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ESI-MS and MALLS analysis of quaternary structure of molluscan hemocyanins†

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The understanding of the function of macromolecular complexes is mainly related to a precise knowledge of their structure. Recently, the development of suitable mass spectrometric techniques (electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)) and multi-angle laser light scattering has enabled mass determination of native complexes and of their subunits. By these techniques, the structure and association/dissociation behavior of huge molecules of molluscan *Octopus vulgaris, Sepia officinalis* and *Rapana venosa* have been characterized. Molecular masses of the native and dissociated molecule of cephalopodan Hcs *O. vulgaris* (3545 and 359.3 kDa, respectively) and *S. officinalis* (4134 and 443.8 kDa, respectively) revealed that only one type subunit organizes their molecules, while the presence of two isoforms with different masses (422.8 and 400.0 kDa) has been determined for gastropodan *R. venosa* Hc, aggregated into didecamers. The difference of their structural subunits was also established after limited proteolysis with TPCK-trypsin. Eight functional units (FUs) with masses of ~ 50 kDa were isolated from both subunits of RvH and isoform of *Sepia officinalis*, while seven FUs were purified from OvH. Further characterization of proteins by ESI-mass spectrometry (MS) and MALDI-MS, methods gave insights into post-translational modifications such as glycosylation. Glycosylation of *O. vulgaris* and *S. officinalis* Hcs was suggested based on the differences (11.6 and 40.0 kDa, respectively) between the masses measured by ESI-MS and those calculated by their gene sequences.

Keywords: Electrospray ionization mass spectrometry (ESI-MS); Glycoproteins; Hemocyanin; Multi-angle laser light scattering (MALLS); Quaternary structure

INTRODUCTION

Hemocyanins (Hcs) are oxygen-binding proteins, freely dissolved in the hemolymph, of many arthropods and molluscs.[1–4] Although the Hcs of both phyla use a binuclear copper site for oxygen binding, they differ substantially for their molecular architecture and arrangement of the subunits constituting the native aggregates. Arthropodan Hcs are hexamers or aggregates of hexamers, built up of about 75-kDa subunits containing a pair of copper atoms.[2,3] On the contrary, molluscan Hcs are high molecular weight oligomeric proteins arranged into decamers or didecamers with molecular mass of 4000 to 9000 kDa.[5–7] They form hollow cylinder shape with diameter of 18 nm and a five- or tenfold symmetry axis. In cephalopods, the decamers are arranged by ten structural subunits with molecular weight about 350 kDa.[8,9] However, in gastropods, two decamers, each arranged by ten structural subunits with molecular **weight of about** 350–450 kDa, are assembled face to face to form the so-called didecamer.[10–12] Electron microscopy revealed that native molluscan Hcs exhibit a predominant decameric and didecameric structure.[11–14] Upon increasing the pH in the absence of divalent cations, all molluscan Hcs dissociate in subunits with sedimentation coefficient 11S and molecular weight range between 250 and 450 kDa according to the species.[12–14] The number of subunits present in a molluscan species varies between 1 and 3. One structural subunit was identified in *Sepia officinalis* (SoH),[15] *Aplycia californica* (ApH)[14] and *Murex fulvascens* (MfH), while two structurally and functionally distinct Hc isoforms were isolated from *Megathura crenulata* (KLH),[8] *Haliotis tuberculata* (HtH),[10,13,16] *Nucula nucleus* (NnH),[17] *Concholepas concholepas* (CcH)[18] and *Rapana venosa* (RvH)[19–21] Hcs. In the case of Hc of the Roman snail *Helix pomatia* (HpH)[22] and *Helix lucorum* (HlH),[23] three components have been identified (β-HlH, α_D-HlH and α_N-HlH). They all formed didecamers of homogeneous decamers, **as it was** observed for *M. crenulata*, *R. venosa*

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Abbreviations: *Hemocyanins, (Hcs); Functional, units (FUs); Multi-angle, laser light scattering (MALLS); Electrospray, ionization mass spectrometry (ESI-MS); Electrospray, ionization mass spectrometer (LCT, Micromass, Altrincham, UK); (ESI-TOF), Octopus vulgaris* (OvH); *Sepia, officinalis* (SoH); *Rapana, venosa* (RvH) and *Helix lucorum* (HlH); *Carcinus, aestuarii* (CaH); Scanning, transmission electron microscopy (STEM); Matrix, assisted laser desorption/ionization mass spectrometer Ultraflex II (Bruker Daltonics, Bremen, Germany) (MALDI-TOF)

and *H. tuberculata* Hcs.[12,24,25] They all show the cylindrical quaternary structure (diameter 35 nm, height 38 nm), typical for gastropodan Hcs, comprising 20 subunits with a molecular mass of approximately 450 kDa each. Structural subunits are single polypeptide chains folded as to form several globular functional units (FUs), with molecular masses in the range of 45–65 kDa and each containing one active site.[26]

On the basis of the mass provided by scanning transmission electron microscopy (STEM) analysis and subunit masses estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the authors were able to propose a structural model where the one or two decamers organized the structure of the native Hcs. However, the methods such as multi-angle laser light scattering (MALLS) and electrospray ionization mass spectrometry (ESI-MS) can detect more subunits than PAGE. **MS is an effective method to measure molecular masses with high precision** and accuracy. Therefore, MS was used to analyse the arthropodan and molluscan Hcs[27–29] as the methods of choice for complete investigation of subunit composition.

