Comp. Biochem. Physiol. Vol. 106A, No. 2, pp. 271–275, 1993
Printed in Great Britain

0300-9629/93 $6.00 + 0.00

HAEMOLYTIC ACTIVITY IN THE BROWN SHRIMP (*PENAEUS CALIFORNIENSIS* HOLMES) HAEMOLYMPH.

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(*Received* 23 *November* 1992; *accepted* 15 *January* 1993)

Abstract—1. Natural haemolytic activity in brown shrimp (*Penaeus californiensis*) haemolymph was detected using mouse erythrocytes as target cells. This activity is unrelated to agglutinating and phenoloxidase activity, but it is another probable component of the shrimp defence system.
2. The haemolytic reaction is time and dose dependent, and a serine–protease is involved.
3. The haemolytic factor is thermolabile and has an apparent molecular weight of 23.5 kDa.

INTRODUCTION

Haemolytic activities have been demonstrated in the haemolymph of many classes of invertebrates including echinoderms (Canicatti *et al.*, 1987; Canicatti, 1988; 1991), mollusks (Bertheussen, 1983; Anderson, 1981; Leippe and Renwrantz, 1988), annelids (Roch *et al.*, 1981; Tuckova *et al.*, 1986; Kauschke and Mohrig, 1987) and arthropods (Weinheimer *et al.*, 1969). Bertheussen (1984) hypothesized that the invertebrate lytic system is analogous to the vertebrate complement system; however, the crucial step of lytic activation was not considered. The complement system of the vertebrates closely resembles the prophenoloxidase (proPO) system of the invertebrates because both are enzymatic cascade reactions involving proteolytic cleavage, serine–proteases, opsonin generation and have Ca^{2+} requirements. Nevertheless, lytic activity has not been detected in the proPO system.

Haemolytic activity can be induced by wounding (Anderson, 1981) or injecting erythrocytes into the animal (Canicatti and Parrinello, 1985). The haemolytic system of *Eisenia foetida andrei*, for example, plays a destructive role in second graft rejection (Roch, 1979) and was also found to have antibacterial properties (Valembois *et al.*, 1982). These responses, nevertheless, vary from animal to animal in their activities and biochemical characteristics, and their role in the immune response of the animal remains unclear.

In this work, we report on the natural haemolytic activity of the haemolymph of the brown shrimp (*Penaeus californiensis*). We describe their main properties, which could help to explain the role of this activity in the shrimp immune system.

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MATERIALS AND METHODS

Haemolymph extraction

Shrimp (*Penaeus californiensis*) of both sexes were collected from San Carlos Bay, B.C.S., México, and maintained in an aquarium (24 ± 4°C, salinity 36‰). Only the haemolymph from intermoult, apparently healthy shrimp was used. To avoid coagulation, we used 2–3 parts of anticoagulant solution (Vargas-Albores, 1992): 450 mM NaCl, 10 mM KCl, 10 mM EDTA.Na_2, 10 mM HEPES, pH 7.3, 850 mOsm/Kg. This solution had pH, osmolality, sodium and potassium concentration equivalent to the shrimp haemolymph (Vargas-Albores and Ochoa, 1992). The haemolymph (50–200 μl) was obtained from the pleopod base of the first abdominal segment. The pooled samples were centrifuged for 10 min (10°C) at 2000 *g*. The cell pellet was discarded and the supernatant (plasma) was frozen until use. The shrimp plasma was dialysed overnight against TBS-Ca (Tris buffered saline: 50 mM Tris–HCl, 100 mM NaCl, pH 7.5, plus 10 mM $CaCl_2$), and clarified by centrifugation at 40,000 *g* (20 min, 10°C).

Chemical Analysis

The total protein content was measured according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Haemolytic assay

The mouse blood was obtained by cardiac puncture, then collected and stored in sterile Alsever's solution. Before use, the cells were washed twice by centrifugation (800 *g*, 10°C, 10 min) with saline solution (NaCl 0.15 M) and twice with TBS-Ca. Finally, the red blood cells (RBC) were re-suspended to 8% (v/v) in TBS-Ca. The haemolytic assays were performed by mixing 850 μl of TBS-Ca, 100 μl of 8%

mouse erythrocytes and 50 μl of haemolymph sample. The mix was incubated at 25°C for 30 min before removing the cellular pack by centrifugation in a microfuge (Eppendorf) for 30 sec. The released haemoglobin was measured spectrophotometrically at 540 nm. The control used buffer instead of haemolymph. The 100% haemolysis tube was prepared by adding 900 μl of ammonium chloride (0.15 M) to 100 μl of mouse erythrocytes (8%). The results are expressed as percentage of haemolysis.

