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Phenoloxidase Activity of *Helix aspersa* Maxima (Garden Snail, Gastropod) Hemocyanin

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Abstract The oxygen-transporting protein, hemocyanin (Hc), of the garden snail *Helix aspersa* maxima (HaH) was isolated and kinetically characterized. Kinetic parameters of the reaction of catalytic oxidation of catechol to quinone, catalyzed by native HaH were determined: the V_{max} value amounted to 22 nmol min^{-1} mg^{-1}, k_{cat} to 1.1 min^{-1}. Data were compared to those reported for other molluscan Hcs and phenoloxidases (POs). The *o*-diphenoloxidase activity of the native HaH is about five times higher than the activity determined for the Hcs of the terrestrial snail *Helix pomatia* and of the marine snail *Rapana thomasiana* (k_{cat} values of 0.22 and 0.25 min^{-1}, respectively). The K_m values obtained for molluscan Hcs from different species are comparable to those for true POs, but the low catalytic efficiency of Hcs is probably related to inaccessibility of the active sites to potential substrates. Upon treatment of HaH with subtilisin DY, the enzyme activity against substrate catechol was considerably increased. The relatively high proteolytically induced *o*-diPO activity of HaH allowed using it for preparation of a biosensor for detection of catechol.

Keywords Hemocyanin · *Helix aspersa* maxima · Phenoloxidase activity · Optical biosensor

Abbreviations

DMF	*N*,*N*-dimethylformamide
L-Dopa	3,4-Dihydroxy-L-phenyl-alanine
EDTA	Ethylenediaminetetraacetic acid
MBTH	3-Methyl-2-benzothiazolinone hydrazone
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecyl sulphate
Tris–HCl	Tris (hydroxymethyl) amino-methane hydrochloride

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1 Introduction

Hemocyanins (Hcs) are oligomeric copper-containing proteins that function as oxygen carriers in the hemolymph of several mollusks and arthropods. Molecules of molluscan Hcs are structured as decamers (in cephalopods) or didecamers (in gastropods) of subunits with molecular mass of 350–450 kDa. The polypeptide chains of subunits contain seven or eight globular functional units (FUs), depending on the species. FUs are generally termed FU-a to FU-h, starting from the N-terminus of the respective subunit. The FUs have a binuclear copper active site, capable of binding reversibly one oxygen molecule. The active site consists of two closely spaced copper ions (Cu_A and Cu_B) each coordinated by three histidine ligands (type 3 copper site) [23].

Hcs are related to phenoloxidases (POs). Both are type-3 copper proteins and show similarities in their structure, sequence and active site architecture, although they perform different functions in organisms [27, 28, 31]. POs, which include the enzymes tyrosinase and catechol oxidase, are present in almost all organisms. In the invertebrates POs mediate three major physiologically important processes: sclerotization, defence reactions and wound healing. It was found that Hcs, isolated from several species, catalyze the oxidation reaction of *o*-diphenols to *o*-quinones and thus show catecholase-like or *o*-diphenoloxidase (*o*-diPO) activity [4]. Moreover, the intrinsic *o*-diPO activity of Hcs can be enhanced by in vivo and in vitro activation [4, 5, 24,

25]. In contrast to many studies on the PO activity of Hcs from arthropod Hcs, only a few reports address the enzymatic activity of molluscan Hcs [12, 15, 24, 25].

Helix aspersa, known by the common name garden snail, is a terrestrial pulmonate gastropod mollusk. Species *H. aspersa* and *Helix pomatia* belong to the family Helicidae. In addition to the *H. aspersa*, which received its name from Fritz Müller, there is a subspecies *H. aspersa* maxima. It differs by its larger size and higher fecundity. *H. aspersa* maxima are of interest to industrial farming, with the needs of the food and pharmaceutical industries. Compared to the numerous studies on the structural organization, protein stability and immunological properties of molluscan Hcs, and in particular of *H. pomatia* Hc, the oxygen-transport protein from *H. aspersa* has been investigated less. The only scientific report, concerning the identification of three types of Hc components in the hemolymph of *H. aspersa*, is from Gielens et al. [10].

The aim of the present study was to isolate the oxygen-transporting protein of garden snails *H. aspersa* maxima and to investigate its intrinsic and inducible PO activity. Comparison of the data with those obtained for other molluscan Hcs gives further insight in the enzymatic properties of these multifunctional proteins.

2 Materials and Methods

2.1 Reagents

L-Dopa, L-tyrosine, tyramine hydrochloride, MBTH, chitosan from crab shells, DMF, ammonium molybdate, and trehalose were purchased from Sigma-Aldrich. Catechol was obtained from Merck. All other chemicals used are of analytical grade.

2.2 Hemocyanin

Hemolymph of the snails *H. aspersa* maxima was extracted by a cardiac puncture, filtered and centrifuged at 5,000×*g* for 10 min at 4 °C to remove the cellular fraction. The supernatant was centrifuged at 180,000×*g* for 4 h (ultracentrifuge Beckman LM-80, rotor Ti 45) and pellets containing Hc were dissolved in 50 mM phosphate buffer, pH 7.2, and stored at 4 °C, in the presence of 0.02 % NaN_3. The isolated HaH was further purified by gel filtration chromatography on a Sepharose 4B column (90 × 2.4 cm) equilibrated and eluted with 50 mM phosphate buffer, pH 7.2.