MALLS and ESI-MS **exhibit enough high resolution and are useful** techniques to investigate the heterogeneity of the Hc complexes. Therefore, in this study, we **report the MALLS and ESI-MS data for the analysis of the quaternary** structure of large complexes (Hcs) from mollusks.

EXPERIMENTAL

Hemolymph collection and purification of hemocyanins

The Hc was obtained from the hemolymph collected from marine snail *R. venosa* (gastropod) from the Black sea (Varna, Bulgaria),[12,20] while the hemolymph of *O. vulgaris, S. officinalis* (cephalopod) **were collected** at the Zoological Station of Napoli (Italy). The marine living animals were stored in running sea water before hemolymph collection. In the case of gastropods, the hemolymph was collected by cutting the foot muscles of animals, whereas the cephalopods and arthropod hemolymphs have been obtained by incannulation of the aorta vessel. Phenylmethylsulfonyl-fluoride (1% w/v) and Cocktail complete™ (Boheringer, Mannhein) (1 tablet/50 ml) were added as protease inhibitors. The hemolymph was then filtered on gauze and dialysed against 10-mM Tris/HCl buffer pH 7.5, containing 20-mM $CaCl_2$ for 24 h. To remove blood cells and precipitated material, after dialysis, the hemolymph was centrifuged at 20 000 g for 30 min in a Beckman centrifuge (J2-21). From the clear solutions, Hcs were obtained by sedimentation in a preparative Beckman XL70 ultra-centrifuge (rotor 70TI) at 450 000 g for 2 h. The dark blue pellets were re-dissolved in 50-mM Tris/HCl buffer pH 7.5 containing 20-mM $CaCl_2$.

Preparation of structural subunits

Structural subunits of Hcs were obtained after dissociation by dialysing of the native protein against a 0.13 M glycine/NaOH buffer, pH 9.2. Structural subunits RvH1 and RvH2 were purified by means of an ion exchange Resource 6 ml (Pharmacia) column using an FPLC system. Elution was performed as described by Dolashka-Angelova *et al.*[12]

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.

Isolation of functional units of RvH2 subunit

Multiunit fragments as well as individual FUs were obtained by limited proteolysis of 20 mg of RvH2 with TPCK-trypsin in a ratio ~400/1 (w/w), performed in 50-mM Tris–HCl, pH 8.0, containing 1-mM EDTA for 4 h, at a temperature of 37 °C. The components of the obtained hydrolysate were separated by anion exchange on a FPLC system using a Pharmacia Q Sepharose High Performance Column (HR 16/10 mm), applying a stepwise NaCl gradient (0–1.0 M) in 50-mM Tris–HCl buffer, pH 8.2. 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-PAGE.

MALLS and ESI- mass spectrometry

100 μL of each Hc was desalted on Centricon PM10 (cut off 100 kDa) micro-concentrators (Amicon, Millipore, Bedford MA, USA) in 10-mM ammonium acetate (pH 6.8). Samples were diluted two to three times in the previous buffer to a final concentration of about 20 pmol/μL for a 1000-kDa complex and continuously infused into the ESI ion source at a flow rate of 5 μL/min. Great care was exercised so that the noncovalent interactions survive the ionization/desorption process. Particularly in order to preserve the integrity of the noncovalent assemblies and to enhance the sensitivity of the detection, the pressure in the interface between the atmospheric source and the high vacuum region was increased to 6.5 mbar by throttling the pumping line. The accelerating voltage applied on the sample cone ranged from 120 to 200 V (optimal value was 200 V), and both source and desolvation temperatures were 100 °C. A solution of 1 mg/mL CsI in 50% aqueous isopropanol was used for the calibration of the extended mass range in the high m/z region. Calibration and sample acquisitions were performed in the positive ion mode on the mass range m/z 2000–25 000, with a manual pusher value of 250 ns. Data were accumulated over 5 min, smoothed with the Savitzky Golay method, the background subtracted and the masses finally calculated. Molecular species were assumed to be represented by series of peaks corresponding to multiply protonated ions. The mass of each species is expressed as a mean of the masses calculated from the series of ions **plus** standard deviation (SD). Charge state assignments were those that gave minimum SD. The maximum entropy-based software (MaxEnt) was used only to find the approximate mass of each subassembly and hence the charge on each multiply charged peak. Because the MaxEnt software fits symmetrical Gaussian peak shapes to the experimental data, it could not be used to establish the accurate mass, since the peaks were asymmetrical due to adduct formation.

