Secretion of a Cytoplasmic Lectin from *Xenopus Laevis* Skin


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**Abstract.** The skin of *Xenopus laevis* contains a soluble β-galactoside-binding lectin with a ~16,000-mol-wt subunit. It resembles similar lectins purified from a variety of tissues from other vertebrates, and differs from two other soluble *X. laevis* lectins from oocytes and serum that bind α-galactosides. The skin lectin is concentrated in the cytoplasm of granular gland and mucous gland cells, as demonstrated by immunohistochemistry with the electron microscope. Upon injection with epinephrine, there is massive secretion of the cytoplasmic lectin from the granular gland cells.

**Materials and Methods**

**Lectin Purification**

Skin was obtained from female *Xenopus laevis*, purchased from Xenopus 1 (Ann Arbor, MI). Animals were killed and 7-10 g of skin were obtained from the trunk of a single specimen. Dissections and subsequent procedures were done at 4°C.

Soluble lectins are widely distributed in vertebrates (1). Immunohistochemical studies of their distribution generally demonstrate both intracellular and extracellular lectin in a given tissue (1). These and other studies suggest that the intracellular lectin may function only upon secretion to an extracellular site, where it could associate with complementary glycoconjugates on the cell surface or in the extracellular matrix (1).

Secretion of soluble vertebrate lectins has been demonstrated directly only in the case of chicken lactose lectin II (3). It is localized along with mucin in the secretory vesicles of the goblet cells of the intestinal mucosa, and both are secreted upon cholinergergic stimulation (3). In contrast, other soluble vertebrate lectins are found in the cytoplasm, not in vesicles, making it hard to accept that they are truly secretory proteins. For example, the intracellular localization of a rat β-galactoside-binding lectin, which accumulates extracellularly in the elastic fibers of lung, is cytoplasmic, not vesicular (8). Yet it must be secreted, since it is ultimately concentrated in extracellular matrix.

In the course of studies of the distribution of α-galactoside-binding of soluble lectins in *Xenopus laevis* (24, 25) we found β-galactoside-binding lectin activity in skin extracts, previously detected by Nitta et al. (19). Since skin is a major secretory organ in *Xenopus* (14), this provided a potential opportunity to study lectin secretion. In the present report we describe purification and immunohistochemical localization of a β-galactoside-binding lectin from *Xenopus* skin. The lectin is highly concentrated in the cytoplasm of the granular gland, from which it is secreted upon systemic injection with epinephrine. The results provide a dramatic example of secretion of a cytoplasmic lectin, without packaging in vesicles.

**Electrophoresis and Amino Acid Analysis**

Lectin preparations were evaluated by polyacrylamide slab gel electrophoresis.
in SDS under reducing conditions as described previously (8) using a 15% sample gel. Protein bands were visualized with silver nitrate (18).

Amino acid analysis was performed at Scripps Clinic and Research Foundation, La Jolla, CA, on a Beckman 121 M amino acid analyzer following hydrolysis in 6 N HCl for 24 and 48 h at 100°C under reduced pressure. Methionine was estimated after 24 h. Cysteine was determined by performic acid oxidation of the sample prior to hydrolysis, and quantitation as cysteic acid.

**Gel Filtration Chromatography.** The native molecular weight of the purified lectin was determined by gel filtration chromatography in 10 mM Tris-HCl, 10 mM lactose, 100 mM NaCl, 4 mM β-mercaptoethanol, pH 6.8, using a Perkin-Eimer TSK-G-3000 column with a flow rate of 0.5 ml/min. Standards used were bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome C (12,400), and aprotinin (6,500).

**Isoelectric Focusing.** Polyacrylamide slab gel isoelectric focusing was performed on a LKB horizontal slab gel apparatus using pre-poured 3.5 to 9.5 gels (LK8 PAG plates, LKB Instruments, Inc., Rockville, MD). Gels were fixed with 10% aqueous trichloroacetic acid for 1 h, rinsed with distilled H2O, and stained overnight with Coomassie Brilliant Blue G-250 (Sigma Chemical Co., St. Louis, MO) and destained in 10% acetic acid.