Following isolation by ultracentrifugation, the HaH was also separated into its isoforms by ion exchange chromatography on a DEAE-Sepharose CL-6B column (32 × 1.2 cm), equilibrated and eluted with buffer 50 mM Tris–HCl, pH 8.0, using a linear gradient 0.1–0.45 M NaCl.

2.3 SDS-PAGE and Native PAGE Analysis

The HaH was analyzed by SDS-PAGE in 7.5 and 10 % polyacrylamide gels as described [17].

Native PAGE was conducted using the same conditions, but omitting the SDS from all buffers. The samples for native PAGE were dialyzed against 50 mM Tris–HCl buffer, pH 8.5, containing 10 mM EDTA. In order to determine bands with PO activity, the gel was washed with water and covered with 20 mM catechol. After the spots with PO activity were developed and scanned, the gel was washed with water and stained with Coomassie brilliant Blue R-250 for total proteins.

2.4 PO Activity Assay

The assay of the intrinsic PO activity of total HaH, isoforms β-HaH and α_{D+N}-HaH, as well as the proteolytically induced PO activity of HaH, was carried out according to the procedure described in [15]. Kinetic parameters, reported as mean ± standard deviation, were determined from three experiments.

2.5 PO Activation by Limited Proteolysis

Limited proteolysis was used to enhance the PO activity of HaH as previously described [15]. Subtilisin DY was added to samples of HaH in 25 mM sodium phosphate buffer, pH 7.2, at an E/S ratio of 1/260 (w/w) and 1/100 (w/w). The proteolysis was carried out for 24 h, at 25 °C. The reaction was stopped by addition of PMSF in 1,000-fold molar ratio with respect to subtilisin.

2.6 UV–Vis Absorption Spectroscopy

UV–Vis spectrophotometer model Evolution 300, Thermo Electron Corporation, equipped with a Peltier temperature control accessory, was used for the kinetic measurements. The temperature of the protein solution in the cell was monitored by a sample temperature probe accessory.

Specific absorption coefficient $a_{278nm} = 1.413$ ml mg^{-1} cm^{-1} for HpH [10] was used for determining the protein concentration.

2.7 Steady-State Fluorescence Measurements

The fluorescence spectra of HaH were recorded by means of a spectrofluorimeter Perkin Elmer model LS55, at an excitation wavelength of 295 nm. In order to avoid inner filter effects, the optical density of protein samples was <0.05.

2.8 Transmission Electron Microscopy

The structure of Hc molecules was investigated by transmission electron microscopy (TEM) with an electron microscope JEOL 1200 EX, at an accelerating voltage of 80 kV and instrumental magnification of 2,000–75,000×. Negative staining with 5 % (w/v) ammonium molybdate, containing 1 % (w/v) trehalose at pH 7.2, was used for better visualization of the HaH samples.

2.9 Immobilization of HaH in Chitosan

An example of a sensor, based on the immobilization of HaH (treated with subtilisin) in chitosan film, for optical detection of catechol was prepared as described in [1]. Chitosan solution (1 %, w/v) was prepared in 0.05 M acetic acid. HaH in 25 mM sodium phosphate buffer, pH 7.2 (a protein concentration of 43 mg/ml), was subjected to limited proteolysis with subtilisin DY (E/S = 1/100, w/w), for 1 h 30 min (see 2.5). A homogeneous Hc/chitosan solution was prepared by mixing of 150 µl of 1 % (w/v) chitosan solution and 150 µl of subtilisin-treated HaH. Following this, 20 µl of the mixture was overlaid uniformly on a clean glass plate (20 mm × 9 mm) and allowed to dry at room temperature.

The glass plate covered with subtilisin-treated HaH, immobilized in a chitosan film, was immersed in a plastic cuvette containing 25 mM sodium phosphate buffer, pH 7.2, 0.2 mM MBTH, 2 % (v/v) DMF and catechol. Absorption spectra were recorded between 300 and 800 nm at an interval time of 5 min.

3 Results and Discussion

3.1 Purification and Characterization of Total HaH

The hemolymph was collected from farmed snails *H. aspersa* maxima (Fig. 1). The HaH, isolated by ultracentrifugation of the hemolymph, was subjected to gel filtration chromatography on Sepharose 4B. The protein eluted as a single symmetrical peak (Fig. 2), indicating that this fraction contains a Hc homogeneous in size. Absorption bands at 280 and 345 nm,

Fig. 1 Snails *Helix aspersa* maxima

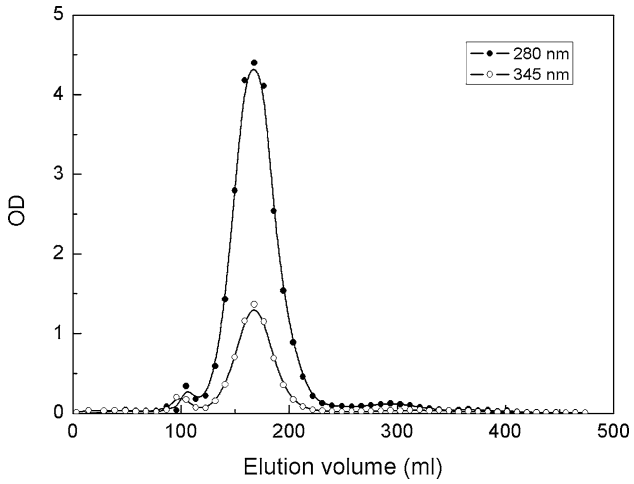

Fig. 2 Purification of HaH by gel filtration chromatography on a Sepharose 4B column (90 × 2.4 cm). The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7.2, at a flow rate of 0.3 ml/min; (*filled circle*) OD at 280 nm, (*open circle*) OD at 345 nm

