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SUGAR SPECIFIC CELLULAR LECTINS OF *PHALLUSIA MAMILLATA* HEMOCYTES: PURIFICATION, CHARACTERIZATION AND EVIDENCE FOR CELL SURFACE LOCALIZATION

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☐Abstract—Cellular lectins (CLs) of *Phallusia mamillata* were demonstrated in protein preparations obtained by salt fractionation from hemocytes sonicated in a suitable medium. Since the lectins from the precipitated fraction bind sugars containing D-galactosyl groups, they were purified by affinity chromatography on Sepharose. SDS-PAGE under reducing conditions showed that CLs are formed of two components of apparent MWs approximately 36,900 and 35,090 and thus differ from serum lectins (SLs) (MW about 62,200). The "shrinkage" observed when SLs were examined under nonreducing conditions suggest the presence of intrachain disulphide bonds which can affect the molecular structure of the SLs. CL-SL differences were also revealed by the nonidentity reaction of the immuno-precipitate in immunodiffusion using an anti-SL immune serum. The capacity of hemocytes to form rosettes or clumps with erythrocytes demonstrated that they possess α-lactose specific CLs on their surfaces.

☐Keywords—Tunicates; Hemocytes; Lectins.

Introduction

Invertebrate lectins are sugar specific glycoproteins which are present in the hemolymph of all the examined species and, in many cases, possess opsonic functions (1–12). Lectins have also been found on hemocyte surfaces in some species (11,13,14,15). The plasma of tunicates contains lectins characterized by binding of specific sites to substances such as sialic acid and, more frequently, D-galactosyl residues. Lectins have been detected by their capacity to agglutinate untreated and enzyme-treated vertebrate erythrocytes and so far their biological role has not been determined. We recently reported that D-galactose specific lectins of *Ascidia malaca* can be found both free in the serum and present on hemocyte surface (4,16). This finding suggests that lectins on cell surfaces might act as receptors which can bind directly with surface sugars on foreign particles or identify self-markers on cell membranes.

Cellular recognition in invertebrates has been attributed to molecular mechanisms based on protein-carbohydrate interactions at cell surface levels (1,11,17–21). It is known that invertebrates lack immunoglobulins and their self nonself recognition systems differ from those of vertebrates inasmuch as they cannot be defined in terms of specific antibody-antigen complementarity (1,22). However, lectins are common to both invertebrates and vertebrates, having also been identified in several vertebrate tissues (23,24).

Since evolutionary relationships between invertebrate and vertebrate lectins remain an open question, studies of tunicates, considered to be the ancestral chordates (25), might yield information on homologs or functional analogs

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of certain vertebrate recognition molecules. Vasta and Marchalonis (2) reviewed the macromolecular properties of purified tunicate plasma lectins, and reported variations in subunit size and carbohydrate specificities, although structural similarities were evident when the $S\Delta Q$ index of the amino acid composition was calculated. Moreover, they suggested that these lectins resemble lamprey μ chains and vertebrate C reactive proteins (2).

In a previous study we showed that *Phallusia mamillata* serum contains anti-rabbit and anti-human ABO lectins, specific for α-lactose and lactulose, respectively. They were isolated by biospecific affinity chromatography on Sepharose (15). The present paper concerns the isolation and characterization of α-lactose-specific lectins of *Phallusia mamillata* hemocytes, and the molecular weights and structure of the serum lectin subunits.

Materials and Methods

Animals

Specimens of *Phallusia mamillata* Traust. were collected in the Gulf of Naples.

Blood Collection and Serum Preparation and Assays

The blood obtained by cardiac puncture forms a coagulum which was separated by centrifuging at 800 g for 15 min at 4°C. The serum was dialyzed with phosphate buffered saline solution pH 7.4 (PBS) and stored at −75°C. The microhemagglutinations and sugar inhibition tests were performed using rabbit erythrocytes (RE), as previously described (4,5).

