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Phenoloxidase activity of intact and chemically modified functional unit RvH1-a from molluscan *Rapana venosa* hemocyanin

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ABSTRACT

o-Diphenol oxidase activities (*o*-diPO) of chemically modified functional unit RvH1-a of molluscan hemocyanin *Rapana venosa* were studied using *L*-Dopa and dopamine as substrates. With *L*-Dopa as substrate the native FU RvH1-a did not show any *o*-diPO activity. Therefore the native FU RvH1-a was converted to enzymatic active form, after treatment with SDS, trypsin, urea and different values of pH when its *o*-diPO activity was studied. The highest artificial induction of *o*-diPO activity was observed after incubation of FU with 3.0 mM SDS, and RvH1-a shows both, dopamine ($K_M = 6.53$ mM, $k_{cat}/K_M = 1.29$) and *L*-Dopa ($K_M = 2.0$ mM, $k_{cat}/K_M = 2.1$) activity due to a more open active site of the enzyme and better access of the substrates. It was determined that the K_M value of SDS-activated RvH1-a against dopamine is higher compared to those of hemocyanins from *Helix vulgaris*, *Helix pomatia* and native tyrosinase from *Ipomoea batatas* but much lower than that from *Illex argentinus* (ST94) tyrosinase and arthropodan hemocyanin from *Carcinus aestuarii*. The *K*m value of SDS-activated RvH1-a against *L*-Dopa is higher than those of hemocyanins from *H. vulgaris* and *Cancer magister*, but lower than that of the tyrosinase from *Streptomyces albus*.

1. Introduction

Hemocyanins (Hcs) are extracellular proteins found in the hemolymph of arthropods and molluscs. Hcs are devoted to molecular oxygen binding and transport (van Holde and Miller, 1995; van Holde et al., 2001; Burmester, 2002). Their characteristic dicupric cluster, the oxygen binding site of Hcs, closely resembles in structure and function the active sites of other proteins, which also contains type III dicupric clusters (Eicken et al., 1999; Decker and Tuczek, 2000; Olianas et al., 2005) such as tyrosinases (TY) (monophenol, dioxygen oxidoreductases, hydroxylating, EC 1.14.18.1) and catecholases (CO) (*o*-diphenol: dioxygen oxidoreductases, dehydrogenating, EC 1.10.3.1). TYs have both of these activities, whereas COs are unable to catalyze monophenol hydroxylation. TYs and COs as a whole are known as phenoloxidases (POs). The striking difference between Hcs and POs resides in the oxygen-carrying function of the former, whereas the latter bind oxygen to use it for catechol dehydrogenation (CO) or for both, catechol dehydrogenation and monophenol hydroxylation (TY).

By contrast to the strict sequence similarity between the dicupric regions of Hcs and POs, the secondary and tertiary structures of these proteins strongly diverge. Mollusc Hcs form either decamers or didecamers (~350 to ~450 kDa per monomer) whereby each monomer forms a single polypeptide chain consisting of seven or eight functional units, capable of binding seven or eight dioxygen molecules. Each cupric ion is co-ordinated by three nitrogen atoms pertaining to the imidazole rings of three histidine residues. Hemocyanin from *Rapana venosa* (RvH) consists of eight functional units (FUs), each of a molecular mass of 45–50 kDa, namely RvH-a to RvH-h, all of them constituting structural subunit RvH1 (Dolashka-Angelova et al., 2007) as well as structural subunit RvH2 (Dolashka et al., 1996; Dolashka-Angelova et al., 2003).

Sodium dodecylsulphate (SDS), commonly known as a denaturant, has been used for decades in an assay to detect PO activity of proteins, such as plant polyphenoloxidases and tyrosinase, even though the mechanism is unknown. In this process inactive PO (Robb et al., 1964; Moore and Flurkey, 1990; van Gelder et al., 1997; Kanade et al., 2006; Marusek et al., 2006; Cong et al., 2009) is converted to a functionally active PO by incubation with SDS at a concentration above the critical micelle concentration (CMC) (Baird et al., 2007; Jaenicke and Decker, 2008). In recent years weak PO activity was observed in Hcs from both, arthropods and molluscs using the above assay (Decker and Rimke, 1998; Salvato et al., 1998; Decker et al., 2001; Pless et al., 2003;

Abbreviations: CO, catechol dehydrogenation; CD, circular dichroism; FU, functional unit; Hcs, hemocyanins; HvH, *Helix vulgaris* Hc; K_M, Michaelis–Menten constant; POs, phenoloxidases; RvH, *Rapana venosa* Hc; RvH1 and RvH2, structural subunits 1 and 2 of *Rapana venosa* hemocyanin; RvH1-a, FU "a" of subunit "1"of *Rapana venosa* Hc; T_d, temperature denaturation; Tm, melting temperature; TY, tyrosinase; v_{max}, maximum kinetic velocity.