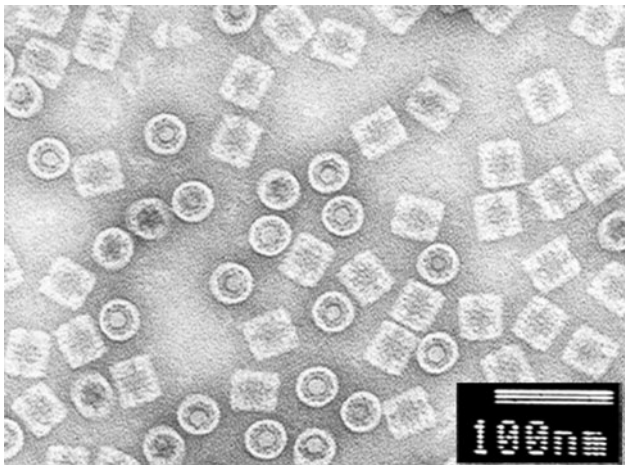

Fig. 3 TEM image of Hc molecules of purified HaH. The sample was negatively stained with 5 % (w/v) ammonium molybdate, containing 1 % (w/v) trehalose at pH 7.2. The *scale bar* indicates 100 nm

characteristic for aromatic residues and Cu^{II}–O_2^{2-}–Cu^{II} complexes at the active sites, respectively, appear in the absorption spectrum of HaH (data not shown). The ratio $OD_{345nm}/OD_{280nm} = 0.25$, found for the peak fraction of HaH is typical for fully oxygenated Hc and indicates that the protein is isolated with preserved active sites. TEM also provides evidence of molecular homogeneity of the isolated HaH (Fig. 3). On the electron micrograph only hollow cylindrical didecameric molecules, characteristic for gastropodan Hcs, can be observed without any detectable dissociation or tubular structures.

3.2 Isolation and Characterization of HaH Isoforms

The total HaH, isolated by ultracentriftype of subunits, while eachugation of the hemolymph, was separated into its

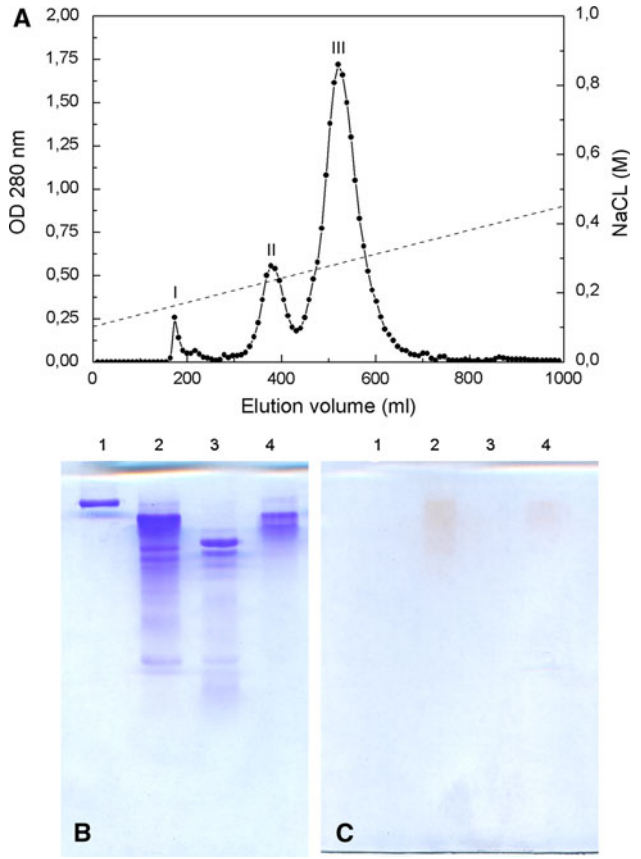

Fig. 4 **a** Chromatography of total HaH on a DEAE-Sepharose CL-6B column (32 × 1.2 cm), equilibrated and eluted with buffer 50 mM Tris–HCl, pH 8.0; linear gradient 0.1–0.45 M NaCl; flow rate 32 ml/h. Collection in fractions of 8 ml. **b** Native PAGE in 7.5 % acrylamide gel under non-denaturing conditions (Coomassie staining); *lane 1* fraction I; *lane 2* fraction III; *lane 3* fraction II; *lane 4* total HaH. **c** The same gel stained with 20 mM catechol. An *o*-diPO activity was detected for the α-isoforms of HaH (*lane 2*) and for the total HaH (*lane 4*)

isoforms by anion exchange chromatography (Fig. 4a). The elution profile is similar to that obtained by Gielens et al. [10] suggesting that the hemolymph of *H. aspersa* maxima also contains the three Hc isoforms (named β-Hc, α_D-Hc and α_N-Hc). The pI 5.2 has been obtained for the β-Hc from *H. aspersa* and pI 4.6 for the two electrophoretic similarly two α-isoforms [10]. Most likely, the protein eluted as fraction II corresponds to the β-Hc. The other isoforms, α_D-Hc and α_N-Hc, elute together as fraction III (Fig. 4a). We will further study this fraction as α_{D+N}-Hc isoforms.