ESI-MS measurements were performed either on a ESI-TOF (LCT, Micromass, Altrincham, UK) or on a ESI-Q-TOF (Q-TOF II, Micromass, Altrincham, UK) mass spectrometer, and five concentrations in ammonium acetate buffer were performed at 4 °C and 6000 trs/min. Purity and homogeneity of the Hcs samples

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were estimated by mass analysis in denaturing conditions: Hcs were diluted to 10 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% formic acid. Mass spectra were recorded on the LCT in the positive ion mode, after calibration with horse heart myoglobin diluted to **2 pmol/μL** in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% formic acid.

MALDI-TOF MS

Matrix-assisted laser desorption/ionization MS (MALDI) analysis were performed using a MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany) operating in linear positive ion mode. Ions were formed by a pulsed UV laser beam (nitrogen laser $\lambda = 337$ nm). The instrumental parameters were the following: ion source voltage 1: 25 kV; ion source voltage 2: 22.20 kV; delay time: 210 ns. Five μL of each sample (native molecule of Hcs from *S. officinalis, O. vulgaris* and *R. venosa* and their isoforms) was mixed with 5 μL of sinapinic acid matrix solution (saturated solution in H_2O/Acetonitrile (50/50; v/v) containing 0.1 % trifluoracetic acid). About 1 μL of this mixture was deposited on the stainless-steel sample holder. External mass calibrations were done using the Protein Calibration Standard II (Bruker Daltonics), based on the average values of $[M+H]^+$ of Trypsinogen, Protein A, Albumin-Bovine (BSA) and the average values of $[M+H]^+$ and $[M+2H]^{2+}$ of Protein A and Albumin-Bovine (BSA) at 'mass/charge' (*m/z*) **23 892, 44 413, 66 432, 22 307 and 33 216, respectively**.

RESULTS AND DISCUSSION

Determination of protein mass and structure has been a central field of investigation since the beginning of biochemistry. Numerous methods have been developed along the decades to obtain information about mass, size, composition, subunit arrangements and physical or biochemical constants.[30] Commonly used methods in this field are sedimentation velocity (SV), sedimentation equilibrium (SE), gel electrophoresis, size-exclusion chromatography (SEC), STEM, cryoelectron microscopy (cryo-EM), crystallography, primary sequence determination and nuclear magnetic resonance.[8,11,23,30–34] All the cited techniques are of major interest for investigation of protein mass and structure: they can either provide high quality information (SE, SV, STEM, crystallography and cryo-EM) or provide rapid and convenient analysis (gel electrophoresis, SEC). These techniques were applied to determine the molecular masses of several molluscan and arthropodan Hcs. A molecular weight of 3580 kDa was determined for the native molecule of *O. dofleini* Hc by the SE and a sedimentation coefficient, $S^0_{20,w}$, of 51.0S. The sedimentation coefficient at about of 51S and 63S is very rapid for the Hcs with one and two subunits, respectively, with molecular mass about 3000 and 9000 kDa. This was confirmed by dissociation of the native molecule of OvH in 10-mM EDTA buffer, at pH 8.0, to a single subunit with a molecular weight of 359 kDa and a sedimentation coefficient, S_{20},w^0, of 11.1S.[32]

The size of the native Hc complexes was also studied by SEC, consistently provided a value of 400–450 kDa for several isoforms.[26] After dissociation of the native Hcs, isolated from the hemolymph of molluscs *S. officinalis, O. vulgaris* and *R. venosa*, against 0.13 M glycin buffer, pH 9.2, they were applied on a Superose 6 column. Only one fraction was eluted with 50-mM Tris–HCl buffer, pH 8.5 on the chromatogram applying dissociated molecule of Octopus Hc and two fractions for Rapana Hc (Figure 1A and B, respectively). The absorption spectrum of all isolated isoforms showed three peaks, at 278, 344 and 550 nm, corresponding to aromatic residues, Cu^{2+}-O^{2-} and Cu^{2+}-histidine coordination centers, respectively (data not shown). However, the masses of subunits are very close, and these techniques give approximate values. In this context and to study high molecular mass noncovalent complexes such as invertebrate respiratory proteins, one would expect to use methods enabling relatively rapid analysis of samples in native and denaturing conditions, to obtain information about quaternary structure with a high precision, able to detect slight variations in close molecular species.

MALLS and MS are two complementary approaches permitting to satisfy most of these requirements. MALLS and ESI-MS have proved to be useful techniques for monitoring interactions between proteins and ligands, association and dissociation kinetics, protein folding and enzymatic mechanisms.[33,34] The complementarity of MALLS solvent versatility and ESI-MS high mass accuracy by Q-TOF instrumentation allows new and original approaches for investigation of large noncovalent protein structures. Therefore, using these techniques, OvH, SpH and RvH were analysed.

Determination of macromolecular complex mass of molluscan hemocyanins by mass spectrometry

Measurements of the molecular masses of the native molecules and isolated isoforms from above mentioned Hcs **were performed by ESI**-TOF, ESI-Q-TOF, MALLS and MALDI-MS. MALLS provides mass and size estimation in various aqueous solvents. Before analysis, 100 μL of each Hc and isoforms was desalted on 100 kDa micro-concentrators in 10-mM ammonium acetate (pH 6.8). In non-denaturing conditions, the mass measurements of native Hcs were performed in the same buffer to preserve their native conformation in solution.

