Biochem. J. (1981) **195**, 435–439
Printed in Great Britain

Mitogenicity and binding properties of β-galactoside-binding lectin from chick-embryo kidney

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(*Received 24 November 1980/Accepted 19 January 1981*)

The β-galactoside-binding lectin binds to glucosamine, mannosamine and galactosamine in addition to β-galactoside, as determined by the inhibition of haemagglutination. Haemagglutination is further extended to examine the interaction of the binding sites for hexosamines and β-galactosides, indicating that the binding of hexosamine and β-galactoside is competitive. The lectin also shows strong mitogenic activity toward lymphocytes from mouse lymph node, as determined by the stimulation of thymidine incorporation.

A family of animal lectins are found to bind β-galactosides and have been isolated from several different tissues of diverse species (Teichberg *et al.*, 1975; DeWaard *et al.*, 1976; Nowak *et al.*, 1977; Den & Malinzak, 1977). The β-galactoside-binding lectins from different sources share similar sugar-binding properties, similar molecular properties and possibly subcellular location (Briles *et al.*, 1979). In addition, they are developmentally regulated (Briles *et al.*, 1979; Kobiler & Barondes, 1977). Despite their wide occurrence and extensive structural studies in some cases (DeWaard *et al.*, 1976; Beyer *et al.*, 1980), the functions of the β-galactoside-binding lectins remain unknown. No mitogenic activity of the lectins has been reported. Receptors of the lectins have not been isolated. Inasmuch as the functions of lectins likely result from their binding properties, closer examination of the binding properties of the lectins is evidently needed to probe the structural features of the receptors and thus provide a route toward the elucidation of the functions of the lectins.

In the present paper we report some unusual binding properties of the embryonic β-galactoside-binding lectin and its mitogenic activity. In addition, the purification of the lectin by conventional purification procedures is reported. A preliminary report on this work has appeared (Pitts & Yang, 1980*a*).

Materials and methods

Materials

White Leghorn chick embryos were obtained from Truslow Farm (Chestertown, MD, U.S.A.). Simple saccharides were purchased from Sigma or from Pfanstiel Laboratories (Waukegan, IL, U.S.A.). Crystalline trypsin, *Clostridium perfringens* neuraminidase (14 units/mg), Pronase and aprotinin were obtained from Sigma. Medium RPMI-1640 and foetal-calf serum were from Gibco and [^{3}H]thymidine (2 mCi/μmol) was from New England Nuclear Corp. Other chemicals were analytical grade or the purest form available from standard sources. Lymph nodes were dissected from female C3H/mai mice (Microbiological Associates, Bethesda, MD, U.S.A.). Embryonic carbohydrates were isolated as described by Pitts & Yang (1980*b*).

Analytical methods

Assay of haemagglutination activity was performed as previously described (Pitts & Yang, 1980*b*). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Laemmli (1971). Protein was stained with Coomassie Brilliant Blue R-250 by using the procedure described by Vesterberg (1971).

Derivation of binding properties by haemagglutination

Owing to the limited amount of purified lectin from the embryonic tissues, the binding parameters were derived indirectly from the inhibition of haemagglutination. Haemagglutination was assumed to be slow enough to reflect the equilibrium of binding of saccharide. To derive usable equations, second-order terms were ignored. The inhibition constant (K_a) can then be determined from the following equations:

$$f = \frac{T_o - T_f}{T_o} = \frac{K_a[A]}{1 + K_a[A]} \quad (1)$$

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where f is the extent of inhibition of haemagglutination by saccharide A, and T_0 and T_f are the agglutination activities of the lectin at a given concentration in the absence and the presence of the saccharide respectively; K_a is the inhibition constant of the saccharide and [A] is the molar concentration of the saccharide. The inhibition constant for binding of α-methyl mannoside to concanavalin A was found to be $1.3 \times 10^4 M^{-1}$, which is in good agreement with that obtained by equilibrium dialysis (So & Goldstein, 1968).

The theoretical binding curves for the competitive and the independent binding in the presence of two different saccharides were derived as follows.

For competitive binding:

$$f_a = \frac{K_a[A]}{1 + K_a[A] + K_b[B]}$$

$$f_T = \frac{K_a[A] + K_b[B]}{1 + K_a[A] + K_b[B]} = f_a + f_b \quad (2)$$

and for independent binding:

$$f_a = \frac{K_a[A]}{1 + K_a[A]}$$

$$f_T = \frac{K_a[A] + K_b[B] + K_a K_b[A][B]}{1 + K_a[A] + K_b[B] + K_a K_b[A][B]}$$

$$= f_a + f_b - f_a f_b \quad (3)$$

where $f_a f_b$ represents the correction for the fraction due to the binding of both saccharides to the same molecules, which is not observed by haemagglutination in terms of decrease of haemagglutination activity.

