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Structure of hemocyanin from garden snail *Helix lucorum*

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ABSTRACT

Hemocyanins are giant extracellular oxygen carriers in the hemolymph of many molluscs and arthropods with different quaternary structure. They are represented in the hemolymph of molluscs with one, two or three isoforms, as decameric, didecameric, multidecameric and tubules aggregates. We describe here the structure of the hemocyanin *Helix lucorum* (HlH), species in the series of molluscan hemocyanins. In contrast with other molluscan hemocyanins, three different hemocyanin isopolypeptides were isolated from the hemolymph of the garden snail *H. lucorum*, named as β-HlH, α_D-HlH and α_N-HlH. Their molecular masses were determined by size exclusion chromatography to be 1068 kDa (β-HlH) and 1079 kDa (α_D-HlH, and α_N-HlH). Native HlH exhibits a predominant didecameric structure as revealed by electron microscopy and additionally few tridecamers are shown in the electron micrographs of HlH resulting from the association of a further decamer with one didecamer. The three isoforms are represented mainly as homogeneous didecamers, but they have different behaviour after dissociation and reassociation in the pH-stabilizing buffer, containing 20 mM $CaCl_2$. All isoforms were reassociated into didecamers and tubules with different length, but in contrast to α_D-HlH isoform, longer tubules were observed in β-HlH. Moreover the structure of β-HlH was analysed after limited proteolysis with trypsin followed by FPLC and HPLC separation of the cleavage products. Eight different functional units were identified by their N-terminal sequences and molecular masses. The protein characteristics, including UV absorption at 340 nm, fluorescence and CD spectra of the native molecule and its units confirmed the structure of multimer protein complexes.
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1. Introduction

The constituents of multimer protein complex can interact non-covalently through electrostatic forces, hydrogen bonds, or hydrophobic forces or by disulfide bonds. Hemocyanins (Hcs) are oligomeric blue copper-containing respiratory proteins with extremely high molecular weight and complex quaternary structure that play a role as dioxygen carriers in the hemolymph of different species of molluscs and arthropods (Van Holde et al., 2001; Burmester, 2004; Salvato and Beltramini, 1990). The structure of arthropodan Hcs (Stoeva et al., 1995; 1999; Dolashka-Angelova et al., 2000a; 1999; 2005a, b; Ali et al., 2000; Paoli et al., 2007) is quite different from the structure of molluscs. Molluscan Hcs result in hollow cylinders from the oligomerisation of the 11 S basic units (structural subunit) with a molecular mass of ca. 400 kDa in gastropods and ca. 350 kDa in cephalopods (Schütz et al., 2001; Dolashki et al., 2005; Cuff et al., 1998; Miller et al., 1998; Lieb and Todt, 2008). Their exact molecular masses were also determined by mass spectrometry multi-angle laser light scattering (MALLS) and Electrospray ionization (ESI-MS) (Bruneaux et al., 2008). As elucidated by 3D cryoelectron microscopy, the decamer of *Haliotis tuberculata* (HtH) contains five subunit dimers; within each dimer the two subunits are in an antiparallel arrangement (Gatsogiannis and Markl, 2009).

The basic assembly of the native molecule of molluscan Hcs is a hollow cylindrical decamer of ca. 35 nm in diameter and 18 nm in height, of similar topology, but with different dimensions and sedimentation coefficients (105 S, 57 S, and 49 S) (Salvato and Beltramini, 1990). The largest hemocyanin quaternary structure of cephalopods such as *Octopus dofleini* (OdH), *Nautilus pompilius* (NpH) and *Sepia officinalis* (SoH) and chiton *Lepidochiton* sp. (Lambert et al., 1994) are represented as a single decamer arranged by one isoform (Miller et al., 1998; Bergmann et al., 2006; Lamy et al., 1998). However, quite a different structure was observed for hemocyanins of gastropods and the few hemocyanins from bivalves containing didecamers formed by face-to-face assembly of two decamers. In many marine species they are accompanied by tubular multidecamers

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of varying length (Gatsogiannis and Markl, 2009; Bergmann et al., 2007; Lieb et al., 2004).

Although most hemocyanins of marine gastropods occur in the hemolymph as didecamers, they have different structures (Gatsogiannis and Markl, 2009; Lieb et al., 2000; Bergmann et al., 2007; Lieb et al., 2004; De Ioannes et al., 2004; Dolashka-Angelova et al., 2003a). For example the native molecules of *Aplycia californica* (ApH) and *Murex fulvascens* (MfH) are didecamers, containing only one structural subunit, in contrast to *Megathura crenulata* (KLH), *H. tuberculata* (HtH), *Nucula nucleus* (NnH), *Concholepas concholepas* (CcH) and *Rapana venosa* (RvH) hemocyanins, which revealed differentially expressed two structurally and functionally distinct hemocyanin isoforms (Lieb et al., 2000; Bergmann et al., 2007; Lieb et al., 2004; De Ioannes et al., 2004; Dolashka-Angelova et al., 2000b; 2003a; 2007; Stoeva et al., 1997; Dolashka et al., 1996; Altenhein et al., 2002). Moreover, two isoforms in the structure of Nautilus hemocyanin was confirmed by the recent comparative cryoEM three-dimensional (3D) analysis at 9-Å resolution (Gatsogiannis et al., 2007). Also, two hemocyanin variants were detected in *O. dofleini*, but they share 98% sequence identity (Miller et al., 1998).

Until now three hemocyanin isoforms have been reported only in one mollusc. In the case of hemocyanin of the Roman snail *Helix pomatia*, three components have been identified, one β-component and two α-components: α_D-Hc (dissociating α-Hc) and α_N-Hc (non-dissociating α-Hc) (Lambert et al., 1995; Lontie, 1983; Wood et al., 1985). The didecameric molecule of α_D and β_C–hemocyanins, are very similar and are composed of a cylindrical wall, typical for gastropodan hemocyanins, comprising 20 subunits with a molecular mass of approximately 450 kDa each. The comparison between the α_D-hemocyanin and the β_C-di-decameric hemocyanin at high thresholds suggests that in the β_C-hemocyanin the oblique wall units of each half molecule may be linked by two connections, whereas in α_D-hemocyanin there may be only one. This difference in the number of connections may be responsible for the lower stability of the αD molecule at high salt concentration (Lambert et al., 1995).

Despite the difference in the quaternary structure of molluscan hemocyanin, the subunits (11 S units) are composed of seven or eight covalently linked functional units (FUs). The functional units contain about 400 amino acid residues and are different in their sequences and genomic pattern (Bergmann et al., 2006; 2007; Lieb et al., 2000; 2004). The domains (the FUs) with molecular masses of about 50 kD contain two copper atoms and are able to bind one dioxygen. Hemocyanin sequences and properties of several FUs of Vetigastropoda (*Haliotis*), Caenogastropoda (*Rapana*), Heterobranchia (*Aplysia*) and from the cephalopod *O. dofleini, N. pompilius* and *S. officinalis* Hcs, are very well studied (Lieb et al., 2000; 2004; Bergmann et al., 2007; De Ioannes et al., 2004; Dolashka-Angelova et al., 2003a; 2007; Dolashka et al., 1996; Altenhein et al., 2002).

