

Comparative Proteomic Analysis of Hemocyanins in *Dinocras cephalotes* and *Perla marginata* (Plecoptera)

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ABSTRACT Hemocyanins are large oligomeric respiratory proteins found in many arthropods and mollusks. The overall expression of hemocyanin mRNA, revealed by studies on Plecoptera hemocyanin sequencing, has raised the question of whether the protein is expressed or not. In fact, the presence of expressed hemocyanin has only been reported in the literature for one species, *Perla marginata* (Panzer, 1799). In this paper, we report the presence of hemocyanin and hexamerin proteins in *Dinocras cephalotes* (Curtis, 1827), a species closely related to *P. marginata*. To assess the presence of hemocyanin, we used a reproducible and highly sensitive method based on liquid chromatography tandem mass spectrometry. We conclude that regardless of its putative function (respiratory, immune defense, storage protein), the hemocyanin is actually expressed in species in which its mRNA is present.

KEY WORDS copper binding proteins, nano-RP-HPLC-ESI-MS, insects

Hemocyanin, hemoglobin, and hemerythrin are the respiratory proteins of the animal kingdom (Mangum 1985). The term “hemocyanin” usually encompasses both the mollusc and arthropod proteins. They were originally given the same name because both are oxygen transport proteins with the basic motif of type 3 copper proteins, suggesting a very ancient common ancestor (van Holde et al. 2001). However, the mollusc and arthropod hemocyanins have very different molecular structures (Decker et al. 2007).

Arthropod hemocyanins are large multimetric (nx6), copper-containing proteins composed of subunits of ≈ 75 kDa (Mangum 1985, van Holde et al. 2001, Decker et al. 2007). Sequence analyses have shown that these hemocyanin macromolecules can contain several variants of monomers (Markl 1986, Voit et al. 2000, Hagner-Holler et al. 2004). The presence of the N-terminal signal peptide is typical of arthropod hemocyanins, presumably because they are secreted into hemolymph by hepatopancreas cells or fat body cells (Fochetti et al. 2006; Kutsche and Burmester, 2001a, b; Sánchez et al. 1998; Pick et al. 2008), and they are freely dissolved in the hemolymph. Although its O₂-binding capacity has earned hemocyanin the common name of “respiratory protein,” there is a large body of literature showing that, under experimental conditions, the protein is multifunctional in Chelicerata and Crustacea. Hemocyanin can potentially act as phe-

noloxidase after proteolytic cleavage (Decker and Rimke 1998, Nagai and Kawabata 2001, Decker et al. 2001, Pless et al. 2003, Lee et al. 2004), as an antibacterial or antimycotic agent (Destoumieux-Garzón et al. 2001, Lee et al. 2002, Pan et al. 2008) and thus participate in the immune defense system, or take part in the molting process and work as a hexamerin (Jaenicke et al. 1999).

The presence of hemocyanin in insects is the subject of ongoing scientific debate. The first insect hemocyanin was reported for the stonefly *Perla marginata* (Panzer, 1799) (Hagner-Holler et al. 2004) and two subunits were identified: subunit one (hc1) of 77 kDa and subunit two (hc2) of 76.3 kDa (Fochetti et al. 2006, Hagner-Holler 2004). Subsequently three-dimensional studies were carried out on a closely related species, *P. grandis* Rambur 1842 (Fochetti et al. 2006). Later, it was assumed that hemocyanin is present across the entire order (Burmester and Hankeln 2007).

In the last few years, we have investigated the presence of this protein across Plecoptera, analyzing 32 species belonging to European, African and Oriental families by RT-polymerase chain reaction (PCR) of total mRNA. We considered ecological and biological parameters that could induce variations in the physiological requirements of specimens and constitute differences in adaptive responses reflecting stonefly biodiversity (Fochetti et al. 2006, Amore et al. 2009, Amore and Fochetti 2009, our unpublished data). We noted that, while hemocyanin is lacking in Nemouroidea, one of the two European superfamilies, and in *Cryptoperla*, a Peltoperlidae belonging to Pteronarcyzoidea, hemocyanin mRNA is expressed in many, albeit not all, of the studied Perloidea. These data led us to infer multifunctionality of hemocyanin

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