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Purification and some characteristics of a β-galactoside binding soluble lectin from amphibian ovary

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Soluble extracts of *Bufo* ovaries agglutinate sialidase-treated rabbit erythrocytes. Unlike other amphibian lectins this agglutination activity does not require the presence of calcium ions. It is specifically inhibited by D-galactose and its derivatives. Thiodi-D-galactoside is the most potent saccharide inhibitor followed by lactose and methyl-β-D-galactoside, respectively. D-Fucose, D-glucose and D-mannose do not inhibit the activity at concentrations at or above 100 mM. The lectin has been purified 500-fold to apparent homogeneity from the ovaries by salt extraction and affinity chromatography on lactose-aminophenyl-agarose, with a yield of about 0.2%. The molecular mass determined by gel filtration under native conditions was 30 kDa; polyacrylamide gel electrophoresis in SDS gave a molecular mass of 15 kDa, suggesting that the lectin is a dimer. The lectin has an isoelectric point of 40 and contains a high proportion of acidic amino acids.

Endogenous lectin; β-Galactoside-binding lectin; Amphibian development; Lectin purification; (Amphibian ovary)

1. INTRODUCTION

Lectins are a class of carbohydrate-binding proteins and glycoproteins which are widely distributed in nature [1–5]. Although they were identified in plants and animals a long time ago, their functions are poorly known. Soluble lectins from animal tissues are considered to be developmentally regulated and studies on slime molds and chicken tissues showed higher lectin levels during definite stages of development [6–8].

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In amphibians, lectins were found in oocytes, embryos and tadpoles of different species [9–13]. We also reported the presence of a β-galactoside-specific lectin in *Bufo arenarum* oocytes and embryos the level of which was higher at early developmental stages [14–16]. This report describes the affinity purification of a lectin from *B. arenarum* ovaries and presents some of its characteristics.

2. MATERIALS AND METHODS

2.1. *Crude extract preparation*

B. arenarum ovaries were obtained from pithed females captured in Buenos Aires (Argentina) during their reproduction period without any kind of stimulation. Homogenates were prepared in 150 mM NaCl, 10 mM Tris, 300 mM lactose, 4 mM β-mercaptoethanol, pH 7.6 (4 g of ovaries per ml of buffer). After centrifugation at

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100000 × *g* for 60 min they were dialyzed and lyophilized.

2.2. *Hemagglutination assay*

Hemagglutinating activity was determined with sialidase treated and fixed human group A and rabbit erythrocytes as described [17]. Titer is defined as the reciprocal of the highest dilution giving a visible agglutination. Sugars and glutaraldehyde were from Sigma (St. Louis, USA) and sialidase (*V. cholerae*) from Behring (Marburg, FRG).

2.3. *Affinity chromatography*

The lactose-aminophenyl-agarose gel (Lactogel) was from E.Y. Laboratories (San Mateo, USA). The ovary extract (50 ml at a concentration of 4 mg of protein/ml) was applied at room temperature to a column (0.9 cm × 8 cm) containing the lactose-aminophenyl-agarose gel (5 ml) equilibrated with MTB (50 mM Tris-HCl, pH 7.6, 4 mM β-mercaptoethanol and 25 mM PMSF). After recycling the excluded fraction at least two times, the unbound material was eliminated by washing with MTB until no protein was detectable in the effluent. The bound material was eluted at 4°C with either 100 or 300 mM lactose in MTB at 4°C. The protein content of 2 ml fractions was assayed according to Bradford [18] as well as for hemagglutinating activity.

2.4. *Polyacrylamide gel electrophoresis in SDS*

Molecular masses of SDS-denatured proteins were determined using the Phastsystem (Pharmacia, Sweden). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in PhastGel gradient, 8–25% (Pharmacia), was carried out according to the instructions of the manufacturer. α-Lactalbumin (M_r 14400), trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 30000), ovalbumin (M_r 43000), serum albumin (M_r 67000) and phosphorylase *b* (M_r 94000) were used as calibration proteins. Gels were stained by the silver method.

2.5. *Isoelectric focusing*

This was performed with the Pharmacia Phastsystem using the PhastGel IEF 3–9 and pI calibration kit for PhastGel IEF (pH range covered: 3.50–8.65). The purified lectin was diluted in 2% Tween 20. After electrophoresis, the protein bands were stained by the silver method and the pI point of the lectin was interpolated from the pH profile of the gel.

2.6. *Gel filtration*

The molecular mass of the native lectin was determined by gel filtration on a column (1.5 × 30 cm) of Ultrogel ACA 44 equilibrated with MTP. Ribonuclease (M_r 13700), chymotrypsinogen A (M_r 25000), ovalbumin (M_r 43000) and albumin (M_r 67000) were used as calibration standards. The lectin was eluted with MTB at a flow rate of 4 ml/cm^2 per h. Molecular masses were calculated by comparing the K_{av} (elution volume – void volume/bed volume – void volume) of the lectin with the values obtained for the calibration standards. The same procedure was performed with crude extracts to determine possible molecular associations.

