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RESEARCH REPORT

Presence of a low molecular weight lectin in the coelomic fluid of the sea urchin *Paracentrotus lividus*

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Abstract

A low molecular weight (MW) lectin (*Paracentrotus lividus* small lectin, *Pl*SL) has been found in the sea urchin, *Paracentrotus lividus*. After gel electrophoresis under denaturing conditions, *Pl*SL exhibits a MW of 13 kDa, while its hemagglutinating activity is Ca^{2+}-independent and inhibited by D-Glucose, L-Rhamnose, D-Arabinose, L-Fucose and *N*-Acetyl-D-glucosamine. Electrophoretic analysis of the coleomic fluid of *P. lividus* reveals that the presence of *Pl*SL increases following immune challenge with bacteria, whereas it is annulled as a consequence of osmotic stress. Interestingly, two other putative inducible hemagglutinins of an approximate MW of 11 and 32 kDa were retrieved in concomitance with the stress-promoted disappearance of *Pl*SL.

Key Words*: Paracentrotus lividus;* sea urchin; lectin; immunity; stress

Introduction

Lectins are carbohydrate binding proteins other than immunoglobulins that display no enzymatic activity towards the recognized sugars (Loris, 2002). Lectins have been retrieved in almost all forms of life, and animal lectins, though fulfilling a variety of functions, have been implicated in defense against pathogens, immune regulation and prevention of autoimmunity (Kilpatrick, 2002).

In marine invertebrates, lectins have been thought to participate in immune response by inducing bacterial agglutination or by acting as opsonins to enhanced phagocytosis by coelomocytes (Yui and Bayne, 1983; Bayne, 1990).

An extensive discussion regarding the physiological significance of lectins in both vertebrates and invertebrates has been reported (Turner, 1994; Kilpatrick, 2002). In the case of invertebrate humoral lectins, it has been postulated more than twenty years ago that one of their physiological functions is distinction of self from non-self in cooperation with cellular and humoral defense processes (Renwrantz, 1986).

A large number of lectins has been isolated and characterized from deuterostomian and protostomian invertebrates, such as tunicates (Vasta *et al*., 1986; Belogortseva *et al*., 1998), insects (Racliffe *et al*., 1985; Murdock and Shade, 2002), crustaceans (Beisel *et al*., 1999) and molluscs (Racliffe *et al*., 1985; Bulgakov, 2004; Ottaviani, 2006). As far as the presence of lectins in echinoderms is concerned, lectins have been found in the sea urchins *Anthocidaris crassispina* (Giga *et al*., 1985, 1987) and *Paracentrotus lividus* (Canicatti *et al*., 1992). In the latter species, the reported authors described the occurrence of a natural C-type lectin with a molecular weight (MW) of more than 200 kDa in the coelomic fluid.

In the present study, a low MW lectin was purified from the coelomic fluid of the sea urchin *P. lividus* by ion-exchange chromatography on DEAE-Sephadex. This molecule has a MW of 13 kDa and displays a Ca^{2+}-independent hemagglutinating activity.

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Materials and Methods

Animals

Adult specimens of the sea urchin *Paracentrotus lividus* Lamarck 1816 (Echinodermata) were collected along the coast of Catania (Sicily, Italy) and transferred to the laboratory in aerated tanks with sea water (salinity: 35 psu, temperature range: 15°-18 °C) and immediately utilized as controls or for either bacterial challenge or osmotic-stress.

Bacteria injection

Escherichia coli (TB1) (Invitrogen) was reconstituted in LB broth (Luria-Bertani) (Sambrook, 1989) and grown overnight under constant agitation at room temperature (RT) as indicated by the supplier. The bacteria density in LB was quantified at the spectrophotometer by evaluating the optical absorbance at 550 nm. A 500 μl suspension of *E. coli* ($3x10^6$ living bacteria/ml) was injected into the perivisceral coelom through the peristomal membrane. The coelomic fluid taken from each specimen was examined 3 h and 24 h after injection. In order to discriminate between the effects of the injection and that of the bacterial challenge, controls for these experiments were performed by injecting an identical volume of sterile LB broth.