Purity and homogeneity of the Hcs samples were estimated by mass analysis in denaturing conditions: Hcs were diluted to 10 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) and 1% formic acid. In these conditions, the noncovalent interactions are disrupted, **allowing** the measurement of the molecular weight of the constitutive monomeric polypeptidic chains with a good precision.

SEC-MALLS analyses were performed with a Superose 6-C column for the native and dissociated molecule of Hc of *S. oficinalis*. In Figure 2, an optical density at 280 nm (full curve) and estimated molecular weight (dots) are represented as a function of the eluted volume. At the native condition, only one fraction was eluted on the column with a mass of Mw = 4134

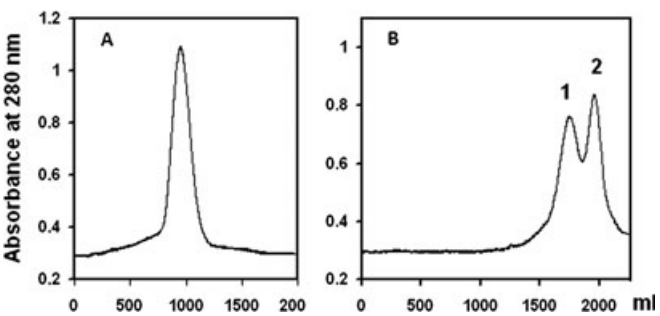

Figure 1. Chromatograms of the dissociated molecule of (A) Octopus Hc, (B) Rapana Hc on a Sepharose 6 column, eluted by 10-mM Tris/HCl buffer, pH 9.0.

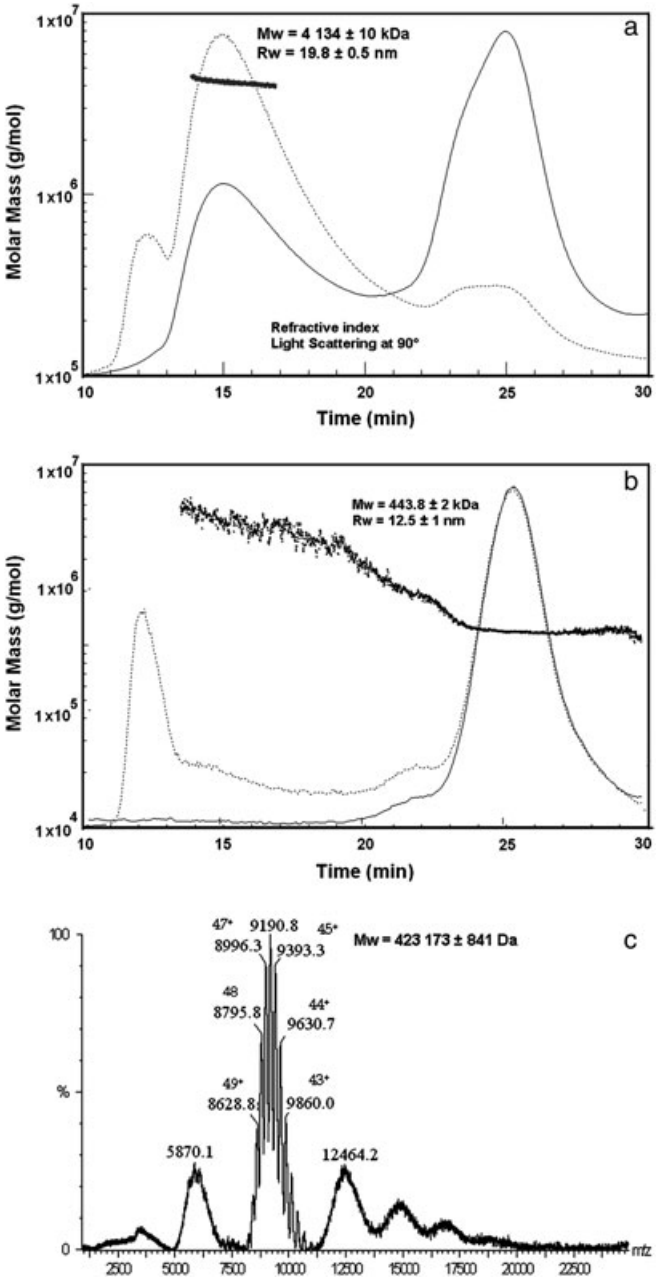

Figure 2. SEC-MALLS analysis obtained for (A) the native and (B) dissociated molecule in 130-mM Gly/NaOH, pH 9.6 buffer of hemocyanin *S. oficinalis*. Analysis was performed with a Superose 6-C columneluted with 50-mM Tris–HCl buffer, pH 8.5. **Optical density at 280 nm (full curve), light scattering at 90° (dash) and estimated molecular weight (dots).** (C) ESIM-MS spectra of dissociated molecule of SoH.