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Lee et al., 2004; Siddiqui et al., 2006; Fan et al., 2009; Kim et al., 2011), suggesting a dual role for Hc *in vivo*.

As mentioned above SDS induces PO activity in Hcs (Zlateva et al., 1996; Salvato et al., 1998; Decker et al., 2001; Nillius, 2002; Pless et al., 2003; Jaenicke and Decker, 2004a,b; Lee et al., 2004; Kim et al., 2011). Proteolysis and modeling studies of arthropod and mollusc Hcs suggest that the natural activation of PO activity in Hcs involves the removal of an N-terminal peptide which subsequently enhances access to the dicupric center for phenolic substrates (Decker and Rimke, 1998; Decker and Tuczek, 2000); This hypothesis has been supported by recent structural studies (Klabunde et al., 1998; Decker and Tuczek, 2000; Decker et al., 2006; Matoba et al., 2006; Baird et al., 2007; Decker et al., 2007).

To date, there has been no direct biophysical characterisation of the conformational changes associated with the artificial induction of PO activity in Hcs by SDS. Protein folding/unfolding and a change in the secondary structure of hemocyanins induced by different concentrations of SDS were studied by CD spectroscopy (Baird et al., 2007).

Some examples of native Hcs showing intrinsic *o*-diPO activity have been described for the whole molecule of RvH (Hristova et al., 2008). Based on the depicted knowledge on Hc activation to PO-like proteins and for additional information about this topic, the molluscan *R. venosa* Hc has been studied in the present work with concern to its possible activation. This will be done based on studies of the catalytic activities of the functional unit of hemocyanin RvH1-a isolated from *R. venosa* against *L*-Dopa and dopamine as substrates in comparison to those of other Hcs from molluscs and arthropods and tyrosinases.

2. Material and methods

2.1. Purification and induction of phenoloxidase activity of functional unit RvH1-a

R. venosa Hc was isolated from the hemolymph of marine snails living in the west coast of the Black Sea near Varna. The two structural subunits, RvH1 and RvH2, were separated on a Resource Q column using a FPLC system (Dolashka-Angelova et al., 2003). The functional unit RvH1-a was purified as described by Dolashka-Angelova et al.(2000).

All experiments in this study were replicated three times.

Limited proteolysis was performed with bovine trypsin (Sigma) in 0.1 M Tris/HCl buffer, pH 7.8 (in the case of the native 24-mer) and pH 9.6 (in the case of subunits), at room temperature. The ratio between hemocyanin and protease varied between 1:1 and 10:1 (w/w). Proteolysis was terminated by addition of soybean trypsin inhibitor (Sigma).

SDS, at a final concentration of 3.0 mM, was added to RvH1-a (1 $mg.mL^{-1}$ final concentration) at 25 °C in 50 mM Tris/HCl buffer, pH 8.0. Absorption spectra of RvH1-a samples in the same buffer were recorded over the range 240–450 nm after incubation for 3 min with different concentrations of SDS.

o-Diphenol oxidase activity was measured after keeping of the functional unit RvH1-a for 3 min in 50 mM Tris/HCl buffer, pH 8.0, containing different concentrations of urea (0.1 M, 0.5 M, 1 M, 2 M) and cocktail buffers (0.1 M Tris/HCl buffer) at different pH (pH 4.5; 6.0; 7.0; 8.5).

2.2. SDS activation of Hc

For activation of RvH1-a we used different concentrations (2.4–3.3 mM) of SDS. A stock Hc solution (20 $mg^{-1}.mL^{-1}$) was diluted with an SDS buffer solution (0.1 M Tris–HCl, pH 7.6 in 3.0 mM SDS) at a ratio of at least 1:30 (v/v), avoiding also the precipitation of Ca–SDS. The Hc is activated after a very short time (3 min) and is stable for several hours (Baird et al., 2007; Jaenicke and Decker, 2008; Nillius et al., 2008).

2.3. Kinetic measurements of o-diphenol oxidase activity

o-Diphenol oxidase activities were determined spectrophotometrically with the substrates *L*-Dopa and dopamine, respectively, both at 25 °C, using a Shimadzu UV-2100 spectrophotometer. The dopachrome assay was performed according to Fling et al. (1963). For the measurements, a cuvette was filled with 1 mL 0.1 M sodium phosphate buffer, pH 7.0 containing 5 mM *L*-Dopa or 20 mM dopamine, respectively, between 0.31 and 25 mM RvH1-a dissolved in 1 mL of 0.4 M Tris/HCl buffer, pH 7.6 were added, and the solutions were mixed for 5 s. Then the increase in absorption at 475 nm, due to the formation of dopachrome ($\varepsilon_{475} = 3600\ M^{-1}.cm^{-1}$), was monitored as a function of time. The enzyme activity is expressed as mol of *L*-Dopa, respectively dopamine oxidized to dopachrome per 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 native and enzymatically activated functional unit RvH1-a. For calculation of dopachrome, a molar absorption coefficient of 3600 $M^{-1}.cm^{-1}$ was used (Zlateva et al., 1996).