In eqns. (2) and (3), f_T is the extent of inhibition of haemagglutination; K_a and K_b are the respective inhibition constants for A and B; [A] and [B] are the molar concentrations of A and B respectively. Knowing the inhibition constant for each saccharide, f_T, f_a and f_b can be calculated and compared with the extent of inhibition to distinguish the two cases. A typical case of such a comparison is shown in Fig. 1 (below). The theoretical binding curves for competitive and independent binding are shown in the lower panels of Figs. 1(*a*) and 1(*b*). Double-reciprocal plots of $1/f_a$ against $1/[A]$ gave converging curves for competitive binding and overlapping curves for independent binding. The experimental binding curves were determined from the extent of inhibition of haemagglutination, f_T, in the presence of both saccharides. To compare with the theoretical binding curves, $f_T - f_b$ and $f_T - f_b + f_a f_b$ were used for competitive and independent binding respectively, as shown in the upper panels in Figs. 1(*a*) and 1(*b*). An alternative plot, of $1/f_T$ against $1/[A]$, gave the same conclusion, although both competitive and independent binding showed converging curves with differences in slopes, which depended on the difference of the respective binding constants.

Assay of mitogenesis

The mitogenic activity of the lectin was assayed by the stimulation of the thymidine incorporation (Osawa & Toyoshima, 1972) by lymphocytes. Lymph nodes were minced, gently pressed through a 100-mesh Teflon screen and dispersed in RPMI-1640 medium containing 10% foetal-calf serum (Medium A). The cell suspension was passed through a loosely packed cotton pad and was then counted for viable lymphocytes by using Trypan Blue exclusion and was diluted with Medium A to give a final concentration of 3.0×10^6 viable lymphocytes/ml. Solutions of all non-sterile reagents were passed through Millipore filters (0.2 μm pore size; Millipore Corp., Bedford, MA, U.S.A.). Each well contained 100 μl of the lymphocyte suspension and 100 μl of lectin in Medium A at a specified concentration. Cultures were kept at 37°C in a humid atmosphere of air/CO_2 (19:1). After being pulsed with 50 μl of [^{3}H]thymidine (100 μCi/μmol) in Medium A for 6h, the lymphocytes were then collected on to a glass-fibre filter (Reeve Angel 934 AH) and counted for radioactivity in 5ml of Beckman HP scintillation fluid.

Purification of the lectin

All steps were performed at 0–4°C, unless otherwise stated.

The homogenate of embryonic tissues from 600 14-day-old chick embryos was sedimented at 2000rev./min ($r_{av.} = 11$cm) for 10min. The pellet was resuspended in 0.3M-lactose and 1.0M-urea and incubated at 37°C for 30min. The postmicrosomal fraction was extensively dialysed before loading to a column of asialo-ovomucoid–Sepharose. After extensive washing of the column, the lectin was eluted with 0.3M-lactose in phosphate-buffered saline (8.0g of NaCl, 0.2g of KCl, 0.2g of KH_2PO_4, and 0.15g of Na_2HPO_4/litre, pH 7.2).

The active fractions were extensively dialysed and chromatographed on hydroxyapatite (Bio-Gel HTP from Bio-Rad) equilibrated with 50mM-potassium phosphate, pH 7.0. The lectin was eluted with a linear gradient of potassium phosphate (50–600mM) at pH 7.0. The haemagglutination activity emerged at 0.3M-phosphate and was maximal at 0.35M-phosphate. The active fractions were further chromatographed on DEAE-cellulose (Whatman DE-52) equilibrated with 50mM-NaCl in 30mM-potassium phosphate, pH 8.0. The lectin was eluted with a linear gradient of NaCl (50–600mM) in 30mM-potassium phosphate, pH 8.0. The haemagglutination activity emerged as a single symmetrical peak close to 0.2M-NaCl. The active

Table 1. *Purification of the lectins from embryonic kidney*
Results shown are for a typical preparation of lectins from 600 14-day-old chick embryos.

	Total protein (mg)	Activity (units)*	Specific activity (units/mg)	Yield (%)†	Overall purification (fold)†
1. 100000*g* supernatant	105	12000	114	100	1
2. Asialo-ovomucoid–Sepharose	2.85	144000	5.1×10^4	1200	442
3. Hydroxyapatite gradient	0.1	33600	3.4×10^5	280	2950
4. DEAE-cellulose gradient	0.01	13400	1.34×10^6	112	11700

* The titre unit is the highest dilution giving a visible agglutination in the standard haemagglutination assay.
† Yield and purification were not corrected for the increase of activity incurred during purification.

fractions were pooled and stored at 4°C. The purified lectin remained fully active for at least 2 weeks under these conditions.

