Studies on *Holothuria polii* (Echinodermata) antibacterial proteins. I. Evidence for and activity of a coelomocyte lysozyme

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Received 7 November 1988; accepted 20 April 1989

Summary. Lysozyme activity has been detected in coelomocyte lysate of the echinoderm *Holothuria polii*. The bacteriolytic reaction was stable when the lysate was heated in acidic buffer but heat-labile in alkaline medium. An incubation temperature of 35 °C, acidic pH values (5.2 and 6.2) and an ionic strength of 0.175 were found to be the best conditions for the coelomocyte enzymatic activity. A low level of lysozyme was also evidenced in cell-free coelomic fluid where it could represent a basal defense level of bacteriolytic molecules released by the coelomocytes.
Key words. Echinoderm; *Holothuria polii*; coelomocytes; antibacterial proteins; lysozyme.

Antibacterial molecules have been described in many invertebrates[2,3]. Among them, the best-characterized antimicrobial agent is lysozyme. This enzyme is ubiquitous in the living world and much information has been acquired about its structure and biological role[4]. Several lysozymes have been characterized. They differ in various physico-chemical criteria such as amino acid sequence, molecular weight and enzymatic properties. Four different types have been recognized; lysozyme c, lysozyme g, lysozyme from bacteriophages and lysozyme from plants[4].

Among the invertebrates, lysozyme activity has been reported in annelids, insects, molluscs and echinoderms[4]. In the annelid *Eisenia fetida andrei*, a lysozyme has been isolated from the coelomic fluid of normal adults. Its low background activity can be enhanced by injection of both gram-positive and gram-negative pathogenic and non-pathogenic bacteria[5]. Enhancement of lysozyme activity has also been reported in insects[6,7] and molluscs[8,9]. In the echinoderms, lysozyme activity was found in the whole homogenate of the sea star *Asterias rubens*[10]. Complete analysis of the molecule indicated a mol.wt of 15,500 ± 1000 dalton with a long N-terminal sequence not related to other known N-terminal lysozyme sequences. However, the presence of lysozyme-like activity, or more generally of antibacterial molecules, has not been explored in the echinoderm *Holothuria polii*, though protective responses have been described. *H. polii* responds to antigenic stimulation with a number of reactions, of which phagocytosis is the most evident[11]. Phagocytosis is mediated by amoebocytes which are also hemoylsin producer cells[12]. These molecules are active lytic proteins with peculiar characteristics[13], probably involved in the clearance of not-self material[11,14]. Because of the important defensive role of *H. polii* coelomocytes, we hypothesized that they could be a site of synthesis and/or storage of antibacterial proteins. We therefore investigated the presence of antibacterial substances in *H. polii*. The present report shows evidence for the presence of a lysozyme, and describes its activity.

Materials and methods

Adult specimens of *Holothuria polii* were collected from Porto Cesareo (Lecce). The coelomic fluid from 10 animals was obtained by longitudinal incision of the body wall, then pooled and centrifuged at 400 × g for 30 min at 4 °C to remove the cells (cell-free coelomic fluid). The pelleted coelomocytes were gently washed 5 times in TRIS-NaCl 0.05 M, pH 8.0, resuspended in 20 ml of distilled water and sonicated in a Branson Sonifier model B-15P until complete lysis was observed (4 min). The sonicate was centrifuged at 12,000 × g for 1 h at 4 °C and the supernatant collected, divided into 2-ml aliquots and stored at − 20 °C (coelomocyte lysate). Aliquots of cell-free coelomic fluid (protein content about 0.09 ± 0.045 mg/ml) were concentrated 10–50-fold by lyophilization. The concentrated samples were then extensively dialyzed against phosphate buffer (PB) 0.05 M, pH 5.2, before testing lysozyme activity.