2.4. Determination of the protein concentration

The protein concentration of RvH1-a was determined by measuring the absorbance at 280 nm using the molar extinction coefficient [ε_{280} (nm) = 1.1 $mL.mg^{-1}.cm^{-1}$] (Dolashka-Angelova et al., 2000, 2007).

2.5. CD measurements

Circular dichroism (CD) spectra were recorded on a J-720 spectropolarimeter (Jasco, Tokyo, Japan). Cylindrical temperature-controlled quartz cells with a path length of 10 mm were used in all experiments. CD spectra were recorded in the range between 200 and 250 nm at 0.2 nm intervals with a bandwidth of 1 nm, a scan speed of 50 $nm.min^{-1}$, and a time constant of 8.0 s. Protein solutions in 20 mM Tris/HCl, 10 mM $CaCl_2$ buffer of different pH values (from 1.5 to 12.0) were thermostatically-controlled using a NESLAB thermostat model RTE-110, connected to a digital programming controller and a thermocouple placed inside the optical cell. Spectra recorded in the presence of increasing concentrations of SDS required a 10 min incubation with SDS prior to CD measurements in order to allow signals to reach steady values. Temperature denaturation studies for the samples at different pH (from 1.5 to 12.0) were measured from 15 up to 95 °C, and $[\theta]_{222}$ values were recorded in intervals of 5 °C. Thereafter, temperature was decreased at the same rate down to 25 °C. The thermal equilibrium of samples was confirmed at each temperature by the constancy of their ellipticity. Each experimental spectrum was obtained by averaging two or three separate scans and was corrected for baseline, recorded with buffer as blank. The CD spectrum of a protein was very roughly considered as the sum of the CD spectra of each secondary structure component of the protein.

3. Results

3.1. Intrinsic phenoloxidase activity of functional unit RvH1-a

Kinetic analysis of phenol oxidase activity of intact functional unit RvH1-a with increasing *L*-Dopa concentrations (from 0.5 to 10.0 mM) was performed and RvH1-a did not show any intrinsic *o*-phenoloxidase activity. Using dopamine as substrate, a very weak intrinsic activity was measured for the native RvH1-a. The initial reaction rate, v_i, increased only up to 0.7 nmol min^{-1} mg^{-1} (at 10 mM dopamine concentration).

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3.2. Induction of phenoloxidase activity of RvH1-a

Several methods were applied to convert RvH1-a into enzyme. Two substrates, *L*-Dopa and dopamine, were used to analyze the *o*-diphenol oxidase activity of native and activated RvH1-a. After treatment with SDS, trypsin and buffers of different pH values, an increasing *o*-diphenol oxidase activity was observed for the both substrates (Fig. 1).

The phenol oxidase activity with *L*-Dopa as substrate was increased by treatment of RvH1-a with trypsin (E/S = 1/500, w/w) in 50 mM Tris/HCl buffer, pH 8.2, at 25 °C. A study of the phenol oxidase activity as a function of *L*-Dopa concentration indicated maximal activation with 5 mM *L*-Dopa. In contrast to the native protein, the RvH1-a sample after 24 h treatment with trypsin showed a relatively high *o*-diphenol oxidase activity against *L*-Dopa ($v_i \approx 1.4$ nmol min^{-1} mg^{-1}).

The *o*-diphenol oxidase activity with dopamine as substrate was increased by treatment of RvH1-a with trypsin (E/S = 1/500, w/w) in 50 mM Tris/HCl buffer, pH 8.2, at 25 °C. A study of the *o*-diphenol oxidase activity as a function of dopamine concentration indicated maximal activation with 20 mM dopamine. In contrast to the native protein, the RvH1-a sample, after 24 h treatment with trypsin, showed an even higher *o*-diphenol oxidase activity against dopamine ($v_i \approx 2.4$ nmol min^{-1} mg^{-1}), than the *o*-diphenol oxidase activity against *L*-Dopa.

RvH1-a was also activated by adjusting the pH value at 5.5. Under these conditions, the activity was relatively high and similar for both substrates (5 mM *L*-Dopa and 20 mM dopamine) (Fig. 1).