Preparation of Hemocytes

To separate hemocytes, blood was withdrawn by means of a sterile syringe in presence (1:5) of Ca^{++} and Mg^{++} free seawater containing 0.1M disodium EDTA (FSW-EDTA, pH corrected to 7.4); the cells were centrifuged at 800 g for 15 min at 4°C, and washed three times in 20 times their volume of FSW-EDTA.

Purification of Serum Lectins

α-Lactose specific serum lectins (SL) were purified by affinity chromatography on a Sepharose-HCl column (2 × 20 cm) according to Parrinello and Canicatti (5).

Purification of Cellular Lectins from Hemocytes by Affinity Chromatography

Hemocytes from individual blood samples were washed and suspended in ten volumes of the extraction medium (150 mM NaCl, 10 mM EDTA, 5 mM benzamidine, 10 mM Tris-HCl pH 7.4, 200 mM lactose). Extraction medium containing 0.05% Triton X-100 and 10 mM 2-mercaptoethanol was also used. Hemocytes were sonicated (Sonifier Branson, model B-15P) for 1 min at 0°C (1 pulse per second, duty cycle was 70%) and centrifuged at 27,000 g for 30 min at the same temperature. Ammonium sulphate was gradually added to the supernatant to 50% concentration, and the mixture was gently stirred overnight. Then the concentration of ammonium sulphate was increased to 90%. The precipitate was removed by centrifugation at 27,000 g for 30 min, and dissolved in 2.5 ml of medium. The solution was extensively dialyzed against α-lactose less medium with repeated changes to eliminate lactose. After dialysis with PBS the protein solution was tested against RE for agglutinating activity. The crude hemocyte lectin extract (protein content 0.5–1.0 mg/ml) was applied to a HCl-treated Sepharose CL-6B column (2 × 20 cm). Affinity chroma-

tography was carried out as described for purification of serum lectins.

Preparation and Absorption of Immune Serum

The schedule for preparing rabbit antiserum against purified lectin was performed as previously described (16). Aliquots of the immune serum were absorbed by mixing them with purified 5 fold concentrated lectin solution. The reaction mixture was incubated at room temperature for 1 hour and 12 hours at 4°C and centrifuged at 10,000 g for 30 min at 4°C. The absorbed sera were tested by immunodiffusion. To control if aspecific precipitation reactin occurred between lectins and immunoglobulin-linked carbohydrates, immunoglobulins from nonimmunized rabbit were assaied with lectins.

Absorption was also performed with lectins inactivated by heat at 100°C. Assays of the absorbed antisera never showed precipitation lines.

Immunodiffusion and Immunoelectrophoresis

Immunodiffusion was carried out on 75 × 25 mm slides covered with 1% agarose gel in Tris-buffered (0.03 M) saline (NaCl 0.1 M) pH 7.2 final concentration of 0.05 M D-galactose to prevent binding of the lectin to agarose; merthiolate was added (0.01%) as preservative (27). Microimmunoelectrophoresis was performed as previously described (13,14).

SDS-Polyacrylamide Gel Electrophoresis

Before electrophoresis the samples were concentrated to a tenth of their previous volume. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in slab gel was performed according to Laemmli (26). Gels were calibrated with molecular weight marker proteins (Bio-Rad), phosphorilase B (97,500), bovine serum albumin (66,200), ovalbumin (42,600), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). The log molecular weights of standard proteins were plotted against their electrophoretic migration to obtain standard curves. The average values were obtained from six independent experiments, and the standard deviations estimated.

Hemocyte Specific Binding to Erythrocytes

Hemocytes ($0.5-1.0 \times 10^5$) and rabbit erythrocytes ($0.5-1.0 \times 10^5$) were mixed to form a 0.3 ml suspension. The reaction mixture was incubated for 30 min at room temperature.

At the end of this time the settled cells were gently mixed with a pipette and transferred to plastic discs for microscopic observations at a Diavert microscope (Leitz).

