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Phenoloxidase activity in the hemolymph of the spiny lobster *Panulirus argus*

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Abstract

The prophenoloxidase activating system plays a major role in the defense mechanism of arthropods. In the present study, the phenoloxidase activity and its location in the hemolymph of the spiny lobster *Panulirus argus* is presented. Phenoloxidase activity was observed in the hemocyte lysate supernatant (HLS) and plasma after their incubation with trypsin. Higher amounts of trypsin were required to activate the HLS prophenoloxidase, due to the presence of a trypsin inhibitor in this fraction. Activation of prophenoloxidase was found when HLS was incubated with calcium, with an optimal pH between 7.5 and 8. This spontaneous activity is due to the prophenoloxidase activating enzyme, a serine proteinase that activates the prophenoloxidase once calcium ions were available. SDS was able to induce phenoloxidase activity in plasma and hemocyte fractions. Prophenoloxidase from HLS occurs as an aggregate of 300 kDa. Electrophoretic studies combining SDS–PAGE and native PAGE indicate that different proteins produced the phenoloxidase activity found in HLS and plasma. Thus, as in most crustaceans, *Panulirus argus* contains a prophenoloxidase activating system in its hemocyte, comprising at least the prophenoloxidase activating enzyme and the prophenoloxidase. Finally, it is suggested that phenoloxidase activity found in plasma is produced by hemocyanin.

Keywords: Hemolymph; Immunity; Phenoloxidase; Prophenoloxidase; Spiny lobster; Crustacean

1. Introduction

Invertebrate animals lack an adaptive immune response. Instead, they have developed efficient defense mechanisms based on innate immune responses [1–3]. For a long time it has been recognized that the prophenoloxidase activating system (proPO system) has an important role as a non-self recognition system in arthropods, and participates in the innate immune response through melanization, cytotoxic reactions, cell adhesion encapsulation, and phagocytosis [1,3–5].

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The proPO system is composed, among other proteins, of the prophenoloxidase enzyme (proPO), which is the zymogen of phenoloxidase (PO; EC 1.14.18.1). The conversion of proPO into PO occurs through a proteolytic attack by a serine proteinase named prophenoloxidase activating enzyme (ppA) [1,3,4,5]. Phenoloxidase catalyses the hydroxylation of monophenols to *o*-diphenols (monophenoloxidase or cresolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenoloxidase activity), leading to the synthesis of melanin [6,7]. In addition to its role in the immune response [6,7], the formation of melanin is also involved in other important physiological processes such as wound healing and sclerotization [6].

Whereas in insects the proPO may be located in either the blood cells or the plasma, in crustaceans the proPO is mainly located inside the hemocytes [1,3]. Conversely, it has been recently described that prophenoloxidase from the spiny lobster *Panulirus interruptus* is located in the plasma [8], being the first plasmatic proPO reported in crustaceans.

In the spiny lobster *Panulirus argus*, phenoloxidase activity has been found in the cuticle [9], but there are no reports concerning the occurrence of this activity in its hemolymph. Diseases of aquatic organisms are mostly due to exposure to pathogens like bacteria, fungi and viruses or to inadequate nutrition. Diseases are of a minor importance in lobster's post harvest handling as it is currently practice worldwide for live export, but could be of importance in overseas lobster industries where harvested stocks are held for long periods in ponds for grow-out [10]. Growing of wild-caught postlarvae or juvenile spiny lobsters to market size is an emerging aquaculture industry with the tropical (*Panulirus ornatus*) and the Caribbean (*P. argus*) spiny lobster being the best candidates for aquaculture [11]. Several aspects of nutrition and grow-out methods for *P. argus* are now being studied [12], but unlike other crustaceans, immunity in spiny lobster remains poorly studied. Understanding the immune response at a basic level is crucial to prevent the outbreak and spread of diseases during culture. The aim of the present study was to locate and to characterize the phenoloxidase activity in the hemolymph of the spiny lobster *P. argus.*

2. Material and methods

2.1. Animals and experimental conditions

Adult spiny lobsters *Panulirus argus* collected in the gulf of Batabanó, in the south-western part of the Cuban archipelago, were transported to the laboratory and placed individually in 90-L flow-through tanks connected to a recirculated aquatic system with biological-mechanical filtration and aerated seawater. Lobsters were fed with fresh squid (9% fresh body weight) twice a day. Experiments were carried out after 2 weeks of conditioning to the laboratory environment. The lobster maintenance conditions were (mean ± standard error): temperature of 24.2 ± 0.20 °C, salinity of 36.1 ± 0.09‰, ammonia concentration of 0.07 ± 0.009 mg/L, oxygen concentration of 5.57 ± 0.010 mg/L and pH of 7.9 ± 0.02. Light was automatically controlled to provide a 12-h light and 12-h dark photoperiod. Only intermolt and apparently healthy lobsters were selected for hemolymph extraction. Determination of molt stage was achieved by examining a pleopod tip under a low-power microscope according to Lyle and MacDonald [13].

2.2. Preparation of the hemocyte lysate supernatant (HLS) and plasma samples

All glassware used was depyrogenated by dry heat at 250 °C for 30 min. To obtain hemocytes, the hemolymph was extracted using the Citrate-EDTA buffer pH 4.6 as anticoagulant [14] and was composed of 0.4 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA. Five milliliters of hemolymph was extracted from the fourth walking leg coxa using a 10 ml pyrogen free disposable syringe containing 5 ml of precooled anticoagulant solution (AC). The mixture was then centrifuged at 700 × *g* for 10 min at 4 °C. The cell pellet was washed twice with anticoagulant, suspended in 0.145 M NaCl and homogenized using a glass piston homogenizer. The lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C to obtain the clarified hemocyte lysate supernatant (HLS).

Because the pH of the AC used for obtaining HLS causes the isoelectric precipitation of plasma proteins [15], the plasma samples were obtained from hemolymph collected using 0.2 M *N*-ethylmaleimide (NEM) in 0.45 M NaCl (1 NEM: 4 hemolymph) [16]. The hemocytes were separated by centrifugation as described above and the plasma was immediately used in the assays.