3.3. Induction of phenoloxidase activity of RvH1-a by SDS

Different concentrations of the detergent SDS (2.4–3.3 mM; final concentrations) were used in our study to induce phenol oxidase activity in RvH1-a. It was shown that the micellar form of SDS (present at concentrations > 1 mM in 100 mM sodium phosphate buffer, pH 7.5) is required to induce optimal conformational transitions in the protein, which may result in increasing accessibility to the catalytic site allowing bulky phenolic substrates to access the catalytic site. It was found that incubation with SDS caused a significant increase in *o*-diphenol oxidase activity of RvH1-a. The activation of the *o*-diPO activity of RvH1-a was achieved after a 2–3 min incubation with 3.0 mM SDS using *L*-Dopa as a substrate (Fig. 2).

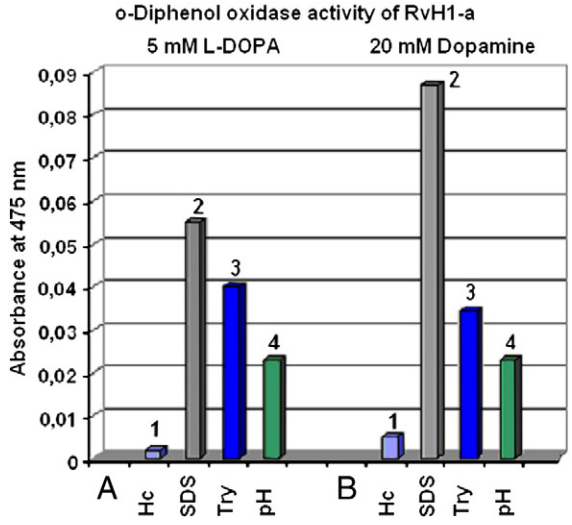

Fig. 1. *o*-Diphenoloxidase activity of native and activated RvH1-a using as substrates (A) 5 mM *L*-Dopa and (B) 20 mM dopamine. Absorbance at 475 nm of: (1) native RvH1-a; (2) 3.0 mM SDS-activated form; (3) 0.1 mM trypsin-activated form; (4) at pH value 5.5 after 60 min of incubation.

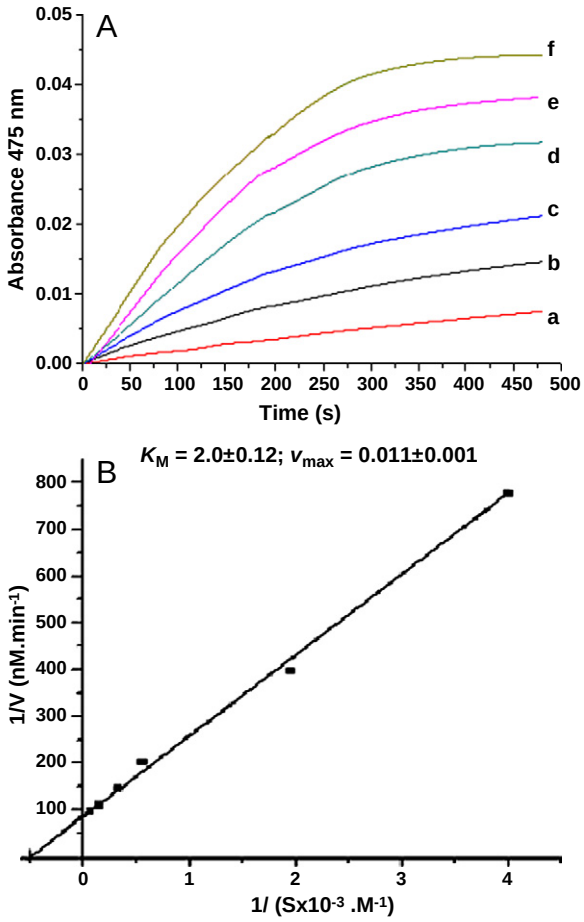

Fig. 2. *o*-Diphenol oxidase activity of activated RvH1-a by 3 mM SDS, expressed as initial velocity (ΔA_{475} nm.min^{-1}) of dopachrome formation in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of dopachrome formation in the presence of RvH1-a at different concentrations of *L*-Dopa: (a) 0.25 mM, (b) 0.5 mM (c) 2 M (d) 5 M (e) 7 M, (f) 10 M. (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate *L*-Dopa.

Highest activation of the *o*-diPO activity in RvH1-a was achieved after the 2–3 min incubation with 3.0 mM SDS and using dopamine as a substrate (Fig. 3).

Absorption spectra taken for RvH1-a at increasing concentrations (2.4–3.3 mM) of SDS showed that the copper–dioxygen band at 350 nm gradually decreased as a function of SDS concentration in the RvH1-a samples. This indicates a disintegration of the dicupric complex at the active site due to the SDS-induced conformational changes. Analysis of the copper content of the protein on incubation with 3.0 mM SDS, however, showed that, although the copper–oxygen band at 350 nm was affected, no copper was liberated from the protein, meaning that the copper atoms are still tightly bound.