Protein Estimation

Quantitative protein analysis was performed by the method of Lowry *et al.*, (27) using bovine serum albumin as a standard.

Sample Concentration

The samples were concentrated by a Diaflo equipped with a UM2 membrane, Amicon Corp., Livington, MA.

Results

Cellular Anti-Re Lectin Purified from Hemocytes

Anti-RE lectin was found in the crude hemocyte lectin extract. Values of the

hemagglutinating titers obtained when the protein solution was treated against RE for agglutinating activity ranged from 8 to 128 according to the various sample preparations. α-Lactose (25 mM) inhibits the agglutination reaction, and this property allows the CLs to be purified by biospecific affinity chromatography on an acid-treated Sepharose gel bed, as previously reported for the serum lectins (4).

Figure 1 shows a typical protein elution profile in which cellular lectins were purified from 15 ml of sample (0.5–1.0 mg/ml protein content). The fractions showing anti-RE agglutinating activity were eluted by a competitive reaction with D-galactose, the monosaccharide component of Sepharose. After exhaustive dialysis with PBS, hemagglutinating titers of the second peak fractions, which showed the highest optical density, ranged from 8 to 64 depending on the various sample preparations.

Sugar-binding Specificity of the Anti-RE Cellular Lectin

The ability of various monosaccharides and oligosaccharides to inhibit hemagglutinating activity of the lectin isolated from hemocytes was tested. Results showed that the specificity of the lectin from both preparations was towards α-lactose, which inhibits the agglutination up to a final concentration of 25 mM; lactulose was an inhibitor at higher concentrations (100 mM). No significant inhibition was observed between the monosaccharides D-galactose, 2-deoxygalactose, D-glucose, fucose and mannose, and the oligosaccharides melibiose and raffinose, up to a final concentration of 200 mM.

Molecular Weight Determination of the Cellular Lectins

The lectins isolated from sonicated hemocytes were analyzed by SDS-PAGE and compared with serum lectins purified by affinity chromatography.

Under nonreducing conditions the isolated CLs were resolved in two components of apparent MWs 37,533 ± 858 and 35,842 ± 811, (Fig. 2b), while the SLs moved in the gel showing a single component of about 41,635 ± 920. (Fig. 2a).

When the samples were subjected to electrophresis under reducing conditions the apparent MWs of the CLs were slightly lower, 36,900 ± 405 and 35,060 ± 680, respectively, while the SLs appeared to consist of a major component of 63,613 ± 804, and a minor component of 20,308 ± 713. Two feeble bands, whose mobilities corresponded to those of the CLs, were occasionally found in the SL pattern (Fig. 2a,d).

Immunological Properties of the Isolated Cellular Lectins

The SLs and CLs purified by affinity chromatography, all produced single precipitation arcs when tested in immunoelectrophoresis with an anti-SL antiserum. All the lectin samples were 10 fold concentrated.

The immunoelectrophoretic pattern shows the anodal properties of SLs, while the purified CLs do not migrate under such experimental conditions, but precipitate near the walls (Fig. 3a). Ouchterlony comparative immunodiffusion technique produced a precipitation pattern showing no interaction between SLs and CLs (Fig. 3b). The asymmetric arcs suggest that the systems tested were probably unbalanced; however, clearly the precipitation lines extend far enough from either side and intersect (Fig. 3b). This type of reaction indicates a nonidentity reaction between the two lectins. A precipitation line occurs where excess lectins contained in the absorbed immune serum (Fig. 3b, well 4) diffuse against specific antibodies intersecting the cellular lectins immunopre-