Protein concentration was determined by Lowry's method[15] with bovine serum albumin as reference standard. Lysozyme activity was determined by recording spectrophotometrically at 584 nm the decrease of absorbance occurring during the lysis of a suspension of *Micrococcus luteus* cell walls (Sigma). 10 µl of a suspension of *M. luteus* (5 mg/ml) in PB 0.05 M, pH 5.2, were added to 800 µl of coelomocyte lysate or cell-free coelomic fluid. The mixtures were placed at 35 °C and the absorbance measured after 45 min. Alternatively, a standard assay on a petri dish was used. Briefly, 700 µl of 5 mg/ml of *M. luteus* were diluted in 7 ml PB agarose (pH 5.2) then spread on a petri dish. When the agarose had solidified, 4-mm diameter wells were sunk and filled with 30 µl of coelomocyte lysate or cell-free coelomic fluid. Lysis diameters were measured after overnight incubation at 35 °C.

Thermal inactivation, and dependence on pH, ionic strength and temperature were the physico-chemical parameters tested. Coelomocyte lysate or cell-free coelomic fluid were extensively dialyzed against PB

0.05 M (I = 0.175) pH 4.5, or PB 0.05 M (I = 0.175) pH 8.5, before thermal treatment. Samples of both dialysates were heated for 5 min at 100 °C, and the pH was then adjusted to 5.2 by overnight dialysis against PB 0.05 M (I = 0.175) at 4 °C, before spectrophotometric or petri dish assays. pH-dependence was tested in PB 0.05 M (I = 0.175) at the pH values 5.2, 6.2, 7.2 and 8.2. Dependence on ionic strength was tested in PB (pH 6.2) at I = 0.0175, 0.175 and 1.75. The effect of incubation temperature on lytic activity of the lysate was tested at 5, 15, 22 and 35 °C.

Results

As demonstrated by the standard assay in petri dishes (fig. 1 A), an activity able to lyse *M. luteus* cell wall was present in the coelomocyte lysate of *H. polii*. After overnight incubation at 35 °C, a clear ring of lysis 12 mm in diameter was observed. With lysate which had been heated at 100 °C for 5 min in pH 4.5 medium, a similar clear ring of lysis (diameter 11 mm) was produced. When the lysate was heated in pH 8.5 medium, no ring of lysis appeared. No lytic activity was observed in normal cell-free coelomic fluid (protein content 0.09 ± 0.045 mg/ml) nor in 10-fold concentrated fluid. However, when 50-fold concentrated cell-free coelomic fluid was used, a ring of lysis of 10 mm in diameter was detected, though in this case, the lytic area was unclear, with edges not well defined (fig. 1 B). An equivalent diameter of lysis was produced by 200 µg/ml of hen eggwhite lysozyme used as standard. In some individuals the coelomic fluid protein content was higher (0.200 mg/ml), and this fluid gave a ring of lysis of about 5.6 mm in diameter.

Following the absorbance at 584 nm of a solution of *M. luteus* cell wall incubated with coelomocyte lysate showed that the lysis was almost complete (63 % of lysis) after 45 min at 35 °C. The kinetic curve then became inflexed, and complete lysis (97 %) occurred after 120 min (fig. 2).

Measurements after 45 min incubation at normal environmental temperatures of 15 and 22 °C showed a slight decrease in absorbance, 38 % and 35 % respectively (fig. 3). A similar result was obtained at 5 °C (28 %). On the other hand, incubating the reaction mixture at 35 °C resulted in a substantial (67 %) decrease of absorbance. No further increase of lysozyme activity was registered at 45 °C.

As depicted in figure 4 a, the lytic activity of the coelomocyte lysate was strictly dependent on the pH of the incubation medium. After 45 min, at acidic pH values of 5.2 and 6.2 the initial (0 min) absorbance decreased by 60 % and 53 % respectively. At the neutral pH value of 7.2, the *H. polii* coelomocyte lysate produced a decrease of absorbance of about 40 %; whereas at the basic pH 8.2, only a decrease of 25 % was registered.