The calculated kinetic parameters of native Rvh1-a and modified RvH1-a with 3 mM SDS are summarized in Table 1.

The obtained kinetic parameters of RVH1-a are compared with known data for other molluscan hemocyanins and tyrosinases (Table 2).

3.4. Comparison of the stability of native and activated RvH1-a by CD measurements

Thermostability and stability in the presence of chemical denaturants are important properties of biomolecules, especially regarding their practical application. Thermal denaturation of SDS-activated RvH1-a was studied by circular dichroism (CD) spectroscopy and compared

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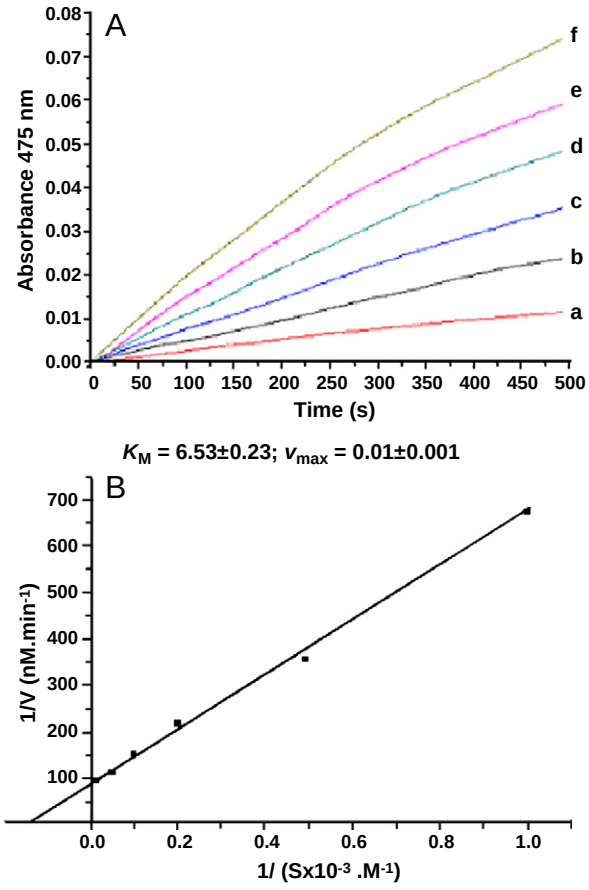

Fig. 3. *o*-Diphenol oxidase activity of SDS-activated RvH1-a, expressed as initial velocity (ΔA_{475} nm.min^{-1}) of quinone formation in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of quinone formation in the presence of RvH1-a at different concentrations of dopamine: (a) 1 M, (b) 2 M, (c) 5 M, (d) 10 M, (e) 20 M, (f) 50 M; (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate dopamine.

with the native RvH1-a (Dolashka-Angelova et al., 2000). The far-UV CD spectra of the native and activated proteins in 50 mM Tris/HCl, pH 8.2, are dominated by the negative dichroic band between 250 and 202 nm with a minimum at 210 nm and a shoulder around 220 nm, since α-helices display negative ellipticities at this wavelength range. β-sheet also displays negative ellipticities down to approximately 205 nm (Fig. 4).

Sigmoidal curves for SDS-activated RvH1-a are obtained when the ellipticity at 222 nm is plotted as a function of the temperature at different pH values. Melting temperatures (Tm), the midpoint in the sigmoidal denaturation curves for acid and basic pH, are in the region from 51 to 61 °C (Fig. 5).

For the native RvH1-a the investigation of the thermal unfolding was limited (Dolashka-Angelova et al., 2000). Unfolding experiments with SDS, carried out close to the isoelectric point of the protein (pH = 5.5), showed only limited reversibility. A two-state nature unfolding transition was supposed. In an acidic pH region, Tms are in the range from 51.6 to 58.2 °C, while in a basic region Tms are found between 54.3 and 61.0 °C.

Table 1
Kinetic parameters of native RvH1-a and modified RvH1-a with 3 mM SDS.

RvH1-a	Substrate	v_{max} (mM min^{-1})	K_M (mM)
Native	*L*-Dopa	0.01 ± 0.0014	0.01 ± 0.003
	Dopamine	0.01 ± 0.0016	0.02 ± 0.003
Modified with 3 mM SDS	*L*-Dopa	0.01 ± 0.0012	2.0 ± 0.004
	Dopamine	0.01 ± 0.0012	6.53 ± 0.004

The pH dependence (4.0 to 10.0) of the melting temperature is calculated from Fig. 5 and represented by a pH-phase diagram (Fig. 6). At pH values between 7.0 and 8.5 T_m is more or less independent of pH and in this region the unfolding process of the protein depends on ionization of different groups, but not on temperature denaturation (T_d). The T_d (ΔG)–pH phase diagram shows that the temperature of denaturation decreases rapidly at pH values lower than 6.0 and higher than 9.5. In these regions the unfolding process is caused by temperature unfolding.