A typical purification is summarized in Table 1. Although affinity purification of the lectin from muscle has been reported (Den & Malinzak, 1977; Nowak *et al.*, 1977), additional steps were evidently needed for the purification of kidney lectin. When analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the purified lectin showed a major protein band with a subunit mol.wt. of 15000. The sedimentation coefficient and the Stokes radius of the lectin were 2.7S and 2.56nm (25.6Å) respectively, giving an apparent mol.wt. of 30000. The molecular properties of kidney lectin are the same as those of the lectins purified from other chick embryonic tissues (Den & Malinzak, 1977; Nowak *et al.*, 1977).

Results and discussion

Binding of carbohydrates

The binding properties of the lectin were initially examined by determining the concentration of simple saccharides that inhibited haemagglutination by 50%. As Table 2 shows, the binding properties of the kidney lectin were similar to those from other embryonic tissues (Den & Malinzak, 1977; Nowak *et al.*, 1977), with the additional new data for the binding of glucosamine, mannosamine and galactosamine, which have not been examined previously for any β-galactoside-binding lectins. The inhibitory effect of glucosamine, mannosamine and galactosamine could not be due to simple electrostatic interaction, since NaCl at up to 0.4M did not inhibit the haemagglutination. The presence of a minor contaminant lectin (Pitts & Yang, 1980*b*) or the physical association of two different lectins was unlikely, since β-galactoside or hexosamine alone completely inhibited the haemagglutainated. We tentatively concluded that the β-galactoside-binding lectin bound hexosamines in addition to β-galactosides.

To study the binding of hexosamines in more detail, the inhibition constants were determined for lactose, thiodigalactoside, glucosamine, mannosamine and galactosamine (Table 2). Knowing the inhibition constants, we calculated the theoretical binding curves for competitive and independent binding, and compared them with the experimental values. The quantitative relation between the extent of inhibition and the concentration of inhibitors were derived and are shown in the Materials and methods section. When the binding of hexosamine and lactose was examined, the results were only consistent with those for competitive binding (Fig. 1). Competitive binding was also observed for lactose and thiodigalactoside, and for glucosamine and mannosamine (results not shown). We thus concluded that β-galactoside-binding lectin also bound hexosamine.

Table 2. *Effect of saccharides on haemagglutination activity*

Saccharide	Concentration that inhibits haemagglutination by 50% (mM)	Inhibition constant (M^{-1})
Thiodigalactoside	0.6	5×10^3
Lactose	1.1	2.5×10^3
Glucosamine	11.3	250
Galactosamine	22.5	130
Mannosamine	22.5	130
Galactose	22.5	
Galactose 6-phosphate	22.5	
Stachyose	22.5	
α-Methyl galactoside	45.0	
β-Methyl galactoside	187	
Raffinose	187	
Others*	>187	

* Saccharides that did not inhibit haemagglutination at 187mM included glucose, mannose, talose, galose, ducitol, tagatose, sorbitol, *N*-acetylglucosamine, *N*-acetylgalactosamine, melibiose, maltose, sucrose.

Embryonic carbohydrates were also isolated (Pitts & Yang, 1980*b*) in an attempt to examine the possible presence of receptor activities. Preliminary results suggest that the β-galactoside-binding lectin