$\pm$10 kDa and Rw = 19.8 $\pm$ 0.5 nm, corresponding to the native molecule of *S. oficinalis* Hc (Figure 2A). After partial dissociation of the native molecule in 130-mM Gly/NaOH, pH 9.2 buffer, again one fraction with a mass of 443.8 $\pm$ 2 kDa and Rw = 12.5 $\pm$ 1 nm (Figure 2B) was obtained.

Concurrent ESI-MS investigations of Hc were performed to obtain the molecular masses of their constituent chains and subunits as well as their relative proportions. While MALLS provides masses in a physiological buffer, ESI-MS measures masses of gas-phase ions. Thus, MALLS and ESI-MS are complementary methods to explore structure heterogeneity and its relevance in biological conditions. Moreover, the experiments were performed on both instruments, ESI-TOF and ESI-Q-TOF. Because the quality of the data was far better on the ESI-Q-TOF than on the **ESI-TOF, only the mass spectra obtained by ESI-Q-TOF are presented here**.

Samples were diluted two to three times in 10-mM ammonium acetate (pH 6.8) buffer to a final concentration of about 20 **pmol/μL** for a >1000-kDa complex and continuously infused into the ESI ion source at a flow rate of 5 μL/min. Figure 2C represents the spectrum of dissociated molecule of *S. oficinalis* Hc. Deconvolution of the ESI mass spectrum gives a Mw = 423 173 $\pm$ 841 Da for the subunit. The good agreement between the masses determined by ESI-MS and **MALLS shows the reliability of the method and validates the use of ESI-MS for investigation of noncovalent assemblies representative of the native state in solution. Since the known mass of a single subunit is 443 kDa, these masses fit** unambiguously with the mass of decameric form, organized by 10 subunits, which confirms that only one isoform organizes the molecule of *Octopus* Hc.

The representative masses of the native and dissociated molecule of SoH are reported in Table 1. The mass spectra of the native Sepia in combination with those of dissociated allowed to analyse the quaternary structure of the molecule, represented in the hemolymph as a decamer.

The same behavior was observed for *O. vulgaris* Hc. At the native condition, only one fraction with a Mw = 3545 $\pm$ 9 kDa and Rw = 16.6 $\pm$ 0.5 nm (Figure 3A) and, after partial dissociation, of 359.3 $\pm$ 1 kDa and Rw = 7.9 $\pm$ 1 nm (Figure 3B) were **eluted on the column. The same mass value was obtained by ESI-MS (Mw = 343 564 $\pm$ 159 Da) after dissociation of the native molecule (Figure** 3**C). Considered together, the results from the various approaches to mass determination of Octopus and Sepia Hcs convincingly suggested that the native molecules of both Hcs are decamers, composed of identical subunits with molecular masses** of 359.3 and 423.2 kDa, respectively.

As MALLS gives very precise measurements of molar mass with a 1% error or smaller, it was used as a rapid and convenient way to determine the mass of Rapana Hc complex, which is dissolved in

Table 1. The molecular masses of ***Octopus, Rapana and Sepia*** hemocyanins measured by different mass spectrometric techniques

Hemocyanin	**Subunits/*FUs***	**Masses measured by: (kDa)**				
		Native		***Dissociated***		***Difference***
		MALLS		**ESI-MS AAS**		
O. vulgaris	17	3 545 $\pm$ 9	359.3 $\pm$ 1	343.6 $\pm$ 0.2	332.011	**11.6**
S. oficinalis	18	4 134 $\pm$ 10	443.8 $\pm$ 2	423.2 $\pm$ **0.8**	383.189	**40.0**
R. venosa	2	8 455 $\pm$ 51	566.0 $\pm$ 2	211.9 $\pm$ 0.8 -		
RvH1	8		422.8 $\pm$ 1	315.1 $\pm$ 0.6		
RvH2	8		400.0 $\pm$ 2			

