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Further studies on vertebrate S-type lectins: cross-reactivity between toad and human lectins

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Galectins (S-type or S-Lac lectins) are a well-defined family of β-galactoside animal lectins characterized by a high sequence homology in the carbohydrate-binding domain. We have previously purified and characterized the S-type lectin from the ovary of the toad ***Bufo arenarum****. In this study, we purified the S-type lectins from* ***Bufo arenarum*** *ovary and human spleen by an improved method which included ion exchange and affinity chromatography. Antibody cross-reactivities between both lectins and some other S-type lectins showed that they share epitopes. Glycosylation studies carried out with detection/ differentiation kits suggested that both lectins are not glycosylated.*

Key terms: *cross-reactivities, glycosylation, ovary, spleen, galectins.*

INTRODUCTION

Galectins, formerly referred to as S-type or S-Lac lectins, exhibit β-galactoside-binding activities in a Ca^{++}-independent fashion and share a consensus sequence in the carbohydrate recognition domain (CRD) (4). Eight mammalian members of this family have been sequenced and well-characterized in different species. Galectin-1 has a subunit molecular weight of 14.5 kDa and although it was supposed to be a homodimer (4), some evidence indicated the existence of a monomer-dimer equilibrium (6). It was previously sequenced from, for example, human placenta (8), lung (12), hepatoma (11), bovine heart and a fibroblast cell line (1), rat lung and uterus (7), porcine heart (17), murine 3T3 cells (22) and Chinese hamster ovary cells (6). A partial amino acid sequence from the human spleen lectin showed high homology to the human placenta galectin-1 (19), demonstrating that the former is a member of this subfamily of galectins. In non-mammalian vertebrates such as amphibians, two galectins have been sequenced: one from *Xenopus laevis* skin (16) and the other from *Rana catesbeiana* oocytes (18).

In the present study, we demonstrate antibody cross-reactivity between the S-type lectins from ovary and blastula embryos of *Bufo arenarum,* human spleen and brain. These two lectins (toad ovary and human spleen) were chosen for this work because we have acquired experience about them for several years. We also performed glycosylation studies which failed to find carbohydrate moieties in the lectins from *Bufo arenarum* ovary and from human spleen.

METHODS

Tissues.

Sexually mature *Bufo arenarum* specimens were collected in the neighborhood of La

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Plata, Argentina, and were kept in a moist chamber until used. Toad ovaries were obtained from pithed females without any kind of stimulation. Human spleens were obtained at splenectomy from healthy volunteer donors of organs for transplantation. Tissues were kept frozen at -20°C until used.

Lectin purification.

S-type lectins from *Bufo arenarum* ovary and human spleen were purified following a protocol slightly modified (18) from Ahmed *et al* (unpublished observations). Frozen tissues were cut into fragments and homogenized in 4 mM 2-mercaptoethanol (2-ME)/2 mM EDTA/PBS (MEPBS), containing 300 mM lactose and 25 mM phenylmethylsulfonylfluoride (PMSF). After centrifugation at 100,000 x g for 1 h, supernatants were incubated with DEAE-Sepharose pre-equilibrated in MEPBS diluted 1:10. The ion-exchange gel was washed with MEPBS 1:10 and the bound protein was eluted with MEPBS containing 500 mM NaCl.

Eluates were applied on columns containing 25 ml of lactose-agarose gel (Lactogel; E Y Lab, San Mateo, USA) which were previously equilibrated with MEPBS. The unbound materials were eliminated by washing with MEPBS containing 500 mM NaCl, followed by MEPBS until no protein was detectable in the effluent. The bound materials were eluted with 300 mM lactose in MEPBS. Each fraction (2 ml) was tested for hemagglutinating activity (10); the protein content was assayed by the method of Bradford, using bovine serum albumin as a standard. Purified lectins were dialyzed against distilled water-2-ME, lyophilized, and stored at -20°C.

Polyacrylamide gel electrophoresis (PAGE).

PAGE was performed in the presence of 0.1 % sodium dodecylsulphate (SDS) using 12% acrylamide minigels (0.75 mm thick), as we previously described (9).