Analyses of additional sequences from other hemocyanins will supply additional information about the quaternary structure of these large complexes. Therefore the aim of this study was to isolate a new hemocyanin from the garden snail *Helix lucorum* (HlH) and elucidate its structure.

2. Materials and methods

2.1. Isolation of the hemocyanin from garden snail H. lucorum

Hemolymph was collected from the foot of *H. lucorum* garden snails (25 g), centrifuged at 1000 *g* and 4 °C for 20 min for removal of rough particles. The crude heamocyanin in the clear supernatant was siphoned off and diluted with an equal volume of 0.4 M sodium acetate buffer, pH 5.2, precipitated with half-saturated ammonium sulphate and centrifuged at 8000 *g* for 60 min. After decantation of the supernatant liquid, the sediment, containing the total heamocyanin (Hc), was solubilized at a concentration of about 5% in 0.1 M sodium acetate buffer, pH 5.7, containing 0.02% NaN_3 to avoid microbial growth. The solution was dialysed at 4 °C against the same buffer for 24 h and then dialysed against a solution containing 1.5 M NaCl and 50 mM sodium acetate buffer, pH 5.7, for 24 h at 4 °C in order to eliminate the ammonium sulphate, the buffer was removed once after 12 h.

2.2. Isolation of the isoforms of H. lucorum hemocyanin

Insolubilized β-hemocyanin was obtained by 4–5 days dialysis against 10–20 vol. of 10 mM sodium acetate buffer, pH 5.2, at 4 °C and the buffer was renewed every 12 h. β-hemocyanin was sedimented by centrifugation of the solution at 15,000 g, at 4 °C for 30 min. The pellet was redissolved in 0.1 M sodium acetate buffer, pH 5.7, and Hc was purified by anion-exchange chromatography on a DEAE-Sepharose CL-6B column in 50 mM Tris–HCl buffer, pH 8.0. Elution was performed with a linear gradient of 0.2–1.0 M NaCl with an elution rate of 2 mL/min. Finally, the pure β-hemocyanin fraction was desalted, concentrated by ultrafiltration (30 kDa Amicon® PM membranes), dialyzed against 0.1 M phosphate buffer (pH 6.5) and stored at 4 °C.

After removal of the sedimented β-fraction, (see above), both, α_D-Hc and α_N-Hc, which were dissolved in the supernatant, were separated from each other on a FPLC system, equipped with an anion-exchange Fast Flow Sepharose Q column, using a stepwise NaCl gradient (0.0–1.0 M) in 50 mM Tris–HCl buffer, pH8.2. The isolated components were concentrated by ultrafiltration (10 kDa Amicon® PM membranes) and further purified by gel filtration chromatography on a Sephacryl S 300 column.

2.3. Electron microscopic measurements

Studies of EM specimens were performed using a Philips® CM10 Transmission 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.

2.4. Polyacrylamide gel electrophoresis (PAGE) and 2D-gel electrophoresis

The purity and approximately masses of the isoforms of HlH were analysed by polyacrylamide gel electrophoresis, after boiling the proteins at 100 °C for 5 min. Electrophoresis was carried out on 5% acrylamide gels. The protein used as a standard for molecular mass determination was ferritin (MW = 440 kDa) and the gel was stained by Coomassie blue R-250.

Second-dimension IEF was performed on a Flat Bed Aparatus FBE 3000, Pharmacia LKB. 10 μg protein from each of the isolated three fractions was resuspended in 2-DE rehydration buffer (350 μL, 8 M urea, 2% CHAPS, 18 mM DTT, 0.5% immobilized pH gradient [IPG] buffer, bromophenol blue) and applied on the 8% gel. Gels were run for 4–6 h, fixed, stained with Coomassie blue R-250 (10 h) and then destained overnight and then scanned.

2.5. Gel filtration

The molecular mass determination of isoforms of *H. lucorum* hemocyanin was performed by gel filtration chromatography on a Sephacryl S300 column, equilibrated with 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 M NaCl. The proteins were eluted with the same buffer at a flow rate of 1 mL min^{-1}. The fractions of 3.5 mL/tube were collected and the absorption was measured on a Shimadzu spectrophotometer at 280 nm. The masses of the subunits of *H. lucorum*

Fig. 1. Shell of mollusc garden snail *Helix lucorum.*

hemocyanin were determined by standard plot. The standard curve plotting, displaying elution time of the standards against their molecular weight logarithm, was performed based on the elution times of the standards, as follows: Ferritin (480 kDa), $t = 00:36:06$ min, catalase from bovine liver (240 kDa), $t = 00:52:17$ min, albumin (66 kDa), $t = 01:02:08$ min.

2.6. Isolation of functional units of the structural subunit β-HlH

Multiunit fragments as well as individual FUs were obtained by limited proteolysis of β-hemocyanin with TPCK-trypsin at a ratio — 400/1 (w/w), performed in 50 mM Tris, pH 8.0, containing 1 mM EDTA for 4 h at a temperature of 37 °C. The components of the obtained hydrolyses were separated on an anion exchange FPLC system applying on a Q Sepharose high performance column (HR 10/10, Pharmacia) using a stepwise NaCl gradient (0–1.0 M) in 50 mM Tris–HCl buffer, pH 8.2. Some fragments were additionally purified by rechromatography on the same column.

Isolated FUs were additionally purified on a Hypersil column (250 mm × 4.6 mm; 5 μm HyPURITY C18, Thermo Quest), eluted with eluent A (0.1% TFA in water) and eluent B (80% acetonitrile in buffer A), using a gradient program of 0% B for 5 min and then 0–100% B in 60 min; the flow rate was 0.6 mL/min. All fragments (FUs) were characterized by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins used as standards for molecular mass determination were: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), ovotransferrin (78 kDa), glutamate dehydrogenase (56 kDa), and ovalbumin (42.7 kDa).

2.7. Mass spectrometric analyses (MALDI-TOF)

Mass spectrometric analyses of the isolated functional units from β-HlH were performed by MALDI-TOF (Voyager, PerSeptive Biosystems, Wiesbaden, Germany). The sample (10–50 pmol), obtained after additional purification on a HPLC system, was dissolved in 0.1% (v/v) TFA. Analysis was carried out using 1 μl α-cyano-4-hydroxycinnamic acid (α-CHCA) as matrix (α-cyano-4-hydroxycinnamic acid MALDI matrix was prepared by dissolving recrystallized α-CHCA (Sigma) in water-acetonitrile (50:50) containing 0.1% TFA and 0–50 mM ammonium phosphate) and 1 μl of the sample to be applied to the target. A total of 4500 shots were acquired in the MS mode. Chicken egg ovalbumin (44,400 Da) and bovine serum albumin (66,430 Da) were used for mass scale calibration.

2.8. Amino acid sequence determination

Peak fractions were dried and dissolved in 40% methanol and 1% formic acid prior to subject on to an automated Edman N-terminal sequencer (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

2.9. Spectroscopic properties

Fluorescence measurements of β-HlH were performed with a Perkin Elmer model LS 5 spectrofluorimeter. The optical absorbance of the solutions was lower than 0.05 at the excitation wavelength to avoid inner filter effects. Excitation at 295 nm was used for measurement of tryptophyl fluorescence.

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 on a Jasco 720 dichrograph. Concentration of the protein solutions was around 0.2 mg mL^{-1} in 20 mM Tris/HCl, 10 mM $CaCl_2$ buffer, pH 8.2.

