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Origin and evolution of arthropod hemocyanins and related proteins

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Abstract Arthropod hemocyanins are large, multimeric, ($n \times 6$) copper-containing proteins that deliver oxygen in the haemolymph of many chelicerate, crustacean, myriapod, and also possibly some insect species. The arthropod hemocyanins belong to a large protein superfamily that also includes the arthropod phenoloxidas, certain crustacean and insect storage proteins (pseudo-hemocyanins and hexamerins), and the insect hexamerin receptors. Here I summarise the present knowledge of the origin, functional adaptations, and evolution of these proteins. Arthropod and mollusc hemocyanins are, if at all, only distantly related. As early as in the arthropod stem line, the hemocyanins emerged from a phenoloxidase-like enzyme. The evolution of distinct hemocyanin subunits, as well as the formation of multi-hexamers occurred independently within the arthropod subphyla. Hemocyanin subunit evolution is strikingly different in the Chelicerata, Myriapoda and Crustacea. Hemocyanins individually gave rise to two distinct copper-less storage proteins, the insect hexamerins and the crustacean pseudo-hemocyanins (cryptocyanins). The receptor responsible for the uptake of hexamerin by the larval fat body of the insects emerged from a hexamerin-precursor. Molecular phylogenetic analyses show a close relationship of the crustacean and insect proteins, providing strong support for a pancrustacean taxon, while structural data suggest a myriapod-chelicerate clade.

Keywords Arthropoda · Hemocyanin · Hexamerin · Hexamerin receptor · Phenoloxidase

Abbreviations *MYA* million years ago · *PPOs* prophenoloxidas

Introduction

The methods of molecular phylogeny have revolutionised our knowledge of animal systematics, as well as our understanding of the evolution of proteins and protein functions (e.g. Swofford et al. 1996; Pagel et al. 1999). It is well established that proteins that serve very different functions may share significant identities at the molecular level. Such sequence similarities reflect a relationship in evolution (Doolittle 1981, 1989). Groups of proteins that share a common ancestry can be classified in families and superfamilies (Dayhoff et al. 1975). The phylogeny of the members of such protein (super-) families can be inferred analogous to those of living species (e.g. Swofford et al. 1996). Protein functions and adaptations at the molecular level cannot be understood without considering species phylogeny. In fact, the proteins of an organism share its phylogenetic history, and physiological adaptations that have occurred during evolution have driven and have been driven by the changes in the protein sequences.

The hemocyanins are large copper-containing proteins that transport oxygen in the haemolymph of many arthropod and mollusc species (Markl and Decker 1992; van Holde and Miller 1995). The arthropod hemocyanin superfamily includes four other classes of proteins that share significant sequence similarities but serve distinct functions (Table 1; Beintema et al. 1994; Burmester and Scheller 1996; Burmester 2001). The phenoloxidas are enzymes involved in the melanisation pathway. The non-respiratory crustacean pseudo-hemocyanins (cryptocyanins) and the insect hexamerins serve mainly as storage proteins. The insect hexamerin receptors are responsible for the uptake of hexamerins by the larval fat body. Previous reviews on arthropod hemocyanins (van Holde and Miller 1982; Ellerton et al. 1983; Linzen et al. 1985; Salvato and Beltramini 1990; Markl and Decker 1992;

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Table 1 Structure and properties of arthropod hemocyanins, phenoloxidases, pseudo-hemocyanins, hexamerins and hexamerin receptors

	Main function	Occurrence	Structure	Cu ²⁺	Length (aa) ^a	Signal peptide	Evolution rate ^c
Phenoloxidase	Tyrosinase	Crustacea	1×6?	yes	~660–690	no ^b	1.2–1.6×10 ⁻⁹
		Myriapoda	1×6?	yes		?	
		Insecta	n.d.	yes		no ^b	
Hemocyanins	Oxygen transport	Chelicerata	1–8×6	yes	~630	no	0.5–0.6×10 ⁻⁹
		Crustacea	1–4×6	yes	~660	yes	1.2–1.5×10 ⁻⁹
		Myriapoda	6×6	yes	~640	yes	0.9–1.2×10 ⁻⁹
		Insecta	n.d.	yes	~650	yes	~1.0×10 ⁻⁹
Pseudo-hemocyanins (cryptocyanins)	Storage protein	Crustacea (Decapoda)	1×6	no	~650–660	yes	~2.0×10 ⁻⁹
Hexamerins	Storage protein	Insecta	1×6 or 2×6	no	~660–750	yes	2.4–2.9×10 ⁻⁹
Hexamerin receptors	Hexamerin uptake	Insecta (Diptera?)	n.d.	no	~1010–1240	yes	~7.5×10 ⁻⁹

^aSubunits; excluding signal peptides^bSynthesised as pro-phenoloxidases^cSubstitutions per site and year

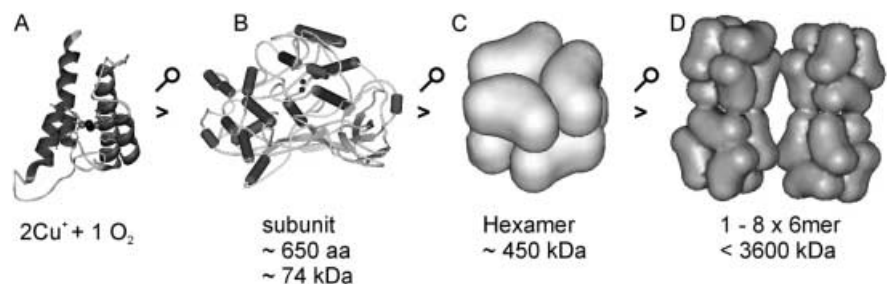
van Holde and Miller 1995), phenoloxidases (Söderhäll and Cerenius 1998), hexamerins (Telfer and Kunkel 1991; Haunerland 1996; Burmester 1999a) or hexamerin receptors (Burmester and Scheller 1999) have mainly focussed on protein function. Markl and co-workers (Markl 1986; Markl et al. 1986) have carried out an immunological approach to elucidate hemocyanin relationship and evolution within the Crustacea and Chelicerata. With the amount of sequence data that have accumulated in recent years, as well as the advances in the development of molecular phylogenetic methods, comparative sequence studies have gained much attention (e.g. Beintema et al. 1994; Burmester and Scheller 1996; Burmester et al. 1998; Durstewitz and Terwilliger 1997a; Burmester 2001). Here I will discuss these findings and will present a comprehensive review of the current knowledge on the evolution of the arthropod hemocyanin superfamily.