**Antibody Preparation and Characterization**

**Antibody Production.** Lectin used for immunization was purified by affinity chromatography followed by ion exchange chromatography to assure freedom from trace contaminants. Initial immunization of a rabbit was carried out by subcutaneous injection at multiple sites of 200 μg of lectin in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The rabbit was boosted with 25 μg of lectin in incomplete Freund's adjuvant beginning at 4 wk after initial injection and continuing at 2-wk intervals. A total of six boosts were given and serum was collected at 5 and 7 d after the final injection.

**Affinity Purification of Antibody.** Purification of the antibody for use in immunohistochemistry was done by immunoaffinity chromatography on a column containing 200 μg X. laevis skin lectin that had been coupled to 2 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. 2 ml of rabbit antiserum was recirculated through the column which was then extensively washed with 75 mM NaCl, 75 mM Na2HPO4/KH2PO4, pH 7.2 (PBS), and eluted with 0.2 M HCl adjusted to pH 2.2 with 2 M glycine. Fractions of 0.6 ml were collected directly into tubes containing 0.15 ml 1 M K2HPO4 and dialyzed against PBS overnight at 4°C. Bovine serum albumin was added to a final concentration of 0.1 mg/ml and the affinity-purified antibody was stored at 4°C in 0.02% sodium azide.

**Immunoblotting.** Antigens that react with the antisera were identified by electrophoresis and immunoblotting procedures, using peroxidase-anti-peroxidase and 4-chloro-1-naphthol to detect bound primary antibody, all as previously described (8).

**Dot-Immunobinding Assay.** Quantitation of X. laevis lectin in skin washings was performed by directly blotting twofold dilutions onto nitrocellulose sheets (Bio-Rad Laboratories, Richmond, CA) and reaction with antisera, peroxidase-anti-peroxidase, and 4-chloro-1-naphthol, all as described for immunoblotting.

**Electron Microscope Immunohistochemistry**

**Tissue preparation.** 2-3-mm thick slices of skin were fixed for 1 h at room temperature in freshly prepared 3% paraformaldehyde and 0.5% glutaraldehyde. 1% OsO4 in 0.1 M sodium phosphate buffer, pH 7.4. Tissue was then rinsed in two changes of buffer for 10 min and then post fixed for 1 h at room temperature in 1% OsO4 in 0.1 M sodium phosphate buffer, pH 7.4. Samples were then dehydrated in ethanol and embedded in Epon 812 by standard procedures. Thin sections were cut and picked up on carbon-laden-coated nickel grids for immunostaining.

**Immunostaining.** Colloidal gold with a mean diameter of 9 nm was purchased from Janssen Life Sciences Products (Piscataway, NJ). Goat anti-rabbit IgG (heavy chain- and light chain-specific) was purchased from Coopers Biomedical Inc. (Malvern, PA). Colloidal gold–IgG complexes were prepared as described previously (8).

Grids containing thin sections of frog skin were immunostained as described previously (8). The grids were incubated with solutions containing 3 μg/ml affinity-purified antibody for 2 h at room temperature. Control grids were incubated with 10 μg/ml of preimmune IgG. After reaction with the colloidal gold goat anti-rabbit IgG complex, grids were stained with saturated aqueous uranyl acetate followed by lead citrate before examination with a Zeiss EM 10 electron microscope.

**Results**

**Lectin Purification and Properties**

Lectin was purified from extracts of *Xenopus laevis* skin by affinity chromatography on agarose gel beads conjugated with lactose. After extensive washing of the beads, pure lectin was eluted with lactose and migrated as a single band with an apparent molecular weight of ~16,000 upon SDS PAGE (Fig. 1). Upon gel filtration, the lectin migrated as a single peak with an apparent molecular weight of ~32,000. Like many related lectins (1) it is, therefore, a dimer. The skin of one adult female yielded from 1 to 1.5 mg of pure lectin.

In some preparations, no impurities were detectable even with a highly sensitive silver stain (Fig. 1). However, in several preparations, trace contaminants were detected, which could be removed by DEAE ion exchange chromatography. The purified lectin behaved as a single entity on ion exchange chromatography. Like soluble lactose-binding lectins from other vertebrates (7, 9, 17, 26), it is rich in aspartic and glutamic acid (Table 1). Upon isoelectric focusing in a slab gel, it can be resolved into three prominent bands with approximate pI 6.2, 6.4, and 6.5.

