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Comparative Biochemistry and Physiology, Part B 149 (2008) 439–446
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o-Diphenol oxidase activity of molluscan hemocyanins

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Received 28 September 2007; received in revised form 17 November 2007; accepted 19 November 2007
Available online 24 November 2007

Abstract

Diphenoloxidase activities of two molluscan hemocyanins, isolated from the marine snails *Rapana venosa* and garden snails *Helix vulgaris* were studied using *o*-diphenol and L-Dopa as substrates. The dimers of *H. vulgaris* Hc show both, diphenol (K_m=2.86 mM and K_{cat}=4.48) and L-Dopa activity due to a more open active sites of the enzyme and better access of the substrates. The K_m value of molluscan *H. vulgaris* Hc is very close to those of *Helix pomatia* and *Sepia officinalis* Hcs, but several times higher compared to those of *Rapana* and *Octopus* Hcs. Also HvH has a very high enzyme activity compared with other molluscan Hcs. Kinetic measurements with native RvH and both structural subunits, RvH1 and RvH2, show that RvH and only one structural subunit, RvH2, exhibited only *o*-diphenol activity, but no L-Dopa oxidizing activity.

Keywords: *Rapana venosa* hemocyanin; *Helix vulgaris* hemocyanin; *o*-Diphenoloxidase activity; kinetic constants

1. Introduction

Type-3 copper proteins have evolved from a dinuclear processor which segregated into hemocyanins (Hcs), tyrosinases (Ty) and phenoloxidases. Molluscan and arthropodan Hcs as well as tyrosinases are copper-containing proteins with similar active sites, but they differ largely with respect to size, primary, tertiary and quaternary structure and their physiological functions (Dolashka et al., 1996; Dolashka-Angelova et al., 2005; Jaenicke and Decker, 2004a,b; Van Holde et al., 2001). While hemocyanins transport dioxygen, phenoloxidase uses one oxygen of the dioxygen molecule for chemical transformations.

Hemocyanins are large multisubunit copper proteins composed of different subunit types and found freely-dissolved in the hemolymph of arthropods and molluscs (Markl and Decker, 1992; Salvato and Beltramini, 1990; Van Holde and Miller 1982; Van Holde and Miller, 1995). Their dioxygen transport and storage function is based on their capability to bind reversibly molecular oxygen at their active sites to which a pair of copper ions is attached (dinuclear-coupled copper site; Van Holde and Miller, 1995).

Phenoloxidases catalyze *o*-hydroxylation of monophenols (cresolases), oxidation of *o*-diphenols to quinones (catecholoxidase) or both substrates (tyrosinases) (Ashida and Brey, 1995; Decker et al., 2000; Decker and Terwilliger, 2000; Hearing and Tsukamoto, 1991; Itoh and Fukuzumi, 2007; Johansson and Söderhäll, 1996; Sanchez-Ferrer et al., 1995; Söderhäll and Cerenius, 1998; Solomon et al., 1996). So far, only one crystal structure of a phenoloxidase is available, that of a catecholoxidase from sweet potato (*Ipomoea batatas*; Klabunde et al., 1998) which is related to a molluscan hemocyanin with respect to sequence similarity (about 25%) and its active site (Klabunde et al., 1998). Two different types of tyrosinases can be classified based on their sequences. One type is more related to molluscan hemocyanins, and the other type, found in arthropods together

Abbreviations: EM, electron microscopy; Hcs, hemocyanins; HvH, *Helix vulgaris* Hc; K_m, Michaelis–Menten constant; RvH, *Rapana venosa* Hc; RvH1 and RvH2, subunit isoforms of *Rapana venosa* Hc; SDS, Sodium dodecyl sulphate; Ty, tyrosinase; V_{max}, maximum velocity.

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doi:10.1016/j.cbpb.2007.11.004

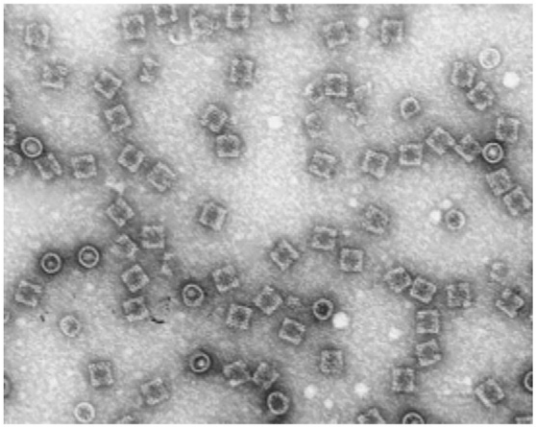

Fig. 1. Electron micrograph of native *Rapana venosa* hemocyanin (RvH) in 50 mM Tris/HCl buffer, pH 7.0, containing 20 mM $CaCl_2$ and 5 mM $MgCl_2$. Staining with 1% uranyl acetate was performed as described in Materials and methods. The scale bar indicates 100 nm.

with hemocyanins is very similar to them (Decker et al., 2001; Nakahara et al., 1983; Salvato and Beltramini, 1990; Salvato et al., 1983; Solomon, 1981; Van Gelder et al., 1997; Wilcox et al., 1985).

Besides exhibiting similar copper oxygen-binding sites, phenoloxidases and hemocyanins have another feature in common: their enzymatic properties can be activated in similar ways by proteolitic cleavage of N- or C-terminal fragments and disturbing their protein structures. Hemocyanins of arthropods and molluscs also exhibit *o*-diphenoloxidase activity after exposure to chaotrophic salts like sodium dodecyl sulphate (Decker et al., 2001; Pless et al., 2003), urea (Morioka et al., 2006) or low pH values (Baird et al., 2007; Salvato et al., 1998; Zlateva et al., 1996).

Several physicochemical properties and functions of Hcs are very similar to those of phenoloxidases (Decker and Rimke, 1998; Decker and Tuczek 2000; Salvato and Beltramini, 1990; Salvato et al., 1998). For example, some Hcs have monophenolase and diphenolase activities (Nakahara et al., 1983), but with much lower efficiency as compared to tyrosinases. Studies on the interaction of *o*-diphenols with the Hc from the molluscs *Octopus vulgaris, Helix pomatia* and *Sepia officinalis* give information on the specific properties of the active site which are relevant for the appearance to catalytic activity (Siddiqui et al., 2006). A reaction mechanism is based on oxy-Hc as active species and on substrate radical formation as a result of substrate interaction.

