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AN ANTICOAGULANT SOLUTION FOR HAEMOLYMPH COLLECTION AND PROPHENOLOXIDASE STUDIES OF PENAEID SHRIMP (*PENAEUS CALIFORNIENSIS*)

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Abstract—1. An anticoagulant solution was designed from data on osmolality, ionic concentration and pH to resemble shrimp haemolymph.

2. This Shrimp Salt Solution (SSS) prevents coagulation and prophenoloxidase system activation during the extraction of shrimp haemolymph.

3. The location of the the proPO system in the brown shrimp (*Penaeus californiensis*) was determined using this anticoagulant solution.

INTRODUCTION

Research into arthropod defence mechanisms has been growing because many insects are vectors for pathogens of man and livestock, and because of the increasing economic importance of crustacean aquaculture. The prophenoloxidase (proPO) system is a constituent of this immune system and is probably responsible, at least in part, for the non-self recognition process of the defence mechanism in crustaceans and insects (Smith and Söderhäll, 1983a; Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985a). Phenoloxidase (PO, *O*-diphenol-oxygen oxidoreductase; E.C. 1.10.3.1), the major enzyme produced during the activation of this system, is necessary for the melanization process observed in response to foreign matter invading the haemocoele and during wound healing. In all arthropod species studied to date, phenoloxidase is present in the haemolymph as an inactive proenzyme called prophenoloxidase (proPO), located in the haemocyte of many insects (Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985a, b; Fisher and Brady, 1983; Dularay and Lackie, 1985; Iwama and Ashida, 1986; Brookman and Ratcliffe, 1989; Rowley and Rahmet-Alla, 1990) and crustaceans (Söderhäll *et al.*, 1979, 1986; Söderhäll and Smith, 1983; Johansson and Söderhäll, 1985). However, this enzyme has also been detected in the plasma of some insects (Ashida, 1981; Sugumaran *et al.*, 1985; Saul *et al.*, 1987; Saul and Sugumaran, 1988; Brehelin *et al.*, 1989). The proPO system is specifically activated by β 1,3-glucans (Söderhäll and Unestam, 1979; Ashida *et al.*, 1983), bacteria cell walls (Pye, 1974; Ashida *et al.*, 1983), and lipopolysaccharides (Söderhäll and Häll, 1984).

For the collection of marine crustacean haemolymph, Söderhäll and Smith (1983b) proposed an anticoagulant solution which contains glucose, sodium citrate, citric acid, NaCl and EDTA. This citrate–EDTA solution has been widely used in studies on the proPO system to determine its location, its activation and to isolate involved proteins (Ashida *et al.*, 1983; Johansson and Söderhäll, 1985, 1988; Saul *et al.*, 1987; Söderhäll *et al.*, 1986). To obtain insect haemolymph, another solution has been used (Leonard *et al.*, 1985b) which is based on the same formula (citrate-EDTA), but with a lower NaCl concentration, thus reducing the osmolality. For example to extract haemolymph from *Blaberus*, the osmolality was adjusted to 370 mOsm/kg; for *Clitummus*, to 440 mOsm/Kg; and for *Locusta* and *Schistocerca*, to 400 mOsm/kg (Brookman and Ratcliffe, 1989). However, if haemolymph from the shrimp species *Penaeus stylirostris* or *P. californiensis* is collected using the original citrate–EDTA solution proposed by Söderhäll and Smith (1983), precipitation of some proteins is observed at less than 15°C (Vargas-Albores, 1992). This phenomenon does not occur at 25°C, but in order to minimize cell activation, 10–15°C is the preferred working temperature.

To resolve this problem, the ionic, osmotic and pH characteristics of the *Penaeus* haemolymph was determined (Vargas-Albores, 1992; Vargas-Albores and Ochoa, 1992), and a solution was designed to simulate these characteristics. In this paper we discuss the application of this anticoagulant solution, called SSS (Shrimp Salt Solution), which prevents protein precipitation and preserves cell viability during the collection of haemolymph from the brown shrimp *Penaeus californiensis*. In addition, we test the efficiency of SSS by using it to locate the prophenoloxidase system in this same species.