The origin of the arthropod hemocyanin superfamily

The delivery of oxygen within the animal's body is an essential process that is accomplished by three different types of metal-containing proteins. While haemoglobins are widespread throughout the animal kingdom, haemerythrins and hemocyanins are restricted to only a few phyla (Vinogradov 1985; Terwilliger 1998; Kurtz 1999).

Hemocyanins are large proteins that may assemble to complexes of up to several million Daltons. These copper-proteins only occur in the body fluid of some arthropod and mollusc species (Markl and Decker 1992; van Holde and Miller 1995). Hemocyanins bind oxygen by means of two Cu⁺ ions, each of which is co-ordinated by three histidines in two distinct binding sites (CuA and CuB) (Fig. 1). There are striking structural and functional similarities among such "type III" copper-binding centres, and also certain sequence resemblance in the CuB site. It is not surprising that some confusion arose due to these apparent similarities. The common nomenclature of mollusc and arthropod hemocyanins even led to the misinterpretation of a close relationship of these two phyla within the Prostostomia (Kim et al. 1996). A common origin of these two different classes of hemocyanins from an oxygen-binding ancestor has been repeatedly proposed in the literature (e.g. Müller et al. 1988; Kawabata et al. 1995; Durstewitz and Terwilliger 1997a; Terwilliger et al. 1999), while others have emphasised the fundamental differences in the construction of the native hemocyanins of the two phyla (e.g. Markl and Decker 1992; van Holde and Miller 1995; van Holde et al. 2001). In fact, statistical analyses have demonstrated that even the sequence similarities between the mollusc and arthropod hemocyanin superfamily are not significant (Burmester 2001). Thus the mollusc and arthropod hemocyanins

Fig. 1A–D Structure of arthropod hemocyanin. **A** O₂-binding site. **B** subunit. **C** simplified hexamer structure. **D** simplified multi-hexamer (arachnid 4×6 hemocyanin)



emerged independently, most likely from existing copper-proteins (Burmester 2001; van Holde et al. 2001). The mollusc hemocyanins share significant sequence similarities with the tyrosinases found in many bacteria, fungi, plants, and animals (Drexel et al. 1987; Müller et al. 1988; Markl and Decker 1992; Cuff et al. 1998; Burmester 2001; Lieb et al. 2001), suggesting that these tyrosinases and the mollusc hemocyanins represent an ancient protein superfamily that had probably already occurred in the stem line of all living cells (for review see: van Gelder et al. 1997). Although the arthropod hemocyanins are related to copper-based tyrosinases as well (see below), these arthropod-specific phenoloxidasases do not closely resemble their non-arthropod paralogues. The origin of the arthropod hemocyanin superfamily is essentially unknown, because no other proteins outside this phylum could be identified that share unambiguous sequence similarities. However, although “non-arthropod” tyrosinases are apparently absent within the Arthropoda, it appears to be unlikely that the arthropod ancestor did not contain such proteins. It is possible that the ancestor of the arthropod hemocyanin superfamily experienced such fundamental changes in its early evolution that its connection to the non-arthropod proteins is now obscured in the sequences (Markl and Decker 1992; Decker and Terwilliger 2000; Burmester 2001; van Holde et al. 2001).

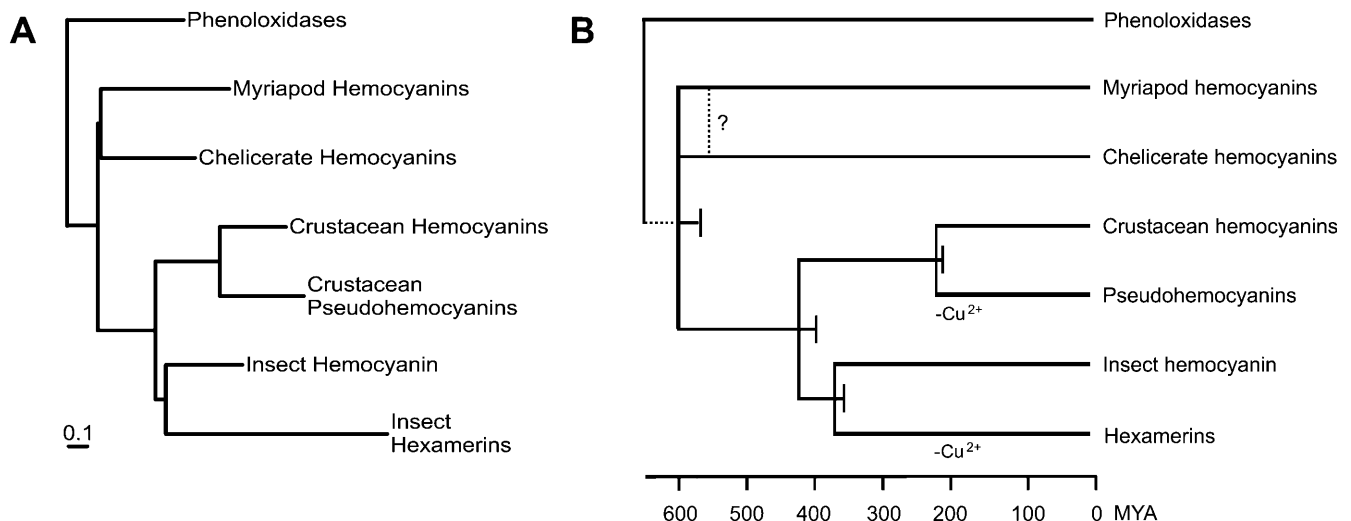
Phylogenetic relationships within the arthropod hemocyanin superfamily

The exclusive occurrence of hemocyanins and related proteins in arthropod species provides additional strong evidence in favour of the monophyly of the Arthropoda. The relationships among different members of the hemocyanin superfamily can be deduced by comparing their sequences using the established molecular phylogenetic methods (Beintema et al. 1994; Burmester and Scheller 1996; Durstewitz and Terwilliger 1997a; Burmester et al. 1998; Burmester 1999a, 2001; Kusche and

Burmester 2001b). Most branching points within the resulting phylogenetic tree are well resolved, with the exception of the positions of the myriapod and insect hemocyanins. Because no natural outgroup is available, the direction of evolution must be deduced by defining the most ancient branching event by alternative considerations. Distinct hemocyanins and phenoloxidasases occur in all arthropod subphyla, with the possible exception that such an enzyme may be absent in the Chelicerata (Nagai and Kawabata 2000; Decker et al. 2001). This suggests that hemocyanins and phenoloxidasases diverged before the radiation of the arthropod subphyla, which occurred at least in the Cambrian period, if not earlier (e.g. Conway-Morris 1993; Gu 1998; Valentine et al. 1999). Thus the phylogenetic tree may be rooted along this fundamental split [Fig. 2A; see Burmester (2001) for a detailed justification of outgroup selection].