Comparative studies on the interaction of Hc and Ty with exogenous molecules showed that the active site of both proteins reacts in the same way with respect to exogenous species. Within Hcs, the proteins from mollusks are more reactive toward exogenous ligands as compared to arthropod Hcs (Nakahara et al., 1983; Nellaiappan and Vinayakam, 1993).

Here we investigated the catalytic activities of oxy-Hcs isolated from the mollusc *Rapana venosa* and *Helix vulgaris* against *o*-diphenol and 3,4-dihydroxy-L-phenylalanine (L-Dopa) as substrates in comparison to those of other Hcs from molluscs and arthropods.

2. Materials and methods

2.1. Purification procedures

R. venosa Hc was isolated from the hemolymph of marine snails living in the west coast of the Black Sea near Varna. The hemolymph was centrifuged at 5000 ×*g* at 4 °C and phenylmethanesulfonyl fluoride (PMSF) was added to inhibit proteases. Hemocyanin was isolated by preparative ultracentrifugation, using a Beckman L-80 ultracentrifuge, equipped with a Ti 45 UZ rotor, at a speed of 24 000 rpm for 4 h at 4 °C. The protein was stored at −20 °C in the presence of 20% sucrose.

The two subunits, RvH1 and RvH2, were isolated by the procedure described in (Dolashka-Angelova et al., 2003). The native Hc was dialyzed in 0.13 M glycine/NaOH buffer, pH 9.6, for 24 h. The dissociated fractions were loaded on an ion exchange chromatogramphy DEAE-Sepharose CL-6B column, equilibrated with 50 mM Tris/HCl buffer, 10 mM EDTA, pH 8.2. The structural subunits were separated by elution with the same buffer and a linear sodium chloride gradient (0.15–0.40 M NaCl) with a flow rate 0.2 mL min^{-1}.

H. vulgaris hemolymph was collected from garden snails from the Sofia area and centrifuged at 5000 ×*g* for 15 min to remove hemocytes. Hc was sedimented in a Beckman L-80

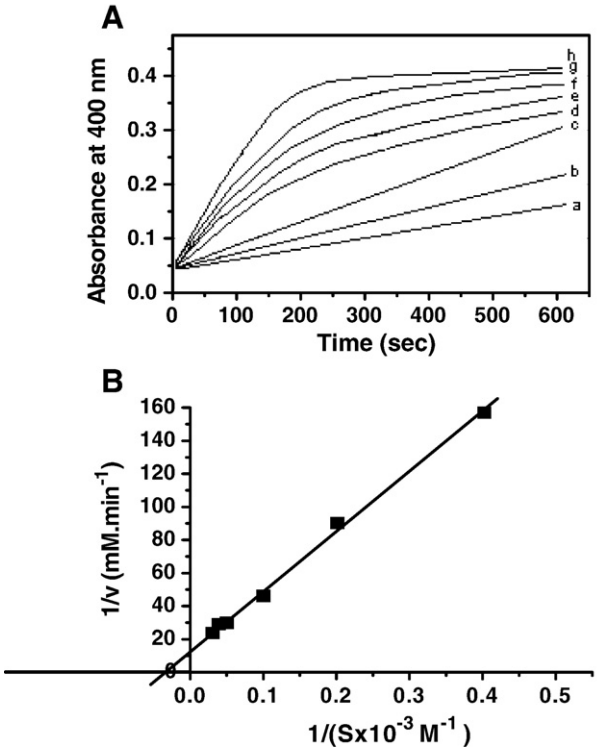

Fig. 2. *o*-Diphenoloxidase activity of native (didecamers) *Rapana venosa* hemocyanin, expressed as initial velocity (ΔA_{475} nm/min) of quinone formation in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of quinone formation in the presence of *R. venosa* Hc at different concentrations of *o*-diphenol: (a) 1.25×10^{-3} M, (b) 2.5×10^{-3} M, (c) 5.0×10^{-3} M, (d) 10×10^{-3} M, (e) 15.0×10^{-3} M, (f) 20.0×10^{-3} M, (g) 25.0×10^{-3} M, (h) 30.0×10^{-3} M. (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate *o*-diphenol.

Table 1
Kinetic parameters of *o*-diphenol oxidase activity of molluscan *Rapana venosa* and *Helix vulgaris* hemocyanins in comparison with other molluscan and arthropodan Hcs, and tyrosinases

Substrates	*o*-diphenol				L-Dopa			
	K_m (mM)	V_{max} (mM min^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m ($mM^{-1}min^{-1}$)	K_m (mM)	V_{max} (mM min^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m ($mM^{-1}min^{-1}$)
Molluscan Hcs								
R. venosa								
Native (didecamers)	28.57	0.08	2.66	0.093	g	g		
RvH2 (subunits)	23.81	0.105	3.5	0.147	g	g		
H. vulgaris								
Native (didecamers)	2.86	0.137	4.48	1.57	0.77	0.018	1.8	2.32
Octopus vulgaris								
Native (decamers)	35[a]	0.37[a]	10[b]	0.28[b]				
Helix pomatia								
Native (didecamers)	3.1[b]	0.01	0.21[b]	0.068[b]	g			
Dimers of subunits	2.6[b]	0.05	0.62[b]	0.23[b]	g			
Sepia officinalis								
Dimers of subunits	3.8[b]	0.08	1.0[b]	0.26[b]				
Arthropodan Hcs								
Carcinus aestuarii								
(16S native)	142[c]	0.023[c]	1.7[b]	0.012[b]				
Induced by $NaClO_4$	182[c]	0.091[c]	6.8[b]	0.037[b]				
16S *CmSS 3*	250[c]	0.15[c]						
5S *CmSS2*	219[c]	1.01[c]						
Cancer magister	0.02[d]	0.16 [d]			1.70 [d]	0.236[d]		
H. americanus	200[c]	0.15[c]	11.0[b]	0.055[b]				
Modified Hcs								
Limulus polyphemus	SDS[e]							
Euripelma califonicum	SDS[e]							
H. pomatia	Subtilisin[f]							
Helix aspersa	SDS[e]							
O. vulgaris	SDS[b]							
Tyrosinases								
Illex argentinus								
(Native ST94)	9.3[d]		2000[b]	215[b]				
Ipomoea batatas								
(Native)	2.5[e]		138.000[b]	55.000[b]				

References: [a] (Salvato et al., 1998), [b] (Siddiqui et al., 2006), [c] (Zlateva et al., 1996), [d] (Terwilliger and Ryan, 2006), [e] (Decker et al., 2001), [f] (Bhagvat and Richer, 1938). g — do not show activity.

ultracentrifuge, equipped with a Ti 45 UZ rotor, at a speed of 24 000 rpm for 4 h, at 5 °C. The blue pellet of native hemocyanin was resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$.