Immunoblotting.

Antibodies against S-type lectins from toad ovary and human spleen were prepared in New Zealand White rabbits, as described elsewhere (9). After performing SDS-PAGE of the purified lectins, minigels were electroblotted onto nitrocellulose sheets at 0.4 A for 1 h in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. Membranes were blocked for 1 h with PBS-0.05% (v/v) Tween 20, 3% (w/v) gelatin and incubated with the anti-toad ovary lectin or the anti-human spleen lectin antisera (1:50-1:1,000) for 1 h. Antisera against other S-type lectins, such as those from human brain and from *Bufo arenarum* blastula embryos, were also tested at the same dilutions. After 3 washings, membranes were incubated with peroxidase-conjugated goat anti-rabbit antibody (Sigma) (1:500) for 1 h. Development was performed as described before (9). Reactivity scores were assigned according to Ahmed *et al* (2).

Reduction and alkylation.

Reduction and iodoacetamide alkylation of the purified galectins was carried out according to Whitney *et al* (21). Briefly, the lectins were denatured and reduced in 6 M guanidinium chloride/100 mM Tris-HCl buffer (pH 8.5)/5 mM dithiothreitol for 1 h, and carboxamidomethylated with 20 mM iodoacetamide in a nitrogen atmosphere at room temperature for 1 h. Finally, samples were dialyzed against distilled water/5 mM dithiothreitol and freeze-dried.

Digoxigenin (DIG) glycan detection/differentiation assay.

Both S-type lectins were run in 10% SDS-PAGE minigels and electrotransferred onto nitrocellulose. For the detection of carbohydrate moieties, immobilized proteins were analyzed using the DIG glycan detection method (Boehringer Mannheim), based on the introduction of the steroid hapten DIG into sugars. Samples bound to the membranes were oxidized by mild periodate treatment to convert sugar hydroxyl groups into aldehyde groups. Then, DIG was co-

valently attached to these aldehydes via hydrazide groups, and the filters were incubated with anti-DIG antibodies conjugated to alkaline phosphatase. Recombinant creatinase from *E. coli* was employed as a negative control protein, and transferrin as a control glycoprotein. To investigate sugar linkage, the DIG glycan differentiation method (Boehringer Mannheim) was used. Briefly, electrotransferred proteins were incubated with 5 plant lectins which selectively recognized specific terminal sugars and were labeled with DIG. Then, the filters were reacted with anti-DIG antibodies conjugated to alkaline phosphatase. Control glycoproteins (carboxypeptidase Y, transferrin, fetuin and asialofetuin) were tested in parallel to evaluate the specificity of the lectins and the function of the kit. The sugar specificities of each lectin provided with the kit were: Manα1-3 (or 1-6, or 1-2) Man for *Galanthus nivalis* (GNA); NeuNAcα2-6Gal for *Sambucus nigra* (SNA); NeuNAcα2-3Gal for *Maackia amurensis* (MAA); Galβ1-3GalNAc for *Arachis hypogaea* (PNA); Galβ1-4GlcNAc and GlcNAc for *Datura stramonium* (DSA).

RESULTS

Purification.

S-type lectins from toad ovary and human spleen were purified according to the same protocol. In these experiments, the yield was 10 and 1 μg/g of wet tissue for the toad ovary and the human spleen lectin, respectively. In SDS-PAGE under reducing conditions, the approximate subunit molecular weights obtained for both lectins were 14.5 kDa (Fig 1).

Immunological cross-reactivities.

Antibodies were developed against the S-type lectins from adult ovary of *Bufo arenarum* and from human spleen. Specificities of these antisera were tested in immunoblotting, using 1:50-1:1,000 dilutions. In cross-reactivity studies, antibodies against the toad ovary lectin, diluted 1:50, showed cross-reaction with the human spleen galectin by immunoblotting (Table I). Similarly, antibodies against the human spleen galectin, diluted 1:50, showed cross-reaction with the toad ovary lectin. Preimmune sera were tested in each experiment and did not show any reaction. Antibodies against the S-type lectin from human brain showed cross-reactivity with the toad ovary and the human splenic lectins (Table I).

