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Review

Diverse immune functions of hemocyanins

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ABSTRACT

Substantial evidence gathered recently has revealed the multiple functionalities of hemocyanin. Contrary to previous claims that this ancient protein is involved solely in oxygen transport within the hemolymph of invertebrates, hemocyanin and hemocyanin-derived peptides have been linked to key aspects of innate immunity, in particular, antiviral and phenoloxidase-like activities. Both phenoloxidase and hemocyanin belong to the family of type-3 copper proteins and share a high degree of sequence homology. While the importance of phenoloxidase in immunity and development is well characterised, the contribution of hemocyanin to biological defence systems within invertebrates is not recognised widely.

This review focusses on the conversion of hemocyanin into a phenoloxidase-like enzyme and the array of hemocyanin-derived immune responses documented to date.

Contents

1. Introduction

The species richness and habitat diversity of invertebrates suggest that the absence of acquired immunity has in no way hindered their successes. The robust innate immunity of invertebrates holds great complexity and is analogous to the innate immunity of vertebrates (Kanost et al., 2004; Coates et al., 2012a; Criscitiello and de Figueiredo, 2013). Of the many immune-related proteolytic and signal transduction cascades explored so far, the prophenoloxidase (proPO) activation cascade is one of the best understood (Cerenius et al., 2008, 2010a). The resultant activated phenoloxidase (PO) possesses functions in both immunity and development. Latent PO activity has also been observed in hemocyanin (Hc), a transport protein which belongs to the same family as PO. Initially, the immune significance of Hc was dismissed until the late 90's when

Abbreviations: AMP, antimicrobial peptide; ERK, extracellular regulated kinase; FU, functional unit; Hc, hemocyanin; Hc-d PO, hemocyanin-derived phenoloxidase; HSC, horseshoe crab; IgSF, immunoglobulin superfamily; LPS, lipopolysaccharide; PAMP, pathogen associated molecular pattern; PO, phenoloxidase; PS, phosphatidylserine; PRR, pathogen recognition receptor; ROS, reactive oxygen species.

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Decker and Rimke demonstrated unequivocally the PO activity of tarantula Hc (Decker and Rimke, 1998). Since then, a plethora of studies have recorded broad, non-specific immune properties of Hc in response to pathogenic/environmental stimuli (Zhang et al., 2004a; Terwilliger et al., 2006; Jiang et al., 2007; Zhang et al., 2009). We present the first review on the significant progress made over the last decade in characterising the immunological versatility of Hc.

2. Hemocyanins and type-3 copper proteins

Type-3 copper proteins are distinguishable by the presence of a di-copper centre where each copper atom (Cu-A & Cu-B) is coordinated by three strictly conserved histidine residues, facilitating the reversible binding of peroxide Cu^{II}-O_2^{2}-Cu^{II} in a side-on bridging coordination (μ: $\eta^2 - \eta^2$) (Solomon et al., 1996; Decker and Terwilliger, 2000; Claus and Decker, 2006; Halaouli et al., 2006; Selinheimo et al., 2007; Panzer et al., 2010; Maria et al., 2011; Rolff et al., 2011). Members of this large protein family include: arthropod and mollusc Hcs, plant, microbial and invertebrate POs, insect hexamerins and crustacean cryptocyanins (Burmester, 1999, 2001; Terwilliger et al., 1999). Hexamerins and cryptocyanins act as storage proteins (with the loss of oxygen binding abilities), whereas POs are catalytic proteins. Generally, the term PO refers to tyrosinase (EC 1.14.18.1) and/or catecholoxidase (EC 1.10.3.1) activities; while the former generates reactive quinonoids from the *ortho*-hydroxylation of monophenols into *o*-diphenols and their further oxidation to *o*-quinones, catecholoxidases perform the second reaction only. Although Hcs and POs differ considerably across all structural levels and have been associated traditionally with separate biological functions (respiration and immunity, respectively), they share almost identical active site architecture (Fig. 1). In a manner similar to POs studied thus far, Hcs incubated in the presence of selected denaturants/proteases can be activated to accommodate bulky phenolic substrates (reviewed by Decker et al., 2007a,b).

3. Fundamental properties of hemocyanins

Hcs are large, extracellular proteins with a predominantly negatively charged electrostatic surface which improves the solubility and hydration of Hc within the hemolymph and limits Hc from associating with other negatively charged surfaces, such as the glycocalyx of cells (Jaenicke and Decker, 2003). Hcs are known to maintain structural and functional integrity over a broad temperature range, withstanding temperatures up to 90 °C, or as low as −20 °C (Sterner et al., 1995; Idakieva et al., 2012; Coates and Nairn, 2013).