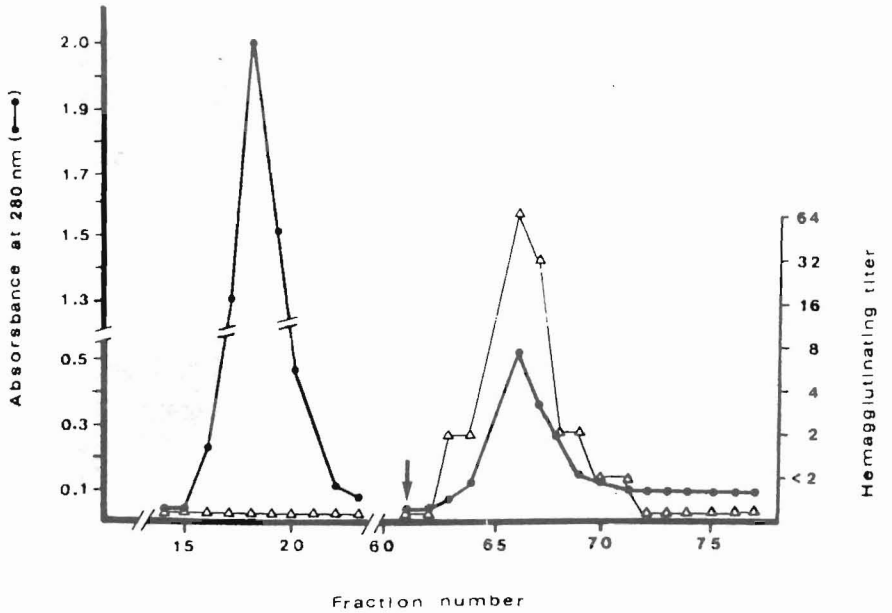

Figure 1. Affinity chromatography of crude hemocyte lectin extract on acid-treated Sepharose CL-6B column (2 × 20 cm). The column was washed with 1 M NaCl until the absorbance became negligible, then the eluting medium was changed to 0.2 M D-galactose in 1 M NaCl at the point indicated by the arrow. Hemagglutinating activity was tested against rabbit erythrocytes.

cipitate (Fig. 3b, well 1). An additional precipitation line runs down from well 3 (Fig. 3b) and might be due to prolonged diffusion (several days) which may cause antibodies (Fig. 3b, well 1) and antigens (Fig. 3b, well 2) to meet in an unusual position.

Hemocyte Sugar-specific Binding to Erythrocytes

The reaction products were as follows: (a) clumps of aggregates composed of hemocytes and REs (Fig. 4a); (b) clumps of agglutinated erythrocytes; (c) rosettes formed of hemocytes with not less than three erythrocytes attached. The rosettes were mainly found at the periphery of the disc, where clumps were rare (Fig. 4b). The intensity of the reactions (larger clumps) depended on the hemocyte numbers, as confirmed by tests in which serial twofold dilutions were used. Mixed clumps and rosettes were more frequent in reaction mixtures containing 0.5×10^4 cells

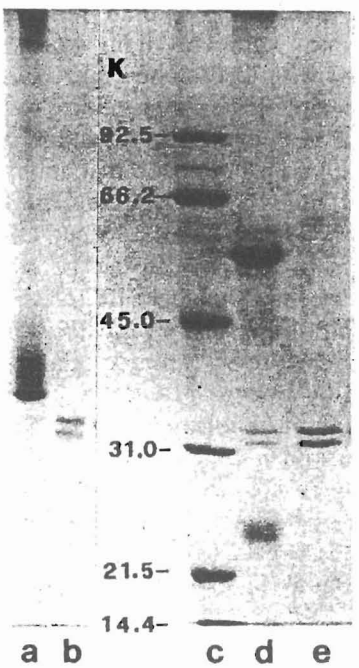

Figure 2. SDS-polyacrylamide gel electrophoresis of *Phallusia mamillata* α-lactose binding lectins isolated by affinity chromatography. (a, b) under nonreducing conditions; (d, e) under reducing conditions (2-mercaptoethanol). (a, d) serum lectins; (b, e) hemocyte lectins; (c) standard proteins (Bio Rad).