Heterogeneity at the molecular level is characteristic for the Hcs from *Helix* species. [11]. Three types of Hc molecules have been identified in the hemolymph isolated from the Roman snail *H. pomatia*: β-Hc, α_D-Hc (dissociating α-Hc) and α_N-Hc (non-dissociating α-Hc) [18]. The β-Hc precipitates or crystallizes during dialysis at pH 5.3 (the isoelectric pH of β-Hc) at low ionic strength, whereas the α-Hcs stay soluble [18]. Another important difference

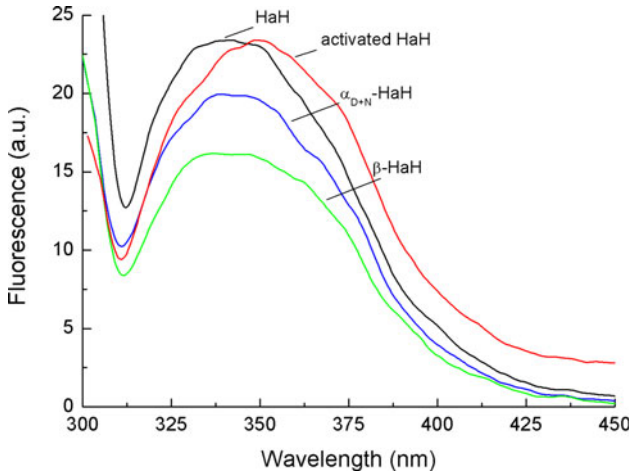

Fig. 5 Fluorescence spectra of the total HaH; α_{D+N}-Hc and β-HaH isoforms; HaH, treated with subtulisin DY [E/S = 1/100 (w/w)], for 1 h 30 min. The spectra are recorded at λ_{ex} 295 nm, at 25 °C

between these Hc isoforms is that the didecameric molecule of β-Hc consists of one type of subunits, while each of the two α-Hcs is an heterooliogomer of two types α and α' subunits [9]. Three isoforms (β-HlH, α_N-HlH and α_D-HlH) have also been isolated from the garden snail *Helix lucorum* [32].

The fractions collected (Fig. 4a) were characterized by absorption spectroscopy and native PAGE under non-denaturing conditions (Fig. 4b). Samples, taken from fractions II and III, showed absorption spectra characteristic for Hcs with bands at 280 and 345 nm. The small fraction I, however, did not show the band at 345 nm, corresponding to the Cu^{II}–O_2^{2-}–Cu^{II} complexes at the Hc active sites. The additional, faster moving components observed in fractions II and III, which are not present in the original HaH sample, are indicative of a partial degradation of the isolated isoforms β-HaH and α_{D+N}-HaH. Enzyme activity was registered for the α_{D+N}-Hc isoforms (lane 2) and for the total HaH (lane 4) (Fig. 4c). The staining of the band corresponding to the β-HaH was very slight, probably this isoform possesses lower *o*-diPO activity (Fig. 4, lane 3).

The total HaH and isolated isoforms exhibited fluorescence properties similar to those of other studied gastropod Hcs [13, 14, 32]. After excitation at 295 nm, the fluorescence spectra of native total HaH, α_{D+N}-HaH and β-HaH possess maxima at 337 ± 1, 339 ± 1 and 334 ± 1 nm, respectively (Fig. 5). These fluorescence maxima are characteristic for tryptophans "buried" in the hydrophobic interior of the protein molecule.

3.3 Identification of an α-Macroglobulin

It has been shown that in addition to the Hc, a minor component, a proteinase inhibitor α-macroglobulin (αM),

Table 1 Kinetic parameters of *o*-diphenoloxidase activity towards catechol and L-Dopa of *Helix aspersa* maxima hemocyanin in comparison with those reported for other molluscan Hcs, molluscan tyrosinase, and a plant catechol oxidase

Hemocyanins/ phenoloxidase	Catechol			L-Dopa			References
	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})	
Molluscan Hcs							
Helix aspersa maxima							
Native (didecamers)	11.8	1.1	0.093				
β-Hc (didecamers)	13.3	0.85	0.064				
α_{D+N}-Hc (didecamers)	17.6	10.25	0.58				
Proteolytically induced	6.8	40.15	5.9	17.3	12.45	0.72	
Helix pomatia (β-Hc)							
Native (didecamers)	3.1	0. 22	0.068				Siddiqui et al. [25]
Dimers of subunits	2.6	0.62	0.23				
Proteolytically induced	2.6	1.4	0.54				
Helix vulgaris							
Native (didecamers)	2.86	4.48	1.57	0.77	1.8	2.32	Hristova et al. [12]
Rapana thomasiana							
Native (didecamers)	30.0	0.25	0.008				Idakieva et al. [15]
Proteolytically induced	12.8	0.49	0.039				
Sepia officinalis							
Dimers of subunits	3.8	1.0	0.26	2.4	0.1	0.04	Siddiqui et al. [25]
Proteolytically induced	4.2	1.5	0.36				
Octopus vulgaris							
Native (didecamers)	35.0	10	0.28				Salvato et al. [24]
Molluscan tyrosinase							
Illex argentinus							
Native (ST94)	9.3	2,000	225				Naraoka et al. [19]
Trypsin induced (ST94t)	7.3	12,500	1,700				
Plant catecholoxidase							
Ipomoea batatas							
Native	2.5	138,000	55,000				Eicken et al. [7]

k_{cat} expressed per dinuclear copper active site

is present in the hemolymph of *H. pomatia* [38]. It is believed that both proteins, Hc and αM, are involved in the innate and humoral immunity system of animals: Hc via its potential PO activity, αM through inactivation of proteinases [26]. The isolated αM from *H. pomatia* has molecular mass 697 kDa and 20.3 % (w/w) carbohydrate [38]. During the isolation from the hemolymph by means of ultracentrifugation the total Hc is sedimented while the αM mainly remains in the supernatant.