Before use, Hcs were dialyzed in 20 mM phosphate buffer, pH 6.0. Protein concentration was determined spectrophotometrically from the absorption at 280 nm, using the value $E = 1.36$ mL/mg/cm for *Rapana* Hc and $E = 1.416$ mL/mg/cm for *Helix* Hc. Absorption spectra were recorded on a Shimadzu spectrophotometer, model Mini-1240.

2.2. Electron microscopic measurements

Studies on EM specimens were performed using a Philips CM 10 electron microscope with a 30 mm objective aperture. Samples were adsorbed for 60 s to a glow-discharged pistoform/carbon-coated support film, washed three times with droplets of distilled water to remove buffer salts and then negatively stained with 1% uranyl acetate. Electron micrographs were routinely recorded at an instrumental magnification of 52,000.

2.3. Amino acid sequence determination

HPLC-purified fractions were dissolved in 40% methanol, 1% formic acid and subjected to automated Edman degradation (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

2.4. Kinetic measurements

The *o*-diphenol oxidase activity of Hcs was assayed at 25 °C. Proteins were dissolved in 20 mM phosphate buffer, pH 6.8. This pH was chosen to minimize autoxidation of the substrate. The reaction was initiated by adding the substrate solutions,

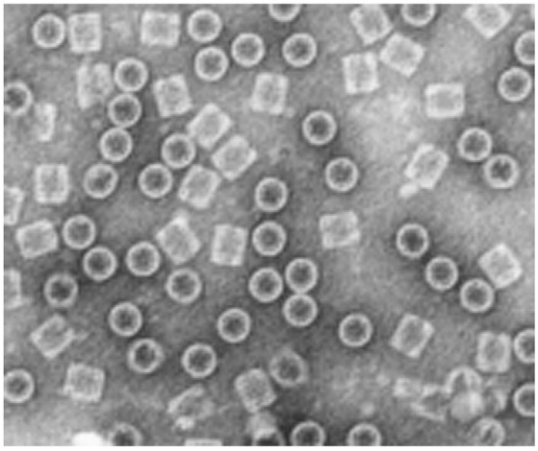

Fig. 3. Electron micrograph of native *Helix vulgaris* hemocyanin (HvH) in 50 mM Tris/HCl buffer, pH 7.0, containing 20 mM $CaCl_2$ and 5 mM $MgCl_2$. Staining with 1% uranyl acetate was performed as described in Materials and methods. The scale bar indicates 100 nm.

where the concentrations of *R. venosa* Hc and its structural subunits RvH2 ranged between $1.25–30\times10^{-3}$ M and those of *H. vulgaris* Hc between $0.31–25\times10^{-3}$ M. Quinone formation was followed *via* a Shimadzu UV-2100 double-beam spectrophotometer monitoring the absorbance at 400 nm for *o*-diphenol and 475 nm for 3,4-dihydroxy-L-phenylalanine for 10–40 min. The reaction velocity was measured from the initial quasi-linear portion of the curves (usually 0–2 min) and Lineweaver–Burk plots were used to determine the kinetic parameters K_m, K_{cat} and V_{max} for the Hc species. For calculation of *o*-quinone concentration a molar absorption coefficient of 1417 M^{-1} cm^{-1} was used (Zlateva et al., 1996).

3. Results

Recently, quite a few reports appeared about the phenoloxidase activity of hemocyanins. Therefore, the diphenoloxidase activity of two hemocyanins, isolated from *R. venosa* (RvH) marine snails and *H. vulgaris* (HvH) garden snails, as well as the structural subunits from RvH1 and RvH2, were studied using *o*-diphenol and L-Dopa as substrates.

R. venosa Hc and its structural subunits RvH1 and RvH2 were isolated as described before (Dolashka-Angelova et al., 2003) and the purities of the samples were controlled by 7.5% polyacrylamide gel electrophoresis (Laemmli, 1970). Electron microscopic investigations confirmed that the isolated biomolecules from native aggregates form didecamers or decamers as shown in Fig. 1.

The capability of *R. venosa* Hc to catalyze the oxidation of *o*-diphenol to the corresponding *o*-quinone was measured at different concentrations of *o*-diphenol (from 1.25×10^{-3} M to 30.0×10^{-3} M) (Fig. 2) following the reaction by the increase of the absorbance at 400 nm, observed upon incubation of the substrate with native oxy-Hc at a concentration of 1.25 mg mL^{-1} in 20 mM phosphate buffer, pH 6.8. The time courses of quinone formation versus a special protein concentration of the native molecule of *R. venosa* Hc are presented in Fig. 2A. Based on these parameters, the Lineweaver–Burk plot was drawn (Fig. 2B), and the kinetic parameters (K_m=28.57 mM, K_{cat}=2.66 min^{-1}, K_{cat}/K_m=0.093 mM^{-1} min^{-1} and V_{max}=0.08 mM min^{-1}) for *R. venosa* Hc were determined (Table 1) and compared with other molluscan (*O. vulgaris, H. pomatia* and *S. officinalis*) and several arthropodan Hcs (Solomon et al., 1996; Van Gelder et al., 1997).

Applying the same method, diphenoloxidase activity of molluscan *H. vulgaris* Hc was analysed. The hemocyanin was isolated from the hemolymph of garden snails after centrifugation at 5000 ×*g* for 15 min and sedimentation a Beckman L-80 ultracentrifuge, equipped with a Ti 45 UZ rotor at a speed of 24 000 rpm for 4 h, at 5 °C. The native hemocyanin was resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$ and investigated by electron microscopy. The native molecule was additionally purified on Superdex 300 column (date not shown). As seen from Fig. 3, only didecameric forms are observed which confirm that the isolated protein adapted a native structure.