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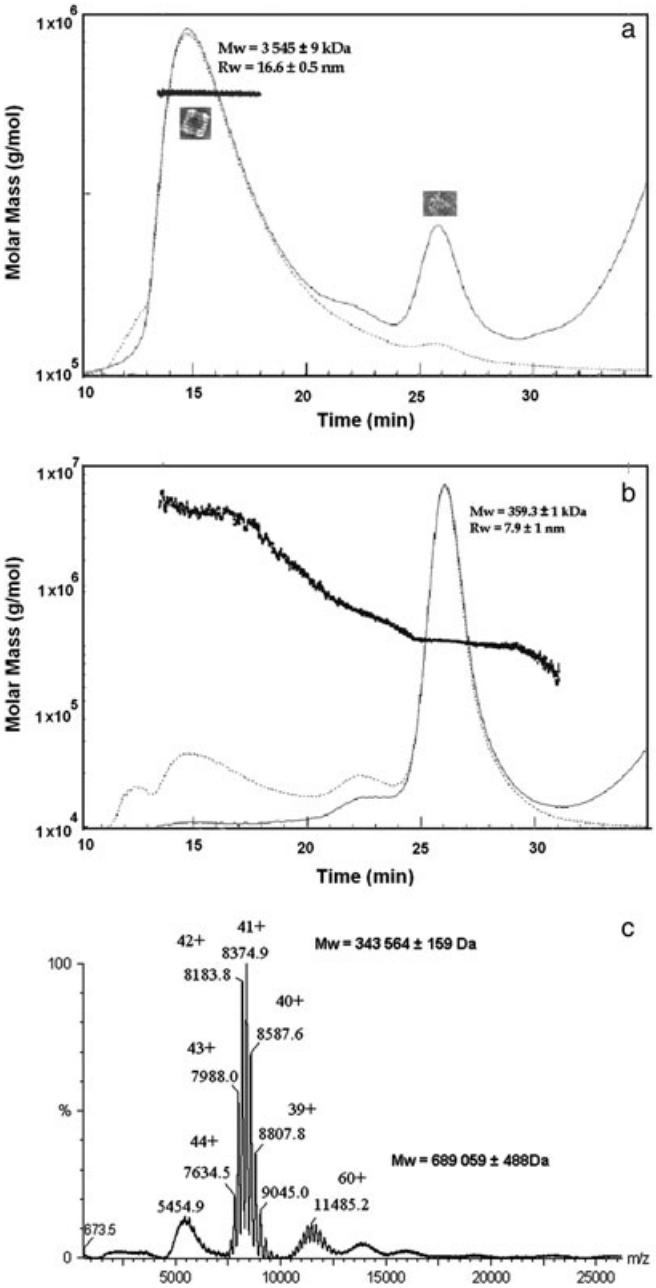

Figure 3. SEC-MALLS analysis obtained for the native (A) and dissociated molecule in 130-mM Gly/NaOH, pH 9.6 buffer of hemocyanin *O. vulgaris*. Analysis was performed as described in Figure 2. (C) ESIM-MS spectra of dissociated molecule of OvH.

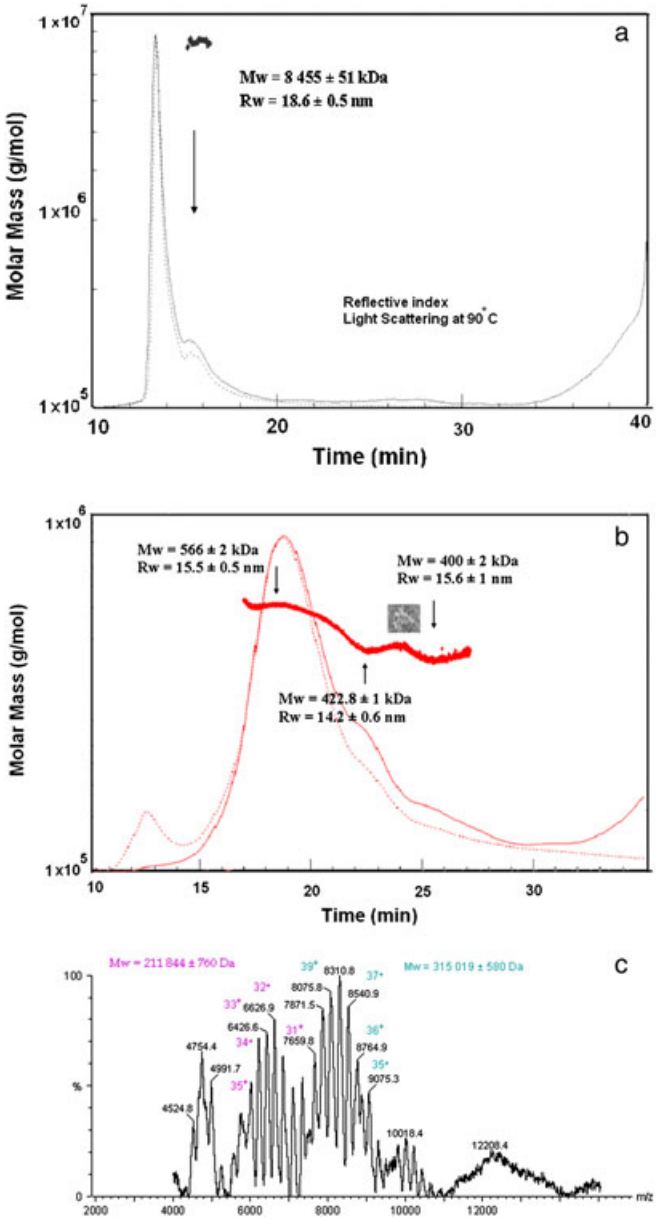

Figure 4. SEC-MALLS analysis obtained for the native (A) and dissociated molecule in 130-mM Gly/NaOH, pH 9.6 buffer of hemocyanin *R. venosa*. Analysis was performed as described in Figure 2. (C) ESIM-MS spectra of dissociated molecule of RvH.