Osmotic stress

The sea urchins were placed in aquaria containing sea water mixed with distilled water (70:30) (salinity 24.5 psu). Sea water was well-aerated and maintained at a temperature varying between 15 °C and 18 °C. The sea urchins were collected 3 (short exposure) and 12 h (long exposure) after osmotic stress. The health of sea urchins was checked visually by monitoring if they tended to crawl up on the sides of the aquaria, as unhealthy animals usually remain on the bottom. Furthermore, healthy animals eat algal food avidly.

Coelomic fluid collection

The coelomic fluid was obtained from untreated, bacterial-challenged and osmotic-stressed animals by cutting a slit into the peristomal membrane and allowed to clot for 1 h at 0 °C and clotted proteins were discarded. The resulting supernatant (serum) was dialyzed against 50 mM Tris-HCl (pH 7.5) and stored at 0 °C or at -80 °C.

Purification of hemagglutinin

The hemagglutinin was purified according to Giga (1985), with minor modifications. Briefly, the dialyzed serum was applied to a DEAE-Sephadex (Sigma, St. Louis, MO, USA) column equilibrated with 50 mM Tris-HCl (pH 7.5). The column was washed with a serially increasing concentration of NaCl (0.5 M, 0.6 M and 0.7 M) diluted in 50 mM Tris-HCl (pH 7.5). Protein concentration was determined in all fractions according to the BCA Protein Assay Reagent (bicinchoninic acid) (Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin (BSA, ranging from 0.003 mg/ml to 0.125 mg/ml) as a standard with both spectrophotometry ND1000 (Nanodrop Technologies, USA) and Microplate Reader Opsys MR™ (ThermoLab system Franklin,NA, USA). After protein measurement, the hemagglutinating activity (HA) against human erythrocytes was analyzed in all fractions. Only those fractions displaying HA were retained for the experiments described below.

Gel electrophoresis

Density-gradient SDS-PAGE electrophoresis (4-12 %) was carried out in denaturing conditions according to Laemmli (1970). Before loading, the protein sample was denatured at 100 °C for 2 min in a solution of 1 % SDS Laemmli buffer. After protein separation (approximately 10 μg/lane), the gel was Silver-stained following, with slight modification, the protocol of Mortz *et al.* (2001). The Multimark-Multi-Colored Standard (1X) (Invitrogen, Carlsbad, CA, USA) was used as standard MW.

Assay of HA with whole and trypsinized erythrocytes

HA assay of the dialyzed serum was performed as indicated by Giga *et al.* (1985). Samples of human blood groups A, B and 0, were washed three times by centrifugation at 500x*g* for 10 min in Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl,