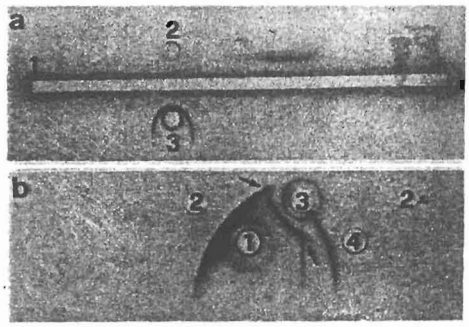

Figure 3. (a) Immunoelectrophoresis analysis of *Phallusia mamillata* α-lactose binding lectins isolated by affinity chromatography. (b) Ouchterlony immunodiffusion of anti-serum lectin-immuneserum before or after absorption. well 1: anti-SL immuneserum; well 2: serum lectin; well 3: purified cellular lectin; well 4: anti-SL immuneserum absorbed with SL. Arrow: spurs. All the lectin samples were 10 fold concentrated.

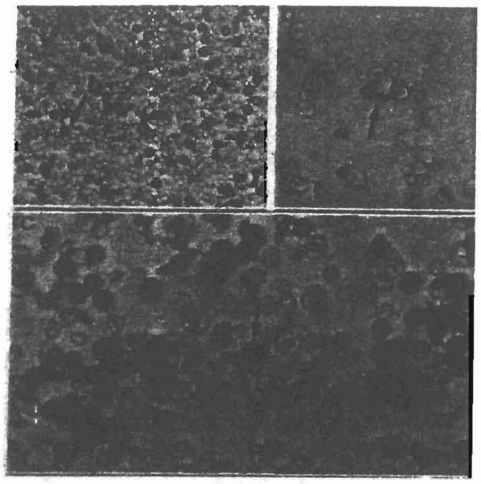

Figure 4. *Phallusia mamillata* hemocytes binding to rabbit erythrocytes. (a) Hemocytes (h) and rabbit erythrocytes in the presence of α-lactose (25 mM), ×204, vital stain; (b) hemocyte forming rosette with RE (arrow) ×410; (c) hemocytes forming rosettes with RE (arrow), clumps of aggregates composed of hemocytes and REs ×410, vital stain.

and an equal number of erythrocytes in 0.3 ml medium. Although precise morphological distinctions were not made, it was obvious that rosette-forming cells could be different in dimension (or type) and that, some of them were vitally stained with 0.001% neutral red.

No significant interactions were observed between hemocytes and Sepharose spherules mixed in reaction suspension containing 1×10^5 cells and 0.1 ml packed Sepharose-HCl. Since CLs had been shown to bind α-lactose or lactulose, attempts were made to determine whether these sugars were able to inhibit the hemocyte-erythrocyte bindings indicated by clumps or rosette formations. Repeated experiments demonstrated that 6 mM α-lactose and 25 mM lactulose were able to inhibit any hemocyte-erythrocyte interactions (Fig. 4c), while 100 mM D-galactose and D-glucose were not able to do so.

Discussion

The results show that, in addition to the previously reported serum lectins (5), the blood of *Phallusia mamillata* also contains cellular lectins, and that both contain binding sites specific for D-galactosyl groups. Cellular lectins are found in the protein preparations obtained by salt precipitation from hemocytes sonicated in medium without detergent. This result suggests that cellular lectins are water-soluble molecules non-integrated on hemocyte membranes. We do not know the nature of the lectin-membrane linkages. The possibility exists that multiple binding lectins are associated with oligosaccharides on membrane surface from which they may be eventually released.

The D-galactosyl group specificity allows us to isolate lectins by affinity chromatography on Sepharose, as reported for serum lectins (5). The inactive proteins were eluted with PBS, whereas the D-galactose binding lectins could only be eluted by a competitive reaction. The separation patterns of CLs and SLs correspond, and D-galactose was able to compete with the Sepharose D-galactose exposed groups by binding lectins. The two lectin types have certain different

features: the pattern obtained by SDS-PAGE reveals that CLs are composed of two components which have similar molecular weights, of approximately 36,900 and 35,060, respectively.