3. Results

3.1. Isolation of HlH and its structural subunits

Hemolymph was collected from the garden snail *H. lucorum*, living in Bulgaria (Fig. 1). Three isoforms, one β-HlH and two α-HlH (α_N- and α_D-hemocyanin), were isolated. The difference between these isoforms to precipitate or crystallize during dialysis against sodium acetate buffer, at a low ionic strength, was used to isolate β-HlH from the hemolymph of *H. lucorum*. The β-Hc component was less crystallized after the fourth to fifth change of the dialyzing buffer (10 mM Na-acetate buffer, pH 5.2). After centrifugation small crystals of β-Hc were obtained which were dissolved in 100 mM acetate buffer, pH 5.8, and purified by anion exchange chromatography on a DEAE Sepharose CL-6B column in 50 mM Tris–HCl buffer, pH 8.0, by linear gradient elution of 0.2–1.0 M NaCl (Fig. 2).

After removal of the β-Hc component from the supernatant, the α-components (α_D-Hc and α_N-Hc) were isolated by anion exchange chromatography on a Flow Sepharose Q column. Two fractions were separated with a stepwise gradient of NaCl (0.0–1.0 M) in 50 mM Tris–HCl buffer, pH 8.2, corresponding to pure α_D-isoform and α_N-isoforms. In the chromatogram, shown in Fig. 3, the first eluted fraction contains a pure α_D-isoform, as α_D- is dissociated ("α_D-" refers to dissociated α-isoform) and therefore could be isolated at a lower ionic strength. The second one contains a pure α_N-isoform which corresponds to a non-dissociated α-isoform which needs stronger ionic strength for isolation. The absorption spectra of the isolated

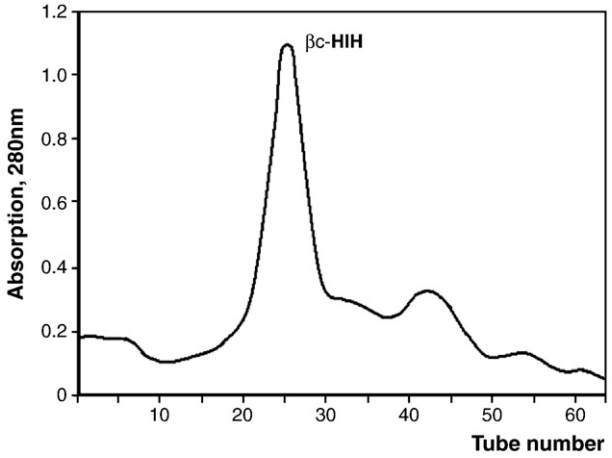

Fig. 2. Purification of the β-HlH component by an anion exchange chromatography on a DEAE-Sepharose CL-6B column, 50 mM Tris–HCl buffer, pH 8.0. Elution was performed with a linear gradient of 0.2–1.0 M NaCl, with an elution rate of 1 mL min^{-1}. Collected in fractions of 2 mL/tube.

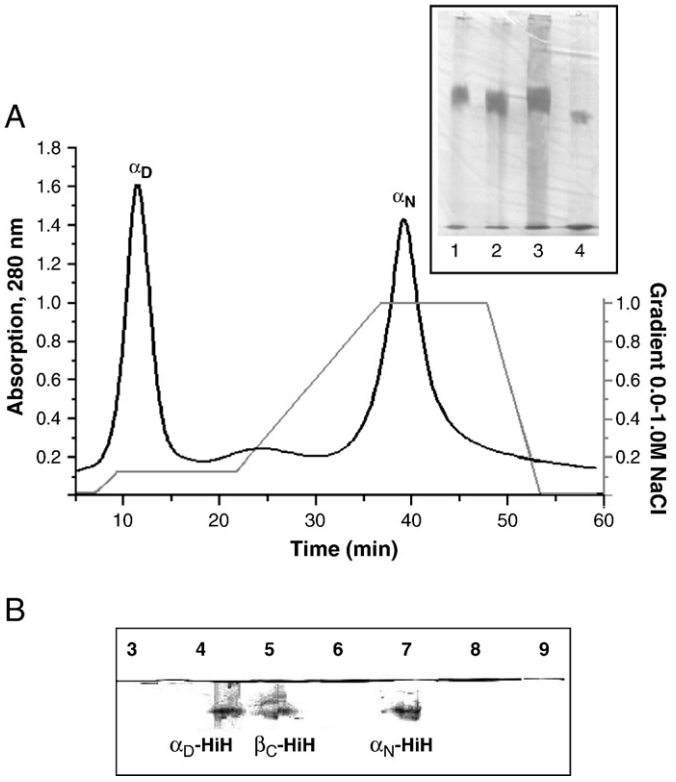

Fig. 3. A) Purification of dissociated structural subunits α_D-HlH and α_N-HlH on an anion-exchange Fast Flow Sepharose Q column, equilibrated with 50 mM Tris/HCl buffer, pH 8.2, using FPLC system. Subunits were separated with a stepwise gradient of NaCl (0.0–1.0 M), at an elution flow rate of 1.5 mL min^{-1} (inset figure). Native 5% – polyacrylamide gel electrophoresis of the isolated isoforms from *H. lucorum* hemocyanin: 1) β-HlH; 2) α_D-HlH; 3) α_N-HlH; 4) molecular weight standard – ferritin. B) 2-D gel electrophoresis of isolated isoforms of HiH. 10 μg protein from each fractions were applied on the 8% gel. Fraction 1 – α_D-HiH, Fraction 2 – β_C-HiH and Fraction 3 – α_N-HiH.

isoforms of HlH were similar to those of other hemocyanins and showed three bands, at 278, 344, and 550 nm corresponding to aromatic residues, Cu^{2+}–O^{2-}, and Cu^{2+}-histidine coordination centers, respectively.

3.2. Molecular weight determination of the structural subunits of HlH

More recently, supramolecular mass spectrometry (MS) and multiangle laser light scattering (MALLS) were used to obtain the masses of large molecules (Bruneaux et al., 2008). However, gel electrophoresis methods can be relatively easily developed in a laboratory and provide rapid analysis of samples. The resolution and accuracy are low, but give preliminary results about the masses of larger molecules. The three isolated isoforms of *H. lucorum* hemocyanin were further analysed using 5% native PAGE. Single bands were observed on PAGE for the structural subunits β-HlH, α_D-HlH, and α_N-HlH (Fig. 3 inset).

These different isoforms were also confirmed by 2D-gel electrophoresis (Fig. 3B) and by their N-terminal amino acid sequences, analysed by Edman degradation (Table 1). Their pI values were determined by 2D-gel electrophoreses (Fig. 3B) to be 4,5 (α_D-HiH), 5,2 (β_C-HiH) and 7.0 (α_N-HiH). Alignments of N-terminal sequences of the isolated isoforms β-HlH and α-HlH with N-terminal sequences of functional units d and g of *H. pomatia* hemocyanin (P12031, P56823), *R. venosa* (Stoeva et al., 1997; Dolashka-Angelova et al., 2007), *H. tuberculata* (CAC20588.1), *O. dofleini* (AAK28276), *N. pompilius* (Bergmann et al., 2006), *A. californica* (Lieb et al., 2004) revealed about 50–67% identity. This suggests that three single hemocyanin polypeptides are expressed in the hemolymph of *H. lucorum*, which differs from the hemocyanins of Aplysia and Octopus (with single isoform), and from Rapana, Haliotis and KLH (with two isoforms).