Within the hemocyanins, three distinct clades diverged with the separation of the extant arthropod subphyla (Fig. 2). The split between the crustacean and chelicerate hemocyanins was calculated to have occurred around 600 million years ago (MYA; Burmester 2001). The first branch represents the chelicerate hemocyanins, the second the myriapod hemocyanins, and the third the crustacean and insect hemocyanins, as well as their non-respiratory descendants. Assuming that protein phy-

Fig. 2A–B Molecular phylogeny of the arthropod hemocyanin superfamily. **A** A simplified neighbour-joining tree was calculated based on the amino acid distances according to Dayhoff's PAM001 matrix (Dayhoff 1979). It comprises a total of 116 proteins: 26 phenoloxidasases, 2 myriapod hemocyanins, 19 chelicerate hemocyanins, 12 crustacean hemocyanins, 3 crustacean pseudo-hemocyanins, 1 insect hemocyanin and 53 insect hexamerins. **B** A timescale of the molecular evolution in the hemocyanin protein superfamily. The tree was linearised assuming the following divergence times: Merostomata – Arachnida 450 MYA (Dunlop and Selden 1997), Astacura – Palinura 180 MYA, Brachyura – Palinura 150 MYA (Briggs et al. 1993), Orthoptera – Neoptera 320 MYA and Brachycera – Nematocera 210 MYA (Kukalová-Peck 1991). The bars are the SEs



logeny reflects species evolution, this topology strongly supports a pancrustacean taxon that includes the insects (Zrzavý et al. 1997; García-Machado et al. 1999; Wilson et al. 2000), but rejects the integrity of the Tracheata (Myriapoda + Insecta; cf. Friedrich and Tautz 1995; Boore et al. 1998; Kusche and Burmester 2001b). There is modest statistical support and some structural arguments (common deletion of an alpha-helix in the first domain) for a relationship of the myriapod and chelicerate hemocyanins. Interestingly, other molecular studies tend to support a sistergroup-position of these taxa (Friedrich and Tautz 1995; Giribet and Ribera 2000; Hwang et al. 2001).

While there is presently no non-respiratory, hemocyanin-like protein known in the Myriapoda or Chelicerata, the insect and crustacean hemocyanins both gave rise to at least three types of proteins that serve functions other than oxygen-binding. In the decapod Crustacea, the copper-less pseudo-hemocyanins evolved (Burmester 1999a; Terwilliger et al. 1999). After the emergence of the insects (of which some species may have retained a hemocyanin; Sánchez et al. 1998), the hemocyanins gave rise to the storage hexamerins and the peculiar, membrane-bound hexamerin-receptors (Beintema et al. 1994; Burmester and Scheller 1996; Burmester et al. 1998; Burmester 1999b; Burmester and Scheller 1999).

The arthropod phenoloxidases

The first members of the hemocyanin superfamily, which might have evolved in the arthropod stem line some 600 MYA, most likely acted as phenoloxidases (monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1; Burmester 2001). These enzymes catalyse the hydroxylation of monophenols to o-diphenols and/or the oxidation of the o-diphenols to o-quinones. Phenoloxidases are involved in the melanin-forming pathway and play a key role in the sclerotisation of the cuticle, in wound healing and in the humoral immune system (e.g. Ashida and Yoshida 1988; Åspan and Söderhäll 1991; Söderhäll and Cerenius 1998). It is conceivable that the emergence of the unique arthropod phenoloxidases was linked to the evolution of a hard exoskeleton or of an effective immune response in the late Precambrian period (Fig. 3).

All known arthropod phenoloxidases are synthesised as zymogens (prophenoloxidases; PPOs) that require activation via cleavage of an N-terminal peptide (e.g. Åspan et al. 1995; Hall et al. 1995). This is achieved in vivo by limited proteolysis with specialised proteases, e.g. in response to a bacterial infection (Ashida and Yoshida 1988; Åspan et al. 1995; Söderhäll and Cerenius 1998). The removal of the peptide, which may correspond to a large fraction of the first structural domain, opens access of the substrate to the active site (Decker and Tuczec 2000). Functionally similar tyrosinases occur in phyla other than the Arthropoda, but these differ

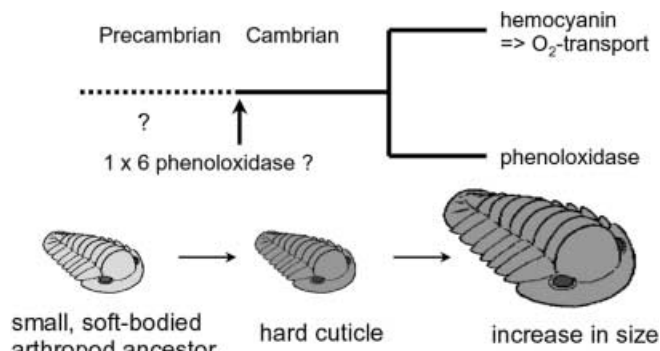


Fig. 3 Origin of the arthropod hemocyanins. Hypothetical course of events during the early evolution of the arthropod hemocyanin superfamily

essentially in their sequence and structure (see above). However, arthropod phenoloxidases display significant similarities with the arthropod hemocyanins and other members of this protein superfamily (Fujimoto et al. 1995; Hall et al. 1995; Kawabata et al. 1995; Burmester and Scheller 1996). The two copper-binding sites of the PPOs are closely related to that of the hemocyanins, and the six copper-binding histidines are strictly conserved (Fujimoto et al. 1995; Burmester and Scheller 1996; Terwilliger et al. 1999).