Effect of temperature

Samples of haemolymph were incubated at 25, 37, 56, or 70°C, and the haemolytic activity was monitored at different times.

Inhibition by PMSF

The haemolymph (100 μl) was incubated with 10 μl of 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature before determining their haemolytic activity.

Gel filtration

The apparent molecular weight of the haemolytic activity was estimated by gel filtration performed at 18°C on a column (1.6 × 85 cm) of Sephacryl S-200 (Pharmacia-LKB Biotech, Sweden) in TBS–Ca buffer. The column was calibrated with catalase (240 kDa), BSA (67 kDa) and cytochrome *c* (12.5 kDa). The exclusion volume (Vo) was determined by dextran blue (Pharmacia-LKB Biotech, Sweden) and fractions of 1 ml were collected. The protein fractions were detected by the Bradford method (Bradford, 1976), and the haemolysin activity by the haemolytic assay described above using mouse erythrocytes. The fractions with haemolytic activity were also tested for haemagglutinating activity against mouse erythrocytes, as described elsewhere (Vargas-Albores *et al.*, 1993).

Phenoloxidase activity

The phenoloxidase activity was measured spectrophotometrically (in triplicate) by recording the formation of dopachrome from L-dihidroxyphenylalanine (L-DOPA) according to (Leonard *et al.*, 1985). The cell suspension (100 μl) was pre-incubated with 50 μl of laminarin (1 mg/ml in cacodylate buffer) or, in the case of controls with cacodylate buffer, for 1 hr at 20°C before 50 μl of L-DOPA (3 mg/ml of cacodylate buffer) were added. The reaction was allowed to proceed for 10 min at 20°C, then 800 μl of cacodylate buffer were added and the absorbance at 490 nm was measured. Enzyme activity was expressed as the change in absorbance at 490 nm/min/mg of protein.

Haemocyte lysate

The haemolymph from 10 shrimps was collected in Shrimp Salt Solution (SSS) as described above. The cell pellets were pooled and re-suspended in cacodylate buffer, then sonicated for 2 min at 20 watts. The haemocyte lysate was centrifuged at 40,000 *g* for 30 min at 10°C, and the cell debris was separated from the supernatant. Both haemolytic and proPO activity were determined in the supernatant.

RESULTS

Because coagulation is a fast process in shrimp and other crustaceans (Durliat and Vranckx, 1981), it is necessary to use an anticoagulant solution to obtain a cell-free plasma. Although one anticoagulant solution has been used previously to obtain haemocytes from marine crustacea (Smith and Söderhäll, 1983), this was not useful for our shrimps because precipitation of plasmatic protein was observed (Vargas-Albores, 1992). From earlier data on sodium concentration, potassium concentration, pH and osmolality of shrimp haemolymph (Vargas-Albores and Ochoa, 1992), an anticoagulant solution was designed and tested. With this solution the haemolymph (50–200 μl) was obtained without protein precipitation or coagulation. The integrity of the cells was verified by microscopic observation to ensure that no cell contamination was present in our plasma preparation.

Since the osmolality of the shrimp haemolymph is higher than the isotonic point for erythrocytes, and because calcium ions are depleted by the anticoagulant, it was necessary to dialyse the shrimp plasma against TBS-Ca before using it in haemolytic tests. For the dialysis, some proteinaceous material without haemolytic activity was precipitated.

Cell-free haemolymph from sub-adult brown shrimp (*P. californiensis*) was found to cause lysis of mouse erythrocytes in a tube assay. Different volumes (0–200 μl) of haemolymph were mixed with TBS–Ca to a final volume of 900 μl before adding 100 μl of

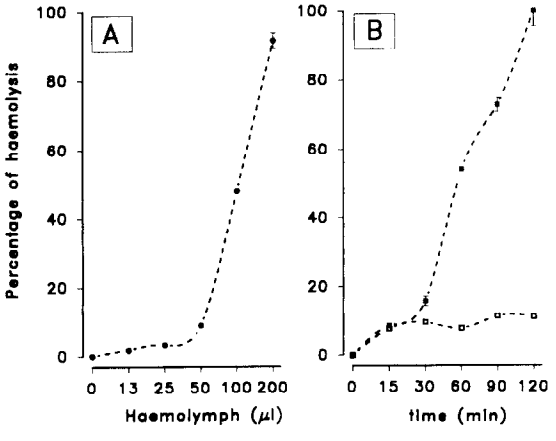

Fig. 1. Direct haemolytic activity detected in the Brown shrimp haemolymph and its correlation with dose (A) and incubation time (B). Tubes containing buffer instead of haemolymph were included to determine the spontaneous haemolysis (open square). Each point was determined in triplicate and represents the mean ±SE.

