ISOLATION AND CHARACTERIZATION OF A MANNAN-BINDING PROTEIN ASSOCIATED WITH THE EARLY CHICK EMBRYO

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1. Introduction

There is much interest in the role of oligomannosyl structures in embryonic development. Evidence of their potential importance comes from 3 separate systems: teratocarcinoma stem cells maintained in the undifferentiated state possess a cell-surface carbohydrate-binding protein that recognizes oligomannosyl residues [1]; oligomannosyl residues have been implicated in cell–cell interactions occurring in the gastrulation of sea urchin embryos [2]; and an extract of cleavage-stage *Rana pipiens* embryos containing an as yet unpurified lectin blocked by a yeast mannan and mannose-containing glycoproteins has been found [3].

As part of a study on the development of the chick embryo we have detected a haemagglutinating activity associated with the vitelline membrane, which is inhibited by yeast mannan and other glycoproteins containing high levels of mannose. In addition, haemagglutinating activity inhibitable by yeast mannan was recovered from the medium in which primitive streak embryos had been suspended.

To study the role of the mannan-binding activity in embryonic development it is necessary to purify and characterize the lectin, which has proved difficult; however, we have been able to effect a 27-fold purification using hydroxyapatite in a batch procedure, this method and some of the properties of the resulting material are described.

2. Materials and methods

Vitelline membranes were removed from unincubated hen's eggs, washed in 150 mM NaCl, 5 mM phosphate (pH 7.2) (PBS) to remove yolk, drained and washed rapidly in PBS containing 0.1% Triton X-100. The complete membrane from each egg was homogenized in PBS containing 0.1% Triton X-100 (3 ml) using a glass Dounce homogenizer. The homogenate was centrifuged at 100 000 × *g* for 1 h at 4°C and aliquots (1 ml) of the supernatant fluid were mixed with 25% (w/v) hydroxyapatite (Sigma type I) in PBS containing 0.1% Triton X-100 (0.3 ml) and shaken at 90 rev. min^{-1} for 30 min at room temperature on a gyratory shaker. The hydroxyapatite was sedimented by bringing the mixture to 900 × *g* for 10 s and washed 4 times with an equal volume of 150 mM NaCl, 100 mM phosphate (pH 7.2) containing 0.1% Triton X-100. Some lectin was eluted by shaking the hydroxyapatite at 90 rev. min^{-1} for 30 min at room temperature in 150 mM NaCl, 2 mM EDTA, 20 mM Tris buffer (pH 7.8) containing 0.1% Triton X-100 and made 36 mM with respect to phosphate; the bulk of the lectin was eluted by shaking the hydroxyapatite at 90 rev. min^{-1} for 30 min at room temperature in 150 mM NaCl, 2 mM EDTA, 20 mM Tris buffer (pH 7.8) containing 0.1% Triton X-100 and made 72 mM with respect to phosphate.

Type I, trypsin-treated, glutaraldehyde-stabilized rabbit erythrocytes were prepared as in [4] and used in a microtitre plate procedure [5] to monitor lectin activity in haemagglutination units (HU). Experiments showed that the titre was not sensitive to changes in Triton X-100 concentrations.

Gel filtration on Sephadex G-200 (65 × 1.5 cm) was performed in PBS containing 0.1% Triton X-100, the column being calibrated with appropriate standard proteins. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate followed the methodology in [6,7]. Gels were stained with Coomassie brilliant blue (CBB)

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or with the periodic acid/Schiff technique [8]. Hydrophobic interaction chromatography was conducted using a column (5.1 × 1.0 cm) of diaminohexyl–Sepharose 4B [9].

Components in the vitelline membrane extracts were fractionated on the basis of differences in isoelectric points either by electrofocusing in a bed of 4% (w/v) Ultrodex (LKB) containing 3–10 Pharmalyte (Pharmacia) diluted 1:18 (v/v) and mounted in the preparative attachment of an LKB Multiphor apparratus, or, by chromatofocusing [10] on a column (28 × 0.9 cm) of polybuffer exchanger PBE 94 (Pharmacia). Samples were fractionated in the presence of 0.1% Triton X-100; in the case of chromatofocusing separations the pH gradient was established using 25 mM imidazole–HCl (pH 7.5) as start buffer and eluting with polybuffer 74 (Pharmacia)–HCl (pH 4.0) both containing 0.1% Triton X-100.