The effect of a series of concentrations of several sugars on binding of pure lectin to the affinity column was determined to evaluate the specificity of its carbohydrate-binding site. Binding could be completely inhibited with 15 mM lactose, 150 mM melibiose, and 300 mM galactose had no detectable inhibitory effect. Therefore this lectin is similar to many other vertebrate β-galactoside binding lectins (1), and differs markedly from lectins purified from *Xenopus* oocytes (24) or serum (25), both of which react well with α-galactosides such as melibiose. Removal of β-mercaptoethanol by dialysis of purified lectin against repeated

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**Figure 1.** PAGE of lectin purified from *Xenopus laevis* skin extracts by affinity chromatography on lactose-conjugated agarose. The pure lectin (A) was eluted with lactose and electrophoresed on a polyacrylamide slab gel, as described in Materials and Methods, along with the indicated standards (B). The gel was stained with silver nitrate. The standards used were: lysozyme (14,300); β-lactoglobulin (18,400); trypsinogen (24,000); carbonic anhydrase (29,000); pepsin (34,700); egg albumin (45,000); bovine albumin (66,000).
Table I. Amino Acid Composition (Residues/100 Residues) of X. laevis Skin Lectin with Mr ~ 16,000

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/100</th>
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<tbody>
<tr>
<td>Asp</td>
<td>14.5</td>
</tr>
<tr>
<td>Thr</td>
<td>5.3</td>
</tr>
<tr>
<td>Ser</td>
<td>6.5</td>
</tr>
<tr>
<td>Glu</td>
<td>10.0</td>
</tr>
<tr>
<td>Pro</td>
<td>5.0</td>
</tr>
<tr>
<td>Gly</td>
<td>7.0</td>
</tr>
<tr>
<td>Ala</td>
<td>4.2</td>
</tr>
<tr>
<td>Cys</td>
<td>0.9</td>
</tr>
<tr>
<td>Val</td>
<td>6.4</td>
</tr>
<tr>
<td>Met</td>
<td>1.8</td>
</tr>
<tr>
<td>Ileu</td>
<td>8.3</td>
</tr>
<tr>
<td>Leu</td>
<td>7.3</td>
</tr>
<tr>
<td>Tyr</td>
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</tr>
<tr>
<td>Phe</td>
<td>5.3</td>
</tr>
<tr>
<td>His</td>
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</tr>
<tr>
<td>Lys</td>
<td>6.6</td>
</tr>
<tr>
<td>Arg</td>
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changes of buffer without this reducing agent greatly inhibited the binding of the lectin to the affinity column.

Specificity of Antiserum

A rabbit antiserum was raised against lectin that had been purified by affinity chromatography followed by DEAE cellulose chromatography, to assure removal of all trace contaminants. Immunoblots of whole skin extracts that had been electrophoresed and transferred to nitrocellulose showed only a single band (Fig. 2 a) that migrated exactly with the purified lectin. Preimmune serum gave no reaction on immunoblots (Fig. 2 b).

Epinephrine-induced Lectin Secretion

It has been established that injection of epinephrine induces marked secretion of biologically active small peptides from X. laevis skin (14). Since we were interested in the possibility that skin lectin was also secreted, we collected cutaneous washings after injection either of epinephrine or of saline, as described in Materials and Methods. We estimated the amount of lectin in the washings by the dot-immunoblot procedure described in Materials and Methods, using serial twofold dilutions of the washings bound to nitrocellulose (Fig. 3). The washings from the epinephrine-injected animal gave a clear positive result out to dilutions of 1:128, whereas those from the saline-injected animal gave a faint positive only at 1:2 (Fig. 3). This indicates that there was massive secretion of lectin upon epinephrine injection.

We also analyzed the nature of the secreted material by staining nitrocellulose transfers prepared after PAGE. Amido black staining of the transfers showed several major bands, but the lectin was not one them (Fig. 4 a). Immunoblotting showed a major band with an apparent molecular weight of ~16,000 which migrated exactly with purified lectin (Fig. 4 b). We also detected a very faint antibody-binding band with an apparent molecular weight of ~32,000. It is possible that this is an undissociated dimeric form of the lectin. However, it is also possible that it represents yet another lectin which is immunologically cross-reactive with the ~16,000-mol-wt lectin, by analogy with recent findings in the rat (9). The faint 32,000-mol-wt band was never observed on immunoblots of whole skin, and would not be expected to make a major contribution to the total immunoreactive material observed in immunohistochemical studies.