The molecular masses of the structural subunits of HlH were determined by size-exclusion chromatography on a Sephacryl S300 column. A calibration curve for Mw determination of alpha and beta isoforms of *H. lucorum* hemocyanin were analysed using the standard curve (Fig. 4). The molecular masses of the isoforms were determined to be 1068 kDa (β-HlH) and 1079 kDa (α_D-HlH, and α_N-HlH), taking into account their elution times, i.e.: 0:28:20 min for α-HlH, 0:28:29 min for β-HlH. The molecular weight of the HlH isoforms, correlates very well with the masses of structural subunits of *H. pomatia* Hc which is from the same family Helicidae (Lambert et al., 1995; Lontie, 1983; Wood et al.,1985).

3.3. Electron microscopic measurements

The native HlH and its isolated structural subunits were analysed at different conditions by electron microscopy. Electron microscopy of the negatively-stained native hemocyanin molecules revealed top views and side views of Hc. Mostly, didecamers and few decamers are observed (Fig. 5A, native HlH). Electron micrographs from purified *H. lucorum* hemolymph show, besides a few decamers mostly didecamers, what is typical for all gastropods, and, in addition, very short multidecamers. The multidecamers consist of a "nucleating" didecamer with attached decamers at one or two sides (Fig.5A). After overnight dialysis against 0.13 M Gly buffer, pH 9.6, the decameric forms dissociate in subunits as shown in Fig. 5B. After changing the conditions with the stabilizing buffer, pH 7.0, containing 20 mM $CaCl_2$ (Fig. 5C) and 50 mM $CaCl_2$ (Fig. 5D), the obtained structural subunits reassociate to decamers, didecamers and short multidecamers.

Table 1
Alignments of N-terminal sequences of the isolated isoforms β-HlH, α_N- and α_D-HlH with N-terminal sequences of functional units d (HpHd, P12031) and g (HpHg, P56823) of *H. pomatia* hemocyanin, *Rapana venosa,* RvH1 (26,28), *Haliotis tuberculata* (HtH1, CAC20588.1), *Octopus dofleini* (OdH, AAK28276), *Nautilus pompilius,* NpH1 (18), *Aplysia californica,* AcH (23). Conserved amino acid residues are shaded in dark gray.

Hemocyanin			Idencity with β-HlH (%)																						
			1				5					10					15						20		
β-HlH			S	**L**	**V**	**R**	**K**	**N**	**V**	**D**	**K**	**L**	**T**	K	D	**E**	**L**	Y	N	**L**	Q	K	100	(20)
α_D-HlH			S	**L**	**V**	**R**	**K**	**N**	**V**	**D**	**H**	**L**	**T**	P	E	**E**	**L**	Y	N	**L**	Q	K	85	(20)
α_N-HlH	A	P	A	**L**	**V**	**R**	**K**	**N**	**V**	E	**K**	**L**	L	P	Q	L	**N**	Y	Q	**L**				
HpH d	V	A	S	H	**V**	**R**	**K**	**D**	L	**D**	T	**L**	**T**	A	G	**E**	I	E	S	**L**	R	S	50	(18)
HpH g	A	G	V	G	**V**	**R**	**K**	**D**	**V**	T	R	**L**	**T**	V	S	**E**	T	E	N	**L**	R	E	47	(20)
RvH1			S	L	L	**R**	**K**	**N**	**V**	**D**	T	**L**	**T**	E	Q	**E**	I	L	R	**L**	Q	N	61	(20)
HtH1		D	N	V	**V**	**R**	**K**	**D**	**V**	S	H	**L**	**T**	V	D	**E**	Q	A		**L**	H	G	67	(20)
OdH			N	L	**I**	**R**	**K**	**D**	**V**	**D**	A	**L**	S	E	D	**E**	V	L	N	**L**	Q	V	59	(20)
NpH1			L	L	**V**	**R**	**K**	**D**	**V**	**D**	S	**L**	**T**	T	A	**E**	V	L	A	**L**	Q	E	65	(20)
AcH	N	A	A	L	**V**	**R**	**K**	S	**V**	**D**	Q	**L**	**T**	S	E	**E**	I	L	N	**L**	Q	K	59	(20)

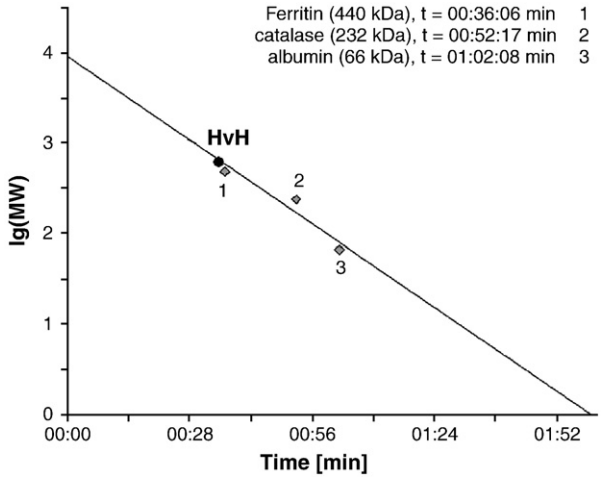

Fig. 4. Mw determination of a-HlH and β-HlH by gel filtration chromatography on a Sephacryl S300 column. Subsequent Mw determination by a standard curve plotting displaying elution time of the standarts used against their Molecular Weight logarithm. Standards used and elution time: Ferritin (480 kDa), $t = 00:36:06$ min; Catalase from bovine liver (240 kDa), $t = 00:52:17$ min; Albumin (66 kDa), $t = 01:02:08$ min.

The three isoforms of HlH, β-HlH, α_D-HlH and α_N-HlH were isolated and studied by electron microscopy. Electron micrographs from the purified β-subunit of *H. lucorum* Hc in stabilizing buffer showed, besides a few decamers and subunits, mostly didecamers, typical for all gastropods (Fig. 6A). After reassociation of β-HlH in the stabilizing buffer, containing 10 mM $CaCl_2$, in addition, short tubules were observed (Fig. 6B). The tubules consist of a didecamer with attached decamers at one or both sides. In the presence of 10 and 20 mM $CaCl_2$ in the buffer, subunits and didecamers associated to tubules of different length (Fig. 6B and C, respectively).

Also, the subunits-dissociated into α-component exhibited the same behavior in the pH-stabilizing buffer as β-HlH. As is shown in Fig. 6D, under the influence of 20 mM $CaCl_2$, α-HlH reassociated into didecamers and tubules of different length but shorter ones than in reassociated β-HlH (Fig. 6C). A similar capacity for the formation of tubules was observed for other molluscan hemocyanins, as much those from Rapana and *H. pomatia* (Dolashka-Angelova et al., 2003a; Lambert et al., 1995), however, no long multidecamers were identified in the isoforms of HlH.