the hemolymph of the marine snail as two isoforms. The native molecule of Hc *R. venosa* was eluted as a single peak on the chromatogram with a molecular mass of Mw = 8455 ± 51 kDa and Rw = 18.6 ± 0.5 nm (Figure 4A). Since the known masses from the literature of Rapana subunits (RvH1 and RvH2) are about 420 kDa, the measured mass of 8455 kDa fits with didecameric form, organized by 20 subunits.[12] This is confirmed by TEM observation of hemolymph samples in which globular particles are didecamer, associated by two decamers.[12] However, several values were measured for structural subunits of Rapana Hc, using different techniques as molecular mass of ~250, 150, 420 and 450 kDa.[21] The masses of 400 kDa and 280 kDa were determined by PAGE for the natural fragments of *Rapana thomasiana* (renamed as Rapana venosa) Hc.[21] In our measurements after dissociation on the native RvH, three fractions were eluted on a Sepharose 6 column with masses of Mw = 566 ± 2 kDa (Rw = 15.5 ± 0.5 nm), Mw = 400 ± 2 kDa (Rw = 15.6 ± 1 nm) and Mw = 422.8 ± 1 kDa (Rw = 14.2 ± 0.6 nm), as determined by MALLS (Figure 4B**). The obtained results by MALLS for the second** (400 ± 2 kDa**) and the third fractions** (422.8 ± 1 kDa) **correlate with the published data of RvH1 and RvH2**[12] **but distinguish then the measured masses by ESI-MS. Analysis of RvH with denaturing ESI-MS reveals fraction with molecular masses of Mw = 211 844 ± 760 Da and Mw = 315 019 ± 580 Da (Figure** 4**C and Table 1). They divers from the subunits, and the sum of the masses of both fractions determined by ESI-MS (Mw = 211.8 + 315.0 =526.8 kDa) for Rapana Hc correlates to the mass of mega-Hc**.

Comparison of the measured masses by different methods of the native and dissociated molecules of Octopus, Sepia and Rapana Hcs is shown in Table 1. **The determined masses of structural subunits of molluscan Hcs by MALLS are about 400 kDa. The 400-kDa subunit has existed for ~740 million years and is widespread among the Mollusk.**[35,36] **Moreover, a fraction with mass of 566 kDa, detected for Rapana Hc on MALLS measurements (Figure** 4**B), was identified until now only as a larger mega-Hc subunit in *Melanoides*** tuberculata **Hc.**[37] The unique 550-kDa Hc subunit is one of the largest polypeptides ever reported, **occurring** probably only in the superfamily Cerithioidea which appeared in the Paleozoic.[38] The wall of the mega-decamer is apparently constructed according to the typical scheme, which means that the six wall FUs FU-a to FU-f that exist in the 400-kDa subunit are also present in the 550-kDa subunit.[21]

For additional information, RvH was analysed by electron microscopical (EM) technique. Typical gastropodan Hcs appearing as didecamers tend to bind additional decamers at one or both ends, thereby forming tridecamers, tubuls and larger multidecamers.[6,12,13] Representations of the RvH, in Figure 5A, as didecamers and tridecamer derived from EM analyses. Moreover, the results clearly show that additional decamer linking to the didecamer forms a tridecamer. The exact association mode of the additional decamers as a novel mega- Hc tridecamer (Figure 5B) in the Hcs of cerithioid snails Leptoxis, Melanoides and Terebralia was explained by Lieb *et al.*[37] **Electron microscopy revealed a variable mixture of mega-Hc oligomers in *Melanoides tuberculata* (Thiaridae)(MtH), with variable proportions of the two subunits, however with a clear excess of the 550-kDa subunit. The 'typical' tridecamer structures were also found in KLH2, but in this case, the tridecamer is partially hollow, whereas the cerithioid tridecamer is almost completely filled with material; it was therefore termed 'mega-Hc'.**

Due to the high accuracy of molecular mass determination of the mass spectrometric techniques, they also allow determination of molecular masses of FUs and post-translational modifications, such as glycosylation.[38–40] **Until now, several FUs were isolated and analysed after enzymatic cleavage of Hcs.**[14,23,31,41,42] **The molecular masses of the fractions obtained after tryptic digestion of Rapana with trypsin and separated by a FPLC system using a Q sepharose high-performance column were measured by MALDI-TOF-MS (Figure** 6**). The ions at m/z 50 585, 55 978 and 59 123 are due to three FUs, while the ions at m/z 97 431 and 108 150 are due to two linked FUs. Some of the eluted peaks on the column contained only one pure FU. These data confirmed that MS is a very rapid and useful technique not only to determine the masses of the proteins, but also to gain information on the composition of mixture of several fractions. Therefore, the molecular masses of the obtained fractions after tryptic digestion with trypsin of the other Hcs as Octopus and Sepia were also analysed by MALDI-TOF to be around 50 kDa (data not shown).**