In preliminary studies we found that cutaneous secretions collected without β-mercaptoethanol were much more viscous than those collected with this reducing agent. We wondered if the lectin was normally linked by disulfide bonds to other secreted materials. To test this we collected epinephrine-induced secretions without β-mercaptoethanol, and examined the released material by gel electrophoresis also done without mercaptoethanol. The lectin was located on immunoblots of a nitrocellulose transfer. All the lectin on these immunoblots had an apparent molecular weight of ~16,000 and, aside from faint staining of a 32-kD band, no other immunoreactive...
bands were found. Therefore, the lectin is not linked by disulfide bands to either large glycoconjugates in the secretions or to toxic peptides of a size large enough to produce a detectable shift in apparent molecular weight of the 16,000-mol-wt band.

Because secretion of lectin with $M_r \sim 16,000$ was so prominent after epinephrine injection, we used skin washings as the starting material for purification of several preparations of lectin. We obtained $\sim 2$ mg of pure lectin from the washings of a single epinephrine-injected animal, more than could be obtained from skin homogenates. This is, therefore, the procedure of choice for obtaining crude lectin for purification. The smaller yield obtained from skin homogenates probably reflects both inefficient dissection and failure to completely disrupt the skin even with vigorous homogenization.

Immunohistochemical Studies. Frog skin contains two prominent glandular structures, granular glands, and mucous glands (Fig. 5). Upon epinephrine injection, the morphology of the granular glands is changed radically, reflecting an outpouring of the contents of this gland, including intact peptide-containing granules, as described by Dockray and Hopkins (14). Since the granular glands are so affected by epinephrine injection, which also releases so much lectin, we expected that lectin would be concentrated in these structures. This was confirmed by immunohistochemistry with the electron microscope, which showed substantial binding of affinity-purified antibody in the cytoplasm of an unstimulated granular gland cell (Fig. 6a). Reaction with antibody raised against the lectin was specific, since exposure of parallel sections to preimmune immunoglobulin gave no significant staining (Fig. 6b).

Secretion of lectin from the granular glands was also demonstrated directly by immunohistochemistry. In an animal injected with epinephrine, there was substantial lectin in the secreted material within the duct of the granular gland (Fig.

![Figure 4. PAGE and immunoblotting of cutaneous washings from an epinephrine-injected X. laevis.](image)

![Figure 5. Glands in the skin of X. laevis.](image)
Figure 6. Immunohistochemical localization of lectin in the cytoplasm of a granular gland cell. Sections through a granular gland were reacted either with affinity-purified antibody raised against purified lectin (A) or with three times as much preimmune IgG (B). The sections were then stained with colloidal gold-conjugated goat anti-rabbit immunoglobulin. There is abundant lectin in the cytoplasm, but granules (gr) do not contain lectin. Bar, 0.1 μm.
Again, staining was specific, since reaction with preimmune immunoglobulin gave no significant staining (Fig. 7b). Lectin was also detected in the cytoplasm of mucous gland cells (Fig. 8). Globules of mucous in other regions of this cell contained no lectin (not shown). The morphology of the mucous gland showed no obvious change upon injection with epinephrine, so it is not known if cytoplasmic lectin is also secreted from mucous gland cells.

**Discussion**

The lectin that we purified from *Xenopus laevis* skin and its secretions closely resembles soluble $\beta$-galactoside-binding lec-
Figure 8. Immunohistochemical localization of lectin in the cytoplasm of a mucous gland cell. The section was reacted with affinity-purified anti-lectin followed by colloidal gold-labeled goat anti-rabbit IgG. Controls stained with preimmune serum were negative. Bar, 0.1 μm.

tins from other vertebrates, including eel (17), chicken (5, 13, 20), snake (16), and several mammals (7-10, 12, 23, 26). Like these, it does not require detergents for solubilization (which distinguishes it from lectins integrated in membranes [1]), has a subunit Mr in the range of 15,000, and binds lactose. It differs strikingly from two other soluble lectins already purified from *Xenopus laevis* (24, 25). The *Xenopus* lectin from oocytes (24) has two subunits with apparent molecular weights of ~43,000 and 45,000 and binds α-galactosides as well as β-galactosides, as does the lectin made in *Xenopus* liver and secreted into the serum (25), which has a subunit in the molecular weight range of ~69,000. Although the *Xenopus* skin lectin is probably closely related to a rat 14,500-mol-wt lectin, potent antisera raised against the rat lectin (9) did not cross-react with nitrocellulose transfers of the *Xenopus* skin lectin.