3.4. Analysis of the subunit organization of β-HlH

Our sequence data show that β-*H. lucorum* hemocyanin contains eight FUs that correspond structurally to the eight different FUs of *H. pomatia* hemocyanin. For the purified β-HlH cleavage we used low concentrations of trypsin (400:1) which in the case of RvH1 and RvH2 allowed to characterise several functional units. Using a FPLC system with a Q Sepharose High Performance column, tryptic cleavage products were obtained in sufficient purity (Fig. 7) consisting of a mixture of single FUs and smaller and larger fragments containing two, three or more FUs. The cleavage products were additionally

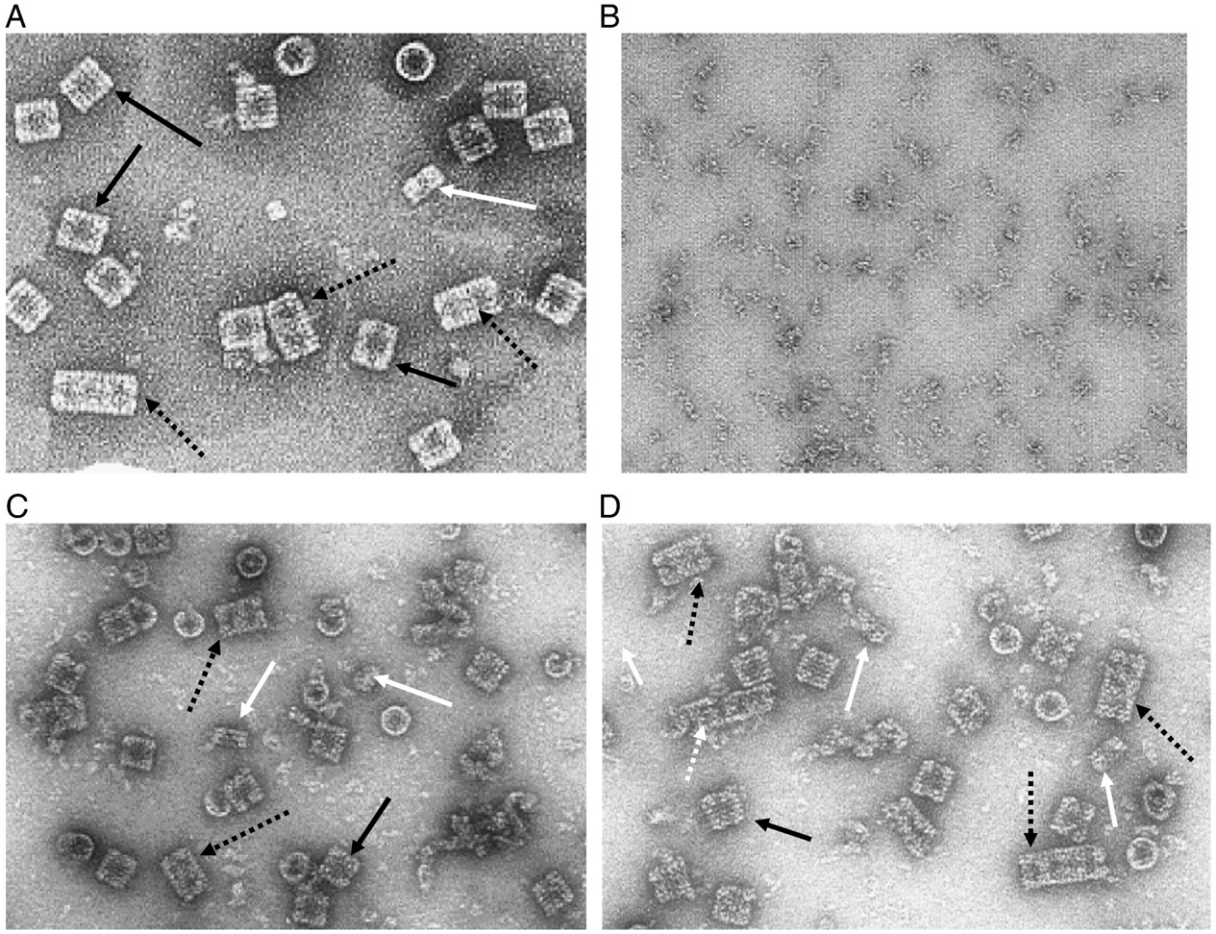

Fig. 5. Gallery of transmission electron microscopy of HlH. A) Negatively stained native HlH didecamers visible in side views (rectangular) and in top views (circles). Didecamers in side view (black arrow) and threedecamers (dash arrows); B) dissociated protein in 0.13 M Gly/NaOH buffer at pH 9.6; C) reassociated HlH after dialysis against SB, pH 7.0, containing 20 mM $CaCl_2$; D) against the SB, containing 50 mM $CaCl_2$. Decamers (dash arrows), didecamers (black arrow) and multidecamers of varying length (width arrow) are observed in C and D. Staining with 1% uranyl acetate was performed as described in Materials and methods.