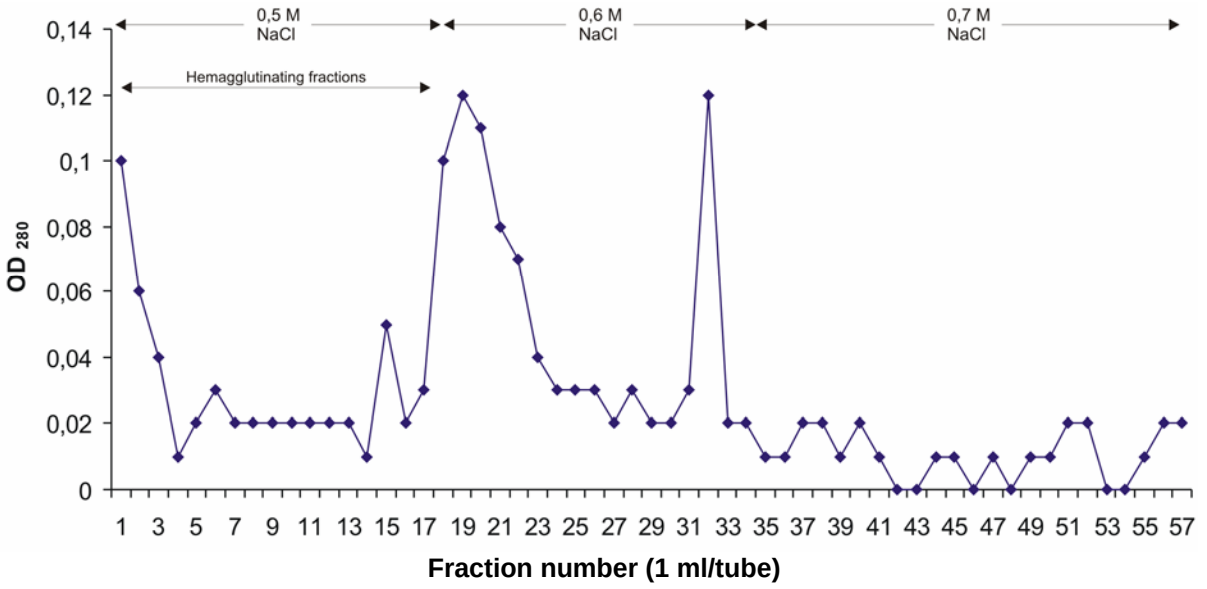

Fig. 1 Pattern of column chromatography of the whole coelomic fluid of *P. lividus*, displaying the fractions (from fraction 2 to fraction 18) with hemagglutinating activity.

Table 1 Effect of different sugars on hemagglutining activity of *P. lividus* hemagglutinin

Sugar	50 mM	100 mM	200 mM
D-Glucose	+	-	-
Sucrose	-	-	-
D-Lactose	-	-	-
D-Melibiose	-	-	-
L-Rhamnose	++	-	-
D-Sorbitol	-	-	-
D-Arabinose	++	-	-
L-Fucose	+	-	-
D-Mannose	-	-	-
D-Galactose	-	-	-
N-Acetyl-D-Galactosamine	-	-	-
N-Acetyl-D-Glucosamine	+	-	-

+, Partial inhibition of the hemagglutinating activity
++, Total inhibition of the hemagglutinating activity
-, No hemagglutination

pH 8.0). HA assays were carried out on U-bottom microtiter plates (Corning Incorporated, Corning, NY, USA). Serial dilutions (from 1:2 to 1:10) of the serum were made in 25 µl of test buffer (50 mM Tris-HCl, 150 mM NaCl and 1 mM $CaCl_2$, pH 7.5). Twenty-five µl of a suspension of 3 % human erythrocytes in test buffer were then added to each well. After gently mixing, the microplates were allowed to stand for 1 h at RT and macroscopic agglutination was recorded. The maximum dilution causing hemagglutination was referred to as the hemagglutination titer. The HA assays were also performed with trypsinized erythrocytes, since it has been reported that proteolytic treatment increases the agglutinating activity. The removal of surface proteins gives the lectins greater accessibility to the receptors (Kapáček *et al*., 1993). The preparation of the erythrocytes followed the same procedure described above, but before tests, the red blood cells were incubated with 0.1 mg/ml trypsin in TBS for 30 min at 37 °C and then washed three times in TBS.

Hemagglutination inhibition assay

To assess their effects on the agglutination of trypsinized erythrocytes, the following sugars were assayed: D-Sorbitol, D-Glucose, D-Galactose, D-Mannose, *N*-Acetyl-D-glucosamine, *N*-Acetyl-D-Galactosamine, D-Galactose, L-Fucose, Sucrose, D-Lactose, D-Melibiose, L-Rhamnose and D-Arabinose. All sugars were purchased from Sigma (except L-Fucose and *N*-Acetyl-D-Glucosamine, which was obtained from United States Biochemical Corporation, Cleveland, OH, USA). Sugars were added to TBS to yield 0.4 M storage solutions. A total of 25 µl of hemagglutinating serum fractions were then added to an equal volume of various dilutions (50 mM, 100 mM, 200 mM) of carbohydrates in the wells of U-bottomed microtiter plates and incubated for 30 min at 37 °C. Erythrocyte suspension was then added, and after a further 60 min of incubation at 37 °C, the lowest carbohydrate concentrations able to inhibit agglutination were recorded.

The dependence of agglutinating activity on divalent cations was assessed by performing the hemagglutination assay in the presence of 1 and 2 mM EGTA or by adding 10 mM $CaCl_2$.

Results

Purification of hemagglutinin and evaluation of its HA

The different eluate fractions collected were tested with erythrocytes to highlight their agglutinatining capacity (Fig. 1). The HA of coelomic fluid from *P. lividus* was assayed against human erytrocytes A, B and 0, and no differences referable to the human blood groups were observed. The analysis of the protein content of the agglutinating fractions by means of SDS-PAGE revealed that HA was invariably related to the presence of a protein with an electrophoretic mobility corresponding to a MW of 13 kDa (Fig. 2).