In order to prove the presence of αM in the hemolymph of *H. aspersa* maxima, a caseinolytic assay was performed on the fraction I (Fig. 4a). The low trypsin activity (~29 % of the basic value) in the region of the first protein peak indicates the presence of a proteinase inhibitor, in this case αM, which interferes with trypsin activity. The electrophoretic pattern for fraction I most probably corresponds to the proteinase inhibitor αM (Fig. 4b, lane 1). We plan further structural investigations on the αM from *H. aspersa* maxima.

3.4 Intrinsic phenoloxidase Activity of HaH: Comparison with Other Molluscan Hcs

Kinetic analysis of the reaction of catalytic oxidation of catechol to quinone, catalyzed by native HaH, as well as by the isoforms β-HaH and α_{D+N}-HaH, was carried out. Initial reaction rates (v_i) were determined at different concentrations of substrate catechol (from 1 to 30 mM). Kinetic parameters K_m and V_{max} were calculated by analysis of the dependence of the initial rate of substrate concentration using the program HYPER (Table 1). The table also summarizes data for other molluscan Hcs and POs. The V_{max} value for total HaH was found to be 22 ± 0.001 nmol min^{-1} mg^{-1}, corresponding

with a $k_{cat} = 1.1$ min^{-1} (expressed per dinuclear copper active site). The V_{max} value for β-HaH amounted to 17 ± 0.001 nmol min^{-1} mg^{-1}, $k_{cat} = 0.85$ min^{-1}. The α_{D+N}-Hc isoforms were found to oxidize catechol with the highest rate—V_{max} reached 205 ± 0.002 nmol min^{-1} mg^{-1} and $k_{cat} = 10.25$ min^{-1}.

Under identical experimental conditions of PO assays, the β-Hc isoform of terrestrial snail *H. pomatia* ($k_{cat} = 0.22$ min^{-1}) [25] and the Hc from marine snail *Rapana thomasiana*) ($k_{cat} = 0.25$ min^{-1}) [15] have shown less activity. The affinity of β-HpH towards substrate catechol, however, is higher than that of HaH ($K_m = 3.1$ mM, vs. $K_m = 11.8 \pm 0.5$ mM). The HaH isoforms also showed low affinity in comparison with β-HpH towards catechol, respectively $K_m = 13.3 \pm 0.5$ mM for β-HaH and $K_m = 17.6 \pm 0.6$ mM for α_{D+N}-HaH. The lowest affinity towards catechol was shown by the Hcs from *R. thomasiana* ($K_m = 30$ mM) and cephalopod *Octopus vulgaris* ($K_m = 35$ mM) [24]. Higher enzyme activity ($k_{cat} = 4.48$ min^{-1}) and affinity ($K_m = 2.86$ mM) towards substrate catechol has been reported for *Helix vulgaris* Hc [12]. The native Hc from *O. vulgaris* has shown the highest *o*-diPO activity ($k_{cat} = 10$ min^{-1}). The K_m values obtained for molluscan Hcs from different species are comparable to that of the tyrosinase from *Illex argentinus* [19] and the catechol oxidase from *Ipomoea batatas* [7] (Table 1). This fact indicates that at least some of the active sites of the native Hc molecules possess high affinity towards catechol. The low catalytic activity of Hcs, when compared to true PO, most probably is associated with difficult access of substrates to the active sites. The crystal structure of deoxygenated FU RtH2-e from *R. thomasiana* Hc shows that the active site is completely buried in the hydrophobic interior of the protein globule [22]. It seems that in molluscan Hcs only small oxygen molecules have access to the active sites, thus maintaining their primary function of oxygen carriers. The hydrophobic microenvironment of the active site repels amino acid substrates containing highly polar ionic functional groups, like dopamine and L-Dopa. Catechol is the smallest diphenol and does not contain polar functional groups, so it is able to enter the active site pocket and thus is the preferred substrate of molluscan Hcs.

Native HaH as well as β-HaH did not show any *o*-diPO activity with L-Dopa and dopamine as substrates. The α_{D+N}-Hc isoforms, however, showed ability to oxidize L-Dopa and dopamine with low v_i of 1.6 nmol min^{-1} mg^{-1} (at 20 mM L-Dopa concentration) and v_i of 3.3 nmol min^{-1} mg^{-1} (at 12 mM dopamine concentration). Taking into account the activation of HaH on limited proteolysis (see Sect. 3.5), this is further evidence that upon isolation of the isoforms some proteolytic degradation occurred. Neither total HaH nor its isoforms showed an intrinsic mono-PO activity towards substrates L-tyrosine and tyramine. Similar results are reported for Hcs from *H. pomatia* and *R. thomasiana* [15, 25]. Native *H. vulgaris* Hc has shown an intrinsic activity towards L-Dopa ($K_m = 0.77$ mM, $k_{cat} = 1.8$ min^{-1}) [12]. Hc dissociated into dimers of subunits from the cephalopod *Sepia officinalis* has also shown a weak *o*-diPO activity with substrate L-Dopa ($K_m = 2.4$ mM, $k_{cat} = 0.1$ min^{-1}) [25]. Suzuki et al. [30] reported mono-PO activity towards phenol of native *O. vulgaris* Hc and FU Ov-g, which was enhanced by an addition of urea.