Hc is distributed within the hemolymph of arthropods, molluscs and larval stages of certain insects (Pick et al., 2008, 2009; Decker and Jaenicke, 2004). Depending on the organism, Hc can be synthesised in organs such as the crustacean hepatopancreas (Lee et al., 2004; Ward et al., 2010) or secreted by specialised cells known as cyanocytes in chelicerates (Fahrenbach, 1970; Kuhn-Nentwig et al., 2014) and rhogocytes in molluscs (Albrecht et al., 2001; Beuerlein et al., 2004). Hc is the major protein component of invertebrate hemolymph, c.a. 50% to >90%, and in some species is present in concentrations exceeding 100 mg mL^{-1} (Coates et al., 2012b). Primarily, Hc transports molecular oxygen to respiring tissues within invertebrates. Vertebrate blood is red in colour due to the oxygenation of haemoglobin via iron within the heme cofactor, whereas chelicerate, and to a lesser extent crustacean hemolymph is blue in colour due to the oxygenation of Hc resulting in a change from a Cu I state to a Cu II state. Oxygenated Hc can be detected readily by an intense absorption peak at ~340 nm (ε = 20,000 M^{-1} cm^{-1}) (Fig. 1). Aside from oxygen transport, Hcs are also known to participate in homeostatic and physiological processes: moulting (Adachi et al., 2005a,b; Kuballa and Elizur, 2008; Kuballa et al., 2011; Glazer et al., 2013), hormone transport (Jaenicke et al., 1999), osmoregulation and protein storage (Paul and Pirow, 1998).

The basic structure of arthropod Hc is hexameric in nature and is usually found as integer multiples of hexamers (2 × 6mer to 8 × 6mer). Each arthropod Hc subunit (~70–75 kDa) consists of three domains: domain I contains five to six α-helices, domain II contains a four α-helix bundle and domain III is a seven stranded anti-parallel β-barrel. Domain II encompasses the di-copper centre (Volbeda and Hol, 1989; Hazes et al., 1993; Magnus et al., 1994; van Holde et al., 2001; Jaenicke et al., 2012; Rehm et al., 2012). The majority of mollusc Hcs are observed as hollow cylindrical decamers, didecamers or tridecamers (Fig. 2); mega-Hc from cerithioid snails are the exception, as the internal structure holds a more complex arrangement (Lieb et al., 2010; reviewed by Markl, 2013). Each mollusc subunit (~350–400 kDa) is made up of seven or eight paralogous functional units (FUs: FU-a to FU-h). Mollusc

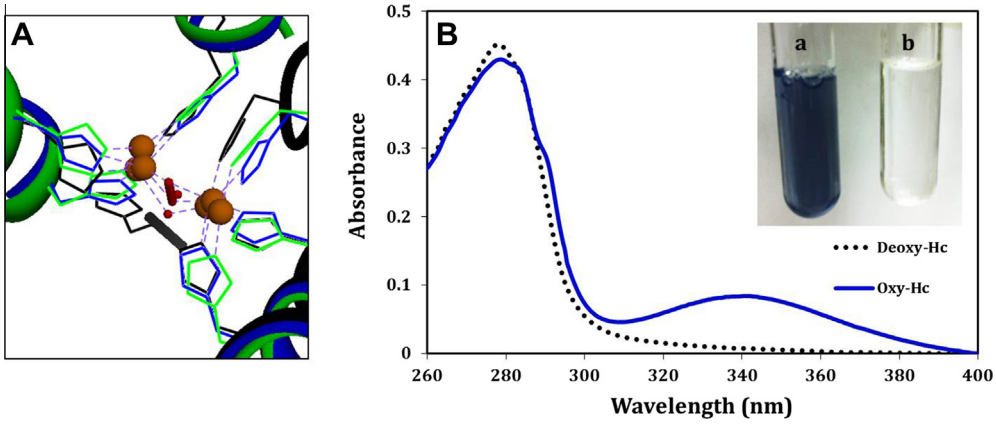

Fig. 1. (A) Superimposed active sites of *Limulus polyphemus* hemocyanin (blue, PDB 1OXY), *Manduca sexta* prophenoloxidase (green, PDB 3HHS) and *Agaricus bisporus* tyrosinase (black, PDB 2Y9W). The dicopper atoms are depicted as orange spheres. Images were produced using UCSF Chimera 1.6.2. (B) Absorption spectra of 0.3 mg mL^{-1} *L. polyphemus* hemocyanin in 100 mM Tris–HCl buffer, pH 7.5. The absorbance peak at ~340 nm observed for hemocyanin with dioxygen bound is in contrast to the deoxygenated hemocyanin spectrum. Inset, an aliquot of oxygenated (blue) horseshoe crab hemolymph (containing ~80 mg mL^{-1} Hc) and an aliquot of deoxygenated (clear) hemolymph. Deoxy-hemocyanin was prepared using dialysis. Oxy-Hc in 100 mM Tris–HCl, pH 7.5 was dialysed against 100 mM Tris–HCl, pH 7.5 containing 20 mM EDTA, overnight at 4 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)