PPO sequences have been identified in insects (Diptera, Lepidoptera, Hymenoptera, and Coleoptera; e.g. Fujimoto et al. 1995; Hall et al. 1995; Kawabata et al. 1995; Jiang et al. 1997; Lee et al. 1999; Parkinson et al. 2001) and the decapod Crustacea (Åspan et al. 1995; Sritunyalucksana et al. 1999). Phenoloxidases are also present in the diplopod Myriapoda (e.g. Xyländer 1996); these enzymes have a molecular weight similar to that of the crustacean and insect PPOs (E. Jaenicke and H. Decker, personal communication). However, specific phenoloxidases may be absent in the Chelicerata (Nagai and Kawabata 2000; Decker et al. 2001). In this taxon, a clotting enzyme transforms the hemocyanin into a functional phenoloxidase without cleavage (Nagai and Kawabata 2000; Nagai et al. 2001). It is uncertain whether this mirrors the ancient phenoloxidase-function of the hemocyanins that is retained in chelicerate evolution (Zlateva et al. 1996; Decker and Rimke 1998; Decker et al. 2001), or whether it is an invention of the Chelicerata that recycles the phenoloxidase motif preserved in the hemocyanins.

Up to six PPO genes, which exhibit differential expression patterns, are present within certain insect species (Müller et al. 1999). Some of the PPO genes are recent developments that have occurred only within the insect orders or even specific genera, but there is also evidence that some of the genes diverged before the radiation of the holometabolous insects (Burmester 2001). The physiological relevance of the existence of multiple PPOs in insects and their functional differences are not clear. The information on PPO structure is poor and conflicting, either indicating monomers (Hall et al.

1995; Chase et al. 2000), dimers (Gollas-Galván et al. 1999) or tetramers (Åspan and Söderhäll 1991; Kopáček et al. 1995). However, from the point of view of conservation of inter-subunit contact sites and other structural features (Hall et al. 1995; Kawabata et al. 1995; Burmester and Scheller 1996), a hexameric arrangement of the subunits, similar to that of the hemocyanins, appears more likely. In fact, recent studies have shown that crustacean PPOs form hexamers *in vivo* that closely resemble the hemocyanin quaternary structure (E. Jaenicke and H. Decker, personal communication).

Origin and structure of arthropod hemocyanins

Hemocyanins have been found in all euarthropod subphyla. Thus they were present in the last common ancestor of the Chelicerata, Crustacea, Myriapoda and Insecta (Markl and Decker 1992; van Holde and Miller 1995; Burmester 2001). A putative hemocyanin also occurs in the Onychophora (K. Kusche and T. Burmester, unpublished observation). The emergence of hemocyanins in the arthropod stem line from oxygen-consuming phenoloxidases might be correlated with the animal's increase in size and the formation of the cuticle in the early Cambrian period (Figs. 2B, 3). Simple oxygen diffusion became inefficient to sustain the animal's metabolic needs and the evolution of efficient O₂-carriers probably provided significant selective advantages (Burmester 2001). It is noteworthy that many arthropods, e.g. the chironomid mosquitoes or the branchiopod crustaceans (Weber and Vinogradov 2001), apparently lost their hemocyanins later in evolution and recruited intracellular globins for this task.

Arthropod hemocyanins either form hexamers or multi-hexamers of six similar or identical subunits, each of which may bind one oxygen molecule (Markl and Decker 1992; van Holde and Miller 1995; Fig. 4). Although the functional relevance is unclear, a hexa-

meric structure probably first evolved in the phenol-oxidases and may be considered as a pre-adaptation for an optimal oxygen-transport with low osmotic impact. A typical arthropod hemocyanin subunit consists of about 630–660 amino acids (ca. 70–80 kDa protein) (Table 1), which are divided into three structural domains (Gaykema et al. 1984; Volbeda and Hol 1989). The first domain comprises the N-terminal 150–180 amino acids and is formed mainly by α -helices that build a super-secondary structure of a stable helical bundle. The second domain (about 220 amino acids) contains the two copper-binding sites, CuA and CuB, each of which consists of two α -helices with three histidine residues that co-ordinate the copper ions. The third domain (about 260 amino acids) is mainly built by β -sheets that form the super-secondary structure of a seven-stranded beta-barrel. X-ray structures of the hemocyanin subunits or hexamers have been resolved from *Panulirus interruptus* (Gaykema et al. 1984; Volbeda and Hol 1989) and *Limulus polyphemus* (Hazes et al. 1993).

One of the most striking feature of the arthropod hemocyanins is their enormous subunit diversity (Markl 1986), which is in contrast to the universal $\alpha 2\beta 2$ -scheme of the vertebrate haemoglobins (e.g. Dickerson and Geis 1983). However, the distinct hemocyanin subunit types are not orthologous among the subphyla, but evolved independently. The mode of assembly of the hexamers to higher quaternary structures also differs markedly among, and even within, the arthropod subphyla (Markl 1986; Markl and Decker 1992). Thus the evolution of the distinct subunits that occur in the Crustacea, Chelicerata and probably also the Myriapoda, from a single hemocyanin subunit type clearly occurred independently (Markl 1986; Burmester 2001; Fig. 2). Also, the formation of the multi-hexamers, which may aggregate up to 8×6-mers, occurred convergently, probably in response to osmotic requirements (Markl 1986; Markl and Decker 1992).

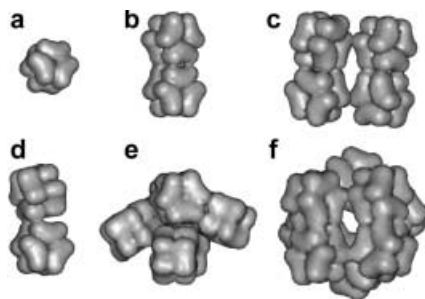


Fig. 4a–f Simplified quaternary structures of arthropod hemocyanins. **a** 1×6 hemocyanin found in many arthropod taxa; **b** 2×6 of many entelegyne spiders (e.g. *Cupiennius salei*); **c** arachnid 4×6 hemocyanin (e.g. *Eurypelma californicum*); **d** crustacean 2×6 hemocyanin (e.g. *Homarus americanus*); **e** myriapod 6×6 hemocyanin (e.g. *Scutigera coleoptrata*); **f** 8×6 hemocyanin of the Xiphosura (e.g. *Limulus polyphemus*). Adapted from Markl and Decker (1992)

Hemocyanin subunit diversity in Chelicerata

In the chelicerates, hemocyanins have been found in many spiders (Arachnida), Scorpiones and Xiphosura (horseshoe crabs), but are apparently absent in the Pantopoda (“sea spiders”), Solifugae (sunspiders) and Acari (mites) (Markl 1986; Markl et al. 1986). The Scorpiones and most arachnids possess a typical 24-mer (4×6) hemocyanin, the Xiphosura 48-mers (8×6). By comparative immunological studies, Markl and co-workers (for review, see: Markl 1986; Markl et al. 1986) identified up to eight distinct subunit types (*a–h*) that may occur in the different chelicerate classes (Fig. 5). The eighth subunit type (*h*), which is most likely a variant of the *a*-type, is only present in the Scorpiones, while the others (*a–g*) are homologous among the Arachnida, Xiphosura and Scorpiones (Markl 1986).