Previous studies have indicated that soluble β-galactoside-binding lectins from vertebrates may be secreted (1). As already noted, this was clearly shown in the case of chicken-lactose lectin II in chicken intestine (3). In this case, the lectin is packaged in secretory vesicles (3). However, evidence for secretion of other β-galactoside-binding soluble vertebrate lectins has been less direct. Chicken lactose lectin I is intracellular in immature muscle but is later concentrated extracellularly, suggesting secretion (2). Chicken lactose lectin I is also found extracellularly in pancreas (4). The ~14,500-mol-wt rat β-galactoside-binding lectin is found in the cytoplasm of several lung cell types, but its prominent extracellular location in elastic fibers (8) suggests that it is secreted there. In these instances secretion from the cytoplasm, by an unknown mechanism, has been inferred but not directly demonstrated. Given the general view that secretion proceeds by packaging in vesicles and not directly from the cytoplasm, it has been difficult to accept that cytoplasmic lectin is actually secreted.

The present experiments show secretion of a cytoplasmic (nonvesicular) β-galactoside-binding lectin from *Xenopus laevis* granular gland by both immunohistochemistry and by collection of copious lectin in the cutaneous washings of epinephrine-injected animals. This case is, of course, unusual because it involves massive secretion from granular gland cells by rupture of plasma membranes at the base of their ducts. This is induced by contraction of the surrounding myoepithelial cells which squeeze out cytoplasm and granules (14). The membranes then reseal so the cells can resynthesize these contents (14). Although such a specialized and dramatic mechanism would not appear to be responsible for the secretion of cytoplasmic lectins in all cases, these results raise the possibility that more subtle breaking and rescaling of plasma membranes may be involved in the secretion of cytoplasmic lectins in other tissues, such as lung (8) and skeletal muscle (2). It is also possible that those cases do involve some massive cellular rupture like that seen here, or even destruction of some cells rich in the lectin and release of cytoplasmic contents.

The function of the lectin secreted by the granular glands...
is not known. Indeed we cannot rule out the possibility that the function of the lectin is in the cytoplasm, and that it just happens to be released along with other cellular contents. The secretion is rich in a variety of biologically active peptides and amines (11, 14, 15), many of which are also found in mammalian gastrointestinal tract and brain (15), and which can act as toxins. Massive release of these compounds is believed to act as a defense against predators (11) who frequently drop frogs that have secreted their cutaneous toxins, and learn to avoid them. This raises the possibility that lectin in Xenopus skin may itself have a toxic effect, as may those found in snake venoms (16, 21). The lectin could also serve to organize glycoconjugates in cutaneous mucins, providing a meshwork to hold the toxin granules on the skin.

Another possibility is that it acts like the carbohydrate-binding subunits of the plant lectins ricin and abrin which combine with toxins (22). Association of the carbohydrate-binding sites of these lectins with glycoconjugates on the surface of cells serves to deliver the toxins to their targets. In the case of the plant lectins, toxin association is via disulfide bonds. These would be cleaved by -mercaptoethanol present in our media and might, have escaped our notice. However in our studies of crude cutaneous secretions isolated from the Department of Biology, University of Waterloo, Waterloo, Ontario, Canada.

This work was performed while N. C. Bols was on sabbatical leave from the Department of Biology, University of Waterloo, Waterloo, Ontario, Canada.

This work was supported by grants from the United States Public Health Service (HD18729, to M. M. Roberson), the National Science Foundation (DCB85-02662, to S. H. Barondes), a travel grant from the Natural Sciences and Engineering Research Council of Canada (to N. C. Bols) and by the Veterans Administration Medical Center.

Received for publication 24 June 1985, and in revised form 16 October 1985.

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