After treatment of the native Hc (concentration 1.15 mg mL^{-1}) with *o*-diphenol as substrate, *o*-diphenoloxidase activity was determined. Different concentrations of *o*-diphenol (from 0.31×10^{-3} M to 25.0×10^{-3} M) were used for the oxidation experiments and, both, time courses and dependence of the rate of quinone formation in the presence of *H. vulgaris* Hc are

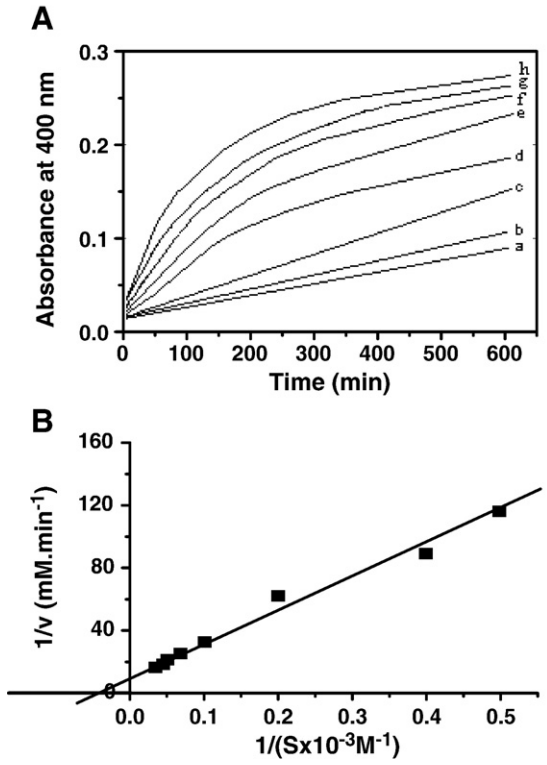

Fig. 4. *o*-Diphenoloxidase activity of native (didecamers) *Helix vulgaris* hemocyanin, expressed as initial velocity (ΔA_{475} nm/min) of quinone formation in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of quinone formation in the presence of *Helix vulgaris* Hc at different concentrations of *o*-diphenol: (a) 0.31×10^{-3} M, (b) 0.625×10^{-3} M, (c) 1.25×10^{-3} M, (d) 2.5×10^{-3} M, (e) 5.0×10^{-3} M, (f) 15.0×10^{-3} M, (g) 20.0×10^{-3} M, (h) 25.0×10^{-3} M. (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate *o*-diphenol.

shown on Fig. 4A, B. The calculated kinetic constants K_m, K_{cat} and V_{max} give evidence for a very high enzymatic activity of *H. vulgaris* Hc (K_m=2.86 mM, K_{cat}=4.48 min^{-1}, K_{cat}/K_m=1.57 mM^{-1} min^{-1} and V_{max}=0.137 mM min^{-1}) (Fig. 4B).

When the assay was performed with the decameric structural subunits RvH1 and RvH2 (see Fig. 5A, B), only the second structural subunit RvH2 exhibited phenoloxidase activity, while RvH1, did not catalyze *o*-diphenol oxidation. The activity of RvH2 against this substrate is higher compared to the native molecule, but still lower than that observed for the native *H. vulgaris* hemocyanin (Table 1). The reaction kinetics for oxidation of *o*-diphenol in the presence of 1.5 mg mL^{-1} of RvH2 is shown in Fig. 5A and B using different concentrations of *o*-diphenol (from 2.0×10^{-3} to 30.0×10^{-3} M). From the Lineweaver–Burk plot the kinetic values K_m=23.81 mM, K_{cat}=3.5 min^{-1}, K_{cat}/K_m=0.147 mM^{-1} min^{-1} and V_{max}=0.105 mM min^{-1} were calculated (Table 1).

Quinone production from L-Dopa was also studied as a function of Hc concentration with *R. venosa* Hc, its structural subunits RvH1 and RvH2, and *H. vulgaris* Hc, confirming that only *H. vulgaris* Hc, but neither RvH nor its structural subunits show any *o*-diphenol activity. Time courses of quinone formation from L-Dopa in the presence of *H. vulgaris* Hc at different concentrations of substrate (from 0.15×10^{-3} M 10.0×10^{-3} M)

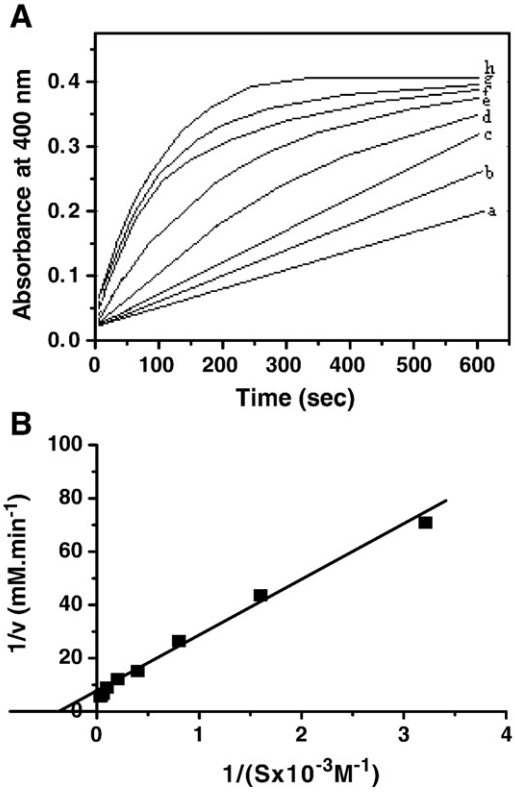

Fig. 5. *o*-Diphenoloxidase activity of structural subunit RvH2 isolated from *Rapana venosa* hemocyanin, expressed as initial velocity (ΔA_{475} nm/min) of quinone formation in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of quinone formation in the presence of Hc (1.5 mg mL^{-1}) at different concentrations of *o*-diphenol: (a) 2.0×10^{-3} M, (b) 2.5×10^{-3} M, (c) 5.0×10^{-3} M, (d) 10×10^{-3} M, (e) 15.0×10^{-3} M, (f) 20.0×10^{-3} M, (g) 25.0×10^{-3} M, (h) 30.0×10^{-3} M. (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate *o*-diphenol.