2.7. *Protein determination and amino acid analysis*

Protein was assayed by the method of Bradford using bovine serum albumin as the standard [18]. Amino acid analysis was performed on a Liquimat III (Labotron) amino acid analyzer following hydrolysis of the lyophilized samples in 6 N HCl for 24, 48 and 72 h at 110°C under reduced pressure. Cysteine was determined by hydrolysis after performic acid oxidation and quantitation as cysteic acid.

3. RESULTS

3.1. *Presence of a β-galactoside-binding activity in crude ovary extracts*

Crude extracts of *B. arenarum* ovaries agglutinate both desialated glutaraldehyde-fixed rabbit and human erythrocytes. The reactivity was higher for rabbit erythrocytes than for human erythrocytes (table 1). Ionic concentration increase and temperature decrease resulted in lower specific activity. This activity was insensitive to the presence of divalent cations.

When the soluble lectin activity was examined by gel filtration on a ACA 44 column, hemagglutinating activity eluted as a polydisperse continuum in the third peak corresponding to a molecular mass between 23 and 48 kDa.

Table 1

Hemagglutinating activity of crude extracts from *B. arenarum* ovaries

Ionic concentration in NaCl (M)	Red blood cells	Temperature			
		20°C		4°C	
		Titer	Specific activity	Titer	Specific activity
0.15	rabbit	64	16000	2	500
	human	4	1000	0	0
0.20	rabbit	2	500	0	0
	human	0	0	0	0
0.40	rabbit	4	1000		
	human	0	0	0	0

The crude extracts were used diluted at a protein concentration of 4 μg/ml. Titers were expressed as the reciprocal of last dilution giving a visible hemagglutination. Specific activity was calculated as the rate of titer to protein concentration (titer × mg/ml^{-1})

3.2. *Lectin purification*

Fig.2 represents results of affinity chromatography of a crude ovary extract on immobilized lactose. A 100 mM lactose solution proved to be appropriate for the complete elution of the lectin.

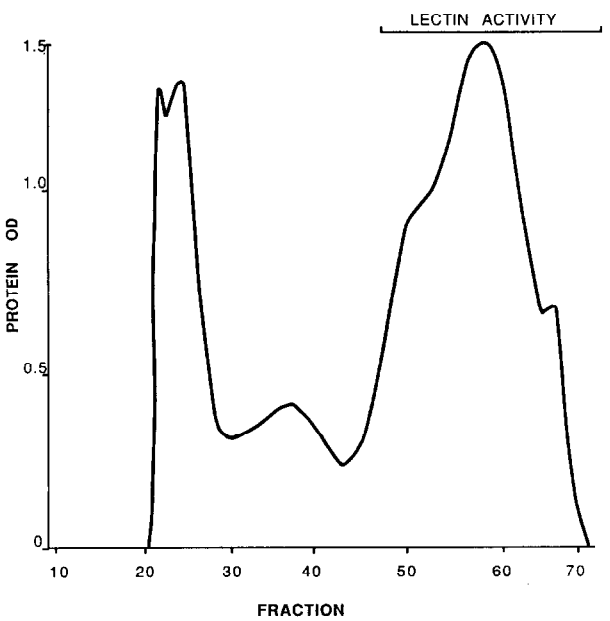

Fig.1. Chromatography of crude oocyte extract on Ultrogel ACA 44. Absorbance was monitored at 280 nm.

In several independent purifications on 200 mg of starting material the average yields of purified soluble lectin were 300–400 μg which represent approx. 0.2% of the total soluble proteins in the extracts. The specific activity of the purified soluble lectin was approx. 500-times higher than that of the crude extracts.

3.3. *Lectin characterization*

The inhibitory effect of saccharides on the ag-

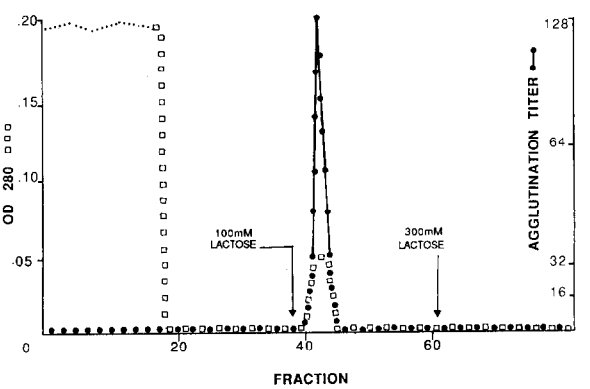

Fig.2. Affinity chromatography of the soluble lectin from *B. arenarum* oocytes on a D-lactose-aminophenyl-agarose column. Extract was applied and after recycling the excluded fraction twice the unbound material was eluted with MTB. Arrows from left to right indicate successive addition of 0.1 M lactose and 0.3 M lactose.

