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Expression of a serine proteinase homolog prophenoloxidase-activating factor from the blue crab, *Callinectes sapidus*

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Abstract

Tanning, or sclerotization, of crustacean cuticle provides initial reinforcement by cross linking cuticular proteins attached to the cuticle chitin–fiber matrix. This process is catalyzed in part by phenoloxidase, which is under the control of a serine protease activation cascade. The cDNA of a prophenoloxidase-activating factor (PPAF) was cloned and sequenced from the hypodermal tissue of the blue crab, *Callinectes sapidus*. It codes for a serine proteinase homolog containing a single clip domain. If it is involved in sclerotization, its transcription might be expected to be molt-cycle related. Expression patterns were determined by quantitative PCR and Northern blotting in hypodermis underlying both arthrodial and dorsal (calcifying) cuticles. Transcript levels in pre-molt RNA from both hypodermis types were high, suggesting that the PPAF produced may be incorporated into the pre-ecdysial cuticle layers and then activated at ecdysis to regulate tanning. After a decrease at ecdysis, a second increase in PPAF mRNA occurred at three to four hours post-molt in arthrodial membrane hypodermis but not dorsal hypodermis. This suggests that cuticle deposited after ecdysis may tan in the non-calcifying regions but may not tan where calcification occurs. The PPAF gene is also transcribed at low levels in the hemocytes of intermolt crabs, but not in the hepatopancreas.

Keywords: *Callinectes sapidus*; Clip domain; Prophenoloxidase-activating factor; Sclerotization; Serine protease domain

1. Introduction

Phenoloxidase (PO) catalyzes both the *o*-hydroxylation of monophenols and the oxidation of diphenols to quinones. The highly reactive *o*-quinone products of PO have at least two important physiological functions in arthropods. First, when PO is activated by a cascade of events initiated by the presence of low quantities of microbial cell wall components, the resulting *o*-quinones may polymerize to form melanin as a part of the innate invertebrate immune system (Söderhäll and Cerenius, 1998). Second, the activation of PO in the cuticle in conjunction with the molt cycle (Ferrer et al., 1998) results in *o*-quinones that cross link histidine and lysine residues of cuticular proteins in the process of sclerotization, or tanning (Andersen et al., 1996). Though enzymatic and immunological evidence suggests that the POs found in insect hemolymph and cuticle are not the same (Mills et al., 1968; Hiruma and Riddiford, 1988), in both cases, the reactivity and the specialized function of the quinone products mean that the activity of the enzyme must be carefully controlled.

PO is produced by proteolytic cleavage from an inactive precursor, prophenoloxidase (proPO). The enzyme responsible is a serine proteinase, hereafter referred to as prophenoloxidase-activating enzyme (PPAE). This enzyme is the final step in a cascade leading to PO activation and is itself under tight control. At least four mechanisms for PPAE regulation have been recognized: gene induction, activation by another proteinase, a requirement for non-catalytic serine proteinase homologues (SPH) as cofactors, and inactivation by serine proteinase inhibitors (Wang and Jiang, 2004). All PPAEs, their cofactor SPHs, and any proteinases that activate them can properly be referred to as

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prophenoloxidase-activating factors (PPAF). The PPAFs described to date all contain a highly conserved carboxyl-terminal proteinase domain and one or more amino-terminal clip domains. The proteinase domains of SPHs are non-catalytic because the typical active-site serine residue is replaced by glycine. Clip domains are features found only in arthropod proteinases. They are cleaved from the proteinase domain upon the activation of the pro-protein but remain attached by a disulfide bridge. Clip domains have been implicated in regulating the function of the enzymes through their interactions with other proteins (Jiang and Kanost, 2000).