The intrinsic PO activity of the hemolymph from *H. aspersa* maxima was also considered. With catechol as substrate the hemolymph (at a final protein concentration of 0.5 mg/ml) showed *o*-diPO comparable to that observed for the purified HaH. This does suggest that the hemolymph of *H. aspersa* maxima, apart from Hc, does not contain a phenoloxidase enzyme. Molluscan tyrosinase, however, has been found in the ink of the cephalopod *I. argentinus* [19].

3.5 Phenoloxidase Activation by Limited Proteolysis

It has been established that limited proteolysis with proteolytic enzymes is capable of inducing PO activity in molluscan Hcs [15, 25]. Between FUs with compact globular structure, there is an interval of 10–15 amino acids, susceptible to proteolytic cleavage. Depending on the nature of the amino acids that compose these fragments in different Hc molecules a mixture of proteolytic enzymes for the separation of all FUs is required. With subtilisin and elastase it is possible to isolate all FUs that build up one structural subunit.

HaH was treated with subtilisin DY at a ratio E/S = 1/260 (w/w) and E/S = 1/100 (w/w), at pH 7.2, at 25 °C, for 24 h. The change in *o*-diPO activity was studied with increasing time of incubation of HaH with different amounts of subtilisin DY, using as substrates 8 mM catechol. By means of limited proteolysis of HaH with subtilisin DY (E/S = 1/100, w/w), after an incubation time of 1 h 30 min, almost a hundredfold increase in enzyme activity against 8 mM catechol was achieved ($v_i = 1.2$ μmol min^{-1} mg^{-1}) (Fig. 6). There were no changes in the *o*-diPO activity of HaH in 25 mM sodium phosphate buffer, pH 7.2, incubated at 25 °C, for 24 h. Hence, modified reactivity of HaH is a result of limited proteolysis of the protein molecule by subtilisin and is not due to other factors such as a non subtilisin-dependent degradation of the protein or conformational alterations due to the prolonged incubation at room temperature.

SDS-PAGE shows that as a result of limited proteolysis of HaH, fragments from FUs and components with lower molecular mass have been formed, whereby the access to the active sites of the molecules of the substrate significantly increases (Fig. 7). The native PAGE of *H. aspersa* maxima hemolymph and Hc, treated with subtilisin DY,

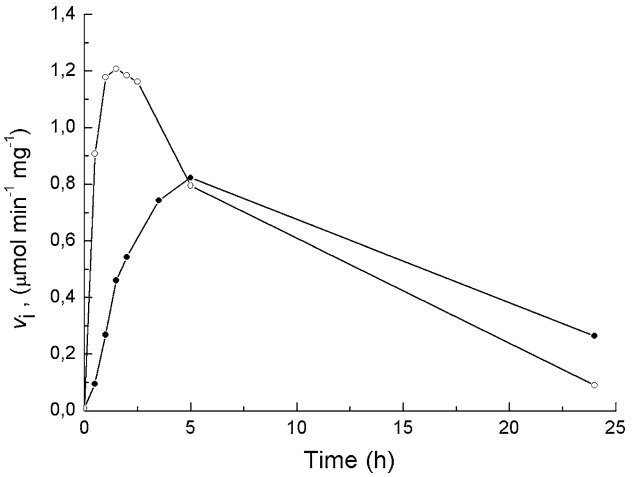

Fig. 6 *o*-diPO activity of HaH, activated with subtilisin DY at a ratio E/S = 1/260 (w/w) (*filled circle*) and E/S = 1/100 (w/w) (*open circle*), towards 8 mM catechol as substrate

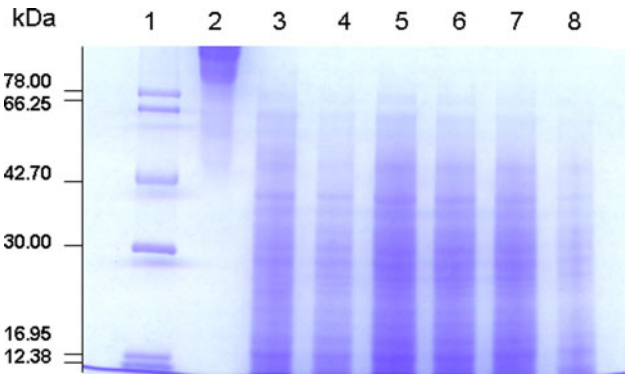

Fig. 7 SDS-PAGE on 10 % running gel; *lane 1* protein markers (from the *top*): ovotransferrin (78.00 kDa), albumin (66.25 kDa), ovalbumin (42.70 kDa), carboanhydrase (30.00 kDa), myoglobin (16.95 kDa), cytochrome c (12.38 kDa) (Merck); *lane 2* total HaH; *lanes 3–8* HaH, treated with subtilisin DY [E/S = 1/100 (w/w)], for 30 min (*3*), 1 h (*4*), 1 h 30 min (*5*), 2 h (*6*), 3 h (*7*) and 4 h (*8*), at 25 °C