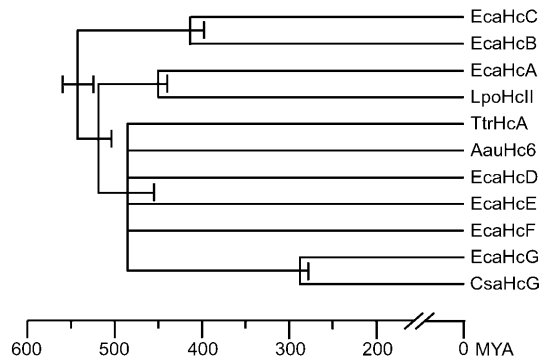


Fig. 5 Evolution of hemocyanins in the Chelicerata. The branch lengths were calculated by the neighbour-joining method using the PAM distance matrix (Dayhohh et al. 1979). The molecular clock was calibrated under the assumption that the Xiphosura and Arachnida diverged about 450 MYA (Dunlop and Selden 1997). (*EcaHcA–EcaHcG* *E. californicum* hemocyanin subunits *a* to *g*, *LpoHcII* *L. polyphemus* hemocyanin, *AauHc6* *Androctonus australis* hemocyanin 6, *TtrHcA* *Tachypleus tridentatus* hemocyanin α , *CsaHcG* six *C. salei* g-type hemocyanin subunits)

The different hemocyanin subunits each have distinct physico-chemical properties and sequences, but similar oxygen-binding behaviour (Decker et al. 1979; Markl et al. 1981a). Full biological activity such as cooperativity requires that each subunit occupies a distinct position within the native hemocyanin (Markl et al. 1981b). Several subunits have been sequenced on the protein level in the Xiphosura (horseshoe crabs) *Limulus polyphemus* and *Tachypleus tridentatus* (Linzen et al. 1985; Nakashima et al. 1986), the scorpion *Androctonus australis* (Buzy et al. 1995), and the North American tarantula *Eurypelma californicum* (Schartau et al. 1983; Schneider et al. 1983; Schartau et al. 1990). In addition, the cDNA sequences of all seven subunits (*a–g*) of *E. californicum* (Voit and Schneider 1986; Voit and Feldmaier-Fuchs 1990; Voit et al. 2000), as well as the gene of subunit *e* of the same species (Voll and Voit 1990), are known. Sequence comparison of the sequences confirms the earlier immunological classification of the different subunits (Markl 1986; Voit et al. 2000; Burmester 2001). The chelicerate hemocyanins are very conserved proteins, with a mean amino acid replacement rate of $0.5\text{--}0.6 \times 10^{-9}$ changes per site per year. The subunit sequences share about 53–64% of the amino acids. In phylogenetic analyses, the relative relationship among the subunit types is not well resolved, suggesting a rapid diversification from a single primordial subunit type. There is, however, strong evidence that the *E. californicum* subunits *b* and *c*, which are responsible for the interhexamer contact, are closely related and only diverged in the Arachnida around 410 MYA. Two distinct *b/c*-like contact subunits also exist in the horseshoe crab *Limulus*, but these are closely related and diverged independently from each other only within the Xiphosura (Lamy et al. 1983; Markl 1986). Estimates assuming a clock-like evolution suggest that the earliest divergence of the subunits occurred around 550 MYA (*b/c* versus other), indicating that the

speciation of distinct subunits occurred very early in the evolution of the Chelicerata. The radiation of the other subunits took place before the divergence of the Xiphosura and Arachnida, which occurred around 450 MYA (Dunlop and Seldon 1997). Thus it is likely that the ancestor of the chelicerate hemocyanins was a hexamer composed of single, but distinct *a*, *b/c*, *d*, *e*, *f* and *g*-type subunits. The multimerisation of the hemocyanin hexamers into a 4×6 multimer had probably already occurred in the chelicerate stem line, because the xiphosuran 48-mer structure is an exact duplication of the arachnid 24-mer hemocyanin (Bijlholt et al. 1982; Markl and Decker 1992).

The Scorpiones, the Uropygi (whipscorpions), Amblypygi (whipspiders), and most Aranaea (true spiders) have 4×6 hemocyanins (Markl 1986; Markl et al. 1986). The Opiliones (harvestmen) possess a 2×6 hemocyanin. The typical 24-mer hemocyanin of a spider such as *E. californicum* consists of $4 \times a$, $2 \times b$, $2 \times c$, $4 \times d$, $4 \times e$, $4 \times f$, and $4 \times g$ subunits (Markl et al. 1981b, 1986; Markl 1986). Variations of this standard scheme of the Aranaea have been observed in the Cribellata, the haplogyne spiders, and some entelegyne spiders. In these taxa, the 1×6 or 2×6 hemocyanin is composed of only one to five distinct subunits, which nevertheless correspond to the above-mentioned subunit types (Markl 1986; Markl et al. 1986). For example, the 2×6 hemocyanin of the hunting spider *Cupiennius salei* possess six g-type subunits, which differ by about 20% of the amino acids (Markl 1980; P. Ballweber, T. Burmester and J. Markl, unpublished results; Fig. 5). These structurally more simple hemocyanins can be considered as secondary modifications resulting from recent subunit loss (Markl 1986; Voit et al. 2000).

The hemocyanins of the Myriapoda

Like the insects, the Myriapoda possess a tracheal system. Therefore, specialised respiratory proteins have long been considered unnecessary in this taxon. The presence of hemocyanins in the centipedes *Scutigera longicornis* and *S. coleoptrata* (Rajulu 1969; Mangum et al. 1985) was attributed to their peculiar blind-ending trachea. However, recently it became evident that such proteins occur in the Spirostreptidae (Diplopoda) as well (Jaenicke et al. 1999; Kusche and Burmester 2001b). Although information concerning the minor myriapod taxa (Symphyla and Pauropoda) is still missing, these findings suggest that oxygen-transporting hemocyanins may be rather widespread within this taxon. The hemocyanins of the Spirostreptidae and Scutigeraformia form quaternary structures of 6×6 subunits (Fig. 4). While the arrangement of the hexamers within the chelicerate and crustacean hemocyanins is variable (Markl 1986; Markl et al. 1986; Markl and Decker 1992), this unique 36-mer hemocyanin is a common feature of the Diplopoda and Chilopoda. The conservative architecture of the myriapod hemocyanins