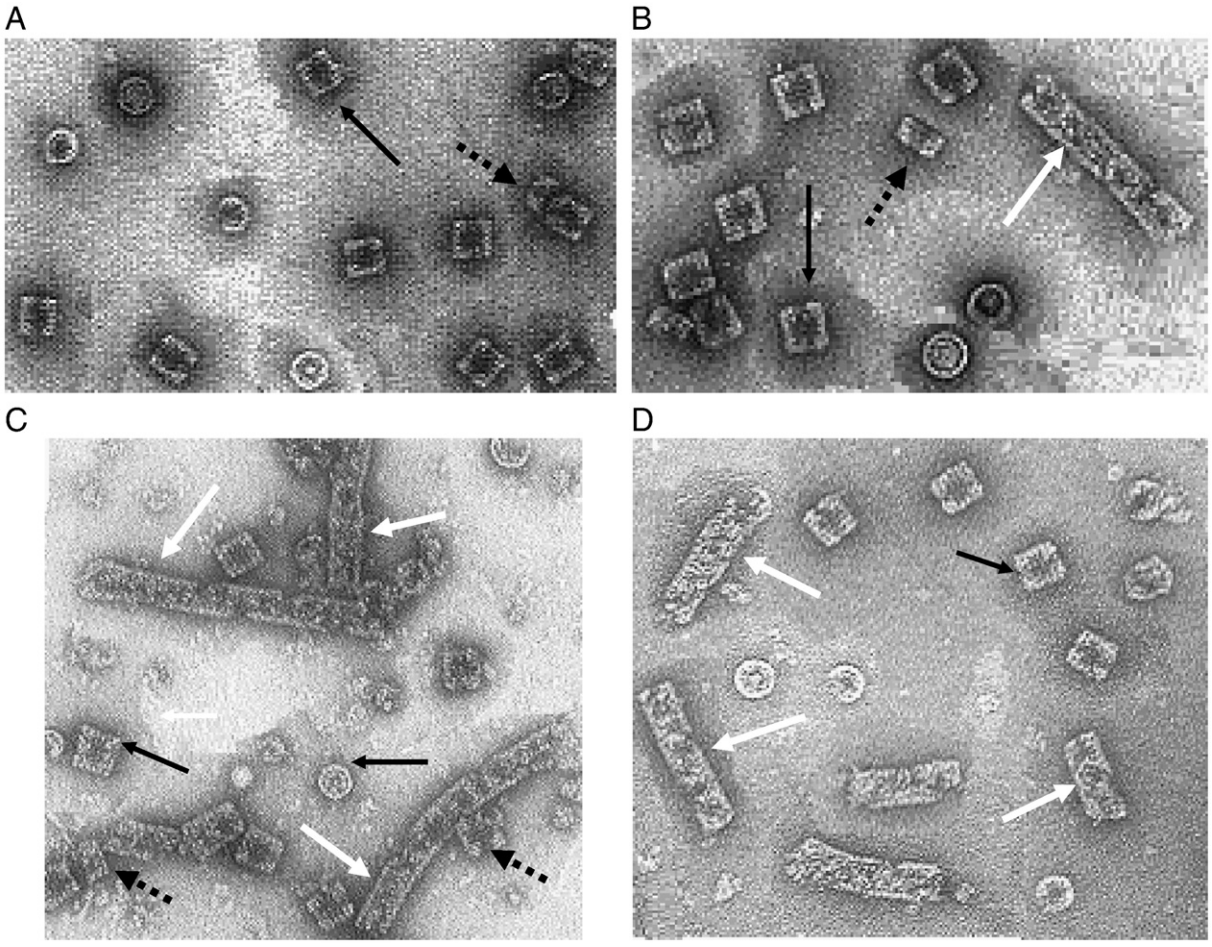

Fig. 6. Electron microscopy of negatively stained of A) β-HlH and α_D-HlH showing didecamers in side view and top view and multidecamers of varying length (white arrow). Note that multidecamers consist of a central didecamer (black arrows) to which single decamers (black dash arrows) are attached. B) Reassociation of β-HlH in the stabilizing buffer, containing 10 mM $CaCl_2$. C) By increasing the concentrations of both divalent ions, 20 mM Ca^{2+} and Mg^{2+}, didecamers (black arrows) and multidecamers (white arrows) are observed. D) Reassociation of α-HlH in the stabilizing buffer, containing 20 mM $CaCl_2$ into didecamers and tubules of different length.

purified by a HPLC system, equipped with a Nucleosil C18 column (data not shown) that enabled their identification in their corresponding SDS/PAGE and MALDI pattern. SDS/PAGE showed eight different fragments with masses between 47 and 55 kDa (Fig. 8, inset). The molecular masses of the isolated fractions were measured by MALDI/MS, and two ions at *m/z* 47635.69 Da and 49184.02 Da were observed for two functional units eluted in fraction N5 (Fig. 8).

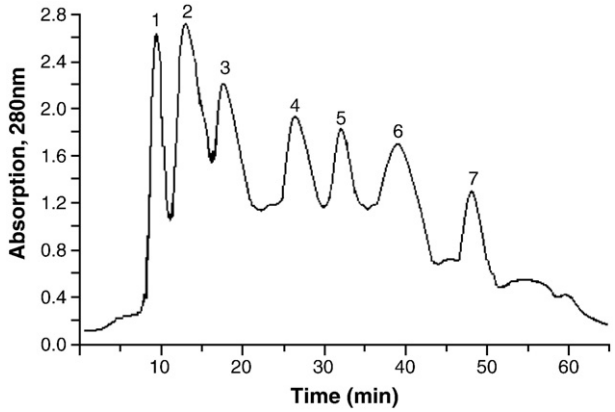

Fig. 7. FPLC profile on trypsin-digested to the β-Hc, applied to a Q Sepharose High Performance column and eluted with 50 mM Tris–HCl buffer, pH 8.2, with gradient NaCl (0.0–1.0 M) in 70 min at a flow rate of 1.5 mL min^{-1}.

Also, a number of isolated components of β-HlH were N-terminally sequenced. HPLC-purified fractions were identified by N-terminal sequence similarities to corresponding FUs in Haliotis, Aplysia and *H. pomatia* Hcs (Fig. 9). In all of them a typical molluscan hemocyanin

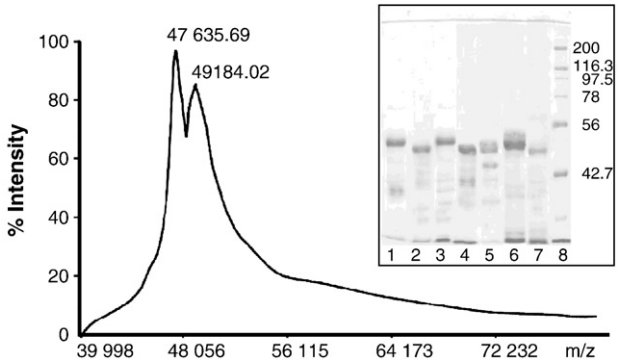

Fig. 8. MALDI spectrum to a fraction no. 5, isolated by FPLC system on a Q Sepharose High Performance column after limited proteolysis of β-Hc with trypsin. After additional purification on a Nucleosil column RP C18, the sample was measured by MALDI/MS, showing a molecular mass at *m/z* 47635.69 Da and 49184.02 Da. Chicken egg ovalbumin (44,400 Da) and bovine serum albumin (66,430 Da) were used for mass scale calibration (inset). 10% SDS-polyacrylamide gel electrophoresis of produced fragments after limited proteolysis with trypsin of β-hemocyanin: from no. 1 to no. 7, which correspond to the isolated ones by FPLC separation system. Lane 8 – protein markers with following molecular masses (from the top): myosin, 200 kDa; L-galactosidase, 116.3 kDa; phosphorylase b, 97.4 kDa; ovotransferrin, 78 kDa; glutamate dehydrogenase, 56 kDa, ovalbumin and 42.7 kDa .

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HlH-a                    SLVRKNVDKLTKDE
ApH-a                    ALVRKSVDQLTSEE
HtH-a                  DNVVRKDVSHLTV

HlH-b        LEHKEEVHEGVSI RKDVD
ApH-b     HGESEHGHEVHDGVSI RKDIDTLT
HtH-b         PPVHHRHDDDLIVRKNID

HlH-c         KQTKKPADVEVHVRKEVSQL
ApH-c         DILPEAQIDEVTVRKNVDSL
HtH-c          PTFEDEKHSLRIRKNVD

HlH-d       YGQEYRPVVAASQVRR
ApH-d       YGQEYREAVTAASYVRRDLSTL
HtH-d    GTRDRDNYVEEVTGASHIRKNLN
HpH-d                  VASHVRKDLDTLTAGEIESLRS

HlH-e            DQHGTTSTYLVRKNVESL
ApH-e             ADGQVGNYLVRKNIASLSPQ
HtH-e       DTHILDHDHEEEILVRKNIIDL

HlH-f                KVPLNKIRRNIDSLEERDIQ
ApH-f             DFKREHVAPNHVRRNLDTLEERDL
HtH-f           HKLNSRKHTPNRVRHEL

HlH-g                SIAGVGVRKDVSSLST
ApH-g           EVSSSSSLAGVGVRKDVS
HtH-g           DDHQSGSIAGSGVRKDVNTL
HpH-g                  AGVGVRKDVTRLTVSETENLRE

HlH-h                  GSGVLLRKNVNQLSQDE
ApH-h    YYERVAAKTAKSSASVL RKDVNDL
HtH-h HRGGKHEDEHHDDRLADVLIRKEVD
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Fig. 9. Alignment of N-terminal sequences of functional units, isolated from *Helix lucorum* (HlH), *Helix pomatia* (HpH), *Haliotis tuberculata* (HtH) and *Aplysia californica* (ApH) hemocyanins.

fragment (-Val Arg Lys Asp-) was identified. Only five N-terminal sequences of the FU (HlH-a, HlH-b, HlH-d, HlH-f and HlH-g) from β-HlH revealed high homology with the sequences of Aplysia Hc. Our results proved that the hemocyanin sequences of *H. lucorum* are significantly more closely related to *A. californica* and *H. pomatia* (Lieb et al., 2004; Altenhein et al., 2002; Wood et al., 1985).