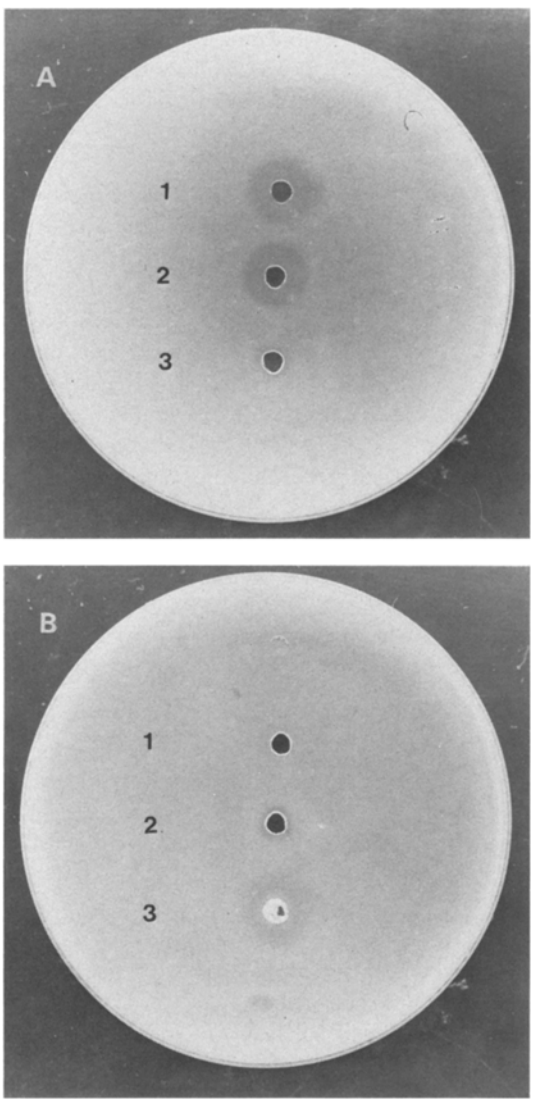

Figure 1. Standard assay of lysozyme activity in petri dish. *A* 40 µl of normal (1), heated in PB 0.05 M, pH 4.5 (2) and heated in PB 0.05 M, pH 8.5 (3) coelomocyte lysate. *B* 40 µl of normal (1), 10- (2) and 50- (3) fold concentrated cell-free coelomic fluid.

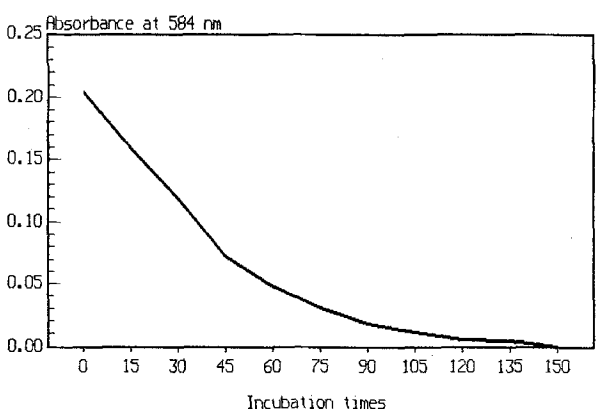

Figure 2. Kinetics of the lytic activity of *Holothuria polii* coelomocyte lysate at constant pH (5.2) and ionic strength (I = 0.175). The activity is recorded as decrease of absorbance at 584 nm of 10 µl of *M. luteus* suspension (5 mg/ml) in 0.8 ml of coelomocyte lysate.

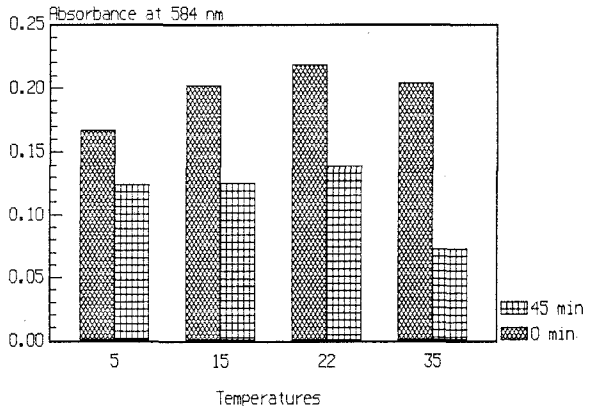

Figure 3. Effect of incubation temperature on the bacteriolytic activity of coelomocyte lysate. Activities recorded as initial absorbance (0 min) and after 45 min incubation (45 min) at pH 5.2 and I = 0.175. Mean values with SEM below 3.1; n = 3.