is noteworthy, because Diplopoda and Chilopoda diverged at least 400 MYA (Shear 1997), and there is molecular evidence that this split may be even deeper (Friedrich and Tautz 1995). However, recent data show that the centipede *Lithobius forficatus* possesses a simple 6-mer hemocyanin (K. Kusche and T. Burmester, unpublished observation), suggesting that different quaternary structures may also occur in the myriapod hemocyanins. Immunological distinct subunit types exist in the chilopod *S. coleoptrata* and the diplopod *Spirostreptus* sp., although it is unknown how they are related (Gebauer and Markl 1999; Jaenicke et al. 1999). A hemocyanin subunit sequence from *Spirostreptus* sp. and two subunit sequences from *S. coleoptrata* have been determined (Kusche and Burmester 2001b; K. Kusche, W. Gebauer, and T. Burmester, unpublished results). The diplopod and chilopod hemocyanins share about 50% of their amino acids. The rate of amino acid evolution was estimated to be $\sim 0.9\text{--}1.2 \times 10^{-9}$ substitutions per site per year (Table 1). One most striking feature is the deletion of alpha-helix 1.2 of the first structural domain in the myriapod hemocyanins; this has been found so far only in the chelicerate hemocyanins and may be considered as support for a close relationship of these taxa (Kusche and Burmester 2001b).

Hemocyanin evolution in the Crustacea

Hemocyanins are present in the malacostracan Crustacea, including the Hoplocarida (mantis shrimps etc.), the Eucarida (Decapoda) and the Pericarida (Isopoda, Amphipoda, Mysidacea and Euphausiacea) (Mangum 1983; Markl 1986). There are two reports on the presence of hemocyanins in non-malacostracan Crustacea ("Entomostraca"). However, it is uncertain whether the hemocyanin of the cirriped *Sacculina carcini* is an endogenous protein or may derive from the decapod host, *Carcinus maenas* (Herberts and de Frescheville 1981). In the haemocytes of the Remipedia, large crystals have been detected that resemble those of the *Limulus polyphemus* 8x6 hemocyanin (Yager 1991). This observation also requires confirmation by biochemical means. In several entomostracan Crustacea, multimeric haemoglobins serve as oxygen carriers (Mangum 1983). It is uncertain whether any of these species also possess hemocyanin. Nevertheless, the presence of hemocyanins in the other arthropod subphyla strongly suggests that such proteins occurred in the crustacean stem line. Malacostraca are a rather recent clade of the Crustacea, which only emerged from an uncertain crustacean ancestor about 360 MYA, while the other classes may have diverged as early as 550 MYA (Schram 1982; Briggs et al. 1993). It seems possible that most Entomostraca have lost hemocyanins, which have been replaced in many species by haemoglobins.

Within the Malacostraca, there is little information on hemocyanin subunit composition of the Hoplocaridae and Pericaridae (Mangum 1983). These species mostly possess hexameric hemocyanins (1x6), although in the mantis shrimp *Squilla mantis* (Hoplocaridae) a unique 2x6-mer has been observed (Bijlholt and van Bruggen 1986). The eucarid taxa of the Isopoda and Euphausiacea have 1x6 hemocyanins, which are composed of immunological identical subunits (Markl 1986). However, recent data suggest multiple subunit types also exist in amphipod and isopod species (Hodgson and Spicer 2001). Most hemocyanins of the Decapoda are either hexamers or dihexamers (Markl 1986; Markl et al. 1986; Markl and Decker 1992), although there is at least one exception: the thalassinid shrimp *Callinassa californiensis* possess a 4x6 hemocyanin (Miller et al. 1977). Subunit composition and evolution of the decapod hemocyanin-hexamer differ strikingly from those of Chelicerata (Markl 1986; Markl et al. 1986; Burmester 2001). While the structure of the chelicerate hemocyanins is highly conserved and may have been retained for more than half a billion years, there is much more variability in terms of subunit organisation in the Crustacea. First, subunit composition and quaternary structures may differ even between closely related species, and may vary even within populations (Markl 1986; Markl et al. 1986; Stöcker et al. 1988; Mangum and Joy 1997). Second, some subunits may be expressed only in certain developmental stages or under specific physiological conditions (Markl and Decker 1992; Durstewitz and Terwilliger 1997b; Terwilliger 1998). This has never been observed in the Chelicerates or Myriapoda.

Immunological studies have demonstrated three types of subunits to occur in the Decapoda (α , β , and γ ; Markl 1986; Markl et al. 1986; Fig. 6). These differentiated from a common ancestor after the Decapoda diverged from the other Eucarida around 250 MYA (Briggs et al. 1993). Subunits β and γ may be absent in some species, but the α -subunit is apparently common to all decapod hemocyanins. Amino acid or cDNA sequences are only known from α - and γ -type subunits of various Decapoda (Bak and Beintema 1987; Jekel et al. 1988, 1996; Neuteboom et al. 1992; Durstewitz and Terwilliger 1997b; Kusche and Burmester 2001a; Kusche and Burmester, unpublished observations). The rate of amino acid replacement in the crustacean hemocyanins is about two times higher than that of the chelicerate proteins (Burmester 2001; Table 1). The γ -subunits evolved about 25% faster than the α -subunits, consistent with the proposed central role in the crustacean hemocyanins of the latter (Kusche and Burmester 2001a). Phylogenetic analyses show that α - and γ -subunits diverged about 200–210 MYA (Fig. 6). This is much more recent than the time of divergence of the chelicerate hemocyanin subunits (see above), suggesting that either the early crustaceans had only a single hemocyanin gene or that at least one severe hemocyanin-bottleneck occurred during crustacean evolution.