Phospholipids were extracted as in [11] and measured as inorganic phosphate [12] following digestion at 180°C for 2 h in 72% (w/v) $HClO_4$.

Mannan was prepared from baker's yeast [13] and contained 4% protein. Following extensive digestion of this preparation (25 g) with pronase B (Calbiochem) (2 mg followed by a further 2 mg after 24 h) over 4 days in 50 mM Tris–HCl (pH 7.8) 5 mM $CaCl_2$ (100 ml) at 37°C, mannan with a decreased protein content was isolated by gel filtration on Sephadex G-75 (45 × 2.6 cm). The pronase-digested material used here contained 0.6% protein and on hydrolysis in 1 M H_2SO_4 at 100°C for 6 h mannose was the only neutral sugar detectable by chromatography [14]. Various manno-oligosaccharides were isolated by a combination of gel filtration on Bio-Gel P2 (200 × 1.2 cm) and preparative paper chromatography (Whatman 3 MM) following the controlled acetolysis of acetylated native and pronase-digested yeast mannon [15,16]. Periodate oxidation of thyroglobulin (Sigma type II) and invertase (Sigma type X) was done as in [1].

Blastoderms from 22 h incubated eggs were removed into Pannett and Compton's saline (PCS) [17] as in [5], placed singly on to glass cover slips, floated in PCS (200 μl) and incubated at room temperature for various times. The supernatant fluid was removed, centrifuged at 12 000 × *g* for 5 min at room temperature and examined for haemagglutinating activity, lactic dehydrogenase activity [18] and protein concentration [19] using bovine plasma γ-globulin as the standard. The blastoderms were homogenized in PCS using a glass Dounce homogenizer, centrifuged at 12 000 × *g* for 5 min at room temperature and the supernatant fluid examined for lactic dehydrogenase activity (EC 1.1.1.27) [18].

The reagents were of the highest purity commercially available. Triton X-100 was further purified by passage through Ambelite mixed-bed resin before use. Trypsin grade XI was obtained from Sigma.

3. Results and discussion

Table 1 summarizes a typical 27-fold purification in 50% yield of the mannan-binding activity of a vitelline membrane extract using hydroxyapatite. Hydroxyapatite when used in a column mode was unsuccessful. The haemagglutinating activity present in vitelline membrane extracts made with 5 mM with respect to NaCl and phosphate (pH 8.0) adsorbed to DEAE-cellulose but could not be eluted by a gradient of 5–50 mM NaCl in 5 mM phosphate buffer (pH 8.0) nor by washing with 600 mM NaCl containing 30 mM phosphate (pH 8.0) although haemagglutinating activity was demonstrably stable in these solutions, all of which were made 0.1% with respect to Triton X-100. For isolating developmentally regulated lectin from chick embryo kidney, which binds to desialated embryonic carbohydrates, hydrophobic interaction chromatography was employed in [9]. Whilst the agglutinating activity present in extracts of the vitelline membrane readily adsorbed the diaminohexyl–Sepharose this activity could not be recovered with either 0.5% deoxycholate in PBS [9] or using 50% ethanediol in 10 mM Tris–HCl (pH 7.0) to reduce the polarity of the eluent; the lectin activity being stable in these eluting media. Recovery of active material (pI 3.8–4.4) by isoelectric focusing and chromatofocusing was low (3.6% and 0.6%, respectively) though the latter procedure resulted in a 7-fold purification. The agglutinating activity readily adsorbed to type I erythrocytes, but elution from these cells could not be effected by reducing the pH to 2.5 with 0.2 M glycine. Polarity reducing agents (50% ethanediol) chaotropic ions (3 M isothiocyanate; pH 7.4), 0.5% deoxycholate or 0.3 M D-mannose were also ineffective as eluting agents.