soaked in a catechol solution (20 mM), demonstrates *o*-diPO activity of the fragments obtained (Fig. 8a, b). Data obtained to date for various FUs of molluscan Hcs show that they differ in their enzymatic efficiency [15, 25]. Detailed analysis of the FUs, isolated from the Hc of *S. officinalis* and β-Hc of *H. pomatia*, allowed for the first time to identify those among them responsible for the intrinsic *o*-diPO activity as well as for the enhanced activity achieved by limited proteolysis [25]. Campello et al. [3] have reported a considerable increase of tyrosinase-like activity as a result of internal proteolytic cleavage between the α-helix and β-sheet domain of FU d of *O. dofleini* Hc.

The absorption spectrum of HaH, activated by subtilisin DY, showed the typical band at 345 nm, but with slightly

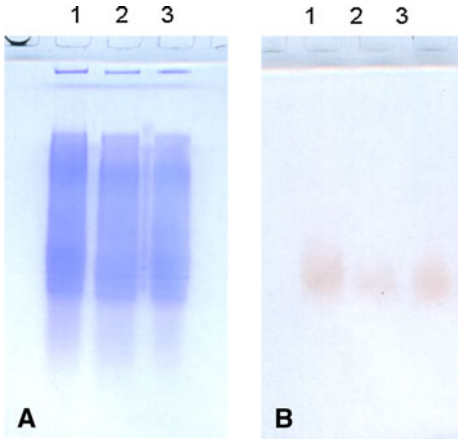

Fig. 8 **a** Native PAGE in 10 % acrylamide gel: *lane 1 Helix aspersa* maxima hemolymph, treated with subtilisin DY [E/S = 1/100 (w/w)] for 1 h 30 min; *lane 2 and 3* HaH, treated with subtilisin DY [E/S = 1/100 (w/w)] for 1 h 30 min and 2 h, respectively. **b**. The same gel stained with 20 mM catechol

reduced intensity (data not shown). Therefore, changes as a result of limited proteolysis did not affect the integrity of the copper active sites in the Hc molecule. However, the fluorescence maximum of HaH, treated with subtulisin DY [E/S = 1/100 (w/w)] for 1 h 30 min, was shifted to 350 ± 1 nm (Fig. 5). It appears that after limited proteolysis certain tryptophan side chains turn out to be exposed at the surface of the protein molecule.

Kinetic analysis of the *o*-diPO activity of HaH, after treatment with subtilisin DY [E/S = 1/100 (w/w)] for 1 h 30 min, with increasing concentration of substrate catechol (from 1.0 to 20.0 mM) was performed. High level of induced activity was detected for HaH—V_{max} increased up to 803 ± 0.002 nmol min^{-1} mg^{-1}, corresponding with a k_{cat} = 40.15 min^{-1} (Table 1). The enzymatic activity of HaH, after its activation by subtilisin DY, is nearly thirty times higher than that observed with proteolytically induced Hcs from *H. pomatia* (k_{cat} = 1.4 min^{-1}), *S. officinalis* (k_{cat} = 1.5 min^{-1}) and *R. thomasiana* (k_{cat} = 0.49 min^{-1}). The difference in the levels of proteolytically induced activity may be due to different conditions in which the limited proteolysis of the respective Hcs is held. Thus, Siddiqui et al. have reported for maximal activation after ~24 h treatment of Hcs from *H. pomatia* and *S. officinalis* with subtilisin Carlsberg (E/S = 1/500 (w/w) in 50 mM Tris–HCl, pH 8.2, at 25 °C) [25]. Such an activation of the Hc from *R. thomasiana* was achieved by means of treatment with subtilisin DY at a ratio E/S = 1/200 (w/w) in 50 mM Tris–HCl, pH 8.2, at 25 °C, for 5 h [15]. The affinity of proteolytically activated HaH towards substrate catechol is higher than the affinity shown by the native HaH (K_m = 6.8 ± 0.2 mM). Highest affinity towards the substrate catechol (K_m = 2.6 mM) has been obtained for β-HpH treated with subtilisin.

After activation with subtilisin DY, HaH showed *o*-diPO towards the more bulky substrate L-Dopa. The highest activity was obtained at the second hour, after treatment with subtilisin in a ration E/S = 1/100 (w/w) ($v_i = 31 \pm 0.001$ nmol min^{-1} mg^{-1} at 9 mM L-Dopa concentration). Kinetic analysis of the *o*-diPO activity induced by limited proteolysis of HaH with increasing L-Dopa concentrations (from 2.0 to 22.0 mM) was performed and kinetic parameters are presented in Table 1. The V_{max} value amounted to 98 ± 0.001 nmol min^{-1} mg^{-1}, $k_{cat} = 12.45$ min^{-1}. K_m value of 17.3 ± 0.5 mM characterizes the affinity of proteolytically activated HaH towards substrate L-Dopa.

3.6 Optical Sensor

Catechol, commonly generated in factory processes, has been proven to have detrimental health effects [16, 29, 36]. Therefore, the detection of low concentrations of catechol is of fundamental importance. Several tyrosinase-based biosensors for determination of phenols have been proposed [1, 33–35, 37–39]. Recently, Yang et al. [37] have reported for a tyrosinase biosensor for detection of catechol based on chitosan-carbon-coated nickel nanocomposite film. Detection limit of 0.083 nM catechol has been achieved.