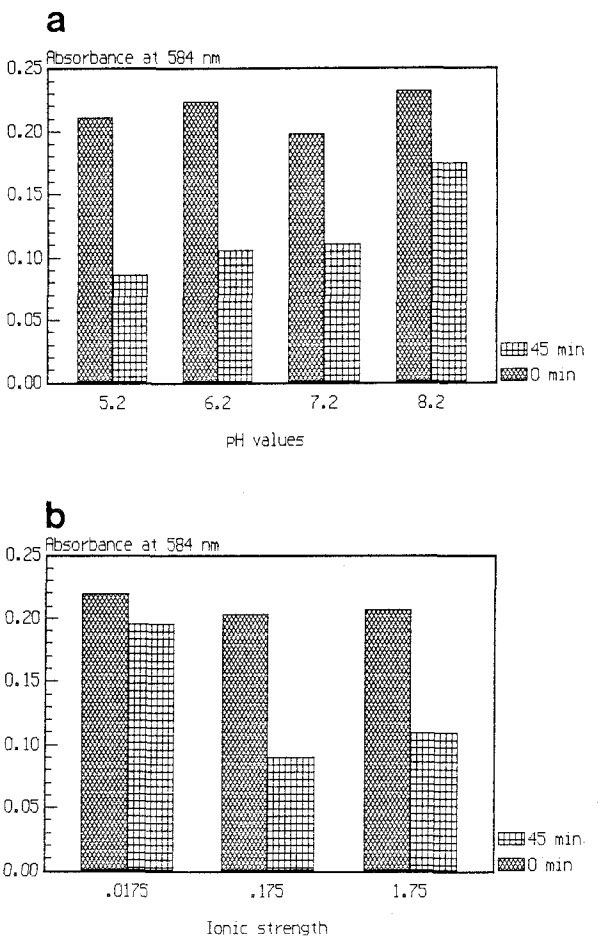

Figure 4. Effect of pH (*a*) and ionic strength (*b*) on the bacteriolytic activity of coelomocyte lysate. Activities recorded as initial absorbance (0 min) and after 45 min incubation (45 min) at 35 °C. Mean values with SEM below 3.1; n = 3.

Coelomocyte lysate was almost non-lytic in the absence of salt (fig. 4b). After dialysis against PB at low ionic strength (I = 0.0175), the bacteriolytic activity was as low as 11%. The maximum activity (59%) was attained at a 10-fold increased ionic strength (I = 0.175); a further 10-fold increase of the ionic strength (I = 1.75) produced a slightly lower activity (49%).

Discussion

An examination of the literature on lysozyme gives the following properties for this enzyme; it is a basic protein of low molecular weight, heat-stable at acidic pH, labile at alkaline pH, and able to lyse a suspension of *M. luteus* cell wall. From the experiments reported here, it can be concluded that the lytic activity of *H. polii* coelomocyte lysate towards *M. luteus* depends on a lysozyme-like enzyme, stable when heated at 100 °C in acidic pH but unstable when heated in alkaline medium. In contrast to hen eggwhite lysozyme, in which 2 pH optima of 6.2 and 7.5–8.0 were found[16], the *H. polii* lysozyme presents a maximum activity only at an acidic pH (5.2–6.2). It has been well established for hen eggwhite lysozyme[17] that the ionic strength of the assay buffer markedly influences the activity of lysozyme towards an *M. luteus* cell suspension. Similar results were obtained in *Asterias rubens*[10] but not in *Nephthys hombergi*[16]. In *H. polii*, we found that a buffer with an ionic strength of 0.175 was the best medium to obtain lytic activity. At a lower ionic strength (I = 0.0175) very low activity occurred. This may be a result of modification of the electrical characteristics of the proteins. As suggested for hen eggwhite lysozyme, the lysis of *M. luteus* could be mediated by electrostatic forces responsible for the binding of *H. polii* lysozyme on the surface of the bacteria. Favorable electrostatic interactions depend on appropriate pH and ionic strength[17]. In this respect, amino acid composition plays a prominent role in the stability of the molecules. Also, a direct relationship exists between the stability of lysozyme and the number of disulfide bonds cross-linking the molecules[18].

