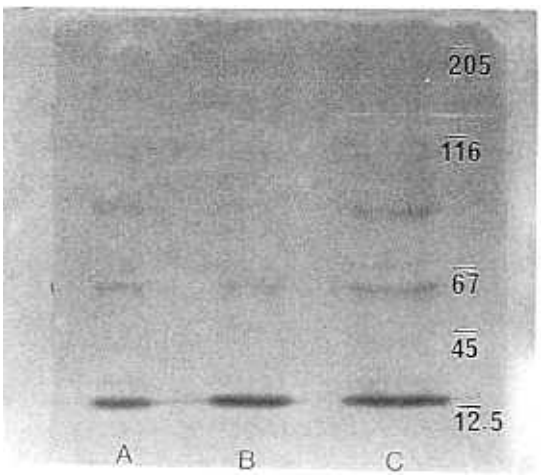

Figure 1. SDS-PAGE in 7·5% gel of BHL-T prepared by affinity chromatography on lactose-Sepharose (A), asialofetuin-Sepharose (B) and lactose Sepharose (C) in the presence of 1% Triton X-100. Bands were stained with Coomassie blue. Positions of molecular weight markers are indicated.

At the same time probing Western blot of BHL-T proteins after SDS-PAGE using rabbit antibody to purified 14 kDa galectin revealed that no other protein band was immunologically cross-reactive with the 14 kDa band (data not shown).

Further resolution of BHL-T by PAGE at pH 8·3 at 4°C yielded mainly two bands (figure 2). The upper (BHL-U) and lower (BHL-L) bands were separately electroeluted under non-denaturing conditions and on SDS-PAGE, and it was observed that BHL-L was considerably enriched in 14 kDa bands and that high molecular weight bands were nearly absent in this sample (figure 3). On the other hand BHL-U contained most of the high molecular weight proteins along with traces of the 14 kDa lectin. Interestingly agglutinating activity of BHL-L was nearly 65 times higher than that of original BHL-T, while BHL-U had little agglutinating activity (table 1).

When protein bands in Western blot of BHL-T were probed with concanavalin A/HRP almost all the bands other than 14 kDa were recognized by the lectin sugar-dependently (figure 4), indicating that non-14 kDa proteins were glycoproteins. That BHL-T samples contain glycoproteins having galactose moieties recognized by galectin is proved by the sugar-dependent binding of HRP conjugate of BHL-L to heat-inactivated BHL-T on dot blots

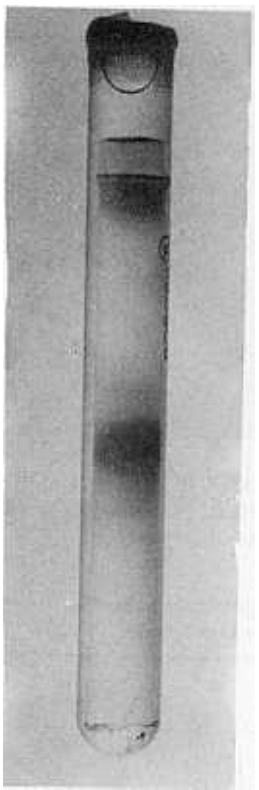

Figure 2. Alkaline (pH 8·2) electrophoresis in 7% gel of BHL-T stained with Coomassie blue.

(figure 5). In addition, the observed 65-fold increase in agglutinating activity from BHL-T to BHL-L could not be attributed solely to enrichment of the 14 kDa lectin content in BHL-L. Taken together, these observations suggest that during preparation of BHL-L, the galectin has been freed from a group of associated endogenous proteins which had actively inhibited its agglutinating activity. The glycoprotein nature of the non-galectin proteins in BHL-T confirmed using concanavalin A support this contention. It appears that the sugar-specific association of galectin with high molecular weight endogenous glycoproteins yielded a large complex which still retained enough sugar-binding sites to bind to lactose-Sepharose. The fact that galectin remaining with the slow moving BHL-U fraction in alkaline electrophoresis defying lactose mediated dissociation had negligible agglutinating activity supported this conclusion. The marked difference in mobility between BHL-T and BHL-L in cross-over immunoelectrophoresis (figure 5) also underline this possibility. Sugar-specific binding of galectin I to laminin has been shown *in vitro* (Zhou and Cummings 1990). However, since laminin requires 500 mM NaCl for extraction from tissue as against the 150 mM NaCl used here and has negligible electrophoretic mobility in non-reduced SDS-PAGE (Timpl *et al* 1982), the presence of this protein among the copurified glycoproteins is ruled out.