Effect of divalent cations on HA

Experiments performed with trypsinized erythrocytes treated with EGTA and mixed with hemagglutinating fractions demonstrated that EGTA cannot influence HA activity. Moreover, the addition of Ca^{2+} to the serum did not affect the hemagglutination.

Effect of sugars on HA

HA against trypsinized human erythrocytes was inhibited by the presence of L-Rhamnose and D-Arabinose. Also D-Glucose, L-Fucose and *N*-Acetyl-D-Glucosamine showed weak inhibition. Conversely, the oligosaccharides, Sucrose, D-Lactose, D-Melibiose, D-Sorbitol, D-Mannose, *N*-Acetyl-D-Galactosamine and D-Galactose, did not inhibit the HA (Table 1).

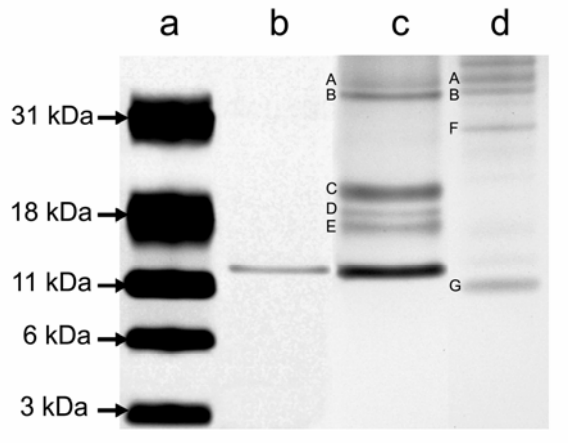

Fig. 2 SDS-PAGE of hemagglutinating fractions. Lane **b**) lectin in controls; lane **c**) 3 h after bacterial injection; lane **d**) 12 h after injection. Lane **a**) MW marker. Bands observed in treated sea urchins and not retrievable in controls were the followings: A= 34 kDa; B= 32 kDa; C= 21 kDa; D= 18 kDa; E= 16 kDa; F= 29 kDa; G= 11 kDa.

Effect of bacterial injection

Three h after the injection of the bacterial suspension, SDS-PAGE showed a band at 13 kDa of higher intensity than controls (Fig. 2). In the same agglutinating fractions, bands at approximately 16, 18, 21, 32 and 34 kDa were also evident, although with a lower intensity than the 13 kDa band. Surprisingly, 12 h after the injection, the band at 13 kDa was no longer visible, while the samples maintained the HA and displayed bands at 11, 29, 32 and 34 kDa (Fig. 2).

Osmotic stress

After a short exposure (3 h) to osmotic stress, all the hemagglutinating fractions showed six bands at a MW of approximately 11, 14, 26, 28 and 32 kDa after SDS-PAGE. The highest intensity was seen at bands 11, 14 and 32 kDa (Fig. 3). It should be noted that the 13 kDa band typical of unstressed samples was not present in the hemagglutinating fractions. Also, after long exposure (12 h) to osmotic stress, the SDS-PAGE analysis of the hemagglutinating fraction revealed the absence of the 13 kDa band retrieved in the controls. On the other hand, two bands of approximately 11 and 32 kDa were visible.

Discussion

The present investigation shows that in the coelomic fluid of the sea urchin *P. lividus* a 13 kDa band is present in all the hemagglutinating fractions. This observation let us to hypothesize that the band corresponds to a lectin able to agglutinate human red blood cells. The carbohydrate recognition domain of the protein can bind D-Glucose, L-Rhamnose, D-Arabinose, L-Fucose and *N*-Acetyl-D-Glucosamine that, in turn, are able to inhibit hemagglutination. It is well-known that erythroagglutination by hemagglutinins is generally inhibited by monosaccarides, which presumably are part of or closely related to saccaride receptor sites on the erythrocyte surface (Giga *et al*., 1985). We named the newly discovered lectin *Pl*SL (*Paracentrotus lividus* small lectin). The proposed name is based on the observation that *Pl*SL is smaller than the other lectin found in *P. lividus*, which has a MW of more than 200 kDa (Canicattì *et al*., 1992).