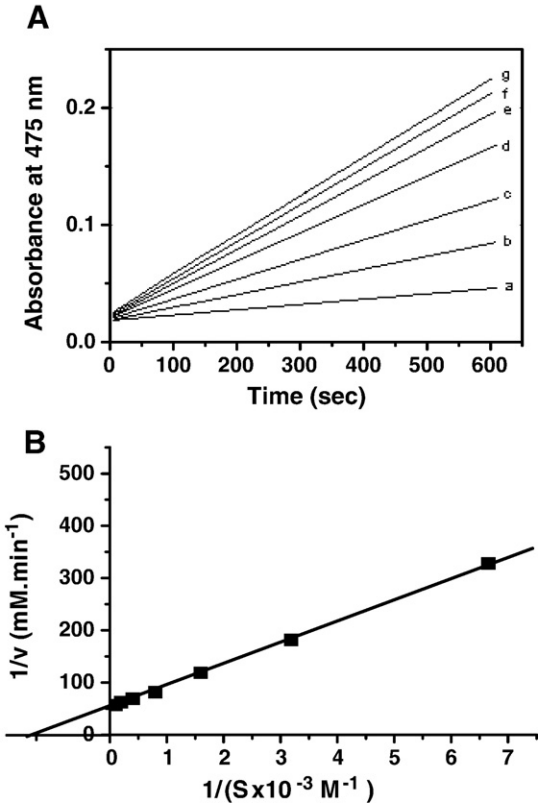

Fig. 6. *Helix vulgaris* Hc-catalyzed oxidation of L-Dopa in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of quinine formation in the presence of *H. vulgaris* Hc at the different concentrations of L-Dopa: (a) 0.15×10^{-3} M, (b) 0.31×10^{-3} M, (c) 0.625×10^{-3} M, (d) 1.25×10^{-3} M, (e) 2.5×10^{-3} M, (f) 5.0×10^{-3} M, (g) 10.0×10^{-3} M. (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate L-Dopa.

are shown in Fig. 6A. The kinetic values K_m (0.77 mM), V_{max} (0.018 mM min^{-1}), K_{cat} (1.8 min^{-1}) and K_{cat}/K_m (2.32 mM^{-1} min^{-1}) present in Table 1 were calculated from the Lineweaver–Burk plot (Fig. 6B).

4. Discussion

The structural and spectroscopic properties of the active sites of Hcs were shown to be remarkably similar to those of tyrosinases (Decker et al., 2004, 2007; Decker and Jaenicke, 2004; Solomon et al., 2001). In both proteins, each metal ion is bound by imidazole ligands to a dinuclear site. However, the two proteins have different biological functions. Based on the recent structure of tyrosinase (Matoba et al., 2006), the mechanism of the catalysis of Ty was proposed (Decker et al., 2000; Land et al., 2007).

In the case of Hcs, in addition to the oxygen transport and/or storage function, it is conceivable that Hc exhibits other reactivities in the presence of suitable exogenous molecules, to be consider as a side property of the metal-dioxygen active site adduct. Quite a few reports confirm the possible conversion of the oxygen-binding functions of hemocyanins to phenol oxidase activity as has recently been observed for arthropodan Hcs from

Homarus americanus, *Limulus polyphemus*, *Carcinus maenas* and the molluscan Hcs from *O. vulgaris, H. pomatia* and *S. officinalis* hemocyanins (Nellaiappan and Sugumaran, 1996; Salvato et al., 1983; Salvato et al., 1998; Terwilliger and Ryan, 2006; Zlateva et al., 1996).

Our study, presented here, clearly shows that the molluscan hemocyanins from *H. vulgaris* and *R. venosa* also exhibit phenoloxidase activity. The dependence of the initial rate of quinone production from *o*-diphenol and L-Dopa for *Rapana* and *Helix* Hcs, together with the kinetic parameters calculated from Lineweaver–Burk plots (Table 1) give a clear indication of their *o*-diphenoloxidase activity. The calculated Michaelis–Menten constant of the native *Rapana* hemocyanin (K_m=28.57 mM) is approximately the same as that of the *O. vulgaris* Hc (K_m=35.0 mM), but its magnitude is, about 10 times larger compared to the Hc of *H. vulgaris* Hc (K_m=2.86 mM) (Table 1) and thus close to those of Hcs of the molluscs *H. pomatia* and *S. officinalis* (Siddiqui et al., 2006). Also the native molecule of *H. vulgaris* hemocyanin showed a larger enzymatic activity (K_{cat}/K_m=1.57 mM^{-1} min^{-1}) compared to those of *Rapana venose*, *O. vulgaris, H. pomatia* and *S. officinalis* (K_{cat}/K_m=0.093; 0.28; 0.068 and 0.26, respectively) (Table 1).

In general, it was found that the molluscan Hcs are far more efficient than the arthropodan Hcs (Salvato et al., 1998; Zlateva et al., 1996). These differences in their activities between of molluscan and arthropodan Hcs could be based on their structures. As shown from the X-ray structures, the arrangement of the domains to each other differs in arthropodan and molluscan hemocyanins (Decker et al., 2007; Decker and Terwilliger, 2000; Jaenicke and Decker, 2004; Van Holde et al., 2001). Each functional unit in molluscan hemocyanins consists of two domains N- and C-terminal (Cuff et al., 1998), which are similar to the second and third domains of arthropodan hemocyanins. In both species the access to the active site is covered by the C-terminal domain in a functional unit of mollusc hemocyanin or by the N-terminal domain I in arthropodan hemocyanins (Decker and Terwilliger, 2000; Decker et al., 2004). This could be explained a very low catalytic activity of molluscs *Rapana* and *Octopus* Hcs or arthropods *Carcinus aestuarii* and *L. polyphemus* hemocyanins where the entrance to the active site is probably blocked by Leu or Phe residues, respectively (Table 2) (Decker and Tuczek, 2000).

Recently a hypothesis was presented that activation of hemocyanin opens a large entrance for phenolic substances to the active site by removal of an N-terminal or C-terminal peptide of the arthropods or molluscs, respectively (Decker and Rimke, 1998). This phenoloxidase activation has been attributed to a conformational change in the hemocyanin molecule, creating access to the active site for phenolic substances (Decker and Rimke, 1998, Decker et al., 2006). As indicated from X-ray structures, the active sites of native Hcs are only well accessible for small-sized molecules like dioxygen (e.g., (Cuff et al., 1998) while that of sweet potato catecholoxidase (Eicken et al., 1999) allows an open entrance for phenolic substrates.