4. Discussion

Hemocyanin and phenoloxidase (EC 1.10.3.1) are members of the type 3 copper proteins, containing binuclear copper centers (van Holde and Miller, 1995; Solomon et al., 1996; van Holde et al., 2001). Despite the close relationship between the two proteins, Hcs function as oxygen transporters in many molluscs and arthropods, whereas POs initiate the synthesis of melanin and are involved in immune responses, wound healing, browning and the sclerotization processes in arthropods after molting. Several studies have indicated that it is possible to convert oxygen-binding Hcs to a functionally active POs (Zlateva et al., 1996; Decker and Rimke, 1998; Decker et al., 2001; Cerenius and Söderhäll, 2004; Decker and Jaenicke, 2004; Jaenicke and Decker, 2004a; Hristova et al., 2008).

The obtained results for native RvH1-a demonstrate a low intrinsic phenol oxidase activity in comparison with the data obtained for other hemocyanins (Table 2). The values of the Michaelis–Menten constant of native RvH1-a against substrate dopamine (K_M = 0.02 mM, k_{cat} = 1.12) are lower compared to values obtained for native *Helix pomatia* Hc (K_M = 3.1 mM, k_{cat} = 0.21), *Helix vulgaris* Hc (K_M = 2.86, k_{cat} = 4.48) and tyrosinase from *Illex argentinus* native (ST94) (K_M = 9.3, k_{cat} = 215). Most probably, the lower k_{cat} and catalytic efficiency are results of subtle differences in the geometry and accessibility of the dinuclear copper centers (Siddiqui et al., 2006).

By means of limited treatment with trypsin, which may produce cleavages between and inside the FUs, we achieved an activation of the phenol oxidase activity, similarly as described from Siddiqui et al.(2006).

Our study, presented here, clearly shows that the functional unit "a" from RvH1 also exhibits phenol oxidase activity after activation by SDS. Micellar concentrations of SDS have promoted induction of *o*-phenol oxidase activity, suggesting that there is an enhanced substrate access to the dicupric center. The values of the Michaelis–Menten constant of SDS-activated RvH1-a against the substrate dopamine (K_M = 6.53 mM, k_{cat} = 8.48) are higher compared to values obtained for native *H. pomatia* Hc (K_M = 3.1 mM, k_{cat} = 0.21) and *H. vulgaris* Hc (K_M = 2.86, k_{cat} = 4.48). In contrast, the K_M values of SDS-activated RvH1-a against the substrate dopamine (K_M = 6.53 mM, k_{cat} = 8.48) are lower compared to those of hemocyanins from *Carcinus aestuarii* (K_M = 142 mM, k_{cat} = 1.7) and tyrosinase from *I. argentinus* native (ST94) (K_M = 9.3, k_{cat} = 215).

The values of the Michaelis–Menten constant of SDS-activated RvH1-a against the substrate *L*-DOPA (K_M = 2.0 mM, k_{cat} = 4.2) are higher compared to values obtained for native *H. vulgaris* Hc (K_M = 0.77, k_{cat} = 1.8), but lower than values obtained for tyrosinase from *Streptomyces albus* native (K_M = 7.8, k_{cat} = 1263).

The kinetic parameters determined for FU RvH1-a are consistent with the fact that RvH1-a is part of the subunit RvH1. Although it can be expected that the access of the substrate *L*-Dopa to the dicopper center is easier for a functional unit than for the didecameric RvH and its structural subunits, the catalytic activity of FU RvH1-a towards *L*-Dopa is still very weak (K_M = 2.0 mM), comparable with that towards dopamine (K_M = 6.5 mM). Recently, PO activity from the hair crab *Erimacrus isenbeckii*, which inhabits very cold regions (2.4−3.4 °C) of the Bering Sea was analyzed after treatment of the native molecule and isolated fraction with 75 kDa with SDS and trypsin. Both *L*-Dopa

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Table 2
Kinetic parameters of *o*-diphenol oxidase activity of RvH1-a in comparison with other molluscan and arthropodan hemocyanins and tyrosinases.