Additionally, α-HlH and β-HlH isoforms and isolated functional units were analysed by UV, fluorescence spectroscopy and circular dichroism. Each functional unit contains about 7–8 tryptophan residues and one active site with two copper ions. Therefore, two maxima, at 278 and 347 nm, were observed in the absorption spectra of isoforms and FUs of HlH, originating from aromatic residues and Cu^{2+}–O^{2-}, respectively (data are not shown). These absorption spectra are similar to other molluscan and arthropodan hemocyanins and the bands at 347 nm disappeared with the addition of reducing agents (Dolashka et al., 1996; Fan et al., 2009). Also, the native molecule and isolated isoforms were analysed by fluorescence spectroscopy after excitation at 295 nm, where the tryptophyl side chains are selectively excited. The fluorescence parameters of the oxy-forms of the native *H. lucorum* hemocyanin and functional units are shown in Fig. 10A. The fluorescence spectrum for HlH has a maximum at $(335) \pm 1$ nm, which is typical for deeply buried tryptophans in a hydrophobic environment. However, the fluorescence maximum of FU was shifted to 342 ± 1 nm, due to the exposed tryptophyl side chains on the surface of the molecule.

Two negative Cotton effects at 222 and at 208 nm were digitalized in the CD spectra of the hemocyanin solutions, recorded from 190 to 250 nm, as observed for Rapana hemocyanin and due to the α-helix and β-sheet structures of the proteins (Velkova et al., 2009a; Dolashki et al., 2008).

4. Discussion

Hemocyanins are dissolved in the hemolymph of molluscs and aggregated in various complexes, which still are not very well understood. Hemocyanins from the marine gastropod *M. crenulata*, *C. concholepas* and *R. venosa* occur in the hemolymph in two isoforms and their protein structures and disassembly/reassembly behavior have been extensively studied (Gatsogiannis and Markl, 2009; De Ioannes et al., 2004; Velkova et al., 2009a; Dolashki et al., 2008; Hristova et al., 2008). At present, there is a growing interest in hemocyanins because of their immunological properties and potential application as promising tumor vaccine carriers (Dolashka-Angelova et al., 2009; Velkova et al., 2009b; Yossifova et al., 2009; Iliev et al., 2008; Toshkova et al., 2006; Toshkova et al., 2007; Dolashka-Angelova et al., 2008; Moltedo et al., 2006; Wuhrer et al., 2004). Structure, evolution, and diversity of hemocyanins is of scientific interest and the relationship between structural features and immunotherapeutic effects is of biomedical concern. Therefore, we present in this communication structural characteristics of hemocyanin, dissolved in the hemolymph of garden snail *H. lucorum* and demonstrate that its special structure differs from the other molluscan hemocyanins.

In contrast to the marine gastropods of the genera, *Megathura, Haliotis, Aplicia, Nucula, Concholepas*, or *Rapana* (Gatsogiannis et al., 2009; Lieb et al., 2000; Bergmann et al., 2007; Lieb et al., 2004; De Ioannes et al., 2004; Dolashka-Angelova et al., 2003a; Lambert et al., 1995), revealing the presence of one, or two structurally and functionally distinct hemocyanin isoforms, three different isoforms (β-HlH, α_D-HlH and α_N-HlH) were identified to be dissolved in the hemolymph of *H. lucorum*. The evidence for three isoforms, α_D-HiH, β_C-HiH, and α_N-HiH are based on different pI values (4,5, 5,2 and 7.0, respectively), determined by 2D-gel electrophoreses (Fig. 3B). α_D-HiH and β_C-HiH show very clos pI values (4,5 and 5,2, respectively) compared to the α- and β-isoforms of *Helix aspersa* Hc (4,6 and 5,2, respectively) but different ones to those of *H. pomatia* (5,3 and 5,4, respectively).

In fact, the presence of three different isoforms in only two molluscs, *H. lucorum* and *H. pomatia*, raises the question of how they assemble into the functional molecules. Therefore, three isoforms β-HlH, α_D-HlH and α_N-HlH have been isolated and characterized by different methods in

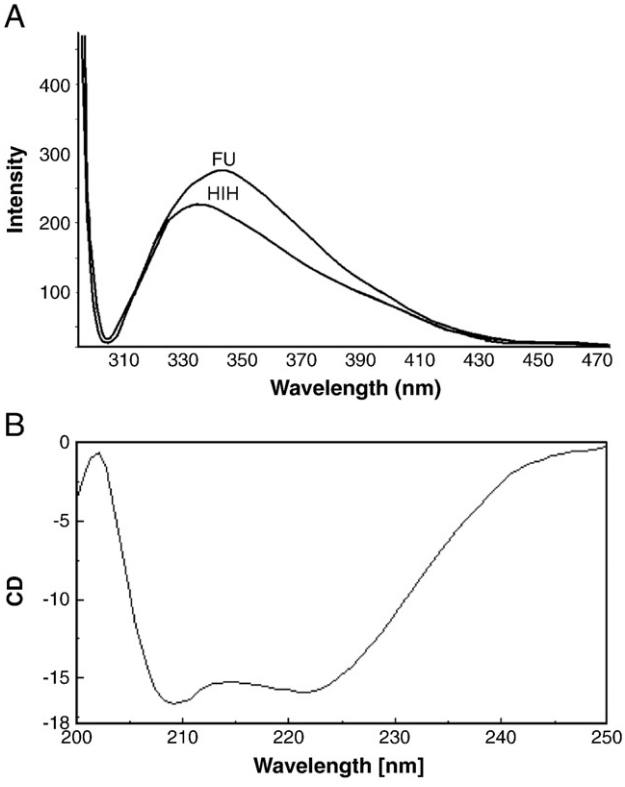

Fig. 10. A) Fluorescence spectra of the native HlH and isolated functional unit were measured at λ_{ext} 295. The absorptions of both proteins were at 280 nm = 0.05, B) CD spectrum of the native molecule of HlH in the region 195 nm to 250 nm. Absorption of protein was 0.20 and the path length of the cuvette was 0.2 cm.