Removal of the N-terminal or C-terminal peptides from arthropodan or molluscan pulls a Phe 49 in *Limulus* (arthropodan Hc) or Leu 2830 (in mollusc Octopus Hc), respectively, out of the substrate-binding pocket, which are highly conserved among arthropods and molluscs, respectively. As a result of this cleavage, a channel in Hcs is opened and the binuclear copper active site becomes accessible. The opening of the entrance to the active site of Hcs could be achieved by proteolysis or a conformational

Table 2
Alignment of the N-terminal or C-terminal peptides of the arthropodan or molluscan hemocyanins

Molluscs					
H. vulgaris	DCTFGGRFSL	**LGG**PLEAP**W**A	YNRLYKREIT	QYLGNIHI	
O. vulgaris	ECTFGGTFCI	**LGG**EHEMF**W**A	**F**DRLFRYDIT	TSLKHLHLDA	HDDFDIKVT
RvH 2-E	ECTHAGYFDV	**LGG**SLETP**W**Q	**F**DRLYKYEIT	DVLESKGLDV	HDVFDIKIT
Helix G	HCEFAGTFAI	**LGG**PLEHP**W**A	**F**DRLFKYDVT	DVFSKLHLRP	DSEYHFNIH
Helix D	CNHKAGVFSV	**LGG**ELEMPFT	**F**DRLYKLQIT	DTIKQLGLKV	NNAASYQLK
Sepia 1-A	CDNYAGEFFI	**LGG**IHEMP**W**D	**F**SYPYLHEIT	DTVNSLGLPL	SGNYYVQAI
Sepia 1-B	NEFMAGSIAV	**LGG**SKEMS**W**R	**F**DRVYKYEIT	AALAALGVDK	YAEYTLRVD
Sepia 1-C	NKMKAGEFYV	**LG**SENEMP**W**K	**F**DRAYKSDIT	HVMDEMKLHY	TDKYHVEYK
Sepia 1-D	CDHYAGIFSV	**LGG**ETEMP**W**Q	**F**DRLFRYEIS	HALNALELTH	KSDFTIKVE
Sepia 1-E	NCHDGSHFSV	**LGG**STEMP**W**A	**F**DRLYRIEIT	DILKDMGLQF	DSHFTIKVN
Sepia 1-F	DCKEAGTIFI	**LGG**ETEMA**W**H	**F**DRNYRFEIT	SVLEEMKIPF	DKLFEHESK
Sepia 1-G	ECHFGGTFCV	**LGG**QHEMA**W**A	**F**DRLFLYDIT	KALNKLHLDA	YDDFLINVS
Sepia 2-A	CDNYAGEFFI	**LGG**IHEMP**W**D	**F**AYPYLHEIT	DTVNSLGLKL	DSNYYVIAE
Sepia 2-B	NEFSVGSIAI	**LGG**SKEMT**W**R	**F**DRVYKHEIT	HALESLGVDK	FAEYTLRVD
Sepia 2-C	NKMKAGEFYV	**LG**SENEMP**W**K	**F**DRAYKSDIT	HVMDEMKLHY	TDKYHVEYK
Sepia 2-D	CGNYAGIFSV	**LGG**VTEMP**W**R	**F**DRLFRYEIT	NELKKLSLNQ	NSHFRVAME
Sepia 2-E	CDNYAGTFAV	**LGG**ETEMP**W**N	**F**DRLFHYEIT	DYMNKLHLTQ	ESKFHLTTK
Sepia 2-F	DCHEGSHFSV	**LGG**SAEMP**W**A	**F**DRLYKMEIT	DILHDMKLNF	DSHFTIKTK
Sepia 2-G	DCHEAGVVFV	**LGG**ETEMP**W**H	**F**DRNYKMDIT	DVLHEMHIPM	EALFENDSK
Sepia 2-H	ECKFGGTFCV	**LGG**QHEMA**W**A	**F**DRLFLYDIS	RTLLQLRLDA	HDDFDVKVT
Arthropods					
CaeSS 2	LS**F**PDLSGHY	DDDGVSAKRL	MKE**L**NEHRL**L**	QQSHW**F**SL**F**N	
LpII	HL**F**EQLSSAT	VIGDG DKKH	SDR**L**KNVGK**L**	QPGAI**F**SC**F**H	

transition of the first domain in arthropods carrying Phe49 or N/terminal domain in molluscs carrying Leu2830 (Baird et al., 2007; Jaenicke and Decker, 2004a,b). In evidence for the hypothesis of a conformational transition was the pH dependent activation of *O. vulgaris* hemocyanin (Salvato et al., 1998). Based on this hypothesis could be explained the different reaction after testing for phenoloxidase activity of the native molecule of RvH and subunits RvH1 and RvH2. Both structural subunits were isolated as described before (Dolashka-Angelova et al., 2003) after dissociation of the native molecule in 0.13 M Gly/NaOH buffer, pH 9.6. Only structural subunit RvH2 exhibits higher diphenoloxidase activity than the native molecule using *o*-diphenol as a substrate, which most probably could be ascribed to a better accessibility of the active sites when the protein is in the dissociated state. The different behavior which was identified for the first structural subunit RvH1 not catalyzing *o*-diphenoloxidation fits to the published results for *L. polyphemus, H. pomatia, H. aspersa* that after further depolymerisation of structural subunits, also not all subunit types exhibit phenoloxidase activity (Table 2). Only three of the eight bands of dissociated *L. polyphemus* hemocyanin appeared to be capable of *o*-diphenol activity (Decker et al., 2001; Nellaiappan and Sugumaran, 1996). While in *E. californicum* hemocyanin, only subunits *b* and *c* showed SDS-activated *o*-diphenoloxidase activity. Analogous result was detected for molluscan hemocyanins where only FU f of *H. pomatia* Hc showed *o*-diphenoloxidase activity after subtilisin treatment and also a significantly induced limited proteolysis for *o*-diphenoloxidase activity was observed only for FU g, from the eight FUs of subunit 2 of Sepia Hc (Siddiqui et al., 2006).