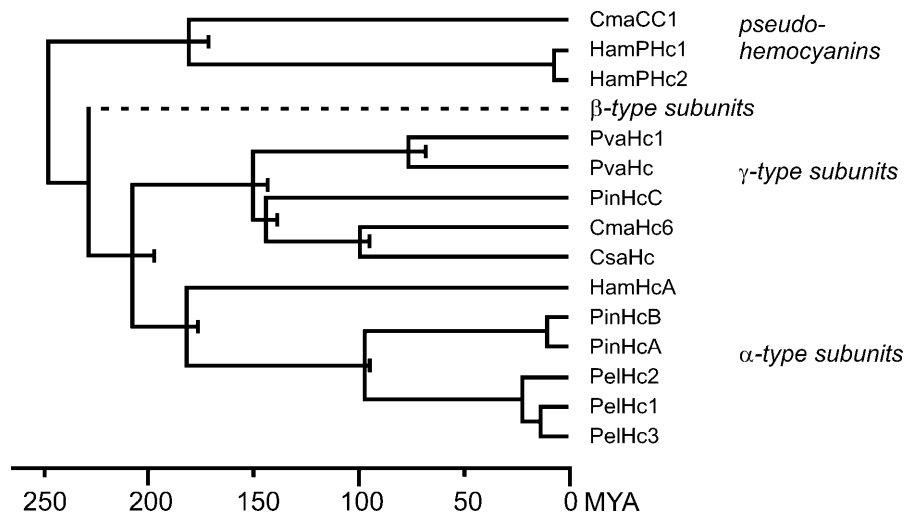


Fig. 6 Evolution of crustacean hemocyanins and pseudo-hemocyanins. The tree was estimated as in Fig. 6, assuming that the Astacura and Palinura diverged 180 MYA, and the Brachyura and Palinura 150 MYA (Briggs et al. 1993). The position of the β -type subunits was inferred on the basis of N-terminal sequences and immunological data (Markl 1986). (*CmaHc6* *Cancer magister* hemocyanin subunit 6, *PvaHc* and *PvaHc1* *Penaeus vannamei* hemocyanin subunits, *PinHcA-C* *Panulirus interruptus* hemocyanin subunit *a* to *c*; *HamHcA* *H. americanus* hemocyanin A, *CsaHc* *Callinectes sapidus* hemocyanin, *PelHc1-3* *Palinurus elephas* hemocyanin subunits 1 to 3; *CmaCC1* cryptocyanin, *HamPHc1* and *HamPHc2* *H. americanus* pseudo-hemocyanin 1 and 2)

Pseudo-hemocyanins – the storage proteins of the decapod Crustacea

Hexameric proteins that closely resemble the hemocyanins in their structure, but do not bind oxygen, have been observed in the haemolymph of some Decapoda (Markl et al. 1979). These proteins were termed non-respiratory proteins (NRPs; Markl et al. 1979; Markl 1986). After recognition of their actual relationship with the hemocyanins, they have been renamed as “cryptocyanin” in case of *Cancer magister* (Terwilliger et al. 1999) or “pseudo-hemocyanin” in *Homarus americanus* (Burmester 1999b). At least one of the copper-binding histidine residues of these proteins is replaced by another amino acid, so these proteins do not contain any copper. Because of their stage-specific synthesis (Terwilliger et al. 1999), it is not clear whether these proteins are a common feature of all Decapoda, or whether they have been lost in some taxa such as the Palinurae (Markl et al. 1979).

A substitution rate of about 2.0×10^{-9} per site and year has been determined for the pseudo-hemocyanins, which is significantly higher than that of the hemocyanins (Table 1). This suggests a relaxation of selective pressure due to the loss of oxygen binding properties. Molecular phylogenetic analyses show their emergence only within the lineage leading to the higher decapod Crustacea (Burmester 1999a, 2001), probably about 230–240 MYA. Thus their universal occurrence in the

Arthropoda or even Ecdysozoa, as suggested by Terwilliger et al. (1999) is rather unlikely. Pseudo-hemocyanins are synthesised either in the connective tissue of the hepatopancreas (*Cancer magister*; Terwilliger et al. 1999) or in the heart tissue and the ovaries (*Homarus americanus*; Burmester 1999b). The concentration of pseudo-hemocyanin changes with the moulting cycle (Terwilliger et al. 1999), indicating that that these proteins act as storage proteins. Although the pseudo-hemocyanins are not evolutionarily homologous to the insect storage hexamerins, their similar function suggests an interesting case of parallel evolution that uses the same protein motive.

Hemocyanins in the insects?

Sánchez et al. (1998) described a potential hemocyanin in the haemolymph of an embryonic grasshopper, *Schistocerca americana* (Orthoptera). Although its function as a true oxygen-carrier protein has not been demonstrated yet, the strict conservation of the copper-binding sites implies that this protein is involved in oxygen-metabolism. In phylogenetic analyses, the insect hemocyanin tends to cluster with the hexamerins (Burmester 2001; Fig. 2A). The insect proteins share a recent common ancestry with the crustacean hemocyanins and pseudo-hemocyanins, supporting the theory of a pan-crustacean taxon (see above). The *S. americana* hemocyanin is exclusively synthesised in the haemocytes of the embryos and disappears in later stages. Its physiological role in *S. americana* is mysterious, but it is possible that oxygen-supply in some insects is more complex than thought. Nevertheless, other functions such as a hemocyanin-derived phenoloxidase involved in the embryonic immune response are conceivable. However, no hemocyanin was found in the complete genomic sequence of the fruit fly *Drosophila melanogaster* (Adams et al. 2000), suggesting that the “higher” Neoptera may have lost any hemocyanin-based oxygen carrier. This notion is supported by the observation that in several neopteran

insect orders, a haemoglobin evolved (e.g. Weber and Vinogradov 2001), most likely from an intracellular globin-ancestor (Burmester and Hankeln 1999).

The insect storage hexamerins

Hexamerins have been found in the haemolymph of all insect species that have been investigated so far (Telfer and Kunkel 1991; Burmester 1999b). Most of these prominent proteins are built by 1×6 identical or closely related subunits (Telfer and Kunkel 1991), although a 2×6 quaternary structure exists as well (Markl et al. 1992). They resemble the hemocyanins in structure and sequence (Fujii et al. 1989; Willot et al. 1989), although they do not bind copper or oxygen. Most hexamerins are considered to be storage proteins. This assumption is based on the observation that these proteins accumulate to extraordinarily high concentrations in the haemolymph of larvae or nymphs (Scheller et al. 1990; Telfer and Kunkel 1991). These proteins provide energy and amino acids to build adult structures (Levenbook and Bauer 1984). Some hexamerins also fulfil other functions (for review, see: Scheller et al. 1990; Telfer and Kunkel 1991; Haunerland 1996; Burmester 1999b): they may serve as carrier proteins for small organic compounds like steroid hormones (Enderle et al. 1983), riboflavin (Magee et al. 1994), juvenile hormone (Braun and Wyatt 1996), or – probably in a more non-specific manner – also for insecticides (Haunerland and Bowers 1986). Other studies have demonstrated that hexamerins are incorporated into the pupal cuticle (Peter and Scheller 1991) and the developing antenna (Danty et al. 1998), or may be involved in the humoral immune response (e.g. Hayakawa 1994; Beresford et al. 1997). A hexamerin may also be a component of the venom of the braconid wasp *Bracon hebetor* (Quistad et al. 1994).