PPAFs have been purified, their cDNAs cloned, and their regulation studied in a number of insect species, usually from hemocytes and generally in relation to their roles in immune defense. Three PPAFs have been described and cloned from *Holotrichia diomphalia*. PPAF-I is the actual proteolytic activator of proPO (Lee et al., 1998), PPAF-II is the SPH cofactor (Kwon et al., 2000), and PPAF-III is a catalytic serine proteinase that activates PPAF-II by cleavage (Kim et al., 2002). In *Manduca sexta*, three prophenoloxidase-activating proteinases (PAP) have been isolated and their cDNAs cloned. All three do, in fact, directly activate proPO. PAP-1 (Jiang et al., 1998) was originally isolated from cuticle, whereas PAP-2 (Jiang et al., 2003a) and PAP-3 (Jiang et al., 2003b) are from hemocytes. Two SPH molecules have also been isolated from *M. sexta* plasma and cloned (Yu et al., 2003). Both SPHs can serve as cofactors for each of the PAPs. Two non-catalytic cofactor proteins are known from the beetle *Tenebrio molitor* as well, one designated PPAF (Kwon et al., 2000) and the other SPH (Lee et al., 2002). As yet, no actual PPAEs are described from this species. The only PPAF published from the silkworm *Bombyx mori* is an actual PPAE (Satoh et al., 1999), though unpublished sequences for an SPH and a second catalytic serine proteinase called PPAE-3 exist in the NCBI database (accession numbers AF514468 and AY061936, respectively). A PPAE from *Drosophila melanogaster* was shown to be a serine proteinase but was not sequenced (Chosa et al., 1997). However, no less than 37 serine proteinases or serine proteinase homologs from the *Drosophila* genome contain clip domains and could be involved in proPO activation or other proteinase cascades (Ross et al., 2003).

Presumably, crustaceans, like insects, use PO both for immune-related melanization and for cuticle sclerotization. The crayfish *Pacifastacus leniusculus* is the only decapod for which a PPAF sequence has been published. Aspán et al. (1990) purified PPAE from hemocytes, abbreviating it ppA. Wang et al. (2001) cloned the cDNA for this enzyme. An SPH has also been cloned from crayfish hemocytes (Huang et al., 2000), but this protein binds bacteria and appears to function as an opsonin rather than a PPAF (Lee and Söderhäll, 2001). Its sequence is remarkable for the fact that it contains seven clip domains. Recently, an unpublished sequence for an SPH from the hemocytes of shrimp *Marsupenaeus japonicus* and an expressed sequence tag similar to insect PPAEs from the American lobster *Homarus americanus* have been added to the NCBI databases (accession numbers AB161692 and CN950293, respectively).

Crustaceans must molt periodically in order to grow and presumably must tan the new cuticle at each molt. Immediately after ecdysis, their cuticle consists only of the previously deposited outer layers, the epicuticle, and the exocuticle. (The biology of the cuticle during decapod molting has been reviewed by Roer and Dillaman, 1984, 1993) The cuticle of an ecdysial blue crab, *Callinectes sapidus*, is extremely soft and pliable. It has to be easily deformed at this time because it stretches as the animal swells via absorption of water across the gills and digestive epithelium. We have observed that by 30 min after ecdysis, the cuticle has become more leathery, though it is still easily deformed. Sclerotization is thought to accomplish this change, strengthening the cuticle and providing a structured environment for the nucleation of calcium carbonate. Mineralization, one feature that distinguishes the crustaceans from the insects, begins at 3 to 4 h after the molt in *C. sapidus* in a clearly defined pattern (Dillaman et al., in press). The deposition of the thick endocuticle begins sometime within the first 24-h post-molt and continues for several days. This layer mineralizes as it is deposited, but it is unclear whether sclerotization occurs in the endocuticle.

The presence of a calcified exoskeleton affords crustaceans protection, support, and sites for muscle attachment. However, a stiff cuticle resists movement and the joints must remain flexible. Uncalcified cuticle, known as the arthrodial membrane, is located at the joints. Though it is morphologically similar and is deposited by immediately adjacent hypodermal cells, the arthrodial membrane has been shown histologically to have distinctly different carbohydrate and protein compositions from the calcified cuticle (Williams et al., 2003). Andersen (1999) proposed important differences between the proteins of flexible and stiff cuticle. His model suggests that arthrodial membrane is more flexible because of a higher proportion of hydrophilic proteins and, thus, greater water content. Conversely, inflexible cuticle contains mostly hydrophobic proteins that pack more tightly. It is unknown whether sclerotization plays any role in the difference between the cuticle types.

Large decapod crustaceans have not been used to study the proPO activation mechanism as it relates sclerotization. Yet, the activation is molt related, being maximal at late premolt in the spiny lobster *Panulirus argus* (Ferrer et al., 1998). Furthermore, ample amounts of cuticle and underlying hypodermis, the epithelium responsible for the synthesis of many cuticular proteins, are available for study at any point in the molt cycle. In this paper, we report the primary structure of a SPH cloned from the hypodermis of the blue crab, *C. sapidus*. It is the first putative PPAF cDNA obtained from crustacean hypodermis tissue. Northern blot analysis and quantitative PCR using RNA extracted from