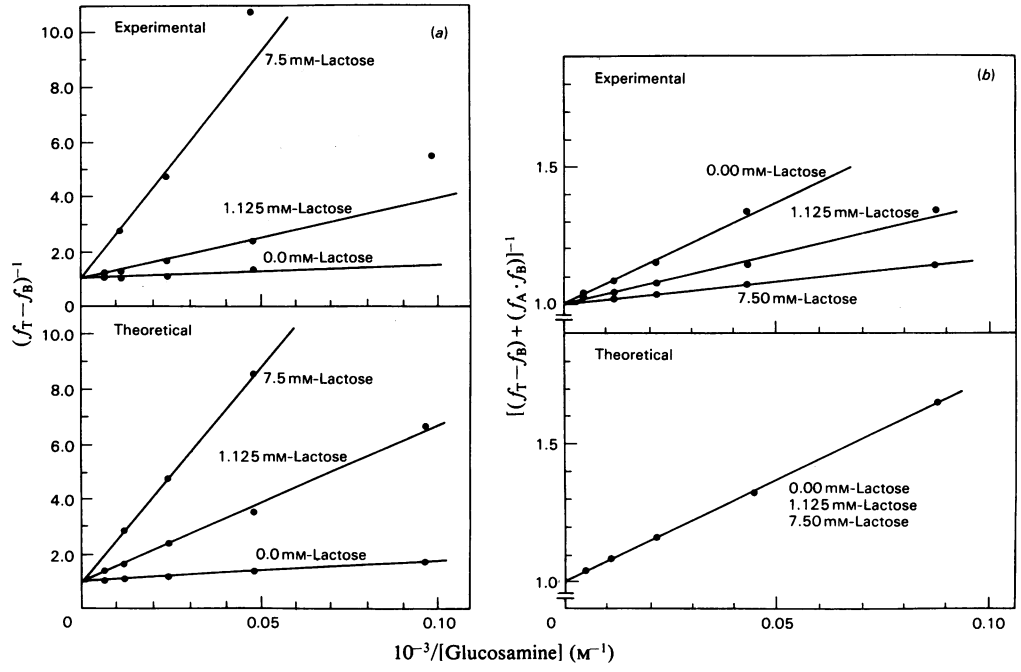

Fig. 1. *Comparison of experimental and theoretical binding curves for (a) competitive and (b) independent binding of lactose and glucosamine*

In (*a*), f_T was determined from the extent of inhibition or calculated by assuming competitive binding; in (*b*), f_T was determined from the extent of inhibition or calculated from eqn. 3. f_B, the fraction of sites occupied by lactose, was calculated by assuming independent binding.

strongly binds embryonic carbonhydrates only after neuraminidase treatment. The binding of desialylated carbohydrates by the β-galactoside-binding lectin was substantiated by the binding of the lectin to asialo-ovomucoid– but not to ovomucoid–Sepharose (Pitts, 1979).

On the basis of the binding properties of the lectin, the lectin receptors may contain β-galactoside and hexosamine, but not terminal sialic acid.

Mitogenicity of the embryonic lectins

The lectin-mediated stimulation of [^{3}H]thymidine incorporation by the lymphocytes from mouse lymph node was used to determine the mitogenic activity of the embryonic lectin. As Table 3 shows, the thymidine incorporation was stimulated more than 40-fold by the embryonic lectin at 0.1 μg/ml. The mitogenic activity of the embryonic lectin was highly dependent of the concentration of the lectin. It should be noted that phytohaemagglutinin stimulated the thymidine incorporation 80-fold and showed broader concentration-dependency under these conditions. Whether a different population of lymphocytes was stimulated by the lectin is not known. The concentration of the lectin *in vivo* is estimated to be within the effective concentrations for mitogenesis. Although the role of the lectin in the regulation of cell proliferation is yet to be determined, such a possibility should not be precluded.

Table 3. *Lectin-mediated stimulation of thymidine incorporation of lymphocytes from mouse lymph node*

[Lectin] (μg/ml)	Thymidine incorporation (% of stimulation)*
0	0
1.6	45
0.8	80
0.4	190
0.2	320
0.1	4100
0.05	370
0.025	180
0.012	140
0.006	51

* Average for triplicate observations at each concentration of lectin. Stimulation (%) represents the thymidine incorporation above that in the absence of the lectin.

We acknowledge the excellent technical assistance of Mrs. Karen M. David. This work was supported by

grant no. GM-25363 from the National Institute of General Medical Sciences.

References

Beyer, E. C., Zweig, S. E. & Barondes, S. H. (1980) *J. Biol. Chem.* **225**, 4236–4239

Briles, E. B., Gregory, W., Fletcher, P. & Kornfeld, S. (1979) *J. Cell Biol.* **81**, 528–537

Den, H. & Malinzak, D. Z. (1977) *J. Biol. Chem.* **252**, 5444–5448

DeWaard, A., Hickman, S. & Kornfeld, S. (1976) *J. Biol. Chem.* **251**, 7581–7587

Hudgin, R. C., Pricer, W. E., Ashwell, G., Stockert, R. J. & Morell, A. G. (1974) *J. Biol. Chem.* **249**, 5536–5543

Kobiler, D. & Barondes, S. H. (1977) *Dev. Biol.* **60**, 326–330

Laemmli, U. D. (1971) *Nature (London)* **227**, 680–685

Nowak, T. P., Kobiler, D., Roel, L. E. & Barondes, S. H. (1977) *J. Biol. Chem.* **252**, 6026–6033

Osawa, T. & Toyoshima, S. (1972) *Methods Enzymol.* **28**, 328–332

Pitts, M. J. (1979) Ph. D. Thesis, Georgetown University, Washington, DC

Pitts, M. J. & Yang, D. C. H. (1980*a*) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1748

Pitts, M. J. & Yang, D. C. H. (1980*b*) *Biochem. Biophys. Res. Commun.* **95**, 750–757

So, L. L. & Goldstein, I. J. (1968) *Biochim. Biophys. Acta* **165**, 398–404

Teichberg, V. I., Silman, I., Beitsch, D. C. & Besheff, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1383–1387

Vesterberg, O. (1971) *Biochim. Biophys. Acta* **243**, 345–348