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MATERIALS AND METHODS

All chemicals were purchased from the Sigma Chemical Co. (St Louis, MO). The glassware was washed with Toxa-Clean (Sigma) to avoid the spontaneous activation of the proPO system by endotoxins. For the same reason, all solutions were prepared using pyrogen-free water.

Solutions

The anticoagulant citrate–EDTA solution for the collection of marine invertebrate haemolymph (100 mM glucose, 30 mM trispodium citrate, 26 mM citric acid, 510 mM NaCl and 10 mM EDTA.Na_2; pH = 4.6) was prepared according to Söderhäll and Smith (1983). From this, two solutions with different pHs (5.6 and 6.6) were prepared by adding NaOH to modify the citrate–citric acid ratio. The shrimp salt solution (SSS) was prepared to correspond to the ionic and osmotic values of shrimp haemolymph (Vargas-Albores, 1992; Vargas-Albores and Ochoa, 1992): 450 mM NaCl, 10 mM KCl, 10 mM EDTA.Na_2, 10 mM HEPES, pH 7.3, 850 mOsm/Kg. A cacodylate buffer (100 mM cacodylate, 10 mM $CaCl_2$, pH 7.0) was used to determine phenoloxidase activity.

Animals

The shrimps, *Penaeus californiensis*, were grown in an experimental tide pond and donated by F. Magallón M.Sc. (Center of Biological Research, La Paz, B.C.S, México). The animals were maintained in an aquarium (24 ± 4°C, salinity: 36‰) 2 days before the experiment. Only the haemolymph from intermolt, apparently healthy shrimps was used.

Extraction and separation of the haemolymph

Groups of eight animals were used for each anticoagulant solution. The haemolymph was extracted from the pleopod base of the first abdominal segment near the genital pore. Haemolymph (100 μl) was obtained using 0.5 ml syringe, 27 gauge needle, containing 200 μl of precooled (10°C) anticoagulant solution. The haemolymph was then centrifuged in a Eppendorf microfuge for 20 sec and the supernatant was separated. The cells were resuspended in 300 μl of cacodylate buffer for the determination of phenoloxidase activity. Protein content and phenoloxidase activity were determined in the supernatant. For the pH 4.6 anticoagulant solution, precipitated proteins were dissolved in SSS and centrifuged again to recover the cells. Protein content and phenoloxidase activity were also determined in the re-suspended protein.

Protein content determination

The total protein content was measured according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Enzymatic 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 was 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 SSS as described above. The cell pellets were pooled and resuspended in cacodylate buffer, then disrupted by sonication for 2 min at 20 W. The haemocyte lysate was centrifuged at 40,000 *g* for 30 min at 10°C, the cell debris was separated from the supernatant, and the proPO activity was determined.

RESULTS AND DISCUSSION

The use of an anticoagulant solution is necessary for shrimp haemolymph collection to prevent cell lysis, degranulation and clotting which occur when shrimp haemocytes are harvested directly into a saline buffer. When the penaeid shrimp (*Paenaeus californiensis* or *P. stylirostris*) haemolymph was collected using the anticoagulant solution recommended for other marine crustaceans (Söderhäll and Smith, 1983), protein precipitation was observed at temperatures less than 15°C (Vargas-Albores, 1992). Although this phenomenon does not occur at 25°C, 10°C is the preferred working temperature to minimize cell activation.

The anticoagulant solution currently used for the collection of marine invertebrate haemolymph (100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 510 mM NaCl and 10 mM EDTA.Na2; pH = 4.6) appears to be based on Alsever solut which is used for the collection of vertebrate blood (114 mM glucose, 26.6 mM trisodium citrate, 2.65 mM citric acid and 71 mM NaCl; pH 6.5). One difference is the NaCl content, which is important for maintaining adequate osmolality of the cells, and varies in anticoagulant solutions for other arthropods such as crayfish (140 mM NaCl) (Johansson and Söderhäll, 1985) and the insect *Gallonella mellonella*(93 mM NaCl) (Leonard *et al.*, 1985b). This means that the osmolality of the solution can be modified to resemble that of the haemolymph. However, another difference between citrate-EDTA and Alsever is the pH (2 units), this parameter could be the most important for cell stability.