Occasionally, when immunoblotting was performed with antibodies against the toad ovary lectin, the presence of a higher molecular weight band (~ 30 kDa) was detected. That band disappeared when the samples were reduced and carboxyamidomethylated

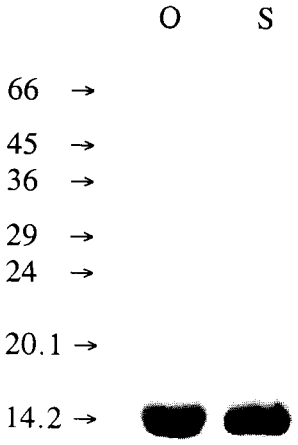

Fig 1. SDS-PAGE of S-type lectins from *Bufo arenarum* ovary (O) and human spleen (S), performed under reducing conditions, as described in Methods. Bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) were employed as molecular weight standards (arrows).

Table I

Antibody cross-reactivities between different S-type lectins by immunoblotting.

Antisera	Lectins from		
	Toad ovary	Humans spleen	Toad embryo (blastula)
Anti-toad ovary lectin	++	+	++
Anti-human spleen lectin	+	++	ND
Anti-human brain lectin	+	+	ND

All antisera used at dilution of 1:50.
Cross-reactivity: ++, strong; +, weak; ND, not determined.

prior to SDS-PAGE and immunoblotting, demonstrating to be the lectin dimer.

Glycosylation studies.

Carbohydrate chains were not found in the toad ovary and the human spleen S-type lectins employing the DIG glycan detection or differentiation methods (Fig 2). Therefore, under the resolution of the methods employed, these lectins do not seem to be glycosylated. In both protocols, positive and negative control proteins showed the expected results, according to their sugar contents. (Results with differentiation method not illustrated).

DISCUSSION

In the present paper, we found immunological cross-reactivity between the human splenic lectin and *Bufo arenarum* ovary lectin. We also detected partial cross-reactivities of these lectins with another S-type lectin (human brain lectin). Cross-reactions between S-type lectins were previously demonstrated, for example, in muscle tissues from pig, rabbit, chicken and rat and different bovine tissues (5) or between human placenta, chicken and mouse lectins (13).

The above reactivities are explained on the basis of the primary sequence homology between galectins. For example, *Xenopus laevis* galectin shows high sequence homology with human galectins-1 from lung, placenta and hepatoma (39% for the whole molecule), the chicken 14 kDa galectin (37%), the human galectin-2 (31%), the chicken 16 kDa galectin (31%), and the eel *Electrophorus electricus* galectin (28%) (16). The short peptide sequence available for *Rana catesbeiana* oocyte galectin (18) was 48% identical to the *Xenopus laevis* galectin (16). Sharma *et al* (19) partially determined the amino acid sequence of the human splenic galectin-1 identifying 94 of 134 residues; the sequences could be completely aligned with those corresponding to the human placenta galectin-1. Peptide sequencing of the galectin from *Bufo arenarum* ovary has recently been performed (3) showing higher