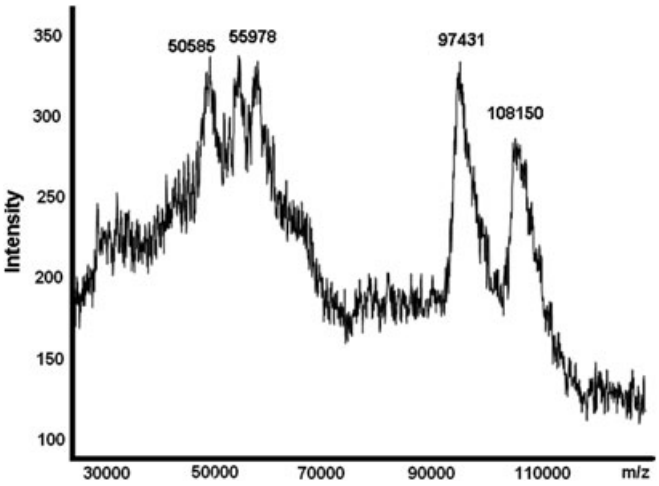

Figure 6. MALDI-TOF spectra on isolated fragment after treatment of RvH2 subunit of Rapana hemocyanin with trypsin. Chicken egg ovalbumin (44 400 Da) and bovine serum albumin (66 430 Da) were used for mass scale calibration.

Molluscan Hcs are glycosylated, but their glycan content is generally not high enough to cause irregular migration on SDS gels. For example, in case of *Haliotis tuberculata* Hc, ~400 kDa was determined by SDS-PAGE, and 392 kDa was later predicted from the amino acid sequence.[13,16] From their total yield, the glycans might well be determined by MS. Therefore, the obtained results by ESI-MS and MALLS were successfully applied to predict the mass of the oligosaccharides in above mentioned Hcs. The molecular masses of the isoforms of Octopus and Sepia measured by MS differ from the calculated masses determined by the gene sequences

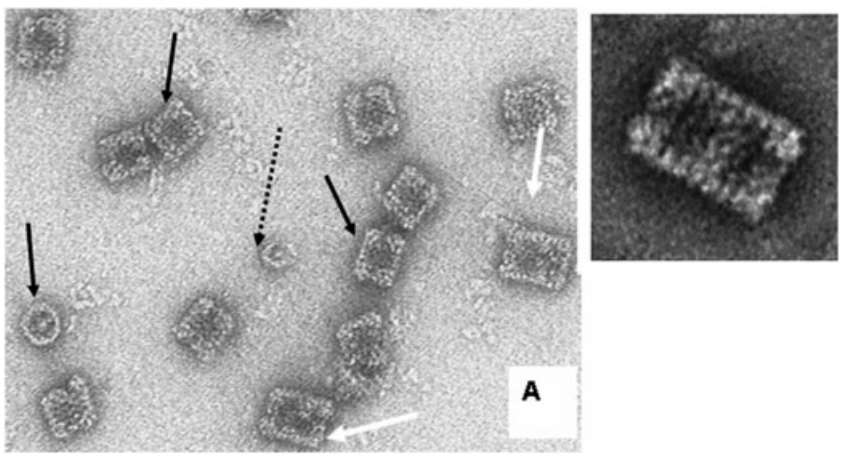

Figure 5. Transmission electron microscope images of negatively stained gastropod hemocyanins. (A) RvH, showing typical didecamers (black arrowhead), typical tridecamers build from a decamer (white arrowhead) and subunit (dash arrowhead); (B) mega-tridecamers.

of *S. oficinalis (*DQ388569) and *O. vulgaris* (AY751301) (Table 1). The mass differences of approximately 11.6 and 40.0 kDa in the case of Octopus and **Sepia, respectively**, suggest the presence of glycoforms. The oligosaccharide structures of OvH, SoH and RvH were determined as complex structures.[38,39]

CONCLUSION

Hcs are glycoproteins, freely dissolved in the hemolymph, of many arthropods and molluscs with huge molecular masses of 4000 to 9000 kDa.[6–8] The Hcs of both phyla, mollusks and arthropods differ substantially for their molecular architecture and arrangement of the subunits constituting the native aggregates often represented as noncovalent multimeric proteins.[2,3,5] Understanding the function of macromolecular complexes is related to a precise knowledge of their structure. Therefore, in this study, the structure, association and dissociation behavior of molluscan Hcs from gastropod (*R. venosa*), cefalopods (*O. vulgaris, S. officinalis*) **were studied by MS and electron spectroscopy methods. This allowed to determine the molecular masses of native and dissociated molecules. ESI-MS, MALLS and MALDI-MS techniques revealed one structural subunit in Sepia and Octopus Hcs, while two or three components with different masses were measured from Rapan Hc. Based on the mass values determined by MS, the structure of the above mentioned Hcs was much more precisely determined. Likewise, the carbohydrate content of molluscs OvH and SoH (11.6 and 40.0 kDa, respectively) was also suggested by the differences of calculated masses of subunits by their gene sequences and confirmed by the by MS measurements**.

Understanding the function of macromolecular complexes is related to a precise knowledge of their structure. Therefore, the obtained results confirmed that using ESI-MS, MALLS and MALDI-MS techniques and also in combination with other methods and techniques as electron microscopy, gene sequences and modeling precise information of Octopus, Sepia and Rapana Hcs was obtained.

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