We have already reported that, in SDS-PAGE, SLs appear to contain a major component 61,000–65,000 (5), and the electrophoretic method used in the current experiment indicates a molecular weight of approximately 63,613 in reducing conditions. This component moves more rapidly (apparent MW 41,600) under nonreducing conditions, as a result of a "shrinkage" which might depend on intramolecular disulphide links. This great difference suggests that the lectin molecule contains numerous cysteine residues, perhaps spaced along the peptide chain. Such a finding would agree with the high resistance of SLs to thermal denaturation (inactivated at 100°C) and the previously demonstrated inactivation by mercaptoethanol (12). The small molecular weight difference observed for the CLs when evaluated in reducing and nonreducing conditions could depend on the different experimental conditions.

An SL minor component of 20,308 was only found in reducing conditions; it could originate from a complex heteropolymeric molecular structure in which two subunit types are linked by disulphide linkage. Occasionally, in concentrated samples, two components corresponding to the cellular lectins could be found in the SDS-PAGE pattern of the isolated serum lectins. We do not know if the CLs are spontaneously released from hemocytes into the plasma, or derived from occasional hemocyte breakage during serum preparation.

The immune serum prepared towards the SLs also reacts with purified CLs, thus allowing comparative analysis. In agarose gel immunoelectrophoresis the mobility of the SLs contrasts with the neutrality of the CLs under the same electrophoretic conditions.

Finally the CL/SL differences are indicated by the nonidentity reaction of the immuno precipitate obtained in immunodiffusion. This method demonstrated that the immune serum also contains antibodies against CLs; this may depend on the SL preparations used as antigens which, as discussed below, can occasionally contain a small quantity of CLs isolated with the SLs, undetectable in the unconcentrated sample used for the immunization. In fact, in concentrated samples, two components corresponding to the cellular lectins could occasionally be found in the SDS-PAGE pattern of the isolated serum lectins.

In spite of such structural differences both lectins showed the same sugar specificity. Inhibition experiments indicated that the anti-RE CL possess site(s) which interact(s) with α-lactose. The whole of this sugar seems to be involved in binding, in fact neither D-galactose nor D-glucose alone are capable of inhibition activity in soluble form. The arrangement of the galactosyl groups of the Sepharose bed could explain the binding of the multimeric lectin and the competitive binding capacity of the D-galactose used in the affinity chromatography.

In this respect anti-RE CLs are similar to anti-RE SLs whereas they differ because the former are also inhibited by lactulose.

The capacity of hemocytes to form rosettes or to aggregate with erythrocytes and the inhibition experiments carried out demonstrate that these cells may possess α-lactose specific lectins on their surface. Despite its competitive capacity to bind Sepharose-linked lectins, D-galactose was not able to inhibit the binding of the hemocyte associated lectins to RE.

This behaviour could suggest that the multiple binding sites of a lectin at cell surface level may cause light affinity binding with the receptor molecules in a cellular site such as a membrane, where

they can form clusters. This interaction pattern at cell surface level is also indicated by the fact that hemocytes are not able to bind to the galactosyl groups of free Sepharose sphaerules. There is no evidence of any involvement of ascidian hemocyte membrane lectins in recognition or communication between cells.

The demonstration that cellular lectins can be found on the hemocyte surface helps to clarify their role as receptors. Vasta and Marchalonis (2) have suggested that lectins from tunicates belong to an extended family of recognition molecules in which they are ancestral to immunoglobulins, C reactive proteins, complement components, MHC products and lectins from vertebrate species. In this respect it should be remembered that lectins with binding specificity for several different saccharides have been detected on the membranes of a variety of mammalian cells including lymphocytes (19,20). Research is in progress to reveal hemocytes bearing surface lectins by using immunofluorescent methods.

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