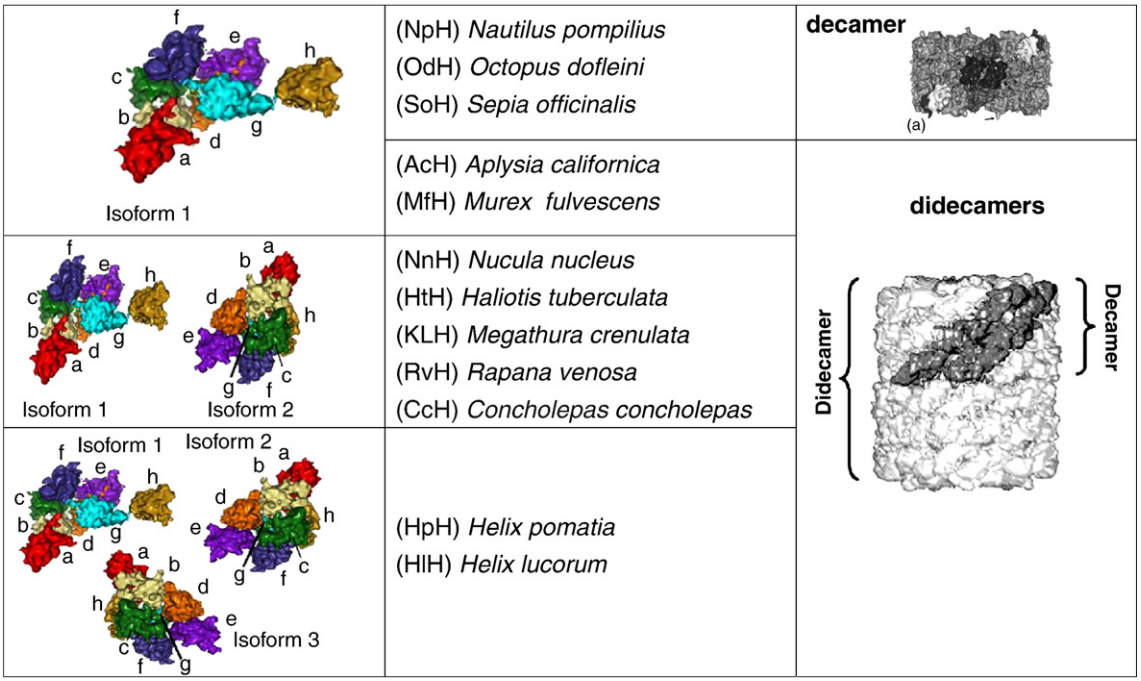

Fig. 11. Organization of the quaternary structure of molluscan hemocyanins into decamers (Hcs with one isoform) and didecamers (Hcs with one, two and three isoforms).

comparison with other molluscan Hcs (Fig. 11). Analyzing the purity and apparent molecular weights of the eluted proteins by gel electrophoresis only one band at ≈450 kDa was detected for each isoform (β-HlH, α_D-HlH and α_N-HlH). However, the calculated results by MALDI-TOF and SDS gel electrophoresis gave more precise results about the molecular mass of β-HlH. It was determined to be about 420 kDa. All of the isolated isoforms are present in the hemolymph mainly as didecamers that resemble the quaternary structure of the hemocyanin. However, only in the electron micrographs of the native HlH a few tridecamers are shown, resulting from the association of a further decamer with one didecamer. It was confirmed that although the hemocyanins from Vetigastropoda (*Haliotis*), *Caenogastropoda* (*Rapana*), Heterobranchia (*Aplicia* and *H. pomatia*) and bivalve Nucula contain one, two or three isoforms, all of them occur as didecamers (Lieb et al., 2000; Bergmann et al., 2007; Lieb et al., 2004; Dolashka-Angelova et al., 2003a; Meissner et al., 2000; Lambert et al., 1995). Their didecameric structure is formed by homogeneous or heterogeneous decamers. The only examples to date of hemocyanins with a hetero-didecameric structure are those of the molluscs Murex, Concholepas and Rapana (De Ioannes et al., 2004; Dolashka-Angelova et al., 2003a).

The three isoforms of *H. lucorum* hemocyanin (β-HlH, α_D-HlH and α_N-HlH) are composed of only one type of organized as subunit, formed didecamers by homogeneous decamers, as was observed for KLH, RvH, HtH (Harris et al., 2004; Dolashka et al., 1996; Altenhein et al., 2002). However, they reveal a different behaviour after dissociation and reassociation. In pH-stabilizing buffer with 20 mM $CaCl_2$, dissociated subunits reassociate into didecamers and tubules with different length. Long tubules are observed in β-HlH, in contrast to α_D- and α_N-HlH, which reassociate into shorter tubules. Although, a similar capacity for the formation of tubules is observed in Rapana and *H. pomatia* hemocyanins (Dolashka et al., 1996; Lambert et al., 1995), a striking different behaviouer of HlH isoforms was observed. No long multidecamers were identified in the isoforms of HlH.

So far, the analyses have given no clue to an explanation for why certain hemocyanins form very long multidecamers, tubules, others form very short ones, and still others are restricted to didecamers, or just decamers, as in the gastropod chitons. Strictly didecameric hemocyanins are observed for those from *H. pomatia* and *S. officinalis* which are heavily glycosylated (Gielens et al., 2004). Studies on the oligosaccharide structures of other hemocyanins, such as RvH, *Arion lusitanicus*, Aplysia, *M. crenulata* and partially *H. lucorum* hemocyanins show the existence of a variety of glycan side chains, exposed on different positions in the molecule (Gielens et al., 2004; Gutternigg et al., 2004; Dolashka-Angelova et al., 2004; Kurokawa et al., 2002; Lommerse et al., 1997; Beck et al., 2007; Sandra et al., 2007; Dolashka-Angelova et al., 2003b). It was found that hemocyanins Haliotis2, Aplysia, Megathura, and Octopus, which form long multidecamers, potential N-glycosylation sites are missing in the case of FU-c. In contrast to HtH2, the isoform HtH1 showed different reassociated behaviour and no long multidecamers were found. However, HtH1-h has two potential N-glycosylation sites in quite unusual positions, localized on the surface of the decamer (Lieb et al., 2000). In all other FUs, including HtH2-h, these two positions are not N-glycosylated. Based on these data, it may be speculated that on the surface of the HlH decamer there is a putative glycosylated site, which may be a reason that decamers do not interact to longer multidecamers.

Additionally, the structure of one isoform, β-HlH, was analysed after enzymatic digestion with trypsin. The obtained fractions were analysed and compared with other Hcs. On the basis of limited proteolytic cleavage, SDS/PAGE and N-terminal sequencing, we identified eight different functional units with molecular masses in the region of 47 to 55 kDa for β-HlH hemocyanin. In phylogenetic trees, derived from sequence alignments, topologically corresponding FUs of different molluscan hemocyanins from discrete branches lead to the concept of an early origin of the eight functional units (Lieb et al., 2000; Bergmann et al., 2007). We have clearly demonstrated, that *H. lucorum* hemocyanin contains eight FUs (Fu-a to FU-h), in contrast to the FUs of *N. pompilius* and *O. dofleini* hemocyanins, consists of seven FUs only. The multiple sequence alignment of N-terminal sequences of the separated FUs shows that HlH shares a higher identity with Aplysia Hc than with Haliotis.

Additionally, analysed by UV, fluorescence spectroscopy and circular dichroism, which are suitable tools for the identification of aromatic residues and studying the structure and conformation in

solution of the proteins, α-HlH and β-HlH isoforms exhibited very similar properties to other molluscan and arthropodan hemocyanins.

In summary, we describe here the structures of hemocyanins isolated from the gastropod *H. lucorum*, emphasizing some attributes that make it interesting among molluscan hemocyanins. This, in turn, would provide essential information on the path of the elongated subunits within the didecamers and tubules which is still largely unknown for any molluscan Hc. Further studies on the gene sequence and oligosaccharide structure of the three isoforms of HlH are required to better understand the structure and molecular basis of the specific association pattern.

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