The relatively high proteolytically induced *o*-diPO activity of HaH prompted us to use it, similarly to tyrosinase, for preparation of a sensor for detection of catechol. The subtilisin-treated HaH, was immobilized in a chitosan film thin layer on a glass plate. The catalytic oxidation of catechol was performed in the presence of the reagent MBTH, which was reported to interact with *o*-quinone products and produced a pink colored *o*-quinone-MBTH adduct [8]. An increase in the optical density at 510 nm with time was observed (Fig. 9a). The peak at 510 nm was absent in sensor lacking Hc (Fig. 9a, blank). The Hc-based sensor showed a linear response at different concentrations of catechol, ranging from 0.05 to 0.9 mM, and a response time of 5 min (Fig. 9b). The sensitivity of this sensor is around 10 times higher than that reported for the optical biosensor for detection of catechol based on immobilized laccase [2]. Nearly simultaneously with us, Naresh et al. [20] report for a similar sensor for detection of phenols using Hc isolated from a freshwater gastropod *Pila globosa*. The *o*-diPO activity of *P. globosa* Hc has been enhanced by treatment with SDS. Linear response of the sensor, towards catechol at concentrations from 0.1 to 0.9 mM catechol, has been obtained. Evidently, the PO activity of Hcs could be utilized for preparation of Hc-based biosensors. Further studies are necessary to improve the sensitivity of this first prototype of sensor based on immobilized Hc from *H. aspersa* maxima.

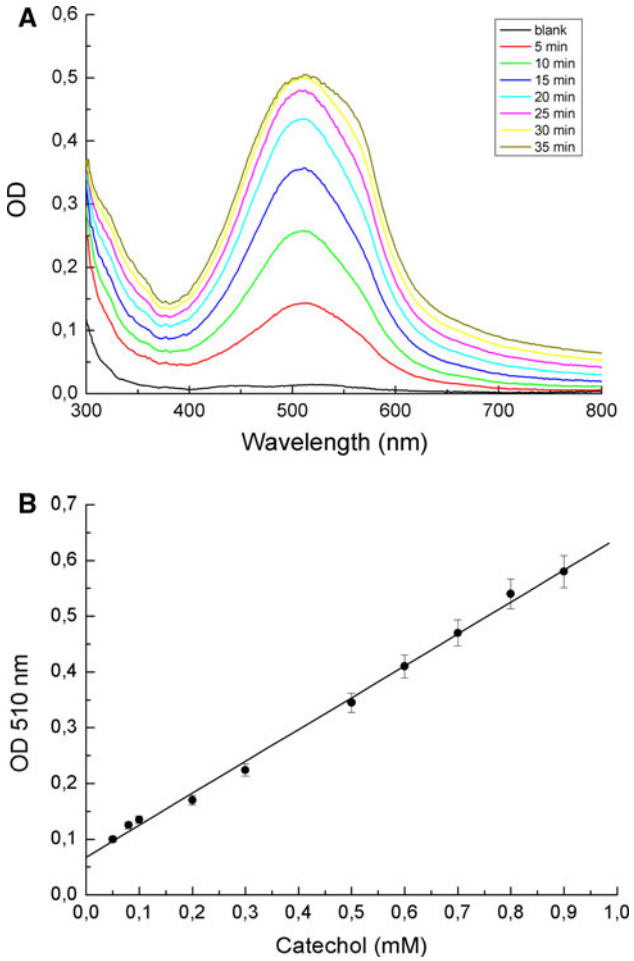

Fig. 9 **a** Absorption spectra of the immobilized HaH in 1 % (w/v) chitosan, in the presence of 0.2 mM MBTH, 2 % (v/v) DMF, and 1 mM catechol. Spectra were recorded at an interval of 5 min for 35 min reaction time. **b** The response curve of the Hc-based sensor at different concentration of substrate catechol (0.05–0.9 mM) and response time of 5 min

4 Conclusion

Most molluscan Hcs show low intrinsic *o*-diPO activity and catechol is the preferred substrate. The enzymatic efficiency of investigated native HaH is low ($k_{cat}/K_m = 0.093$ mM^{-1} min^{-1}), as well as that obtained for other molluscan Hcs, compared with true PO e.g. tyrosinase from the cephalopod *I. argentinus* ($k_{cat}/K_m = 225$ mM^{-1} min^{-1}). It seems that Hcs cannot perform a physiological role as POs and the existing PO activity rather is a residue of the process of their evolution from the ancient tyrosinase. However, the big difference between a true PO enzyme and Hc is the concentration. The low catalytic activity can be compensated by the relatively high concentration of the oxygen-transport protein in the hemolymph of invertebrates (10–50 mg/ml). Therefore, it can be concluded that Hcs are multifunctional proteins and

in addition to the oxygen transport they partake in the innate and humoral immunity of mollusks through their potential PO activity. After activation of Hcs, making the active sites more accessible to the molecules of the substrate, they can function as POs. Thus, upon treatment of HaH with subtilisin DY more than 60-fold increase in the enzymatic efficiency was achieved ($k_{cat}/K_m = 5.9\ mM^{-1}\ min^{-1}$). This fact reveals a potential use of PO activity of Hcs for the preparation of biosensors.

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