In common with hepatic binding protein [7] the highly aggregated state of the vitelline membrane lectin made characterization difficult and Triton X-100 was incorporated into the solutions in an effort

Table 1
Purification of the mannan-binding activity of the vitelline membrane[a]

Stage of purification	Titre[b] (HU . ml^{-1})	Protein (mg . ml^{-1})	Spec. act. (HU . mg protein^{-1})	Relative spec. act.
Vitelline membrane extract (100 000 × *g* supernatant)	40 960	10.61	3860	1
Supernatant fluid after binding to hydroxyapatite	206 ± 78	5.30 ± 0.34	39 ± 14	0.01
1st wash[b]	183 ± 60	4.06 ± 0.1	46 ± 17	0.01
2nd wash[b]	0	0.65 ± 0.05	–	–
3rd wash[b]	0	0.22 ± 0.02	–	–
4th wash[b]	0	0.15 ± 0.01	–	–
Elution with buffer plus 36 mM phosphate[c]	2560	0.22 ± 0.02	11 542 ± 848	3
72 mM phosphate[d]	20 480	0.19 ± 0.01	105 876 ± 5653	27

[a] Seven 2 ml batches of vitelline membrane extracts were subjected to the fractionation scheme. The results are the mean ± SD of the 7 samples
[b] 150 mM NaCl, 0.1 M phosphate (pH 7.2) containing 0.1% Triton X-100
[c] 150 mM NaCl, 2 mM EDTA, 20 mM Tris-buffer (pH 7.8) containing 0.1% Triton X-100 and 36 mM with respect to phosphate
[d] 150 mM NaCl, 2 mM EDTA, 20 mM Tris-buffer (pH 7.8) containing 0.1% Triton X-100 and 72 mM with respect to phosphate

The highest dilution giving definite agglutination is regarded as containing 1 haemagglutination unit

to covercome its tendency towards selfassociation [7].

Even in the presence of detergent only 3% of the haemagglutinating activity applied to a column of Sephadex G-200 penetrated the gel (M_r 3 × 10^5) with a total recovery of 76%. The bulk of the activity being in the void volume.

SDS–Polyacrylamide gel electrophoresis [6] of the vitelline membrane extract revealed with CBB, 3 heavily stained bands of app. M_r 4.35 × 10^4, 5.25 × 10^4 and 7.7 × 10^4 (fig.1) all 3 being positive for periodate/Schiff reagent [8]. Examination of equal quantities of protein recovered from hydroxyapatite demonstrated that these 3 components were absent. This latter material possessed a major CBB-stained band of app. M_r 4.9 × 10^4, together with 2 faint bands of app. M_r 8.4 × 10^4 and 1.13 × 10^5. These 3 bands were negative for periodate/Schiff reagent [8]. Both vitelline membrane extracts and material eluted from hydroxyapatite had small quantities of perhaps highly aggregated material [7] (app. $M_r > 1.26 \times 10^5$) close to the point of application. Inorganic phosphate analysis of the fraction recovered from hydroxyapatite indicated that the maximum phospholipid content of the material recovered from hydroxyapatite was ⩽5%.

The haemagglutinating activity eluted from hydroxyapatite was inactivated by heating at 100°C for 1 min and by digestion with trypsin (100 μg . ml^{-1}, 37°C, 3 h, pH 7.3). This activity was inhibited by thyroglobulin, invertase and yeast mannan, glycoproteins containing oligomannosyl groups, but not

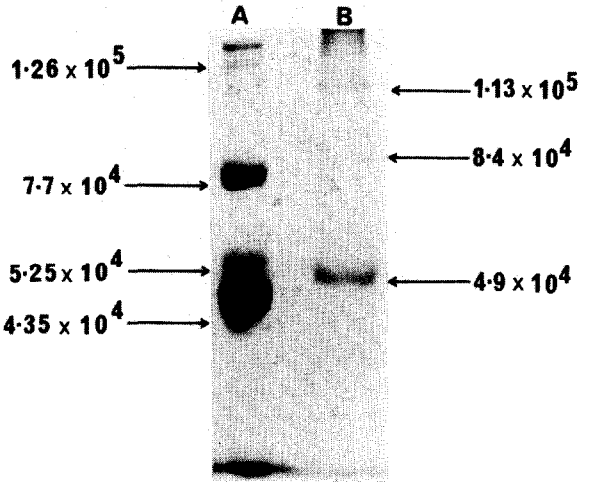

Fig.1. Gel electrophoresis in the presence of sodium dodecyl sulphate and 8% acrylamide of (A) vitelline membrane extract (100 μg protein) and (B) material eluted from hydroxyapatite (100 μg protein).