The influence of pH on protein precipitation was determined by changing the citrate–citric acid ratio of the original formula to obtain solutions with pHs of 4.6, 5.6 and 6.6, and comparing these to SSS which has a pH of 7.3, similar to peanid shrimp haemolymph (Vargas-Albores and Ochoa, 1992). In earlier results (Vargas-Albores, 1992), protein precipitation was observed with an anticoagulant solution of pH 4.6. As shown in Table 1, protein precipitation was not observed using the same anticoagulant solution at pH 5.6 or 6.6, nor with the SSS. The protein precipitated with pH 4.6 anticoagulant, dissolved easily in SSS or citrate/EDTA anticoagulant at pH 6.6, indicating that this protein precipitation is induced mainly by the effect of pH. The resuspended proteins were measured in the same way as done for plasma and presented in Table 1. In all cases, the protein concentration determined in the diluted haemolymph was corrected by the dilution factor (1:3).

It has been difficult to study arthropod proPO in its proenzyme form because the pathway is frequently activated during, or [illegible]diately after, collection of the haemolym[illegible] By [illegible] the anticoagulant solution (EDTA-citrat[illegible] pH 4[illegible] proposed for crustaceans (Söderhäll and Smith, 1983), *Limulus polyphemus* (Dularay and Lackie, 1985) and *Blamerius craniifer* (Leonard *et al.*, 1985b), it is possible to extract the haemolymph cells intact. Since the crustacean proPO system has been detected only in the haemocytes, cell integrity and non-activation of this system could be verified by the absence of phenoloxidase activity in the cell-free haemolymph fraction (plasma and/or resuspended protein). These supernatants were tested with and without laminarin activation. Phenoloxidase activity was not detected in any of the supernatants obtained with the different anticoagulants. Apparently, all the formulae allow for the extraction of intact shrimp haemocytes, without proPO system activation.

However, when the cellular phenoloxidase activity was measured, a significant difference was observed. The phenoloxidase of the shrimp cells obtained using anticoagulant solutions at pH 4.6 or 5.6 was roughly 25% less than that obtained at pH 6.6 or when using SSS (Table 2). The reduction in the PO activity detected in the cell fraction is apparently independent of plasmatic protein precipitation, because when the pH 5.6 solution was used, a similar reduction in enzymatic activity was observed. In addition, this reduction in the phenoloxidase activity could not be due to the pH during the detection of PO activity because all samples were processed in cacodylate buffer (pH = 7.0). On the other hand, PO activity was not detected in the supernatants, indicating that both pH and buffer composition do not induce the activation and/or degranulation of the cells. Therefore, the reduction of the enzymatic activity could be related to an effect of pH on the degranulation process.

Table 1. Total haemolymph protein of the brown shrimp (*P. californiensis*) obtained using different coagulants

pH of the anticoagulant	Precipitated protein (mg/ml)	Total protein (mg/ml)
Citrate–EDTA pH 4.6	36.3 ± 6.8	106.8 ± 15.6
Citrate–EDTA pH 5.6	0.0	116.7 ± 15.9
Citrate–EDTA pH 6.6	0.0	119.8 ± 20.4
SSS pH 7.3	0.0	117.3 ± 12.8

Only the pH 4.6 anticoagulant showed protein precipitation. No significant difference was observed in total protein. Phenoloxidase activity was not detected in plasma.

Table 2. Effect of the anticoagulant pH on the phenoloxidase activity

Anticoagulant	Phenoloxidase units
Citrate–EDTA pH 4.6	51.5 ± 6.7
Citrate–EDTA pH 5.6	50.8 ± 7.2
Citrate–EDTA pH 6.6	72.3 ± 6.9
SSS pH 7.3	73.2 ± 7.3

The haemolymph obtained using anticoagulants with pH 4.6 and 5.6 showed a significant decrease in enzymatic activity ($P < 0.01$).