	Substrates							
	Dopamine				*L*-Dopa			
	K_M (mM)	v_{max} (mM min^{-1})	k_{cat}	k_{cat}/K_M	K_M (mM)	v_{max} (mM min^{-1})	k_{cat}	k_{cat}/K_M
Hemocyanins from:								
R. venosa native (didecamers)	0.02	0.01	1.12	56	0.01	0.01	1.1	11
RvH1-a (modified with SDS)	6.53	0.01	8.48	1.29	2.0	0.01	4.2	2.1
Helix vulgaris	2.86[a]	0.137[a]	4.48[a]	1.57[a]	0.77[a]	0.018[a]	1.8[a]	2.32[a]
Helix pomatia	3.1[b]	0.01[b]	0.21[b]	0.068[b]				
Carcinus aestuarii	142[c]	0.023[c]	1.7[b]	0.012[b]				
Cancer magister	0.02[e]	0.16[e]			1.70[e]	0.236[e]		
Tyrosinase from:								
Illex argentinus native (ST94)	9.3[d]		2000[b]	215[b]				
Ipomoea batatas	2.5[e]		138.0[b]	55.0[b]				
Streptomyces albus					7.8		1263	157

References: a (Hristova et al., 2008), b (Siddiqui et al., 2006), c (Zlateva et al., 1996), d (Terwilliger and Ryan, 2006), and e (Decker et al., 2001).

and catechol were efficient substrates for the unit of 75 kDa with 216.7-fold higher PO activity than that of the whole molecule (K_m) = 0.96 and 1.15 mM, respectively (Kim et al., 2011).

It was found that the molluscan Hcs are far more efficient than the arthropodan Hcs in term of *o*-diphenol oxidase activity (Zlateva et al., 1996; Salvato et al., 1998). Each functional unit in molluscan hemocyanins consists of two domains N- and C-terminal (Cuff et al., 1998). The access to the active site is covered by the C-terminal domain in a functional unit of molluscan hemocyanin (Decker and Terwilliger, 2000). This could explain a very low catalytic activity of molluscan *Rapana* hemocyanins where the entrance to the active site is probably blocked by Leu or Phe residues, respectively (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 molluscs (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 molluscan Hcs pulls Leu2830 (in molluscan Octopus Hc), out of the substrate-binding pocket, which is highly conserved among molluscs. 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 transition of the N-terminal domain of molluscan Hc carrying Leu2830 (Jaenicke and Decker, 2004a,b; Baird et al., 2007). 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 the different reactions could be explained after testing for phenoloxidase activity of the native RvH and its functional unit RvH1-a. RvH1-a exhibits higher diphenoloxidase activity than the native molecule using *L*-Dopa and dopamine as substrates, which most probably could be ascribed to a better accessibility of the active sites when the protein is in the dissociated state. Interesting results were detected for the molluscan hemocyanin from *H. pomatia*, where only FU f showed *o*-diphenol oxidase activity after subtilisin treatment. Moreover, among the eight FUs of subunit 2 of *Sepia officinalis* Hc, only one FU, namely FU g, could be induced by limited proteolysis for *o*-diphenol oxidase activity.

The activation of these functional units could be explained with pulling out a Leu residue and opening the entrance to the active site. Comparison of the C-terminus of FUs of several molluscs indicates that Leu2830 residues in molluscan Hcs are conserved. This suggests that after pulling out this specific residue from the active site, each FU should exhibit activity. However, only few of these FUs show activity

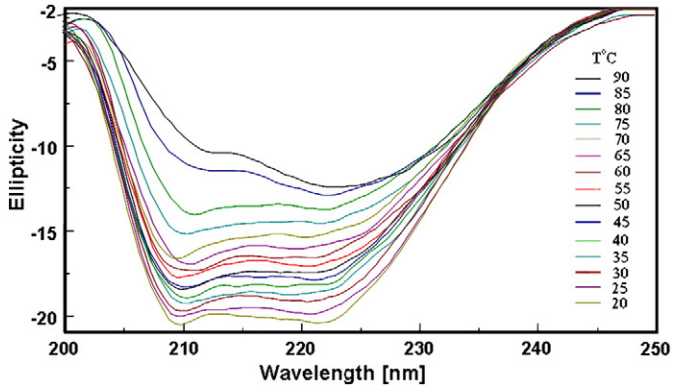

Fig. 4. CD spectra of activated RvH1-a at different temperatures (20–90 °C). Temperature denaturation was followed by recording the 200–250 nm CD spectra of Hc (0.4 mg.mL^{-1}). Quartz cylindrical cells with 0.05 cm path length were used throughout.