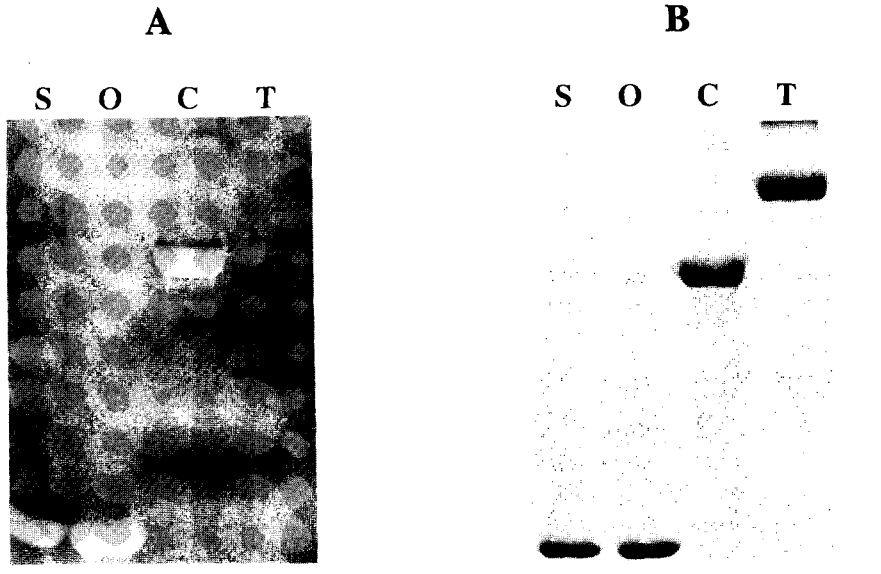

Fig 2. Glycosylation studies on S-type lectins from human spleen and *Bufo arenarum* ovary. Carbohydrate content of lectins from human spleen (S) and *Bufo arenarum* ovary (O) was analyzed. **A**. DIG glycan detection kit. Control proteins: creatinase (C), and transferrin (T). Toad ovary and human splenic lectins and creatinase are seen as white (negative) bands; transferrin is observed as a purple (positive, see arrow) band. **B**. SDS-PAGE minigel (10 %) stained with Coomassie brilliant blue.

identity with the bovine spleen (15) and heart (1) galectins-1 (48% for the whole molecule and 77% for the CRD) than with the human lung galectin-1 (14) [45% for the whole molecule and 70% for the CRD (3)]. Surprisingly, less identity was found between the galectins from *Bufo arenarum* ovary and from *Xenopus laevis* skin (38% for the whole molecule and 47% for the CRD) (3). An homology comparison between the amino acid sequences from the human splenic galectin-1 and *Bufo arenarum* galectin is not available, mainly because the complete sequence of this human galectin has not been yet determined. The amino acid sequence of the human placenta galectin-1 (8, 12) agrees completely with the sequence of the human lung galectin-1 (14), which was included for the comparison of identities with *Bufo arenarum* galectin by Ahmed *et al* (3), and with the predicted sequence of the human hepatoma galectin-1 (11).

Based on the above, we speculate that the amino acid sequences of the human galectins-1 from spleen and lung may probably be completely aligned. If so, the comparison between the sequences from *Bufo arenarum* galectin and human lung galectin-1 would also be extended to the comparison between *Bufo arenarum* galectin and human splenic galectin-1. Therefore, we presume that the antibody cross-reactivity between the galectins from *Bufo arenarum* ovary and human spleen that we found herein is a consequence of a probable high percentage of primary structure similarity.

The human spleen galectin has a consensus N-glycosylation site at the amino acid residues 95-97, but the presence of an N-glycoside at this site could be neither confirmed nor ruled out (19). Other studies determined that different galectins are not glycosylated. The presence of an N-linked glycosylation site was also reported for the human placenta galectin-1, but glycosylated forms were not observed (12). The galectin-1 purified and sequenced from human promyelocytic leukemia HL-60 cells was treated with N-glycanase (N-glycosidase F), an enzyme which cleaves N-asparagine-linked oligosaccharides, but the treatment did not show any effect on the electrophoretic mobility of the lectin (8). Another galectin-1 from normal murine embryo fibroblasts which has no N-glycosylation sites was also unaffected by tunicamycin and by N-deglycosylating enzymes. Nevertheless, a non-covalent linked complex glycan was shown to be associated to this lectin, using the same enzyme immunoassay method employed in this paper for the detection of carbohydrates (20). O-deglycosylating enzymes failed to alter the electrophoretic mobility of this galectin-1. In contrast, by removing sialic acid residues with neuraminidase and thus allowing access to a competing sugar, the glycosidic complex was dislodged, regardless of whether the O-glycanase enzyme was present or not (20). In the present study, we show that the galectins from *Bufo arenarum* ovary and human spleen are not glycosylated.

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