Table 2

Effect of different saccharides on the hemagglutinating activity of *B. arenarum* ovary lectin, assayed with fixed sialidase-treated rabbit erythrocytes

Saccharide[a]	Concentration for 50% inhibition (mM)
Thiodigalactoside	0.02
Lactose	0.1
Methyl-β-D-galactoside	6.2
p-Nitrophenyl-β-D-galactoside	12.5
Melibiose	12.5
Methyl-α-D-galactoside	12.5
D-Galactose	25.0
D-Galactosamine	50.0
D-Glucosamine	50.0
N-Acetyl-D-galactosamine	50.0
Stachyose	75.0

[a] The following saccharides were tested and had no inhibitory activity at 100 mM: D-fucose, L-arabinose, D-glucose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-mannosamine

Table 3

Amino acid composition of *B. arenarum* ovary lectin

Amino acid	Residues/100
Asp	8.4
Thr	6.0
Ser	17.9
Glu	13.2
Pro	n.d.
Gly	13.6
Ala	8.4
Val	5.0
Cys	4.7
Met	0.3
Ile	2.4
Leu	4.4
Tyr	1.9
Phe	5.0
Lys	3.6
His	2.1
Arg	2.9

n.d., not done

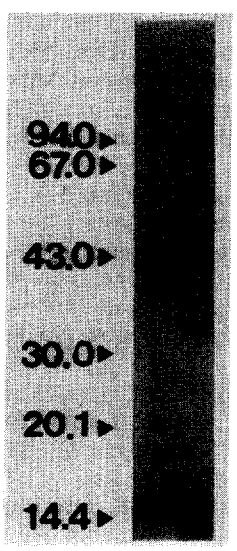

Fig.3. PAGE of lectin purified from oocyte extract. The lectin was electrophoresed on PhastGel gradient, 8–25%. The standards used were: α-lactalbumin (M_r 14400); soybean trypsin inhibitor (M_r 20100); carbonic anhydrase (M_r 30000); ovalbumin (M_r 43000); bovine serum albumin (M_r 67000) and phosphorylase *b* (M_r 94000). The gel was stained by the silver method.

glutination activity of the purified lectin was tested using a constant lectin concentration (0.5 ng/ml). The most potent inhibitors were sugars bearing a β-D-galactoside configuration such as thiodigalactoside and lactose (table 2). A somewhat weaker reactivity against α-D-galactosides was also detected.

Use of SDS-PAGE demonstrated that, under reducing conditions, the subunit molecular mass of the ovary lectin was 15000 ± 500 Da (fig.3). With gel filtration under non-reducing conditions, it was eluted as dimers of apparent M_r of 30000.

Isoelectric focusing in polyacrylamide gel and amino acid analysis showed that the lectin was an acidic protein. On isoelectric focusing, a single band corresponding to a pH of 4.0 was observed. The amino acid composition (table 3) showed that serine, glutamic acid and glycine were the predominant amino acids.

4. DISCUSSION

In a previous work, we described the hemagglutinating activity of crude extracts of *B.*

arenarum embryos at different developmental stages, using trypsinized red blood cells, which have receptors for lectins with a β-galactoside specificity [16]. As we also detected the same lectin activity in the ovary, we decided to employ this material as the source for the purification studies.

The results presented in this report indicate that a soluble lectin can be extracted and purified from *B. arenarum* ovaries. The lectin appears to be a small molecule in common with other soluble galactoside-binding lectins from vertebrates [8]. It is present in solution as a dimer, and in this respect it appears to differ from the lectins of the blastula and gastrula [11] and of the oocytes [28] of *Xenopus laevis*. Saccharide inhibition studies with additional sugars to those previously employed [14] show that the sugars with high affinity are thiodigalactoside, lactose and methyl-β-D-galactoside. The α-anomers of galactose are somewhat weaker inhibitors, although it seems that specificity is not quite restricted to β-anomers.

Several lectins have been purified from amphibian oocytes of different species (*Rana catesbiana* [12,19], *R. japonica* [20], *R. pipiens* [21] and *X. laevis* [9,22]). They differ in their localization, solubility, molecular mass, number of units, cationic requirement, saccharide specificity and amino acid and carbohydrate composition. This marked variability is of unknown significance. The presence of specific β-galactoside lectins in different organs and fluids of adults and embryos demonstrates that these lectins may play different roles during development as well as in adult life [8]. For instance, in chicken and also in amphibians, lectins are implicated in cell-cell adhesion and in cell-extracellular matrix interactions [8,23–25]. Another role proposed is related to the mechanism which prevents the polyspermy by means of lectins exuded by cortical granules [10,13].

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