To test the efficiency of SSS, we used it to determine the location of the prophenoloxidase system. When the cells were obtained in SSS, separated from the plasma by centrifugation, and washed twice with SSS, the phenoloxidase activity was not detected in the plasma or in the supernatant, thus all the phenoloxidase activity was located in the haemocytes for [illegible] shrimp species.

When the shrimp haemocyte lysate was obtained by sonication, centrifuged, and the supernatant separated, all PO activity was detected in the supernatant, indicating that the proPO system was not associated with membranes. In addition, when the haemocyte lysate was made using cells obtained with citrate–EDTA anticoagulant at pH 4.6, a similar amount of phenoloxidase activity was detected (Table 3). This result supports the idea that pH affects the degranulation process more than it affects phenoloxidase activity.

Our data confirmed that the brown shrimp proPO system is located in the haemocytes, similar to other crustaceans (Söderhäll *et al.*, 1979, 1986; Söderhäll and Smith, 1983; Johansson and Söderhäll, 1985) and some insects (Fisher and Brady, 1983; Ratcliffe *et al.* 1984; Leonard *et al.*, 1985a, b; Dularay and Lackie 1985; Iwama and Ashida, 1986; Brookman and Ratcliffe, 1989; Rowley and Rahmet-Alla, 1990). The proPO system has also been found in the plasma o[illegible] some insects (Ashida, 1981; Sugumaran *et al.*, 1985

Table 3. Phenoloxidase activity in lysate of haemocytes from the brown shrimp (*Penaeus californiensis*)

	Citrate–EDTA (pH = 4.6)	SSS
Supernatant	88.3 ± 7.3	90.5 ± 8.4
Sediment	9.1 ± 0.8	8.4 ± 1.2
Total	94.4 ± 7.2	95.9 ± 7.9

The cells were obtained using either EDTA–Citrate (pH = 4.6) or SSS (pH = 7.3) anticoagulants.

Saul *et al.*, 1987; Saul and Sugumaran, 1988; Brehelin *et al.*, 1989). In the silkworm, *Bomyx mori*, Ashida *et al.*(1981) claimed that the proPO system was contained in the plasma. They injected insect larvae with a cane sugar factor to obtain a stable proPO system. However the resulting total haemocyte count dropped from *ca* 1×10^4 cells to *ca* 1×10^3 cells/mm^3 of haemolymph (Ashida, 1981). Thus it is plausible that most of the cells (90%) were lost from circulation, either by lysis or differential sticking. Working with *Manduca sexta*, Saul and coworkers (1987) demonstrated that this reduction in the number of cells from using the cane sugar factor had no effect on the total phenoloxidase activity, and no degranulation occurred. It is difficult to draw valid conclusions from tl · two methods of phenoloxidase location. "ince c :ean haemocytes are extremely sensitive 'ow :s of β 1,3-glucans (Smith and Söderhäll, 1983) or LPS (Johansson and Söderhäll, 1985), it is likely that the cane sugar factor may have caused extensive degranulation of the haemocyte to result in the release of the proPO system, as detected in the plasma.

The *Penaeus* proPO system also showed sensitiv. to minute amounts of β 1,3-glucans, thus supporting the idea that this system participates in non-self recognition. This activation mechanism has been found in other crustacean defence systems (Söderhäll and Unestam, 1979; Ashida *et al.*, 1983; Pye, 1974; Söderhäll and Häll, 1984), in the *Limulus* clotting system (Dularay and Lackie, 1985) and in the proPO system of insects such as *Blaberus* (Leonard *et al.*, 1985b). We did not study the specific biochemical mechanism for shrimp proPO system activation by laminarin, assuming similarities to previous studies of other crustaceans.

In summary, while SSS was proven to be an effective anticoagulant at 10°C and useful for locating the proPO system, further studies should be done to complete our understanding of the activation and regulation of this system.

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