Cellular localization of co-purified glycoproteins *vis a vis* the lectin may reveal whether these glycoproteins remain associated with the lectin *in vivo* or only meet the lectin consequent to tissue homogenization. However

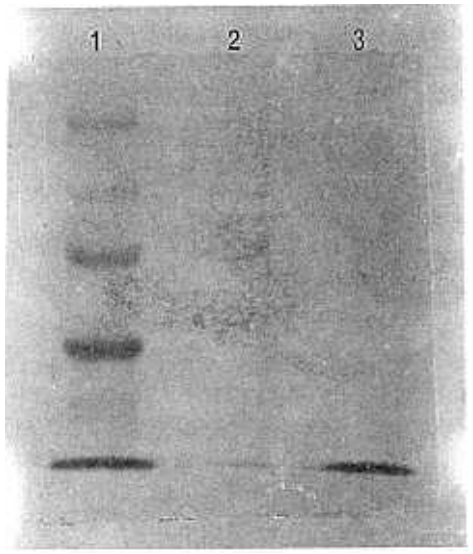

Figure 3. SDS-PAGE in 7·5% gel of BHL-T (1) along with BHL-U (2) and BHL-L (3) obtained by electroelution of bands from alkaline electrophoresis.

Table 1. Hemagglutinating activity of bovine heart galectin at various stages of purification.

Sample	Minimum hemagglutinating amount
BHL(T)	540 ng
BHL(L)	8 ng
BHL(U)	NA*

*No agglutinaton by maximum amount tested namely 3000 ng.

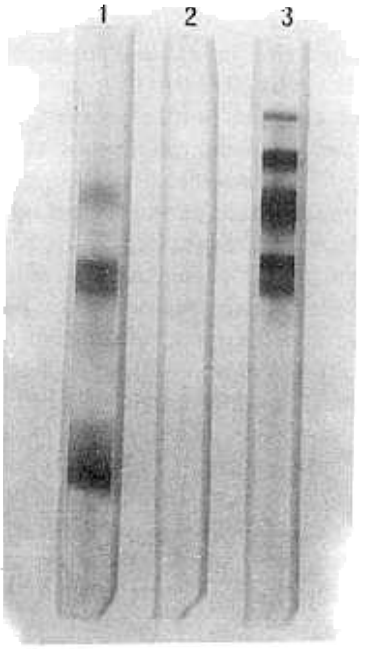

Figure 4. Western blot of BHL-T proteins on nitrocellulose. (1), Amidoblack staining for proteins. (2 and 3), Concanavalin A treatment followed by HRP in the presence (2) and absence (3) of inhibiting sugar (0·5 M 1-0-Methyl-α-D-mannoside).

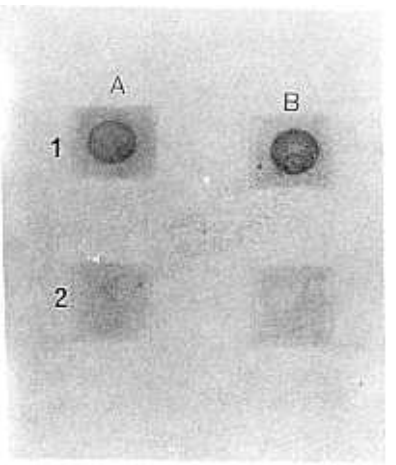

Figure 5. Binding of HRP conjugate of BHL-L to dot blotted heat-inactivated BHL-T samples (A and B). (1) In the absence and (2), in the presence of 100 mM lactose.

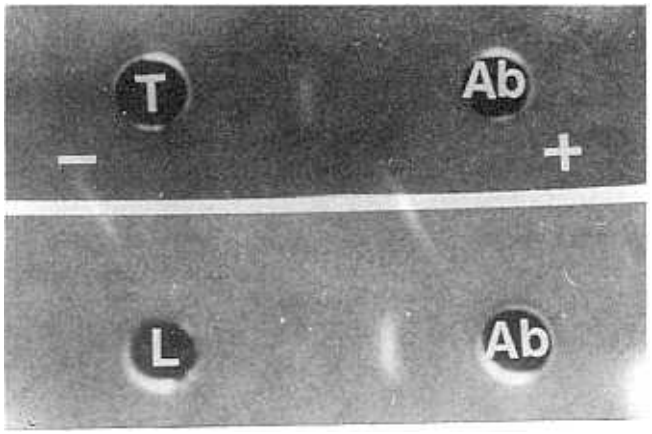

Figure 6. Cross-over immunoelectrophoresis in agarose gel of BHL-T (T) and BHL-L (L) against rabbit antibody prepared against bovine brain galectin.

their fast association with the lectin and the consequent considerable reduction of its agglutination activity observed here suggest that these glycoproteins are potential regulators of lectin activity. Tumour cell adhesion *in vitro* as well as cancer cell metastasis *in vivo* that are mediated by endogenous lectins have been successfully controlled using sugars and sugar analogues (Beuth *et al* 1995; Woynarowska *et al* 1994). Search for synthetic glycoconjugates capable of blocking pathological cell-cell recognition are reported (Paulson 1996). In so far as the co-purified glycoproteins offer high affinity ligands for the 14 kDa galectin, they are candidate therapeutic molecules for preventing tumour spread.

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