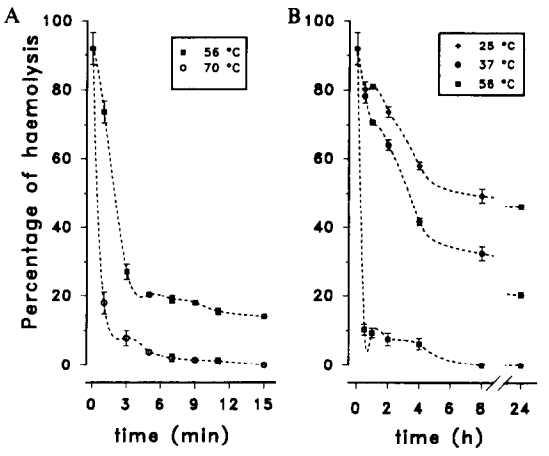

Fig. 2. Effect of temperature on the haemolytic activity of the brown shrimp haemolymph. Each point was determined in triplicate and represents the mean ± SE.

mouse erythrocyte (8%) and determining the haemolysis percentage. The results in Fig. 1A show that the haemolysis increased proportionally to the amount of haemolymph. In the same way, this reaction was found to be time dependent (Fig. 1B), as determined by incubating 50 μl of haemolymph and monitoring the haemolysis at different times (0–120 min). The spontaneous haemolysis which occurred in the control was also monitored (Fig. 1B). The effect of the serine–protease inhibitor, PMSF, was assayed in the haemolytic system. Only 32 ± 2% of the original activity was detected after the haemolymph was incubated with PMSF. This serine–protease inhibitor alone, only reduced the haemolytic activity in 2.0%. Thus, nearly 65% of this activity was lost in the presence of PMSF, suggesting that a serine–protease is involved in the haemolytic reaction.

The temperature lability of the haemolytic factor was investigated by incubating, at different times, samples of haemolymph at 25, 37, 56 and 70°C. As observed in Fig. 2A, the haemolytic activity was totally abolished after 3–5 min of incubation at 70°C. Although 8 hr were necessary for complete inactivation at 56°C, 20% of the original activity was detected after 5 min of incubation, indicating the sensitivity of the haemolytic factor to temperature. At other temperatures tested (25 and 37°C), changes in the activity were also observed. After 24 hr of incubation, 20% of the original activity was detected at 37°C, but at 25°C, the remaining activity was 50%. Under refrigeration (10°C) and freezing conditions (−5°C), no significant changes in activity were detected, for as long as 1 month.

In order to determine the apparent molecular weight of the brown shrimp haemolytic factor, 500 μl of the cell-free haemolymph from brown shrimp were fractionated by gel filtration on a Sephacryl S-200 column (1.6 × 85 cm). The major portion of haemolymph proteins was detected (280 nm) in, or near, exclusion volume (Fig. 3). Haemolytic activity was detected in the zone with a molecular weight of less than 50 kDa. According to the molecular weight markers used, the active fraction was of 23.5 ± 4 kDa. The ve/vo values of marker proteins were: catalase, 1.13; BSA, 1.36; cytochrome *c*, 1.89; and haemolytic factor, 1.70.

When the shrimp haemocyte lysate was obtained by sonication, centrifugation, and subsequent separation of the supernatant, only PO activity was detected, indicating that the haemolytic activity is not in the haemocytes and consequently not associated with the proPO system.