In phylogenetic analysis, the hexamerins form a stable monophyletic clade, which is most likely in sister-group position with the only known insect hemocyanin (Burmester 2001). This suggests that storage hexamerins evolved within the insect stem line, probably around 400 MYA (Fig. 7), close to the appearance of the first hexapods in the fossil record (Kukalová-Peck 1991). Two different levels of hexamerin evolution have been observed within the insects (Burmester et al. 1998; Burmester 1999b, 2001) (Fig. 7). The basic scheme follows the generally accepted phylogeny of the insects. Thus, on the superorder level, the diversification of the hexamerins followed the evolution of taxa (Fig. 7). The only exceptions are the lepidopteran riboflavin-binding hexamerins, which may have been lost in other Holo-metabola. Within the insect orders, a broad scheme of diversification into different hexamerin-types has been observed. Some of the hexamerins have accumulated distinct types of amino acids (Telfer and Kunkel 1991; Burmester et al. 1998; Burmester 1999a). These are the highly aromatic hexamerins (“arylphorins”), which have evolved convergently in the Coleoptera, Diptera, Lepidoptera and possibly also other insect orders (Burmester et al. 1998). Similarly, some lepidopteran hexamerins have accumulated high amounts of methionine. These features may reflect specific needs of the developing insect for these amino acids (Telfer and Kunkel 1991; Haunerland 1996; Burmester 1999b), or may enhance the cross-linking-ability of the arylphorins required in the humoral immune response (Hayakawa 1994; Beresford et al. 1997). In some cases, the distinct hexamerins that exist within a single insect species had diverged more than 200 MYA, obviously reflecting their particular functions (Burmester 1999b).

Origin of the insect hexamerin receptor from its own ligand

In the late insect larval stages, hexamerins are incorporated into the cells of the fat body (e.g. Ueno and Natori 1982; Burmester and Scheller 1992). Hexamerin uptake is controlled by the arthropod moulting hormone 20-hydroxyecdysone and requires the presence of a specific hexamerin receptor at the cell surface (Ueno et al. 1983; Ueno and Natori 1984; Burmester and Scheller 1995a, 1997; Burmester et al. 1999). Receptor sequences are only known from the Diptera, notably from the blowfly *C. vicina* (Burmester and Scheller 1995b), the flesh fly *Sarcophaga peregrina* (Chung et al. 1995), and the fruit fly *Drosophila melanogaster* (Maschat et al. 1990; Burmester et al. 1999). These proteins show remarkable similarities to the hexamerins as well as to the other members of the hemocyanin-superfamily (Burmester and Scheller 1995b). The similarities are mainly restricted to the N- and C-terminal ends, and cover approximately the first and third structural domains of hemocyanins (Burmester and Scheller 1996). The middle

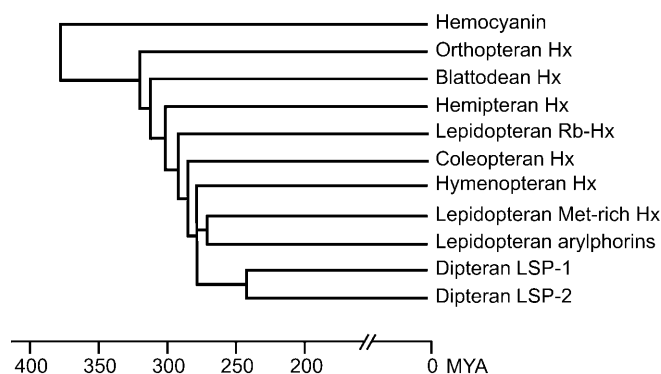


Fig. 7 Evolution of insect hexamerins. This simplified phylogenetic tree was constructed using the neighbour-joining method based on 53 hexamerin sequences. The tree was linearised under the assumption that the Orthoptera diverged from the other Neoptera 320 MYA, and that the Brachycera and the Nematocera split 220 MYA (Kukalová-Peck 1991). (*Hx* hexamerins, *LSP* larval serum proteins, *Met-Hx* methionine-rich hexamerins, *Rb-Hx* riboflavin-binding hexamerins)

region is highly variable, resulting in a high overall replacement rate of 7.5×10^{-9} substitutions per site (Table 1). Although only the N- and C-terminal regions can be aligned with high confidence, phylogenetic studies clearly show an ancient position of the receptors relative to all known insect hexamerins, indicating that they are common to all insects (Burmester and Scheller 1996; Burmester et al. 1998). This suggests that the receptors emerged after the hexamerins differentiated from the hemocyanins some 400 MYA, but before the radiation of the neopteran insects 350 MYA. The striking sequence similarities may be explained by the assumption that it is likely that the same sites which are responsible for the interaction of the multi-hexameric hemocyanins have evolved to ligand-receptor interaction regions (Burmester and Scheller 1996). According to this model, the ligand part of this pair remained soluble in the haemolymph but the hexamerin receptors became associated with the protein uptake machine by some means, which consequently allowed the intracellular storage of hexamerins (Burmester and Scheller 1996, 1999).

Perspectives

Despite recent advances in the understanding of the evolution of the arthropod hemocyanins superfamily, many challenging questions remain to be solved. In future studies, hemocyanins and phenoloxidases must be looked for in several minor, but evolutionarily important taxa such as the Onychophora, Paurpoda or Symphyla. Similarly, the large assembly of the entomostracan Crustacea clearly requires attention. In other taxa, hemocyanins have already been identified, but not yet sequenced. In addition, new members of the hemocyanin-superfamily may be discovered that serve functions other than those reported above, and new functions of known members may be reported. Most notably, several recent studies suggest that hemocyanins and hexamerins are involved in the arthropod immune system (Hayakawa 1994; Beresford et al. 1997; Nagai and Kawabata 2000; Decker et al. 2001). These findings may eventually provide a new functional link between phenoloxidases, hemocyanins, pseudo-hemocyanins and hexamerins. Moreover, the subunit diversity of phenoloxidases, hemocyanins and hexamerins requires further studies. Finally, the sequencing of new genes, anonymous cDNAs ("expressed sequence tags", ESTs) or complete genomes may eventually provide an answer to the still unresolved question of the ancestry of the arthropod hemocyanin superfamily.

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