The activation of these functional units could be explained with pulling out Leu residue and opening the entrance to the active site. The comparison of the C-terminus of FUs of several molluscs and N-terminus of domain I in arthropodan hemocyanins, the positions of Phe49 in arthropods and Leu2830 in molluscan Hcs are conserved as shown in Table 2. This suggests that after pulling out these two specific residues from the active site each FUs should exhibit activity. However only few of these FUs show activity which could suggest that not only Phe49 and Leu2830 are related to the activity of arthropodan and molluscan hemocyanins. In *Cancer magister* (Cm) Hc all subunits exhibited some *o*-diphenoloxidase activity, but subunits Cm IV and Cm V gave the strongest reactions (Durstewitz and Terwilliger, 1997; Jaenicke and Decker, 2004a,b) (Table 2). Also the arthropodan hemocyanins from a chelicerate, the tarantula *Eurypelma californicum*, *L. polyphemus* and *C. magister* (Decker et al., 2001) have shown phenoloxidase activity after proteolysis with SDS (Table 2). Hemocyanins from the two chelicerate species, develop *o*-diphenoloxidase activity in response to submicellar concentrations of SDS, a reagent commonly used to identify phenoloxidase activity (Nellaiappan and Sugumaran, 1996; Nellaiappan and Vinayakam, 1993). The functional conversion of Hc may be induced by a conformational change. This activation is restricted to only a few of the various subunit types of each hemocyanin (Baird et al., 2007; Decker et al., 2001).

In Table 1 it is shown that even Leu2830 is present in the polypeptide chain of *H. vulgaris* Hc (Table 2), this hemocyanin exhibits not only the high *o*-diphenol activity, but it was also found to react with L-Dopa as a substrate, which is unusual for other Hcs (Table 1). Only the dimers of subunits of Sepia Hc showed a small intrinsic activity (Siddiqui et al., 2006). In case of hemocyanins of *L. polyphemus* and *E. californicum* they converted L-Dopa, dopamine, and catechol after activation by SDS (Table 2). No activity was observed for *H. pomatia* Hc when L-Dopa was used as a substrate but after treatment with subtilisin hemocyanin showed a small *o*-diphenol activity against L-Dopa (Table 2).

Based on the obtained results it could be suggested that not only Leu2830 is responsible for the high phenoloxidase activity of *H. vulgaris* Hc as well as the different behavior of hemocyanins. It may depend on the C-terminus of the domains in HvH which are not so close to the entrance of the active site.

Acknowledgments

This work was supported by NATO grants LST.CLG and (PDD (CP)-(CBP.EAP.RIG 982552) and Ministry of Sciences and Education (VU-607/07). Dr. P. Dolashka-Angelova would like to thank DFG and DAAD for granting a scholarship.

References

Ashida, M., Brey, P., 1995. Role of the integument in insect defense: prophenol-oxidase cascade in the cuticular matrix. Proc. Natl. Acad. Sci. U. S. A. 92, 10698–10702.

Baird, Sh., Kelly, Sh., Price, N., Jaenicke, E., Meesters, Ch., Nillius, D., Decker, H., Nairn, J., 2007. Hemocyanin conformational changes associated with SDS-induced phenol oxidase activation. Biochim. Biophys. Acta. 1774, 1380–1394.

Bhagvat, K., Richer, D., 1938. Animal phenolases and adrenaline. Biochem. J. 2, 1397–1406.

Cuff, M.E., Miller, K.I., van Holde, K.E., Hendrickson, W.A., 1998. Crystal structure of a functional unit from *Octopus hemocyanin*. J. Mol. Biol. 278, 855–870.

Decker, H., Rimke, T., 1998. Tarantula hemocyanin shows phenoloxidase activity. J. Biol. Chem. 273, 25889–25892.

Decker, H., Terwilliger, N., 2000. Cops and robbers: putative evolution of copper oxygen-binding proteins. J. Exp. Biol. 203, 1777–1782.

Decker, H., Tuczek, F., 2000. Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends. Biochem. Sci. 25, 392–397.

Decker, H., Jaenicke, E., 2004. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. Dev. Comp. Immunol. 28, 673–687.

Decker, H., Dillinger, R., Tuczek, F., 2000. How does tyrosinase work. Angew. Chem., Int. Ed. Engl. 39, 1591–1595.

Decker, H., Ryan, M., Jaenicke, E., Terwilliger, N., 2001. SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister*. J. Biol. Chem. 276, 17796–17799.

Decker, A., Rohde, J.-U., Que Jr., Lawrence, Solomon, E., 2004. Spectroscopic and quantum chemical characterization of the electronic structure and bonding in a non-heme FeIV=O complex. J. Am. Chem. Soc. 126, 5378–5379.

Decker, H., Schweikardt, T., Tuczek, F., 2006. The first crystal structure of tyrosinase: all questions answered? Angew. Chem. 45, 4546–4550.

Decker, H., Schweikardt, T., Nillius, D., Salzbrunn, U., Jaenicke, E., Tuczek, F., 2007. Similar enzyme activation and catalysis in hemocyanins and tyrosinases. Gene 398, 183–191.

Dolashka-Angelova, P., Schwarz, H., Dolashki, A., Stevanovic, S., Fecker, M., Saeed, M., Voelter, W., 2003. Oligomeric stability of *Rapana venosa* hemocyanin (RvH) and its structural subunits. Biochim. Biophys. Acta 1646, 77–85.

Dolashka-Angelova, P., Dolashki, A., Savvides, S.N., Hristova, R., Van Beeumen, J., Voelter, W., Devreese, B., Weser, U., Di Muro, P., Salvato, B., Stevanovic, S., 2005. Structure of hemocyanin subunit CaeSS2 of the crustacean Mediterranean crab *Carcinus aestuarii*. J. Biochem. 138, 303–312.

Dolashka, P., Genov, N., Pervanova, K., Voelter, W., Geiger, M., Stoeva, S., 1996. *Rapana thomasiana grosse* (gastropoda) haemocyanin: spectroscopic studies of the structure in solution and the conformational stability of the native protein and its structural subunits. Biochem. J. 315, 139–144.