by fetuin which lacks these moieties. Polysaccharides with an oligoglucosyl (dextrans) or oligogalactosyl (agarose) structure were without inhibitory activity. Thiodigalactoside, lactose, galactose and asialofetuin, which inhibited the β-galactoside binding lectin from early chick embryos [5] failed to inhibit the activity obtained from vitelline membrane (table 2).

Several lines of evidence indicate that it is the carbohydrate rather than the protein component of the glycoprotein inhibitors which is responsible for the inhibition of haemagglutination. Heat treatment (100°C, 10 min), which may be expected to denature proteins without modifying carbohydrates, had no effect on the inhibitory activity of thyroglobulin and invertase. Periodate oxidation abolished thyroglobulin-mediated inhibition and reduced the inhibitory activity of invertase. A yeast mannan fraction with a lowered protein content after extensive pronase-digestion of the native glycoprotein was equally effective as the untreated mannan as an inhibitor of haemagglutination.

The invertase of *Candida utilis* used here is known to be a manno-protein yielding mannose exclusively in a neutral sugar fraction [20] though detailed structural information is lacking. However, the structure of the carbohydrate groups of yeast mannan, which consists almost entirely of mannose residues, and thyroglobulin is known with close structural similarities existing between the A units of thyroglobulin [22] and the inner core structure of yeast mannan [23]. Di-*N*-acetylchitobiose was a weak inhibitor suggesting that this linkage region alone is not sufficient for inhibition but that mannosyl residues are an important feature recognized by this lectin. Of the mannosaccharides available to us, penta-, hexa- and heptasaccharides were the most active, though these saccharides are mixtures of isomers of different ratios [16] making direct comparison difficult. All 3 saccharides possess one or more α(1→6) linkages which, as in the case of mannan-binding protein from rabbit liver [24], may be an important structural requirement for inhibition. By comparison with the glycoproteins the mannosaccharides are much less potent possibly reflecting the multivalency of the inhibitory glycoproteins. It is interesting that D-mannose-6-phosphate, a potent inhibitor of the uptake of lysosomal hydrolases by fibroblasts [25] and D-mannose, an inhibitor of the lectin from *E. coli* [26] were without effect on the agglutinating activity associated with the vitelline membrane.

Table 2
Effect of glycoproteins and saccharides on purified haemagglutination activity of vitelline membrane

Inhibitor	Specific inhibition activity[a]		
	HIU . mg^{-1}	HIU . μmol^{-1}	
Thyroglobulin	20	13 380	(12)
heat-treated	20	13 380	(3)
periodate-oxidized	0	0[b]	(3)
control without periodate	20	13 380	(3)
Invertase	320	96 000[c]	(12)
heat-treated	320	96 000	(6)
periodate-oxidized	20	6000	(3)
control without periodate	320	96 000	(3)
Yeast mannan	128	7552[d]	(12)
pronase treated	160	7520[e]	(3)
Mannobiose	8	3	(3)
Mannotriose	16	8	(3)
Mannotetraose	16	11	(3)
Mannopentaose	40	33	(3)
Mannohexaose	80	79	(6)
Mannoheptaose	40	46	(2)
Di-*N*-Acetylchitobiose	2	1	(4)

[a] Specific inhibition activities are expressed as haemagglutination inhibition units (HIU). One HIU is defined as the minimum concentration of inhibitor necessary to inhibit completely 4 haemagglutination units of lectin activity, where the highest dilution giving definite agglutination is regarded as containing 1 HU [5]

[b] Tested to a final concentration of 0.15 nmol/assay

[c] Assuming M_r 3 × 10^5 [20], for *Candida utilis* invertase, periodate oxidised material was not corrected for any change in M_r-value

[d] Assuming M_r 5.9 × 10^4 [21]

[e] Calculated using M_r 4.7 × 10^4, determined by gel filtration

The numbers in parentheses refer to the number of assays performed; thyroglobulin, invertase, mannan and thiodigalactoside were tested in triplicate on 4 separate batches of purified lectin and an identical specific inhibition was achieved in all cases.