Such a difference in the molecular size of lectins belonging to the same organism is not new, at least not in echinoderms. Indeed, in the sea cucumber, *Stichopus japonicus*, lectins of 400, 60, 15 and 13 kDa have been reported (Hatakeyama *et al*., 1993; Matsui *et al*., 1994). Four lectins with an estimated MW of 27, 35, 45 and 68 kDa have been retrieved in *Cucumaria echinata* (Hatakeyama *et al*., 1994), whereas only one 44 kDa lectin was found in *Cucumaria japonica* (Bulgakov *et al*., 2000). In other echinoderms, lectins with a MW ranging from 220 kDa in the sea cucumber *Holothuria polii* (Canicattì and Rizzo, 1991) and starfish *Asterina pectibifera* (Kamiya *et al*., 1992) to 182 kDa in the sea cucumber *Holothuria scabra* (Gowda *et al*., 2008), have been recorded.

On the whole, these findings indicate that the echinodermal lectins are a very heterogeneous group, at least with regards molecular size, and therefore deserve a more detailed biochemical characterization.

While the HA of *Pl*SL is Ca^{2+}-independent, most echinodermal lectins are Ca^{2+}-dependent (Hatakeyama *et al*., 1993, 1994; Himeshima *et al*., 1994; Matsui *et al*., 1994; Hatakeyama *et al*., 1995; Bulgakov *et al*., 2000), and this is also true for the other lectin found in *P. lividus* (Canicattì *et al*., 1992). Interestingly, from *A. crassispina* eggs it has been identified a Ca^{2+}-independent lectin named SUEL, whose MW is 11.5 kDa and 23 kDa under reducing and non-reducing conditions, respectively (Ozeki *et al*., 1991). SUEL is suggested to be important for sea urchin development, but at present no definitive conclusions have been reported in this sense (Ozeki *et al*., 1995).

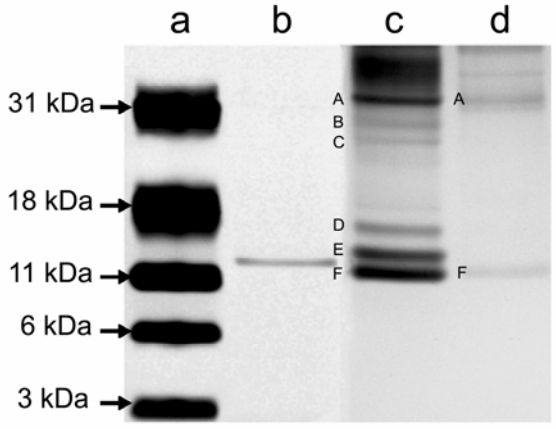

Fig. 3 SDS-PAGE of hemagglutinating fractions. Lane **b**) lectin in controls; lane **c**) 3 h after osmotic stress; lane **d**) 12 h after osmotic stress. Lane **a**) MW marker. Bands observed in treated sea urchins and not retrievable in controls were the followings: A= 32 kDa; B= 28 kDa; C= 26 kDa; D= 16 kDa; E= 14 kDa; F= 11 kDa.

As far as the role of *Pl*SL as a humoral component of the immune system of *P. lividus* is concerned, our data show the increase of the density of the13 kDa band 3 h after the injection with *E. coli.* Unexpectedly, *Pl*SL is no longer visible 12 h after the injection, even if the collected fractions maintain their hemagglutinating capacity. Similarly, in the osmotic stress assays, *Pl*SL is no longer visible either 3 or 12 h after injection, but the coelomic fluid of the sea urchin continues to agglutinate red blood cells. An increased HA has been observed as a consequence of bacterial challenge in the sea cucumber *H. scabra*, and it has been suggested that this finding is related to an increased expression in lectins (Gowda *et al*., 2008). Moreover, a relationship between lectins and bacterial clearance has been proposed (Gowda *et al*., 2008), indicating that data collected at different time points after injection of bacteria could present some discrepancies.

An interesting outcome of our experiments is that even if the band corresponding to *Pl*SL is no longer visible under most of the stressful conditions tested, the HA of coleomic fluid is maintained. When comparing the electrophoretic profiles of hemagglutinating fractions collected 12 h after bacterial injection or osmotic stress, it seems reasonable to impute the HA to the 11 and/or 32 kDa bands. Further studies are needed to verify if these proteins correspond to two new lectins, with slower synthesis, especially because it cannot be excluded that the 11 and 32 kDa bands represent post-traslational modifications in *Pl*SL.

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