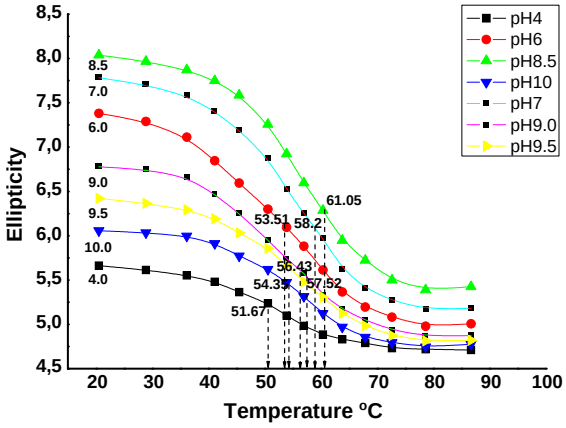

Fig. 5. Influence of pH and temperature on the intensity of the Cotton effect at 222 nm of RvH1-a. Ellipticity at 222 nm is plotted as a function of the temperature at different pH values. Temperature denaturation studies were performed at a temperature increase rate of 2 °C per min from 25 to 90 °C in each buffer and the ellipticity $[\theta]_{222}$ was recorded for 5 °C intervals. The RvH1-a solution was kept for 20 min in buffers (0.05 M carbonate/bicarbonate) of the pH range 4.0–10.0. pH and temperature denaturation were followed by recording the 200–250 nm CD spectra of Hc (0.4 mg.mL^{-1}). Quartz cylindrical cells with 0.05 cm path length were used throughout.

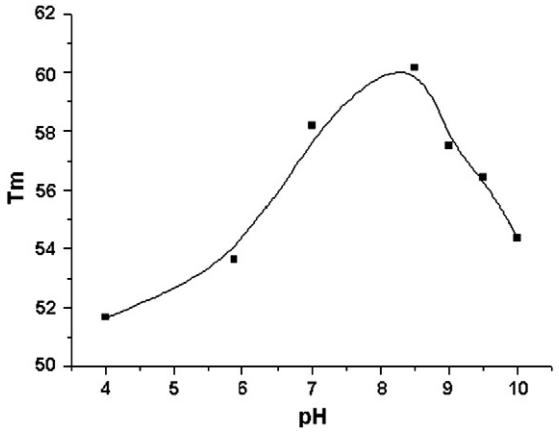

Fig. 6. $T_d(\Delta G)$–pH phase diagram of SDS-activated functional unit RvH1-a. pH-dependence of melting temperature (Tm). The protein is completely denaturated at pH 4 and 10 at 25 °C and stable at pH 8.5.

which could suggest that not only Leu2830 hinders *o*-diphenol oxidase activity of molluscan hemocyanins.

Thermal stability of activated RvH1-a was studied by circular dichroism (CD) spectroscopy and compared with the native FU. However, for the native RvH1-a the thermal unfolding was studied only in 50 mM Tris/HCl buffer, 10 mM $CaCl_2$, pH 8.2 by CD and fluorescence spectroscopy. The melting temperature ($T_m = 51$ °C) of RvH1-a was determined by CD (Dolashka-Angelova et al., 2000), while the pH dependence (4.0 to 10.0) of the T_m was calculated of SDS-activated RvH1-a. In acidic pH region, Tms are in the range from 51.6 to 58.2 °C, while in basic region Tms are found between 54.3 and 61.0 °C.

The pH dependence (4.0 to 10.0) of the melting temperature is calculated from Fig. 5 and is represented by a pH-phase diagram (Fig. 6). At pH values between 7.0 and 8.5 T_m is more or less independent of pH and in this region the unfolding process of the protein depends on ionization of different groups, but not on temperature denaturation (T_d). The T_d (ΔG)–pH phase diagram shows that the temperature of denaturation decreases rapidly at pH values lower than 6.0 and higher than 9.5. In these regions the unfolding process is caused by temperature unfolding.

The far UV CD spectra revealed a small but significant change in the secondary structure content of all three Hcs *E. Californicum, L. polyphemus* and *P. imperator* when incubated with 5 mM SDS (Baird et al., 2007). Incubation in SDS to elicit these small but significant changes in secondary structure, denaturation was not observed.

The PO activity of *E. isenbeckii* Hc was maximal at 4 °C, decreased slightly at temperatures up to 60 °C, and fell rapidly at 80 °C (Kim et al., 2011). The results suggest that cold-adapted hemocyanin-derived PO activity is important to the survival of these crabs. This is the first report of a crab PO activity with broad temperature stability extending into the cold environment.

In conclusion, because of its very low intrinsic activity, Hc of *R. venosa* does not seem to play a physiological role as a PO enzyme in the hemolymph. Our results also show that treatment of protein with SDS and trypsin increases significantly the enzymatic activity. Based on the obtained results it could be suggested that not only Leu2830 hinders the high *o*-phenol oxidase activity of RvH1-a but also the different behaviors of different hemocyanins. So, our results demonstrate that although the type-3 copper proteins share structurally similar active sites, differences in accessibility of the active sites to the substrates determine their different function.

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