Durstewitz, G., Terwilliger, N., 1997. cDNA cloning of a developmentally regulated hemocyanin subunit in the crustacean *Cancer magister* and phylogenetic analysis of the hemocyanin gene family. Mol. Biol. Evol. 14, 66–76.

Eicken, C., Krebs, B., Sacchettini, J., 1999. Catechol oxidase—structure and reactivity. Curr. Opin. Struck. Biol. 9, 677–683.

Hearing, V., Tsukamoto, K., 1991. Enzymatic control of pigmentation in mammals. FASEB J. 5, 2902–2909.

Itoh, S., Fukuzumi, S., 2007. Monooxygenase activity of type 3 copper proteins. Acc. Chem. Res. 40, 592–600.

Jaenicke, E., Decker, H., 2004a. Conversion of crustacean hemocyanin to catecholoxidase. Micron 35, 89–90.

Jaenicke, E., Decker, H., 2004b. Functional changes in the family of type 3 copper proteins in evolution. Chem. Biol. Chem. 5, 163–176.

Johansson, M., Söderhäll, K., 1996. The prophenoloxidase activating system and associated proteins in invertebrates. Prog. Mol. Subcell. Biol. 15, 46–66.

Klabunde, T., Eicken, C., Sacchettini, J., Krebs, B., 1998. Crystal structure of a plant catechol oxidase containing a dicopper center. Nat. Struct. Biol. 5, 1084–1090.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Land, E.J., Ramsden, C.A., Riley, P.A., 2007. The mechanism of suicide-inactivation of tyrosinase: a substrate structure investigation. Tohoku J. Exp. Med. 212, 341–348.

Markl, J., Decker, H., 1992. Molecular structure of arthropod hemocyanins. Adv. Comp. Environ. Physiol. 13, 325–376.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., Sugiyama, M., 2006. Crystallographic evidence that dinuclear copper center of tyrosinase is flexible during catalysis. J. Biol. Chem. 281, 8981–9003.

Morioka, C., Tachi, Y., Suzuki, S., Itoh, S., 2006. Significant enhancement of monooxygenase activity of oxygen carrier protein hemocyanin by urea. J. Am. Chem. Soc. 128, 6788–6789.

Nakahara, A., Suzuki, S., Kino, J., 1983. Tyrosinase activity of squid. Hemocyanin. Life Chem. Rep. 1 (Supl. 1), 319–322.

Nellaiappan, K., Vinayakam, A., 1993. A method for demonstrating prophenoloxidase after electrophoresis. Biotech. Histochem. 68, 193–195.

Nellaiappan, K., Sugumaran, M., 1996. On the presence of prophenoloxidase in the hemolymph of the horseshoe crab, *Limulus*. Comp. Biochem. Physiol. 113B, 163–168.

Pless, D.D., Aguilar, A., Falcon, E., Lozano-Alvarez, E.P., 2003. Heimer de la Colera, Latent phenoloxidase activity and N-terminal amino acid sequence of hemocyanin from *Bathynomus giganteus*, a primitive crustacean. Arch. Biochem. Biophys. 409, 402–410.

Salvato, B., Beltramini, M., 1990. Hemocyanin: molecular architecture, structure and reactivity of the binuclear copper active site. Life Chem. Rep. 8, 1–47.

Salvato, B., Jori, G., Piazzese, A., Ghiretti, F., Beltramini, M., Lerch, K., 1983. Enzymatic activities of type 3 copper pair in *Octopus vulgaris* haemocyanin. Life Chem. 1, 313–317.

Salvato, B., Santamaria, M., Beltramini, M., Alzuet, G., Casella, L., 1998. The enzymatic properties of *Octopus vulgaris* hemocyanin: *o*-diphenol oxidase activity. Biochemistry. 37, 14065–14077.

Sanchez-Ferrer, A., Rodriguez-Lopez, J., Garcia-Canovas, F., Garcia-Carmona, F., 1995. Tyrosinase: a comprehensive review of its mechanism. Biochim. Biophys. Acta 1247, 1–11.

Siddiqui, N.I., Akosung, R.F., Gielens, C., 2006. Location of intrinsic and inducible phenoloxidase activity in molluscan hemocyanin. Biochem. Biophys. Res. Commun. 348, 1138–1144.

Söderhäll, K., Cerenius, L., 1998. Role of the prophenoloxidaseactivating system in invertebrate immunity. Curr. Opin. Immunol. 10, 23–28.

Solomon, E.I., 1981. In: Spiro, T.G. (Ed.), Binuclear copper active site: hemocyanin, tyrosinase and type 3 copper oxidases, Copper proteins. J. Wiley, New York, pp. 43–108.

Solomon, E.I., Sundaram, U.M., Machonkin, T.E., 1996. Multicopper oxidases and oxygenases. Chem. Rev. 96, 2563–2605.

Solomon, E.I., Chen, P., Metz, M., Lee, S.-K., Palmer, A., 2001. Oxygen binding, activation, and reduction to water by copper proteins. Angew. Chem. Int. Ed. 40, 4570–4590.

Terwilliger, N.B., Ryan, M.C., 2006. Functional and phylogenetic analyses of phenoloxidases from brachyuran (*Cancer magister*) and branchiopod (*Artemia franciscana*, *Triops longicaudatus*) crustaceans. Biol. Bull. 210 (1), 38–50.

Van Gelder, C., Flurkey, W., Wichers, H., 1997. Sequence and structural features of plant and fungal tyrosinase. Phytochemistry 45, 1309–1323.

Van Holde, K.E., Miller, K.I., 1982. Haemocyanins. Q. Rev. Biophys. 15, 1–129.

Van Holde, K.E., Miller, K.I., 1995. Haemocyanins. Adv. Protein Chem. 47, 1–81.

Van Holde, K.E., Miller, K.I., Decker, H., 2001. Haemocyanins and invertebrate evolution. J. Biol. Evol. 276, 15563–15566.

Wilcox, D.E., Porras, A.G., Hwang, Y.T., Lerch, K., Winkler, M., Solomon, E.I., 1985. Substrate analog binding to the coupled binuclear copper active site in tyrosinase. J. Am. Chem. Soc. 107, 4015–4027.

Zlateva, T., di Miro, P., Salvato, B., Beltramini, M., 1996. The *o*-diphenol oxidase activity of arthropod hemocyanin. FEBS Lett. 384, 251–254.