DISCUSSION

The direct lytic action of invertebrate lysins has received less attention than the cellular immune defence functions such as: opsonization, chemotaxis and activation of haemocytes. Consequently, there are few reports about lysis, and the physiological significance of haemolytic activities (cellular and humoral), therefore the defence repertoire of invertebrates is still unclear. Although non-proteinaceous haemolytic compounds such as saponins have been described in invertebrate fluids (Nigrelli *et al.*, 1967; Nigrelli and Jakowska, 1960; Cenini, 1983), the most important activity seems to be associated to proteins. These proteins represent one of the several cellular and humoral mechanisms that might damage intruding or abnormal cells (Ratcliffe *et al.*, 1985). A recent report has shown that haemocytes of the mussel *Mytilus edulis*, release a potent cytotoxin that produces lysis to mammalian erythrocytes (Leippe and Renwrantz, 1988). In *Galleria mellonela*, haemolytic activity appears to be a part of the cell-free haemolymph and closely associated with the immune response of insects (Phipps *et al.*, 1989). Similarly, in

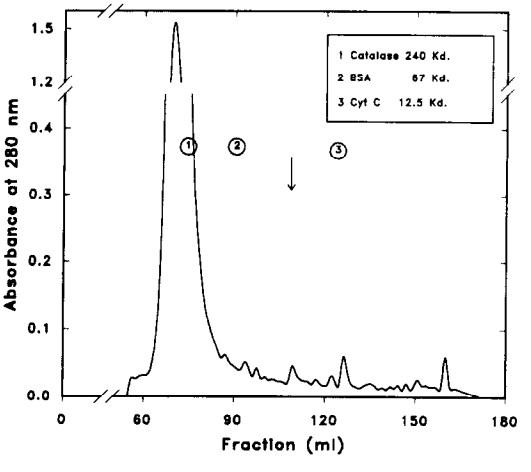

Fig. 3. Gel filtration of the *Penaeus californiensis* haemolymph on Sephacryl S-200. Protein profile was obtained from the absorbance of the individual fractions at 280 nm. Haemolytic active fractions were detected by haemolytic assay. The protein markers were detected by Bradford.

the echinoid *Spherechinus granularis*, a lytic factor has been described (Stabili *et al.*, 1992) which is capable of disrupting erythrocytes and malignant cells (mouse mastocytome P815, human leukaemia K562 and mouse fibrosarcoma WEHI 164/B).

The cell-free haemolymph of the brown shrimp (*Penaeus californiensis*) contains haemolytic activity against mouse erythrocytes. This activity was still detected after exhaustive dialysis against TBS–Ca, the haemolytic activity was not due to osmotic or ionic effects. The reaction is time and dose dependent, suggesting the participation of an enzyme. Furthermore, the haemolytic reaction in brown shrimp haemolymph involves a thermolabile molecule (Fig. 2), similar to the vertebrate complement system (Cooper, 1978). Other thermosensitive haemolytic factors have been isolated from invertebrates like *Eisenia foetida* (Andrews and Kukulinsky, 1975), although Cenini (1983) reported a thermostable factor from the same animal. A thermolabile lysin, which is also opsonic, has been described in sea urchin coelomic fluid (Bertheussen, 1983). Thus, invertebrate haemolytic activity may be ascribed to different compounds with varying molecular properties and mechanisms.

The brown shrimp haemolytic activity is inhibited by PMSF, suggesting that a serine–protease is involved. Serine-proteases are involved in other invertebrate physiological reactions such as coagulation (Durliat and Vranckx, 1981) and proPO activation (Johansson and Söderhäll, 1989). Since in brown shrimp, the proPO system is located in the cells (Vargas-Albores, 1992), it is unlikely that this haemolytic factor is part of the proPO system. Furthermore, when using the adequate anticoagulant solution to prevent cell activation (Vargas-Albores, 1992), phenoloxidase activity was not found in the cell-free haemolymph used in the haemolytic assays. In addition, the shrimp haemocyte lysate showed phenoloxidase, but not haemolytic activity.

Haemagglutinating activity is also related to the defence system of the brown shrimp, and is found in the free-cell haemolymph (Vargas-Albores *et al.*, 1993). This LPS-binding agglutinin was retained in a *N*-acetyl galactosamine–agarose column and had a molecular weight of 180 kDa. The haemolytic factor is not related to the serum agglutinin because it is not retained by the affinity column and has an apparent molecular weight of 23.5 kDa, as determined by gel filtration. The haemolytic fractions obtained from gel filtration also showed no haemagglutinating activity.

In summary, for the recognition and elimination of foreign particles, the haemolymph of the brown shrimp (*Penaeus californiensis*) has several molecular elements such as a haemagglutinin (Vargas-Albores *et al.*, 1993), a proPO system (Vargas-Albores, 1992), and haemolytic activity (this study), all of which could help to explain the efficient defence system of this penaeid.

Acknowledgements—We are grateful to Dariel Tovar for technical assistance, and to Roy Bowers for a grammatical review.

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