The temperature of incubation influenced the activity of *H. polii* lysozyme. Surprisingly, the bacteriolytic activity, tested at temperatures in which the animals usually live (15–22 °C), was not as high as at 35 °C. The best temperature of incubation was similarly found to be 37 °C for another kind of biological activity, hemolytic activity[19]. In both cases, this phenomenon could depend on the in vitro stability of the molecules.

Of interest is the observation that coelomocytes contain lysozyme activity. This is in agreement with similar findings in other invertebrates. In molluscs, lysozyme is present in phagocytes that, after antigenic stimulation, release it into the serum[8,9]. In the insect *Locusta migratoria*, two types of granular hemocytes are the sites of synthesis and storage of lysozyme[20]. Both cells are associated with defense mechanisms, either directly by encap-

sulation of foreign substances or through coagulation processes. Although the exact role of the *H. polii* lyzosyme is still unknown, the observed level in coelomocytes, the cells involved in defensive processes[11], substantiates the hypothesis that the enzyme could participate in antibacterial defense mechanisms.

Lysozyme activity was also detected in *H. polii* cell-free coelomic fluid, though it was evident only when 50-fold concentrated coelomic fluid was used. This suggests that a low level of enzyme is naturally released by coelomocytes, probably to supply a basic defensive concentration of the antibacterial substance. As demonstrated by Cheng[8], lysozyme occurs in both serum and cells of *Mercenaria mercenaria*. The enzyme is normally released by the cells, but its hemolymph content is enhanced during phagocytosis[8,9]. As yet, we do not know whether any change in the lysozyme level of cell-free coelomic fluid occurs after injection of micro-organisms. It is possible that, as in other invertebrates[5–9], the holothuroid bacteriolytic enzyme could be enhanced by injections.

1 Acknowledgments. This work was supported in part by a grant from MRES (C. C.). The authors thank M. Lasségues and F. Lassalles for their help and criticism.

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0014-4754/89/080756-04$1.50 + 0.20/0

Necessity of adjuvants for inducing effective antibody response to zona pellucida antigens

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Received 17 January 1989; accepted 30 March 1989

Summary. Rhesus monkeys evoked a vigorous antibody response to a single heteroimmunization dose of zona pellucida antigens, when these were administered along with complete Freund's adjuvant. The antisera recognized all the three major porcine zona glycoprotein families and the animals were rendered amenorrheic after such immunization. Monkeys immunized with zona without adjuvant, however, failed to show any anti-zona antibody response and had normal menstrual cycles. Zona pellucida glycoproteins are thus not effective immunogens unless administered along with a powerful adjuvant.
Key words. Zona pellucida glycoproteins; adjuvant; immunoblotting; enzyme-linked immunosorbent assay; autoantibodies; infertility.

Zona pellucida (ZP), the extracellular glycoprotein coat enveloping the mammalian oocyte, has been the subject of extensive investigations as a candidate for immunological fertility control. A number of studies have emphasized the strong antigenicity and immunogenicity of this structure, and recently, studies on ZP have gained added importance since it has been established beyond doubt that active heteroimmunization with ZP glycoproteins results in the production of anti-zona antibodies which can inhibit fertility in many species[1–4]. The porcine ZP in particular has become the focus of such research owing to its cross-reactivity with human ZP and ready availability.

Autoantibodies to ZP have also been implicated in the development of infertility in women. Several studies employing immunofluorescence methods have demonstrated anti-ZP antibodies in the sera of infertile women[5,6]. Similar autoantibodies were also found in the sera of aging women as well as aging animals[6,7]. These studies led to the conclusion that such an autoimmune phenomenon might occur naturally in aging animals.

It has been postulated that breakdown products of ovum and zona may initiate an immune response due to their accessibility to the immune system for a long period[8]. Since zona is formed rather late in ontogeny, it may be recognized as foreign. Some reports dismissed this hy-