No inhibition was achieved when fetuin and asialofetuin were tested to a final concentration of 0.012 μmol, with mannose, mannose-6-phosphate, maltose, thiodigalactoside, lactose and galactose to a final concentration of 5 μmol, and dextrans with av. M_r 2 × 10^6, 5 × 10^5 and 2.43 × 10^5 to a final concentration of 0.5 nmol, 2 nmol and 4.25 nmol, respectively.

Agarose (electrophoretically pure) was tested to a final amount of 0.1 mg/assay

The physiological role of the vitelline membrane lectin has yet to be established. In view of the finding that a mannose-specific lectin from *E. coli* agglutinates a number of cell types, this sugar may be of special importance at the cell periphery [21]. High manno-oligosaccharides are associated with transformed and rapidly growing fibroblasts but not density-inhibited cells [27] so it is of interest that mannan binding activity is associated with the gastrulating chick embryo. Fig.2 shows that increasing quantities of haemagglutinating activity were found in media in which isolated blastoderms [5] had been suspended. This activity was inhibited by mannan but insensitive to inhibitors of the β-galactoside binding lectin obtained from early embryos [5]. Lactic dehydrogenase, a useful marker for the soluble component of cells, could not be detected. However this enzyme was present in homogenates of blastoderms (6.77 μmol NADH oxidized . min^{-1} . mg $protein^{-1}$) suggesting that the mannan-binding activity detected was not the result of cellular damage.

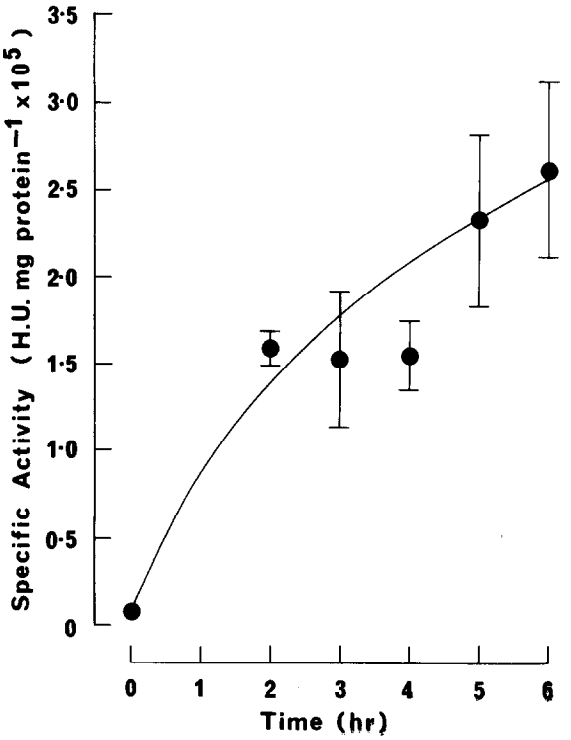

Fig.2. Time course of release of mannan-binding activity (HU . mg $protein^{-1}$) from isolated blastoderms. Each point represents the results obtained from 4 separate embryos. The values are the mean ± SEM.

There is considerable interest in developmentally regulated vertebrate lectins [28] with particular attention having been given to a β-D-galactoside inhibitable lectin of chick embryo pectoral muscle [4]. Some controversy exists whether this activity is involved in myoblast fusion [4,29]. A similar or identical lectin has been identified at the pregastrula and gastrula stages in chick embryos [5] and it is suggested that this lectin may be involved in cellular adhesion [30]. The finding that a mannan-inhibitable lectin as opposed to a β-D-galactoside sensitive lectin is released from gastrulating embryo cells urges caution in regard to the latter suggestion. The purification procedure described here should be of interest where mannan-binding lectins have been identified but not as yet purified [1,3].

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